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Impacto de stressores naturais e/ou químicos no enquitraídeo tolerante ao frio e eurihalino, *Enchytraeus albidus*

Impact of natural and/or chemical stressors on the freeze-tolerant and euryhaline enchytraeid, *Enchytraeus albidus*

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palavras-chave

Fatores abióticos, salinidade, contaminantes, tolerância ao frio, ciclos de congelamento e descongelamento, variação intra-específica, osmolalidade, crioprotectores, conteúdo em gelo, biomarcadores de stress oxidativo, alocação de energia, sobrevivência, reprodução, absorção e eliminação de contaminantes.

resumo

As alterações climáticas estão a atingir rapidamente as regiões do Ártico, Sub-Ártico e as regiões temperadas, apontando as previsões para um aumento de eventos de congelamento-descongelamento, bem como mudanças nos padrões de precipitação, evaporação e de salinidade. Estas alterações climáticas poderão resultar em impactos francamente negativos no funcionamento e dinâmica de ecossistemas, especialmente quando associados à presença de contaminantes resultantes da intensa atividade antropogénica. Embora a incorporação de stressores múltiplos em estudos de ecotoxicidade tenha recebido um crescente interesse pela comunidade científica, o seu número é ainda reduzido. Particularizando, o conhecimento dos efeitos de eventos de congelamento-descongelamento e de flutuações de salinidade permanecem desconhecidos, especialmente quando se consideram espécies supra-litorais. Neste contexto, o objetivo geral da presente tese consistiu em investigar os efeitos das flutuações de temperaturas e salinidade, individualmente ou em combinação com contaminantes, no enquitraídeo tolerante ao frio e eurialino - o *Enchytraeus albidus*.

A avaliação de parâmetros populacionais (sobrevivência, reprodução e bioacumulação), fisiológicos (níveis de crioprotetores, conteúdo em gelo / água, temperatura de fusão e sobrecongelamento) e bioquímicos (biomarcadores de stress oxidativo, alocação de energia celular) permitiu compilar novas e valiosas informações sobre os efeitos dos stressores físicos e químicos selecionados no enquitraídeo e compreender quais os reajustes nos mecanismos de resposta primários que lhes permitem manter a homeostasia e sobrevivência em ambientes inóspitos como as regiões Polares e temperadas-frias.

A presença de níveis moderados de salinidade aumentou significativamente a tolerância a temperaturas congelantes (essencialmente avaliada como sobrevivência, crioprotecção e fracção de gelo extracelular) e a reprodução do *E. albidus*. Além disso, contribuiu para a regulação de crioprotectores, restauração dos níveis de antioxidantes nestes organismos e alterou significativamente o efeito e a incorporação/absorção de substâncias químicas (cádmio, cobre carbendazim e 4-nonilfenol). As flutuações de temperatura (simuladas como ciclos diários de congelamento-descongelamento, com temperaturas entre 2ºC e -4ºC) causaram um efeito substancialmente negativo na sobrevivência de organismos previamente expostos a concentrações não letais de 4-nonilfenol, quando comparados com organismos expostos a uma temperatura congelante constante (-4ºC) ou à temperatura controlo (2ºC). A diminuição na crioproteção, o aumento no consumo de energia e a maior concentração de 4-nonilfenol nos tecidos vieram sublinhar o elevado gasto energético e o nível de toxicidade sofrido pelos organismos expostos à combinação de contaminantes e eventos de congelamento e descongelamento.

Os resultados apresentados nesta tese demonstram, assim, que a presença de stressores naturais (físicos) e químicos, isoladamente ou em combinação, podem alterar a dinâmica do *E. albidus*, afetando não só a sua sobrevivência e reprodução (e consequente presença / distribuição), mas também as suas adaptações fisiológicas e bioquímicas. Essas alterações podem levar a consequências graves para o funcionamento dos ecossistemas do Ártico, sub-Ártico e regiões temperadas-frias, uma vez que estes organismos desempenham um papel importante para a decomposição de matéria orgânica morta. Esta tese fornece ainda uma base científica para melhorar a atribuição de coeficientes de segurança para os ecossistemas naturais do solo, alertando para a integração de investigações semelhantes em ecotoxicologia, e, eventualmente, para a avaliação de risco ecológico de contaminantes

keywords

Abiotic factors, salinity, contaminants, freeze-tolerance, freeze-thaw cycles, intra-specific variation, osmolality, cryoprotectants, ice-content, oxidative stress biomarkers, cellular energy allocation, survival, reproduction, uptake, elimination.

abstract

Rapid climatic changes are taking place in Arctic, subarctic and cold temperate regions, where predictions point to an increase in freeze-thaw events, changes in precipitation, evaporation and salinity patterns. Climate change may therefore result in large impacts in ecosystem functioning and dynamics, especially in the presence of contaminants due to intense anthropogenic activities. Even though multiple stress approaches have received increasing interest in the last decades, the number of such studies is limited. In particular, knowledge on the effect of freeze-thaw events and salinity fluctuations on ecotoxicology of soil invertebrates is lacking, especially important when considering supralittoral species. Therefore, the aim of this thesis was to investigate the effects of low temperature and salinity fluctuations, singly and in combination with contaminants, in the freeze-tolerant and euryhaline enchytraeid *Enchytraeus albidus*.

The assessment of population level endpoints (survival and reproduction), along with physiological and biochemical parameters such as levels of cryoprotectants, ice/water content, oxidative stress biomarkers, cellular energy allocation, and tissue concentration of chemicals (when applied), provided new and valuable knowledge on the effects of selected physical and chemical stressors in *E. albidus*, and allowed the understanding of adjustments in the primary response mechanisms that enable worms to maintain homeostasis and survival in harsh environments such as polar and temperate-cold regions.

The presence of moderate levels of salinity significantly increased freeze-tolerance (mainly evaluated as survival, cryoprotection and ice fraction) and reproduction of *E. albidus*. Moreover, it contributed to the readjustments of cryoprotectant levels, restoration of antioxidant levels and changed singnificantly the effect and uptake of chemicals (copper cadmium, carbendazim and 4-nonylphenol). Temperature fluctuations (simulated as daily freeze-thaw cycles, between -2ºC and -4ºC) caused substancial negative effect on survival of worms previsouly exposed to non-lethal concentrations of 4-nonylphenol, as compared with constant freezing (-4ºC) and control temperature (2ºC). The decrease in cryoprotectants, increase in energy consumption and the highest concentration of 4-nonylphenol in the tissues have highlighted the high energy requirements and level of toxicity experienced by worms exposed to the combined effect of contaminants and freezing-thawing events.

The findings reported on this thesis demonstrate that natural (physical) and chemical stressors, singly or in combination, may alter the dynamics of *E. albidus*, affecting not only their survival and reproduction (and consequent presence/distribution) but also their physiological and biochemical adaptations. These alterations may lead to severe consequences for the functioning of the ecosystems along the Arctic, subarctic and cold temperate regions, where they play an important role for decomposition of dead organic matter. This thesis provides a scientific basis for improving the setting of safety factors for natural soil ecosystems, and to underline the integration of similar investigations in ecotoxicology, and eventually in risk assessment of contaminants.

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Context and outline of the thesis

Soil organisms are often exposed to multiple stressful situations during their lifetime, and among these are sub-optimal and occasionally stressful environmental conditions that are becoming more extreme and frequent due to climate change. These stressful environmental conditions may significantly modify organisms' tolerance to a given contaminant (and vice-versa) or even to other natural factors. Even though "multiple stress" approaches have received increasing interest in the last decades, such studies are still relatively scarce concerning soil organisms. Up to now, a gap still persisted on the effect of freeze-thaw events and salinity fluctuations on ecotoxicology of soil invertebrates, particularly the ones that inhabit challenging environments as Artic, sub-arctic and cold temperate regions.

The objective of this thesis was, therefore, to examine the biological, physiological and biochemical responses mechanisms to temperature and salinity fluctuations, singly and in combination with contaminants in a representative and keystone species of many terrestrial and supralittoral ecosystems along the Arctic and Temperate regions – the enchytraeid *Enchytraeus albidus* (Annelid, Oligochaeta). This species, also known as potworms or white worms, has also been extensively used as model on freeze-tolerance studies and a standard organisms in terrestrial ecotoxicology and risk assessment for which several tools are available as, for instance, ISO and OECD standardized guidelines for reproduction, survival and avoidance; and optimized protocols to assess energy reserves and oxidative stress levels. This thesis also intends to contribute with a scientific basis for improving the setting of safety factors required when extrapolating results from optimal laboratory conditions (often used in soil ecotoxicology tests) to natural soil ecosystems.

The structure of this thesis has the format of a series of publications (accepted/published, unpublished) as follows:

Chapter I: "Adaptations of enchytraeids to single and combined effects of physical and chemical stressors" published on the web 29 September 2015 by *Environmental Reviews*. It provides an integrated overview of the investigations carried out on this PhD and on the literature regarding the influence of natural stressors and contaminants on enchytraeids.

Chapter II: "Soil salinity increases survival of freezing in the enchytraeid *Enchytraeus albidus"* published in the *Journal of Experimental Biology* 216 (Pt14) (2013), 2732-2740. This investigation aimed to study the importance of salinity on the freeze tolerance capacity of two different populations of *E. albidus* – one collected on decaying seaweeds along the coast of Nuuk, Greenland; and another from a compost soil in Jena, Germany. The experiment consisted in a short-term exposure to environmental relevant salinity concentrations and decreasing (low to frost) temperatures; where survival, osmolality capacity, cryoprotectant levels, super-cooling and melting points, water and ice-contents, fresh and dry weights were

evaluated as main endpoints. Complementarily, it was assessed the effect of salinity on *E. albidus* reproduction.

Chapter III: "Worms from the Arctic are better adapted to freezing and high salinity than worms from temperate regions: Oxidative stress responses in *Enchytraeus albidus*", published in the *Journal of Comparative Biochemistry and Physiology - Part A* 163 (2013), 582-589. This investigation is complementary to Chapter II, by assessing oxidative stress responses under similar experimental design. Various enzymatic and non-enzymatic oxidative stress markers, as well as neurotransmission activity, were used as endpoints.

Chapter IV: "Salinity changes impact of hazardous chemicals in *Enchytraeus albidus*", published in the *Journal of Environmental Toxicology and Chemistry* (2015). This study evaluates the influence of low levels of salinity on the effect of four contaminants with known mode of action – 2 metals (copper and cadmium) and 2 organic contaminants (4-nonylphenol and carbendazim). Survival and reproduction were selected as endpoints using the international guidelines for this species (ISO, 2004; OECD, 2004), to allow comparisons with previous investigations and to evaluate how appropriate these guidelines are for the model species. The tissue concentration of the contaminants was also evaluated in order to provide new evidences on the effect of salinity on uptake of chemicals by *E. albidus*.

Chapter V: "Importance of Freeze−Thaw Events in Low Temperature Ecotoxicology of Cold Tolerant Enchytraeids", published in the *Journal of Environmental Science and Technology* 48(16) (2014), 9790-9796. This study evaluates how *E. albidus* respond to a short-term exposure to constant freezing and freeze−thaw cycles when combined with environmentally relevant (and sub-lethal) concentrations of 4-nonylphenol (a known lipophilic compound and widely spread contaminant). Survival, number of cryoinjuries, levels of cryoprotectants and internal concentration of 4-nonylphenol were used as endpoints.

Chapter VI: "Effect of freeze-thaw cycles and 4-nonylphenol on cellular energy allocation in the freeze-tolerant enchytraeid *Enchytraeus albidus*" accepted for publication in Environmental Science and Pollution Research. This investigation is complementary to Chapter V. Energy available (lipids, carbohydrates and proteins), energy consumed (ETS) and cellular energy allocation were evaluated during several set points during exposure to combined effect of 4-nonylphenol and temperature treatments, under the same experimental design as described on Chapter V.

Chapter VII: "Uptake and elimination of 4-nonylphenol in the enchytraeid *Enchytraeus albidus"* accepted for publication in the *Bulletin of Environmental Contamination and Toxicology.* This investigation evaluated the uptake and elimination of 4-nonylphenol in the key species *Enchytraeus albidus*.

Chapter VIII - Supplementary research: "Does salinity affect phospholipid fatty acids composition of the enchytraeid *Enchytraeus albidus?*" (*unpublished*). This small investigation tries to further understand the positive influence of salinity on worms' cold tolerance (as observed in Chapter II), by evaluating the potential effect of this abiotic factor on membrane composition of the organisms, which is a crucial adaptation to ensure survival to low temperatures.

Chapter IX - Final remarks.

Photo by Karina Fisker

Chapter I

Adaptations of enchytraeids to single and combined effects of physical and chemical stressors.

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SUMMARY

Climate changes are expected to be greatest in the polar and temperate areas, where predictions point to an increase in freeze-thaw events and changes in precipitation, evaporation and salinity patterns. These events will therefore affect biological activity of the soil compartment that may result in large impacts in ecosystem functioning and dynamics therein. This concern becomes even more important when considering the presence of contaminants due to intense anthropogenic activity, which may lead to synergistic or antagonistic effects and increase or decrease the impact on natural ecosystems. This paper reviews the effect of physical and chemical stressors on enchytraeids, with special emphasis on *Enchytraeus albidus* because most relevant studies have involved this species. *E. albidus* is a freeze-tolerant and euryhaline organism and several studies suggest that the absence of salinity may have important (negative) consequences not only for their freeze tolerance ability but also for their reproduction and capacity to deal with the presence of contaminants, such as metals and fungicides. Single and joint effects of constant freezing or freeze-thaw cycles and surfactants such as 4-nonylphenol affected negatively *E. albidus* freeze tolerance by decreasing the levels of cryoprotectants, membrane fluidity and interfering with cellular energy allocation. Because enchytraeids are of ecological significance in many important habitats along the Artic and cold-temperate environments, a reduction in abundance may result in disturbances of the decomposition processes in soils. The knowledge of the biological, physiological and biochemical limits of enchytraeids to combined effect of physical and chemical stressors are crucial in order to provide a scientific basis for improving the setting of safety factors when extrapolating from controlled (and optimal) laboratory conditions to natural soil ecosystems. Therefore, there is a need to expand and evolve experiments that more realistically mimic the situation in the field, where interactions between factors are highly relevant. The synergistic or antagonistic interactions identified in the present review may also represent a stepping-stone in the evaluation and possible inclusion of natural factors, like cold and salinity, in standardized enchytraeid test guidelines and consequently in risk assessment of chemicals.

Key words: oligochaeta, enchytraeids, supralittoral, cold hardiness, salinity, contaminants.

1.INTRODUCTION

Climate change is affecting natural ecosystems all over the globe. However, effects of climate change are expected to be more drastic and rapid in the Arctic and in temperate regions (Bates et al., 2008; Rhein et al., 2013). The frequency of freeze−thaw events in subarctic soils are reported to increase, mainly because of the slight rise in the average temperature that is leading to the reduction (or absence) of an insulating snow cover during frost periods (IPCC, 2013). Frost periods

are becoming shorter in duration, but more frequent, with impacts on temperate regions (Bates et al., 2008). In parallel, the mean sea level rise has significantly accelerated through the last two centuries, mainly due to glaciers and ice sheets melting, inducing floods and causing shifts in precipitation, evaporation and salinity patterns along the shoreline (Rhein et al., 2013). Therefore, littoral and supralittoral ecosystems along the Arctic and temperate regions are among the most challenging environments to soil organisms, where climate change may alter their dynamics in time and space, and subsequent consequences for the functioning of these and related ecosystems.

The climatic changes that lie ahead are superimposed by hazardous chemicals present in nature. Several studies have shown that natural stressors can significantly modify the response mechanisms of organisms to toxicants, and vice-versa (Noyes et al., 2009; Holmstrup et al., 2010). Such combinations may be of concern if their effects interact synergistically, that is, with higher risk than the sum of the toxic and natural stressors (Coming, 1983; Holmstrup et al., 2010). Therefore, knowledge of the biological, physiological and biochemical limits of soil organisms to combined effects of natural and chemical stressors are crucial in order to provide a scientific basis for improving the setting of safety factors when extrapolating from controlled (and optimal) laboratory conditions to natural soil ecosystems in environmental risk assessment.

Among soil organisms, enchytraeids have received special attention from the scientific community because of their ecological relevance, distribution, plasticity, and easy manipulation and maintenance. The Enchytraeidae family, commonly known as potworms or white worms because of to their pale colour and small size (~ 3 to 45 mm), belongs to the phylum Annelida, and class Oligochaeta. They form a large group of saprophagous mesofauna living in the litter layer and the upper mineral soil or sediments of many terrestrial and seashore ecosystems (Didden, 1993; Giere, 2006; Boxshall et al., 2014). Enchytraeids are distributed from the Arctic to tropical areas (Kairesalo, 1978; Healy and Bolger, 1984; Martinez-Ansemill and Giani, 1987; Standen, 1988; Verdonschot et al., 1992; Vliet et al., 1995; Schenkova et al., 2001; Peralta et al., 2002; Christensen and Dozsa-Farkas, 2006). Their presence in soil ecosystems is ecologically important because enchytraeids are actively involved in the decomposition of organic matter, nutrient cycling and soil structure formation and maintenance (Petersen and Luxton, 1982; Didden, 1993; Swift et al., 1998).

The Enchytraeidae family contains 27 genera and about 500 species, with new species being described each year (Boxshall et al., 2014). However, Enchytraeidae also seems to contain more variability and more species (cryptic species) than traditional morphological studies have been able to establish (see Erséus and Gustafsson, 2009). Most enchytraeid species are hermaphrodites and reproduce sexually, although parthenogenesis, self-fertilization, and asexual reproduction (fragmentation) occur as well (e.g. *Cognettia sphagnetorum* and *Enchytraeus bigeminus*) (Christensen, 1964). Despite their wide distribution, it is mostly in harsh environments that they have

the highest ecological importance, because they often become the dominant group (in terms of biomass) in such environments, taking over the role of earthworms. For instance, the members of the genus *Cognettia* are the keystone species and dominant group of soil fauna in acidic and nutrient-poor ecosystems such as temperate heathland and boreal forests (Abrahamsen, 1972; Lundkvist, 1982). While marine and brackish supralittoral areas along the northern hemisphere are mainly dominated by the genera *Enchytraeus, Lumbricillus* and *Marionina*, sandy beaches (wrack zone) are exclusively inhabited by *Enchytraeus albidus* and *Lumbricillus lineatus* (Giere and Pfannkuche, 1982; Giere, 2006). As small permeable soil invertebrates, enchytraeids are very susceptible to natural stressors and contaminants. Thus, they have been extensively used as model on cold tolerance studies (Block and Bauer, 2000; Bauer, 2002; Holmstrup et al., 2002b; Slotsbo et al., 2008; Patrício Silva et al., 2013a, 2013b, 2014; Fisker et al., 2014a, 2014b) and as standard organisms in terrestrial ecotoxicology and risk assessment of chemicals (e.g. Römbke and Moser, 2002). Because of their wide distribution and ecological relevance, with sufficient sensitivity to a wide range of environmental stress (chemical and natural), enchytraeids are also used as indicators in monitoring biological soil quality (Graefe and Schmelz, 1999; Didden and Römbke, 2001).

Even though the effects of physical stressors and contaminants on enchytraeids have been extensively studied, the interactions between different physical stressors and between physical and chemical stressors, need more attention.

The objective of this review is to synthesize the existing literature that addresses interactions between physical stressors and between physical and chemical stressors that characterize the actual environmental scenario in different habitats (e.g. supralittoral, soil compost) along the Arctic, Sub-arctic and temperate regions, on cold tolerant enchytraeids. This review is structured by firstly addressing the adaptations and stress response of enchytraeids to cold environments and how these responses are affected by geography, salinity, drought, food sources, and contaminants. Following this, relevant studies addressing the interactions between physical stressors and contaminants and the potential of enchytraeids to acclimate to these new conditions will be discussed. A special emphasis will be given to the supralittoral enchytraeid, *E. albidus,* because most relevant studies have involved this species.

2. COLD HARDINESS OF ENCHYTRAEIDS

From the Arctic to temperate regions, soil invertebrates often face frost temperatures during winter that may cause freezing of their body fluids. Extracellular ice formation potentially leads to an elevation of salt concentrations to toxic levels (Zachariassen, 1985), while (more rarely) intracellular ice formation seems to cause osmotic swelling of the cell compartments with eventual rupture of the cell membranes (Meryman, 1971). To guarantee survival, cold hardy invertebrates opt between two

major strategies: freeze avoidance or freeze tolerance (Zachariassen, 1985; Ramløv, 2000; Zachariassen and Kristiansen, 2003). Freeze-avoiding species have developed mechanisms to stay unfrozen even at temperatures much below the melting point of body fluids, by increasing their supercooling ability (Zachariassen, 1985) and/or through cryoprotective dehydration until the melting point of their body fluids is lowered to the ambient temperature (Holmstrup et al., 2002b). On the other hand, freeze tolerant species seek to establish controlled and protective freezing of the extracellular body fluids at high sub-zero temperatures (Zachariassen, 1985). Each cold hardiness survival strategy is characterized by several physiological and biochemical adaptations that present advantages under certain scenarios such as freeze-thaw events, short or long-term exposure, or at extreme sub-zero temperatures (see Table 1).

Table 1: Comparison between the two main survival strategy of cold hardiness invertebrates – Freeze-avoidance and Freeze tolerance.

Freeze-avoidance	*VS*	Freeze tolerance
Similarities		
Production of cryoprotectants (such as sugars and polyols) (Zachariassen, 1950)		
Stabilization of membranes (by changing composition of cellular lipids to increase membrane fluidity) and proteins (Crowe et al., 1987; Hazel and Williams, 1990; Bindesbøl et al., 2009; Fisker et al., 2015).		
Differences		
Avoid Ice formation, by removing ice-nucleating particles and producing antifreeze proteins, which associated with cryoprotectants leads to a depression of the freezing point (Zachariassen and Husby, 1982; Zachariassen, 1985; Zachariassen and Kristiansen, 2000; Ramløv, 2000).		Allow extracellular freezing by reducing the amount of ice formed due to an increase of cryoprotectants, and by increasing ice-nucleating agents to induce early (but controlled) crystallization (Zachariassen and Kristiansen, 2000).
Allow cryoprotective dehydration i.e., losing water (dehydrate) until the melting point of their body fluid and the water vapor pressure in the surrounding atmosphere reaches equilibrium, so that no freezing can occur (Holmstrup and Sømme,1998).		Decrease the cellular metabolism to save energy (Storey and Storey, 1988; Irwin et al., 2003; Calderon et al., 2009; Fisker et al., 2014).
Main advantages		
High rate of surviving during a short-term exposure to freeze-thaw events (considering high sub-zero temperatures) (Bale et al., 2001).		No (low) mortality of inoculative freezing (Holmstrup and Zachariassen, 1996; Patrício Silva et al., 2013b). At sub-zero temperatures, the vapor pressure remains in equilibrium (no dehydration) (Zachariassen, 1985; Lundhein and Zachariassen, 1993). Tolerance of extremely low temperatures (Block, 1982).
Main disadvantages		
Probability of freezing is proportional to time. Longer periods, higher risk of inoculative freezing (Sømme, 1995) At sub-zero temperatures, vapour-pressure of organisms is in deficit compared to the air causing severe dehydration. Some species can deal with such water loss by cryoprotective dehydration (Holmstrup and Sømme,1998; Holmstrup and Sjursen, 2001).		Long-term medium/high risk of mortality during freeze-thaw events (Churchill and Storey, 1989; Bale et al., 2001; Brown et al., 2004; Marshall and Sinclair, 2011).

Cold hardiness of enchytraeids in Arctic and temperate environments has been reported in several studies (e.g. Kähler, 1970; Bauer, 2002; Slotsbo et al., 2008; Patrício Silva et al., 2013b). When ambient temperatures decrease below the melting point of the body fluids, enchytraeids, like any other ectothermic animal, face the risks associated with the freezing of body fluids (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003; Slotsbo et al., 2008). Because enchytraeids are small hygrophilic soil organisms with high cuticular permeability for water, they are not likely to use supercooling as a freeze-avoiding cold tolerance strategy because the intimate contact with environmental ice will result in inoculative freezing of body fluids (Pedersen and Holmstrup, 2003). Instead, they use cryoprotective dehydration if facing high sub-zero temperatures (between -2 and -4ºC) in relatively dry soils (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003), or freeze tolerance if facing lower sub-zero temperatures (lower than -4ºC) in moist soils (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003; Slotsbo et al., 2008). Other studies indicate that some species, such as *Henlea perpusilla, Enchytraeus variatus* and *Fridericia spp*., may survive frost in the cocoon stage, but very little is known about the cold hardiness of enchytraeid cocoons (Birkemoe, 1995; Klungland, 1997; Bauer et al., 1998, 2001; Bauer, 2002). At least 15 enchytraeid genera are reported from Arctic, sub-Arctic and temperate regions (Healy and Bolger, 1984; Erséus et al., 1999; Christensen and Dozsa-Farkas, 2006; Coulson, 2013; Coulson et al., 2014), but the cold hardiness of the majority of them has not been studied yet (Table 2).

Table 2: List of Enchytraeidae species distributed along the arctic and temperate regions, with known cold hardiness strategy.

Species	Cold hardiness strategy	Reference
Bryodrilus ehlersi glandulosus Dózsa-Farkas, 1990 [1]	CD	Sømme and Birkemoe (1997)
Bryodrilus parvus Nurminen 1970 [2]	CD	Sømme and Birkemoe (1997)
Enchytraeus albidus Henle, 1837 [1, 2, 3, 4]	FT	Slotsbo et al. (2008)
Enchytraeus kincaidi Eisen 1904 [1]	FT	Sømme and Birkemoe (1997)
Fridericia hegemon Dóska-Farkas 1975 [1]	Low-no FT	Dozsa-Farkas (1973)
Fridericia galba Hoffmeister, 1843 [1]	Low-no FT	Dozsa-Farkas (1973)
Fridericia ratzeli Eisen, 1872 s.lat. [1]	FT and CD	Pedersen and Holmstrup (2003)
Henlea perpusilla Friend, 1911 [4]	CD	Sømme and Birkemoe (1997)
Henlea ventriculosa Udekem, 1854 [2, 3, 4]	CD	Sømme and Birkemoe (1997)
Henlea similis Nielsen & Christensen, 1959 [1]	CD	Sømme and Birkemoe (1997)
Mesenchytraeus flavus Levinsen, 1884 [1]	CD	Sømme and Birkemoe (1997)
Mesenchytraeus argentatus Nurminen 1973 [1]	CD	Sømme and Birkemoe (1997)
Mesenchytraeus solifugus Emery, 1898 [1]	FT and FA/HSCA	Edwards (1986)
Stercutus niveus Michaelsen, 1888 [1]	FT and FA/HSCA	Bauer et al. (1998) Dozsa-Farkas (1973)

[1] can be found in inland soils, [2] can be found along littoral and supralittoral habitats; [3] brackish water habitats; [4] marine environments. CD: cryoprotective dehydration, FT: freeze tolerance; FA/HSCA: Freeze-avoidance by high supercooling ability.

Considering the enchytraeids that can be found along the coastal areas (from marine to supralittoral habitats), *Bryodrilus parvus* and *Henlea ventriculata* are known to resort to cryoprotective

dehydration (Sømme and Birkemoe, 1997), and only *E. albidus* seems to resort to freeze tolerance as a main survival strategy to freezing temperatures. This species has become a model organism for more detailed studies (Slotsbo et al., 2008; Patrício Silva et al., 2013a, 2013b; 2014; Fisker et al., 2014a, 2014b, 2015).

2.1. PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS IN FREEZE TOLERANCE

Importance of cryoprotectants

During cold or frost exposures, there are important readjustments that are needed to prevent lethal osmotic shock and freeze damage. Two of the crucial features of adaptation to tolerate frost periods are to avoid severe cellular dehydration and/or to control internal ice formation, mainly at high sub-zero temperatures (Ramløv, 2000). To control these processes, enchytraeids accumulate high levels of glucose (e.g. up to 180 µg mg^{-1} dry weight as observed in *E. albidus,* during 21 days of exposure to -14ºC as reported by Slotsbo et al., (2008)), probably because it is the main blood sugar of oligochaetes with non-toxic and non-reactive effects on cells and proteins (Holmstrup and Zachariassen, 1996; Slotsbo et al., 2008). This main cryoprotectant can either act by colligative effects, diluting the concentration of potentially toxic solutes (e.g. salts) of the unfrozen body fluids and decreasing the melting point and consequently the ice fraction at a given temperature (Zachariassen, 1985; Ramløv, 2000; Slotsbo et al., 2008; Fisker et al., 2014b). In addition, non-colligative effects can stabilize the structure of membranes and labile proteins at low temperature (Anchordoguy et al., 1987; Crowe et al., 1987), maintain the phospholipid membrane in a fluid phase in the absence of water and prevent damage from low temperatures or dehydration (Anchordoguy et al., 1987; Fisker et al., 2014b).

Membrane readjustments

Another important adaptation of cold-tolerant organisms during frost exposure is the maintenance of cell membrane fluidity at its full functioning, also called homeoviscous adaptation (Hazel and Williams, 1990). A fully functioning membrane is in the liquid-crystalline state and with the decrease of temperature it becomes gradually ordered and stiffened and may ultimately undergo transition to a gel phase (a more rigid state), causing a rupture of membranes, and consequent loss of intracellular metabolites and ions (Hazel and Williams, 1990; Cantor, 1999; Kostal, 2010). Therefore, cold-tolerant organisms must modify membrane composition to counteract a phase transition, and some of these adjustments are associated with a desaturation (increase of unsaturated fatty acids or unsaturated/saturated fatty acid ratio), shorting of the average fatty acid chain length, polar head group restructuring and change of the cholesterol ratio (Russell, 1989; Hazel and Williams, 1990; Kostal, 2010). Studying *E. albidus* populations from different climatic zones, Fisker et al. (2015)

reported differences in phospholipid fatty acid (PLFA) composition that were correlated with cold tolerance. The PLFA C18:2, which is highly related with cold tolerance in ectothermic invertebrates (e.g. (Holmstrup et al., 2007), was highest in the most cold adapted populations. These authors also observed a significant decrease in PLFA length (which have lower melting temperature compared with longer PLFA) and an increase of the unsaturation index, which is important for the maintenance of proper membrane fluidity (in a liquid-crystalline state) and cold tolerance as previously mentioned (Hazel and Williams, 1990; Holmstrup et al., 2007; Kostal, 2010).

Metabolism depression, antioxidant defenses and energy allocation

A decrease in temperature and, in some cases, contact with ice crystals may also induce a decrease in mobility or even partial immobilization of enchytraeids and other ectothermic invertebrates inhabiting the topsoil layer (Holmstrup, 2002, 2003; Slotsbo et al., 2008; Storey and Storey, 2012). Living in soil, these organisms are also likely to deal with reduced available oxygen (hypoxia), increased carbon dioxide (hypercapnia) and low nutrient availability and uptake (Storey and Storey, 2010). To ensure survival at frost temperatures, enchytraeids tend to reduce consumption of energy stores, decrease cellular metabolic rate, increase antioxidant defenses, and readjust the protein, carbohydrates and lipid budget allocation (Patrício Silva et al., 2013a; Fisker et al., 2014a, 2014b, Patrício Silva and Amorim, 2015). Fisker et al. (2014b) recorded a metabolic depression (measured as CO_2 production) around 30% – 40% in *E. albidus* from Germany and Svalbard exposed to constant -2ºC, which was significantly lower compared with bigger annelids such as *Dendrobaena octaedra* that revealed a metabolic depression around 90% (Calderon et al., 2009). The high metabolic depression observed in *D. octaedra* was linked to a switching to mainly anaerobic metabolism, as opposed to *E. albidus* where a modest metabolic depression was likely aerobic (Fisker et al., 2014b). During frost exposure, Patrício Silva et al. (2013a) observed some oxidative stress level in *E. albidus* worms, as indicated by high variation on superoxide dismutase (SOD), catalase (CAT), glutathione-S-reductase (GST) activities during the first four days of exposure to saline soils (15‰, 35‰ and 50 ‰ NaCl), followed by a slight recovering within the next 7 days. These researchers also observed that worms from Greenland maintained relatively high and stable antioxidant defenses and large cellular pools of glutathione compared to worms from Germany. Analysing the response at energy basal levels and allocation on *E. albidus* during a short-term exposure (11-17 days), Patrício Silva and Amorim (2015) observed a significant increase in lipid content, accompanied by a decrease (or consumption) in proteins and carbohydrates budgets as a preparation for freezing. Extending the period of exposure (up to 42 days), Fisker et al. (2014a) observed a slight increase in lipids but with a significant decrease in carbohydrates, suggesting that, during long-term exposures to frost temperatures, carbohydrate fuel resources are highly essential, and even more important than lipids, for the cold tolerance of enchytraeids.

2.2. PHYSICAL AND CHEMICAL FACTORS THAT AFFECTS COLD TOLERANCE OF ENCHYTRAEIDS

As previously mentioned, the capacity of cold tolerance of enchytraeids clearly differs in terms of geographical distribution, which is mainly determined by the temperature regime, salinity, soil moisture, substrate (food type and availability) and contaminants.

Temperature regimes and geography

Temperature regime is probably the most important physical factor influencing cold tolerance of enchytraeids, because they inhabit the topsoil layer and seem not to seek thermal refuge during cold exposures (Didden, 1993; Slotsbo et al., 2008). Therefore, it is likely that different populations have adapted to their local thermal conditions, characterized by different cooling rates, exposure periods and even changes in acclimation temperature. Research carried out by Slotsbo et al. (2008) revealed that cold tolerance and cryoprotection (glycogen catabolization and glucose accumulation) of *E. albidus* was higher in populations from cooler environments (e.g Greenland *versus* Germany). Similar evidence was highlighted in studies of Fisker et al. (2014b), where intraspecific variation was found in cold hardiness of *E. albidus* from Germany and Svalbard, considering inland and littoral habitats. During a prolonged cold exposure experiment (27-57 days), enchytraeids from Arctic or sub-Arctic regions, e.g. Svalbard, Greenland and Iceland, had lower LTemp50 (lethal temperature with 50% observed mortality) and higher long-term tolerance compared to enchytraeids from temperate regions, e.g. Norway, Sweden and Germany. In parallel, worms from the Arctic or sub-Arctic regions had higher glucose accumulation (Fisker et al., 2014b; Slotsbo et al., 2008), higher metabolic depression (Fisker et al., 2014b) and higher, more stable antioxidant defenses and larger cellular pools of glutathione (Patrício Silva et al., 2013a) than the worms from the temperate regions.

Although some enchytraeid species, such as *E. albidus*, are able to survive freezing, the transition between the frozen and unfrozen states during freeze-thaw events seems to imply higher physiological consequences. A short-term laboratory simulation of daily freeze-thaw cycles (10 days of exposure, with temperatures ranging from +2ºC to -4ºC within 24h) did not affect *E. albidus'* survival, nevertheless, it had significant implications in terms of cellular metabolism, where an increase in energy consumption, decrease on protein budget and low glucose accumulation was evident as compared with enchytraeids exposed to constant freezing (Patrício Silva et al., 2014; Patrício Silva and Amorim, 2015). When increasing the period and number of cycles (14 cycles, 3 days at 0ºC followed by 3 days at -5ºC) the enchytraeids' survival was negatively affected, as well as glycogen reserves and glucose accumulation (Fisker et al., 2014a), which are important both as cryoprotectants and as fuel for metabolism during frost exposure. While *E. albidus* from Arctic locations seems to be better adapted to prolonged freeze periods as compared with temperate locations, a different scenario was observed when considering the effects of freeze-thaw events.

Fisker et al. (2014a) observed that enchytraeids from arctic locations had a higher survival rate at prolonged freeze periods compared with populations from temperate regions, but when exposed to freeze-thaw events both populations revealed the same survival rate, indicating that worms from temperate regions are primarily adapted to repeated freeze–thaw cycles. The divergences on cold-tolerance, during prolonged exposures to frost or to freeze-thaw cycles, on *E. albidus* populations (and probably other enchytraeids) reinforce the physiological and biochemical heterogeneity among enchytraeid populations, and lead us to believe that genetic adaptations may be acting. On the other hand, it could be argued that these differences may also be related with the existence of cryptic species, frequently overlooked when comparing studies worldwide. Although *E. albidus* (*sensu lato*) is known to consist of several species (Erséus and Gustafsson, 2009; Schmelz and Collado, 2010), the worms used in the mentioned studies belong to the same phylogenetic clade (and consequently, same species) based on the cytochrome oxidase I of the mitochondrial genome (mtDNA) (K. Fisker personal communication, 2014).

Salinity

Along with cold temperature regimes, the shorelines from the Arctic Circle to temperate regions are also influenced by the presence of salinity fluctuations associated with tidal movements and precipitation/evaporation patterns. Therefore, the influence of salinity on cold tolerance has been the subject of several studies, with main focus on intertidal and brackish invertebrates (e.g. Kähler, 1970; Aarset, 1982; Murphy, 1983; Patrício Silva et al., 2013b). The available literature on enchytraeids is limited to the species *E. albidus*, and reveals that acclimation to even modest salinities of soil water improved survival considerably during freezing at low temperature (e.g. Fig.1) (Kähler, 1970; Patrício Silva et al., 2013b).

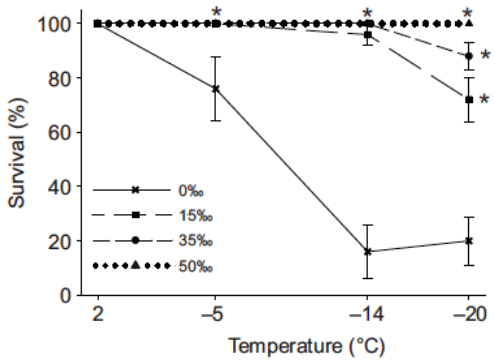

Fig. 1. Freeze survival of *E. albidus* when exposed to soil with a range of salinities (0‰, 15‰, 35‰ or 50‰ NaCl). Results are shown as means ± s.e.m. (N=5). Significant difference from 0‰ salinity is shown (Dunnett, *P<0.05). Adapted with permission from "Patrício Silva et al. 2013b". Copyright 2015 The Company of Biologists Ltd.

The increased survival of freezing in saline soils was highly related to the amount of extracellular ice formed, as shown by Patrício et al (2013b). According to this study, worms that were acclimated in saline soils and frozen had a significantly lower ice content compared with those in non-saline soils (Fig.2), as a result of the decrease in melting point (by ~1.03–1.29°C in 50‰ saline soil) due to the

passive influx of Na^+ and Cl^- ions across the body wall, and also to some degree from a decrease in water content. The reduction in ice fraction caused by salinity was more evident at high sub-zero temperatures, meaning that worms exposed to salinity would be subjected to a slower freezing of body fluids during cooling to very low temperatures (−14 or −20°C) than worms frozen in control soil (Patrício Silva et al., 2013b). The reduced ice content in the early phase of the freezing process seems to be important for physiological mechanisms to prevent severe cellular dehydration, and may promote the stabilization of proteins and cell membranes by cryoprotectants (Zachariassen, 1985; Crowe et al., 1987) as discussed in previous sections.

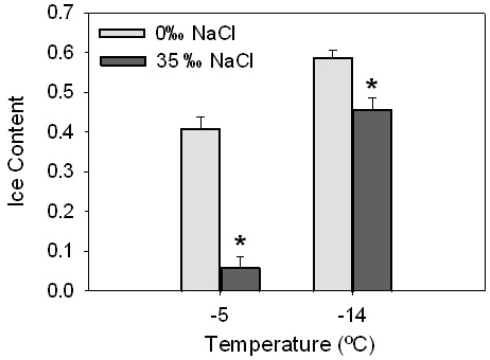

Fig.2. Relative ice content of *E. albidus*, determined by differential scanning calorimetry, when cooled to target temperatures of (A) −5°C and (B) −14°C, after 48h exposure to salinities of 0 and 35‰ NaCl. Results are shown as mean values ± s.e.m. (N=10 and 5 for target temperatures of −5 and −14°C, respectively). Significant difference from 0‰ salinity is shown (Dunnett, *P < 0.05). Adapted with permission from "Patrício Silva et al. 2013b". Copyright 2015 The Company of Biologists Ltd.

The role of glycogen and glucose as cryoprotectants was also affected by the presence of salinity. Previous studies on enchytraeids have pointed to a positive correlation between glycogen reserves and the accumulation of glucose with the ability to survive extreme freeze events (Pedersen and Holmstrup, 2003; Slotsbo et al., 2008). However, in the presence of salinity, glucose concentration decreased to a level such that its contribution to lowering the ice fraction (by colligative effects) was negligible (Fig.3) (Patrício Silva et al., 2013b). Despite the low contribution of glucose to osmolality and reduced ice fraction, this cryoprotectant may have importance in other processes such as stabilization of membranes and proteins as pointed out by Anchordoguy et al. (1987) and Crowe et al. (1987).

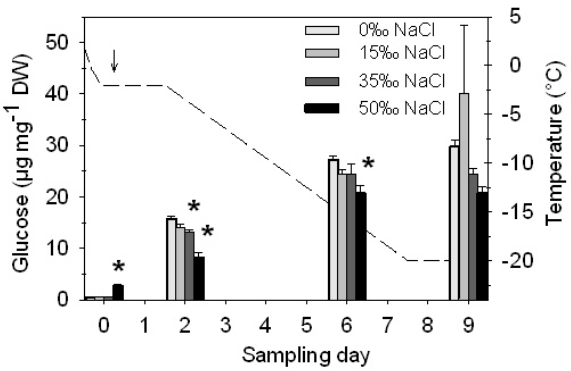

Fig. 3. Glucose content of *E. albidus* exposed to soil with different salinities, during a decrease in temperature down to -20ºC. The arrow marks the point in time when ice crystals were added to the soil surface to induce freezing. Results are shown as means ± s.e.m. (N=5). Significant difference from 0‰ salinity is shown (Dunnett, *P<0.05). Adapted with permission from "Patrício Silva et al. 2013b". Copyright 2015 The Company of Biologists Ltd.

During cold-frost exposures, and being a freeze-tolerant species, *E. albidus* also experienced some level of oxidative stress during internal ice formation (Patrício Silva et al., 2013a). Yet, in the presence of salinity (15‰, 35‰ and 50‰ NaCl), these worms revealed a quick restoration of their antioxidant defenses, by increasing GST levels and GSH, GR and CAT activities after 4 days under freezing temperatures, emphasizing once more the positive effects of salinity on worm tolerance to freezing (Patrício Silva et al., 2013a).

The presence of salinity also influenced the PLFA composition (Patrício Silva – Chapter VIII) without apparently resulting in a significant change in the unsaturated index (UI) or in a shortening of the average fatty acids length as expected, which plays a significant role in homeoviscous adaptation in earthworms and enchytraeids (Holmstrup et al., 2007; Fisker et al., 2015) and is crucial in adaptation to cold temperatures and high levels of salts (Russell, 1989; Hazel and Williams, 1990). Therefore, these results suggest that other physiological traits triggered by the presence of salinity (than those concerning osmolality and depression of ice fraction) play important roles for cold tolerance of these worms.

Desiccation

Soil invertebrates in Polar Regions must cope with daily and seasonal fluctuations in thermal and hygric conditions that constitute some of the most hostile environments on Earth. Inhabiting the topsoil, enchytraeids are therefore cooled to temperatures far below the regular melting point of their body fluids, which associated with the low availability of liquid water during most of the year, may result in desiccating conditions (Coulson et al., 1995). It is likely that enchytraeids must have adapted to persist under such conditions; otherwise they would be facing extreme cellular shrinkage, denaturation of cellular proteins, and loss of the original conformation of cellular membranes (Crowe, 1992).

To avoid dry conditions (as main or only stressor), enchytraeids normally migrate to deeper and moister zones or increase aggregation of worms in moist topsoil microhabitats (reviewed by Maraldo et al., 2009). Some enchytraeids that reproduce sexually were even found to cover their cocoons with sand and debris to avoid dehydration (Lagerlöf and Strandh, 1997). However, very little is known about when dry conditions meet extreme cold temperatures. Sømme and Birkemoe (1997) recorded higher survival rates of enchytraeids from Spitsbergen (*Mesenchytraeus flavus* and *Enchytraeus kincaidy*) during cooling to temperatures below zero in dry soils, compared with freezing in a moist environment. Survivors were also able to tolerate a high degree of water loss (42-55% of fresh weight) through cryoprotective dehydration, and able to increase their osmolality probably associated with the production of protective substances such as glucose and alanine as revealed in *E. albidus* when exposed to drought as their only stressor (Maraldo et al., 2009). In

freeze-intolerant enchytraeids, Bauer (2002) also found that the quantity of osmotically active (frozen) water was significantly reduced after acclimation at – 3 °C in cocoons of *Enchytraeus crypticus* and *Enchytraeus sp*. with consequent decrease of SCPs, probably for the same reason mentioned in the previous paragraph. The slow cooling rate of 1°C day^{-1} was comparable with natural field conditions indicating that an acclimation process occurs in cocoons of both these species.

The mobilization of compatible osmolytes during desiccation periods is therefore important, and the combined effect of cold and desiccation seems even to lead to synergistic (positive) effects. Previous investigations on springtails (*Megaphorura arctica* and *Folsomia candida*) revealed that drought acclimation also confers tolerance of these species to cold temperatures, and in particular combined with accumulation of cryoprotectants, drought-induced PLFA desaturation and the induction of proteins such as heat shock proteins 70 (HSP70) and trehalose-6-phosphate synthase (TPS), which are important protective systems induced to prevent damaging effects of both desiccation and cold (Bayley et al., 2001; Holmstrup et al., 2002a; Sørensen et al., 2010). Therefore, it would also be interesting to study the levels of cryoprotectants, PLFA adjustment and the temporal gene expression profile of candidate genes (e.g. HSP, TPS, aquaporines, among others) under drought conditions (as only stressor) and under combined effect with low temperatures in enchytraeids.

Substrate, food source and starvation

Enchytraeids are in permanent contact with the physical and chemical properties of the surrounding substrate through their moist and permeable body surface, which seems to affect eco-physiological capabilities of the worms to cope with sub-zero temperatures. Cold hardiness of worms and cocoons of *Enchytraeus variatus* seemed to be influenced by the ingested substrate particles and by the particles attached to the surface (Bauer, 2005). Cocoons cultured on nettle leaves decreased the SCP by 5ºC when compared to cocoons cultured on compost plus nettle leaves (Bauer, 2005). In addition, SCP of cocoons was lower than the SCP of worms, indicating that *E. variatus* may be better protected against frost in the cocoon stage than in later life stages (Bauer, 2005).

Likewise, *Stercutus niveus* fed only on leaves devoid of mineral particles were significantly more cold resistant than the ones fed with a mixture of compost (Bauer, 1998, 2002), which may explain why most enchytraeids are found in the topsoil layer during cold periods. Similar results were found for cocoons cultured in different substrates (leaves of *Urtica dioica* and compost), indicating that cocoons survive better in a substrate with a high amount of dead organic material, such as leaves and roots, than in a substrate with a high mineral particle content. It also seems that food or substrate particles may act as ice nucleators, and depending on their quality, may therefore interfere

with supercooling abilities of worms. *S. niveus* without gut content had higher supercooling abilities than the ones with gut content (Bauer, 1998; Bauer et al., 1998). Similarly, Bauer (2002) reported that *C. sphagnetorum*, *Buchholzia sp.* and *Buchholzia fallax* had better survival at temperatures below zero when they had few or no food particles in their guts.

Contaminants

Because freeze tolerance and freeze avoidance depend on the accumulation of cryoprotectants (such as sugars and polyols) and also on membrane adjustments, it is expected that toxicants interfering with these processes could potentially reduce survival at low temperatures (Holmstrup et al., 2010). Likewise, toxicants interfering with ice-nucleating agents as well as anti-freeze proteins may reduce freeze tolerance and freeze avoidance respectively, and, in freeze-tolerant organisms specifically, freezing may also potentiate the effect of toxicants by concentrating them in the fluid fraction of the frozen body fluids (Aarset and Zachariassen, 1982).

The effect of contaminants on cold hardiness is well documented for earthworms, springtails and insects (e.g. Holmstrup et al., 2010; Fisker et al., 2011). Only recently, enchytraeids have been subjects of similar studies but the information available is still relatively limited.

An investigation using *E. albidus* revealed that sublethal concentrations of phenanthrene (PHE) and 4-nonylphenol (4-NP) (both are lipophilic contaminants commonly found in sludge-amended soil) influenced worms' cold tolerance in opposite directions. While 4-NP significantly reduced the survival rate after freezing at −6 °C, from 80% to 33%, PHE significantly increased survival after freezing at -8ºC, from 75% to 100% (Holmstrup et al., 2014). The opposite effects of 4-NP and PHE, may be related to the membrane fluidity. In vitro studies, using artificial multilamellar membranes consisting of DMPC (1,2-dimyristoyl-sn-glycero-3- phosphocholine) and giant unilamellar vesicles made from reconstituted phospholipid extracts of the worms, revealed that the interaction of PHE with the membranes or vesicles caused a decrease in phase transition temperature and a reduction of the bending rigidity, indicating that the interactions with PHE molecules stabilize (and even increase) the fluid (liquid-crystalline) phase. In contrast, 4-NP (at the same molar concentration) increased the phase transition temperature and increased the bending rigidity of the membranes or vesicles (Holmstrup et al., 2014). These results provide clear indications that membrane fluidity is indeed one of the primary targets of lipophilic substances for the changes in cold tolerance.

Other studies on the effect of 4-NP on *E. albidus* cold tolerance underlined its negative effect on worms' survival, but also evidenced that a pre-exposure to sublethal concentrations of this chemical induced a significant decrease of glycogen reserves and protein budgets with higher impact in frozen worms (Patrício Silva et al 2014, Patrício Silva and Amorim, 2015). Worms exposed to the highest tested 4-NP concentration (250 mg kg^{-1} dry soil) and to frost temperatures also revealed

significantly lower energy levels available and cellular energy allocation than unfrozen worms (Patrício Silva and Amorim, 2015), which might partially explain the lower survival of these worms.

A knowledge gap in the literature still persists regarding the effect of metals on cold tolerance of enchytraeids, however, pilot studies have been performed and many others are still ongoing (at the Institute of Biosciences - Aarhus University). A 7 d pre-exposure to increased concentrations of zinc (Zn) and mercury (Hg) (0, 20, 40, 60, 80, 100 mg of chemical per kg of dry soil) decreased significantly the survival of *E. albidus* in a subsequent 7 d exposure to -4ºC, by 10%-40% and 30%-60% respectively, which points to synergistic (negative) effect (Furey, Patrício Silva and Holmstrup, unpublished). Measurements of cryoprotectants, concentration of contaminants in the tissue, up-regulation of metallothioneins, changes in membrane composition and properties will bring more insight to the subject.

3. INTERACTION BETWEEN FREEZE-THAW CYCLES AND 4-NONYLPHENOL ON COLD HARDINESS OF ENCHYTRAEIDS

Because of to the predicted climate scenario and dispersion of chemicals through anthropogenic activities, it is not unlikely to find interactions between freeze−thaw events and contaminants along the Arctic and cold temperate regions. These interactions may have ecological concern, especially when it leads to synergistic negative effects (Holmstrup et al., 2010).

The investigations carried out by Patrício Silva et al (2014) provided evidence regarding this subject for enchytraeids, using 4-nonylphenol as a model contaminant. According to these studies, combinations of sublethal concentrations of 4-NP and daily freeze-thaw cycles (from 2ºC to -4ºC) caused stronger effects than combinations including constant freezing (Fig. 4).

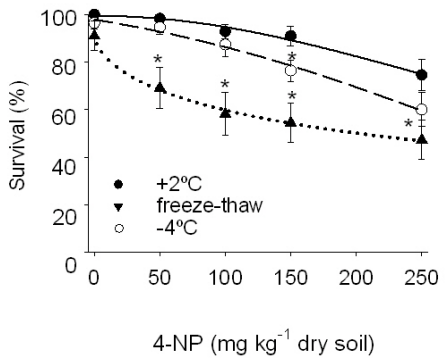

Fig. 4. Survival of *E. albidus* exposed to combined effect of 4-NP and three temperature regimes (constant +2ºC, constant -4ºC and daily freeze-thaw cycles) for 10 days. (*) Statistical significant differences compared to control temperature (2ºC), per 4-NP concentrations, Dunnett, $p < 0.05$. Results are shown as mean ± standard error (N = 15) and described with a log-logistic concentration response model (2ºC = solid line, -4ºC = broken line, daily freeze-thaw cycles = dotted line). Adapted with permission from "Patrício Silva et al., 2014". Copyright 2015 American Chemical Society.

The higher mortality observed in worms exposed to 4-NP and daily freeze-thaw cycles seemed partially related to levels of glycogen and glucose in the worms (Patrício Silva et al., 2014). Glycogen depletion increased significantly with the increase of 4-NP concentrations, without resulting in the equivalent accumulation of cryoprotective glucose (or even to an increment),

indicating that this sugar may be used as fuel to fight 4-NP toxicity and to restore the metabolic system during thawing. In addition, worms exposed to daily freeze-thaw cycles also revealed the highest concentrations of 4-NP in the tissues (Fig. 5) and higher number of cryoinjuries with increase with 4-NP concentration (Fig. 6) (Patrício Silva et al, 2014). It is possible that repeated freezing and thawing of soil influenced the sorption and desorption processes of 4-NP. As a lipophilic compound, this organic surfactant partitions favourably to organic matter (Soares et al., 2008), and because consecutive freezing periods seems to affect the sorption of 4-NP by leading to a partial destruction of organic macromolecules or organo-mineral structures present in the soil through ice crystals (as observed by Yu et al. (2010) and Shchegolikhina et al. (2012)), it is possible that bioavailability of 4-NP is higher in the repeated thawing periods and this could lead to higher concentrations in the tissues of worms and consequently higher mortality.

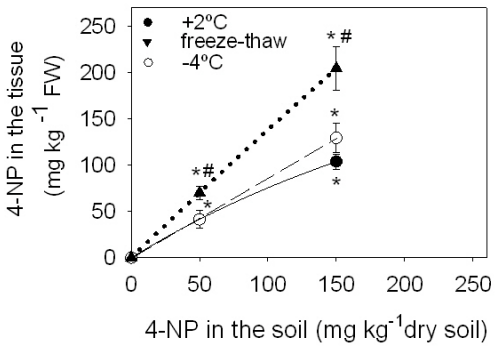

Fig. 5. Concentration of 4-nonylphenol in tissues of *E. albidus* exposed for 10 days to NP and different temperatures regimes (day 17) (B). Data were described with an exponential function (2 °C = solid line, −4 °C = broken line, daily freeze−thaw cycles = dotted line). (*) Statistically significant differences compared to control soil (0 mg 4-NP kg−1 soil dry soil), Dunnett, $p < 0.05$; (#) Statistically significant differences compared to control temperature (2 °C), Dunnett, $p < 0.05$. Results are shown as mean ± standard error (N = 5). Adapted with permission from "Patrício Silva et al., 2014". Copyright 2015 American Chemical Society.

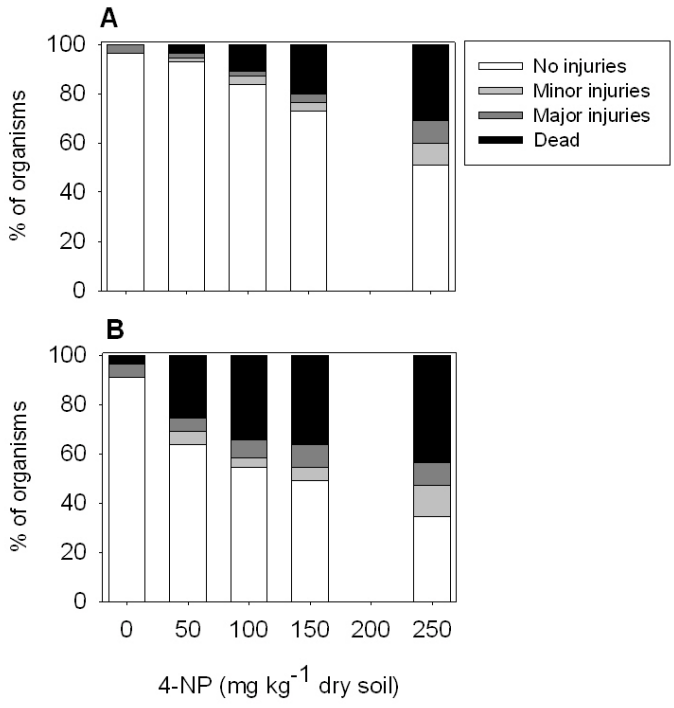

Fig. 6. Condition of *E. albidus* in terms of cryoinjuries ("no injuries", representing healthy worms with intact integument; "minor injuries", representing worms with one injury, characterized by a small physical disruption of the integument; "major injuries", representing worms with two or more injuries but still able to move and "dead" were completely immobilized by the high number of injuries) after 7 days pre-exposure to 4-NP followed by 10 days at constant −4 °C (A) or daily freeze−thaw cycles (B). Adapted with permission from "Patrício Silva et al., 2014". Copyright 2015 American Chemical Society.

Likewise, the high hydrophobicity properties of 4-NP ($logK_{ow}$ of 4.5) makes it also liable to accumulate in cell membranes (Ekelund et al., 1993; Jacobsen et al., 2004; Shan et al., 2010), and because membrane-partitioning of 4-NP seemed to reduce fluidity of model membranes in vitro, Patrício Silva et al. (2014) assumed that the number of cryoinjuries observed in the worms as a sign of 4-NP interference with membrane fluidity and cell integrity.

To go deeper into this subject, Patrício Silva and Amorim (2015) analysed the combined effect of 4-NP and freezing on the energy basal levels and allocation. Results showed that worms exposed to freeze-thaw cycles had higher energy consumption than worms exposed to constant freezing, suggesting a possible higher investment of energy when shifting between freezing and thawing events, which could also have contributed to increase worms' vulnerability compared with frozen worms. On the other hand, worms exposed to continuous freezing presented relatively stable and positive levels of energy available and low levels of energy consumed, which is congruent with a decrease in metabolism that could have saved the worms against the negative (toxic) effect of 4-NP. The interpretation of the data in terms of cellular energy allocation was not so clear, indicating that its interpretation must be carefully carried out and compared with the individual energy reserves and energy consumption parameters, in particular if these show opposite balances.

4. INTERACTION BETWEEN SALINITY AND CONTAMINANTS IN SUPRALITTORAL ENCHYTRAEIDS

The influence of salinity on the toxicity of various classes of chemicals is well documented for aquatic, especially marine, biota (Hall and Anderson, 1995; Noyes et al., 2009). However, the same is not applied to soil organisms, with only three main studies on earthworms (Owojori et al., 2008, 2009; Owojori and Reinecke, 2010) and only one on enchytraeids whose test species is commonly found in supralittoral ecosystems (Patrício Silva et al., 2015). The latter study in particular evaluated the influence of salinity on toxicity of four contaminants with well-known modes of action (two metals – copper and cadmium, and two organic contaminants – 4-NP and carbendazim).

Commonly found in supralittoral habitats along the Arctic and temperate regions, it is not surprising that *E. albidus* has a broad salinity tolerance, with no mortality in salinities up to 60‰ (Kähler, 1970; Patrício Silva et al., 2013b), and a capacity for osmoregulation in low salinities (< 20‰) by remaining hyperosmotic (Schöne, 1971; Generlich and Giere, 1996; Patrício Silva et al., 2013b). The presence of low salinity in the soil (up to 30‰ in soil water) was shown to have a positive effect on reproduction of *E. albidus*, by increasing the number of juveniles by a factor of ca. 10 (Patrício Silva et al., 2013b).

In order to understand how salinity influences the effect of contaminants on *E. albidus*, Patrício Silva et al. (2015) exposed the worms to increased concentrations of copper, cadmium, carbendazim and 4-NP in non-saline soils and in soils containing 15‰ NaCl (most common salt present in seawater).

Results revealed a generally higher number of juveniles in saline soil than in control soil, but the effects on toxicity of each chemical depended on their nature. For example, the presence of salinity decreased the effects of carbendazim, copper and cadmium on reproduction increasing the EC50 by a factor of ca. 8, 2.6 and 1.6, respectively. It also decreased the effect of 4-NP on worms' survival (LC50 increased by a factor of ca. 1.2), but increased toxicity for reproduction (EC50 decreased by a factor of ca. 1.8). The impact on reproduction observed in worms exposed to 4-NP and metals in saline-soil may, however, be partially explained by the tissue concentrations. Patrício Silva et al. (2015) observed that worms exposed to 4-NP in saline soils had higher concentration of this contaminant in the tissues compared with worms exposed to non-saline soils. The opposite occurred for metals, where worms in saline soils had lower tissue concentration of the metals than worms exposed to non-saline soils (Fig. 7). According to the authors, the presence of NaCl in low concentrations decreases metal bioavailability probably by competing and occupying the ligands at the organism's point of entry. Because the presence of 15‰ NaCl is not deleterious for survival or reproduction (on the contrary, it stimulates reproduction), its combination with metals resulted in some form of an additive or antagonistic effect. Negative correlations between salinity and contaminants (toxicity increasing with decreasing salinity) was also observed with high frequency for estuarine and marine annelids and other organisms, which is also related to higher bioavailability of free metal ions at lower salinities (reviewed by Hall and Anderson, 1995). Regarding the effect of salinity on 4-NP uptake by worms' tissues, the authors could not present a plausible explanation because there are no previous studies on 4-NP toxicity, uptake, elimination, and bioaccumulation in soil enchytraeids (or other oligochaetes). Knowledge about the uptake and depuration kinetics of copper, cadmium and nonylphenol in both soils types (non-saline *versus* saline), could contribute to a better understanding of this subject.

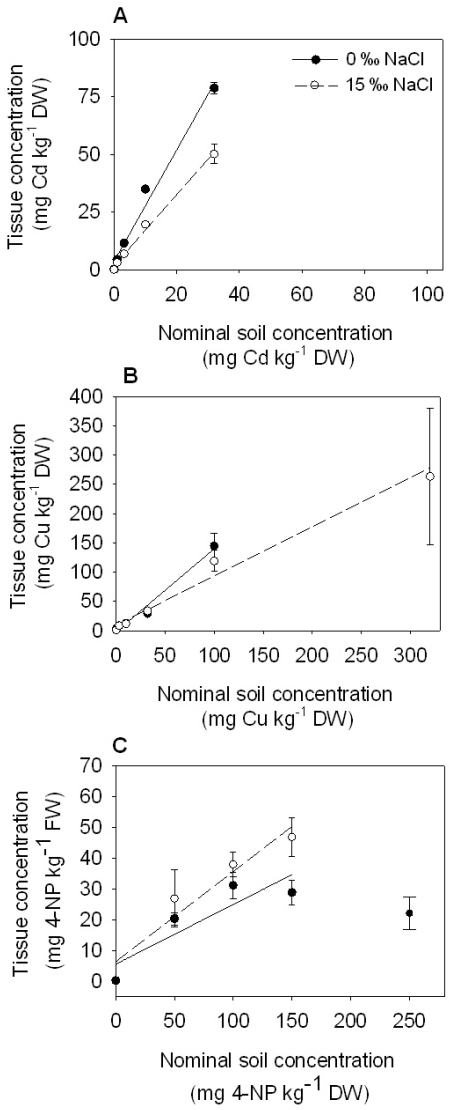

Fig. 7. Tissue concentration of Cd (A), Cu (B) and 4-NP (C) in *E. albidus* fresh weight (FW) or dry weight (DW), after 21 days of exposure to different soil concentrations and two salinities – 0 and 15 ‰ NaCl. Results are shown as mean ± standard error (N= 3-8). Adapted with permission from "Patrício Silva, et al. 2015". Copyright 2015 John Wiley & Sons, Inc.

Because *E. albidus* is a model species in international standardized guidelines for risk assessment of chemicals (e.g. ISO, 2004; OECD, 2004), Patrício Silva et al. (2015) also discussed the relevance of such standards. Results revealed from 1.5 to 8-fold changes in the effect of contaminants in the presence of low salinities, thus it may be argued that the effects of salinity on toxicity should be acknowledged, although these results remain within the applied uncertainty factor in terms of risk assessment of chemicals (Römbke and Moser, 2002), and therefore revisions of the currently agreed enchytraeid standardized test may not be necessary.

5. CONCLUSIONS AND PERSPECTIVES

This review shows that interactions between environmental factors and between physical and chemical stressors are a common occurrence, which can take unexpected directions in enchytraeid populations. Furthermore, it also indicates that one natural/physical factor can modify the effects of other natural factors and chemicals, meaning that traditional laboratory studies where the organisms

are exposed to one single stressor (physical and chemical) at a time with the other variables under otherwise optimal conditions, may underestimate their effect in the field.

Although the available literature on the importance of natural factors in eco-physiology and ecotoxicology of cold tolerant enchytraeids is limited, some general patterns seem to emerge. Cold tolerance of enchytraeids seemed negatively affected (pointing towards synergism) by temperature fluctuations and long-term exposure to freezing. These negative effects can even become worse when combined with sublethal concentrations of contaminants, as for instance 4-NP. On the other hand, the presence of salinity, drought and substrates with a high amount of dead organic material has a positive influence on cold tolerance of enchytraeids. For freeze-tolerant species as *E. albidus*, the control of internal ice formation is crucial, where accumulation of cryoprotectants and maintenance of membrane fluidity seems to play important roles to avoid severe dehydration, cell shrinkage and membrane (and integument) disruption. These processes, associated with a depression of metabolism, increase in antioxidant defenses, readjustments in lipids, proteins and carbohydrate budgets during freezing constitute the primary lines of response mechanisms to maintain homeostasis and warrant survival in harsh environments such as Polar and cold-temperate regions.

Along the shoreline, the salinity seems to be essential for marine or littoral enchytraeids such as *E. albidus.* Even moderate salinity not only increased the reproductive rate of *E. albidus* but also decreased the effect of some contaminants such as copper, cadmium and carbendazim for the same endpoint. These effects on metals seemed to be correlated with the tissue concentrations of contaminants in the worms, confirming that ion exchange (and balance) is the primary factor determining effects of metals in the presence of salinity. The effect of salinity on toxicity of 4-NP was, however, less clear, but the reproduction capacity and tissue concentration of this chemical were negatively related.

Enchytraeids may, therefore, respond in unpredictable ways to environmental changes (involving climate factors and presence of contaminants), which imposes an even larger challenge when extrapolating from laboratory conditions to the field. Therefore, it is important to expand and develop experiments that more realistically mimic the conditions in the field, where interaction between factors is highly relevant. Additionally, it is also important to include more representative enchytraeid species when considering particular habitats (e.g. *Cognettia sphagnetorum* for acidophilic soils and boreal forests).

The synergistic or antagonistic interactions between natural factors and chemicals highlighted in the present review may also represent a stepping-stone in the evaluation (or adaptation) and possible inclusion of natural and physical factors in standardized toxicity tests involving enchytraeids, and consequently in ecological risk assessment. Cumulative risk assessment procedures including

mixtures of natural stressors and chemicals are currently being discussed and developed (see e.g. Løkke et al., 2013). Some authors have proposed that such improved risk assessments requires a paradigm shift from the traditional stressor- and source-oriented assessments toward receptor-oriented assessment (e.g. Schlink and Ragas, 2011). This paradigm shift has implications for the way exposure and effects are assessed and require a broader regulatory context than currently provided.

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Photo by Karina Fisker

Chapter II

Soil salinity increases survival of freezing in the enchytraeid *Enchytraeus albidus*

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SUMMARY

Enchytraeus albidus is a freeze-tolerant enchytraeid found in diverse habitats, ranging from supralittoral to terrestrial and spanning temperate to arctic regions. Its freeze tolerance is well known but the effect of salinity in this strategy is still poorly understood. We therefore studied the combined effect of salinity (0, 15, 35, 50‰ NaCl) and sub-zero temperatures (−5, −14, −20°C) on the freeze tolerance of *E. albidus* collected from two distinct geographical regions (Greenland and Germany). A full factorial design was used to study survival, and physiological and biochemical end points. The effect of salinity on the reproduction of German *E. albidus* was also assessed. Exposure for 48 h to saline soils prior to cold exposure triggered an increase in osmolality and decrease in water content. Worms exposed to saline soils had an improved survival of freezing compared to worms frozen in non-saline soils, particularly at −20°C (survival more than doubled). Differential scanning calorimetry measurements showed that the fraction of water frozen at −5 and −14°C was lower in worms exposed to 35‰ NaCl than in control worms. The lowering of ice content by exposure to saline soils was probably the main explanation for the better freeze survival in saline-exposed worms. Glucose increased with decreasing temperature, but was lower in saline than in non-saline soils. Thus, glucose accumulation patterns did not explain differences in freeze survival. Overall, the physiological responses to freezing of *E. albidus* from Greenland and Germany were similar after exposure to saline soils. Soil salinity up to 30‰ improved reproduction by a factor of ca. 10.

Key words: Ice content, freeze tolerance, osmolality, cryoprotectants, glucose.

INTRODUCTION

Enchytraeids (Oligochaeta), commonly known as potworms, form a large and widely distributed group of saprophagous organisms, which inhabit the litter layer and the upper mineral soil of many terrestrial and supralittoral ecosystems (Didden, 1993; Giere, 2006). They play important functions, namely as soil structure promoters, in the decomposition of dead organic matter and nutrient mobilization (Petersen and Luxton, 1982; Didden, 1993). *Enchytraeus albidus* Henle 1837 can be found in organic matter-rich soils as well as in decaying seaweed at the supralittoral zone along the coast, e.g. in Germany and Greenland (Christensen and Dózsa-Farkas, 2006; Giere, 2006). This species shows a large physiological tolerance range to salinity (Kähler, 1970; Generlich and Giere, 1996) and freezing temperatures (Slotsbo et al., 2008) of its habitat.

Cold hardiness in enchytraeids originating from temperate or arctic environments has been reported in several studies (Sømme and Birkemoe, 1997; Block and Bauer, 2000; Bauer et al., 2001; Holmstrup and Sjursen, 2001; Holmstrup et al., 2002a; Pedersen and Holmstrup, 2003; Christensen and Dózsa-Farkas, 2006; Slotsbo et al., 2008). When ambient temperatures decrease below the melting point of the body fluids, enchytraeids, like any other ectothermic animal, face the risks

associated with the freezing of body fluids (Zachariassen, 1985; Ramløv, 2000). As enchytraeids are small hygrophilic soil organisms with a high cuticular permeability for water, they are not likely to use supercooling as a cold tolerance strategy. Instead, two other cold tolerance strategies have been adopted by enchytraeids, namely cryoprotective dehydration (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003) and freeze tolerance after inoculative freezing of body fluids (Sømme and Birkemoe, 1997; Slotsbo et al., 2008). Cryoprotective dehydration and freeze tolerance are assisted by the accumulation of cryoprotectants such as glycerol, sorbitol and trehalose as well as free amino acids (Zachariassen, 1985; Storey, 1997; Holmstrup et al., 2002b). Enchytraeids accumulate glucose as a cryoprotectant, probably because it is the main blood sugar of oligochaetes (Holmstrup et al., 1999; Slotsbo et al., 2008), but glucose may also serve as an energy resource in the frozen organism (Calderon et al., 2009). The accumulation of cryoprotectants lowers the melting point, and with that the ice fraction at a given temperature, and dilutes the concentration of potentially toxic solutes (e.g. salts) in the unfrozen body fluids (Zachariassen, 1985; Ramløv, 2000).

With regard to the effects of salinity on enchytraeids, the published data has mainly been related to survival and osmoregulatory capacity. These studies show that *E. albidus* can rapidly adjust body fluid osmolality to changes in environmental salinity and remain hyperosmotic at salinities up to about 25‰; at higher salinities the worm osmoconforms (Kähler, 1970; Schöne, 1971; Generlich and Giere, 1996). A study reported that reproduction can occur under full-strength seawater salinity but did not indicate an optimal salinity for fitness (Schöne, 1971).

Physiological mechanisms in cold or salinity tolerance of enchytraeids have been examined in several studies (Schöne, 1971; Block and Bauer, 2000; Bauer et al., 2001; Generlich and Giere, 1996; Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003; Slotsbo et al., 2008; Owojori et al., 2009), but very little information is available on the combined effect of cold and salinity and the interactions between these two factors. One study has addressed this topic in a short-term experiment, indicating that acclimation to low temperature interacts with salinity in *E. albidus* (Kähler, 1970).

In the present study, we exposed *E. albidus* from two populations (Greenland and Germany) to a range of ecologically relevant salinities and low temperatures in a full-factorial experimental design. We assessed survival, glucose and glycogen levels, osmolality, supercooling point and estimated the ice content. Additionally, the effects of salinity on reproduction were assessed for the German population.

MATERIALS AND METHODS

Test species

Enchytraeus albidus (Oligochaete: Enchytraeidae) from Germany were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany; coordinates: 51°51′N, 9°50′E). These worms were originally collected from garden compost, and cultured for several years in the laboratory in agricultural (loamy) soil at 20.0±1°C and fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly Fucus spp., collected near Aarhus, Denmark). Worms from Greenland (Kobbefjord, about 20 km from Nuuk; coordinates: 64°8′N, 51°23′W) were collected in 2010 from decaying seaweed near the sea shore and kept in the laboratory at 5°C in agricultural soil (as used for German worms) for about 1 year prior to experiments. Before the experiments began, the organisms were cold acclimated at 5°C for 6 weeks and then at 2°C for 1 week.

Test soil and salt spiking procedure

All experiments were conducted with the natural standard soil LUFA 2.2 (Speyer, Germany). In short, this soil has ca. 6% clay, 17% silt, 77% sand and 4.4% organic matter. The pH (CaCl2) of LUFA soil is 5.5. This soil is within the optimum range of pH in natural soils where *E. albidus* are found (Jänsch et al., 2005).

Salt spiking was performed using NaCl (99.5% purity, Merck, Darmstadt, Germany), added as aqueous solution to the dry soil. Soil water content was 22 ml 100 g^{-1} dry soil, which is equivalent to 50% of the water-holding capacity. For the survival test we used the following NaCl concentrations: 0‰, 15‰, 35‰ and 50‰ NaCl. For the reproduction test the concentration range was 0‰, 2‰, 4‰, 6‰, 8‰, 10‰, 20‰, 30‰ and 40‰ NaCl. The NaCl-spiked soil was transferred to 1 l cylindrical plastic vessels and allowed to equilibrate for 1 day before being used in tests.

Experimental setup and survival assays

Each replicate consisted of a test vial (3 cm height, 2 cm diameter) containing 5 g of test soil and 15 mg oatmeal. Five worms per replicate were used. The vials were covered with a perforated lid to allow ventilation. For survival assessment, five replicates were used; for physiological and biochemical measurements, three to six additional replicates were prepared.

Vials with worms were kept at 2°C for 48 h, after which the vials were transferred to −2.0±0.2°C (a subset of vials was kept at 2°C as non-frozen controls). Once at −2°C, an ice crystal was added after 6 h to induce freezing of the soil water. This procedure has been shown to ensure inoculative freezing of enchytraeids once the temperature becomes lower than the body fluid melting point (Slotsbo et al., 2008). After 24 h at −2°C, when the soil was frozen (verified by visual inspection), the vials were transferred to programmable cooling cabinets in which temperature was gradually

lowered by 3°C day^{-1} (0.125°C h^{-1}) until it reached −5, −14 or −20°C depending on the required treatment/sampling. Worms were kept at their target temperature until 2 days after the coldest cabinet had reached its final temperature (−20°C). Hence, each group remained at sub-zero temperatures for 9 days. Mortality was assessed 1 day after thawing at 5°C. Only the worms that reacted normally to tactile stimuli and showed no freezing damage (deformations and rupture of the skin) were scored as surviving.

Water content, osmolality and supercooling point measurements

Fresh and dry mass, water content, osmolality and supercooling point were determined for worms that were acclimated to 2°C for 48 h, i.e. just before exposure to sub-zero temperature. The water content of individual enchytraeids was calculated from measurements of fresh mass and of dry mass after drying at 60°C for 24 h (*N*=6) using a ±1 µg accuracy scale (Sartorius AG, Goettingen, Germany). The body fluid osmolality of single individuals was measured by placing an enchytraeid in a sample holder, quickly crushing it with a pestle in order to expose the body fluids, and then placing it in a Wescor C-52 sample chamber connected to a Wescor HR 33 T Dew Point Microvoltmeter (Wescor, Logan, UT, USA) operated in the dew point mode (*N*=6). Soil water osmolality was also measured, by quickly filling the bottom of the sample holder with soil particles and following the same measurement procedure as for enchytraeids (*N*=3). Melting point was calculated using the osmolal melting point depression constant (1.86°C $osmol^{-1}$ kg water). The supercooling point was measured using copper–constantan thermocouples as described elsewhere (Pedersen and Holmstrup, 2003). The worms were gently surface dried with filter paper and carefully attached to a thermocouple with adhesive tape. The cooling rate was ~1°C min^{-1}. Supercooling points of *N*=8–14 individuals from each population and treatment were determined.

Quantification of glycogen and glucose

In order to assess the glycogen reserves and glucose concentration after acclimation at +2°C in test soil for 48 h (but before exposure to sub-zero temperature; day 0), five samples of three representative worms were taken from both control soil and treated soils. Glucose concentration was also determined for frozen worms (−5, −14 and −20°C). The sampling of frozen worms was carried out during the frost exposure, on days 2, 3, 6 and 8. Another sampling was made at the end of the freezing exposure, on day 9, at all target temperatures (+2, −5, −14 and −20°C). Groups of 15 worms were quickly thawed with deionized water and cleaned of excess soil; three organisms were pooled per sample in Eppendorf tubes, snap-frozen in liquid nitrogen and thereafter kept at −80°C until analysis. Because it was not possible to discriminate between dead and alive worms after rapid thawing, glucose measurements were (for some treatments) based on a mixture of surviving and dead worms. Glucose and glycogen analysis was carried out as previously described (Overgaard et al., 2007) using spectrophotometrically based enzymatic test kits (Gluc-DH FS from DiaSys

Diagnostic Systems, Holzheim, Germany).

Direct measurement of ice content

Ice content (fraction of water that was frozen) was measured using differential scanning calorimetry (DSC). Because of the extensiveness and complexity of our experiment, we only measured ice content in a subset of the treatments: worms acclimated to two salinities (0‰ and 35‰ NaCl), and frozen at two target temperatures (−5 and −14°C).

Before DSC analysis, the worms were quickly and gently cleaned with filter paper pre-moistened with distilled water and weighed to determine fresh mass (Mettler Toledo, Lisbon, Portugal; accuracy ±10 µg). Each worm was then placed in a hermetically sealed 50 µl aluminium test-pan and the combined mass of the worm and pan was determined (total fresh mass). Thermal analyses of whole worms were conducted using a DSC4000 calorimeter (Perkin Elmer,Waltham, MA, USA) as described previously (Koštál et al., 2012). For measurement of ice content at −5°C the following temperature program was used: (i) hold for 1 min at 10°C; (ii) cool to −20°C at a rate of 5°C min^{-1}; (iii) hold for 5 min at −20°C; (iv) heat to −5°C at a rate of 5°C min^{-1}; (v) hold at −5°C for 30 min; and (vi) heat to 5°C at a rate of 1°C min^{-1} (*N*=9). For measurement of ice content at −14°C, the same temperature program was followed, except in for steps iv–vi: (iv) heat to −14°C at a rate of 5°C min^{-1}; (v) hold at −14°C for 30 min; and (vi) heat to 5°C at a rate of 1°C min^{-1} (*N*=4). After thermal analysis, the test-pans were perforated to allow the worms to be dried for 3 days at 60°C. Test-pans containing dried worms were weighed (total dry mass). The amount of frozen water was calculated from the area under the melt endotherm using the value of 334.5 J g^{-1} for the enthalpy of water. The amount of unfrozen water was determined by subtracting the mass of frozen water from the total water mass (calculated from the difference of total fresh mass and total dry mass). The relative amount of total osmotically active water (OAW) and total osmotically inactive water (OIW, or 'bound water') was determined as described elsewhere (Holmstrup and Westh, 1994) by cooling worms to −70°C at a rate of 10°C min^{-1}, holding them here for 30 min and then heating them to 30°C at a rate of 5°C min^{-1} (*N*=5 for each population).

Estimation of ice content

The relative ice content of frozen worms was estimated at each combination of sub-zero temperature (−5, −14, −20°C) and salinity (0‰, 15‰, 35‰ and 50‰ NaCl), by using the values of melting point and water content (measured before freezing) and glucose concentrations (measured after freezing). These values were randomly paired before calculations, leading to five replicated estimates of ice fraction. The osmotic contribution of glucose was based on the assumption that all glucose is osmotically active, resulting in 1 mol l^{-1} glucose being equivalent to ~1 osmol kg^{-1}, and that 60% of the worm's water content could be regarded as OAW (see Results). The melting point depression of accumulated glucose was calculated using the osmolal melting point depression

constant, and added to the measured melting point of unfrozen individuals. The ice fraction, F, at a given temperature was calculated according to the formula: F=1–(MP/T), where MP is the melting point and T is ambient temperature (Zachariassen and Husby, 1982).

Reproduction test

The test was performed according to the standardized guideline for the enchytraeid reproduction test used in ecotoxicological studies (ISO, 2004; OECD, 2004), with minor adjustments. Because of the large number of worms required for testing and in order to spare the natural population from Greenland, only worms from Germany were used.

In short, eight adult worms with well-developed clitellum were introduced into glass vessels (50 ml) containing 25 g of test soil (moistened to 50% of the water-holding capacity) plus food supply (50 mg of finely ground and autoclaved rolled oats, half of the amount supplied every week). Four replicates per salinity treatment and eight controls were used. The tests were run at 20±1°C with a 16 h:8 h light:dark photoperiod, for 6 weeks. Soil moisture content was checked each week and mass loss replenished with the appropriate amount of deionized water. At the end of the test, the juveniles were immobilized with 80% alcohol and counted under a dissection microscope.

Statistical analysis

Comparisons between treatments were tested using ANOVA. Dunnett's, Holm–Sidak and Tukey tests were used to assess significant differences after one-way or two-way ANOVA. All statistical analyses were performed using Sigmaplot for Windows Version 11.0 (Systat software Inc., Chicago, IL, USA).

RESULTS

Survival

Worms from both populations showed a significant improvement in survival in saline soils within the tested range (for salinity, Germany: $F_{3,64}$=73.90, P<0.001; Greenland: $F_{3,64}$=51.14, P<0.001) (Fig. 1). This effect was more pronounced in worms from Germany than in those from Greenland, where significant differences were already apparent at −5°C between 0‰ and all other salinities (Dunnett, P<0.05). At −20°C, 20% of German worms survived in non-saline soils (0‰) compared with 70%, 85% and 100% in soils with 15‰, 35‰ and 50‰ salinity, respectively. Comparison between the two populations in non-saline soil showed that the Greenland population had higher survival after freezing than the German population ($F_{1,24}$=6.13, P<0.05) for all freezing temperatures (−5, −14 and −20°C).

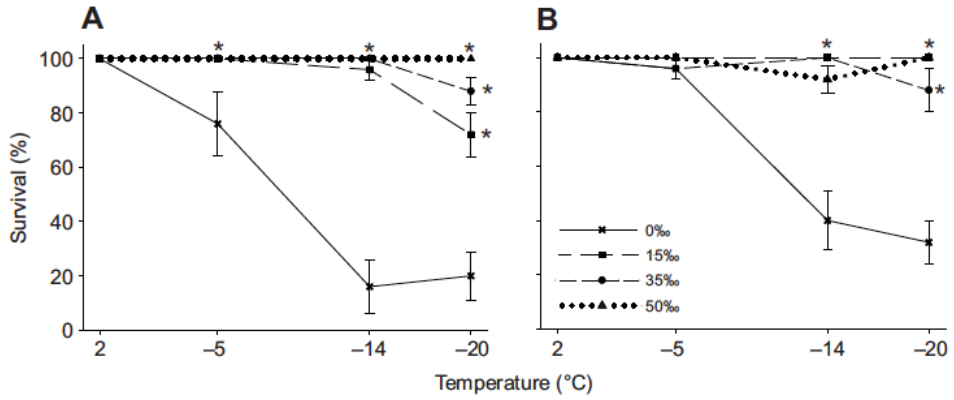

Fig. 1. Freeze survival of *E. albidus* from Germany (A) and Greenland (B) when exposed to soil with a range of salinities (0‰, 15‰, 35‰ or 50‰ NaCl). Results are shown as means ± s.e.m. (*N*=5). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

Osmolality, melting point, supercooling point, water content and dry mass

Both populations showed an increase in the osmolality of body fluids with exposure to increasing salinity in soil (Fig. 2). Despite similar osmoregulatory responses, there was a significant interaction between population and soil salinity ($F_{3,40}$=10.78, *P*<0.001). At lower salinities (0‰ and 15‰) both populations were hyperosmotic in relation to the soil medium. At higher salinities (35‰ and 50‰) worms from Greenland became osmoconforming, whereas the worms from Germany remained slightly hyperosmotic to the soil medium.

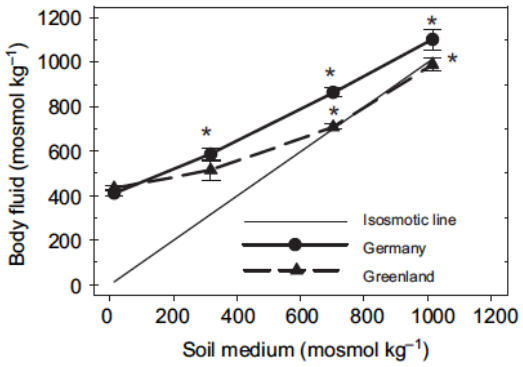

Fig. 2. Osmolality of body fluids of *E. albidus* from Germany and Greenland after 48 h exposure to different salinities. Results are shown as means ± s.e.m. (*N*=6). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

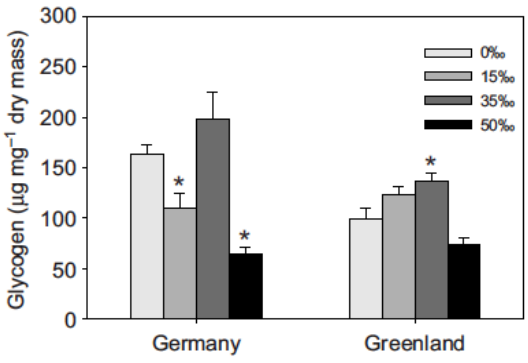

Fig. 3. Glycogen content of *E. albidus* from Germany and Greenland after 48 h exposure to different salinities. Results are shown as means ± s.e.m. (*N*=5). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

The corresponding melting points are shown in Table 1, along with supercooling point, water content and dry mass measurements. Water content and supercooling point decreased with the salinity increase in both populations; however, this decrease was not of statistical significance (Table 1). Water content differed significantly between populations in non-saline soils, being lowest in worms from Greenland. The worms from Greenland were also significantly larger than the worms from Germany, as shown by their higher dry mass (Table 1).

Table 1. Body fluid melting point, supercooling point, water content and dry mass of *E. albidus* from Germany and Greenland sampled after 48 h at 2°C.

		Salinity (NaCl ‰)			
Physiological parameter	Population	0	15	35	50
Melting point (°C)	Germany	–0.76±0.03^{a}	–1.09±0.05^{a,*}	–1.61±0.04^{a,*}	–2.05±0.09^{a,*}
	Greenland	–0.81±0.02^{a}	–0.96±0.08^{a}	–1.31±0.02^{b,*}	–1.84±0.06^{b,*}
	Soil	–0.02±0.01^{b}	–0.59±0.01^{b,*}	–1.30±0.01^{b,*}	–1.89±0.01^{b,*}
Supercooling point (°C)	Germany	–3.50±0.47^{a}	–4.50±0.50^{a}	–5.55±0.66^{a}	–5.52±0.55^{a}
	Greenland	–4.18±0.42^{a}	–5.24±0.66^{a}	–5.17±0.80^{a}	–5.91±0.71^{a}
Water content (mg mg^{-1} dry mass)	Germany	4.40±0.66^{a}	3.52±0.10^{a}	3.25±0.13^{a}	2.84±0.05^{a}
	Greenland	2.86±1.04^{b}	2.68±0.28^{a}	2.22±0.11^{a}	2.13±0.14^{a}
Dry mass (mg)	Germany	0.80±0.08^{a}	0.75±0.08^{a}	0.42±0.05^{a,*}	0.69±0.07^{a}
	Greenland	2.10±0.24^{b}	1.87±0.20^{b}	1.49±0.19^{b}	2.55±0.28^{b}

Results are shown as means ± s.e.m. (N=6–8).
Significant differences between 0‰ and other salinities are shown (Dunnett, *P<0.05).
Significant differences between populations are indicated by different superscript letters (t-test, P<0.05).

Glycogen and glucose

Salinity (without freezing) had an effect on glycogen reserves (Fig. 3), causing a significant decrease at 15‰ and 50‰ in worms from Germany. In the worms from Greenland, no significant decrease was observed; instead, a small but significant increase in glycogen was observed at 35‰. Temperature and salinity had an effect on glucose accumulation (Fig. 4). At the end of freeze exposure (day 9), worms from Germany had the highest levels of glucose at the lowest sub-zero temperatures (20- to 30-fold increase compared with +2°C, independently of soil salinity), revealing a significant positive effect of sub-zero temperature ($F_{3,64}$=484.51, P<0.001), a negative effect of salinity ($F_{3,64}$=38.07, P<0.001) and also an interaction between temperature and salinity ($F_{9,64}$=10.80, P<0.001). At −5°C we observed an increase in glucose with an increase in the time of exposure (from 3 to 9 days) ($F_{1,32}$=49.02, P<0.001). At −14°C the interaction between time of exposure and salinity was significant ($F_{3,32}$=3.03, P<0.05). Similar to worms from Germany, worms from Greenland also showed a negative effect of salinity ($F_{3,64}$=8.77, P<0.001), a positive effect of sub-zero temperature ($F_{3,64}$=419.41, P<0.001) and a significant interaction between temperature and salinity on glucose accumulation ($F_{9,64}$=5.15, P<0.001). Time of exposure generally increased the glucose levels in worms at 2°C ($F_{1,32}$=5.55, P<0.05), −5°C ($F_{1,32}$=74.19, P<0.001) and −14°C ($F_{1,32}$=9.01, P<0.05). There was no significant interaction between time of exposure and salinity. Comparing the two populations, worms from Greenland tended to accumulate more glucose than worms from Germany, especially at −5 and −14°C in soils with 0‰ ($F_{1,32}$=39.50, P<0.001), 35‰ ($F_{1,32}$=19.21, P<0.001) and 50‰ salinity ($F_{1,32}$=40.09, P<0.001). At these salinities, the effects varied with

temperature depending on the population source (0‰ $F_{1,3}$=7.51, P<0.001; 35‰ $F_{1,3}$=7.13, P<0.001; and 50‰ $F_{1,3}$=6.18, P<0.05). There was an interaction between population and salinity at +2°C ($F_{3,32}$=10.34, P<0.001) and −14°C ($F_{3,32}$=3.67, P<0.05).

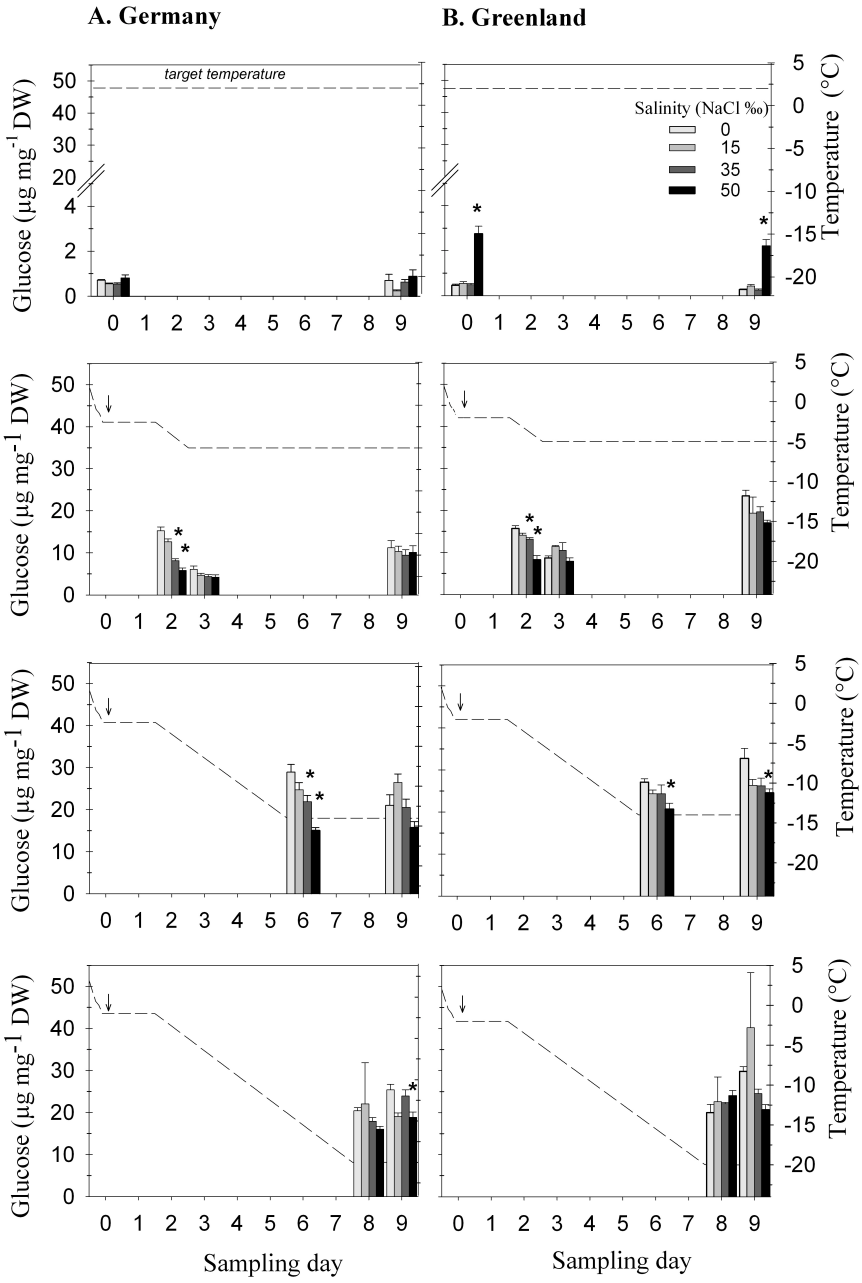

Fig. 4. Glucose content of *E. albidus* from Germany (A) and Greenland (B) measured at different sampling times, when exposed to soil with a range of salinities (0, 15, 35 or 50‰ NaCl) under different temperature regimes (2, −5, −14 and −20°C). The arrow marks the point in time when ice crystals were added to the soil surface to induce freezing. The dashed line indicates the target temperature. Results are shown as means ± s.e.m. (N=5). Significant difference from 0‰ salinity is shown (Dunnett, *P<0.05).

Ice content determined by DSC

The total fraction of bound water (OIW, unfreezable water) was determined by DSC to be 38.6±2.3% (mean ± s.e.m.) for the worms from Germany (*N*=5) and 41.2±0.9% for the Greenland population (*N*=4). As these values were not significantly different, we used a mean value of 40% for OIW and 60% for OAW (freezable water) for both populations. At the lower target temperature of −14°C, the ice content measured by DSC was significantly higher than that at −5°C ($F_{1,44}$=9.91, P<0.001), particularly in worms exposed to saline soils (by ~30%; Fig. 5). At each target temperature, the ice content was significantly lower in worms exposed to 35‰ salinity than in control worms (Fig. 5). Exposure to 35‰ salinity reduced the ice fraction of worms by 35% at −5°C in both populations (Fig. 5A). At −14°C, salinity reduced the ice fraction by 13% and 6%, in worms from Germany and Greenland, respectively (Fig. 5B). There was a significant interaction between temperature and salinity ($F_{1,44}$=22.70, P<0.001); however, no differences between populations were revealed.

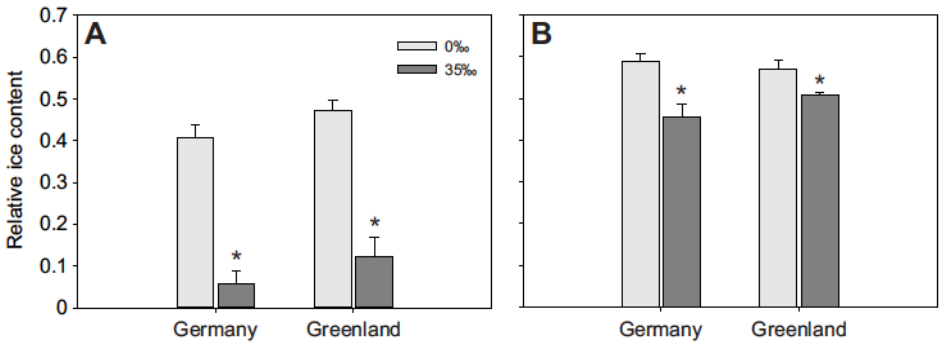

Fig. 5. Relative ice content of *E. albidus* from Germany and Greenland determined by differential scanning calorimetry when cooled to target temperatures of (A) −5°C and (B) −14°C, after 48 h exposure to salinities of 0 and 35‰ NaCl. Results are shown as mean values ± s.e.m. (*N*=10 and 5 for target temperatures of −5 and −14°C, respectively) (Dunnett, **P* < 0.05).

Estimated ice content

At each target temperature the calculated relative ice content decreased with increasing salinity of the soil (Fig. 6). Comparing 0‰ and 50‰ salinity, the estimated ice content of worms from Germany was reduced by 32%, 8% and 7% at −5, −14 and −20°C, respectively. A similar pattern was found for worms from Greenland, with a reduction of 24%, 7% and 5% at −5, −14 and −20°C, respectively. The relative ice content increased with the decrease of temperature in both populations ($F_{2,48}$=892.26 in Germany, $F_{2,48}$=675.17 in Greenland; P<0.001). There was a significant interaction between salinity and temperature ($F_{6,48}$=41.58 in Germany, $F_{6,48}$=17.62 in Greenland; P<0.001). Differences between populations were observed at the target temperature of −5°C ($F_{1,32}$=403.13, P<0.001), −14°C ($F_{1,32}$=433.80, P<0.001) and −20°C ($F_{1,32}$=51.60, P<0.001).

The estimated ice content was in fairly good agreement with the DSC-determined values, except at relatively high sub-zero temperatures (−5°C), where the estimated ice content was much higher than

the DSC-determined one. Relating the survival data to the estimated ice content showed that lethal effects occurred when the ice fraction reached a threshold between 0.56 and 0.57 (Fig. 7).

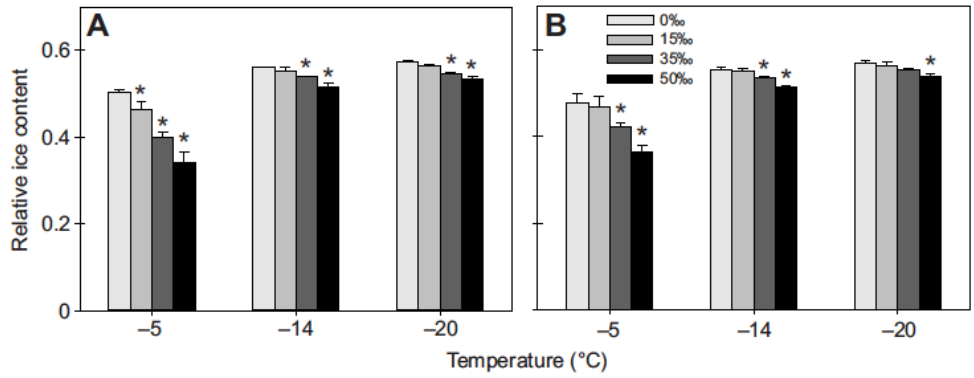

Fig. 6. Estimated relative ice fraction of *E. albidus* from Germany (A) and Greenland (B) when exposed to combinations of sub-zero temperature and salinity. Results are shown as mean values ± s.e.m. (*N*=5) (Dunnett, **P*<0.05).

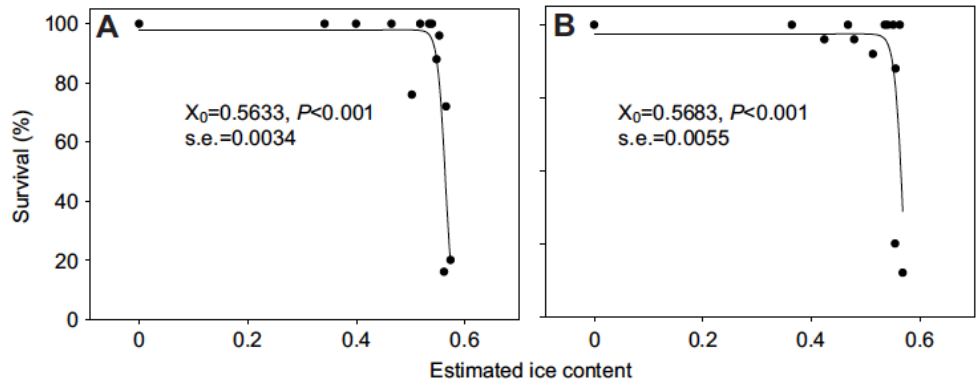

Fig. 7. Relationship between the relative ice content and survival of freezing in *E. albidus* from Germany (A) and Greenland (B). Each point represents the mean survival and the corresponding estimated mean ice fraction. A sigmoidal regression curve was fitted to the data. X_0 denotes the ice fraction causing 50% mortality. Standard error of the X_0 estimate is shown.

Reproduction

The reproduction test was valid according to the guidelines (ISO,2004; OECD, 2004). The validity criteria of adult survival of 80% and a minimum of 25 juveniles per 10 adults (coefficient of variance <50%) were fulfilled. Reproduction was positively influenced by salinity from 2‰ to 40‰ salinity with a significant increase (Fig. 8). The results indicate a typical normal distribution curve for salinity, where the optimum seems to range between 10‰ and 30‰. To provide the estimate of the effect of salt concentration on reproduction, because the data describe an optimum distribution curve instead of a linear dose–response, calculations were done using 20‰ salinity as the optimum point in a two-parameter logistic curve fit. The 90% upper limit of NaCl was predicted to be 72.9‰ (39<CI<106).

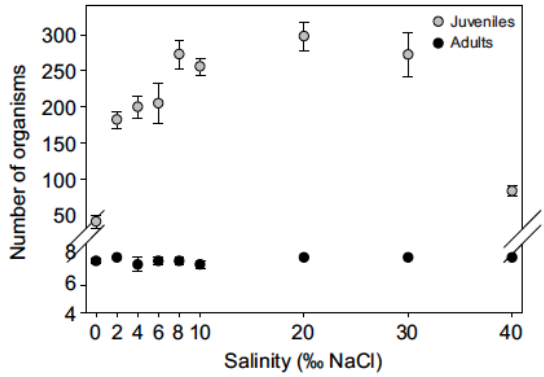

Fig. 8. Results from a reproduction test performed with *E. albidus* from Germany when exposed to a range of salinities in LUFA 2.2 soil, showing the number of surviving adults and juveniles. Results are shown as means ± s.e.m. (*N*=4).

DISCUSSION

Effect of salinity on freeze-tolerance

The most important finding of our study is that pre-acclimation to even modest salinities of soil water improved survival during freezing at low temperature considerably. For example, worms from Germany kept in 15‰ NaCl survived (>90%) freezing at −14°C whereas less than 20% of the worms kept in soil of 0‰ NaCl survived this temperature. Thus, *E. albidus* resembles many intertidal invertebrates in which changes in salinity can modify cold survival considerably (Aarset, 1982; Murphy, 1983; Elnitsky et al., 2009). The survival of freezing is probably related to the amount of extracellular ice formed. Extracellular ice formation causes cell dehydration and shrinking, which at very low temperatures may reach a lower critical volume that affects membrane integrity, leading to irreversible cell collapse (Meryman, 1971; Zachariassen, 1985). It is also likely that extensive ice formation causes mechanical damage to tissues in the frozen organism. Thus, keeping ice formation to a minimum is crucial for cold tolerance and cellular homeostasis. When relating estimated ice content to survival rate it appears that lethal effects emerge when the ice fraction crosses a threshold between 0.56 and 0.57. In our case, worms that were pre-acclimated in saline soils and frozen had a significantly lower ice content compared with those in the 0‰ soil (not salt acclimated), which can at least partly explain their much higher survival at subzero temperatures. The decrease in ice content with increasing salinity was observed in both estimated and DSC-measured ice content, despite lower values revealed in the latter at −5°C. However, at −14°C the estimated ice content matched the DSC-determined ice content quite well. The ice content measured by DSC must be carefully interpreted as inoculation at high temperatures by ice crystals was not possible and cooling rates were much more rapid than those used in the survival experiment. This probably prevented the accumulation of cryoprotectants during freezing. However, the estimated ice content also depends on assumptions that may not be accurate.

The main contribution to the lower ice fraction observed in worms exposed to saline soils was the decrease in melting point (by ~1.03–1.29°C in 50‰ saline soil) and to some degree from a decrease

in water content. This decrease in melting point was probably due to the passive influx of Na^+ and Cl^- ions across the body wall, as demonstrated in other annelids, such as *Lumbricus* sp. (Ramsay, 1949; Dietz and Alvarado, 1970; Dietz, 1974; Prusch and Otter, 1977) and *Nereis* sp. (Smith, 1976). According to these previous results, the uptake of Na+ and Cl– increases with salinity, although the velocity of this process tends to diminish with time of exposure. At lower temperatures such as −14 and −20°C, the melting point-driven effect on ice fraction becomes less prominent; however, even small reductions in ice fraction at these temperatures may improve survival if the lethal threshold (~0.6) is not reached. At a relatively high sub- zero temperature (−5°C), the reduction in ice fraction caused by salinity is considerable, meaning that worms exposed to salinity would be subjected to a slower freezing of body fluids during cooling to very low temperatures (−14 or −20°C) than worms frozen in control soil. This reduced ice content in the early phase of the freezing process could be important for physiological mechanisms to prevent severe cellular dehydration, and would promote the stabilization of proteins and cell membranes by cryoprotectants (Zachariassen, 1985; Crowe et al., 1987) as discussed below.

The results of the freeze tolerance experiment showed that *E. albidus* from Germany and Greenland are highly tolerant to saline soils. No mortality due to salinity was observed, even at 50‰. Moreover, our results show that up to 30‰ salinity is beneficial for *E. albidus* reproduction, which is perhaps not so surprising considering that this species is commonly found in decaying seaweed on the seashore. Taken together, our study shows that non-saline soils are in fact sub-optimal for *E. albidus*.

Role of glycogen and glucose

Previous studies on enchytraeids and other annelids have pointed to a positive correlation between glycogen reserves and the accumulation of glucose with the ability to survive extreme freeze events (Pedersen and Holmstrup, 2003; Holmstrup and Overgaard, 2007; Slotsbo et al., 2008). Glycogen is the principal source for mobilization of carbohydrate cryoprotectants in terrestrial oligochaetes (Holmstrup et al., 2007; Overgaard et al., 2007), and glucose acts as their main cryoprotectant, probably because it is the primary blood sugar of these animals (Prentø, 1987).

Previous work (Slotsbo et al., 2008) has shown that *E. albidus* from Greenland accumulated glucose to between 50 and 110 μg mg^{-1} dry mass upon freezing at −2 and −14°C, respectively, equivalent to concentrations in OAW of ~120–250 mmol l^{-1}. As glucose is a reducing sugar and may cause damaging glycation of proteins at 10-fold lower concentrations in vertebrates (MacDonald et al., 2009), it is surprising that enchytraeids can tolerate such high glucose concentrations. The glucose concentration accumulated at −2°C was enough to reduce the ice fraction by ~20% at this temperature, which will help to ensure controlled tissue dehydration and glucose transportation across tissues. In our investigation, we also observed an increase in glucose concentration with the decrease in temperature. However, considering the relatively low glucose concentrations of worm

tissue (20–30 µg mg^{-1} dry mass), the contribution of glucose to lowering the ice fraction was negligible (<1%). Thus, in this case, the colligative effects of glucose were not of importance for freeze survival. This was also underlined by the fact that the worms exposed to saline soils (e.g. 50‰) had lower glucose concentrations than the worms exposed to non-saline soils, but still had a higher freeze survival. In addition, as we were not able to discriminate between live and dead specimens at the time of sampling, the glucose content of worms exposed to non-saline soils at −14 and −20°C could be underestimated and thus corroborate the observed negative effect of salinity on glucose accumulation. These observations suggest that glucose synthesis is somehow triggered and regulated (via feedback mechanisms) by changes in body fluid osmolality. Even though glucose was the only cryoprotectant measured in the present study, the possibility of other carbohydrate cryoprotectants being of importance seems unlikely. Slotsbo and colleagues explored different types of low molecular weight cryoprotectants that were produced by *E. albidus*, and glucose was clearly the dominant cryoprotectant (Slotsbo et al., 2008).

Despite the low contribution of glucose to osmolality and reduced ice fraction, its importance in other processes such as stabilization of membranes and proteins is crucial (Anchordoguy et al., 1987; Crowe et al., 1987). It is believed that such sugars maintain the phospholipid membrane in a fluid phase in the absence of water, preventing damage from low temperatures or dehydration. They are also effective at preserving the structure and function of labile proteins that are important for maintaining vital functions (Crowe et al., 1987). Lastly, glucose may also alleviate the potentially toxic effect of high Na^+ and Cl^- concentrations during freezing. It should be noted that these and glucose will increase to at least 10-fold higher concentrations in the unfrozen parts of the body fluids bathing the cells when worms are frozen at very low temperatures. Worms from Greenland had the highest glucose levels, and this may possibly explain their better freeze survival.

Glucose also increased as a response to osmotic stress caused by salinity without freezing, as has also been shown in other invertebrates (Elnitsky et al., 2009). However, the glucose concentrations were low under these conditions compared with glucose levels in frozen worms. Furthermore, the decrease in glycogen levels due to salinity exposure was not matched by similar glucose concentrations, which points to the conversion of glycogen to other low molecular weight carbohydrates in addition to glucose.

CONCLUSIONS

Despite the similar overall physiological and biochemical response mechanisms of the two populations, our study indicates significant differences in their survival, absolute values of physiological parameters and the dynamics of these responses. Taken together, these differences led to an overall superior survival at sub-zero temperatures in the worms from Greenland. It seems reasonable to assume that genetically based adaptations to extreme environmental conditions of

worms from Greenland are the reason to these population differences.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S., M.H. and V.K. designed the experiments measuring ice content. A.L.P.S. and V.K. performed the ice content measurements. A.L.P.S. carried out data analysis. All authors wrote the paper.

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Photo by Karina Fisker

Chapter III

Worms from the Arctic are better adapted to freezing and high salinity than worms from temperate regions: Oxidative stress responses in *Enchytraeus albidus*

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SUMMARY

Enchytraeus albidus is a freeze-tolerant enchytraeid found in diverse habitats, from supralittoral to terrestrial, and spanning temperate to arctic regions. Thus, this worms often exposed to sub-zero temperatures and fluctuating salinity regimes that can lead to physiological stress. We therefore studied the oxidative stress by measuring lipid peroxidation, anti-oxidant defenses and neurotransmission activity in *E. albidus* from arctic (Greenland) and temperate (Germany) regions during a short-term exposure to saline conditions (0, 15, 35 and 50‰ NaCl) and low temperatures (+2, −2 and −5 °C). Various enzymatic and non-enzymatic oxidative stress markers were analyzed. Results have shown that both salt and freezing caused oxidative stress in *E. albidus*, particularly from Germany, as confirmed by catalase, glutathione-S-transferase and superoxide dismutase activities and lipid peroxidation levels. Neurotransmission (as judged from acetylcholinesterase activity) was reduced by saline conditions at +2 °C, but stimulated at −2 and −5 °C. Worms from Greenland had relatively higher and more stable levels of antioxidants than worms from Germany, reflecting their higher tolerance of freezing and saline conditions.

Keywords: Enchytraeids, natural stressors, antioxidants, oxidative stress, biomarkers.

INTRODUCTION

Enchytraeus albidus (Oligochaeta: Enchytraeidae) is a small terrestrial earthworm common in soils with high organic content and in decomposing seaweed. It may reach a size of 3 cm and a fresh weight of about 5 mg. *E. albidus* can be found in very diverse habitats from supralittoral to terrestrial, and is spanning contrasting climatic zones from temperate regions to the Arctic (Christensen and Dózsa- Farkas, 2006; Giere, 2006). Consequently, this species must have mechanisms to tolerate rapid shifts in salinity and fluctuations in temperatures between freezing and thawing. Recent studies have shown that *E. albidus* survives sub-zero temperatures down to −20 °C by freeze-tolerance, and that acclimation to salinities up to 50‰ of the substrate increases the cold tolerance primarily by reducing the amount of ice formed during freezing (Patrício Silva et al., 2013). Freezing of the worms' body fluids triggers synthesis of glucose in concentrations that are probably high enough (20–80 μg mg^{-1} dry mass) to provide some cryoprotection (Slotsbo et al., 2008; Patrício Silva et al., 2013).

Freeze-tolerant animals may suffer cellular damage due to oxidative stress resulting from the production of reactive oxygen species (ROS) if the rate of ROS production is higher than the capacity of antioxidant systems (Joanisse and Storey, 1996a, 1996b; Hermes-Lima et al., 1998; Hermes-Lima, 2004). Oxidative stress occurring in freeze tolerant animals is likely due to effects of ischemia followed by reperfusion during freezing and thawing events. In addition to this, the use of

glucose as a cryoprotectant has the potential to cause glucose-mediated oxidative damage (Storey and Storey, 2004).

The overproduction of ROS, e.g. the superoxide anion ($O2^{\bullet-}$), hydroxyl (OH•), singlet oxygen ($1O_2$) and hydrogen peroxide (H_2O_2) causes cellular damage, including lipid peroxidation (LPO), protein oxidation and DNA damage (Hermes-Lima, 2004). Cells trigger antioxidant defense systems to prevent ROS formation, suppressing ROS using free radical scavengers or antioxidant enzymes. In this way, for instance an increase in the level of oxidized glutathione (GSSG) at the expense of reduced glutathione (GSH) is generally viewed as an indication of oxidative stress. The enzymes superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), selenium-dependent glutathione peroxidase (GPx; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7) and the glutathione-S-transferases (GST; EC 2.5.1.18) are interacting key line of defense against ROS and their products of attack (Chaudière and Ferrari-Iliou, 1999). GSH can act as a substrate for antioxidant enzymes (e.g. GPx) and as an independent scavenger of electrophilic xenobiotics (GST). Glutathione is also involved in the reactivation of some enzymes inhibited under oxidizing conditions (Reddy et al., 1982; Meister and Anderson, 1983).

In the present study we used two source populations of *E. albidus*, from Greenland and Germany, with known difference in cold tolerance (Slotsbo et al., 2008; Patrício Silva et al., 2013). We tested the following hypotheses: i) the oxidative defense capacity of worms from Greenland is superior to worms from Germany since the former is adapted to a colder climate; ii) freezing in soil with high salinity is associated with low oxidative stress since the ice content of frozen worms is lower here than in non-saline soils (Patrício Silva et al., 2013). The aim was therefore to assess the oxidative stress in *E. albidus* when exposed to a range of salinities (0–15–35–50‰ NaCl), temperature regimes (2°C, −2 and −5°C) and different exposure times. We measured the following markers: lipid peroxidation (LPO), total, reduced and oxidized glutathione content (TG, GSH and GSSG), catalase activity (CAT), glutathione reductase activity (GR), glutathione peroxidase activity (GPx), glutathione-S-transferase activity (GSTs) and superoxide dismutase activity (SOD). Acetylcholinesterase (AchE; EC 3.1.1.7) activity was also measured to assess the effects on neurotransmission, since environmental variables may have a direct or indirect effect on its inhibition (Pfeifer et al., 2005).

MATERIALS AND METHODS

Test animals

Echytraeus albidus (Oligochaeta, Enchytraeidae) from Germany was obtained in 2011 from a commercial supplier (Büchner Zierfischfutter, Jena, Germany; coordinates: 51° 51′N 9° 50′ E). Worms from Greenland were collected in 2010 from decaying seaweed on the seashore of Kobbefjord (about 20 km from Nuuk; coordinates: 64° 8′ N 51° 23′ W). Both cultures were kept in

moistened natural (loamy) soil at 15°C and weekly fed with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). The lifespan of this species is approximately 6 weeks at 18°C in standard soils, but it can be delayed for 4 more weeks if the temperature drops to 12°C (ISO, 2004).

Before the experiments began, the worms were cold acclimated at 5°C for 6 weeks and then at 2°C for one week.

Test soil and spiking procedures

The tests were performed in the natural standard soil LUFA 2.2 (Speyer, Germany). Main characteristics of the soil are as follows: organic carbon 1.7%, grain size distribution 7.3% clay, 13.8% silt, 78.9% sand and a maximum water holding capacity (WHC) of ca. 44% of fresh weight. The pH (CaCl2) of LUFA soil is 5.5. This soil is within the optimum range of pH in natural soils where *E. albidus* is found (Jänsch et al., 2005).

To test a saline environment, soil was spiked using NaCl. We are aware that salinity in soils should be represented by many other ions (see e.g. Owojori and Reinecke, in press) than Na^+ and Cl^- but these are the major ions in seawater (Cl^- = 18.9 mg L^{-1}; Na^+=10.6 mg L^{-1} in a total dissolved solids = 34.5 mg L^{-1}) hence resembling the study habitat. NaCl (99.5% purity, Merck, Germany) was added as aqueous solution to the dry soil at 50% of soil water holding capacity (corresponding to 22 mL per 100 g dry soil). For the exposure we used the following NaCl concentrations: 0, 15, 35 and 50‰. Soil was allowed to equilibrate for 1 day before test start.

Experimental setup and sample handling

Test procedure followed the standard guidelines (ISO, 2004; OECD, 2004) with few adaptations. Each replicate consisted of a plastic test vial (3 cm height, 2 cm diameter) containing 5 g of test soil and 15 mg oatmeal. Fifteen worms per replicate were used. The vials were covered with a perforated lid to allow ventilation. Seven replicates per treatment were used. Test setup consisted of exposing *E. albidus* (from 2 populations) to salinity and to a gradual temperature decrease as shown in Fig. 1.

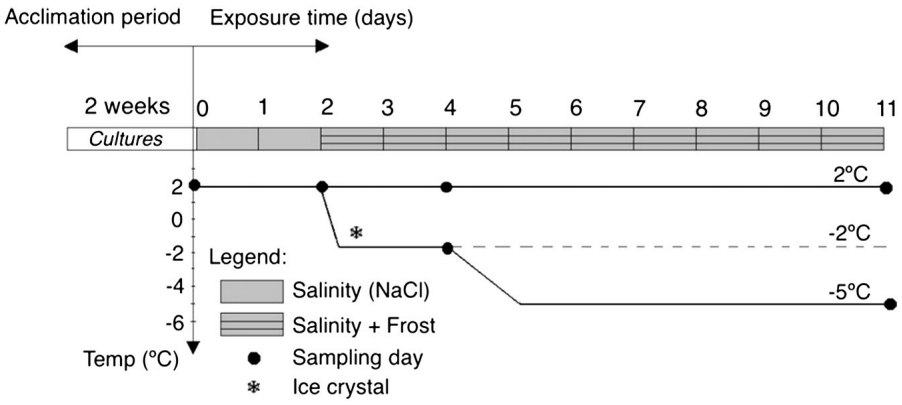

Fig. 1. Schematic representation of the experimental setup, which consisted to expose *E. albidus* organisms to a salinity (NaCl) range and decreasing temperature. The various sampling moments are signed with a dot.

In short, the procedure consisted of acclimation of the worms in treated soils at 2°C for 48 h. After this, vials were transferred to a walk-in freezer where the temperature was lowered to −2.0 ± 0.2°C within 2 h. A subset of vials was kept at 2°C as non-frozen controls. Once at −2°C, an ice crystal was added after 6 h to induce freezing of soil water. This procedure has been shown to ensure inoculative freezing of enchytraeids (Slotsbo et al., 2008). After 48 h at−2°C, when the soil and worms were frozen (manually checked), the vials were transferred to automatic cooling cabinets where temperature was gradually decreased by 3°C per day (0.125 °C/h) until −5°C. Worms were kept at their target temperature for 6 more days. Hence, worms remained at −5°C for 6 days. At sampling, the soil of each vial was rapidly thawed in deionized water, and the worms were collected, quickly rinsed and transferred to microtubes, snap-frozen in liquid nitrogen and kept at −80°C until further analysis.

Biochemical analysis — biomarkers

Preparation of samples for biochemical analysis was carried out following previously described procedures (Howcroft et al., 2009; Gomes et al., 2011; Novais et al., 2011). In short, each replicate was homogenized in 1500 µL K-Phosphate 0.1 M buffer, pH 7.4. Part of the tissue homogenate (150 µL) was separated into a microtube with 2.5 µL of 2,6-Di-tert-butyl-4-methylphenol (BHT) 4% in methanol for endogenous lipid peroxidation (LPO) determination. The extent of LPO was measured as thiobarbituric acid-reactive substances (TBARS) at 535 nm (Ohkawa et al., 1979; Bird and Draper, 1984). The remaining tissue homogenate was centrifuged for 20 min at 10,000 g (4 °C) to isolate the post-mitochondrial supernatant (PMS). The PMS was divided into eight microtubes and stored at −80 °C for later analysis of biomarkers and protein quantification. Protein concentration was assayed using the Bradford method (Bradford, 1976), adapted from Bio-Rad's Bradford micro-assay set up in a 96-well flat bottom plate, using bovine γ-globuline as a standard. Acetylcholinesterase activity (AChEs) was measured using the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), at 414 nm. Glutathione reductase (GR) activity was assayed by monitoring the decrease of NADPH levels at 340 nm (Cribb et al., 1989). Glutathione-S-transferase (GST) activity was determined following the conjugation of GSHwith 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). Total glutathione (TG, GSH + GSSG) and oxidized glutathione (GSSG) were measured at 412 nm, using the recycling reaction of reduced glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenczoic acid), DTNB, in the presence of GR excess (Tietze, 1969; Baker et al., 1990). 2-Vinyl-pyridine was used to conjugate GSH for the GSSG determination (Griffith, 1980). The GSH content was calculated by subtraction of GSSG from the total glutathione. Measurements were recorded at 550 nm (McCord and Fridovich, 1969) adapted to microplate. Glutathione peroxidase (GPx) activity was determined by measuring the decrease in

NADPH at 340 nm, using H2O2 as a substrate (Mohandas et al., 1984). Total superoxide dismutase (SOD) activity was measured photochemically at 560 nm, according Giannopolitis and Ries (1977) and adapted to microplate. All spectrophotometric measurements were performed at 25 °C.

Data analysis

The experimental design included four variables: population (Germany, Greenland), temperature (2°C, −2°C, −5°C), NaCl (0, 15, 35 and 50‰) and exposure period (0, 2, 4, 11 days). Comparisons between variables were made within overlapping conditions of other exposure variables, using t-test or analysis of variance (ANOVA), for two or more than two groups of variables respectively. Dunnetts' and Holm–Sidak were used to assess significant differences after one-way and two-way ANOVA respectively. Outliers (outside average ± 2* standard error) were excluded from analysis. All statistical analyses were performed using Sigmaplot for Windows Version 11.0 (Systat software Inc., San Jose, CA, USA).

RESULTS

Antioxidant and detoxification enzyme activities

The results on enzymatic antioxidant biomarkers (SOD, CAT, GPx, GR) and on the detoxification (GST) are shown in Fig. 2. SOD activity in worms from Greenland was higher and more stable than in worms from Germany. The presence of NaCl had a significant positive effect in worms from Germany, and no effect in worms from Greenland. Freezing resulted in significantly lower SOD activity (less than 20 to 30 nmol min^{-1} mg^{-1} protein) in the worms from Germany as compared to control worms kept at 2°C. At 2°C, SOD was influenced by time of exposure ($p < 0.05$). CAT activity was also more stable in worms of Greenland than in worms from Germany. In worms from Germany, the presence of NaCl caused a significant decrease in CAT activity after 4 days of exposure at 2°C. A positive effect of NaCl was observed in worms exposed at −5°C. When worms were frozen, CAT activity increased with temperature decrease ($p < 0.05$). At 2°C, worms from Germany showed a decrease of CAT with increase in time of exposure ($p < 0.05$).

At 2 °C, there was also a significant interaction between NaCl and time of exposure ($F_{6,70} = 4.8$; $p < 0.001$). There was a significant interaction between NaCl and temperature at day 4 ($F_{3,48} = 11.6$, $p < 0.001$) and day 11 ($F_{3,46} = 3.1$, $p < 0.05$). GPx activity was very variable in both populations. In worms from Germany, changes were less variable at freezing temperatures. The highest NaCl concentration (50‰) seemed to have a stabilizing effect with little fluctuation over time, whereas at NaCl lower concentration there was a fluctuating activity. No significant interactions were observed between variables within each population. GR activity changed significantly over time. In worms from Greenland kept at 2 °C, GR activity decreased over the 11-day exposure ($p < 0.05$), whereas there was no clear pattern in worms from Germany. In frozen worms the GR activity increased with

the decrease in temperature ($p < 0.05$). The effect of NaC was more apparent in the worms from Germany at 2°C, e.g. at day 4 and 11 there was respectively an increase and decrease that seems dose related. At freezing temperatures the opposite tendency occurred, with an increase in GR activity as temperature decreased ($p < 0.05$). There was a significant interaction between the presence of NaCl and temperature ($F_{2,69} = 2.3$, $p < 0.05$). GST activity decreased as temperature decreased from 2 °C to −2 °C, except for the highest NaCl concentration (50‰). Yet, at negative temperatures there was a significant increase from −2 to−5°C (except in control salinity) accompanied by a small positive effect of NaCl ($p < 0.05$). In worms from Germany kept at 2 °C, GST activity decreased with time of exposure ($p < 0.05$). In worms from Greenland, GST presented a tendency to decrease with increase in time of exposure at 2 °C. At 2 °C, the presence of NaCl had a general negative effect on GST. At the same temperature, there was a significant interaction between NaCl and time of exposure ($F_{6,70} = 5.4$; $p < 0.001$). Interaction between NaCl and temperature was significant at day 4 ($F_{3,47} = 14.9$, $p < 0.001$).

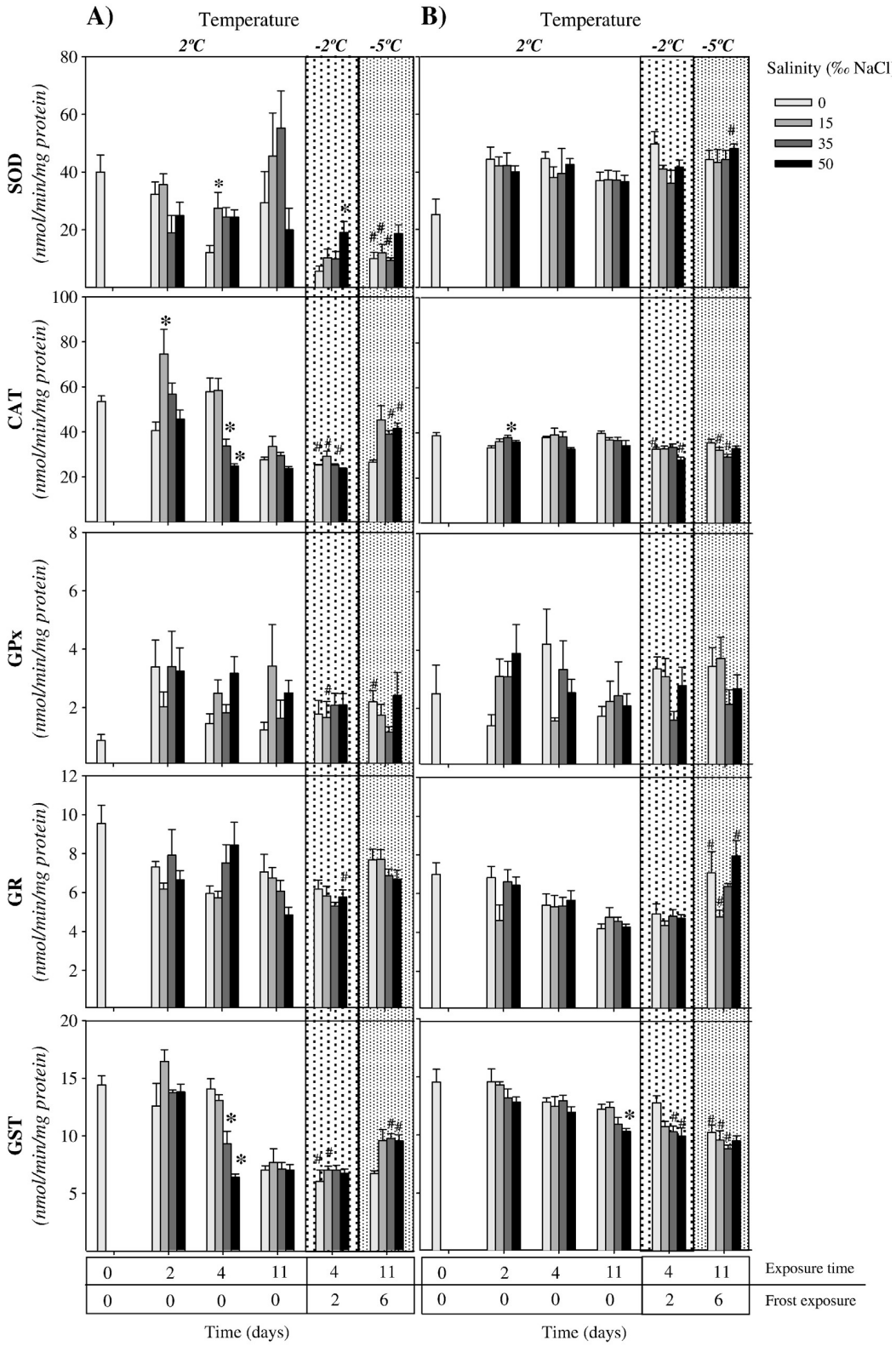

Fig. 4. Results on enzymatic antioxidant biomarkers (SOD, CAT, GPx, GR) and on the detoxification (GST) of *E. albidus* Germany (A) and Greenland (B). Worms were exposed in LUFA 2.2 soil under different temperatures (2,−2 and−5°C), exposure periods (0, 2, 4, 6 and 11 days) and salinities (0, 15, 35 and 50‰ NaCl). Day 0 samples are organisms sampled from culture boxes kept at constant 5°C. Frost exposure scale indicates the time that the organisms were frozen within the total exposure time. Results are expressed as average ± standard error (N = 7). Significant differences ($p < 0.05$) between salinities are indicated (*) (Dunnetts' test) and between temperatures (#) within days 4 and 11 (t-test).

Non-enzymatic antioxidants levels

Results on the non-enzymatic antioxidant biomarkers (TG, GSSG and GSH) are shown in Fig. 3. Glutathione levels varied in both populations, but overall, worms from Germany had lower glutathione levels than worms from Greenland, especially the GSSG levels. In worms from Germany, TG and GSH levels showed a tendency to increase with temperature decrease.

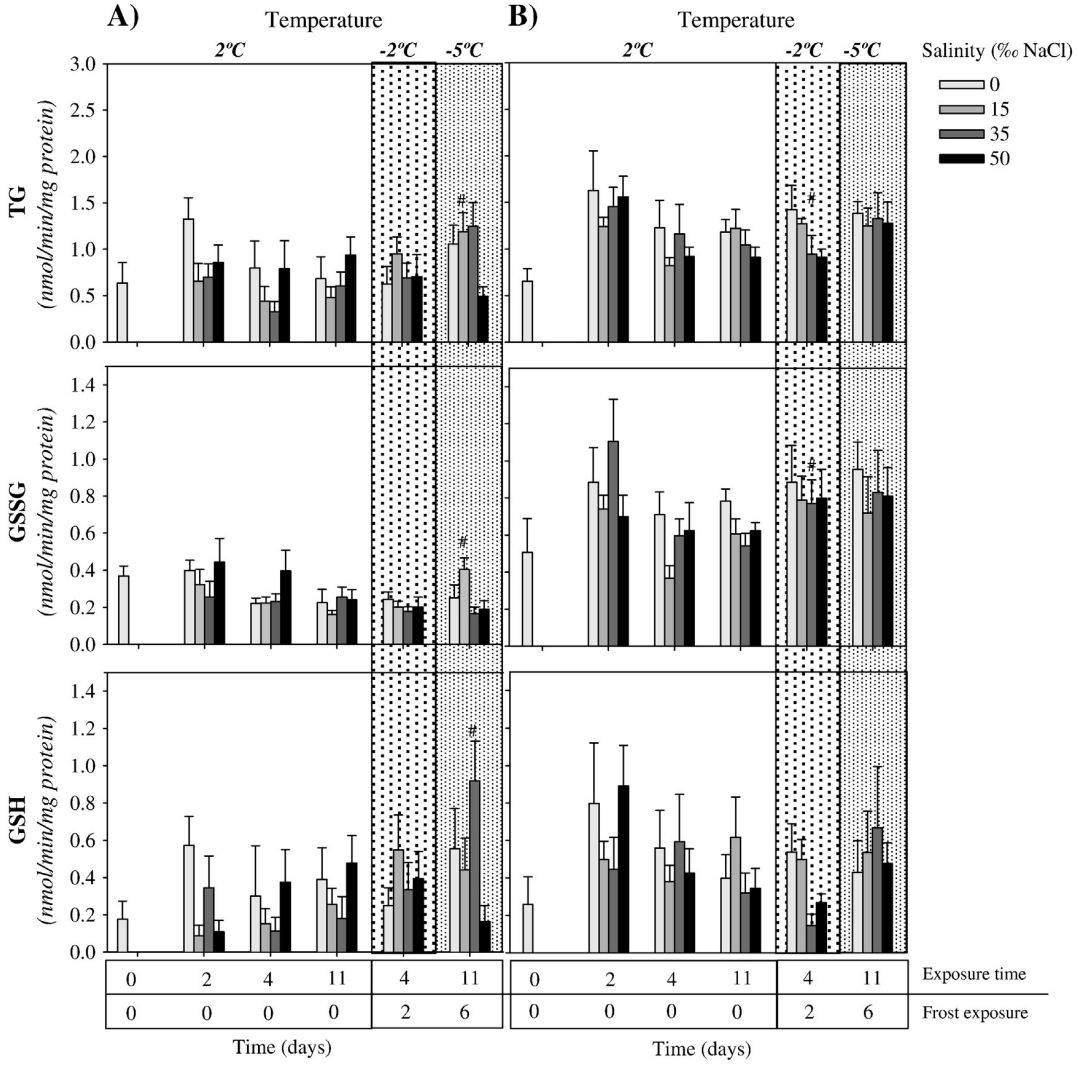

Fig. 3. Results on the non-enzymatic antioxidant defenses (TG, GSSG and GSH) of *E. albidus* from Germany (A) and Greenland (B). Worms were exposed in LUFA 2.2 soil under different temperatures (2,−2 and−5°C), exposure periods (0, 2, 4, 6 and 11 days) and salinities (0, 15, 35 and 50‰ NaCl). Day 0 samples are organisms sampled from culture boxes kept at constant 5°C. Frost exposure scale indicates the time that the organisms were frozen within the total exposure time. Results are expressed as average ± standard error (N = 7). Significant differences ($p < 0.05$) between salinities are indicated (*) (Dunnetts' test) and between temperatures (#) within days 4 and 11 (t-test).

Lipid peroxidation

Worms from Germany had 2–3 times higher levels of LPO, and were significantly more affected by exposure time and the presence of NaCl than worms from Greenland (Fig. 4). In worms from Greenland kept at 2°C, the presence of NaCl (35–50‰) prevented LPO whereas at −2/−5°C no further LPO occurred. In worms from Germany kept at 2°C (and also at −2/−5°C, to a lesser extent) LPO increased mainly with increase in time. In worms from Greenland, LPO at day 0 (i.e. worms in culture) was significantly higher than all other.

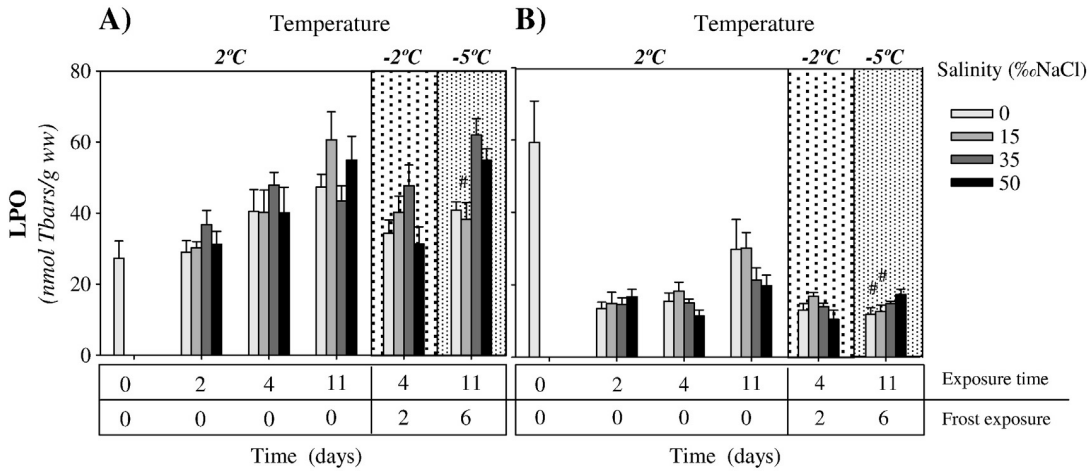

Fig. 4. Results of LPO levels of *E. albidus* Germany (A) and Greenland (B). Worms were exposed in LUFA 2.2 soil under different temperatures (2,−2 and−5ºC), exposure periods (0, 2, 4, 6 and 11 days) and salinities (0, 15, 35 and 50‰ NaCl). Day 0 samples are organisms sampled from culture boxes kept at constant 5°C. Frost exposure scale indicates the time that the organisms were frozen within the total exposure time. Results are expressed as average ± standard error (N = 7). Significant differences ($p < 0.05$) between salinities are indicated (*) (Dunnetts' test) and between temperatures (#) within days 4 and 11 (t-test).

Acetylcholinesterase activity

Overall, worms from Greenland had relatively stable AChE activity when exposed to increasing NaCl concentrations and/or decreasing temperatures (Fig. 5). In worms from Germany kept at 2°C, time and NaCl (significant interaction: $F_{6,69} = 7.02$; $p < 0.001$), caused an initial increase followed by a decrease with time. An increase in AChE is observed from −2 to −5°C in the presence of NaCl ≥15‰.

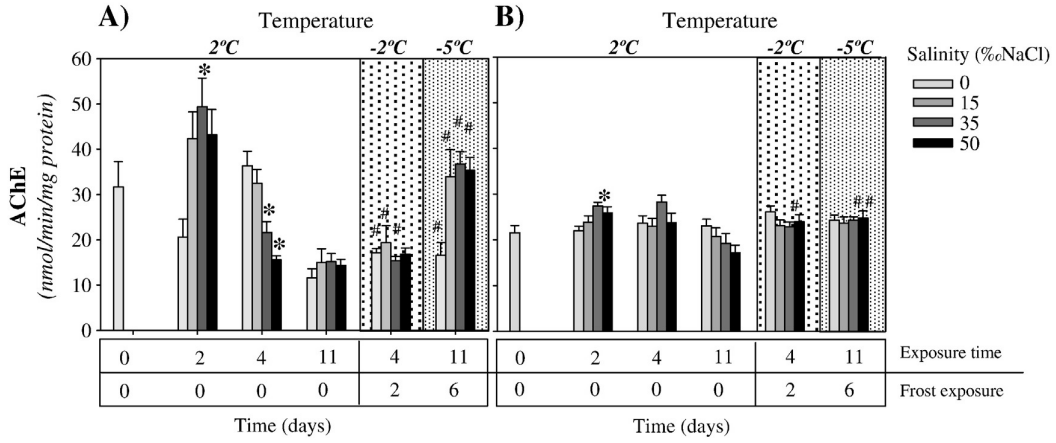

Fig. 5. AChE activity of *E. albidus* from Germany (A) and Greenland (B). Worms were exposed in LUFA 2.2 soil under different temperatures (2,−2 and−5°C), exposure periods (0, 2, 4, 6 and 11 days) and salinities (0, 15, 35 and 50‰ NaCl). Day 0 samples are organisms sampled from culture boxes kept at constant 5°C. Frost exposure scale indicates the time that the organisms were frozen within the total exposure time. Results are expressed as average ± standard error (N = 7). Significant differences ($p < 0.05$) between salinities are indicated (*) (Dunnetts' test) and between temperatures (#) within days 4 and 11 (t-test).

DISCUSSION

Worms from Germany and Greenland presented significant differences in their responses to freezing and salinity (i.e. presence of NaCl in the soil). Overall, worms from Greenland had relatively higher and more stable levels of antioxidants than worms from Germany. Hence, worms from Germany suffered comparatively more from oxidative stress when exposed to increased salinity and during freezing. These results are probably reflecting both the higher cold tolerance of worms from Greenland and perhaps also a greater tolerance of shifts in salinity. The worms from Greenland were collected on fjord shores of the Nuuk region where salinity changes on a regular basis due to tidal inundation and precipitation, and where winter temperatures are low for a substantial part of the year. Comparing only two populations from contrasting environments may not be sufficient to prove adaptative differences. However, recent studies in our laboratory including seven populations distributed along a south-north gradient from Germany/Scandinavia to Iceland and Greenland/Svalbard confirm that cold tolerance is highest in Arctic populations (Fisker et al., in press).

It could also be argued that the differences between Germany and Greenland worms observed here may reflect the long period of acclimation to laboratory conditions, particularly in worms from Germany. In fact, these worms were previously collected from a compost soil near Jena (where they naturally occurred), and cultured by the supplier in the same agricultural soil for several years at approximately 19°C. However, the environmental conditions of the cultures were kept similar to the ones in compost soils (relatively high temperature, high content of organic matter, pH range between 4 and 6). This may imply that the worms have kept their natural capacity to respond to changing environmental conditions. Furthermore, in our previous investigation (Patrício Silva et al.,

2013), worms from the supplier revealed similar physiological and biochemical response mechanisms to changes in salinity and freezing temperatures.

The overall higher activity of SOD in worms from Greenland indicates a higher capacity for the removal of O^{2-} radicals, when compared with worms from Germany which showed an inhibition in SOD activity (more than 50%) during freezing, possibly caused by interaction with peroxides and oxygen derived free radicals (Pigeolet et al., 1990) accumulated during internal ice formation. In addition to SOD inhibition, worms from Germany showed also an initial decrease in CAT activity (after 2 days at freezing temperatures), probably also due to the increase of hydroxyl radicals and superoxide anions in the cells (Kono and Fridovich, 1982), which could indicate a problem in removing H_2O_2. The same tendency was observed in GST levels, suggesting a direct negative interaction of ROS species on its enzymatic activity, a possible complexation with the Na^+ or Cl^- ions (in the case of soils with NaCl) and/or a depletion of its substrate (GSH). The two-fold increase in GPx levels observed in worms from Germany could be an indication of an increase in H_2O_2 during exposure to freezing and the respective detoxification. Another difference between populations observed in the response to oxidative stress was the levels of total, oxidized and reduced forms of glutathione. Glutathione levels were much higher in worms from Greenland compared with Germany, also indicating a higher antioxidant potential in these worms, particularly by the higher GSH (reduced form of glutathione) levels since GSH is both an antioxidant (by its own) and a substrate for a number of antioxidant enzymes. Our results are in agreement with previous studies carried out in cold-hardy insects, showing that the freeze tolerant *Eurosta solidaginis* undergoing frequent events of freezing and thawing during winter maintains high and stable levels of antioxidant defenses, whereas the freeze avoiding *Epiblema scudderiana* that never experiences seasonal or sporadic stress from freezing shows more responsive antioxidant defenses (Joanisse and Storey, 1996a, 1996b; Storey and Storey, 2010). Worms from Germany exposed to saline soils (15, 35 and 50‰ NaCl) quickly restored their antioxidant levels, by increasing GST levels and GSH, GR and CAT activities 4 days after freezing. Such increase has also been reported in cold-hardy insects, although only observed after long-term exposure (overwintering) to low temperatures (Joanisse and Storey, 1996). The partial inhibition followed by an increase in GST, GR and CAT activity in frozen worms could probably be related with direct regulation of ROS and readjustments of their levels. During exposure to freezing temperatures and subsequent freezing of soil and internal ice formation, the availability of oxygen to the tissues may become limited, leading to higher levels of ROS and consequent increase in the defenses to suppress oxidative stress and to maintain homeostasis (Storey and Storey, 2010). Thus, worms exposed to freezing in soil with high salinity showed a relatively lower oxidative stress, probably related to reduced ice content of worms frozen in saline soils (Patrício Silva et al., 2013).

Salinity and frost temperatures also interfered with the neurotransmission in worms from Germany.

Acetylcholinesterase (AChE) is known to be inhibited not only by various insecticides such as dimethoate in e.g. *E. albidus* (Novais, 2011) or *Sorex araneus* (Dell'Omo et al., 1999) but also by salinity in marine organisms e.g. *Nereis diversicolor* (Scaps and Borot, 2000), Mytilus sp. (Pfeifer et al., 2005), *Cragon cragon* (Menezes et al., 2006), *Eurytemora affinis* (Cailleaud et al., 2007) or *Carcinus maenas* (Rodrigues et al., 2012;). Our results showed a negative effect of salinity on AChE activity after 4 days in non-frozen worms. Inhibition of AChE due to salinity was also observed in *Mytilus* sp. (salinity ranging between 5 to 20‰, after exposures of 2 hrs to 10 days) as reported by Pfeifer et al. (2005). According to the authors, inhibition of the AChE might be related to a shift in the extra and intracellular inorganic ion concentration, which occurs along the process of osmotic regulation. When considering the combined effect of salinity and subzero temperatures, the low AChE activity after ca. 2 days at −2°C could be interpreted as an initial response to internal ice formation together with an overall decrease in metabolism; being freeze-tolerant, *E. albidus* seems to restore AChE activity (as well as antioxidant defenses) during frost exposure, especially at higher NaCl concentrations where frozen worms showed lower internal ice contents.

Time of exposure affected the controls but this has been commonly observed within similar time intervals (Gomes et al., 2011; Novais et al., 2011). It is not fully clear why this happens but it seems to be related with physiological adjustments to the test conditions. This highlights of course the importance of having controls per exposure time. Overall, the effect of salinity was most distinct when in the 50‰ NaCl level. Results for exposure to salinity in the 15–35‰ range seem to be in agreement with the results obtained for reproduction, where an optimal 20–30‰ NaCl effect was observed (Patrício Silva et al., 2013), after which it decreased if salinity was higher (despite the fact that survival to freeze was improved up to 50‰).

CONCLUSIONS

E. albidus from Greenland appears to deal with oxidative stress during salinity and frost exposures by maintaining relatively high and stable antioxidant defense and large cellular pools of glutathione. In comparison, worms from Germany were more susceptible to challenges by saline soils and freezing, as indicated by higher variability in levels of SOD, CAT GST and AChE. The divergence of these oxidative stress responses during short-term exposure to different salinity and temperature regimes reinforces the physiological and biochemical heterogeneity among populations, and lead us to believe that genetic adaptations may be acting. Furthermore, worms exposed to saline soils showed a higher capacity to restore antioxidant levels and AChE activity during exposure to frost temperatures than worms exposed to non-saline soils.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S. carried out data analysis. All authors wrote the paper.

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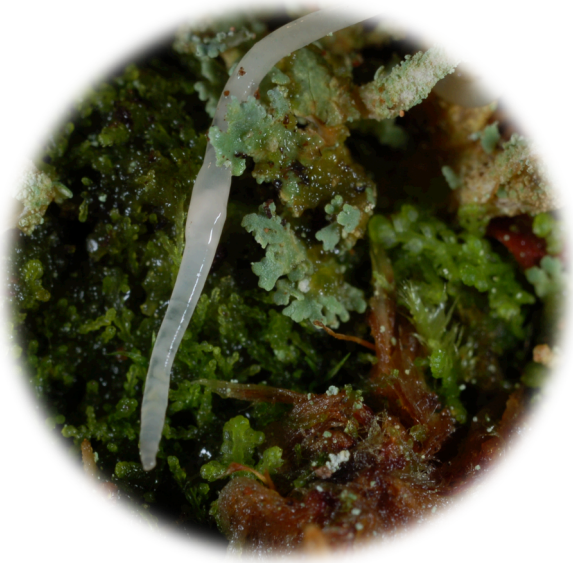

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Chapter IV

Salinity changes impact of hazardous chemicals in *Enchytraeus albidus*

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SUMMARY

Supralittoral ecosystems are among the most challenging environments for soil organisms, particularly when salinity fluctuations are involved, frequently combined with the presence of contaminants as a result of intense anthropogenic activities. Knowledge of how salinity influences the effect of contaminants in supralittoral species is crucial for determining the safety factors required when extrapolating results from optimal laboratory conditions to these natural ecosystems. The present study therefore evaluated the effects of 2 metals (copper and cadmium) and 2 organic compounds (carbendazim and 4-nonylphenol) in the absence or presence of 15‰ NaCl in the potworm *Enchytraeus albidus*, a model organism for ecotoxicology studies commonly found in supralittoral ecosystems, The potworms had a higher reproduction in saline soil than in control soil. Moreover, the effects of copper and carbendazim on reproduction were smaller than when they were tested in nonsaline soil. Potworms exposed to nonsaline soils also had significantly higher tissue concentrations of metals, which partly explains the effects on reproduction. The influence of salinity on effects of 4-nonylphenol was, however, less clear; effects on survival decreased in saline soil, but effects on reproduction were highest in saline soil. The latter slightly correlated with tissue concentrations of the chemical. The present study provides the first evidence that soil salinity has a significant influence on the impact of contaminants evaluated with the enchytraeid reproduction test.

Keywords: Salinity, enchytraeids, contaminants, survival, reproduction.

INTRODUCTION

The rise in the mean sea level has significantly accelerated throughout the last 2 centuries, mainly because of the melting of glaciers and ice sheets (Rhein et al., 2013), causing flooding along coastal areas. Tidal movement of water, associated with shifts in precipitation and evaporation patterns, are constantly changing salinity along littoral and supralittoral ecosystems, imposing a physiological challenge to the species living there. The ability of these organisms to tolerate rapid and significant changes in salinity may be impaired by exposure to contaminants, thereby affecting the osmotic and ionic balance, as well as other physiological and biochemical response mechanisms, and their ability to maintain homeostasis (Depledge, 1987; Edwards, 2002).

The influence of salinity on the toxicity of various classes of chemicals is well documented for aquatic biota (Hall and Anderson, 1995; Noyes et al., 2009), but a knowledge gap persists for soil species, particularly those that inhabit supralittoral habitats, in which salinity can change dramatically. To our knowledge, only a few studies have been conducted on soil species (earthworms) that address the combined effects of salinity (NaCl salt) and metals on survival, reproduction and bioaccumulation, revealing additive to synergistic negative interactions (Owojori and Reinecke, 2010; Owojori et al., 2009a). However, these earthworms did not tolerate salinity

higher than 4‰ NaCl (Owojori and Reinecke, 2014; Owojori et al., 2009b), whereas supralittoral species such as certain enchytraeids have large physiological plasticity to shifts in salinity (from 0 to 50‰ NaCl) (Patricio Silva et al., 2013). In addition, some of these enchytraeids have optimal growth and reproduction in soils with some salinity, as observed for instance in the worm *Enchytraeus albidus* (Patricio Silva et al., 2013). Thus, interactions between effects of salinity and contaminants may have a different trend in supralittoral species. Studies with estuarine species (e.g., crustaceans, mollusks, fish, and annelids) have revealed negative correlations between salinity and impacts of various chemicals (metals and organic compounds), mainly because salinity decreases bioavailability and bioaccumulation of some pollutants (Hall and Anderson, 1995; Noyes et al., 2009). Therefore, knowledge of how salinity influences the impact of contaminants on supralittoral species becomes crucial for improving science-based risk assessments, and setting safety factors required when one is extrapolating from standard and optimal laboratory conditions to these natural ecosystems.

In this context, we have investigated the interactive effects of salinity (0 and 15‰ NaCl) and contaminants in a supralittoral species, the potworm *E. albidus*, using survival, reproduction, and bioaccumulation of contaminants as endpoints. This species is commonly found in supralittoral ecosystems along temperate and subarctic regions, where it plays important roles in soil structure and the decomposition of organic matter (Didden, 1993). Furthermore, it is used as a test organism in the environmental risk assessment of contaminants and polluted soil (e.g. Römbke and Moser, 2002). Standardized tests are currently available to evaluate the toxic effects of contaminants on *E. albidus* (ISO, 2004). However, these guidelines recommend the use of nonsaline soils for this species even though they are in fact euryhaline, with optimal performance in soils with 15 to 25 ‰ salinity (Patricio Silva et al., 2013).

From among the hazardous chemicals present in the environment, we selected 4 common contaminants with known modes of action for our study: 2 metals (copper [C_u] and cadmium [Cd]) and 2 organic compounds (4-nonylphenol, an amphiphilic, yet lipophilic, organic compound) and carbendazim (a fungicide often used as a reference compound in toxicity tests with earthworms and enchytraeids). The presence of free Cu^{2+} and Cd^{2+} in organisms can result in the production of reactive oxygen species, can cause lipidic peroxidation and induce significant changes in antioxidant defenses, leading to a decrease in survival and reproduction (Gomes et al., 2011; Novais et al., 2011). In addition, free metal ions and other cations (such as Na^{+}) may compete for binding at the organism's point of entry, interfering with osmoregulation and uptake, which may either reduce or enhance the negative effects of the chemicals (Lee et al., 2010; Lock et al., 2006). Because of the lipophilic nature of 4-nonylphenol, it is readily accumulated in membranes causing toxicity by

narcosis (Holmstrup et al., 2014). Carbendazim is highly toxic to annelids, strongly affecting biomass, reproduction, and energy allocation (Holmstrup, 2000; Novais et al., 2010).

MATERIAL AND METHODS

Test species

Enchytraeus albidus (Henle, 1837) were obtained from a commercial supplier (Büchner Zierfischfutter), and cultivated in the laboratory in an agricultural (loamy) soil at 19 ± 1°C. The worms were fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark).

Test soil

All experiments were conducted with a natural standard soil, LUFA 2.2 (Speyer). This soil has 7% clay, 14% silt, 79% sand, 3.1% organic matter, and only trace concentrations of sodium and chloride. The soil pH ($CaCl_2$) is 5.5, which is within the optimum range of the pH in natural soils where *E. albidus* are found (Jänsch et al., 2005). Before use, the soil was dried for 24 h at 80°C.

Test substances and test procedures

The following tests aimed to evaluate the effect of low salinity (15‰) on reproduction and survival of chemically treated *E. albidus*. The selected chemicals included 2 metals (copper chloride [$CuCl_2$; ACROS Organics; Cas No. 7447.39.4, 99% pure] and cadmium chloride [$CdCl_2$; Fluka; Cas. No 10108-64-2, 99% pure]), and 2 organic compounds (carbendazim; BASF Agro, Bavistin; 50% pure) and 4-nonylphenol (Aldrich; Cas. No. 29.005.8; 100% pure). The range of concentrations of the test substances were based on previous investigations (Amorim et al., 2005; Novais et al., 2011; Novais et al., 2010; Widarto et al., 2004) and was as follows: 0, 3.2, 10, 32, 100, 320, and 1000 mg Cu kg^{-1} dry soil; 0, 1, 3.2, 10, 32, 100, 320 mg Cd kg^{-1} dry soil; 0, 50, 100, 150, 250, 400 and 750 mg 4-nonylphenol kg^{-1} dry soil; 0, 0.032, 0.1, 0.32, 1, 3.2 and 10 mg Carbendazim kg^{-1} dry soil. To simulate salinity in the soil, we used sodium chloride salt (NaCl) because it is the most common salt present in seawater. In the following we refer to nominal concentrations of NaCl when stating the use of 0 or 15 ‰ salinity. The carbendazim, Cu and Cd were added alone or combined with 15‰ of NaCl (Merck, Cas. No. 7647.14.5, 99.5% pure) in aqueous solution and mixed thoroughly with the soil. The amount of deionized water (alone or with 15‰ of NaCl) was equivalent to 50% of the water holding capacity (WHC) of the test soil (21 mL water was added per 100 g of dry soil). Soils spiked with metals were allowed to equilibrate for 3 d before test animals were added. The 4-nonylphenol was dissolved in acetone, mixed thoroughly with the soil and kept in a fume-hood for 24 h to allow evaporation of the acetone. Then water was added equivalent to 50% of WHC with deionized water (corresponding to 0‰ of NaCl), or saline-water (15‰ of NaCl).

The reproduction tests were performed according to the standardized guideline ISO (2004), with some adjustments. Briefly, 8 adult potworms with well-developed *clitellum* were introduced into glass vessels containing 25 g of test soil plus food supply (50 mg of finely ground and autoclaved rolled oats). Four replicates per chemical concentration plus 8 controls were used. Each control set consisted of clean soil moistened with deionized water or saline water (15 ‰ of NaCl aqueous solution) but otherwise treated as described. The tests ran at 19 ± 1°C with 16:8h light:dark photoperiod, for 6 wk. Soil moisture content was checked each week, and weight loss was replenished with the appropriate amount of deionized water. After 3 wk, live adults were removed, rinsed in deionized water, weighed, and snap-frozen in liquid nitrogen to analyze the concentrations of each chemical in the tissues. Weighing and tissue analysis of enchytraeids were carried out without purging, as this is technically difficult due to the small size of these worms (as recommended in the Organization for Economic Cooperation and Development [OECD] guideline for bioaccumulation in terrestrial oligochates (OECD, 2010). At the end of the test (after 6 wk), the juveniles were immobilized with 70% alcohol and counted under a dissection microscope. All potworms used for the tests were collected from the same culture batch, and the tests ran simultaneously, with only 1 d between metals and organic contaminants.

Measurements of metals and 4-nonylphenol in tissues

Concentrations in soil are presented as nominal and were not measured; tissue concentrations were measured in the adults. Determination of tissue concentrations of 4-nonylphenol was performed on potworms fresh weight by solid-phase extraction followed by gas chromatography – mass spectrometry (GC-MS) analysis as previously described (Patricio Silva et al., 2014). Tissue concentration of carbendazim in potworms could not be measured, because of the lack of an optimized protocol.

Tissue concentrations of Cu and Cd were analyzed by atomic absorption spectrometry (Perkin-Elmer 4100). The enchytraeid samples were freeze-dried for 24 h and the dry weight was determined to the nearest 0.01 mg (Sartorius, Ultra Microbalance SC-2). Worm tissues were acid-digested using 1 mL of 65% nitric acid at 80ºC. After digestion, the acid was allowed to evaporate with increasing temperature (80–135ºC). When all fluid had evaporated, the samples were re-dissolved in 2% of nitric acid and analyzed by atomic absortion spectrometry. Certified reference material (oyster tissue material from the National Institute of Standards and Technology, U.S. Department of Commerce and lobster hepatopancreas from National Research Council Canada) was analyzed to verify the efficiency of the digestion and atomic absorption spectrometry procedure. The measurements were 97% of the certified values for Cu and 87% for Cd.

All samples were analyzed in 1 run. The detection limit for 4-nonylphenol, Cd and Cu were 0.1, 0.001 and 0.002 mg L^{-1}, respectively. Because soil concentrations of the test chemicals were not measured, all the tissue concentrations are presented in relation to nominal soil concentrations.

Statistical analyses

All statistical analyses described below were performed using the statistical software R Ver 2.12.9. Toxicity data for reproduction were described using a log-logistic concentration - response model (Seber and Wild, 1989) with the drc (dose-response curve) package (Ritz and Streibig, 2005),

$$y = d/(1 + \left(\frac{x}{EC50}\right)^{b}) \quad (1)$$

where *y* is the number of offspring per adult, *d* is the number of offspring per adult of the control, *x* is the nominal concentration of each chemical in mg kg^{-1} dry soil, EC50 is the concentration where the response is reduced by 50%, and *b* is the slope of the curve. When hormesis was observed (carbendazim), data were fitted using a modified 4-parameter logistic model (Cedergreen et al., 2005) given by the expression:

$$y = c + \frac{d - c + f \exp\left(-\frac{1}{x^{\alpha}}\right)}{1 + exp\{b[ln(x) - ln(e)]\}} \quad (2)$$

where *α* gives the rate of increase of the hormetic response (numeric value between 0 and 1), *d* represents the control, *c* is the response at infinitive dose, *b* determines the steepness of the curve after the maximal hormetic effect, and *e* provides a lower bound on the EC50 level.

Survival data were described using the same model as described in Equation 1, substituting EC50 with LC*n* (where *n* is the concentration where the response is reduced by *n*%), assuming binomial data and a *d* value of 1. Effect concentrations were calculated according to lethality (lethal concentration [LC]) or effect [EC] of 10%, 30% and 50% compared with controls. Comparisons between nonsaline and saline ECx values from survival and reproduction were performed using the generalized likelihood ratio test (Sokal and Rohlf, 2012). Significant differences between EC*x* values indicate significant interactions (positive/negative) between salinity and contaminants. A linear model described tissue concentration of metals as a function of salinity, and the significant differences between the slopes were tested by comparing the regression pairwise with an analysis of covariance (ANCOVA).

When appropriate, comparisons between variables (chemical and ‰ of NaCl) were made using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiples comparison using 95% confidence level.

RESULTS

Combined effect of salinity and contaminants on survival and reproduction

Tests fulfilled the validity criteria as described by the standard guidelines (ISO, 2004). Soil pH did not change significantly as a result of chemical spiking or test duration (5.0<pH<5.8). Increased concentrations of all tested contaminants caused a significant decline in survival in soils regardless of salt content, causing 100% mortality at the highest concentrations tested (Figure 1A). Carbendazim was the most toxic of the test compounds, with the lowest LC values, followed by Cd, Cu and 4-nonylphenol (Table 1). Survival of potworms exposed to 4-nonylphenol was significantly higher in saline soil than in nonsaline soil, whereas survival of potworms exposed to Cd was significantly lower in saline soil (Figure 1A and Table 1). Salinity had no significant effect on the survival of adults in the carbendazim and Cu experiments.

Reproduction in control soil was 4-6 fold higher in 15‰ saline soils than in nonsaline soils (Figure 1B). Potworms exposed to 4-nonylphenol revealed a clear stimulatory response at 50 mg 4-nonylphenol kg^{-1} dry soil in nonsaline soils (0‰ NaCl), with a significantly higher number of juveniles (one-way ANOVA, Dunnett test, $p < 0.05$). Because of the high stimulatory response at the lowest 4-nonylphenol concentration, various hormesis models were tried, but the fit to data was very poor; hence the EC estimates were obtained using the regular log-logistic 3P and with the hormesis data points removed from the calculation (Figure 1). The hormetic phenomenon was absent in the saline soils (containing 15‰ NaCl). The presence of salinity seemed to increase the negative effect of 4-nonylphenol on potworm reproduction, with a significant decrease in the EC10 and EC30 values but not the EC50 values. Potworms exposed to carbendazim revealed a stimulatory response to lower doses (0.0032, 0.1, 0.32 mg carbendazim Kg^{-1} dry soil; one-way ANOVA, Dunnett test, $p < 0.05$) in soil with 15‰ NaCl that was absent in potworms exposed to nonsaline soils. Regarding metals, potworm reproduction was higher in saline soils, with statistical significance for Cu (Table 2, Figure 1B).

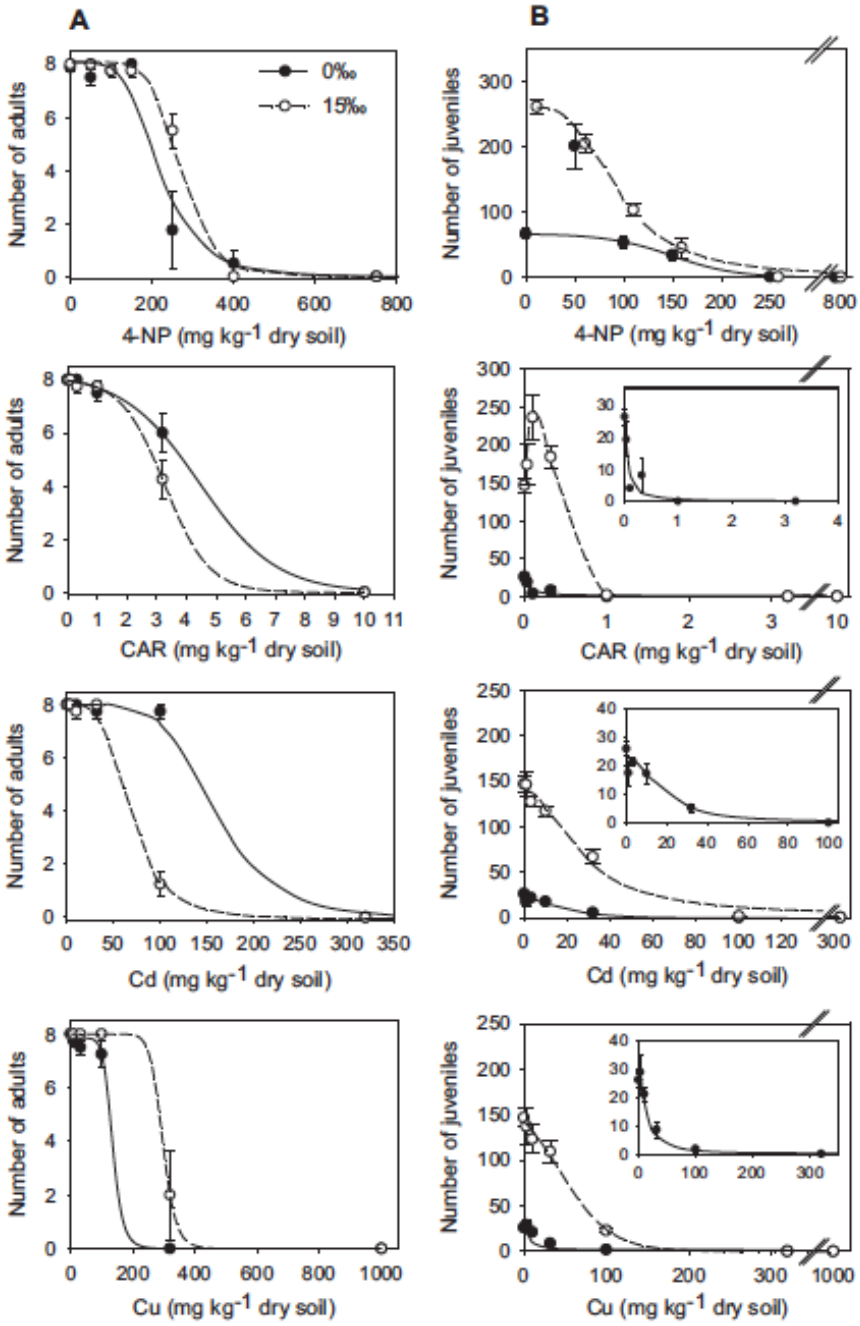

Fig. 1. Effect of organic compounds (4-nonylphenol [4-NP], and carbendazim [CAR]) and metals (Cd and Cu), combined or not with 15‰ NaCl, on *Enchytraeus albidus* survival (number of alive adults) (**A**) and reproduction (number of juveniles) (**B**). Results are shown as mean ± standard error (N=4-8).

Table 1: Effect concentrations on survival (LCx) of *Enchytraeus albidus* exposed to chemicals alone or combined with 15‰ of NaCl[a].

		0 ‰ NaCl				15 ‰ NaCl			
Compound		10	30	50	Model and parameters	10	30	50	Model and parameters
4-NP		129.1	176.0	213.7	Log-logistic 2P	188.5*	231.8*	264.0*	Log-logistic 2P
	95%CL	105.3-152.9	153.4-198.5	188.8-238.7	*b*=4.36; Y_0= 8	160.0-217.0	208.0-255.6	239.5-288.5	*b*=6.53; Y_0= 8
Car		1.9	3.0	3.9	Log-logistic 2P	1.4	2.3	3.1	Log-logistic 2P
	95%CL	1.3-2.6	2.3-3.6	3.1-4.7	*b*=3.15; Y_0= 8	0.9-1.9	1.7-2.8	2.4-3.8	*b*=2.67; Y_0= 8
Cd		101.8	133.7	158.7	Log-logistic 2P	37.3*	53.5*	67.0*	Log-logistic 2P
	95%CL	77.7-125.9	105.9-161.5	124.7-193.4	*b*=4.95; Y_0= 8	25.1-49.5	40.8-66.1	53.1-80.8	*b*=3.77; Y_0= 8
Cu		64.4	105.0	142.7	Log-logistic 2P	n.d.	266.8	288.8	Log-logistic 2P
	95%CL	42.6-86.1	79.5-130.4	110.3-175.1	*b*=2.76; Y_0= 8	n.d.	n.d.	64.6-513.0	*b*=10.7; Y_0= 8

[a]Values are in mg of compound per kg of dry weight LUFA 2.2 soil. * Statistically significant difference between salinities (generalized likelihood ratio test). LCx=lethal concentration that affected x%of survival; 95% CL=95% confidence limits; n.d.=not determined; 4-NP=4-nonylphenol; CAR=carbendazim; 2-, 3-, and 4P=number of parameters of the log-logistic [26] or the modified log-logistic [28] models; b=slope; Y_0=highest Y value of the dose–response model in control soils.

Table 2: Effect concentrations on reproduction (ECx) of *Enchytraeus albidus* exposed to chemicals alone or combined with 15‰ of NaCl[a].

		0 ‰ NaCl				15 ‰ NaCl			
		10	30	50	Model and parameters	10	30	50	Model and parameters
4-NP		92.5	123.2	147.5	Log-logistic 3P	37.4	61.2*	83.4	Log-logistic 3P
	95%CL	46.4-138.5	87.4-159.0	117.2-177.9	*b*=4.7.0; Y_0= 66.1	27.7-47.1	51.4-71.1	73.7-93.2	*b*=2.74; Y_0= 260.7
Car		0.01	0.03	0.05	Log-logistic 3P	0.3*	0.4*	0.4	Modif. Log-logistic 4P
	95%CL	n.d.-0.03	n.d.-0.06	0.02-0.09	*b*=1.22; Y_0= 26.2	0.2-0.5	0.2-0.6	0.04-0.8	*b*=4.49; Y_0= 152.6
Cd		5.1	10.6	16.8	Log-logistic 3P	7.5	16.3	26.4	Log-logistic 3P
	95%CL	n.d.-12.7	1.6-19.6	7.1-26.4	*b*=1.84; Y_0= 23.1	1.0-13.9	8.0-24.5	17.5-35.4	*b*=1.74; Y_0= 142.2
Cu		7.98	12.99	20.21	Log-logistic 3P	20.9	36.6*	52.2*	Log-logistic 3P
	95%CL	2.3-13.7	5.8-20.2	10.3-30.1	*b*=1.91; Y_0= 26.1	6.0-35.7	21.1-52.2	34.9-69.5	*b*=2.39; Y_0= 139.5

[a]Values are in mg of compound per kg of dry weight LUFA 2.2 soil. * Statistically significant difference between salinities (generalized likelihood ratio test). LCx=lethal concentration that affected x%of survival; 95% CL=95% confidence limits; n.d.=not determined; 4-NP=4-nonylphenol; CAR=carbendazim; 2-, 3-, and 4P=number of parameters of the log-logistic [26] or the modified log-logistic [28] models; *b*=slope; Y_0=highest Y value of the dose–response model in control soils.

Effect of salinity on the tissue concentration of contaminants in potworms

Concentrations of Cd and Cu in potworm tissue samples increased linearly with the nominal concentration in soil of these metals irrespective of absence or presence of 15‰ NaCl (Figure 2A and 2B). Based on the slopes of these relationships, potworms accumulated more Cd and Cu in the tissues when exposed in the nonsaline soils than in soils containing 15‰ NaCl. Both regressions (0‰ *vs* 15‰ NaCl, for Cd and Cu) were significantly different (ANCOVA, $p < 0.001$).

Tissue concentrations of 4-nonylphenol also increased significantly with soil nominal concentrations (slopes of 0.29 ± 0.06 and 0.19 ± 0.04 with r^2 of 0.6 and 0.7 in saline and nonsaline soils, respectively, $p < 0.001$; Figure 2C). However, the slopes were not statistically different (ANCOVA, $p > 0.05$; Figure 2C).

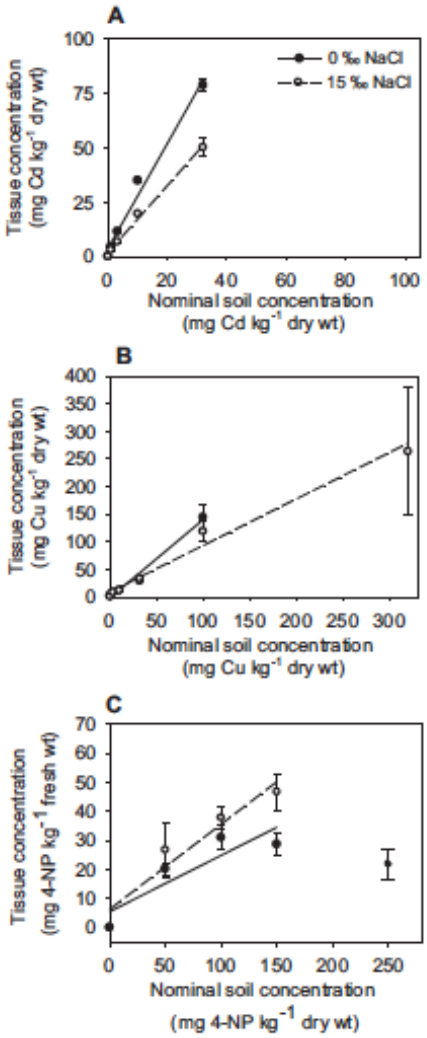

Figure 2: Tissue concentration of Cd (**A**), Cu (**B**) and 4-nonylphenol [4-NP] (**C**) in *Enchytraeus albidus* fresh weight or dry weight, after 21 d of exposure to different soil concentrations and two salinities – 0 and 15 ‰ NaCl. Results are shown as mean ± standard error (*N*= 3-8). Fit parameters are given in the text.

DISCUSSION

Our results from the present study showed that effects and tissue concentrations of contaminants in several cases were influenced by soil salinity. Soil salinity also influenced the presence or absence of hormetic effects in organic compounds.

Interactions between salinity and organic contaminants

In nonsaline soil, low concentrations of 4-nonylphenol had a clear stimulatory effect on reproduction (at ~50 mg kg^{-1} dry soil). Stimulation of reproduction at lower doses of 4-nonylphenol has also been observed in the collembolan *Folsomia candida* (Widarto et al., 2007), the polychaete *Capitella* sp. (Hansen et al., 1999), and the nematode *Caenorhabditis elegans* (Hoss et al., 2002). These authors often note that this stimulation is related to estrogenic effects, as observed for instance in fish (Yadetie et al., 1999). However, many other substances can also cause hormesis without any clear relationship to hormonal mechanisms (e.g.Hansen et al., 1999; Widarto et al., 2007). The positive effects on reproduction shown in the present study may or may not be associated with estrogenic characteristics that have been reported in fish for this contaminant (e.g. Yadetie et al., 1999).

The presence of 15‰ NaCl seemed to offset the hormetic response observed in potworms exposed to 4-nonylphenol in nonsaline soils, possibly because of the presence of a high number of juveniles in the saline controls (soil with 15‰ NaCl; Supplemental Data, Figure S1). Furthermore, 15‰ salinity also seemed to increase the negative effects of 4-nonylphenol on potworm reproduction despite the significantly lower effect on survival. The higher impact on reproduction observed in potworms exposed in saline soil may be partially explained by the tendency of higher tissue concentrations of 4-nonylphenol (and probably higher toxicity) as compared with potworms exposed to non-saline soils (despite lack of statistical significance). The effect of NaCl on uptake, bioaccumulation and elimination of 4-nonylphenol remains undocumented, so it is difficult to provide a plausible explanation for the differences observed in tissue concentrations, in the absence or presence of NaCl. Furthermore, 4-nonylphenol seems to decrease salinity tolerance in, for example, Atlantic salmon (McCormick et al., 2005), so perhaps similar negative effect might occur on *E. albidus,* resulting in a decreased egg viability and thus number of juveniles.

The effect of carbendazim was more pronounced on potworm reproduction. Potworms exposed in saline soil showed hormetic responses at doses lower than 0.32 mg kg^{-1}, but hormesis was absent in potworms exposed in nonsaline soils. This stimulatory response at low concentrations of carbendazim resulted in a approximately 10-fold decreased effect (from EC30=0.01 to 0.36 mg carbedazim kg^{-1} dry soil with statistical significance; from EC50=0.05 to 0.43 mg carbendazim kg^{-1} dry soil, with lack of statistical significance). Even though fact the EC50 values observed in both soils (saline and nonsaline) are within the range observed in previous investigations of *E. albidus*

according to OECD and International Organization for Standardization (ISO) guidelines (Novais et al., 2010; Römbke and Moser, 2002), we need to emphasize the decrease in the effect of carbendazim in the presence of 15‰ NaCl, particularly on *E. albidus* reproduction. It is known that carbendazim induces lipidic peroxidation, decreases the reduced glutathione, and changes the total protein and the activity of the enzymes superoxide dismutase, catalase, selenium-dependent glutathione peroxidase, glutathione reductase and acetylcholinesterase activity in the invertebrate *Donax faba* (JanakiDevi et al., 2013). In *E. albidus*, a short exposure to the fungicide induced transcripts encoding for intermediate filament proteins that are involved in deoxyribonucleic acid (DNA) ligation during DNA repair and interfered with the assembly of microtubules in *E. albidus* that are crucial for reproduction (Novais et al., 2012). Thus, it is likely that the increase in reproduction observed in the present study might be related to the decrease in genotoxicity and proteotoxicity as a result of the fungicide itself. Furthermore, *E. albidus* may suffer higher osmotic stress in nonsaline soils than in soils containing 15‰ NaCl, and thus be less resistant to carbendazim toxicity.

Interactions between salinity and metals

The effects of Cu and Cd shown in the present study contrast with those of previous studies. For instance, Novais et al. (2010) and Amorim et al. (2005) studied the effects of Cu and Cd on *E. albidus* using same methodology and soil type (natural LUFA soil 2.2) as we did, but found significantly different LC50 (10 times less for Cd and 2.2 times higher for Cu) and EC50 (2.7 times less for Cd and 5.7 times higher for Cu) values (Amorim et al., 2005; Novais et al., 2011). Lock and Janssen (Lock and Janssen, 2002) studied the effect of Cu and Cd (among other metals) on *E. albidus* according to OECD guidelines (with artificial OECD soil), and found lower toxicity levels for both metals (LC50= 554 mg Cd kg^{-1} dry soil and 671 mg Cu kg^{-1} dry soil). Thus, an intraspecific variation in sensitivity can be observed from laboratory to laboratory (same species, different cultures, different soil cultures, suppliers, maintenance, and so on).

Interestingly, we found that Cd caused greater mortality when combined with 15‰ NaCl, with significantly lower LC10, LC30 and LC50 values (2 to 2.5-fold) despite a lower tissue concentration than in nonsaline soil. Nevertheless, the surviving adults exposed to soils containing 15‰ NaCl reproduced better and had lower tissue concentration of Cd than potworms exposed in nonsaline soil (see also Supplemental Data, Figure S1). Potworms exposed to Cu had a higher reproductive performance in saline soil, also in agreement with the lower tissue concentration of Cu found in adult tissues. The lower tissue concentrations of metals in potworms exposed to saline soil confirm previous studies in aquatic species, where ion exchange (and balance) is the primary factor determining metal toxicity (Hall and Anderson, 1995; Lee et al., 2010). This could explain the lower effect of metals on *E. albidus* reproduction, but not necessarily their survival. Knowledge about the

uptake and depuration kinetics in both soils types (nonsaline vs saline) could contribute to a better understanding of this subject.

Considering the combined effect of salinity and metals, particularly for Cu, our results from the present study contrast the additive to synergistic negative interactions observed previously using the earthworms *Eisenia fetida* and *Aporrectodea caliginosa* as test species (Owojori and Reinecke, 2010; Owojori et al., 2009a). These observations were probably related to the osmotic and ion regulation capacity of earthworms to deal with salinity and contaminants as individual stressors. Thus, salinity and metals as individual stressors seemed to be very deleterious for *E. fetida* and *A. caliginosa* that are commonly found in compost and/or agricultural soils. When combined, salinity increased bioavailability of the tested metals (by reducing the partitioning of metals/increasing values of labile metals), but it may be that the competition between Na^+ and free metal ions at the organisms´ point of entry (because Na^+ occupies for instance the biotic ligand with metals (Calamari and Alabaster, 1980; Santore et al., 2001)) resulted in higher toxicity. The Na^+ ions seem to occupy the ligand to such an extent that it causes a toxic effect by its own, as is observed when salinity was tested as the only stressor in both earthworms' species. Lock and Janssen (Lock and Janseen, 2006) reported that for *E. albidus*, the presence of Na^+ did not significantly affect the toxicity of, for instance, cobalt through competition at the biotic ligand, but the concentration of sodium ion tested was 10 times lower than that used in the present study. Thus, it seems reasonable to assume that the presence of NaCl in low concentrations decreased metals toxicity to *E. albidus* by competing and occupying the ligand at the organism's point of entry. Because the presence of 15‰ NaCl is not deleterious for survival or reproduction (on the contrary, it stimulates reproduction (Patricio Silva et al., 2013)), combination with metals results in some form of an additive to antagonistic effect. Negative correlations between salinity and contaminants (toxicity increasing with decreasing salinity) was also observed with high frequency for aquatic (mainly estuarine or marine) annelids and other organisms, which is also related to higher bioavailability of free metal ions at lower salinities (reviewed by Hall and Anderson, 1995).

The negative effect of NaCl on the uptake of Cu and Cd was later confirmed with 2 more tests, in which adult potworms were exposed for 3 wk to the EC50 of each chemical (12.4 mg Cd kg^{-1} dry soil and 23.2 mg Cu kg^{-1} dry soil) in LUFA 2.2 soil containing different NaCl concentrations (0‰,10‰,20‰,30‰,40‰,60‰ and 80‰ NaCl). In these tests, we observed that potworms exposed to increased concentrations of NaCl had significantly lower tissue concentrations of these metals (Supplemental Data, Figure S2).

Are the international standardized tests (ISO, OECD) lacking a more realistic approach for intertidal species like *Enchytraeus albidus*?

The observed significant differences between effects of contaminants in the presence and absence of NaCl, and the consistent relationship between tissue concentration of chemicals and reproductive output, provide a new insight into the effect of salinity on contaminants in soil invertebrates, particularly in euryhaline species. Furthermore, and considering specifically the test species *E. albidus,* which is widely used for the risk assessment of contaminants, the presence of low levels of salinity (15 to 20‰ NaCl) clearly improves their reproduction as observed in our control data (present study) and on previous studies (Patricio Silva et al., 2013). These observations may raise the question: Is the standardized enchytraeid reproduction test incomplete because it lacks an optimal saline content in soil for this test species? Clearly, our results revealed 1.5 to 8-fold changes in the effect of contaminants in the presence of low salinities. However, in terms of risk assessment, the results presented remain within the applied uncertainty factor. It may therefore be argued that the effects of salinity on toxicity should be acknowledged, but revisions of the currently agreed enchytraeid reproduction test are not deemed necessary.

CONCLUSIONS

Our results from the present study demonstrate that salinity has an influence on results of standardized toxicity tests using *E. albidus*, and that the effects seemed to be correlated with the tissue concentrations of contaminants in potworms. The lower tissue concentrations of metals found in potworms exposed to saline soil confirms what is known from previous works on estuarine, marine and intertidal species, where ion exchange (and balance) is the primary factor determining effects of metals. For 4-nonylphenol, the effect of salinity is less clear; however the reproduction capacity and tissue concentration of this chemical are negatively related.

SUPPORTING INFORMATION

Figure S1. Effect of tissue concentration of Cd, Cu and 4-NP in *E. albidus* reproduction, when exposed in LUFA 2.2, in the absence or presence of 15‰ NaCl.

Figure S2: Tissue concentration of Cd and Cu in *E. albidus*, after exposure to EC50 concentrations and increasing range of salinities, for 3 weeks at 20ºC.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S. and MH. carried out data analysis. All authors wrote the paper.

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SUPPORTING INFORMATION

This supporting information contains the following:

- Effect of tissue concentration of Cd, Cu and 4-NP in *E. albidus* reproduction when exposed in LUFA 2.2, in the absence or presence of 15‰ NaCl (Figure S1).
- Tissue concentration of Cd and Cu in *E. albidus,* after exposure to EC50 concentrations (12.4 mg Cd kg^{-1} dry soil and 23.2 mg Cu kg^{-1} dry soil) and increasing range of salinities (0-10-20-30-40-60‰ NaCl), for 3 weeks at 20ºC (Figure S2).

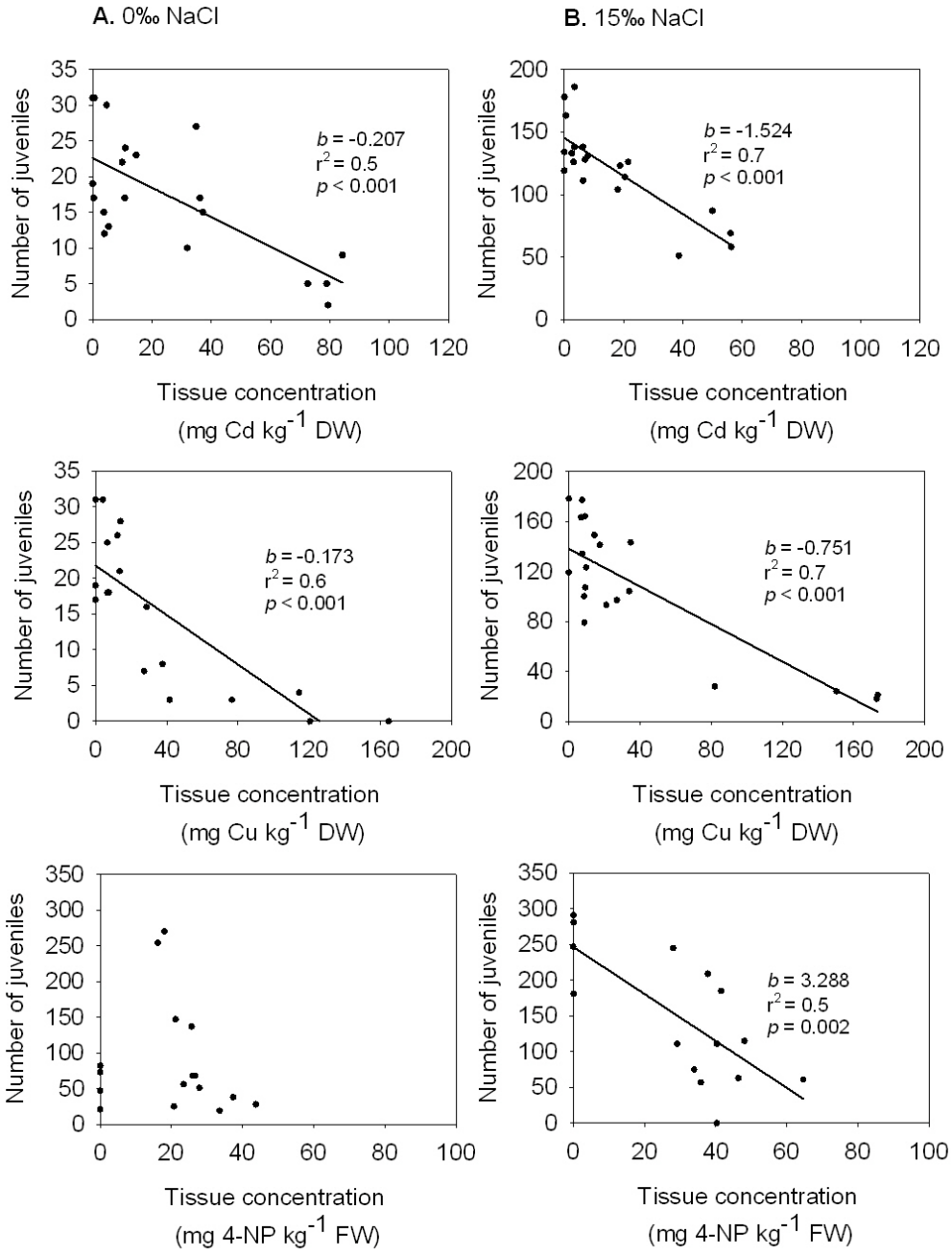

Figure S1: Effect of tissue concentration of Cd, Cu and 4-NP in *E. albidus* reproduction (number of juveniles) when exposed in LUFA 2.2, in the absence (A) or presence (B) of 15‰ NaCl. Solid line represents linear regression (due to hormesis, no regression was performed for 4-NP in non-saline soil). Slopes of regression lines for both Cd and Cu were significantly different in 0 and 15‰ (ANCOVA, $p < 0.001$).

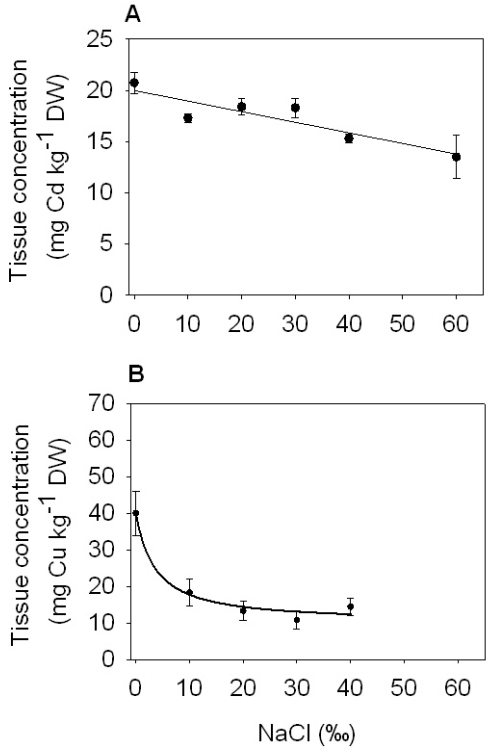

Figure S2: Tissue concentration of cadmium (A) and copper (B) in *E. albidus,* after exposure to EC50 concentrations (12.4 mg Cd kg^{-1} dry soil and 23.2 mg Cu kg^{-1} dry soil) and increasing range of salinities (0-10-20-30-40-60‰ NaCl), for 3 weeks at 20ºC. Results are shown as mean ± standard error (*N*= 3-8). Cd data is described by a linear regression and Cu data is described with an exponentially decreasing function (r^2 = 0.85).

Photo by Karina Fisker

Chapter V

Importance of Freeze−Thaw Events in Low Temperature Ecotoxicology of Cold Tolerant Enchytraeids

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SUMMARY

Due to global warming it is predicted that freeze−thaw cycles will increase in Arctic and cold temperate regions. The effects of this variation becomes of particular ecological importance to freeze-tolerant species when it is combined with chemical pollutants. We compared the effect of control temperature (2°C), daily freeze−thaw cycles (2 to −4°C) and constant freezing (−4 °C) temperatures on the cold-tolerance of oligochaete worms (*Enchytraeus albidus*) and tested how survival was influenced by pre-exposure to 4-nonylphenol (4-NP), a common nonionic detergent found in sewage sludge amended soils. Results showed that combined effect of 4-NP and daily freeze−thaw cycles can cause higher mortality to worms as compared with sustained freezing or control temperature. Exposure to 4-NP caused a substantial depletion of glycogen reserves, which is catabolized during freezing to produce cryoprotective concentrations of free glucose. Further, exposure to freeze−thaw cycles resulted in higher concentrations of 4-NP in worm tissues as compared to constant freezing or control temperature (2°C). Thus, worms exposed to combined effect of freeze−thaw cycles and 4-NP suffer higher consequences, with the toxic effect of the chemical potentiating the deleterious effects of freezing and thawing.

Keywords: Freeze-thaw cycles, nonylphenol, enchytraeids, cryoprotectants, tissue concentration.

INTRODUCTION

In Arctic and subarctic regions, the effects of climate change are taking place rapidly. It is predicted that a slight increase in the average temperature can lead to the reduction (or absence) of an insulating snow cover during frost periods, and thus to an increase in frequency of freeze-thaw events (IPCC, 2001). Changes to soil freezing dynamics may impose a great challenge to soil invertebrates, particularly the ones that resort to freeze-tolerance as main survival strategy to endure harsh winters. Freeze-tolerant organisms initiate and control extracellular ice formation and survive for weeks or months typically with up to 65% of total body water as ice (Zachariassen, 1985). This process is assisted by the accumulation of cryoprotectants such as glycerol, glucose and trehalose (Storey, 1997; Zachariassen, 1985), which lowers the melting point and the ice fraction at a given temperature. As a consequence, the concentration of potentially toxic salts in the unfrozen body fluids tends to decrease (Ramløv, 2000; Zachariassen, 1985) and the membranes and proteins are stabilized at low temperatures (Anchordoguy et al., 1987; Crowe et al., 1987). Rapid switching between freezing and thawing events may require much higher metabolic rates and, depending on the extent of freezing, impair the organisms' survival. Previous studies on freeze-tolerant invertebrates reported that continuous exposure to freeze-thaw events caused a cumulative damage in cell structures (Bale et al., 2001; Brown et al., 2004; Churchill and Storey, 1989; Marshall and Sinclair, 2011; Sinclair and Chown, 2005), decrease in feeding activity with loss of body mass

(Sinclair and Chown, 2005), alteration of cryoprotectant levels (Marshall and Sinclair, 2011) and increase in energy expenditure (Churchill and Storey, 1989; Sinclair and Chown, 2005). Some species even lost their ability to survive freezing, by switching to a strategy of freeze avoidance (Bale et al., 2001; Brown et al., 2004).

The climatic changes that lie ahead are superimposed by hazardous chemicals present in nature. Several studies have shown that the combination of toxicants and natural stressors can significantly modify the response mechanisms of organisms to toxicants (Holmstrup et al., 2010). Such combinations when having synergistic effects on populations, i.e. with higher risk than the sum of the toxic and natural stressors (Bindesbøl et al., 2005; Holmstrup et al., 2010), pose high concern. Among the chemicals released in the environment, nonylphenol (NP) (a breakdown product of nonionic nonylphenol ethoxylate surfactants) is of particular interest for terrestrial organisms due to application of sewage sludge for soil amendment and waste disposal. Concentration of NP in bulk soil are relatively low (1.4–1.6 mg kg^{-1}) (Soares et al., 2008), but lumps of sewage sludge which are often colonized by soil fauna due to the nutritional value of sludge reach much higher amounts (3 mg to 10 g kg^{-1} dry sludge) (Hojer et al., 2001; TemaNord, 1996). Additionally, the high hydrophobicity properties of NP ($logK_{ow}$ of 4.5) makes it liable to accumulate in cell membranes (Ekelund et al., 1990; Jacobsen et al., 2004; Shan et al., 2010) and to decrease membrane fluidity which is likely to reduce cold tolerance of the organism (Holmstrup et al., 2014). The ability to adjust and reach appropriate membrane fluidity is crucial for ectothermic animals living in cold environments since low temperature leads to increase and detrimental membrane rigidity (Hazel and Eugene Williams, 1990). Furthermore, exposure to NP also impacts negatively the synthesis of sugar and polyols in soil invertebrates (Hojer et al., 2001), affecting negatively the osmoregulating capacity that also reduce tolerance to drought and freezing stress (Zachariassen, 1985).

Combined effect of NP and natural stressors, such as temperature, on freeze-tolerant invertebrates in soil ecosystems remains poorly documented (Holmstrup et al., 2010). In particular, there is an absence of knowledge regarding the combined effect of contaminants and freeze-thaw cycles that, in our point of view, may impose a much higher challenge to freeze-tolerant soil organisms (Bale et al., 2001; Brown et al., 2004; Churchill and Storey, 1989; Marshall and Sinclair, 2011; Sinclair and Chown, 2005).

In the present study we used the model species *Enchytraeus albidus* (Oligochaeta, Annelida). This species is widely distributed in temperate and sub-Arctic regions; inhabiting the upper layer of many terrestrial and supralittoral soils where they play an important role in organic matter breakdown (Didden, 1993). This worm has been used as a standard organism for risk assessment of chemicals (ISO, 2004; OECD, 2004) and as a model for freeze-tolerance studies (Bauer, 2002; Bauer et al., 2001; Block and Bauer, 2000; Patricio Silva et al., 2013a; Patricio Silva et al., 2013b; Slotsbo et al.,

2008).

The purpose of this investigation was to assess the effects of constant freezing and freeze-thaw cycles on *E. albidus*, when combined with exposure to environmentally relevant concentrations of 4-nonylphenol (4-NP) in a full factorial design. We attempted to answer the following questions: i) are there negative costs of freezing and daily freeze-thaw cycles per se? ii) are the effects of nonylphenol more deleterious when combined with daily freeze-thaw cycles as compared to continuous freezing or control treatments? and iii) is lower freeze-tolerance capacity caused by nonylphenol associated with lower levels of cryoprotectants?

MATERIAL AND METHODS

Test species

Enchytraeus albidus (Henle, 1837) were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany) in 2011, and cultured since then in agricultural (loamy) soil at 5.0 ± 1°C. The worms were fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). Prior to experiments, the organisms were cold acclimated at 2ºC for two weeks.

Test soil and spiking

Experiments were performed using the natural standard soil, LUFA 2.2 (Speyer, Germany). Main characteristics of the soil are as follows: organic carbon 1.7%, pH ($CaCl_2$) 5.5, grain size distribution 7.3% clay, 13.8% Silt, 78.9% sand and maximum water holding capacity (WHC) of ca. 44% of fresh weight.

The soil was spiked with 4-nonylphenol (4-NP) (Aldrich, Cas. No. 29.005.8, 100% pure), dissolved in acetone (J.T. Barker, Hayward, CA, HPLC quality) to obtain the concentration range of: 0, 50, 100, 150, and 250 mg kg^{-1} dry soil. The choice of concentrations was based on preliminary toxicity tests, in which we exposed worms to increasing concentrations of 4-NP (0-50-100-150-250-500 mg kg^{-1} dry soil) for 7 days at +2ºC or at -5ºC. Only the concentrations revealing worms' survival higher than 80 and 50% at +2ºC and -5ºC, respectively, were selected (data not shown).

The spiked soil was thoroughly mixed and left in a fume hood for 24 h to evaporate acetone. Afterwards, soil humidity was restored with 15‰ NaCl (Merk, Cas. No. 7647.14.5, 99.5% pure) solution till about 50% of WHC (205 ml salty water was added per 1 kg of dry soil). This salinity level was used since it represents normal conditions in the typical habitat of *E. albidus*, and to ensure freeze-tolerance as previously reported (Patrício Silva et al., 2013b).

Experimental procedures and setup

The following experimental procedure was adapted from a method previously described (Slotsbo et al., 2008). Each replicate consisted of five worms in a test vial (3.5 cm high, 2.5 cm diameter) containing 10 g of test soil and 20 mg of oatmeal. The vials were covered with a perforated lid to allow ventilation. A total of twenty-one replicates for each treatment combination was prepared for survival assessment and biochemical analysis (see later sections for details). Worms were exposed to 4-NP and different thermal regimes as shown in Fig. 1.

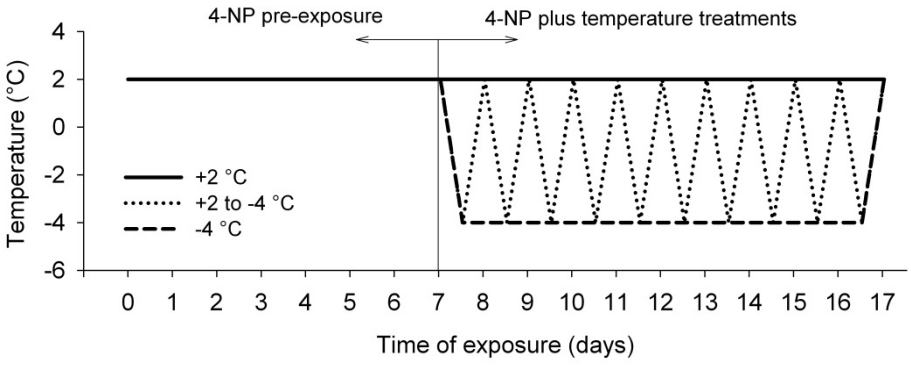

Figure 1. Schematic representation of the experimental setup consisting of pre-exposing *Enchytraeus albidus* to 4-NP in LUFA 2.2 soil for 7 days, followed by 10 days under 3 different temperatures regimes: i) continuous +2°C regime; ii) daily freeze-thaw cycles, shifting between +2°C to −4°C, with a decrease/increase rate of 0.5°C h^{-1}; iii) continuous −4°C regime.

In short, the worms were kept at 2°C for 7 days in soil spiked with different concentrations of 4-NP. Subsequently, the test was split into 3 temperature regimes: i) daily freeze-thaw cycles – with temperature from +2°C to −4°C, with a decrease/increase rate of 0.5°C h^{-1}; ii) continuous temperature regime of +2°C and iii) continuous temperature regime of −4°C. The vials in which worms were exposed to freeze-thaw cycles were briefly sprayed with freeze-spray used for treatment of sport injuries (Select ice spray, SportMaster, Skanderborg, Denmark) every day when the temperature was lowered and reached -2.5°C. This procedure is important to induce nucleation of the soil and ensure inoculative freezing of enchytraeids once the temperature becomes lower than the body fluid melting point (Slotsbo et al, 2008) After 10 days, at either of the temperature regimes and further 12 h thawing at 2ºC, survival was assessed for all treatments. Only the worms that reacted normally to tactile stimuli, with no or minor injuries, were scored as surviving. Damages caused by freezing were recorded using a semi-quantitative scale: i) “no injuries”, representing healthy worms with intact integument; ii) “minor injuries”, representing worms with one injury, characterized by a small physical disruption of the integument; iii) “major injuries”, representing worms with two or more injuries but still able to move and iv) “dead” were completely immobilized by the high number of injuries.

Quantification of glycogen and glucose

Glycogen and glucose contents were determined before (day 7) and after (day 17) exposure to freezing. The optimal amount of biological tissue to perform the analysis was 2-4 mg of dry weight (DW) (corresponding approximately to 3 worms). Thus, from each treatment, 15 worms (corresponding to three replicates of five worms of the experimental setup) were rinsed with deionized water, cleaned of excess soil, pooled in groups of three individuals in Eppendorf tubes and snap-frozen at -80°C. Five replicates of 3 worms were then generated for each treatment.

In worms exposed to constant -4°C and to daily freeze-thaw cycles, samples for glycogen were collected when the temperature reached +2°C (day 17), since this would allow time for conversion of most free glucose to glycogen when the worms were thawed. Samples for glucose were collected when the temperature was at -4°C and the worms were frozen (i.e. 12h before the end of the exposure, at day 17).

Glucose and glycogen analysis was carried out as previously described (Overgaard et al., 2007) using spectrophotometrically based enzymatic test kits (Gluc-DH FS from DiaSys Diagnostic Systems GmbH, Holzhein, Germany).

Measurement of 4-NP in worms by gas chromatography – mass spectrometry (GC-MS)

Internal concentration of 4-NP in worms was measured for all 4-NP concentrations at day 7, but only for three target 4-NP concentrations at day 17: 0, 50 and 150 mg kg^{-1} dry soil, for all tested temperature treatments.

This analysis required a minimum fresh weight of 40-50 mg (corresponding to 15-20 worms). Thus, surviving worms collected from at least 15 replicates from the original experimental setup were rinsed with deionized water, cleaned of excess soil, pooled in groups of 15 (originating from 15 replicates of 5 worms) and snap-frozen at -80°C until chemical analysis.

Samples were homogenized in Eppendorf tubes with 1.5 ml 70% ethanol and a steal ball using a Tissuelyser II (Qiagen Gmbh, Hilden, Germany). The homogenate was transferred to a glass tube, where 10 µl of 6.4% NaOH, 250 µl of 0.2 M K_2CO_3 and 20 µl Ac_2O was added. After vortexing for 10 min, the homogenate was left for 3 h in darkness to allow glycation. This procedure was followed by solid phase extraction, where 4-NP was isolated and eluted on LiChrolut columns (bottom EN 100 mg, top RP-18 200 mg) (Merck KGaA, Darmstadt, Germany). After washing the columns with 2 ml Elga water followed by 2 ml of 96% and 70% ethanol, respectively, the homogenate was filtered and added to the columns. The retained 4-NP was then eluted with 1 ml of acetonitrile (super gradient for HPLC, Prolabo, Graumanngasse, Vienna; CAS: 75-05-8). The remaining water in 4-NP extract was removed by adding a tip of a spatula (2-3 mg) of sodium sulfate (Na_2SO_4). Extracts were centrifuged for 3 min at 3000 g, and the supernatant was transferred to GC-MS sample vials.

A Shimadzu GCMS-QP2010 with autosampler was used to perform the analysis. The GC was equipped with a FactorFour™ capillary column VF-5ms (length 30 m, inner diameter 0.25 mm, film thickness of 0.25 µm; Varian, Netherlands). The injection volume was 2.0 µl and the autosampler was operating in split mode with a split ratio of 3. Helium was used as carrier gas with a total flow of 5.3 ml min^{-1} and a column flow at 0.57 ml min^{-1}. The injection temperature was maintained at 260°C and the oven was programed as follows: initial temperature of 120°C was held for 2 min, then increased to 150°C with a rate of 20°C min^{-1}, then increased to 170°C with a rate of 6.0°C min^{-1} and held for 1 min, finally increased to 190°C with a rate of 6°C min^{-1} and held for 1 min. The mass spectrometer was operated in electron ionization mode at 70 eV. The ion source temperature was 300°C and the interface temperature 280°C.

Identification of 4-NP was based on external standards that had been treated like worm samples. The area of identified peaks was calculated using GCMS Solution software and the concentration was calculated based on specific standard curves.

Statistical analysis

Comparisons between variables were made using analysis of variance (ANOVA), with Dunnetts' and Holm-Sidak post hoc tests to assess significant differences after one-way and two-way ANOVA respectively ($p < 0.05$) (Sigmaplot for Windows Version 11.0, Systat software Inc., San Jose, CA). Survival data were arcsin-square root transformed prior to analysis. and when non-normality was observed, a chi-square test was applied. A one-way anova was performed specifically on survival data at 150 mg/kg since this sub-dataset passed on both normality and equal variance tests. Glucose and glycogen data were log transformed to correct for non-normality or heteroscedasticity. A few outliers were excluded, being calculated as the mean ± 2*standard deviation.

Survival data were described with a log-logistic concentration response model using the statistical software R version 2.12.9 (http://www.r-project.org) with the drc package (Ritz and Streibig 2005) assuming binomial data,

$$y = 1/(1 + \left(\frac{x}{LCn}\right)^{b})$$

where y is the fraction of survivors, x is the nominal 4-NP concentration in mg kg^{-1} dry soil, LCn is the concentration where the response is reduced by n%, and b is the slope of the curve in LCn.

Our experimental design did not provide data for full analysis of the dose-response surface defined by effects of thermal regime and 4-NP, which has been used for indications of synergistic/antagonistic interactions between natural stressors and contaminants (Bindesbøl et al., 2005; Hojer et al., 2001). However, significant interaction in 2-way ANOVA may indicate significant synergistic/antagonistic interactions by inspecting the trends in mean values (Laskowski et al.,

2010).

RESULTS

Survival

The effect of 4-NP on worms was different depending on the temperature regime. Significant differences in survival were revealed at all exposures, with worms exposed to freeze-thaw cycles showing a decrease in survival of 29% when compared to worms exposed to +2°C and a decrease of 25% compared to the ones exposed to sustained freezing (Fig 2). Significant differences between the three temperature treatments were observed in worms exposed to 150 mg kg^{-1} dry soil (one-way ANOVA, $F_{2,32}$=10.67, $p < 0.001$). The estimated lethal concentration (LCx) causing 10, 20 and 30% reduction of survival was significantly lower in worms exposed to freeze-thaw cycles than for constant freezing (Fig. 3).

The percentage of worms revealing severe cryoinjuires (that caused partially or total immobilization/death) increased with the increase of 4-NP concentration in the soil, being worst in worms exposed to freeze-thaw cycles (Fig. 4).

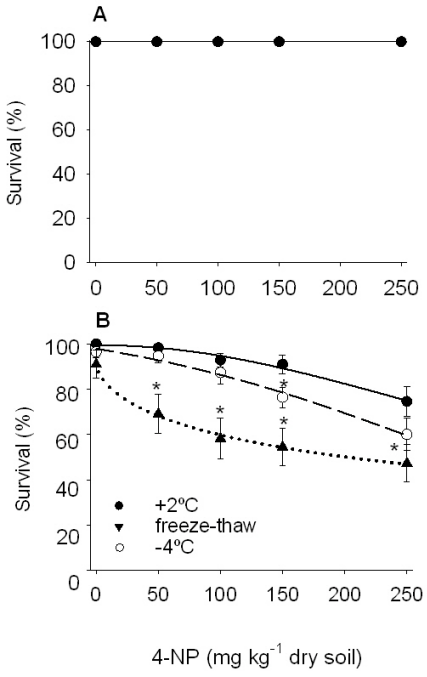

Figure 2. Survival of *Enchytraeus albidus* exposed to 4-NP before (day 7) (A) and after (day 17) (B) exposure to three temperature regimes (constant +2ºC, constant -4ºC and daily freeze-thaw cycles). (*) Statistical significant differences compared to control temperature (2ºC), per 4-NP concentrations, Dunnett, $p < 0.05$. Results are shown as mean ± standard error (N = 15) and described with a log-logistic concentration response model (2ºC = solid line, -4ºC = broken line, daily freeze-thaw cycles = dotted line).

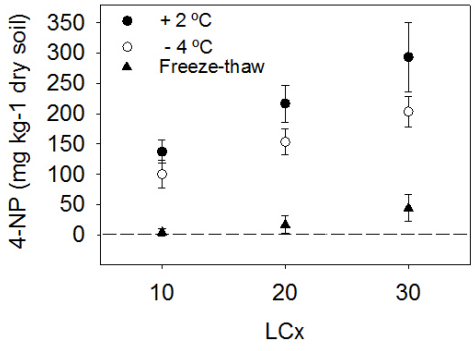

Figure 3. Lethal concentration (LCx) for 10, 20 and 30% effect in worms exposed to 4-NP and three temperature regimes. Bars represent standard error.

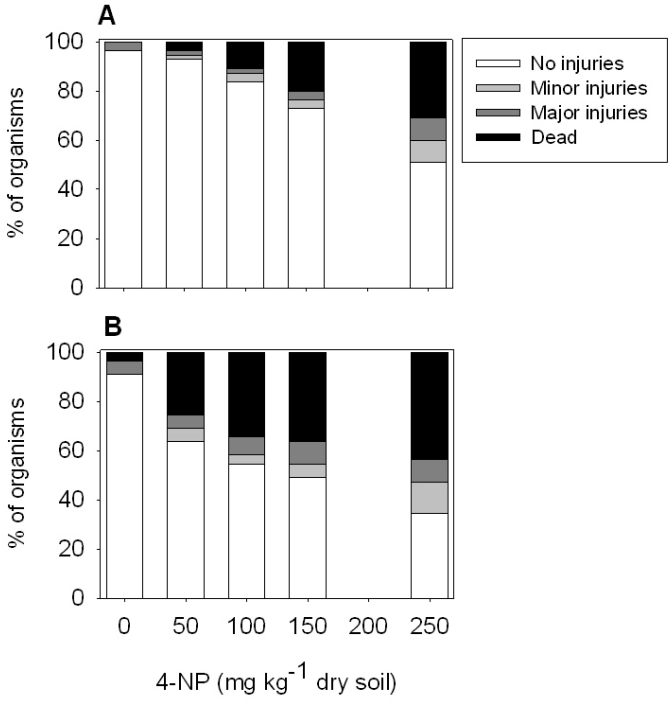

Figure 4. Condition of *Enchytraeus albidus* in terms of cryoinjuries ("no injuries", representing healthy worms with intact integument; "minor injuries", representing worms with one injury, characterized by a small physical disruption of the integument; "major injuries", representing worms with two or more injuries but still able to move and "dead" were completely immobilized by the high number of injuries) after 7 days pre-exposure to 4-NP followed by 10 days at constant −4 °C (A) or daily freeze−thaw cycles (B).

Glycogen depletion and Glucose accumulation

Exposure to 4-NP had a significant overall negative effect on glycogen reserves (Fig. 5) in all temperature treatments (2-way ANOVA, $F_{4,73}$=12.1, p<0.001). The glycogen content of worms exposed to 250 mg kg^{-1} dry soil was reduced to less than 50% of control levels (Fig. 5). Freezing *per se* (i.e. no exposure to 4-NP) also significantly reduced glycogen content (2-way ANOVA, $F_{2,73}$=37,7, p<0.001). Within each 4-NP concentration, worms exposed to -4ºC had the lowest glycogen reserves, equivalent to 30-60% of the reserves observed in worms exposed to 2ºC. Similar tendency was observed in worms exposed to daily freeze-thaw cycles, with 50 to 75% of the reserves observed in worms exposed to 2ºC. The negative effect of 4-NP was less prominent in constantly frozen or freeze-thaw exposed worms, reflecting a significant interaction between 4-NP and the temperature regime on glycogen (2-way ANOVA, $F_{8,73}$=3.02, p<0.05).

Freezing caused an increase of body glucose concentration (Fig. 6). Worms exposed to continuous -4ºC for 10 days had the highest levels of glucose (2 to 20-fold higher than unfrozen worms, depending on the 4-NP concentration), followed by worms exposed to 10 daily freeze-thaw cycles. 4-NP significantly affected the glucose levels (2-way ANOVA, $F_{4,74}$= 9.07, p<0.001), particularly the ones exposed to constant freezing and daily freeze-thaw cycles but with no clear dose response. There was a significant interaction between 4-NP and temperature regime, but not with any clear trend (2-way ANOVA, $F_{8,74}$=6.81, p<0.001). Worms kept at 2ºC maintained both glycogen and glucose at relatively similar levels.

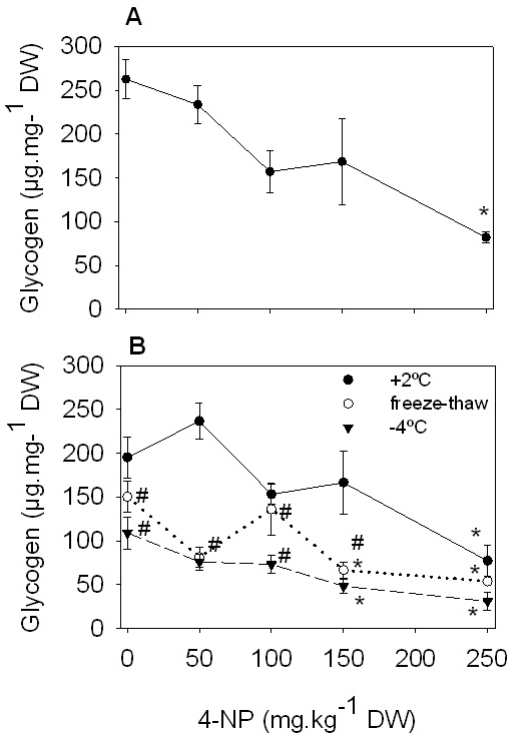

Figure 5. Glycogen contents of *Enchytraeus albidus* exposed to 4-NP before (day 7) (A) and after (day 17) (B) exposure to three temperature regimes (constant +2 °C, constant −4 °C and daily freeze−thaw cycles). (*) Statistically significant differences compared to control soil (0 mg 4-NP kg−1soil DW), Dunnett, $p < 0.05$; (#) Statistically significant differences compared to control temperature (2 °C), Dunnett, $p < 0.05$. Results are shown as mean ± standard error (N = 5).

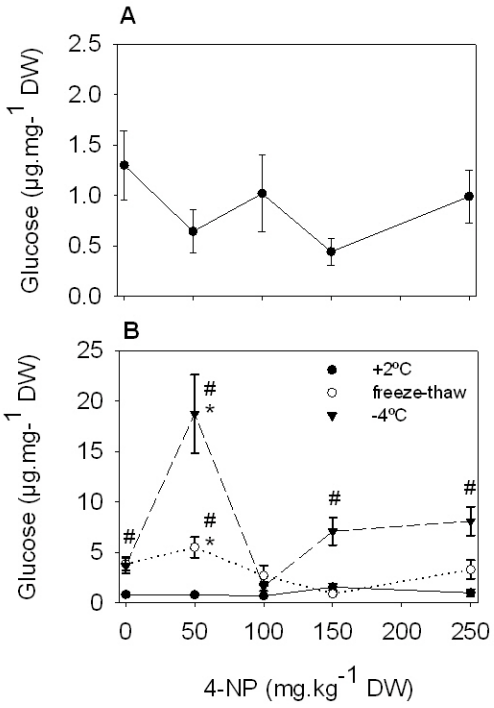

Figure 6. Glucose contents of *Enchytraeus albidus* exposed to 4-NP before (day 7) (A) and after (day 17) (B) exposure to three temperature regimes (constant +2 °C, constant −4 °C and daily freeze−thaw cycles). (*) Statistically significant differences compared to control soil (0 mg 4-NP kg−1soil DW), Dunnett, $p < 0.05$; (#) Statistically significant differences compared to control temperature (2 °C), Dunnett, $p < 0.05$. Results are shown as mean ± standard error (N = 5).

Internal concentration of 4-NP in worms

At day 7, the internal concentration of 4-NP in worm tissues increased with soil concentrations up to 150 mg kg^{-1} dry soil (Fig. 7). At 250 mg 4-NP kg^{-1} dry soil the concentration in tissues was lower than the nominal concentration used to spike the soil. At the end of the exposure (day 17, figure 7B), 4-NP continued to increase linearly with soil concentration, but was significantly higher in worms that had been under daily freeze-thaw cycles than in worms exposed at continuous temperatures of -4ºC or +2ºC (One-way ANOVA, *p* <0.001), resulting in a significant interaction between 4-NP concentrations in the soil and temperature regime (2-way ANOVA; $F_{4, 36}$=4.12; p<0.05).

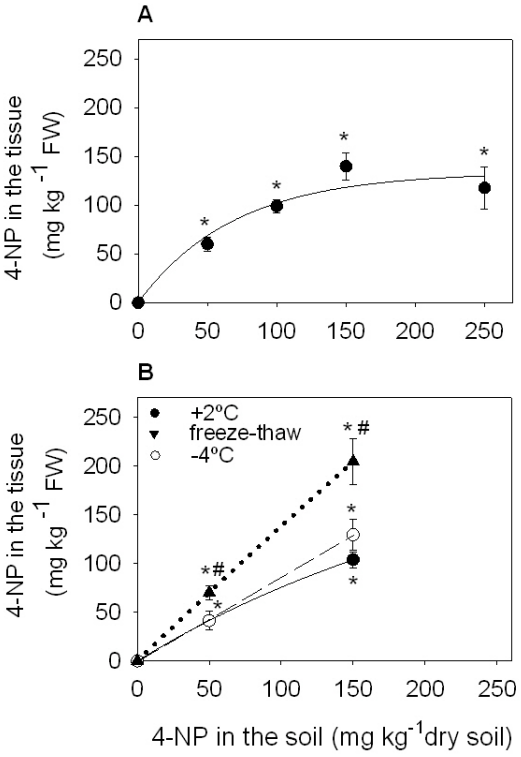

Figure 7. Concentration of 4-nonylphenol in tissues of *Enchytraeus albidus* exposed for 7 days at +2 °C (A) and at the end of the exposure to different temperatures regimes (day 17) (B). Data were described with an exponential function (2 °C = solid line, −4 °C = broken line, daily freeze−thaw cycles = dotted line). The r2 of the fit was 0.89 for +2 and −4 °C and 0.90 for freeze−thaw cycles. (*) Statistically significant differences compared to control soil (0 mg 4-NP kg−1 soil dry soil), Dunnett, p < 0.05; (#) Statistically significant differences compared to control temperature (2 °C), Dunnett, p < 0.05. Results are shown as mean ± standard error (N = 5).

DISCUSSION

Effect of temperature regimes

In the present study, we observed none or very little lethality from freezing or daily freeze-thawing per se, since survival of worms in control soil (soil not spiked with 4-NP) did not differ significantly between temperature treatments. There was no difference in the glycogen or glucose contents of worms exposed to sustained freezing and freeze-thaw cycles. These results are, in part, not surprising, since *Enchytraeus albidus* possesses a considerable tolerance to freezing temperatures over extended periods of time, by controlling internal ice formation and accumulating glucose (by a

rapid catabolism of glycogen) as the main cryoprotectant (Patricio Silva et al., 2013b; Slotsbo et al., 2008). It should be noted that in this study we used a saline soil since this ensures tolerance to freezing in *E. albidus* (Patricio Silva et al., 2013b). Thus, using non-saline soil in the experiments could have resulted in a poor survival when exposed to constant freezing or daily freeze-thaw cycles. Other field studies have shown that freezing and thawing events can have drastic and negative consequences for enchytraeid populations if temperatures become very low or are repeated over longer time-scales (Sulkava and Huhta, 2003).

High frequency (hours to days) in freeze-thaw events seems to interfere with feeding processes in other freeze-tolerant invertebrates. For example, the sub-Antartic caterpillar, *Pringleophaga marioni*, survived after a short exposure of 5 days to repeated freezing events (between 2ºC to -5ºC), but it led to decreased mass, largely accounted for by a decreased gut mass caused by cessation of feeding (Sinclair and Chown, 2005). Despite not having measured the mass of *E. albidus* during exposure to daily freeze-thaw cycles, we could observe that these worms had much less soil particles in their gut compared to worms exposed to +2ºC (unpublished observations). Thus, switching between frozen and unfrozen state is likely also causing a decrease of feeding in *E. albidus*.

Freeze-thaw events with large amplitude (+3 to -16ºC) had cumulative detrimental effects on organism physiology (increase in cryoinjuries) and cellular energy in the freeze-tolerant insects *Eurosta solidaginis* and *Pyrrharctia isabella*, (Churchill and Storey, 1989; Marshall and Sinclair, 2011). Some species even seemed to change cold-tolerance strategy to cope with such weather events. The sub-Antarctic beetle *Hydromedion sparsutum* lowered the supercooling point, increasing its ability of freeze-avoidance (Bale et al., 2001). This was also observed for the hoverfly *Syrphus ribesii* (Brown et al., 2004). It seems unlikely that this change in cold tolerance strategy occurred in *E. albidus* since they remained freeze-tolerant throughout the experiment, and would have little capacity to avoid inoculative freezing.

Combined effect of 4-nonylphenol and temperature regimes

As we hypothesized, the potentially negative effects of daily freeze-thaw cycles or constant freezing on *E. albidus* were indeed augmented in the presence of 4-NP, pointing towards possible synergism. These results are in line with a recent study by Holmstrup et al. (2014) who also observed that *E. albidus* exposed to 4-NP had reduced survival in short-term freeze experiments. The present results also suggest that the effects of daily freeze-thaw cycles were even more deleterious to worms pre-exposed to 4-NP, as compared with sustained freezing or control temperature. An identical experiment was later carried out confirming similar tendency in terms of negative effects of 4-NP on cold tolerance. However, the replicated experiment did not indicate significant differences between effects of daily freeze-thaw and constant freezing even though a

similar trend was seen (Supporting Information Fig. S1 and S2). The worms used in the repeated experiment (using animals from a different batch) seemed to be less sensitive to 4-NP, which could perhaps explain why interactions between thermal regime and 4-NP was less obvious than in the first experiment.

The higher mortality observed in worms exposed to 4-NP and to freezing seemed partially related to levels of cryoprotective glucose in the worms. Pre-exposure to an increased concentration of 4-NP negatively affected glycogen reserves, with a decrease of more than 50% in worms exposed to the highest concentration of 4-NP (250 mg kg^{-1} dry soil). This reduction in glycogen levels did not result in increased accumulation of glucose in 4-NP exposed worms, suggesting that glucose may be mobilized and used as energy resource to detoxify 4-NP, or deal with possible oxidative stress resulting from 4-NP toxicity (Patricio Silva et al., 2013a). Nevertheless, worms exposed to both freeze-thaw events and sustained freezing did accumulate glucose although the concentrations were relatively low. Even though the colligative effects of glucose in such low concentrations are negligible and hardly of importance for reduction of ice fraction during exposure to -4ºC for 10 days, it may be effective in stabilization of membrane fluidity and labile proteins (Anchordoguy et al., 1987; Crowe et al., 1987) and thus preventing damage from low temperatures or dehydration. Since worms exposed to constant freezing had higher glucose concentrations than worms exposed to freeze-thaw cycles we may speculate that this contributed to a better overall survival in the former group. It should, however, be stressed that these suggested physiological mechanisms are based on correlations and not direct evidence. More studies are therefore needed to explore these ideas further.

Another explanation for the different mortality observed in worms exposed to combined effect of 4-NP and temperature regimes may be related to the internal concentration of 4-NP in the tissues. Within each 4-NP concentration, worms exposed to +2ºC had the lowest internal concentration in the tissues, followed by the worms exposed to sustained freezing and daily freeze-thaw cycles. The observed lower bioaccumulation is probably related with the degradation of 4-NP in the soil. In the first 7 days of exposure at +2ºC the internal concentration of 4-NP in worm tissues was similar to the nominal soil concentration, followed by a slight decrease after 17 days suggesting that some degradation of 4-NP may have occurred. Biodegradation of 4-NP in the soil is dependent on the quantity of organic matter, aerobic conditions and microbial activity (Hesselsøe et al., 2001; Soares et al., 2008), but it is the strong sorption of this surfactant that limits the biodegradation rates (Hollrigl-Rosta et al., 2003). The half-life of 4-NP can be between 4 days to approximately one month, and shorter at higher temperature (Chang et al., 2005; Hesselsøe et al., 2001). At low temperature (e.g. -4ºC as used here) the microbial activity is limited and it seems reasonable to expect that the half-life is considerably longer under these conditions, which can explain the

relatively higher bioaccumulation in the worms compared to the ones exposed to +2ºC. Interestingly, worms exposed to daily freeze-thaw cycles had twice as high concentrations of 4-NP in the tissues (Fig. 5B) as compared to worms exposed to constant -4ºC or +2ºC. It is possible that repeated freezing and thawing of soil influenced the sorption/desorption processes of 4-NP. Freezing of moist soil affects the sorption of 4-NP since it leads to a partial destruction of organic macromolecules or organo-mineral structures through ice crystals (Shchegolikhina et al., 2012; Yu et al., 2010). Thus, bioavailability of 4-NP could be higher in the repeated thawing periods and this could lead to higher concentrations in the tissues of worms and consequently higher mortality.

Worms exposed to daily freeze-thaw cycles also revealed a higher number of cryoinjuries (Fig. 4 and Supporting Information Fig. S3), that is, physical damage observed in the worms' integument during frost exposure as a results of severe cellular dehydration, and consequently rupture of cell membranes (Holmstrup and Zachariassen, 1996). The number of cryionjuries was strongly and positively related with 4-NP concentration in the soil and in the tissues. Membrane-partitioning of 4-NP can reduce fluidity of model membranes in vitro (Holmstrup et al., 2014), thus it seems reasonable to infer that the number of cryoinjuries observed in the worms may be a sign of 4-NP interfering with membrane fluidity and cell integrity.

The information available on combined effects of contaminants in the environment and natural stressors such as drought, heat, and subzero (freezing) temperatures is scarce and almost always based on rather simple laboratory experiments. There is an urgent need to expand and evolve experiments that more realistically mimic the situation in the field. Our study is the first to explore the combined effects of contaminants and freeze−thaw cycles, which may provide a first step guiding the development of such investigations. Since freeze− thaw cycles is probably a very important environmental constraint for organisms living in thermally fluctuating habitats, we suggest that this is an important topic in ecotoxicology, and eventually also in risk assessment of contaminants, deserving further consideration and scientific work.

ASSOCIATED CONTENT

* Supporting Information

Experiment II was conducted in order to confirm the results obtained from the first experiment. Figure S1 shows the average in survival ± SE of *E. albidus*. Figure S2 shows the Lethal Concentration (LC) of 10, 20, and 30% reduction in *E. albidus* exposed to 4-NP and different temperature regimes. Figure S3 shows the percentage of organisms according to the type of damage caused by freezing, after exposure to 4-NP and different temperature regimes. This material is available free of charge via the Internet at http://pubs.acs.org.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S. and MH. carried out data analysis. All authors wrote the paper.

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SUPPORTING INFORMATION

This supporting information contains the following:

- Description of the experiment II.
- Average in survival ± SE of *E. albidus* (Figure S1).
- Lethal Concentration (LC) of 10, 20 and 30% reduction in *E. albidus* exposed to 4-NP and different temperature regimes (Figure S2).
- Physiological state of *E. albidus* in terms of cryoinjuries after exposure to 4-NP and different temperature regimes (Figure S3).

Experiment II

The experiment II was conducted in order to confirm the results obtained from the first experiment. The experimental setup was performed as the first experiment, with the following exceptions:

- ten worms were used per replicate (instead of 5);
- ten replicates were used to assess survival.

Data is shown in the figure S1, S2 and S3.

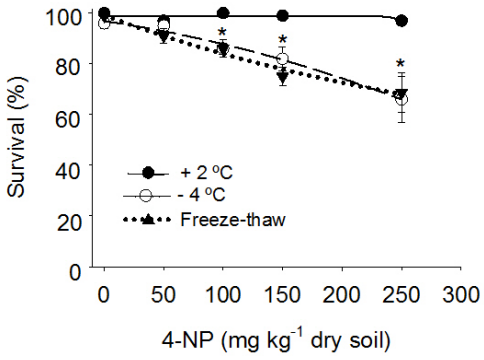
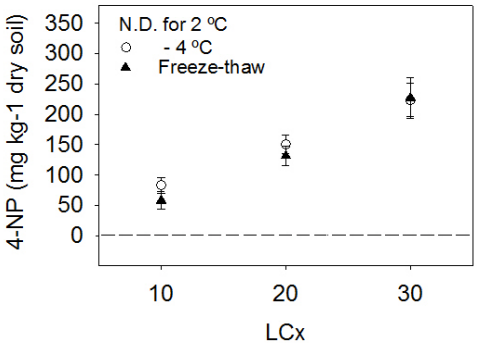


Figure S1: Survival of *Enchytraeus albidus* exposed to 4-nonylphenol and three temperature regimes (constant +2ºC, constant -4ºC and daily freeze-thaw cycles). (*) Statistically significant differences with qui-square test ($p<0.05$). Results are shown as mean ± standard error (*N* = 10) and described with a log-logistic concentration response model (2ºC = solid line, -4ºC = broken line, daily freeze-thaw cycles = dotted line).

Figure S2: Lethal concentration (LC) for 10, 20 and 30% reduction in worms exposed to 4-nonylphenol and two temperature regimes (constant -4ºC and daily freeze-thaw cycles). No mortality occurred for worms exposed to 2ºC. Results are shown as mean ± standard error.



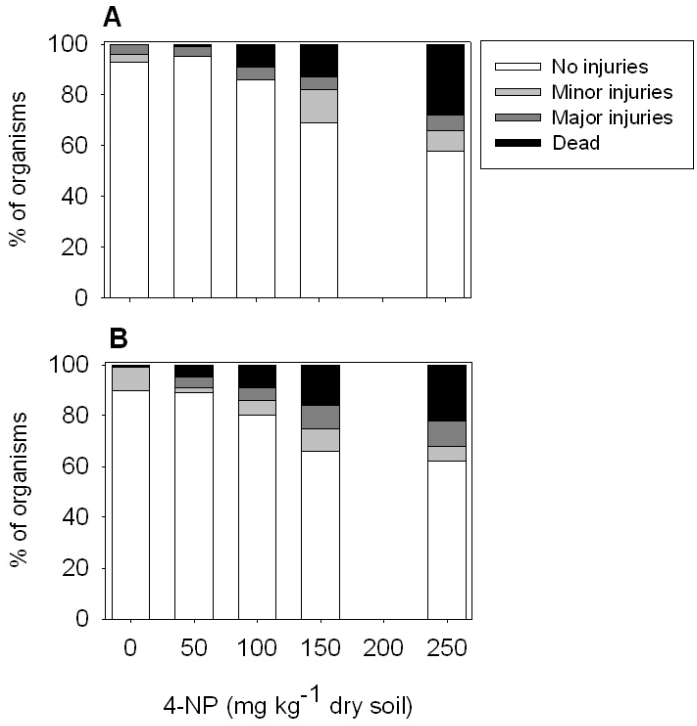


Figure S3: Condition of *Enchytraeus albidus* in terms of cryoinjuries ("no injuries", representing healthy worms with intact integument; "minor injuries", representing worms with one injury, characterized by a small physical disruption of the integument; "major injuries", representing worms with two or more injuries but still able to move and "dead" were completely immobilized by the high number of injuries) after 7 days pre-exposure to 4-NP followed by 10 days at constant -4ºC (A) or daily freeze-thaw cycles (B).

Photo by Zdenek Gavor

Chapter VI

Effect of freeze-thaw cycles and 4-nonylphenol on cellular energy allocation in the freeze-tolerant enchytraeid *Enchytraeus albidus*

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SUMMARY

Due to climate change and intense anthropogenic activity, organisms from cold regions are often exposed to combined effects of temperature fluctuations and contaminants. In this investigation, we assessed the lipid, protein and carbohydrate energy budgets, the energy available (Ea), consumed (Ec) and cellular energy allocation (CEA) of the freeze-tolerant *Enchytraeus albidus*, when exposed to sub-lethal concentrations of 4-nonylphenol (a lipophilic contaminant) for 7 days, followed by exposure to different temperature regimes (continuous 2ºC, continuous -4ºC, and daily freeze-thaw cycles (FTC) (2 to -4ºC) for additional 10 days. Results showed that a pre-exposure to 4-NP induced important changes in the worms' energy budgets and CEA and increased mortality with most severe effects observed for the FTC events. For FTC, lipids were the most accumulated energy source, whereas during freezing (-4ºC) proteins were the most used. FTC caused the highest Ec, indicating the higher energy requirements for organisms when shifting between freezing and thawing events. This is also in line with the higher mortality observed in FTC compared to continuous -4ºC or 2ºC. Worms exposed to continuous freezing presented relatively stable and positive levels of Ea and low levels of Ec, possibly related with the decrease in metabolism.

Keywords: Freeze-tolerance, cellular energy allocation, energy budgets, freeze-thaw cycles, 4-nonylphenol.

INTRODUCTION

The potworm *Enchytraeus albidus,* Henle 1837, belongs to a large and ecologically relevant group of saprophagous organisms, which inhabit the litter layer and the upper mineral soil of many terrestrial and supralittoral ecosystems in temperate and subarctic regions (Didden, 1993; Giere, 2006). The presence and abundance of *E. albidus* in harsh environments such as Greenland and Iceland is due to its tolerance of freezing of its extracellular compartments (Patricio Silva, et al., 2013; Slotsbo, et al., 2008), also known as freeze-tolerance capacity (Zachariassen, 1985). This process is assisted by the accumulation of glucose as main cryoprotectant, which lowers the melting point and the ice fraction at a given temperature (Patricio Silva, et al., 2013; Slotsbo, et al., 2008). As a consequence, the concentration of potentially toxic salts in the unfrozen body fluids tends to decrease (Zachariassen, 1985; Ramløv, 2000), and the membranes and proteins are stabilized at low temperatures (Anchordoguy, et al., 1987; Crowe, et al., 1987).

Due to global warming it is predicted that freeze-thaw cycles will increase in sub-arctic and cold temperate regions due to the reduction (or absence) of an insulating snow cover during frost periods (IPCC, 2013). Several studies point to the potential negative effect of freeze-thaw events on survival chances of freeze-tolerant invertebrates by, for instance, causing a cumulative damage in cell

structures (Churchill and Storey, 1989; Brown, et al., 2004; Bale, et al., 2001; Sinclair and Chown, 2005; Marshall and Sinclair, 2011), decrease in feeding activity with loss of body mass (Sinclair and Chown, 2005), alteration of cryoprotectant levels (Marshall and Sinclair, 2011) and increase in energy expenditure (Churchill and Storey, 1989; Sinclair and Chown, 2005). In parallel, the increase of contaminants in cold regions may also affect the freeze-tolerance capacity of several species (reviewed by Holmstrup, et al., 2010). A particular attention has been given to some lipophilic compounds, such as 4-nonylphenol, a surfactant commonly found in sludge-amended soils. This model compound seems to cause a decrease in the cryoprotection levels (Patricio Silva, et al., 2014) and tends to accumulate in cell membranes (Jacobsen, et al., 2004; Shan, et al., 2010; Ekelund, et al., 1993) and decreasing membrane fluidity, which is likely to reduce cold tolerance of the organism (Holmstrup, et al., 2014).

Despite concerns about the interactive effect of contaminants and natural stressors (Holmstrup, et al., 2010; Noyes, et al., 2009), combination between contaminants and freeze-thaw events, particularly in freeze-tolerant species, remains poorly documented. There is only one study that addressed the combined effect of a contaminant (4-nonylphenol) and daily freeze-thaw cycles on a freeze-tolerant soil species - the enchytraeid *E. albidus* (Patricio Silva, et al., 2014). According to this study, worms that were previously exposed to sub-lethal concentrations of 4-nonylphenol revealed a significant decrease in survival and cryoprotection after exposure to daily freeze-thaw cycles (+2ºC to -4ºC), as compared with worms exposed to constant freezing temperatures (-4ºC) or to control temperature (+2ºC). In order to increase the knowledge on this subject, we investigated the combined effect of 4-nonylphenol and freeze-thaw cycles on the basal energy levels and allocation in *E. albidus*, under the same experimental conditions simulated by Patrício Silva et al. (2014). The cellular energy allocation (CEA) approach integrates the energy available (Ea) (proteins, lipids and carbohydrates) and energy consumed (Ec) (via electron transport system activity, ETS) at different time-points, and has proven to be a sensitive stress marker (De Coen and Janssen, 1997; De Coen and Janssen, 2003; Amorim, et al., 2011).

MATERIAL AND METHODS

Test species

Enchytraeus albidus (Henle, 1837) were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany), and cultured for three years in the laboratory in agricultural (loamy) soil at 5.0 ± 1°C and fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). Prior to experiments, the organisms were cold acclimated at 2ºC for two weeks.

Test soil and spiking

All experiments were conducted using the natural standard soil, LUFA 2.2 (Speyer, Germany). Main characteristics of the soil are as follows: Organic Carbon 1.7%, grain size distribution 7.3% clay, 13.8% Silt, 78.9% sand and maximum water holding capacity (WHC) of ca. 44% of fresh weight, pH ($CaCl_2$) of 5.5. This soil is within the optimum range of pH in natural soils where *E. albidus* are found (Jänsch, et al., 2005).

The soil was spiked with 4- nonylphenol (4-NP)(Aldrich, Cas. No. 29.005.8, 100% pure), dissolved in acetone (J.T. Barker, Hayward, CA, HPLC quality) to obtain the following concentration range: 0 - 50 - 250 mg kg^{-1} dry soil. After spiking with acetone-NP, the soils were kept in a fume-hood for 24 h to allow evaporation of the solvent. Soil humidity was restored with 15‰ saline-water till 50% of WHC (205 ml saline water was added per 1kg of dry soil). Saline water was used in order to improve survival in frozen soil (Patricio Silva, et al., 2013); this salinity level is commonly present in supralittoral soils in temperate and subarctic coastal regions.

Experimental procedures and setup

The experimental setup was adapted from a method previously described (Patricio Silva, et al., 2014). Briefly, each replicate consisted of 10 worms in a test vial (3.5 cm high, 2.5 cm diameter) containing 10 g of test soil. The vials were covered with a perforated lid to allow ventilation. Test concentrations used were 0 – 50 – 250 mg 4-NP kg^{-1} dry soil, selected based on previous investigations, corresponding to the approximate LC30 and LC50, respectively, for worms exposed to FTC (Patricio Silva, et al., 2014). Worms were exposed to 4-NP and 3 different temperature regimes as shown in Fig. 1. Five replicates were performed per treatment; to ensure sufficient number of surviving worms for analysis (N=10) in the highest concentration and FTC treatments the number of replicates was ten in these conditions.

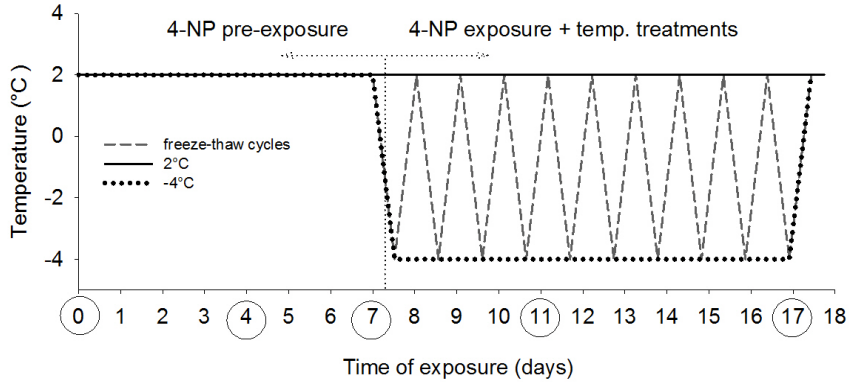

Fig. 1: Schematic representation of the experimental setup used, which consisted to expose *Enchytraeus albidus* to 4-NP for 7 days, followed by 10 days under 3 different temperatures regimes: i) daily freeze-thaw cycles (+2 °C to −4 °C, with a decrease/increase rate of 0.5ºC h^{-1}; ii) continuous −4 °C and iii) continuous +2 °C. The circles in the days mark the sampling points for analysis.

In short, vials with worms were kept at 2 °C for 7 days. After this period, the vials were split into 3 groups, each subjected to a particular temperature regime: i) daily freeze-thaw cycles – with temperatures from 2 °C to −4 °C, with a decrease/increase rate of 0.5ºC h^{-1}; ii) continuous temperature regime of 2 °C and iii) continuous temperature regime of −4 °C. The FTC treatment test vessels were daily sprayed with a freeze-spray when the temperature lowered and reached -2.5 °C. This procedure is important to induce nucleation of the soil and ensure inoculative freezing of enchytraeids once the temperature becomes lower than the body fluid melting point (Slotsbo et al., 2008). At day 0, 4, 7, 11 and 17 worms were collected, briefly rinsed in demineralized water and snap-frozen in liquid nitrogen.

CEA analysis

CEA was measured following the procedures described in Novais and Amorim (2013). Each replicate (i.e. 10 worms combined) was homogenized in 1 ml deionized water and divided into 3 Eppendorf tubes (300 µl each) to measure: 1) protein and carbohydrates; 2) lipids and 3) electron transport system activity (ETS).

Energy available – Ea

Energy available (Ea) reserves were measured by determining spectrophotometrically the total protein, carbohydrate and lipid content at each time point and transforming them into energetic equivalents using enthalpy of combustion (24 kJ g^{-1} proteins, 17.5 kJ g^{-1} carbohydrates and 39.5 kJ g^{-1} lipids) as described in De Coen and Janssen (1997). Protein content was determined according to the Bradford method (Bradford, 1976) at 600 nm using bovine serum albumin as standard. Total carbohydrate content was determined with 5% phenol and concentrated H_2SO_4 at 490 nm using

glucose as standard. Total lipids were extracted according to the method described by Bligh and Dyer (1959). Total lipid content was determined by measuring the absorbance at 400 nm using Tripalmitine (Sigma) as standard. All measurements from each replicate were made in triplicate.

Energy consumption – Ec

The energy consumption (Ec) (consumed oxygen rate) was determined based on the measurement of the electron transport system activity (ETS) (King and Packard, 1975) over several exposure periods, following the methodology described in detail by (De Coen and Janssen, 1997). The electron transport activity was measured by adding NADPH solution and INT (p-IodoNitroTetrazolium, Sigma) and following the increase in absorbance at 490 nm for 3 min. The oxygen consumption rate was determined based on the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system (De Coen and Janssen, 1997). The quantity of consumed oxygen was then transformed into energetic equivalents using the specific oxyenenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ mol 1^{-1} O_2 (Gnaiger, 1983).

Cellular Energy Allocation – CEA

The CEA consists of the difference between the budget of energy reserves and energy consumption. The budget of each energy reserve (proteins, carbohydrates, and lipids) was calculated by integrating the changes in energy values over the exposure periods. The total Ea value was calculated by integrating the change in the summed energy reserves fractions over corresponding exposure periods (0-4, 4-7, 7-11, 11–17d in worms exposed to 2ºC; 7–11, 11–17d in worms exposed to sub-zero temperature treatments, i.e. constant freezing and freeze-thaw cycles, respectively). The Ec value was similarly calculated, integrating the change in energy consumption over the same exposure periods.

The CEA, representing the net cellular energy budget, was calculated for each time interval as described in De Coen and Janssen (1997), using the following equation:

$$CEA\ (mJ\ mg^{-1}\ mg\ fresh\ weight^{-1}) = \frac{\int_{t-1}^{t} Ea.dt - \int_{t-1}^{t} Ec.dt}{t-(t-1)}$$

with *t* being the exposure time and *t-1* being the previous time in which measurements were done.

Statistical analysis

Comparisons within each variable: 4-NP (0, 50 and 250 mg kg $^{-1}$ dry soil) and exposure period (0, 4, 7, 11 and 17 days), were performed using one-way analysis of variance (ANOVA). Dunnetts' test was used to assess significant differences after one-way ANOVA ($p< 0.05$). A t-test was performed to check significant differences in worm's weight, before and after exposure to temperature treatments. The outliers were excluded before statistical analysis, being the outliers calculated as the mean ± 2*standard error. All statistical analyses were performed using Sigmaplot for Windows Version 11.0 (Systat software Inc., San Jose, CA).

RESULTS

For all sampling points 10 organisms per replicate were obtained (mortality was compensated by the extra replicates in treatments of FTC and 250 mg NP kg^{-1} dry soil). For details on survival data please see (Patricio Silva, et al., 2014) and supplementary information (Fig. S1).

No significant differences were observed in terms of weight along time, with exception of worms exposed to 0 and 250 mg 4-NP kg^{-1} dry soil to continuous -4ºC, where a significant increase was observed; and worms exposed to control in FTC, where a decrease was observed (supplementary information, Table S1).

Results for Ea can be observed in Fig. 2.

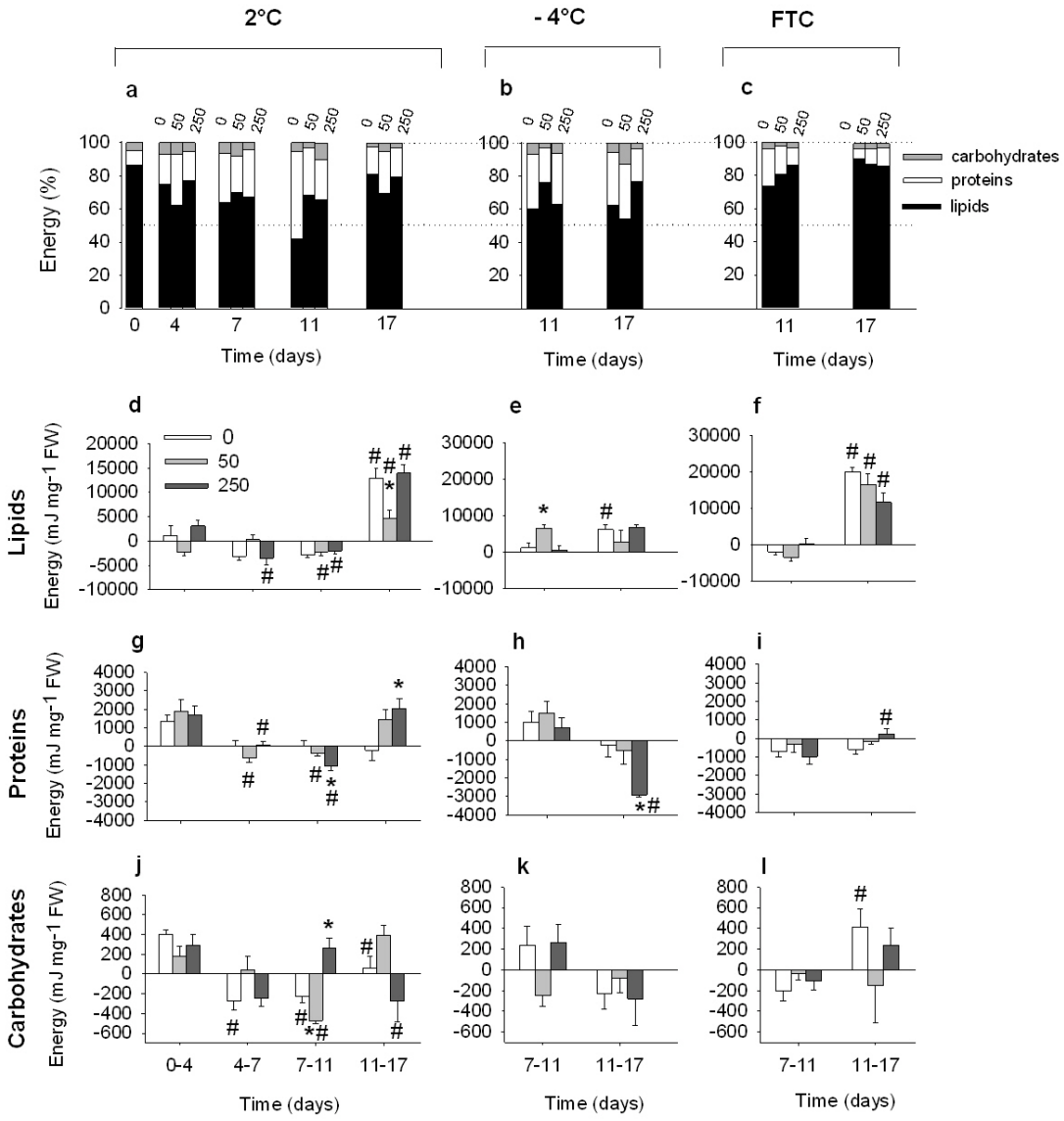

Fig. 2: Energy available in *Enchytraeus albidus* when exposed to 4-NP (0 - 50 - 250 mg kg^{-1} dry soil) to 2ºC, -4ºC and freeze-thaw cycles (FTC) along various exposure periods (0-4-7-11-17 days). a-c: Proportion of the absolute energy reserves levels in each treatment; d-l: Protein, Carbohydrate and Lipid budgets integrated over exposure periods (0-4, 4-7, 7-11, 11-17 days); Results are expressed as average ± standard error; *Dunnett's test, $p<0.05$, for differences between 4-NP and control groups within each exposure period. # Dunnett's test, $p<0.05$, for differences between 0-4d and the other exposure periods.

Lipids represented the main energy resource of *E. albidus,* with relative portions around 60-80%, followed by proteins (20-40%) and carbohydrates (5-15%) (Fig 2a-c). Results show variation along time and with concentration but without a clear pattern, except for the FTC where a tendency to accumulate lipids is apparent.

The main lines, in terms of Ea, at 2ºC, the lipid budget shows an increase after 17 days, whereas proteins and carbohydrates show a decrease followed by increase along the time points (Fig. 2 d, g, j). At -4ºC lipids increase with exposure time for control and highest concentration, whereas proteins

show significant decrease and carbohydrates fluctuate (Fig.2 e, h). For FTC a clear increase in lipids is observed with longer time, this being a dose-response decrease pattern; proteins show a minor increase and carbohydrates fluctuate (Fig. 2 f, i, l).

The Ea, Ec and CEA results are shown in Fig. 3.

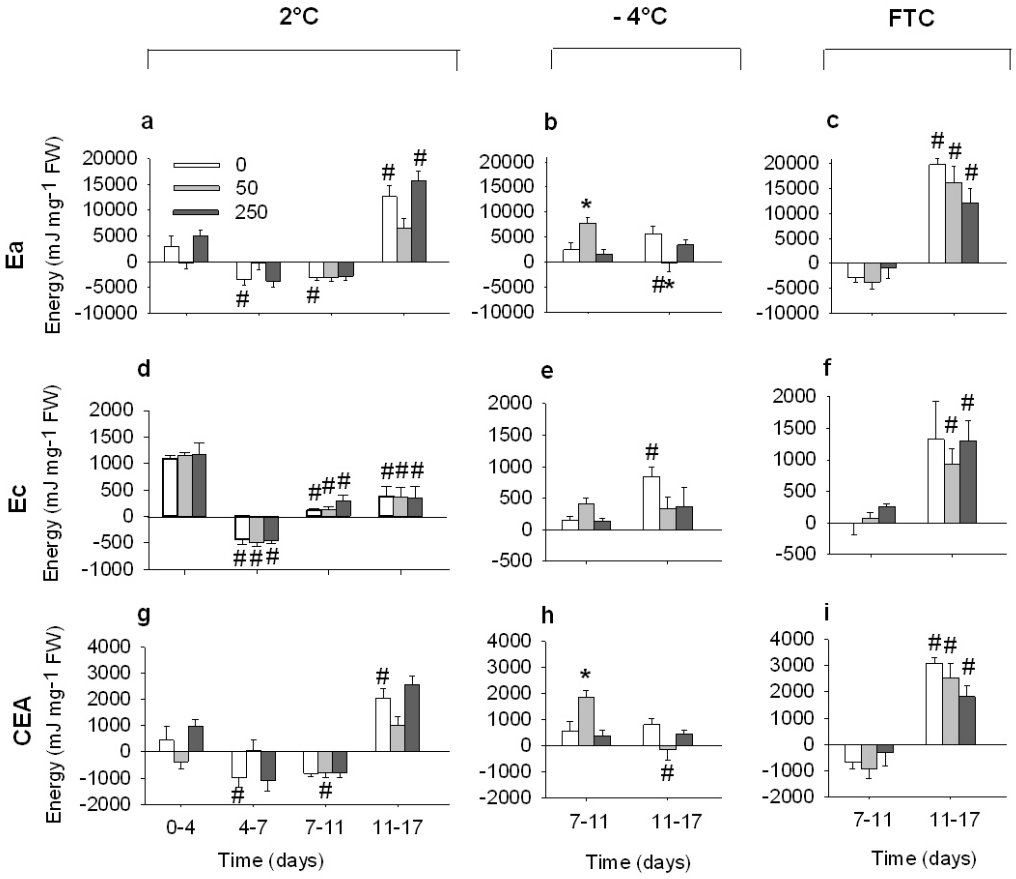

Fig. 3: Energy available (Ea) (a-c), energy consumed (Ec) (d-f) and Cellular Energy Allocation (CEA) (g-i) in *Enchytraeus albidus* when exposed to 4-NP (0 - 50 - 250 mg kg^{-1} dry soil) to 2ºC, -4ºC and freeze-thaw cycles (FTC) along various exposure periods (0-4-7-11-17 days). Results are expressed as average ± standard error (*N*=5). *Dunnett's test, $p<0.05$, for differences between exposed and control groups within each exposure period. # Dunnett's test, $p<0.05$, for differences between 0-4d and the other exposure periods.

In terms of total Ea, the trend follows the one described for the lipids since these are the major absolute source.

In terms of Ec, at 2ºC there is an initial decrease from 4 to 7 days, followed by an increase from 7-17 days. At -4ºC there is an increase of Ec with time, this being significantly larger for the FCT treatment.

The CEA, overall, shows a pattern of decrease followed by increase along time for 2ºC and -4ºC. For the FTC a clear increase with time, this being a dose-response decrease with concentration.

DISCUSSION

In terms of relative proportions of Lipids, Proteins and Carbohydrates (L-P-C) the organisms showed a relatively higher L (60-80%) from the beginning of the exposure, when compared to previous studies on the same test species (Amorim, et al., 2011; Novais and Amorim, 2013; Novais, et al., 2013). This is likely related with the acclimation to a lower temperature in the present study (cultures were kept at 5ºC instead of 20ºC). Accumulation of lipids during cold acclimation (e.g. overwintering) is common, for instance as observed in the freeze-tolerant gall fly *Eurosta solidaginis* (Lee, et al., 1995).

It was observed that as the exposure period became longer the changes became more pronounced, even 17 days is probably a short term for major changes to be captured. One of the major differences compared to the majority of other studies, is related with the lipids. Lipids are normally the first energy resource to be used when exposed to chemicals, as reported e.g. for *Daphnia magna* when exposed to metals or organic compounds (De Coen and Janssen, 2003; De Coen and Janssen, 1997), for *Brachynema germari* exposed to pyriproxyfen (Bagheri, et al., 2010) and in *E. albidus* exposed to metals (Amorim, et al., 2011; Novais and Amorim, 2013). Here we observed an increase in lipid budget towards the last period of exposure in all temperature treatments, although most pronounced at 2ºC and FCT.

The increase in lipids (itself) could be due "lipogenesis". The conversion of carbohydrates, a major component of worms and insect's diet and also major cryoprotectants (Slotsbo, et al., 2008; Zachariassen, 1985; Holmstrup and Zachariassen, 1996), to lipid in the fat body are well documented in insects as reviewed by Arrese and Soulages (2010). In higher species such as fish or mice, lipogenesis was shown affected by increased concentrations of metals (Song, et al., 2014) and 4-NP (which acts as an endocrine disruptor) (Hao, et al., 2012). A previous study on *E. albidus* showed an increase of lipid budgets with decrease of carbohydrates, when exposed to an increased concentration of carbendazim and dimethoate (Novais and Amorim, 2013), suggesting that lipogenesis could be playing a role in enchytraeids. Nevertheless, in the present study this does not seem to be the case, as the measured increase in lipids is not corresponding to a decrease in carbohydrates.

It is known that *E. albidus* accumulates 4-NP in their tissues (Patrício Silva, et al., 2015). Further, worms exposed to FTC have higher concentrations of 4-NP in the tissues than worms exposed to continuous -4ºC or +2ºC (Patricio Silva, et al., 2014) hence the lipid increase highlights the increased risk of bioaccumulation of 4-NP at FTC events. This means that during thawing and

higher temperatures, e.g. during seasonal variation, worms will increase their metabolism hence metabolize lipids and have increased exposure to internal 4-NP.

In comparison, worms at -4ºC for the same period (11-17 d) increased their lipids much less. On the one hand it could be due to the lower metabolism (metabolic rate (MR)) as reported by Fisker, et al. (2014a) in frozen worms. On the other hand, the Ec as measured in the present study does not decrease. Whether such difference is due to the different parameters (i.e. metabolism versus consumed energy) or due to experimental design is unknown. The mechanisms to respond freezing and freeze thaw seem to be differentiated or of different level. This is in line with results on survival previously reported by Patricio Silva, et al. (2014), who observed that worms exposed to combined effect of 4-NP and freeze-thaw cycles had higher mortality, followed by worms exposed to continuous freezing and to continuous +2ºC.

As mentioned, whereas lipid budget was very sensitive in FTC, at -4ºC proteins' were the most changed, showing a significant decrease with exposure time and dose related. The combined effect of 4-NP and low temperatures (lower metabolism) could be interfering negatively (and antagonistically) with proteins synthesis, e.g. detoxifying enzymes, increasing 4-NP toxicity and freezing damage, although, the mechanisms remain unclear.

Worms exposed to +2ºC show an increase on protein budget during the last exposure period (11-17d) with the increase of 4-NP. Such increase in proteins has been observed for *E. albidus* exposed to dimethoate (Novais and Amorim, 2013) and in other species, e.g. *Daphnia magna* exposed to Cd, TBT, linear alkylbenzene sulfonic acid, 2,4- dichlorophenoxy acetic acid (De Coen and Janssen, 2003), lindane (De Coen and Janssen, 1997), *Danio rerio* exposed to different effluent concentrations (Smolders, et al., 2003), and in *Neomysis integer* exposed to chlorpyrifos (Verslycke, et al., 2004). Again, and as discussed by Smolders, et al. (2003), the increase in protein levels could be the result of an induction in protein synthesis for detoxification or other defense mechanisms. During FTC, proteins do not increase which can indicate a decrease in the ability to detoxify and increased risk of toxicity.

Results in terms of carbohydrates were quite variable without a clear pattern, which could be due to the fact that the method is not sensitive enough in this experimental design (the absolute values are the lowest, accurate variations may be compromised) or, that there is fast mobilization. For instance, *E. albidus* acclimated to cold temperature environments are known to have higher content in carbohydrates (particularly glucose since it is the main cryoprotectant) than worms acclimated to warmer temperatures (e.g. 15-20ºC) (Slotsbo, et al., 2008; Fisker, et al., 2014b). Furthermore, exposure to increased concentrations of 4-NP showed significant decrease of glycogen reserves (the major carbohydrate reserves in *E. albidus*) without glucose accumulation (Patricio Silva, et al., 2014). The current is a measure of the total carbohydrates hence this cannot be discriminated.

In terms of Ec a clear decrease is observed at 2ºC and an increase at FTC. The decrease in Ec observed in the first 7 days to 2ºC (prior exposure to temperature treatments) can be a consequence of the decrease in worms' metabolism to lower temperature (5 to 2ºC). The subsequent increase in worms exposed to FTC can be related with the increase in the metabolism during thawing periods required to restart important cellular mechanisms. For worms exposed to -4ºC, the increase can be linked to the proteins mobilization and consumption to control extracellular ice formation.

Ec budgets tended to be lower in worms exposed to higher 4-NP (e.g. 11-17d at -4ºC). The tested 4-NP is a lipophilic compound ($logK_{ow}$ of 4.5), which makes it liable to accumulate in cell membranes (Jacobsen, et al., 2004; Shan, et al., 2010; Ekelund, et al., 1993) and to decrease mitochondrial enzymatic activities related to complex I (the first and major entry point of electrons into the mitochondrial respiratory chain, through NADH-coenzyme Q reductase) and complex III (cytochrome b complex) (Belaiche, et al., 2009; Bragadin, et al., 1999). 4-NP is probably interfering with the electron transport system (ETS), particularly on the oxidation of the coenzyme Q-cytochrome B complex, which seems to be rate-limiting (King and Packard, 1975), hence the lower activity measured. A decrease on ETS activity was also observed by De Coen and Janssen (2003) in Daphnia magna exposed to LAS and by Smolders, et al. (2004) in *Dreissena polymorpha* (mussel).

Despite the increase in Ec observed in the longer period of exposure in FTC, Ea was still substantially higher, hence the observed increase in CEA. The CEA results are in agreement with results obtained in other organic contaminant, atrazine, where an increase was also observed (Novais and Amorim, 2013). As opposed to this, CEA decreases when exposed to metals (Amorim et al., 2011; Novais et al., 2013), i.e., this is chemical specific and indicates the different mechanisms of response. Effects at later stages (e.g. survival, reproduction) are not only related with decrease in CEA, e.g. De Coen and Janssen (1997) also failed to find this relationship between CEA. The authors found that when *D. magna* was exposed to lindane causing effects at the population levels (decrease in survival, growth and reproduction) the CEA increased.

CONCLUSION

The measured parameters (Ea, Ec, CEA) were sensitive to assess effects of cold temperature and chemical stress. For FTC lipids were the most accumulated energy source, whereas during freezing (-4ºC) proteins were the most used energy. Worms exposed to FTC showed the highest Ec, indicating the higher energy requirements for organisms when shifting between freezing and thawing events. This is also in line with the higher mortality observed in FTC compared to continuous -4ºC or 2ºC. Worms exposed to continuous freezing presented relatively stable and positive levels of Ea and low levels of Ec, likely due to a decrease in metabolism. Moreover, the risk of bioaccumulation of 4-NP (and probably other lipophilic compounds) is comparatively increased in FTC.

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AUTHOR CONTRIBUTIONS

A.L.P.S., and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S. and M.A. carried out data analysis. Both authors wrote the paper.

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SUPPORTING INFORMATION

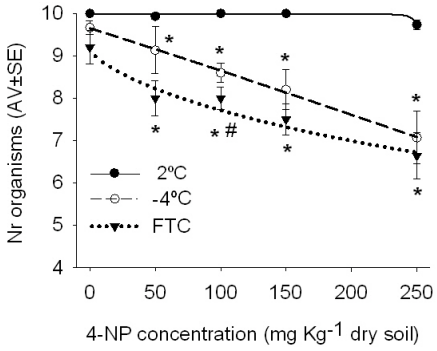

Fig. S1 Survival of *Enchytraeus albidus* exposed to 4-nonylphenol and three temperature regimes (constant +2oC, constant -4oC and daily freeze-thaw cycles). (*) Statistically significant differences with qui-square test ($p<0.05$). Results are shown as mean ± standard error (N = 10) and described with a log-logistic concentration response model (2oC = solid line, -4oC = broken line, daily freeze-thaw cycles = dotted line) (as in Patrício Silva et al., 2014; as supporting information).

Table S1 Fresh weight (mg) of *Enchytraeus albidus* (pool of 10 worms, AV±SE), before and after exposure to the various treatments.

Temperature	Day	4-Nonylphenol (mg kg^{-1} dry soil)		
		0	50	250
+2ºC	0	42.4±3.6	26.4±4.3	32.7±3.7
	17	37.2±9.3	26.2±1.2	28.1±2.1
-4ºC	7	26.3±2,3	22.1±2.2	21,1±1.0
	17	44.9±2.5	40..0±9.2	43.8±3.6
		t-test, $P < 0.05$		*t-test,* $P < 0.05$
FTC	7	48.8±3.6	43.1±3.3	40.9±3.9
	17	30.3±1.7	35.4±4.6	44.5±4.5
		t-test, $P < 0.05$		

Photo by Zdenek Gavor

Chapter VII

Uptake and elimination of 4-nonylphenol in the enchytraeid *Enchytraeus albidus*

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SUMMARY

We determined the uptake and elimination kinetics of 4-nonylphenol (4-NP) in *Enchytraeus albidus*. A relatively fast degradation of 4-NP in test soil occurred at 20 °C (λ = 0.11 day^{-1}). The concentration of 4-NP in worm tissue followed a three-phase kinetics model, with a short phase of fast 4-NP accumulation shortly after exposure start (k_u = 0.97 mg kg^{-1} day $^{-1}$), followed by partial elimination (K_{e1} = 1.47 day^{-1}) until reaching the equilibrium phase (A = 44.7 mg kg^{-1} dry tissue), and finally the elimination upon transfer to uncontaminated soil (K_{e2} = 0.67 day^{-1}). During uptake, the internal concentration was similar to the concentration found in the soil, with a BAF ~1. In un-spiked soil, elimination took place within the first 24 h (elimination $t_{1/2}$ ~1 d).

Keywords: Terrestrial enchytraeids, surfactant, bioaccumulation, toxicokinetics

INTRODUCTION

Sewage sludge is frequently applied as a fertilizer to cultivated land. However, municipal sewage sludge often contains organic contaminants including nonylphenol (NP), a breakdown product of the widely used nonionic nonylphenol ethoxylate (NPEO) surfactant (Soares et al., 2008). Due to the potentially harmful effects of NP and other degradation products of NPEOs, their use and production have been restricted in the European Union (Directive 2003/53/EC) and substituted by other surfactants (mainly alcohol ethoxylates) considered to be environmentally safer with higher degradation rate (Campbell, 2002). Nevertheless, NP is still present in environmental samples and its hydrophobicity (logK_{ow} of 4.5) results in much higher bioaccumulation than the parent compounds (NPEO), which poses high concern in terms of ecological risk assessment (Ekelund et al., 1993; Jacobsen et al., 2004; Shan et al., 2010; Soares et al., 2008).

4-Nonylphenol (4-NP) is the most widespread branched nonylphenol found in natural ecosystems (US-EPA, 2010). The occurrence, toxic effects and bioaccumulation of 4-NP in the aquatic environment is well documented in comparison to soil ecosystems, for which the literature is still relatively scarce (reviewed by (Mao et al., 2012; Soares et al., 2008). Concentrations of 4-NP in bulk soil are relatively low (1.4−1.6 mg kg^{-1}) (Soares et al., 2008), but lumps of sewage sludge may contain much higher concentrations (3-10,000 mg kg^{-1} dry sludge (TemaNord, 1996), and sludge lumps are often colonized by soil fauna such as enchytraeids due to their nutritional value (Krogh et al., 1997). In a field study it was demonstrated that an initial and fast degradation of NP occurred in sludge-treated soil and landfills (> 80% of the initial input, 4.7 mg kg^{-1}, within the first month), but the remaining part was found to be persistent (Marcomini et al., 1989), indicating that other parameters in addition to biodegradation and sorption rates were implicated in the removal of NP. In sludge-soil mixtures, 4-NP was almost totally degraded in less than 30 days in aerobic homogenized mixtures, sandy-clay and silty-clay soils (Chang et al., 2007; Chang et al., 2005a, b; Hesselsøe et al., 2001;

Jacobsen et al., 2004), but degradation was not completed within 3 months in nonhomogeneous mixtures containing sludge aggregates (Hesselsøe et al., 2001). These studies were, however, performed without assessing the uptake of 4-NP or its toxicity to soil organisms. On the other hand, studies on the toxicity of 4-NP to soil organisms have been performed with the enchytraeids *Enchytraeus crypticus* (Domene et al., 2009) and *E. albidus* (Gejlsbjerg et al., 2001; Patricio Silva et al., 2015), the collembolans *Folsomia fimetaria* (Krogh et al., 1997) and *Folsomia candida* (Gejlsbjerg et al., 2001; Sørensen and Holmstrup, 2005; Widarto et al., 2007), and in the earthworms *Dendrobaena octaedra* (Widarto et al., 2004) and *Aporrectodea caliginosa* (Krogh et al., 1997), without assessing 4-NP bioaccumulation or soil concentrations. Shan et al. (2010) studied bioaccumulation of the most common branched isomer of technical grade 4-NP (4-NP_{111}) and its metabolites in the earthworm *Metaphire guillelmiin*. These authors found that earthworms rapidly accumulated 4-NP, but that much of this was biotransformed to bound residues in the earthworm body.

Ecotoxicological test organisms should represent species that play key roles in functioning of ecosystems, be abundant and widely distributed, and be easily culturable under laboratory conditions (Didden and Römbke, 2001). The species *Enchytraeus albidus* fullfills all of these criteria and has thus been extensively used in ecotoxicological effect assessment studies (Didden and Römbke, 2001; Didden, 1993; Römbke and Moser, 2002). The aim of the present study was to assess the uptake and elimination of 4-NP in *E. albidus*.

MATERIAL AND METHODS

Test Species

Enchytraeus albidus (Henle, 1837) were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany) in 2011, and cultured since then in agricultural (loamy) soil at 5.0 ± 1 °C. The worms were fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). Prior to experiments, the organisms were acclimated to 20°C for 2 months.

Test Soil and Spiking

The experiment was performed using the natural standard soil, LUFA 2.2 (Speyer, Germany). Main characteristics of the soil are as follows: organic carbon 1.7%, pH ($CaCl_2$) 5.5, grain size distribution 7.3% clay, 13.8% silt, 78.9% sand and maximum water holding capacity (WHC) of ca. 44% of fresh weight.

The soil was spiked with 4-NP (Aldrich, Cas. No. 29.005.8, 100% pure) to a nominal concentration of 100 mg kg^{-1} dry soil, in acetone-solution (J.T. Barker, Hayward, CA, HPLC quality). The test concentration was selected based on a previous study showing that reproduction was slightly reduced while no mortality was observed (Patrício Silva et al., 2015). The solvent was allowed to evaporate under a fume hood overnight. Soil moisture content was then adjusted to approximately 50% of the water holding capacity (WHC) (205 mL deionized water per 1 kg of dry soil).

Bioaccumulation test procedures

The bioaccumulation test was adapted from the OECD guideline for bioaccumulation in Terrestrial Oligochaetes (OECD, 2010). Methods utilized in this experiment were similar to those described by Patricio Silva et al. (2014). In brief, the experiment included a 14-d uptake phase followed by a 14-d elimination phase. At the beginning of the test, groups of 20 adult worms were selected at random from our laboratory mass culture and introduced into each of 70 test vessels (250 mL glass containers) with 25 g of moist soil spiked with 100 mg 4-NP kg^{-1} dry soil. Containers were covered with parafilm with a few holes for aeration and incubated in a climate room at 20 ºC, with a 16:8 h light:dark cycle. Once per week, the animals were fed with oat flakes, and water loss was replenished. Samplings were performed at days 1, 2, 4, 7, 9, 11, and 14 for the uptake phase. After the 14 d uptake phase, the remaining animals were transferred into clean soil for the elimination phase. Worms were then sampled after 6 h, 1, 2, 4, 7, 10, and 14 d during the elimination phase. Five replicates were used for each sampling time. At each sampling time, organisms were sorted from the test soil, rinsed in deionized water, gently dried on filter paper, weighed, and kept frozen at -80ºC until analysis. Similarly, 5 replicates of 5 g soil were collected at days 0, 7 and 14 and stored at -80ºC until analysis.

4-NP quantification by Gas Chromatography−Mass Spectrometry (GC−MS)

Analysis of 4-NP in tissue samples was performed by solid phase extraction followed by gas chromatography – mass spectrometry (GC-MS) as previously described by Patrício Silva et al (2014), and reported on a fresh weight basis. In brief, the chemical analysis of the tissues required a minimum fresh weight of 40−50 mg (corresponding to ~20 worms). Samples were homogenized in Eppendorf tubes with 1.5 mL 70% ethanol and a steal ball using a Tissuelyser II (Qiagen Gmbh, Hilden, Germany). The homogenate was transferred to a glass tube, where 10 µL of 6.4% NaOH, 250 µL of 0.2 M K_2CO_3 and 20 µL Ac_2O was added. After vortexing for 10 min, the homogenate was left for 3 h in darkness to allow acetylation. This procedure was followed by solid phase extraction, where 4-NP was isolated and eluted on LiChrolut columns (bottom EN 100 mg, top RP-18 200 mg) (Merck KGaA, Darmstadt, Germany). After washing the columns with 2 mL Elga water followed by 2 mL of 96% and 70% ethanol, respectively, the homogenate was filtered and added to the columns.

The retained 4-NP was then eluted with 1 mL of acetonitrile (super gradient for HPLC, Prolabo, Vienna; CAS: 75−05−8). The remaining water in 4-NP extract was removed by adding a tip of a spatula (2−3 mg) of sodium sulfate (Na_2SO_4). Extracts were centrifuged for 3 min at 3000 *g*, and the supernatant was transferred to GC−MS sample vials. A Shimadzu GCMS-QP2010 with autosampler was used to perform the analysis. The GC was equipped with a FactorFour capillary column VF-5 ms (length 30 m, inner diameter 0.25 mm, film thickness of 0.25 µm; Varian, Netherlands). The injection volume was 2.0 µL and the autosampler was operating in split mode with a split ratio of 3. Helium was used as carrier gas with a total flow of 5.3 mL min^{-1} and a column flow at 0.57 mL min^{-1}. The injection temperature was maintained at 260 °C and the oven was programmed as follows: initial temperature of 120 °C was held for 2 min, then increased to 150 °C with a rate of 20 °C min^{-1}, then increased to 170 °C with a rate of 6.0 °C min^{-1} and held for 1 min, finally increased to 190 °C with a rate of 6 °C min^{-1} and held for 1 min. The mass spectrometer was operated in electron ionization mode at 70 eV. The ion source temperature was 300 °C and the interface temperature 280 °C. Identification of 4-NP was based on external standards that had been treated like worm samples. The area of identified peaks was calculated using GCMS Solution software and the concentration was calculated based on specific standard curves.

Concentration of 4-NP in soil was quantified with the same method, but with some readjustments in the beginning of the procedure. In brief, 8 mL 70% ethanol were added to each soil sample (5 g) and shaken for 45 min. Afterwards, soil samples were briefly centrifuged (2000 *g* for 3 min). From the soil suspension, 2 mL were transferred to a glass tube, where 10 µL of 6.4% NaOH, 250 µL of 0.2 M K_2CO_3 and 20 µL Ac_2O was added. After vortexing for 10 min, the homogenate was left for 3 h in darkness to allow acetylation. From this point, analysis of soil samples followed the same procedure as the tissue samples.

Data analyses

The decrease constant of 4-NP concentration in soil during the uptake phase was calculated assuming a first-order kinetic model according to, as follows:

$$C(t) = C_{exp}.e^{-\lambda.t} \quad (1)$$

where *C(t)* is the 4-NP concentration in the soil at time *t* (mg kg^{-1} dry soil); C_{exp} is the initial 4-NP concentration used to spike the soil (100 mg kg^{-1} dry soil); λ is the rate constant for the decrease of 4-NP in the soil (day^{-1}). Most studies on toxicokinetics of pollutants have used a “classic” two-phase model with constant uptake and elimination rates. However, data of the present study did not fit such a model well (see later discussion), and we therefore adopted a three-phase model to describe 4-NP concentrations in worm tissue. The three-phase model was adapted from Laskowski et al. (2010), as follows:

$$C_t = C_0 + \frac{k_u}{k_{e1-\lambda}} . C_{exp}.\left(1 - e^{-k_{e1}t}\right) \qquad for\ t \leq b \qquad (2)$$

$$\mathrm{C_t = A + \left[(C_0 + \frac{k_u . C_{exp}}{k_{e1} - \lambda}\left(e^{-\lambda . b} - e^{-k_{e1} b}\right)\right) e^{-k_{e2}(t-b)}\right]} \qquad \text{for } b < t < t_c \qquad (3)$$

$$C_t = C_f + A \times e^{-k_{e2}(t-t_c)} \qquad for\ t > t_c \qquad (4)$$

This model assumes a first phase described by the classic one-compartment model (eq. 2), where worms exposed to an initial concentration C_{exp}, with a decrease rate of λ, assimilates the chemical with a constant rate k_u which can (but may not) be partly balanced by the animal's ability for excretion of the toxicant at a rate k_{e1}, which gives at time *t* an internal body concentration, *Ct*, until the breakpoint, *b*, is reached, after which further 4-NP accumulation effectively stops. Following the breakpoint, *b*, the model assumes one first elimination phase (eq. 3), considering a second elimination constant rate (k_{e2}), leading to an equilibrium concentration, A. The third phase of final depuration is considered after transferring an animal to un-spiked medium, following the simple decay kinetics (eq. 4). The asymptotic final concentration after elimination, C_f, was also estimated. The bioaccumulation factors, BAF1 and BAF2, were determined as the ratio of k_u and k_{e1} and K_u and K_{e2}, respectively. The half-life ($t_{½}$) of 4-NP and elimination were calculated according to (eq. 5):

$$t_{1/2} = \frac{Ln(2)}{\lambda (or\ k_e)} \qquad (5)$$

Additionally, the biota-soil accumulation factors (BSAF) normalized for soil organic carbon and enchytraeid lipid contents were calculated as described by Shan et al. (Shan et al., 2010). BSAF was calculated at the breakpoint, b, (BASF1) and at equilibrium (BSAF2). For this we used an average dry weight:fresh weight ratio of 0.27 and a lipid content of 0.24 g g^{-1} fresh tissue (Holmstrup et al., 2014). All the statistical analysis was performed with IBM SPSS statistics, version 21 (IBM, 2012). All the statistical analysis was performed with IBM SPSS statistics, version 21 (IBM, 2012).

RESULTS AND DISCUSSION

Validity criteria were fulfilled, with less than 2% of mortality, indicating that the tested 4-NP concentration did not cause lethality during the experiment. No major changes occurred in soil pH ($CaCl_2$) from start until the end of uptake in spiked soil (pH 5.4 ± 0.1) and elimination in control (pH 5.7 ± 0.3), remaining within the optimum range of pH in natural soils where *E. albidus* are found (Jansch et al., 2005). Solid phase extraction followed by gas chromatography – mass spectrometry proved to be an appropriate method for tissue samples as shown in previous investigations (e.g. Patricio Silva et al., 2014; Patricio Silva et al., 2015), but also for soil analysis as tested here, despite the relatively high variability observed in soil data.

The three-phase model including degradation of 4-NP in soil fitted the data well (Figure 1; R^2 = 0.90), whereas a "classic" two-phase model gave a poorer fit and did not describe data as well as the three-pase model (R^2 = 0.70; fitted curve not shown). During the uptake phase, the concentration of 4-NP in worm tissue increased rapidly within the first 24 h (reaching a breakpoint), followed by a significant decrease (elimination) within the next 3 days to almost 50% of the concentration observed after 24 h, and then staying at the equilibrium concentration during the following 10 days (~44.7 mg kg^{-1} FW) (Figure 1).

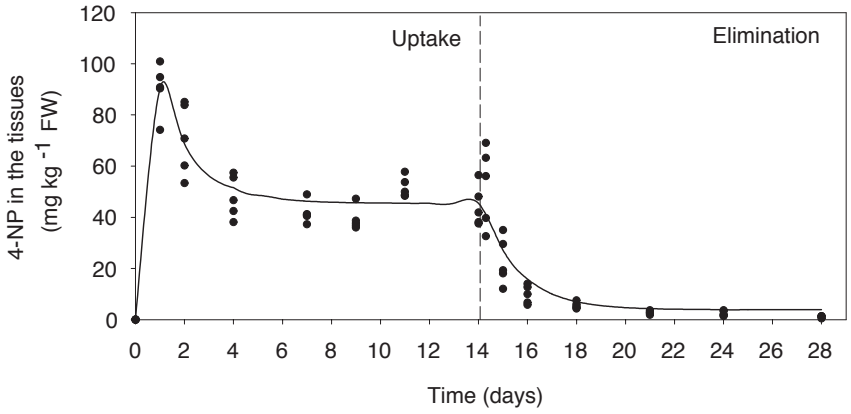

Figure 1: Concentration of 4-nonylphenol in tissue of *Enchytraeus albidus* during uptake phase (14 days) when exposed to 100 mg of 4-nonylphenol (4-NP) kg^{-1} dry soil and elimination phase (14 days) in un-spiked soil. Line represents the model fit to data.

Laskowski et al. (2010) observed a similar kinetics model as we did for 4-NP, when testing nickel in carabid beetles and earthworms, with a short phase of fast accumulation immediately after exposure, followed by partial elimination to an equilibrium concentration at a later stage of exposure, and by final elimination upon transfer to an uncontaminated food/soil – showing a three-phase kinetics. Therefore, same model was used to fit our data with the addition of the decay constant for soil concentration (Table 1) since 4-NP degraded during the test period; this is known to occur in natural soils with aeration (Soares et al., 2008). In the beginning of the uptake phase, the measured concentration of 4-NP in soil was 102.5 ± 14.3 mg kg^{-1} dry soil, decreasing to 45.0 ± 11.7 mg kg^{-1} dry soil after 7 days and to 22.6 ± 3.02 mg kg^{-1} dry soil after 14 days (Figure 2).

Table 1: Estimated parameters of the three-phase kinetic model as based on raw data (± SE). for 4-NP toxicokinetics in *Enchytraeus albidus* exposed to LUFA soil spiked with 100 mg 4-NP kg^{-1} dw.

Parameters	***Values***
C_0	0 [b]
K_u (mg kg^{-1} day^{-1})	0.970 [a]
K_{e1} (day^{-1})	1.470 [a]
K_{e2} (day^{-1})	0.665 ± 0.13
b (days)	0.952 [b]
A (mg kg^{-1})	44.7 ± 2.12
Cf (mg kg^{-1})	3.853 ± 1.023
C_{exp} (mg kg^{-1})	100 [b]
λ (day^{-1})	0.110 [b]
R^2	0.89
BAF1	0.7
BAF2	1.5
$t_{½}$ soil (day^{-1})	6.27
$t_{½}$ elimination phase 3 (day^{-1})	1.042

K_u= assimilation constant rate; Ke_1 and Ke_2 = elimination rates; *b* = time breakpoint; *A* = equilibrium concentration in the tissues; C_f = final concentration in the tissues; C_{exp} = initial concentration of 4-NP in the soil (100 mg kg^{-1} dw); BAF = Bioaccumulation factors; $t_{1/2}$ = half-life. [a] SE for some of the parameters was rather large due to the limited number of input data, [b]. fixed values.

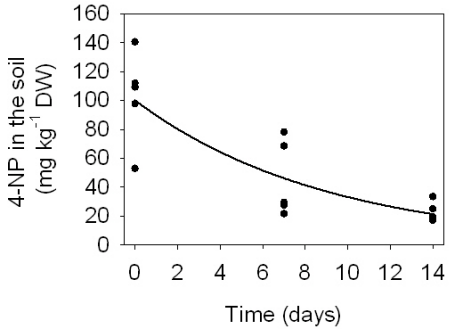

Figure 2: Concentration decrease of 4-NP in LUFA 2.2 soil during the uptake phase, when exposing *Enchytraeus albidus*. Line represents the model fit to data.

Data on 4-NP soil concentration during the uptake phase (3 time points) allowed to estimate a half-life of 6.3 days (Table 1), which is in agreement with the range observed by other authors in homogeneous sludge-soil mixture with half-life of 4-NP varying between 4 - 6 d (Hesselsøe et al., 2001; Jacobsen et al., 2004). However, increased half-life of 4-NP were reported in other types of natural soil, which is not surprising since 4-NP concentrations and persistence are highly dependent of various factors such as organic matter content (to which it binds strongly through non-specific lipophilic interactions), oxygen availability (soil aeration), microbial activity, temperature and pH (Amorim et al., 1999; Soares et al., 2008). Recent, and more detailed, studies have shown that degradation of different isomers of technical grade 4-NP does not happen at similar rates. The most common isomer (4-NP111) is degraded considerably slower than other isomers, and bound residues may persist for longer periods in the soil (Shan et al., 2011).

During the uptake phase, the concentrations of 4-NP observed in *E. albidus* tissues seemed to be similar to the soil concentration at each time-point, with a BAF ~ 0.7 – 1.5 (Table 1). The corresponding BASF was 0.26 after one day (i.e at the breakpoint, b) and 0.52 at equilibrium after 14 days. Patricio Silva et al. (2014) also observed that *E. albidus* exposed to 4-NP at +2ºC for 7 days had tissue concentrations similar to the soil concentration (BAF ~1). In both studies, the BAF values was lower than expected, considering the lipophilic nature (high hydrophobicity) of 4-NP and its high bioaccumulation demonstrated in aquatic invertebrates, like in the oligochaetas *Lumbriculus variegatus* and *Metaphire guillelmi*, and in other invertebrates such as *Mytilus edulis*, *Gasterosteus aculeatus* and *Crangon grangon* (Croce et al., 2005; Ekelund et al., 1990; Maenpaa and Kukkonen, 2006; Shan et al., 2010). The BAF of 4-NP in *E. albidus* is even lower when compared with BAF of other lipophilic compounds, such as phenanthrene and Lindane (with BAF values > 6), for the same species (Amorim et al., 2002a; Amorim et al., 2002b; Amorim et al., 2011).

The differences in uptake rates and BAFs between terrestrial *E. albidus* and benthic/aquatic oligochaetes are related, not only with the habitat (e.g. organic matter and organomineral contents), lipid content, but also with distinct mechanisms or uptake pathways. *L. variegatus*, for instance, possess cloragogen tissue that seems to store excretion products and to accumulate micropollutants, which could explain the lack of or low elimination of 4-NP during the uptake phase, and therefore explain the continuous increase in tissue concentration of 4-NP and its metabolites (Croce et al., 2005; Maenpaa and Kukkonen, 2006; Shan et al., 2010). In the present study we analysed only the parent compound, but not the metabolites emerging from detoxifying biotransformation. In the earthworm, *Metaphire guillelmi*, it was shown that bound metabolites of 4-NP constituted a much larger fraction than extractable 4-NP, and the resulting BSAF in this species was as high as 120 at equilibrium (Shan et al., 2010). It should therefore be noted that the BSAF calculated in the present study represents un-metabolised 4-NP only.

Detoxification of 4-NP involves induction of both phase I (e.g. cytochrome P450, carboxyl esterase) and phase II (e.g. glutathione-S-transferase) detoxification enzymes, some of which are induced by the toxicant itself (Hughes and Gallagher, 2004). It could be speculated that the detoxification and elimination mechanisms of *E. albidus* require some time to be activated and increased, and that this is indicated by the breakpoint, b, (occurring after one day). In un-spiked soil, the steep elimination phase took place within the first 48 h, during which a 75% reduction of 4-NP concentration in the tissues was observed (half-life for elimination: ~1 d, Table 1). After 14 days, concentration was lower than 2 mg kg-1 FW (Figure 2). A fast elimination response was also observed in *Metaphire guillelmi* within the first 24-48 h, after which it became slower, with earthworms still containing 51% of the initially accumulated total 4-NP residues (Shan et al., 2010), showing that the parent 4-NP may be rapidly eliminated, whereas bound residues may persist in the body for long periods.

The fast 4-NP eliminating response by *E. albidus* does not rule out potential long-term effects, e.g. in reproduction and survival, especially if combined with natural stressors such as low temperature, drought or salinity (Højer et al., 2001; Holmstrup et al., 2014; Patricio Silva et al., 2015). The presence of sub-lethal concentrations of 4-NP reduced the cold tolerance of *E. albidus* (Patricio Silva et al., 2014b), and affected negatively their reproduction in the presence of low levels of salinity (Patricio Silva et al., 2015). Therefore, detailed examination of the toxic effects of 4-NP and interaction with natural stressors (such as temperature) is relevant. Further, the formation of conjugates and bound residues are two pathways for the detoxification of xenobiotics in organisms (Hughes and Gallagher, 2004; Shan et al., 2010), thus the effect of biotransformation and metabolization of 4-NP in *E. albidus* or other soil organisms should also be addressed in future studies.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S. carried out data analysis. All authors wrote the paper.

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Chapter VIII: Supplementary Research I

Does salinity affect phospholipid fatty acid composition of the enchytraeid *Enchytraeus albidus*?

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SUMMARY

Our previous investigation revealed that a pre-exposure of 48 h to saline soil increased significantly the freeze-tolerance capacity of the supralittoral enchytraeid *Enchytraeus albidus*. In order to further understand the positive influence of salinity on cold tolerance of worms, we evaluated the potential effect of this abiotic factor on membrane composition of the organisms, a crucial adaptation to ensure survival at low temperatures. With this purpose, adult worms were exposed to non-saline (0‰ NaCl) and saline soils (50‰ NaCl, the highest salinity tested in our previous investigation) for one week at 2ºC. Results showed a significant influence of NaCl salt in some of the phospholipidic fatty acids, but without significant effects on the overall unsaturation index or on average fatty acid length. Therefore, salinity did influence membrane composition in *E. albidus* but apparently it was not enough to influence membrane physical characteristics such as fluidity, suggesting that other physiological traits play more important roles on cold tolerance of these worms. Further studies are recommended to enhance the understanding, e.g. combined effect of NaCl and temperature in a full factorial design, the inclusion of cholesterol concentrations as endpoint, or using other laboratory methodologies/measurements.

Keywords: Enchytraeids, salinity, phospholipid fatty acids.

INTRODUCTION

During frost exposures, the freeze-tolerant enchytraeid *Enchytraeus albidus* (Annelidae, Oligochaeta) accumulates glucose as main cryoprotectant, to levels that can sometimes reach 15% of total body dry weight (dw), corresponding to 200 mmol/l in body fluids, without known toxic effects (Slotsbo et al., 2008). These concentrations can be high enough to significantly decrease, by colligative effects, the amount of ice formed at ecologically relevant temperatures (Slotsbo et al., 2008), and to improve stability of protein and membrane function during freeze-induced cellular dehydration (Crowe et al., 1987). However, in the presence of salinity, the colligative effects of glucose seem to lose importance. One of our previous investigations showed that pre-acclimation to even modest salinities of soil water improved survival during freezing at low temperature considerably, by significantly lowering the melting point, and by decreasing considerably the amount of ice in their body fluids (Patricio Silva et al., 2013). The mobilization of glucose during freezing in saline soil was modest and became lower with the increase of soil salinity. The contribution of glucose to lowering the ice fraction was therefore negligible (<1%) whereas salinity drastically reduced the ice fraction. So, are the positive effects of salinity on survival of freezing based on other mechanisms than mere reduction of the ice fraction?

Changes in the membrane composition and properties represent an important factor in the adaptation to high salt concentrations and to low/frost temperatures as shown for halophilic and euryhalophilic bacteria (e.g. (Russell, 1989; Imboff and Thiemann, 1991; Srivastava et al., 2013), marine fish (e.g. (Cordier et al., 2002)), and for freeze-tolerant ectothermic organisms (Hazel and Williams, 1990), since the salt-stress and low-temperature stress seems to reduce membrane fluidity, which in turn leads to lethal osmotic (and ionic) shock and cell damaging (Hazel and Williams, 1990; Los and Murata, 2004). Membrane lipid composition is, therefore, important in controlling membrane fluidity and phase. Several factors are involved in the maintenance of proper membrane fluidity: the type of fatty acyl chains (their length and unsaturation), the amount of sterols and, to a lesser extent, the nature of the polar phospholipid head-groups (Russell, 1989; Hazel and Williams, 1990; Kostal, 2010).

The influence of salt stress on lipid composition and membrane fluidity has been studied predominantly in bacteria (e.g. Russell et al., 1995), yeast (e.g. Turk et al., 2004), and marine fish (Cordier et al., 2002) but nothing has been published on soil invertebrates. Hence, in the present study, we exposed *E. albidus* to non-saline (0‰ NaCl) and saline soils (50‰ NaCl) for 7 days, in order to understand what is the influence of shifts in salinity on membranes and how worms respond (adaptively).

MATERIAL AND METHODS

Test species

Enchytraeus albidus (Henle, 1837) were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany) in 2011, and cultured since then in agricultural (loamy) soil at 5 ± 1 °C. The worms were fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). Prior to experiments, the organisms were cold acclimated at 2 °C for 2 weeks.

Soil preparation and experimental setup.

All experiments were conducted with the natural standard soil LUFA 2.2 (Speyer, Germany). In short, this soil has *ca*. 6% clay, 17% silt, 77% sand and 4.4% organic matter. The pH (CaCl2) of LUFA soil is 5.5, which is within the optimum range of pH in natural soils where *E. albidus* are found (Jänsch et al., 2005). Salt spiking was performed using NaCl (99.5% purity, Merck, Darmstadt, Germany), added as aqueous solution to the dry soil in order to obtain the concentrations of 0 and 50‰ NaCl. Soil water content was 22 ml 100 g^{-1} dry soil, which is equivalent to 50% of the water-holding capacity.

Each replicate consisted of a test vial (3 cm height, 2 cm diameter) containing 5 g of test soil, 15 mg

oatmeal and 5 adult worms. Five replicates per salinity treatment were used. The vials were covered with a perforated lid to allow ventilation. Vials with worms were kept at 2°C for one week. After this period, survivors were scored, cleaned in moistened filter paper, pooled in 2 ml Eppendorf tubes, weighted, snap frozen at -80ºC. Prior analysis, samples were freeze-dried for 24 h.

Phospholipid fatty acids (PLFA) analysis.

Homogenization of worms' tissues, extraction and separation of PLFA were performed as described by Fisker et al. (2015). Identification and data analysis of PLFA was performed as described by Waagner et al. (2013).

Calculations and Statistical analysis

The mol percentage of each sample was determined by dividing the amount of each peak by the total amount of PLFAs recovered in each sample. The degree of unsaturation (UI) was calculated as: ∑ (% monoenes + 2 x % dienes + 3 x % trienes...)/100 (Kates, 1986).

A t-test was used to evaluate significant differences between 0 and 50‰ (NaCl) salinity, using the statistical software Sigmaplot for Windows Version 11.0 (Systat software Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

All the worms survived after one week of exposure to both soil treatments (non-saline and saline). A total of 19 fatty acids were identified in the PLFA fraction of all samples (Table 1).

Table 1: Molar percentage distribution of phospholipid fatty acid and calculated PLFA variables in *Echytraeus albidus,* after exposure to 0 and 50‰ NaCl in LUFA soil 2.2, for one week at 2ºC.

Phospholipids fatty acids (PLFAs)	Shorthand	0 ‰ NaCl			50 ‰ NaCl			*P value*
Tridecanoic acid, 12-methyl-, methyl ester	**C13:0**	**0.50**	**±**	**0.09**	**0.81**	**±**	**0.07**	**0.035**
Tetradecanoic acid methyl ester	C14:0	1.24	±	0.07	1.71	±	0.20	n.s.
Tetradecenoic acid methyl ester	**C14:1**	**0.48**	**±**	**0.02**	**0.79**	**±**	**0.08**	**0.032**
Pentadecanoic acid, methyl ester	C15:0	0.65	±	0.04	0.94	±	0.09	n.s.
Hexadecanoic acid methyl ester	**C16:0**	**1.31**	**±**	**0.06**	**0.96**	**±**	**0.02**	**<0.001**
Hexadecenoic acid methyl ester	C16:1	1.28	±	0.07	1.55	±	0.09	n.s.
Heptadecanoic acid, methyl ester	C17:0	0.33	±	0.02	0.33	±	0.02	n.s.
Octadecanoic acid, methyl ester	C18:0	7.90	±	0.27	7.87	±	0.11	n.s.
9-Octadecenoic acid (Z)-, methyl ester	**C18:1**	**3.07**	**±**	**0.03**	**2.07**	**±**	**0.06**	**<0.001**
cis-13-Octadecenoic acid, methyl ester	C18:1	1.62	±	0.04	1.72	±	0.09	n.s.
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	**C18:2**	**8.36**	**±**	**0.48**	**6.71**	**±**	**0.22**	**0.011**
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	g C18:3	1.16	±	0.12	1.06	±	0.06	n.s.
cis-11-Eicosenoic acid, methyl ester	C20:1	6.36	±	0.05	6.49	±	0.06	n.s.
cis-11,14-Eicosadienoic acid, methyl ester	C20:2	17.18	±	0.28	17.87	±	0.17	n.s.
Methyl 5,11,14-eicosatrienoate	**C20:3**	**0.47**	**±**	**0.01**	**0.36**	**±**	**0.01**	**0.002**
Methyl 8,11,14-eicosatrienoate	C20:3	0.54	±	0.06	0.48	±	0.05	n.s.
5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	C20:4	26.69	±	0.33	26.82	±	0.72	n.s.
cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester	C20:5	17.30	±	0.57	17.90	±	0.65	n.s.
cis-10-Nonadecenoic acid, methyl ester	C22:2	3.56	±	0.05	3.55	±	0.17	n.s.
Calculated variables	UI	2.71	±	0.01	2.71	±	0.01	n.s.
	SFA	11.92	±	0.02	12.62	±	0.01	n.s.
	MUFA	12.81	±	0.01	12.62	±	0.01	n.s.
	PUFA	75.27	±	0.01	74.76	±	0.01	n.s.
	LENGTH	19.34	±	0.02	19.32	±	0.03	n.s.
	UFA/SFA	7.39	±	0.11	6.94	±	0.22	n.s.

Significant effects of salinity are indicated in bold numbers. All values are mean ± sem (*N* = 5). UI is unsaturation index, SFA is Mol% saturated fatty acids, MUFA is Mol% mono-unsaturated fatty acids, PUFA is poly-unsaturated fatty acids, LENGTH is average number of c-atoms of the PLFA; UFA/SFA is the proportion of unsaturated fatty acids divided by the proportion of saturated fatty acids. n.s. is non-significant.

Six fatty acids; 18:0, 18:2, 20:1, 20:2, 20.4, 20.5, accounted for more than 83% of the total amount of PLFA. The long-chain unsaturated fatty acids, 22:n, 20:n and 18:n, were abundant whereas shorter chain fatty acids were much less abundant. The dominance of these PLFA corroborates the findings from Fisker et al., (2015) regarding *E. albidus*, and is also similar to what was observed in other earthworm species such as *Dendrobaena octaedra* (Holmstrup et al., 2007; Bindesbøl et al., 2009), *Lumbricus terrestris* (Albro et al., 1992), *L. rubellus* and *Eisenia nordenskioldi* (Petersen and Holmstrup, 2000).

Considering the effect of salinity per se, only the PLFA species 13:0, 14:1, 16:0, 18:1, 18:2 and 20:3 varied significantly between treatments. However, the change in PLFA did not result in a significant change in the unsaturated index (UI) or in a shortening of the average fatty acids length as

expected, which plays a significant role in homeoviscous adaptation in earthworms and enchytraeids (Holmstrup et al., 2007; Fisker et al., 2015). Such effect of salt on membrane composition is also common in marine fish (e.g. Cordier et al., 2002), bacteria (e.g. Imboff and Thiemann, 1991; Srivastava et al., 2013) and fungi species (e.g. Turk et al., 2004) to better cope with the changed turgor pressure, but without resulting necessarily in a change in fluidity, as also observed in our study. In contrast, some species such as the yeast *Candida membranefaciens* grown at high NaCl concentrations exhibited an increase in fatty acid unsaturation and in the content of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) (not object of our study), resulting in higher membrane fluidity (Khaware et al., 1995). Since hyperosmotic stress seems to reduce membrane fluidity similarly to low-temperature stress (as reviewed by (Los and Murata, 2004), some organisms (such as *C. membranefaciens*) must modify membrane composition to counteract a phase transition. In our study the salt-stress induced to *E albidus* has resulted in a change in membrane composition (not affecting necessarily its fluidity) to maintain homeostasis during osmotic (and ionic) stress.

Despite knowing that membrane cholesterol concentrations and the distribution among different phospholipid head-groups (e.g. phosphati-dylethanolamine or phosphatidylcholine, not the object of study on this investigation) may also influence membrane fluidity (Hazel and Williams, 1990), we are inclined to conclude that one week of exposure to high salinity (as single factor) did not influenced membranes fluidity, as initially hypothesized. Therefore, the role of salinity on the increased freeze-tolerance in *E. albidus* still relies on other physiological traits, such as increasing of osmolality and MP readjustments due to the passive influx of Na^+ and Cl^- ions across the body wall (Ramsay, 1949; Patricio Silva et al., 2013).

This study represents a small step to understand the influence of salinity on cold tolerance of invertebrates and its results provide us with further research question options. As future research directions we suggest to include for instance the combined effect of salinity with low temperatures, adding cholesterol concentrations as additional endpoint, and using other methodologies/measurements such as bending rigidity, scanning calorimetry and/or electron paramagnetic resonance spectroscopy measurements, which may give a more clear indication of the role of salinity on membrane characteristics of ectothermic organisms.

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Chapter IX: Final remarks

Artic, sub-arctic and cold temperate environments are being strongly affected by the intense anthropogenic activity and climate change. Despite a growing research focus on the effect of climatic change in organisms, the combined impact of contaminants on these environments remains poorly understood and documented. The findings reported in this thesis showed that organisms living in stressful or extreme environments, such as *Enchytraeus albidus*, might experience profound interaction effects between natural stress factors and between natural and chemical stressors, even considering non- to sub-lethal levels. The evaluation of "one stressor at the time" commonly used in the majority of studies are under and overestimating risks, and can lead to considerable environmental and/or economic undesirable consequences. Thus, the integration of "multistressors" approaches (natural x chemical; chemical x chemical) should be a priority in Environmental Risk Assessment (ERA) framework. Equally, the need of re-evaluation/adaptation of the standardized guidelines (e.g. OECD and ISO) commonly used in ecotoxicology can be important, especially when considering species that inhabit different types of environments. For instance, *E. albidus* is a model organism for ecotoxicological tests, (e.g. ISO, 2004; OECD, 2004; OECD, 2010). According to these guidelines, two of the experimental (optimal) conditions to apply are a temperature of 19-20ºC and a negligible concentration of salts (e.g. NaCl) in the soil. These test conditions are probably "sub-optimal" for *E. albidus* populations that live along the shoreline or/and in cold environments (e.g. Greenland and Iceland) (e.g. see chapter IV). This raises the question of whether the current risk assessment procedures and guidelines cover the risk in natural fluctuating environments when considering that there are several natural stressors, such as salinity and temperature, in soil ecosystems. In chapters II, III and IV, the positive influence of low-levels of salinity in *E. albidus* populations was observed, and the presence of such abiotic factor can change the effect of metals and organic contaminants on this species. Furthermore, in the standardized guidelines, the toxic effects are based mainly on sensitive biological indicators (e.g. behavior, mortality and reproduction), which lack in information regarding the underlying mechanisms of the observed stress response.

The research carried out during this PhD provides a step towards the development and integration of natural factors in ecotoxicology of cold-tolerant and euryhaline soil species. Using a series of experimental setups, and by analyzing various biological endpoints such assurvival/reproductionphysiological and biochemical responses (e.g. oxidative stress biomarkers, level of cryoprotectants, cellular energy allocation), the obtained results allowed the recognition of some of the key mechanisms and pre-requisites that are crucial to insure survival in harsh environments. However, the ecological realism of these investigations can be increased or complemented with the use of higher tier testing methods, such as model ecosystems, field and semi-field studies and mesocosms. The identification of key response mechanisms will bring new insight to establish strategies and methodologies of control/management/conservation of species.