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

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

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Abstract

Climate changes are expected to be greatest in the polar and temperate areas, where predictions points to an increase in freeze-thaw events, change 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/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/freezing-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. Since enchytraeids are of ecological significance in many important habitats along the Arctic 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

40 in the field, where interactions between factors are highly relevant. The
41 synergistic/antagonistic interactions identified in the present review may also represent a
42 stepping-stone in the evaluation and possible inclusion of natural factors, like cold and
43 salinity, in standardized enchytraeid test guidelines and consequently in risk assessment of
44 chemicals.

45 **Keywords:** Oligochaeta, enchytraeids, supralittoral, cold hardiness, salinity, contaminants.

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66 **1. Introduction**

67 Climate change is affecting natural ecosystems all over the globe. However, effects of
68 climate change are expected to be more drastic and rapid in the Arctic and in temperate
69 regions (Bates et al., 2008; Rhein et al., 2013). The frequency of freeze–thaw events in
70 subarctic soils are reported to increase, mainly due to the slight rise in the average
71 temperature that is leading to the reduction (or absence) of an insulating snow cover during
72 frost periods (IPCC, 2013). Frost periods are becoming shorter in duration, but more
73 frequent, with impacts on temperate regions (Bates et al., 2008). In parallel, the mean sea
74 level rise has significantly accelerated through the last two centuries, mainly due to
75 glaciers and ice sheets melting, inducing floods and causing shifts in precipitation,
76 evaporation and salinity patterns along the shoreline (Rhein et al., 2013). Therefore, littoral
77 and supralittoral ecosystems along the Arctic and temperate regions are among the most
78 challenging environments to soil organisms, where climate change may alter their
79 dynamics in time and space, and subsequent consequences for the functioning of these and
80 related ecosystems.

81 The climatic changes that lie ahead are superimposed by hazardous chemicals present in
82 nature. Several studies have shown that natural stressors can significantly modify the
83 response mechanisms of organisms to toxicants, and vice-versa (Noyes et al., 2009;
84 Holmstrup et al., 2010). Such combinations may be of concern if their effects interact
85 synergistically, i.e. with higher risk than the sum of the toxic and natural stressors
86 (Comins, 1983; Holmstrup et al., 2010). Therefore, knowledge of the biological,
87 physiological and biochemical limits of soil organisms to combined effects of natural and
88 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 due to their ecological relevance, distribution, plasticity and easy manipulation and maintenance. The Enchytraeidae family, commonly known as potworms or white worms due 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 since 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, since 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; Patrício Silva et al., 2013b; Fisker et al., 2014a; Fisker et al., 2014b; Patrício Silva et al., 2014) and as standard organisms in terrestrial ecotoxicology and risk assessment of chemicals (e.g. Römcke and Moser, 2002). Due to 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 of biological soil quality (Graefe and Schmelz, 1999; Didden and Römcke, 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, needs more attention.

The objective of this review is to synthesize the existing literature that addresses interactions between physical stressors and between natural 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

137 enchytraeids to cold environments and how these responses are affected by geography,
138 salinity, drought, food sources and contaminants. Following this, relevant studies
139 addressing the interactions between physical stressors and contaminants and the potential
140 of enchytraeids to acclimate to these new conditions will be discussed. A special emphasis
141 will be given to the supralittoral enchytraeid, *E. albidus*, because most relevant studies
142 have involved this species.

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2. Cold hardiness of enchytraeids

From the Arctic to temperate regions, soil invertebrates are often facing 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 (and 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).

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). Since 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 -4°C) in relatively dry soils (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003), or freeze tolerance if facing lower sub-zero temperatures (lower than -4°C) 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; Bauer et al., 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).

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 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; Patrício Silva et al., 2013b; Fisker et al., 2014a; Fisker et al., 2014b; Patrício Silva et al., 2014; Fisker et al., 2015).

2.1. Physiological and biochemical mechanisms in freeze tolerance

Importance of cryoprotectants

During cold/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 $\mu\text{g mg}^{-1}$ dry weight as observed in *E. albidus*, during 21 days of exposure to -14°C 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 (HVA) (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 UFA/SFA 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, that 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).

Metabolic depression, antioxidant defences and energy allocation

A decrease in temperature and, in some cases, the 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; Holmstrup, 2003; Slotsbo et al., 2008; Storey, 2012). Living in soil, these organisms are also likely to deal with reduced oxygen available (hypoxia), increased carbon dioxide (hypercapnia) and low nutrient availability and uptake (Storey and Storey, 2010). To ensure survival at frost temperature, enchytraeids tend to reduce consumption of energy stores, decrease cellular metabolic rate,

increase antioxidant defenses, and readjust the protein, carbohydrates and lipid budgets allocation (Patrício Silva et al., 2013a; Fisker et al., 2014a; Fisker et al., 2014b, Patrício Silva, 2015). Fisker et al. (2014b) recorded a metabolic depression (measured as CO₂ production) around 30 – 40% in *E. albidus* from Germany and Svalbard exposed to constant -2°C, 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 probably linked to sustained aerobic metabolism that was also underlined by oxygen consumption rates (Fisker, unpublished). 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 seven 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 (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, since 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 vs 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/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/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 +2°C to -4°C 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, 2015). When increasing the period and number of cycles (14 cycles, 3 days at 0°C followed by 3 days at -5°C) 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 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 on the above mentioned studies belongs to the same phylogenetic clade (and consequently, same species) based on the cytochrome oxidase I (COI) 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 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).

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 $\sim 1.03\text{--}1.29^{\circ}\text{C}$ 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 subzero temperatures, meaning that worms exposed under 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.

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 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 by Anchoroguy et al. (1987) and Crowe et al. (1987).

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's tolerance to freezing (Patrício Silva et al., 2013a).

The presence of salinity also influenced the PLFA composition (Patrício Silva, 2015) 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/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, when dry conditions meet extreme cold temperatures, very little is known. 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 only stressor (Maraldo et al., 2009). In freeze-intolerant enchytraeids, Bauer (2002) also found that the quantity of osmotically

active (frozen) water (OAW) was significantly reduced after acclimation at -3°C in cocoons of *Enchytraeus crypticus* and *Enchytraeus sp.* with consequent decrease of SCPs, probably due to the same reason as mentioned in the previous paragraph. The slow cooling rate of $1^{\circ}\text{C day}^{-1}$ 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 underline that, 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 5°C 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* might 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; Bauer, 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 might act as ice nucleators, and depending on their quality, might 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

Since freeze tolerance and freeze avoidance depends on the accumulation of cryoprotectants (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 (INAs) as well as anti-freeze proteins (AFPs) 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. Fisker et al., 2011; Holmstrup et al., 2010). Only recently, enchytraeids have been subject 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 -8°C, from 75 to 100% (Holmstrup et al., 2014). The opposite effects of 4-NP and PHE, might 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 (GUV) made from reconstituted phospholipid extracts of the worms, revealed that the interaction of PHE with the membranes/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/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, 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, 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 zinc (Zn) and mercury (Hg) decreased significantly the survival of *E. albidus* in a subsequent 7 d exposure to -4°C, 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

Due 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-nonylphenol (4-NP) and daily freeze-thaw cycles (FTC) (2°C to -4°C) caused stronger effects than combinations including constant freezing (Fig. 4).

The higher mortality observed in worms exposed to 4-NP and daily FTC 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 might be used as fuel to fight 4-NP toxicity and to restore the metabolic system during thawing. In addition, worms exposed to daily FTC 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/desorption processes of 4-NP. As a lipophilic compound, this organic surfactant partitions favourably to organic matter (Soares et al., 2008), and since 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.

Likewise, the high hydrophobicity properties of 4-NP ($\log K_{ow}$ 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 since 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

496 observed in the worms as a sign of 4-NP interference with membrane fluidity and cell
497 integrity.

498 To go deeper into this subject, Patrício Silva (2015) analysed the combined effect of 4-NP
499 and freezing on the energy basal levels and allocation. Results showed that worms exposed
500 to freeze-thaw cycles had higher energy consumption than worms exposed to constant
501 freezing, suggesting a possible higher investment of energy when shifting between freezing
502 and thawing events, which could also have contributed to increase worms' vulnerability
503 compared with frozen worms. On the other hand, worms exposed to continuous freezing
504 presented relatively stable and positive levels of energy available and low levels of energy
505 consumed, which is congruent with a decrease in metabolism that could have saved the
506 worms against the negative (toxic) effect of 4-NP. The interpretation of the data in terms of
507 cellular energy allocation was not so clear, indicating that its interpretation must be
508 carefully carried out and compared with the individual energy reserves and energy
509 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; Owojori et al., 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 mode 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. Since 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 since 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 vs saline), could contribute to a better understanding of this subject.

Since *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 1.5 to 8-fold changes in the effect of contaminants in the presence of low salinities, thus it may be argued that the

559 effects of salinity on toxicity should be acknowledged, although these results remain
560 within the applied uncertainty factor in terms of risk assessment of chemicals (Römbke and
561 Moser, 2002), and therefore revisions of the currently agreed enchytraeid standardized test
562 may not be necessary.

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5. Conclusions and perspectives

This review shows that interactions between environmental factors and between natural and chemical stressors are a common occurrence, which can take unexpected directions in enchytraeid populations. Furthermore, it also indicates that one natural 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, might 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 carbohydrates 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/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 the 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/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/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 assessments (e.g. Schlink and Ragas,

609 2011). This paradigm shift has implications for the way exposure and effects are assessed
610 and require a broader regulatory context than currently provided.

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Draft

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

Freeze-avoidance	VS	Freeze tolerance
Similarities		
Production of cryoprotectants (such as sugars and polyols) (Zachariassen, 1985)		
Stabilization of membranes (by changing composition of cellular lipids to increase membrane fluidity) and proteins (Bindesbøl et al., 2009; Crowe et al., 1987; Fisker et al., 2015; Hazel and Williams, 1990)		
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 (Ramløv, 2000; Zachariassen, 1985; Zachariassen and Husby, 1982; Zachariassen and Kristiansen, 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 an equilibrium, so that no freezing can occur (Holmstrup and Sømme, 1998).		Decrease the cellular metabolism to save energy (Calderon et al., 2009; Fisker et al., 2014a; Irwin and Lee, 2003; Storey and Storey, 1988)
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) (Lundheim and Zachariassen, 1993; Zachariassen, 1985)
		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)		Long-term medium/high risk of mortality during freeze-thaw events (Bale et al., 2001; Brown et al., 2004; Churchill and Storey, 1989; Marshall and Sinclair, 2011; Sinclair and Chown, 2005)
At sub-zero temperatures, vapor-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 Sjørnsen, 2001; Holmstrup and Sømme, 1998)		

3

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

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

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

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 $\pm$ 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.
63x48mm (300 x 300 DPI)

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 $\pm$ 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.
70x57mm (300 x 300 DPI)

Fig. 3. Glucose content of *E. albidus* exposed to soil with different salinities, during a decrease in temperature down to -2°C . The arrow marks the point in time when ice crystals were added to the soil surface to induce freezing. Results are shown as means $\pm$ 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

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64x47mm (300 x 300 DPI)

Fig. 4. Survival of *E. albidus* exposed to combined effect of 4-NP and three temperature regimes (constant +2°C, constant -4°C and daily freeze-thaw cycles) for 10 days. (*) Statistical significant differences compared to control temperature (2°C), per 4-NP concentrations, Dunnett, $p < 0.05$. Results are shown as mean $\pm$ standard error ($N = 15$) and described with a log-logistic concentration response model (2°C = solid line, -4°C = broken line, daily freeze-thaw cycles = dotted line). Adapted with permission from "Patrício Silva, A.L., Enggrob, K., Slotsbo, S., Amorim, M. J. and Holmstrup, M. Importance of freeze-thaw events in low temperature ecotoxicology of cold tolerant enchytraeids. *Environmental Science and Technology*. 2014. 48: 9790-9796". Copyright 2015 American Chemical Society. 75x63mm (300 x 300 DPI)

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 $\pm$ standard error (N = 5). Adapted with permission from "Patrício Silva, A.L., Enggrob, K., Slotsbo, S., Amorim, M. J. and Holmstrup, M. Importance of freeze-thaw events in low temperature ecotoxicology of cold tolerant enchytraeids. *Environmental Science and Technology*. 2014. 48: 9790-9796". Copyright 2015 American Chemical Society.

140x142mm (300 x 300 DPI)

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 $-4\text{ }^{\circ}\text{C}$ (A) or daily freeze-thaw cycles (B). Adapted with permission from "Patrício Silva, A.L., Enggrob, K., Slotsbo, S., Amorim, M. J. and Holmstrup, M. Importance of freeze-thaw events in low temperature ecotoxicology of cold tolerant enchytraeids. Environmental Science and Technology. 2014. 48: 9790-9796". Copyright 2015 American Chemical Society.
129x137mm (300 x 300 DPI)

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 $\pm$ standard error (N= 3-8). Adapted with permission from "Patrício Silva, et al. 2015. Copyright 2015 John Wiley & Sons, Inc. 218x513mm (300 x 300 DPI)