ENDOCRINE DISRUPTION IN SOIL INVERTEBRATES: Assessing Multigeneration Effects of Insect Growth Regulators on *Folsomia candida* and Developing a Toxicoproteomic Approach

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Summary

In the past years, it has been observed that some compounds present in our environment can disturb the reproduction and development of animals like fishes, birds, or reptiles by interfering with their endocrine system. Indeed, these endocrine disrupting compounds (EDC) can mimic or antagonize the effects of hormones, alter the pattern of synthesis and metabolism of hormones or modify hormone receptor levels. These substances represent a risk for wildlife, and possibly for humans. Up to now, endocrine disruption was mainly evaluated for vertebrates and aquatic organisms and for oestrogenic-like substances. However, soil invertebrates, which play an important role in soil functioning, have rarely been considered. Moreover, as their endocrine system differs substantially from those of vertebrates (estrogens do not seem to regulate endocrine functions in invertebrates), other substances than can mimic invertebrate hormones should be taken into account. In this sense, insect growth regulators (IGR), which are third generation insecticides specially developed to interfere with insect endocrine system, are interesting compounds. These substances are supposed to have a high specificity for insect pest and a low toxicity for non-target organisms.

In the first part of this study, the sublethal effects of six IGR (methoprene, fenoxycarb, precocene II, tebufenozide, hexaflumuron and teflubenzuron) were evaluated on the non-target soil arthropod *Folsomia candida*.

The collembola *F. candida* represents an integral and beneficial part of the soil ecosystem. It is an euedaphic (subsurface) species which plays an important role in soil respiration and decomposition processes and is therefore vulnerable to the effects of soil contamination. This ecologically relevant organism is one of the most appropriate invertebrate test species for the assessment of environmental quality. It is recommended as test organism by the international standard ISO 11267.

The 28-days reproduction tests conducted according to this protocol show that *F. candida* is affected by the chosen IGR. The most toxic compounds were the two chitin synthesis inhibitors, teflubenzuron and hexaflumuron, with an EC50 of 0.05 mg/kg (dw) for teflubenzuron and an EC50 of 0.6 mg/kg for hexaflumuron. These concentrations are probably environmentally relevant (toxicity/exposure ratios <5) and thus show that Collembola

population are at risk.

Endocrine disruptive damage induced by pollutants are often not detected by classical toxicity test based on acute or chronic exposure as they can be latent and not manifested until later in life (damage can even arise in the second or third generations of individuals). Therefore, multigeneration tests were developed and conducted with the collembola *F. candida*. Effects of four of the chosen IGR were evaluated on the second generation of collembola according to different types of exposure of the parental generation F_0. In the first set-up, the F_0 generation was exposed for 28 days to the pollutant. Eggs and juveniles from the F_1 generation (generated by the F_0) were also in contact with the pollutant. In the second set-up, only the F_0 generation was exposed to the compound for 10 days. Neither the eggs nor the juveniles from the F_1 generation were exposed to the pollutant. In both cases, the F_2 generation was never in contact with the toxic substance.

Multigeneration tests conducted show that some of the tested IGR (methoprene and teflubenzuron) have an impact on several generations of collembola, although only the F_0 generation was exposed. Moreover, the complementarity of the two experimental set-up (28 days and 10 days) provide important data on the endpoints affected by these compounds which cannot be obtained with classical reproduction or mortality tests.

In the second part of this study, as it is established that a specific and consistent protein response is given by organisms exposed to chemical stressors, a toxicoproteomic approach was conducted with *F. candida* to assess the impact of the chosen IGR on its protein pattern. Two-dimensional gels of *F. candida* were realized and the protein expression profile of 10-day old juvenile collembola was established. As the reproducibility of the protein pattern was quite good, reference mapping was possible and the protein expression profiles of collembola unexposed and exposed to precocene II and methoprene was compared. Only weak alterations of protein expression were observed. It is possible that the use of pools of whole organisms to realize the 2-D gels have masked some of the induced or repressed proteins. The feasibility to identify collembola proteins by MS analysis was determined. Three proteins were identified but the scores obtained were relatively low, probably because of the lack of homologous sequences in the protein databases. The sequencing of *F. candida* genome would facilitate further identifications.

In conclusion, the classical reproduction and multigeneration tests conducted show that the non-target soil arthropod *Folsomia candida* is affected by the chosen insect growth regulators at concentrations that are probably close to environmental levels. Moreover, the transgenerational effects of some of these IGR was demonstrated. This can have crucial consequences on the populations of collembola or other non-target soil invertebrates. The

disappearance of beneficial soil organisms may affect soil fertility and soil community balance and could lead to serious environmental damage. This should be considered in environmental risk assessment. However, there is no evidence that the observed effects can be considered as endocrine disruption. The elucidation of this question would need molecular approaches. The use of proteomics to obtain a specific response (a "protein expression signature") of *F. candida* exposed to chemicals seems to be a promising method to acquire early warning response of exposure to identify toxicants in environmental risk assessment. Moreover, as *F. candida* is a widely used test organism to assess the impact of pollutants in the environment, the obtention of specific protein response to pollutant exposure and in a further time the identification of these proteins would complement the data already available with classical ecotoxicological testing.

Version abrégée

Ces dernières années, on a constaté la présence dans notre environnement de substances ayant la capacité d'interférer avec le système endocrinien des organismes vivants, perturbant ainsi la reproduction et le dévloppement d'animaux tels que les poissons, les oiseaux ou les reptiles. Ces composés, également connus sous le terme de perturbateurs à effets endocrininens, peuvent mimer les effets des hormones, altérer les processus de synthèse et de métabolismes hormonaux ou modifier le niveau des récepteurs hormonaux. Ces substances représentent donc un risque pour l'environnement, la faune et potentiellement pour l'être humain. Jusqu'à présent, la disruption endocrinienne a été principallement étudiée chez les vertébrés et les organismes aquatiques ainsi que pour les substances immitant les oestrogènes. Cependant, les invertébrés du sol qui jouent un rôle prépondérant dans son fonctionnement, n'ont que rarement été pris en considération. De plus, de par le fait que leur système endocrinien diffère substantiellement de celui des vertébrés (les oestrogènes ne semble pas réguler de fonctions endocriniennes chez les invertébrés), d'autres substances pouvant immiter les hormones des invertébrés doivent également être considérées. Les régulateurs de croissance des insectes, insecticides de "troisième générations" spécialement conçus pour intéreférer avec leur système endocrinien semblent donc représenter des substances d'intérêt. Ces insecticides sont considérés comme étant hautement spécifiques pour supprimer les insectes nuisibles et faiblement toxiques pour les organismes non-cibles.

Dans la première partie de ce travail, les effets subléthaux de six régulateurs de croissance des insectes (le méthoprene, le fenoxycarb, le précocène II, le tébufénozide, l'hexaflumuron et le téflubenzuron) ont été évalué sur un organisme non-cible du sol, l'arthropode *Folsomia candida*. Le collembole *F. candida* est partie intégrante et bénéfique de l'écosystème du sol. C'est une espèce euedaphique qui joue un role important dans les processus de respiration et de décomposition du sol. Il est donc vulnérable aux effets de contamination des sols. Cet organisme, représentatif pour l'écologie, est l'espèce test d'invertebrés la plus appropriée pour évaluer la qualité de l'environnement. Il est recommandé en tant qu'organisme de test par la norme ISO 11267.

Les tests de reproduction "28 jours" conduits selon ce protocole ont mon-

tré que *F. candida* est affecté par les régulateurs de croissance des insectes choisis. Les composés les plus toxiques sont les deux inhibiteurs de la synthèse de la chitine, le téflubenzuron et l'hexaflumuron, avec une EC50 de 0.05 mg/kg pour le téflubenzuron et une EC50 de 0.6 mg/kg pour l'hexaflumuron. Ces concentrations sont proches de celles que l'on pourrait trouver dans l'environnement et indiquent que les populations de collemboles courrent un risque.

Les dommages relatifs à la disruption endocrinienne induits par des polluants ne peuvent généralement pas être détéctés par des tests de toxicité classiques basés sur la toxicité aigue ou chronique. En effet, ces dommages sont souvent latents et ne se manifestent que tardivement dans un cycle de vie. Ils peuvent même n'apparaître qu'au cours de la deuxième ou troisième génération d'individus. Pour cette raison, il nous a paru important de réaliser des tests multigénérationnels avec le collembole *F. candida*. Les effets de quatre régulateurs de croissance des insectes ont été évalués sur la seconde génération de collemboles selon différents modes d'exposition de la génération parentale (F_0). Dans le premier mode expérimental, la F_0 a été exposée durant 28 jours au polluant. Les oeufs et les juvéniles de la génération F_1 (générés par la F_0) ont également été en contact avec le polluant. Dans le second mode opératoire, seul les individus de la génération F_0 sont exposés au composé toxique pendant 10 jours. Ni les oeufs ni les juvéniles de la génération F_1 ne sont exposés au polluant. Dans les deux cas, la géneration F_2 n'a jamais été exposée à la substance toxique.

Les tests multigénérationnels menés ont montré que certains des régulateurs de la croissance des insectes testés (le méthoprène et le téflubenzuron) pouvaient avoir un impact sur plusieurs générations de collemboles, bien que seule la génération parentale (F_0) aie été exposée. De plus, la complémentarité des deux modes opératoires (28 jours et 10 jours d'exposition de la F_0) permet de fournir des informations importantes sur ces composés. Ces informations ne peuvent pas être obtenues par des tests de reproduction ou de mortalité classiques.

Dans la seconde partie de ce travail, une étude de toxicoprotéomique a été conduite avec le collembole *F. candida* afin d'évaluer l'impact des substances choisies sur son pattern de protéines. En effet, il a été démontré qu'une réponse consistante et spécifique des protéines était fournie par les organismes exposés à des facteurs de stress environnementaux. Des gels d'électrophorèse en deux-dimensions ont donc été établis pour le collembole *F. candida*. Le profil d'expression de protéines d'individus âgés de 10 jours a été constitué. La reproductibilité du pattern de protéines étant relativement bonne, des cartes de référence protéiniques ont pu être établies, permettant ainsi de comparer les patterns de protéines de collemboles exposés au précocène II et méthoprène. Seules de faibles altérations du profil d'expression des protéines ont été observées. Il n'est pas improbable que le fait d'avoir utilisé un groupe d'organismes entiers pour la réalisation des gels 2D ai pu masquer certaines

des protéines induites ou réprimées. La faisabilité d'identifier des protéines de *F. candida* par spectrométrie de masse a été déterminée. Trois protéines ont pu être identifiées mais les scores obtenus étaient relativement faibles, probablement à cause du manque de séquences homologues dans les bases de données de protéines. Le séquençage du génome de *F. candida* pourrait faciliter les identifications futures.

En conclusion, les tests de reproduction classiques et les tests multi-générationnels réalisés ont permis de démontrer que l'arthropode et organisme non-cible du sol *Folsomia candida* est affecté par les régulateurs de la croissance des insectes testés, et ceci pour des concentrations probablement proches de celles qui pourraient être retrouvées dans l'environnement. De plus, l'effet transgénérationnel de certaines de ces substances à également été démontrés. Ceci pourrait avoir des conséquences dramatique pour les populations de collemboles et autres invertébrés non-cibles du sol. La disparition d'organismes bénéfiques pour le sol pourrait affecter sa fertilité ainsi que l'équilibre de la communauté du sol, conduisant à de sérieux dommages environnementaux. Il serait judicieux d'en tenir compte lors d'une évaluation du risque environnemental. Cependant, il n'y a pas de preuves que les effets observés soient liés à de la disruption endocrinienne. L'élucidation de cette question nécessiterait des études moléculaires. L'utilisation de la protéomique pour l'obtention d'une réponse spécifique (une "signature des protéines") de *F. candida* exposé à des substances toxiques semble être une approche prometteuse. Elle permettrait d'acquérir des marqueurs d'exposition précoces pour l'identification de substances toxiques dans une évaluation du risque environnemental. De plus, de par le fait que *F. candida* est un organisme de test fréquement utilisé pour évaluer l'impact de polluants sur l'environnement, l'obtention d'un pattern de protéines spécifiques en réponse à une exposition à des substances toxiques et dans un autre temps, l'identification de ces protéines permettrait de compléter les données déjà établies par les tests d'écotoxicité classiques.

Contents

List of Tables

List of Figures

Glossary

Ametabolous Undergoing slight or no metamorphosis.

Arthropods Any of numerous invertebrate animals of the phylum Arthropoda, including the insects, crustaceans, arachnids, and myriapods, that are characterized by a chitinous exoskeleton and a segmented body to which jointed appendages are articulated in pairs.

Balsam fir North american tree (Abies balsamea, Pinacea) having a pyramidal shape and flattened needles. Also called Canada balsam, as is the resin it produces, which is used as an adhesive in optical lenses and glass slides.

Cnidaria Any of various invertebrate animals of the phylum Cnidaria, characterized by a radially symmetrical body with a saclike internal cavity, and including the jellyfishes, hydras, sea anemones, and corals. Also called coelenterate.

EC50 Effect concentration, concentration causing 50% of effects.

ECx Effect concentration, concentration causing x% of effects.

Ecdysis The shedding of an outer integument or layer of skin, as by insects, crustaceans, and snakes; molting.

Ecdysone A steroid hormone produced by insects and crustaceans that promotes growth and controls molting.

Ecdysteroid Arthropod steroids derived from cholesterol.

Endocrine disrupter Exogenous substance that cause adverse health effects in an intact organism or its progeny, consequent to changes in endocrine function.

Euedaphic organism Soil dwelling organism.

Hemimetabolous Undergoing a metamorphosis that lacks a pupal stage.

Holometabolous Undergoing complete metamorphosis.

Instar A stage of an insect or other arthropod between molts.

Juvenile Hormone In insects, juvenile hormone refers to a group of hormones which ensure growth of the larva, while preventing metamorphosis. It is secreted by a pair of endocrine glands behind the brain called the corpora allata. Juvenile hormone is also important for the production of eggs in female insects.

LC50 Concentration that causes 50% of mortality relatively to the control

LOEC Lowest Observed Effect Concentration, lowest concentration out of the tested concentrations at which a statistically significant difference from the control group is observed.

NOEC No Observed Effect Concentration, tested concentration just below the LOEC (no statistically significant difference is observed compared to the control)

Paracrine signalling Form of signalling in which the target cell is close to the signal releasing cell. The signal chemical is broken down too quickly to be carried to other parts of the body.

Pheromones A chemical substance secreted externally by some animals (especially insects) that influences the physiology or behavior of other animals of the same species, often functioning as an attractant of the opposite sex.

Proteomics Large-scale study of proteins, particularly their structures and functions.

Xenobiotics A xenobiotic (Greek, xenos “foreign”; bios “life”) is a compound that is foreign to a living organism.

Chapter 1

General Introduction

1.1 Overview

These last decades, protection of the environment from contamination by natural or synthetic substances emitted by human activities have become matters of growing concern. Ecological disasters are often discussed in newspapers and other media. One of the main characteristics of human pollution is voluntary or involuntary spreading of substances, such as pesticides, hydrocarbons or metals which can contaminate many of the biosphere compartments like atmosphere, hydrosphere or lithosphere and this even far away from their initial site of emission. At the present time, no more unpolluted ecosystem can be found on the globe and even areas without human activities are contaminated by pollutants carried by airstreams or by ocean currents (Lagadic *et al.*, 1998).

Although pollution of ecosystems is a current topic, this phenomenon is not recent and seems to have become more important at the beginning of the 19th century when demographic growth and new technologies from Industrial Revolution have led to an increase of the impact of human activities on the environment (Lagadic *et al.*, 1998). It was only at the beginning of years 1960 that significant effects of industrial and agricultural pollutants on the environment were noticed. Nowadays, the quantity of substances released in the biosphere is still increasing. At the beginning of the 21st century, it was estimated that around 70'000 chemicals were commonly used for a wide variety of purposes in our global community and that the rate of introduction of news substances was in the order of 200-1000 compounds per year (Connell *et al.*, 1999).

Moreover, it has been observed in the past years that some pollutants can disturb the reproduction and development of animals like fishes, reptiles and birds. The observed abnormalities vary from subtle changes to permanent alterations, including disturbed sex differentiation with feminized or masculinized sex organs, changed sexual behavior, and altered immune function (Vos *et al.*, 2000). It is thus an urgent need to protect the environment and

to regulate industrial and human activities which may cause pollution and adverse health effects on living organisms.

1.2 Ecotoxicology

The task of ecotoxicology is to assess, monitor and predict the fate of foreign substances in the environment. This definition encompasses the chemical characterization of contaminants that are present in our environment, the need to monitor them in order to assess whether environmental loads are increasing or decreasing and the need to predict their impacts through studies involving modern methods of toxicology and ecology (Moriarty, 1983).

As said above, one of the major concerns of ecotoxicology are the interactions between living organisms and toxic chemicals in the environment (Connell *et al.*, 1999). Assessment of environmental quality can be evaluated by two main methods. In the first one, a chemical approach is used for the detection and quantification of pollutants in physical and biological matrices (analytical chemistry). The second method deals with the assessment of the effect of pollutants on individuals or on communities (biological approach). These two approaches are complementary and should be used together for a good environmental risk assessment.

Regarding the biological approach, different methodologies can be used to evaluate the impact of substances on living organisms. For example, *in situ* observations of presence or absence and abundance of some species, defined as bioindicators, can give information on the quality of an ecosystem (Lagadic *et al.*, 1998). Other methods like ecotoxicity tests can also provide important information on the impact of substances on the environment.

1.2.1 Ecotoxicity testing

Ecotoxicity tests are biological experiments performed to examine if either a potentially toxic compound, or an environmental sample (e.g., effluent, sediment or soil sample) causes a biologically important response in test organisms, and if so to determine the concentration that produces a given level of effects (ECx, LCx, LOEC ...) or produces an effect that cannot be distinguished from background variation (NOEC) (OECD, 2003). One of the most important criteria for the choice of test organisms is their representativeness of ecosystem.

The great majority of ecotoxicity tests are done in the laboratory where they are easier to conduct and effects easier to measure (but unfortunately less representative) than tests realized *in situ*. From an historic perspective, aquatic ecotoxicology was developed earlier and therefore more intensively than soil ecotoxicology.

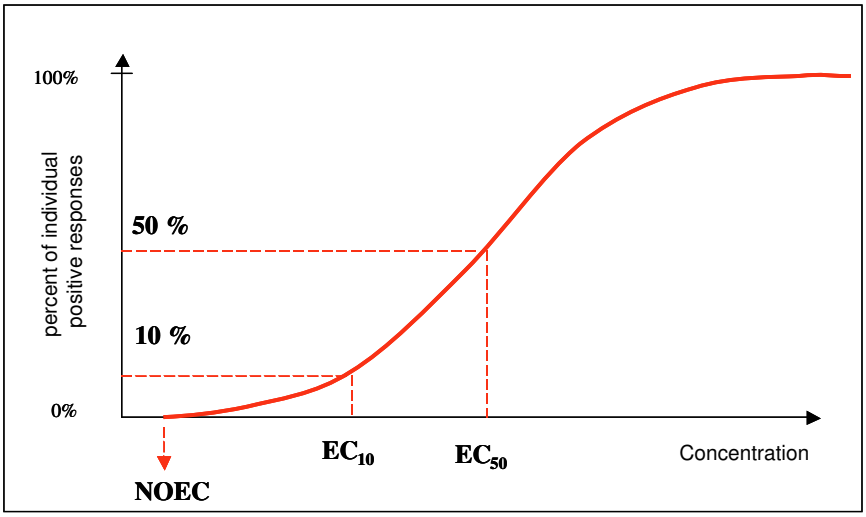

Figure 1.1: Dose response curve of a pollutant with some remarkable values (EC_{10}, EC_{50}, NOEC)

Principle of a test

In a test, organisms are exposed to different concentrations or doses of a test substances or a test substrate (e.g., water, sludge, or a contaminated soil or sediment), generally diluted in a test medium. Typically, at least one group of test organisms (the control group) is not exposed to the test substance or the substrate, but is otherwise treated in the same way as the exposed organisms.

The endpoint observed or measured in the different batches may be the number of surviving organisms, size, growth of organisms, number of eggs or offspring produced or any relevant biochemical or physiological variable that can be reliably quantified. Observations are made after one or several predefined exposure times. The endpoint's relationship with the concentration of the tested chemical or substrate is examined (OECD, 2003).

Test duration varies; acute toxicity tests are conducted over a short period of time (relative to the organism generation time) and measure effect such as mortality. Chronic toxicity tests are performed over a more longer period. Reproduction is the most commonly measured parameter of this kind of test.

When conducting ecotoxicity tests, biomarker responses can also be measured in individuals exposed to pollutants (see 1.3).

1.2.2 Soil ecotoxicology

Soil is an important habitat that upholds rich communities of organisms. Its contamination is mainly due to chemical compounds that are spread by pesticide application, fertilization, recycling of waste water or atmospheric deposition. By direct action or by interaction with other impact factors, flora, fauna and micro-organisms can be affected. Thus, the biodiversity may be changed. Soil fertility, agricultural products, non-food crops, surface water and groundwater may be impacted. Indeed, even though soil has a very limited function as a transport medium, it may be an important source for contamination of groundwater or for emission of volatile chemicals to the atmosphere (Løkke & van Gestel, 1998).

There is thus an increasing need for appropriate test methods as tools for measuring soil quality and the effects of xenobiotics. Soil ecotoxicology being a relatively young science, there are only three standard bioassays with soil invertebrates described in international standards and used for routine determination: one concerning the earthworm *Eisenia fetida* (OECD, 1984; ISO, 1993), one with the enchytraeids and one with the springtail *Folsomia candida* (Collembola) (ISO, 1999). However, several OECD and ISO standards with other organisms are under development and some of them will be available soon. Collembolla can be exposed to contaminants via the soil and/or food in tests that examine life-history parameters, bioaccumulation, and/or effects on behavior. Such tests assess the toxicity of a wide range of organic and inorganic pollutants and have been used as bioassays to monitor the success of remediation of contaminated soil (Fountain & Hopkin, 2001). More details on tests with collembola are given in chapter 2

1.3 Biomarkers

The effects of chemical contaminants can be viewed as occurring at different levels of biological organization, extending from the molecular or biochemical level, to the physiology of the individual, and ultimately to the levels of population and ecosystem (Stegeman *et al.*, 1992). Biochemical or molecular alterations are usually the first detectable and quantifiable responses to environmental change. Moreover, alterations in biochemical systems are often more sensitive indicators than those at higher levels of biological organization, such as organisms and populations . In this sense, a difference is made between the term biomarker and the term bioindicator in as much as the first one normally refers to lower levels of biological organization, whereas with the latter one, emphasis is generally at the species level (Bucheli & Fent, 1995).

A biomarker can thus be described as a parameter reflecting the interaction between a biological system and an environmental agent and is detected at the molecular, biochemical, cellular and physiological level of an organism

following it's exposure to pollutants (Peakall & Shugart, 1992; Depledge & Fossi, 1994).

Biomarkers represent initial responses to environmental perturbations and contamination (de Lafontaine *et al.*, 2000) and relate in a dose- or time-dependent manner the degree of dysfunction that the contaminant has produced (Mayer *et al.*, 1992). By showing a short response time and a high sensitivity to exposure, they permit a rapid detection and evaluation of the impact of pollution on living organism and may therefore be used as an "early warning system" for assessing environmental health.

Moreover, conventional tools of environmental monitoring may assess levels of contamination, as well as state of environmental health, but they can hardly serve as a link in between. The use of biomarkers may bridge the gap between cause and effect, thus filling the lack of knowledge about causal relationships between environmental contamination and biological response (Bucheli & Fent, 1995).

Biomarkers can be classified into three different groups:

- Biomarkers of exposure: level of a toxic substance, or its metabolite, in a biological fluid of an organism; measurement of an early reversible biochemical change in a biological fluid that reflects exposure
- Biomarkers of effect: assessment of the potential adverse effects of a chemical on a physiological process or organ system (e.g. mutation in response to a mutagen exposure)
- Biomarkers of susceptibility: innate or induced capability of the individual to respond to the exposure to an environmental toxicant (e.g detoxification mechanism) (Ward Jr & Henderson, 1996; Lowry, 1995)

These divisions can be considered as somewhat arbitrary, all types representing a continuum of responses of organisms to chemicals (Bucheli & Fent, 1995).

1.4 Endocrine disruption

In the past years, observations and scientific evidences have demonstrated that some environmental pollutants can impair fertility and development of animals, and possibly of humans, by interfering with their endocrine system. These xenobiotics are known under the term of "endocrine disruptors" and are defined as "exogenous substances that cause adverse health effects in an intact organism or its progeny, consequent to changes in endocrine function" (OECD, 2001a).

Endocrine disrupting compounds (EDC) may disturb directly or indirectly the hormonal system of organisms. They may act to (Depledge *et al.*, 1994):

- mimic the effects of hormone
- antagonize the effects of hormones
- alter the pattern of synthesis and metabolism of hormones
- modify hormone receptor levels

In consequence, alteration of endocrine function caused by an endocrine disruptor may be through interference of processes such as the synthesis, secretion, transport, binding, action or elimination of natural hormones that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior of the organism (Vos *et al.*, 2000).

Up to now, diverse substances are known to have an endocrine disrupting action. For example, some of them like PCB, DDT and alkylphenols can disrupt estrogen receptor function of vertebrates. Related reproductive effects to such compounds on aquatic and terrestrial mammals could be sterility, hermaphroditism, decreasing fecundity, feminization or masculinization. Many other reproductive and also non-reproductive effects of endocrine disrupting compounds are also reported for other vertebrates like fishes, amphibians or birds (Vos *et al.*, 2000). However, invertebrates and particulary soil invertebrates have rarely been considered.

Different kind of methods are available to establish endocrine effects of a chemical. *In vivo* methods (e.g., reproductive and developmental studies) allow to determine the endocrine effects of substances in the intact organism. *In vitro* methods are used to analyze effects at the level of cells and molecules (OFEFP, 1999).

Endocrine disruption can show unusual dose-response profiles and markedly greater sensitivity compared with other toxic mechanisms (OECD, 2001a). Many endocrine compounds seem to act at very low concentrations, probably close to hormone levels. Moreover, dose, body burden, timing, frequency

and duration of exposure at critical periods of life are important considerations for assessing adverse effects of an endocrine disrupters (Vos *et al.*, 2000).

1.5 Objective of the work

The aim of this project was to investigate the effect of endocrine disrupting compounds (EDC) on the non target soil arthropod *Folsomia candida* which is a very useful organism for soil fertility and soil decomposition processes. In order to reveal these effects on the endocrine system of this organism, a toxicoproteomic approach was developed aiming to identify potential "early warning" biomarkers for endocrine disruption.

Endocrine disruption (ED) by man-made chemicals in vertebrates is well established through both laboratory and field studies. Knowledge of endocrine disruption in invertebrates is much more limited even though ED in aquatic invertebrates has become matter of growing concern. As soil invertebrates play an important role for the function and fertility of a soil, it seemed therefore very important to evaluate a potential effect of these chemicals on their reproduction.

In this sense, the first part of this work is focused on the impact of third generation insecticides, the insect growth regulators (IGR), on the ecologically relevant model organism, the collembola *F. candida*. These insecticides have been specially developed to disrupt insect endocrine functions in order to suppress their population by stopping their proliferation.

In **chapter 4**, the toxicity of several IGR was evaluated on the survival and reproduction rate of *F. candida*. Effects of the juvenile hormone analogs methoprene and fenoxycarb, the anti-juvenile hormone precocene II, the ecdysone agonist tebufenozide and the chitin synthesis inhibitors teflubenzuron and hexaflumuron were determined after 28 days of exposure according to the ISO standard 11267. The toxicity of diuron, an herbicide known to potentially act as an endocrine disruptor in vertebrates, was also evaluated.

As EDC can potentially have an effect on several generations of individuals, which could have a consequence for the survival of a species and thus for the soil ecosystem, multi-generation test methods were developed for *F. candida*. Two-generation tests were conducted with some of the choosen IGRs and effects of these compounds on the F_2 generation of *F. candida* were evaluated. Different protocols that allows or not exposure of eggs or juveniles of the F_1 generation were established. All these data are presented in **chapter 5**.

The second aim of this project was to detect and analyze possible changes in the protein synthesis of *F. candida* induced by exposure to IGR. An attempt was made to find protein biomarkers that could highlight exposure

of *F. candida* to EDC and the effects of these substances on the endocrine system of these invertebrates.

Chapter 6 presents the methods developed for the realization of two-dimensional gel electrophoresis with whole organisms of *F. candida*. The protein pattern of *F. candida* was established for various ages, exposure times and exposure concentrations with methoprene, fenoxycarb and precocene II. The impact of the type of substrate on the protein expression profile was also evaluated. Different kind of gel staining methods were tested to maximize the possibilities of protein identification and characterization in *F. candida* by mass spectrometry technics. Attempts were made to identify some of the most important proteins by LC-MS-MS.

Chapter 2

Studied parameters

2.1 Collembola

Collembola, commonly known as springtails, are among the most abundant arthropods on Earth. They are very profuse in soil ecosystems but they have also radiated into many other niches, from the littoral zone to mountaintops, and are particulary affluent in epiphytes of tropical rain forest (Fountain & Hopkin, 2005).

The systematic position of collembola in Arthropoda phylum is not well defined. It seems now that they are no longer considered as primitive apterous insects but as Hexapoda or Crustacea. Following the conventional hypothesis of Collembola as basal Hexapoda, they are considered to form the largest of the three orders of modern Hexapods that are no longer considered to be insects along with Protura and Diplura (Fig. 2.1). An alternative hypothesis regard them as a unique and ancient group of well adapted terrestrial crustaceans (Janssens, 2005).

Approximately 7'600 different species are known to exist (Janssens, 2005). Their population densities can reach up to 200'000 individuals per square meter in soil and leaf litter layers (Wiles & Krogh, 1998; Massoud, 1971) where they play an important role for soil functioning such as decomposition processes and soil respiration. Springtails occupy a key position in the soil food web being consumers of fungi, detritus and nematodes (Laskowski *et al.*, 1998; Lee & Widden, 1996). They are also prey animals for a wide variety of invertebrates such as mites, centipedes, spiders, carabid and rove beetles (Wiles & Krogh, 1998). Study of bioaccumulation of chemicals could thus be important for predicting food chain transfer (Hopkin, 1997).

As they are an integral part of soil ecosystems, collembola are vulnerable to the effects of soil contamination. The abundance and diversity of collembola have been used widely to assess the environmental impact of a range of pollutants on soils (Fountain & Hopkin, 2005). They possess many attributes of the ideal experimental organism for ecotoxicological research. They are ecologically important, widely distributed, often highly abundant,

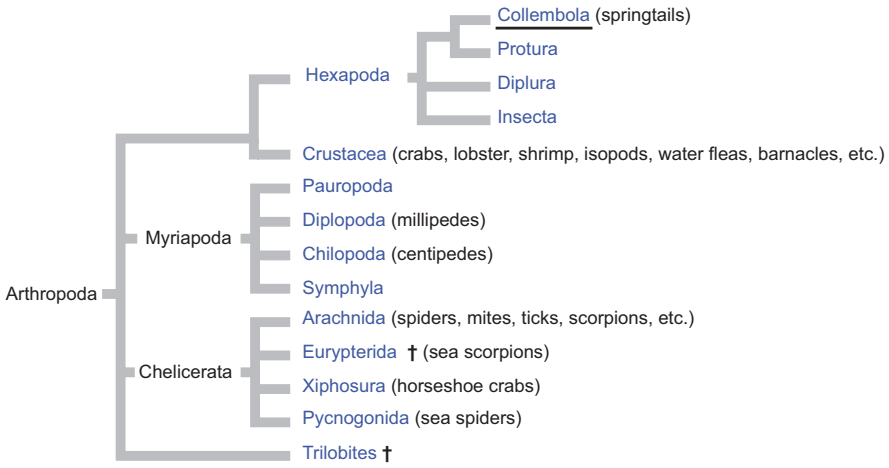

Figure 2.1: Systematic position of collembola within arthropod taxa following the conventional hypothesis (adapted from "tree of life web project" (http://tolweb.org/tree/phylogeny.html))

easily sampled in the field, can be cultured or maintained in laboratory and have a rapid life cycle with a high reproduction rate (Spahr, 1981). The springtail *Folsomia candida* is the most intensively studied species of Collembola. It is a routinely used model arthropod for ecotoxicity test and an international standard is available (ISO, 1999).

2.1.1 *Folsomia candida*

Distribution

Folsomia candida (Isotomidae) is a white or faintly yellowish in color species from 1.5 to 3.0 mm in length at maturity and which does not bear ocelli. It is often found in caves and mines. Elsewhere, this euedaphic species inhabits agricultural systems, soils with a high level of organic matter, forests and edges of streams (Fountain & Hopkin, 2005). It has been observed in most biogeographic regions except Africa and India (Fig. 2.2)(Hopkin, 1997).

External anatomy and morphology

The body of *F. candida* is divided into a head, a thorax with three segments, each bearing a pair of legs, and an abdomen with six segments (Fig. 2.3). The head bears two antennae that are often waved in the air to pick up airborne chemicals as the animal walks along (Hopkin, 1997). Like all other collembola, *F. candida* has a pair of thin-walled, closely apposed, eversible vesicles on the ventral side of the first abdominal segment. This structure is commonly known as the ventral tube, or collophore, and is involved in electrolyte balance and fluid exchange with external environment. The ventral tube is an important exposure route for chemicals dissolved in soil pore water (Fountain & Hopkin, 2005). A well developed furca (springing organ) is present and is used for rapid locomotion and to escape from predators (Hopkin, 1997). Most Collembola respirate through a mechanism of cutic-

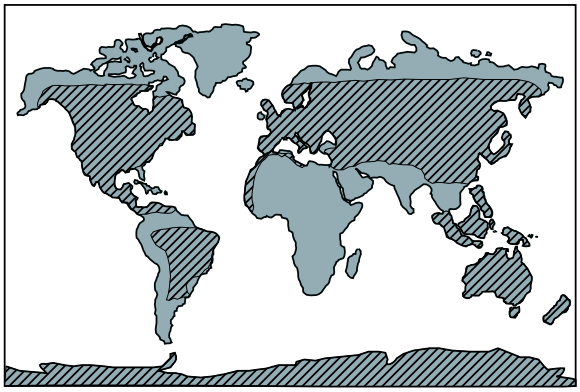

Figure 2.2: Worldwide geographic distribution of *F. candida* (hatched area) (adapted from Janssens (2005))

ular gas diffusion, in which the eversible vesicles of the collophore play an important role (Janssens, 2005). Their multilayered cuticle is hydrophobic due to the presence of a waxy layer.

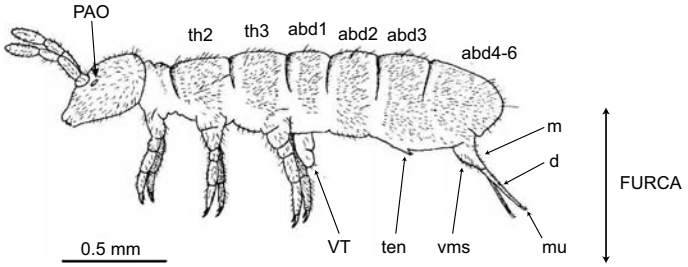

Figure 2.3: Adult female *F. candida*. th2, th3: thoracic segment (the first one is reduced dorsally); abd1 to abd6: abdominal segments (abd4-6 are fused together); PAO: post-antennal organ; VT: ventral tube; ten: tenaculum (holds the furca in place under the body)(Fountain & Hopkin, 2005)

Internal anatomy

All collembola possess a full complement of internal organs. The basic arrangement in all springtails is a head containing salivary glands and labial nephridia or kidneys and a digestive tract which is surrounded by the other thoracic and abdominal organs. A neurosecretory system and a brain are also present. Salivary glands secrete enzymes onto the food whereas labial nephridia collect fluids that can be regarded as urine (Hopkin, 1997). The digestive system of *F. candida* is essentially cylindrical and consists in a cuticle lined foregut and hindgut, and a midgut derived from endodermal cells that produce enzymes and absorb products of digestion. The gut passage time of *F.*

candida at 20 °C is approximately 35 min. The lining of the midgut is shed and voided in the feces during molting. This provides an important route of excretion of waste products and pollutants stored in the midgut cells as part of a storage detoxification system. Collembolans probably follow this strategy due to their lack of Malpighian tubules (insects main organ of excretion and osmoregulation helping them to maintain water and electrolyte balance) (Fountain & Hopkin, 2005). Neurosecretory system is similar to that of insects and is involved in the secretion of hormones (see 2.2). However, secretory cells and *Corpora cardiaca*, present in insects, are laking in collembola (Hopkin, 1997; Janssens, 2005).

Development and reproduction

The population of *F. candida* consists of parthenogenetic females. At 20 °C (ISO standard test temperature), they reach the adult instar and are sexually mature at the age of 21 to 24 days. Eggs are often laid in communal heaps, in which females add to previously laid batches. There are approximately 30 to 50 eggs per batch, which take 7 to 10 days to hatch. The eggs are white when laid, spherical and 80 to 110 μm in diameter. The optimal temperature for hatching success is 21 °C. At lower temperatures, the time span for each developmental stage is extended. Crowding (>1 animal cm^2) reduces the number of eggs laid. Stress from jostling, pheromones or contamination of substrate by waste products may be the causes of reduced fecundity. Although *F. candida* is blind, it seems that more eggs are produced in constant darkness than in a light:dark cycle, which suggests the presence of internal photoreceptors (Fountain & Hopkin, 2005).

The development of *F. candida* is direct, without metamorphose (ametabolous), adults differing from juveniles in proportion and size. Molting occurs during it's whole life span which is 136 days in average. An adult female may go through 45 molts in her lifetime with short reproductive instars (duration 1.5 days) alternating with longer nonreproductive instars (duration 8.5 days) (Snider, 1973; Fountain & Hopkin, 2001). During molting, springtails secrete an exuvial fluid which fills the space between the old and new cuticle. The fluid may contain enzymes which dissolve the inner layer of the old cuticle (Hopkin, 1997).

2.2 Insect endocrinology

Although collembola are no more considered as insects and although their neurosecretory system is not exactly the same, a brief introduction to insect endocrinology will be made as the compounds (IGR) tested in our experiments were specifically developed to interfere with insect hormonal systems. Furthermore, relatively little information is available on the endocrine system of collembola.

2.2.1 Introduction

Any multicellular organism requires coordinating mechanisms that involve various chemical messengers. Nervous coordination has appeared very early during evolution, and complex neuronal networks already exist among Cnidarians. Controls over longer distances involving diffusible substances (paracrine and endocrine mechanisms) have been progressively superimposed to these ancestral mechanism. Indeed, during evolution, the endocrine system increased in complexity and appearance of endocrine cells in annelids was followed by the appearance of endocrine glands in mollusks. In the same way, appearance of neurohormones has preceded that of true hormones. For invertebrates, the complexity of the endocrine system seems to culminate in Arthropods (however, there can be a lack of knowledge for the other groups) (Lafont, 2000a). Some of the major invertebrate hormones are given in table 2.1.

Two sets of hormones exist in invertebrates, those which show a good similarity between vertebrates and invertebrates and clearly arise from common ancestral molecules, and invertebrates-specific ones which must have arisen more recently (Lafont, 2000a). Some xenobiotics can alter the endocrine system by simulating hormones (see 1.4 and 4.1) . They could thus mimic a common ancestral hormone, endangering several invertebrate and/or vertebrate taxa. However, as hormone structure is more conserved than hormone function through evolution, the risk seems to be low.

2.2.2 Insect endocrine system and hormones

The insect endocrine system is composed of different categories of hormone-secreting structures (Fig. 2.4) that help to maintain homeostasis, coordinate behavior and regulate growth, development and other physiological activities. The major types of hormones regulating insect life are ecdysteroids (ecdysone, 20-hydroxyecdysone) (Fig. 2.5), terpenoids (Juvenile hormone (JH))(Fig. 2.6) and neurosecretory peptide hormones (Riddiford, 1994). For more details on insect hormone-secreting structures, see table 2.2

Ecdysteroids and JH control both growth and development and later reproductive maturation. The neurohormones regulate the release of these two

Group	Hormone	Source
Annelids		
Polychaetes	Feed-back substance	Submature Oocytes
Molluscs		
Gastropods	Gonadotropic Hormone	Dorsal bodies
	Schitosomin (Lymnaea)	Brain/hemocytes?
	"Vertebrate-type" steroids	Gonads
Cephalopods	Gonadotropic hormone	Optic glands
Arthropods		
Insects	Juvenile Hormones (JHs)	*Corpora allata* (male accessory gland?)
	Ecdysteroids	Molting glands, gonads (other tissues?)
	ETH (ecdysis-triggering hormone)	Epitracheal glands (Inka cells)
	TMOF (trypsin-modulating oostatic factor)	Ovaries
Crustaceans	Methyl farnesoate (MF)	Mandibular organs
	Ecdysteroids	Y organ and gonads
	Androgen hormone	Androgen glands

Table 2.1: Major invertebrate hormones (Lafont, 2000a)

hormones and also a variety of homeostatic activities and behavior (Riddiford, 1994; Lafont, 2000b) (see section 2.2.3).

Ecdysteroids and JHs act at the genomic level by binding specific nuclear receptors where they stimulate the transcription of inducible genes (Lafont, 2000a,b).

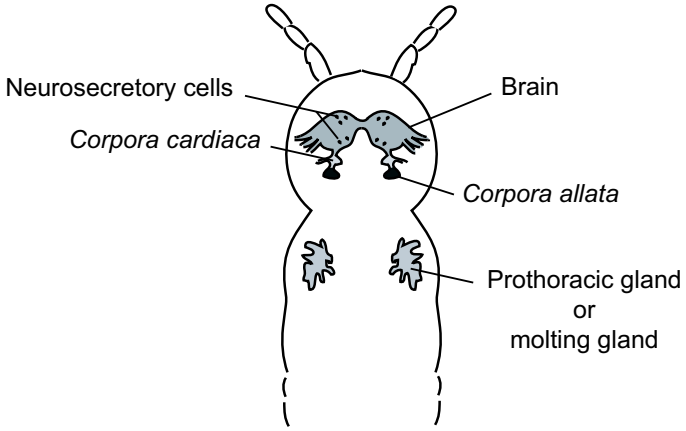

Figure 2.4: Overview of insect endocrine system

Ecdysteroids

Ecdysteroids are arthropod steroids derived from cholesterol (Lafont, 2000b). Indeed, as insects and other arthropods are unable to *de novo* synthesize cholesterol, they rely on dietary sterols (either cholesterol or phytosterol).

Ecdysteroids source is different between larvae and adults (Lafont, 2000a). In the larval stage, they are produced by the prothoracic glands (Fig. 2.4) (or by epidermal cells in some insect species), and they coordinate and trigger events such as molting and metamorphosis. In adult insects, molting glands (or prothoracic glands) degenerate but ecdysteroids are still present. They are produced by endocrine cells of the gonads and are involved in the control of several reproductive processes (Lafont, 2000b,a).

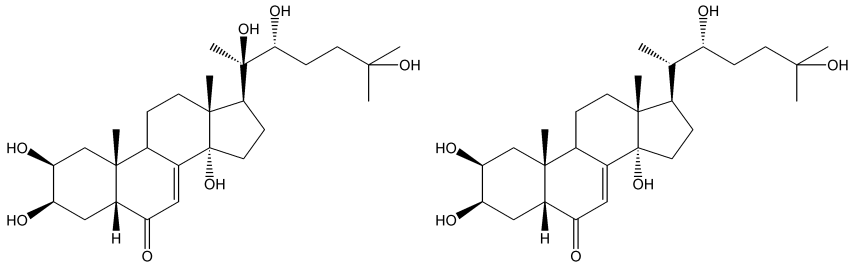

Figure 2.5: Chemical structures of the major ecdysteroids in insects

Juvenile hormone

Juvenile hormones are sesquiterpenes produced by the *Corpora allata* (Fig2.4). Juvenile hormones maintain insects in the larval stage by interacting with ecdysteroids and by controlling the type of molt. JH disapearance allows insects to reach the adult stage, either directly (hemimetabolous insects) or through a metamorphosis (holometabolous insects). Like ecdysteroids, they also have a gonadotropic role in adult insects and are involved in adult insect reproduction (vitellogenesis induction). A major difference to ecdysteroids is that the biosynthetic site (the *corpora allata*) is the same in larva and adults (Lafont, 2000b)

Different structurally related molecular forms of JH occur in insects such as JH I, JH II, JH III. The most common of them is JH III (Fig. 2.6).

2.2.3 Role of hormones in molting and metamorphosis

The cyclical processes of growth and molting in insects are brought about by two hormones, one produced by the neurosecretory cells (NSC; specialized nerve cells (neurons) found in the brain, see Figure 2.4) in the brain and the other by the prothoracic glands (PG). The product of the NSC, prothoracicotropic hormone (PTTH), is secreted periodically in response to environmental "signals" and activates the PG. Activation of the PG results in an increased rate of synthesis of the prohormone, ecdysone, which is transported by hemolymph and converted in the molting hormone (20-hydroxyecdysone (20E)) in peripheral tissues and fat body. The action of 20E on the epidermal cells results in the initiation of the molting process: retraction of the epidermis from the old cuticle (apolysis); deposition of a new cuticle; and ultimate shedding of the remnant of the old cuticle (ecdysis). A third hormone, the juvenile hormone (JH), is secreted by the *corpora allata* (CA), paired endocrine glands located near the insects brain. The role of JH

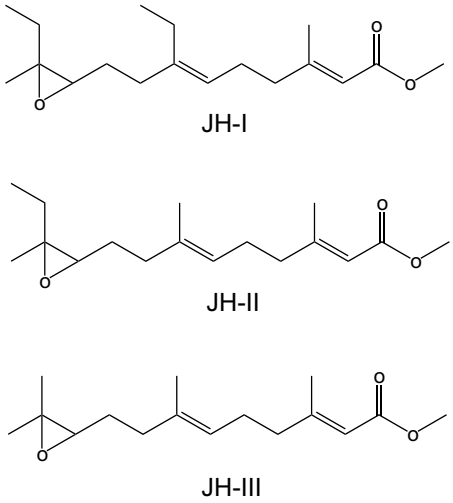

Figure 2.6: Chemical structures of the major juvenile hormones in insects

in the molting process is to determine the character of the molt initiated by 20E. It is generally believed that a high JH titer dictates a larval-larval molt, while a low JH titer permits a larval-pupal molt (first metamorphic molt); in the presumed absence of JH, pupal-adult metamorphosis occurs (Fig. 2.7). In many adult insects, JH is again synthesized by the CA and this time functions as a gonadotropin. Precise quantities of these three hormones must be secreted at critical stages and titers maintained for specific periods of time in order for normal development to proceed. Although only the three hormones controlling post-embryonic development (PTTH, 20E, JH) are considered, these and a variety of largely uncharacterized hormones, presumably peptides, control an array of homeostatic and behavioral events in the life of the insect (Gilbert *et al.*, 1980).

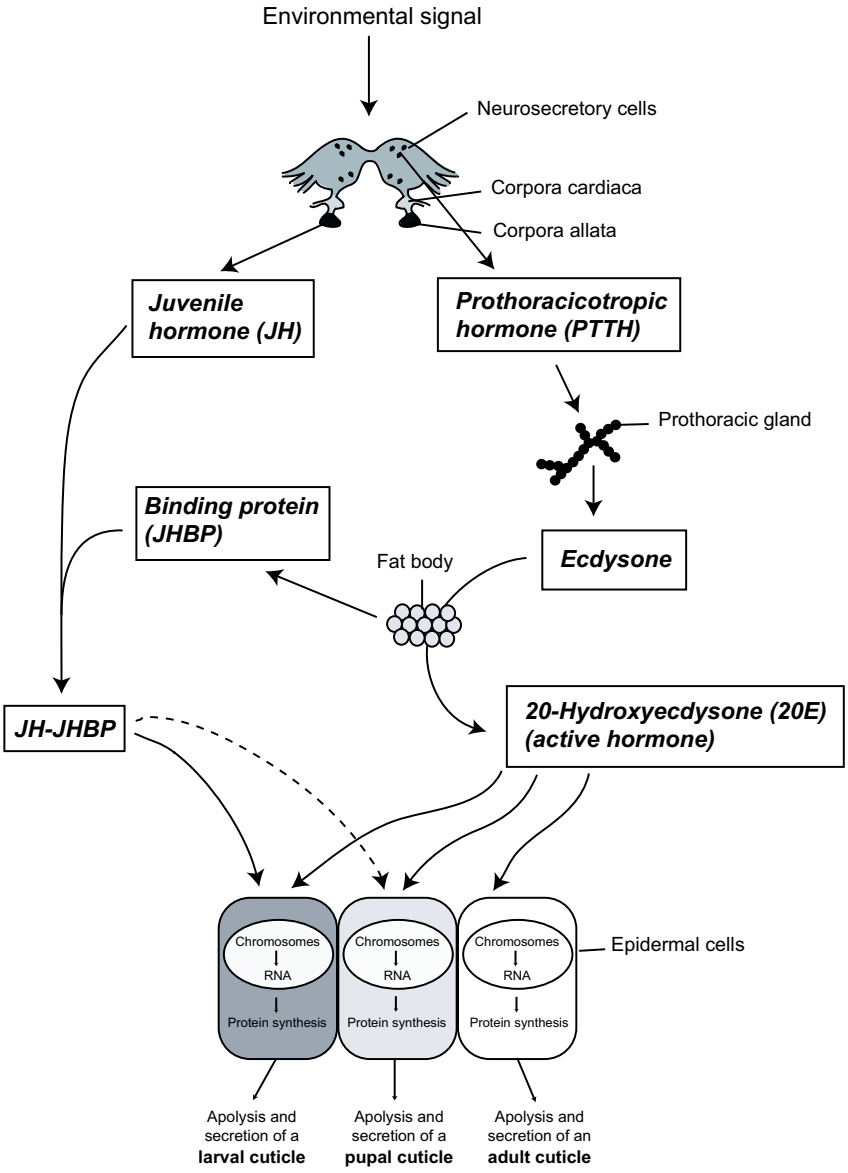

Figure 2.7: Control of molting and metamorphosis in insects (Lafont, 2000a)

Hormone-secreting structure categories	**Function**	**Type of cell, glands or organs**	**Secreted Hormones**	**Chemical type**
Endocrine glands	Secretory structure adapted exclusively for producing hormones and releasing them into the circulatory system	Prothoracic glands	Ecdysteroids	Steroid
Neurohemal organs	Similar to gland, but they store their secretory product in a special chamber until stimulated to release it by a signal from the nervous system (or another hormone)	*Corpora cardiaca*	Prothoracicotropic hormone (PTTH)	Peptide
		Corpora allata	Juvenile hormone(JH)	Terpenoid
Neurosecretory cells	Specialized nerve cells (neurons) that respond to stimulation by producing and secreting specific chemical messengers	-	Brain hormones	Peptide
Internal organs	Hormone-producing cells are associated with numerous organs of the body, including the fat body, and parts of the digestive system	Gonads	Gonadal hormones	?

Table 2.2

2.3 Insect growth regulators

Insect growth regulators (IGR) are chemicals that affect the ability of insects to growth and mature normally. They are referred to as "third generation" pesticides, the "first generation" being constituted by inorganic pesticides such as lead arsenate and the "second generation" by chlorinated hydrocarbons such as DDT (Grenier & Grenier, 1993). This new class of biorational compounds were specially developed to reduce undesirable effects on man, wildlife and the environment and for their compatibility with modern pest management principles (Staal, 1975).

The first account of IGR was reported in year 1965 with the discovery of the natural compound juvabione that was found in paper products derived from balsam fir pulpwood (Tunaz & Uygun, 2004; Staal, 1975).

IGR were developed to deliberately inhibit or disrupt insect growth during oval, larval or pupal stages. They are likely less hazardous for adult stages than for other insect developmental stages. They usually exert their effect through ingestion and typically take several days to have an effect on insect populations.

IGR act on insects by various mechanisms such as inhibition of chitin synthesis or by mimicking their hormones. Substances that simulate insect hormones may work as juvenile hormone analog or antagonist or as ecdysone agonist or antagonist, for example. Common insect growth regulators and their major characteristic are described at the end of this chapter. Their main modes of action are described below.

2.3.1 Mode of action

Chitin synthesis inhibitors

Insects and crustacean cuticle is primarily composed of protein and chitin fractions. Chitin is an amino polysaccharide and is equally present in cells wall of fungi and protozoa or in nematode eggshell but is absent in vertebrates. Insect growth and morphogenesis is strictly dependent on the capability to remodel chitin-containing structures.

Chitin formation is catalyzed by chitin synthetase (see Fig. 2.8), a highly conserved enzyme found in every chitin synthesizing organism, which converts chitin precursors (e.g. glucose, glucosamine, UDP-N-acetylglucosamine; depending on insect species) in the polymer of chitin (Tunaz & Uygun, 2004; Merzendorfer & Zimoch, 2003). This enzyme is first produced in an inactive forme, a zymogen, and has to be activated by proteases.

In the presence of chitin synthesis inhibitors, the final step of the chitin biosynthesis pathway is inhibited and the precursor is not converted into chitin. The precise mode of action is still unknown but hypothetical target sites for chitin synthesis inhibitors have been proposed:

- the chitin synthetase, or its biosynthesis, is inhibited,
- the transport of UDP-N-acetylglucosamine through the plasma membrane is inhibited.

A third mode of action was proposed which stated that the protease (the zymogen activator) or its biosynthesis could be inhibited but some studies seem to refute this hypothesis (Tunaz & Uygun, 2004).

Insect growth regulators are primarily used as larvicides. Treated larvae develop until molting, but fail to ecdysis. Insects are thus unable to reach the

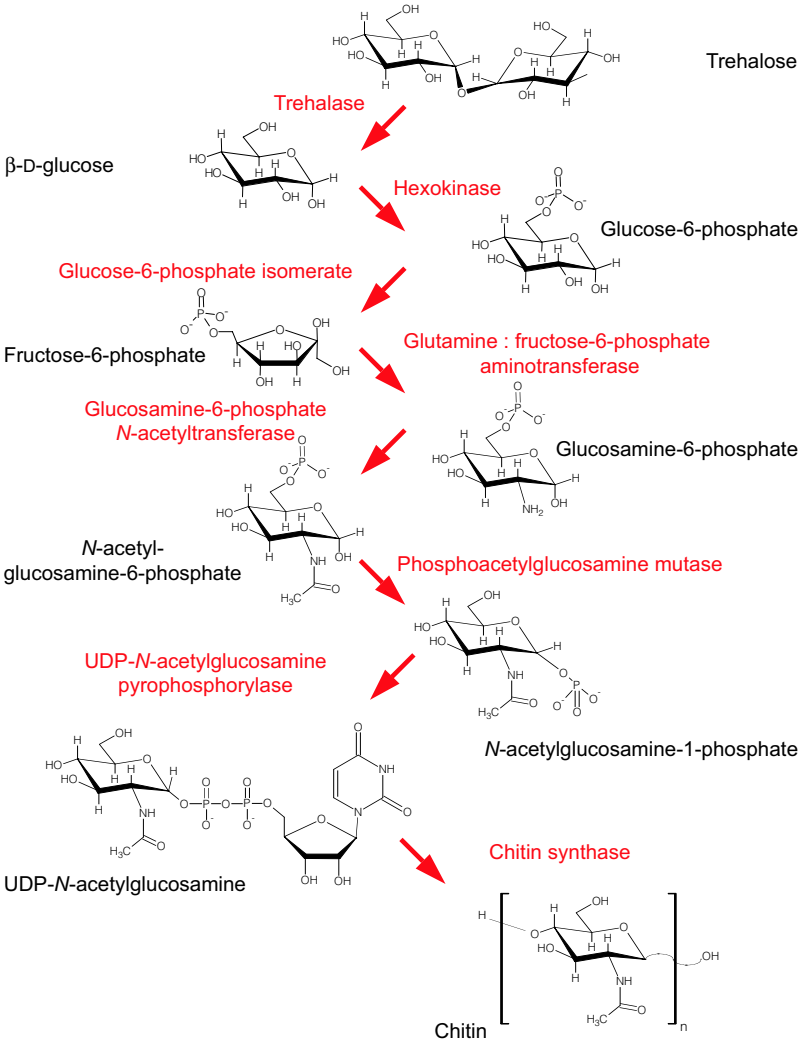

Figure 2.8: Biosynthesis of chitin in insects. The pathway starts with trehalose, the main hemolymph sugar in most insects, and ends with the chitin polymer (Merzendorfer & Zimoch, 2003)

adult stage which prevent them from reproducing. Poisoned insects eventually die.

Since molting process is similar among arthropods, chitin synthesis inhibitors are less species specific than other IGR compounds like juvenile hormone analogs. Crustacean and non-target arthropods species could then be endangered by their application (Tunaz & Uygun, 2004). However, as vertebrates do not produce chitin, they should not be affected by these compounds and hypothetical toxic effects would follow other mode of action.

Hormone mimicking substances

Some IGR can mimic the action of insect growth and developmental hormones, the juvenile hormone and the ecdysone. These compounds can interact with hormone receptors or with hormone metabolism.

Mimicking substances can be hormone agonists or antagonists. Agonists can bind the target receptor of the endogenous hormone and trigger a cell response. If the agonist is a "partial agonist", the receptor will be activated but it will not cause as much of a physiological change as would do a full agonist. Antagonists inhibit the normal physiological function of the receptor by preventing the endogenous hormone or the agonist from binding to the receptor. Antagonists can compete with an agonist for a receptor (competitive antagonists). As a single hormone may have multiple targets and cause different effects, it could be supposed that mimicking substance could act in the same way.

Hormone mimicking substances can lead to various abnormalities that impair insect development and survival. Indeed, abnormal morphogenesis is the first and the most readily observed aspect of mimicking substances action upon insects. Insect adults that have developed with certain morphogenetic abnormalities show a reduced reproductive potential. These substances can also disrupt insects metamorphosis ensuring that no reproductive adults are formed (Tunaz & Uygun, 2004; Staal, 1975).

In general, hormone mimicking substances are more effective at the early stages of metamorphosis and embryogenesis, such as freshly ecdysed last larval instars, freshly ecdysed pupal instars and deposited eggs (Tunaz & Uygun, 2004). Most insect species respond to mimicking substances treatment by producing extra larval, nymphal or pupal forms which may range from giant, but almost perfect forms, to a scale of intermediates between immature and adult forms (Staal, 1975). Some of the main effects of hormone mimicking substances on insects according to their developmental stage (holometabol cycle) are shown in figure 2.9. As the resultant activity of a given compound can show heterogenic effects and depends on several different factors, this figure is not exhaustive and only presents a small part of possible insect responses to mimicking substances exposure. Indeed, factors such as the difference of susceptibility of a particular insect species towards

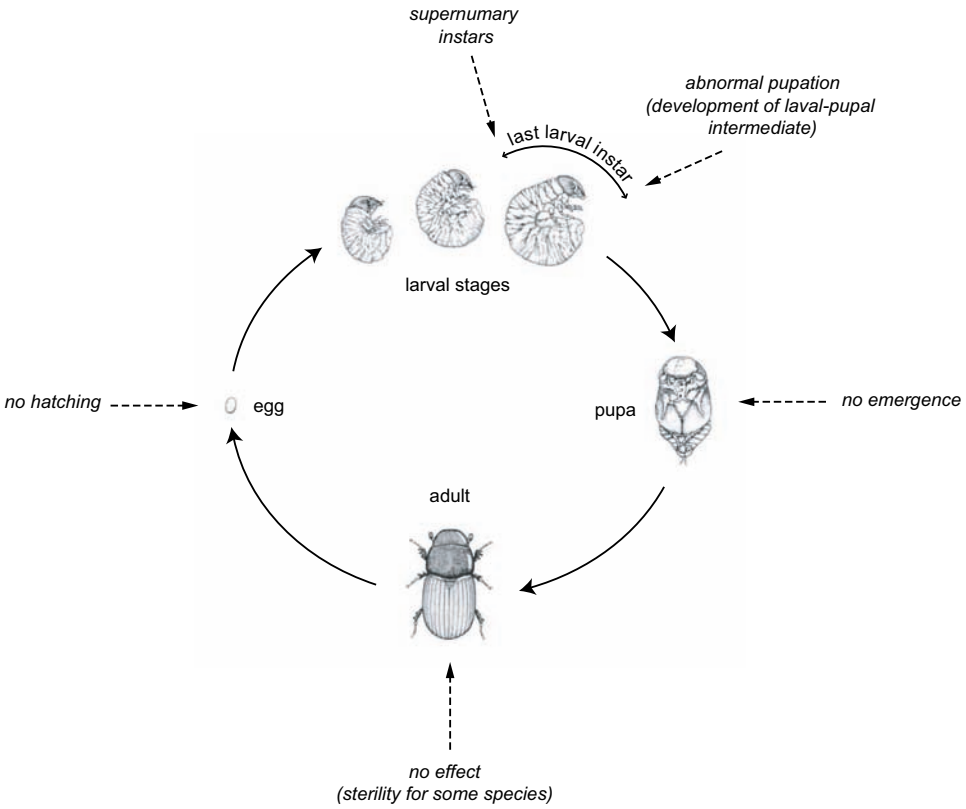

Figure 2.9: Major effects of hormone mimicking substances on insect developmental stages during holometabolous cycle

different types of compounds, the variation within the effects of one type of compound for different insect species, as well as developmental stage sensitivity to the substance can lead to great disparity in the type of response (Staal, 1975). More influencing response factors are given in figure 2.10

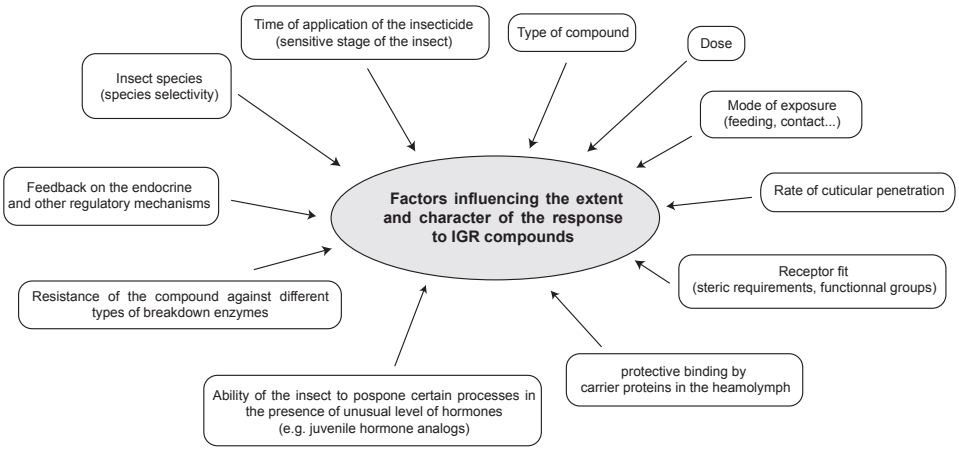

Figure 2.10: Main factors influencing insect response to hormone mimicking substances

The use of insect hormone mimicking substances may be impractical under field condition in some species since the most damaging stage of some insect pests is in the entire larval stage, while mimicking substances are most effective at the last larval instar.

Insect hormone mimicking substances are generally selective, but the last stage of some non target organisms will potentially suffer morphogenetic effects or anomalies, while crustaceans will probably have defective reproductive systems (albeit reversible). However, their effects are considered as transient and thus acceptable due to their high degradability and non-lethal and reversible effects on most aquatic arthropods (Tunaz & Uygun, 2004).

2.4 Studied insect growth regulators[1]

2.4.1 Methoprene

Juvenile hormone analog

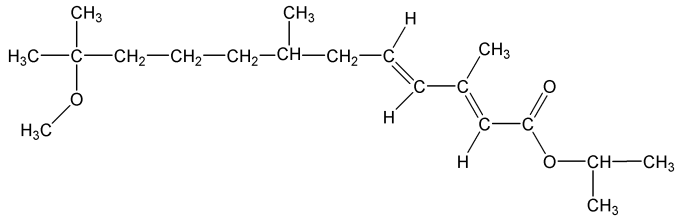

Figure 2.11: Methoprene

Product identification

Common name	methoprene
IUPAC	isopropyl (E,E)-(RS)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate
CAS RN	40596-69-8
Formula	$C_{19}H_{34}O_3$
Chemical family	terpene

Physical data

Molecular weight	310.48
Physical state	amber liquid
Water solubility	1.4 mg/l (20 °C)
Organic solvent solubility	miscible
log K_{ow}	5.212
Stability	Sensitive to U. V. light

Mode of action

Prevents metamorphosis to viable adults when applied to larval stage

[1]source of information (for all IGR data sheets): (Pesticide Manual, 1997; http://www.inra.fr/agritox/, 2005; Extoxnet, 2003); [1] data from http://www.inra.fr/agritox/ (2005) eclusively; [2] data from Pesticide Manual (1997) exclusively; source of information for data concerning application in Switzerland: Index Phytosanitaire (2002)

Applications

General stage effectiveness	larvae (not toxic to pupal and adult stages)
Target species	Diptera (e.g., flies, mosquitoes), Coleoptera (e.g., beetle), Siphonaptera (e.g., fleas), Homoptera, and other insects like pharaoh's ants, lice, moths
Worldwide uses	aquatic area (to control mosquitoes), insect pests in public health (e.g., pharaoh's ants, fleas), stored commodities (e.g., tobacco moth in stored tobacco), stored product pests, animals (flea collars), plants (e.g., leaf miners on glasshouse chrysanthemums)

Applications in Switzerland

Uses	stored crops protection (tobacco)
Formulations	Dianex, Kabat
Manufacturers	Dianex, Kabat: Tybolin, (Syngenta)
Solution concentration	Dianex: 598.3 g/l Kabat: 41 g/l
Application rate	Dianex: 10 ml solution in 1 to 25 l of water for 1000 m^3 Kabat: 250 ml solution per 1000 kg tobacco
Toxicity category	Dianex: 5S Kabat: free

Ecotoxicity

Eisenia fetida	no data
Daphnia magna	LC_{50} (48h) 360 μg/l [1,2]
Bees	Non toxic to adult bees: LC_{50} (oral and topical) >1000 μg/bee [2] Bee larva are sensitive at 0.2 μg/bee

Environmental fate

DT_{50} in soil	field: 10 days [1]

2.4.2 Fenoxycarb

Juvenile hormone analog

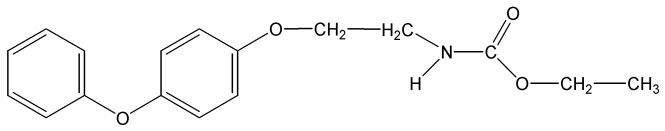

Figure 2.12: Fenoxycarb

Product identification

Common name	fenoxycarb
IUPAC	ethyl 2-(4-phenoxyphenoxy)ethylcarbamate
CAS RN	79127-80-3
Formula	$C_{17}H_{19}NO_4$
Chemical family	carbamate

Physical data

Molecular weight	301.3
Physical state	colorless to white crystals
Water solubility	6 mg/l (25 °C)
Organic solvent solubility	acetone: > 250 g/l [1]
log K_{ow}	4.07 (25 °C)
Stability	Stable to light

Mode of action

Inhibit metamorphosis to the adult stage by contact and stomach action. Interfere with the molting of early instars larvae.

Applications

General stage effectiveness	larvae, other?
Target species	Lepidoptera, Coleoptera, fire ants, mosquito larvae, fleas, cockroaches
Worldwide uses	stored products, insect pests in public health (e.g., fire ants, cockroach...), sucker on fruit, cotton, olives, vines and ornamentals

Applications in Switzerland

Uses	stone fruit, pipe fruit, vines, ornamentals
Formulations	Insegar DG
Manufacturers	Coop, Maag
Solution concentration	25 % (wet powder)
Maximal field application rate	0.64 kg/ha
Toxicity category	free
Remarks:	toxic to fishes and bees

Ecotoxicity

Eisenia fetida	LC_{50} (7 days) > 1000 mg/kg; LC_{50} (14 days) > 850 mg/kg [1]
Daphnia magna	LC_{50} (48h) 0.4 μg/l [1]
Bees	According to the Pesticide Manual: non toxic to bees: oral LC_{50} (24h) >1000 ppm [2]
Fish (*Onchorynchus mykiss*)	LC_{50} (96h) 1.6 mg/l [1]

Environmental fate

DT_{50} in soil	laboratory: 51 to 75 days [1,2] field: 17.6 to 31 days [1]

2.4.3 Tebufenozide

ecdysone agonist

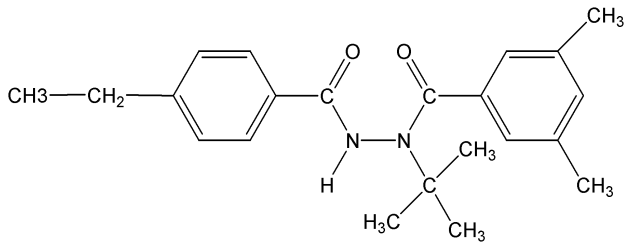

Figure 2.13: Tebufenozide

Product identification

Common name	tebufenozide
IUPAC	N-tert-butyl-N'-(4-ethylbenzoy)-3,5-dimethybenzohydrazide
CAS RN	112410-23-8
Formula	$C_{22}H_{28}N_2O_2$
Chemical family	diacylhydrazine

Physical data

Molecular weight	352.5
Physical state	Off-white powder
Water solubility	0.83 mg/l (25 °C)
Organic solvent solubility	acetone: 74.8 g/l [1]
log K_{ow}	4.25 (pH 7)
Stability	stable to light (pH 7 aqueous solution)

Mode of action

Lethally accelerates molting process

Applications

General stage effectiveness	larvae
Target species	Lepidoptera
Worldwide uses	rice, fruit, row crops, nut crops, vegetables, vines and forestry

Applications in Switzerland

Uses	stone fruit, pip fruit, vines, cabbages
Formulations	Mimic
Manufacturers	Omya
Solution concentration	240 g/l
Maximal field application rate	0.8 l/ha (application rate in year 2004)
Toxicity category	5

Ecotoxicity

Eisenia fetida	LC_{50} (14 days) > 1000 mg/kg [1]
Daphnia magna	LC_{50} (48h) 3.8 mg/l [2]
Bees	LD_{50} (96h, contact) > 234 μg/bee [1,2]

Environmental fate

DT_{50} in soil	laboratory: 7 to 105 days [1] field: 98 days [1]

2.4.4 Teflubenzuron

chitin synthesis inhibitor

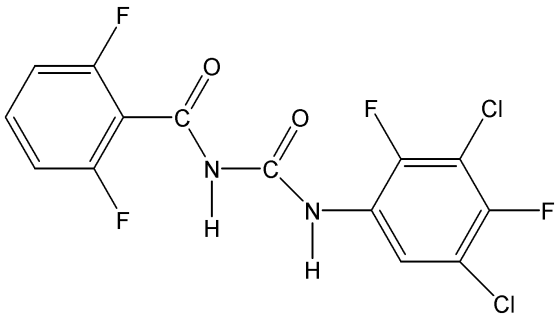

Figure 2.14: Teflubenzuron

Product identification

Common name	teflubenzuron
IUPAC	1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)urea
CAS RN	83121-18-0
Formula	$C_{14}H_6Cl_2F_4N_2O_2$
Chemical family	benzoylurea

Physical data

Molecular weight	381.1
Physical state	white to yellowish crystal
Water solubility	0.019 mg/l (23 °C) [2]
Organic solvent solubility	acetone: 10 g/l [2]
log K_{ow}	4.3 (20 °C)
Stability	Stable for more than 2 years at room temperature

Mode of action

Affects molting. May affect fertility of female insect after contact or ingestion.

Applications

General stage effectiveness	larvae
Target species	Lepidoptera, Coleoptera, Diptera, Aleyrodidae, Hymenoptera, Psyllidae and Hemiptera
Worldwide uses	vines, pome fruit, stone fruit, citrus fruit, cabbages, potatoes, vegetables, soya bean, trees, sorghum, tomatoes and cotton

Applications in Switzerland

Uses	vines, vegetables, potatoes, cabbages, ornamentals, trees
Formulations	Nomolt agro, Nomolt
Manufacturers	Nomolt agro: Bayer Nomolt: Stähler
Solution concentration	150 g/l (Nomolt agro, Nomolt)
Maximal field application rate	0.6 l/ha (Nomolt agro, Nomolt)
Toxicity category	free (Nomolt agro, Nomolt)

Ecotoxicity

Eisenia fetida	LC_{50} (14 days) > 1000 mg/kg [1]
Daphnia magna	IC_{50} (24h) > 1 mg/l; IC_{50} (48h) > 0.00044 mg/l [1]
Bees	Non toxic to bees when used at recommended rates; LD_{50} (topical) > 1000 μg/bee

Environmental fate

DT_{50} in soil	laboratory: 14 to 42 days [1] field: 2 to 12 weeks [2]

2.4.5 Hexaflumuron

chitin synthesis inhibitor

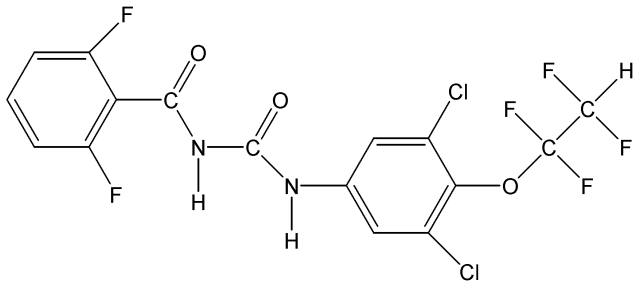

Figure 2.15: Hexaflumuron

Product identification

Common name	hexaflumuron
IUPAC	1-[3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl]-3-(2,6-difluorobenzoyl)urea
CAS RN	86479-06-3
Formula	$C_{16}H_8Cl_2F_6N_2O_3$
Chemical family	benzoylurea

Physical data

Molecular weight	461.1
Physical state	Crystalline solid
Water solubility	0.027 mg/l (18 °C) [1,2]
Organic solvent solubility	acetone: > 100 g/l (20 °C) [1]
log K_{ow}	5.68
Stability	Photolysis DT_{50} 6.3 days (pH 5, 25 °C)

Mode of action

Inhibit chitin synthesis. Ingested, systemic insecticide.

Applications

General stage effectiveness	larvae
Target species	Lepidoptera, Coleoptera, Homoptera, Diptera, termites
Worldwide uses	top fruit, cotton, potatoes

Applications in Switzerland

Uses	pip fruit, potatoes
Formulations	Consult SC
Manufacturers	Omya
Solution concentration	100 g/l
Maximal field application rate	1.2 l/ha
Toxicity category	free
Remark	toxic to bees, hazardous for aquatic organisms

Ecotoxicity

Eisenia fetida	LC_{50} (14 days) > 1000 mg/kg [1]
Daphnia magna	LC_{50} (48h) 0.11 μg/l [1]
Bees	LD_{50} (48h, oral and contact) > 100 μg/bee [1,2]

Environmental fate

DT_{50} in soil	laboratory: 36 to 160 days [1] field: no data

2.4.6 Precocene II

anti-juvenile hormone

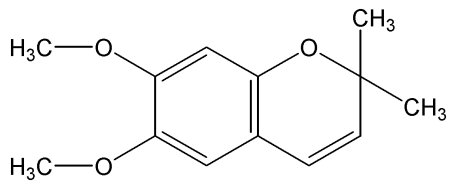

Figure 2.16: precoceneII

Product identification

Common name	precocene II
IUPAC	6,7-dimethoxy-2,2-dimethylchromene
CAS RN	644-06-4
Formula	$C_{13}H_{16}O_3$
Chemical family	no data

Physical data

Molecular weight	220.3

no other data

Mode of action

Prevent *corpora allata* from producing juvenile hormone

Applications

This insecticide was not commercialized on the market because of its lack of activity against most holometabolous insect (Tunaz & Uygun, 2004). However, it is often used as positive control for IGR testing.

Applications in Switzerland

none

Ecotoxicity

no data

Environmental fate

no data

Chapter 3

Methods

3.1 Collembola ecotoxicity testing

As effects of chemicals can not be tested on all animals before being released in environment, ecotoxicology studies have to focus on representative species used as screening tools with the aim of highlighting substances that are particularly toxic. Because of their ease of culture in laboratory and their relatively short generation time at room temperature, earthworms (*Eisenia* sp.), enchytraeids (*Enchytraeus* sp., *Cognettia* sp.) and collembolla are the most widely used groups in soil ecotoxicology. Although several species of collembola including the sexually reproducing *Folsomia fimetaria* have been employed over the years, most researchers have used *Folsomia candida* leading to the publication in 1999 of a recommended protocol by the International Standard Organization (ISO) (Fountain & Hopkin, 2001). *F. candida* has many characteristics required for an ecotoxicity test species (Riepert & Kula, 1996). Leon and van Gestel (1994, cited in van Gestel 1998) developed test selection criteria for the comparison and scoring of different test protocols. Among the tested species, *Eisenia fetida*, *F. fimetaria* and *F. candida* obtained the best scores of 72/100, 63/100 and 60/100, respectively (van Gestel & Hensbergen, 1997).

A variety of routes of exposure of *F. candida* to chemicals have been studied. These include food, gas, water, contaminated leaf surfaces over which the collembolans were forced to walk, and topical application of substances onto individual springtails. However, the ISO test deals exclusively with contact with contaminated soil, as this appears to be the most toxic route of exposure (Fountain & Hopkin, 2001). When conducting reproduction tests with *F. candida* according to the ISO standard, the organisms are exposed to contaminated standard artificial soil for 28 days before recording the number of offspring. For mortality testing, springtails are exposed for 7 or 14 days and surviving individual are counted.

3.1.1 Laboratory culture of *Folsomia candida*

The parthenogenetic collembola *Folsomia candida* has been bred in our laboratory since 1996. The organisms were generously provided by Dr. Frank Riepert from the Biologische Bundesanstalt fr Land- und Forstwirtschaft, Berlin, Germany, who was the principal author of the ISO standard (ISO, 1999). *F. candida* cultures were maintained according to the ISO standard in plastic boxes (160*110*60 mm with transparent covers) containing a substrate of plaster of Paris and activated charcoal (mass ratio 8:1). The plaster (120 g per box) and the charcoal (15 g per box, Merck 1.02186.1000, analytical grade) were mixed in a plastic flask with tightly closing lid by vigourous shaking of the flask. Bidistilled water (120 ml per box) was added and mixed homogeneously by energetic shaking of the flask for approximately 30 second (the more the mixture is agitated and the more the solidification will be rapid). The mixture was then rapidly poured into the plastic boxes, obtaining a depth of about 1 cm. The box was tapped to remove air bubbles (bubbles leave holes in which collembola deposit their eggs, increasing the difficulty of their recovery). The substrate was air dried for approximately 3 hours, bidistilled water was added to almost saturation (no water should be standing on the surface). Approximately 30 mg of granulated dry yeast (Dr. Oetker, Germany) was added in three heaps in each box. Adult collembola were then introduced in each containers.

Plaster substrate avoid burying of collembola which facilitate eggs recovery and springtails transfer to new breeding containers. Some formulations of plaster of Paris contain zinc as bactericide which may interfere with results (Fountain & Hopkin, 2001). Active charcoal absorbs excretion products and waste gases. Moreover, the dark color of the substrate makes the observation of whitish collembola easier.

Laboratory conditions were constant (20 $\pm$ 2 °C and 600 lux with a light:dark cycle of 16:8 h). As the covers closed tightly, humidity inside the boxes was kept between 70 and 80 %. Twice per week the containers were aerated, some drops of bidistilled water were added to maintain humidity. Unconsumed yeast was removed to avoid development of bacteria and fungi, which might be harmful to collembola and new food was added. Every 4 to 8 weeks the collembola were transferred into new boxes taking care to mix populations from the different boxes.

3.1.2 Synchronization of the culture

In order to reduce the variability of the response given by organisms exposed to pollutants, they have to be synchronized in age. For that, adults *F. candida* were placed on fresh substrate in new containers in order to induce oviposition which occurred in general two days after the transfer. Seven days later (9 days after the transfer of the adults), clusters of eggs were re-

sistant enough to be transferred with a wetted fine-bristled paintbrush to wet "egg papers". "Egg papers" consist of isosceles paper filter (basal length 3 cm, height 5 cm) coated with a layer of plaster and charcoal mixture, brought to saturation with bidistilled water and deposited in a circle in a high Petri dish (diameter 10 cm, height 5 cm) containing the same substrate as the breeding containers. Eggs begin to hatch 0 to 3 days after their transfer. Three days after the transfer, unhatched eggs were removed from the petri dishes by taking off the "egg papers". Remaining eggs that fell of from the "egg papers" on the petri dishes substrate were removed with a wetted fine paintbrush under a stereomicroscope. Juvenile collembola were fed with some granulated baker yeast and kept under the same laboratory conditions as the breeding containers. After three weeks these juveniles are mature and begin to lays eggs of their own. For ISO reproduction or mortality tests with *F. candida*, juveniles were used 10 days after hatching at the age of 10 to 12 days.

3.1.3 Preparation of the artificial soil

The artificial soil was prepared according to the ISO earthworm standard (ISO, 1993), which has the same composition as the OECD soil (OECD, 1984). The soil was composed of 70 % quartz sand (50 % 40-100 mesh, Fluka 84878 and 50 % $\geq$ 230 mesh, Fluka 83340), 20 % of kaolinite clay (Fluka N° 60609) and 10 % of sphagnum peat. The peat was air dried for at least one week and visible plant pieces were removed. When totally dry, the peat was ground with a commercially available electric mixer and sieved to 1 mm with a metal sieve. The main type of peat used for experiments was the "Norddeutscher Hochmoortorf", pH 3.3, obtained from ESG-Busch GmbH, Rastede, Germany. Pulverized carbonate of calcium ($CaCO_3$, Fluka N° 21060)) was added to bring the pH to 6 $\pm$ 0.5. The exact amount of $CaCO_3$ to be added was determined by measuring the pH of subsamples of the hydrated artificial soil (see 3.1.5 and 3.1.6). A quantity of bidistilled water corresponding to 50 % of the water holding capacity (see 3.1.4) was added to the different soil constituents which were mixed thoroughly. For the contamination of the soil the toxicants were added using acetone as solvent carrier (see 3.1.7).

3.1.4 Determination of the water holding capacity

Soil structure and soil moisture are important parameters for the survival and reproduction of collembola. A "crumbly" structure which depends on the aggregates formed by the soil is generally recommended for springtails. As soil moisture is one of the parameters determining soil structure, it seemed important to standardize the moisture content of the soil by determining its water holding capacity (WHC). Soil moisture is then expressed as a function

of the WHC (e.g. % of WHC). The determination of the WHC was done according to annex C of the *F. candida* ISO standard 11267 (1999) and was made in triplicate.

The extremity of a glass tube (internal diameter 3.5 cm, height 10 cm) was plugged with an unfolded paper filter (diameter 150 mm, Schleicher & Schuell (MicroScience) LS 17 1/2) fixed with a string or rubber band. The plugged glass tube was weighed (T) and filled with approximately 6 cm of uncompressed soil (about 30g (dw) artificial soil; depending on the density of the soil). The tube was then placed inside a glass beaker (capacity 600 ml), the paper filter plugged extremity directly standing on the bottom of the beaker. Water was gradually added to fill the space between the glass tube and the beaker inner side. The porosity of the paper filter will allow water infiltration into the glass tube, progressively immersing the soil. When the water level was approximately 1 cm above the top of the soil, no more water was added. The glass tube was left like this for 3 hours at room temperature. The tube was then removed from the beaker and placed into a Petri dish containing wet finely ground sand (quartz 40-100 mesh) for at least 2 hours to drain the non-absorbed water. The tube containing the mass of water-saturated substrate was weighed (S) and dried at 105 °C until no change in weight was observed. The glass tube was weighed (S') again and the dry mass of the substrate (D) was then determined as

$$D = S' - T \tag{3.1}$$

The water holding capacity was calculated using the following equation:

$$WHC = \frac{(S - T - D)}{D} \cdot 100 \tag{3.2}$$

where:

WHC = water holding capacity, expressed as a percent of dry mass

S = mass of water-saturated substrate + mass of glass tube + mass of filter paper

T = tare (mass of glass tube + mass of filter paper)

D = dry mass of the substrate

For the natural soils, the initial moisture content of the soils had to be determined. The soils were dried for 24 h at 105 °C and the percent moisture content was calculated by expressing the dry mass (weight) as a percentage of the wet mass.

3.1.5 Adjustment of soil pH to 6 ± 0.5

Soil pH is an important parameter for collembola survival and reproduction. It seems that *F. candida* achieve its highest level of reproduction in soils with pH 5.6 compared with more acidic or alkaline conditions (Fountain and Hopkin). The pH of the artificial soil was set to 6 ± 0.5 by the ISO standard

11267 (1999). The estimation of the amount of $CaCO_3$ to be added to obtain this pH was realized according to this standard. Before adjusting the pH, the water holding capacity was determined as described above. The pH was determined for small soil portions (approximately 10 g dry weight; quantity necessary for 3 replicates) containing different amounts of $CaCO_3$ (0.2, 0.4, 0.5, 0.6 % dw). Each soil portion was introduced into a plastic Petri dish (diameter 10 cm, height 5 cm). On day 0, the soil moisture content of the soil was adjusted to 25 % of the WHC (by adding bidistilled water), the soil was well homogenized and the petri dishes were closed with their lids. After 24 hours (day 1), a water quantity corresponding to 25 % of WHC (same quantity as on day 0) was added to the soil. The soil was well homogenized and the dishes again closed with their lids. On day two, the pH was measured as described below (3.1.6). The results were plotted as a graph of pH versus the amount of $CaCO_3$. From this graph, the estimated amount of $CaCO_3$ necessary to obtain a pH of 6 ± 0.5 was estimated.

3.1.6 Determination of the pH (KCl)

The pH was determined according to the ISO standard 10390 (ISO, 1994) and to recommendations from Dr. Frank Riepert, Biologische Bundesanstalt fr Land- und Forstwirtschaft, Berlin, Germany.

Five ml of uncompressed soil (with a soil moisture content of about 50 % of WHC) was sampled with a graduated cylinder and placed in a glass container (50 ml). 25 ml of KCl 1M was added, the sample was agitated with a magnetic stirrer for 5 minutes. After approximately 20 hours (not more than 24 hours) the sample was agitated for 5 minutes and the pH was measured with a calibrated electrode (Metrohm 691).

For each experiment, the pH was determined in triplicate at the beginning and at the end of the experiment in the control and in the highest concentration of the tested substance. The soil sample used for the measurement of the pH at the end of the experiment contained the same number of collembola and was kept under the same conditions as the test containers.

3.1.7 Soil contamination

Soil contamination was made as described in the ISO standard 11267 (ISO, 1999). Depending on the solubility of the substance, water or acetone were used as solvent carrier. Stock solutions of the tested chemical and needed for soil contamination were prepared prior to each experiment. The required quantity of the test substance was dissolved in an appropriate volume of solvent to obtain the desired concentration of the stock solution. Prior soil contamination, all glass dishes was rinsed with acetone and hexane in order to avoid external contamination.

Substances insoluble in water

Substances insoluble in water were dissolved in acetone and added to the required quantity of quartz sand which was then mixed with other soil constituents in order to contaminate artificial soil in a homogeneous way as describe below.

The quantity of the stock solution required to obtain the desired concentrations for experimental setup was introduced in a 250 ml round bottom flask out of glass. A constant acetone volume equivalent to the total mass of quartz sand was added to all concentrations and to one control (21 ml for 21 g of quartz for 30 g of soil). As the volume of the stock solution was generally lower than this volume, more acetone was added with a graduated cylinder to obtain the constant acetone volume. The total amount of quartz sand needed per concentration was then added in the flask and mixed thoroughly. The mixture was rotary evaporated (Rotavapor Buchi R114, pressure 500 mmbar, temperature 40 °C, medium speed of rotation; conditions chosen according to the physico-chemical characteristics of the compounds) and then placed under a fume hood for at least 3 hours to allow all acetone residues to evaporate. Finally the contaminated quartz sand was mixed with the other soil constituents. All soils were prepared one day in advance and stored at room temperature.

Water soluble substances

For water soluble substances the required volume of stock solution needed to obtain the desired test concentration was directly added to the prepared soil sample. Soil samples were prepared by mixing all the constituents, except the bidistilled water. The volume of stock solution required to obtain the desired concentration for testing was put in a glass beaker and bidistilled water was added in order to obtain the total volume of water needed to reach the desired WHC percent (e.g. 50%). The liquid was then added to the corresponding soil sample and mixed homogeneously with a metallic spoon until a "crumbly" structure was obtained. The soil were stored for 24 h at room temperature before the beginning of the test.

If experiments were carried out with natural soil, soil samples were acclimated to room temperature (20 °C) 48 h prior the beginning of the experiment (if stocked at cooler temperature, e.g., 4 °C). Twenty-four hours after the beginning of the acclimatization period, natural soil samples were contaminated by adding stock solution of the test substance and bidistilled water as for artificial soil. The initial water moisture content of the natural soil has to be considered before adding water for WHC adjustment.

3.1.8 Acute mortality and chronic reproduction testing

Folsomia candida 28-days reproduction tests

The 28-days reproduction tests with *Folsomia candida* were conducted according to ISO 11267 (ISO, 1999). Thirty g ww of uncompressed soil were introduced into glass containers (diameter 4.5 cm, height 9.5 cm, with plastic covers closing tightly). The glass containers were previously rinsed with acetone and hexane for decontamination. Ten individuals were then poured from the Petri dish containing the synchronized 10 to 12 days old juveniles onto a folded black cardboard by tapping the Petri dish so that the organisms fall off. Once counted, they were then transferred to the test containers (by tapping the cardboard) and approximately 10 mg of dry yeast were added onto the soil. The containers were kept under the same conditions as the culture (see 3.1.1). They were opened twice per week to allow aeration (the lid was opened slowly so that individuals hiding under the lid will fall back into the container) and another 10 mg of yeast was added after 14 days. In general, five replicate containers were used for the control or control with acetone and three replicates were used for each concentration. Five additional test containers were prepared: four for the pH measurement containing collembola and one for the water content control. The latter with no collembola was weighed at the beginning of the experiment and then once per week until the end of the test. The water loss was not compensated as it did not exceed 2 % of the initial water content. The pH was determined (in the presence of 1M KCl, see 3.1.6) in triplicate according to the ISO standard 10390 (ISO, 1994). For each experiment, the pH was determined at the beginning and at the end of the test in the control and in the highest concentration.

After 28 days, the adults and juveniles were recovered and counted according to the following method. The soils were poured into 500 ml glass beakers (diameter 9 cm). Approximately 150 ml of tap water was added to the beaker and the containers were rinsed with water which was added to the beaker. After gentle stirring with a spatula, approximately 250 ml tap water and blue bromophenol was added (the latter to improve the contrast between the whitish collembola and the soil). The mixture was stirred thoroughly before counting the adults by eye. The beakers were then completed with tap water to 500 ml and a picture was taken of the surface of the water with a digital camera (Nikon Coolpix 880, 3.34 megapixels) fixed on a tripod. Three spots were used to improve the light conditions. The pictures were transferred to a computer and the number of juveniles and adults were counted on the screen or on a paper-printed photograph. The use of the free image analysis software, "Image Tool" (http://biotec.casaccia.enea.it/GIC/MicroSoftware.html) with the function "tag and count", facilitates the counting.

Mortality tests

Acute toxicity of a substance can be tested for *F. candida* by conducting 7-day or 14-day mortality tests. Tests were conducted as for the 28-day reproduction test but only 20 g (ww) of soil were introduced per test containers instead of 30 g (ww). As the test duration was shorter, 10 mg of granulated beaker yeast was added as food supply at the beginning of the test only (day 0). At the end of the test (7-day or 14 day), collembola were recovered using the flotation method describe for the 28-day reproduction test but surviving springtails were counted by eye without taking a photograph. Other test conditions were the same.

3.1.9 Pollutants exposure through artificial compressed soil to perform proteomic experiments

In order to easily recover collembola to observe eventual changes in their protein pattern (over or under expressed proteins), they were exposed to compressed artificial soil contaminated at the desired concentration as described below.

Seventy g (ww) of artificial soil, contaminated to the desired concentration (see 3.1.7), were introduced in a petri dish (diameter 10 cm, height 5 cm). The substrate was evenly distributed in the petri dish and compressed using a teflon piston (slightly smaller in diameter than the petri dish). The soil surface was smoothed with the fingers (with gloves!) to avoid holes where collembola could bury. The compressed substrate should reach a height of approximately 1 cm in the petri dish. Aged synchronized collembola were introduced in each petri dish using a folded black cardboard (see 3.1.8). Springtails were fed with dry beaker yeast and the petri dishes were closed with their lids. Laboratory condition and maintenance were the same as for *Folsomia candida* breeding containers. Juvenile or adult collembola were exposed between 1 to 12 days at different concentrations of the chosen substance, in general at the EC25, EC50 and EC75, determined with the 28-day reproduction test according to the ISO standard. Twenty-four hours before the end of the exposure, remaining food was removed to avoid presence of yeast proteins in two-dimensional gel electrophoresis of collembola. Individuals exposed for only 24 h or 48 h to pollutants were not fed.

At the end of the exposure period, the lid of the petri dish was rapidly opened and the petri dish was turned down over a black cardboard (A4 size) so that the organisms fell off. They were then counted and the desired number of collembola were gathered in the middle of the cardboard and poured in an eppendorf tube using a glass funnel. All the material used for two-dimensional gel electrophoresis was autoclaved and manipulated with gloves to avoid external protein contamination. The eppendorf tube were closed and put in liquid nitrogen in order to kill the collembola. They were

	Folsomia candida	
	Acute mortality test	Chronic reproduction test
Duration	7 or 14 days	28 days
Endpoint	Mortality	Mortality and number of juveniles
Age at beginning of test	10-12 days after eclosion	10-12 days after eclosion
Number of individuals per replicates	10	10
Number of replicates	5 per control and 3 per concentration	5 per control and 3 per concentration
Type of containers	100 ml glass containers with tightly closing plastic covers	100 ml glass containers with tightly closing plastic covers
Transfer system	Black folded cardboard	Black folded cardboard
Soil quantity	20 g ww	30 g ww
Light	600 lux; 16:8h	600 lux; 16:8h
Feeding	10 mg yeast	10 mg yeast day 0 and 10 mg day 14
Temperature	20 ± 2 °C	20 ± 2 °C
Aeration	Twice per week	Twice per week
Humidity	No need to verify	Verify after 7, 14, and 21 days; add water if loss > 2 %
Counting	Pour in 500 ml beaker, add 150 ml tap water, stir, add 100 ml water + blue bromophenol, stir, count by eye	Pour in 500 ml beaker, add 150 ml tap water, stir, add 250 ml water + blue bromophenol, stir, add water (total 500 ml), take photograph and count on screen (Image analysis software Image Tool)

Table 3.1: Recommended conditions and procedures for *Folsomia candida* testing

then stored at -80 °C until freeze-drying (see 3.2.1).

3.1.10 Statistical analysis

Reproduction tests

Reproduction data from chronic toxicity tests were analyzed with the linear interpolation method which calculates a point estimate, referred to as an inhibition concentration (IC) of a toxicant that causes a specific percent effect (e.g., 25 %, 50 %). The program used is a linear interpolation method for sublethal toxicity estimate "The Inhibition Concentration (ICp) Approach (Version 2.0)" from U.S. EPA (http://www.epa.gov/nerleerd/stat2.htm, 2004). The 95 % confidence intervals for the ICp linear interpolation technique are calculated by the bootstrap method. This linear interpolation method generates concentration data that represent the effect level for a test method using a continuous model. The nonparametric monotonic technique and smoothing methodology used in the ICp approach is sufficiently flexible to accommodate a wide range of data with a single, unified formulation.

Mortality tests

Mortality data were analyzed with the probit program version 1.5 of the U.S. EPA (http://www.epa.gov/nerleerd/stat2.htm, 2004). This program was used to calculate a lethal concentration causing 50% of mortality (LC50) and the associated 95 % confidence limits. Computations performed by the program are based on (Finney, 1952). The effect (probit transformation) was plotted against the log of the concentration. The LC50 was then determined by a linear regression.

The fit of the probit line is shown by the computed value of chi-square, which must not exceed a critical value if the line and the estimates are to be accepted. Otherwise the data would be significantly heterogeneous and the line would not be an acceptable fit. If the mortality of the adults in the control(s) was lower or equal 20 %, the number of responding (dead) organisms in the control group was set to zero. Otherwise, the program would automatically correct the mortality obtained for the different concentration with the Abbott's formula which is going to increase the LC50 estimate. This correction should only be done if the mortality in the control group is higher than 20 %, which was not the case.

3.2 Proteomics

Xenobiotic exposure has the potential to cause alterations at different organizational levels of a cell or tissue: in the genome (chromosomal DNA information), in the transcriptome (messenger RNA from actively transcribed genes), in the proteome (entire protein complement of a biological sample) and in the metabolome (constituent metabolites in a biological sample). Potentially, xenobiotics can have a primary mode of action that affects any of these compartments although it is unlikely that a phenotypic change can take place without measurable alterations of all compartments downstream of the genome (ecetoc, 2001).

In many cases, mRNA transcripts do not directly correlate with protein expression (Phillips & Bogyo, 2005). Posttranscriptional splicing and recombination of RNA can lead to various protein products which will multiply the number of components and functions (Kellner, 2000). Therefore, the field of proteomics is challenged with the task of providing both quantitative and functional data to further complement genomics effort (Phillips & Bogyo, 2005).

Proteomics is widely used for the global analysis of protein expression patterns. The set of proteins expressed by a cell, the proteome, is a dynamic entity, varying in response to the effects of the environment of the genome. Proteomics enables a snapshot to be taken of the current state of protein expression, providing both qualitative and quantitative information on protein profiles and can be used to investigate changes in protein expression patterns in differing situations. For example, proteomics can be used to study the effects of genetic changes (e.g. mutation, gene knockout), the effects of environmental challenge (e.g. pollution, disease, drug intervention) or to investigate the components of protein complexes involved in biochemical pathways or signal transduction. The application of proteomics in toxicology is referred to as toxicoproteomics. The core technology involves for example 2-dimensional electrophoresis (2DE) for highresolution separation of proteins, coupled to mass spectrometry for high-throughput protein identification. The aim is to separate highly complex protein mixtures into discrete components, generating an expression profile for each condition under investigation that can be used to select proteins displaying differential expression. Proteins of interest can then be analyzed independently to provide their identification. This usually involves digestion of the protein and analysis of the peptide mixture using mass spectrometry. The results are used to search databases to provide the identity of the parent protein. This knowledge can then be related to the biological situation, improving our understanding of how cells or organisms respond to differing situations (Keen, 2005).

3.2.1 Protocol for two-dimensional gel electrophoresis with collembola

Overall information and guidance on two-dimensional gel electrophoresis and mass spectrometry methods are provided in appendix B.

Chemicals used for buffers, solutions and gels preparations are listed in table 3.2.

Protocols for two-dimensional gel electrophoresis were adapted from the online available protocols from the "SWISS-2DPAGE" (http://www.expasy.ch/-ch2d/protocols/protocols.fm4.html) and from the protein analysis facility (PAF) from the University of Lausanne (http://www.unil.ch/paf).

Organisms recovery and preparation

Collembola exposed to pollutant were recovered as explained in section (3.1.9) and placed in eppendorf tubes (1.5 ml capacity). The tubes were put in liquid nitrogen to kill the collembola. Eppendorf tubes were then placed in a freeze-dryer with lids open and the collembola were freeze-dried for 24 h. Freeze-drying helps to prevent protein degradation. Collembola can be kept at -80 °C for a few days before freeze-drying if needed. After collembola were freeze-dried, eppendorf tubes were closed and stored at -80 °C before use to avoid protein degradation. Eppendorf tubes were kept on ice during transportation steps. Gloves were worn during all the manipulation and also for further steps of the 2D gel electrophoresis to avoid skin keratin contamination of the sample.

Sample preparation and protein solubilization

The entire collembola were solubilized in a solution containing 7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 65 mM DTT, 4 mM AEBSF, 2 % (v/v), ampholine L 3-10 and a trace of bromophenol blue. The volume of solution to add to the eppendorf tube depends on the age and on the number of collembola present in the tube. A final concentration of 0.66 μg/μl (200 μg of protein for 300 μl of solution) must be reached. Only half of the total volume of the solution required was first added. Using an eppendorf piston, springtails were blended until no more solid parts were seen. The second half of the solution was used to rinse the piston in the eppendorf tube. All the work was done on ice (when possible) to avoid protein degradation. The solution with blended collembola was well homogenized using a mini shaker.

IPG gel strips rehydratation

Immobilized pH gradient (IPG) strips, pH 4-7, length 7 cm from BioRad were used for isoelectric focusing (IEF). Fifty μg of collembola proteins were loaded per strip for a total rehydration solution volume of 125 μl.

The indicated volume of sample was pipeted as a line in the channel of a rehydratation/equilibration tray. The line of the sample should be approximately as long as the length of the strip. Air bubbles must be avoided as they may interfere with the even distribution of the sample in the strip. The strip was then placed gel side down onto the sample. Each strip was overlaid with paraffin oil to prevent evaporation during the rehydration process. Strips were rehydrated overnight (11-16 h) to allow loading of the protein sample.

Preparation of IPG strips for first dimension (isoelectric focusing)

Using forceps, strips were removed from the rehydratation/equilibration tray, held vertically for a few seconds and rinsed with ultra pure water to remove paraffin oil. This washes the surface of the gel, removing unabsorbed proteins and reducing horizontal streaking. Strips were then transferred to the focusing tray of the Multiphor II system (Pharmacia-Amersham) prepared for the circumstance (constant temperature of 16 °C, kerosene layer under the focusing tray to allow a better thermic conduction). The correct polarity between the tray and the strip must be observed. Humid electrode wicks were placed perpendicularly to both ends of the strip, the middle of the wick on the gel extremity. The strip must be parallel to the focusing tray channels. The electrodes were placed in the middle of the wicks, perpendicularly to the strip. After everything was correctly positioned, the assembly was covered with low viscosity paraffin oil. Up to 4 strips were run in parallel.

IEF running conditions

The voltage was increased linearly from 0 to 300 V during 5 minutes, and then from 300 to 3500 V for 30 min followed by 90 minutes at 3500 V.

IPG gel strips equilibration

After the first dimension run, the strips were equilibrated in order to resolubilize the proteins (with urea) and to reduce -S-S bonds (with DTT). Strips were removed from the focusing tray channels of the Multiphor II system and held vertically for a few seconds to allow paraffin oil to drain. Strips were first equilibrated in the rehydratation/equilibration tray (using one channel per strip) in 2 ml of a solution containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 2 % (w/v) DTT for 12 min. Strips were then equilibrated for another 12 minutes (one channel per strip) in 2 ml of a solution with 50 mM Tris-HCl pH 6.8, 6 M urea, 30 % (v/v) glycerol, 2.5 % (w/v) iodoacetamide and a trace of bromophenol blue.

SDS-PAGE composition and preparation

Gels for second dimension were prepared during the first dimension to allow polymerization of the gel. Mini gels 13.2 % were made using the Mini-PROTEAN II cell from BioRad. They are composed of Acrylogel-PIP 2.6 (33 %), Tris-HCl 1.5 M pH 8.8, sodium thiosulfate 1M, APS 0.1 % and TEMED 0.05 %. After the gel apparatus was mount, the gel solutions were poured until 0.5 cm from the top of the plates and overlayered with ultra pure water until the polymerization occurred. The water was then replaced by a solution of 25 mM Tris, 192 mM Glycine and 0.1 % SDS until the gel was needed. Gels were not polymerized in the presence of SDS. This seems to prevent the formation of micelles which contain acrylamide monomers, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerized monomers in the polyacrylamide. The SDS used in the gel running buffer is sufficient to maintain the necessary negative charges on proteins. Sodium thiosulfate was used as an additive to reduce background in the silver staining gels (http://www.expasy.ch/ch2d/protocols/protocols.fm4.html, 2005).

IPG strips transfer for second dimension

After the equilibration, the solution overlaying the top of the gel was removed and replaced by a solution containing agarose 0.5 % and Tris-glycine-SDS (25 mM - 192 mM - 0.1 % w/v) pH 8.3 heated at about 70 °C to dissolve the agarose. The IPG strips were immediately loaded through it. Agarose was allowed to solidify for a few minutes.

Second dimension running condition

One hundred ml of running buffer solution composed of 25 mM Tris, 192 mM Glycine and 0.1 % SDS were mixed with 400 ml of ultra pure water (= diluted 5X) and kept at 4 °C before use. The Mini-PROTEAN glass plates with the gels were mounted for the second dimension and the diluted running buffer was added in the reservoir of the apparatus. Gels were run at a constant voltage of 200 V, with a maximal current of 30 mA per gel for approximately 1 hour at 8-12 °C (the Mini-PROTEAN II cell apparatus was put in a plastic box filled of ice).

Silver staining procedure

At the end of the second dimension run, the gels were removed from the glass plates and washed in ultra pure water for 5 min on an orbital shaker (Heidolph UNIMAX 1010) at 63 rpm. Gels were then fixed in 40 % (v/v) ethanol, 10 % (v/v) acetic acid and 50 % ultra pure water for 1 hour. They were fixed overnight in 5 % (v/v) ethanol, 5 % (v/v) acetic acid and 90 % ultra pure water. Gels were washed 5 minutes in ultra pure water and

then soaked in a solution containing 1 % glutharaldehyde and 0.5 M sodium acetate for 30 minutes. They were washed 3 times 10 minutes in ultra pure water. In order to obtain homogenous dark brown staining of proteins, gels were soaked twice in a 0.05 % (w/v) 2,7 naphtalene disulfonic acid solution for 30 min. Gels were stained for 30 minutes in a freshly made ammoniacal silver nitrate solution (0.8 % silver nitrate, 1.3 % (v/v) ammonium hydroxyde (25 %) and 20 mM sodium hydroxyde (10 M)). To prepare 40 ml of this solution (volume required for 1 mini-gel), 0.32 g silver nitrate were dissolved in 400 μl of ultra pure water, which was slowly mixed in a solution containing 39 ml of ultra pure water, 80 μl sodium hydroxyde (10M) (NaOH) and 520 μl of ammonium hydroxyde (25 %) (NH_4OH). A transient brown precipitate might form. After staining, gels were washed 4 times in ultra pure water for 4 minutes. Gels were then developed in a solution containing 0.01 % (w/v) citric acid and 0.1 % (v/v) formaldehyde (added just before use) until a slight background stain appeared (between 2 to 10 minutes). The development was stopped by soaking the gel in a solution containing 5 % (w/v) Tris and 2 % (v/v) acetic acid for approximately 5 minutes. Gels were then rinsed 3 times in ultra pure water for about 5 minutes. Gels were stored in water at 4 °C. This staining procedure is 100-fold more sensitive than Coomassie Brilliant Blue staining but unfortunately not compatible with mass spectrometry technics.

Colloidal Coomassie Brilliant Blue G250 staining procedure

The colloidal Coomassie G250 staining is more sensitive than regular Comassie R250. Moreover, this staining procedure is compatible with MS analysis.

Gels were fixed overnight in 50 % ethanol, 3 % phosphoric acid (H_3PO_4). They were then washed 3 times 15 min in ultra pure water. In the meantime, an equilibration solution containing 34 % methanol, 3 % phosphoric acid (H_3PO_4) and 17 % ammonium sulphate was prepared. The ammonium sulphate was first dissolved in water (which takes time) and the methanol was then added slowly. Gels were equilibrated in equilibration solution for 30 min. For staining, CBB G250 powder (0.35 g/l of equilibration solution) was directly added to the equilibration solution containing the gel and rocked 2 days in well closed boxes. Gels were then destained 2 times 30 min or until the desired background was obtained in 10 % acetic acid and stored at 4 °C.

Product	Company	Catalog number
2, 7 naphtalene disulfonic acid	Acros	41523-0250
Acetic acid glacial (100 %) anhydrous	Merck	1.00063.1000
Acrylamide/PIPERAZINE di acrylamide 37.5:1	Biosolve	26051
Agarose	Bio-Rad	162-0100
Ammonium hydroxide	Fluka	09858
Amonium persulfate (APS)	BioRad	161-0700
Ampholine (3.5-10)	Amersham	80-1125-87
Bromophenol blue	Merck	8122
CHAPS	Calbiochem	220201
Citric acid monohydrate	Sigma	C 7129
Coomassie brilliant blue R-250	BioRad	161-0400
Coomassie brilliant blue G-250	BioRad	161-0406
DTT (1,4-Dithiothreitol)	Roche	10 197 777 001
Ethanol	Merck	1.00983.2500
Formaldehyde	Fluka	47630
Glutaraldehyde	Fluka	49626
Glycerol (87%)	Fluka	49782
Glycerol anhydrous	Fluka	49767
Glycine	Sigma	G 8898
Hydrochloric acid (32%)	Merck	1.00319.2500
Iodoacetamide	Fluka	57670
IPG Strips (ReadyStrips), 7 cm, pH 4-7	BioRad	163-2001
Parrafine oil	Fluka	76234
Pefabloc SC (AEBSF)	Roche	11 429 868 001
Silver nitrate	Merck	1.01512.0025
Sodium acetate anhydrous	Merck	1.01539.0500
Sodium dodecyl sulfate (SDS)	Fluka	71728
Sodium hydroxide	Merck	106498
Sodium thiosulfate	Fluka	72049
TEMED	Bio-Rad	161-0800
Trizma base	Sigma	T 1503
Thiourea	Fluka	88810
Urea	Fluka	51456

Table 3.2: Products, company and reference number of the used chemicals

Chapter 4

Effects of Insect Growth Regulators on the non-target soil arthropod *Folsomia candida* (Collembola)

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Abstract

The aim of this study was to assess the effect of several Insect Growth Regulators (IGR) on the non-target soil arthropod *Folsomia candida* (Collembola). The survival and reproduction rate of *F. candida* was evaluated after 28 days of exposure to six IGR (methoprene, fenoxycarb, precocene II, tebufenozide, hexaflumuron and teflubenzuron), and to a herbicide (Diuron) in artificial soil. The difference of sensitivity of *F. candida* to these different substances is high. The chitin synthesis inhibitors teflubenzuron and hexaflumuron were the most toxic compounds with an EC50 of 0.05 mg/kg dw for teflubenzuron and an EC50 of 0.6 mg/kg for hexaflumuron. Teflubenzuron is toxic for *F. candida* at concentrations which are probably close to environmental levels of this insecticide. Inhibition of reproduction is strongly related to adult survival for the juvenile hormone agonist methoprene and for the anti-juvenile hormone precocene II, with an EC50 of 173 mg/kg and a LC50 of 178 mg/kg for Methoprene and an EC50 of 15 mg/kg and a LC50 of 26 mg/kg for precocene II. Fenoxycarb, another juvenile hormone analog, showed a different dose-response curve for mortality than methoprene; at concentrations such as 3052 mg/kg no effect on adult survival was observed.

However, the EC50 value of 113 mg/kg is in the same order of magnitude than the one obtained for methoprene. A test with compressed soil contaminated with fenoxycarb was conducted in order to observe parameters such as number of laid eggs and hatched juveniles. No differences were observed between these two endpoints for fenoxycarb. An EC50 of 109 mg/kg was obtained for the ecdysone agonist tebufenozide. The herbicide diuron showed a relatively high toxicity for *F. candida* with an EC50 of 20 mg/kg. Our results show that some of the tested IGR can have an effect on collembola at environmentally relevant concentrations (toxicity/exposure ratios <5 for teflubenzuron, hexaflumuron and diuron).

4.1 Introduction

Insect Growth Regulators (IGR) are third generation insecticides less toxic and compatible with insect pest management that were developed to reduce the pollution of food and environment. These compounds have a specific mode of action on insects and a lower toxicity against vertebrates than conventional insecticides (Grenier & Grenier, 1993). For this purpose, the endocrine system of insects has been intentionally targeted for insecticidal activity. The developed insecticides are used to suppress insect populations, stopping their proliferation by disrupting their normal endocrine functions (EDSTAC, 1998).

Juvenile hormone mimics (e.g., methoprene, fenoxycarb), anti-juvenile hormone (e.g., precocene II), ecdysone analogs (e.g., tebufenozide) are some examples (Fig. 4.1). Other IGR compounds like teflubenzuron and hexaflumuron act preferentially by interfering with the chitin synthesis metabolism (chitin synthesis inhibitors). These substances appear to have a high target pest specificity and their effects can differ significatively among insect species (Dhadialla *et al.*, 1998; Grenier & Grenier, 1993). Some of the IGR are considered as highly toxic (LC50 100-1000 μg/l for tebufenozide) to very highly toxic (LC50 < 100 μg/l for fenoxycarb and methoprene) for aquatic insects (http://docs.pesticideinfo.org/Index.html, 2005). Their other major characteristics are summarized in Table 4.1.

"Endocrine disrupting compounds "(EDC) is a commonly used term to describe these substances that potentially interfere with hormones. Until now, observations on impact of endocrine disruptor compounds mainly focused on vertebrates and steroid substances (Vos *et al.*, 2000; OECD, 2001a). Endocrine disruption has also been relatively well studied in aquatic invertebrates (OECD, 2005; LeBlanc *et al.*, 1999; Depledge *et al.*, 1994; Hutchinson, 2002) but hardly any information is available on soil invertebrates.

Studies on the toxicity of IGR are mainly conducted by chemical companies producing or marketing the product, or with their financial support and focused principally on target pest organisms (Dhadialla *et al.*, 1998; Grenier

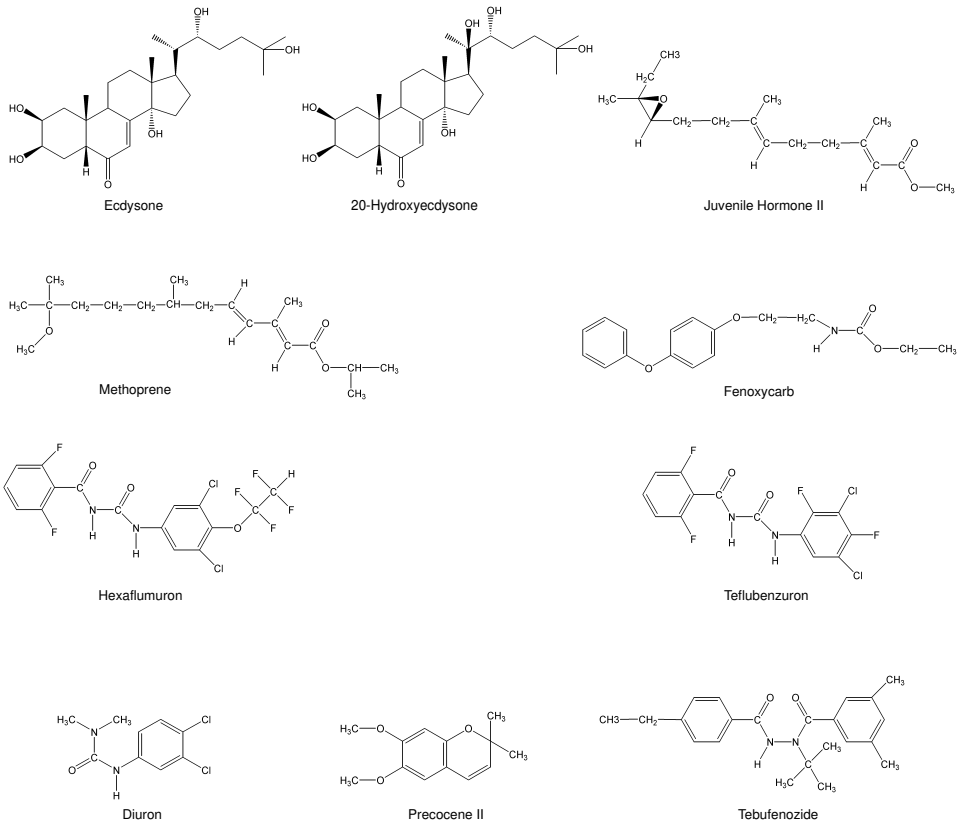

Figure 4.1: Chemical structures of insect hormones, Insect Growth Regulators and Diuron

& Grenier, 1993). Some of them concern aquatic invertebrates but very few of them deal with soil invertebrates (Kreutzweiser & Thomas, 1995; Addison, 1996; Hahn *et al.*, 2001; Preston *et al.*, 2000; Beck *et al.*, 2004). As after application many of these substances end up in the soil, which constitutes an essential resource of our environment, they can have a significant impact on soil organisms. According to annex 2 of the EU directive 91/414/EC on plant protection products (EU, 1991), the impact of active substances must be tested on earthworms and non-target terrestrial arthropods. However, testing of arthropods living in the soil is not always required although these organisms are essential to maintain fertility and functioning of the soil. The evaluation of potential effects of these chemicals on the reproduction of beneficial arthropods living in the soil should certainly be part of a global risk assessment.

In this sense, the objective of this study was to investigate the sublethal effect of six IGR on the springtail *Folsomia candida* (Willem) (Collembola; Isotomidae). Effect of methoprene, fenoxycarb, precocene II, tebufenozide,

hexaflumuron and teflubenzuron was evaluated on the survival and reproduction rate of *F. candida*. The impact of fenoxycarb on the number of laid eggs and hatched juveniles was also evaluated. Furthermore the effect of diuron, a herbicide of the urea chemical family, was tested on *F. candida* as this substance is known to potentially interfere with the androgen receptors of vertebrates (Bauer & Rombke, 1997; http://docs.pesticideinfo.org/Index.html, 2005).

Hormone action or mechanism	**Active substances**	**CAS number**	**Chemical family**	**Formulations**	**Effects noted (aquatic insects)**
Juvenile hormone agonist	Methoprene	40596-69-8	Terpene	ALTOSID, KABAT, PRECOR	DIANEX, MANTA, Development, growth, morphology, mortality, population, reproduction
Juvenile hormone agonist	Fenoxycarb	79127-80-3	Carbamate	INSEGAR, TORUS, VARIKILL	LOGIC, PICTYL, Development, mortality, population
Anti-juvenile hormone	Precocene II	644-06-4			
Ecdysone agonist	Tebufenozide	112410-23-8	Diacyl-hydrazine	MIMIC, ROMDAN	CONFIRM, Development
Chitin synthesis inhibitor	Hexaflumuron	86479-06-3	Benzoylurea	RECRUIT, CONSULT, CONSOL	SONET, Development
Chitin synthesis inhibitor	Teflubenzuron	83121-18-0	Benzoylurea	TEFLURON, NOMOLT, NEMOLT	Development, reproduction

Table 4.1: Insect Growth Regulators: their mechanisms of action, effects on aquatic insects and other properties.

4.2 Material and methods

4.2.1 Test organism

Folsomia candida is an apterygote (wingless) arthropod belonging to the Collembola classis which includes approximately 6500 different species. It is a parthenogenic, unpigmented and eyeless organism which can be found in most regions of the world except for Africa and India (Hopkin, 1997). Collembola have no metamorphosis and molt continuously throughout the entire life cycle. *F. candida* can reproduce after 12 to 16 days, the species has a high reproductive rate and can easily be bred in the laboratory (Spahr, 1981; Wiles & Krogh, 1998). Due to its importance to soil biology and to the key position in the soil food web (prey and consumer), *F. candida* is a relevant species for ecotoxicological testing (Laskowski *et al.*, 1998; Fountain & Hopkin, 2005) and is the collembola species used in the ISO standard 11267 (1999). The Guidance document on terrestrial ecotoxicology (EC, 2002) and the Standards of the European and Mediterranean Plant Protection Organization (EPPO, 2003) recommend the use of this reproduction test with *F. candida* under certain circumstances as part of the EU pesticide assessment scheme.

4.2.2 Breeding of *Folsomia candida*

The collembola *Folsomia candida* used for the experiments were generously provided by Dr. Frank Riepert from the Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany and have been bred in our laboratory since 1996.

According to the ISO standard 11267 (ISO, 1999), *F. candida* were cultured in plastic containers (160*110*60 mm with transparent cover). Recipients were filled with a layer (ca. 1 cm depth) of mixed plaster of Paris (Pharmacie du Closelet, Lausanne, Switzerland) and activated charcoal (Merck N°1.02186.1000 (Dietikon, Switzerland), analytic grade) (mass ratio 8:1). To maintain a high relative humidity (70-80%) in the container, the substrate was saturated with bidistilled water (without water standing on the surface). Granulated dry yeast (Dr. Oetker, Germany) was used as food supply.

Laboratory conditions were maintained constant (20 ± 2° C and 600 lux, with a light:dark cycle of 16h:8h). Twice a week, containers were aerated and unconsumed yeast was removed to avoid spoilage by fungi and bacteria. New food and some drops of bidistilled water were added. Once per month the collembola were transferred into new containers.

4.2.3 Synchronization of the culture

To synchronize the age of the organisms, oviposition was stimulated by placing adult collembola on a new breeding substrate. Oviposition occurred generally two days after transfer. Seven days later the clusters of eggs were transferred with a wetted fine-bristled paintbrush to a fresh substrate in new containers. The eggs hatched 2-3 days later. Three days after the transfer the remaining unhatched eggs were removed from the containers. The juveniles were used for experiments 10 days later, at the age of 10-12 days.

4.2.4 Chemicals

Methoprene (98.0 % purity), fenoxycarb (98.5 % purity), hexaflumuron (99.5 % purity), teflubenzuron (98.0 % purity) and diuron (97.5 % purity) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Precocene II and Tebufenozide (99.9 % purity) were obtained from Sigma-Aldrich (Seelze, Germany). As their water solubility is low, stock solutions were prepared by dissolving the substances in acetone prior to each experiment.

4.2.5 Preparation and contamination of the substrate

Testing was conducted with artificial OECD soil (OECD, 1984). The soil was composed of 70 % quartz sand (50 % 40-100 mesh, Fluka N° 84878 (Buchs, Switzerland) and 50 % $\geq$ 230 mesh Fluka N° 83340), 20 % of kaolinite clay (Fluka N° 60609) and 10 % of sphagnum peat ("Norddeutscher Hochmoortorf", pH 3.3, ESG-Bush GmbH, Rastede, Germany). The peat was air dried, ground and sieved to 1 mm. Sufficient amount of $CaCO_3$ was added to reach a pH of 6$\pm$0.5 (ISO, 1994). A quantity of bidistilled water corresponding to 50 % of the water holding capacity (ISO, 1999) was added to the different soil constituents, which were mixed thoroughly.

For each experiment, the quantity of the stock solution required to obtain the desired concentrations was added to a volume of acetone equivalent to the total mass of quartz sand. The acetone was added to the quartz sand and mixed thoroughly. The mixture was rotary evaporated and then placed under a fume hood for 3 hours to allow all acetone residues to evaporate. Finally, the contaminated quartz sand was mixed with the other soil constituents. All soils were prepared one day in advance and stored at room temperature.

4.2.6 Survival and reproduction testing

According to the ISO standard 11267 (ISO, 1999), substances were tested in a 28-day reproduction test. Preliminary range finder tests were conducted in order to determine the relevant test range of each substances. The concentrations which were finally tested are shown in Figure 4.2. For each test, a control containing acetone was run in parallel. For fenoxycarb only, a test

with compressed soil was conducted in addition to the ISO standard testing (see below).

Experiments according to the ISO standard were conducted in glass containers (diameter 4.5 cm, height 9.5 cm, with plastic covers closing tightly). Each container was filled with 30 g wet weight (ww) of artificial soil, without compressing the substrate. Ten juvenile springtails aged of 10-12 days were introduced into each container. The containers were kept under the same conditions as the culture. They were opened twice per week to allow aeration. Approximatively 10 mg of dry yeast was added at the beginning of the test (day 0) and 14 days later.

Five replicate containers were used for the control. Three replicates were used for each concentration (except for methoprene, hexaflumuron and teflubenzuron were five replicates were used per concentration). Five additional test containers were prepared: four for the pH measurement containing collembola and one for the water content control. The latter with no collembola was weighed at the beginning of the experiment and then once per week until the end of the test. The water loss was not compensated as it never exceeded 2% of the initial water content. The pH was determined (in the presence of 1M KCl) in triplicate according to the ISO standard 10390 (ISO, 1994). For each experiment, the pH was determined at the beginning and at the end of the test in the control and in the highest concentration.

After 28 days, the adults and juveniles were recovered and counted to evaluate the effect of the test substance on the mortality and on the reproduction. For this purpose, the soils were poured into 500 ml glass beakers (diameter 9 cm) and bromophenol blue colored water was added. The mixture was stirred gently with a spatula to allow drift of collembola to the water surface. Adults were counted by eye. A picture of the surface of the water was taken with a digital camera. Pictures were transferred to a computer and the number of juveniles was counted on the screen or on a paper-printed photograph.

4.2.7 Compressed soil experiment

For fenoxycarb, further testing was conducted with a semi-static set-up with compressed soil to prevent the collembola from burying into the soil. This protocol enables to record number of eggs and number of hatched juveniles per adult. The substrate was prepared and contaminated as for the experiments described above. Approximatively 1.5 g of soil was introduced in mini Petri dishes with lids (25 mm x 25 mm, internal diameter 22 mm, Semadeni N° 3390 (Ostermundigen, Switzerland)) and compressed to 0.5 to 1 cm height. One juvenile springtail aged of 2-3 days was introduced to one Petri dish.

The following three concentration series were tested (in mg/kg soil dry weight): 0, 0.16, 0.8, 4, 20, 100 for experiment 1; 0, 4, 20, 100, 500, 2500

for experiment 2; 0, 0.8 for experiment 3. Five replicates per concentration were used for experiments 1 and 2, whereas 15 replicates were prepared for experiment 3. For each experiment, a control containing acetone was run in parallel.

Test containers were maintained in the same conditions as for breeding. Each week, collembola were transferred to new Petri dishes contaminated on day 0 and kept at -20 °C in order to prevent fenoxycarb degradation (containers were acclimated to room temperature approximatively 3 hours before use).

Once a week (before changing of Petri dishes), the number of laid eggs and hatched juveniles was recorded. Observations were made over a period of 30 ± 1 days.

4.2.8 Statistical analysis

The concentrations causing 50 or 10 percent of reduction in the number of juveniles produced (EC50 or EC10, respectively) were calculated using a linear interpolation method based on the inhibition concentration approach using the ICp 2.0 software from the USEPA software (http://-www.epa.gov/nerleerd/stat2.htm, 2004). Mortality of the adults (LC50) was calculated with Probit analysis or Trimmed Spearman-Karber method using the USEPA software (http://www.epa.gov/nerleerd/stat2.htm, 2004). Level of significance was evaluated with the non-parametric Mann-Whitney-Wilcoxon U test. As there was no significant difference between the control and the control containing acetone, both controls were grouped. The only exception are the tests carried out with compressed soil, where a significant difference was observed between the two controls. In this case, treatments were compared to the control containing acetone.

4.3 Results

The following three validity criteria are required for collembola testing according to the ISO standard 11267 (ISO, 1999):

- the mortality of the adults in the control shall not exceed 20% at the end of the test;
- the reproduction rate shall reach a minimum of 100 instars per control vessel;
- the coefficient of variation of reproduction in the control shall not exceed 30%

These three validity criteria are respected for each test except for Diuron testing where the first validity criteria was not met with 25% mortality of

the adults in the control (see below). Dose-response curves were established for all substances (Fig. 4.2). Reproduction rate in the controls was quite constant over all tests, with a mean of 311 (± 95) individuals per test container after 28 days.

The 28-day data regarding the effect on reproduction (EC50 and EC10) and survival (LC50), as well as the corresponding no effect (NOEC) and lowest effect (LOEC) concentrations for each substance tested are shown in Table 4.2.

	LC50	**EC50**	**EC10**	**NOEC**	**LOEC**
Methoprene	178.0 (168.2-194.7)	172.6 (159.5-185.5)	70.0 (33.6-147.2)	130.0	175.0
Fenoxycarb	>3052	113.2 (9.3-174.0)	2.4 (1.4-11.1)	12.5	31.5
Precocene II	25.7 (22.5-29.3)	14.8 (13.2-16.4)	3.7 (0.3-10.5)	8.8	12.8
Tebufenozide	>730	109.2 (n.c.)	9.2 (0-63.3)	9	20
Hexaflumuron	1.8 (1.5-2.6)	0.6 (0.4-0.7)	0.1 (0.03-0.23)	0.1	0.15
Teflubenzuron	0.22 (0.19-0.27)	0.05 (0.03-0.06)	0.004 (0-0.02)	0.007	0.02
Diuron	703.0 (451.1-1095.6)	19.7 (13.2-23.6)	0.7 (0.4-12.9)	10.0	25.0

n.c.= not calculable

Table 4.2: 28-day effect and no effect concentrations with 95% confidence intervals of the tested compounds on *F. candida* (mg/kg soil dry weight)

In the case of methoprene and precocene II, an effect on the mortality of the adults was observed at the same order of magnitude as the effect on the reproduction with an EC50 of 173 mg/kg soil dw and a LC50 of 178 mg/kg for methoprene and an EC50 of 15 mg/kg and a LC50 of 26 mg/kg for precocene II.

Hexaflumuron and especially teflubenzuron show steep dose-response curves and a high toxicity with an EC50 of 0.6 mg/kg and a LC50 of 1.8 mg/kg and an EC50 of 0.05 mg/kg and a LC50 of 0.2 mg/kg, respectively.

Diuron also shows a relatively high toxicity with an EC50 of 20 mg/kg and a LC50 of 703 mg/kg. Although the first validity criteria was not re-

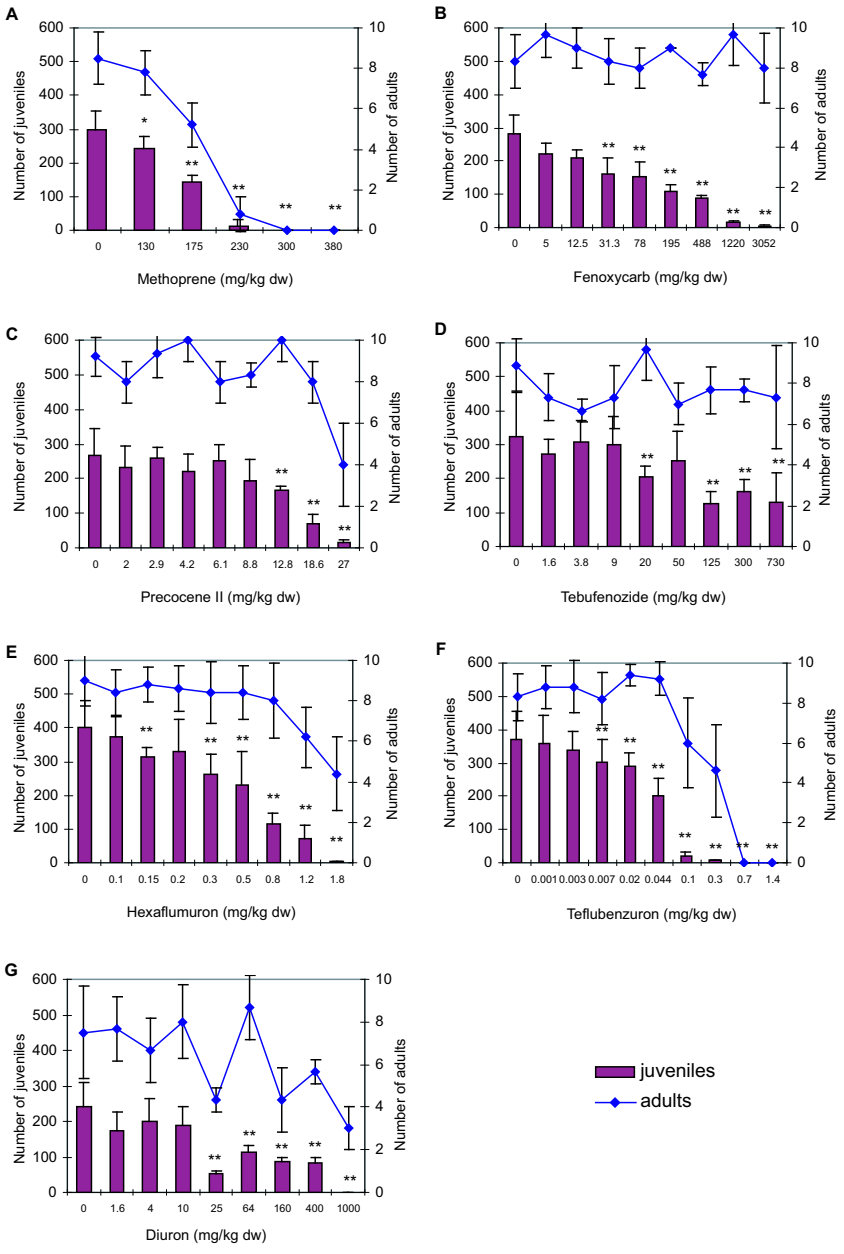

Figure 4.2: Number of adults and juveniles of *F. candida* after a 28-day exposure to the different compounds (* p<0.1, ** p<0.05)

spected due to a high mortality of the adults (80%) in one of the 10 control replicates, effect concentrations were calculated as for the other compounds (without discarding the replicate). The same EC50 and a slightly lower LC50 (587 mg/kg) are obtained when discarding the incriminated replicate.

For fenoxycarb, an EC50 of 113 mg/kg is obtained but no effect on the mortality of the adults was observed at the highest concentration tested (LC50 > 3052 mg/kg). Tebufenozide shows an EC50 of 109 mg/kg and as for fenoxycarb, no mortality of the adults occurs at the highest concentration tested (LC50 > 730 mg/kg). The EC50s of these two compounds are in the same order of magnitude as the EC50 obtained for methoprene.

Testing with compressed soil contaminated with fenoxycarb (Fig. 4.3) showed a decrease in reproduction at low concentrations (0.16 mg/kg), no effect in the medium range concentrations and, again, a decrease in reproduction at higher concentrations (> 500 mg/kg). Mortality of the adults was induced at the highest concentration (2500 mg/kg). No difference was observed between the number of eggs and the number of hatched juveniles (data not shown).

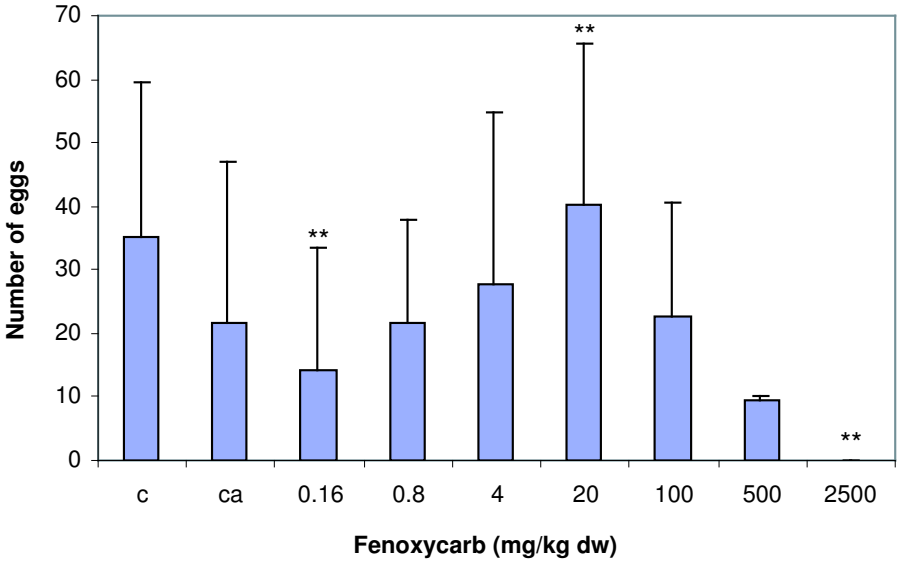

Figure 4.3: Number of total eggs laid per individual of *F. candida* during a 30-day exposure on compressed soil contaminated with Fenoxycarb (** $p<0.05$)

4.4 Discussion

F. candida showed high difference of sensitivity to the different compounds tested. The chitin synthesis inhibitor teflubenzuron has the highest toxicity for *F. candida* for both mortality and reproduction of all the tested substances. No measured soil concentrations could be found in the literature for the tested compounds. In order to evaluate the obtained effect concentrations, an initial worst-case predicted environmental concentration (PEC_i) was estimated and was compared to ecotoxicity values (Table 4.3). According to the EU directive on plant protection products (EU, 1991), it was assumed that 50% of the amount of the applied substance (field application rate) reaches the soil, that the bulk density of soil was 1.5 g/cm^3 (dry weight) and that the depth of the soil layer was 5 cm. Long-term PECs could not be calculated because, to our knowledge, the degradation data needed for such an estimation is not available. Therefore, the toxicity/exposure ratios (TER) were determined by dividing the NOEC by the initial PEC (Table 4.3). For hexaflumuron, teflubenzuron and diuron the TER is below the trigger value 5 used for earthworms in the EU Directive 91/414 (EU, 1991), which shows that collembola populations are at risk.

As said above, degradation time was not taken into consideration for this estimation. According to Pesticide Manual (1997), the DT50 of teflubenzuron is between 2 and 12 weeks in soil which means that the DT90 could

	Maximal field application rate	**Solution concentration**[a]	**PEC_i**	**TER**
Fenoxycarb	0.64 kg/ha[b]	25% (WP)	0.11	113.6
Tebufenozide	1 l/ha[a]	240 g/l	0.16	56.3
Hexaflumuron	1.2 l/ha[a]	100 g/l	0.08	1.3
Teflubenzuron	0.6 l/ha[a]	150 g/l	0.06	0.1
Diuron	5 kg/ha[a]	80% (WP)	2.67	3.8

PEC_i = initial predicted environmental concentration (mg/kg).
TER = toxicity/exposure ratio (NOEC/PEC_i.)
WP = wet powder.
[a] Index Phytosanitaire (2002).
[b] C. Linda, Swiss agricultural research station, Changins, Switzerland, personal communication.

Table 4.3: Worst-case predicted environmental concentrations and toxicity/-exposure ratios

be above the persistence trigger value of 100 days used in the EU Directive 91/414 (according to this directive, the testing of effects on soil non-target macro-organisms other than earthworms is not required with DT90 < 100 days; EU (1991)). For this compound, the initial worste-case PEC (0.06 mg/kg) is slightly higher than the EC50 (0.05 mg/kg) and the TER is 0.1. This shows that the potential environmental concentrations of teflubenzuron could have an important impact on the reproduction rate of soil arthropods like *F. candida*.

Hexaflumuron is the second most toxic substance tested on *F. candida* in this study. EC50 obtained for this compound is ten fold higher than the worst-case PEC_i value of 0.08 mg/kg.

For fenoxycarb and tebufenozide, the PEC values are considerably smaller than the measured EC50s and the TERs are far above 5, indicating a low environmental risk for *F. candida*. As precocene II is not an insecticide and as methoprene is not applied in the field (used to protect stored crops), no PECs could be estimated for these two compounds.

The very high observed toxicity of benzoylphenyl urea (teflubenzuron and hexaflumuron) for *F. candida* is probably due to an inhibition of chitin synthetase, the final enzyme in the pathway by which chitin is synthesized from glucose (Hammock & Quistad, 1981). Such an inhibition prevents the molting process and leads to collembola mortality. This could also explain the strong relation between mortality and reproduction dose response curves, the reduction in number of adults explaining the low reproductive output. If the regulatory effect described above is the mode of action of benzoylphenyl ureas in collembola, it can not be considered as endocrine disruption.

Methoprene shows a steep dose-response curve and, as for teflubenzuron, inhibition of reproduction is strongly related to adult survival. A different picture is obtained with fenoxycarb, where even at the highest concentration tested (3052 mg/kg) no effect on adult survival was recorded while reproduction is strongly inhibited. In the case of methoprene and teflubenzuron, it could be assumed that the development, molting or the morphology of the adults could be impaired which prevents the adults from reproducing.

It is surprising that two juvenile hormone analogs like fenoxycarb and methoprene show such big differences of toxicity for *F. candida*. These differences in toxicity might be due to two distinct molecular structures which do not have the same affinity for the receptor. The apparent similarity of methoprene to juvenile hormone is greater than the apparent similarity of fenoxycarb to juvenile hormone (Fig. 4.1). Methoprene is considered as one of the most active juvenile hormone analogs for insects (Dhadialla *et al.*, 1998). However, according to Edwards & Abraham (1985), fenoxycarb is 100 times more effective than methoprene for the coleoptera *Alphitobius diaperinus*, which is not the case for *F. candida* with our results. Considering the wide variety of effects noted for these juvenile hormone disrupting substances on insects (see Table 2.3), it is difficult to predict the exact mode of

action in *F. candida*.

With compressed soil experiments, two different effects could be observed. The first effect occurs at low concentrations where a diminution of the reproduction could be noted. The second effect occurs at higher concentrations, where a decrease in the number of laid eggs and a mortality of the adults was observed. Edwards & Abraham (1985) showed that low doses of fenoxycarb have a morphogenetical effect on the mealworm *Alphitobius diaperinus*, whereas higher doses result in direct toxic action. Therefore, it is possible that the two effects observed for *F. candida* are due to two different modes of action of fenoxycarb. No difference was observed between the number of eggs produced and the number of hatched juveniles, indicating that the substance does not influence the hatching. This is not the case for other substances such as dinoseb, which reduces the number of hatched juveniles but not the number of eggs at a concentration of 20 mg/kg dw (Staempfli, 1999).

The higher toxicity of fenoxycarb for collembola in the compressed soil experiment than in the uncompressed soil set-up could be explained by several factors. Average fenoxycarb concentrations were probably higher in the compressed soil experiments, as the experimental design was semi-static (less degradation due to renewal of the mini Petri dishes each week). The phenomenon of isolation (one individual per recipient) can also increase the sensibility of the organisms to the pollutant (Staempfli, 1999). Furthermore, it has been observed for other substances that collembola are more sensitive to a contamination of a compressed soil than to an uncompressed soil (Staempfli, 1999).

Precocene II is often used as a positive control for IGR testing. This compound inactivates the *corpora allata* which looses the ability to secrete juvenile hormone. Effectiveness of precocene II depends on the timing of application; maximal response is obtained if a treatment occurs when the *corpora allata* are active (Hammock & Quistad, 1981). In our experiments, this anti-juvenile hormone shows a higher chronic toxicity than the juvenile hormone agonists fenoxycarb and methoprene and the ecdysone agonist Tebufenozide. The latter showed a toxicity in the same order of magnitude as the two tested juvenile hormone agonists. According to soil microcosms studies (Addison, 1996), population growth of collembola was not affected by a concentration of 70 g/ha of tebufenozide (72 mg/kg organic material), which is not in contradiction with our results. However, our data is not in accordance with the considerations from Dhadialla *et al.* (1998) regarding the high specificity of tebufenozide for lepidopteran pests and no toxicity for non-target organisms.

Diuron inhibits plant photosynthesis but its exact mode of action on soil invertebrates is not known. Although it is not an insecticide, it shows a relatively high toxicity for *F. candida* (EC50 = 20 mg/kg). Jiang *et al.* (2000) demonstrated that 3,4-dichloroaniline (DCA), one of diuron's transformation

products metabolized by the organism, is more toxic for *F. candida* than diuron itself. Diuron is also transformed to DCA in soils and in water (Cox, 2003). Therefore, high toxicity of diuron for *F. candida* is probably due to DCA or other degradation products rather than to diuron itself. This is in accordance with a recent study conducted by Hund-Rinke & Simon (2005) where the EC50s obtained for DCA exposure of *F. candida* with three different natural soils were between 10 and 90 mg/kg.

In spite of the relatively high toxicity of the tested substances (teflubenzuron, hexaflumuron, fenoxycarb, tebufenozide, and diuron) for *F. candida*, they show a low acute toxicity for the compost worm *Eisenia foetida* (LC50 $>$ 800 mg/kg) (http://www.inra.fr/agritox/, 2005; Bauer & Rombke, 1997). However, the compost worm *Eisenia foetida* is the only soil invertebrate test required under all circumstances by the EU directive for plant protection products (EC, 2002).

4.5 Conclusion

Although Insect Growth Regulators (IGR) were specifically developed to suppress pest insects, our results show that non-target arthropods such as the collembola *F. candida* are affected by these compounds. The two chitin synthesis inhibitors teflubenzuron and hexaflumuron were the most toxic compounds at concentrations which are probably environmentally relevant (toxicity/exposure ratios $<$5). IGRs having apparently the same mode of action, as the two juvenile hormone analogs methoprene and fenoxycarb, show different dose response curves; inhibition of reproduction is strongly related to adult survival for methoprene while for fenoxycarb, at the highest concentration tested, reproduction is inhibited but no adult mortality was observed. Another test conducted with fenoxycarb on compressed soil showed that this insecticide has no effect on hatching of eggs. Like methoprene, the anti-juvenile hormone precocene II shows an effect on adult survival at the same order of magnitude as the effect on reproduction. The herbicide diuron, known to potentially interfere with the androgen receptors of vertebrates, has a relatively high toxicity for *F. candida*.

Juvenile hormone or ecdysone disrupting substances have a wide variety of effects on insects (see Table 4.1). We have shown that compounds targeting the same hormone do not necessarily have the same effect. The wide range of effect and difference of sensitivity noted for these compounds seem to depend strongly on parameters such as receptor affinities, regulatory pathways, life stages and taxa.

Our results do not enable to elucidate the exact mode of action of the tested compounds in *F. candida*. It is not clear whether the effect can be considered as endocrine disruption, which usually occurs at lower than sublethal concentrations. The elucidation of this question would need molecular

approaches. However, effects of endocrine disruption may be ecologically irrelevant in the presence of other mechanisms of acute and/or chronic toxicity (Preston *et al.*, 2000). The chronic effects observed in this study at environmentally relevant concentrations are of concern and could have consequences for the population of *F. candida* or other soil arthropods.

Acknowledgments

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Chapter 5

Multigeneration effects of Insect Growth Regulators on the springtail *Folsomia candida*

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Abstract

Multigeneration test are very useful for the assessment of long term toxicity of pollutants as the whole life cycle of several generations of organism can be integrated. With the emergence of endocrine disruptor compounds, such tests can provide very helpful information about damage produced by these substances. In this study, multigeneration reproduction tests adapted from the ISO standard 11267 were conducted with the collembola *Folsomia candida*. Springtails were exposed to artificial soil contaminated with four insect growth regulators (methoprene, fenoxycarb, teflubenzuron, and precocene II) according to two different experimental set-ups. In the first set-up, the parental generation (F_0) of collembola was exposed to a pollutant for 28 days. Juveniles from the F_1 generation were transferred to uncontaminated soil for another 28-day period to generate the F_2 generation. In the second set-up, the F_0 generation was exposed to a pollutant for 10 days before being transferred to uncontaminated soil to reproduce. After 18 to 28 days, juveniles from the F_1 were transferred to clean soil to generate the F_2 generation. An effect on the number of hatched juveniles of the F_2 generation was observed for methoprene after exposure of the F_0 for 28 days and hatching

of F_1 in contaminated soil. For methoprene and teflubenzuron, significant effects were even observed on the F_2 generation with the 2nd experimental set-up, when only the F_0 generation was exposed for 10 days. This shows that the impact of these substances is transgenerational, which can have important consequences for the population of these or other organisms. No effect on the F_2 generation was observed with fenoxycarb and precocene II with the 10-day exposure experiment. Our results show that the developed experimental procedures are appropriate to assess the long term effects of endocrine disrupting compounds on reproduction of the non target species *F. candida*.

5.1 Introduction

These last decades, hormonally active agents have emerged that are susceptible to interfere with the endocrine system of organisms. These endocrine disruptor compounds (EDC) can have adverse effects on wildlife and humans such as abnormal reproductive organs or sterility. Several *in vitro* test systems were developed to evaluate and characterize the impact of such substances on living organisms. It was finally recognized that *in vitro* testing was not sufficient to assess endocrine disruption on wildlife and that endocrine-disruptors assessments should primarily focus on *in vivo* studies. Strategy for ecological effects characterization of endocrine disruptors needs to be integrated into the exposure characterization components of a risk-based laboratory and field approach (Hutchinson, 2002).

As traditional risk assessment employing standard toxicity tests based on acute or chronic exposure seems to be insufficient for detecting reproductive damage mediated by an endocrine modulation mechanisms (Patyna *et al.*, 1999), new toxicity test guidelines for endocrine disruption have to be developed or currently used protocols adapted. For instance, multigeneration reproduction testing seems to be a suitable test method to detect and characterize endocrine disrupting chemicals (EDC)(OECD, 2005). Indeed, effects associated with potential endocrine disrupters can be latent and not manifested until later in life, or may not be apparent until reproductive processes occur in an organism's life history (OECD, 2005). In this sense, tests specially developed for endocrine disruption often encompass two generation to address effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity (OECD, 2005).

There are several ways to conduct a multigeneration test. Different strategies can be adopted, such as exposure of all generations or only exposure of the parental F_0 generation. The exposure can be static or semi-static, continuous exposure being impossible to realize in soil with a degradable substance. Such multigeneration tests can also be used for other substances

and should not exclusively be linked to substances with potential endocrine activity.

For vertebrates, particulary for rodents and fish, EDC detection is mainly performed through screening assays or two-generation toxicity studies where test procedures are defined by international standards (OECD, 2001b, 2004; USEPA, 2002). For invertebrates, however, although they represent about 90 % of worldwide animal species and although many invertebrate toxicity test protocols are routinely used in regulatory ecotoxicity testing, few of these methods have been designed with endocrine-specific endpoints in mind. Therefore, new methods for testing effects of endocrine disrupters compounds will be needed to assess the adverse consequences of such chemicals on invertebrates development and reproduction (OECD, 2005).

The Organization for Economic Co-Operation and Development (OECD) has recently published a review paper on "Aquatic arthropods in life cycle and two-generation toxicity tests" were candidate protocols for EDC testing are listed. In most of cases it is proposed to adapt or modify for EDC testing chronic testing protocols developed by several regulatory agencies for species such as daphnids, chironomids, copepods or mysids (OECD, 2005).

If few EDC testing procedures are available for aquatic invertebrates even less exist for soil invertebrates. However, soil arthropods, like springtails (Collembola) which play an important role in soil respiration and decomposition processes, are also concerned by endocrine disruption. In previous work we have shown that insect growth regulators (IGR) have an important effect on the reproduction of the collembola *Folsomia candida* at environmentally relevant concentrations. By mimicking the juvenile hormone, Methoprene prevents metamorphosis to viable adults when applied to larval stages in most insect species. It is a widely employed insecticide throughout the world (North America, Europe, Asia and Pacific region) where it is used against mosquitoes in aquatic areas or in some domestic and commercial products against other insect pests (http://docs.pesticideinfo.org/Index.html, 2005). Fenoxycarb is another juvenile hormone analog acting by contact or stomach action. The chitin synthesis inhibitor teflubenzuron inhibits molting processus. When used as larvicide, insects are unable to reach the adult stage which prevents them from reproducing. Fenoxycarb and teflubenzuron are applied as insecticides in agriculture. Precocene II is an anti-juvenile hormone which prevents the corpora allata from producing juvenile hormone. It is not commercialized as insecticide because of its lack of activity against most holometabolous insects (Tunaz & Uygun, 2004) but is often used as positive control for IGR testing (Pesticide Manual, 1997; http://www.inra.fr/agritox/, 2005; Extoxnet, 2003). The chemical formulas of the described IGR are shown in Figure 5.1.

It is conceivable that these substances specifically developed to inhibit insect growth and maturation interfering with their endocrine system by mimicking insect hormones like juvenile hormone or ecdysone, could also have

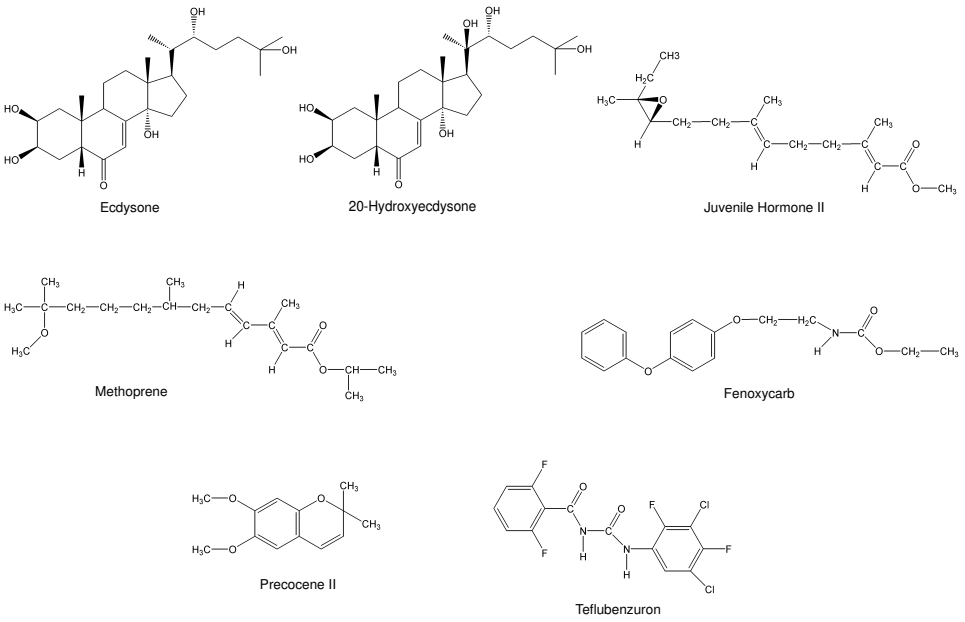

Figure 5.1: Chemical structures of insect hormones and Insect Growth Regulators

endocrine disrupting effects on other invertebrates like *F. candida* which is phylogenetically close to insects. As *F. candida* is already a widely used soil invertebrate in ecotoxicology testing (ISO 11267), it seemed of interest to us to modify existing protocols or to develop new ones for EDC testing with this organism.

The aim of this study was to adapt the Collembola ISO standard 11267 for multigeneration reproduction testing. Effects of four IGR, the juvenile hormone analogs methoprene and fenoxycarb, the chitin synthesis inhibitor teflubenzuron and the anti-juvenile hormone Precocene II were evaluated on the second generation of *F. candida* according to different types of exposure of the parental F_0 generation.

5.2 Material and methods

5.2.1 Test organism

The springtail *Folsomia candida* is a small soil arthropod belonging to the Collembola classis. It is an unpigmented and eyeless organism which can be found in most regions of the world except for Africa and India (Hopkin, 1997). It reproduces by parthenogenesis and is sexually mature after approximately 22 days. *F. candida* grows by molting continuously throughout its entire life cycle without metamorphosis. It is a relevant species for

ecotoxicological testing and is used in the ISO standard 11267 (ISO, 1999) due to its importance to soil biology, key position in the soil food web (prey and consumer), easiness of breeding in the laboratory and high reproductive rate (Spahr, 1981; Wiles & Krogh, 1998; Laskowski *et al.*, 1998; Fountain & Hopkin, 2001).

5.2.2 Breeding of *Folsomia candida*

The collembola *Folsomia candida* used for the experiments were generously provided by Dr. Frank Riepert from the Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany and have been bred in our laboratory since 1996.

According to the ISO standard 11267 (ISO, 1999), *F. candida* were cultured in plastic containers (160*110*60 mm with transparent cover). Recipients were filled with a layer (ca. 1 cm depth) of mixed plaster of Paris (Pharmacie du Closelet, Lausanne, Switzerland) and activated charcoal (Merck N°1.02186.1000 (Dietikon, Switzerland), analytic grade) (mass ratio 8:1). To maintain a high relative humidity (70-80%) in the container, the substrate was saturated with bidistilled water (without water standing on the surface). Granulated dry yeast (Dr Oetker, Germany) was used as food supply.

Laboratory conditions were maintained constant (20 ± 2° C and 600 lux, with a light:dark cycle of 16h:8h)

Twice a week, containers were aerated and unconsumed yeast was removed to avoid spoilage by fungi and bacteria. New food and some drops of bidistilled water were added. Once per month the collembola were transferred into new containers.

5.2.3 Synchronization of the culture

To synchronize the age of the organisms, oviposition was stimulated by placing adult collembola on a new breeding substrate. Oviposition occurred generally two days after transfer. Seven days later the clusters of eggs were transferred with a wetted fine-bristled paintbrush to a fresh substrate in new containers. The eggs hatched 2-3 days later. Three days after the transfer the remaining unhatched eggs were removed from the containers. The juveniles were used for experiments 10 days later, at the age of 10-12 days.

5.2.4 Chemicals

Methoprene (98.0 % purity), fenoxycarb (98.5 % purity) and teflubenzuron (98.0 % purity) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Precocene II was obtained from Sigma-Aldrich (Seelze, Germany). As their water solubility is low, stock solutions were prepared by dissolving the substances in acetone prior to each experiment.

5.2.5 Preparation and contamination of the substrate

Testing was conducted with artificial OECD soil (ISO, 1993). The soil was composed of 70 % quartz sand (50 % 40-100 mesh, Fluka N° 84878 (Buchs, Switzerland) and 50 % $\geq$ 230 mesh Fluka N° 83340), 20 % of kaolinite clay (Fluka N° 60609) and 10 % of sphagnum peat ("Norddeutscher Hochmoortorf", pH 3.3, ESG-Bush GmbH, Rastede, Germany). The peat was air dried, ground and sieved to 1 mm. Sufficient amount of $CaCO_3$ was added to reach a pH of 6±0.5 (ISO, 1994). A quantity of bidistilled water corresponding to 50 % of the water holding capacity (ISO, 1999) was added to the different soil constituents, which were mixed thoroughly.

For each experiment, the quantity of the stock solution required to obtain the desired concentrations was added to a volume of acetone equivalent to the total mass of quartz sand. The acetone was added to the quartz sand and mixed thoroughly. The mixture was rotary evaporated and then placed under a fume hood for 3 hours to allow all acetone residues to evaporate. Finally the contaminated quartz sand was mixed with the other soil constituents. All soils were prepared one day in advance and stored at room temperature.

5.2.6 Exposure conditions and test set-up

Testing was carried out according to an adaptation of the ISO standard 11267 (ISO, 1999). Test set-up was divided into two cycles of approximately 28 days each. During the first cycle, parental generation (F_0) of springtails was allowed to reproduce (generating the F_1 generation) in contaminated soil after different exposure times. The first generation of collembola (F_1) was then allowed to reproduce in uncontaminated soil (generating the F_2 generation) during the second cycle (28 days).

Effect concentrations of methoprene, fenoxycarb, teflubenzuron, and precocene II were determined in preliminary experiments according to the ISO standard 11267 (ISO, 1999) in a 28-day reproduction test (Campiche *et al.*, 2006). Tested concentrations are shown in Figures 5.4, 5.5, 5.6 and 5.7. For fenoxycarb (28-day F_0 exposure) a control and a control containing acetone were run in parallel. As there was no significant difference between these two controls, only a control containing acetone was prepared for the other experiments.

For the first cycle, five to ten replicate containers were used for the control (containing acetone). Artificial soil was contaminated according to the chosen concentrations and three replicates were made for each concentration. Five additional test containers were prepared: four for the pH measurement containing collembola and one for the water content control. The latter with no collembola was weighed at the beginning of the experiment and then once per week until the end of the test. The water loss was not compensated as it did not exceed 2 % of the initial water content. The pH was determined (in

the presence of 1 M KCl) in triplicate according to the ISO standard 10390 (ISO, 1994). For each experiment, the pH was determined at the beginning and at the end of the test in the control and in the highest concentration.

For the second cycle, the number of replicates per control and concentrations were the same than for the first part (5 and 3, respectively) but all the containers contained uncontaminated soil, this means that for each replicate from the first cycle of the experiment (even those with contaminated soil), a new container with uncontaminated soil was prepared). Only two containers for pH measurement were needed (for the beginning and the end of the experiment).

Two different multigeneration test set-ups, differing by their first cycle were conducted. For the first one, parental generation (F_0) of springtails was exposed during 28 days to contaminated soil. Laid eggs and hatched juveniles (F_1 generation) were also exposed until the end of the cycle. In the second test set-up, only the parental generation (F_0) was exposed to pollutants. They were exposed for 10 days to contaminated soil, then recovered and placed in uncontaminated soil to allow eggs laying without exposure of the F_1 generation. They stayed in uncontaminated soil for 18 to 28 days, until juveniles of the F_1 generation were aged of approximately 10 days and were big enough to be transferred to clean soil for the second cycle.

5.2.7 28-day exposure

Experiments were conducted in glass containers (diameter 4.5 cm, height 9.5 cm, with plastic covers closing tightly). Each container was filled with 30 g wet weight (ww) uncompressed and contaminated (except the control) artificial soil. Ten juveniles springtails (F_0; parental generation) aged of 10-12 days were introduced into each container and exposed for 28 days to the pollutant. Approximatively 10 mg of dry yeast was given as food supply. The containers were kept under the same conditions as the culture. They were opened twice per week to allow aeration. At the end of the 28 days (first cycle), the hatched juveniles (F_1) and adults (F_0) were recovered by flotation. Soil was poured into 500 ml glass beakers (diameter 9 cm), tap water was added to almost the top of the beaker and the mixture was gently stirred with a spatula to allow drift of the collembola to the water surface. Bromophenol blue was added to color the water and to allow a better contrast with the whitish collembola. Adults were counted by eye. A picture of the surface of the water was taken with a digital camera. Pictures were transferred to a computer and the number of juveniles was counted on the screen using an image analysis software (UTHSCSA Image Tool 3.0) or on a paper-printed photograph. For each replicate, 10 juveniles collembola from the F_1 generation were removed from the water surface using a teaspoon and transferred in non contaminated soil (30 g ww per replicate) for a new 28 days reproduction period (second cycle). To avoid introduction of contami-

nated substrate in the new test containers, springtails were first transferred from the teaspoon to a A4 black cardboard. This step was realize by blowing them from the teaspoon to the cardboard by using a tip equipped with a rubber bulb. They were then counted and transferred from the cardboard to glass containers to allow reproduction. Juveniles were chosen according to their size and only those that had the approximate size of 10 day old collembola were taken (generally the biggest)(Figure 5.2). This set-up was used for methoprene and fenoxycarb only.

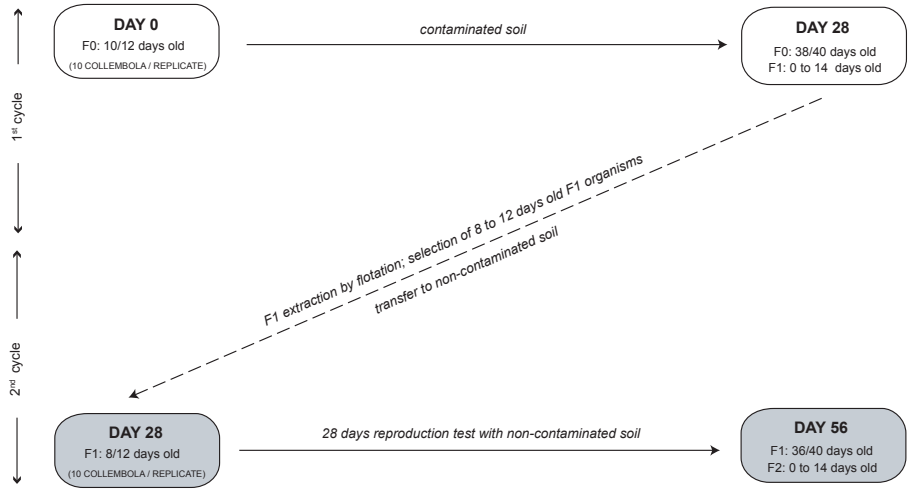

Figure 5.2: Multigeneration test with 28-day F0 generation exposure to pollutants

5.2.8 10-day exposure

Soil and test containers were prepared as for the 28 days exposure experiment. Fifteen to twenty juveniles springtails (F_0; parental generation) aged of 10-12 days were introduced into each container. Feeding and laboratory conditions were the same as for the 28-day exposure experiment. After 10 days of exposure, adults (F_0) were recovered by flotation but without adding bromophenol blue (see above). For each replicate, 10 adults collembola were recovered from the water surface using the "teaspoon method" and transferred to uncontaminated artificial soil (30 g ww) in glass containers to allow reproduction. Baker's yeast was added as food supply. Eighteen to twenty-eight days after the transfer, the first cycle of the experiment was stopped

and adult (F_0) and juvenile (F_1) collembola were recovered by flotation using the same method as described above. Adults (F_0) and juveniles (F_1) were counted. Ten juveniles from the F_1 generation were then removed from the water surface of each replicate according to the method described above and transferred to non-contaminated soil in new containers. Juveniles were chosen according to their size and only those that have the approximate size of 10 day old collembola were taken (generally the biggest). The second cycle was run like a standard ISO test for 28 days. After 28 days, the adults (F_1) and juveniles (F_2) were recovered by flotation again and counted (Figure 5.3). This set-up was used for the four tested substances.

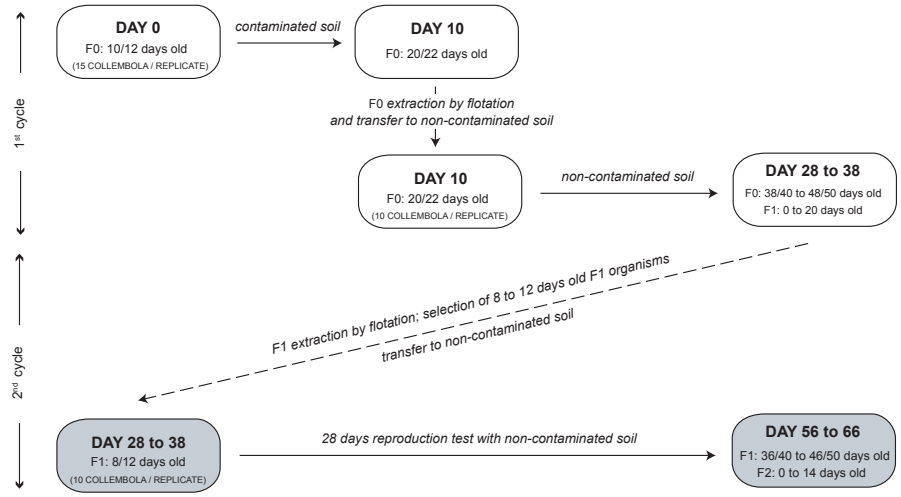

Figure 5.3: Multigeneration test with 10-day F0 generation exposure to pollutants

5.2.9 Statistical analysis

To enable a better comparison between the results obtained for the different generations, the number of juveniles are expressed in percent of the control set to 100 %. The concentration causing 50 percent of reduction in the number of juveniles produced (EC50) was calculated using a linear interpolation method based on the inhibition concentration approach using the ICp 2.0 software from USEPA (http://www.epa.gov/nerleerd/stat2.htm, 2004). Mortality of the adults (LC50) was calculated with Probit analysis or Trimmed Spearman-Karber method using the USEPA software (http://-

www.epa.gov/nerleerd/stat2.htm, 2004). Level of significance was evaluated with the non-parametric Mann-Whitney-Wilcoxon U test.

As for fenoxycarb (28-day F_0 exposure) no significant difference between the control and the control containing acetone was observed, both controls were grouped.

5.3 Results

Reproduction dose response curves were established for each collembola daughter generation (F_1 and F_2) after the different exposures (28-day or 10-day) of the parental generation (F_0) to each compound. They are shown in Figures 5.4, 5.5, 5.6 and 5.7. If more than ten adults (F_0 or F_1 generation) were recovered at the end of the experiment, meaning that an error had occurred during counting and introduction of collembola in test containers at the beginning of test, the replicate was discarded and not included in the results. The effect concentrations (EC50) obtained for the two generations of *F. candida*(F_0 generating the F_1 and F_1 generating the F_2 generation) are shown in Table 5.1 for the different exposure times. For comparison, 28-day EC50s obtained in previous experiments (Campiche *et al.*, 2006) are also shown in Table 5.1. All effect concentrations are expressed as exposure concentrations of the F_0 generation.

	EC50[a] 28-day exp.		**EC50[a] 10-day exp.**	
	F_0	**F_1**	**F_0**	**F_1**
Methoprene	162 (89 - 191)	191 n.c.	no data	201 n.c.
Fenoxycarb	122 (11 - 191)	no significant effect at 488	no effect at 3052	no effect at 3052
Teflubenzuron	0.05[b] (0.03 - 0.06)	no data	0.11 n.c.	> 0.12 n.c.
Precocene II	15[b] (13-16)	no data	no data	no effect at 18.3

n.c.= not calculable.

[a] EC50 are expressed in exposure concentrations of the F_0 generation.

[b] data: Campiche et al., 2006.

Table 5.1: Effect concentrations (mg/kg) for F_0 and F_1 generation (28-day and 10-day exposure; with 95% confidence intervals)

After a 28-day exposure of the F_0 generation to methoprene, a similar dose-response reproduction curve was obtained for the two generations of collembola. Indeed, for both the F_1 and the F_2 generation, the percentage of reproduction decreases to less than 20% compared to the control (0.6 % for F_1 and 19 % for F_2) at the highest concentration tested (300 mg/kg). The EC50 obtained for the F_0 generation confirms the EC50 obtained in previous work (162 and 173 mg/kg, respectively) (Campiche *et al.*, 2006). An EC50 of 191 mg/kg was obtained for the F_1 generation, which was transferred after hatching from a contaminated to a clean soil (see Figure 5.4).

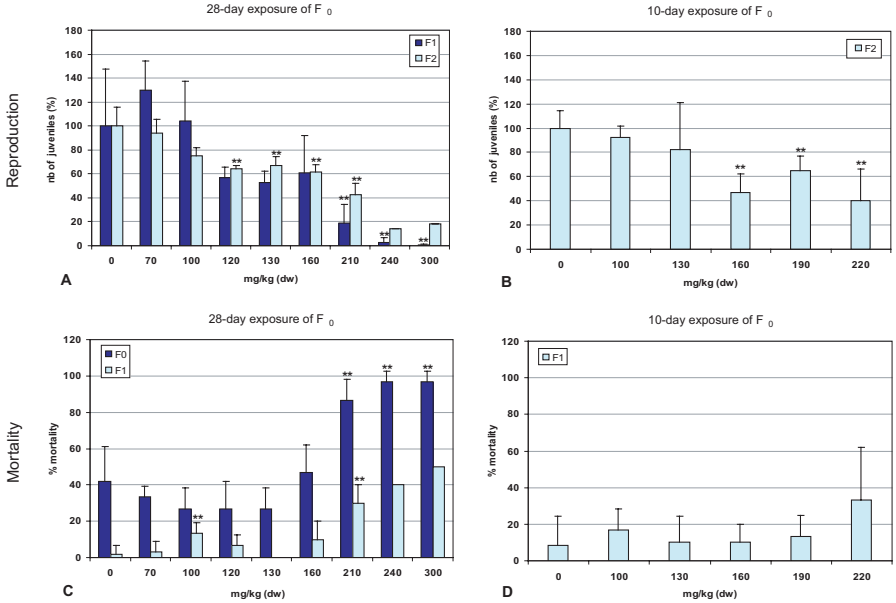

Figure 5.4: Number of juveniles (%) (A and B) and adult survival (C and D) after 28-day (A and C) and 10-day exposure (B and D) of the F_0 generation to methoprene (** $p < 0.05$, * $p < 0.1$)

When the F_0 generation is exposed for 10 days to methoprene and transferred to uncontaminated soil for reproduction, a smoother dose-response curve than for the 28-day exposure was observed. At the highest concentration tested (220 mg/kg), the number of juveniles generated by the F_1 was 40 % compared to the control. An EC50 of 201 mg/kg was obtained for the F_1 generation after a 10-day exposure.

When the F_0 generation was exposed for 28 days to methoprene, 97 % mortality of the adults (F_0) was reached for the highest concentration tested (300 mg/kg) and a LC50 of 135 mg/kg was estimated. In previous work, a LC50 of 178 mg/kg was obtained with a classic 28-day reproduction test (Campiche *et al.*, 2006), which is in accordance with this result. As for F_0, adult mortality of the F_1 generation also increased with the tested

concentrations with 30% of mortality at 210 mg/kg and 50 % at 300 mg/kg (one replicate). The LC50 for the adults of the F_1 generation was 309 mg/kg. However, no mortality of the adults of the F_1 generation was observed after a 10-day exposure of the F_0 generation for all the tested concentrations.

For fenoxycarb, the two daughter generations showed different dose-response reproduction curves after the 28-day exposure experiment (Fig. 5.5). An EC50 of 122 mg/kg was estimated for the F_0. For the F_1, a slight but insignificant effect at 488 mg/kg was observed with a reduction in the number of F_2 offsprings. This tendency could not be confirmed as not enough F_1 juveniles were available for the second experimental cycle in the higher concentrations. After a 10-day exposure of the F_0 generation to fenoxycarb, no reduction could be observed on the number of juveniles of the F_1 or the F_2 generation. There was no mortality of adults of the F_0 or the F_1 generation after a 28-day and 10-day exposure to fenoxycarb.

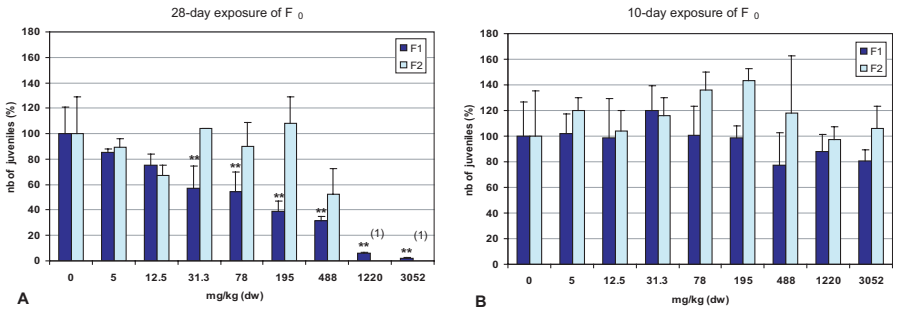

Figure 5.5: Number of juveniles (%) after 28-day (A) and 10-day exposure (B) of the F_0 generation to fenoxycarb ((1) = no data available; see text for explanation) (** $p < 0.05$)

For teflubenzuron, an EC50 of 0.11 mg/kg was obtained for the F_0 generation after the exposure for 10 days. However, as a high variability was observed between the replicates, only the highest concentration (0.12 mg/kg) showed a significant effect. A similar dose-response curve was observed between the two daughter generations (Fig. 5.6), but an EC50 could not be determined for the F_1 (> 0.12 mg/kg). In this case, a significant effect was observed at 0.036 mg/kg, but not at the two subsequent concentrations (0.08 and 0.12 mg/kg). No explanation could be found for this observation. No significant effects of teflubenzuron was observed on the survival of the adults from the F_0 generation when exposed for 10 days to this pollutant.

In the case of precocene II, no effect was observed on the juveniles generated by the F_1 generation of springtails after a 10-day exposure of the F_0 generation (Fig. 5.7). No adult mortality of the F_1 was observed.

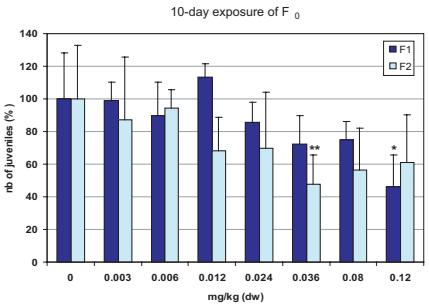

Figure 5.6: Number of juveniles (%) after 10-day exposure of the F_0 generation to teflubenzuron (** $p < 0.05$, * $p < 0.1$)

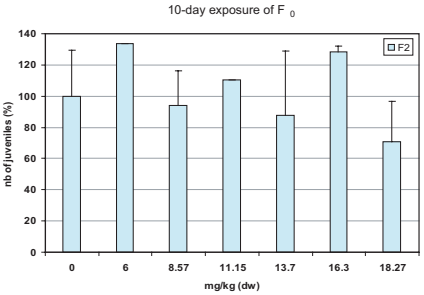

Figure 5.7: Number of juveniles (%) after 10-days exposure of the F_0 generation to precocene II

5.4 Discussion

The different exposure set-ups (10 days or 28 days) and the various compounds tested show great differences in response of *Folsomia candida*. Methoprene shows an effect on the F_2 juveniles generated by the F_1 for both exposure modes (28-day or 10-day) and teflubenzuron for the 10-day exposure mode. Fenoxycarb has a slight but insignificant effect on the F_2 juveniles in the 28-day exposure experiment whereas precocene II had no effect on the F_2 juveniles. The comparison between the results obtained with the different experimental set-ups can enable us to deduce important information on the mode of action of these compounds.

The main difference between the two exposure modes is that in the 28-day exposure experiment the F_1 eggs and hatched juveniles are in contact with the pollutant before being transferred to uncontaminated soil. In the 10-day exposure experiments, the F_0 is transferred to clean soil before oviposition. This means that the F_1 eggs and juveniles are never exposed to the tested substances. However, the exposure time of the F_0 before oviposition

is only slightly shorter in the 10-day exposure experiment that in the 28-day exposure experiment. In both cases, the F_2 is not exposed and all effect concentrations are expressed according to the exposure concentrations of the F_0.

The EC50s obtained for methoprene for the F_0 (162 mg/kg) and F_1 (191 mg/kg) generation for the 28-days exposure set-up are very close and the dose-response curve very similar. In the 28-day exposure experiment, the F_1 eggs are laid after approximately 10 days, the eggs hatch after approximately 20 days, which means that the F_1 juveniles are exposed for about 8 days. However, the methoprene concentration is probably much lower for the F_1 than for the F_0, as the estimated DT50 of methoprene is 10 days ((Pesticide Manual, 1997; http://www.inra.fr/agritox/, 2005; Extoxnet, 2003)). On the other hand, the exposure of the F_1 occurs in an earlier life stage than the exposure of the F_0 generation (starting at the age of 10 days), therefore F_1 might be more sensitive to the tested pollutant. This can explain the very similar EC50s obtained for the two generations.

The fact that an effect was observed on the reproduction of the F_1 shows that methoprene cannot only have an effect on egg eclosion or on mortality of juveniles - in that case the surviving F_1 individuals would have reproduced normally. Methoprene seems to reduce the reproduction capacity of the adults of the F_0 and F_1 generations. This hypothesis is confirmed by the following observation.

The LC50 for adults survival of F_0 (135 mg/kg) is very close to the EC50 obtained for reproduction of F_0 (162 mg/kg) (comparable results were obtained in previous experiments realized in our laboratory in a standard ISO reproduction test (Campiche *et al.*, 2006)). According to these results, it could be conceivable that the main effect of methoprene is an effect on adult survival, which would explain the diminution of the number of juveniles. But, although the LC50 of F_0 is more than two fold higher than the LC50 of the F_1 (309 mg/kg), which might be linked to the lower methoprene concentrations, the EC50 for reproduction is in the same order of magnitude for the two generations. This confirms that methoprene has an effect on fecundity.

In insects, Methoprene is usually not effective against adult organisms and it is generally used against the larval/nymphal form. However, sublethal effects on fecundity or toxic effects on eggs as well as direct "adulticidal" actions are observed in some cases. Adult dying results in abnormal morphology characteristics leading to mortality but is not directly related to a direct toxic action (Glare & O'Callaghan, 1999). It seems that for *F. candida* two modes of action can be outlined as methoprene affects adults viability and fecundity.

For the 10-day exposure set-up, a very similar EC50 was obtained for the F_1 generation (201 mg/kg) as for the F_0 of the 28-day exposure set-up (191 mg/kg) although the F_1 eggs and juveniles were not exposed to methoprene. This confirms that methoprene does not exert an effect on

eggs or on survival of hatched juveniles, but that there is a reduction in the reproductive capacity which is transmitted from the F_0 to the F_1. This result is quite astonishing, and, to our knowledge, it is the first time that such observations are made in nontarget invertebrates.

This transgenerational effect could be explained by a reduction in fitness of the F_0 due to the methoprene exposure, which will generate a weak F_1. Another explanation could be bioaccumulation of methoprene (or it's metabolites) in fat tissues of the organisms. Walker *et al.* (2005) showed in a recent study that methoprene, which has a log Kow of 5.12, can concentrate in hepatopancreas, gonadal tissue, nervous tissue and epidermal cells of the adult crustacea *Homarus americanus*. Assuming that methoprene can bioaccumulate in gonadal tissues of *F. candida*, the substance or it's metabolites could be transferred to eggs and then to the next generations of individuals disturbing further reproduction. Indeed, as oocytes of collembola are composed of lipid droplets and other materials such as yolk spheres and granular material (Hopkin, 1997), it is possible that methoprene (or it's metabolites) can be transferred with these lipids. In some insect species (*Manduca sexta*), up to 40 % of the dry weight of the mature egg is lipid. The majority of the lipids are stored in the fat body (insects analogous organ to vertebrates adipose tissue and liver) and then delivered to oocyte by lipophorin (Canavoso *et al.*, 2001). An other hypothesis explaining this cross-generational effect could be an alteration of the genetic material or of the chromatin structure. In the case of a genetic alteration in germinal cells, as *F. candida* is a parthenogenic organism, the entire genome with all alterations is transmitted from one generation to the other.

Fenoxycarb shows a different response pattern than methoprene. With the 28-day exposure set-up, a clear effect is observed on the reproduction of the F_0 generation. For the reproduction of the F_1 generation a tendancy is observed with a slight effect at 488 mg/kg, but this effect is not statistically significant. Unfortunately, not enough F_1 individuals could be recovered at the highest concentrations (1228 and 3052 mg/kg) to enter the second experimental cycle. Degradation of the compound cannot explain these results, as fenoxycarb has a DT50 of 17 to 31 days (field values) ((Pesticide Manual, 1997; http://www.inra.fr/agritox/, 2005; Extoxnet, 2003)). No mortality of the adults from the F_0 generation was observed at the end of the first experimental cycle (end of the 28-day period) even at the highest test concentrations. As no significant effect on the number of hatched F_2 juveniles could be observed, it can be presumed that fenoxycarb does not affect the reproductive capacity of the adult *F. candida* but rather the hatching of eggs or the survival of juveniles. This result is confirmed by the 10-day exposure set-up where the F_1 eggs and juveniles were not exposed to fenoxycarb and where no effect on the number of hatched juveniles could be observed. As in previous experiments no ovicidal effects of fenoxycarb were observed (Campiche *et al.*, 2006) it can be assumed that this substance only affects the early

juvenile stage of *F. candida*. Many studies on insects have already reported that the effects of fenoxycarb strongly depend on the developmental stage of the organisms, affecting mainly early larval instars (Grenier & Grenier, 1993; Pesticide Manual, 1997).

Teflubenzuron, just as methoprene, has an effect on the reproduction of the F_1 generation after 10 days of exposure of the F_0 generation (no exposure of the F_1). The number of hatched F_1 juveniles decreased by 50 % compared to the control for the highest concentration tested (0.12 mg/kg). A similar dose-response curve was obtained for F_2 as the number of juveniles enumerated at 0.036 mg/kg corresponded to 60 % of the control. As for methoprene, it seems that the damages induced by teflubenzuron on *F. candida* are transmitted from one generation to another, leading to the observation of a comparable effect on the two daughter generations. To our knowledge, there is no evidence of bioaccumulation of this compound. In insects, teflubenzuron inhibits chitin synthesis, affecting molting processes. Treated organisms fail to ecdysis and are thus unable to reach the adult stage which prevents them from reproducing (Tunaz & Uygun, 2004). We assumed in previous work where a strong relation between mortality and reproduction dose-response curves was observed that the compound has the same mode of action in collembola (Campiche *et al.*, 2006). However, in this experiment no mortality of the adults of the F_0 generation occurred, showing that the reduction in the number of juveniles cannot be simply due to a lower number of adults. As for methoprene, the fecundity of the parental generation F_0 of *F. candida* seems to be affected, which might be due to another mode of action than chitin synthesis inhibition. In some insect species such as hemiptera exposure to teflubenzuron led to a reduced fertility (Scott Brown *et al.*, 2003).

No measured soil concentrations could be found in the literature for the tested compounds. However, for teflubenzuron the observed effects occur at concentrations which are above the predicted initial worst-case environmental concentrations (0.06 mg/kg; (Campiche *et al.*, 2006)).

Precocene II showed no significant effect on the reproduction of the F_1 generation with the 10-day exposure set-up. As no data on the reproduction of the F_0 generation was available (number of hatched F_1 juveniles not recorded), it is difficult to predict the mode of action of precocene II. In insects, precocene II acts by inhibiting the synthesis of the juvenile hormone by the *corpora allata*. However, we can conclude that in our experimental conditions the effect is not transgenerational.

5.5 Conclusion

Multigeneration tests conducted with the collembola *F. candida* show that some of the tested IGR (methoprene and teflubenzuron) have an impact

on several generations, although only the F_0 generation was exposed. This can have crucial consequences on the populations of collembola or other nontarget soil invertebrates and should be considered in risk assessment of these compounds. Although these multigeneration experiments do not allow to predict the exact mode of action of the tested IGR, they provide important indications on the affected endpoints which cannot be provided by classical reproduction or mortality tests. The two experimental set-ups (28-day exposure or 10-day exposure of F_0, with or without exposure of F_1 eggs and juveniles, respectively) are complementary and permit to accept or refute hypothesis on the affected endpoints. As in the second experimental set-up the F_1 eggs and juveniles are clearly not exposed, the observed transgenerational effects can be confirmed.

Even though there is no incontestable evidence that the decrease of the number of hatched juveniles observed in the daughter generations is linked to reproductive damages caused by endocrine disruption mechanisms of the tested IGRs, multigeneration tests conducted with *F. candida* provide useful data on these compounds. The duration of these tests is at least twice as long as classical reproduction tests, but the time invested is not much more. Thus, it seems appropriate to use the developed multigeneration tests as starting points to characterize the effects of potential endocrine disruptor compounds on collembola.

Acknowledgments

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Chapter 6

Two dimensional gel electrophoresis of *Folsomia candida*: differences in protein expression profiles of collembola exposed to IGR

Authors: S. Campiche, K. Becker-van Slooten, J. Tarradellas, E. Felley-Bosco.

To be submitted.

6.1 Introduction

The collembola *Folsomia candida* is a widely used test species for the assessment of environmental quality. Indeed, as springtails are among the most abundant and widespread terrestrial arthropods throughout the world, and as their role in decomposition process is considered beneficial for the long term well-being of soils (Hopkin, 1997), they are regarded as ecologically relevant organisms. *F. candida* is a parthenogenic reproducing species easy to maintain in laboratory. Up to now, it was mainly used for the evaluation of biological effects of pollutants at the individual or population level. Studies conducted according to the ISO standard 11267 evaluate effects of toxicants on the reproduction. Other endpoints can be used as a response to environmental pollution such as mortality or growth. Collembola lipid or protein content were also investigated (Staempfli *et al.*, submitted). Moreover, as protein expression depends on the state of development (stages of the life cycle), the tissue and the physiological status of an organism, and

its response to environmental conditions (Shepard *et al.*, 2000), an attempt was made to use heat shock proteins (HSP70) as indicators to assess the toxicity of substances such as pesticides (Staempfli *et al.*, 2002).

In order to analyze and characterize the changes in protein expression of a given cell or organism, proteomics can be used. Classical proteomic techniques provide methods to determine the identity and abundance of proteins within a given set of proteins (proteome) which are most commonly visualized by two-dimensional gel electrophoresis and analyzed by mass spectrometry (Quadroni & James, 1999; Phillips & Bogyo, 2005). One of the purposes of two dimensional electrophoresis-MS methods are reference mapping and protein expression profiling. Reference mapping permits to define the location and identity of as many proteins of an organism as possible on an index map. This index map then provides a point of reference for experiments aimed at studying the response of a given organism to various external stimuli. Reference maps can be used as the basis of protein expression profiling (PEP). PEP compares either differences between proteomes of different organisms or differences in protein expression of a single organism under different stimuli. Both up- or downregulated proteins can be visualized under different conditions (Phillips & Bogyo, 2005).

Up to now, the major use of proteomics has been in the pharmaceutical industry or to discover novel protein markers for disease (Kuperman *et al.*, 2003). It has also been applied in the clinical and biomedical field to measure and correlate the alterations in the proteome with the action of drugs and the occurrence of disease (Olsson *et al.*, 2004).

However, although it is established that different chemical stressors can induce specific proteins, proteomic is practically not applied to environmental assessment. Nevertheless, when an organism is exposed to a toxicant, an unique and consistent response to the xenobiotic is rapidly given by the proteins. As each chemical exposure condition produces a different impact on the profile of that organism, the specific signature (also called "protein expression signature" (PES)) created by the induced and repressed proteins can be isolated and analyzed to identify a toxic agent or to signal the presence of chemicals of concern (Kuperman *et al.*, 2003).

Specificity in protein expression was already studied for a variety of organisms and stressors, but principally for aquatic organisms. To our knowledge, protein profiles changes in response to chemical was not studied for soil arthropods such as *F. candida*. (Table 6.1).

By using proteomic, the specific and consistent response given by the "protein expression signature" of organisms exposed to chemicals could thus be used as potential biomarker for early warning response to identify toxicants in environmental risk assessment.

As *F. candida* is already a well established test organism to assess the impact of pollutant at the ecotoxicological level (reproduction or mortality tests), it seemed interesting to us to find new endpoints at the molecular level

Organisms		Stressors	Reference
Algae	*Nannochloropsis oculata*	Cadmium	Kim *et al.* (2005)
Copepod	*Eurytemora affinis*	salinity and temperature variation	Kimmel & Bradley (2001)
Mollusc	Blue mussel (*Mytilus edulis*)	PCB, copper, salinity stress	Shepard *et al.* (2000)
Mollusc	Blue mussel (*Mytilus edulis*)	PCB, PAH	Olsson *et al.* (2004)
Mollusc	Bivalve (*Chamaelea gallina*)	Aroclor 12554, Cu(II); As(II), TBT	Rodriguez-Ortega *et al.* (2003)
Fish	Rainbow trout (*Oncorhynchus mykiss*)	Zinc	Hogstrand *et al.* (2002)
Fish	Zebrafish (*Danio rerio*)	17 B-estradiol, 4-nonylphenol	Shrader *et al.* (2003)
Insect	Hemiptera (*Rhodnius prolixus*)	Fenoxycarb	Kelly & Huebner (1987)
Earthworm	*Eisenia fetida*	Chemical warfare agents	Kuperman *et al.* (2003)

Table 6.1: Realized studies on specific protein expression of organisms exposed to environmental stressors

that can complete or correlate these observations. A link between ecotoxicological and molecular data in response to pollutant exposure could permitt a better understanding of the impact of toxic substances on this organism. Moreover, parameters such as reproduction or mortality are generally long to obtain (e.g., 28 days for reproduction testing). Protein alteration in response to pollutants would generally be observed before any physiological effects and thus biomarkers of exposure that could be found with proteomic methods would be more predictive in an environmental risk assessment than ecotoxicological testing. The changes in protein expression in response to environmental stress exposure can be analyzed by mass spectrometry and proteins of interest can be identified. This could permit to obtain more details on the impact and mechanisms of action of toxic substances on an organism.

The aim of this study was to establish proteome reference maps for *Fol-*

somia candida and to follow the evolution of its protein pattern over time by realizing 2-dimensional gel electrophoresis. Alterations in the protein expression profile of *F. candida* exposed to three insect growth regulators (known to be potential endocrine disruptors for some insect species), the juvenile hormone analogs methoprene and fenoxycarb, and the anti-juvenile hormone precocene II were also investigated for ages, exposure concetrations and time intervals. Specific protein expression signatures were established for each of these exposure conditions. The feasibility of identification by MS analysis was determined.

6.2 Materials and methods

6.2.1 Collembola breeding and culture synchronization

The collembola *Folsomia candida* were generously provided by Dr. Frank Riepert from the Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany. They were kept in plastic containers (160 * 110 * 60 mm with transparent plastic cover). Recipients were filled with a layer (ca. 1 cm depth) of mixed plaster of Paris (Pharmacie du Closelet, Lausanne, Switzerland) and activated charcoal (Merck N°1.02186.1000 (Dietikon, Switzerland), analytic grade) (mass ratio 8:1). To maintain a high relative humidity (70-80%) in the container, the substrate was saturated with bidistilled water (without water standing on the surface). Granulated dry yeast (Dr Oetker, Germany) was used as food supply.

Laboratory conditions were maintained constant (20 ± 2° C and 600 lux, with a light:dark cycle of 16h:8h). Twice a week, containers were aerated and unconsumed yeast was removed to avoid spoilage by fungi and bacteria. New food and some drops of bidistilled water were added. Once per month the collembola were transferred into new containers.

To synchronize the age of the organisms, oviposition was stimulated by placing adult collembola on a new breeding substrate (plaster of Paris and activated charcoal, mass ratio 8:1). Oviposition occurred generally two days after transfer. Seven days later the clusters of eggs were transferred with a wetted fine-bristled paintbrush to a fresh substrate in new containers. The eggs hatched 2-3 days later. Three days after the transfer the remaining unhatched eggs were removed from the containers. The synchronized juveniles or adults were used at the age required for the experiment (see Table 6.2).

6.2.2 Exposure experiments

Collembola were exposed to pollutants through compressed artificial OEDC soil (OECD, 1984), contaminated with the following substances: methoprene (98.0 % purity), fenoxycarb (98.5 % purity), obtained from Dr. Ehrenstorfer (Augsburg, Germany) and precocene II obtained from Sigma-Aldrich (Seelze, Germany).

Concentrations for exposure trials were chosen in accordance to concentration-response relationship determined in 28-days reproduction tests conducted in prior experiment by Campiche *et al.* (2006). According to the chosen experimental set-up, springtails were exposed to different effect concentrations for different ages and for different time of exposure to the chosen compounds (Table 6.2).

The artificial soil was composed of 70% quartz sand (50 % 40-100 mesh, Fluka N° 84878 (Buchs, Switzerland) and 50 % $\geq$ 230 mesh Fluka N° 83340), 20 % of kaolinite clay (Fluka N° 60609) and 10 % of sphagnum peat ("Norddeutscher Hochmoortorf", pH 3.3, ESG-Bush GmbH, Rastede, Germany). Sufficient amount of $CaCO_3$ was added to reach a pH of 6$\pm$0.5 (ISO, 1994). A quantity of bidistilled water corresponding to 50 % of the water holding capacity (ISO, 1999) was added to the different soil constituents, which were mixed thoroughly. As the test substances were weakly soluble in water, stock solutions were prepared by dissolving the compounds in acetone prior to each experiment. The quantity of the stock solution required to obtain the desired concentrations was added to a volume of acetone equivalent to the total mass of quartz sand. The acetone was added to the quartz sand and mixed thoroughly. The mixture was rotary evaporated and then placed under a fume hood for 3 hours to allow all acetone residues to evaporate. Finally the contaminated quartz sand was mixed with the other soil constituents. All soils were prepared one day in advance and stored at room temperature.

70 g (ww) of artificial soil, contaminated to the desired concentration, were introduced in a petri dish (diameter 10 cm, height 5 cm, with lids). The substrate was compressed to avoid holes where collembola could bury (a height of approximately 1 cm should be reached). Between 100 to 400 (depending on their age) age synchronized collembola were introduced in each petri dishes. Springtails were fed with dry beaker yeast. Laboratory conditions and maintenance were the same as for *Folsomia candida* breeding containers. Forty-eight hours before the end of the exposure, remaining food was removed to avoid presence of protein yeast in two-dimensional gel electrophoresis of collembola. Individuals exposed for only 24 h or 48 h to pollutants were not fed. As a sufficient number of collembola was present per petri dishes, only one replicate was used per exposure time and concentration.

Complementary experiments were realized in order to follow the evolution of the protein pattern of *F. candida* according to their age. Age synchronized collembola kept on a substrate made of plaster and charcoal were recovered each day from the age of 0 to 30 days.

Substances	**Age at the beginning of the exposure**		**Exposure time**	***Exposure concentrations**
Precocene II	Juveniles	7 days	3 days	EC5 (1 mg/kg) and EC30 (10 mg/kg)
(anti-juvenile hormone)	Adults	30 days	3 days	EC50 (13 mg/kg)
Methoprene	Juveniles	1 day	1, 4, 5, 7 days	EC25 (140 mg/kg), EC50 (175 mg/kg) and EC75 (200 mg/kg)
(juvenile hormone agonist)	Juveniles	7 days	3 days	EC50 (175 mg/kg)
	Adults	38 days	1, 4, 5, 7, 12 days	EC25 (140 mg/kg), EC50 (175 mg/kg), EC75 (200 mg/kg)
Fenoxycarb	Juveniles	2 days	1, 3, 5 days	<EC1 (0.8 mg/kg)
(juvenile hormone analog)	Juveniles	10 days	1, 3, 5 days	EC25 (13 mg/kg), EC50 (113 mg/kg) and EC75 (685 mg/kg)

*corresponding to effect concentrations (ECx, concentration causing x % of effects) determined in 28-day reproduction tests according to ISO 11267

Table 6.2: Exposure time and concentrations of the tested IGR

6.2.3 Sample preparation

Chemicals used for buffers, solutions and gels preparations are listed in table 6.4. At the end of each experiment (see table 6.2), a sufficient number of collembola to obtain 50 ug of proteins (see table 6.3) was collected per replicate. Springtails were poured in an eppendorf tube using a glass funnel. The eppendorf tubes were closed and put in liquid nitrogen to kill the collembola. They were then freeze-dried for 24h and stored at -80 °C before use. The entire collembola were solubilized in a solution containing 7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 65 mM DTT, 4 mM AEBSF, 2 % (v/v), ampholine L 3-10 and a trace of bromophenol blue. Using an eppendorf piston, springtails were blended in the solution to solubilize the proteins. All steps were carried out on ice when possible.

The total protein content available per collembola was determined in previous experiments by Staempfli (1999) using the Bradford assay with BSA as a standard. Great variations in the total quantity of proteins per individual of the same age were obtained (see Figure 6.1). Moreover, depending of the method used to extract and denature the proteins (i.e., with or without 2-mercaptoethanol), results obtained for protein quantification with Bradford assay can vary by a factor 6.

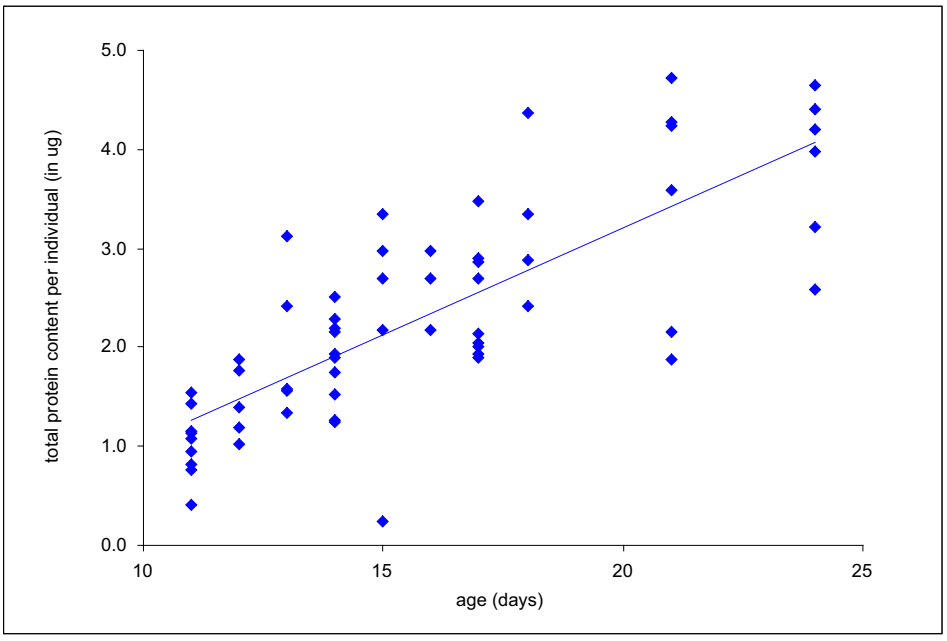

Figure 6.1: Total protein content available per collembola (Bradford assay determination)(Staempfli, 1999)

For the first 2D gel assays and in accordance with the data obtained by Staempfli (1999), it was decided to take a mean value of 8 μg of protein per collembola aged of more than 20 days, 4 μg for collembola between 10 and 20 days and 2 μg for collembola aged under 10 days (Table 6.3).

Age	**Total quantity of protein (μg) per collembola**	**Number of collembola per 2D gel**
$<$ 10 days	2 μg	25
10 to 20 days	4 μg	12-13
$>$ 20 days	8 μg	6-7

Table 6.3: Mean protein content of collembola as a function of the age

Product	Company	Catalog number
2, 7 naphtalene disulfonic acid	Acros	41523-0250
Acetic acid glacial (100 %) anhydrous	Merck	1.00063.1000
Acrylamide/PIPERAZINE di acrylamide 37.5:1	Biosolve	26051
Agarose	Bio-Rad	162-0100
Ammonium hydroxide	Fluka	09858
Amonium persulfate (APS)	BioRad	161-0700
Ampholine (3.5-10)	Amersham	80-1125-87
Bromophenol blue	Merck	8122
CHAPS	Calbiochem	220201
Citric acid monohydrate	Sigma	C 7129
Coomassie brilliant blue R-250	BioRad	161-0400
Coomassie brilliant blue G-250	BioRad	161-0406
DTT (1,4-Dithiothreitol)	Roche	10 197 777 001
Ethanol	Merck	1.00983.2500
Formaldehyde	Fluka	47630
Glutaraldehyde	Fluka	49626
Glycerol (87%)	Fluka	49782
Glycerol anhydrous	Fluka	49767
Glycine	Sigma	G 8898
Hydrochloric acid (32%)	Merck	1.00319.2500
Iodoacetamide	Fluka	57670
IPG Strips (ReadyStrips), 7 cm, pH 4-7	BioRad	163-2001
Parrafine oil	Fluka	76234
Pefabloc SC (AEBSF)	Roche	11 429 868 001
Silver nitrate	Merck	1.01512.0025
Sodium acetate anhydrous	Merck	1.01539.0500
Sodium dodecyl sulfate (SDS)	Fluka	71728
Sodium hydroxide	Merck	106498
Sodium thiosulfate	Fluka	72049
TEMED	Bio-Rad	161-0800
Trizma base	Sigma	T 1503
Thiourea	Fluka	88810
Urea	Fluka	51456

Table 6.4: Products, company and reference number of the used chemicals

6.2.4 2-D electrophoresis

Immobilized pH gradient (IPG) strips pH 4-7 were passively rehydrated for at least 12 h. Fifty μg proteins were loaded per strip for a total rehydration volume solution of 125 μl (the composition of the rehydratation solution was the same than the solubilization solution). Isoelectric focusing (IEF) was carried out at 16 °C, using a Multiphor II system (Pharmacia-Amersham). The voltage was increased linearly from 0 to 300 V during 5 min, then from 300 V to 3500 V for 30 min and finally held at 3500 V for 90 min. After the first dimension was completed, the strips were equilibrated in a solution containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 2 % (w/v) DTT for 12 min. Strips were then soaked for another 12 minutes equilibration in a solution containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30 % (v/v) glycerol, 2.5 % (w/v) iodoacetamide and a trace of bromophenol blue.

A 13.2 % acrylamide mini-gel (6 x 7 cm) composed of Acrylogel-PIP 2.6 (33%), Tris-HCl 1.5 M pH 8.8, sodium thiosulfate 1M, APS 0.1 % and TEMED 0.05 % (without SDS) was used for the second dimension analysis. "SDS-PAGE" was carried out in a Mini-PROTEAN II cell (BioRad) at a constant voltage of 200 V with a maximal current of 30 mA per gel until the bromophenol blue dye reached the bottom of the gel.

6.2.5 Gel staining and image analysis

After electrophoresis, proteins were visualized by silver staining according to the online available protocol from the "SWISS-2DPAGE" (http://www.-expasy.ch/ch2d/protocols/protocols.fm4.html) (Table 6.5).

Each gel was scanned as a TIFF image using a "HP ScanJet 6300C" scanner at 600 dpi. The spot pattern was analyzed with the PDQuest software, version 7.4 from BioRad. Spot detection and edition were performed on each gel, producing a two dimensional-scan, filtered and gaussian image for each gel. These three images were used to create a matchset (a grouping of gels with a standard incorporating protein on all gels). In order to reduce variation in spot size and intensity between gels that is not due to differential protein expression, gels were normalized according to the PDQuest normalization option "Total density in gel image" (used as 1 saturated spot was found). The raw quantity of each spot in a member gel was divided by the total intensity value of all the pixels in the image. The molecular weights and isoelectric points of individual proteins were estimated using the carbamylyte standard (GAPDH protein) from Pharmacia-Amersham and the protein standards (conalbumin, bovine serum albumin, actin, GAPDH, carbonic anhydrase, trypsin inhibitor and myoglobin) from BioRad (ref n° 161-0320).

For MS protein identification, different staining techniques were tried such as silver staining without glutaraldehyde or Coomassie R250, but only

Steps	Solutions	Time of treatement
Wash	bidistilled water	5 min
Fix	40 % EtOH; 10 % acetic acid	1 h
Fix	5 % EtOH; 5 % acetic acid	over night
Wash	bidistilled water	5 min
Sensitize	1 % glutaraldehyde (25 %); 0.5 M sodium acetate	30 min
Wash	bidistilled water	3 x 10 min
Wash	0.05 % naphtalene disulfonic acid	2 x 30 min
Wash	bidistilled water	4 x 15 min
Stain	0.8 % silver nitrate (AgNO3); 1.3 % NH4OH (25 %); 20 mM NaOH	30 min
Wash	bidistilled water	4 x 4 min
Develop	0.01 % citric acid; 0.1 % formaldehyde (37 %)	5-10 min
Stop	5 % tris base; 2 % acetic acid	5-10 min
Wash	bidistilled water	3 x 5 min

Table 6.5: Silver staining procedure for 2D gels

satisfactory results were obtained with colloidal Coomassie Brilliant Blue G-250 (CBB G250). This staining procedure is more sensitive than regular Coomasssie (CBB R250) (no protein spot were visible with CBB R250). Silver staining without glutaraldehyde was not as convenient for protein identification than coomassie staining. For CBB G250 staining, gels were equilibrated 30 min in a solution containing 3 % phosphoric acid, 17 % ammonium sulphate, 34 % methanol and stained for 2 days with 0.035 % CBB G250 (powder). Gels were destain in 10 % acetic acid until the desired background was obtained and stored in water at 4 °C.

6.3 Results and discussion

6.3.1 Protein profile of juvenile *F. candida*

Figure 6.2 shows the reference protein map of *Folsomia candida* (whole organism) established for 10-day old juvenile collembola (kept on uncontaminated and compressed artificial soil). To ensure a good representation of the proteome, five replicate gels were included in a primary matchset to create a master gel (Figure 6.3). Although manual matching was required for some raw gels, matching between replicate gels was quite good with correlation coefficients (r^2) comprised between 0.51 and 0.71 for any of the pair members of the matchset. An overall mean coefficient of variation of 45.2 % was obtained for the five gel replicates indicating that a quite reproducible protein pattern was obtained between the five replicates. This indicates that the protein expression profile of 10-day old juvenile collembola is repeatable over time and can be used as reference map for further experiments.

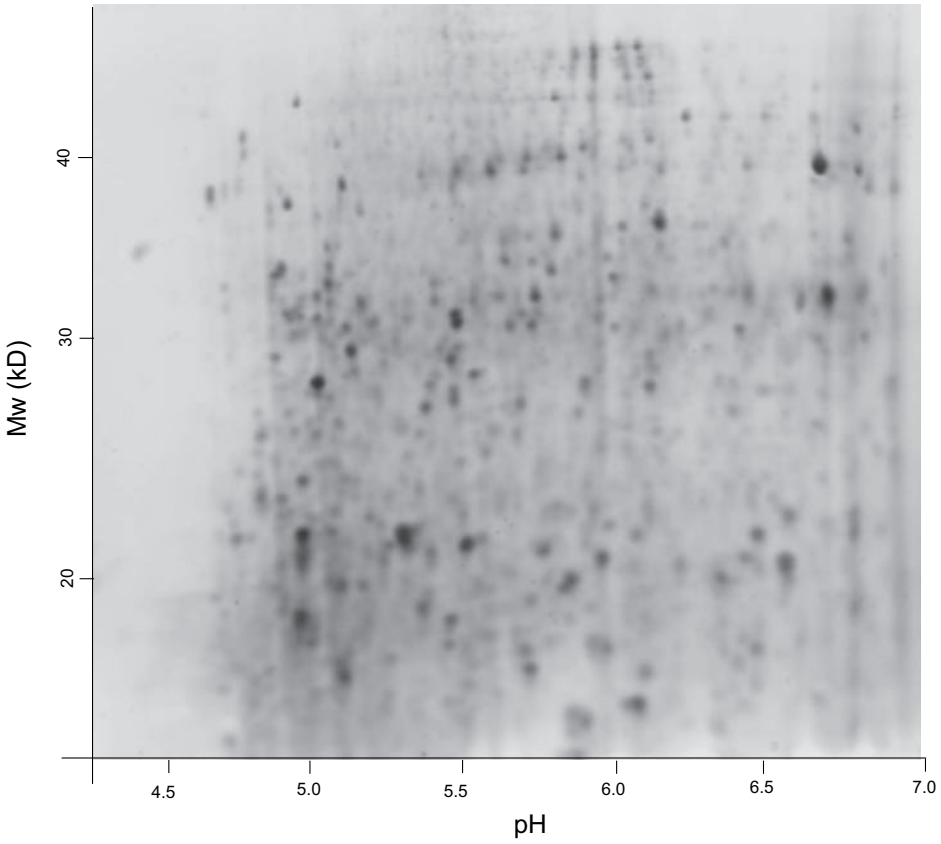

Figure 6.2: Reference map of 10-day old juvenile *F. candida*

An average of 376 spots (see Table 6.6) with pIs between pH 4-7 and Mw between 15.5 and 50.6 kDa were obtained by silver staining for each 2-D mini-gel protein pattern of the 10-day old *F. candida*. 126 protein spots can be found on all the five replicates, which are shown in Figure 6.3. As these proteins were found on every 2D-gel, they can be used as reference proteins for further experiments.

Replicate gel number	**Total protein spots per gel**
r1	299
r2	343
r3	428
r4*	506
r5	310

* gel serving as master template.

Table 6.6: Total number of proteins expressed by replicate 2D-gel of 10-day old *F. candida*

The estimated minimal pI obtained for the observed 126 proteins of the 10-day old juvenile collembola was 4.35 (Mr = 34.2) whereas the maximal pI observed was 6.93 (Mr = 27.53). The protein spot with the lowest molecular weight observed was 16.98 kDa (pI = 5.86) and the protein spot with the highest molecular weight was 45.6 kDa (pI = 6.11). The pI range of 4.4 - 6.93 obtained for 126 proteins of the 10-day old *F. candida* is comparable to the pI range obtained for other invertebrates like the nematode *Caenorhabditis elegans* (http://www.bio mol.unisi.it/2d/2d.html, 2005)

It seemed that proteins expressed in the proteome of *F. candida* are much more basic than acidic as the pI of the first grouping of proteins expressed start at a pI of about 4.6. This was confirmed by the realization of 2D gels with pIs between 3 and 10 and (image not shown) where no proteins with a pIs between 3 and 4 were found.

The most expressed protein (Mr = 31.8; pI = 6.75) is shown on Figure 6.3 (black arrow) and represents approximately 2.2 % (normalized according to the quantity in valid spots and discarding saturated spots [1]) of the total amount of proteins expressed in the protein profile of 10-day old *F. candida*.

[1]The raw quantity of each spot in a member gel is divided by the total quantity of all the spots in that gel that have been included in the Master

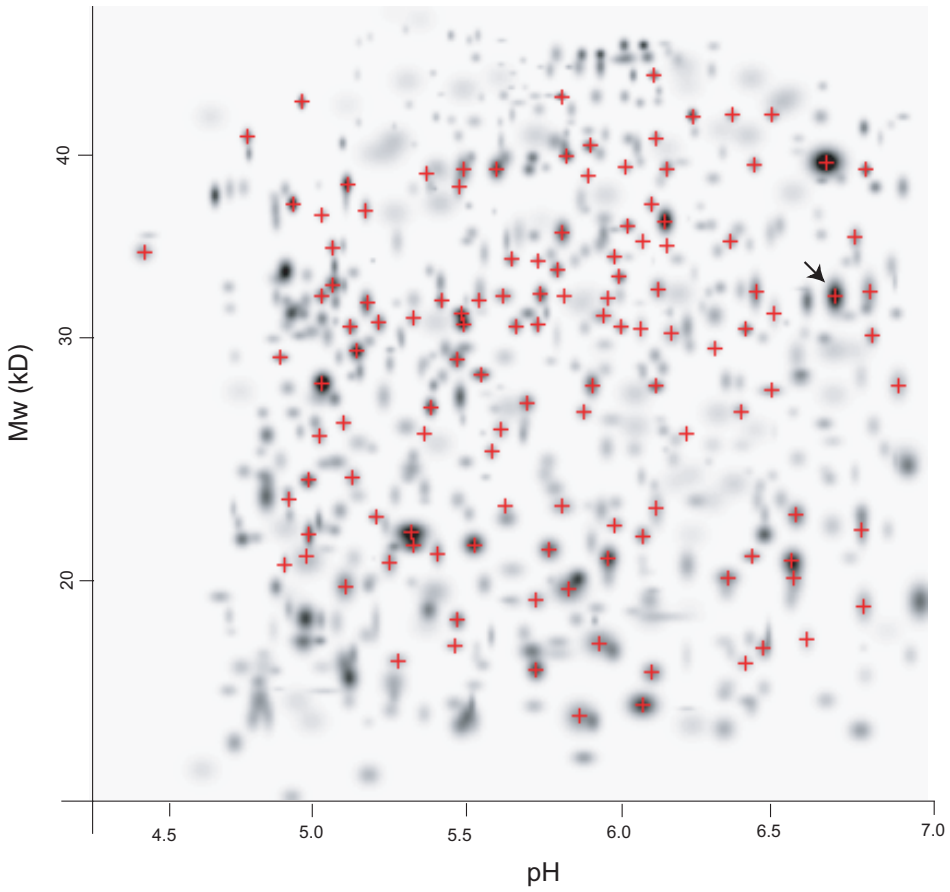

Figure 6.3: Master 2D gel of 10-day old collembola (+ = 126 proteins of reference found on all the 5 gel replicates; black arrow = protein the more expressed of the total protein pattern of 10-day old *F. candida*)

6.3.2 Evolution of the protein pattern as a function of age

As the protein profile of *F. candida* seemed to vary with the age, the evolution of its protein pattern was followed over time. Gels for collembola aged between 0 to 30 days and kept on plaster-charcoal substrate were realized for each day (Fig. 6.4, 6.5, 6.6, 6.7). Master gels were created for 5 age classes (see Table 6.7). The number of proteins increased with the age. In average, 558 proteins were found to be expressed in the protein profile of 0 to 4 days old juvenile collembola whereas 794 proteins were found to be expressed in adults (24 to 30 days old) (Table 6.7).

Age (days)	**Mean total protein spots per master gel**
0 to 4	558
5 to 11	533
12 to 14	595
16 to 22	692
24 to 30	794

Table 6.7: Variation of the total number of proteins expressed as a function of the age

As already mention, protein expression depends on the stage of development, the tissue and the physiological status of an organism (Rodriguez-Ortega *et al.*, 2003). It is surprising that the number of proteins expressed in juvenile collembola is much lower than the one expressed in adults as synthesis of new proteins is usually needed to permit growth and to reach critical stages of development such as sexual maturity.

Nineteen proteins were already present at day 0 and were also found on all the gels realized until the age of 30 days. As these proteins seemed to be "conserved" throughout *F. candida* life cycle, they probably play a basic role in physiological processes, in metabolism or in physiological structures.

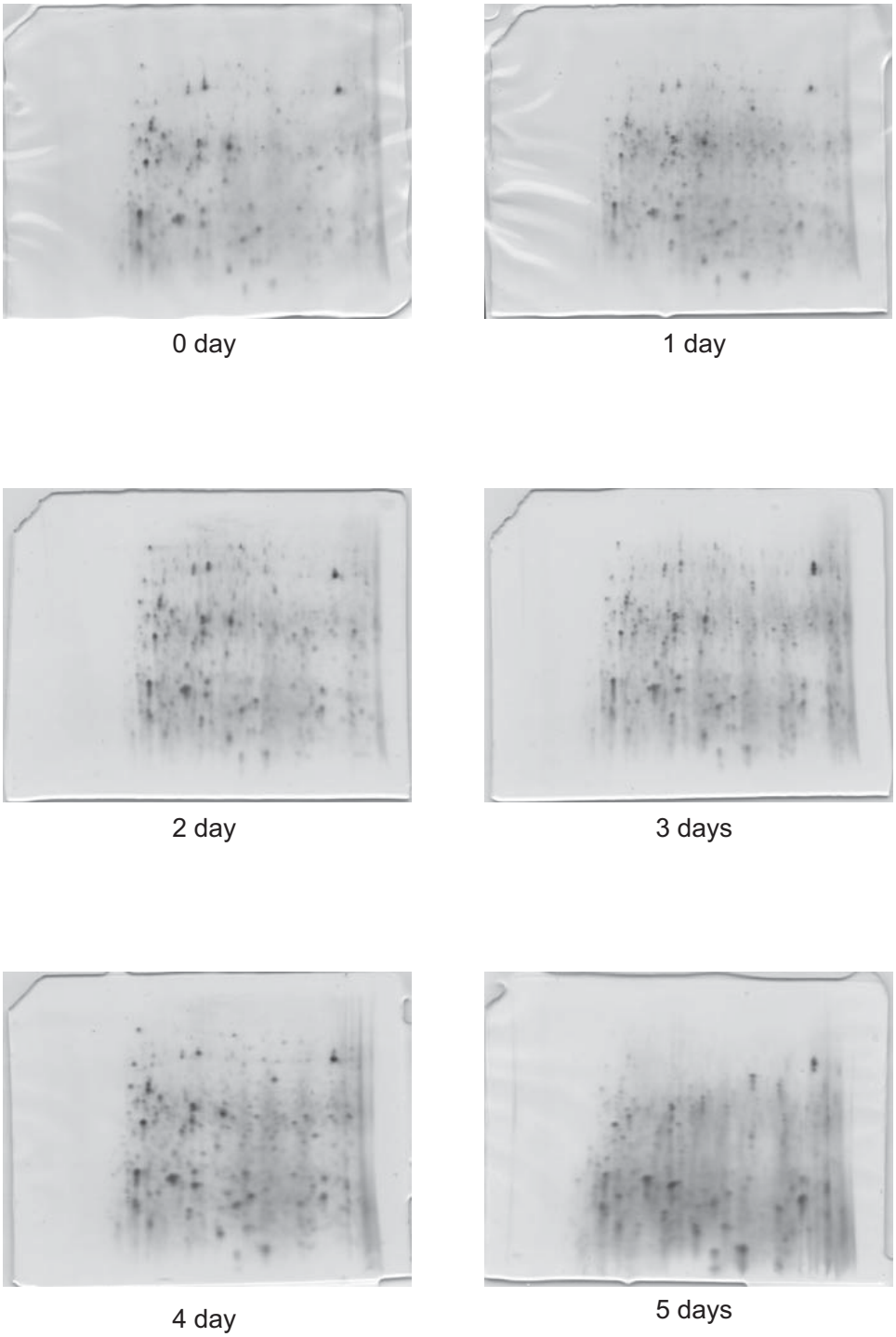

Figure 6.4: Protein expression profiles of 0 to 5 days old *F. candida*

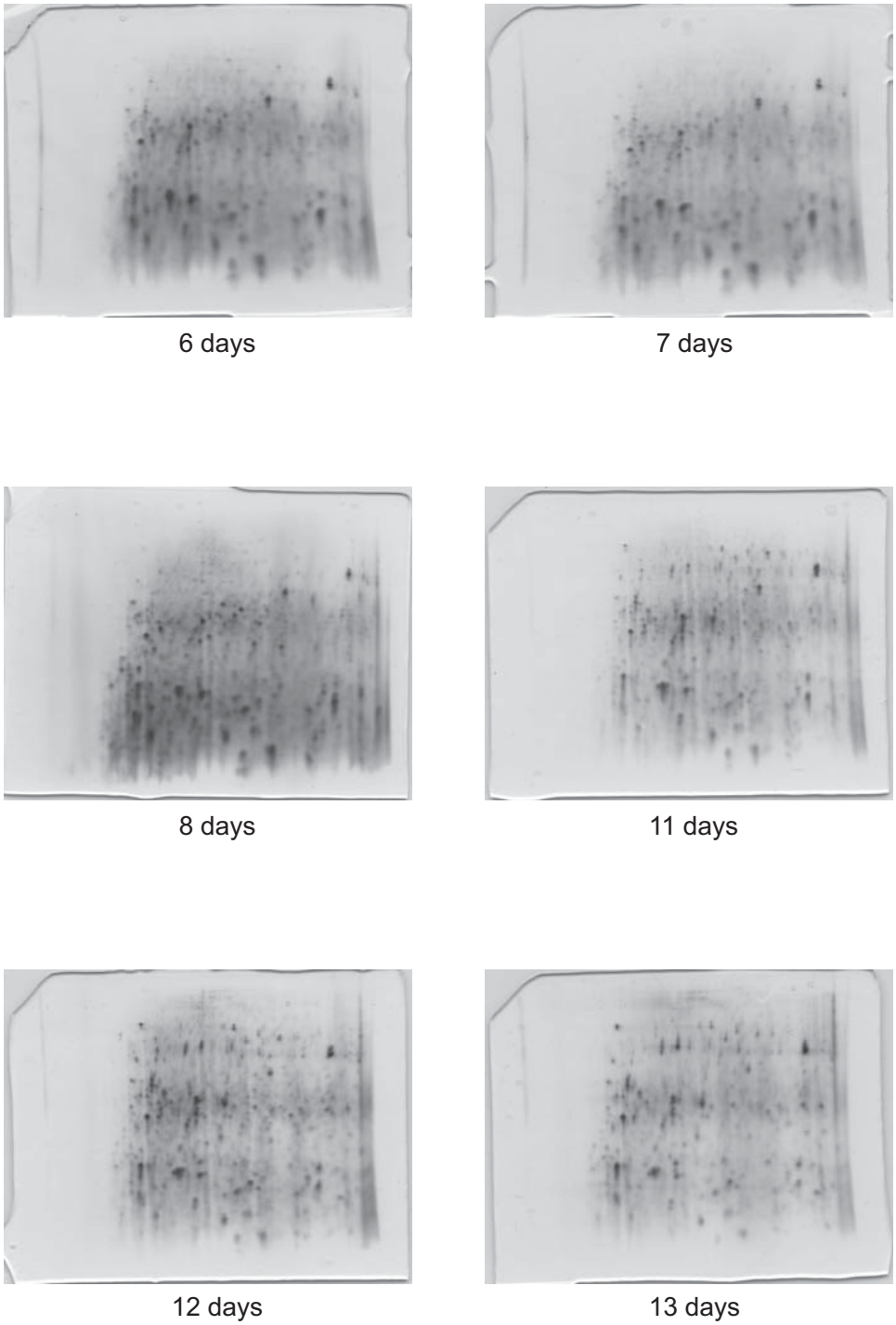

Figure 6.5: Protein expression profiles of 6 to 13 days old *F. candida*

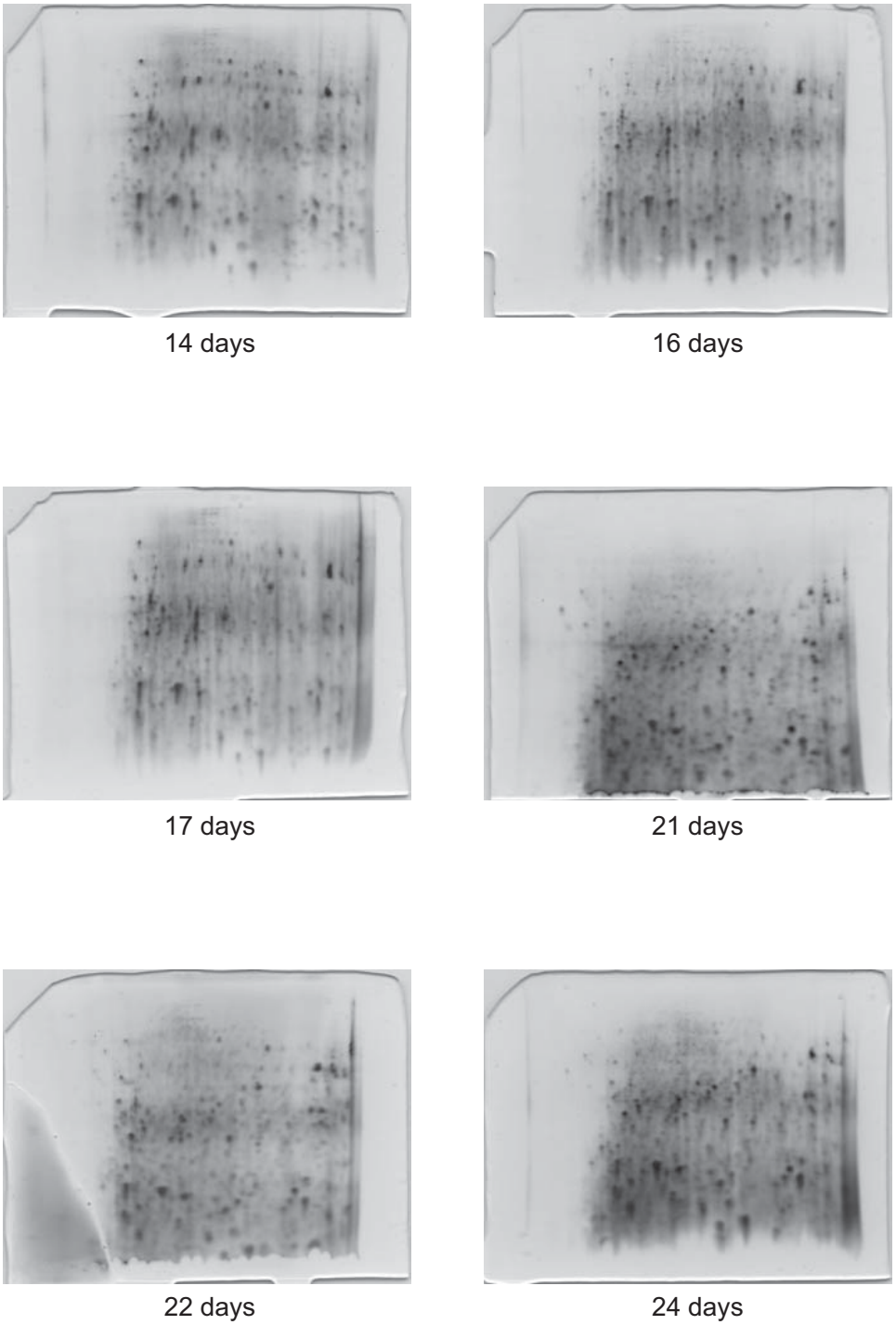

Figure 6.6: Protein expression profiles of 14 to 24 days old *F. candida*

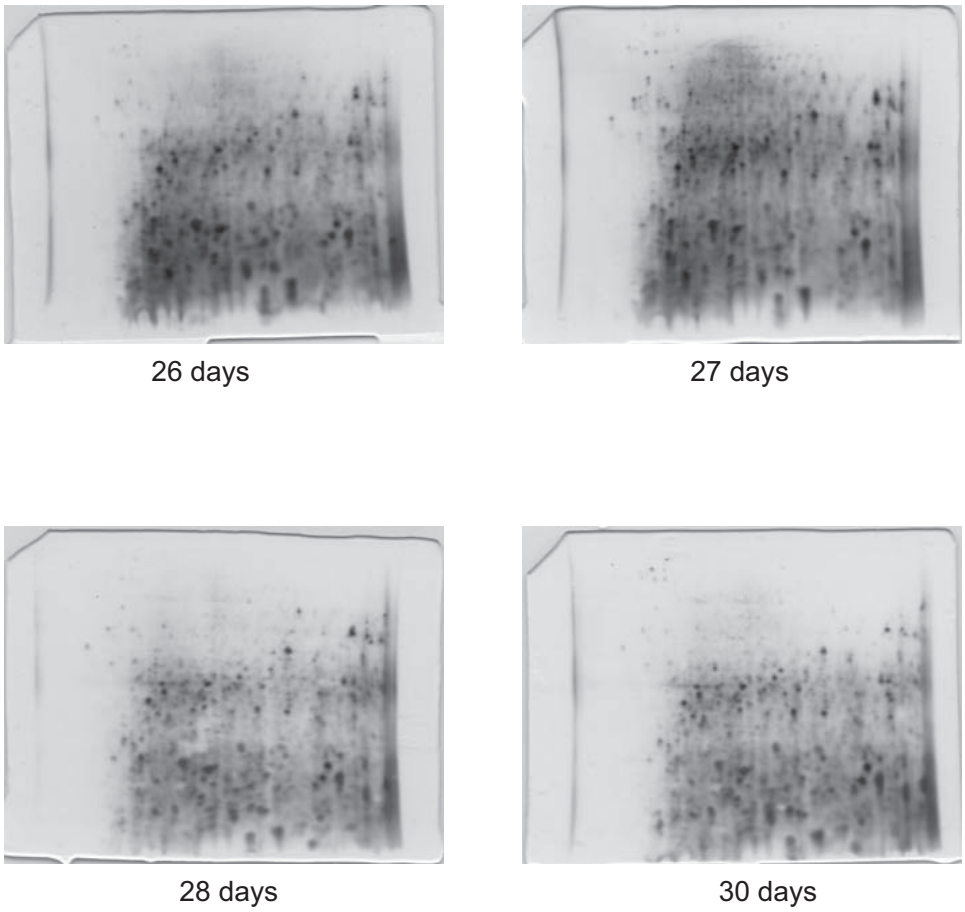

Figure 6.7: Protein expression profiles of 26 to 30 days old *F. candida*

6.3.3 Protein profile expression of collembola exposed to contaminants

Qualitative differences in protein expression profiles of *F. candida* exposed to methoprene, fenoxycarb and precocene II were studied by 2D-gels electrophoresis. On all gels, no easily visible change in protein pattern could be observed without computer analysis. As the number of replicates was not sufficient for fenoxycarb to ensure a good analysis quality, the changes in protein expression profiles were only analyzed for one exposure time and concentration of methoprene and precocene II with the PDQuest software. Quantitative differences in protein expression were not analyzed as not exactly the same amount of proteins was loaded per 2-D gel (see above).

For Precocene II, gels of *F. candida* exposed for 3 days to the substance were compared to unexposed individuals. Springtails were aged of 10 days at the end of the experiment. For methoprene, springtails aged of 1 day were exposed for 5 days to the substance before realizing 2D gels. Two replicate gels were used for each test condition (2 for the control and 2 for the exposure) and for each substance.

For precocene II, qualitative differences in the protein expression pattern were observed between control and exposed organisms. A total of 13 proteins were up- or down-regulated by this substance after 3 days of exposure of 7-day old juvenile collembola to the EC30 (10 mg/kg of soil (dw)). Two proteins were induced (up-regulated) and 11 disappeared after the exposure (down-regulation). The 13 up- and down-regulated proteins are shown in Figure 6.8 (master gel). The pI and Mr of the 13 up and down-regulated proteins is given in Table 6.8. However, the expression of the changing proteins is very weak as these proteins represent in average only 0.16 % percent of the total quantity of the expressed protein spots. It would thus be very difficult to analyze these proteins by LC-MS-MS. Furthermore, they are not visible on a gel stained with Commassie. Indeed, Coomassie staining, which is one of the most widely used technics for protein detection in MS analysis, does not permit to detect a quantity of proteins inferior to 100 ng. By considering that the effective quantity of protein loaded per 2D gel was 50 μg, proteins expressed at 0.16 % of the total protein quantity would represent approximately 80 ng of protein. This is insufficient to be detected on a gel stained with Commassie. An alternative that could enable the visualization of these protein spots would be to use other more outstanding staining technics compatible with MS analysis or to load more proteins at the beginning of the first dimension (IPG strips rehydratation) but in this case more expressed protein spots would be saturated.

For methoprene, after 5 days of exposure of newly hatched juvenile collembola (1 day old) to the EC25 (140 mg/kg of soil (dw)) a total of 27 proteins spots were found to be up- or down-regulated (Figure 6.9). Six proteins (+ in fig. 6.9) were induced after exposure of the juvenile *F. candida*

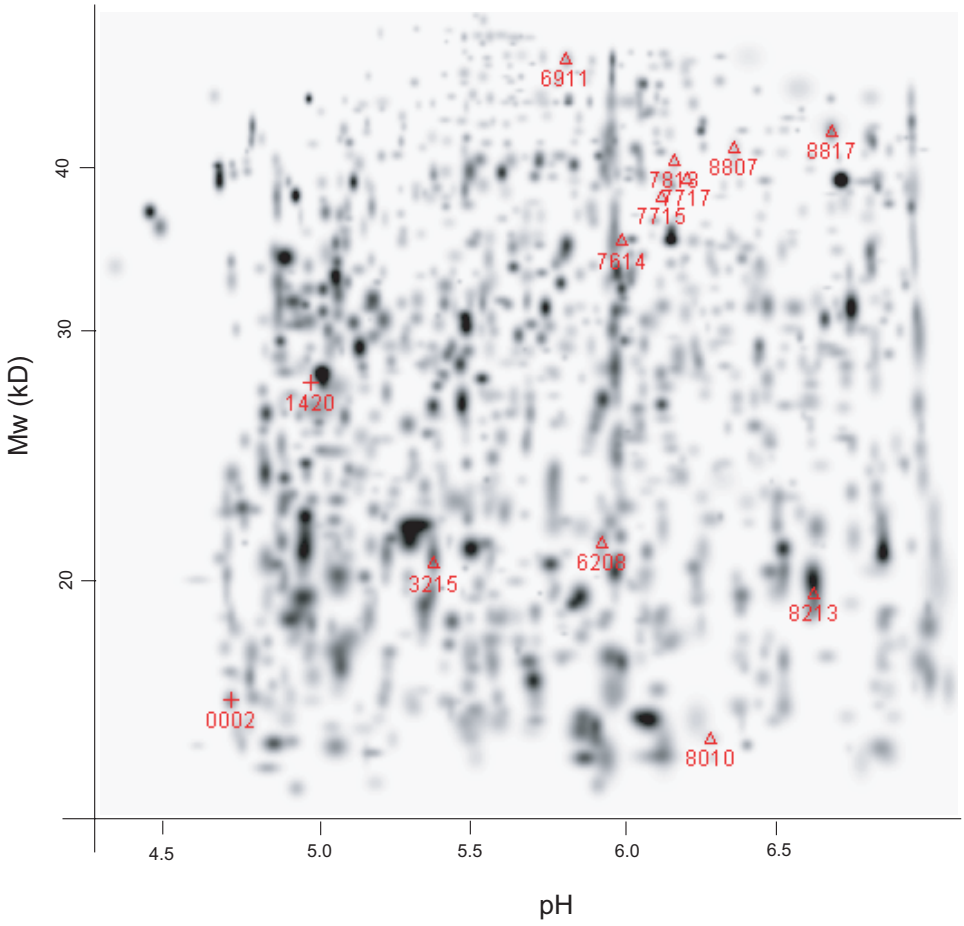

Figure 6.8: Protein expression signature of 7-day old *F. candida* exposed for 3 days to a precocene II concentration of 10 mg/kg (+ = up-regulated proteins; △ = down-regulated proteins)

to methoprene and 21 proteins were down-regulated (△ in fig. 6.9). As for precocene II, in average these proteins showed a very low expression rate (0.12 % of the total expressed proteins) which will increase the difficulties of their identification by LC-MS-MS. The pI and Mr of these 27 proteins are given in Table 6.8.

In both cases, for springtails exposed to precocene II and methoprene, the number of proteins that were down-regulated by these substances is greater than the number of up-regulated proteins. It is already known that some proteins like stress proteins, also referred to as heat shock proteins, are up-regulated in response to a stress like an exposure to toxic compounds.

Moreover, by considering the observed induced and repressed proteins as protein expression signatures (PES), this demonstrates the specificity of

the protein response to the type of IGR tested, the number and the identity of the changing proteins being not the same for the anti-juvenile hormone precocene II and for the juvenile hormone analogue methoprene.

precocene II		**methoprene**	
identification number	**Mr/pI**	**identification number**	**Mr/pI**
0002	17.57/4.72	2011	17.73/5.45
1420	27.24/4.96	3111	20.59/5.62
3215	20.51/5.37	5511	32.03/5.89
6208	21.20/5.92	5512	31.69/5.97
6911	46.21/5.80	6208	25.58/6.07
7614	35.89/5.98	6410	30.69/6.11
7715	36.96/6.12	0212	23.98/5.00
7717	39.38/6.20	0403	31.15/4.85
7813	40.35/6.16	0602	37.14/4.98
8010	16.83/6.28	0804	44.11/5.04
8213	19.72/6.61	1805	43.96/5.22
8807	41.06/6.35	2405	29.09/5.37
8817	42.00/6.68	2807	44.46/5.45
		3501	34.15/5.53
		4204	24.20/5.75
		5805	45.80/5.88
		6108	20.98/6.15
		6302	28.54/6.07
		7107	21.36/6.55
		7202	24.29/6.37
		7302	26.46/6.28
		8004	17.96/6.63
		8105	20.69/6.70
		9003	17.45
		9103	20.54
		9303	26.83
		9501	34.51

Table 6.8: Molecular weight (Mr), isoelectric point (pI) and identification number of the proteins induced and repressed by precocene II and methoprene

6.3.4 Variation of the protein pattern between collembola of the same ages kept on two different substrates

At first sight, we thought that the type of substrate (artificial soil or plaster with charcoal) on which the collembola were kept can induce differences in the protein expression profile of *F. candida*. However, by using the PDQuest software, approximately the same coefficient of correlation was found between the protein expression profile of these two type of substrates than between the five control gels used for reference mapping (see above). This seems to indicate that the observed differences have no correlations with the different type of substrates but are mainly due to the intrinsic variation of the 2D gels.

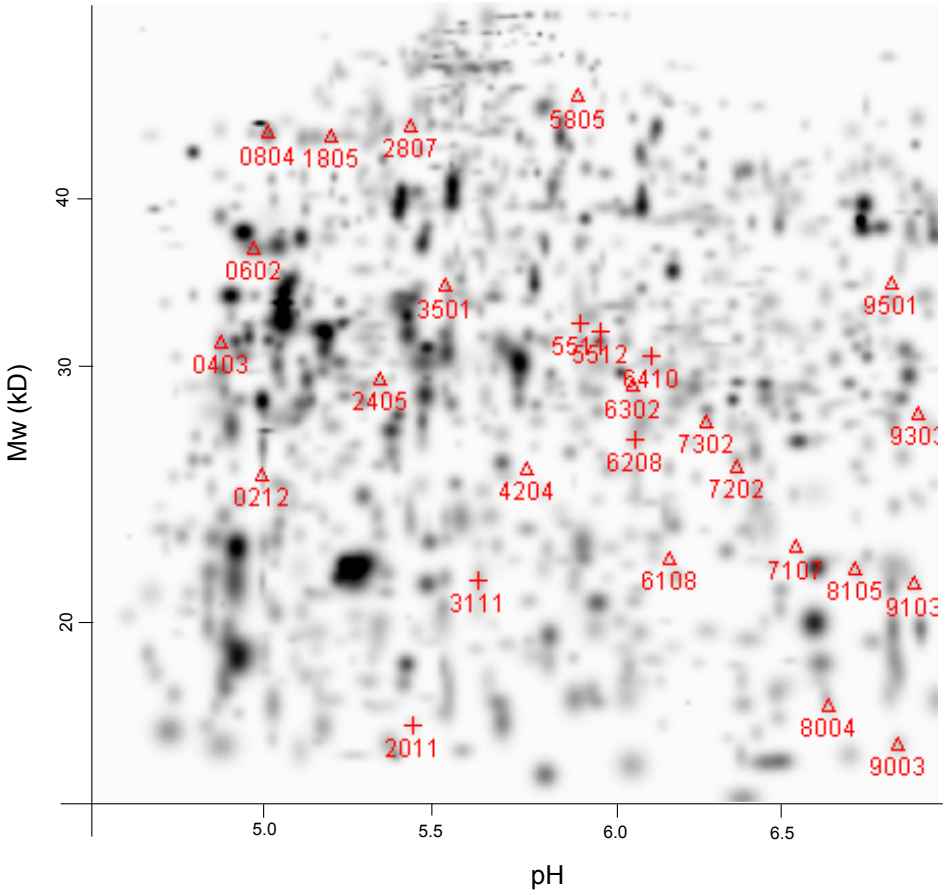

Figure 6.9: Protein expression signature of 1-day old *F. candida* exposed for 5 days to a methoprene concentration of 140 mg/kg (+ = up-regulated proteins; △ = down-regulated proteins)

6.3.5 Protein identification

In order to explore the feasibility of protein identification, an attempt was made to identify some spots of the protein expression profile of *F. candida* by LC-MS-MS. As the protein spots up- and down-regulated by precocene II and methoprene were too weakly expressed to be visible with CBB G250 staining, 4 of the more strongly expressed proteins were chosen for identification (see Figure 6.10). Unfortunately, no significant MS signal was obtained for neither of the 4 spots analyzed.

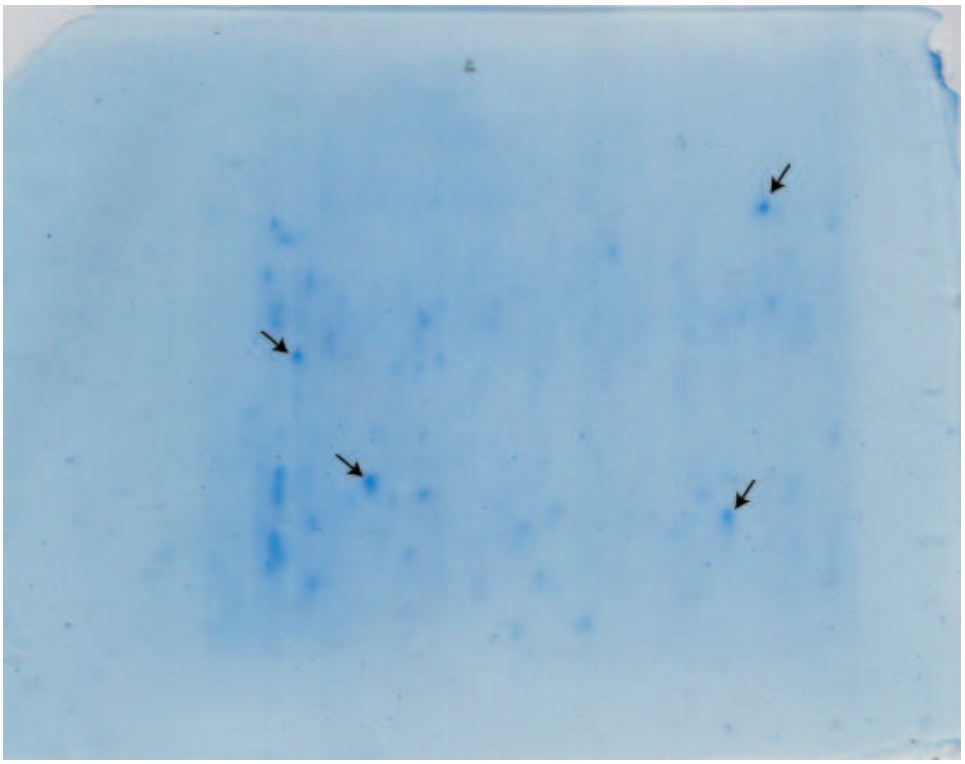

Figure 6.10: 2D gels stained with CBB G250 (black arrow = proteins chosen for LC-MS-MS identification)

However, by realizing an one dimensional gel electrophoresis of adult collembola, a good signal intensity was obtained for a major protein band at approximately 40 kDa. The sequences obtained were analyzed using MASCOT search engine software (and arthropods proteins database (110'604 entries)). Three significant matches were obtained but the scores were relatively low, probably because of the lack of homologous sequences in the database. Indeed, the genome of *F. candida* is not yet sequenced and only very few fragments of protein sequences are identified for this organism (for more details see the Swiss-Prot and TrEMBL databases or http://www.expasy.org/cgi-

bin/sprot-search-de?Folsomia%20candida). The closest matched sequence was for the ribosomal protein (L8e) with a MASCOT score of 56. MASCOT score is given as S= -10*log(P), where P is the probability that the observed match is a random event. In our case, scores > 42 indicate identity or extensive homology ($p<0.05$) and are thus considerate as significant for our analysis (the statistical significance depends on the size of database, which is small in this case). This result demonstrates the feasibility of the approach. However, since only abundant proteins were used it remains to be demonstrated for less abundant proteins and for protein spots from the 2D protein gels. The entry name of the closest matched sequences for the other identified peptides, protein name, animal species, MASCOT score and mass of the peptide are given in Table 6.9.

Entry name	**Protein name**	**Animal species**	**MASCOT score**	**Mass of the identified protein (Da)**
Q4GXL2_9CUCU (Q4GXL2)	Ribosomal protein L8e	*Mycetophagus quadripustulatus* (Coleoptera)	56	28352
Q4GXL1_9CUCU (Q4GXL1)	Ribosomal protein L8e (Fragment)	*Timarcha balearica* (Coleoptera)	53	27592
ACTA_LIMPO (P41339)	Actin, acrosomal process isoform (Actin-5)*	*Lymulus polyphemus* (Chelicerata)	46	-

* Actin matches for many species (highly conserved sequence)

Table 6.9: Major characteristics of the three identified peptides.

6.4 Conclusion

By realizing 2D gels with the collembola *F. candida*, quite reproducible protein maps were obtained despite that pools of individuals had to be used to establish the protein expression profiles of this organisms. Indeed, although *F. candida* are clones due to their parthenogenic mode of reproduction, variability can occur between individuals as a consequence of environmental conditions.

Collembola exposed to the insect growth regulators Precocene II and Methoprene through compressed artificial soil did not show great alterations of their protein expression profile. Only some very weakly expressed proteins were induced or repressed after exposure to these substances. As said above, as a pool of collembola was used to realize one 2D-gel and not an individual alone, alterations in the protein expression of *F. candida* can be masked. Indeed, it is conceivable that an alteration of the expression of a particular protein does not occur at exactly the same time in individuals. In this case, the change in protein expression that could be observed for only some individuals remains invisible because biased by individuals not yet affected by the pollutant. Moreover, by using the whole organism, it seems that too much information is available in the protein expression profile which makes the detection of protein alteration much more difficult. The small size of *F. candida* (2 mm) unfortunately does not permit to collect part of the organism like hemolymph. However, some techniques such as differential extraction permit to sequentially extract proteins and to load each extract on a 2D gel. This enables the reduction of the complexity of the sample which can increase the visibility of minor proteins and simplify the pattern of 2D gels.

As 2D gels are not able to resolve several classes of proteins such as very large or small proteins, membrane associated proteins, extremely hydrophobic, acidic or basic proteins and proteins found in low abundance in the cell (Phillips & Bogyo, 2005), the detection of a possibly altered expression of these proteins would fail. Low copy number proteins might represent key regulatory molecules within cells or signalling molecules in tissues and organs, and the inability to measure changes in expression levels of these important proteins should be considered (Lee, 2001).

On the other hand, it is possible that precocene II and methoprene, which interfere with the insect endocrine system, do not cause much greater alterations of the protein pattern of *F. candida* than the ones observed.

Further information and investigations are required to bring the developed method to completion. The sequencing of the *F. candida* genome (ongoing project of the Institute of Ecological Science of the Vrije University Amsterdam, directed by Prof. Nico van Straalen) will be a critical step for further advancement. Other substances like metals or other pesticides could have greater effects and should be tested with the established method. As

the differences in protein expression between collembola exposed and unexposed to precocene II or methoprene were weak, radiolabelling with 35S-methionine could permit to increase the sensitivity of the detection of weak protein alterations. This work is ongoing in our laboratory (master thesis of Cynthia Cochet).

The use of proteomic to obtain a specific response ("protein expression signature") of *F. candida* exposed to chemical seems to be a promising method to obtained early warning response of exposure to identify toxicants in environmental risk assessment. Moreover, as *F. candida* is a widely used test organism to assess impact of pollutants in the environment, obtaining a specific protein response to pollutant exposure and in a further time the identification of these proteins will complement the data already available with classical ecotoxicological testing.

Chapter 7

Synthesis and perspectives

7.1 Synthesis

With the emergence of substances that can adversely impact the endocrine system of living organisms and their progeniture, there is an urgent need to develop suitable methods that can precociously detect and evaluate their effects and prevent deleterious consequences for the environment. Up to now, endocrine disruption was mainly studied for vertebrates or aquatic organisms and for estrogenic-like substances. However, soil invertebrates which play an important role in soil functioning have rarely been considered. Moreover, as their endocrine system differs substantially from that of vertebrates (estrogens do not seem to regulate endocrine functions in invertebrates), other substances that can mimic invertebrate hormones have to been investigated.

In this sense, the aim of this study was to evaluate the effects of insect growth regulators (IGR), which are considered as endocrine disrupting compounds (EDC) for insect species, on the ecologically relevant soil arthropod *Folsomia candida*. The first part of this project aimed to investigate the sublethal effects of six IGR on the collembola *F. candida*. A multigenerational experimental design was developed to assess the impact of four of the chosen IGR on the reproduction of two generations of collembola. In the second part of the project, a toxicoproteomic approach was developed to reveal the alterations in the protein expression profile of *F. candida* that could result from the exposure to these substances. Ideally, these induced or repressed proteins could be used as "early-warning" biomarkers for environmental risk assessment.

7.1.1 Assessment of toxic effects of insect growth regulators on *Folsomia candida*

Springtails, which are among the most abundant arthropods on Earth, are an integral and beneficial part of the soil community. The collembola *F. candida* is one of the most appropriate invertebrate species for soil ecotoxicity testing

(van Gestel & Hensbergen, 1997). It is an euedaphic (subsurface) species that can inhabit agricultural systems. Therefore, it is directly concerned by the application of insecticides in agriculture as most of these chemicals end up in the soil. However, the impact of active substances is mainly evaluated on earthworms, other soil invertebrates being rarely considered.

Insect growth regulators are third generation insecticides that were specifically developed to interfere with the endocrine system of insects. They can mimic juvenile hormone or ecdysone, or inhibit chitin synthesis. Even though *F. candida* is not an insect, its endocrine system is close to those of insects and it may be affected by these compounds. Nevertheless, according to chemical companies, IGR have a high target pest specificity and are only little toxic for non-target species or beneficial organisms.

When conducting 28-day reproduction tests according to the ISO standard 11267, the obtained results show that the non-target arthropod *F. candida* was affected by the chosen IGR. The most toxic compounds were the two chitin synthesis inhibitors, teflubenzuron and hexaflumuron, with an EC50 of 0.05 mg/kg (dw) for teflubenzron and an EC50 of 0.6 mg/kg for hexaflumuron. The toxicity exposure ratios (TERs) obtained for these two substances was below the trigger value 5 used for earthworms in the EU Directive 91/414 (EU, 1991). The chronic effects observed at these environmentally relevant concentrations thus show that Collembola populations are at risk and that these concentrations could have consequences for other populations of soil arthropods. Disappearance of beneficial soil organisms could have devastating results on soil fertility.

The conducted 28-day reproduction tests do not allow to elucidate the mode of action of the chosen substances and it remains unclear if the observed effects are linked to endocrine disruption. However, this classical reproduction test permits to show that compounds (methoprene and fenoxycarb) mimicking the same hormone, the juvenile hormone, do not necessarily have the same effects. The inhibition of the reproduction was strongly related to the adult survival for methoprene while for fenoxycarb, no mortality of the adults was observed at the highest concentration tested (3052 mg/kg) whereas the reproduction was almost totaly inhibited. The complexity of the hormonal pathways and the differences in the chemical structures of these two compounds can explain the variations of the observed effects.

7.1.2 Multigenerational effects of IGR

Endocrine disruptive damages induced by pollutants are often not detected by classical toxicity tests based on acute or chronic exposure. Indeed, effects associated with potential endocrine disrupters can be latent and not manifested until later in life. They can even arise only in the second or third generation of individuals. Therefore, research on more than one generation seems to be appropriate. By conducting multigeneration testing, the whole

life cycle of the organism is integrated and effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity can be addressed (OECD, 2005). A study of more than one generation will also allow confirmation of any effects seen in the first generation and may give an identification of the effects due to bioaccumulation. Up to now, very few EDC testing procedures are available for aquatic invertebrates and, to our knowledge, none exist for soil invertebrates. Therefore, new toxicity test guidelines for endocrine disruption with soil invertebrates have to be developed or currently used protocols adapted.

The existing *F. candida* ISO standard 11267 was thus modified for multigenerational reproduction testing. Effects of the chosen IGR were evaluated on the second generation of collembola according to different types of exposure of the parental generation (F_0).

In the first set-up, the F_0 generation was exposed for 28 days to the pollutant. Eggs and juveniles from the F_1 generation (generated by the F_0) were also in contact with the pollutant. In the second set-up, only the F_0 generation was exposed to the compound for 10 days. Neither the eggs nor the juveniles from the F_1 generation were exposed to the pollutant. In both cases, the F_2 generation was never in contact with the toxic substance. Both scenarios (28-day or 10-day exposure to pollutant) could be envisaged in an environmental risk assessment as the collembola could stay in the contaminated area or migrate towards uncontaminated regions (avoidance of contaminants in the soil) to reproduce.

The multigeneration experiments conducted demonstrate that the different exposure set-ups and the compounds tested show great differences in response of *F. candida*. Indeed, depending on the type of exposure (28 days or 10 days) of the F_0 generation to the chosen compound, a different response pattern was obtained for the F_2 generation. As an example, it was found that fenoxycarb can affect (28-day exposure), or not (10-day exposure), the number of hatched juveniles of the F_2 generation. Moreover, as already shown with the classical 28-day reproduction test, different dose response patterns were obtained for substances mimicking the same hormone such as the two juvenile hormone agonists methoprene and fenoxycarb. This demonstrates that by comparing two substances with the same predicted mode of action (e.g. juvenile hormone analog), no correlation concerning the effects of these compounds can be made. The observed effects depend of many other factors.

Used in complementarity, these two experimental set-ups can bring important indications on the affected endpoints which cannot be provided by classical reproduction or mortality tests. Indeed, precious information on the mode of action of these compounds can be deduced by comparison of the results obtained between the two experimental set-up. For fenoxycarb, for example, by comparing the results obtained between the two exposure

set-ups (28 days and 10 days), it can be presumed that this substance only affects the early juvenile stage of *F. candida*. Methoprene, however, does not exert an effect on eggs or on survival of hatched juveniles, but induces a reduction in the reproductive capacity of the adults.

Moreover, the multigeneration tests developed with the collembola *F. candida* can demonstrate transgenerational effects of a pollutant. Indeed, the conducted tests show that two of the tested IGR, methoprene and teflubenzuron, have an impact on several generations of individuals, although only the F_0 generation was exposed. This can have crucial consequences on the populations of collembola or other nontarget soil invertebrates and should be considered in risk assessment of these compounds. The observed transgenerational effects could be explained by hypothesis such as bioaccumulation of the substances in fat tissue of the organisms or alterations of the genetic material. In this case, as *F. candida* is a parthenogenic reproductive species, the genetic damage could be more problematic than for a sexually reproductive species as a population of clone with the same alterations of the genomic material could be created. In sexually reproducing species, this risk is reduced due to genetic shuffling.

7.1.3 Use of a toxicoproteomic approach to evaluate the effects of IGR on the protein expression profile of *F. candida*

Folsomia candida is a widely used test species for the assessment of environmental quality. The impact of toxic substances on this organism is mainly evaluated at the ecotoxicological level and few is done at the molecular level. However, the use of early warning biomarkers of exposure could permit a more rapid detection of toxicants in an environmental risk assessment. Therefore, it seemed of interest to us to find new endpoints at the molecular level for *F. candida*. Moreover, this information can complete the observations obtained with reproduction or mortality testing. A link between ecotoxicological and molecular data in response to pollutant exposure could enable a better understanding of the impact of toxic substances on this organism.

As it is established that a specific and consistent protein response is given by organisms exposed to chemical stressors, a toxicoproteomic approach was conducted with the collembola *F. candida*.

A reproducible protein pattern was obtained for two-dimensional gel electrophoresis realized with 10 days old juvenile springtails even though a pool of individuals had to be used. Indeed, the use of a pool of individuals can increase the variability of the protein expression profile compared to one individual alone and, although *F. candida* has a parthenogenic mode of reproduction, variability can occur between individuals as a consequence of the surrounding conditions. As the reproducibility of the protein pattern

was quite good, reference mapping was possible and the toxicoproteomic approach was applied. *F. candida* exposed to sublethal concentrations of precocene II and methoprene only show very weak alterations of their protein expression profile. The expression of approximately 20 spots of proteins were found to be altered. More proteins were found to be repressed than induced for both of the tested substances. Identification of proteins by LC-MS-MS remains difficult due to the low level of expression of the altered proteins and to the lack of identified protein sequences for collembola species. The genome of *F. candida* is not yet sequenced and very few studies are made at the molecular level for this organism.

Although the observed changes in protein profiles were weak, the use of proteomics to obtain a specific response (a "protein expression signature") of *F. candida* exposed to chemicals seems to be a promising method to acquire early warning response of exposure to identify toxicants in environmental risk assessment. Moreover, as *F. candida* is a widely used test organism to assess the impact of pollutants in the environment, obtaining a specific protein response to pollutant exposure, and in a further step, the identification of these proteins would complement the data already available with classical ecotoxicological testing.

7.1.4 Conclusions and perspectives

Although insect growth regulators were specially developed to suppress insect pests, the non-target soil arthropod *Folsomia candida* is affected by these compounds. Depending of the type of tested substances, effect concentrations were found to be environmentally relevant. The disappearance of *F. candida* may affect soil fertility and soil community balance. Moreover, as other non-target soil arthropods may be impacted, this can lead to serious environmental damage. Up to now, the compost worm *Eisenia foetida* was the only soil invertebrates test required under all circumstances by the EU directive for plant protection products. However, the substances tested in this study show a low acute toxicity for this organism compared to the one observed on *F. candida*. This ecologically relevant soil arthropod should therefore also become an integral part of environmental risk assessment schemes and guidance documents.

The *F. candida* reproduction test (ISO standard 11267) is well adapted to evaluate the impact of toxic substances for environmental risk assessment. Nevertheless, by developing and conducting multigeneration tests, essential and complementary data can be obtained on the tested compounds. These tests permit to assess the transgenerational effects of the toxicants. Moreover, the comparison between to test designs (with and without exposure of eggs and juveniles of the F_1 generation) give more precision on the affected endpoints and therefore on the mode of action of the substances. Multigenerational tests are longer to conduct but not much more labor intensive

than classical reproduction tests. Therefore, due to the valuable and additional source of information they provide, they should be integrated in risk assessment for long term toxicity evaluation of substances such as EDC. In further experiments, multigenerational tests with methoprene and teflubenzuron could be conducted beyond the second generation of individuals to assess the evolution of the observed effects.

The classical 28-day reproduction and multigeneration tests conducted do not permit to elucidate if the observed effects are linked to endocrine disruption. In most cases, endocrine disruption occurs at much lower than the sublethal concentrations. Moreover, no correlations can be made between the effects observed for *F. candida* and data on endocrine disruption found in the literature for insect species and other organisms. Indeed, the observed effects can differ significantly among species for the same substance. The wide range of effects and sensitivity noted can depend on parameters such as receptor affinities, regulatory pathways, life stage, and taxa and can lead to great disparity in the type of response. The elucidation of the question whether the observed effects can be considered as endocrine disruption would need molecular studies.

Nevertheless, the toxicoproteomic approach conducted could help to resolve this uncertainty. The identification of some of the induced or repressed proteins responding to an IGR exposure could permit to comfort the hypothesis of endocrine disruption if the proteins identified are clearly linked with *F. candida* endocrine system (e.g., induction or repression of protein such as vitellogenin). However, further investigations and information are required to improve the proteomic method.

The protein quantity to load on the 2D-gels should always be the same. As the total protein amount available per collembola can vary greatly from one individual to another, a Bradford assay should be conducted before each protein loading. This would enable to obtain very reproducible protein expression profiles where quantitative and not only qualitative differences in protein expression can be searched for.

It could be very helpful if more data on *F. candida* proteins identity or peptide sequences was available in protein databases. The sequencing of *F. candida* genome will be an essential step for further proteins identification.

By loading greater quantities of proteins per 2D-gel (e.g. 100 μg instead of 50 μg), the altered and weakly expressed proteins could be seen with CBB G250 staining technics which could enable their detection by LS-MS-MS.

Subtle changes of protein expression can be masked by the complexity of the sample of collembola whole organism load on 2D-gel. By sequentially extracting the proteins of the sample, protein fractions could be loaded on 2D-gels. The reduction of the complexity of the sample would increase the visibility of minor proteins and simplify the pattern of 2D-gels, which could allow to detect alterations of protein expression not visible before.

It could be interesting to observed the "protein expression signature" of

F. candida proteome in response to an exposure to other toxicants with a mechanism of action not linked to endocrine disruption. This could enable to see if altered proteins are as weakly expressed as for IGR or if stronger alterations of the protein pattern can be observed.

The collembola *Folsomia candida* seems to be a promising organism to evaluate the toxicity of chemicals from the ecotoxicological to the molecular level. This organism could permit to obtain a better understanding of the mode of action of toxic substances by assessing the effects at the individual or population level and then by characterizing these effects at the molecular level. The whole toxicity processes from observed effects to mechanism of action could be encompassed.

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Appendices

Contents

Appendix A

Protocols for ecotoxicity testing

A.1 Protocol for the breeding

A.1.1 General

Breeding of *Folsomia candida* was done according to the ISO standard 11267 (ISO, 1999).

A.1.2 Products

- Plaster of Paris (Pharmacie/Droguerie du Closelet, chemin du Closelet 1, 1006 Lausanne, Switzerland)
- Activated charcoal (Merck N° 1.02186.1000. analytic grade, pH 4 to 7)
- Bidistilled water
- Granulated dry baker yeast (Dr.Oetker, Germany)

A.1.3 Material

- Polystyrene plastic boxes, 160*110*60 mm, capacity 1000 ml, Thermoflex N° 1590.0
- Transparent covers (PVC), Thermoflex N° 1459.2
- Plastic flask, capacity 2.5 l, with lid closing tightly
- Fluorescent light (neon) 18 W, Philips Ecotone, "soft light" (827), 59 cm, 1350 lumen
- Spatulas
- Pasteur pipettes, short form 150mm (Assistent N° 567/1)

A.1.4 Preparation of the breeding containers

Breeding containers are plastic boxes filled with 1 cm layer of plaster and charcoal.

- A plaster-charcoal mass ratio of 8:1 was used to prepared the substrate of the breeding containers. In order to obtain a substrate layer of 1 cm depth, 120 g of plaster of Paris, 15 g of activated charcoal and 120 ml of bidistilled water were needed per plastic boxes.
- The total mass of plaster and charcoal required for all the breeding boxes desired was weighed in a plastic flask. The flask was closed tightly with the lid. The two constituents were mixed homogeneously by energetic shaking of the flask.
- The required volume of bidistilled water needed for the total number of breeding boxes was added in the flask and the mixture was well homogenized for about 20 to 30 seconds by shaking the flask vigorously (the more the mixture is agitated and the more the plaster solidification will be short).
- The mixture was then rapidly poured and shared out in the different breeding boxes before plaster solidification. Substrate should reach a depth of about 1cm per boxes. The walls of the boxes must be clean without splashes of plaster on them (collembola will hide and deposit their eggs behind them).
- The walls of the breeding boxes were gently tapped with the fingers to remove air bubbles (bubbles leave holes in the substrate in which collembola deposit their eggs, increasing the difficulty of their recovery)
- The substrate was air dried for approximately 3 hours.
- Bidistilled water was added to the substrate, using a pasteur pipette, to almost saturation (but without water standing on the surface).
- Collembola were then introduced in the breeding boxes.
- Approximately 30 mg granulated dry beaker yeast (split into three parts and distributed among the substrate) was added as food supply.
- Boxes were closed with transparent lids (to allow light to enter) to avoid collembola to escape and to prevent a to great loss of humidity.

Note: Plaster substrate stop collembola burying which facilitate eggs recovery and springtails transfer to new breeding containers. Active charcoal adsorbs waste gases and excretion products. Moreover, by coloring the substrate in black, it make the observation of whitish collembola easier.

Laboratory conditions

- The temperature of the laboratory chamber must be maintained at 20 ± 2 °C
- The relative humidity inside the boxes should be between 70% and 80%.
- Light conditions must be constant: 600 lux and a light:dark cycle of 16h:8h

Breeding cares

Twice per weeks:

- Breeding containers were aerated by lifting the cover for a short time.
- Unconsumed food was removed and the surface of the substrate (where the yeast was laying) was gently scraped with a spatula to avoid development of fungi.
- If needed, bidistilled water was added using a pasteur pipette, until almost saturation of the substrate.
- After use, material should be rinsed with ethanol and bidistilled water for disinfection.

Every 4 to 8 weeks: To avoid overcrowding and to induce oviposition, springtails were transferred in new breeding boxes containing fresh substrate. Transfer was done by gently tapping the old box with the fingers over the new one to let the collembola fall. Only a small part of the individuals contained in the old box were transferred to the new one (the others could be discarded). Collembola from several old boxes should be mixed together to create a new one.

A.2 Protocol for the obtention of aged-synchronized juvenile collembola

A.2.1 General

Aged-synchronized collembola were obtained as described in the ISO standard 11267 (1999).

A.2.2 Products

- Plaster of Paris (Pharmacie/Droguerie du Closelet, chemin du Closelet 1, 1006 Lausanne, Suisse)
- Activated charcoal (Merck N° 1.02186.1000. analytic grade, pH 4 to 7)
- Bidistilled water
- Granulated dry baker yeast (Dr.Oetker, Germany)

A.2.3 Materials

- Polystyrene petri dishes (PS), diameter 101 mm, 54 mm in height, Semadeni N° 1999, with lids
- Plastic flask, capacity 1l, with lid closing tightly
- Glass beakers
- Spoons or spatulas
- Pasteur pipettes, short form 150 mm (Assistent N° 567/1)
- Paper filters, diameter 125 mm, Schleicher & Schuell (MicroScience) LS 14 1/2
- Plastic sheets
- Fine paintbrushes N° 000 and N° 002

A.2.4 Equipment

- Stereomicroscope (Olympus SZ40)

A.2.5 Preparation of the plaster-charcoal substrate and containers for the obtention of aged-synchronized juveniles

The substrate was prepared the same way as that of the breeding containers (appendix A.1.4)

- 40 g of plaster of Paris, 5 g of activated charcoal (mass ratio 8:1) and 40 ml of bidistilled water were needed per petri dishes (diameter 101 mm) in order to obtain a depth of the substrate from approximately 1 cm
- The total mass of plaster and charcoal required for all the petri dishes desired was weighed in a plastic flask. The flask was closed tightly with the lid. The two constituents were mixed homogeneously by energetic shaking of the flask.
- The required volume of bidistilled water needed for the total number of petri dishes was added in the flask and the mixture was well homogenized for about 10 to 20 seconds by shaking the flask vigorously (the more the mixture is agitated and the more the solidification will be short).
- The mixture was then rapidly poured and shared out in the different petri dishes before plaster solidification. Substrate should reach a depth of about 1cm per container.
- Petri dishes walls were gently tapped with the fingers to remove air bubbles (bubbles leave holes in which collembola deposit their eggs and which will increase the difficulty of their recovery)
- The substrate was air dried for approximately 3 hours.
- Just before use, bidistilled water was added to the substrate, using a pasteur pipette, to almost saturation (but without water standing on the surface).

A.2.6 Preparation of "egg papers"

"Egg papers" are small pieces of paper filter covered with a layer of plaster and charcoal. They were used as egg support and facilitated removing of unhatched eggs.

- Paper filters were cut in small pieces (isosceles triangles, basal length 3 cm, height 5 cm).
- In a beaker, plaster of Paris and activated charcoal (mass ratio 8:1) were mixed homogeneously.

- A volume of bidistilled water, slightly higher than the mass of plaster added (1 g = 1 ml), was poured in the beaker and mixed with the other constituents using a spatula (the mixture should be neither too thick nor too liquid).

- Paper filters pieces were rapidly plunged into the mixture and allowed to dry on a plastic sheet.

- Just before use, egg papers were brought to saturation with bidistilled water.

A.2.7 Obtention of aged-synchronized juvenile springtails

- Adult collembola were placed in new breeding boxes (see appendix A.1.4 for their realization) which will stimulate the oviposition. In general, 2 days after the transfer a new batch of eggs was laid.

- Seven days after the eggs were laid (9 days after the transfer in new breeding boxes) they were resistant enough to be transferred in petri dishes prepared for the circumstance (see A.2.5). Using a fine paintbrush wetted with bidistilled water, batch of eggs were delicately removed from the breeding boxes and settled on wet "egg papers" (see A.2.6), which were placed on the plaster substrate in the petri dishes before.

- Petri dishes were kept under the same laboratory conditions as the breeding boxes (A.1.4).

- Eggs began to hatched 0 to 3 days after their transfer (10 to 12 days after the transfer of the adult collembola to new breeding boxes). Three days after the transfer, unhatched eggs were removed from the petri dishes by taking off the "egg papers". Generally, collembola should have moved on the petri dishes substrate. The back side of the "egg papers" could be gently brushed with a dry paintbrush in order to remove juvenile collembola present on this side and to keep a maximum of them.

- Eggs that fell off the "egg papers" on the petri dishes substrate should be removed with a wetted fine paintbrush under a stereomicroscope.

- Collembola were fed with granulated dry beaker yeast as described in appendix A.1.4. Breeding conditions were the same as for the breeding boxes (A.1.4).

- Juvenile springtails were used at the age required by the experimental setup (e.g. 10 to 12 days for a reproduction or mortality test).

Note: If a lot of juvenile collembola are needed or if several experiments should be done in a short period of time, the removed "egg papers" could be placed in new petri dishes for another 24h to let more collembola hatch.

A.3 Protocol for the introduction of the springtails in test containers

A.3.1 General

Collembola were transferred from their breeding container in test containers by using a folded black cardboard

A.3.2 Methods

- A black cardboard, dimension 9 cm x 9 cm, was folded in the middle to form a gutter.
- By gently tapping the petri dishes containing aged-synchronized collembola, they fall off on the black cardboard.
- Collembola were then gathered in the gutter in the middle of the cardboard by gently tapping this one with the fingers.
- Springtails were observed to verify if they are healthy. Any collembola that didn't move (not walking or jumping, no movement of the antennae) or that were in a suspicious position (curved, rolled up in ball) were removed.
- Collembola were then counted and the desired number was introduced in test recipients by gently tapping the cardboard.

Note: If more than 10 collembola should be introduced in a test container, the size of the black cardboard could be increased (e.g., A4 size).

A.4 Protocol for the preparation of the artificial soil

A.4.1 General

The artificial soil was prepared according to the ISO earthworm standard ((ISO, 1993)).

A.4.2 Products

- Quartz sand, $\geq$ 230 mesh, Fluka N^{o} 83340
- Quartz sand, 40-100 mesh, Fluka N^{o} 84878
- Sphagnum peat, "Norddeutscher Hochmoortorf", pH 3,3, ESG-Busch GmbH, Rastede, Germany
- Kaolinite clay, Fluka N^{o} 60609
- Calcium carbonate ($CaCO_3$), USP, $\geq$99%, Fluka N^{o} 21060
- Bidistilled water

A.4.3 Material

- Polystyrene petri dishes (PS), diameter 101 mm, 54 mm in height, Semadeni N^{o} 1999, with lids
- Metallic sieve, 1 mm mesh size
- Plastic basins
- Metallic spoons or spatulas

A.4.4 Equipment

- Balance Metler Toledo, PR 503 Delta Range®, precision d= 0.01 g/0.001 g
- Balance Metler Toledo, AT 261 Delta Range®, precision 0.01 mg
- Electric mixer (Miostar, Migros, Switzerland)

A.4.5 Methods

Sphagnum peat preparation

- The peat was air dried at room temperature in a plastic basin for at least one week. It was mixed from time to time to allow the lower layers to dry.
- When totaly dry, the peat was ground with an electric mixer and sieved to 1 mm. Remaining large vegetal fragments were removed.

Artificial soil preparation

The composition of the artificial soil is given in the table below (Table A.1):

Artificial soil constituents	**Dry mass fraction**
Sphagnum peat	10 %
Kaolinite clay	20 %
Quartz sand ($\geq$ 230 mesh)	35 %
Quartz sand (40-100 mesh)	35 %
Calcium carbonate ($CaCO_3$)	0.37 % (sufficient quantity of $CaCO_3$, usually 0.5 % to 1 %) to bring the pH of the soil to 6 $\pm$ 0.5;(A.7)
Bidistilled water	50% of the Water Holding Capacity of the artificial soil (A.5)

Table A.1: Artificial soil components expressed as a dry mass fraction

Example of the component masses needed to obtain 30 g of artificial soil (dw)

- *3 g of peat*
- *6 g of kaolinite clay*
- *10.5 g of quartz sand ($\geq$ 230 mesh)*
- *10.5 g of quartz sand (40-100 mesh)*
- *0.42 g of $CaCO_3$ (specific to the sort of peat used)*
- *13.64 ml of bidistilled water (specific to the sort of peat used)*

- All the soil constituents, except the bidistilled water, were put in a petri dishes. The petri was closed with the lid and hand shaken for 30 to 60 seconds.
- The bidistilled water was then added and mixed homogeneously with the soil using a spoon or spatula for approximately 1 minute to obtain a crumbly structure.

Note: If the soil sample was prepared in order to determine the Water Holding Capacity, all the soil constituents were mixed together EXCEPT the $CaCO_3$ and the bidistilled water (see appendix A.5).

A.5 Protocol for the determination of the Water Holding Capacity (WHC) of a soil

A.5.1 General

The WHC was determined according to annex C of the ISO standard 11267 ((ISO, 1999)).

A.5.2 Products

- Bidistilled water
- Soil samples (artificial soil see appendix A.4, or natural soil)
- Quartz sand, 40-100 mesh Fluka N^{o} 84878

A.5.3 Material

- Glass tubes, internal diameter 3.5 cm, 10 cm height
- Paper filters, diameter 150 mm, Schleicher & Schuell (MicroScience) LS 17 1/2
- Glass dishes
- Glass beakers, capacity 600 ml
- Metallic spoons
- String or rubber band

A.5.4 Equipment

- Drying chamber
- Balance Metler Toledo, PR 503 Delta Range®, precision d= 0.01 g/0.001 g

A.5.5 Methods

- The paper filter was unfolded and attached with a string or rubber band to one of the extremity of the glass tube, covering and plugging this one. The WHC determination was made in triplicate.

- Each glass tube plugged with paper filter was weight (T) and filled with approximately 6 cm of uncompressed soil (about 30 g (dw) of artificial soil; depending on the density of the soil).

- Glass tubes were placed in 600 ml beakers and gradually submerged with water until the level was above the top of the soil.

- Tubes were let in water for 3 hours at room temperature

- They were then placed for 2 hours in glass dishes containing a layer of humid quartz sand to allow non-absorbed water to drain.

- Glass tubes containing the mass of water-saturated substrate were weighed (S) and dried at 105 °C in a drying chamber until no change in weight was observed.

- The tube was weighed (S') and the dry mass of the substrate (D) was then determined as

$$D = S' - T \tag{A.1}$$

The Water Holding Capacity was calculated using the following equation (EQU.3.1.4):

$$WHC = \frac{(S - T - D)}{D} \cdot 100 \tag{A.2}$$

where:

WHC= Water Holding Capacity, expressed as a percent of dry mass

S = mass of water-saturated substrate + mass of glass tube + mass of filter paper

S' = mass of the dry substrate + mass of glass tube + mass of filter paper

T = tare (mass of tube + mass of filter paper)

D = dry mass of substrate

Note: When determining the WHC of artificial soil, $CaCO_3$ was not added to the other soil constituents (the WHC must be known to determine pH and the mass of $CaCO_3$ to be added for pH adjustment).

A.6 Protocol for the determination of the pH (KCl method) of a soil (artificial or natural)

A.6.1 General

The pH of the soil was determined as described in the ISO standard 10390 (ISO, 1994). It is necessary to know the Water Holding Capacity of the soil for the pH determination (see appendix A.5).

A.6.2 Products

- Bidistilled water
- KCl 1M
- Soil samples (natural or artificial)

A.6.3 Material

- Polystyrene petri dishes (PS), diameter 101 mm, 54 mm in height, Semadeni N° 1999, with lids
- Glass containers, capacity 50 ml
- Glass beaker, capacity 25 ml
- Graduated cylinder out of glass, capacity 25 ml
- Parafilm®
- magnetic stirrer
- metallic spoons

A.6.4 Equipment

- pH meter Metrohm 691
- combined pH and temperature electrode Metrohm 6.0238.000
- Magnetic agitator, Gerhardt (N° LSSI1)

A.6.5 Methods

The method for the determination of the pH differs slightly if artificial or natural soil were used (natural soil already possess an initial water content while artificial soil is dry).

Preparation of a 1M KCl solution

- In a round flask out of glass (capacity 1000 ml), 74.56 g of KCl were dissolved in approximately 60 ml of bidistilled water by gentle stirring.
- The solution was then brought to 1 l by adding bidistilled water, stirred homogeneously and stored at room temperature (20 °C).

Artificial soil pH determination

- Approximately 20 g of an artificial soil sample (dw) (appendix A.4) was placed in a polystyrene petri dish.
- A volume of bidistilled water corresponding to the 25 % of the Water Holding Capacity of the soil sample was added and mixed homogeneously to the soil with a metallic spoon. The polystyrene dish was closed with the lid and soil sample was let at rest for 24 hours at room temperature.
- After this period of time, another volume of bidistilled water corresponding to the 25 % of the Water Holding Capacity was added, mixed with the soil and let at rest for another 24 hours period.
- Using a graduated cylinder or a beaker, 5 ml of uncompressed soil was introduced in a 50 ml glass container.
- 25ml of KCl (5 volume of solution for 1 volume of soil) were added to the soil.
- The mixture was agitated for 5 minutes with a magnetic stirrer. Containers were recovered with Parafilm®to avoid evaporation and let at room temperature.
- After approximately 20 hours (at minimum 2h and at maximum 24h), the sample was agitated again for 5 minutes and the pH was measured with a calibrated electrode and pH meter.
- For each soil sample, the pH was measured in triplicate and the pH value was calculated on the average of these 3 measurements.

Natural soil pH determination

The pH of natural soils was determined as for artificial soil but the initial water content of the soil must be considered. The quantity of bidistilled water to add to the soil was then equivalent to the 50 % of WHC minus the water content of the soil. Only half of this volume of bidistilled water was added at first. The second half was added 24 h later (as for artificial soil).

All other steps of pH determination were made as described for artificial soil.

Example of calculation of the volume of bidistilled water to add to natural soil samples with an initial water content

Initial water content of the natural soil: 20 %
WHC of the natural soil: 86 %
50% of WHC: 43 %
Percent of bidistilled water to add to the soil: 43 % - 20 % = 23 % (11.5 % was added first and 11.5 % after 24h)

Note: When the pH had to be determined at the beginning or at the end of a mortality or reproduction test, the soil is already at 50 % of WHC moisture content. The pH determination protocol could then directly start at the step where 25 ml KCl are added to 5 ml soil.

A.7 Protocol for pH adjustment of artificial soil

A.7.1 General

Adjustment of pH of artificial soil was done according to the ISO standard 11267. To estimate how much $CaCO_3$ is needed to obtain the optimum pH of 6 $\pm$ 0.5 required for *F.candida*, different amounts of this compound were added to the other constituents of artificial soil samples (see appendix A.4).

A.7.2 Products

- Artificial soil constituents (see appendix A.4)

A.7.3 Materials

- Polystyrene petri dishes (PS), diameter 101 mm, 54 mm in height, Semadeni N^o 1999, with lids
- Metallic spoons or spatulas

A.7.4 Equipment

- Balance Metler Toledo, PR 503 Delta Range®, precision d= 0.01 g/0.001 g
- Balance Metler Toledo, AT 261 Delta Range®, precision 0.01 mg

A.7.5 Methods

- Artificial soil was prepared by mixing peat, sand, kaolin and bidistilled water as described in appendix A.4.
- Small portion of artificial soil were taken and mixed with different amounts of $CaCO_3$. In general, 7 samples of artificial soil (each of 30 g (dw)) were prepared and different concentrations of $CaCO_3$ were added as described in the table A.2. Soil samples were mixed thoroughly with a spoon or spatula.

% of $CaCO_3$ to add to the of soil	**Corresponding amount of $CaCO_3$ (g) to add to 30 g (dw) of soil**
0	0
0.2	0.06
0.4	0.12
0.5	0.15
0.55	0.165
0.6	0.18
0.7	0.21

Table A.2

- The pH of each soil sample was then determined as described in appendix A.6.
- The results obtained for each soil sample were plotted on a graph of pH values versus amounts of $CaCO_3$. From this graph, the amount of $CaCO_3$ necessary to obtain a pH of 6 ± 0.5 was estimated.

A.8 Protocol for soil contamination (artificial or natural soils)

A.8.1 General

Soil contamination was made as described in the ISO standard 11267 ((ISO, 1999)). Method for soil contamination is different if the test substances are soluble in water or soluble in organic solvents.

A.8.2 Products

- Stock solutions of the test substances (A.9)
- Artificial soil samples (see appendix A.4) or natural soil samples
- Bidistilled water
- Acetone (Super Purity Solvent), Romil-SpS N° H031
- Hexane (Super Purity Solvent), Romil-SpS N° H389

A.8.3 Materials

- Round bottom flasks out of glass, capacity 250 ml, with lids
- Glass funnels
- Polystyrene petri dishes (PS), diameter 101 mm, 54 mm in height, Semadeni N° 1999, with lids
- Glass dishes
- Graduated cylinder out of glass, capacity 100 ml
- Metallic spoons or spatulas

A.8.4 Equipment

- Rotavapor Buchi R114
- Balance Metler Toledo PR 503 DeltaRange®, precision d= 0.01 g/0.001 g
- Micropipettes

A.8.5 Methods

Substances insoluble in water but soluble in organic solvents

Stock solutions needed for soil contamination are prepared as described in appendix A.9.

Artificial soil In order to contaminate the soil in a homogeneous way, quartz sand is contaminated at the desired concentrations and then mixed with the other artificial soil constituents.

- Prior the soil contamination, all glass dishes was rinsed with acetone and hexane in order to avoid external contamination.
- The quantity of the stock solution required to obtain the desired concentration for experimental setup was introduced in a 250 ml round bottom flask with a micropipette.
- A constant acetone volume equivalent to the mass of quartz sand was added to all concentrations and to one control (21 ml to 21 g of quartz to 30 g of soil). As the volume of acetone of the stock solution was generally lower than this volume, more acetone was added with a graduate cylinder to obtain the constant volume.
- The flask was agitated to mix the stock solution and the newly added volume of acetone.
- The amount of quartz sand 40-100 mesh and quartz sand $\geq$ 230 mesh needed (see A.4) was weighed and mixed in a polystyrene petri dishes for each concentration to be tested.
- Using a glass funnel, the total mass of quartz sand (40-100 mesh and $\geq$ 230 mesh) required for each concentration was transferred in the flask containing the stock solution-acetone mixture.
- The mixture was mixed thoroughly and rotary evaporated (pressure 500 mmbar, temperature 40 °C, medium speed of rotation) until no more acetone was present (the quartz sand should begin to take off from the flask wall).
- By gently taping the round flask (or using a metallic spatula), quartz sand was recovered in a glass dishes and placed under a fume hood for approximately 3 h to allow all acetone residues to evaporate (quartz sand should not smell of acetone).
- Finally, the contaminated quartz sand was mixed homogeneously with the other artificial soil constituents and with the required volume of bidistilled water(A.4). All soils were prepared 1 day in advance and stored at room temperature until beginning of testing.
- Soil samples and collembola could then be introduced in test containers as described in A.10 and A.3.

  Note: As the acetone volume varies highly with temperature, it is better to work with weight than with volume.

Substances soluble in water

Stock solutions were prepared as described in appendix A.9.

If the test substance is water soluble, the required volume of stock solution needed to obtain the desired test concentration was directly added to the prepared soil sample.

Artificial soil

- Soil samples were prepared by mixing all the constituents, except the bidistilled water, in polystyrene petri dishes as described in appendix A.4.
- The volume of stock solution required to obtain the desired concentration for testing was put in a glass beaker with a micropipette. Bidistilled water was added in order to obtain the total volume of water needed to reach the desired WHC percent (e.g., 50 %).
- The liquid was then added to the corresponding soil sample and mixed homogeneously with a metallic spoon until a "crumbly" structure was obtained. The soils were stored for 24 h at room temperature before the beginning of the test.

Natural soil

- Forty-eight hours prior the beginning of the experiment, natural soil samples were acclimated to room temperature (20 °C) (if stocked at cooler temperatures (e.g., 4 °C)).
- Twenty-four hours after the beginning of the acclimatization period, natural soil samples were put in polystyrene petri dishes and contaminated by adding bidistilled water and stock solution of the test substance as for artificial soil.

  Note: The initial water moisture content of the natural soil has to be considered before adding water for WHC adjustment

A.9 Protocol for the realization of the stock solutions

A.9.1 General

Stock solutions were prepared as described in the ISO standard 11267 (ISO, 1999)

To prepare the stock solutions, the different test substances were dissolved in water or acetone according to their solubility. Each stock solution was prepared before starting the test. All glass dishes were rinsed with acetone and hexane before the preparation of the solutions to avoid organic contamination.

A.9.2 Products

- Acetone (Super Purity Solvent), Romil-SpS N° H031
- Hexane (Super Purity Solvent), Romil-SpS N° H389
- Bidistilled water
- Test substances

A.9.3 Material

- Round flasks out of glass
- Metallic spatulas
- Magnetic stirrer

A.9.4 Equipment

- Balance Metler Toledo, PR 503 Delta Range®, precision d= 0.01 g/0.001 g
- Magnetic agitator, Gerhardt (N° LSSI1)

A.9.5 Methods

Water soluble substances

- The quantity of the substance required to obtained the desired concentration of the stock solution was weighed and introduced in a round flask of appropriate capacity.
- The required volume of bidistilled water was added (bring to gauge capacity).

- The solution was stirred until the substance was entirely dissolved.

Acetone soluble substances

The stock solutions were prepared as for water soluble substances but acetone was added instead of bidistilled water (see A.9.5)

Example of preparation of a stock solution (concentration 1 mg/ml):

If 10 ml of solution are required, weight 10 mg of substance, introduce in a 10 ml round flask and complete with the solvent (water or acetone) to 10 ml (bring to gauge capacity).

Note If different concentration of the same stock solution are required for a test, the solution with the highest concentration was diluted to prepare the other solutions.

A.10 Protocol to start a reproduction or mortality test

Experiments were carried out according to the ISO standard 11267 (ISO, 1999)

A.10.1 Products

- Natural or artificial soil samples, contaminated at desired concentrations
- Granulated dry baker yeast (Dr.Oetker, Germany)
- Acetone (Super Purity Solvent), Romil-SpS N^{o} H031
- Hexane (Super Purity Solvent), Romil-SpS N^{o} H389

A.10.2 Materials

- Transparent glass containers, capacity 100 ml, with lids
- Metallic spoons or spatulas

A.10.3 Equipment

- Balance Metler Toledo PR 503 DeltaRange®, precision d= 0.01 g/0.001 g

A.10.4 Methods

Each experiment was composed of a control (= control containing acetone in case of soil contamination with acetone as carrier), the chosen concentration range of pollutant, a pH control and a humidity control. The number of replicates varied between the different experiments, but generally and according to the ISO protocol, there were five replicates for the control with acetone and three replicate for each concentration tested. The pH control was composed of four replicates and the humidity control of one replicate.

- Glass containers were rinsed with acetone and hexane prior to each experiment to prevent external contamination. They were then placed under a fume hood to allow all solvent residuals to evaporate.
- Soil samples were contaminated at the desired concentration of pollutant as described in appendix A.8.
- Using a spoon or spatula, each container was filled with 30 g wet weight of soil (20 g for a mortality test), without compressing the substrate to allow burying of the collembola.
- Ten juvenile springtails aged 10-12 days were introduced in each replicate, except in the humidity control, as described in appendix A.3. To avoid loss of humidity, containers were closed tightly with their plastic lids.
- Collembola were fed with 10 mg granulated yeast at the beginning of the test (days 0) and 14 days later.
- Containers were kept under the same conditions as the culture (A.1). They were opened twice per week to allow aeration.
- The humidity control was weighed at the beginning of the experiment (day 0) and then once per week until the end of test. If the water loss exceeds 2 % of the initial water content, it must be compensated by adding bidistilled water.
- pH was determined in the control and in the highest concentration at the beginning (day 0) and at the end (day 28) of the test.

A.11 End of test: Protocol for the extraction and counting of juvenile or adult collembola in uncompressed soil

A.11.1 General

Extraction and counting of juvenile and adult collembola was done according to the ISO standard 11267 (ISO, 1999). This method was used to extract springtails from the soil at the end of a reproduction or mortality test for counting.

A.11.2 Products

- Bromophenol blue

A.11.3 Materials

- Glass beaker, capacity 500 ml, diameter 9 cm.
- Metallic spatula

A.11.4 Equipment

- Digital camera (Nikon Coolpix 880, 3.34 megapixels)
- Tripod
- Spots
- Image analysis software (UTHSCSA Image Tool 3.00)

A.11.5 Methods

At the end of a test (28 days for reproduction test and 7 or 14 days for a mortality test), adult and/or juvenile collembola present in test containers were recovered and counted according to the following method:

- The substrate of the test container was poured in a 500 ml glass beaker
- The container walls were rinsed with tap water and the liquid was added to the beaker content.
- Approximately 150 ml of supplementary tap water was added to the beaker. The mixture was gently stirred with a spatula to allow collembola to rise to the surface.

- Approximately 250 ml of tap water colored with bromophenol blue were added (colored bromophenol blue water improves the contrast with the whitish collembola and allows a better distinction between them and the soil paritcles).
- The mixture was stirred another time for approximately 1 minute to allow the remaining collembola to drift to the surface.
- Adult springtails were counted by eye.

  Note: If a mortality test was realized, the protocol ended there.
- For reproduction tests, the beaker was completed with tap water to 500 ml and a picture of the surface of the water was taken with a digital camera. The camera was fixed with a tripod and spots were used to improve the light conditions.
- Pictures were transferred to a computer and the number of juvenile springtails were counted on a paper-printed photograph or on the screen using an image analysis software (Image Tool®with the function "count and tag").

A.12 Protocol for pollutant exposure of collembola to perform proteomic experiments

A.12.1 General

In order to easily recover collembola to observed eventual changes in their protein pattern (over or under expressed proteins), they were exposed to compressed artificial soil contaminate at the desired concentration as describe below.

A.12.2 Products

- Soil samples contaminated at the desired concentration of pollutant (A.8)

A.12.3 Materials

- Polystyrene petri dishes (PS), diameter 101 mm, height 54 mm , Semadeni N^{o} 1999, with lids
- Teflon piston, slightly smaller than the petri dishes diameter (< 101mm)
- Metallic spoons or spatulas
- Pasteur pipettes, short form 150mm (Assistent N^{o} 567/1)

A.12.4 Methods

- In order to obtain a compressed substrate height of approximately 1 cm, 70 g (ww) of artificial soil (contaminated to the desired concentration) were introduced in a petri dish using a spoon or spatula.
- The substrate was evenly distributed in the petri dishes and compressed using the teflon piston. The surface was smoothed with the fingers (with gloves!) to avoid holes where collembola could bury. The compressed substrate should reach a height of approximately 1 cm in the petri dish.
- Aged synchronized collembola were introduced in each petri dish using a folded black cardboard as describe in appendix A.3.
- Springtail were fed with dry beaker yeast and the petri dishes were closed with their lids. Laboratory condition and maintenance were the same as for the breeding containers.

A.13 Protocol for recovery and freeze-drying of collembola for proteomic methods

A.13.1 General

After the exposure period to the chosen substance, collembola were recovered and prepared for two-dimensional gel electrophoresis. The time of exposure depend of the experiment conducted (generally collembola were exposed between 1 and 12 days).

All the material used for two-dimensional gel electrophoresis was autoclaved and manipulated with gloves to avoid skin keratin contamination of the samples. Gloves were worn during all the manipulation and also for further steps of the 2D gel electrophoresis.

A.13.2 Products

- liquid nitrogen

A.13.3 Sterile Materials

- black cardboard (A4 size)
- glass funnel
- eppendorf tubes (1.5 ml capacity)
- forceps

A.13.4 Equipment

- Ultra-low temperature freezer (-80 °C) (FROILABO)
- freeze dryer (”Lyolab C” LSL SECFROID)

A.13.5 Methods

- Twenty-four hours before the end of the exposure on compressed soil, remaining food was removed to avoid presence of yeast proteins in two-dimensional gel electrophoresis of collembola. Individuals exposed for only 24 h or 48 h to pollutants were not fed.
- At the end of the exposure period, the lid of the petri dish was rapidly opened and the petri dish was turned over a black cardboard (A4 size) so that the organism fell off. These two movements (opening of the lids and turning over a black cardboard) have to be done simultaneously. This will ensure that collembola fall down, as the opening of the lid will make them jump.

- Collembola were then counted to the desired number. The number of collembola should be sufficient to obtain a total protein quantity of 50 μg (quantity required for one 2D gel). See table A.3 for more details. Collembola were gathered in the middle of the cardboard and poured in an eppendorf tube using a glass funnel.
- The eppendorf tubes were closed and put in liquid nitrogen using forceps in order to kill the collembola. Collembola can be stored at -80 °C for a few days before freeze-drying if needed.
- Eppendorf tubes containing the collembola were then placed in freeze-dryer apparatus with lids open and the collembola were freeze-dried for 24 h.
- After collembola were freeze-dried, eppendorf tubes were closed and stored at -80 °C before used to avoid proteins degradation.

Eppendorf tubes were kept on ice during transportation steps.

Age	Total quantity of protein (μg) per collembola	Number of collembola per 2D gel
$<$ 10 days	2 μg	25
10 to 20 days	4 μg	12-13
$>$ 20 days	8 μg	6-7

Table A.3: Mean protein content of collembola as a function of the age

Appendix B

Overall information and guidance for proteomic methods

Two of the main proteomic tools are two-dimensional electrophoresis (2-DE) and mass spectrometry. 2-DE permit to resolve complex mixtures of proteins first by isoelectric point and then by size. In general, 2-DE is capable of separating proteins within an isoelectric point (pI) range of 3.5-10 and of molecular masses ranging from 6 to 300 kDa (Ashcroft, 2003). Combined with mass spectrometry, 2-DE allows identification of proteins of a specific cell, tissue or organism.

In classical proteomics methods (2DE-MS), proteins are separated based on net charge by isoelectric focusing (IEF) in the first dimension. During this phase, proteins migrate towards the charged electrode through the gel strip containing a specific pH gradient until they reach their isoelectric point (pI), at which point they carry no net charge. Following isolectric focusing, proteins are further separated in the second dimension based on their molecular weight using standard SDS-PAGE. Gels are then stained to identify protein spots and spots of interest are excised from the gel, trypsin-digested and extracted peptides are sequenced using tandem mass-spectrometry. In the first MS analysis, peptides are ionized and resolved on the basis of mass to charge ratio (MS). From the resulting mass spectrum, parent ions are selected for MS/MS analysis. These ions are passed through a collision cell where they undergo fragmentation along the peptide backbone to produce a predictable pattern of fragments (MS/MS). Differences in fragment ion masses in the MS/MS spectra can be used to determine the sequence of peptides from the target protein. MS/MS fragment profiles can be used to directly search databases for predicted peptide matches (Phillips & Bogyo, 2005).

Some of the main steps of classical proteomic methods are detailed below. The first step begin with the solubilization of proteins from samples of interest.

B.1 Two-dimensional electrophoresis

Source of information: "methods and product manual" from BioRad and course documentation "Applied Mass Spectrometry in proteomics" given by the Biomedical Proteomics Research Group of Geneva and the Protein Analysis Facility of Lausanne (May 2004) (Corthals *et al.*, 2004; BioRad, n.d.).

B.1.1 Sample preparation

Sample preparation is an important step for reducing the complexity of a protein mixture. Factors such as the solubility, size, charge and isoelectric point (pI) of the protein are considered. The protein fraction to be loaded must be in a low ionic strength denaturing buffer that maintains the native charges of the proteins and keeps them soluble.

Solubilization

Solubilization of proteins is achieved by the use of chaotropic agents, detergents, reducing agents, buffers and ampholytes. Variations in the concentrations of these reagents can affect 2-D pattern. Moreover, they must be chemically and electrically compatible with isoelectric focusing (IEF)) in order not to increase the ionic strength of the solution to allow high voltage to be applied during focusing without producing high currents.

Chaotropic agents Urea is the most commonly used chaotropic agent in sample preparation for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). It maintains sample proteins in their denatured state and keeps them soluble. Thiourea can be used to help solubilize many otherwise intractable proteins. Urea and thiourea disrupt hydrogen bonds and are used when hydrogen bonding causes unwanted aggregation or formation of secondary structures that affect protein mobility. Urea is typically used at 8 M. As thiourea is weakly soluble in water but is more soluble in high concentrations of urea, a mixture of 2 M thiourea and 5-8 M urea is used when strongly chaotropic conditions are required.

Detergents Detergents are added to disrupt hydrophobic interactions and increase solubility of proteins at their pI. Protein aggregation is minimized. Detergent must be non ionic (e.g. octylglucoside) or zwitterionic (e.g. CHAPS) to allows proteins to migrate according to their own charges. Concentrations of 1-2 % are generally recommended. Some proteins, especially membrane proteins, require detergents for solubilization during isolation and to maintain solubility during focusing.

Carrier ampholytes Carrier ampholyte mixtures (sometimes referred to as IPG buffer) improve protein solubility and ensure uniform conductivity during IEF without altering the pH gradient of the IPG strip. They are usually included at concentration of ≤ 0.2 %.

Reducing agents Reducing agents such as dithiotreitol (DTT) are used to disrupt disulfide bonds. They maintain protein sulfhydryls in their reduced state and prevent oxidative crosslinking through disulfide bonds. Bond disruption is important for analyzing proteins as single subunits.

Prefractionation

In order to increase the visibility of minor proteins, techniques such as sequential extraction, subcellular fractionation chromatography or prefocusing can be used to reduce the complexity of the sample loaded on a 2-D gel. In sequential extraction for example, proteins are sequentially extracted in increasingly powerful solubilizing solutions. More protein spots are resolved by applying each solubility class to a separate gel. An increase in the total number of proteins is detected using this approach.

B.1.2 First-dimension separation: isoelectric focusing (IEF)

Protein are first separated on the basis of their pI, the pH at which a protein carries no net charge and will not migrate in an electric field. The technique is called isoelectric focusing (IEF). For 2-D PAGE, IEF is best performed in an immobilized pH gradient (IPG).

Isoelectric point (pI)

Differences in proteins' pI are the basis of separation by IEF. The pI is defined as the pH at which a protein will not migrate in an electric field and is determined by the number and type of chargedd groups in a protein. Proteins are amphoteric molecules. As such, they can carry positive, negative, or zero net charge depending on the pH of their environment. For every protein there is a specific pH at which its net charge is zero; this is its pI. Proteins show considerable variation in pI, although pI values usually fall in the range of pH 3-12, with the majority falling between pH 4 and pH 7. A protein is positively charged in solution at pH values below its pI and negatively charged at pH values above it.

Isoelectric focusing

When a protein is placed in a medium with a pH gradient and subject to an electric field, it will initially move towards the electrode with the opposite charge. During migration through the pH gradient, the protein will either

pick up or lose protons. As it migrates, its net charge and mobility will decrease and the protein will sloe down. Eventually, the protein will arrive at the point in the pH gradient equal to its pI. There, being uncharged, it will stop migrating. If this protein should happen to diffuse to a region of lower pH, it will become protonated and be forced back toward the cathod by the electric field. If on the other hand, it diffuses into a region of pH greater than its pI, the protein will become negatively charged and will be driven toward the anode. In this way, proteins condense, or are focused, into sharp bands in the pH gradient at their individual characteristic pI values. figure

IPG strips

A stable, linear, and reproducible pH gradient is crucial to IEF. pH gradient for IPG strips are created with sets of acrylamido buffers, which are derivatives of acrylamide containing both reactive double bonds and buffering group. These acrylamide derivatives are covalently incorporated into polyacrylamide gels at the time of casting and can form almost any conceivable pH gradient. Commercial IPG strips are dehydrated and must be rehydrated to their original thickeness before use. This allow flexibility in applying sample to the strips. There is 3 methods for sample loading: passive in-gel rehydratation with sample, active in-gel rehydratation with sample or cup loading of sample after IPG rehydratation. Introducing the sample while rehydrating the strips is the easiest and in most cases the most efficient way to apply sample. As the strips hydrate, protein in the sample are absorbed and distributed over the entire length of the strip. When rehydrating strips actively or passively, it is very important that they be incubated with sample for at least 11 hours prior to focusing. This allow the high molecular weight proteins time to enter the gel after the gel has become fully hydrated and the pores have attained full size. This sample application methods work because IEF is a steady state technique, so protein migrate to their pI independent on their initial position.

Power conditions and resolution in IEF

During an IEF run, the electrical conductivity of the gel changes with time, especially during the early phase. When an electrical field is applied to an IPG at the beginning of an IEF run, the current will be relatively high because of the large number of charge carriers present. As the proteins and ampholytes move towards their pIs, the current will gradually decrease due to the decrease in the charge on individual proteins and carrier ampholytes. At the completion of focusing, the current drops to nearly zero since the carriers of the current have stopped moving. Electrical conditions and time needed to achieve the run of the strips depends of the pH range of the IPG

strip used as well as sample and buffer characteristics.

B.1.3 Equilibration

Transition from first-dimension to second dimension gel electophoresis required equilibration of the resolved IPG strips in sodium dodecyl sulfate (SDS) reducing buffer. Equilibration step ensure that proteins are coated with dodecyl sulfate. Moreover this process reduces disulfides bonds and alkylates the resultant sulfhydryl groups of the cysteine residues.

B.1.4 Second-dimension separation

Second-dimension separation is by protein mass, or MW, using SDS-PAGE. The proteins resolved in IPG strips in the first dimension are applied to second-dimension gels and separated by MW perpendicularly to first dimension. The pores of the second-dimension gel sieve proteins according to size because dodecyl sulfate coats all protein essentially in proportion to their mass. The net effect is that proteins migrate as ellipsoids with a uniform negative charge-to-mass ratio, with mobility related logarithmically to mass.

Gel composition

Gel can be composed of single percentage or increasing gradient concentration of acrylamide (gradient gels).

Single-percentage gel The percentage of acrylamide, often referred to as $\%T$ (total percentage of acrylamide plus crosslinker) determine the pore size of a gel. The higher the $\%T$, the smaller the pore size. Most protein separation use 37.5 parts acrylamide to 1 part bis-acrylamide (bis). Piperazine diacrylamide (PDA) is sometimes subtitued to bis, which can reduce silver staining background and give higher gel strength.

Gradient gels In gradient gels, acrylamide concentrations increased from top to bottom so that the pore size decreases as the proteins migrate further in the gels. As proteins move through gradient gels from region of relatively large pores to region of relatively small pores, their migration rates slow. However, a gradient gel cannot match the resolution obtainable with a chosen single concentration of acrylamide.

Migration

After equilibration, IPG strips are placed on top of the gel and fixed with molten agarose solution to ensure good contact between the gel and the

strip. The migration rate of a polypeptide in SDS-PAGE is inversely proportional to the logarithm of its MW. The larger the polypeptide, the more slowly it migrates in gel. MW is determined in SDS-PAGE by comparing the migration of protein spots to migration of standards.

B.1.5 Staining

In order to visualize proteins in gels, they must be stained in some manner. Protein visualization is an important step since it directly influences protein detection and the subsequent processing of excised proteins. The choice of staining methods is determined by several factors, including desired sensitivity, ease of use, expense and the type of imaging equipment available. It is most common to make proteins in gels visible by staining them with organic dyes (Coomassie Brilliant Blue (CBB), SYPRO or Amino-Black) or metallic ions (silver or zinc). Each type of protein stain has it own characteristics and limitations with regard to the sensitivity of the detection and the type of proteins that stain best. The sensitivity that is achievable in staining is determined by

- the amount of stain that bind to proteins
- the intensity of the coloration
- the difference in coloration between stained proteins and the residual background in the body of the gel (the signal-to-noise ratio)

A high-sensitivity stain such as silver stain or fluorescent stain combined with a high protein load (0.1-1mg) permit identification of low-abundance proteins. For use of protein as antigen or for protein sequence analysis, a high protein load should be applied to the gel and the proteins visualized with a staining procedure that does not fix proteins in the gel.

Silver staining

Silver staining is a widely used protein visualization technique and is considered as a protein modifying method. The principle is to use the ability of the carboxylic groups of the proteins to bind silver ions, which are then reduced to metal, by treatment with alkaline formaldehyde, producing a brown-black metallic staining at the position of the focalized protein. Color development is stopped with acetic acid when the desired staining intensity has been achieved. Staining methods such as silver staining using glutaraldehyde as a sensitizer and cross linker agent could detect down to 1-10 ng of separated proteins. This staining protocol can be as much as 100 times more sensitive than Coomassie Blue staining R250. But to be compatible with mass spectrometric analysis, such staining methods must be conducted without

glutaraldehyde, since this reagent crosslinks the amino groups of the protein, producing complex and non-identifiable peptides .

Coomassie staining

Coomassie staining is one of the most common stain for protein detection in polyacrylamide gel. Two dyes can be used, Coomassie Brilliant blue (CBB) G250 or R250, which differ only by one methyl group. The "R" and the "G" designations indicate red and green hues, respectively. The dye binds to proteins thanks to an interaction of its sulfonate group with basic residues, e.g. the E- amino groups of Lys residues. Indeed, the staining response is more likely linked to concentration of basic sites at the surface of the proteins as well as the hydrophobicity of the protein. Staining by CBB is quite reproducible but however it is not possible to use this technique for absolute protein quantitation due to a large variability of staining intensity observed for different proteins. Limit detection of these staining techniques is 8-10 ng for colloidal CBB G250 and 50-100 for R250. CBB staining is compatible with MS for protein characterization.

In the past few years, new staining procedure have been developed based on fluorescent probes. These staining technique offer the double advantage to be mores sensitive than coomassie blue and to be compatible with mass spectrometry. SpyroRuby staining is one of the most popular staining methods at the moment with a limit of detection of 0.25-8 ng of proteins.

B.2 Mass spectrometry

Source of information: "methods and product manual" from BioRad and course documentation "Applied Mass Spectrometry in proteomics" given by the Biomedical Proteomics Research Group of Geneva and the Protein Analysis Facility of Lausanne (May 2004) (Corthals *et al.*, 2004; BioRad, n.d.).

B.2.1 Spot detection

2D-gel have to be first digitized before to be analyzed with an image evaluation system. The most commonly used devices are camera systems, densitometers, phosphor imagers and fluorescence scanner. 2-D gel are then evaluated using computer-assisted image analysis software. In general, most of image analysis software allows spot detection, spot quantitation, gel comparison or statistical analysis of digitized gel images and thus permit collection, interpretation and comparison of proteomics data. Series of 2-D gels can for example be compared to evaluate qualitative (presence or absence) or quantitative (over- or under expression) variations in protein expression after different experimental conditions. Once interesting proteins have been selected, they can be excised from gel and identified using mass spectrometry

(MS) techniques. MS enables protein structural information, such as peptide masses or amino acid sequences, to be obtained. This information can be used to identify the protein by searching nucleotide and protein databases. It also can be used to determine the type and location of protein modifications. The harvesting of protein information by MS can be divided into three stages: sample preparation, sample ionization and mass analysis.

B.2.2 Protein identification

Sample preparation for MS analysis

In order to analyze protein or its constituents peptides by MS, they have first to be extracted from the gel and the sample purified. The extraction of whole protein from gels is inefficient. However, if a protein is "in-gel" digested with a protease such as trypsin, chymotrypsin or pepsin, many of the peptides can be extracted from the gel. Indeed, the objective of digestion protocols prior to MS identification of proteins is to obtain sufficient enzymatic or chemical cleavage to successfully extract peptides from the matrix in a form that is directly compatible with MS analysis. Moreover, the conversion of a protein into its constituent peptides provides more information than can be obtained from the whole protein itself. For many applications, the peptides recovered following in-gel digestion need to be purified to remove gel contaminants such as salts, buffer and detergents that can interfere with MS.

Mass spectrometry

In MS analysis, peptides are ionized and resolved on the basis of mass (m) to charge (z) ratio (m/z)(Phillips & Bogyo (2005)). Ions are usually detected by a photomultiplier and MS data are recorded as "spectra" which display ion intensity versus the m/z value. The accurate mass measurement of enzymatically derived peptides combined to specific bioinformatics tools lead to protein identification and characterization by either mass fingerprinting analysis (PMF) or sequence analysis by peptide fragmentation.

Mass spectrometers are usually composed of three principal components:

- an ion source that produces ions either from solid-phase samples in the case of matrix-assisted laser desorption/ionization (MALDI-MS) or from liquid samples in the case of electrospray ionization (ESI-MS)

- A mass analyzer that separates the ions by their masses (m) to charge (z) ratio. Common mass analyzer are ion trap (IT), quadrupole (Q) and time of flight (TOF).

- A detector, usually a photomultiplier, used to sequentially detect ions

Various types of sources, analyzers and detector can be mixed and matched in various configurations but some combinations are more convenient that others. Currently most proteins are identified by MS using ESI-MS or MALDI-TOF-MS.

Appendix C

Curriculum Vitæ

Name :	Sophie Campiche
Date of Birth :	24 August 1977
Adress :	Av. de Châtelaine 91
	1219 Châtelaine
Nationality :	Swiss
Civil state :	Single
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Professional achievements

2002-2006 PhD thesis in the laboratory of Environmental Chemistry and Ecotoxicology (CECOTOX) at the EPFL and under the supervision of Prof. J. Tarradellas. Study of endocrine disruption in soil invertebrates. Development of multigeneration test experiments and of a toxicoproteomic approach to assess the effects of insect growth regulators on the collembola *Folsomia candida*

- Laboratory work: soil ecotoxicity testing and development of test protocols, molecular biology (proteomic methods, 2d gel electrophoresis)
- Diploma thesis supervision
- Research in support of the Environment Canada collembolan toxicity test method with *Folsomia candida* for assessment of contaminated soil.

September 1998 Stage at "Laboratoire du Service de l'Eau", Services Industriels de Genève (SIG)

Scientific publications and Proceedings

- Campiche S., Becker-van Slooten K., Tarradellas J., Two dimensional gel electrophoresis of *Folsomia candida*: differences in protein expression profiles of collembola exposed to IGR, 63, 216-225.
- Campiche S., Becker-van Slooten K., L'Ambert G., Tarradellas J., Multigeneration effects of insect growth regulators on the springtail *Folsomia candida*, Environmental Toxicology and Chemistry *(submitted)*.
- Campiche S., Becker-van Slooten K., Ridreau C., Tarradellas J., Effects of insect growth regulators on the nontarget soil arthropod *Folsomia candida*, Ecotoxicology and Environmental Safety *(available on line)*.
- Cochet C., Campiche S., Felley-Bosco E., Tarradellas J., Becker-van Slooten K., Improvement of sensitivity of 2D protein detection in collembola by radiolabelling. National Research Programme 50 "Endocrine Disruptors" Workshop of the Swiss National Science Foundation, Cadro-Lugano, October 2-4 2005 *(oral presentation)*
- Becker-van Slooten K., Campiche S., Ridreau C., L'Ambert G., Cochet C., Felley-Bosco E., Tarradellas J., Effekte von Insektwachstumsregulatoren auf Collembolen: Reproduktionsstudien und Toxikoproteomik. Proceedings of the Society of Environmental Toxicology and Chemistry (German Language Branch), Basel, September 28-30 2005 *(oral presentation)*
- Campiche S., Becker-van Slooten K., Felley-Bosco E., Tarradellas J., Effects of insect growth regulators on the soil invertebrate *Folsomia candida*: a toxicoproteomic approach, Proceedings of the Society of Environmental Toxicology and Chemistry, Lille, May 22-26 2005 *(oral presentation)*.
- Becker-van Slooten K., L'Ambert G., Campiche S., Ridreau C., Tarradellas J., Toxicity of insect growth regulators for *Folsomia candida*: effects on reproduction in single- and multigeneration tests, Proceedings of the Society of Environmental Toxicology and Chemistry, Lille, May 22-26 2005 *(oral presentation)*.
- Becker-van Slooten K., Campiche S., Felley-Bosco E., Tarradellas J., New findings regarding endocrine disruption in soil invertebrates, National Research Programme 50 "Endocrine Disruptors" Workshop of the Swiss National Science Foundation, Gurten-Berne, October 10-12 2004 *(oral presentation)*
- Becker-van Slooten K., Campiche S., Tarradellas J., Advances in soil invertebrate ecotoxicity testing, Proceedings of the Asian

International Conference on Ecotoxicology and Environmental Safety, Songkla-Thailand, September 26-29 2004 *(oral presentation)*.

- Becker-van Slooten K., Campiche S., Feisthauer N., Stephenson G., Tarradellas J., Scroggins R., Research in support of quality assurance requirements for new soil toxicity tests with collembola, Proceedings of the Society of Environmental Toxicology and Chemistry, Prague, April 18-22 2004 *(poster)*.
- Becker-van Slooten K., Campiche S., Tarradellas J. 2003. Research in support of the Environment Canada collembolan toxicity test method with *Folsomia candida* for assessment of contaminated soil. Report prepared for Environment Canada.
- Campiche S., Becker-van Slooten K., Felley-Bosco E., Andre M., Tarradellas J., Development of a proteomic biomarker approach to study endocrine disruption in soil invertebrates, Proceedings of the Swiss Proteomics Society, Basel, December 2-4 2003 *(poster)*.
- Campiche S., Becker-van Slooten K., Felley-Bosco E., Tarradellas J., Endocrine disruption in soil invertebrates: assessing multigeneration effects and developing a proteomic biomarker approach, National Research Programme 50 "Endocrine Disruptors" Workshop of the Swiss National Science Foundation, Zermatt, October 16-17 2003 *(oral presentation)*
- Campiche S., Becker-van Slooten K., Tarradellas J., Effect of fenoxycarb, a juvenile hormone analog, on the soil invertebrate *Folsomia candida*, Proceedings of the Society of Environmental Toxicology and Chemistry, Hamburg, April 27-May 1 2003 *(poster)*.

Educations

2002-2004 Certificate of complementary studies in toxicology (CECT) organised by the "Lemanic Network of Toxicology" (Lausanne and Geneva Universities and EPFL). Certificates in Ecotoxicology, Neurotoxicology, Genetic toxicology and Analytical toxicology.

2001-2002 Master in biology University of Geneva. Research work realized in the Laboratory of Environmental Chemistry and Ecotoxicology (CECOTOX) from Prof. J. Tarradellas at the EPFL and under the direction of Dr. K. Becker van Slooten.

1996-2001 Bachelor in biology University of Geneva

Computer skills

LaTeX, Excel, Word, EndNote, PowerPoint, Illustrator, Photoshop

Languages

French	Native language
English	Fluent. Public presentations, articles
German	School knowledge
Spanish	Notions

Others

Horse riding, Music (tenor violin in an orchestra), Indoor cycling