Universidade de Aveiro Departamento de Biologia
Ano 2013

Vanessa Bezerra de Menezes Oliveira

FUNÇÃO E BIODIVERSIDADE DO SOLO - VARIAÇÕES REGIONAIS E ALTERAÇÕES CLIMÁTICAS

SOIL FUNCTION AND BIODIVERSITY – REGIONAL VARIATIONS AND CLIMATE CHANGES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, professor catedrático do Departamento de Biologia da Universidade de Aveiro, e co-orientação científica da Doutora Mónica João de Barros Amorim, investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor Janeck James Scott-Fordsmand, investigador sénior do Departamento de Biociências da Universidade de Aarhus, Dinamarca.

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“Nature is not only more complex than we think. It is more complex than we can think” (Egler, 1970).

o júri

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Palavras-chave

Densidades, Mesocosmos, Cobre, Colêmbolos, Enwuitraídeos, Ácaros, Funções do solo, Alterações climáticas

resumo

Embora o objetivo principal da proteção internacional dos solos seja proteger tanto as funções quanto a estrutura do solo, a atual abordagem trata principalmente da proteção ao nível estrutural. Há uma carência de estudos que contemplem a ligação das funções do solo com os níveis da comunidade. Além disso, é ainda desconhecido se as variáveis ambientais (ex: tipos de solo, condições climáticas) atuam nas funções do solo da mesma maneira que influenciam sua estrutura biológica. Ademais, as alterações climáticas poderão ter sozinhas ou combinadas com os poluentes, um grande efeito nos ecossistemas terrestres. O presente trabalho propõe estudar as funções e a estrutura biológica do solo quando impactados devido a estresse tóxico (poluição por Cu) e/ou alterações a fatores como a temperatura e abundância de organismos, de maneira a simular possíveis variações regionais ou climáticas. Para alcançar os objetivos principais 3 experiências utilizando diferentes densidades de *E. crypticus* e 2 gerações foram feitas (Capítulos II e III). Duas experiências com mesocosmos (SMS) decorreram durante 3 meses sob uma gama de diversas temperaturas (10 – 29°C), que representam temperaturas médias para Portugal e Dinamarca (Capítulos IV e V). Duas experiências de campo também foram realizadas com intuito de validar os SMSs (Capítulo VI). Resultados demonstraram que os efeitos do Cu na reprodução dos enquitraídeos dependem da densidade inicial de organismos, especialmente na 2ª geração. Entretanto, nos SMSs expostos a Cu, a densidade inicial é menos importante nos resultados finais. O aumento da temperatura alterou majoritariamente a fase inicial de crescimento populacional. Em períodos mais longos, a abundância estabilizou tornando-se menos influenciada pelas temperaturas. Períodos longos de exposição reforçaram os efeitos da temperatura, como por ex: diversas espécies foram similarmente afetadas a 29 ou 26°C quando expostas durante 28 ou 61 dias respectivamente. De forma geral, o Cu reduziu a abundância da maioria das espécies ao longo do tempo, com poucas exceções. Os resultados da decomposição da matéria orgânica (MO) e atividade alimentar associaram-se com a abundância de organismos em baixas temperaturas (10-23°C). Entretanto, com o aumento das temperaturas (19-29°C), este comportamento não foi claro e a abundância de espécies e atividade alimentar diminuíram enquanto a decomposição da MO aumentou. Além disso, os resultados observados nos SMSs foram confirmados no campo. Mais especificamente, alterações ocorreram na fase de crescimento (correspondente à Primavera) e a exposição ao Cu diminuiu os efeitos da temperatura. Metodologias mais complexas (ex: mais gerações e experiências com múltiplas espécies) apresentam muitos benefícios, mas também proporcionam respostas mais complexas, as quais exigem um maior "peso" de evidências para serem comprovadas.

keywords Densities, Mesocosms, Copper, Collembola, Enchytraeids, Acari, Soil function, Climate change.

abstract Although the main aim for international soil protection is to protect both the soil structure and the soil function, the current soil protection approach mainly deals with protecting the soil structure level. There is a lack of studies that link the community level with soil function. Additionally, it is unknown if the environmental variables (e.g. soil type, climate conditions) are acting on function in the same way they influence the biological soil structure. On top of this, climate change will alone and in combination with pollution have a strong effect on the terrestrial ecosystem. In the present work the soil biological structure and function were studied when impacted due to a toxic stress (Cu pollution) and due to changes in factors such as organisms' abundance, and temperature, simulating ecological aspects, regional and climate changes. To achieve the main goals 3 experiments using different densities of *E. crypticus* and two generations were performed and culminated in two papers (Chapters II & III). Two multispecies experiments (SMS) were conducted until 3 months and under various temperatures (10-29˚C), representing the span of average temperatures for Denmark and Portugal (Chapters IV & V). Two Field experiments were also performed in order to validate the results of the SMSs (Chapter VI). Results showed that the effect of Cu on reproduction does depend on the density, especially so in the succeeding generation. Nevertheless, in the SMS test with Cu, the initial density is less important for the outcome. Increased temperature in the SMSs caused major changes in the abundance, mainly in the initial phase of population growth. At longer exposures the population abundance stabilized and became less influenced by temperatures. The longer exposure enforced the temperature effects, e.g. for several species effects at 29ºC-28 days were similar to 26ºC-61 days. Copper caused a general depression in abundance over time for most species with a few exceptions. The OM decomposition and feeding activity responses at low temperature (10-23ºC) were associated with the increase in species abundance whereas this was less clear at high temperatures (19-29ºC), here with a decrease in feeding activity and species abundance but increase in OM decomposition. Additionally, responses observed in the SMSs were confirmed in the field. In specific, changes occurred in the growth phase (corresponding to the late spring exposure) and Cu depressed the temperature responses. More complex approaches (i.e. more generations and multispecies approach) has many benefits, but provides also more complex answers that may require more weight of evidence.

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Chapter I – Brief context, aims and outline

Brief context, aims and outline

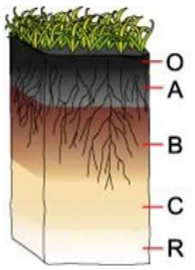
Introductory note

Soil has been defined in several different ways and a very simple definition is found in the Merrian Webster dictionary as "*soil is the upper layer of earth which can be dug or ploughed and in which plants grow*". However, soils are certainly a lot more than this and as defined by the European Environmental Agency "*soil underpins 90% of all human food, fiber, and fuel and is essential for water and ecosystem health. It is a global carbon sink; holding an important role in the potential slowing of climate change. Soil conserves the remains of our past, it is a reservoir for genes and is an important element of our cultural heritage, through the maintenance of landscapes and biodiversity. Nevertheless, soil is being exploited and irreversibly lost and degraded as a result of conflicting demands from most economic sectors.*"

In this thesis the terrestrial ecosystem is going to be studied through its biological structure and some of its functions in different regions in Europe. In this introductory chapter we merely attempt to give a context to the thesis that follows, summarising some aspects of particular importance in a more broad and environmental perspective. Further, we will address the aims and structure of the thesis.

Brief context

Soils are a complex matrix, originated from the interaction of physical, chemical and biological processes between the atmosphere and the parental material (Coleman et al. 2004). Soils can be formed by basic minerals and organic constituents, being commonly divided in four layers (soil horizons): A, B, C and R (Figure 1, Adapted from www.lrss.co.nz.). The A horizon (topsoil) also comprises a leaf litter layer (O horizon), B horizon (subsoil) includes plants' roots and where most of the nutrients are deposited, C horizon is composed of fragments of rocks (regolith) and R is the parental material (solid rock) (Gerrard 2000).



The top soil is mainly constituted by water, air, inorganic (mineral particles derived either from parent materials or depositional input into the soil surface) and organic material (living

and/or dead organisms). The percentiles of the different constituents of the top soil influence its fertility for agricultural purposes (Gerrard 2000). Soil is also the habitat to a broad group of different animals. Numbers and biomass of soil organisms are dominated by the invertebrate mesofauna (0.1-10 mm), which is generally dominated by microarthropods, where mites and springtails account for about 90% of it in most soil systems (Paul 1999). Organisms have a significant role on soil structure (community composition and its interactions) and function (e.g. nutrient cycling and organic matter breakdown). Processing organic wastes and recycling nutrients constitutes one of the most important services to all biota (Costanza et al. 1997).

Ecosystem services, as the name indicates refers to beneficial aspects provided by ecosystems, and has been categorised by the international programme Millennium Ecosystem Assessment (MA) as provisioning, regulatory, cultural and supporting (MA 2005b). In summary, some of the supporting services pointed out by the MA which may indirectly influence the three other categories are e.g. soil formation, nutrient cycling and photosynthesis. They, in general, seek to quality of life and human health. The factors and activities that may influence the supporting services of soil are numerous, of which changes in the biological structure are of high concern. According to MA (2005a), changes in the biological diversity of soils, due to human activities in the past 50 years, were faster than at any time in human history. Threats to services can have serious consequences to the maintenance of life in earth.

Soil is exposed to various threats of different sources, e.g. pollution and climate change. For instance copper (Cu), despite being an essential element it is known to be toxic to a great variety of animals when in excess (Eisler 1997). Some scientists believe that it was the first metal worked by humans more than 80 centuries ago (Schroede et al. 1966). Due to its high usage, essentiality and toxicity, several studies regarding the effects of Cu to organisms have been performed and will be the focus of the present thesis.

Moreover, changes in climate have been identified to have a tremendous impact on ecosystems. One of the major changes is related to the rise in temperature. According to the IPCC (2007) “warming of the climate system is unequivocal” as evidences of the increase in global average temperatures in the air, land and ocean have been observed. Eleven of the

twelve years from 1995 to 2006 ranked among the twelve warmest years recorded in terms of global surface temperature since 1850.

Studies on the combined effect of pollution and temperature or taking regional climate variations into consideration are lacking. Further, significant differences in temperature increase and events were observed for the northern and southern European regions (Raisanen et al. 2004, Alcamo et al. 2007).

Ecosystems require decades to become established and therefore adapt slower to changes in climate. Evidences are that species change their life cycles during the year as a consequence of annual and long-term variations in temperature (Menzel et al. 2006, Rosenzweig et al. 2007). Moreover, they shift their geographic distribution (Chen et al. 2011) and have been observed to change reproduction and developmental rates (Kardol et al. 2011).

Besides climate, there are other factors responsible for differences between ecosystems within distinct geographical regions. Species are known to have specific attributes which improve or allow them to live in diverse habitats, thus are normally differently adapted to changes. Hence, different species and their respective interactions provide distinct responses to changes in ecosystems. Particular physicochemical properties such as soil particles, water content, pH, cation exchange capacity (CEC), organic material (OM), etc., will also influence ecosystem responses to different stressors (Coleman et al. 2004).

To assess effects on the ecosystems, several test guidelines have been optimised for standardisation purposes, allowing e.g. ecotoxicological tests to be comparable between laboratories. This approach has supported regulatory bodies and based deriving concentration limits of substances. Up to date, many organisms have been covered representing ecologically relevant and abundant species; examples of terrestrial organisms include the standard tests with earthworms (OECD 1984), enchytraeids (OECD 2004), collembolan (OECD 2009) or mites (OECD 2008).

However, single species tests lack ecological relevance being difficult to extrapolate to the natural environment, where species interactions occur and have an important role. Hence, multispecies experiments have also been developed using soil mesocosms in laboratorial controlled conditions (Pernin et al. 2006, Scott-Fordsmand and Damgaard 2006, Jensen and Scott-Fordsmand 2012). Further, field studies (Koolhaas et al. 2004, Sousa et al. 2004) are

required to validate results. Experiments as presented by e.g. (Jensen and Scott-Fordsmand 2012) and (Pernin et al. 2006) attempt to combine the laboratorial advantages (i.e. standardized conditions and monitoring) with important field relevant factors, i.e. species interactions.

Further, analysis of functional parameters is important and methodologies assessing e.g. the OM breakdown and feeding activity are available (Kratz 1998, Kula and Rombke 1998, Paulus et al. 1999, Römbke et al. 2002).

Although standardised tests are important for risk assessment of toxic compounds, these are often constrained to optimum conditions and do not cover most ecologically relevant scenarios. In the present studies we included some of these variability factors which occur in nature and are not represented in standard tests for a number of reasons, e.g., organisms density, more than one generation exposure, multispecies testing or large temperature range.

Aims and outline

The main aim of this thesis was to study the combined impact of Cu contamination and climate change (increase in temperature) on terrestrial ecosystems, particularly in terms of species composition and services. Further, to assess differences between two distinct geographic regions representing northern and southern Europe climate and environments.

For that purpose experiments were designed using a multispecies approach covering laboratory and field. Laboratorial soil terrestrial mesocosms were used and experiments were conducted using temperatures ranging from 10-29ºC (10-14-19-23ºC for DK and 19-23-26-29ºC for PT), two different field soil sources along 3 months' time and control versus Cu treatment. Field experiments were performed at the two field sites (Denmark and Portugal) along the period of ca. 1 year. Further, two types of contamination were studied, historically Cu contaminated site (ca. 90 years) and freshly Cu spiked. All experimental data was produced to be comparable

This thesis is divided in seven chapters, including the current thesis contextualization (Chapter I), five chapters in the format of scientific publications (one published and three

submitted status) which present all results (Chapter II to VI), and a final chapter as overview and final remarks (Chapter VII). In detail:

Chapter I: "Brief context, aims and outline".

Chapter II: "Interaction between density and Cu toxicity for *Enchytraeus crypticus* and *Eisenia fetida* reflecting field scenarios", published in Science of the Total Environment. The effects of Cu pollution to *Enchytraeus crypticus* and *Eisenia fetida* were assessed on total population growth when organisms were exposed to different initial densities. The interaction between the effects of Cu toxicity and population density was investigated.

Chapter III: "Interaction between density and Cu toxicity for *Enchytraeus crypticus* – comparing first and second generation effects", submitted. The effects of Cu and the different initial population densities were further investigated in a two generational experiment.

Chapter IV: "Development of ecosystems to climate change and the interaction with pollution – unpredictable changes in community structures", submitted. Combined effects of Cu and temperature changes from 10 to 23ºC on species communities structure and function, assessed at 3 exposure times: 28, 61 and 84 days.

Chapter V: "Global warming and Cu pollution on soil community – previous extreme temperature events become a normal occurrence and can lead to total community extinction", submitted. Combined effects of Cu and temperature changes from 19 to 29ºC on species communities structure and function, assessed at 3 exposure times: 28, 61 and 84 days.

Chapter VI: "Effects of Cu and seasonality on mesofauna communities – northern and southern Europe field studies". Assessment of the soil mesofauna structure and function over seasons in two different geographic regions (Portugal and Denmark), under clean and Cu contaminated (freshly and old contaminated, respectively) soils.

Chapter VII: "Final remarks". Thesis overview, concerns and future research needs.

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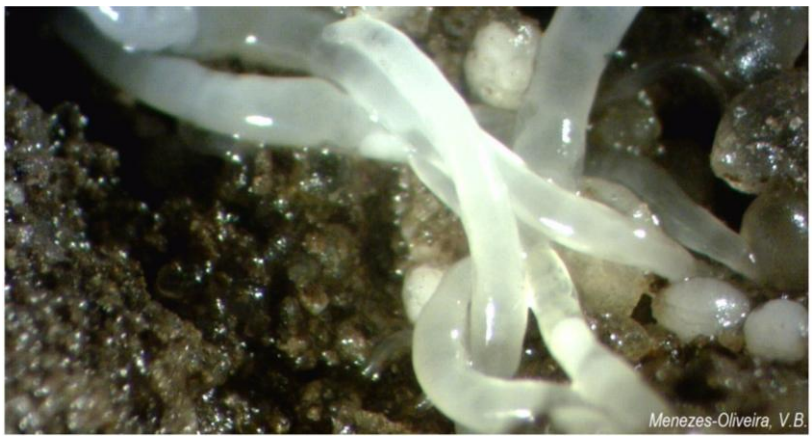

Chapter II - Interaction between density and Cu toxicity for *Enchytraeus crypticus* and *Eisenia fetida* reflecting field scenarios

Interaction between density and Cu toxicity for *Enchytraeus crypticus* and *Eisenia fetida* reflecting field scenarios

Menezes-Oliveira, V.B, Scott-Fordsmand, J. J., Rocco, A., Soares, A.M.V.M. & Amorim, M.J.B.

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Abstract

Effect assessment is usually based on responses obtained from standard tests, in which organisms are well fed and in an optimal population density. For a more thorough assessment of ecotoxicological risk, information is needed for chemical effects in systems that closer reflect the potential exposure in the field systems. Responses measured in standard density experiments do not fully reflect the field scenario, where populations' size fluctuate with environmental conditions, leading to very low organism number in certain season/conditions and high number in other. In the present study, the possible interaction between density and Cu-pollution was investigated in regard to population growth, using *Enchytraeus crypticus,* and for individual juvenile growth, using *Eisenia fetida*. The standardized ISO and OECD guidelines for enchytraeids and earthworms were adapted to test four densities and four Cu concentrations. The final population number was used to assess the effects and possible interaction between densities and Cu toxicity for population responses and the increase in individual organism wet weight was used as growth response. The study showed that although initial density itself had tremendous impact on population and individual growth, organisms under different densities had the same sensitivity to Cu.

Keywords: Population density, *Enchytraeus crypticus*, *Eisenia fetida,* Copper, Stress factors.

1. Introduction

Effect assessment of toxic compounds in the environment is usually investigated through standard experiments using well-fed individuals at one population density (ISO 16387, 2003). An important aim in ecotoxicology is to link the responses obtained in laboratory condition to the field, and, standardized methods do not reflect various densities experienced by organisms in nature. For the individual, different densities can cause stress due to e.g. competition for resources and space (Gui and Grant, 2008).

The joint interactions between densities and toxicant effects on population dynamics has been discussed theoretically and experimentally (Forbes et al., 2003). Results suggest that interaction does occur but that there is no standard outcome (Forbes et al., 2001). The interactions can be classified as additive (independent interactions), antagonistic (the density dependent effects are reduced by an increase in toxicant concentrations) and synergistic (the density-dependent effects are exacerbated by an increase in toxicant concentrations). In line with this, numerous studies have been reported with plants, copepods, rotifers and blowflies among others, showing the different interactions between densities and toxicant exposure. For example, the blowfly *Lucilia sericata* was exposed to cadmium (Cd) for two generations, and when the organisms' density was low the survival of old larvae (three spiracle slits at the third instar) and pupae was drastically reduced by Cd exposure while at high densities the survival was higher in Cd exposed populations in comparison to the control (Moe et al., 2002a).

Earthworms and enchytraeids (potworms) are important organisms in the terrestrial ecosystems. They have a crucial role in the maintenance of the soil structure as well as in the promotion of soil processes (Amorim et al., 2005; Jones et al., 1994; Kammenga et al., 2003). Moreover, they are frequently exposed to chemicals and like all other organisms have population size fluxes (Kapusta et al., 2003; Spurgeon and Hopkin, 1999). Predation, food limitation and crowding effects influence the density of oligochaete populations as observed by Baveco and DeRoos (1996). Nevertheless, there are only a few studies reported in regard to oligochaete worms' populations' response when exposed to a combination of higher and

lower densities [when compared to the standard density used in laboratory tests and/or field scenarios] and toxicants (Lahr et al., 2008; Salminen and Haimi, 2001).

In this study we focused on this aspect by evaluating density dependent population consequences when various densities of potworms and earthworms are exposed to a range of copper (Cu) concentrations. The species used, *Enchytraeus crypticus* and *Eisenia fetida*, are widely used in standard laboratory methods to assess acute or chronic toxicity. Two responses were studied: number of organisms and growth. In the case of enchytraeids, the final population numbers and the population growth rates (PGR) as an instantaneous rate of population increase, r_i (Sibly, 1999) was used. For earthworms, the individual juvenile growth as a measure of population growth (Baveco and DeRoos, 1996) was used. Copper was selected as the test chemical because it is commonly occurring in the environment due to extensive use in agriculture and several industrial activities. Additionally, it is an essential metal, usually regulated internally by organisms although causing injuries at high concentrations. The soil used was collected in a control area of the Hygum site, a fallow field situated in Jutland, Denmark which has a well known Cu contamination gradient (Scott-Fordsmand et al., 2000b). In the present experiment the soil was collected from the non-polluted area and spiked with Cu, to perform the tests as in the standard bioassays.

2. Materials and Methods

2.1. Test organisms

The test species used were *Enchytraeus crypticus* and *Eisenia fetida*. Cultures of Enchytraeids were kept at 20ºC in laboratory agar culture plates, consisting of a sterilized mixture of four different salt solutions ($CaCl_2 \cdot 2H_20$; $MgSO_4$; KCl; $NaHCO_3$) and a Bacti-Agar medium (Oxoid, Agar Nº1) as a substrate and fed on dried oats. Adult organisms with visible clitellum and of approximately same size were selected and used for the experiment. Cultures of earthworms were kept at 20ºC in laboratory using a Danish agricultural loam soil. Earthworms with an initial weight in the range between 20-30mg were selected and

acclimatized for three days prior test start using the test soil and the same conditions as in the experiment. Immediately prior to and after finalizing the experiment earthworms were placed in Petri dishes on wetted filter paper for 24h, in order to depurate their guts to obtain a soil free wet weighed worm.

2.2.Test soil

Test soil was collected from a control area at Hygum site, Jutland, Denmark. This site occupies a total of 7,200m^2 and is known historically for having been exposed to $CuSO_4$ due to activities for timber preservation (Scott-Fordsmand et al., 2000a; Strandberg et al., 2006). The general physico-chemical characteristics of the soil are as follows: 20–32% coarse sand (>200µm), 20–25% fine sand (63–200µm), 11–20% coarse silt (20–63µm), 12–20% silt (2–20µm), 12–16 % clay (<2µm), 3.6–5.5% humus and pH (H_2O) of 6.3 (Scott-Fordsmand et al., 2000a). The soil was sampled to a depth of 20cm. To exclude soil fauna, the soil was dried at 80°C for 24h in an oven (Memmert, Type UL40, Braunschweig, Germany), and then sieved through a 2mm mesh to remove larger particles.

2.3.Test chemical and soil spiking

The chloride salt $CuCl_2 \cdot H_2O$ (99% purity, Merck Pro Analysis, Darmstadt, Germany) was the test chemical used. Spiking was done with $CuCl_2$ as an aqueous solution into pre-moistened soil batches, one per concentration. Soil was manually mixed with a spatula during ten minutes. Final Cu concentrations were not measured and all results are based on the nominal values. The pre-spiking concentration (control/background concentrations) was measured to be 15mg Cu/kg of dry soil. Copper concentrations were measured by an inductively coupled plasma mass spectrometer (PE SCIEX Elan 6000 ICP-MS, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) equipped with a cross flow nebulizer. The oven dried (105°C for 24h) samples were ground into a fine

powder by an agate mortar and pestle and digested by nitric acid (65%, Romil-SpA, Super Purity Acid, Cambridge, United Kingdom), super pure hydrofluoric acid (47.5%, Romil-SpA, Super PurityAcid,Cambridge, United Kingdom) and hydrogen peroxide (30%, Merck,

Darmstadt, Germany) at a ratio of 3:1:1 (v:v:v) in the micro-wave oven (Anton Paar Multiwave, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK).

2.4. Experimental setup

The exposure method in general followed the ISO guideline 16387 (2003) Enchytraeid Reproduction Test (ERT) for enchytraeids and the OECD 207 (1984) for earthworms, with the design adjusted to a density and Cu interaction experiment. For both tests the experimental conditions were: 21 days at constant temperature of 20 ± 1°C and with a 12h:12h light:dark regime. Soil moisture was checked weekly. The pH was measured at test start and test end.

The present study was a 2-factorial experiment; Factor 1: Cu exposure in the range of 0-35-70-120-300mg Cu/kg; Factor 2: Initial organisms' densities of 5, 10, 20 and 50 individuals per 20g (dry weight) of soil for *E. crypticus*, and of 1, 3, 10 and 20 individuals per 250g (dry weight) of soil for *E. fetida*. Concentration range was selected based on the EC_{50} reproduction value for *E. crypticus* (Maraldo et al., 2006) [EC50=341mg/kg].

For the enchytraeids experiment three replicates were used and the different densities were fed with 12.5, 25, 50 and 125mg of oat flakes (corresponding with increasing densities) at the beginning of the experiment and with half of the amount in the two subsequent weeks, as stated in the guideline. The food was correlated to density to avoid a food-limitation effect at high densities and fungi growth at low densities. At test end enchytraeids were extracted by spreading the soil of each test container into four 200ml plastic beakers (diameter 7cm), which were filled with demineralized water, gently shaken, and then left for 24h at 5ºC for sedimentation. Adult and juvenile enchytraeids appeared on the soil surface and were thereafter picked and counted under a dissection microscope within 48h.

For the earthworms experiment, three replicates were used and the food in the form of 2g dried horse manure per earthworm was added to the soil surface once a week for containers with 1 and 3 earthworms, twice a week for containers with 10, and 4 times a week for containers with 20. At the end of the experiment the earthworms were hand-sorted from the

soil, depurated and weighed. Earthworm survival and growth was evaluated for each container.

2.5. Statistical analysis

Effect Concentration (ECx) values were calculated for each density, using the logistic 2 parameter and Weibull models and No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) were derived by the Williams Multiple Sequential t-test Procedure (ToxRat®, 2003).

A multiple comparison using the Bonferroni method (one way ANOVA) was performed to analyze interactions between the density and the Cu exposure (SPSS, 1997).

For enchytraeids the instantaneous population rate of increase was calculated by $r_i = (\ln(nt/no))/t$, where *nt* and *no* are the population sizes at the end and start of the experiment, respectively, and *t* is the time in days (Sibly 1999).

3. Results

3.1. *Enchytraeus crypticus*

Test fulfilled the validity criteria of the standard test in terms of survival (≥80%) and reproduction (≥50 juveniles) in the controls. The soil pH was 6.4 at the test start, except for the 300 mg Cu/kg exposure where it was 0.5-0.7 units lower.

3.1.1. Interactions between density and Cu exposure

Based on the total population number divided by the number of initial adults, a two way ANOVA and pairwise multiple comparison procedures (Holm-Sidak method) showed significant Cu exposure effects (Factor 1: $F=21.854$; $p<0.001$), no density effect (Factor 2: $F=1.656$; $p=0.192$) and no significant interaction ($F=0.360$; $p=0.970$) between the two factors. Results are visualized in figure 1. In regard to the effect of Cu exposure which was significant, see Table 1.

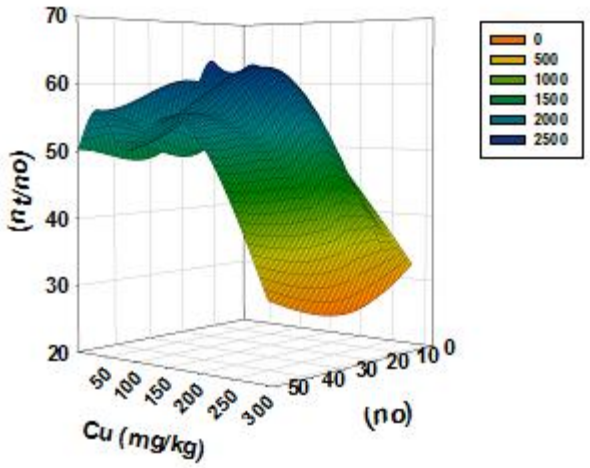

Figure 1 - Results of the reproduction bioassay with *Enchytraeus crypticus* when exposed to $CuCl_2$ (0, 35, 70, 120 and 300 mg Cu/kg) and different initial densities (n_o = 5, 10, 20 and 50). Results are shown as n_t/n_o, the ratio between the total number of organisms per starting initial adults.

To provide a different visualization of results, figure 2 shows data expressed as percentage of the control (set to 100%) within the different tested densities (Fig. 2).

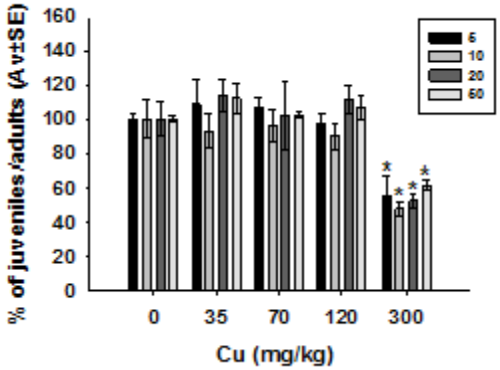

Figure 2 - Results of the reproduction bioassay with *Enchytraeus crypticus* when exposed to $CuCl_2$ spiked Hygum soil, at different initial densities: 5, 10, 20 and 50 organisms. Number of organisms, expressed as number of juveniles per starting adults is shown as percentage of the controls in average ± standard error (Av±SE). The asterisc (*) denotes significant difference (ANOVA, Dunnett's test ($p < 0,05$)).

Organisms exposed to Cu spiked soils in the highest and lowest density (5 and 50 initial adults) showed a tendency to be less affected than the ones exposed in the standard number (10 initial adults), although the difference was not significant.

3.1.2. Individual dose-responses

The results of the effect concentration values calculations can be seen in table 1.

Table 1 - Effect concentration (ECx) values calculated based on final population numbers of *E. crypticus* when exposed to $CuCl_2$ in the individual different densities (CI: 95% confidence interval; n.d.: not determined (due to a weak goodness of fit) ; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration; $p(Chi^2)$ = goodness of fit measure; p(F) = test for regression).

Density	**EC_{10}**	**EC_{20}**	**EC_{50}**	**NOEC**	**LOEC**	**$p(Chi^2)$**	**p(F)**	**Model used**
5	179.9 (169<CI<189)	222.8 (215<CI<229)	321.1 (317<CI<325)	120	300	1.000	0.001	Logistic 2 parameters
10	111.5 (0.6<CI<176)	159.3 (7.1<CI<223)	293.2 (200<CI<937)	120	300	0.998	0.038	Logistic 2 parameters
20	218.6 (n.d.)	247.3 (n.d.)	305.4 (n.d.)	120	300	1.000	0.153	Logistic 2 parameters
50	100.4 (n.d.)	184.1 (n.d.)	459.5 (n.d.)	120	300	0.992	0.057	Weibull

* The effect concentration values (ECs) are expressed as mg Cu/kg soil dw.

3.1.2.1. Growth rate – all – for comparison with literature

Population growth rate (PGR) as an instantaneous rate of increase (r_i) was calculated to evaluate the results. The instantaneous rate of increase is used when the age structure of the experiment is ignored. This measure was chosen because it has been presented as a good link from effects on individuals to effects on population levels (Forbes and Calow, 1999). Mean values of instantaneous rate of increase obtained and the standard deviation is presented (Table 2). The rate of increase was different between all densities for each Cu concentration and in each density only the highest concentration (300mgCu/kg) was different as can also be observed in figure 3.

Table 2: Summary of the instantaneous rate of increase (r_i) values. Results are expressed as the average ± standard deviation (±SD) per concentration at each initial density. Significant differences between growth rate (r_i) between various densities within each Cu concentration is denoted by $^{A-B}$ and significance between concentrations within each density by $^{X-Y}$[ANOVA; Bonferroni ($p<0.05$)].

	Start density			
	5	**10**	**20**	**50**
Cu (mg/kg)	r_i (±SD)			
0	$0.194^{A,X}$ (±0.003)	$0.230^{B,X}$ (±0.009)	$0.255^{C,X}$ (±0.008)	$0.296^{D,X}$ (±0.002)
35	$0.197^{A,X}$ (±0.011)	$0.227^{B,X}$ (±0.009)	$0.261^{C,X}$ (±0.006)	$0.301^{D,X}$ (±0.007)
70	$0.197^{A,X}$ (±0.004)	$0.229^{B,X}$ (±0.008)	$0.254^{C,X}$ (±0.015)	$0.297^{D,X}$ (±0.001)
120	$0.192^{A,X}$ (±0.005)	$0.225^{B,X}$ (±0.007)	$0.260^{C,X}$ (±0.015)	$0.299^{D,X}$ (±0.005)
300	$0.164^{A,Y}$ (±0.016)	$0.196^{B,Y}$ (±0.008)	$0.225^{C,Y}$ (±0.007)	$0.274^{D,Y}$ (±0.003)

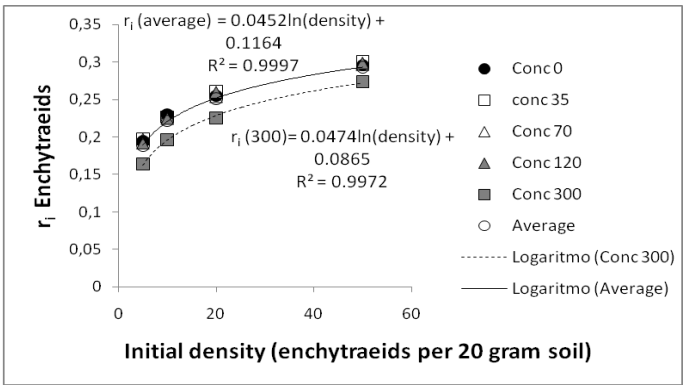

Figure 3 - Rate of increase (r_i) as a function of initial density, displayed for the individual concentration and when the average of r_i for all concentrations within one density (Average) and one concentration (300mgCu/kg). The actual values and significance can be seen in table 2.

3.2. *Eisenia fetida*

Test fulfilled the validity criteria in terms of survival. The soil pH was 6.6 at the test start, except for the 300 mg Cu/kg exposure where it was 0.5-0.7 units lower. During the

experiment it increased approximately 0.5 units in the two lowest densities and 1.1 units in the two highest densities.

Mortality of *E. fetida* was generally low, reaching a maximum value of about 13%, not related neither to soil Cu concentrations nor earthworm density. The growth of the controls was within normal ranges (see Table 3).

Table 3: Results of earthworm weight: values are shown as average final wet weight (g) ± standard deviation (SD) for each density and exposure concentration in mg Cu/kg soil dry weight. Significant differences in weight between the various densities at each Cu concentration is denoted by $^{A-B}$ [ANOVA; Bonferroni ($p<0.05$)]. The Cu concentrations imposed had no significant effect on the mass of earthworms, regardless of density level.

	Start density^{A-B}			
	1	**3**	**10**	**20**
Cu (mg/kg)*	Weight (g) (±SD)			
0	0.210 ^{A} (±0.071)	0.122 ^{AB} (±0.042)	0.106 ^{B} (±0.022)	0.065 ^{B} (±0.031)
35	0.190 ^{A} (±0.056)	0.125 ^{A} (±0.054)	0.115 ^{A} (±0.009)	0.080 ^{A} (±0.018)
70	0.220 ^{A} (±0.052)	0.167 ^{AB} (±0.026)	0.120 ^{B} (±0.007)	0.097 ^{B} (±0.010)
120	0.200 ^{A} (±0.052)	0.173 ^{A} (±0.018)	0.120 ^{AB} (±0.012)	0.079 ^{B} (±0.022)
300	0.225 ^{A} (±0.049)	0.184 ^{A} (±0.017)	0.119 ^{A} (±0.009)	0.089 ^{A} (±0.016)

3.2.1. Individual dose-responses

The increase over time of the earthworms' weight was higher for containers with one individual when compared to those with higher densities. For the containers with 1 organism the earthworms gained on average 210 (± 14) mg, whereas for those with 20 organisms the gain was around 82 (± 12) mg for 21 days. For all concentrations the growth was ln related to density [General equation: Growth (mg wet weight increase per day) = - 0.002 ln (number of earthworm per kg soil) + 0.0156, $R^2 = 0.99$], exponential function has a less good fit.

Within each earthworm density there was no differences in earthworm mean weights with increasing Cu concentration (see Table 3). There was no interaction between density changes

and Cu exposure changes despite the significant differences in weight between the various densities at each Cu concentration.

4. Discussion

Various interactions between densities and toxicant exposure have been shown in previous studies using other organisms (Cecchine and Snell, 1999; Gui and Grant, 2008; Hooper et al., 2003; Lahr et al., 2008; Moe et al., 2002b). In this study we found no significant interaction between the density and the Cu exposure for *E. crypticus* (population number), and there was no interaction effect on individual growth between earthworm density and Cu exposure.

For enchytraeids the reproductive output per adult increased approximately 10% when increasing the density up to 50 initial adults, compared to the density of 5-10 animals. There was no clear pattern in regard to the effect of Cu for different densities. A tendency (10%, non-significant) was observed for the standard density (10 animals per replicate) to have the lowest reproductive output (per adult) in all Cu exposures, hence also providing the lowest mean EC_{20} and EC_{50} values (see Table 1). The better performance (higher reproductive output) with higher densities could be due to a clustering of organisms at higher densities, which could have minimized exposure to the stress environment and perhaps increased interactions. Avoidance of Cu is well known for enchytraeids and earthworms (Amorim et al., 2008). This is of course speculative, and does not explain the similar effect at the lowest density. Kramarz et al. (2005) studied the joint effect of exposure to zinc (Zn) in soil and density of *Enchytraeus doerjesi* and found a significant interaction with population growth parameters. They observed that Zn influenced the total number of animals differently for all densities (except density 10 and 20); this was observed as a difference in slope of linear regression line (Zn concentration versus total population number, plotted per density). The same was observed in the present experiment where the slopes of the linear regressions were different between the tested densities. However, linear regression in the present case was an approximation since the influence of Cu seemed to follow the conventional logistic concentrations response pattern. Fitting of logistic curves to these data (as performed in ECx estimations) showed no clear pattern in the slope of the fitted curves, although the lowest

slope was observed at the highest density (see Fig. 1). It should be noted that although the initial maximum density of 50 used here was lower than the 80 used by Kramarz et al. (2005) the present study resulted in a total number of 3000 animals (control situation), which was more than twice as many (1200-1300 animals) as observed by Kramarz et al. (2005). In both experiments there was food *ad libitum*, so the difference was not due to food limitation. Further, it should be noted that 3000 animals (as was the highest density in the present study) is equivalent to around 150 animals per gram of dry soil which is approximately 300 mg organism (wet weight) per gram of dry soil (to give an idea this is the equivalent body mass of adding 400-500 *E. fetida* in a standard 500 gram soil experiment, which normally contains 10 animals). For instantaneous growth rate Kramarz et al. (2005) found a significant decrease in growth rate with increasing Zn at higher densities, which (when making similar calculations although simpler) was not the case here, where the decrease in growth rate due to Cu was similar for all densities. It is unknown whether in this part of the experiment effects were occurring on the size of the individual animals, as is shown in the other part (*E. fetida*) of the experiment.

As for *Eisenia fetida*, the clear reduction in average juvenile weight with increasing densities followed previous observations (Lahr et al. 2008) on *Lumbricus rubellus*; Lahr et al. (2008) also maintained food *ad libitum*. In the present experiment the worms at density 1 grew almost 3 times more than those at density 20. The density range in Lahr et al. (2008) i.e. 3 and 5 worms per 700 g dry soil, was lower than in the present study i.e. 1, 3, 10 and 20 worms per 250 g dry soil. The average final weight in *L. rubellus* experiment was approx 1600-1800 mg and in the present study 200-300 mg, hence the total biomass in our experiment spanned below and above that in the Lahr et al. (2008) experiment. It is unknown whether the reduced growth was due to physical space limitations or chemotaxis, but probably not due to food limitations as it was available *ad libitum*. As in regard to the growth model observed (see results), it should be noted that steepness of the curve (0.002ln number earthworm per kg soil) only holds for populations with homogenous sizes, as worms of different sizes grow at different rates until reaching a certain age at which weight increase usually terminates.

There was no apparent effect of any of the Cu concentration on the growth, which indirectly indicated that there was not a strong interaction between Cu and density up to 300mg Cu/kg

in Hygum soil. It is clear that this was not the optimal cover of possible interactions as preferably effects should have been observed in at least one exposure concentration; however the test concentrations were chosen just at the edge of where effects were expected (Scott-Fordsmand et al., 2000b).

5. Conclusion

Standardized tests are normally performed with ten organisms, even though one of the major evidences of the ecological imbalance is the increase or decrease in the number of organisms within a certain ecosystem. This study showed that although initial density itself had tremendous impact on population and individual growth, organisms under different densities had the same sensitivity to Cu and hence no interaction was observed on the measured toxicity parameters. Based on the above observations, the density is not of pronounced importance for enchytraeids sensitivity (at least this species) in relation to risk assessment, hence the standard test may represent different densities in the field.

Acknowledgements

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Chapter III - Interaction between density and Cu toxicity for *Enchytraeus crypticus* – comparing first and second generation effects

Interaction between density and Cu toxicity for *Enchytraeus crypticus* – comparing first and second generation effects

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Submitted

Abstract

Density of organisms varies considerably in nature depending e.g. on seasonality or food availability. A recent investigation on interaction between Cu and density using *Enchytraeus crypticus* showed that density itself (5-50 per 20 g dry soil) had an impact on population and individual growth [up to 3000 individuals per test vessel], but the interaction between density and Cu toxicity was not significant. Here, a follow up study was performed, in which the interactions between density and Cu-exposure were investigated along a two-generation exposure using *E. crypticus* (three factorial: 1) density (5-50); 2) Cu (0-300 mg/kg) and 3) generation (1-2)). After the first generation, the juveniles were retrieved and further exposed under the same conditions along a 2nd generation (using a refined density set – 10 and 50). Results showed an interaction between density and Cu in the reproduction of *E. crypticus*, this being significant in the 2nd generation, showing lower toxicity for higher density of organisms. There was a transfer of the density effect from generation 1 to 2, i.e. animals from density 50 in generation 1 when further exposed at density 50 in generation 2 had lower Cu toxicity compared to when further exposed at density 10.

Keywords: generational effect, density, copper, ecology, ecotoxicology

1. Introduction

Investigation on the effects of chemicals for regulatory purposes are usually based on standardized tests (ISO 2003, OECD 2004), where test conditions have been optimized to an optimal success criteria. These include, among others, an "ideal" density of organisms per soil amount and one reproduction cycle alone if reproduction tests. However, in nature populations experience fluctuations in the number of individuals due to various conditions, e.g. temperature or humidity changes (Lokke and van Gestel 1998). Such fluctuations in the population number may cause stress due to competition for resources or space (Gui and Grant 2008). As also described in literature, an increase in the per-capita mortality rate of a population can cause an increase in the population as a response in consequent generations - this phenomenon has been termed "hydra" effect (Abrams 2009) - after the mythological beast that grew two heads to replace each one that was removed. This is not a new concept having been described already by Daniel and Park (1954), although still fairly unknown. There are different underlying mechanisms and parameters, one of which being that density dependent mortality that operates prior to over compensatory density dependence may produce hydra effects that are significant in magnitude.

There are few studies focusing on the importance of invertebrate population-density interacting with toxic-effects, e.g. for arthropods (Moe et al. 2002, Gui and Grant 2008) and for worms (Forbes et al. 2003, Kramarz et al. 2005, Menezes-Oliveira et al. 2011). Even fewer studies have focused on the effect of such interactions in successive generations (Moe et al. 2002, Vogt et al. 2007, Vogt et al. 2010).

In our previous study (Menezes-Oliveira et al. 2011), where *Enchytraeus crypticus* were exposed in a range of densities to copper concentrations, we observed an interaction between organisms density and Cu exposure although not significant - animals exposed to start density of 5 and 50 were less affected by Cu-pollution than animals at density 10 and 20 (per 20 g of dry soil). Within the test, juveniles were continuously "born" throughout the experiment, hence animals were born at lower densities (biomass per soil) in the start than those at the end of the test (i.e. final density up to 3000 animals per 20 g of soil). Our hypothesis is that if juveniles are born in and/or from high/medium/low density populations, this must have an

influence on their performance afterwards. Further, if juveniles grow in different densities this could also influence their and the following generation performance. Such density related stress (if stress) may also indirectly cause changes in the sensitivity of the *E. crypticus* to Cu.

Hence, we performed a 3-factorial study – 1) start density (5-50); 2) Copper (0-320 mg/kg); 3) Generation (1-2) - to test these hypothesis, in an attempt to discriminate and assess the following effects: 1) effect of parental start densities on the reproduction and Cu toxicity; 2) effect of parental start densities on juveniles performance in a second generation; 3) the effect of density combined with Cu in generations 1 and 2; 4) the effect of two generational continuous exposure to Cu on the toxicity; 5) the effect of parental density on the Cu sensitivity for the second generation.

2. Materials and Methods

To test our hypothesis two sequential experiments were performed: Experiment 1 (1^{st} generation) consisted on the exposure of adult animals to a range of Cu concentrations (0-35-70-120-300 mg Cu/kg) in various initial densities (5, 10, 20 and 50 organisms per 20 g of dry soil for each replicate); Experiment 2 (2^{nd} generation), the resulting juveniles from experiment 1 were collected and further exposed to the same Cu concentration at a standard (10 organisms) density. Additionally, density 50 was tested for animals previously exposed in density 50 (maximum initial density).

2.1. Test species

Enchytraeus crypticus (Oligochaete: Enchytraeidae) was used as test organism. The animals were maintained in laboratory at 20ºC in agar plates and fed on dried oats. Culture medium consisted of a sterile mixture of four solutions ($CaCl_2 \cdot 2H_2O$, $MgSO_4$, KCl e $NaHCO_3$) and Bacti-Agar medium as a substrate. Adult organisms with approximately same size and visible clitellum were selected for the experiment 1 (according to the ISO 16387 (2003)). For

experiment 2, juveniles resulting from experiment 1 were used, selected with approximately same size.

2.2. Experimental Soil

The standard natural soil LUFA 2.2 was used. The general characteristics of the soil are: pH of 5.5, 3.9% organic matter, 6% clay, 17% silt and 77% sand.

2.3. Test chemical and soil spiking

Soil was spiked with $CuCl_2 \cdot H_2O$ (99% purity, Merk Pro Analysis, Darmstadt, Germany) three days before the test start. An aqueous concentrated stock solution was prepared and sequential dilutions were made to obtain the required concentrations.

2.4. Experimental setup

Procedures were according to the ISO guideline 16387 (2003) for Enchytraeid Reproduction Test (ERT) with adaptations to test various densities and 2^{nd} generational effects. Experiments were a 2-factorial design: Factor 1: Cu concentration (0, 35, 70, 120 and 300 mg Cu/kg), Factor 2: organisms start density (5, 10, 20 and 50 animals per replicate) (Figure 1).

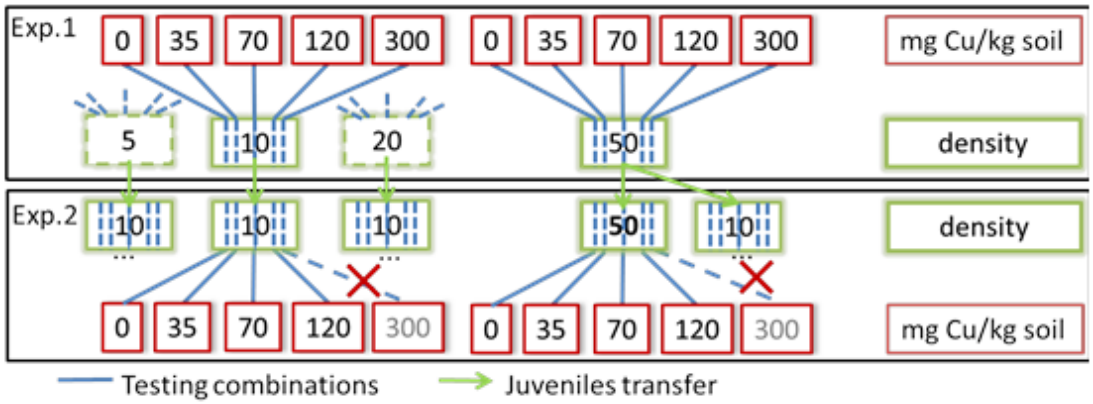

Figure 1 - Experimental design including experiment 1 (Generation 1-G1) and 2 (Generation 2-G2) exposure combinations, red denote $CuCl_2$ concentration range and green starting densities. In G1 densities 5, 10, 20 and 50 were exposed to all concentrations. In G2, juveniles from each G1 treatment were exposure to Cu at a density 10 or 50, as indicated.

Three replicates were used for each experimental condition. Food was added weekly in amounts corresponding to the increasing densities (12.5, 25, 50 and 125 mg dried oat flakes at the beginning of the experiment, and half of that amount in the subsequent weeks). Replicates were checked regularly to avoid food limitation and soil moisture was replenished weekly. Tests were conducted at 20 ± 1ºC and at 16 h: 8 h light: dark regime.

2.4.1. Experiment 1 (Generation 1)

After 21 days, organisms were extracted through two different steps to be able to count the total number of animals and also to retrieve animals to continue in experiment 2. First step consisted of transferring a fraction of soil from each replicate to a Petri dish with deionised water. Using a stereo microscope, ca. 20 juveniles from each test vessel were picked and pooled per treatment in a Petri dish containing deionised water. Juveniles with approximately same size were selected from the pool of replicates and transferred to test vessels of experiment 2. As a second step, all soil from each replicate was divided into two 200 ml plastic beakers, filed with tap water, carefully shaken during 10 seconds and left for 24 h at 5ºC for sedimentation. Organisms laid on the soil surface and were thereafter picked and preserved in alcohol for a posterior total counting.

At test condition of 300 mg Cu/kg the extraction was not successful [due to the few and smaller size organisms] and not enough animals were retrieved for further exposure in Generation 2.

2.4.2. Preliminary experiment deciding for 2nd generation testing (life-cycle assessment):

Sexually mature animals were transferred to new culture boxes and daily observations were performed to assess *E. crypticus* life-cycle, i.e., to follow time for cocoon laying, egg hatching and maturing. Supervised plates were kept under the same conditions as culture plates (e.g., 20ºC, 16: 8 photoperiod). Daily observations showed that *E. crypticus* life-cycle had the following timings at 20ºC: week 1: cocoon laying; week 3: egg hatching/juveniles; and week 5: sexually mature juveniles. Hence, a period of 6 weeks was selected for 2nd generation experiment test duration, where juveniles were used at the test start.

2.4.3. Experiment 2 (2nd generation)

Juveniles resulting from experiment 1, with approximately same size, were used for testing interaction between Cu exposure and densities 10 and 50 (Exp. 2, figure 1) under the exact same conditions as generation 1. The selection of densities 10 and 50 in generation 2 was due to previous indication that major changes in effects occurred at density 10 and 50 (Menezes-Oliveira et al. 2011). Further, density 10 is a mean value, important for comparisons. So this is the result of a compromise between the possible work load within the required time to continue the test.

The Experiment duration was 6 weeks as decided after the preliminary life-cycle assessment test. At test end organisms were extracted and counted as described previously in step 2 of experiment 1.

2.5. Data analysis

Data (total number of animals divided by initial number) was always checked for the required assumptions/validity of standard test guideline.

Data was analysed as follows:

1) Two-way ANOVA (Holm-Sidak) to assess interaction between Cu and density (SPSS 1997); *ANOVA:* One-way ANOVA (Dunnett's), to discriminate differences between the control and treatments for Cu and density separately.

2) Concentration-response curves: The effect-concentrations (EC_x) and the lethal concentration to 50% of the organisms (LC_{50}) were calculated using the Toxicity Relationship Analysis Program (TRAP). For the EC_x determination a 2-parameters non-linear logistic regression model was used on log-transformed density-data.

Additional analyses were performed using a *mixture model*: sigmoid concentration-response curve modeling on combined stress was done as described by Damgaard et al. (2002), with the exception that modelling was based on reproduction instead of survival data, and was consequently assumed to be Poisson distributed with a modeled mean value. The fit of the model was assessed by comparing the expected values to the observed values for each combination of density and Cu concentration.

3. Results

Both experiments 1 and 2 fulfilled the validity criteria as established in the guideline for the standard-test in terms of survival (≥80%), reproduction (≥50 juveniles per 10 initial adult) and coefficient of variation around the mean number of juveniles (not higher than 50% in the end of the experiment) in the controls. To note that at 35 mg Cu/kg at density 50 the final

number of animals reached 3000 animals per container, similar to the reported by Menezes-Oliveira et al. (2011).

3.1. 1st generation – the importance of one generation interaction between density and Cu-exposure.

The interaction between density (5, 10, 20 and 50 animals per 20 g dry soil) and Cu-exposure (0, 35, 70, 120 and 300 mg Cu/kg dry soil) was analysed as follows.

The two-way ANOVA comparison (Holm-Sidak) showed significant density effect (F= 12.284; p≤0.001), Cu exposure effect (F= 27,958; p≤ 0.001) and interaction (F= 6.159; p≤ 0.001) between the two factors, this based on the total number of individuals divided by the number of initial adults n_t/n_0 (see figure 2). The standard initial density (D10) was used as the control.

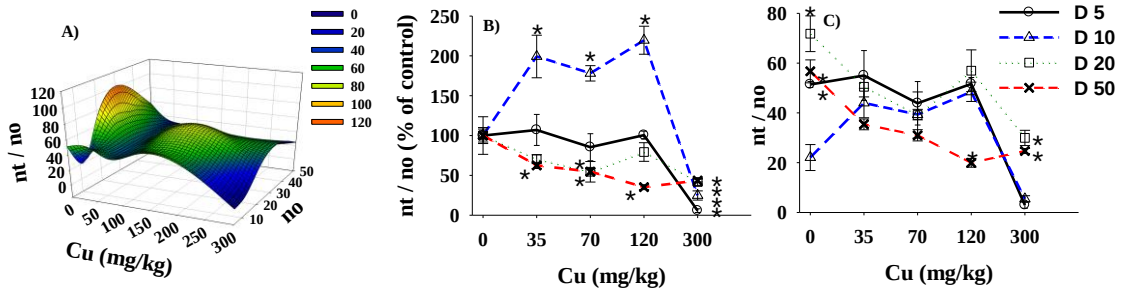

Figure 2 - Results of the reproduction of *E. crypticus* when exposed to $CuCl_2$ under different start densities (n_0). Results are expressed as average ± standard error (Av ± SE) of the total number of organisms (n_t) per starting number (n_0) as: A) 3D plot; B) Percentage of the controls; C) n_t/n_0. *(p<0,05; Dunnett's): compared to the control in the respective density (B); compared to the standard density (10 initial adults) (C).

In regard to the individual concentration-responses at density 5, no differences were observed between control and up to 120 mg Cu/kg, but a decrease of ca. 90% was present at 300 mg Cu/kg (see figure 2B). At density 10 there was a significant increase until 120 mg Cu/kg and then a decrease at 300 mg Cu/kg. To note that for density 10 the end-of-experiment density was lower than for the other densities in the control (Cu-0). At densities 20 and 50 there was a gradual decrease in the total number of organisms with increasing Cu concentrations.

Across the densities, there was no clear trend for effect concentration (EC_x) (see table 1), having all broad overlapping confidence intervals.

Table 1 - Effect concentration (EC_x) for *E. crypticus* when exposed to $CuCl_2$ at different densities. (95% confidence intervals are shown in brackets; n.d.: not determined (due to a weak goodness of fit); S: steepness of the curves; Y_0: starting point. Model: non-linear regression, logistic equation, 2 parameters with no transformation.

Start density	**EC_{10}**	**EC_{20}**	**EC_{50}**	**Model parameters**
		(mg/kg)		
5 *	175 [-97-447]	195 [-35-425]	300 [69-391]	S=0.0099; Y_0=52
10**	175 [168-182]	200 [195-205]	242 [238-245]	S=0.0082; Y0=38
20	168 [-105-442]	222 [51-393]	313 [210-417]	S=0.0038; Y_0=54
50	n.d.	n.d.	152 [66-238]	S=0.0017; Y_0=56

*One outlier (value: 29) replaced by the mean of the remaining replicates (value: 51) to keep statistics (defined as more than 2 times different from the average ± standard deviation of the other replicates); this was done at exposure concentration 70. This did not change the EC values but narrowed the confidence limits.

**The effect data were log-transformed due to a lower reproduction in the control. Several models and non-transformed data were tested and all providing similar values.

Exposure at density 50 (see also figure 2B) showed a more continuous and slow decrease with increase in Cu concentration than at the other densities (as can be confirmed by the steepness); for density 5 the population-number remained similar until 120 mg Cu/kg, where after it dropped drastically. The most different response occurred for density 10, where it could be observed a hormesis-like effect. Given the non consistency of this pattern in any other density we believe that it was likely a poor control performance, reason by which we calculated the EC values using logged data.

3.2. 2nd generation – importance of one generation density range (5 to 50) for the sensitivity to Cu-exposure (0, 35, 70 and 120) in a second generation

To study the transfer (generation 1 to generation 2) of density effect on the Cu-sensitivity it was decided to keep the density constant in the 2nd generation, while exposing the animals to different Cu-concentrations. Again, data analyses results showed the following.

There was a significant density effect (F=20.387; p<0.001), Cu exposure (F=6,272; p=0.001) and interaction (F=2,016; p=0,048) between the two factors, this based on the total number of individuals divided by the number of initial adults n_t/n_0 (Figure 3). Organisms exposed to the initial density of 10 animals per replicate in both generations were used as control.

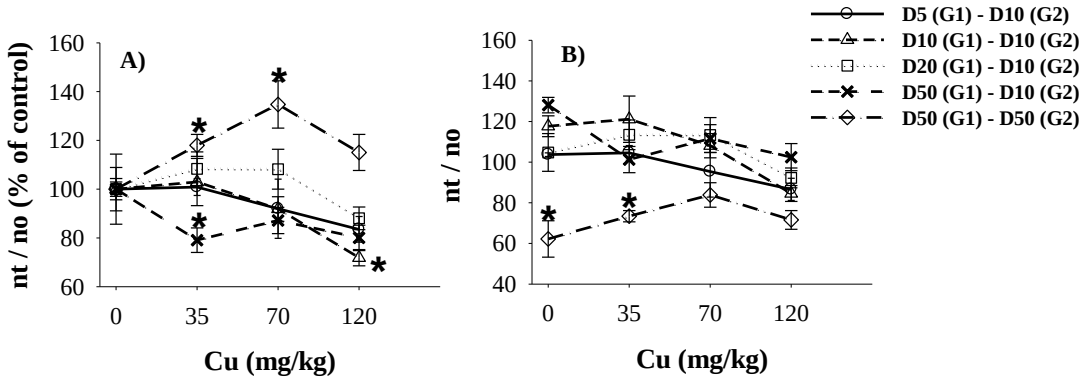

Figure 3 - Results of 2nd generation reproduction of *E. crypticus* when exposed to $CuCl_2$ under different start densities (n_0). The different line patterns indicate the generation 1 (G1) start density and respective testing density in generation 2 (G2). Results are expressed as average ± standard error (Av ± SE) of the total number of organisms (nt) per starting number (n_0) as: A) Percentage of the controls; B) n_t/n_0. *(p<0,05; Dunnett's): compared to the control in the respective density (B); compared to the standard density (10 initial adults) (C).

When D50 (G1) was transferred to D10 (G2) the total number of organisms was significantly higher for the control and the exposure to Cu had most pronounced effects at 120 mg Cu/kg.

When calculating the individual concentration-responses the apparent trend was that organisms which were exposed to higher density (20, 50) in the 1st generation had a lower ECx in the 2nd generation (Table 2).

Table 2 - Effect concentration (EC_x) values based on final population numbers per initial density (n_t/n_0) of *E. crypticus* when exposed to $CuCl_2$ in different starting densities of previous generation. G1 (generation 1 – previous exposure); G2 (generation 2 – current exposure); 95% confidence intervals are shown in brackets; S: steepness of the curves; Y0: starting point.

Density in **Generation** G1 → G2	EC_{10}	EC_{20}	EC_{50}	**Model**
5 → **10**	100	183	324	S = 0.0024; Y_0 = 104
	[-13-213]	[-327-975]	[-327-975]	
10 → **10**	85	113	163	S = 0.0070; Y_0 = 120
	[43-126]	[89-138]	[97-228]	
20 → **10**	115	157	229	S = 0.0048; Y_0 = 110
	[35-195]	[-65-320]	[-132-590]	
50 → **10**	27	88	193	S = 0.0033; Y_0 = 128
	[-62-116]	[-31-207]	[-184-571]	
50 → **50**	n.d.	n.d.	n.d.	*

*****n.d. not determined as number increased with increasing Cu concentration

The decrease in the toxicity was most visible for the D50→D10 at the EC_{50}. Again the differences seem to be mainly due to the more continuous decline with concentration for density 50 (1st generation), than due to a lower final number. Although the EC_x fits are uncertain, it should be noted that the steepness (S) of the concentration-response curves and the starting point (Y_0) is generally higher in the 2nd generation exposure than in the 1st generation (see tables 1 and 2).

3.3. 2nd generation – comparison between first and second generation density 10 and 50

In addition to the previous we also studied the performance of animals from an original "high" initial density (50, generation 1) at "standard" and "high" density in the next generation (10 and 50, generation 2). Results were as follows.

Modelling the 2nd generation density 10 and 50 versus Cu-concentration (0, 35, 70 and 120) showed a significant dependency between density and Cu exposure ($p<0.01$). This means that there was a significant change in copper toxicity with increasing density and vice-versa (Figure 4).

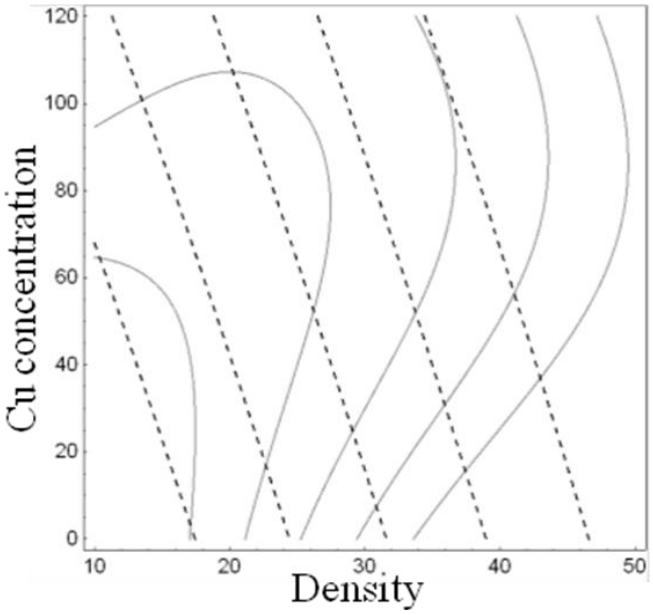

Figure 4 - Population number probability isoclines for stress combinations of copper and density fitted to the 2st generation data by the maximum likelihood approach.

The model was judged to give an adequate description of the data, although be reminded that the model is based on "only" two densities.

The Cu-concentration response curves showed different slopes and ECx values (see figure 5 and table 2).

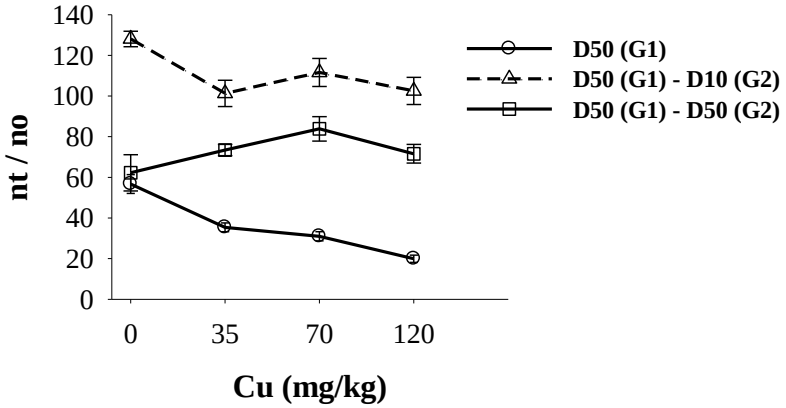

Figure 5 - Results of 1st and 2nd generation reproduction of *E. crypticus* when exposed to $CuCl_2$ with start density of 50 organisms. The different line patterns indicate the generation 1 (G1) and start density (D50) and respective testing density in generation 2 (G2), being D10 or D50. Results are expressed as average ± standard error (Av ± SE) of the total number of organisms (nt) per starting number (n_0).

The animals from D50 and exposed to D50 also in G2 were not affected by Cu exposure. On the other hand, the animals exposed under D10 in G2 were affected by Cu.

In terms of relative number (nt/n_0), reproduction of organisms that originated from an initial density of 50 animals and continued in density 50 in a second generation was approximately 50% lower than the ones that were continued in density 10 (Figures 3 and 5). To note that at the lowest Cu concentration and density 50 [D50 (G1) – D50 (G2)] the final number of animals reached in average 3665 animals per container, approximately 183 animals per gram of dry soil.

4. Discussion

In the 1st generation there was possibly a dependency between density and Cu-exposure; the ANOVA models indicated significant interactions. The indication of a density-dependent effect was neither supported nor disproved by the individual concentration-response estimates, as the models were associated with large standard deviations (possibly due to a low

steepness in the slope). This indication of a possible "weak" (or no) interaction was in line with previous results (Menezes-Oliveira et al. 2011), although less clear here. For comparison, Kramarz et al. (2005) studied the combined effect of density and Zn exposure in soil with *Enchytraeus doerjesi* where they found synergism for low densities (5 and 10 worms), but at density 20 the highest growth rate was obtained independently of the Zn concentration. Studying *Lumbriculus rubellus* Klok (2008) showed that an increase in density (2-4-8 worms) induced an added stress factor to Zn toxicity. Forbes et al. (2001) in a review reported that the combination of density and concentration of toxicants can differ, from additive, less than additive to more than additive effects. There is also evidence that such interaction with density can vary with increase in exposure concentration, shifting from less than additive at low concentrations to more than additive at high concentrations (Linke-Gamenick et al. 1999), such a non-continuous pattern agree with our observations.

In a standard ecotoxicological approach context this raises an additional point: the actual density in the laboratorial culture of the test organisms should be standardized to avoid biasing the test results.

In the 2nd generation (including densities 10 and 50) a significant dependency was observed, where lower toxicity occurred for D50→D50 than for D50→D10 or D10→D10. One possible explanation could be density-dependent overcompensation, also known as hydra effect – "population increasing in response to an increase in its per-capita mortality rate" (Abrams 2009). As follows, for the highest 1st generation density (50) there may have been an increased mortality (than for lower density) which promoted an increase in the reproduction in the 2nd generation. Another possible explanation for this density effect was that at higher densities the organisms are exposed to less Cu than at lower densities; e.g. 1) organisms avoid exposure by lumping/clustering (as was observed at test end) instead of being distributed evenly in the soil which would limit exposure to Cu [the ability to avoid Cu has been described for Enchytraeids (Amorim et al. 2008)] and 2) there would be less available Cu contaminated soil per individual at high density hence less exposure [for animals exposed in density 50 in both generations, the final densities reached ca. 3600 animals, the equivalent to 180 animals per gram of dry soil]. In regard to cause 1, lumping/clustering by its nature also promotes more frequent meetings (between organisms)

and hence potentiates reproduction, while keeping exposure to a minimum. Notable, given the final density of up to 3600 animals per container, the question is probably rather whether they can be without lumping in such numbers. In regard to cause 2, at the end of the experiment (for animals initially exposed at density 50 in 1st and 2nd generation) the biomass became approximately 36.5% of the soil mass (higher than observed by Menezes-Oliveira et al. (2011)), which reduces the actual exposure concentration. Hence, since the final biomass is density-dependent and constitutes a good part of the soil mass, one would actually expect a reduced Cu-effect at high densities. One way of clarifying this latter aspect could be the performance of a study where uptake-kinetics were measured at different densities, as this would possibly quantify actual exposure under different densities of organisms. In generation 2, the higher Cu toxicity at lower density was especially pronounced for the EC_{10} compared to the EC_{50}. EC_{10} and EC_{20} were lower for the 2nd generation than for the 1st generation, however, as the concentration-response fits are relatively poor, the possible difference between 1st and 2nd generation are not fully clear. Further studies would be required to check whether this higher sensitivity at lower concentration is a general generation effect. When comparing all the concentration-response curves for the 2nd generation exposure a significant dependency (antagonism) is observed. The significant antagonism in the 2nd generation exposure (translating the effect of the 1st generation density on the 2nd generation Cu toxicity), was supported by both the ANOVA and the mixture-model, and partially by the individual concentration-response estimates (the latter hampered by broad confidence intervals). The interaction was especially pronounced at lower Cu concentrations i.e. as mentioned the difference between the EC_{10} at various densities was larger than the difference between the EC_{50}. We have no knowledge in regard to the mechanisms for the transfer of interaction between 1st generation density and 2nd generation Cu sensitivity. Nevertheless, Cu is known to be an essential metal and regulated by many organisms, actively accumulating Cu at low doses and able to excrete it at high doses (McGeer et al. 2003). For example, results from Bossuyt and Janssen (2005) who studied acclimation to Cu on daphnia and algae, indicated regulation at low doses after which the mechanisms changed to storage at high dose. Furthermore, in contrast to our results, *Daphnia* was less sensitive to Cu after more five acclimation generations. In our study, it could be that more generations were needed to observe such acclimation effects or it could also be that our tested concentrations were already above the acclimation dose. Nevertheless, it seems more likely that in *E. crypticus* the sensitivity to Cu tends to increase from G1 to G2.

On a more test-regime note, based on results from 1st generation where effects of density were not significantly (or straight forward) affecting toxicity, a discussion issue is the minimum required amount of soil in terms of standardization guidelines (ISO/OECD). If results are further confirmed it suggests that the amount of soil per test vessel could be reduced, which would result in less waste production and smaller test requirements, hence reduced resources and costs are possible. This would also "confirm or reject" whether toxicity results obtained from standard density tests can be extrapolated to other densities. On the other hand, results obtained with G2 indicated that toxicity changed within generations as well as the interaction between density and toxicity, not observed with G1 alone. If bioassays contain more than one generation then toxicity results would be best assessed and ecologically integrated.

5. Conclusion

One generation study showed no significant interaction between density (5-50 starting organisms) and Cu toxicity (0-300 mg Cu/kg), despite a tendency to lower toxicity under higher density of animals. It was noted that the generation 1 Cu-response curves became flatter with increasing densities. In a 2nd generation exposure at density 10 (medium), an overall increase in toxicity was observed – organisms were more sensitive. Moreover, depending on the 1st generation start density this toxicity changed in the 2nd generation, i.e., a previous history of a higher density lead to a more Cu sensitive group of organisms in the 2nd generation [when based on EC_{10} and EC_{20} estimates, but not on EC_{50}]. There was a transfer of the density effect from generation 1 to 2 i.e. animals from the highest density (50) in generation 1 had lower Cu toxicity and when exposed in the:

a) highest density (50) in generation 2 the lower Cu toxicity effect was enhanced, while when exposed in the

b) lower density (10) in generation 2 this lead to higher Cu toxicity. Possibly this is related to the higher likelihood to clustering behavior of organisms at higher densities under stress - indirectly a lower exposure.

From the present results there is an indication that toxicity increased in a two-consecutive generation exposure to Cu. Therefore standard one generation testing extrapolation to field can be underestimating real effects. In regard to the density, continuous high population numbers seem to represent an ecological advantage. On the other hand, for animals under fluctuations in density numbers along generations (10→50, 50→10) the Cu toxicity patterns were less clear as both positive and negative effects were observed along generations. Hence, the conditions organisms are kept in culture previous testing can influence the toxicity results as well.

Acknowledgments

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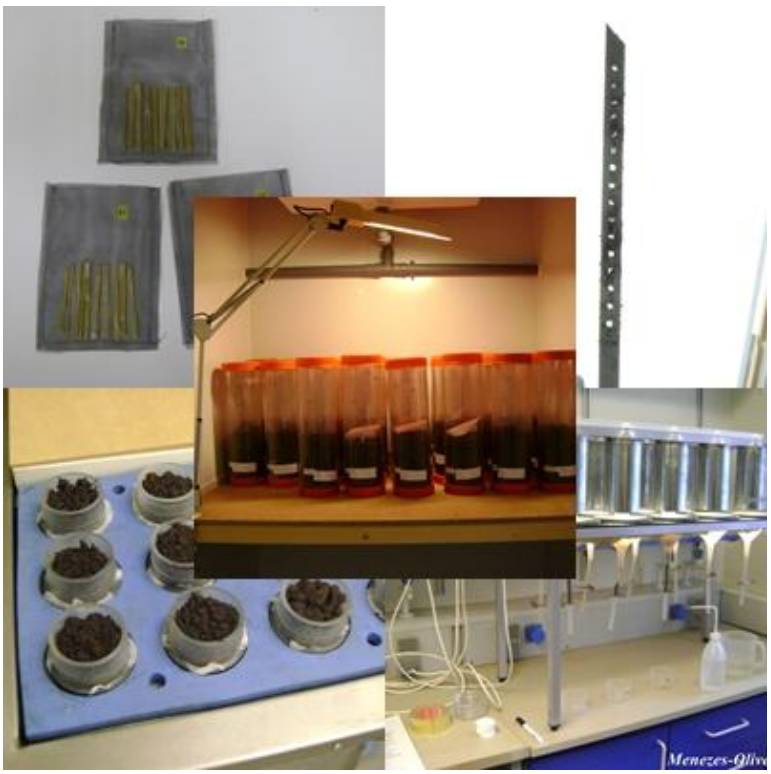

Chapter IV - Development of ecosystems to climate change and the interaction with pollution – unpredictable changes in community structures

Development of ecosystems to climate change and the interaction with pollution – unpredictable changes in community structures

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Submitted

Abstract

Climate change has serious impacts on ecosystems e.g. species' diversity and abundance. It is well known that changes in temperature may have a pronounced influence in the reproductive output, growth and survival of various terrestrial species. However, much less is known in regard to how changes in temperature combined with exposure to pollution will influence biodiversity, the interaction between species, and the resulting change in species composition. In order to understand the effects of changes in temperature and copper pollution (individually and in combination) on soil communities and processes, a factorial multispecies experiment was performed. Six species (representing different functional groups) were exposed in control (30 mg Cu/kg) and copper-contaminated soil (1000 mg Cu/kg) to four temperatures (10, 14, 19, 23°C) - temperatures representing the "summer" range (low to high) for Denmark – and three exposure periods (28, 61, 84 days). The species composition, feeding activity and OM turnover were assessed throughout. Multivariate analysis displayed significant changes in the food-web both with different copper levels and temperatures, resulting in different species composition for each exposure scenario. The most important species were *E. crypticus* (most sensitive to copper and temperature) and *F.candida*, (most abundant). Major changes in abundance due to temperature occurred in the first 28 days of exposure, where population growth was higher. A temperature dependent growth rate was possible to model for an exposure period of 28 days, whereas after 61 and 84 days of exposure data was less fitted, especially when combined with Cu. Further, in nature when climate change occurs in polluted areas, the consequences on population cannot be predicted based on data from non-polluted areas. The risk may be synergistic for certain species, as

indicated in the present study, and the final balance may depend on the particular species composition of that ecosystem.

Keywords: mesocosms, soil mesofauna, ecotoxicology, temperature, soil function, multispecies, ecosystem services.

1. Introduction

Climate change such as increase/decrease in temperature, depending on the geographical location, has caused severe impacts in the environment, e.g. on species abundance and distribution in soils (Messenger 1959, Angilletta et al. 2004, Uvarov et al. 2011). As a consequence, such effects can be transferred into changes at the ecosystem services (Nicolardot et al. 1994, Iglesias Briones et al. 2009, Blankinship et al. 2010).

It is however well known that temperature does influence the development of individual invertebrate species i.e. thermal-time or degree day concept. Interaction between temperature and contamination has been little studied and when done it has been done using single-species test systems (Ma 1984, Sandifer and Hopkin 1997, Khan et al. 2007). The information regarding effects on single-species is essential for present risk prediction, although of course lacking important ubiquitous community components of species-interactions e.g. predation, mutualism, competition, etc. (Beare et al. 1995, Bogomolov et al. 1996). Such interaction combined with known differences in optimum temperatures for each species (Jansch et al. 2005), limits the prediction of climate change community effects from single species tests data.

Species-interaction has been studied partly via field-studies (Briones et al. 1997, Fountain and Hopkin 2004, Holmstrup et al. 2007), partly via laboratory-studies using more than one species systems (Baker and Senft 1995, Bogomolov et al. 1996, Parmelee et al. 1997, Forster et al. 2004, Alonso et al. 2006, Pernin et al. 2006, Scott-Fordsmand et al. 2008, Kools et al. 2009). The multispecies-systems as presented by e.g. Pernin et al. (2006) or Scott-Fordsmand et al. (2008) attempt to combine the advantages of laboratory test-systems, (i.e. standardized conditions and monitoring) with important field relevant factors, i.e. species interaction. There are several multispecies-studies reporting the influence of contaminants, but to our knowledge only very few studies reported on the interaction between chemicals and climate change related parameters, i.e. drought (Holmstrup et al. 2007, Kools et al. 2008).

Many studies have been performed in regard to the impact of increased Copper (Cu) level on ecosystem structure and function. Although essential, Cu is toxic when present in “high” concentration, leading to changes in the community structures and altering the nutrient cycles (e.g. C and N) (Strandberg et al. 2006, Vieira et al. 2009, Amorim et al. 2011, Howcroft et al. 2011).

The aim of the present study was to assess the effects of changes in temperature in combination with Cu pollution at the soil community level and processes/functions. A Soil Multispecies System (SMS) was designed consisting of six species representing different functional groups in a "food-web design" community. This food-web community was tested to a range of temperatures (mean annual values and extreme summer temperatures for Denmark) and to a non-contaminated and a historically Cu-contaminated field soil (Pedersen et al. 1999). The measured parameters included species abundance, also used for diversity interactions and changes (PRC analyses), feeding activity and organic matter turnover.

2. Methods and materials

A laboratory food-web experiment was conducted following a previous mesocosms design (Scott-Fordsmand et al. 2008), using the described Soil Multi-species Test Systems (SMS). In brief (each aspect is explained in detail in the next sections), the species composition used is representative of e.g. a Danish agro-ecosystem (Krogh et al. 1996). Six species were included: four collembolans, one predatory mite and one enchytraeid. These belong to different functional groups - with mutualism, competition, predation and other interactions being represented. The soil used is from a well-studied site with a history of Cu contamination and known Cu gradient. Soil, collected from clean and contaminated area was first defaunated and then inoculated with a controlled microbial substrate. Soil biological structure was followed over time, sampled at days 28, 61 and 84. Soil function parameters were also measured using bait lamina and litterbag tests to assess feeding activity and organic matter turnover, respectively. A total of 124 SMS units were performed.

2.1. Experimental design and set up

The present study was designed as a two-way factorial experiment: factor 1 - Cu exposure (Control (30 mg Cu/kg dw) and contaminated soil (1000 mg Cu/kg dw)); factor 2 - temperature: 10-14-19-23°C. Sampling dates were at 28, 61 and 84 days. Five replicates per treatment were used.

The experimental Soil Multispecies Systems (SMS) units consisted of polyethylene tube (33 cm x 9.3 cm ø), having a surface area of 68 cm^2 and sample volume of 2241 cm^3. The tubes,

closed on the bottom with a lid, were filled with 1000 g moist soil (800 g dry weight plus 200 ml aqueous microbial substrate). Experiments were performed in temperature controlled rooms (variation in temperature was ± 1°C). After the addition of animals, each unit was covered with a perforated lid, weighted and transferred to the temperature test room with a 12h: 12h light: dark cycle. The soil water content was kept by replenishing water loss weekly.

2.2. Test soil

The soil used was from a well-known site in Hygum, Jutland, Denmark. This site has a known history of contamination where from 1911 till 1924 a timber impregnating factory labored, using mainly copper. From 1924 until 1991 the area has been used for agriculture purposes and thereafter it became fallow. This resulted in a Cu gradient, ranging from background levels to approximately 3000 mg Cu/kg (dry weight), with a gradual increase toward the centre of the field (Pedersen et al. 1999, Scott-Fordsmand et al. 2000, Strandberg et al. 2006).

The main physical-chemical characteristics of the soil are as follows: 20–32% coarse sand (>200 µm), 20–25% fine sand (63–200 µm), 11–20% coarse silt (20–63 µm), 12–20% silt (2–20 µm), 12–16 % clay (<2 µm), 3.6–5.5% humus and pH (H_2O) of 6.3. The soil was sampled to a depth of 20 cm. To exclude fauna, the soil was dried at 80°C during 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany), and then sieved through a 2 mm mesh to remove larger particles.

The soil used in the experiment was collected from two different locations, a control and Cu polluted with the concentration of ca. 1000 mg Cu/kg dried soil. The concentration was selected based on Enchytraeids sensitivity, since it was known to be very sensitive to Cu, and was a compromise between the effects on survival (non effect) and reproduction (EC_{50} – 439 ± 130) assessed by Maraldo et al. (2006) together with the previous knowledge that species are more drastically affected when exposed alone than in combination with other species (Scott-Fordsmand et al. 2008).

2.3. Microbial substrate

The procedure to extract the microbial substrate and inoculate it into the experimental chambers was based on Scott-Fordsmand et al. (2008). Freshly collected soil from the control

area of the field site (1 kg wet weight) was mixed with 2 L of deionised water and shaken for 3 hours. After this, the soil-water solution was filtered through a 50 µm mesh and diluted 10 times to use in the experiment. The soil water content was adjusted to 20% of which 10% was carried with the microbial substrate.

2.4. Test organisms

All soil invertebrates used in the SMS were cultured in the laboratory, kept for several generations. The organisms were randomly selected among adult sized animals and added in the SMS at 3 different stages (see Table 1 for details).

Table 1 – Summary information in regard to the biological component of the SMS, including each added test species, respective group, food web and/or associated function, the assumed interaction type between organisms and preferable living layer in the soil ecosystem (corresponding to epedaphic=surface, hemiedaphic=upper, euedaphic=middle; enchytraeids are regarded endogeic). The number of organisms added at test start and the day at which organisms were added is indicated.

Test species	Group	Food web / Function	Assumed interaction type			Living layer	Initial Number	Day
Microbial community	Bacteria Fungi	Decomposer Prey			← Prey / Grazing	all	n.d.	-3
Enchytraeus crypticus	Oligochaete	Decomposer Fungivore Prey	Competition; Mutualism; Neutralism; Amensalism;	Predation →		middle	50	0
Folsomia candida	Collembola	Prey for *H. aculeifer*				upper - middle	30	1
Proisotoma minuta						upper	30	1
Mesaphorura macrochaeta						middle	30	1
Hypogastrura assimilis						upper	30	1
Hypoaspis aculeifer	Acari	Predator				upper - middle	10♀; 5♂	7

Here we attempted to summarize the information in terms of test species, respective group of organisms, known role in the food-web/function and assumed/known interactions. Information regarding the living layer of the organisms is based on Hopkin (1997) and Lokke and van Gestel (1998). As can be seen from table 1, the types of interactions that may occur between collembolans are numerous, e.g. competition, mutualism, neutralism or amensalism (Hopkin 1997), as further discussed in the discussion section. Predation was included via the mites, which prey on collembolans and enchytraeids (Krogh 1995). There are of course some less clear functions, e.g. we know that collembolans can be decomposers to a lesser extent (more important in acidic fields where few earthworms are present) or that some enchytraeid species may feed on fungal hyphae as a relevant substrate but it is not entirely understood if they feed on microorganisms (Lokke and van Gestel 1998).

2.5. Soil function analysis

2.5.1. Bait lamina test

The soil fauna feeding activity was assessed using the bait lamina method (Kratz 1998). In short, bait lamina are plastic lamina sticks, containing 16 holes which are filled with a cellulose-based substrate (70% cellulose, 25% nettle powder (organic grown), 5% activated charcoal). One bait lamina stick was used per SMS, added on days 7, 28 and 61, and removed one week after insertion date. The feeding activity was expressed as the percentage of empty (eaten) plus the partially eaten holes per day. Four extra SMS units were used, under the same test conditions and at temperature of 19ºC to determine the appropriate time period for bait-lamina sampling days. Observations on the feeding activity in the extra units were made daily.

2.5.2. Litterbag test

The soil organic matter turnover was assessed using litterbags. The bags (1.5 mm x 1.5 mm mesh) were filled with approx. 200 mg of dried straw and inserted into the top 5 cm soil layer. In an attempt to minimize/prevent that invertebrates would have many hiding places, the straws were carefully placed side by side, thus promoting exposure to the soil test. The litterbag was always placed in replicate one of each experimental condition and left in until the end of each sampling day.

2.6. Cu concentration, pH and organic matter (OM) measurements

The total soil Cu was determined by flame Atomic Absorption Spectrometry (AAS, Perkin Elmer 4100, Ueberlingen, Germany) before test start. Soil samples were first digested in a heating block using a 70% concentrated HNO_3 solution in 100 mg dry soil. Temperature was increased from 80°C to 110°C until all fluid from the samples became clear and the acid evaporated. Before AAS analysis the samples were dissolved in a 0.1M HCl.

The organic matter (OM) and pH were measured at days 0, 28, 61 and 84. The pH was measured in water. The OM content was measured as loss of organic matter after 3 h at 600°C.

2.7. Soil organisms sampling and extraction

At each sampling time (28, 61 and 84 days – here day 0 is the day that enchytraeids were added) 10 SMS units/replicates (5 control plus 5 contaminated soil) were extracted from each test temperature (10, 14, 19 and 23ºC). The soil from each replicate was divided in 3 layers (top, middle and bottom) with approximately 5 cm height. From each layer ca. 130 g (random sub-samples) was sampled to quantify collembolans and mites, and ca. 60 g to quantify enchytraeids. The microarthropods sub-samples were extracted over 7 days in a high gradient extractor (MacFadyen) (Scott-Fordsmand et al. 2000). Animals were collected in benzoic acid, transferred to glycerol, identified and counted. The enchytraeids were extracted by spreading the soil sample into five 200 ml plastic beakers (ø 7 cm), which were filled with tap water, gently shaken, and then left for 24 h at 5°C for sedimentation. Adult and juvenile enchytraeids get dispersed on the soil surface and were thereafter picked and counted under a stereo microscope within 48 h.

2.8. Data analysis

2.8.1. Univariate analysis

Temperature dependent growth-rates were estimated using sigmoidal, Gaussian and exponential decay regression models (SPSS 1997).

A multiple comparison using the Tukey method (two-way ANOVA) was performed to analyze interactions between the temperatures and contamination effects over time (exposure

duration) (SPSS 1997). Data were ln transformed prior to the multiple comparisons and results may be seen in the supplementary information (Table S1).

2.8.2. Multivariate analysis

The community data was analyzed using Principal Response Curves (PRC) method as described by Van Den Brink and Ter Braak (1998; 1999). This method is based on Redundancy Analysis ordination techniques, the constrained form of Principal Component Analysis (PCA) and is specially designed to evaluate the effects of stressors at the community level taking time into account. The significance of the PRC diagrams was tested by Monte Carlo permutation tests by permuting whole time series in the partial RDA, from which the PRC was obtained. All data were ln transformed. PRC analysis was performed using the software package CANOCO version 4.5 (Ter Braak and Smilauer 2002).

3. Results

3.1. Soil pH and soil OM content

The pH values ranged between 6.0 ± 0.5 in all treatments and exposure periods. The initial OM content for control soils was 5.4% and for the Cu contaminated soil it was 6.7%. For both control and contaminated soils the % of OM content reduced around 10% over the 84 days of exposure in the two lowest temperatures (10 and 14°C). For the two highest temperatures (19 and 23°C) a 10% reduction was seen for systems exposed during 28 days but no reduction was observed when systems were exposed for 61 and 84 days.

3.2. Individual species analyses

The abundance of the different species depends both on time, temperature and contamination level (Cu/Control) (see figure 1 and S1 in the supplementary material).

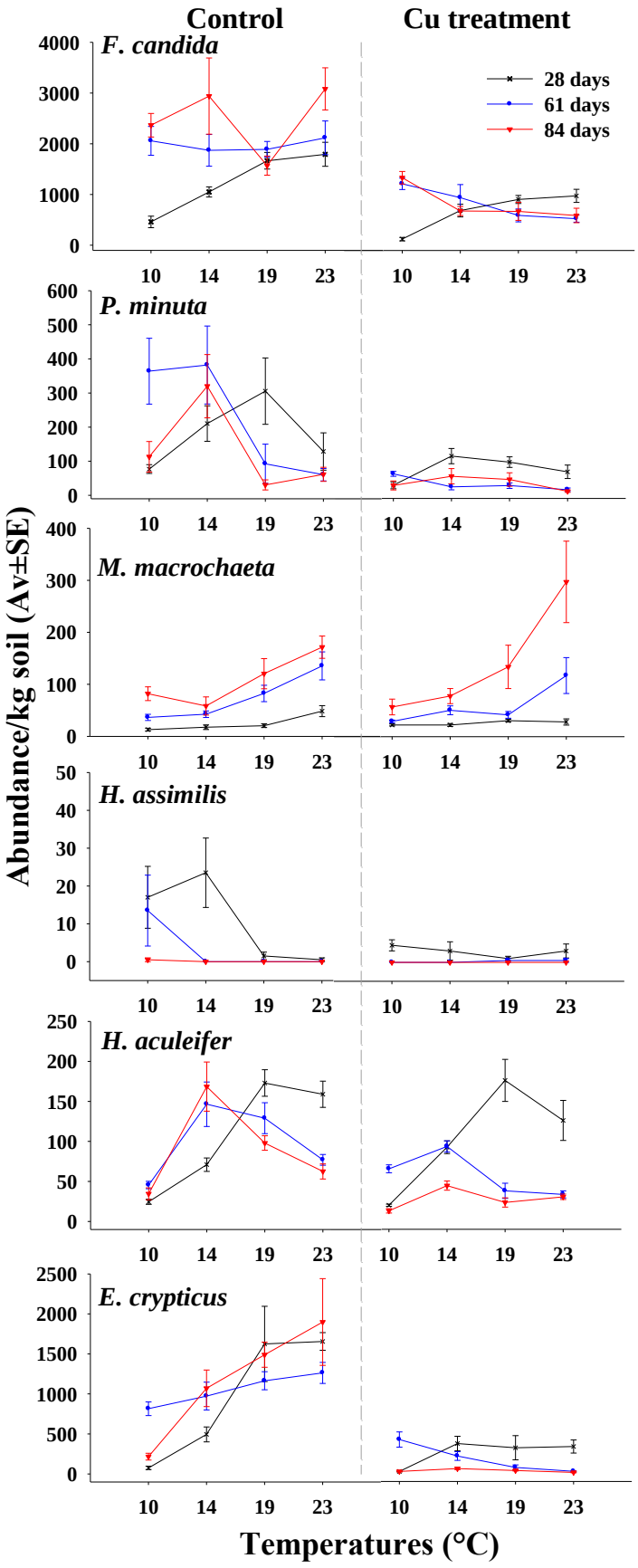
Control
Cu treatment
F. candida
28 days
61 days
84 days
P. minuta
M. macrochaeta
H. assimilis
H. aculeifer
E. crypticus
Abundance/kg soil (Av±SE)
Temperatures (°C)

Figure 1 – Results of species abundance from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. Results are presented as the average number of individual ± standard error (AV±SE) per kg soil. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods of (28, 61 and 84 days).

With increasing temperatures the abundance developed faster for most species, hence at low temperature the population reached a max around 61 days whereas at higher temperature the max was reached at 28 days (Figure S1, Table 2). For some species (see figure S1) the abundance flattened or decreased from 61 to 84 days. The significant effects for the individual species to the different treatments (Ct and Cu contamination), temperatures and the interactions between treatments and temperatures over time were analyzed and results may be seen in the supplementary information (Table S1).

Table 2 – Models describing the temperature dependency of the individual growth-rates, the models are based on the population growth-rate from the SMS (rather than total abundance, would give "same" models) in order to provide values comparable with literature. SMS was performed with four different temperatures (10, 14, 19 and 23°C) over 3 exposure periods (28, 61 and 84 days). Models applied are sigmoid (high fecundity populations), peak Gaussian and exponential decay. The a values represent the estimated top point and b represents the steepness of the curve.

Species	Cu (mg/kg)	28 days				61 days				84 days			
		a	b	r^2	Model	a	b	r^2	Model	a	b	r^2	Model
F. candida	0	60.9 [n.d]	2.36 [n.d]	1	Sig.4 par.*	400.7 [438.5]	0.22 [0.12]	0.98	Exp.3 par.##	-	-	-	-
	1000	33.9 [1.2]	1.31 [0.25]	0.99	Sig.3 par.**	285.0 [58.9]	0.17 [0.02]	1	Exp.3 par.	11.1 [n.d]	-0.16 [n.d]	0.21	Sig.4 par.
E. crypticus	0	59.0 [n.d]	1.02 [n.d]	1	Sig.4 par.	133.3 [46.1]	0.07 [0.19]	0.89	Exp.3 par.	20.9 [19.6]	0.18 [7.62]	0.51	Sig.3 par.
	1000	12.2 [n.d]	0.16 [n.d]	0.98	Sig.4 par.	1066.4 [874]	0.39 [0.08]	1	Exp.3 par.	42.9 [n.d]	3.08 [n.d]	1	Sig.4 par.
M. macrochaeta	0	-0.5 [0.49]	-0.16 [0.9]	0.55	Sig.3 par.	2.0 [n.d]	1.23 [n.d]	1	Sig.4 par.	1.1 [n.d]	-0.06 [n.d]	0	Sig.4 par.
	1000	0.3 [+∞]	0.12 [+∞]	0.94	Sig.4 par.	2.4 [+∞]	0.14 [+∞]	0.94	Sig.4 par.	6.8 [+∞]	0.17 [+∞]	1	Sig.4 par.
H. aculeifer	0	5.2 [+∞]	0.22 [+∞]	0.99	Sig.4 par.	3.9 [+∞]	-0.21 [+∞]	0.9	Sig.4 par.	1.2 [n.d]	-0.06 [n.d]	0.54	Sig.4 par.
	1000	4.9 [+∞]	0.19 [+∞]	0.9	Sig.4 par.	4.9 [+∞]	-0.21 [+∞]	0.95	Sig.4 par.	2.2 [n.d]	1.37 [n.d]	1	Sig.4 par.
P. minuta	0	10.8 [0.48]	3.74 [0.18]	1	Peak, 3par.#	13.0 [+∞]	-0.21 [+∞]	0.93	Sig.4 par.	-0.8 [0.37]	-9.22 [0.36]	0.95	Hyp.2 par.###
	1000	3.5 [1.32]	3.71 [1.54]	0.78	Peak, 3par.	3.3 [+∞]	-0.16 [+∞]	0.92	Sig.4 par.	2.4 [+∞]	0.17 [+∞]	0.74	Sig.4 par.
H. assimilis	0	0.7 [+∞]	-0.08 [+∞]	0.95	Sig.4 par.	-0.4 [0.30]	-0.02 [0.25]	0.45	Sig.3 par.	0.6 [+∞]	0.14 [+∞]	1	Sig.4 par.
	1000	0.1 [+∞]	-0.27 [+∞]	0.68	Sig.4 par.	0.1 [+∞]	0.22 [+∞]	0.62	Sig.4 par.	0.0 [+∞]	-0.21 [+∞]	0.75	Sig.4 par.

* f= y0+a/(1+exp(-(x-x0)/b)); ** f= a/(1+exp(-(x-x0)/b)); #f= y0+a/(1+exp(-(x-x0)/b)); ##f=y0+a*exp(-b*x); ### f=a*x/(b+x)

For *F. candida* in control soil the population increased continuously over all 84 days, except for the 19ºC exposure which remained constant after 28 days. However, the abundance increase was faster for the 19 and 23ºC exposure than for the 10 and 14ºC exposures (see figure S1). The exposure to Cu also showed an initial faster population growth at higher temperature, although at a much lower level. For animals at 14-23°C the population remained constant after 28 days of exposure whereas it continued to increase for the 10°C; the latter reached a higher abundance than the former. The abundance dependency of temperature can be seen in the figure 1 where at 28 days the abundance is clearly temperature dependent, but at day 61 and 84 the abundance is generally independent (see also Table S1 for the significances of the different temperatures over time). The same trend is present for the copper exposed, although abundance decreased with increasing temperatures at day 61 and 84.

The same trend with higher initial population growth rate at higher temperatures was apparent for *E. crypticus*. However, here the population increase stopped at 28 days at 19 and 23°C, while at 14ºC it continued to grow for 61-84 days. At 10°C there was an initial population growth between 28 and 61 days, after which abundance decreased to initial levels. Copper exposure caused the same pattern on growth rates as for *F. candida*, however abundance decreased to initial levels at day 61 (19-23ºC) and day 84 (10-14ºC). The abundance dependency of temperature can be seen from figure 1 where the abundance increased with temperature at all exposure periods. For Cu exposed animals a positive correlation was only observed between 10 and 14°C at day 28. At day 61 and 84 there was a negative correlation with temperature.

The *M. macrochaeta* population increased continuously over all 84 days (Figure S1) for all temperatures tested. The abundance was positively correlated with temperature for all exposure durations, max at 84 days (see figure 1). Animals in control and Cu-treatments showed the same trend; although *M. macrochaeta* seemed to perform better at 23°C in Cu-contaminated soil (10-fold increase) than control soil (6-fold increase, after 84 days).

For *P. minuta* the abundance initially (28 days) increased and then (61-84 days) decreased. At the two highest temperatures the peak was at 28 days, whereas at the two lowest temperatures it was delayed till 61 days. The lowest abundance was observed at the highest temperature (23°C). The same trends were apparent in the Cu contaminated soil, but here the maximum

abundance was 3-4 times lower than in control soil. For both exposures regimes the optimum temperature was 14°C, except for the 28 day exposure where 19ºC was optimum.

For *H. assimilis* a temperature dependent abundance decrease (over time) was observed, being absent after 28 days at 19° and 23°C (both in control and contaminated soil).

For the predatory mite *H. aculeifer* the same increase was observed in the first 28 days of exposure irrespectively of whether it was control or Cu contaminated, showing a 6-fold increase in abundance from 10 to 23ºC (see figure S1). However, after day 28, the organisms' abundance decreased at higher temperatures, especially in the Cu-contaminated soils. There was an optimum dependency between the population growth and the temperature, exposure times of 28 days showed a peak at 19°C whereas exposure times of 61 and 84 days showed a peak at 14°C (Figure 1), the same seen for *P. minuta* in control soil.

Results in terms of vertical distribution in the SMS can be seen in figure S4 (supplementary material). Overall main differences occur for *H. aculeifer*, which is in general more distributed in the lower 5-15 cm layers compared to the other species and, *E. crypticus*, clearly localized in the upper 0-5 cm layer. There was also an indication of a change with increased exposure time and temperature driven, e.g. *H. aculeifer* and *F. candida* at 10ºC between 28 and 61 days onwards compared to 14ºC and higher temperatures.

3.3. Multispecies analysis

Multivariate PRC analyses were performed using the 10°C temperature as the control (i.e. to which other exposure temperatures were compared, figure 2). The choice of the "control" impacted the analyses but not general trend (see example with 19°C in figure S2). The PRC display/analyses (Figure 2), showed a clear separation between contaminated and control soil (Monte Carlo permutation test, $p = 0.002$) becoming most pronounced at 84 days.

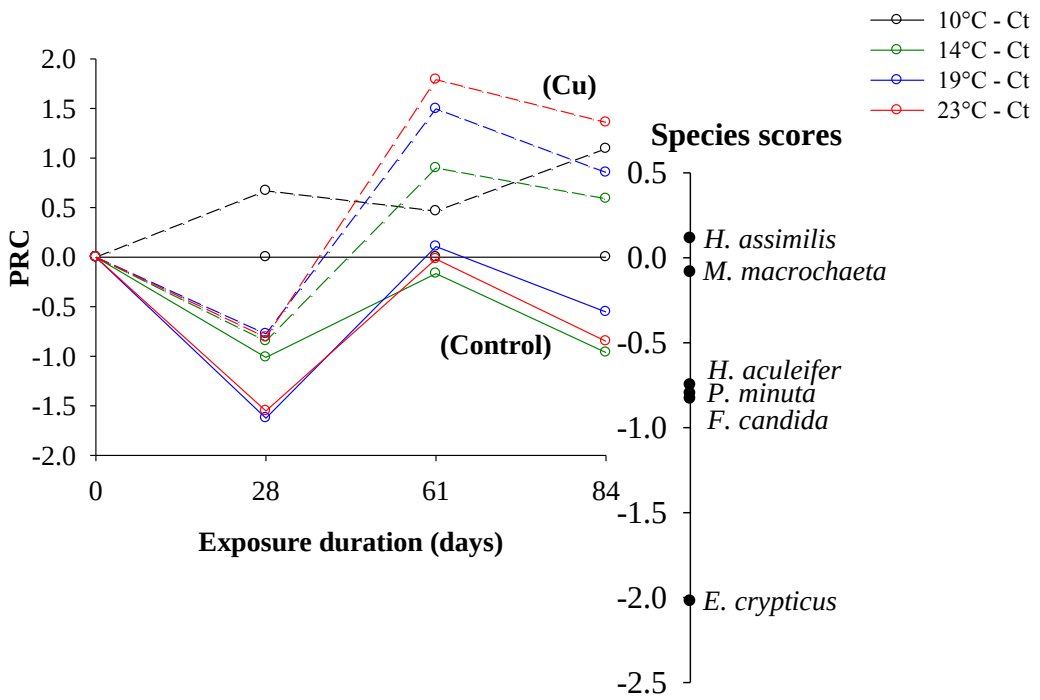

Figure 2 - Principal Response Curves (PRC) with species scores (*bk*) for food-web data set, obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. The solid lines (—) represent the control and the dashed lines (--) represents the Cu contaminated treatment. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods (28, 61 and 84 days). Control temperature was set to be 10°C.

Species scores indicated that *E. crypticus* was most affected by changes in temperature and soil contamination (Figure 2). From the RDA analysis, for control soil, 68.4% of the total variation in species data may be explained by temperature and exposure duration together, of which 7.1% was explained by temperature and 57.8% by exposure time. For Cu contaminated soils, the total variation explained by temperature and exposure time together was 63.4%, temperature and sampling times were responsible for 2.5 and 47.1%, respectively. Monte Carlo permutation test indicated a significant effect for all temperatures at 28 and 61 days for both control and Cu treatments when compared to 10°C. Control soil SMS showed the highest significance over 84 days for the highest temperature and the only situation in which no significant difference occurred was at 19°C. For the Cu contaminated soil the same results were observed until 61 days. After 84 days both 14°C and 23°C showed significant difference

when compared to 10°C and the highest significance was still seen for the highest temperature tested (Table 3).

Table 3 – Significance levels (*p*) as calculated by the Monte Carlo permutation test, done by testing the results of the exposure to every temperature (14, 19 and 23°C) versus the temperature of 10°C in each soil treatment (0 and 1000 mg Cu/kg) and exposure period (28, 61 and 84 days). The lowest possible *p* value is 0.002 for 499 permutations.

Cu (mg/kg)	Temperature	Sampling day		
		28	61	84
		Monte Carlo significance level (p)		
0	14°C	0.006	0.006	0.006
	19°C	0.006	0.006	0.074
	23°C	0.006	0.006	0.018
1000	14°C	0.006	0.006	0.074
	19°C	0.006	0.006	0.11
	23°C	0.006	0.006	0.01

Monte Carlo permutation tests performed using 19°C as the control temperature showed the same trend as using 10°C as the control temperature although less significance was observed. Moreover, Monte Carlo permutation tests revealed that for all temperatures (with 19°C as control) there was a significant effect between control and Cu contaminated SMS ($p<0.05$).

3.4. Functional assessment

3.4.1. Bait lamina

Feeding activity was in general higher in control soil than in contaminated soil (except at day 7), the difference being more pronounced at day 61 than at day 28. Feeding activity in controls was positively related to temperature for the 7 day (both in control and contaminated soil) and 28 day (only in control soil) exposures (One way ANOVA; Holm-Sidak method $p<0.001$). For the longer exposure durations there were no apparent temperature relationship (Figure 3a). The higher feeding activity in the control soil is in agreement with the total abundance of organisms (Figure 3b) mainly during the first 28 days (growing phase), this is presented for comparison purposes.

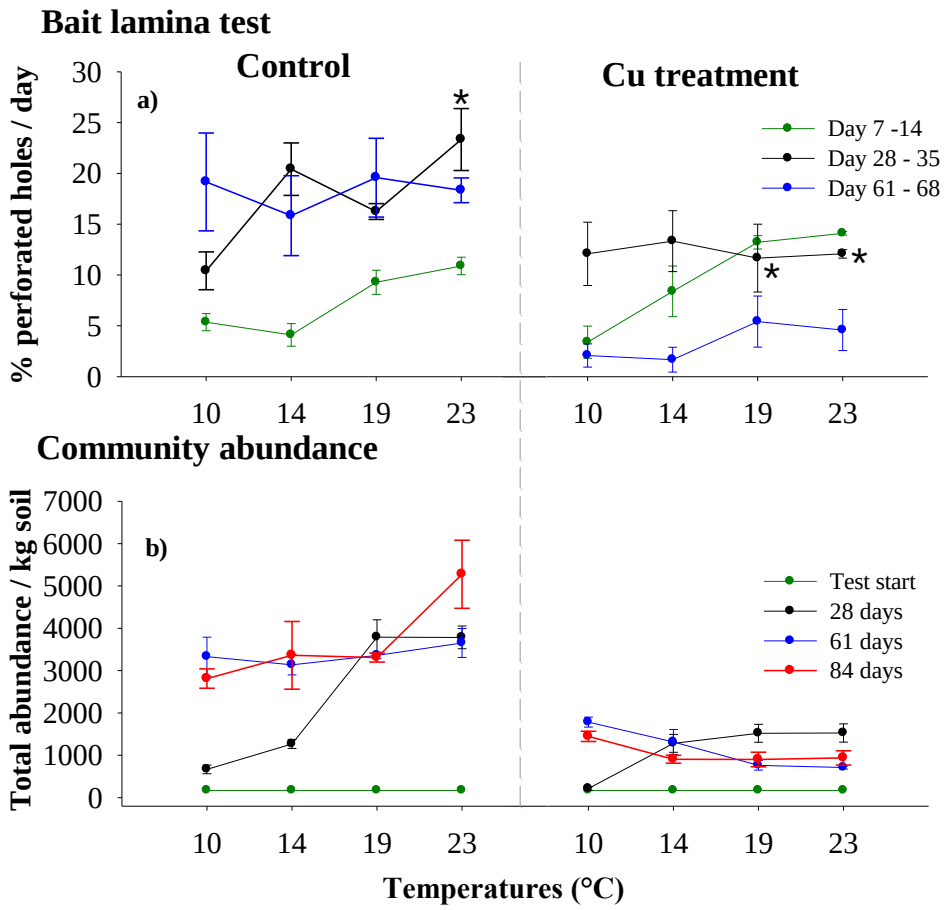

Figure 3 – Results of bait lamina test used as feeding activity measurements and for comparison, invertebrate community abundances, obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. Following treatments were used: four temperatures (10, 14, 19 and 23°C) and three exposure periods (28, 61 and 84 days). a) Feeding activity expressed as average ± standard error (AV±SE) of the % of perforated holes (partially empty (eaten) + totally empty holes) in bait lamina per day; *p<0.05, using 10ºC as control; b) Total abundance of invertebrates (AV±SE) per kg of soil.

3.4.2. Litterbags

Results of litterbag studies showed a positive relation between loss of litter weight, exposure time and temperature. This relation was apparent for both control and Cu contaminated treatments, but the contaminated samples generally had lower litter loss weight than control samples (Figure 4).

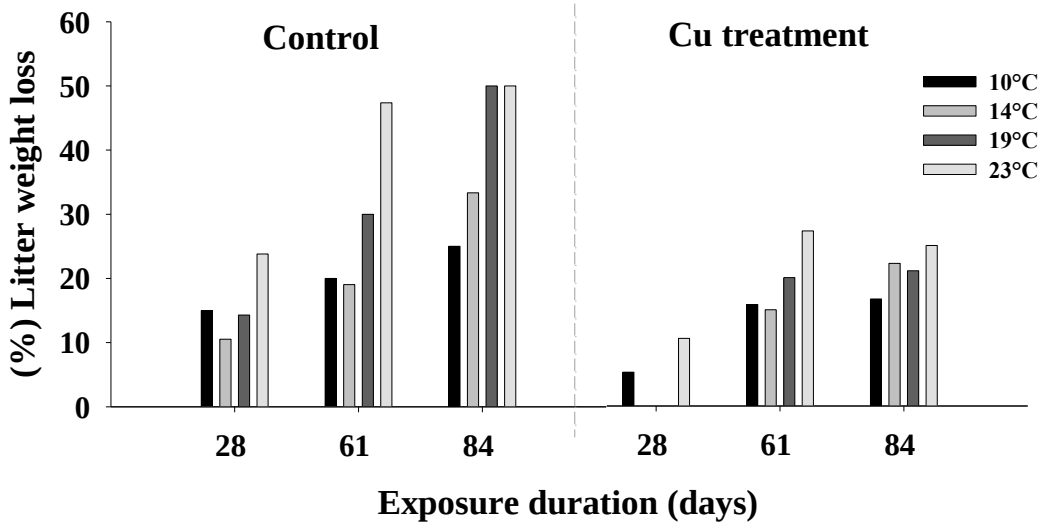

Figure 4 - Results of the litterbag study used as organic matter turnover measurements, obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. Following treatments were used: four temperatures (10, 14, 19 and 23°C) and three exposure periods (28, 61 and 84 days). The graph represents the % loss of litter weight compared to start (n=1).

4. Discussion

The present study showed that the species abundance (and total number) increased with temperature [as in accordance with "thermal-time"], but that in contaminated soils such trends were less pronounced (see table 2). This positive correlation with temperature was mainly observed in the initial stages of population development (28 days of exposure). The initial phase corresponded to a high fecundity phase, which is generally the main stage displaying a positive correlation between abundance and temperature (Amarasekare and Savage 2012). In later phases (day 61-84), when the reproductive activity was lower, the total number of organisms was either independent of or negatively related to temperature; which is also in agreement with the generalizations derived by Amarasekare and Savage (2012).

For Cu-contaminated soil, the abundance was always lower than for the corresponding control temperature/duration exposure, except for *M. macrochaeta* at 23°C. The initial phase populations showed a lower growth-rate increase with temperature, resulting in a flatter temperature-abundance curve (Figure 1, table 2). For the later phases (61 and 84 days)

growth-rates were either negatively or constant related with increasing temperature (except for *M. macrochaeta*) as in control conditions. Differences between control- and Cu-exposure to temperature were not only observed for abundance (above), but also for the development in the species composition (Figure 2 and S3, supplementary material). The percentage of species-variability explained by temperature was about 3 times higher for the community in the control soil than in the Cu-contaminated soil (see PRC results).

Hence, our study indicated that the effects of temperature changes in field populations/communities are easier observed in growing populations, e.g. spring conditions in temperate zones where the fecundity based growth-phase is dominant. It also showed that in contaminated areas temperature increase has a much lower or negative impact on most species.

Although *F. candida* was the most abundant in the experiment, *E. crypticus* had the largest impact on the data analysis, probably because besides being abundant it was also more sensitive to changes in both temperature and Cu (see figure 1 and S1), as confirmed in single species testing for temperature by Briones et al. (1997) and for Cu by e.g. (Amorim et al. 2005a, Maraldo et al. 2006, Scott-Fordsmand et al. 2008). This temperature dependency was in agreement with previous studies where Enchytraeid population size has been shown to depend on temperature and water content (Springet.Ja et al. 1970, Abrahams.G 1971). *E. crypticus* was more affected by Cu at higher than at low temperatures, which is in line with studies by Khan et al. (2007) who showed that the Cu-LT_{50} (lethal time) of *Lumbricus terrestris* was lower with increasing temperatures (from 10 to 22°C).

In our experiment, *F. candida* population number was dependent of temperature at day 28, but not at the 61 and 84 exposure days. Martikainen and Rantalainen (1999) observed a continuous positive (linear or exponential) relationship between population number and temperature (13-19ºC) for up to 56 days, when using *F. candida* in a single species experiment. This indicated that the lack of temperature dependency at 61-84 days in our experiment is related to interaction between species, as it seems unlikely that the temperatures we used would be physiologically inhibiting (Amarasekare and Savage 2012). Although, exposing the same species to 15, 20 and 25ºC (28 days), Sandifer and Hopkin (1997) observed an almost complete reproductive arrest (30 juveniles) at 25ºC compared to 20ºC (800 juveniles). In our experiment, after 28 days the population number in the 23ºC

exposure was similar to the 19ºC exposure, hence no reproductive arrest, indicating little/no physiological inhibition. In regard to interaction with Cu, Sandifer and Hopkin (1997) observed little difference in Cu-toxicity (EC_{50}) when comparing 15 and 20°C; this latter is in agreement with our results for temperatures of 14 and 19°C where population numbers were similar for the two temperatures.

H. assimilis decreased to near zero over time, for both control and Cu-contaminated soil in all temperature conditions. While *H. assimilis* decreased rapidly, *H. aculeifer* was increasing, which could indicate predation. In Cortet et al. (2003) they tested SMSs with and without *H. aculeifer* and it was very pronounced that with *H. aculeifer* the population of *H. assimilis* was totally extinct after 30 days while without *H. aculeifer* the population of *H. assimilis* showed a slow decrease in time. The fact that other potential prey species of *H. aculeifer* (*F. candida*, *E. crypticus*) did not decrease could be due to higher reproduction rates (e.g. for *F. candida* (Amorim et al. 2005b)) masking the predation. Whether the reason accounts for *P. minuta* is uncertain, as *P. minuta* possibly also have a lower reproduction rate than *F. candida* (Dodd and Addison 2010).

The increase of *P. minuta* was influenced by both temperature and Cu-contamination. The almost total lack of population growth for this species in the Cu-contaminated soil is in agreement with the known sensitivity of this species to Cu (Greenslade and Vaughan 2003, Nursita et al. 2005). When exposed to the two lowest temperatures in the control soil, organisms showed the highest abundance after 61 days of exposure while for the two highest temperatures it was seen after 28 days. These temperature-related differences are likely related to the faster individual growth of *P. minuta* at higher temperatures (Park 2007), and probably combined with competition with *F. candida – P. minuta* and *F. candida* had a very similar vertical distribution in the soils (Figure S4) but *F. candida* abundance was at least 7 times higher - or predation by *H. aculeifer*. The latter was not depicted in the dataset i.e. a negative correlation between the two species, possibly balanced because the species depend differently on temperature changes, (Figure 1) (see e.g. (Jiang and Morin 2004, Jansch et al. 2005)). As discussed above, if we for a moment disregard the species interaction then it is noticeable that the population optimum temperature probably depends on the population dynamic, e.g. for growing populations (by day 28) of *H. aculeifer* and *P. minuta* the population optimum temperature was higher than for the stable populations (61 and 84 days).

M. macrochaeta was the only species showing an increased growth rate with the combination of increased temperature and Cu-exposure. The fact that in control soil this species has a continuous (despite slow) increase with time could be due to that, as discussed by Cortet et al. (2003), this species is likely less pressed by competition. *M. macrochaeta* has smaller comparative size (and euedaphic life habits), hence the possibility to hide in small soil pores being less exposed to factors such as competition for space and food. Further, it has morphological characteristics such as complex pseudocellis which, as discussed by Filser et al. (2000), has an advantage for survival in contaminated environments, could contribute to the higher abundance in Cu contaminated soil. This, together with the fact that other species abundances decreased due to Cu, indicates that these must be a pressure of somehow for *M. macrochaeta* as inferred by control results. Additionally, if *M. macrochaeta* is less affected by Cu than the other species, then it will also be more competitive and less prone to be predated.

In terms of vertical distribution (Figure S4), when looking at *H. aculeifer* and *F. candida*, at 10ºC (28 and 61 days onwards) compared to the other temperatures, could indicate that the mobility of these two species is more affected than the others, probably meaning that 10ºC is closer to their lower temperature boundaries.

The feeding activity and organic matter turnover were influenced by both temperature and Cu-contamination. Copper exposure was causing a decrease and the increase in temperature increased the activities (Figures 3 and 4). This trend corresponded with the total abundance of individuals, which has been observed in other studies as well, e.g. Filzek et al. (2004). The positive correlation between soil mesofauna abundance and feeding activity and OM decomposition is a clear indication of the association between soil organisms and ecosystem services, as also referred by others, e.g. Lavelle et al. (2006). Invertebrates are important, not only directly but also indirectly, contributing to the microbial communities' dispersal and hence stimulating the OM decomposition (Rombke 2003). In the present study we could depict that while an increase of temperature from 10 to 23ºC stimulated the activity, the Cu contamination caused a decrease in the OM decomposition, probably due to a direct decrease on the species abundance and/or an indirect change in composition. It is not likely that abundance of mesofauna was responsible alone as it has been described that very high abundances can cause overgrazing of the microflora (fungi, bacteria) and a consequent

reduction in OM turnover (Mebes and Filser 1998). Further, it is known that the species composition is also determining due to functional dissimilarities (Heemsbergen et al. 2004).

5. Conclusion

Temperature increase from 10-23ºC caused an overall increase in total abundance of the invertebrate community, with species interaction clearly visible. The major increase occurred during the first 0-28/-61 days (equivalent to spring), fastest at higher temperatures. We conclude that the effects of temperature changes on populations/communities in the field are best captured in populations with high fecundity e.g. equivalent to spring-time in temperate zones where the fecundity based growth-phase is dominant.

Total abundance was in general reduced with exposure to Cu contaminated soil, except for *M. macrochaeta*, which had a more pronounced population (compared to control) at higher temperatures.

The relative species composition changed both with temperature and pollution. Key species in the community were the collembolan *F. candida* and the enchytraeids *E. crypticus*, due to the high reproduction efficiency for the first and the temperature and Cu sensitivity for the second.

One of the soil services, as measured by OM decomposition function, decreased due to Cu pollution. Although an increase in OM decomposition was observed with temperature increase (10-23ºC), associated with overall abundance decrease, the community changes are less predictable here.

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Supplementary information

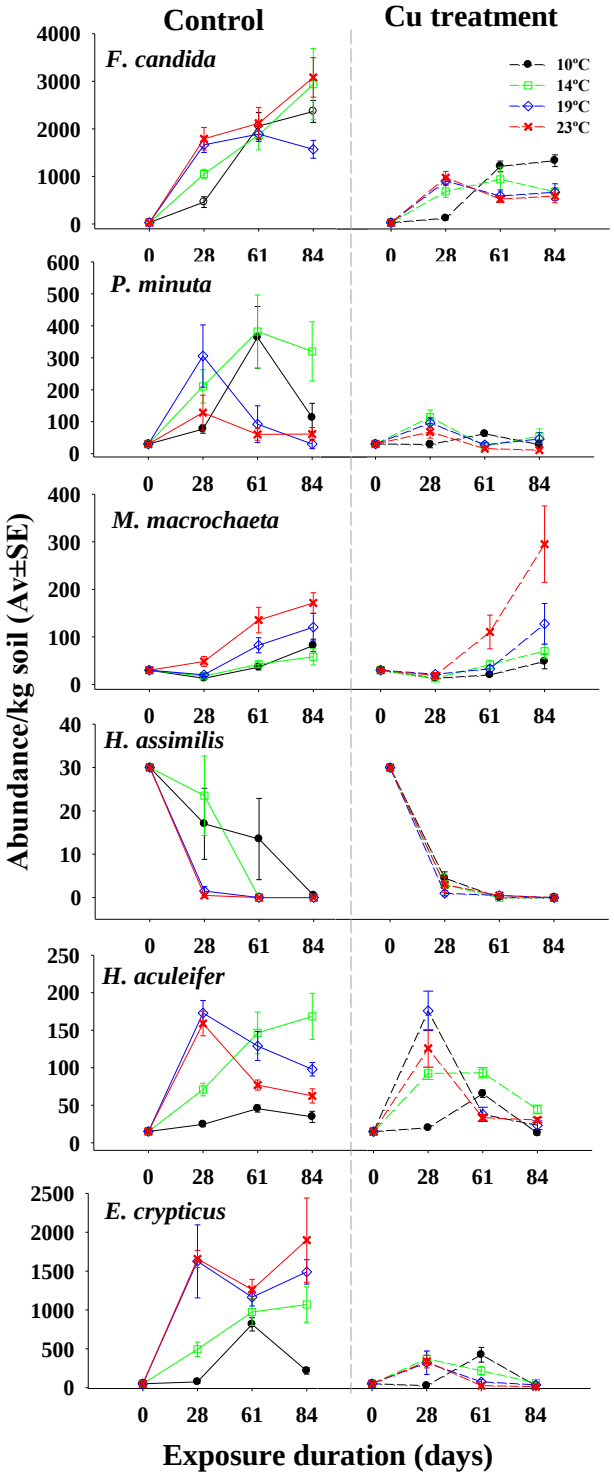

Figure S1- Results of species abundance from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mgCu/kg) site from Hygum, Denmark. Results are presented as the average number of individuals ± standard error (AV±SE) per kg soil. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods of (26, 61 and 84 days).

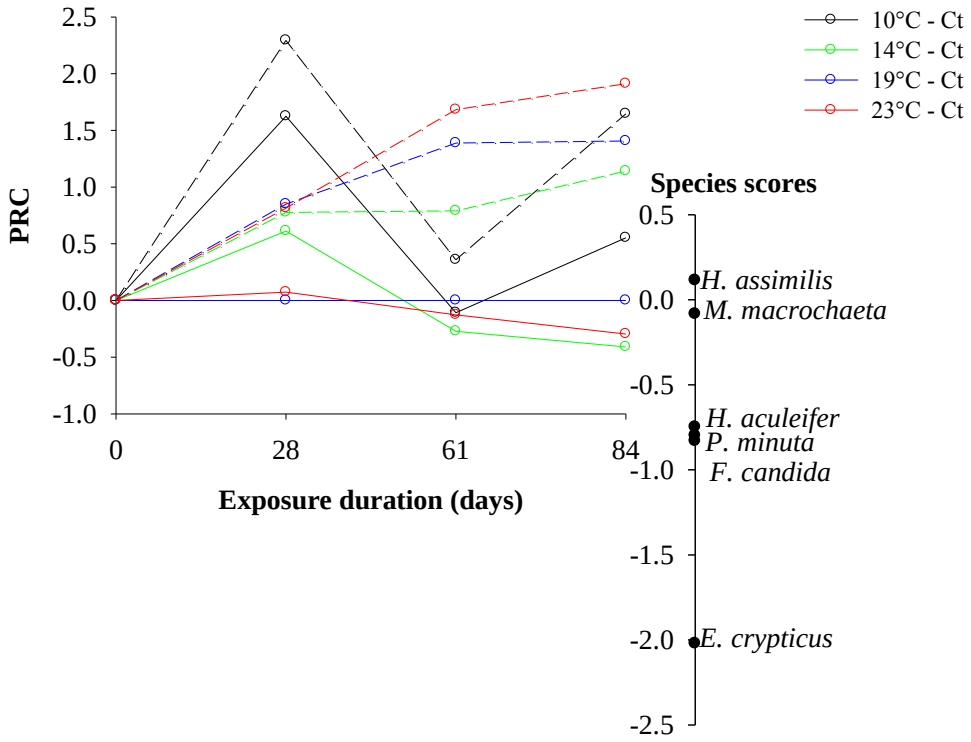

Figure S2 - Principal Response Curves (PRC) with species scores (bk) for food-web data set, obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mgCu/kg) site from Hygum, Denmark. The solid lines (—) represent the control and the dashed lines (--) represent the Cu contaminated treatment. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods (26, 61 and 84 days). Control temperature was set to be 19°C.

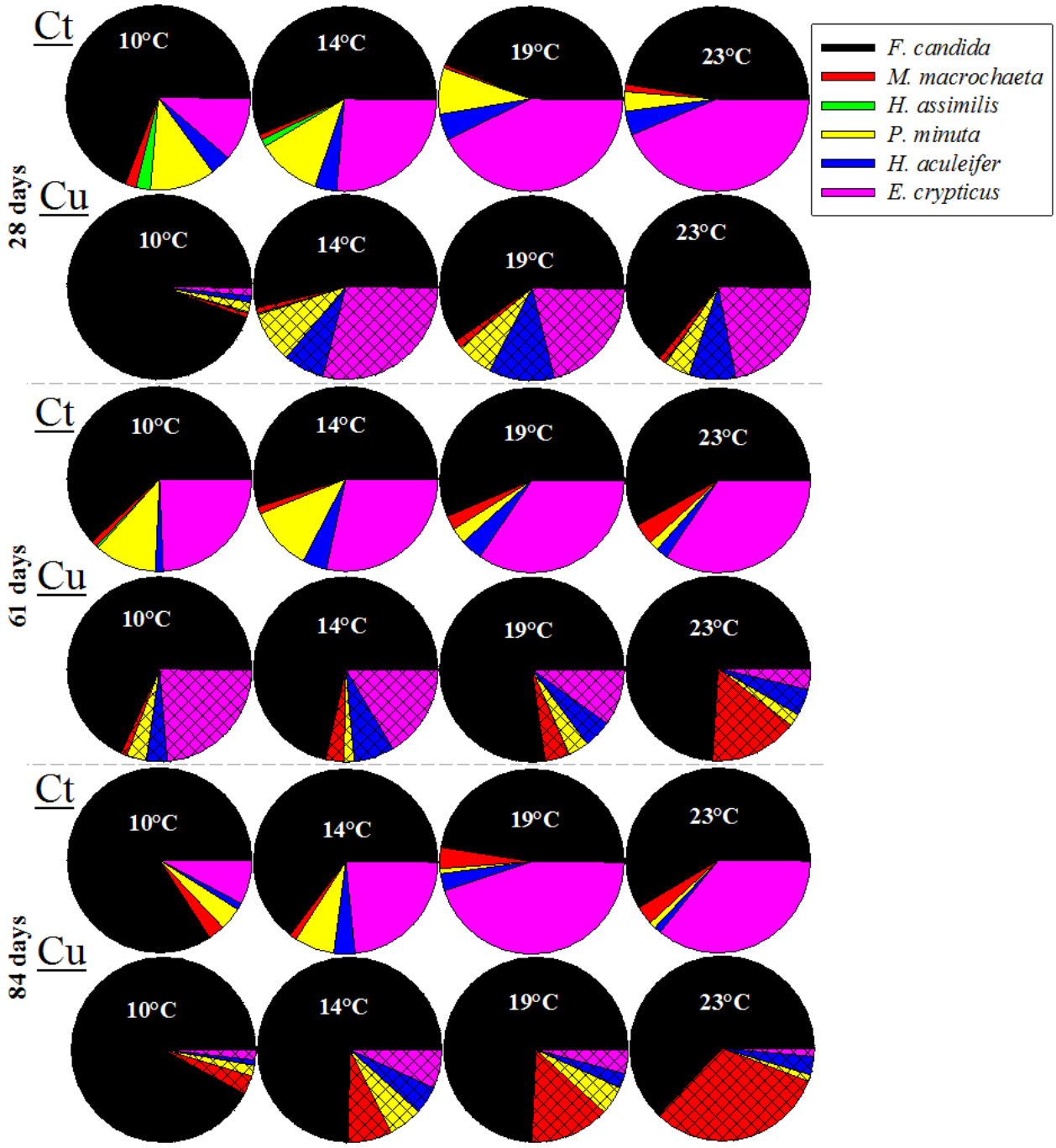

Figure S3 – Pie diagrams representing the species community structure composition obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods (28, 61 and 84 days). Squared pattern represents Cu contaminated soil.

Table S1 – Multiple comparisons of the single species data, which was generated in the SMS experiment with soil from a reference and a contaminated (1000 mg Cu/kg dw soil) at Hygum, Denmark in four different temperatures (10, 14, 19 and 23°C), over three sampling times (28, 61 and 84 days). p-values as calculated by using the Tukey method (two-way ANOVA) to analyze interactions between treatments (Ct and 1000 mg Cu/kg dw soil). Data was ln transformed prior to the analysis.

Species	**Exposure (days)**	**Treatment**	**Temperature**	**Interaction**
			p	
F.candida	28	<0.001	<0.001	0.171
	61	<0.001	0.354	0.343
	84	<0.001	0.101	0.045
M. macrochaeta	28	0.029	0.001	0.016
	61	0.073	<0.001	0.565
	84	0.298	<0.001	0.181
H.assimilis	28	0.021	0.022	0.057
	61	0.193	0.139	0.111
	84	0.325	0.405	0.405
P.minuta	28	0.003	0.013	0.293
	61	<0.001	0.005	0.017
	84	0.002	0.001	0.008
H.aculeifer	28	0.774	<0.001	0.396
	61	<0.001	<0.001	0.002
	84	<0.001	<0.001	<0.001
E.crypticus	28	<0.001	<0.001	<0.001
	61	<0.001	0.97	<0.001
	84	<0.001	0.004	0.003

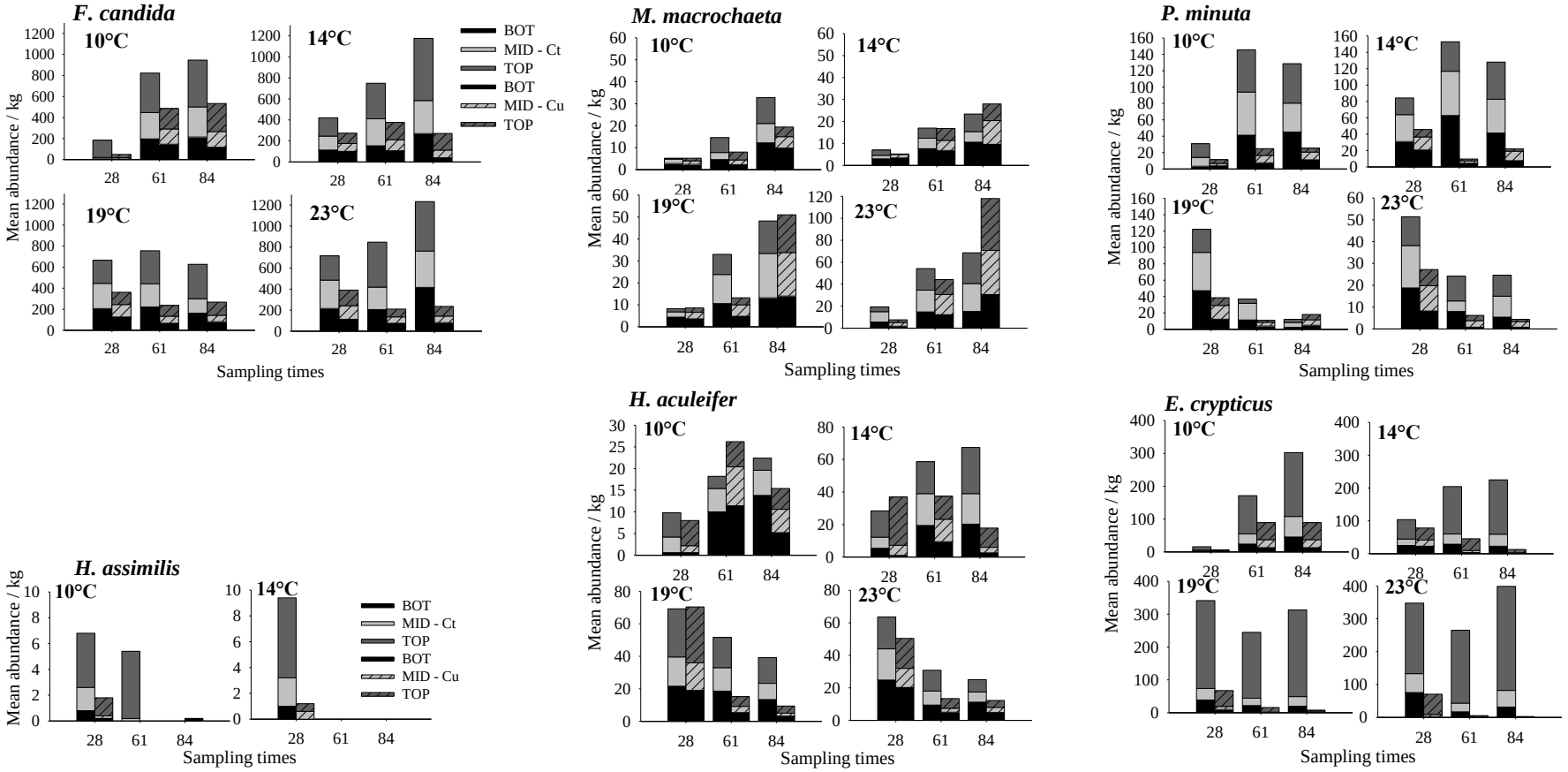

Figure S4 – Vertical distribution [distribution over the soil layers: TOP (0 – 5 cm), MID (5 – 10 cm) and BOT (10 – 15 cm)] of the individual species *Folsomia candida*, *Mesaphorura macrochaeta*, *Proisotoma minuta*, *Hypogastrura assimilis*, *Hypoaspis aculeifer* and *Enchytraeus crypticus* obtained from the SMS experiment performed using soil from a reference and Cu contaminated (1000 mg Cu/kg) site from Hygum, Denmark. Tests were performed at four temperatures (10, 14, 19 and 23°C) and three exposure periods (28, 61 and 84 days). Coarse patterns represent Cu contaminated soil.

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Chapter V – Global warming and pollution on soil community – previous extreme temperature events that now become more normal – can lead to total community extinction.

Global warming and Cu pollution on soil community – previous extreme temperature events become a normal occurrence and can lead to total community extinction

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Submitted

Abstract

Global warming will affect ecosystems and species' diversity, as predicted in e.g. the reports by IPCC (Intergovernmental Panel on Climate Change). Physiological and reproductive activities of individual species are known to be highly influenced by changes in temperature. Effects on species communities are less studied, and virtually unknown when combining pollution and temperature. To assess the effects of temperature and pollution in the soil structure and function, a 2-factorial soil mesocosms multispecies experiment was performed. Three exposure periods (28, 61 and 84 days) and four temperatures (19, 23, 26 and 29°C) were tested, the temperature resembling the mean annual values for southern Europe countries and extreme events. The soil used was from a non-contaminated field site, used as clean and as spiked with Cu (100 mg Cu/kg). Results indicated distinct effects between 29ºC and all other treatments, with a decrease in overall abundance of organisms, further potentiated by the increase in exposure time. *Folsomia candida* was the most abundant species and *Enchytraeus crypticus* was the most sensitive to Cu toxicity. Differences in species optimum temperatures were adequately covered, e.g. 19ºC for *Hypoaspis aculeifer* or 26ºC for *E. crypticus*. The longer the exposure time duration (28, 61, or 84 days) the more pronounced were the temperature effects. Feeding activity (bait-lamina) decreased with higher temperature and exposure time, following the decrease in invertebrate abundance, while for the same conditions the organic matter turnover (litterbags) increased. Potentially negative impacts on ecosystem services due to temperature increase can be expected, e.g. biodiversity loss and soil capacity function.

Keywords: Soil function, climate change, ecosystem services, mesocosms, biodiversity loss.

1. Introduction

The impact of climate change on various ecosystems is a matter of serious concern, e.g. risk is posed to ecosystem services such as food supply. As predicted in the IPCC reports, global warming may affect ecosystems and species' diversity (Alcamo et al. 2007). At an European scale, climate change differs from south to north, and there is indication that the natural resources will be most severely affected in the southern part (Raisanen et al. 2004, Kjellstrom et al. 2007). The rise in temperature during summer in southern Europe are closer to represent the previous definition of extreme events (Kjellstrom 2004). For example, a severe heat-wave took place in the summer of 2003; by then the average temperatures raised 3 to 5°C in most of southern and central Europe (Alcamo et al. 2007). Such extreme conditions already caused a serious reduction in the agricultural production – obviously an important socio-economical ecosystem service. Additionally, for example in Portugal, the hot and dry conditions have potentiated many wildfires (Fink et al. 2004) which lead to a national catastrophe. These environmental changes have been preceded over the last 18 years by an increase in the mean annual temperature e.g. in 2011 five heat-waves were registered (Instituto de Meteorologia 2011d, e, c, b, a).

Temperature is one of the main drivers of biotic systems structure and function (Messenger 1959, Parmesan et al. 2000) via e.g. changes in organisms' development rate (Bale et al. 2002, Robinet and Roques 2010). Such temperature related changes may cause a species/process to increase or decrease, depending on differences between that and its optimum temperature range (Jansch et al. 2005, Iglesias Briones et al. 2009). In species communities, changes will be also the result of species/processess interactions e.g. competition, predation, etc. (Van der Putten et al. 2010). If not enough, pollution also impact ecosystems and this impact will vary depending on temperature (Abdel-Lateif et al. 1998, De Silva et al. 2009).

In the current global warming scenario, it is important to understand how it may affect species communities in combination with pollutants. Multispecies experiments have been used to assess the effects of soil pollutants (Pernin et al. 2006, Scheifler et al. 2006, Jensen and Scott-Fordsmand 2012), but not including its interaction with temperature. The aim of the present study was to assess the effect of temperature in combination with copper toxicity on soil community structure/composition and processes. Copper (Cu) was the selected test substance due to being a widespread pollutant (Abreu et al. 2008, Antunes et al. 2008) with

well-known ecotoxicity (e.g. ScottFordsmand et al. (1997), Pedersen et al. (2000), Maraldo et al. (2006), Strandberg et al. (2006)). Besides this, few single-species studies regarding the interaction between Cu and temperature (Sandifer and Hopkin 1997, Khan et al. 2007) are available. The experiment was done using a Soil Multispecies System (SMS) combined with functional measures (feeding activity, organic matter breakdown). The temperatures used were set to resemble the mean annual values for southern countries and extreme events, the latter actually becoming more common.

2. Materials and Methods

A laboratory food-web experiment was conducted using a mesocosms design type named Soil Multispecies test Systems (SMS) (Scott-Fordsmand et al. 2008). In brief (each aspect is explained in detail in the next sections), six species were included: four collembolans, one predatory mite and one enchytraeid, belonging to different functional groups - mutualism, competition, predation and other interactions represented. The soil used is from a non-contaminated field in Portugal. Soil biological structure was followed over time, from day 28 till 84. Soil function parameters were also measured, assessing e.g. organic matter turnover. A total of 124 SMS units were performed. Concurrently, single species experiments were performed to determine individual Cu toxicity values in the test soil and further define the test concentration to use in the SMS experiment.

2.1. Test soil

Soil was collected from a field site situated at the University of Aveiro, Portugal. The field study is located 200 m from the Ria de Aveiro (N 40°37.538' W 008°39.691'). The site occupies a total of approximately 1600 m² and has not been used for agriculture or other purposes for at least 30 years.

The general physicochemical characteristics are as follows: 37% coarse sand (>200 µm), 11.2% fine sand (63–200 µm), 35% silt (<63 µm). The soil was sampled to a depth of 20 cm and sieved through a 2 mm mesh to remove larger particles. To exclude fauna, the samples were dried at 80°C for 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany).

2.2. Test organisms

All soil invertebrates were cultured in the laboratory at 20 ± 1°C and photoperiod of 16: 8 h of light: dark and kept for several generations. Collembola species (*Folsomia candida*, *Mesaphorura macrochaeta*, *Proisotoma minuta* and *Hypogastrura assimilis*) were cultured in a substrate of Plaster of Paris and charcoal and fed on bread yeast twice a week. The same substrate was used for the *Hypoaspis aculeifer* cultures, fed on *Tyrophagus putrescentiae*, also cultured in the laboratory on beer yeast. Acari used in the SMS experiment were fed on juveniles of *F. candida*. *Enchytraeus crypticus* were kept in agar culture plates, consisting of a sterilized mixture of four different salt solutions ($CaCl_2.2H_2O$; $MgSO_4$; KCl; $NaHCO_3$) and a Bacti-Agar medium (Oxoid, Agar n°1). Food consisted of dried oat flakes.

Individual species tests

Organisms were selected according to the guideline specifications, i.e. for *F. candida,* 10-12 days old synchronised organisms were used, for *H. aculeifer,* 35 days synchronised adult females were used and for *E. crypticus*, adult organisms with visible clitellum were used.

Multispecies test

Organisms were randomly selected among adult sized animals and added in the SMS at 3 different stages (see table 1 for details).

Table 1 - Summary information in regard to the biological component of the SMS, including each added test species, respective group, food web and/or associated function, the assumed interaction type between organisms and preferable living layer in the soil ecosystem (corresponding to epiedaphic=surface, hemiedaphic=upper, euedaphic=middle; enchytraeids are regarded endogeic). The number of organisms added at test start and the day at which organisms were added is indicated.

Test species	Group	Food web / Function	Assumed interaction type			Living layer	Initial Number	Day
Microbial community	Bacteria Fungi	Decomposer Prey			← Prey / Grazing	all	n.d.	-3
Enchytraeus crypticus	Oligochaete	Decomposer Fungivore Prey	Competition; Mutualism; Neutralism; Amensalism;	Predation →		middle	50	0
Folsomia candida	Collembola	Prey for *H. aculeifer*				upper - middle	30	1
Proisotoma minuta						upper	30	1
Mesaphorura macrochaeta						middle	30	1
Hypogastrura assimilis						upper	30	1
Hypoaspis aculeifer	Acari	Predator				upper - middle	10♀; 5♂	7

Here we present the summarized information in terms of known role in the food-web/function and assumed/known interactions of the species tested. As displayed in table 1, the types of interactions that may occur between collembolans are numerous, e.g. competition, mutualism, neutralism or amensalism (Hopkin 1997). Predation was included via the mites, which prey on collembolans and enchytraeids (Krogh 1995). Certain functions are less clear, e.g. we know that collembolans can be decomposers to a lesser extent (more important in acidic fields where few earthworms are present) or that some enchytraeid species may feed on fungal hyphae as a relevant substrate but it is not entirely understood if they feed on microorganisms (Lokke and van Gestel 1998).

2.3. Test chemical and soil spiking

The copper chloride salt $CuCl_2.H_2O$ (99% purity, Merck Pro Analysis, Darmstadt, Germany) was the test chemical used. Spiking was done with $CuCl_2$ as an aqueous solution into pre moistened soil batches (max 4kg dry weight in the SMS experiment) to facilitate a homogeneous mixing. The process was done manually during ca. 30 minutes per batch.

Concentration ranges tested in the individual test species were as follows: for *F. candida* 0-32-100-320-1000 mg Cu/kg, for *H. aculeifer* 0-100-320-1000 mg Cu/kg and for *E. crypticus* 0-3.2-10-32-100-320 mg Cu/kg.

Test concentration in the SMS was 100 mg Cu/kg dry soil, selected based on the individual species tests results using the approximate EC_{50} value for *E. crypticus* – the most sensitive species.

2.4. Test performance

2.4.1. Individual test species

Standard ecotoxicological tests were performed for *E. crypticus*, *H. aculeifer* and *F. candida*, following the respective guidelines (OECD 2004, 2008, 2009). Four replicates per treatment were used. Tests were performed at 20 ± 1°C and photoperiod of 16: 8 h of light: dark. Food and soil water content was replenished weekly.

2.4.2. Multispecies test (SMS)

A two-factorial design experiment was performed: factor 1- Cu exposure: 0 and 100 mg Cu/kg dw; factor 2 - temperature: 19-23-26 and 29°C. Exposure periods were at 28, 61 and 84 days. Five replicates per treatment were used.

The experimental Soil Multispecies Systems (SMS) units consisted of polyethylene tubes (33 cm x 9.3 cm ø), having a surface area of 68 cm^2 and sample volume of 2241 cm^3. The tubes, closed on the bottom with a lid, were filled with 1000 g moist soil (800 g dry weight plus 100 ml aqueous microbial substrate and 100 ml stem Cu contaminated solution or distilled water in the case of control treatment). Experiments were performed in temperature controlled rooms (variation in temperature was ± 1°C). After the addition of animals, each unit was covered with a perforated lid, weighted and transferred to the temperature test room with a 12h: 12h light: dark cycle. The soil water content was kept by replenishing water loss weekly.

2.5. SMS experimental components/details

2.5.1. Microbial substrate

The procedure to extract the microbial substrate and inoculate it into the experimental units was based on Scott-Fordsmand et al. (2008). For microbial inoculation a forest field soil was used (1 kg wet weight), shaken for 3 hours with 2 L of distilled water and then sieved through a 50 µm mesh and diluted 10 times for use in the experiment. The soil water content was 20% being 10% of distilled water and the other 10% the microbial substrate.

2.5.2. Soil function measurements

2.5.2.1. Bait lamina test

The soil fauna feeding activity was assessed using the bait lamina method (Kratz 1998). In short, bait lamina are plastic lamina sticks, containing 16 holes which are filled with a cellulose-based substrate (70% cellulose, 25% nettle powder (organic grown), 5% activated charcoal). One bait lamina stick was used per SMS, added on days 7, 28 and 61, and removed one week after insertion date. The feeding activity was expressed as the percentage of empty (eaten) plus the partially eaten holes per day. Four extra SMS units were used, under the same test conditions and at temperature of 19ºC to determine the appropriate time period for

bait-lamina sampling days. Observations on the feeding activity in the extra units were made daily.

2.5.2.2. Litterbag test

The soil organic matter turnover was assessed using litterbags. The bags (1.5 mm x 1.5 mm mesh) were filled with approx. 200 mg of dried straw and inserted into the top 5 cm soil layer. In an attempt to minimize/prevent that invertebrates would have many hiding places, the straws were carefully placed side by side, thus promoting exposure to the soil test. The litterbag was always placed in replicate one of each experimental condition and left in until the end of each exposure period, i.e., for a total of 28, 61 or 84 days.

2.5.3. pH and organic matter (OM) content

The organic matter (OM) and pH were measured at days 0, 28, 61 and 84. The pH was measured in water. The OM content was measured as loss of organic matter after 3 h at 600°C.

2.5.4. Soil organisms sampling and extraction

At each exposure period (28, 61 and 84 days – here day 0 is the day that enchytraeids were added) 10 SMS units/replicates (5 control plus 5 contaminated soil) were extracted from each test temperature (19, 23, 26 and 29ºC). The soil from each replicate was divided in 3 layers (top, middle and bottom) with approximately 5 cm height. From each layer ca. 130 g (random sub-samples) was sampled to quantify collembolans and mites, and ca. 60 g to quantify enchytraeids. The micro arthropods sub-samples were extracted over 7 days in a high gradient extractor (MacFadyen) (Scott-Fordsmand et al. 2000). Animals were collected in benzoic acid, transferred to glycerol, identified and counted.

The enchytraeids from the first exposure duration were extracted by spreading the soil sample into five 200 ml plastic beakers (ø 7 cm), which were filled with tap water, gently shaken, and then left overnight at 5°C for sedimentation. Adult and juvenile enchytraeids get dispersed on the soil surface and were thereafter picked and counted under a stereo microscope within 48 h. For the last two exposures (for practical reasons) enchytraeids were extracted by using an adapted Tulgreen apparatus with the temperature varying from 25 to

50°C during five hours. The efficiency of the wet extraction and Tullgren apparatus respectively is 98 and 76% for adults and 53 and 75% for juveniles.

2.6. Data analysis

2.6.1. Univariate analysis

Effect Concentration (ECx) values were calculated in the individual species testing using the Logistic 2 parameters model, using log concentrations (best fit approach) (TRAP_1.2 2008); No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) were derived by the Williams Multiple Sequential t-test procedure (ToxRat® 2003).

Temperature dependent growth-rates for each species in the SMS experiment were estimated using sigmoidal, Gaussian and exponential decay regression models (SPSS 1997).

2.6.2. Multivariate analysis

The community data was analyzed using Principal Response Curves (PRC) method as described by Van Den Brink and Ter Braak (1998; 1999). This method is based on Redundancy Analysis ordination techniques, the constrained form of Principal Component Analysis (PCA) and is specially designed to evaluate the effects of stressors at the community level taking time into account. The significance of the PRC diagrams was tested by Monte Carlo permutation tests by permuting whole time series in the partial RDA, from which the PRC was obtained. All data were ln transformed. PRC analysis was performed using the software package CANOCO version 4.5 (Ter Braak and Smilauer, 2002).

3. Results

3.1. Individual species tests

Validity criteria were fulfilled in all tests and according to the respective guideline requirements.

Results in terms of numbers of surviving adults and juveniles can be observed in figure S2. Effect concentration (EC) values were calculated and can be depicted in table 2.

Table 2 - Effect concentration (ECx) values calculated for *Folsomia candida*, *Hypoaspis aculeifer* and *Enchytraeus crypticus* performed using field soil from Aveiro, Portugal, spiked with a range of $CuCl_2$ concentrations. Results are presented as mg Cu/kg of dry soil. In brackets are the lower and upper 95% confidence limits.

Test species	**EC_{10}**	**EC_{20}**	**EC_{50}**	**NOEC**	**LOEC**	**Model**
	(mg Cu/kg)					
	Reproduction					
E. crypticus	55 [4-717]	64 [9-434]	83 [37-185]	32	100	Logistic 2 parameters
F. candida	212 [128-352]	274 [195-387]	426 [309-588]	320	1000	Logistic 2 parameters
H. aculeifer	2 [0-342]	31 [2-427]	>1000 [n.d.]	≤100	≤100	Logistic 2 parameters
	Survival					
E. crypticus	62 [42-77]	77 [57-92]	115 [96-140]	32	100	Probit

Results show the different levels of toxicity of Cu among these 3 species, with *E. crypticus* being the most sensitive. The selected Cu concentration for the SMS experiment, 100 mg/kg, represents an effect on reproduction that is approximately the EC50 for *E. crypticus*, the LOEC for *H. aculeifer* and the NOEC for *F. candida*. The model for *H. aculeifer* provided $EC_{10/20}$ values below 100, although with large standard deviations due to the low slope for reduction in juvenile production as related to increased concentration. It was further observed (not shown) that the reproductive output of *E. crypticus* was highly correlated with the number of adults. For *F. candida* this was not observed indicating that for this species the reproductive capacity is more sensitive than the adults' survival.

3.2. Multispecies test

3.2.1. Soil pH and OM content

The pH ranged between 5.0 and 5.6 independently of the test duration or treatment. Similarly, no changes occurred in the OM content, being ca. 3%.

3.2.2. Species

Results in terms of abundance of the individual species can be seen in figure 1 (see also figure S1, supplementary information).

The major changes in terms of abundance were observed in the first 28 days, i.e. the largest population increase was in the first 28 days for all species (except *M. macrochaeta*). In fact, for 3 species (*H. aculeifer, P. minuta* and *E. crypticus*) the population number decreased from 28 to 61/84 days whereas for *F. candida* and *M. macrochaeta* it remained constant (see figure S1). Temperature dependent growth rates were calculated using changes in abundance along time (Table 3, Figure 1), showing clear logistic decrease with increasing temperatures. There were no major differences between the control and Cu spiked exposure.

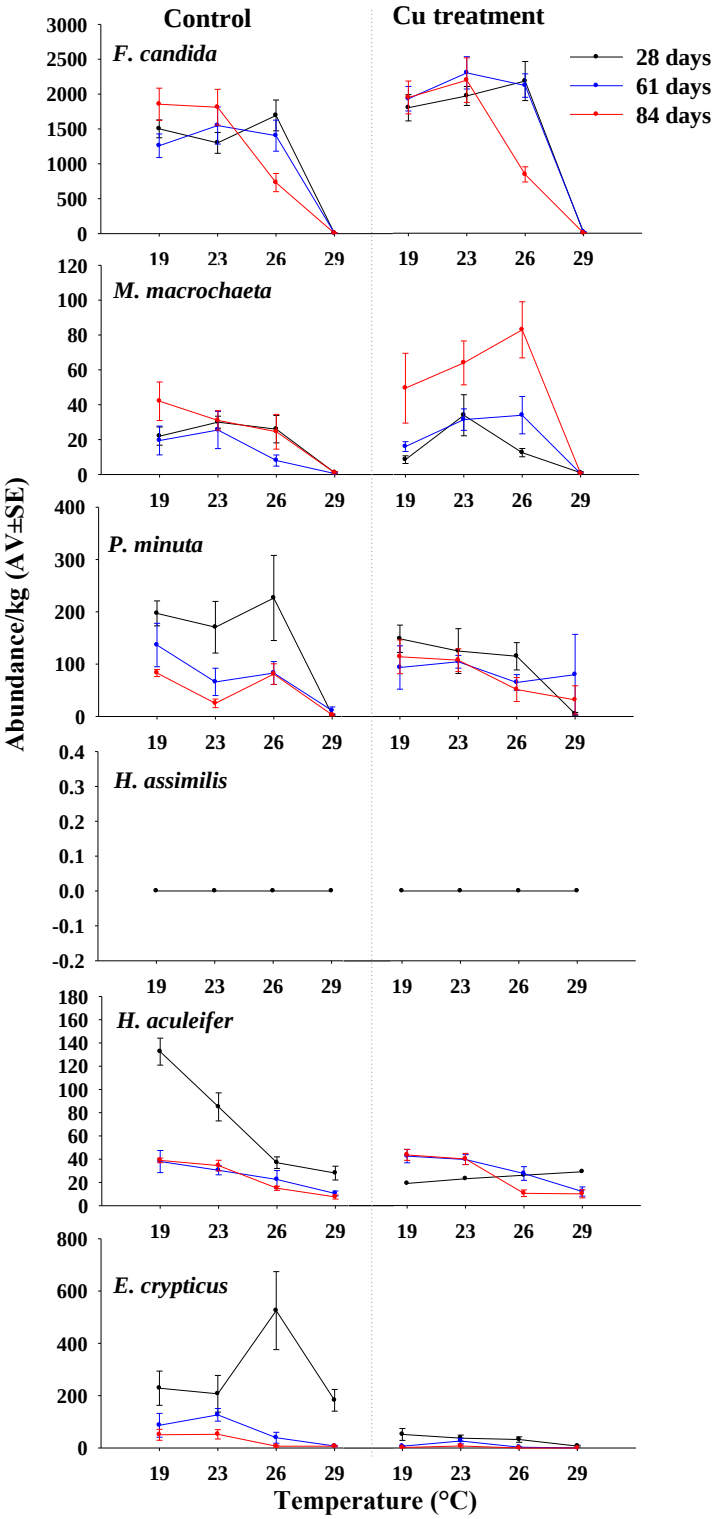

Figure 1 – Results of species abundance from the SMS experiment performed using a laboratory Cu spiked soil from Aveiro, Portugal. Results are presented as the average number of individuals ± standard error (AV±SE) per kg soil. Exposures were performed at four temperatures (19, 23, 26 and 29°C) and three sampling exposure periods of (28, 61 and 84 days). Species composition included *Folsomia candida*, *Mesaphorura macrochaeta*, *Proisotoma minuta*, *Hypoaspis aculeifer* and *Enchytraeus crypticus*. Graphs show results in control soil (left) and 100 mg Cu/kg spiked soil (right).

Table 3 – Models describing the temperature dependency of the individual growth-rates, the models are based on the population growth-rate from the SMS (rather than total abundance, would give "same" models) in order to provide values comparable with literature. SMS was performed with four different temperatures (19, 23, 26 and 29°C) over 3 exposure periods (28, 61 and 84 days). Models applied are sigmoid (high fecundity populations), peak Gaussian and exponential decay.

		Exposure duration (days)											
Species	**Cu**	**0 - 28**				**28 – 61**				**61 – 84**			
	(mg/kg)	**a**	**b**	**r^2**	**Model**	**a**	**b**	**r^2**	**Model**	**a**	**b**	**r^2**	**Model**
F candida	0	58.6 [+∞]	-0.10 [+∞]	0.96	Sig. 4 par.**	12.4 [+∞]	0.44 [+∞]	0.73	Peak 4 par.	40.5 [+∞]	-0.03 [+∞]	0.74	Sig. 4 par.
	100	76.1 [+∞]	-0.12 [+∞]	0.98	Sig. 4 par.	7.3 [n.d.]	-0.05 [n.d.]	0.73	Sig. 4 par.	- 58.4 [n.d.]	1.22 [n.d.]	1.00	Peak, 4 par.
E. crypticus	0	11.8 [+∞]	0.44 [+∞]	0.99	Peak, 4 par. ##	-64.5 [+∞]	0.66 [+∞]	0.98	Peak, 4 par.	-3.5 [n.d.]	2.91 [n.d.]	1.00	Peak, 4 par.
	100	-9.4 [107.3]	3.37 [4.2]	0.96	Sig. 3 par.*	1.0 [+∞]	0.16 [+∞]	0.70	Sig. 4 par.	-0.9 [n.d.]	1.90 [n.d.]	1.00	Peak, 4 par.
M. macrochaeta	0	1.0 [+∞]	-0.17 [+∞]	0.94	Sig.4 par.	-0.6 [0.28]	1.35 [0.68]	0.97	Peak, 3par.	17.5 [43.1]	0.15 [0.12]	0.54	Exp. 2 par.***
	100	1.3 [n.d.]	2.25 [n.d.]	1.00	Peak, 4 par.	0.5 [n.d.]	0.41 [n.d.]	0.79	Peak,4 par.	1.7 [+∞]	-0.03 [+∞]	0.87	Sig. 4 par.
H. aculeifer	0	4.0 [n.d]	-1.24 [n.d.]	1.00	Sig. 4 par.	2.4 [+∞]	0.13 [+∞]	1.00	Sig. 4 par.	-4.2 [+∞]	0.66 [+∞]	0.94	Sig. 4 par.
	100	3.7 [n.d.]	-0.83 [n.d.]	1.00	Sig. 4 par.	2.1 [n.d.]	0.65 [n.d.]	1.00	Sig. 4 par.	-0.8 [n.d.]	1.29 [n.d.]	1.00	Peak. 4 par.
P. minuta	0	7.7 [+∞]	-0.09 [+∞]	0.95	Sig.4 par.	45.1 [+∞]	0.14 [+∞]	0.73	Sig. 4 par.	2.11 [+∞]	0.12 [+∞]	0.99	Sig. 4 par.
	100	4.7 [1.96]	3.97 [3.16]	0.77	Peak.3 par.#	3.8 [+∞]	0.12 [+∞]	0.94	Sig. 4 par.	27.9 [+∞]	0.03 [+∞]	0.92	Peak, 4 par.

*f= a/(1+exp(-(x-x0)/b)); **f= y0+a/(1+exp(-(x-x0)/b)); *** f = a*exp(-b*x); #f=a*exp(-.5*((x-x0)/b)^2); ##f=y0+a*exp(-.5*((x-x0)/b)^2).

In terms of toxicity, among all species *E. crypticus* was the most affected by Cu spiking, showing nearly no animals at any exposure duration or temperature (Figure 1). *H. aculeifer* and *P. minuta* showed also a decrease in abundance after 28 days but at time sampling of 64 and 81 days there were no differences between Cu and control soils. In the case of *F. candida* and *M. macrochaeta*, the abundances were potentiated at day 61 (*F. candida*) and day 84 (*M. macrochaeta*). The effect of temperature was generalised for 29ºC, causing a global decrease in all species abundance, with a close to extinction result after 84 days of exposure. *Hypogastrura assimilis* did not survive any exposure treatment/duration of the experiment.

Folsomia candida was the most abundant species both in the control and spiked soil (Figure 1), and showed the largest increase in total abundance in the first 28 days, stabilizing growth from day 28 till the 61st. Further, exposure to 26ºC from 28 to more than 61 days caused a decrease in abundance.

For *M. macrochaeta* and *P. minuta*, major changes in abundance occurred only at 29ºC. For *H. aculeifer*, 19ºC was the optimum tested temperature to reproduce/survive, with higher temperatures from 23º up to 29ºC showing a decrease in performance. For *E. crypticus*, the optimum temperature here was ca. 26ºC – this pattern was not confirmed when in Cu spiked soil.

Results in terms of vertical distribution in the SMS can be seen in Figure S3 (supplementary material) and show an overall even distribution, except for *E. crypticus*, clearly localized in the upper 0-5cm layer.

3.2.3. Community analysis

Results from the Principal Response Curves (PRC) and analyses of significance by Monte Carlo permutation tests (selecting 19ºC as a standard/control temperature) are displayed in figure 2 and table 4, respectively.

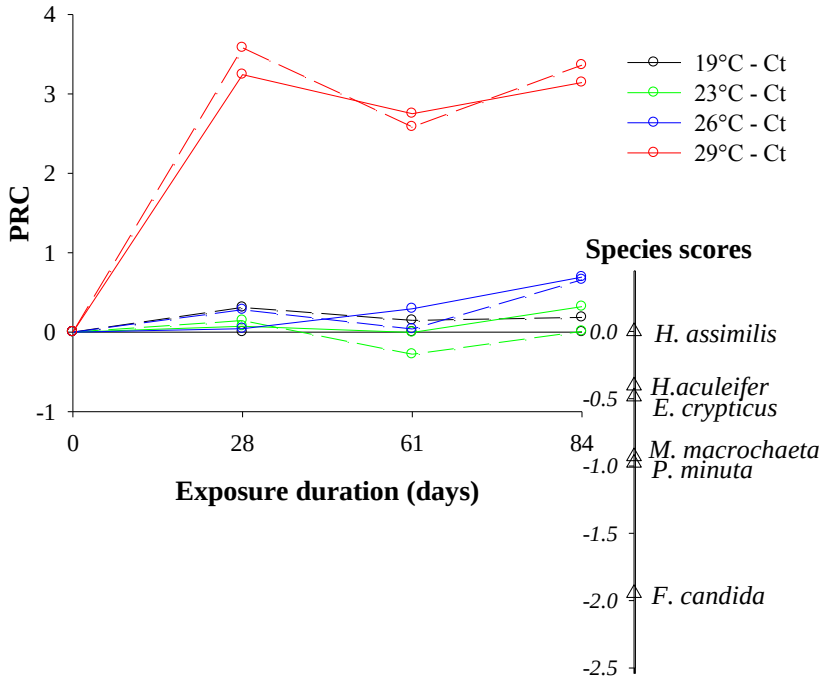

Figure 2 - Principal Response Curves (PRC) with species scores (bk) for food-web data set, obtained from the SMS experiment performed using soil from a field site in Aveiro, Portugal, as a control and Cu spiked (100 mg Cu/kg). The solid lines (—) represent the control and the dashed lines (- -) represent the Cu spiked treatment. Species were exposed for 3 time periods (28, 61 and 84 days) and at four temperatures (19, 23, 26 and 29°C). Control temperature was set to be 19°C.

PRC data analysis showed a separation of the response curves to 29ºC from all other temperatures, being significantly different (Monte Carlo permutation test, ($p = 0.002$) for all exposure periods and independent of the Cu treatment. *F. candida*, showed the highest negative species score, probably driven by its abundance and sensitivity to the highest temperature (29°C). RDA analysis showed that for SMSs exposed to control soil 60.3% of the total variation in species data may be explained by temperature and exposure duration together. Of this variance, 35.1% is explained by temperature and 23.5% by exposure duration.

For the analysis of the Cu spiked soils SMS, RDA showed that 65.6% of the total variation may be explained by temperature and exposure duration together, being 35.4% explained by

temperature and 29.5% by exposure duration. Monte Carlo permutation test indicated the level of significance (Table 4).

Table 4 - Significance levels as calculated by the Monte Carlo permutation tests, done by testing the results of the exposure to every temperature (23, 26 and 29°C) against the temperature of 19°C for the correspondent soil treatment and exposure period (28, 61 and 84 days). The p values for each sampling day are represented bellow. The lowest possible p value is 0.002 for 499 permutations.

Cu (mg/kg)	Temperature	Sampling day		
		28	61	84
		Monte Carlo significance level (p)		
0	23°C	0.438	0.198	0.038
	26°C	0.006	0.600	0.026
	29°C	0.006	0.006	0.006
100	23°C	0.308	0.108	0.390
	26°C	0.528	0.362	0.006
	29°C	0.006	0.006	0.006

Because the analysis was so driven by the results of 29ºC, the PRC was redone without the 29ºC data in an attempt to overview differences due to the other treatments (Figure 3).

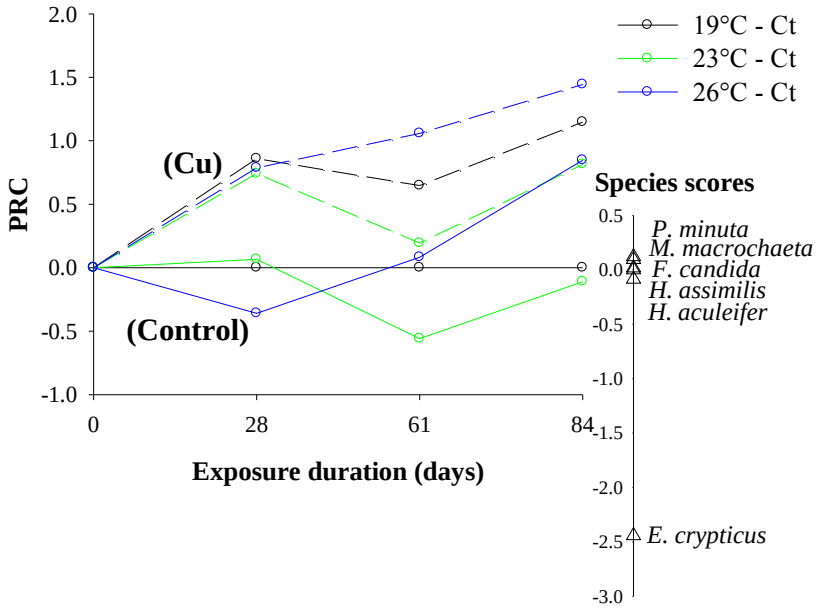

Figure 3 - Principal Response Curves (PRC) with species scores (bk) for food-web data set, obtained from the SMS experiment performed using soil from a field site in Aveiro, Portugal, as a control and Cu spiked soil (100 mg Cu/kg). The solid lines (—) represent the control and the dashed lines (- -) represent the Cu spiked treatment. Species were exposed for 3 time periods (28, 61 and 84 days) and four temperatures (19, 23, 26 and 29°C), but for this analysis data from systems exposed to 29°C were excluded. Control temperature was set to be 19°C.

As can be observed in figure 3, curves indicate differences occurring with time of exposure, from a separation between control and Cu treatments at 28 days to being less separate after 84 days of exposure, as seen by the responses at 26ºC control being more similar to the Cu treatments. *E. crypticus* was the species with highest negative score, probably due to its high sensitivity to Cu.

RDA output showed that for systems exposed to Control soil 67.3% of the total variation in species data was explained by temperature and exposure duration together, of which 2.3% was explained by temperature and 53.9% by exposure period. In the Cu treatment 77.2% of the total variation may be explained by temperature and exposure time together, of which 2.5% is due to temperature and 63.3% due to exposure time.

3.2.4. Soil functional assessment

3.2.4.1. Bait lamina

Overall, the mean feeding activity in the SMS experiments fluctuated between 19-23ºC and decreased when at 26-29ºC in both control and Cu polluted soils (Figure 4). Results indicated that in the exposure at the start of the experiment (days 7-14) there were more perforated holes compared to the last exposure momentum (days 61-68)

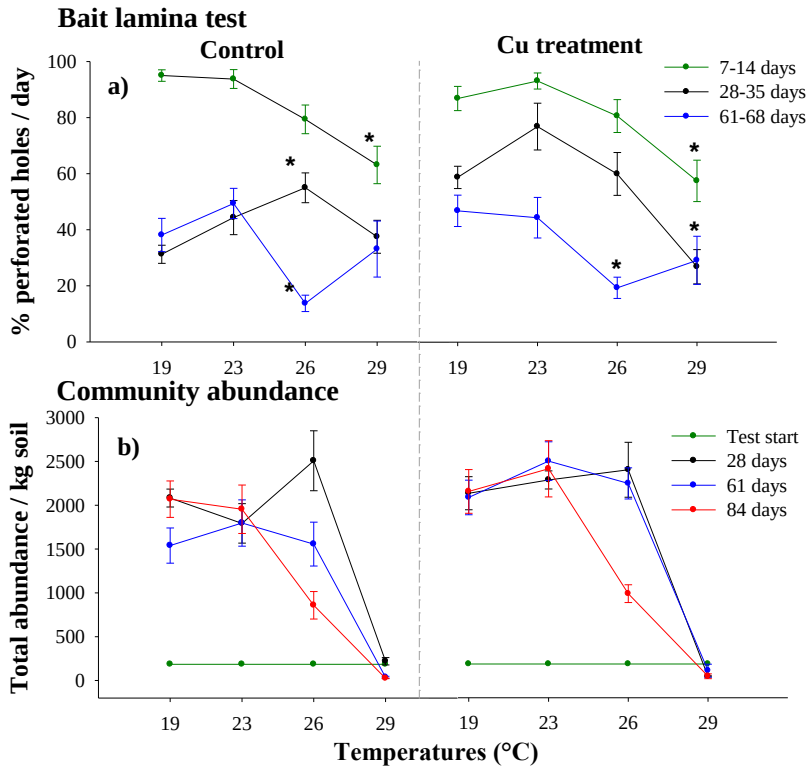

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Figure 4 - Results of bait lamina test used as feeding activity measurements obtained from the SMS experiment and for comparison, invertebrate community abundances. Following treatments were used: control and Cu spiked soil (100 mg Cu/kg), four temperatures (19, 23, 26 and 29°C) and three exposure periods (28, 61 and 84 days). a) Feeding activity expressed as average ± standard error (AV±SE) of the % of perforated holes (partially empty (eaten) + totally empty holes) in the bait lamina per day; *p<0.05, using 19ºC as control; b) Total abundance of invertebrates (AV±SE) per kg of soil.

3.2.4.2. Litterbags

Overall, organic matter turnover, as measured using litterbag experiments, increased with temperature and time for both control and Cu treatments (Figure 5).

Although data has no replication and results cannot be interpreted in the light of significance, they showed a clear trend and were inversely proportional to the mesofauna total abundance.

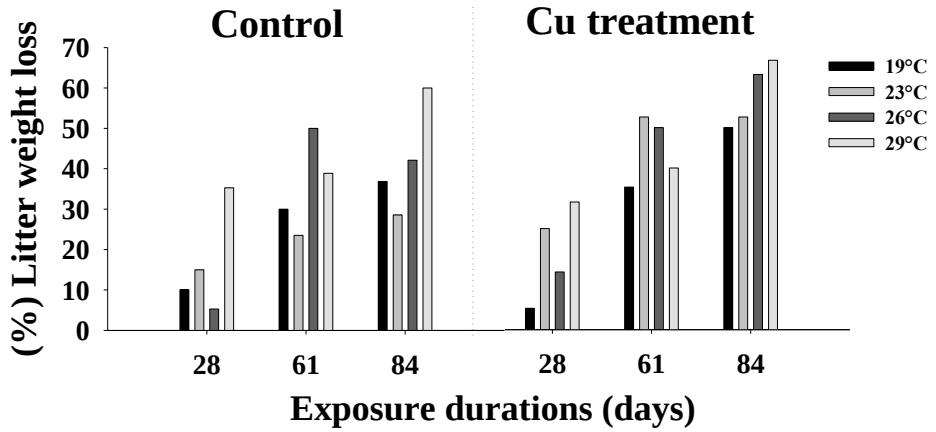

Figure 5 - Results of the litterbag studies as performed in the SMS experiments, using control and Cu spiked (100 mg Cu/kg) soil from Aveiro, Portugal. The graph represents the loss of litter weight, determined using litterbags, at the end of each exposure duration (28, 61 and 84 days) when exposed to four temperatures (19, 23, 26 and 29°C). n=1.

4. Discussion

First of all we believe it is of interest to infer about the data source and design: given we start our experiment with adult organisms, by the time we reach the first sampling - exposure duration of 28 days - we are collecting data that corresponds to the population growth phase curve. After this, and at 61 and 84 days samples, most of the populations have reached a stable growth phase and the results are less species specific and become more multi-species, where time was allowed for interaction. Because the test design included 3 exposure periods overlapping with 4 temperatures, the degree-day concept can also be used (Trudgill et al. 2005, Amarasekare and Savage 2012) as will be further discussed ahead. Further, organisms got distributed vertically in the SMS (Figure S3) in a roughly homogeneous manner, except for *E. crypticus* (discussed ahead). This was independent of temperature and Cu, which is not

so surprising given that temperature and Cu pollution were not different in layers in the soil core.

Discrimination between effects at 29ºC and all other temperatures and treatments (e.g. figure. 2) was clear. If this could perhaps be considered an extreme event in the previous decade, it is at present an ever more frequent occurrence (Alcamo et al. 2007). As could be inferred, a 29ºC temperature in soil will affect – nearly wipe-out - the whole species community, independent of these being in a growth (28 days) or stable phase (61 or 84 days) of their population dynamic. Soil temperatures are at present and normally below 29ºC but can for example reach temperatures above 40ºC in the upper layer (Vieira et al. 2003).

In a more detailed analysis we could see that the large species score yield for *F. candida* in the community analyses may be attributed to its very high abundances and the sharp effect of 29ºC, which dominated the analysis. Further, as well exemplified in the results of *F. candida* (Figure 1), if a longer period of exposure takes place at lower temperatures the effect can be equally damaging as at 29ºC, i.e. 61 days at 26ºC or 84 days at 23ºC. Hence, indicating that not only it is the maximum temperature affecting the system but also the duration, which is in support of the degree-day or thermal time concept, although the latter is not simple. In a previous study (Sandifer and Hopkin 1997) it was observed that reproduction of *F. candida* was having a near total reproductive arrest when exposed for 28 days at 25°C compared to the standard 20ºC. In the SMS the effects obtained at 26ºC were not causing such an effect, hence we believe that we are observing the combined effect of species interaction.

As shown by Martikainen and Rantalainen (Martikainen and Rantalainen 1999) it is assumed that, at least in control conditions, increasing temperatures below the species optimum, would result in increasing population at all time-points – this did not occur here (see result for 19ºC) in all cases, showing as well that species interaction must be playing a role.

If, like in the analysis shown in figure 3, we disregard the results obtained at 29ºC, then we realise that Cu impacted the community, as seen by the separation between control and Cu treatments. The highest temperature (now being 26ºC) to some extend overruled the effect of Cu during the longer exposure durations.

If we look at *M. macrochaeta* individual results, the least abundant species, the positive effect of Cu is apparent after 61 and 84 days. This is not so surprising when we know this species

has a high EC_{50}, i.e. EC_{50s} (32days; egg-laying) of 2382mg Cu/kg and 875mg Cu/kg for parthenogenetic and sexual populations respectively (Martín and Camargo 2001). This, together with the fact that morphological characteristics such as complex post antennal organs and pseudocellis - existing in *M. macrochaeta* – seem to have an advantage for survival in contaminated environments (Filser et al. 2000) partially explain the result.

Moreover, species growth rate dependencies may also vary with their habitat in soils, i.e. epiedaphic (surface) species are more exposed to temperature variations than hemiedaphic (upper layer) species (Van straalen 1994). This could be an explanation for the delayed growing phase of *M. macrochaeta*, a species known to live in natural habitats in the lower soil layers compared with other collembolan species. This species showed the highest abundance after 84 days in either spiked or control soil, although in the mesocosms experiment this was not due to such changes in vertical distribution (Figure S3) or temperature.

The species *P. minuta* showed little Cu toxicity in the SMS. Results were in line with Nursita et al. (2005) who observed reproduction EC_{50} of 696 mg Cu/kg for *P. minuta*.

As mentioned, physiological and reproductive activities of organisms are very influenced by temperature with different optimums (Mertens et al. 1983, Jansch et al. 2005). Results of *H. aculeifer* population indicate it to be very responsive to temperature in the growth phase – 28 day results indicate a temperature effect of 50% (TE_{50}) of 23ºC and TE_{80} of ca. 26ºC (see supplementary info - figure S1). After 28 days (when abundance was lower than during first 28 days) the number of organisms decreased considerably less between temperatures; it is known that *H. aculeifer* have a narrow preference (20ºC) despite the larger tolerance range (10-35ºC) (Jansch et al. 2005). An increase in temperature is known to decrease the time for species development, which consequently affects reproduction and longevity (Kevan and Sharma 1964, Lobbes and Schotten 1980, Chi 1981). Studies on the longevity of a mite species (Chi 1981) indicated a decrease in females longevity between 15ºC and 28ºC of 194 to 23 days respectively. This could be a reason for the lower number of individuals at longer exposure periods.

In regard to *E. crypticus*, its preferable temperature has been described to be ca. 27ºC, while the tolerance limits would be between 15-30ºC (Jansch et al. 2005). Hence, the results are in good agreement. As to the effect of Cu, the toxicity was in good agreement with what was

expected based on own results of single species tests (Figure S2). Compared to testing in other soils for individual species (Posthuma et al. 1997, Menezes-Oliveira et al. 2011) and multi species experiment (Scott-Fordsmand et al. 2008), this soil was more toxic. Differences in soil OM and CEC must be the main cause of variability, to which bioavailability and toxicity of Cu are greatly dependent (Peijnenburg et al. 1999, Amorim et al. 2002, Amorim et al. 2005b, Criel et al. 2008, Howcroft et al. 2011). Further, another particularly different aspect of this species was its vertical distribution in the SMS, being mostly on the upper 0-5cm (Figure S3). This could indicate its lower mobility compared to the other species

H. assimilis mortality must have been partly temperature related – a similar multispecies study (Menezes-Oliveira et al, submitted) testing a temperature range of 10-14-19-23ºC showed nearly zero abundance at 19-23ºC while at 10-14ºC at least some individuals survived. Further, the different soil must have been a conditioning factor, since in e.g. LUFA or OECD soil the species reproduce normally at 20ºC (Amorim et al. 2005a). On the other hand, also predation by *H. aculeifer* may have occurred, as this has been observed by Cortet et al. (2003) who compared species compositions with and without predation.

In regard to the feeding activity, results seemed to be partially following the overall decrease in abundance of the mesofauna community, and to a less extent it must have been influenced by the microbial fraction. Helling et al. (1998) showed that the contribution to feeding activity by invertebrates (collembolans and enchytraeids) was at least 10 times higher compared to the role of microorganisms (e.g. bacteria).

On the other hand, litterbag results indicated higher OM decomposition with temperature increase, despite decrease in invertebrates' abundance. It is known that different species vary in their effects on soil processes (as included in our SMS experiment, e.g. decomposers and detritivores) and also with differentiated strengths. The composition of such species community can result in facilitative or inhibitory interactions, depending on whether species mixtures perform better or worse than would be expected on the basis of the mere additive effects of single species (Heemsbergen et al. 2004b). As suggested by Heemsbergen et al. (2004a) it is not the species number but the degree of functional differences between species that is a driver of ecosystem processes, and this effect in turn is due to facilitative interactions among species. In the present study, the species composition suffered changes (Figure S4) but it is not conclusive which is the optimal combination of species (we cannot exclude the

effect of changing abundances here). Despite that, this rational seems to apply as the nature of these interactions (inhibitory, neutral, or facilitative) is related to the degree in which species differ in their impact on soil processes. The differences between bait lamina and litterbag, reflect also differences in exposure time: bait lamina results indicate feeding activity at a time momentum (resembling actual feeding during 7 days) whereas the litterbags are the result of the sum effects along time (allowing for prolonged organisms activity during 28, 61 or 84 days). Results from litter bags seem to indicate that the primary decomposition process has resilient properties (possibly after an initial promotion induced at test start during ca. 28 days), and following this the OM degradation keeps on-going at lower invertebrates' abundance (until 84 days) possibly due to overtake of microbial activity in the now primarily shredded OM.

In nature, we need to consider that the effect of temperature increase will be tightly linked to at least an increase in drought (Bérard et al. 2011) - which we excluded in the present study since moisture was replenished – hence we expect effects on the community to be even more pronounced than measured here. Kellermann et al. (2012) suggest that factors related to water in the environment are more important than high temperature alone for the upper thermal limits - warm and dry environments select for increased heat tolerance in ectotherms like *Drosophila*. Further, these species have limited evolutionary capacity to evolve and alter the upper thermal limits. Hence, it has been suggested that the ability of terrestrial insect species to evolve or resist the heat are limited. One way to predict responses to climate change is by assessing how close species upper thermal limits are in regard to the current environment (Deutsch et al. 2008).

As considered by Kellermann et al. (2012) organisms like e.g. ectotherm insects, will not be able to tolerate enough for the current IPCC scenarios. As we showed in the present study the exposure to increasing temperatures from 23-29ºC caused a decrease in species community abundance, being higher with the higher temperature and/or exposure duration.

Climate change is predicted to lead to losses in biodiversity (Alcamo et al. 2007). Modelling species diversity with climate change, as published in Nature (Norberg et al. 2012), has demonstrated that high dispersal and lower genetic variability increased the risks of biodiversity alteration/loss. Further, the importance of species interactions, such as competition, was highlighted. So, adaptation, dispersal and community dynamics are key

factors determining biodiversity. While these biodiversity changes will have direct consequences from the associated functions, there is growing concern on whether the provision of goods and ecosystems services will be equally affected (Hairston 1993, de Ruiter et al. 2002, Hunt and Wall 2002). The degree to which biodiversity change will impact services needs to be studied via targeted experimental design.

5. Conclusion

Effect of temperature was linked with exposure duration – degree days – indicating that 29ºC caused decrease in abundance, close to extinction, and the same effect occurred if exposure to 26ºC was longer (61-84 days).

Effect of Cu was mostly dominated by the temperature effects - the use of a higher concentration could had further elucidated on the combined effect of the two, but that would mean that species community would had been more affected by mortality and results would include less species interaction possibilities.

Effects on the soil function indicated a decrease in the organic matter turnover, hence potential impacts on soil capacity are predicted. Other ecosystem services such as e.g. biodiversity loss are expected.

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Supplementary information

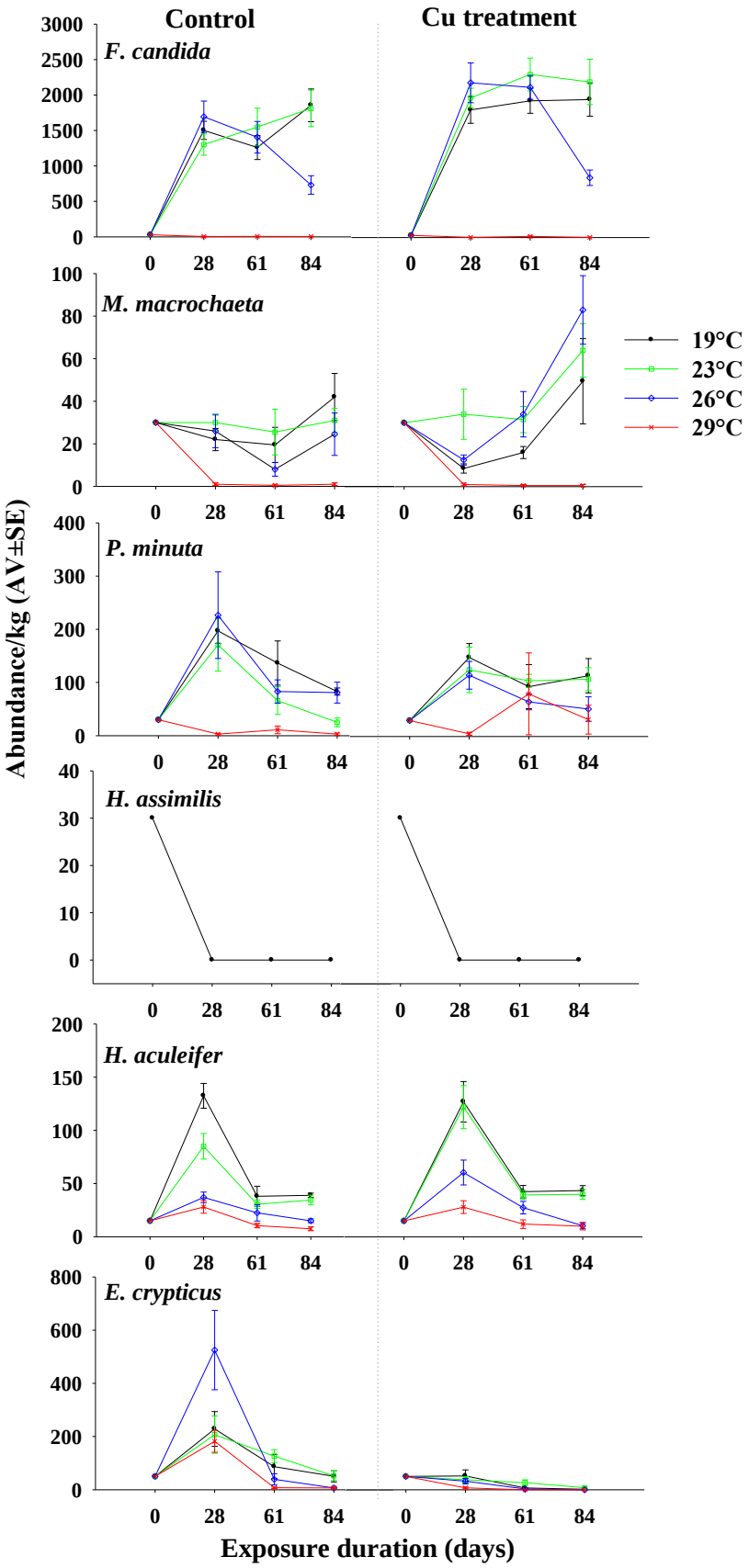

Figure S1– Results of species abundance from the SMS experiment performed using a clean and a laboratory Cu spiked soil from Aveiro, Portugal. Results are presented as the average number of individuals ± standard error (AV±SE) per kg soil at three exposure periods of (28, 61 and 84 days). Exposures were performed at four temperatures (19, 23, 26 and 29°C). Species composition included *Folsomia candida*, *Mesaphorura macrochaeta*, *Proisotoma minuta*, *Hypoaspis aculeifer* and *Enchytraeus crypticus*. Graphs show results in control soil (left) and 100 mg Cu/kg spiked soil (right).

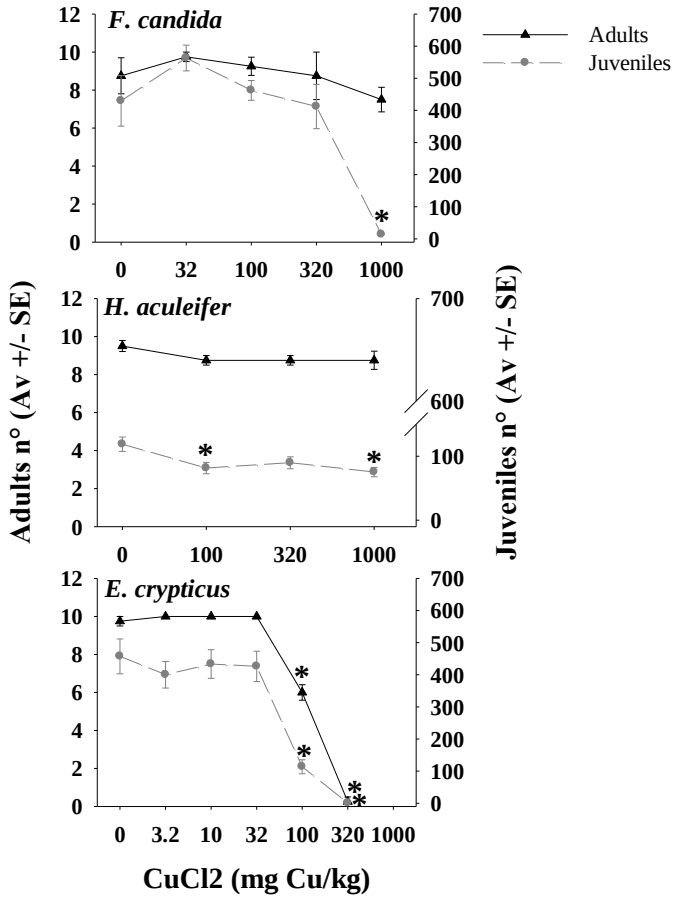

Figure S2 – Results from the reproduction tests with *Folsomia candida*, *Hypoaspis aculeifer* and *Enchytraeus crypticus* performed using field soil from Aveiro, Portugal, spiked with a range of $CuCl_2$ concentrations. Results are presented as average number of organisms (Av) ± Standard Error (SE). *Dunnett's test, *p≤0.05.

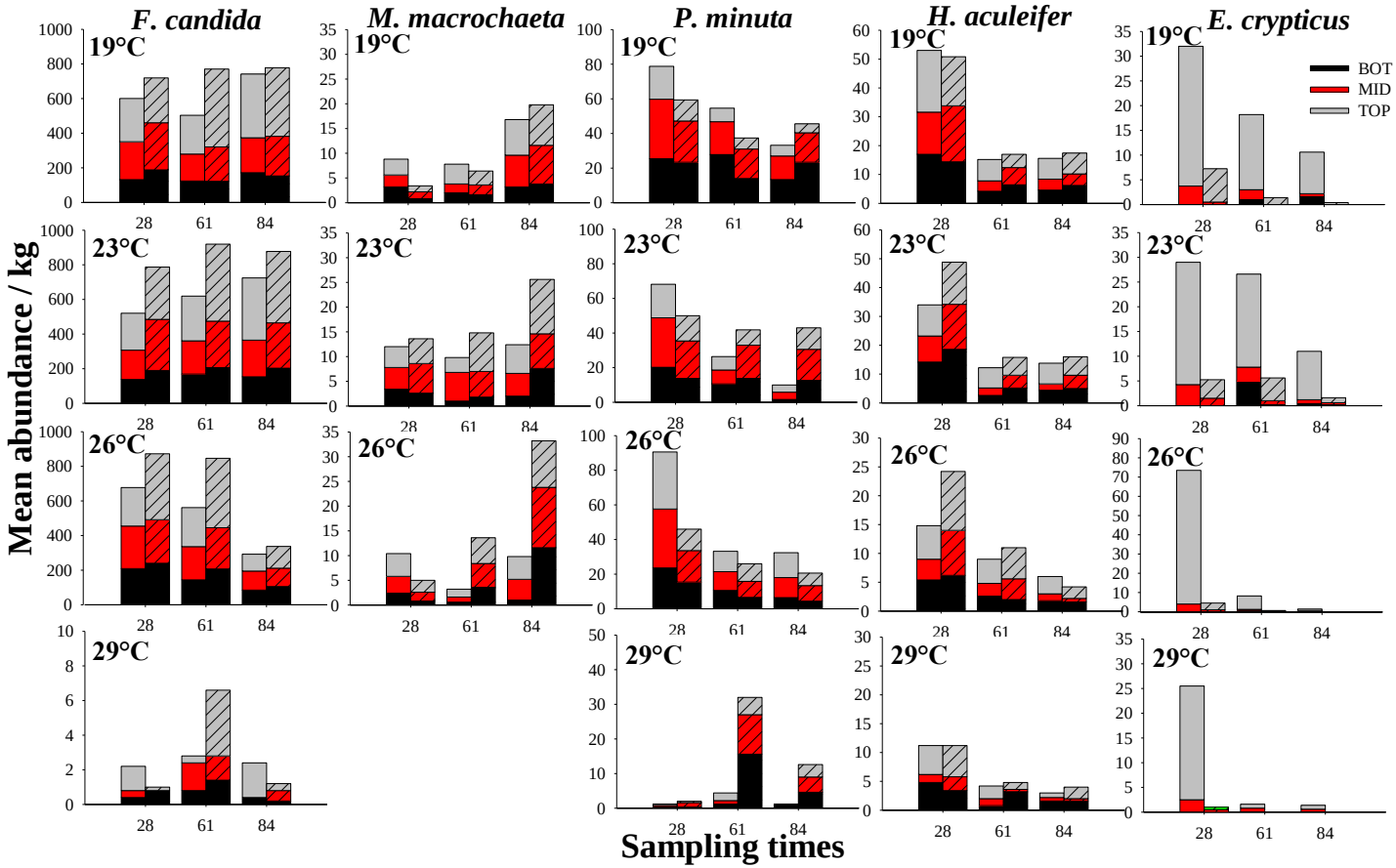

Figure S3 - Vertical distribution [distribution over the soil layers: TOP (0 – 5 cm), MID (5 – 10 cm) and BOT (10 – 15 cm)] of the individual species *Folsomia candida*, *Mesaphorura macrochaeta*, *Proisotoma minuta*, *Hypoaspis aculeifer* and *Enchytraeus crypticus* obtained from the SMS experiment performed using a clean and Cu spiked (100 mg Cu/kg) soil from Aveiro, Portugal. Tests were performed at four temperatures (19, 23, 26 and 29°C) and three exposure periods (28, 61 and 84 days). Coarse patterns represent Cu contaminated soil and non patterns represent control soil.

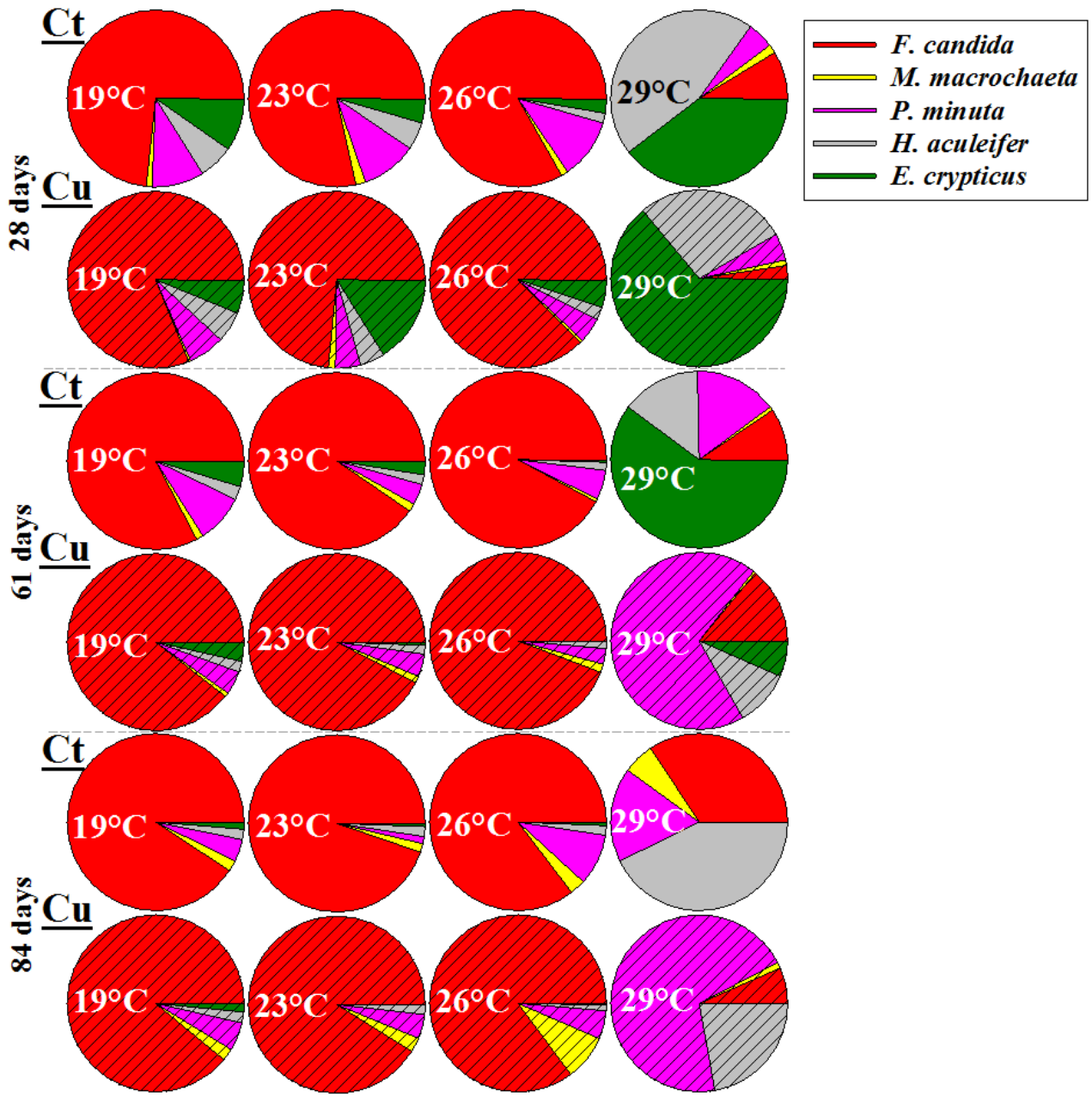

Figure S4 – Pie diagrams representing the species community structure composition obtained from the SMS experiment performed using clean (Ct) and Cu spiked (100 mg Cu/kg) from Aveiro, Portugal. Tests were performed at four temperatures (19, 23, 26 and 29°C) and three exposure periods (28, 61 and 84 days). Coarse line pattern represents Cu spiked soil.

Chapter VI – Effects of Cu and seasonality on mesofauna communities – northern and southern Europe field studies

Effects of Cu and seasonality on mesofauna communities – northern and southern Europe field studies

V. B. Menezes-Oliveira, J.J. Scott-Fordsmand, A.M.V.M. Soares, M.J.B. Amorim

Abstract

Pollutants affect the soil mesofauna community which can have severe consequences for the soil structure and functions. Field studies, integrating the effects of pollutants and seasonal variation are scarce. Moreover, field studies comparing different geographical regions are lacking. In the present study experiments were performed in two European regions [Portugal and Denmark] comparing the effect of Cu contamination on the structure (collembola community) and function (OM turnover and feeding activity) during 1 year (all seasons). Further, two contamination sources were compared: 1) Portugal (UAV) – freshly spiked soil (100 mg Cu/kg dw soil) and 2) Denmark (HYG) historical contamination (1000 mg Cu/kg dw soil). Results were compared between the two sites, using the thermal time (or degree days) concept. Copper suppressed the overall soil community, in particular in the southern site, where also the feeding activity was affected. Effects of Cu on organic matter decomposition were detected on the last sampling period.

Keywords: Species interaction, seasonality, copper, collembola.

1. Introduction

Soil fauna has a main role on soil fertility (e.g. nutrient cycling, OM decomposition) (Lavelle 1997). Organisms (e.g. earthworms, collembolan and mites) may act directly or indirectly onto soil fertility. Direct interaction may be decomposition of organic matter via litter fragmentation and through faeces production (Jones et al. 1994, Lokke and van Gestel 1998, Römbke et al. 2002). Indirect interaction may be for example affecting abundance of primary and secondary decomposers (Cole et al. 2004). Semi-field (Koolhaas et al. 2004, Jensen and Scott-Fordsmand 2012) and field studies (Pedersen et al. 1999, Fountain and Hopkin 2004) have been performed, assessing the effects of pollutants to the community composition, species interaction and distribution.

Soil community composition, distribution and species-interactions are not only altered by the presence of pollutants, but they may be also altered by the physicochemical characteristics which may change the sensitivity of different organisms. Amorim et al. (2005a) found that the collembola species *F. candida* showed different sensitivities to the herbicide Phenmedipham when exposed to different types of soil.

In this study we aimed to compare the seasonal effects on the mesofauna community of a new (UAV) and a historical (HYG) Cu contamination, using two European temperature regimes (geographical regions, Portugal and Denmark). The mesofauna community was identified at four time point across seasons and related to changes in soil temperatures.

2. Materials and Methods

2.1. Experimental design

For both sites a two factorial experiment was designed: factor 1: exposure period (4 sampling times), factor 2: Cu exposure (100 and 1000 mg Cu/kg for UAV and HYG, respectively due to differences between soils and fresh and aged contamination). Effects were assessed on the species community and functional endpoints. Details will follow per section.

2.2. Sites description

Study sites were located in northern and southern Europe (Denmark and Portugal). The Danish site is situated at Hygum (designated HYG), Jutland, Denmark. The site has been well studied (Pedersen et al. 1999, Maraldo et al. 2006b) and has a Cu contamination gradient in the soil as a result of previous (1911 - 1924) timber impregnating factory in which copper was the main compound employed. The area has been used for agriculture purposes from 1924 until 1991 and thereafter, it was permanently fallow. The present Cu concentration ranges from background levels (30 mg Cu/kg) to approximately 3000 mg Cu/kg dry weight (Pedersen et al. 1999, Scott-Fordsmand et al. 2000, Strandberg et al. 2006). Soil was sampled from a clean and 1000 mg Cu/kg fraction.

The Portuguese site is situated in Aveiro (designated UAV), a central region of Portugal, at the university campus (N 40°37.538' W 008°39.691'). The study area occupies a total of approximately 1600 m² and has not been used for agriculture purposes for at least 30 years.

Physicochemical properties of the two soils are summarised in table 1.

Table 1 - Physicochemical characteristics of Portuguese (UAV) and Danish (HYG) soil, including the grain size distribution, OM content and pH.

	UAV	HYG
coarse sand (>200 µm)	37%	20–32%
fine sand (63–200 µm)	11.20%	20–25%
silt (<63 µm)	35%	-
Clay (<2 µm)	-	12–16 %
OM	2.8-4%	3.6–5.5%
pH (H_2O)	5.0	6.3

Copper (Cu) concentration

The Cu concentrations for UAV and HYG were selected based on the toxicity range for the most sensitive species in the respective soil. Hence, for HYG a concentration of 1000 mg Cu/kg was selected within the EC_{50} range for the enchytraeid species (Maraldo et al. 2006a);

Menezes-Oliveira et al., submitted). For UAV, a concentration of 100 mg Cu/kg was selected based on previous experiments (Menezes-Oliveira et al., submitted). Differences in test concentrations can be explained by the differences in terms of bioavailability between the two soils i.e. approximately a factor of 10 was expected based on pH and Cation Exchange Capacity (SCHER 2009). Thus, the test concentrations were leveraged to obtain similar exposure/effect concentration per soil.

Soil collected from HYG was analysed by flame Atomic Absorption Spectrometry (AAS, Perkin Elmer 4100, Ueberlingen, Germany) to confirm Cu concentration within the selected range (Control (background) and 1000 mg Cu/kg). Soil samples were first digested in a heating block using a 70% concentrated HNO_3 solution in 100 mg dry soil. Temperature was increased from 80°C to 110°C until all fluid from the samples became clear and the acid evaporated. Before AAS analysis the samples were dissolved in a 0.1M HCl.

Experimental procedures

2.3. UAV experiment

2.3.1. Soil pre-sampling and spiking

Soil was pre-ploughed one year before the sampling for the experiment. Growing vegetation was removed before sampling. Soil was sampled to a depth of 20 cm and sieved through a 2 mm mesh to remove larger particles. The soil was spiked in the laboratory using a chloride copper salt $CuCl_2.H_2O$ (99% purity, Merck Pro Analysis, Darmstadt, Germany). Spiking was performed adding $CuCl_2$ as aqueous solution into pre moistened soil batches of 4 kg to obtain a 100 mg Cu/kg concentration. The mixing was done manually during 15 minutes per batch.

2.3.2. Field set up

Soil cores were inserted in the field site. Soil cores consisted of PVC tubes (35 cm height, 10 cm diameter). The tubes were closed with a 0.45µm mesh on the bottom and 1.5 mm mesh on the top, this to avoid colonization of "undesirable" animals but still allow exposure. To avoid compaction, soil was taken to the field in batches and introduced into the tubes until the height of 25 cm. Two small holes (1 cm diameter) were made in the upper 25 cm of the tube and closed with 0.45 µm mesh to allow water runoff in case of surface rain/flooding. Tubes were then inserted in the field, displaced as shown in figure 1.

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In short, 8 blocks sized 2 x 2 m were marked, 4 allocated to control and 4 for Cu spiked soil. In each block 12 holes with depth and diameter of approximately 25 and 15 cm, respectively, were made. Samplings were made as indicated in the figure 1 for the various periods.

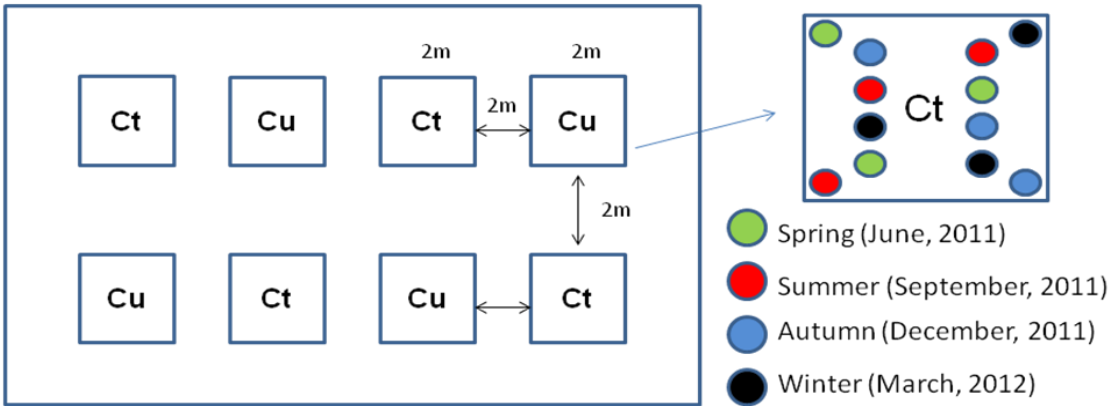

Figure 1 – Experimental design of the samples distribution in the UAV field. Each sampling time is represented by a different colour, being green for spring, red for summer, blue for autumn and black for winter exposures.

Litterbags and bait laminas were added to the systems and the animals were added to the top soil using 100 g of soil containing the previously extracted animals (details are given in the following sections).

2.3.3. Test organisms

Microarthropods were extracted from UAV soil prior adding to the experimental field units. To extract the animals three soil cores weighting (in total) around 1 kg were collected for each replicate. A heat extraction system was setup, composed of a heating lamp (placed 10 cm above the soil cores) focusing on the soil samples, introduced in a 0.45 mm bottom mesh basket. The animals were collected on a tray containing a layer of plaster of Paris to keep the organisms alive. Collections were made daily during 7 days. Organisms were kept in Petri dishes with Plaster of Paris at 5°C to avoid reproduction and predation. At day 7 animals were separated for each replicate and were added to ca. 100 g soil (control or Cu concentration), ready to add in the mesocosms in the field the day after.

2.3.4. Litterbags

Litterbags were used to assess the organic matter turnover in the soil system. The bags (1.5 mm x 1.5 mm mesh) were filled with approx. 200 mg of dried straw and inserted into the top 5 cm soil. The straws were carefully placed side by side to prevent animals to hide themselves in the spaces between them thus avoiding exposure to the test soil. One bag per replicate was added in 4 out of 12 replicates per condition and were sampled in the end of the each exposure period, washed, dried at 50°C overnight and weighted to determine the mass loss.

2.3.5. Bait lamina

The feeding activity of the soil fauna was determined using the bait lamina method (Kratz 1998). One bait lamina stick was inserted per each unit in 8 out of the 12 replicates of each test condition. These sticks contain 16 holes filled with a cellulose-based substrate (70% cellulose, 25% nettle powder, 5% activated charcoal). The sticks were added to the soil system on the 1st day of the experiment and were taken out after 35 days. The feeding activity was expressed by the percentage of empty (eaten) holes per day.

Field sampling

In UAV, the soil cores were inserted during March, 2011 and sampled in June 13th, September 12th, December 19th, 2011 and March 12th, 2012 selected based on the thermal time (1394, 3229, 4518 and 5046°C days) i.e., exposure lasted ca. 3, 6, 9 and 12 months corresponding to spring, summer, autumn and winter respectively.

The temperature in the experimental fields was measured using data-loggers, placed in the soil to a depth of approximately 10-15 cm and data was recorded every 30 minutes.

Organisms' extraction (end of each exposure)

In the end of each exposure period, 24 experimental units (12 control plus 12 Cu spiked) were sampled from the field and transferred to the laboratory. Each soil core was divided in 3 layers (top, middle and bottom) with approximately 6 cm height. From each layer a random sub-sample of approx. 400g was used for collembolans quantification. The collembola sub-samples were extracted over 7 days in a high gradient extractor (MacFadyen), using the

following temperature regimes: 25°C – 24h, 35°C – 24h, 40°C – 48h, 45°C – 24h, 50°C – 24h and 60°C – 24h. Animals were collected in benzoic acid, transferred to glycerol, identified and counted. The reference keys used for the identification of the UAV species were (Bellinger et al. 1996-2012) and (Jordana 1997).

2.4. HYG experiment

2.4.1. Collembola and Enchytraeid sampling

Soil cores were extracted from non-manipulated soil site sampled in 2010 June 6th, July 5th, August 9th and August 25th, selected based on the thermal time (692, 955, 1190 and 1574°C days) and previous studies (Menezes-Oliveira et al, submitted). Sampling times corresponded to late spring and summer where the highest values for temperature and population growth rates may be observed.

In order to collect soil microarthropods any large plant material was removed from the soil surface and soil cores were taken with a metal core sampler to a depth of 5 cm. Samples were then transferred directly to a plastic tube (5 cm height, 6 cm diameter and 28 cm² surface area). Animals were extracted during 7 days in a high gradient extractor (MacFadyen like), collected in benzoic acid, transferred to glycerol, identified and counted. At each sampling time 8 soil cores were sampled from each site. Samples were randomly done in an area of 1 m². The reference keys used for the identification of the HYG species were (Bellinger et al. 1996-2012) and (Fjellberg 1998, 2007).

For the enchytraeids sampling a cylindrical soil corer with an inner diameter of 5.5 cm was used. Eight soil cores were taken within an area of 1 m² in 0-9 cm depth. Immediately after sampling each soil core was divided into layers of 3 cm height with a knife. For the extraction an adapted Tulgreen apparatus using wet funnels with a stepwise increase in temperature from 25 to 50°C during five hours was used. After extraction enchytraeids were collected in tap water and stored for 24-48 h at 5°C, period that they were counted.

2.5. Statistical analysis

2.5.1. Univariate analysis

Significance of differences between physicochemical parameters (pH, % OM content and % relative humidity) of control and contaminated soils in the different sampling times for both

UAV and HYG soils was tested by using One-way Analysis of Variance (ANOVA, Tukey test) (SPSS 1997).

For each sampling time species diversity was calculated by using the Shannon-Wiener index according to (Magurran 1991).

2.5.2. Multivariate analysis

Multivariate statistical program Canoco for Windows 4.5 (Ter Braak and Smilauer 2002) was used. Species data was ln transformed. The length of the gradient, shown by Detrended Correspondence Analysis, was smaller than 3 and thus correlations in the data set were analyzed by Principal Component Analysis (PCA). The contribution explaining the variation of these variables and their significance was analyzed using ReDundancy Analysis (RDA), a constrained linear ordination method using automated forward selection in combination with Monte Carlo test with 499 permutations.

3. Results

Soil temperatures

In the Portuguese (UAV) field soil temperatures were measured from March 2011 to March 2012. In Denmark (HYG) it was measured from March 2010 to August 2010 (see Fig 2).

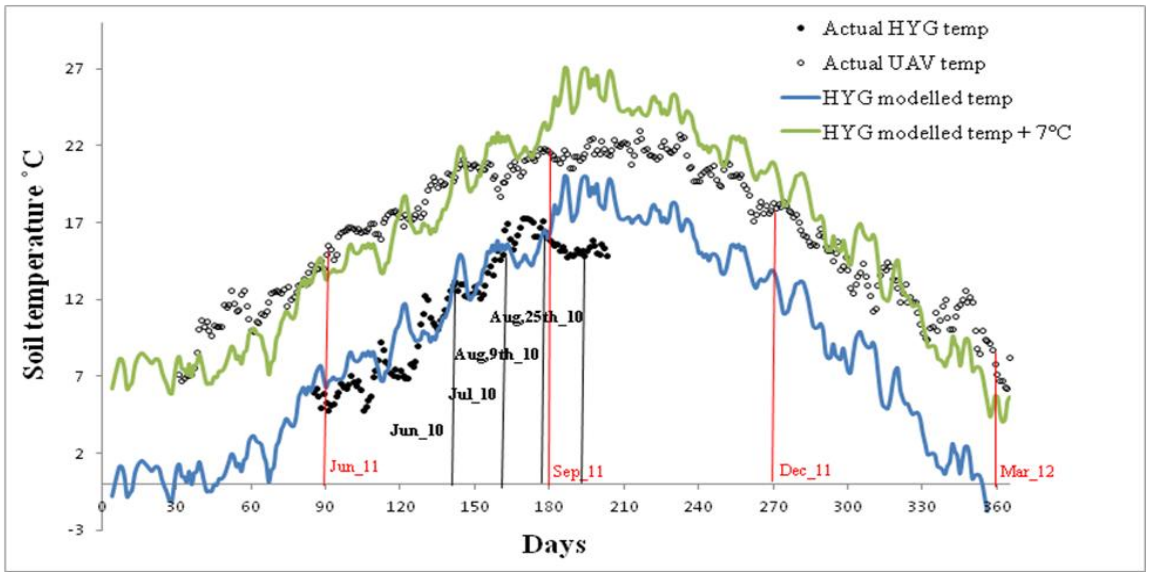

Figure 2 – Temperature °C during the sampling dates, including modelled temperatures. The red lines represent the sampling dates for UAV experiment (June/11, September/11, December/11 and March/12) and the black lines represent the sampling point for the HYG experiment (June/10, July/10, August, 9th/10 and August, 25th/10).

For HYG, the full year temperature curve was "assessed" with a combination of the measured soil temperatures and the modelled temperatures from January to March. The model was based on daily air temperatures from the same area (mean temperature in area of 20*20 km over the sample location see figure 2. For UAV the temperatures was on average 7°C higher than for HYG, the temperature development over the year showed a slightly flatter curvature.

During the exposure periods in the field soil temperatures for UAV reached the maximum value of 25°C and the minimum of 5°C. In HYG temperatures reached a maximum value of 19°C and a minimum of 0 °C.

In order to compare the results obtained in the two field experiments, in terms of warming exposure, the "Degree days" approach was adopted (Trudgill et al. 2005). The "Degree days" is based on the cumulative values of temperature (°C) measured per day, this was done by integrating the equation for the temperature curves. In figure 3 it is displayed the cumulative temperature received for both UAV and HYG field experiments as well as to the laboratory experiments (Chapters IV and V), for comparison.

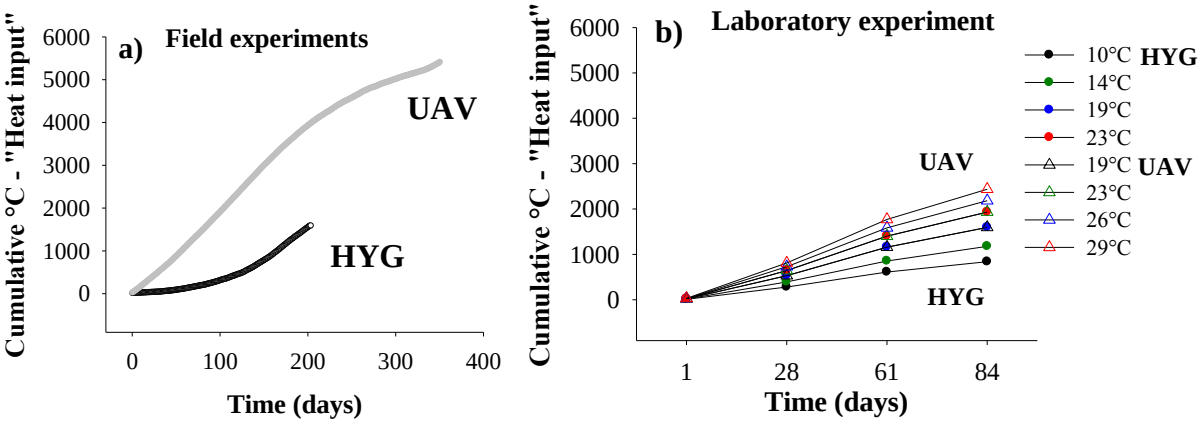

Figure 3 – Heat input as calculated for field and laboratory experiments; a) Field experiments – black dots represent data for HYG from January 1st, 2010 to August 25th, 2012 and grey dots represent values for UAV from March 28th, 2011 to March 12th, 2012; b) Laboratory experiments previously performed (Menezes-Oliveira et al., submitted) – circles represent the values for HYG and triangles represent values for UAV. Different colours represent the distinct temperatures for which systems were exposed during 28, 61 and 84 days.

Soil physicochemical parameters

Soil pH, Organic Matter (OM) content and % of Relative humidity (Rh) did not vary significantly (one way ANOVA) over seasons, for either control or polluted/spiked soil, and contributed similarly (seen by their correspondent sizes of the arrows in the PCA analysis – not shown) to the variation of the species over time. Hence, the results are not shown in the multivariate analysis.

Collembola species composition

The species composition differed at the two sites, hence to enable comparison between the two sites the soil community was compared via taxa or group of species. The groups used (see table 2) were: entomobryomorpha, poduromorpha and symphypleona. Entomobryomorpha, was constituted by three families (Isotomidae, Entomobryidae and Tomoceridae), poduromorpha by four families (Hypogastruridae, Brachystomellidae, Neanuridae and Onychiuridae) and for symphypleona seven families (Neelidae, Sminthurididae, Arrhopalitidae, Katiannidae, Dicyrtomidae, Bourletiellidae and Sminthuridae). All abundances are given per square meter.

The distribution of the collembola groups over time in the two experimental sites was analysed through the ordination method - Principal Component Analysis (PCA), see figure 4.

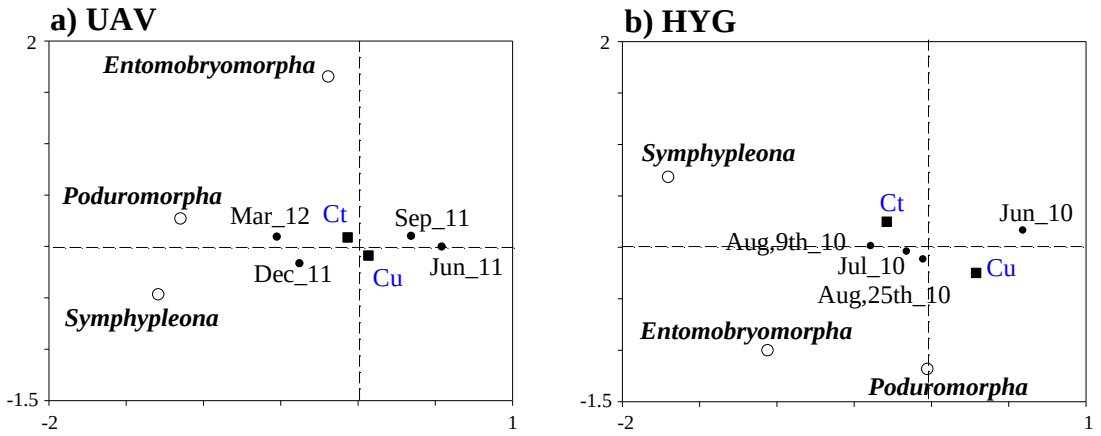

Figure 4 – PCA diagram showing the ordination of the group of species found in the experimental fields in four different sampling dates [Jun_11, Sep_11, Dec_11 and Mar_12 for UAV and Jun_10, Jul_10, Aug,9th_10 and Aug,25th_10 for HYG]; a) Results for UAV experiment. First (horizontal) and second (vertical) axes explain 72.2% and 18.5% of the variation in the group of species, respectively; b) Results for HYG experiment. First (horizontal) axis explains 76.2% and second (vertical) axis explains 16.2% in the group of species.

Changes in abundance of entomobryomorpha species over time was positively correlated with unpolluted (control) samples in UAV and negatively in the Cu contaminated area. In HYG the entomobryomorpha abundance was independent on contamination level. Symphypleona species, in turn, were highly correlated to the control samples in HYG but was independent of Cu contamination in UAV. Poduromorpha species was more correlated to the control samples in UAV but in HYG species showed to be more abundant in the Cu contaminated samples. The sampling times in UAV similarly influenced the analysis, despite being in different directions, what may be observed by their similar locations in the different quadrants of the diagram. In HYG, the first sampling (on June, 2010) showed a higher influence on the species variation, when compared to the other sampling dates, what can be explained by the collembolan' lower abundance in this sampling, especially for control exposure (Figure 5).

Table 2 – Collembola species identified in both UAV and HYG field. The presence or absence of a species in a given soil is marked by an X. The abbreviations were used to describe the species in the diagrams.

		Field				Field				Field	
Group	**Species [code]**	**UAV**	**HYG**	**Group**	**Species [code]**	**UAV**	**HYG**	**Group**	**Species [code]**	**UAV**	**HYG**
Entomobryomorpha	*Cryptopygus nidicola* [C_nid]	X		**Entomobryomorpha**	*Proisotoma ripicola* [P_rip]		X	**Poduromorpha**	*Neanura muscorum* [N_mus]	X	
	Desoria spp [Des_spp]	X			*Proisotoma spp* [Proi_spp]		X		*Onychiurus normalis* [O_nor]	X	
	Entomobrya nivalis [E_niv]		X		*Pseudosinella alba* [P_alb]		X		*Protaphorura fimata* [P_fim]	X	
	Entomobrya spp [Ent_spp]	X	X		*Pseudosinella halophila* [P_hal]		X		*Protaphorura spp* [Prot_spp]		X
	Entomobrydae (juv.) [Ent_juv]	X			*Pseudosinella spp* [Pse_spp]	X			*Pseudochorutes parvulus* [P_par]	X	
	Folsomia candida [F_can]		X		*Sinella curviseta* [S_cur]		X		*Scaphaphorura arenaria* [S_are]	X	
	Folsomia manolachei / quadrioculata [F_man]		X		*Sinella tenebricosa* [S_ten]	X			*Stenaphorura quadrispina* [S_qua]	X	
	Folsomia spp [Fol_spp]	X		**Poduromorpha**	*Bilobella aurantiaca* [B_aur]		X		*Tullbergia spp* [Tul_spp]	X	
	Heteromurus spp [Het_spp]	X			*Brachystomella parvula* [B_par]		X	**Symplypleona**	*Arrhopalites spp* [Arr_spp]	X	
	Isotoma viridis [I_vir]		X		*Ceratophysella denticulata* [C_den]	X			*Deuterosminthurus bicinctus* [D_bic]		X
	Isotomiella minor [I_min]	X	X		*Ceratophysella spp* [Cer_spp]	X			*Dicyrtomidae* [Dicdae]	X	
	Isotomurus alticolus [I_alt]	X			*Ceratophysella succinea* [C_suc]	X			*Dicyrtomina minuta* [D_min]		X
	Isotomurus fucicola [I_fuc]		X		*Fissuraphorura gisini* [F_gis]		X		*Neelus murinus* [N_mur]	X	X
	Isotomurus spp [Iso_spp]	X			*Friesea truncata* [F_tru]	X			*Sminthuridae* [Smdae]	X	
	Lepidocyrtus spp [Lep_spp]	X	X		*Hypogastrura ripperi* [H_rip]		X		*Sminthurides spp* [Smin_spp]	X	
	Parisotoma notabilis [P_not]	X	X		*Mesaphorura betschi* [M_bet]		X		*Sminthurinus spp* [Smth_spp]	X	
	Pogonognathellus longicornis [P_lon]		X		*Mesaphorura spp* (juv.) [Mes_juv]	X			*Sphaeridia spp* [Sph_spp]	X	X
	Proctostephanus spp [Pro_spp]	X			*Mesaphorura* spp* [Mes_spp]		X		*Sphyroteca spp* [Sphy_spp]		X
	Proisotoma minuta [P_min]	X			*Micranurida pygmaea* [M_pyg]	X	X				

*The species *Folsomia manolachei/quadrioculata* is like this represented here because they are very similar and due to the time consuming of this task it was not possible to confirm all the species to the minimum detail. # *Mesaphorura spp* – adult different from *M. betschi*.

When analysing separately the distribution of the different group of species when exposed to clean or contaminated soil over time and plotting the results on top of each other it is possible to observe that the variation in the group of species were similarly influenced by the sampling times when exposed to control or Cu contaminated soil (Figure 5). Apart from the Poduromorpha group at HYG which showed to be highly correlated to the last sampling when exposed to Cu while in Ct samples its abundance was very low in all sampling dates. See also the relative abundance of the different group of species in the supplementary information (Figure S1).

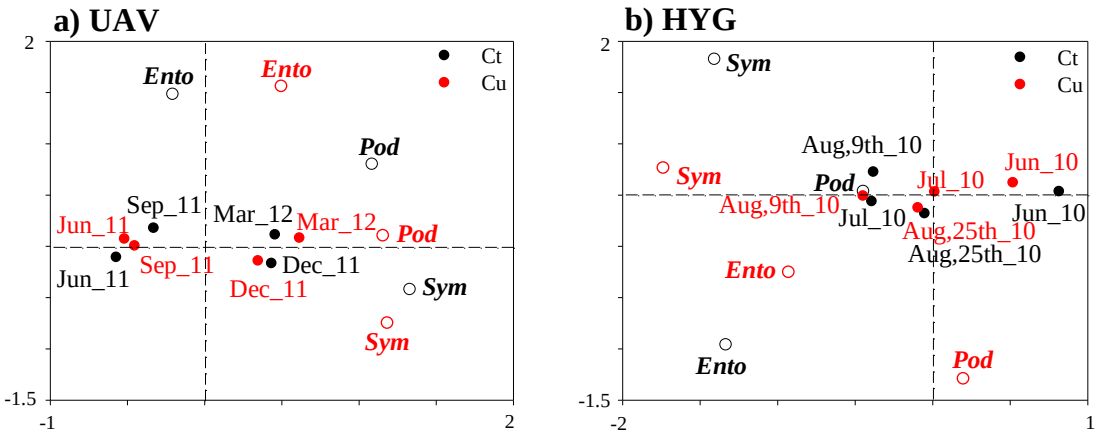

Figure 5 - PCA diagrams showing the ordination of the group of species [entomobryomorpha (ento), Poduromorpha (Pod) and Symphypleona (Sym)] found in the experimental fields in four different sampling dates [Jun_11, Sep_11, Dec_11 and Mar_12 for UAV and Jun_10, Jul_10, Aug,9th_10 and Aug,25th_10 for HYG]. Analysis were separately performed for control (Ct) (Black symbols) and Cu contaminated (Cu) (Red symbols) samples and plotted on top of each other; a) UAV experiment - First (horizontal) and second (vertical) axes explain 70.8% and 19.3% (Ct) and 83.2% and 11.4% (Cu) of the variation in the group of species, respectively; b) HYG experiment - First axis explains 77.8% (Ct) and 71.7% (Cu) and second axis explains 14.3% and 13.4%, for Ct and Cu exposure, respectively, of the variation in the group of species.

The Redundancy Analysis (RDA), which is a constrained form of the Principal Component Analysis (PCA), was used to further understand how the exposure to the contaminated and clean soils over time has influenced the species response. In figure 6 it is possible to

observe that species variation was partly explained by the different samplings and partly by the Cu contamination. For HYG, the variables that best explained the species variation were the exposure treatment (Cu versus control soil) while in the Portuguese (UAV) experiment it was the exposure period.

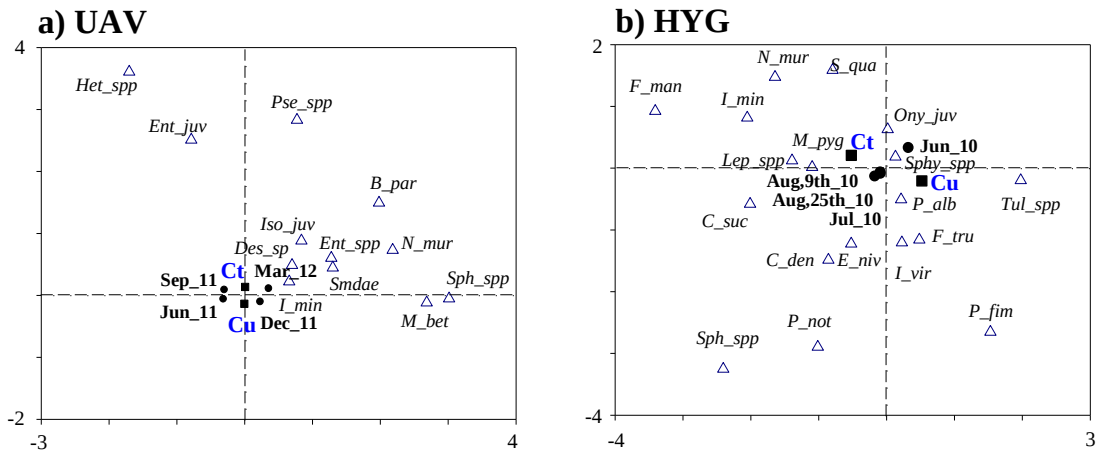

Figure 6 – RDA diagram showing structural variables (triangles up) over time. Data was ln-transformed. a) HYG field: The explanatory variables (sampling times and treatments) explained 52.9% of the variation in species data with 31.3% explained by the treatments (Ct and Cu) and 21.6% explained by the exposures (sampling times); b) UAV field: The explanatory variables (sampling times and exposures) explained 32.7% of the variation in species data with 3.7% and 27.6% explained by the treatments (Ct and Cu) and sampling times, respectively. Only species with the fit range between 20 and 100% are shown in this graph. For the complete name of the species please see table 2.

In the UAV field the entomobryomorpha species *Heteromurus spp.* showed to be highly abundant and correlated to the control samples (Figures 4 and 6). The species contributing with most of the variation in poduromorpha group are *Brachystomella parvula* and *Mesaphorura betschi* while for the symphypleona group are *Neelus murinus* and *Sphyroteca spp*. In HYG, the symphypleona species is most related to the Ct samples and is represented in the RDA diagram by *Neelus murinus* (Figure 6). *Sphaeridia spp.*, which is also symphypleona, showed its average number in both Ct and Cu contaminated soils. The most representative poduromorpha species for the analysis is *Protaphorura fimata*, which showed to be very abundant in the contaminated site.

Differences between control and Cu

Total collembola abundance

A total of 9741 individuals, separated into 41 taxa, were identified over all sampling times in UAV. In HYG the total amount of individuals identified was 14395 and they were separated into 38 taxa. The highest collembola abundance in HYG site was around 10-fold higher than the highest amount of collembola found in UAV. In both soils the peak of animals was seen in the first sampling time for control conditions while for the contaminated soils the reproduction was delayed, peaking in the 3rd sampling time in HYG and only in the last sampling time in UAV.

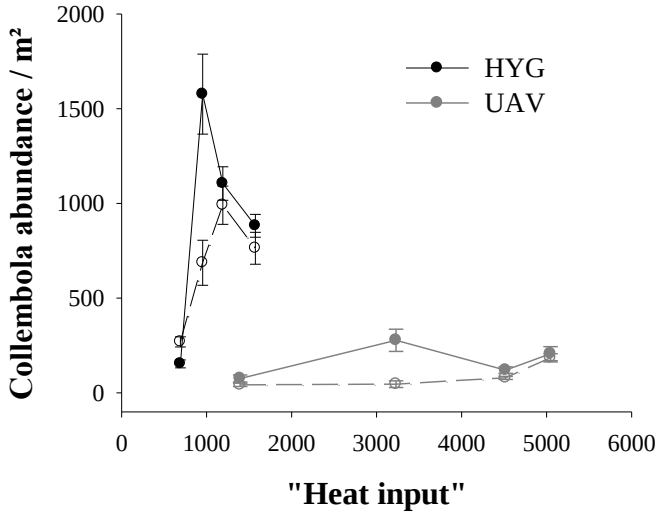

Figure 7 – Collembola overall abundance per m² found in the two different soils (UAV and HYG). Gray circles and lines represent UAV experiment while black circles and lines represent HYG experiment. Solid lines represent the control systems and dashed lines represent Cu contaminated systems.

Species interactions

In general it was observed that the Cu exposure affected the community responses in both UAV and HYG soils and that the species variation depended also on the seasons that they were exposed.

Copper contamination affected differently the organisms' response over time. As it can be seen in figure 8, when separating the species response from the different sampling times, for both experiments, not only the distribution and abundances of the species changed but there were also species which were either not able to live in the contaminated soil or were very rare. There were also species which showed to be less sensitive to copper and were present only in the contaminated sites as for example the hemiedaphic species *Friesea truncata* in HYG and *Hypogastrura ripperi* in UAV soil. The epiedaphic species *Isotomiella minor*, however, is an example of a species only present in the unpolluted soils, for both fields (based on the raw data).

Species abundance changed considerably over time. The species *Mesaphorura betschi* and *Brachystomella parvula*, for example, were the species contributing with most of the variation for the group poduromorpha in UAV field over time (Figure 6). However, looking at the diagram for each exposure separately (Figure 8) it is seen that when these species are present at the same time they are negatively correlated to each other. An opposite pattern occurred for the HYG soil. The species *Protaphorura fimata* and *Tullbergia spp* were the two species contributing most to the variation in the poduromorpha group. They were always present at the same time in the different samplings and positively correlated to each other, however, they altered their abundance being one normally more abundant than the other in a giving time.

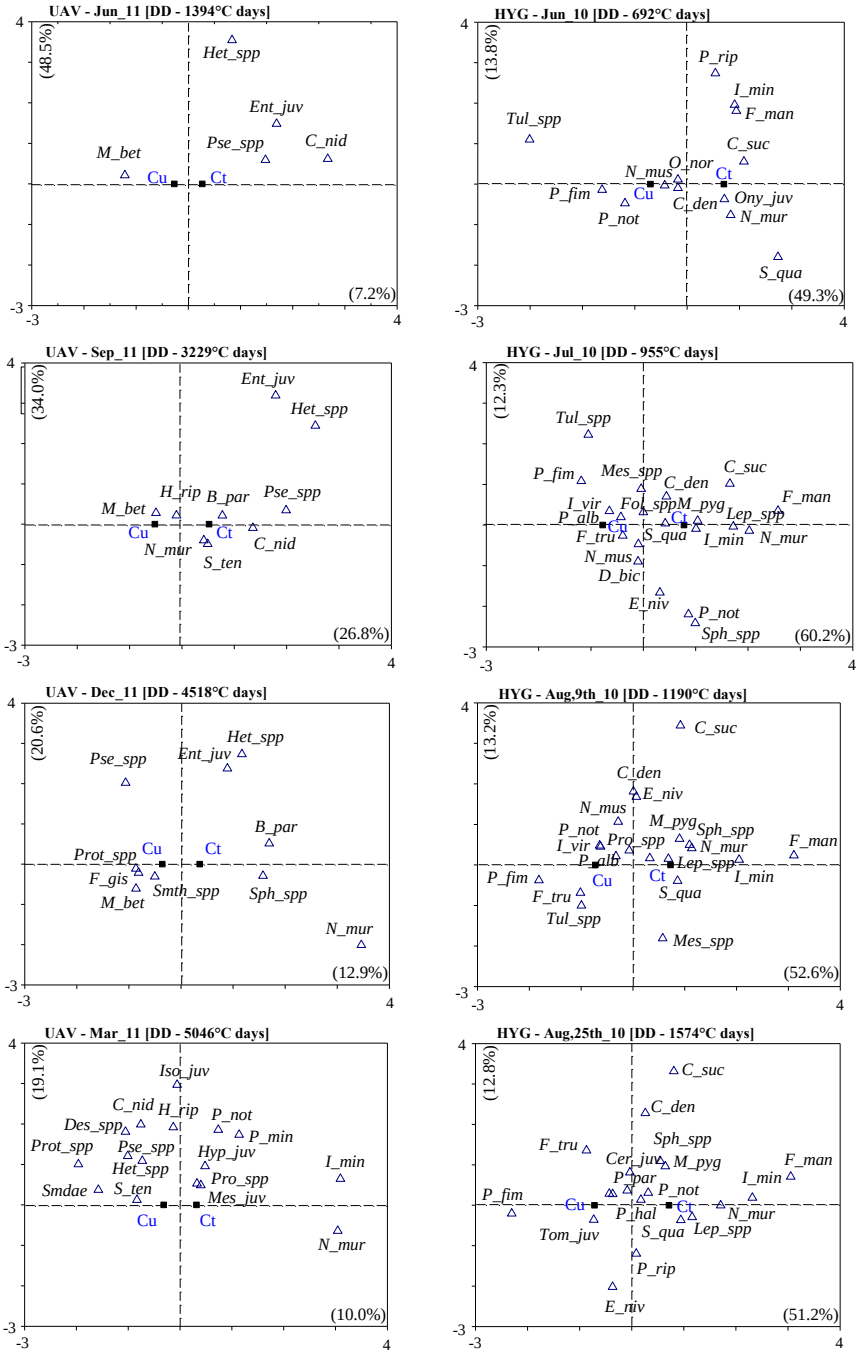

Figure 8 – RDAs diagrams showing the species distribution over control and Cu contaminated samples for the two experimental fields for each sampling time/degree days received by the systems separately. Only species with the fit range between 15 and 100% are presented in the diagram; In the left side are the results from UAV experiment and in the right side are the results from HYG experiment. The percentages of explanation of the 1st (horizontal) and 2nd (vertical) axes for the different analysis are presented in the respective diagrams.

Biodiversity index differences

Shannon Wiener index calculated for the different group of species showed that poduromorpha was the group of species comprising the highest biodiversity in both control and contaminated soils in Denmark (HYG). For Portugal (UAV) entomobryomorpha was the group with the highest biodiversity also for both control and polluted soil. Looking at all species together it may be seen that the biodiversity increased within time in UAV showing it highest number in the 3rd exposure and remaining constant to the 4th exposure. In HYG, the biodiversity decreased within time and it was slightly steeper for control soil (Figure 9).

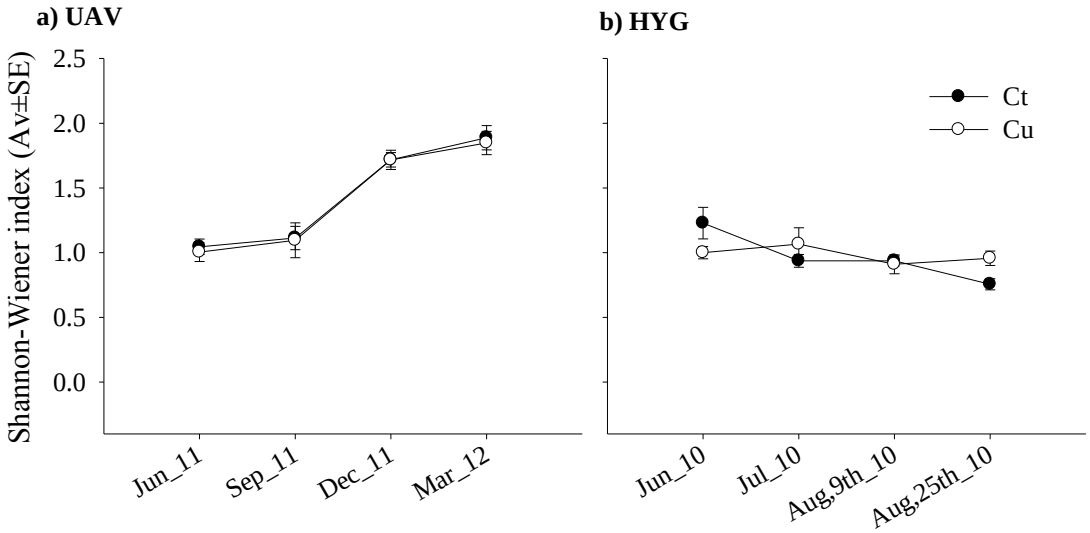

Figure 9 – Collembola species diversity calculated for both HYG and UAV fields, i.e. Shannon-Wiener index (log base e), as a function of sampling times under polluted or unpolluted soils.

Single species differences

Possibly indicator species could be identified. Hence, PCA analyses were performed in order to detect the species contributing most with the variations in species data over the different sampling times. For UAV, species with the fit range between 50 and 100% in the PCA were chosen and for the HYG soil species with the fit range between 60 and 100% were chosen. Animals selected are individually displayed in figure 10 and 11. In general,

euedaphic (including here *M. betschi* and *P. fimata*) and hemiedaphic (*B. parvula*, *C. succinea*) species, showed to be less sensitive to copper with their abundance increasing over time (Figure 10 and 11). The exception in the euedaphic group is the species *Neelus murinus* which showed large variation but was in general more abundant in the control soil. The epiedaphic species, including here *Heteromurus spp* and the juveniles of the Entomobrydae family in the UAV soil were very sensitive to Cu while the *Sphaeridia spp*, which is also epiedaphic, increased the abundance over time for both control and contaminated soils. In HYG, the same trend was observed and the epiedaphic species such as *Isotomiella minor* and *Folsomia manolachei/quadrioculata* were very sensitive to Cu while *Sphaeridia spp* abundance increased within time until the 3rd sampling and then decreased.

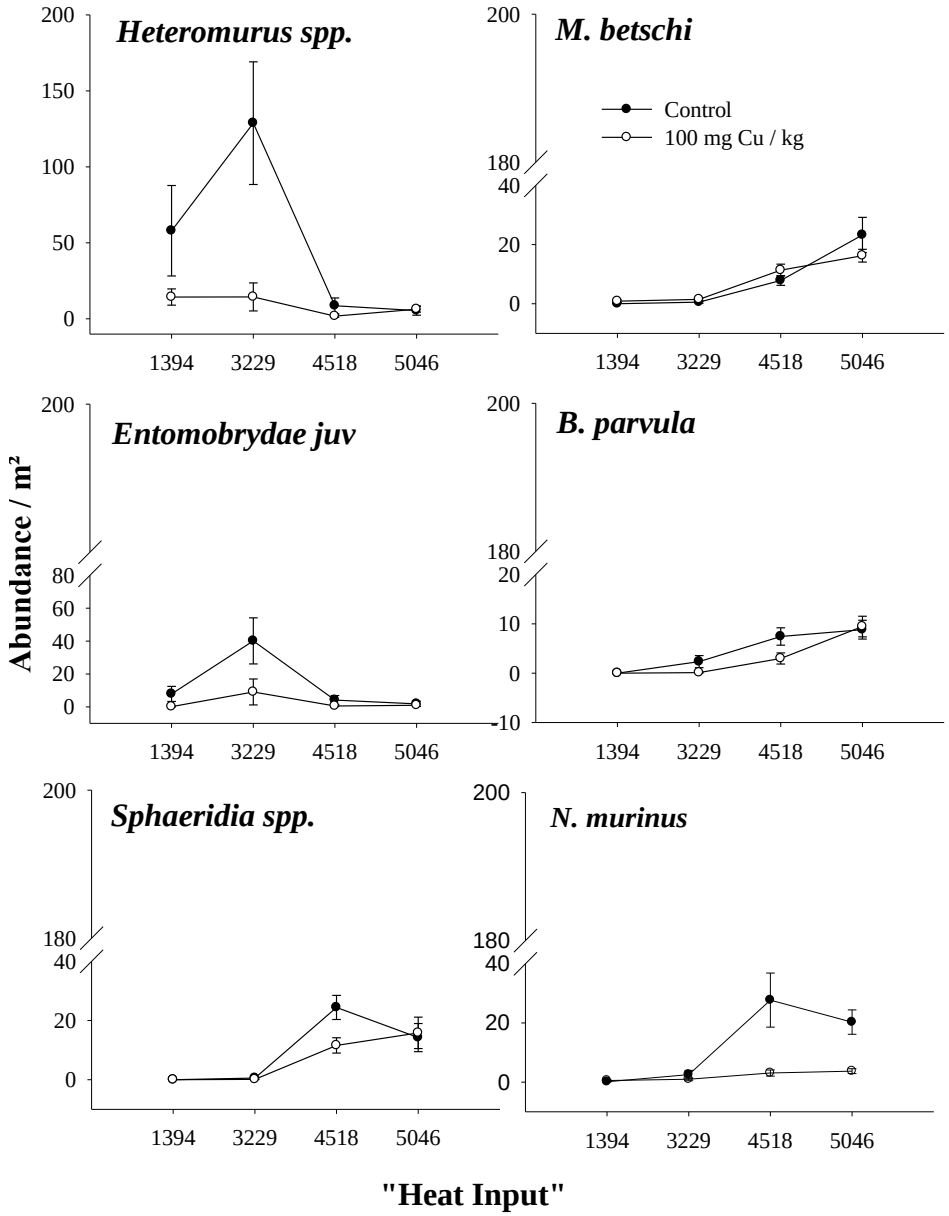

Figure 10 – Abundance of the collembolan species contributing most to dissimilarities in species composition in the UAV field. Results are presented as the average of the total abundance of animals per m² in the control (black circles) and in the Cu spiked soil (100 mg Cu/kg) (open circles) versus the cumulative °C – "heat input".

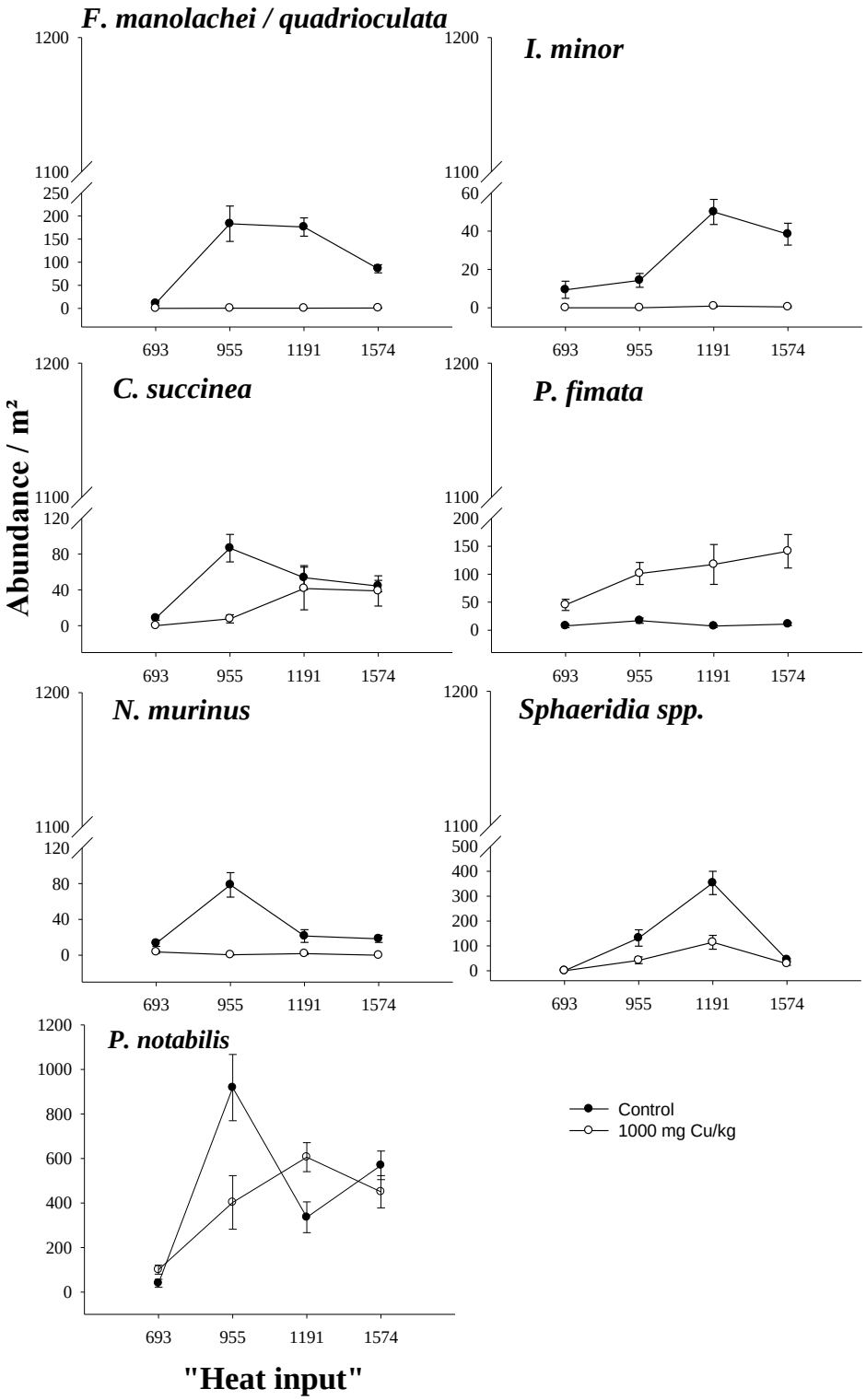

Figure 11 – Abundance of the collembolan species contributing most to dissimilarities in species composition at the Hygum (HYG) field. Results are presented as the average of the total abundance of animals per m² in the reference site (black circles) and the contaminated site (1000 mg Cu/kg) (open circles) versus the cumulative °C – "heat input".

In general, the euedaphic poduromorpha species (*M. betschi*, *P. fimata*) responded similarly to the different treatments over time showing a delay on reproduction in comparison to the other individual species presented in figure 10 and 11. Results corroborates to the laboratory experiment where the similar species *Mesaphorura macrochaeta* showed the same trend slightly increasing its abundance within time for both control and contaminated soils (Menezes-Oliveira et al. submitted) .

Enchytraeid changes

Enchytraeid abundance was only assessed at HYG field since they were not present at UAV, probably due to the characteristics of the soil. Looking at the results of the laboratory experiments performed with both soils (Menezes-Oliveira et al., submitted) it was observed that *Enchytraeus crypticus* abundance was at least three times lower in UAV than at HYG and that they were practically extinct after 84 days for all conditions tested.

The total number of individuals per square meter at HYG decreased over time for both control or Cu contaminated conditions. The decrease in number of enchytraeids showed to be more pronounced in the clean soil. In the contaminated soil broad overlapping standard errors were presented and the number of individuals showed to be nearly the same for the first 3 samplings. The higher difference in abundance between control and contaminated samples was observed in the first sampling date where the number of enchytraeids was three times higher at control conditions (Figure 12).

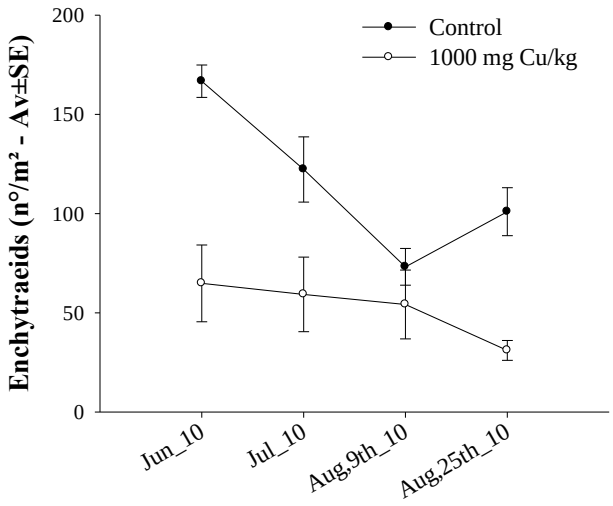

Figure 12 – Total abundance of Enchytraeids at the Danish (HYG) field over four sampling times (from June to August, 2010). Results are presented as the average (Av) number of organisms per square meter ± Standard Error (SE). Control samples are represented by the solid symbols while the Cu contaminated soil (1000 mg Cu/kg) is represented by the opened circles.

Soil function analysis

Soil function analysis was performed only for the UAV field experiment. Feeding activity and OM decomposition assessment were done by using bait lamina and litterbags, respectively.

- Feeding activity

Soil fauna feeding activity is reported in figure 13 a) as the percentages of the empty holes in bait laminas per day. Significant statistical differences were observed between control and Cu contaminated samples for the 3rd and 4th samplings (t-test, $p = 0.038$ and $p = 0.01$ for 3rd and 4th sampling, respectively). Results of One Way ANOVA showed that differences observed over time for control or contaminated samples were not statistically significant. Results from the feeding activity did not follow the same pattern seen for the overall community in the soil. The highest abundance of collembola was seen at the second

sampling time (end of summer) while the highest feeding activity was observed at the 3rd sampling time (end of autumn) (Figure 13 b).

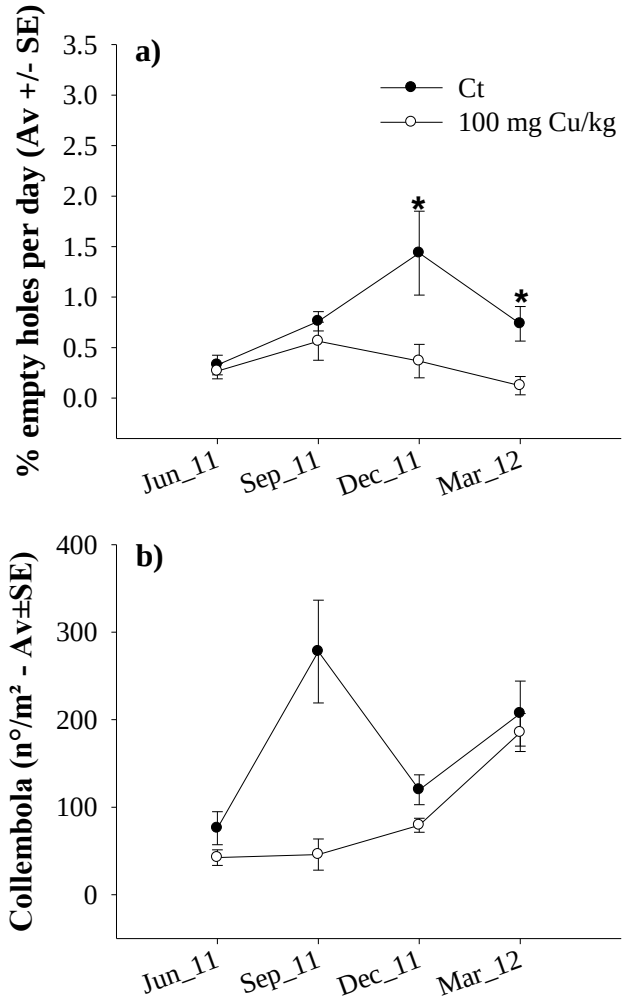

Figure 13 - Results of bait lamina test used as feeding activity measurements obtained from the UAV field experiment. Following treatments were used: control and Cu contaminated soil (100 mg Cu/kg) four temperatures exposure periods. a) Feeding activity expressed as average ± standard error (AV±SE) of the % of empty (eaten) holes in the bait lamina per day; *$p<0.05$, testing control versus spiked; b) Total abundance of collembola (AV±SE) per m².

- Organic Matter (OM) breakdown

Decomposition of the organic material increased with increase in exposure time for both control and Cu contaminated treatments (Figure 15). Statistical significant differences were observed in the last exposure between control and contaminated soils (t-test, $p = 0.009$). One way analysis of variance was used to detect significant differences between the exposures for each control or contaminated treatment. For control soil, significant differences were observed for all groups with the exception for the 3rd and 4th samplings (ANOVA, Holm-Sidak method, $p < 0.005$). In contaminated soil, no differences in OM breakdown were found only between samples from the 2nd and 4th exposure; all other conditions were significantly different (ANOVA, Holm-Sidak method, $p < 0.005$). Unlike feeding activity the OM decomposition followed the same response pattern seen for the overall community (Figure 14).

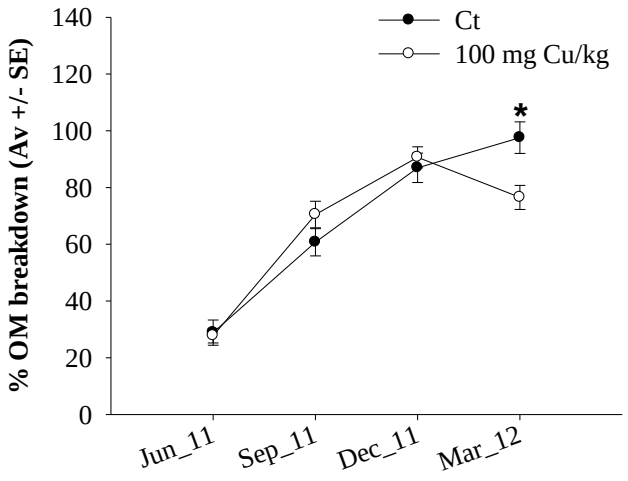

Figure 14 – Results of the litterbag studies as performed in the field experiment, using control and Cu contaminated (100 mg Cu/kg) soil from UAV, Portugal. The graph represents the loss of litter weight, determined using litterbags, at the end of each exposure duration.

4. Discussion

Effects of copper on species composition

For both UAV and HYG fields, in control conditions, the collembola community increased with time from the first to the second exposure (spring and summer conditions in UAV and spring conditions in HYG) and then decreased until the end of the experiments (autumn – winter in UAV and summer in HYG). Under Cu-contamination, overall collembola community also increased with time but abundance was never higher than found at control conditions. The largest difference seen between control and copper samples was immediately after spring (2^{nd} sampling time) for both fields (Figure 7).

Species reacted differently to copper contamination, some species were less occurring in contaminated soil, while other species were present at high abundances: the general decrease in abundance with Cu exposure is in line with several studies (Pedersen et al. 1999, Filser et al. 2000, Holmstrup et al. 2007).

Of the species contributing for the variation on data over time the most tolerant were *M. betschi* and *B. parvula* at UAV, which were not very abundant, but showed positive increase over samplings, and *P. fimata* and *P. notabilis* at HYG, especially *P. fimata* which showed higher abundance under Cu pollution when compared to control.

The results observed here for the euedaphic species (*M. betschi* and *P. fimata*) corroborates with previous finds for *M. macrochaeta*, a species close related to *M. betschi*, in laboratorial multi-species experiments performed with both soils (Menezes-Oliveira et. al., submitted). (Filser et al. 2000) pointed out some morphological characteristics such as complexes post antennal organs and pseudocellis - existing in *M. macrochaeta, M. betschi and P. fimata* - to have an advantage for survival in contaminated environments and may contribute to their general lower sensitivity.

The species slightly favoured by copper were *Sphaeridia spp.* in both soils as well as *C. succinea* at HYG. *Ceratophysella denticulata*, which was also present in our study and very high correlated to *C. succinea* in contaminated samples, were found to be metal tolerant by (Fountain and Hopkin 2004) and (Cole et al. 2001). The most sensitive species were *Heteromurus spp*., the juveniles of Entomobridae and *N. murinus* at UAV and *F.*

manolachei/quadrioculata, *I. minor* and *N. murinus* at HYG. The sensitivity of the species *I. minor* and *F. manolachei/quadrioculata* to Cu at HYG corroborates with previous finds by (Holmstrup et al. 2007) and (Pedersen et al. 1999).

Total abundance of enchytraeids, which was only assessed for HYG soil, decreased with time for both control and contaminated (1000 mg Cu/kg dw soil) systems. The decrease in numbers of enchytraeids was more pronounced at control conditions, which showed 3-fold more abundance in the 1st sampling when compared to the contaminated site but did not differ significantly in the last two samplings (Summer conditions). Results are in line with previous observations (Maraldo et al. 2006a) where the authors found the same inhibition in the enchytraeids abundance when exposed to Cu pollution besides a reduction due to summer drought. (Maraldo et al. 2006a) further discussed that when animals were allowed to recover from summer drought it did not confer any long-term results, neither for control nor for the contaminated site. In this work, a small increase in the enchytraeid abundance with decrease in field temperature could be observed in the last sampling but more samplings would be necessary to properly address this last find.

Effects of copper on species composition over time – Comparisons among the two experimental soils

The overall community for both experiments was compared by means of the thermal time concept (Trudgill et al. 2005) and it was observed that the heat needed to increase, by its maximum, the population in HYG was smaller than the required in UAV and the highest abundance in HYG was 10-fold higher than the maximum abundance seen for UAV in the end of the first exposure. These differences were at first thought to be related with the variation in the systems exposure i.e. UAV experiment was a semi-field approach where soil and animals were manipulated in the laboratory while the HYG was an undisturbed soil. Nevertheless, looking at the previous multispecies experiment performed in the laboratory with both soils and comparing the results, in terms of collembola abundance per square meter, for animals exposed to 14 and 23°C (which is the approximated annual difference in the mean temperature between the two soils – figure 2) at UAV and HYG respectively, a similar trend was observed, maximum overall population was more than 10-fold lower at UAV than at HYG (Figure S2).

The differences in collembola total abundance between both UAV and HYG soils are more likely to be related to the soil physicochemical parameters. The UAV soil has a lower pH and OM content when compared to the HYG soil and these characteristics are known to alter the Cu toxicity to invertebrate species such as *Folsomia candida* (Criel et al. 2008). Besides, even without contamination, the physicochemical characteristics of the soil affect the physiological development of invertebrate species. (Amorim et al. 2005b) found that two species of Enchytraeids were not able to survive in acid soils (i.e. $pH \leq 5$).

For both fields, under control or copper contaminated conditions, the entomobryomorpha group was generally the most abundant. The entomobryomorpha group, which in many studies is divided into Entomobridae and Isotomidae families, is normally reported as a very abundant group (Pedersen et al. 1999, Koolhaas et al. 2004, Sousa et al. 2004) and it may be due to its general easier mobility and, in case of the epiedaphic organisms, which are normally numerous in this group, their faster reaction to the changes in temperature (Hopkin 1997), which make them easier extractable by the conventional methods.

There were few common species for both sites but they were not very representative species in the analysis, with the exception for the epiedaphic species *Sphaeridia spp.* and the euedaphic species *N. murinus*, which are both symphypleona. *P. notabilis* was very abundant at HYG but rare at UAV, which was in line with (Holmstrup et al. 2007) and (Sousa et al. 2004) which found, respectively, a high abundance for *P. notabilis* at HYG and its rare presence in two fields in Portugal.

In Portugal, 2011 was registered as the warmest year since 1931 (Instituto de Meteorologia 2011). The five warmest months were April, October, May, June and September, respectively. The warmest months comprises the 1st to 2nd and 2nd to 3rd samplings in the UAV field and may indicate, by the overall increase in collembola abundance followed by a severe decrease, that a representative number of species may have reached their optimums heat during the 1st exposure and then exceeded it in the 2nd exposure. That is confirmed by the RDA analysis for each sampling date (Figure 8) where species richness increased considerably over time but 60% of the species were represented by only three species, for each exposure. This fact together with the Shannon index, which show an increase in the diversity of species over time, may indicate that there were few species

capable to live in constant heat conditions, but the species present were very abundant (Figure 10 and 11), and for the last two exposures, where temperatures have decreased considerably (Figure 2) the species abundance was lower but a higher biodiversity was observed.

Heteromurus spp was the only species fitted in the range of 60 to 100% in the PCA analysis in any exposure period at UAV. *Heteromurus spp* in this work correspond to only one species and although it has not been identified to the species level it is very similar to *Heteromurus major*, which has been shown to be very abundant in Mediterranean landscape in several soil types (Sousa et al. 2004).

Several factors may influence the distribution and abundance of collembola in the field over time but extreme warm and drought events has been reported as the most critical factors (Jucevica and Melecis 2006, Holmstrup et al. 2007, Xu et al. 2012). Although they are rarely occurring separately, and in this paper the humidity was only measured at the end of each exposure, no significant differences were seen in the % of relative humidity over time or between treatments for both soils. Moreover, the response pattern observed here with an increase in abundance in the growth phase followed by a severe decrease, besides the fact that collembola abundance was largely suppressed by Cu pollution in the growth phase, is in line with the multispecies experiments performed with both soils, controlled moisture conditions and different temperatures in the laboratory (Menezes-Oliveira et al. submitted).

Although the response of the common species have showed that abundance was generally higher at HYG and more sensitive to Cu when it was spiked in the soil (UAV), the comparisons between the effects of the community to the freshly or old contaminated soils cannot be strictly made since the exposure periods, and consequently the heat received by both experiments was very different. However, comparing the overlapping temperatures in the laboratorial multispecies experiments, an indication of a greater sensitivity to the UAV soil may be seen (total abundance in HYG was around 10-fold higher than at UAV).

Functional analysis

Due to practical reasons functional analysis were performed only in the UAV experiment.

The feeding activity seemed to be influenced by both sampling times and Cu exposure. The control samples showed an increase from the 1st to the 3rd sampling (spring to autumn exposure) followed by a decrease in the last exposure (winter) while in the Cu contaminated samples the activity did not differ significantly over time showing a slight bell shape with the highest percentage in the 2nd sampling time (summer). Activities differed significantly between Ct and Cu samples in the last two exposures. Litterbag results only showed a significant separation between Cu and control treatments in the last exposure. For both control and Cu spiked soils the OM turnover was greatly more influenced by the sampling times.

In general, feeding activity was not very well correlated with the community structure i.e. in comparison with the total abundance of individuals. This is in contrary to the observations made by (Filzek et al. 2004) which showed that the feeding activity corresponded with a reduction in the abundance of soil arthropods following exposure to metal contamination. However, although not positively correlated with the community structure, feeding activity showed to be slightly affected by Cu what may indicate that the microorganisms present played a role in the feeding activity (Römbke et al. 2002).

Unlike feeding activity the OM decomposition followed the same response pattern seen for the overall community, increased decomposition with increasing collembola abundance. The positive correlation between soil mesofauna abundance and OM decomposition indicates the association between soil biota and ecosystem services (e.g. food supply), as also referred by other authors, e.g. (Lavelle et al. 2006). Invertebrates contribute directly or indirectly to the OM decomposition process (Römbke et al. 2002).

5. Conclusions

Copper and heat input largely influenced the community composition, especially in the growth phase where overall abundance was severely decreased by copper while in control

conditions the maximum amount of animals was observed. The higher total abundance at HYG, which was exposed to a much lower heat, was 10-fold greater than at UAV in control conditions showing that the high heat lead the species to faster exceed their optimums.

Monitoring experiments over the whole year are very important since the communities change over time and so the species interactions and sensitivities are differently altering the response to the stressors. Together with the different types of interaction between the species, differences in soil pH and OM content, were also very important for the changes in community seen between the two sites. Most of the common species were more abundant in HYG than in UAV and this was also confirmed with the multispecies experiment performed in the laboratory where species in control soil exposed to the same temperatures in both soils presented a 10-fold lower reproduction at UAV when compared to HYG.

Acknowledgements

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Supplementary information

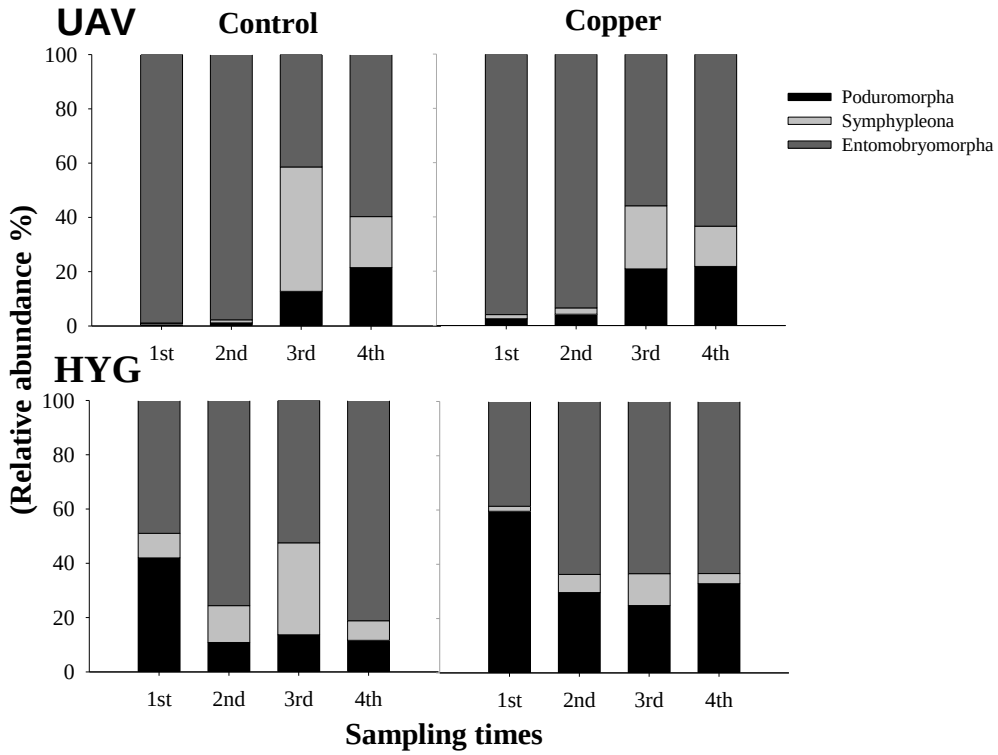

Figure S1 – Community taxonomic profile (Relative percentage of the different groups) from each soil (UAV and HYG) in the four different sampling times.

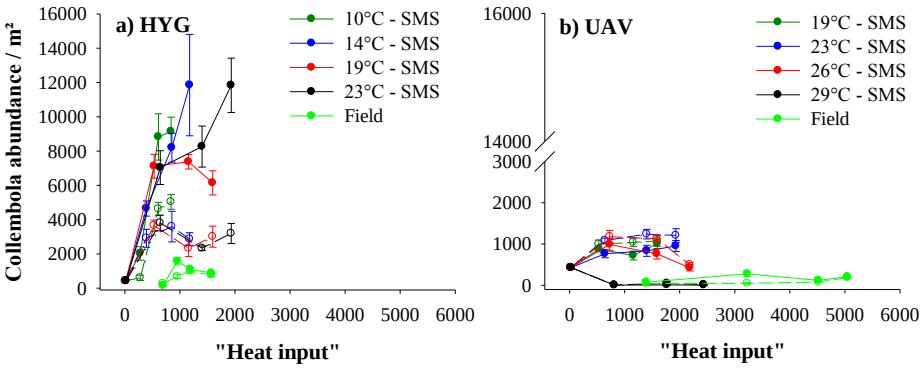

Figure S2 – Collembola abundance as observed in the field and in the laboratory experiments – Soil Multispecies Systems (SMS) (Menezes-Oliveira et al., submitted). The light green lines represent the results of the field experiments while the other colours represent different temperature exposures in the SMS tests. In the left side is the graph from HYG soil and in the right side the graph from UAV soil.

Chapter VII – Thesis Overview and Final Remarks

Thesis overview and final remarks

There is continuously concern regarding the consequences of the release of materials to the terrestrial environment and their potential impact on soil function and structure. One such material is Cu, widely used in several important processes (e.g. electronic devices, timber preservation, wires and plumbing). Wide spread used has caused that Cu may be found in higher concentrations (in certain areas) than required by the soil organisms. The effects caused by Cu to the soil inhabitants have been studied (i) by far most of the studies deal with fixed density single-species experiments (population studies) (ii) less is known about how such pollution affects species when interacting with other species in standard conditions (community studies), and (iii) finally almost nothing is known on how pollution affect species in a community when temperatures changes. The latter is relevant in the current global warming perspective.

In this thesis we intended to explore the effects of Cu to the soil structure and function, with focus on interaction with temperature changes. The structure was studied via the soil community composition and the soil function was studied via the OM turnover and feeding activity. To achieve these goals several experiments were conducted which aimed at determining:

1) The effects of **Cu contamination** to the reproduction of the enchytraeid species ***Enchytraeus crypticus*** and the earthworm species ***Eisenia fetida*** when these were exposed to different **density** conditions (Chapter II);
2) The effects of **Cu contamination** to the reproduction of the enchytraeid species ***Enchytraeus crypticus*** when exposed to different **density** conditions over **two generations** (Chapter III);
3) Soil **function** (OM turnover and feeding activity) and **structure** (mesofauna community) responses to a **historically Cu contaminated** soil from Hygum, **Denmark**, under different conditions of **temperature** (Chapter IV);

4) Soil function (OM turnover and feeding activity) and structure (mesofauna community) responses to a **newly Cu spiked** soil from Aveiro, **Portugal**, under different conditions of temperature (Chapter V).
5) Biological structure (mesofauna community responses) and function (OM turnover and feeding activity) to the same historically Cu contaminated and the freshly spiked soil from Denmark and Portugal, respectively, under **field conditions** over **seasons** (Chapter VI).

The ecotoxicological bioassays using different densities of the enchytraeid *Enchytraeus crypticus* and the earthworm *Eisenia fetida* (Chapter II) allowed the observation of important trends in the response of the oligochaeta species tested, especially for the *E. crypticus*, when exposed to Cu pollution. The different densities and its interaction with Cu pollution did not cause a significant effect in the final *E. crypticus* populations of both organisms tested. Although the Cu concentrations in general negatively impacted the total population, the impact had a tendency to be lower at the highest and lowest densities (5 and 50 animals). The earthworm *E. fetida*, was not affected by the Cu concentrations used in terms of total population number. For each Cu concentration tested significant differences in weight across densities were observed, but no interaction was observed. The observed response trend for the enchytraeids combined with previous observation on interactions, spurred further density studies (Cecchine and Snell 1999, Moe et al. 2002, Hooper et al. 2003, Gui and Grant 2008, Lahr et al. 2008).

The experiment performed in chapter II was repeated in a different soil using *E. crypticus* (Chapter III). The latter was further extended to include a density experiment also with the juveniles. In chapter III, the results of the first generation response was slightly different from the results observed on chapter II in the sense that, besides the Cu exposure, organisms were significantly affected by the densities and by its interaction with Cu exposure. Nevertheless, the effects of Cu across densities showed all broad overlapping confidence intervals, which is in line with Menezes-Oliveira et al. (2011). In the second generation, density dependence was also observed. In addition, high start densities in the first generation lead to more sensitive group of organisms in the 2nd generation, especially those secondly exposed to lower densities. However, this pattern was only observed for the

EC_{10} and EC_{20} and not for the EC_{50} (higher sensitivity at lower concentrations). Organisms exposed to high densities in both generations were not affected by Cu in the second generation although the reproduction was around 50% lower for all Cu concentrations (inclusive control) when compared to organisms exposed to standard density in the 2nd generation. The lower sensitivity under high densities may have been caused by the organisms' behaviour of avoiding exposure to Cu by lumping instead of being evenly distributed in the soil. Lumping could promote more frequent meetings (between organisms), while keeping the exposure to a minimum, or even that the Cu contaminated soil was less available at higher densities due to the large amount of organisms per gram of soil.

It is also important to note that Cu is an essential metal which organisms are capable of regulating, actively accumulating at low doses and excreting at high doses (McGeer et al. 2003), whether this explanation accounts for the enchytraeids we don't know exactly but the mechanisms has been already observed for the earthworms (Morgan and Morgan 1990, Marinussen et al. 1997, Salminen and Haimi 2001). Hence, this may either cause the organisms to be little affected or maybe the energy expenditure to regulate metals uptake/excretion may be affected by temperature changes.

Results found in the chapters II and III neither confirmed nor disproved the possible density dependence toxicity for Cu pollution. More studies are needed, which should include both more generations and metal analysis in order to verify whether the organisms are less exposed to Cu when in high densities or whether the lower sensitivity is a density effect. These density results indicate that in multispecies test with Cu, the initial density is not of major importance for the direct toxicity on the enchytraeids.

As mentioned, temperature changes may affect the community structure and function, which may be different depending on various other factors e.g. soil types or pollution.

Therefore, two experiments were performed (SMS experiments – Chapters IV and V). Experiments were conducted using soil from two different sites (Portugal and Denmark). The two laboratory **Soil Multispecies test Systems (SMS)** included six species, all

cultured in the laboratory, and representative of different functional groups. Microbial activity was also ensured. OM decomposition and soil fauna feeding activities were also assessed. Four different temperatures in the range of 10 to 29°C were chosen for each experiment, based on the mean annual values of the temperature in Denmark and/or Portugal. The exposure covered three sampling periods (28 to 84 days). The Cu effect was assessed via a field contaminated soil (DK) and a field spiked (PT).

Results of the SMS experiments showed that increased temperature caused major changes in the abundance of the individual species exposed to both control and Cu contaminated soils. In DK, the abundance of the individual species was always lower under Cu contamination for all temperatures. In PT, the individual species showed very distinct responses to Cu exposure and the effects were smaller than the predicted with the single tests performed within the same soil. Hence, there was an indication that the species interaction played a role on the Cu toxicity in this system.

The higher temperatures (19-29ºC) tested in the PT experiment severely affected community, which was generally extinct at 29ºC, and longer exposure periods at 26ºC produced similar effects. Hence the period of exposure is as important as the actual soil temperature. For both experiments, most of the community changes were observed in the first 28 days of exposure (also corresponding to the population growth phase), after which, at stable phases, changes in populations dynamics will be more reflecting actual species interactions and matured ecosystem status being both less flexible to changes and better fitted to the exposure scenarios (Cu and temperature).

The soil biological structure and function were also assessed under field conditions (Chapter VI). Collembola communities were monitored and identified to the species level when possible. Four samplings were performed in each soil. In DK, sampling times were chosen based on the thermal time concept, in comparison to the SMS experiment with DK soil. In PT, it was not relevant to use the thermal time as a deciding factor for samples (due to high heat input) here samplings corresponded to the different seasons from March, 2011 to March 2012.The same Cu concentrations and type of contamination were used for both field and laboratory experiments.

In the field, the overall community changes with time (and temperature) and due to Cu exposure was also confirmed. Similarly, major changes occurred during the growth phase (corresponding to the late spring exposure).

It was also clear in both multispecies experiments that the temperature related population changes differed between species, which is in different optimum temperatures (Jansch et al. (2005). Further, for the individual species, the populations did not change monotonic with temperature. This is in contrast to observations in single species tests, e.g. Martikainen and Rantalainen (1999) where the rise in temperature up to 19°C caused an increase in *F. candida* population. The authors Martikainen and Rantalainen (1999) have discussed that, under control conditions, temperatures below the species optimum would result in population increase in all time points, which did not occur for all species in our experiments.

These "discrepancies" in the individual species responses, together with the differences in species composition over time for both field and SMS experiments indicate that the species interaction must be playing a role. Besides the species interactions a soil issue may have occurred. For both field (Chapter VI) and SMS experiments (Chapters IV and V) the overall collembola community was around10-fold higher in DK than in PT, even when comparing the same temperatures in the SMSs. This difference can be explained by differences in optimums for soil physicochemical parameters, such as pH and OM, or by different food availabilities.

The soil functional assessments for the SMS experiments demonstrated that the increase in OM decomposition and feeding activity responses at low temperature were associated with the increase in species abundance whereas at high temperatures this was not so clear, with a decrease in feeding activity and species abundance but increase in OM decomposition. In the Portuguese field experiment (Chapter VI) the same patterns were observed for the OM decomposition and feeding activity when compared to the laboratory experiment (Chapter V). More experiments assessing the combination of soil function and structure under pollution are needed to better understand their relation to different kinds of environments.

Laboratory mesocosm experiments were proved to satisfactory demonstrate ecologically relevant changes in communities for both control and contaminated soils, especially for DK. In the case of PT, overall community was more affected by Cu under field conditions. The use of a higher concentration could had further elucidated on the combined effect of the two, but that would mean that species community would had been more affected by mortality and results would include less species interaction possibilities.

Regarding the differences between the effects of the community to the freshly or old contaminated soils, it could not be straight answered within this work. In the field experiment overall community was greatly affected at PT than at DK while in the lab the opposite occurred.

Such complexity level studies as conducted with the SMS experiments are often requested for (also by Governmental Risk Assessors), but seldom provided as they are here and there is no such study using many temperatures to our knowledge. As shown, more complex approaches (i.e. experiments employing more generations or multispecies) has several merits e.g. being closer to "ecosystems", but as also shown it also provides more complex answers that may require a weight-of-evidence interpretation of the actual outcome.

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