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Recovery period of *Folsomia candida* influence the impact of nonylphenol and phenanthrene on the tolerance of drought and heat shock

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ABSTRACT

Soil organisms are exposed to natural and anthropogenic stressors, such as xenobiotics. However, to simplify and make laboratory experiments easily reproducible, natural stressors are often excluded from ecotoxicological studies and risk assessment. This might underestimate the effect of chemicals, since synergistic interactions between chemicals and natural stressors might occur, creating a more severe impact than expected. Several studies have addressed simultaneous exposure to natural and chemical stressors, but very little is known of about the persistence of these interactions during recovery. Here, we examined if recovery after chemical stress exposure was important for the ability of springtails (*Folsomia candida*) to tolerate subsequent drought- and heat stress. Nonylphenol (NP) and phenanthrene (PHE) was tested and their isolated toxicity resulted in LC₅₀ values of 206 mg NP kg⁻¹ dry soil and 109 mg PHE kg⁻¹ dry soil in a 7-day test. Elimination of NP and PHE was rapid and only trace amounts remained in springtail tissues after 3-7 days of recovery. Isolated studies of drought and heat shock on *Folsomia candida* resulted in a lethal effect for 50% of the animals (LRH₅₀) at a relative humidity (RH) of 97.9%, and 190 minutes at 34° C was shown to be lethal for 50% of the test species (LT₅₀). The results showed, as expected, significant synergistic interactions between the effects of the chemicals and the effects of drought and heat stress. The negative effects of NP and PHE on the drought tolerance disappeared within 7 days post exposure. Springtails exposed to PHE also recovered their heat tolerance within 7 days post exposure, while NP exposed animals had not fully recovered their heat tolerance 14 days after exposure. Overall, a recovery period post chemical exposure was found to be very important for springtails in order to cope with natural stressors like heat and drought.

Keywords: Springtails; Multiple stressors; Synergy; Climatic Stress; Ecotoxicology.

Capsule: Exposure to hazardous substances phenanthrene and nonylphenol is shown to worsen the sensitivity of springtails to subsequent heat and drought stress, but a relative short recovery period of up to two weeks removed synergistic interactions between chemical and climatic stress, most likely due to an almost completely elimination of the chemicals within a week.

37 INTRODUCTION

38 In recent years, climate change has altered the precipitation patterns, increased mean temperature,
39 and enhanced the intensity and frequency of weather extremes. These trends will likely continue
40 and increase in the near future, resulting in prolonged and frequent heat waves and desiccation of
41 soils (IPCC, 2007; EEA 2017). Such stressors are hence a natural part of the challenging living
42 conditions influencing the performance of soil biota in their habitat. However, the soil biota is
43 evolutionary adapted to cope with these types of natural stressors by using several different
44 physiological defence mechanisms (Bayley and Holmstrup, 1999; Bahrndorff et al., 2009a, b;
45 Holmstrup et al., 2002; Menta, 2012). However, soil fauna also experience anthropogenic stressors
46 such as environmental contaminants that have the potential for interacting with the natural stressors
47 (Holmstrup et al., 2010). Multiple stressors present in the soil simultaneously, may result in a more
48 substantial and intricate effect on the soil fauna (Holmstrup et al., 2010; Laskowski et al., 2010).
49 Synergistic interactions between naturally occurring stressors and several toxic substances have
50 already been established (Højer et al., 2001; Sørensen and Holmstrup, 2005; Skovlund et al., 2006;
51 Schmidt, 2014; Sjørnsen et al., 2001a; Slotsbo et al., 2009). Slotsbo et al. (2009), for example, found
52 a highly significant reduction of the heat tolerance in springtails after exposure to sublethal
53 concentrations of mercury. If combining sublethal exposure of mercury and heat, about 65%
54 mortality was observed at levels where no mortality were found if exposed to a single stress factor.
55 These interactions are particularly likely to influence soil-dwelling organisms living in agricultural
56 soils, where substantial amounts of chemicals are deposited for example in the form of sewage
57 sludge, fertilizers and pesticides. Furthermore, the agricultural practices have the potential to
58 modify the distribution of moisture retention and heat in cultivated soils compared to undisturbed
59 soils, making soil fauna living in arable soils even more exposed to heat and drought (McLaughlin
60 and Mineau, 1995; Ross and Malcolm, 1982).

61 The Organization for Economic Co-operation and Development (OECD) has provided several
62 ecotoxicological guidelines, used as terrestrial standard tests. These tests are highly simplified and
63 easily reproducible, but lack the complexity of field conditions (European Commission, 2002). The
64 targeted exclusion of natural stressors may underestimate the toxicity of chemicals to soil fauna
65 under field conditions (Amorim et al., 2012), emphasizing the need for further investigation of
66 interactions between effects of natural stressors and hazardous chemicals, and their potential
67 implementation in risk assessment. Several studies have investigated the interactions between
68 natural and chemical stressors, as listed above. However, only a few of these have exposed

organisms simultaneously to both types of stressors. Furthermore, there are still many relevant combinations of interactions that remain relative un-investigated due to the vast complexity of actual field conditions. For example, the importance of sequence in the exposure to stressors, and in particular long-lasting effects during recovery from an exposure have been only little studied (Holmstrup et al., 2010; Hooper et al., 2013).

Most scientific investigations addressing recovery are field studies showing the recovery of different communities after major disturbances or long-term exposures (Antunes et al., 2009; Maute et al., 2017; Lindberg and Bengtsson, 2005). A few studies have focused on the recovery of particular species exposed to a natural or a chemical stressor (Maraldo and Holmstrup, 2009; Nunes et al., 2017; Vázquez et al., 2016). However, to the knowledge of the authors, no studies have yet investigated the importance of the recovery period in-between different stressors. Springtails (Collembola) are widely abundant in the soil environment, influencing the nutrient cycle by their function as secondary decomposers (Barrios, 2007; Filser, 2002). As many other soil dwelling species, the springtail *Folsomia candida* Willem, inhabits the top soil, which is highly affected by temperature and moisture fluctuations (Fountain and Hopkin, 2005), and it is therefore a suitable model species for multiple stressor studies.

This study aimed to investigate the effect of a recovery period on the interaction between an environmental contaminant and a natural stressor using *F. candida* as a model species. In four separate experiments we analysed the interaction between effects of nonylphenol (NP) or phenanthrene (PHE) and heat or drought stress, respectively, and how the interaction was influenced by recovery time after exposure to each of the contaminants. Exposure concentrations was chosen with the aim to produce a moderate chemical stress, i.e. 20% effect. Such exposure concentrations are still markedly above environmental concentrations likely to be found after e.g. normal sludge application (González et al., 2010). We hypothesized that previous exposure to these chemicals would lower the tolerance of subsequent exposure to drought and heat shock, respectively, resulting from synergistic interactions. Further, we hypothesized that the inherent drought and heat stress tolerance of *F. candida* would gradually be restored when exposure to environmental contaminants was terminated.

MATERIALS AND METHODS

101

102 **Test species**

103 *Folsomia candida* were cultured in Petri dishes (9 cm diameter) with a substrate consisting of
 104 plaster of Paris and charcoal in a 8:1 ratio. The culture was kept at 20 ± 1 °C with a 12:12 hour
 105 light:dark photoperiod. On a weekly basis, the springtails were fed dried baker's yeast *ad libitum*
 106 and water was added to the dishes to secure humid conditions. Each Petri dish contained
 107 approximately 100 animals to reduce negative effects of high density on growth rate. Eggs and
 108 smaller animals were regularly removed to maintain a constant density in each dish. The springtails
 109 were not synchronized according to age before the experiment, due to the significant higher work
 110 load this would have necessitated. However, in order to minimize variation in individual responses
 111 only large mature adults of roughly the same size were used, i.e. mean ($\pm$ SD) fresh weight of adult
 112 springtails of 0.150 ± 0.025 mg ($N = 23$). The relative narrow range in size of test species resulted
 113 in standard deviations not unlike the ones observed in ecotoxicological testing with synchronised
 114 animals.

115

116 **Experimental soil**

117 A pesticide free Danish soil from Askov was used for the experiment. The soil was dried at 80° C
 118 for 24 hours and sieved through a 2 mm mesh screen prior to use. The Askov soil is a sandy loam
 119 with the following particle distribution: coarse sand (200-2000 μ m) 38.4%, fine sand (63-200 μ m)
 120 23.6%, clay (2 μ m) 13.0%, fine silt (2-20 μ m) 12.3%, coarse silt (20-63 μ m) 10.0%. The humus
 121 content of the soil was 2.8%, and the total content of organic carbon was 1.6%. The soil pH (pH
 122 H₂O) was 6.2, the soil density was 1.135 (g cm⁻³ dry soil), and the total cation exchange capacity
 123 (CEC) was 8.14 meq (100 g)⁻¹ (Jensen and Sverdrup, 2002).

124

125 **Toxicity of nonylphenol and phenanthrene**

126 Nonylphenol (4-nonylphenol; NP) was obtained from Sigma Aldrich (CAS No. 104-40-5, 100%
 127 pure). Phenanthrene (PHE) 98% purity was obtained from Sigma Aldrich (CAS No. 85-01-8). NP
 128 and PHE were dissolved in acetone (J.T. Barker, Hayward, CA, HPLC quality) creating stock
 129 solutions corresponding to the highest test concentrations, and then diluted to obtain six soil
 130 concentrations in the range 80-280 mg NP kg⁻¹ dry soil (based on Højer et al., 2001) and 40-160 mg
 131 PHE kg⁻¹ dry soil (based on Sverdrup et al. 2001). Five mL of the different solutions were mixed
 132 with 25 g of the dried and sieved soil, and added to plastic containers (4.7 cm high, 7.2 cm

diameter). Pure acetone was used as controls. The soil was thoroughly mixed with the acetone solutions to ensure even distribution of the chemicals throughout the soil. The containers were placed under a fume hood overnight and stirred once again to evaporate the acetone completely. Long-time in-house experience from numerous studies have verified that no toxicity of solvent is remaining after this procedure eliminating the need of a water-only control. After 24 hours, 5 mL water was added to each container. The water and soil was thoroughly mixed giving a soil water content of 20% of dry weight, which approximately corresponded to 50% water holding capacity. Ten springtails were transferred to each container, representing one replicate (four replicates at each concentration), and the beakers were covered with lids having small holes to allow ventilation. The containers were held in a temperature-controlled room at a temperature of 20 °C with a 12:12 h light:dark cycle. After 7 days, springtails were extracted by flotation in tap water. The springtails of each container were transferred to a separate small water saturated Petri dish and allowed to recover for 24 hours at 20 °C before survival was scored. When counting, dead individuals of *Folsomia candida* was defined as not moving freely when agitated, animals only moving antennas or legs were counted as dead (Sjursen et al., 2001b).

Uptake and elimination of nonylphenol and phenanthrene

The experiment included a 7-day uptake phase followed by elimination for 7 (PHE) or 8 days (NP). At the beginning of the test, groups of 20 adult springtails were selected at random and introduced into each of 30 test vessels (250 mL glass beakers) containing 25 g of moist soil spiked with either 80 mg NP kg⁻¹ or 100 mg PHE kg⁻¹ dry soil. Containers were covered with parafilm with a few holes for aeration and incubated in a climate room at 20 °C, with a 12:12 h light:dark cycle. Springtails were sampled at days 0 (control without NP or PHE), 1, 3, and 7 for the uptake phase. After the 7-day uptake phase, springtails of the remaining beakers were extracted by flotation and placed in clean Petri dishes for the elimination phase. Springtails exposed to NP were then sampled after 6 h, 1, 2, 3, and 8 days during the elimination phase. Springtails exposed to PHE were sampled after 1, 3, and 7 days during the elimination phase. During the uptake phase springtails were collected from soil by flotation in tap water and briefly placed in clean Petri dishes. Shortly after this (<5 min) they were placed in pre-weighed 2-mL Eppendorf vials, weighed and frozen at -80 °C. During the elimination phase, animals were sampled directly from Petri dishes and weighed. Fresh weight was determined using a Sartorius Micro SC 2 balance accurate to ± 1 µg (Sartorius AG, Goettingen, Germany).

The contents of PHE in springtail tissues was determined after extraction in acetonitrile followed by GC-MS analysis as described in details by Holmstrup et al. (2014). The contents of NP was determined after extraction in 70% ethanol followed by glycation, solid phase extraction and finally analysis by GC-MS. The details of the analytical methods are described elsewhere (Holmstrup et al 2014).

Drought tolerance

Based on the results of Højer et al. (2001), the springtails were exposed to relative air humidities (RH) ranging between 97.1 and 99.9% RH representing realistic soil air humidities found during a severe summer drought (Hillel, 1998). Aqueous NaCl solutions in the range 1.0 - 51.7 g L⁻¹ were used to create the desired RH in small vials ("drought chambers"), where the springtails were kept (Holmstrup 1997; Holmstrup and Bayley 2013). Briefly, ten springtails were placed in open-top plastic vials (3 cm high, 1.6 cm diameter) that was covered with 100 µm nylon net preventing escape of the springtails. These vials were glued to the floor of 300-mL plastic cups (4.8 cm high, 9.3 cm diameter). About 35 mL NaCl solution was added to the outer vial, which was then closed with tightly fitting plastic lids with rubber sealing. The air in this small closed system rapidly equilibrates with the salt solution (following Raoult's law) and creates the desired drought level (Bayley and Holmstrup, 1999). Four replicates of each of seven RH levels were used in the experiment. The experiment proceeded over a period of 7 days, and the samples were kept at 20 °C in a Styrofoam box to avoid large temperature fluctuations. After the exposure period, the springtails were transferred to water saturated Petri dishes for 24-hours. After rehydration, surviving individuals were counted, based on their ability to move freely when agitated.

Heat shock tolerance

Ten springtails were transferred to a 2 mL Eppendorf tube (four replicates (tubes) per treatment) and exposed to a constant temperature of 34.0 °C by submerging the tubes in a water bath accurate within ± 0.1 °C (Lauda ECO RE 1225; Pharmacia Biotech AB, Uppsala, Sweden). Groups of springtails were exposed to heat for increasing periods of time (0 to 4 hours). After the desired heat exposure period, springtails were immediately transferred to water saturated Petri dishes kept at 20 °C and allowed to recover for 24 hours before assessment of survival.

The influence of post-chemical exposure duration (recovery during elimination phase) on heat and drought tolerance

Springtails were exposed for seven days in soil to a single concentration of NP or PHE as described above. Based on dose-response curves we chose concentrations of 138 mg NP kg⁻¹ dry soil and 101 mg PHE kg⁻¹, respectively, since these concentrations were predicted to reduce survival by ca. 20% relative to controls (Table 1). A targeted 20% effect was chosen as a moderate stress level. After exposure to the chemicals in soil, the surviving springtails were collected by flotation and placed on clean Petri dishes to recover from the chemical stress (elimination phase). Control springtails were exposed in un-contaminated soil, but otherwise treated as described. At increasing recovery periods (1, 3, 7 and 14 days) the springtails were exposed to either drought or heat stress, testing the effects of the chemicals on the tolerance towards these natural stressors. For heat stress at 34 °C we used a Lethal Time causing approximately 20% lethality (LT₂₀; 164 minutes at 34 °C; Table 1). Control treatment for heat stress used exposure to 20 °C in Eppendorf tubes. For drought stress we used a Lethal RH causing approximately 35% lethality (LRH₃₅; 98.2% RH; Table 1). Control treatment for drought exposure used 99.9% RH. These treatments were chosen to represent a moderate effect of heat and drought stress. At each of the designated recovery times post chemical stress (NP and PHE, respectively), ten randomly selected springtails (in four replicates) were exposed to each combination of stressor and control treatments using a 2×2 full-factorial test design.

Statistical analysis

The dose-response relationships of survival with contaminants and natural stressors were analyzed by non-linear regression fitting data ($N = 28-32$) to a four-parameter logistic regression model:

$$(1) \quad y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b},$$

where y is the survival, x is the intensity of the stressor and a , b , x_0 , y_0 are constants. The fitted model was used to estimate the LC-values (LC₂₀, LC₅₀), LT-values (LT₂₀, LT₅₀) and LRH-values (LRH₃₅, LRH₅₀). Data for uptake and elimination of contaminants were subjected to one-way ANOVA. The elimination kinetics were analysed by non-linear regression fitting data ($N = 16-24$) to an exponential decay model:

$$(2) \quad y = y_0 + ae^{-bx},$$

Where y is the internal concentration of the contaminant and x is time.

The effect of recovery time and chemicals (NP and PHE, respectively), and their interaction, were analyzed by two-way ANOVA and *post hoc* Holm-Sidak pairwise comparisons corrected for multiple testing. A significance level (α) of 0.05 was used in all analyses. All analyses were performed using Sigmaplot 12 (Systat Software, Inc.).

RESULTS

The effect of NP and PHE

Results showed 100% survival in all controls, but survival decreased significantly with increasing concentrations of PHE ($P < 0.0001$; $R^2 = 0.98$; Fig. 1a) and NP ($P < 0.0001$; $R^2 = 0.87$; Fig. 1b). Interpolated values of LC_{20} and LC_{50} for NP and PHE are shown in Table 1. The shapes of the fitted logistic functions for NP and PHE indicates different response pattern by *Folsomia candida*. NP caused an almost linear decrease in survival with increasing concentrations, whereas PHE has a sigmoidal curve with a very sharp decrease in survival above ca. 100 mg PHE kg^{-1} .

Table 1. Lethal concentrations (LC) of nonylphenol and phenanthrene (mg kg^{-1} dry soil) for *Folsomia candida* when exposed in spiked soil for 7 days. Lethal relative humidity (LRH) of drought (% relative humidity) and lethal duration (LT) of heat shock (minutes) at 34° C for *Folsomia candida* when exposed 7 days. Values are given with 95% confidence intervals (N = 28).

Stressor	LC_{20}	LC_{50}	LRH_{35}/LT_{20}	LRH_{50}/LT_{50}
Nonylphenol	133 [96; 160]	206 [186; 224]	-	-
Phenanthrene	100 [98; 103]	109 [107; 111]	-	-
Drought	-	-	98.1 [97.9; 98.4]	97.9 [97.7; 98.1]
Heat shock	-	-	141 [120; 157]	190 [177; 203]

The effect of drought and heat stress

Results showed 100% survival in all controls, but survival decreased significantly with increasing drought stress ($P < 0.0001$; $R^2 = 0.81$; Fig. 1c) and heat stress ($P < 0.0001$; $R^2 = 0.87$; Fig. 1d). Values of LT_{20} and LT_{50} for heat stress, and LRH_{35} and LRH_{50} for drought stress, are shown in Table 1.

Uptake and elimination of PHE and NP

The body content of PHE in springtails increased steadily and significantly throughout the uptake phase, and decreased rapidly during the elimination phase ($F_{32,6} = 148.8$; $P < 0.001$; Fig. 2a). The half-life of body PHE content was about one day during the elimination phase, and after 7 days, only trace amounts were detected.

NP body content increased rapidly during the uptake phase and also decreased rapidly in the elimination phase ($F_{33,8} = 31.74$; $P < 0.001$; Fig. 2b). During the uptake phase, steady state at a body NP content of ca. 0.08 mg g^{-1} dry weight was reached already after one day. Elimination of NP took place at an even higher rate than seen for PHE. Already after 6 hours, body NP was reduced by 75%, however, a low body NP content remained throughout the elimination period.

Influence of recovery time on interactions between effects of chemicals and natural stressors

The survival in all control treatments was in all cases 98-100% (data not shown). Thus, survivors of PHE and NP exposed springtails, and springtails exposed in un-contaminated soil, had practically full survival in the respective control treatments to heat exposure (164 minutes at 20°C) and drought exposure (7 days at 99.9% RH). The statistical analysis of the effects of chemicals on tolerance of heat and drought, respectively, only included effects of the chemical and recovery time.

The heat treatment alone resulted in 60-80% survival, which was in some cases slightly more than the target LT_{20} value (Fig. 3a and Fig. 4a). Both PHE and recovery time had highly significant effect on heat survival, and there was a significant interaction between the two (Fig 3a; Table 2). Comparisons within recovery times showed that the negative effect of PHE gradually was reduced over time, as significant effects were found after 1 and 3 days, but not after 7 or 14 days recovery (Fig 3a). In the NP-heat experiment, both NP and recovery time had highly significant effect on survival, and there was a highly significant interaction between the two (Fig. 4a; Table 2). Comparisons within recovery time showed that the negative effect of NP persisted throughout the 14 days of recovery (Fig. 4a).

The drought treatment alone resulted in 70-80% survival, which roughly corresponds to the target LHR_{35} value (Fig. 3b and Fig. 4b). While PHE had a negative effect of drought survival, recovery time had a positive influence (Fig. 3b; Table 2). Comparisons within recovery time showed that the

negative effect of PHE was significant after 3 days, but not after 1, 7 or 14 days of recovery (Fig 3b). Regarding NP, significant ($p<0.05$) difference in drought tolerance between exposed and non-exposed groups was only observed after one day of recovery. Overall, NP had a weak, but not statistically significant ($p=0.099$) effect on drought survival, whereas recovery time had a significant ($p<0.05$) effect (Fig. 4b; Table 2).

Table 2. Analysis of variance statistics for survival of *Folsomia candida* exposed to drought (7 days at 98.3% Relative Humidity) or heat (164 minutes at 34 °C) following exposure to 4-nonylphenol (NP) or phenanthrene (PHE), respectively. Analysis of the effect of contaminant (NP or PHE), recovery time (RT) and their interaction are shown.

Parameter	Source	DF	Sum of squares	F-value	Statistical significance (p-value)
Survival of drought	NP	1	1374	2.89	0.099
	RT	3	6579	4.612	0.009
	NP×RT	3	3951	2.771	0.058
	PHE	1	2822	6.846	0.013
	RT	3	8088	6.54	0.001
	PHE×RT	3	1602	1.296	0.293
Survival of heat	NP	1	16402	84.115	<0.001
	RT	3	6028	10.303	<0.001
	NP×RT	3	3568	6.098	0.002
	PHE	1	4000	31.068	<0.001
	RT	3	9140	23.663	<0.001
	PHE×RT	3	3500	9.061	<0.001

DISCUSSION

The recovery period in-between stressors

The present study highlights the importance of recovery between multiple stressor incidents. To the knowledge of the authors, this is the first study investigating the importance of a recovery period after chemical exposure in relation to natural stressors like desiccation and heat shock. The results showed that *Folsomia candida* surviving a moderate chemical stress from PHE and NP corresponding to an estimated LC₂₀ had significantly lower tolerance towards subsequent drought and heat shock. The synergistic interactions between drought and both chemicals ceased after day 7 of recovery, showing

that at this point in time, the damaging effects of PHE and NP did no longer influence the physiological defense mechanisms against drought in the organisms. The heat tolerance was fully recovered 14 days after exposure to PHE. In contrast, the results revealed that concerning heat shock, the animals had not yet fully recovered from NP exposure even after 14 days. Compared to drought as a natural stressor, heat shock required at least twice as long recovery period from NP exposure.

It was hypothesized that the recovery of springtails to NP and PHE exposure would be initiated by excretion and/or metabolism of the substances, which turned out to be during the first days after exposure. The fast elimination of NP is in agreement with a previous study showing similarly fast elimination in enchytraeids (*Enchytraeus albidus*) after transfer to uncontaminated soil (Patrício Silva et al., 2016).

Results showed that, although NP was quickly eliminated, some additional time was required before full recovery was achieved (Fig 4a), indicating that the springtails might have experienced initial cellular damage, caused by toxic action of NP, as they were still sensitive to desiccation and heat shock, even though they had excreted or metabolized the most of the chemical. This could indicate a toxic action of remaining non-excreted metabolites and/or persistent cellular damage. A study conducted by Jensen et al. (2009) reported similar synergistic interactions in the earthworm, *Dendrobaena octaedra*, when combining the effects of NP and high temperatures (33°C for one hour). These authors suggested that the synergistic interaction was a result of NP-induced denaturation of proteins, which overwhelmed the cellular defence mechanisms against further heat-induced protein denaturation (Jensen et al., 2009). The prolonged effects of NP, even after the compound had been eliminated from springtail tissues, emphasizes the need for further research to determine the concentration and effect of xenobiotic metabolites.

Both NP and PHE chemicals are known for their interference with the physical properties of the membranes by interacting with the lipid bilayers (Holmstrup et al., 2014a). Embedded in the membranes, PHE increases the fluidity whereas NP decreases fluidity of membranes (Holmstrup et al., 2014a). Based on the ability to decrease fluidity, NP should interact antagonistically with heat shock since high temperatures makes cellular membranes more fluid (Hazel and Williams, 1990). However, the opposite was the case in this study. It seems that both NP and PHE might destabilize the cell membrane, decreasing its ability to cope with the increased fluidity associated with increasing

temperature (van Dooremalen and Ellers, 2010). The evidence of a general destabilization by these chemicals is further supported by the fact that they both have been found to interact synergistically with desiccation stress in *Folsomia candida* (Højer et al., 2001; Holmstrup et al., 2014b; Schmidt et al., 2014). Besides destabilizing the cell membrane, NP and PHE may affect the cell in a number of ways, which might act in synergy with the natural stressors. Both chemicals are known for their induction of enzymes associated with oxidative stress in the cells (de Boer et al., 2011; Nota et al., 2009; Gong and Han, 2006; Slotsbo et al., 2009), as well as heat shock proteins, associated with protein denaturation (Holmstrup et al., 2014b; Jensen et al., 2009; Nota et al., 2009). These negative impacts can cause cellular damage and may explain the need for recovery before the interaction disappears. Depletion of heat shock proteins and glutathione, and interactions with other cellular mechanisms by these narcotic chemicals could severely inhibit the cell's ability to tolerate desiccation and elevated temperatures. It seems hence likely that the cell's shared mechanism of coping with chemical and natural stress might play a role in the synergistic interactions observed in this study. However, the exact interactions of these chemicals with heat shock are still unknown, and further research is needed to uncover these.

The conditions in the soil environment are often very complex adding up multiple possibilities of (synergistic) interactions between hazardous chemical and natural stressor (Holmstrup et al., 2010; Laskowski et al., 2010; Verhoef, 1996). In the soil, springtails may experience a cease in exposure of a single toxicant allowing them to recover, but it is just as likely that they are exposed to a combination of chemicals for a prolonged period, and therefore rarely allowed to recover. The present study may hence potentially underestimate the degree of synergy, since the stressors were only applied individually, sequentially, and not simultaneously. Schmidt et al. (2014), showed an exposure dependent synergy between drought and PHE when exposing *Folsomia candida* to the two stressors simultaneously instead of sequentially by using silicone plates to control the chemical activity and saline solutions to control drought stress. They found that the degree of synergy was exposure dependent with some synergy at higher- and only minor synergy at lower exposure levels. This emphasizes the need for considering exposure levels when concluding on potential synergy observations from studies done at high exposure levels. The present study used markedly higher concentrations of nonylphenol and phenanthrene, than those typically found in arable soils (Schwærter and Grant, 2003; Danish Ministry of the Environment, 2009). On the other hand, recovery from stress response was only monitored at the mortality level and after a short-term stress period.

Højer et al. (2001), combined the effect of NP and drought, and found synergistic effects at much lower concentrations than used in this study. However, the drought treatment resulting in synergistic effects was relatively high, implying that these interactions might only occur at moderate to high drought levels. Nevertheless, it is conceivable that the combination of long-term exposure to multiple chemicals at low concentrations and different low-level natural stressors might be sufficient causing synergistic interactions in a natural soil environment. NP degradation occurs with half-lives between 38 to more than 126 days (Hesselsøe et al., 2001), while PHE has a half-life in soil estimated to 125-420 days (EU, 2008), making these compounds relatively persistent, exposing soil organisms for a prolonged period after entering the soil.

In conclusion, this study has shown that exposure to the hazardous substances phenanthrene and nonylphenol worsened the sensitivity of springtails to subsequent heat and drought stress. On the other hand, it was shown that a relatively short recovery period removed the synergistic interactions between chemical and climatic stress, most likely due to an almost complete elimination of the parent chemicals within a week. Demonstrating synergy between effects of climatic and chemical stress, the present study has emphasised the importance of considering multiple stressors in the ecological risk assessment of contaminants. It would be valuable information to investigate similar interactions between e.g. chemical stress and natural stressors like frost, salinity or oxygen stress, in order to elucidate the potential and importance of recovery. Furthermore, it could be useful to observe if the found results would be reproduced if reversing the order of stress factors. Consideration of multiple stressors are equally important when evaluating the potential impact of future climatic changes causing increasing periods of temperature related stress situations in soil ecosystems. Currently the only feasible approach for considering these interactions is by stressing the need of imposing a margin of safety in our risk assessment procedures, as it adds further to the challenges of extrapolating from simple standardised single species laboratory studies to complex ecosystem relations in realistic field situations. The good news is that results presented here indicate that a relative short recovery period may reduce the impact of subsequent stress.

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Figure Captions.

Fig.1 Fitted response curves (and 95% confidence limits; dashed lines) for springtail survival after: a) Exposure to increasing soil concentrations of phenanthrene; b) Exposure to increasing soil concentrations of nonylphenol; c) Exposure to increasing level of drought (reduced relative humidity for 7 days); d) Exposure to prolonged heat shock (increasing period at 34 °C). Black circles represent mean $\pm$ SE ($N = 4$); open circles represent replicate values (note that some points are overlapping and therefore not visible). The equations of the fitted regression models were:

$$\text{phenanthrene } (R^2 = 0.98; N = 28): y = 1.57 + \frac{96.12}{1 + \left(\frac{x}{109.19}\right)^{16.86}};$$

$$\text{nonylphenol: } (R^2 = 0.87; N = 28): y = -178535 + \frac{178634}{1 + \left(\frac{x}{9316}\right)^{2.15}};$$

$$\text{Relative Humidity: } (R^2 = 0.81; N = 28): = 13.70 + \frac{89.27}{1 + \left(\frac{x}{98.04}\right)^{-309}};$$

$$\text{Time at 34 °C: } (R^2 = 0.87; N = 32): = -93.3 + \frac{193.5}{1 + \left(\frac{x}{4.18}\right)^{3.73}};$$

Where y is survival (%) and x is the intensity of the stressor.

Fig. 2. Internal concentrations of phenanthrene (a) and nonylphenol (b) in springtails during an initial exposure period of seven days (uptake phase; left of vertical dashed line) and subsequent elimination period of 7-8 days (elimination phase; right of vertical dashed line). During the uptake phase, springtails were exposed to a nominal concentration of 100 mg phenanthrene kg^{-1} dry soil and 80 mg nonylphenol kg^{-1} dry soil, respectively. During the elimination phase, springtails were kept on uncontaminated plaster of Paris. Fitted first-order elimination curves (and 95% confidence limits; dashed lines) are shown. Black circles represent mean $\pm$ SE ($N = 3-5$). The equations of the fitted regression models were: Phenanthrene ($R^2 = 0.99; N = 20$): $y = -0.029 + 2.87e^{-0.76x}$; Nonylphenol ($R^2 = 0.87; N = 24$): $y = 0.0074 + 0.078e^{-7.35x}$.

Fig. 3. Survival of springtails exposed to moderate (164 minutes at 34 °C) heat (a) and drought (b) stress (98.2% relative humidity for 7 days) after a seven-day exposure to sub-lethal concentrations (101 mg kg^{-1} dry soil) of phenanthrene (black bars) or seven days in uncontaminated soil (control; white bars). The x-axis represent various recovery periods in-between exposure to toxicants and natural stressor. Significant differences (Holm-Sidak; $p < 0.05$) between control soil and contaminated soil are indicated with asterisks.

Fig. 4. Survival of springtails exposed to moderate (164 minutes at 34 °C) heat (a) and drought (b) stress (98.2% relative humidity for 7 days) after a seven-day exposure to sub-lethal concentrations (138 mg kg^{-1} dry soil) of nonylphenol (black bars) or seven days in uncontaminated soil (control; white bars). The x-axis represent various recovery periods in-between exposure to toxicants and natural stressor. Significant differences (Holm-Sidak; $p < 0.05$) between control soil and contaminated soil are indicated with asterisks.

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