# Effects of Different Temperatures and Exposure Times on the Survival and DNA Damage of Collembolans (Springtails) in Response to Esfenvalerate (Sumi-Alpha) Soil Exposure

**Kun Chen**

**(Master's Study in Ecotoxicology)**

**University of Oslo**

**Department of Biosciences**

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***Abstract***

There are multiple environmental factors that can affect the toxicity of a chemical to an organism in its natural environment. These include temperature, humidity, predation, competition, diseases, and presence of other chemicals. Due to this, extrapolation from laboratory to field conditions in a risk assessment is challenging. While low concentrations of a chemical may cause significant sub-lethal effect, such as DNA damage, which are not visible at the individual performance level, higher concentrations may affect endpoints of direct ecological relevance such as survival and reproduction. By including more than one endpoint in a toxicity study, the different levels of responses of the organisms can also be compared.

Collembolans lives worldwide in the upper layer of soil, they have small body size and a short life cycle. They are generally considered sensitive to chemical changes in the soil, which makes them suitable as test organisms in toxicity studies. Collembola constitute a diverse group when it comes to living environment, taxonomy and physiology, and these differences may manifest as differences in sensitivity to chemicals between species.

To add insight into how temperature and exposure time could affect the toxicity of a commonly used pesticide to collembolans, a series of experiments were performed in which two species of collembola (*Folsomia quadrioculata* and *Onychiurus* sp. separately exposed in soil) were investigated for lethal and sub-lethal responses to the chemical esfenvalerate. Both temperature and exposure time is especially relevant to risk assessment of pesticides used in Norway as soil temperatures here are much lower than that used in standard laboratory tests and most pesticides degrade more slowly at low temperatures (with longer exposure as a result).

Toxicity of a fixed concentration series of esfenvalerate in the form of the commercial product "Sumi-Alpha" was investigated for both collembola species at three different temperatures (5℃, 10℃, and 20℃) for short-term exposure (1 week) and at 20℃ for long-term (5 weeks) exposure. From the short-term exposure experiments, survival was assessed for both species and DNA damage in the *Onychiurus* sp. In long-term experiments, survival and reproduction were measured for both species, so did the DNA damage for *Onychiurus* sp.

Effects on survival (LC50) in short-term studies ranged from 44 to 50 mg/kg soil for *F. quadrioculata* and 53 to 54 mg/kg soil for *Onychiurus* sp. In long-term studies, LC50-values were lower; 30 mg/kg soil for *F. quadrioculata* and 30 mg/kg soil for

*Onychiurus* sp. In long-term experiments, reproduction was insignificantly affected for both species already at the lowest exposure concentration (20 mg/kg soil for *F. quadrioculata* and 25 mg/kg soil for *Onychiurus* sp). DNA damage between different exposure durations on *Onychiurus* sp. was significant ($P<0.001$), and the estimated 50% effect concentration was 30 mg/kg.

There was only a small difference in sensitivity between the two species, for all endpoints and exposure times. LC50 values were lower at long-term exposure compared to short-term exposure for both species (*F. quadrioculata*: P=0.0867, n=4; *Onychiurus* sp.: P=0.0001, n=4).

From the results of this study, exposure temperatures in the range of 5-20 ℃ and exposure times in the range of 1 to 5 weeks do not seem to have a very large influence on the toxicity of esfenvalerate to soil-dwelling collembolans.

## 1. Introduction

In the modern world, chemical pesticides are widely used in agriculture to protect crops and vegetables. Although pesticides are subject to extensive ecotoxicological testing (biodegradability, bioaccumulation and toxicity to aquatic and terrestrial organisms) prior to approval of use, the environmental fate of these pesticides is only partly understood, and sometimes they cause unintended effects. Following their application in agricultural areas, the highest concentrations of pesticides will occur within the crop (on plant material and in the soil underneath), but many chemicals are mobile and can be transported with air or drainage water and potentially expose non-target organisms further away. Effects on the community of organisms inhabiting the crop soil may negatively affect the function of the soil as a growth medium, and effects on non-target soil dwelling organisms is therefore one of the focused areas for assessing pesticide impact.

Chemicals may cause several types of damage to animals. While high concentrations typically cause reduced survival and reproduction of sensitive species, lower concentrations of chemicals may cause sub-lethal effects such as DNA damage. In the environmental risk assessment of pesticides, information on their use pattern is combined with fate parameters and effect studies in order to characterize their expected impact. Local conditions such as temperature, rainfall/humidity, soil type, and topography may significantly influence on degradation rates and chemical migration patterns.

Norway typically has lower average soil temperatures than what is used in standard laboratory testing. Lower temperature typically results in a slower degradation of the chemical, and this effect may be accounted for with a standard approach in fate modeling. However, the net effect of temperature on the toxicity of chemicals is less understood; temperature may affect up-take, metabolism and excretion, as well as the general sensitivity of the organism.

The current study aimed at increasing the understanding of how temperature affects the toxicity of pesticides in soil, and included a range of experiments to characterize the effect of temperature on survival and sub-lethal responses in two species of collembolans (springtails); the small colored species *Folsomia quadrioculata* and the larger, white species *Onychiurus* sp. The pesticide esfenvalerate, in the form of the commercial product Sumi-Alpha, was chosen as a model substance. The original plan of the study included two pesticides and two types of soil – farm soil and forest soil.

However, due to unexpected experimental and methodological challenges – and very limited opportunities to do things over (slow reproduction in springtail cultures and the mere time it takes to run experiments) – the contents of the study was reduced to one pesticide and one soil type.

The experiments included both short-term (1 week) and longer-term (5 weeks) exposure. In longer-term exposures, survival and reproduction was assessed, in addition to sub-lethal effects. I investigated two different measures of sub-lethal effect, DNA damage and membrane stability of hemocytes (lysosomes).

In a pilot experiment, it became clear that it would be very difficult to extract hemolymph from *Folsomia quadrioculata*. Consequently, analysis of DNA damage and membrane stability was only performed using *Onychiurus.* For *F. quadrioculata*, endpoints measured were survival and reproduction following long-term exposure.

### 1.1. Test animals: Collembolans (Springtails)

Springtails are no longer classified as insects, but they belong to the hexapod group of arthropods. They have internal mouthparts, six or fewer abdominal segments, and a special structure called furcula, which is a tail-like appendage under the retinaculum structure on the last body segment. The furcula is used to jump when the animal is threatened. Some species have lost their jumping organ altogether (Hopkin, 1997).

Collembolans are small animals mostly a few millimeters long. They are abundant and live worldwide, from equatorial (Maunsell *et al.*, 2013) to Polar Regions (Krab *et al.*, 2013). Most of them prefer a moist living environment and are sensitive to desiccation. They can be found in the upper layer of soil, rotten woods, grass tufts, and fallen leaves. Springtails are omnivorous, with a diet typically containing fungal hyphae (Hopkin, 1997), bacteria (Haubert *et al.*, 2006; Chamberlain *et al.*, 2006), or decaying organisms (Chamberlain *et al.*, 2006). Most of them are harmless, only a few species are pests of food crops.

Collembolans generally have early breeding, and a short life cycle. As a group, they can live no more than a year (Hopkin, 1997). Because of the short breeding time, small body size, abundance and sensitivity, collembolans are frequently used in the ecotoxicology research – for instance, to investigate the toxicity of organic chemicals (Domene *et al.*, 2008), heavy metals (Meli *et al.*, 2013), as well as in studies of other environmental stressors (Holmstrup *et al.*, 2008). *Folsomia candida* is one of the most comprehensively studied and widely used Collembola species in ecotoxicology. *F. candida* is significantly larger than *F. quadrioculata*. It is even larger than *Onychiurus*.

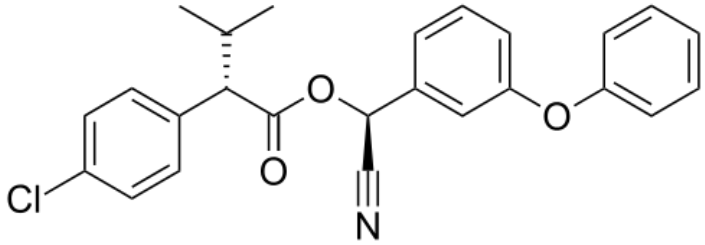
However, *F. candida* and *F. quadrioculata* both belong to the family *Isotomidae*, while *Onychiurus* belongs to *Onychiuridae*.

The Onychiuridae lack pigments and are blind. They also lack the furcula, and are thus unable to jump (Hopkin, 1997). The adult is around 1.5 mm long. They live in the soil for most of their lives, or under stones or wood on the surface of the ground. *F. quadrioculata* is smaller than *Onychiurus* sp. and their pigments give them a gray color. *F. quadrioculata* lives closer to the surface of soil than *Onychiurus* sp. (Hopkin, 1997[fig.9.4 page165]). They have furcula and typically move faster. Because of the differences in living environment, taxonomy and physiology, these two species may differ in their sensitivity to chemicals.

### 1.2. Test substance: Esfenvalerate

For the current study, a pesticide, esfenvalerate, was chosen as the model toxicant.

The chemical name of esfenvalerate is (S)-cyano (3-phenoxyphenyl) methyl-(S)-4-chloro-alpha-(1-methylethyl) benzeneacetate.




It is a synthetic pyrethroid insecticide (Fishel, 2014), which has toxic effects on a wide range of insects, like beetles and flies. It is widely used in agriculture to protect crops, vegetables and fruit trees.

In its pure form, esfenvalerate is a white crystalline solid, which has a melting point at 23 ℃. It has a low vapor pressure (0.067 mPa at 25 ℃), is stable in acid environments, but will decompose in basic solutions. Laskowski (2002) summarized the physical and chemical properties of pyrethroids in the book 'Reviews of Environmental Contamination and Toxicology', and characterizes esfenvalerate as having low water solubility, low volatility, and a high affinity to soil and sediment particles.

In the risk assessment of esfenvalerate by the Norwegian Scientific Committee for Food Safety (Sverdrup *et al.*, 2009), it was concluded that esfenvalerate had a strong sorption to soils. A typical degradation pattern was a rapid initial decomposition followed by a much slower decrease in soil concentrations. The hydrolysis of

esfenvalerate was considered to be low. Photo degradation was indicated as possibly being an important degradation pathway in both water and soil (Norwegian Scientific Committee for Food Safety, 2009).

In studies by Garey and Wolff (1998) and Go (1999), pyrethroid chemicals are reported to have hormonal activities and interactions on endocrine systems of animals. Compared to many other pyrethroid pesticides, esfenvalerate is considered less harmful to the environment (Adelsbach, 2003). From the 'Pesticide Toxicity Profile' Frederick M. Fishel (2014), esfenvalerate shows a high toxicity to mammals, to bees, and to fish. It also shows toxic to the springtail *Folsomia fimetaria*, which is in the same family as *F. quadrioculata* (Schnug, *et al*. 2014).

### 1.3. Exposure Time

Exposure time is the amount of time the organisms will be exposed to the toxicant. A short-term exposure time will show a quick response of animals to the toxicants or environmental stressor (Osler *et al.*, 2001). Modern pesticides (including esfenvalerate) typically degrade rapidly, resulting in a rapid decline of initial concentrations in the sprayed area. Long-term exposure can provide insight in the toxic effect of a chemical or stressor after prolonged time, giving experimental animals more time to adapt and potentially recover (Waagner, *et al.*, 2010), and in springtail it also allows for studying the effect on reproduction (Broerse et al., 2010; Meli et al., 2013).

In the current study, animals were collected from the short-term exposures (1 week) to look at survival rates in both species of springtails, and for the Onychiurus sp., the Comet Assay was used to analyze DNA damage in surviving individuals. The long-term exposure (5 weeks) was used to measure effects on the reproduction of the springtails.

### 1.4. Exposure Temperatures

Collembolans are sensitive to temperature change. Temperature will affect not only activity, reproduction speed and rate of collembolans, but also the toxicity of chemicals in the animal (Martikainen *et al.*, 1999; Everatt *et al.*, 2013). However, adult growth does not seem to be related to temperature change (Martikainen *et al.*, 1999). As collembolans are poikilothermic, temperature has significant effects on its adaptation to toxicants in general, and particularly absorption and metabolism *in vivo*. The net effect of differences in exposure temperatures is complicated by the many

factors involved, and thus difficult to predict. For example, exposure to mercury has been shown to reduce both heat tolerance (Slotsbo *et al.*, 2009) and cold tolerance (Holmstrup *et al.*, 2008) in *Folsomia candida*. A study by Martikainen and Rantalainen (1999) showed a relationship between experimental temperatures and exposure time to the chemical dimethoate, as well as correlations with adult growth and reproduction. However, in a different study, Sjursen and Holmstrup (2004) showed that when the concentration of pyrene was low, the survivals of the springtail – *Protaphorura armata* remained the same at all temperatures.

In the current study, the effects of esfenvalerate at three different temperatures (5 ℃, 10 ℃ and 20 ℃) in short-term exposure for both springtail species were tested to investigate potential differences in toxicity as a result of exposure temperature. For the long-term experiments, only the 20 ℃ exposure temperature was used.

### 1.5. Cell Damage – sub-lethal endpoints

Toxicants can cause many kinds of damage in animals, including damage to cellular structures such as DNA and the phospholipid membranes. DNA damage is a normal process inside the cell, which is continuously compensated by the repair systems of the cell. Toxicants can increase the mutation rate of the genes, which may cause repair function overload, and thus increase the possibility of irreversible changes to the DNA.

Membranes of the cell include the cell membrane, nuclear membrane, membranes of the endoplasmic reticulum, Golgi apparatus, mitochondria, and lysosomes membrane. Cellular organelles are closely integrated as a whole in structure and function and play an extremely important role in the living activity of cells. Toxicants may harm membranes either by connecting to or damaging functional structures of the membrane. When the membrane is damaged and become unstable, the materials and cytosols from different cellular organelles may mix together, resulting in decreased function or loss of function, as well as damage to cell organelles. Membrane stability is thus an indication of cellular health. To analyze potential sub-lethal effects on DNA, I used the Comet Assay. Membrane stability was assessed using the Neutral Red Assay. The two methods are described below.

#### 1.5.1. The Comet Assay

In the Oxford Dictionary of Biomedicine (Lackie, 2010), the Comet Assay is defined as ‘A sensitive method (single cell gel (SCG) electrophoresis) to examine

DNA damage and repair at individual cell level.

In the Comet Assay, single cells are embedded in agarose on a microscope slide. They are lysed and then electrophoresed *in situ*. The slide is then stained with fluorescent dye (e.g. acridine orange) and examined under the microscope. When present in an electric field, damaged DNA fragments will move faster to the anode than undamaged DNA. Intact DNA remains in the cell, as the comet's 'head', while DNA-fragments of different sizes from the 'tail'.

In the present project, the comet assay was used to characterize DNA damage in collembolans. It is a simple and effective testing method, which has previously been used in investigating DNA damage, cell apoptosis, genetic toxicology, radiation injury, risk factors and biomarker identifications (Tice *et al.*, 2000; Speit and Hartmann, 2006; Collins, 2009).

It can also test the DNA damage caused by different pollutants (Zhang *et al.*, 2011). The Comet Assay generally shows good sensitivity to all kinds of DNA damage caused by toxicants. In a study of Ahmed *et al.* (2011), Comet Assay is proved to be a good method to test for genotoxicity at a range of exposed concentrations and exposure times. The Comet Assay has been successfully used in many kinds of animals, like fish (Kammann *et al.*, 2004), rodents (Zhang *et al.*, 2011), humans (Wentzel *et al.*, 2010), mussels (Wilson, *et al.* 1998), and earthworms (Reinecke *et al.*, 2004). There is, however, limited experience with collembolans.

The hemocytes of springtails are very small and the amount of cells is limited due to the small body size. Still the Comet Assay can show us an overview of the DNA damage in the whole cell, which includes all kinds of damage to the DNA strand. In the Comet Assay procedure, the density of the tail relative to the core can be used to compare the damage level of cells. The aim of my study was to compare the different levels of total DNA damage as result of esfenvalerate exposure at different temperatures and between species.

### 1.5.2. The Neutral Red Assay

This assay is used to test the membrane stability of lysosomes. Lysosomes contain many types of acid hydrolases that can decompose useless materials in the cell. If the stability of lysosomal membranes is decreased by toxicants, lysosome might break, and cause cell death. Neutral Red is an alkaline phenazine dye for *in vivo* staining and acid-base indicator. It is dark red powder in its pure form. When dissolved, the pH of the solution will affect the color of dissolved dye, resulting in a color ranging from

red (pH 6.8) to yellow (pH 8.0). In a normal cell, the pH inside and outside lysosomes are different. The pH of plasma inside the lysosome is lower than in the surrounding cytoplasm. When the dye is added to a solution with cells, viable cells will take up the dye, displaying lysosomes in red. Damaged or dead cells will not take up the dye, and their lysosomes will be in lighter color. Therefore, membrane stability can be a suitable method to measure toxicity (Lowe, et al. 1995) and effect of stressors from nature and human activities (Ringwood, et al. 1998).

The Neutral Red Assay has been demonstrated useful in many kinds of animals, such as earthworms (Weeks *et al.*, 1996), kidney tissues of fish (Holth *et al.*, 2011), and mussels (Nesto *et al.*, 2007). The study of Holth indicates there is no difference on membrane stability between genders, and it was responsive at low test concentrations. Membrane damage can be caused by a variety of pollutants, including trace metals, PAHs, and PCBs (Nesto *et al.*, 2007).

Unfortunately, there was no former research on lysosome membrane stability of collembolans, and proper methods for extractions, cell handling and analysis was missing. Few people work on cells of this animal (Ksiazkiewicz *et al.*, 1979). Among the methodological challenges for this part of the project, the small size of collembolans made it very time-consuming to obtain sufficient amounts of biological material. Furthermore, the small size of Collembola hemocytes also made handling and analysis hard for the Neutral Red Assay. Even though significant efforts were put into the Neutral Red Assay, results from the sub-lethal toxicity as measured by lysosome membrane stability measurements were therefore very limited compared to the original scope of the project.

### 1.6. Aims

Based on the background information of species, temperature effects, and effects of exposure time, the main aims and hypotheses of my study were as follows:

- Is there difference in sensitivity between *Onychiurus* sp. and *Folsomia quadrioculata* for the effect of esfenvalerate?
  - For short-term exposure (1 week), there was no difference in effects of esfenvalerate on survival and DNA damage between the two species at any of the three exposure temperatures.
  - For long-term exposure (5 weeks), there was no difference in effects of esfenvalerate on the survival and reproduction between the two species at

20℃.

- Will the sub-lethal effects (dose-response curve for DNA damage) vary between temperatures (5/10/20℃) for the collembolans?

  - There is no difference between temperatures of 5, 10 and 20 ℃ in toxicity (both survival and DNA damage results) of esfenvalerate after 1 week exposure for the *Onychiurus* sp. species.

  - There is no difference between temperatures of 5, 10, and 20 ℃ in toxicity (survivals) of esfenvalerate after 1 week exposure of *Folsomia quadrioculata*.

- Is there a difference in effects on survival between the short-term (1 week) and long-term (5 weeks) exposure of *Onychiurus* sp. and *Folsomia quadrioculata* to esfenvalerate?

  - There is no difference in effects on survival between exposure times – 1 week and 5 weeks -- of esfenvalerate on *Onychiurus* sp. at a temperature of 20℃.

  - There is no difference in effects on survival between exposure times – 1 week and 5 weeks -- of esfenvalerate on *F. quadrioculata* at a temperature of 20℃.

## 2. Materials and methods

### 2.1. Collembolans

The collembolans in my experiments, *Folsomia quadrioculata* and *Onychiurus*, were provided by professor Hans Petter Leinaas, Department of Biosciences, University of Oslo, Norway.

The cultures of springtails were kept in cylinder boxes (r=1.5 cm, h=5 cm). The fixed material in the bottom of the boxes was made of plaster and active carbon powder, and ratio of these two materials was around 9:1. After being mixed with distilled water, the slurry was separated into boxes to provide, each box with a 0.5-1cm layer. The slurry was left to dry for at least 8 hours, and the boxes could then be used as cultivation containers for collembolans. Food for springtails was provided in the form of small pieces of dried bark, which were covered with a layer of fungi. All the cultivation boxes were checked twice per week, and new bark and a few drops of distilled water was occasionally added, to make sure the animals had a moisture and clean environment to live.

All cultures were kept in incubators. *Onychiurus* cultures were kept at 20±1 ℃, while *F. quadrioculata* cultures were kept at 15±1℃.

### 2.2. Preparation of Soil

In my experiment, I used an agricultural soil type collected by Dr. Line Sverdrup from a farm in Ås, Norway.

The original soil was put in a drying machine at about 50℃ for 7 days. Then, it was sieved using a 2 mm sieve to remove stones and large materials. At last, it was stored in a large plastic container with a cover at about 20℃ in the laboratory.

To prepare test concentrations of esfenvalerate, 50 g of dry soil was placed in each of four 300 ml glass beakers. In order to obtain a final soil concentration of 100 mg/kg soil, 0.371 ml Sumi-Alpha (50 g/L) was mixed with $dH_2O$ to 50 ml in a volumetric flask (stock solution of chemical). A three times 1:2 dilution series of Sumi-Alpha (esfenvalerate) was made in 50ml volumetric flasks from the stock solution and $dH_2O$. After shaking each flask to carefully mix content, 13.5 ml of the Sumi-Alpha solution was added to the corresponding beaker, and stirred to mix the liquid and soil. 13.5 ml to 50 g of soil was selected to provide optimal humidity for

collembolans during exposure. With the 1:2 dilution, test concentrations of 100, 50, 25 mg/kg test concentration series was obtained. For the control, 13.5 ml distilled water was added instead of Sumi-Alpha solution.

Different concentration series for experiments were prepared in the same way. For *F. quadrioculata*, the highest test concentration was 80 mg/kg (dry) soil. Thus, 0.297 ml of the original Sumi-Alpha liquid was added to the 50 ml stock solution.

Following addition of the Sumi-Alpha liquid/distilled water, beakers were cover with parafilm and put overnight to let the soil moist and the chemical to distribute evenly. The next day, soil from each exposure concentration was separated into 3 exposure boxes (r=25 mm, h=70 mm) as three replicates for each treatment level.

### 2.3. Chemicals

Pesticide:

Esfenvalerate (Sumi-Alpha 5 FW) with a content of active ingredient of 50 g/L. The product was produced by company DuPont Norge AS (Wæhli gård, 1592 Våler i Østfold).

Chemicals for solutions:

NaCl, $Na_2HPO_4$, $KH_2PO_4$, $Na_2EDTA•2H_2O$, NaOH, Trizma base, Triton X-100, HCl, Tris-HCl, and EDTA were all produced by Sigma-Aldrich.

Neutral Red Powder (Sigma-Aldrich),

LMP Agarose (Thermo Scientific),

SYBR Gold Nucleic Acid Gel Stain (Life Technologies).

### 2.4. Methods

#### 2.4.1 Pilot experiments

In order to find the suitable exposure concentration gradient, I did several pilot experiments. Test concentrations in the pilot experiments were as follows:

*Onychiurus* sp.: 0-2-10-50-250 mg/kg soil; 0-20-40-80-160 mg/kg soil; and 0-30-60-120 mg/kg soil for both long-term (1 week) and short-term (5 weeks)

exposures. These tests used the soil collected in 2013, by Line Sverdrup from a farm in Ås, Norway. Results of sub-lethal effects from these pilot experiments showed that the LC50 of Sumi-Alpha on *Onychiurus* in this farm soil was between 30-60 mg/kg soil. After change to the new farm soil Line collected in 2014 in Ås, which appeared slightly different from the first one, another pilot test was set up using test concentrations of 0-40-45-50 mg/kg soil (three replicates and 20 animals added per beaker). The results indicated a 50% lethal concentration (LC50) of about 50 mg/kg soil (Table 1).

*Folsomia quadrioculata*: After change to the new farm soil, both short-term and long-term exposure tests were set up also for *F. quadrioculata*, and results indicated a short-term LC50 of about 40 mg/kg (Table 2). Long-term exposure indicated an LC50 below the lowest tested concentration (40 mg/kg) and also a long-term 50% effect on reproduction below this concentration (Table 3).

***Table 1 Results of the pilot for Onychiurus sp. Exposure for 1 week at 20℃. three replicates per treatment level; 10 animals added per beaker.***

| *Onychiurus* sp. pilot study | Esfenvalerate concentrations (mg/kg soil) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 40 | | | 45 | | | 50 | | |
| Surviving adults (of 20) | 19 | 18 | 19 | 15 | 16 | 14 | 13 | 12 | 14 | 14 | 11 | 12 |

***Table 2 Results of the pilot test for short-term exposure of Folsomia quadrioculata. Exposure for 1 week at 20 ℃. Three replicates per treatment level; 20 animals added per beaker.***

| *F. quadrioculata* | Esfenvalerate concentrations (mg/kg soil) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 40 | | | 45 | | | 50 | | |
| Survival adults (of 20) | 20 | 17 | 18 | 10 | 13 | 8 | 11 | 7 | 9 | 10 | 6 | 7 |

***Table 3 Results of the pilot test for long-term exposure of Folsomia quadrioculata, measuring effects on survival and reproduction. Exposure for 5 weeks at 20℃. Three replicates per treatment level; 20 animals added per beaker.***

| *F. quadrioculata* | Esfenvalerate concentrations (mg/kg soil) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 40 | | | 45 | | | 50 | | |
| Survival adults (of 20) | 20 | 14 | 17 | 4 | 6 | 5 | 7 | 4 | 2 | 0 | 2 | 0 |
| Juvenile | 46 | 12 | 21 | 0 | eggs | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

### 2.4.2 Experimental set-up

Based on the results of the pilot studies, I set up exposure concentrations as follows (used for all experiments):

- *Onychiurus* sp.: 0-25-50-100 mg/kg soil;
- *F. quadrioculata*: 0-20-40-80 mg/kg soil.

Three replicates were used per treatment level. In each replicate, 20 adult springtails were added. All groups were kept in incubators with temperatures regulated at ±1℃.

For the short-term exposure experiments, separate tests were run for both species at 5, 10 and 20℃. The 5 weeks long-term exposure was run only at 20℃ (both species).

In the first pilot studies, growth of fungi in the soil caused mortality. The problem was partly caused by humidity settling in the lid of the exposure beakers. To reduce the problem, exposure beakers for the remaining tests were specially made for this study; the cover lids for test beakers had a round hole (r=15 mm) in the middle and this hole was covered with a layer of 10μm filter, which could let steam and air to go through. All test beakers were checked daily throughout the exposure period to keep the soil moist.

At the end of the test exposure, animals from each replicate were transferred into a larger beaker. Distilled water was added to the soil, and living individuals could be found located at the surface of the water due to their water-repelling cuticle. Adult and juvenile animals were counted and adults collected for subsequent sub-lethal studies.

### 2.4.3 DNA Damage Test

DNA damage was quantified using the Comet Assay.

After flowing up the surviving individuals, 5 adult collembolans were picked randomly from each replicate. They were beheaded in 75 μl PBS working solution. The hemolymph of Collembola would then leak out. After stirring with a tip for a few seconds, hemolymph and PBS mixture was collected into a 0.5 ml eppendorf tube, and put it on ice.

Hemocytes are sensitive to strong direct light, so to avoid light artifacts the whole procedure was performed with dim light conditions.

Following is the detailed procedure.

**Solutions**

*Phosphate Buffered Saline (Ca+Mg Free) – Stock Solution (PBS Stock Solution):*

8.5 g NaCl

0.85 g $Na_2HPO_4$ – anhydrate

0.54 g $KH_2PO_4$

Add $dH_2O$ to 100 ml. Adjust pH to 7.4.

Fresh before use, prepare a *10% PBS solution containing 10 mM EDTA*:

Dissolve 18.61 g $Na_2EDTA \cdot 2H_2O$ (372.2 g/mol) in 100 ml $dH_2O$

This requires addition of NaOH – set pH to 7.4

2 ml 0.5M EDTA to 98 ml diluted PBS

Stock solution could be held in the fridge at 4℃.

*Lysis Buffer*

Stock solution: Prepare for 1000 ml, and store it in the fridge at 4℃.

Distilled Water 786.5 ml

NaCl (58.44g/mol) 164.2 g

NaOH (40.0g/mol) 8.99 g

$Na_2EDTA \cdot 2H_2O$ (372.2g/mol) 41.80 g

Trizma base (121.2g/mol) 1.40 g

Adjust pH to 10 using NaOH.

Prepare lysis working solution fresh, 30-60 min before use. Mix 89 ml lysis stock solution with 10 ml distilled water and 1ml Triton X-100, and store the working solution in the fridge at 4℃.

*Electrophoresis Buffer*

Stock Solution: For 1000ml stock solution, 120 g NaOH (40.0 g/mol) and 3.72 g $Na_2EDTA \cdot 2H_2O$ (372.2 g/mol) were needed. Adjust volume with distilled water when dissolved. Store the Electrophoresis Stock Solution in the fridge at 4°C.

Working solution: made fresh before use. To prepare 2000 ml Electrophoresis working solution, 200 ml stock solution and 12ml concentrated HCl were required. Adjust volume with distilled water. The pH of the working solution would be about 13.2.

*Neutralising Buffer*

Dissolve 48.48g Trizma Base (121.2 g/mol) in ~1000 ml distilled water, and adjust pH to 7.5 with concentrated HCl. After dissolving the Trizma Base in distilled water, a large amount of HCl was needed to adjust the pH. Therefore, about 800 ml distilled water was added to dissolve Trizma Base, and then pH was adjusted close to 8. After that, volume and pH was adjusted to 1000ml and pH 7.5.

*LMP Agarose (0.75%)*

Mix 75 mg of Low Melting Point Agarose (Thermo Scientific, Product No. R0801) in 10 ml of PBS working solution. Perform watertight boil it in microwave oven machine until all agarose was dissolved in the solution. Loosen the lid during this process. Then cool the LMP agarose on a heating block at 37°C.

*Tris-EDTA (TE) Buffer*

0.5M Tris-HCl (pH8) 20 ml

0.5M EDTA (pH8) 2 ml

$dH_2O$ to 1000 ml

Adjust to pH 8.0

*Staining Solution (SYBR Gold)*

The SYBR Gold Nucleic Acid Gel Stain (10,000x Concentrate in DMSO) I use was produced by Life Technologies (product number S-11494).

Dilute SYBRGold 10x in DMSO – store in 40 ul aliquots.

For 50 ml TE-Buffer, add 40ul pre-diluted SYBR Gold before use.

**Procedure**

- After hemolymph extraction, cells were counted in a counting chamber under a microscope. A living cell concentration of no smaller than 20,000 cells/ml was required. If the cell concentration was high, it was diluted. For adult *Onychiurus*, 3-4 individuals could provide enough cells.

- 225 ul of LMP agarose gel was added into a 1.5 ml eppendorf tube, which was already heated on a heating block at 37℃. Then 25 ul of sample was added into the tube and the content mixed gently. To succeed with analysis, a 1:10 ratio of sample:gel was important and sufficient cell concentration was crucial.

- A GelBond film was placed on a pre-colded aluminum plate (~4℃) and 30ul cell-LMP agarose suspension was then gently spread on the film (d=0.5-1 cm). Absence of air bubbles in/on the gel was important. 12-15 gel could be add to one film. The GelBond Film I used was produced by GE Healthcare (product number is 80-1129-32, Pack of 50 films, 124x258 mm). The film had one hydrophilic side and one hydrophobic side, and the gel must be cast on the hydrophilic side, which is the side protected by a paper sheet.

- 50 ml lysis buffer was added to a suitable container with a cover lid. Film was covered in pre-chilled lysis solution and put in the dark at 4℃ for at least 1 hour. When the film was put in the lysis buffer, all biological reactions in the cell will stop. The film could be left in this way for days, but the remaining steps prior to staining were run in a single operation.

- Rinse film briefly (about 5 minutes) in electrophoresis buffer, then put film in the electrophoresis chamber, which contained 1.4 L buffer, for another 15 minutes. (Total 20 minutes).

- Open the circulating buffer system, and run the horizontal electrophoresis at 4℃ for 20 minutes, applying a voltage of 25V. One electrophoresis chamber took up to 4 films. I usually ran 2 films at a time.

- Transfer the film to neutralizing buffer at room temperature. Briefly rinse it for 5 minutes and soak it for another 10 minutes in new buffer. Total 15 minutes.

- Dip the film in distilled water, and then briefly rinse it in 96% ethanol for 5 minutes. This ethanol could be used for up to 15 times.

- Fix films for at least 2 hours in 96% ethanol (new one).

- Put the film in a dark dry place. Air-dry it. Now the film could be stored for more than 6 month.
- For the staining, add 50ml TE-buffer, which contained 40 µl SYBR Gold. Films were stained for 20 minutes (dark environment was needed). Films were then placed on a gentle rocking table.
- Dip the film in distilled water. Put it on a plexi glass plate and apply a cover slide. Use some $dH_2O$ to avoid air bubbles.
- Use a fluorescence microscope at 20x magnification to score the gels on the film. 50 cells were scored per sample. A real-time camera was connected to the computer to take photos of the cells, and the computer software, 'Comet Assay IV', was used to automatically analyse the fluorescence images of the cells.

### 2.4.4 Neutral Red Assay

The protocol references the procedure of 'Measuring lysosomal stability in marine invertebrates by Neutral Red Retention', written by Steven Brooks (Norwegian Institute for Water Research).

*Phosphate Buffered Saline (Ca +Mg Free) – Stock solution (PBS Stock solution)*

8.5 g NaCl

0.85 g $Na_2HPO_4$ – anhydrate

0.54 g $KH_2PO_4$

Add $dH_2O$ to 100 ml. Adjust pH to 7.4.

Fresh before use, prepare a *10% PBS solution containing 10 mM EDTA*:

Dissolve 18.61 g $Na_2EDTA \cdot 2H_2O$ (372.2 g/mol) in 100 ml $dH_2O$

This requires addition of NaOH – set pH to 7.4

2 ml 0.5M EDTA to 98 ml diluted PBS

Neutral Red Powder: Product number is N4638. CAS Number is 553-24-2; Empirical Formular is C15H17ClN4. Produced by Sigma-Aldrich (3050 Spruce Street, Saint Luois, Missouri, 63103, USA).

Dissolve 28.8 mg neutral red powder into 1ml DMSO. Store the stock solution in a light proof tube in the fridge (4 ℃). This could be left up to 3 weeks. To make 5 ml neutral red working solution, mix 10 µl stock solution with PBS working solution (1:500). The neutral red working solution was also need to be light proof and fresh before use.

Experimental procedure:

Hemolymph from collembolans was extracted in the same way as for the Comet Assay.

Add 30 µl of the hemolymph/PBS mixture on the center of a microscope slide. The slide should be contained in a light proof humidity chamber. Put ice under the slide, without touching it, on the base of the chamber. The chamber I used was thick, so I needed to put the chamber on some ice to keep the temperature sufficiently low inside the chamber.

In order to allow adherence of cells, the slides should be put in the chamber for 40 minutes. After that, the liquid was removed from the slide by putting it laterally upright and let the water drop flow down.

Replace the slide into the chamber and apply 30µl neutral red working solution. Put on a cover slip (22 ×22 mm) on top of the slide. Now time should be 0.

After 15min, slides were examined systematically using light microscopy with 40x magnification. The examination time for each slide should be less than 1min, and the light level during microscopy should be as low as possible.

The next examination was after another 15 minutes, and thereafter 30 minutes.

Slides should be placed back into the light proof humidity chamber when they were not examined.

The theoretical end point was the leaking of the dye (neutral red) into the cytosol. The test for each slide was terminated when dye loss was evident in 50% of the granular hemocytes. Associated with dye loss was the rounding up of the cells and enlargement of lysosomes. I recorded the time when occurred, and the results were expressed as minutes.

**2.5. Statistical Analysis**

Statistical analysis for DNA damage was done with the software ‘Comet Assay IV’

and ‘CometMacro – Comet Assay Spreadsheet Generator, Version 1.3.1’. With this software, I got the data of median tail intensity of all experiment replicates.

The DNA damage data and survival data of individuals were analyzed by ‘GraphPad Prism 5’. With the Nonlinear Regression (curve fit) of XY Analyses, I got the dose-response graphs and LC50 values of DNA damage and survival in sub-lethal effects of esfenvalerate. The equation of the dose-response curve was Asymmetric, which was Richard’s five-parameter dose-response curve. P values were tested by two-way ANOVA model.

The graph of reproduction data of long-term exposure for both species were made with Grouped Analyses, which showing the histogram plot of median with range.

## 3. Results

### 3.1. Neutral Red Assay

The size of the hemocytes was too small to be conveniently used in this assay and it was difficult to measure the changes of lysosomes in the cells. Furthermore, it was challenging to recognize different cell types, and I had great difficulties in selecting the same type of hemocytes for the assay. Therefore, the results of the Neutral Red Assay are not considered reliable. Figures 1 and 2 illustrate the many types of cells present, in typical examinations at 15 and 90 minutes of exposure, respectively.

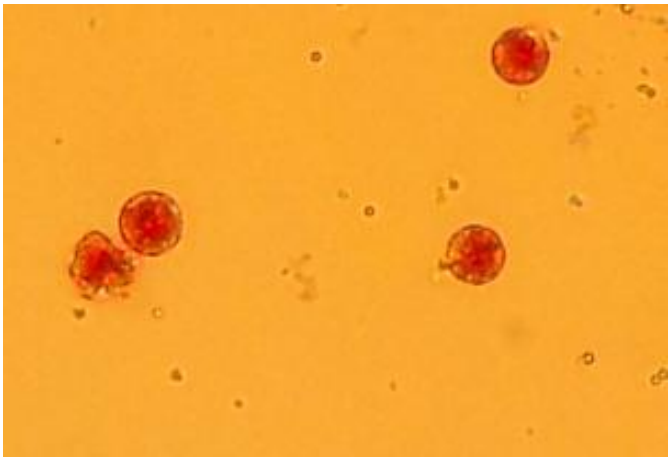


***Figure 1 Hemocytes of collembolans after adding neutral red dye for 15 minutes. Animals were exposed to the chemical esfenvalerate.***

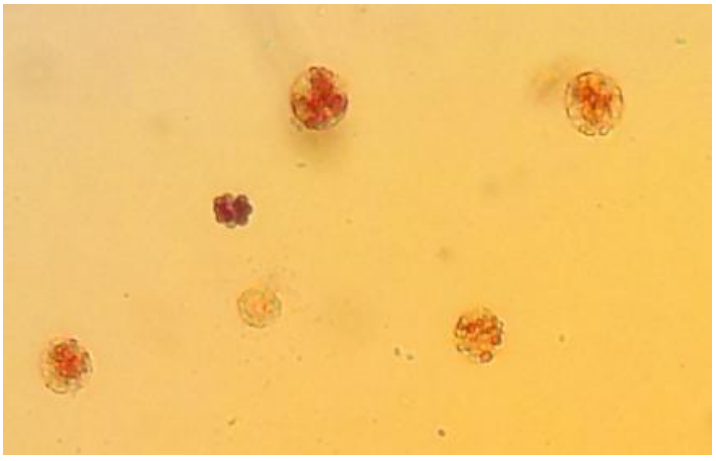


***Figure 2 Hemocytes of collembolans after adding dye for 90 minutes. Animals were exposed to esfenvalerate.***

### 3.2. Sensitivity of the two collembolan species to esfenvalerate

Figure 3 shows the different sensitivity of two species, *Folsomia quadrioculata* and *Onychiurus* sp. exposed at the same exposure temperatures and exposure times. In the short-term (1 week) exposure tests (graphs A, B. C), it is obvious that there is no large sensitivity difference to esfenvalerate between species *F. quadrioculata* and *Onychiurus* sp. at the same temperature. In the long-term (5 weeks) exposure tests, the curves show that the sensitivities of two species are similar to each other. Graph A shows that, *Onychiurus* sp. is more sensitive than *F. quadrioculata* when exposed to high esfenvalerate concentration (80 mg/kg soil) at 5 ℃. However, at other exposure temperatures (10 ℃ and 20 ℃), the curve trends looks similar between species, which means that sensitivities between *F. quadrioculata* and *Onychiurus* sp. are not significant.

Table 4 shows all the LC50 values of the does-response curves in Figure 3. In the short-term exposure tests, when the exposure temperature was 20 ℃, *F. quadrioculata* (LC50=44 mg/kg soil) and *Onychiurus* sp. (LC50=53 mg/kg soil) have the most sensitivity difference. When the exposure temperature was 5 ℃ or 10 ℃, sub-lethal effect of survivals between species had even smaller variations. Therefore, LC50 values show little tolerance difference to esfenvalerate between *Onychiurus* sp. and *Folsomia quadrioculata* at same exposure temperature. In the 5 weeks long-term exposure test, the LC50 values of *Folsomia quadrioculata* and *Onychiurus* sp. were the same – 30 mg/kg soil and 30 mg/kg soil. The difference in toxicity of esfenvalerate between these two species was insignificant after long-term exposure at 20 ℃.

As exposure temperature gets higher, *F. quadrioculata* has stronger toxicity reflection. The LC50 values of *F. quadrioculata* drops from 50 mg/kg soil to 44 mg/kg soil. On the other hand, *Onychiurus* sp. does not show very obvious changes on toxic effect based on temperature changes. The LC50 values changed form 54 mg/kg soil (5 ℃) to 53 mg/kg soil (20 ℃). It shows a little toxic sensitivity difference to esfenvalerate between the two species at different exposure temperatures.

Figure 4 shows that both *F. quadrioculata* and *Onychiurus* sp. can produce breeding and hatching under low esfenvalerate concentration at 20 ℃ after 5 weeks exposure. However, when the exposing concentration gets higher, no reproduction results can be seen. Besides, the figure shows no significant reproduction difference between species.

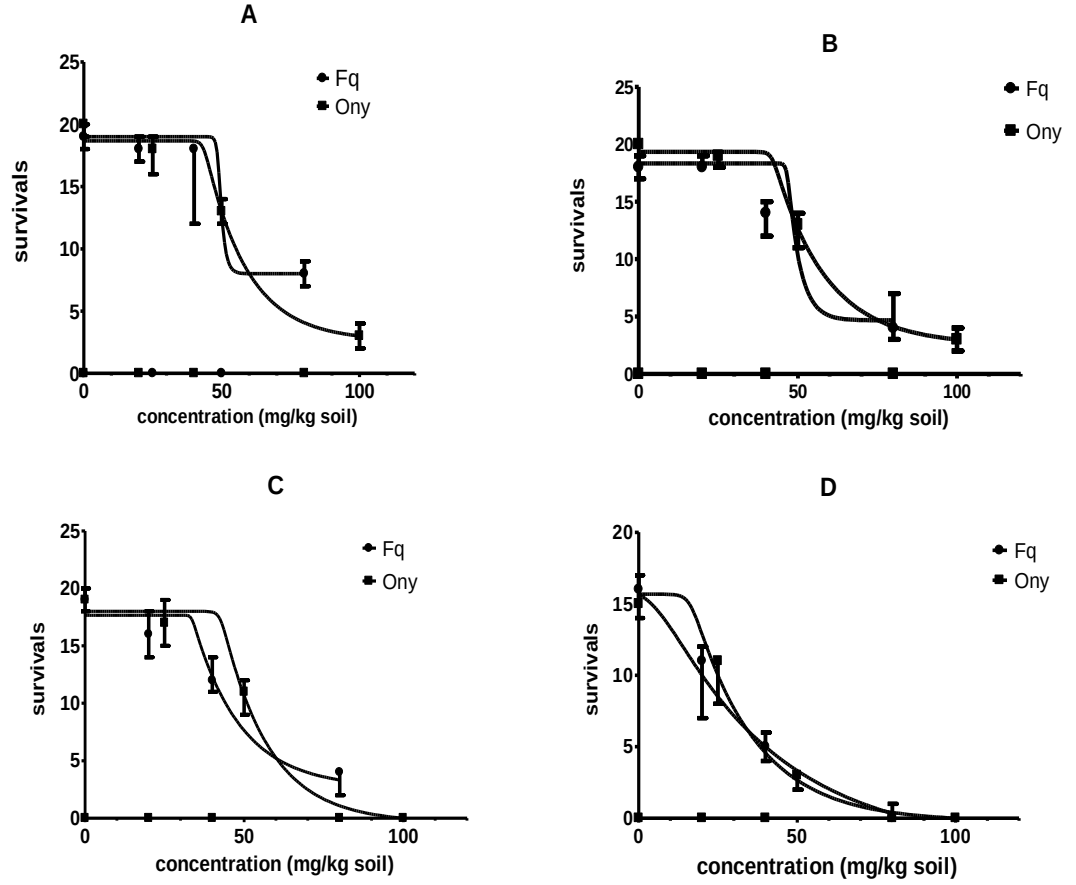



***Figure 3 Short-term dose-response curves of survival of both Folsomia quadrioculata (Fq) and Onychiurus (Ony) at different exposure temperatures – 5℃ (graph A), 10℃ (graph B), and 20℃ (graph C). Graph D is the long-term exposure dose-response curves of the above two species at 20℃. 20 animals were originally added to the test beakers. Three replicates were used per treatment. The graph shows average values ± standard errors. $R^2$ values of the curves are – in graph A, $R_{Fq}^2$=0.76, $R_{Ony}^2$=0.97, P>0.1; in graph B, $R_{Fq}^2$=0.79, $R_{Ony}^2$=0.98, P>0.1; in graph C, $R_{Fq}^2$=0.93, $R_{Ony}^2$=0.97, P>0.1; in graph D, $R_{Fq}^2$=0.95, $R_{Ony}^2$=0.98, P>0.1.***

***Table 4 LC50 values for esfenvalerate for two species of springtails exposed in soil at three different temperatures and two exposure durations. The LC50 values are calculated by GraphPad Prism 5 based on the sub-lethal dose-response curves in Figure 3.***

| Exposure Time | Temperatures | LC50 (mg/kg soil) | |
|---|---|---|---|
| | | *F. quadrioculata* | *Onychiurus* sp. |
| 1 week | 5℃ | 50 | 54 |
| | 10℃ | 49 | 53 |
| | 20℃ | 44 | 53 |
| 5 weeks | 20℃ | 30 | 30 |

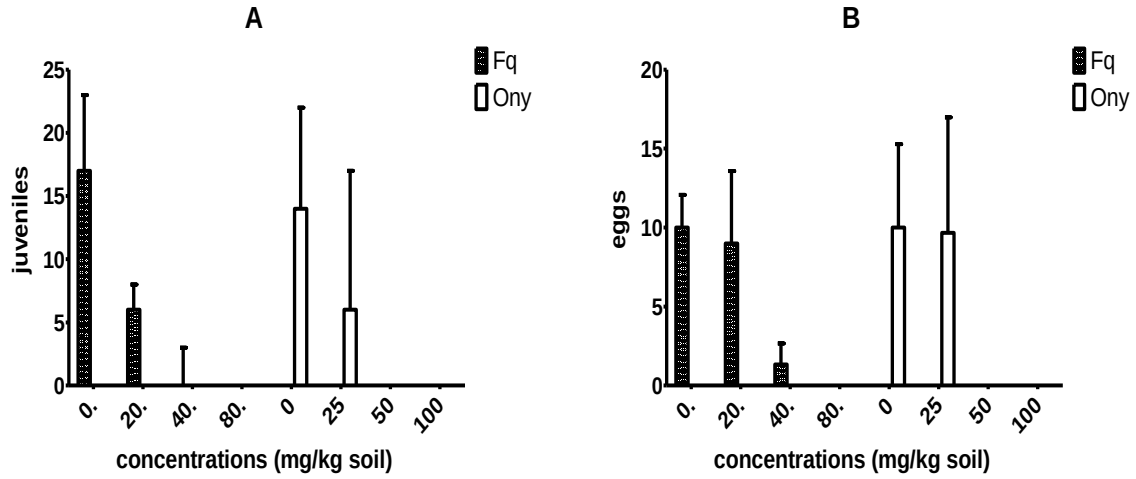



***Figure 4 Reproduction of Folsomia quadrioculata and Onychiurus sp. in response to esfenvalerate exposure in the long-term exposure test at 20℃. Graph A (P>0.1) represents the number of juveniles of the two species, while graph B (P>0.1) shows the number of eggs in the culturing soil. 20 adult animals were originally added to test beakers. Three replicates were used per treatment. The graph shows average values ± standard errors.***

### 3.3. Effects of temperature on toxicity

#### 3.3.1 Effects on survivals

In the current study, differences in toxicity between different temperatures can only be compared for the short-term exposure tests. From the calculated LC50 values in Table 4, we can see that, in general, the rising of temperature has inversely proportional to LC50 values for both species. However, the relationship between temperature and LC50 value is not linear. When temperature rises from 5℃ to 10℃, LC50 of *Folsomia quadrioculata* drops from 50 mg/kg soil to 49 mg/kg soil. The difference is very little. On the other hand, when temperature rises from 10℃ to 20℃, LC50 of *F. quadrioculata* has a larger change, dropping to 44 mg/kg soil. The toxicity changes of *Onychiurus* sp. on esfenvalerate between different temperatures are also very little, LC50 are 54 mg/kg soil at 5℃ and 53 mg/kg soil at 20℃.

In Figure 3, when the exposure concentration is high (80 mg/kg soil for *F. quadrioculata*, and 100 mg/kg soil for *Onychiurus* sp.), the number of survivals gets smaller as temperature goes higher, especially *Onychiurus* sp. When the exposure concentration is 100 mg/kg soil, no springtails is alive at 20℃. Mortality effects of high esfenvalerate concentration exposure on both species are heavier at higher temperature (20℃) than lower temperature (10℃).

### 3.3.2 Effects on DNA damage

In the current study, the DNA damage was only investigated for *Onychiurus* sp. I chose tail intensity of the comet of each cell as a parameter to compare the damage level. Figure 5 shows the images of single cell in Comet Assay in different exposure concentrations.

Because there were no survivals in replicates of concentration 100 mg/kg soil at 20 ℃, the tail intensity results of this concentration were missing for both short-term and long-term exposure. The tail intensities of control groups (concentration of 0 mg/kg soil) were small, which meant that the DNA damage level in the cells of control groups was low. Therefore, the cells of were retrieved in an acceptable way during the experimental operations.

When the concentration of esfenvalerate was low, tail intensities were small and almost the same at different exposure temperatures (Figure 6). The median tail intensity value of 5 ℃, 10 ℃, and 20 ℃ in the concentration of 25 mg/kg soil were 7.45%, 7.06% and 7.02%. However, when the concentration got higher, the DNA damage levels between different temperatures became more obvious. The obvious difference between exposure temperatures in high esfenvalerate concentrations could be seen in Figure 6. When the concentration was 50 mg/kg soil, the intensity of comet tails at 5 ℃ was 8.73%, and the intensity of tails at 20 ℃ is almost the same (8.76%) with at 5 ℃. However, in the same concentration, the tail intensity at 10 ℃ rise to 12.06%, and when the exposure concentration was 100 mg/kg soil, it reached 16.49% which was 10 percent higher than the same concentration at 5 ℃. When the temperature is 20 ℃, tail intensity in the concentration of 50 mg/kg soil was almost the same as 5 ℃, which was 8.76%. Both of them were lower than that at 10 ℃.

With these tail intensity data, I got the dose-response curves (Figure 6) and LC50 values (Table 6) at different temperatures. The DNA damage levels, presents by LC50 of comet tail intensities, were 22 mg/kg soil at 5 ℃, 37 mg/kg soil at 10 ℃, and 21 mg/kg soil at 20 ℃. *Onychiurus* sp. had the most serious damage on DNA strand at 20 ℃ exposure temperature, and least serious damage at 10 ℃ after 1 week esfenvalerate-soil exposure. However, DNA damage level at lower (5 ℃) and higher (20 ℃) temperatures were almost the same, due to the LC50 values.

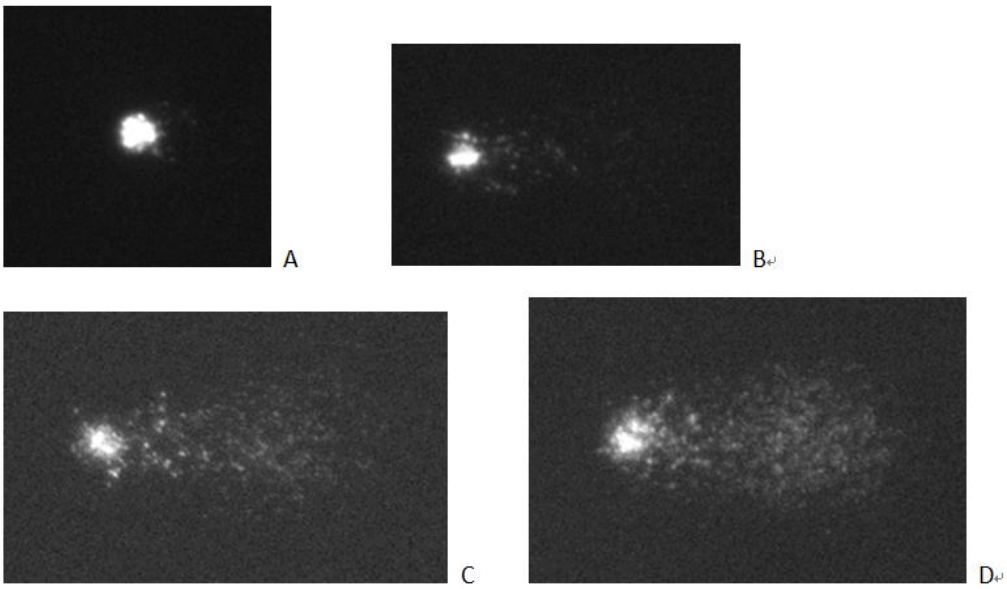



***Figure 5 Comet Assay scoring images of short-term exposure tests at 10℃. Image A is the cell from survival animal in control group, which has exposure concentration of 0 mg/kg soil. Image B is the cell from replicate which has exposure concentration of 25 mg/kg soil. Image C and D are cells in the concentration of 50 mg/kg soil and 100 mg/kg soil.***

***Table 5 Median value of comet assay tail intensity (%) of three experimental replicates at 3 different exposure temperatures in short-term exposure duration (1 week). 3-5 animals were taken from the survivals in each replicates. Their cells were used for the Comet Assay. One LMP gel with cells on the film represented one replicate. 50+ cells were counted in one gel. The median values of the following table were coming from all cells' tail intensities of three replicates in one concentration.***

| Concentrations of esfenvalerate (mg/kg soil) | Median value of comet tail intensity (%) | | | |
|---|---|---|---|---|
| | Short-term (1 week) | | | Long-term (5 weeks) |
| | 5℃ | 10℃ | 20℃ | 20℃ |
| 0 | 1.92 | 3.59 | 6.20 | 4.92 |
| 25 | 7.45 | 7.06 | 7.02 | 13.24 |
| 50 | 8.73 | 12.06 | 8.76 | 15.76 |
| 100 | 10.12 | 16.49 | 0 | 0 |

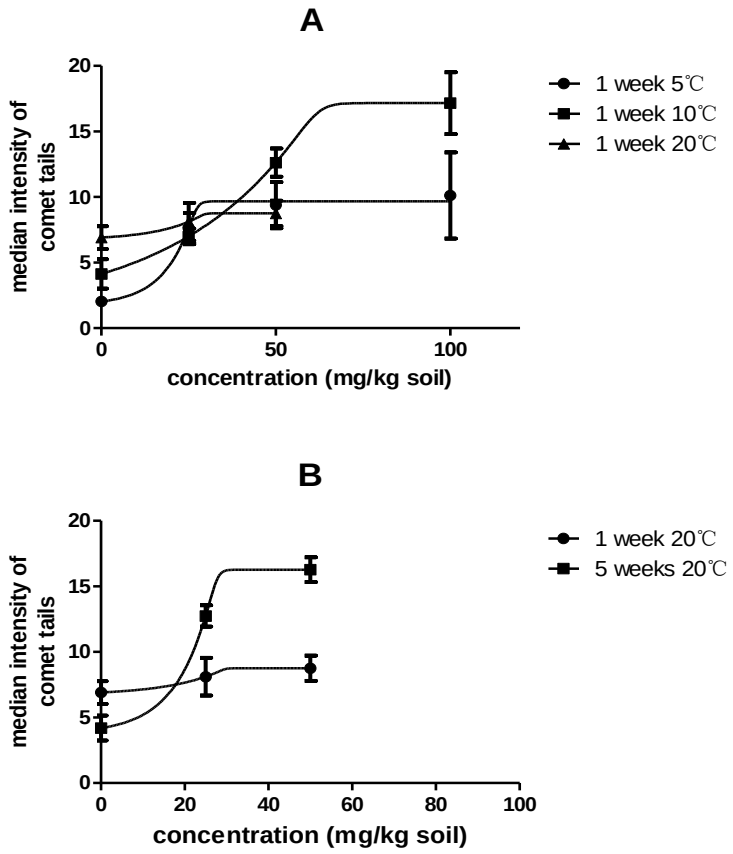



***Figure 6 Dose-response curves of tail intensity of the Comet Assay of Onychiurus sp., including 3 different exposure temperature groups of short-term tests and one long-term exposure group at 20 ℃. $R^2$ values of the curves are – in graph A, $R_5^2$=0.69, $R_{10}^2$=0.86, $R_{20}^2$=0.19, P>0.01; in graph B, $R_{20}^2$= 0.94, P<0.001. Subscripts 5, 10, 20 of R refer to temperatures. Compare the***

***Table 6 LC50 values for effects of esfenvalerate on Onychiurus sp. tail intensity as measured by the Comet Assay at different temperatures in both short-term (1 week) and long-term (5 weeks) exposure studies.***

| exposure time | temperatures | LC50 (mg/kg soil) (based on the tail intensity of Comet Assay) |
|---|---|---|
| 1 week | 5℃ | 22 |
| | 10℃ | 37 |
| | 20℃ | 21 |
| 5 weeks | 20℃ | 22 |

### 3.4. Toxicity at different exposure times on *Onychiurus* sp. at 20 ℃

#### 3.4.1 Survival

In general (Figure 7), esfenvalerate had more slightly toxicity in short-term exposure than long-term for both *Folsomia quadrioculata* and *Onychiurus* sp. at 20 ℃. The LC50 value of esfenvalerate for *Folsomia quadrioculata* and *Onychiurus* sp. at 20 ℃ for both short-term and long-term exposure are shown in Table 4. For *F. quadrioculata*, the LC50 of 1 week esfenvalerate exposure is 44 mg/kg soil, and 30 mg/kg soil in 5 weeks exposure. The LC50 value of *Onychiurus* in 1 week exposure is 53 mg/kg soil, and 30 mg/kg soil in 5 weeks. It was obvious that the exposure time difference had effect on toxicity of esfenvalerate on both *Folsomia quadrioculata* and *Onychiurus* sp. at 20 ℃ exposure temperature. In long-term exposure tests, LC50 has smaller values than in short-term exposure tests, which means the total toxicity of esfenvalerate was stronger in long exposure time than in short exposure time.

#### 3.4.2 DNA damage

For low exposure concentration (25 mg/kg soil) and higher concentration (50mg/kg soil), the comet tail intensities in long-term exposure were higher (nearly two times) than in short-term exposure group (Figure 6 and Table 5). Moreover, the LC50 of tail intensity value of 5 weeks exposure group (22 mg/kg soil) was a little higher than 1 week exposure group (21 mg/kg soil). Therefore, the result showed that the DNA toxicity of esfenvalerate to *Onychiurus* sp. became obvious at almost the same concentration level for both short-term and long-term exposures (LC50 values are very close). DNA damage in long-term exposure was much more serious than short-term when the exposure concentration increased over LC50. However, LC50 values for both short-term (21 mg/kg soil) and long-term (22 mg/kg soil) exposures indicated that exposing durations had no significant effect on the toxicity of esfenvalerate to *Onychiurus* sp.

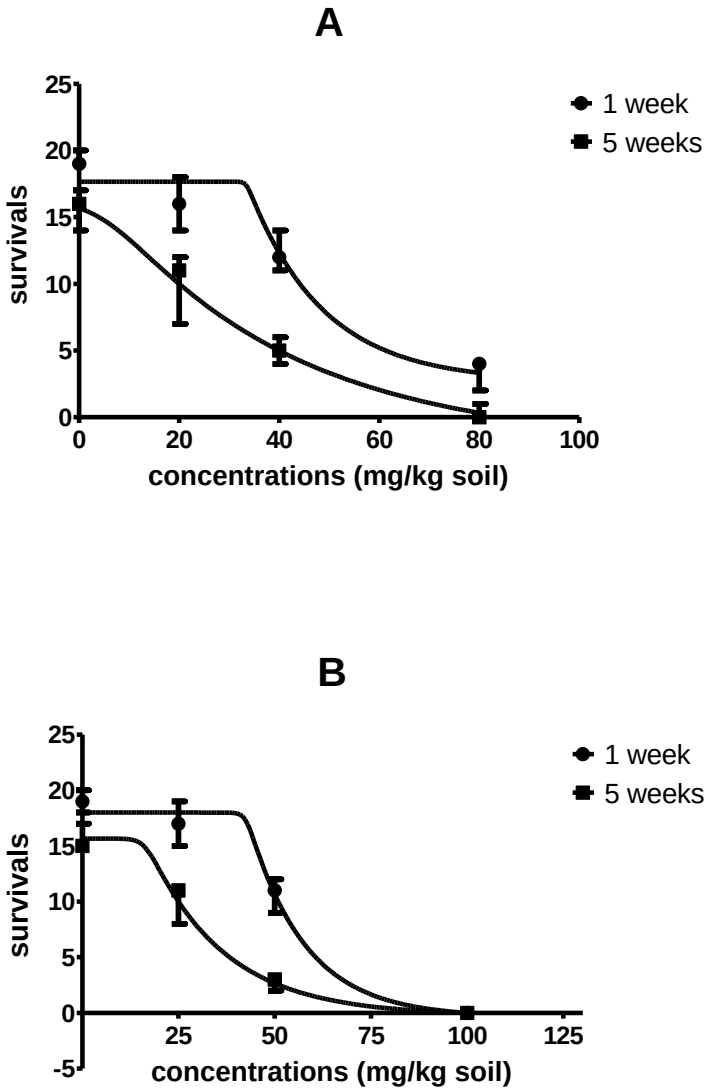



***Figure 7 Survivals-concentrations curves of Folsomia quadrioculata (graph A) and Onychiurus sp. (graph B) for both long-term and short-term exposure at 20 °C. $R^2$ values of the curves are – in graph A, $R_S^2$=0.93, $R_L^2$=0.95, P>0.01; in graph B, $R_S^2$=0.97, $R_L^2$=0.98, P<0.001. Subscripts S and L refer to the exposure durations short-term and long-term.***

## 4. Discussion

### 4.1. Comparison between species

There is no significant species sensitivity difference between *Folsomia quadrioculata* and *Onychiurus* sp. to the toxicant esfenvalerate. The study by Dodd and Addison (2010) indicated a sensitivity difference to the same toxicant between different species of springtails. Survivals in the short-term exposure and reproductions in the long-term exposure differed for *Folsomia candida*, *Onychiurus folsomi*, and *Proisotoma minuta* after being exposed to methyl tert butyl ether (MTBE). In the study by Schnug *et al.* (2013), a significant sensitivity to esfenvalerate was reported between two earthworm species. The current study had a converse result. Unfortunately, there was no esfenvalerate toxicity study on collembolans by other people. Different collembolan species may also have different ratios in uptake, distribution and excretion on esfenvalerate. Biotransformation level may also vary based on different species, circumstances *in vivo*, and environmental conditions. All of them may have independent or synthesized effects on toxicity of esfenvalerate. As a new study in this area, my results showed that there was insignificant variation of general toxicity of the chemical between collembolan species, *F. quadrioculata* and *Onychiurus* sp.

As exposure time increased from 1 to 5 weeks, there was no obvious sensitivity difference of *F. quadrioculata* and *Onychiurus* sp. on esfenvalerate (both species had LC50 values of 30 mg/kg soil). Persistent toxicants will accumulate in the body of the exposed organisms as exposure time gets longer. LC50 decreases as exposure time rises (Broerse *et al.*, 2010). As the effective concentration *in vivo* increases with time, the toxicity difference between species may be insignificant. However, compare the survival results between different exposure durations, *F. quadrioculata* showed insignificant sensitivity on esfenvalerate, while *Onychiurus* sp. had significant interaction between exposure times. At 20 ℃, *Onychiurus* sp. had higher tolerance (LC50=53 mg/kg soil) on esfenvalerate than *F. quadrioculata* (LC50=44 mg/kg soil) in short-term exposure. After 5 weeks exposure, toxicity effects were the same on both species. The results suggested that *Onychiurus* sp. had higher bioaccumulation ratio than *F. quadrioculata*.

In the current study, investigation of species sensitivity in reproduction showed no obvious difference between species (ANOVA, $P>0.01$). There are juveniles and eggs in low exposure concentration for both species, the differences between them were not

significant. However, it is obvious that high exposure concentration of esfenvalerate would reduce the reproduction level. The toxicant may have harmful effect on breeding ability of adult collembolans, or killed the eggs before hatching. Or juveniles had higher sensitivity to esfenvalerate and dead in a very short time after hatching. Santo *et al.* (2012) indicated that agricultural insecticides would reduce the reproductive capacity of collembolan *Folsomia candida.*

### 4.2. Toxicity following exposure at different temperatures

#### 4.2.1. Survival

At higher temperature, biotransformation and other energy-requiring processes in the collembolans will be more active than at lower temperatures. It has been shown that esfenvalerate can bind to sodium channels and change the phase of action potentials in the membranes of neurons in insects (Tomlin, 1994; Xu *et al.*, 2006). Collembolans and insects have the same sodium channel proteins, because all invertebrates have conserved organizations of sodium channel (Xu *et al.*, 2006). At low temperature, enzymes will have low activities, so the toxicity of esfenvalerate will be limited. When the temperature gets higher, the toxicant will have more obvious effects on the target proteins. Serious molecular damage may lead to individual death.

In the study published by Sjursen and Holmstrup (2004), the main reasons of how temperature interact the toxicity of chemicals' entry into the body are affecting the bioavailability and uptake of the toxicants. When the toxicants are internalized in the organism, the body temperature will affect the toxicity by increasing metabolism (Martikainen *et al.*, 1999; Sjursen *et al.*, 2004).

In the current study, there was no big difference between exposure temperatures in short-term exposure for both species. For *Onychiurus* sp., when the temperature changed from 5 ℃ to 20 ℃, LC50 decreased 1 mg/kg dry soil, which was very small change. It indicated *Onychiurus* sp. had small sensitivity on temperature change between 5-20 ℃. *F. quadrioculata* showed a little higher sensitivity on temperature change, which had LC50 value decreased from 50 to 44 mg/kg soil. However, both of their changes were insignificant ($P>0.01$). Therefore, in the current study, temperature range of 5-20 ℃ had no efficient disturbance on toxicity of esfenvalerate for both species in short-term exposures.

This phenomenon may because there was no stress from both the lowest (5 ℃) and

the highest temperature (20 ℃) on the two species. All bioactivities are stable, and no fatal toxicity effects *in vivo* are caused at this temperature range. Temperature change between 5 ℃ and 20 ℃ will not be the main reason for collembola death to esfenvalerate.

### 4.2.2. DNA damage

Using the Comet Assay to test DNA damage in collembolans is a new method. The method has been widely used and shown to be efficient in invertebrates (Reinecke *et al.*, 2004; Lourenco *et al.*, 2011; 2012) and other animals (Tice *et al.*, 2000; Speit *et al.*, 2006). DNA damage can be used as a biomarker to present the toxicity of chemicals *in vivo*. The Comet Assay is a suitable method to quantify general damage in DNA.

The Comet Assay results in the current study represented the DNA damage of all surviving animals in each experimental replicate, rather than each individual. The tail intensity (%) of the comet showed the degree of DNA damage degree. Higher intensity meant more serious damage. Hence, LC50 calculated from tail intensity data had the opposite meaning. Higher LC50 to tail intensity showed lower DNA damage level.

In short-term exposure DNA damage test, *Onychiurus* sp. had lower LC50 value at 5 ℃ (22 mg/kg soil) and 20 ℃ (21 mg/kg soil), which meant that at 10 ℃ (LC50=37 mg/kg soil), esfenvalerate has the least serious toxicity on DNA strand. This result was a little different from survival test in individual level, which showed the most toxic effect at 20 ℃ and least at 5 ℃. DNA damage and repair in the cell are complex processes, and they are controlled by various enzymes and other energy requiring processes, all of which can be affected by temperature. The toxicity of a chemical can be very different at molecular level and individual level. At higher temperature, enzymes for DNA repair may be more active. This may lead to two converse end of an individual. One is that with a well done DNA repair, the damage can be fixed, and the cell is recovered, then the individual will healthily live longer. On the other hand, an inappropriate DNA repair may cause mutation, which can be lethal to the individual. Hence, the toxicity to DNA of esfenvalerate may not relate to the mortality of individual.

In the study by Waagner *et al.* (2010), heat shock protein *Hsp70* was tested in response to short-term thermal change in *Folsomia candida*. The temperature regime was from 8 ℃ to 32 ℃. There was a rapid induction and significant increase of *Hsp70* by the temperature increase. They also tested the metabolism of small molecular

weight compounds. The result showed a significant divergence between heat-shocked group and control. The study indicated that temperature change of the environment has effects on expression of proteins and metabolisms *in vivo*.

The increase of LC50 of tail intensity from 5℃ to 10℃, and the decrease from 10℃ to 20℃ may relate to combination effects of DNA damage by esfenvalerate and the DNA repair program of the cell. When the exposure temperature increased from 5℃ to 10℃, DNA repair enzymes can be more activated. Therefore, it may cause less DNA damage. However, as temperature increased, the toxicant may have higher activities working with DNA strand as temperature rises to 20℃. In the study of an *Apis cerana cerana* glutathione S-transferase gene expression in response to thermal stress by Yan *et al.* (2013). The highest expression level of the enzyme appeared at 15℃, while expression level at either lower (4℃) or higher (25℃, 34℃, 43℃) temperatures resulted in lower expression than that at 15℃.

Although comparison of DNA damage dose-response curves in graph A, Figure 6 were tested as insignificant, difference between different exposing temperatures were obvious. In the highest esfenvalerate concentrations (100 mg/kg soil), DNA damage (tail intensity) at 10℃ was much higher than that at 5℃. This may because, in high toxic concentration environment, the poison efficiency of esfenvalerate is mainly related to enzyme activity and energy supply, which have close relationship with temperature change. As esfenvalerate is a hydrophobic organic compound with a large molecular weight, the transportation of it requires many different enzymes and energy. With a higher exposure temperature (10℃), the activity of related enzymes will have higher activities, which may lead to a higher toxic DNA damage effect of esfenvalerate.

Considering both sub-lethal result and comet assay result, it appeared that temperature difference had complicated effects on the toxicity of esfenvalerate to *Onychiurus* sp. The toxicity on survival and DNA strand were not the same between different exposure temperatures.

### 4.3. Toxicity at different exposure times

The toxicity difference of esfenvalerate related to exposure time was visible in the survival experiment. The LC50 values of concentration for 5 weeks exposure were smaller than those following 1 week exposure for both species, especially on *Onychiurus* sp. This might because of the bioaccumulation of esfenvalerate in the animal body. As reported by Nesto *et al.* (2007) and Lourenco *et al.* (2011), trace metals, micro-organic pollutants, and radionuclides all had bioaccumulation effects in

mussels, fish and earthworms. There were also accumulation studies on collembolans, for example, the copper accumulation study on *Folsomia candida* by Pedersen *et al.* (1997). The study also included fitness research, and pH and soil moisture factors. Overall, the toxicity of chemicals on springtails would increase as extension of exposure time.

The LC50 of tail intensity of Comet Assay results showed significant differences in DNA damage between short-term and long-term exposures on *Onychiurus* sp. ($P<0.001$). It meant that the sub-lethal effect on DNA had relationship with exposure time. When the exposure concentration of esfenvalerate was over 25 mg/kg soil, the tail intensity of long-term exposure was clearly higher than following short-term exposure. It indicated that in long-term exposure, DNA damage caused by esfenvalerate was stronger in high concentrations than in short-term exposure. This may also caused by the accumulation effects. As esfenvalerate could remain in soil for a long time, absorption of the chemical was a long-time and slow event. For short-term exposure, the effective concentration *in vivo* might not saturated before exposure stopped when the concentration was 50mg/kg soil. For the long-term exposure, there was sufficient time for collembolans to accumulate esfenvalerate, which led to more pronounced DNA damage at the higher exposure concentrations. In a genotoxic study on earthworms, DNA damage also showed high level in long-term exposure and low level in short-term exposure (Lourenco *et al.*, 2011).

### 4.4. Integration of factors

In the current study, species difference, temperatures, and exposure times showed independent effects on the toxicity of esfenvalerate. There was no sensitivity difference between the two collembolan species, *Folsomia quadrioculata* and *Onychiurus* sp. In general, the toxicity of esfenvalerate at different temperatures was insignificant. However, compare the dose-response curve trends and LC50 values on survival and DNA damage, the toxicity of esfenvalerate varied following temperature change. The toxicity-temperature relationship was more complicated at the molecular level, which may be related to adaptation, metabolism, and DNA damage and repair progress *in vivo*. Esfenvalerate had higher toxicity after long time exposure than short time. There was a significant toxicity difference between exposure durations (1 week vs. 5 weeks) on esfenvalerate, especially for *Onychiurus* sp. on both survival and DNA damage.

In the natural environment, temperature, chemical concentration, and exposure time will not have separate effects on organisms. The toxicity of a toxicant will be

intricately affected by all kinds of environmental factors. There are also other factors, like soil moistures, pH, and other contaminants in the soil, which may affect the toxicity of a chemical pollutant. Therefore, the toxic effects in the natural world are more complicated than in a laboratory environment.

The toxic properties of esfenvalerate also include effects on cell membranes. Attempts at establishing the Neutral Red Retention Assay for hemocytes was unsuccessful. The cells used in Comet Assay were the hemocytes of collembolans, which are also main cells for immune system of springtails. In order to present the esfenvalerate toxicity on the immune system of collembolans, it is better to include both DNA damage and membrane stability. Hence it needs further work to complete the toxicity study. It was unfortunately not possible to perform the DNA damage test with *Folsomia quadrioculata* hemocytes due to the difficulties in extracting hemocytes from such a small organism.

### 4.5. Risk assessment of esfenvalerate

The results of the study are reliable and have practical meanings regarding to the control of pesticide using management, which needs to include investigation of multiple environmental factors. Considering all factors included in the study, a concentration of 50 mg/kg soil of esfenvalerate cause reduced survival collembolans, at a temperature of around 5 ℃. At 20 ℃, a concentration of 30 mg/kg soil would be sufficient to affect survival following long-term exposure. Considering seasonal temperature change and agricultural activities in Norway, the concentration of 30 mg/kg soil would be the best. Moreover, further investigation on more species, reproduction ratio, higher temperature, and other soil types are needed. It is also important to include more environmental factors and long-term exposure at different temperatures, so that the risk assessment of environment can have more practical references.

## 5. Conclusions

There was no significant species sensitivity difference of esfenvalerate toxicity between the two species, *Folsomia quadrioculata* and *Onychiurus* sp., following both short-term (1 week) and long-term (5 weeks) exposures.

Esfenvalerate became more toxic to *F. quadrioculata* with increasing temperature than to *Onychiurus* sp. Different exposure temperatures had low toxicity variations for both species following short-term exposure.

Esfenvalerate caused genotoxicity to *Onychiurus* sp. DNA damage in *Onychiurus* hemocytes varied with temperature following short-term exposure. The genotoxicity of esfenvalerate was not linearly correlated to survival. At the same exposure temperature, both *F. quadrioculata* ($P>0.01$) and *Onychiurus* sp. ($P<0.001$) showed obvious toxicity difference between short-term and long-term exposures at individual level. DNA damage between different exposure durations had significant effect on toxicity of esfenvalerate on *Onychiurus* sp. at 20℃.

In summary, the toxicity of esfenvalerate may vary between collembolan species at different experimental temperatures and following different exposure times. When the exposure temperature increases there might be higher toxicity on survivals. The genotoxicity change of esfenvalerate will be complicated, due to complex bioactivities *in vivo*. The prolonged exposure time will increase the toxicity of esfenvalerate on collembolans.

## 6. References


Adelsbach TL, Tjeerdema RS. 2003. Chemistry and fate of fenvalerate and esfenvalerate. Reviews of Environtal Contamination and Toxicology 176:137-154.

Ahmed MK, Habibullah-Al-Mamun M, Hossain MA, Arif M, Parvin E, Akter MS, Khan MS, Islam MM. 2011. Assessing the genotoxic potentials of arsenic in tilapia (Oreochromis mossambicus) using alkaline comet assay and micronucleus test. Chemosphere 84(1):143-149.

Broerse M, van Gestel CAM. 2010a. Chlorpyrifos reduces nickel-induced growth retardation of the soil dwelling Collembolan Folsomia candida. Ecotoxicology and Environmental Safety 73(5):1051-1056.

Broerse M, van Gestel CAM. 2010b. Mixture effects of nickel and chlorpyrifos on Folsomia candida (Collembola) explained from development of toxicity in time. Chemosphere 79(9):953-957.

Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP. 2006. Collembolan trophic preferences determined using fatty acid distributions and compound-specific stable carbon isotope values. Soil Biology and Biochemistry 38(6):1275-1281.

Collins AR. 2009. Investigating oxidative DNA damage and its repair using the comet assay. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 681(1):24-32.

Dodd M, Addison JA. 2010. Toxicity of methyl tert butyl ether to soil invertebrates (springtails: Folsomia candida, Proisotoma minuta, and Onychiurus folsomi) and lettuce (Lactuca sativa). Environmental Toxicology and Chemistry 29(2):338-346.

Domene X, Alcaniz JM, Andres P. 2008. Comparison of solid-phase and eluate assays to gauge the ecotoxicological risk of organic wastes on soil organisms. Environmental Pollution 151(3):549-558.

Everatt MJ, Worland MR, Convey P, Bale JS, Hayward SAL. 2013. The impact of salinity exposure on survival and temperature tolerance of the Antarctic collembolan Cryptopygus antarcticus. Physiological Entomology 38(3):202-210.

Fishel FM. 2014. Pesticide toxicity profile: synthetic pyrethroid pesticides. In: The Institute of Food and Agricultural Sciences (IFAS) UoF, editor. UF/IFAS Extension. University of Florida. p 3.

Garey J, Wolff MS. 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochemical Biophysical Research Communication 251(3):855-859.

Go V, Garey J, Wolff MS, Pogo BG. 1999. Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. Environmental Health Perspectives 107(3):173-177.

Haubert D, Haggblom MM, Langel R, Scheu S, Ruess L. 2006. Trophic shift of stable isotopes and fatty acids in Collembola on bacterial diets. Soil Biology and Biochemistry 38(7):2004-2007.

Holmstrup M, Aubail A, Damgaard C. 2008. Exposure to mercury reduces cold tolerance in the springtail Folsomia candida. Comparative Biochemistry and Physiology. Toxicollogy and Pharmacology: CBP 148(2):172-177.

Holth TF, Beckius J, Zorita I, Cajaraville MP, Hylland K. 2011. Assessment of lysosomal membrane stability and peroxisome proliferation in the head kidney of Atlantic cod (Gadus morhua) following long-term exposure to produced water components. Marine Environmental Research 72(3):127-134.

Hopkin SP. 1997. Biology of the springtails (Insecta, Collembola). Oxford ; New York: Oxford University Press. x, 330 p. p.

Kammann U, Biselli S, Huhnerfuss H, Reineke N, Theobald N, Vobach M, Wosniok W. 2004. Genotoxic and teratogenic potential of marine sediment extracts investigated with comet assay and zebrafish test. Environmental Pollution 132(2):279-287.

Krab EJ, Van Schrojenstein Lantman IM, Cornelissen JHC, Berg MP. 2013. How extreme is an extreme climatic event to a subarctic peatland springtail community? Soil Biology and Biochemistry 59:16-24.

Ksiazkiewicz-Ilijewa M, Rościszewska E. 1979. Ultrastructure of the haemocytes of Tetrodontophora bielanensis Waga (Collembola). Cytobios 26(102):9.

Lackie J. 2010. A Dictionary of Biomedicine. Oxford: A Dictionary of Biomedicine. Oxford, New York: Oxford University Press.

Laskowski DL. 2002. Physical and chemical properties of pyrethroids. Reviews of Environmental Contamination and Toxicology. 49-170.

Lourenco J, Pereira R, Silva A, Carvalho F, Oliveira J, Malta M, Paiva A, Goncalves F, Mendo S. 2012. Evaluation of the sensitivity of genotoxicity and cytotoxicity endpoints in earthworms exposed in situ to uranium mining wastes. Ecotoxicology and Environmental Safety 75(1):46-54.

Lourenco JI, Pereira RO, Silva AC, Morgado JM, Carvalho FP, Oliveira JM, Malta MP, Paiva AA, Mendo SA, Goncalves FJ. 2011. Genotoxic endpoints in the earthworms sub-lethal assay to evaluate natural soils contaminated by metals and radionuclides. Journal of Hazardous Materials 186(1):788-795.

Lowe DG, Dizhoor AM, Liu K, Gu Q, Spencer M, Laura R, Lu L, Hurley JB. 1995. Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. Proceedings of the National Academy of Sciences of the United States of America 92(12):5535-5539.

Martikainen E, Rantalainen ML. 1999. Temperature-time relationship in collembolan response to chemical exposure. Ecotoxicology and Environmental Safety 42(3):236-244.

Martikainen EAT, Krogh PH. 1999. Effects of soil organic matter content and temperature on toxicity of dimethoate to Folsomia fimetaria (Collembola Isotomiidae). Environmental Toxicology and Chemistry 18(5):865-872.

Maunsell SC, Kitching RL, Greenslade P, Nakamura A, Burwell CJ. 2013. Springtail (Collembola) assemblages along an elevational gradient in Australian subtropical rainforest. Australian Journal of Entomology 52(2):114-124.

Meli M, Auclerc A, Palmqvist A, Forbes VE, Grimm V. 2013. Population-level consequences of spatially heterogeneous exposure to heavy metals in soil: An individual-based model of springtails. Ecological Modelling 250:338-351.

Nesto N, Romano S, Moschino V, Mauri M, Da Ros L. 2007. Bioaccumulation and biomarker responses of trace metals and micro-organic pollutants in mussels and fish from the Lagoon of Venice, Italy. Marine Pollution Bulletin 55(10-12):469-484.

Osler GHR, Westhorpe D, Oliver I. 2001. The short-term effects of endosulfan discharges on eucalypt floodplain soil microarthropods. Applied Soil Ecology

16(3):263-273.

Pedersen MB, Temminghoff EJM, Marinussen MPJC, Elmegaard N, vanGestel CAM. 1997. Copper accumulation and fitness of Folsomia candida Willem in a copper contaminated sandy soil as affected by pH and soil moisture. Applied Soil Ecology 6(2):135-146.

Reinecke SA, Reinecke AJ. 2004. The comet assay as biomarker of heavy metal genotoxicity in earthworms. Archives of Environmental Contamination and Toxicology 46(2):208-215.

Ringwood AH, Conners DE, Hoguet J. 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters Crassostrea virginica. Marine Ecology Progress Series 166:163-171.

Santos MJ, Ferreira MF, Cachada A, Duarte AC, Sousa JP. 2012. Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei. Ecotoxicology 21(8):2113-2122.

Schnug L, Jakob L, Hartnik T. 2013. The toxicity of a ternary biocide mixture to two consecutive earthworm (Eisenia fetida) Generations. Environmental Toxicology and Chemistry 32(4):937-947.

Schnug L, Leinaas HP, Jensen J. 2014. Synergistic sub-lethal effects of a biocide mixture on the springtail Folsomia fimetaria. Environmental Pollution 186:158-164.

Sjursen H, Holmstrup M. 2004. Cold and drought stress in combination with pyrene exposure: studies with Protaphorura armata (Collembola: Onychiuridae). Ecotoxicology and Environmental Safety 57(2):145-152.

Slotsbo S, Heckmann LH, Damgaard C, Roelofs D, de Boer T, Holmstrup M. 2009. Exposure to mercury reduces heat tolerance and heat hardening ability of the springtail Folsomia candida. Comparative biochemistry and physiology: CBP 150(1):118-123.

Speit G, Hartmann A. 2006. The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. Methods in Molecular Biology 314:275-286.

Sverdrup LEl, Bjørge C, Eklo OM, Källqvist T, Klingen I, Rivedal E, Ropstad E, Skåre JU, Øvrebø S. 2009. Miljørisikovurdering av plantevernmidlet

Sumi-Alpha med det virksomme stoffet esfenvalerat. In: mattrygghet Vf, editor. Vitenskapskomiteen for mattrygghet (VKM): Uttalelse fra Faggruppe for plantevernmidler i Vitenskapskomiteen for mattrygghet. 14 p.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenetics 35(3):206-221.

Tomlin C. 1994. The pesticide manual : a world compendium : incorporating The agrochemicals handbook. Farnham: British Crop Protection Council. 1341 s. p.

Waagner D, Heckmann LH, Malmendal A, Nielsen NC, Holmstrup M, Bayley M. 2010. Hsp70 expression and metabolite composition in response to short-term thermal changes in Folsomia candida (Collembola). Comparative Biochemistry Physiology. Part A, Molecular and Integrative Physiology 157(2):177-183.

Weeks JM, Svendsen C. 1996. Neutral red retention by lysosomes from earthworm (Lumbricus rubellus) Coelomocytes: A simple biomarker of exposure to soil copper. Environmental Toxicology and Chemistry 15(10):1801-1805.

Wentzel JF, Gouws C, Huysamen C, Dyk E, Koekemoer G, Pretorius PJ. 2010. Assessing the DNA methylation status of single cells with the comet assay. Analytical Biochemistry 400(2):190-194.

Wilson JT, Pascoe PL, Parry JM, Dixon DR. 1998. Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, Mytilus edulis L. (Mollusca: Pelecypoda). Mutation Research-Genetic Toxicology and Environmental Mutagenesis 399(1):87-95.

Xu QA, Wang HC, Zhang L, Liu NN. 2006. Sodium channel gene expression associated with pyrethroid resistant house flies and German cockroaches. Gene 379:62-67.

Yan H, Jia H, Wang X, Gao H, Guo X, Xu B. 2013. Identification and characterization of an Apis cerana cerana Delta class glutathione S-transferase gene (AccGSTD) in response to thermal stress. Die Naturwissenschaften 100(2):153-163.

Zhang W, Lei T, Lin Z-Q, Zhang H-S, Yang D-F, Xi Z-G, Chen J-H, Wang W. 2011.

Pulmonary toxicity study in rats with PM10 and PM2.5: Differential responses related to scale and composition. Atmospheric Environment 45(4):1034-1041.