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# High temperature tolerance and heat hardening ability in *Enchytraeus albidus* Henle, 1837 (Oligochaeta) show no interaction with lipophilic organic pollutants

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## Abstract

High temperature stress poses various direct challenges for organisms on earth, but also indirectly affects the simultaneous tolerance to other stress factors. In this study we examined the heat tolerance, heat hardening ability and possible interactions between chemical stress and elevated temperatures in the enchytraeid *Enchytraeus albidus*. Specimens of *E. albidus* were exposed to chemicals and high temperature stress separately, after which they were exposed to a combination of the two stressors to test for a possible interaction. The chemicals used in this study were 4-nonylphenol and phenanthrene. Both of these chemicals reach the environment as a consequence of anthropogenic activities, and both have the potential to interact with elevated temperatures through effects on the cell membrane. We carried out dose-response experiments, including concentrations of the chemicals and high temperatures, having a significant negative effect on survival, however there was no interaction between the two types of stressors. Additionally we found that heat hardening improved survival significantly, but none of the chemicals had an effect on *E. albidus'* ability to heat harden. Thus, we found no evidence for the hypothesized negative interaction between increasing temperature and pollution with the chemicals used here.

Keywords Phenanthrene | Nonylphenol | Synergism | Thermal tolerance | Combination stress

## 1. Introduction

In nature, organisms are forced to deal with suboptimal conditions during most of their lifespan. Adverse environmental conditions might be caused by naturally occurring factors such as temperature and from chemicals being introduced into the environment as a consequence of human activities. The effects of these different stressors may sometimes interact synergistically (Holmstrup et al. 2010). Thus, the effects of a changing climate could be exacerbated by the interaction with pollutants, resulting in severe detrimental effects for a variety of organisms. Although climate models vary in their predictions, there is broad consensus that average temperatures will increase, but, perhaps more importantly, the variability in temperatures will also increase resulting in an increased intensity and frequency of heat waves (Bates et al. 2008). Thus, to realistically evaluate the effects of anthropogenic stressors it is relevant to look at combinations rather than single stressors.

In this study two chemicals were investigated: 4-nonylphenol (NP) and phenanthrene (PH). NP is an alkylphenol that occurs in the environment, where is derived from the anaerobic breakdown of alkylphenol polyethoxylates, which are used as surfactants in cleaning agents and pesticide adjuvants (Giger et al. 1984). NP accumulates in sewage sludge and may be spread into the terrestrial environment if sewage sludge is used as fertilizer in agriculture (Petersen et al. 2003). Since sewage sludge is an attractive food source for many soil invertebrates, these organisms may become particularly exposed to this chemical. NP is an amphiphilic compound


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with a log $K_{ow}$ of ca 4.2 and chemical properties which causes it to accumulate in the cell membrane. When large amounts are accumulated among the membrane lipids, NP likely affects the physical properties of the membrane by affecting the natural movements of the phospholipid molecules. PH is a Polycyclic Aromatic Hydrocarbon (PAH) with a log $K_{ow}$ of ca 4.6, mainly emitted from combustion and other high-temperature industrial processes (Newman 2010). The PAHs are hydrophobic and are therefore accumulated in dead organic material in the soil and in the cell membrane of the organisms that take up the chemical. Due to the persistence of these compounds, the background levels of the PAHs have increased significantly since the industrial revolution (reviewed by Wilcke 2000). PH exerts its toxicity by narcosis. Narcotic chemicals do not bind to specific receptors, but they can be toxic by affecting the fluidity and function of the cell membrane (Di Toro et al. 2000).

A main consequence of elevated temperatures is the disturbance of the homeostasis of the cell. Elevated temperatures affect the stability and integrity of proteins and cellular structures (Lindquist 1986) and will make the membrane more liquid, affecting its properties and correct functioning of the membrane (Hazel & Williams 1990). To counteract the damaging effects of such exposure, most organisms can initiate phenotypic changes (phenotypic plasticity), e.g. membrane modifications and/or the expression of protective stress proteins (Sørensen et al. 2003). There are tremendous benefits from this response in many organisms. It has been shown that many invertebrates increase survival, if they – before heat stress – have been exposed to moderately high (i.e. sublethal) temperatures (Feder & Hofmann 1999).

In this study we used the enchytraeid *Enchytraeus albidus* Henle, 1837 as a surrogate model for enchytraeids in general. Enchytraeids are important in terrestrial food webs as prey for other organisms and, due to their role as microbivores and detritivores, regulate the breakdown of dead organic material (Cole et al. 2000, Didden 1993). Enchytraeids can also in some cases have a direct effect on soil aggregate structure (Marinissen & Didden 1997). Soil dwelling organisms in general might be expected to have a low heat tolerance and/or be less selected for the ability to mount a plastic response, as they have evolved in a relatively stable environment (Bahrndorff et al. 2009a, Liefting & Ellers 2008, van Dooremalen et al. 2013). However, some species also occur in more thermally variable habitats. *E. albidus* is commonly found in decaying seaweed on beaches and in soils with high contents of organic material. Organic soils or patches of sea weed do provide some thermal insulation; individuals may therefore be exposed to moderately fluctuating temperatures in the field and thus be selected for both basal and induced high temperature tolerance. Because of the general importance of the ability to increase heat tolerance following exposure to sublethal high temperatures (i.e. heat harden), and since enchytraeids have never been investigated for this ability, we also examined the ability to heat harden and whether this ability was affected by the simultaneous exposure to the chemicals.

Effects of low temperatures have in previous studies been found to interact with effects of PAHs (Bindesbøl et al. 2009), but only few studies have examined heat stress in combination with either PAHs like phenanthrene or surfactants like 4-nonylphenol (Holmstrup et al. 2010). Exposure to chemicals at elevated temperatures may increase both uptake and detoxification rates due to increased metabolism, but elevated temperatures are also likely to increase microbial degradation of the chemical in the soil, which would probably lessen the exposure to the toxicant. Both chemicals are lipophilic and likely to influence the fluidity of the cell membrane if occurring in high enough concentrations (van Wezel & Opperhuizen 1995). Thus, if membrane property is an important part of heat tolerance, we hypothesize that the two chemicals will have an effect on heat tolerance. Furthermore, the chemicals might affect the ability to induce increased heat tolerance by heat hardening.

## 2. Materials and methods

### 2.1. Test animals

In this experiment, *E. albidus* obtained from a fish-food supplier (Büchner Zierfischfutter, Jena, Germany) in 2011 was used as the test organism. The animals were acclimated at 20°C in containers with LUFA 2.2 soil and fed seaweed for minimally two weeks before they were used in the experiments.

### 2.2. Soil and chemicals

A natural standard soil, LUFA 2.2 (LUFA, Speyer, Germany) was used for the experiments. The water content was in all cases 17.7% of soil dry weight. The soil had an organic carbon content of 1.87%, and it contained 6.8% clay, 12.6% silt and 80.6% sand. pH of the soil was approximately 5.5 and the water holding capacity was 44.4 g/100 g. Before use, the soil was sieved through a 0.9 mm sieve. Phenanthrene (99.5%, Aldrich, CAS no. 85-01-8) and nonylphenol (Technical grade, mixture of isomers, Aldrich, CAS no. 84852-15-3) were used as test chemicals.

### 2.3. High temperature tolerance

To test heat tolerance, the worms were exposed to a series of temperatures in pre-heated waterbaths. The worms were exposed in 1.5 ml Eppendorf tubes without soil and with a piece of wet filter paper to keep the worms from desiccating. This procedure was chosen because it is much easier to control the temperature in an Eppendorf tube than in a container with soil, and this procedure requires less post-treatment handling with the following risk of effects on survival. The worms were exposed for 2 h to 30, 31, 32, 33 or 34°C, respectively, using six replicate tubes (five worms in each) per temperature. After exposure to the elevated temperatures, the worms were carefully transferred to Petri dishes, containing wet filter paper where they recovered for 24 h at 20°C, before survival was assessed. Worms that were able to move around when stimulated were scored as alive.

### 2.4. Effects of heat-hardening

To test whether heat hardening the worms before heat stress had a positive effect on survival, the worms were exposed to a range of moderately increased temperatures before being exposed to a potentially lethal heat stress. These experiments generally followed the protocol described above. In a preliminary experiment, we tested the hardening effect at 28, 29 or 30°C to determine the optimal hardening temperature along with a corresponding handling control group kept at 20°C (preliminary data not shown). According to this result the hardening temperature for subsequent experiments was chosen to be 30°C. The hardening effect of this temperature was investigated at three different recovery periods (0 h, 2 h, 4 h) to assay the short-term heat shock hardening response. For each treatment and recovery time period, we used six replicate Eppendorf tubes with five worms each. The worms were hardened for 2 hours followed by a recovery period of different length at 20°C. After the recovery period, the worms were exposed to 32.5°C for 2 hours. After the heat stress the worms were transferred to Petri dishes and scored for survival as described above.

### 2.5. Effects of 4-nonylphenol and phenanthrene on survival

The two test chemicals were diluted in acetone, producing a stock solution corresponding to the highest concentration wanted. The stock solution and acetone was mixed with dry LUFA 2.2 soil, producing the requested concentrations. The soil intended for control received acetone only. After mixing the chemicals with the soil, the soil was kept in a fume cupboard for 24 hours, allowing the acetone to fully evaporate. The next day distilled water was added to each of the beakers with soil, producing a water content of 17.7 % of soil dry weight. The concentrations produced were: 0, 50, 100, 200, 300, 400, 600, 800 and 1000 mg/kg dry soil for NP and 0, 50, 100, 200, 300, 400, 600, 800, 900, 1000, 1200 and 1500 mg/kg dry soil for PH. The soil was divided into small containers with perforated caps, allowing ventilation. Five individuals and 5.5–6 mg soil were added to each container. Six replicates were exposed for 7 days at 20°C for each concentration, after which survival was tested by visual inspection as described above.

### 2.6. Effects of combination of chemicals and heat stress

To test for a possible interaction between either of the two chemicals and heat, the worms were exposed to one of the two chemicals, after which they were heat stressed. The worms were exposed to NP or PH for 7 days at 20°C. The concentrations chosen for this experiment were for NP 0, 250 and 400 mg/kg dry soil and for PH 0, 500 and 1000 mg/kg dry soil. These concentrations were chosen on basis of the dose-response curves produced from the preceding experiment and represent survival rates of ca 100, 75 and 50 %, respectively. The worms were exposed to the chemicals following the same procedure as in the previous experiment. After the worms had been exposed for 7 days, they were removed from the contaminated soil and transferred to Eppendorf tubes, again with a piece of wet filter paper. Half of the worms were heat stressed at 32.5°C for 2 hours, while the other half remained at 20°C and served as controls. After the heat stress, the worms were transferred to Petri dishes, allowing them to recover for 24 hours before cumulative survival to the combined treatments was tested.

### 2.7. Effects of combination of chemicals and heat hardening

To test if the two chemicals had an effect on the worms' ability to heat harden, the worms were exposed to either of the two chemicals and then heat hardened before being exposed to heat stress. First the worms were exposed to either of the two chemicals for 7 days at 20°C at similar conditions and concentrations as described above. In this experiment half of the worms were heat hardened, while the other half served as non-hardened controls. The hardened worms were first exposed to 30°C for 2 hours,

followed by a 2 hour recovery period. After the 2 hours, all the worms, including the non-hardened controls, were exposed to 32.5°C for 2 hours. Afterwards, all the worms were transferred to Petri dishes, and cumulative survival to the combined treatments was tested after 24 hours.

### 2.8. Data analysis

The effect of the elevated temperatures on survival was fitted to a generalized linear model (glm) assuming a binomial distribution and using a logit-link function (logistic regression) in R (http://www.r-project.org). The effect of the two chemicals on survival was modelled separately using the sigmoid dose-response function as in the previously mentioned temperature survival experiment. Associated lethal temperatures (LT, °C) or lethal concentration (LC, mg/kg soil dry weight) were calculated with standard error using the dose.p application for R. The effect of heat hardening was evaluated using a one-way ANOVA, and *post hoc* comparisons performed with Bonferroni t-tests. These, and all following data that were calculated as proportions, were Arcsin-sqrt transformed to improve equality of variances and normality. The effects of chemical and heat stress and the effects of chemicals on the heat hardening ability were tested using a two-way ANOVA. All analyses of variance and post hoc tests were performed using Sigmaplot 12.0 software (Systat Software Inc.).

## 3. Results

### 3.1. High temperature tolerance

Survival of the worms decreased when temperature increased (Fig. 1). The analysis showed a significant dose-response effect of temperature on survival ($z = -5.40$, $P = 6.8*10^{-8}$). The $LT_{50}$ was estimated to be 32.2°C (± 0.1) , and the $LT_{75}$ was estimated to be 32.5°C (± 0.1). On basis of the $LT_{75}$, 32.5°C was chosen as the stress temperature to be used in the subsequent experiments. A high mortality (75 %) was chosen to allow the potentially beneficial effect of heat hardening to be detected.

### 3.2. Heat hardening ability

Heat hardening at 30°C had a marked effect on subsequent heat stress survival (Fig. 2). The ANOVA showed a significant effect of heat hardening treatment ($F_{3,20} = 14.3$, $P < 0.001$) and post hoc analyses showed a significant effect of hardening at all recovery periods.

### 3.3. Effects of chemicals

The concentration of both chemicals negatively affected survival of *E. albidus* (NP: Fig. 3A, PH: Fig. 3B). The logistic regression analyses showed highly significant dose-response relation between concentration and survival (NP: $z = -9.4$, $P = 2*10^{-16}$; PH: $z = -7.39$, $P = 1.4*10^{-13}$). For NP the $LC_{25}$ was estimated to be 240.7 (± 22.4) mg/kg dry soil, and the $LC_{50}$ was estimated to be 402.0 (± 22.9) mg/kg dry soil. For PH $LC_{25}$ was estimated to be 518.1 (± 67.8) mg/kg dry soil, and $LC_{50}$ was estimated to be 1070.2 (± 91.7) mg/kg dry soil.

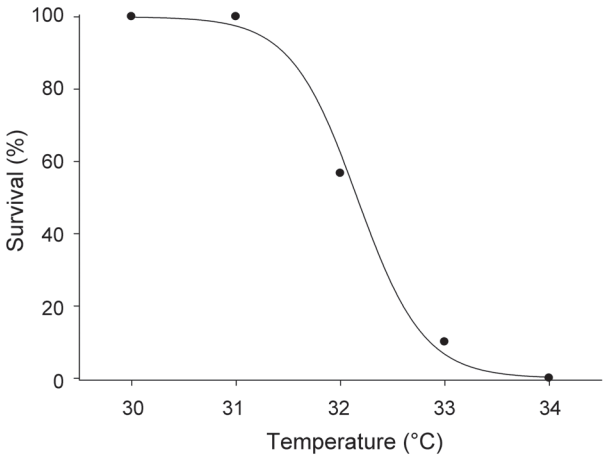


**Figure 1**. Survival of *E. albidus* exposed to a two-hour temperature stress at different temperatures. Each data point represents the average survival of 30 individuals. The curve depicts the fit of the logistic regression.

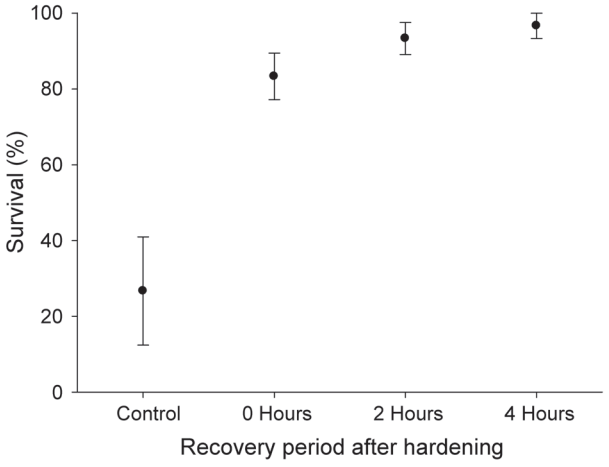


**Figure 2**. Effect of heat hardening on heat shock survival of *E. albidus*. Samples were exposed to a two-hour pre-treatment at either 20°C (control) or at 30°C (heat-hardening) before being exposed to a temperature stress at 32.5°C. Heat-hardened samples were allowed to recover at 20°C for 0, 2 or 4 hours, respectively, before being exposed to the heat shock treatment. The points represent mean survival (6 replicates of 5 individuals each) ± SEM.

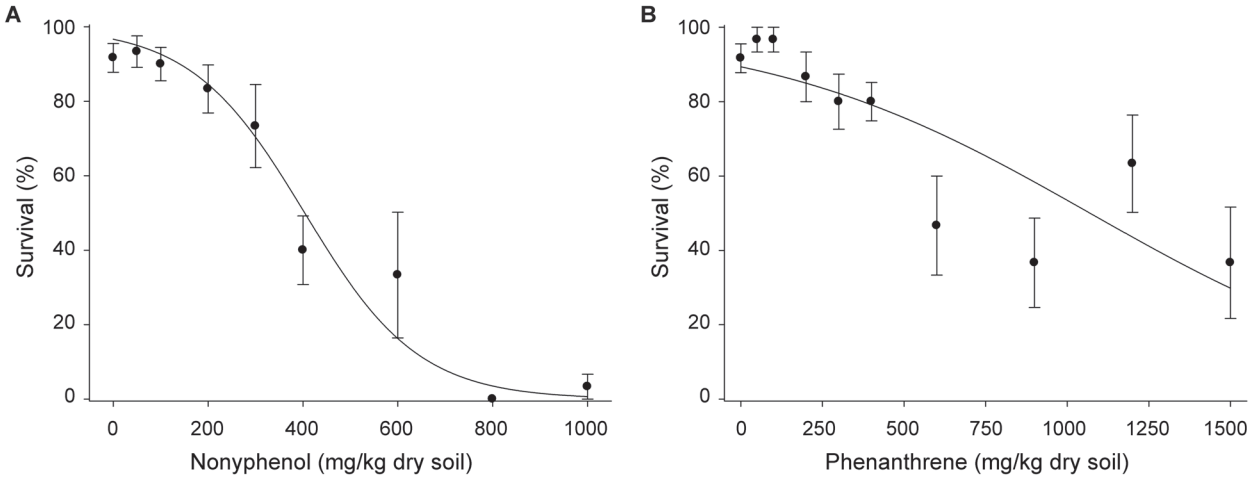


**Figure 3**. The effect of **A**: 4-nonylphenol (NP) and **B**: phenanthrene (PH) on survival of *E. albidus*. Animals were exposed in soil for seven days before scoring survival. The curve depicts the fit of the logistic regression, while points show the mean survival (± SEM) for N replicates with 5 animals each. N = 6, except for NP concentrations: 0, 200 & 400 mg/kg dry soil; and PH concentrations: 0 & 300 mg/kg dry soil, where N = 12.

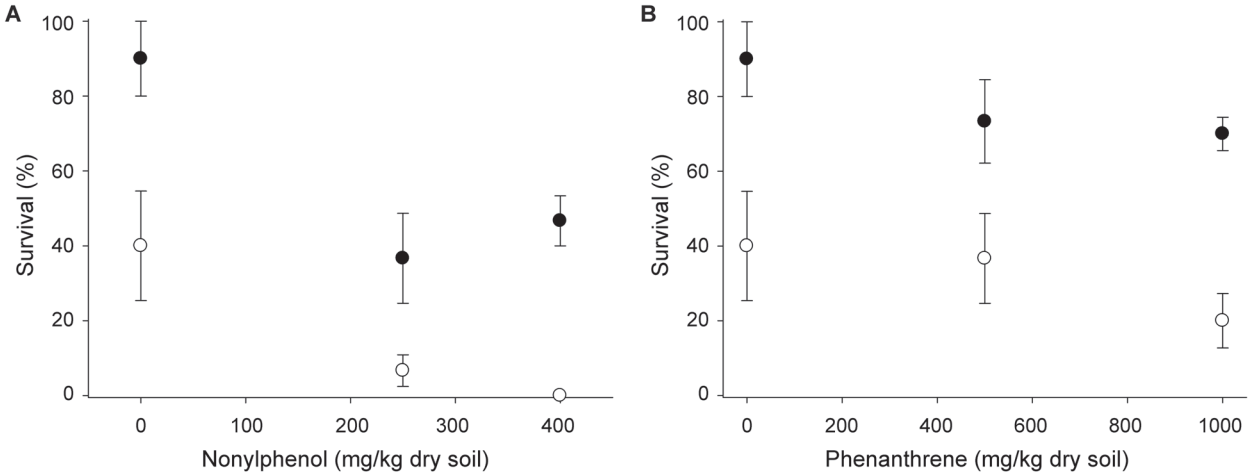


**Figure 4**. The effect of **A**: 4-nonylphenol and **B**: phenanthrene on survival of *E. albidus* exposed to heat stress and a control group. The results are presented as average survival of the combined treatments with corresponding standard errors. Black symbols represent control individuals, while open symbols represent heat stressed individuals.

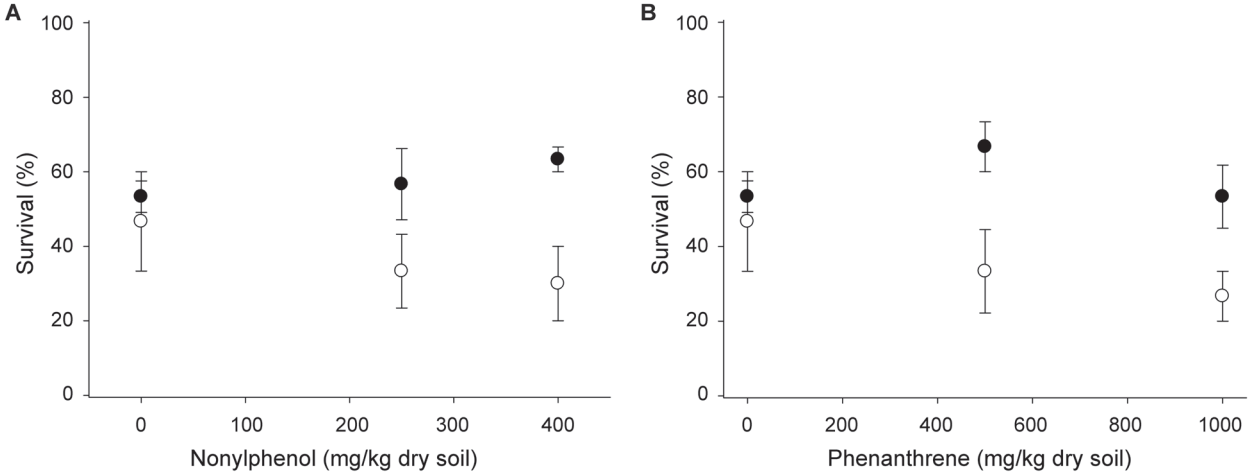


**Figure 5**. The effect of previous exposure to **A**: 4-nonylphenol and **B**: phenanthrene on survival of heat stressed (2 hours at 32.5°C) *E. albidus* who were either heat hardened before being exposed to heat stress (black symbols) or were directly exposed to heat stress without hardening (control; open symbols). The results are presented as average survival of the combined treatments with corresponding standard errors.

### 3.4. Effects of combination of chemicals and heat stress

When the combination of NP and heat stress was applied to the animals, both factors affected the survival negatively, with no apparent interaction (Fig. 4A,B). This was confirmed by the ANOVA analyses, which showed a significant effect of chemical (NP) ($F_{2,30} = 15.7$, $P < 0.001$) and heat stress ($F_{1,30} = 39.8$, $P < 0.001$), but no significant interaction between the two ($F_{2,30} = 1.2$, NS).

For the combination of PH and heat stress there was a similar pattern, although with a less marked effect of the chemical. The corresponding ANOVA analysis showed a significant effect of heat stress ($F_{1,30} = 28.9$, $P < 0.001$), but no significant effect of the chemical (PH) ($F_{2,30} = 2.3$, NS) or the interaction between the two ($F_{2,30} = 0.6$, NS).

### 3.5. Effects of chemicals on heat hardening ability

Survival increased when the worms were hardened before heat stressed (Fig. 5) compared to just heat stressed. The least effect of heat hardening was seen in controls, where the heat hardening showed unusually low survival (as compared to results for 2 hours shown in Fig. 2). Still, the ANOVA analyses showed for both chemicals a significant effect of hardening (NP: $F_{1,30} = 7.9$, $P = 0.01$; PH: $F_{1,30} = 8.7$, $P = 0.01$), no significant effect of the chemical (NP: $F_{2,30} = 0.1$, NS; PH: $F_{2,30} = 0.6$, NS) and no significant interaction between the two stressors (NP: $F_{2,30} = 0.8$, NS; PH: $F_{2,30} = 0.8$, NS).

## 4. Discussion

### 4.1. High temperature tolerance

The survival decreased steeply when temperature increased from 30 to 34°C. Worms died within a narrow temperature interval (31–33°C). At the lower temperature limits, the temperature-lethality curve was much less steep and increased over at least 15°C (Slotsbo et al. 2008). In general, when comparing different (insect) species there is great variation in the lower critical thermal limit, but less in the upper thermal limit (Addo-Bediako et al. 2000, Sgro et al. 2010). Even though there is variation between species from different habitats, the high temperature tolerance seems to be limited by similar physiological or biochemical processes among species. In accordance with this, the heat tolerance of *E. albidus* was found to be roughly similar to what has been reported for this species (Kähler 1970) and to that typically found for other soil dwelling oligochaetes of temperate environments (Lee 1985).

### 4.2. Effect of heat hardening

The survival of the worms increased significantly after exposure to hardening at moderate temperatures prior to heat shock in all recovery periods. This indicates that the worms must have a potent heat-shock response induced during the two-hour exposure. The survival seemed to increase slightly with increasing recovery time, and there might have been an even larger effect on survival, if the recovery period had been longer. Thus, this species showed a distinct heat hardening ability, and there is no indication that the evolution in this species has favoured a reduced plasticity of thermal tolerance. Bahrndorff et al. (2009b) found a much slower hardening response in the springtail *O. cincta*, where the maximum thermal resistance peaked 27 hours after hardening. *O. cincta* lives on the soil surface, where temperature fluctuations might be less thermally buffered than the soil or seaweed, which may represent the habitat of *E. albidus*. However, it is not known if the thermal conditions the two organisms are exposed to in their typical habitats are similar or differ. The dependency of the hardening phenotype of Hsp expression and recovery periods deserve further studies in *E. albidus*.

### 4.3. Effects of the two chemicals

Even though the two chemicals have similar log $K_{ow}$ [NP: 3.8–4.77 for various isomers (U.S. EPA 2005)] and PH: 4.6 (Sverdrup et al. 2002), the $LC_{50}$s were quite different (also on a molar basis), suggesting that NP is more toxic to *E. albidus* than PH, given that uptake kinetics are similar for the two chemicals. Gejlsbjerg et al. (2001) found a $LC_{50}$ of 420 mg/kg sludge-soil mixture when testing survival of *E. albidus* exposed to NP for six weeks at 20°C. This is very similar to the $LC_{50}$ of 402.0 mg/kg dry soil found in this experiment. Amorim et al. (2011) found a $LC_{50}$ of 135 mg/ kg dry LUFA 2.2 soil when exposing *E. albidus* to PH for two weeks at 20°C. This is a much lower $LC_{50}$ than the one found in this study. One week exposure instead of two may give a higher $LC_{50}$, because the worms would not have sufficient time to accumulate the chemical. However, this probably is not the main or only explanation for this large difference in $LC_{50}$-values. When examining the worms after exposure, the worms had a tendency to be on the walls of the container, instead of in the soil. The higher

the concentration of the chemicals was, the less frequent the worms were found in the soil. Amorim et al. (2008) found that *E. albidus*, because of chemoreceptors on their posterior segments, in some cases are able to avoid unfavourable conditions in the soil. It is therefore likely that the worms tried to avoid the spiked soil, by crawling out of the soil and onto the walls of the container. This avoidance from the soil together with a shorter exposure (1 week instead of 2 weeks) could possibly explain the higher $LC_{50}$ than the one found in earlier studies.

### 4.4. Effects of combination of chemicals and heat stress

In the experiment with chemicals and heat stress, there was a significant effect of the heat stress with either of the two chemicals. NP had a significant effect on survival as opposed to the control, but – contrary to what was expected – there was no interaction between the chemical and the heat stress. Jensen et al. (2009) investigated the effect of NP and high temperatures on the earthworm *D. octaedra* and found a synergistic interaction between the effects of the two stressors. We did not observe any interactions between heat stress and NP, nor did we observe this for PH. Our hypothesis that potential interactions between the two toxicants and high temperature could be mediated through effects on the cellular membranes could therefore not be confirmed. This suggests that interactions among chemicals and heat (observed by other authors) act through at least partly different pathways. Possibly, heat targets damage to proteins and cellular structures, while the chemicals target membrane properties. In the field an indirect interaction between chemicals and temperature might arise from a temperature-mediated change in bio-availability, however, this is beyond the scope of this study where chemical exposure was done at a benign (20°C) temperature.

### 4.5. Effects of combination of chemicals and heat hardening

In the experiments with a combined exposure to both a chemical and heat hardening before heat shock, there was a significant effect of hardening, but no significant effect of the chemicals or any interaction. In a study by Slotsbo et al. (2009), the springtail *F. candida* was able to heat harden; however, when the springtails experienced a previous exposure to sublethal concentration of $Hg^{2+}$, heat hardening failed to improve survival of heat shock at 34.5°C. The most important factor in the heat-hardening response is the production of Hsps, but Hsps can also be induced by other stress factors than heat (Lindquist 1986). As a consequence it is possible that the chemicals induced some production of Hsp, thereby giving the worms a small advantage over the control worms by increasing the Hsp production. Lee & Choi (2006) found that NP induces the production of Hsp in larvae of *Chironomus riparius*, while Nota et al. (2009) found phenanthrene to induce hsp70 in adult *F. candida*. We did not observe any significant effect of either of the toxicants on heat tolerance. In this experiment we had expected a more pronounced positive effect of heat hardening as observed in the first hardening trial. Compared to the initial hardening experiment, the survival could have been as high as 0.9 (see Figure 2). Future studies should aim at finding the optimal pre-treatment conditions maximizing the heat hardening effect and then use that in multiple stressor experiments. Despite these needs for exploration of the test design, our present study provides no evidence of an interaction between the two chemicals and heat stress or heat hardening. It is possible that even though NP and PH do influence membrane functioning, the factor that kills the worms is the denaturing of proteins or other injuries. Furthermore, based on the findings of Lee & Choi (2006) and Nota et al. (2009), both chemicals would be very interesting to examine again with respect to expression of heat shock proteins and genes, as both hardening and chemicals could affect the induction of Hsp.

In conclusion, we found that heat stress at temperatures above ~31°C had a significant negative effect on survival, but the survival of *E. albidus* increased significantly if the worms were heat hardened before being heat stressed. Thus, the high temperature tolerance was found to be moderate compared to temperate terrestrial insects and correspond to what is typically found for soil dwelling oligochaetes of temperate environments (Lee 1985). The two chemicals tested in this study, NP and PH, had $LC_{50}$s of 402 and 1070 mg/kg dry soil, respectively. Even though both heat stress and the chemicals had a significant effect on survival in the initial experiments, there was no apparent interaction between the two stressors. The same applies for hardening and the two test chemicals. Neither of the chemicals affected *E. albidus*' ability to mount a heat hardening response.

## 5. Outlook

Gejlsbjerg et al. (2001) found that the concentration of NP likely to be introduced into the environment from sludge is 0.1 mg/kg dry soil, and the worst case scenario would be 1.5 mg/kg dry soil, under the assumption that sludge is mixed homogeneously into the upper 15 cm of

soil. Even though enchytraeids and other soil invertebrates actively seek for and feed upon sludge lumps in soil, the likely exposure scenarios are probably not near the concentrations that were tested in this experiment, and they are not likely to directly affect survival of the worms. Still, the worms might suffer from sublethal effects, e.g on reproduction which is expected to occur at lower stress levels (Fasolo & Krebs 2004, Jørgensen et al. 2006, Zizzari & Ellers 2011). For example, Zizzari & Ellers (2011) investigated nonlethal effects of high temperature stress in the springtail *Orchesella cincta* and found that sublethal high temperatures lead to a decrease in the reproductive performance of males. Corroborating this, reproduction of *E. albidus* may already be affected at temperatures > 22°C according to the ISO guideline 16387 (ISO 16387 2004). Thus, even if naturally occurring soil temperatures and environmentally realistic concentrations of the chemicals do not often approach lethal limits, these factors alone or in combination might still have negative consequences for soil organisms.

Our study shows that enchytraeids do have adaptive phenotypic plasticity enhancing thermal tolerance, and that the effects of chemicals on both high temperature tolerance and heat hardening response can be tested in the laboratory. We suggest that future studies in this line could be rewarding if methods are also elaborated to assess effects on reproduction.

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