Living in a broccoli world Design of a decision matrix for assessing the impact of novel (GM) crops on the soil ecosystem

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Living in a broccoli world: Design of a decision matrix for assessing the impact of novel (GM) crops on the soil ecosystem

ACADEMISCH PROEFSCHRIFT

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geboren te Elburg

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Always surpass the one you were yesterday
昨日の我に今日は勝て

Yagyu Sekishusai
柳生石舟斎

Contents

CHAPTER 1

General introduction

1.1 Earth, civilization and soil

Earth. We live on her, shape her into our fashion and grow crops in her soil. Soil is commonly referred to as Earth's living skin – something which we cannot live without (Logan, 2007). Soil management has been important ever since human civilization began to develop agriculture around 10,000 years ago (McNeill and Winiwarter, 2004). Agricultural practise is essential for human populations and depends largely on healthy and fertile soil. Yet, we rarely realize that maintaining healthy and fertile soil, in turn, depends on a realm which lies below the soil surface. This realm consists of an astonishing number of organisms, chemical reactions and interactions: the soil ecosystem. This thesis focuses on how novel crops, such as genetically modified (GM) crops, can affect the soil ecosystem and how we can assess the potential risks of using novel crops on soil ecosystems. In this chapter, I first introduce soil ecology and inhabitants of the soil, followed by the function of these soil–dwelling organisms and the ways in which they interact with the above and below ground environment. I will further describe what genetic modification of crops entails by outlining the different types of genetically modified crops that have been generated and identifying the risks that are involved in growing GM crops on an agricultural scale. I will then give an overview of current perspectives

on risk assessment and the tools required for risk assessment. This chapter concludes by setting forth the research questions asked and the methods and model systems used in this thesis.

1.2 Soil ecology

Soils come in a wide range of forms, varying in properties such as texture (particle size distribution), water content, pH, minerals contained and organic matter. They depend on soil formation processes, local climate and most importantly geological history (Bardgett, 2005). The layers (horizons) closest to the soil surface contain the majority of soil microbes, soil fauna, nutrient (re–) cycling and mineralization compared to deeper layers (Bardgett, 2005). Decomposition of dead organic matter, is one of the most important soil processes, which depends on the physiochemical environment, litter quality and the presence of a detritivorous community (Hättenschwiler et al., 2005). Decomposition converts dead organic matter into simple organic molecules (e.g. amino acids) or inorganic molecules (e.g. N or C) through the interplay between microorganisms and litter–feeding detritivores. Microbes degrade large complex molecules (e.g. lignin, proteins and cellulose) by using extracellular enzymes, making detritus easier to digest by litter feeding detritrivores, such as isopods and millipedes (Bardgett, 2005). These litter fragmenters disintegrate the litter by producing faecal pellets, which in turn create a fresh surface with increased nutrient availability for soil–dwelling microbes to further enhance the decomposition process (Edwards and Bohlen, 1996; Drobne, 1997). Earthworms can create up to 40 ton casts (i.e. faecal pellet) per hectare in temperate ecosystems (Edwards and Bohlen, 1996). The effect and impact of faecal pellets produced by detritivores can thus be extensive.

Microbes, and especially fungi, are fed or grazed upon by secondary consumers, such as collembolans (Filser, 2002). Grazing enhances decomposition and mineralisation processes (Bardgett et al., 1993; Berg et al., 2001) and remobilizes nutrients that were bound in microbial biomass (Clarholm, 1985). Other key players in the soil are ecosystem engineers, such as earthworms that modify physical structure of the soil by their bioturbation activity (Edwards and Bohlen, 1996). Such physical structures provide habitats for other organisms and further aids in the movement of material through the soil (Brown, 1995; Wardle, 2002). Moreover, drainage, moisture balance and aridity of the soil are enhanced, by creating structures such as canals and macropores (Lavelle et al.,

1995, 1997). Decline of earthworm populations due to the use of pesticides increased leaching of nitrogen and phosphorus as a result of a 2–fold increase in volume of surface runoff (Sharpley et al., 1979), indicating the importance of these soil engineers for soil ecosystem sustainability. Hence, decomposition processes, and in turn primary production, depend on the multitude of soil organisms.

Feeding interactions between various organisms within the soil ecosystem form the soil food web, which can be represented by simplified schemes (Figure 1.1) that give information on 'who–eats–who', or in other words, the trophic relationships between consumers and resources (Hassall et al., 2006). Interactions between species include facilitation (positive effect) and inhibition (negative effect) of other species or processes. For instance, concentrations of ammonium in a grassland soil increase due to the combined presence of collembolan and nematodes species, which in turn facilitate plant nutrient uptake (Bardgett and Chan, 1999). In contrast, inhibitory effects were described in a microcosm study simulating a coniferous forest floor, where the presence of nematodes negatively affected nutrient release from detritus (Setälä and Huhta, 1991).

It has been suggested that soil ecosystem processes are dependent on the identity of species and functional diversity within the community, rather than the number and diversity of species it contains (Bardgett, 2005). Evidence for this idea comes from microcosm studies that manipulated species assemblages of soil macro–detritivores, resulting in altered decomposition processes, such as soil respiration and leaf litter mass loss (Laakso and Setälä, 1999; Heemsbergen et al., 2004; Zimmer et al., 2005). Indeed, Heemsbergen et al. (2004) found inhibition of decomposition when functionally similar species were present, as a result of competition over food. Loss of individual species, therefore, does not necessarily result in changes of ecosystem properties, as other species will replace it, which is known as species redundancy (Setälä et al., 2005). On the contrary, earthworms were found to be irreplaceable key stone species causing overall facilitation of decomposition processes as a single species (Laakso and Setälä, 1999; Heemsbergen et al., 2004; Zimmer et al., 2005). The loss of a single species that represents an essential soil function may, therefore, have serious consequences for soil ecosystem functioning, quality and primary production.

It is important to realise that the 'brown' detritus food web is inextricably linked to the 'green' or aboveground food web, with plants connecting the two (Wardle, 2002). For instance, aboveground producers (e.g. plants) provide resources for the detritus food web and root–associated organisms, while

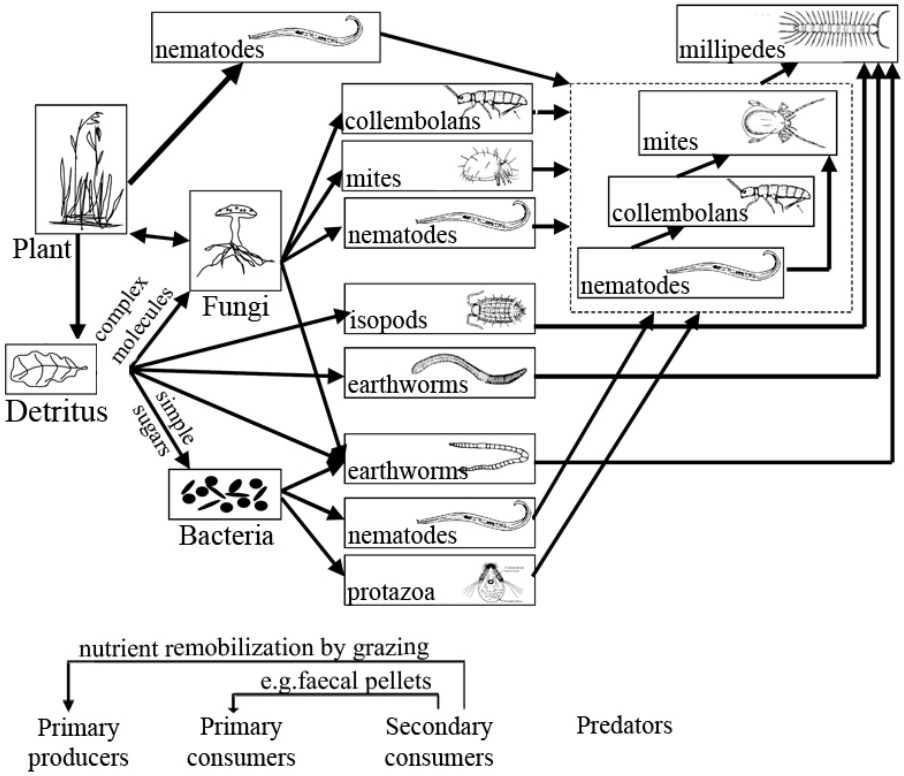

Figure 1.1: A simplified overview of the detritus–based (brown) foodweb. Detritus is degraded into more simple molecules by microbes (bacteria and fungi), making it easier to digest for litter feeding organisms, while others feed on microbes (grazing) or predate on smaller fauna. Feedback loops include faecal pellets and nutrients release by grazing on microbes. Re–modelled after Schouten et al. (2001).

decomposers provide nutrients for plants through their decomposition activities (Wardle et al., 2004). Detritus quantity and quality can affect decomposition rates, for instance because high contents of lignin, phenols, tannins and secondary metabolites are hard to digest (Wardle et al., 2004; Bardgett, 2005). Moreover, soil community composition depends on the litter input of the dominant plant species in an ecosystem (Loranger-Merciris et al., 2007). Root–associated organisms, such as pathogens, herbivores or mycorrhizal fungi have a more direct connection to plants and are for instance able to affect foliar quality via the production of secondary metabolites in plant leaves (Soler et al., 2005). In conclusion, the soil ecosystem is extremely complex and responsible for many 'ecological services' which affect not only soil quality, but also primary production of, for instance, crop cultivation. Functional diversity and a high fitness level (survival and reproduction) of the detritivorous community are therefore of critical importance for maintaining healthy soil ecosystems that can simultaneously be used as an objective measure of soil quality and functioning.

1.3 Crop innovation & genetic modification

It is unclear to what extent introduction of a novel crop will affect the below–ground ecosystem. Genetic modification (GM) is a fast and efficient way to introduce or enhance valuable economic traits in plants. It entails the deliberate incorporation of genetic material across species boundaries and was first successfully applied in 1984 to create a herbicide resistant tobacco (Birch, 1997; Nap et al., 2003; Snow et al., 2005). These genetic alterations cannot be achieved by classical breeding, but make use of molecular techniques, including, direct gene transfer via protoplast, particle bombardment, or treatment with a plasmid of the soil pathogen *Agrobacterium tumefaciens* (Birch, 1997; Snow et al., 2005).

Many commercial crops have been altered for various goals ranging from enhanced crop yield (e.g. resistance to pests, diseases, herbicides, abiotic stress) to crop quality traits (e.g. flavour, colour, ornamentation) (Nap et al., 2003; Singh and Walker, 2006; Chandler and Sanchez, 2012). Especially novel GM crops to improve human health (so–called health foods) draw increasing attention. Traits of interest include anti–carcinogenic activity, nutritional, as well as vitamin A content (Nap et al., 2003; Traka and Mithen, 2009). Agricultural areas containing GM crops took up approximately 160 million hectares in 2011

worldwide, of which 69 million (43% of global production) were established in the U.S.A alone. It is, therefore, one of the fastest growing technologies in modern agricultural practise (James, 2011). The well–known crop Bt maize, which contains the Cry1Ab gene of the bacterium *Bacillus thuringiensis* (Bt) is the most widespread and known cultured GM plant. The incorporated gene allows for the production of an insect–specific toxin in order to become resistant to pest species, such as the European corn borer *Ostrinia nubilalis* (Cortet et al., 2007; James, 2011). Bt maize is one of the few GM crops allowed to be grown in Europe and currently occupies ca. 114,490 hectares of agricultural soil, with an increase of 26% from 2010 to 2011 alone (James, 2011).

As with any new technology, GM raised many concerns about potential undesired environmental or health risks (Pretty, 2001). Other environmental concerns include i) invasion of GM crops into other ecosystems (natural or domesticated), ii) gene transfer of the GM genes to wild related species (hybridisation), bacteria in soil ecosystems or guts of other organisms within the ecosystem, iii) resistance of target organisms, iv) loss of biodiversity and v) secondary ecological impacts, such as effects on non-target (unintentionally affected) organisms (Pretty, 2001; Conner et al., 2003; Snow et al., 2005). It is therefore essential that risks of novel GM crops towards the environment are carefully assessed before they are released into the field.

1.4 Environmental Risk Assessment

Potential ways of exposure of the soil ecosystem to GM crops can be divided into four categories: 1) direct exposure, due to toxicity of a foreign gene product (e.g. Bt toxin), 2) indirect exposure via changes in trophic interactions (e.g. loss of one species affects another species), 3) unintended changes in the metabolism or physiological makeup of the GM crop (e.g. higher lignin content of GM maize), and 4) indirect 'knock on' effects caused by changes in the crop management that comply with the needs of the novel GM crop (Birch et al., 2007; Romeis et al., 2008, 2011). Direct exposure of beneficial (non–target) soil organism to GM material can therefore occur via detritus (e.g. litter or plant remains after harvest), root exudates (leaching) (Wardle et al., 2004) or application of plant material to change soil properties (Morra and Kirkegaard, 2002).

Bt maize, for instance, excretes Bt toxins via plant roots and therefore enters the soil ecosystem via the rhizosphere, where it affects not only root

herbivores, but can also harm beneficial non–target soil organisms. Potential risks of Bt have already been extensively investigated in controlled laboratory experiments as well as in long–term field studies (Krogh and Griffiths, 2007; Cortet et al., 2007). So far, no negative effects of Bt material have been found under laboratory conditions for collembolans, isopods, earthworms and other essential soil organisms (Cortet et al., 2007). In agreement, a vast amount of field studies also concluded that Bt maize does not negatively influence soil ecosystems. Reported detrimental effects are attributed to natural variance between maize varieties and changes in agricultural practises (Krogh and Griffiths, 2007; Cortet et al., 2007). Other, more novel GM crops are, however less studied and scientific knowledge on the risks they pose on the soil ecosystem is currently lacking (Snow et al., 2005).

Current Environmental Risk Assessment (ERA) concerning novel GM crops requires extensive testing of potential risks before and after their commercial introduction (post–market monitoring) (Smit et al., 2010). Emphasis is put on the use of various scales (tiers) to investigate potential risks (Snow et al., 2005; Birch et al., 2007; Romeis et al., 2011). Three main tiers are identified. The first tier involves specific laboratory studies with pure compounds and single ecological model species (Romeis et al., 2011). The second (or intermediate) tier includes semi–field studies, such as, glasshouse or microcosm studies, specifying more natural conditions and multiple species. Finally the third tier describes the broad scale studies, either via field studies or via comparison of lower tier data with natural fluctuations found in the field, so–called baseline data. Each tier and method has its own limitations and benefits to predict direct and indirect effects on the soil ecosystems (Birch et al., 2007). An approach integrating multiple methods and tiers is, therefore, preferred. The use of key species (indicator species), or species that represent key functions in the soil is thus recommended over studying an entire complex ecosystem (Birch et al., 2007; Smit et al., 2010; Romeis et al., 2011).

The European Union and its member countries are very cautious when it comes to the use of GM crops, having a legislation and regulatory framework that rarely allows the introduction of a novel GM crop (Nap et al., 2003). Up to this day, the Dutch government does not allow the use of any GM crop for agricultural or commercial use. In 2001, concerns about GM and biotechnology in general were raised by the Dutch government. To answer the questions raised at the time and in order to develop guidelines necessary to evaluate the potential risks of GM crops, the research program ‘Ecology Regarding

Genetically modified Organisms' (ERGO) was developed in 2007. The aims of ERGO were to study the ecology of GM crops within three specific research fields 1) Multithrophic interactions in GM crops, 2) effects of hybridisation (and introgression) between GM crops and wild relatives and 3) to investigate GM effects on the functioning of soil ecosystems.

1.5 Design of a decision matrix for novel (GM) crops

The aim of my PhD was to develop a practical tool, based on scientific studies that could aid governmental decision makers in the assessment of potential risks of introducing novel (GM) crops to soil ecosystems. *Brassica oleracea* of the family *Brassicaseae* was chosen as model for a novel GM crop due to their well–known anti–herbivore defence strategy (Ratzka et al., 2002), involving the production of glucosinolates (GSL). Glucosinolate hydrolysis products are known for their indistinct toxicity towards insect herbivores (Halkier and Gershenzon, 2006) and potential risks or negative effects on beneficial soil invertebrates can thus be expected. Moreover, GSL biosynthesis is well–studied, making *Brassica* species ideal models for GM and risk assessment. Many of our daily vegetables are part of this family, e.g. broccoli (*B. oleracea* var. *italica*), cabbage (*B. oleracea* var. *capitata*), Brussel sprouts (*B. oleracea* var. *gemmifera*) and the scientific model plant *Arabidopsis thaliana* (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006; van Dam et al., 2009). Glucosinolates are nitrogen and sulphur containing compounds that are hydrolysed into a range products (Figure 1.2) when in contact with the enzyme myrosinase (βb–thioglucoside glucohydrolases). Around 132 GSL are recognized which can be divided into the groups aliphatic, indolic or aromatic, depending on their side–chain (Reichelt et al., 2002; Hopkins et al., 2009; Agerbirk and Olsen, 2012). Within the plant, GSL are stored in vacuoles that are isolated from this enzyme and hydrolysis only occurs upon tissue damage, for instance due to herbivory or microbial infection (Morant et al., 2008; Bednarek and Osbourn, 2009). Glucosinolates thus evolved as a consequence of a co–evolutionary arms–race between plants and their pest species (Ratzka et al., 2002; Agerbirk and Olsen, 2012). Isothiocyanates (ITC) are the most predominant and toxic GSL hydrolysis products, due to their non–specific and irreversible mode of action (Brown and Morra, 1997), causing various detrimental effects ranging from denaturation of proteins to delayed larval development (Ratzka et al., 2002).

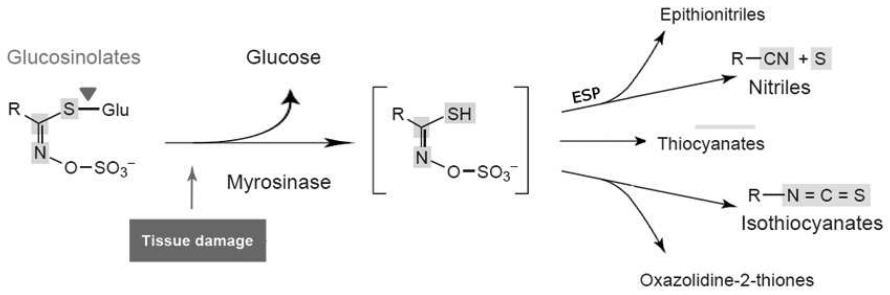

Figure 1.2: The 'mustard oil bomb': schematic overview of glucosinolate hydrolysis by the enzyme myrosinase into several natural toxins upon tissue damage. Focus of this thesis is on isothiocyanates (ITC) and nitriles which are formed in the presence of an epithiospecifierprotein–like factor (ESP). Re–modelled after Wittstock and Halkier (2002) and Halkier and Gershenzon (2006).

This was shown for a wide range of organisms, including fungi, bacteria and soil invertebrates (reviewed by Wittstock et al. (2003)).

Glucosinolates and their hydrolysis products elicit a strong flavour and taste (Halkier and Gershenzon, 2006; Bednarek and Osbourn, 2009; Björkman et al., 2011). For this reason, crop breeding in the past decades was directed to reduce overall GSL concentration in order to obtain crops with a more acceptable taste (Bones and Rossiter, 1996; Mithen et al., 2003). Recent studies have discovered that the toxic nature of ITC shows anti–carcinogenic activity and inhibition of other chronic diseases and inflammation (Jeffery and Araya, 2009). The anti–carcinogenic ability of ITC resides in the general induction of phase I and II detoxification enzymes (e.g. Gluthionine S–transferase) and cell apoptosis of cancer cells (Figure 1.3), aiding in the prevention, detoxification and excretion of potential carcinogens (Jeffery and Araya, 2009; Traka and Mithen, 2009).

Furthermore, GSL are applied as alternative pest management strategy, better known as biofumigation (Halkier and Gershenzon, 2006; Matthiessen and Kirkegaard, 2006). In the case of biofumigation GSL containing *Brassica* plant material is mixed into the soil in order to suppress soil–borne pests and

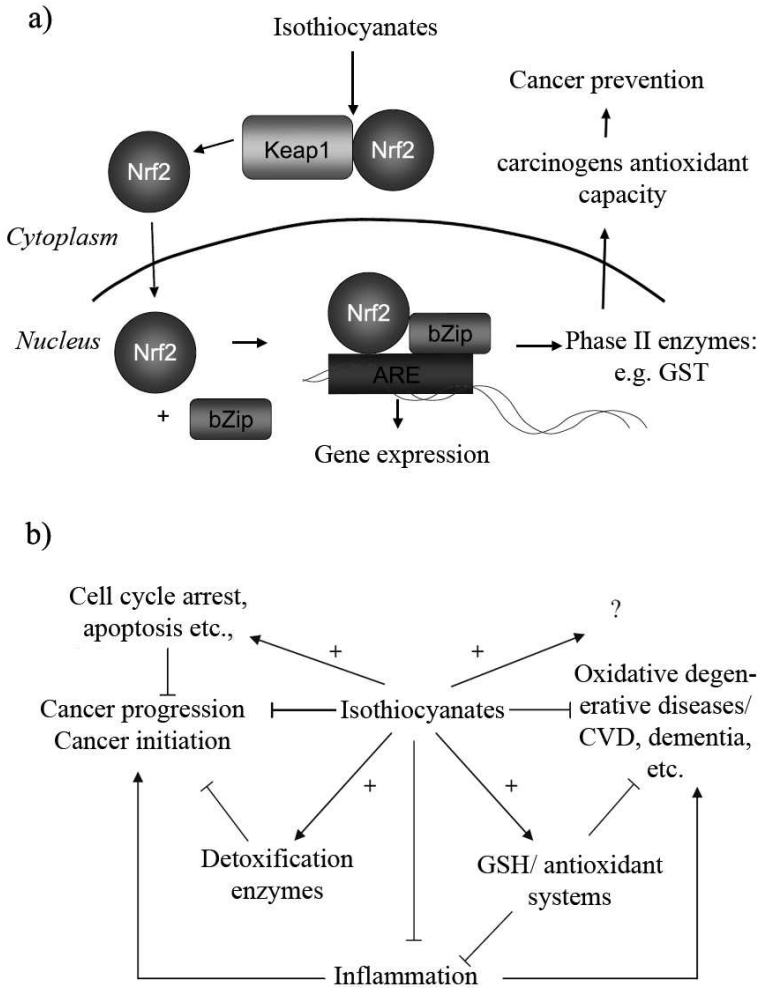

Figure 1.3: Description of the molecular mode of action of isothiocyanates via a) activation of the Nrf2 pathway to facilitate gene expression of phase II detoxifying enzymes within the nucleus such as glutathione S–transferase (GST) to aid inhibition and prevention of cancer. b) Other target sites of ITC induced agents inhibiting e.g. tumor growth and inflammation. Remodelled after Saw et al. (2010) and Jeffery and Araya (2009).

pathogens (Kirkegaard and Sarwar, 1998). This agricultural practise has been shown to effectively reduce potato cyst nematode populations, especially due to the presence of 2–phenylethyl and 2–propenyl ITC (Aires et al., 2009). Clearly, GSL are very beneficial from a socio–economic point of view and GSL expression levels in *B. oleracea* are amenable to genetic manipulation. As such, commercial health crops comprising *Brassica* species with enhanced levels of GSL would be suitable candidates for GM (Traka and Mithen, 2009). Although various researchers endeavoured to create a GM *Brassica* crop (Gigolashvili et al., 2007b; Mithen et al., 2003), a GM variety of *B. oleracea* did not yet exist at the time this project was initiated. Therefore, a collaboration was established with the plant genetics department of the Wageningen University (WUR) to develop a novel (GM) *B. oleracea* crop, with enhanced levels of GSL.

Most studies on GSL and their hydrolysis products have focussed on species that directly interact with *Brassica* plants, such as insect herbivores (Hopkins et al., 2009). To investigate such potential hazardous scenario on non–target organisms in the soil ecosystem a decision matrix was developed, representing a tiered approach to investigate adverse effects of GSL on different levels of biological organisation ranging from the molecular level (transcriptomics) up to the community level. This approach will provide more comprehensive information on the adverse effects of GSL (natural or GM enhanced) on the soil ecosystem (Figure 1.4).

Ecotoxicology is the study of potentially toxic compounds, using standardized laboratory tests (ISO, OECD) that enable the characterization of dose–dependent effects on endpoints such as survival and reproduction of ecologically relevant indicator species (Walker et al., 2001). Ecotoxicogenomics refers to the study of toxic effects on ecologically relevant species on the genome scale (Ankley et al., 2006). Genomic tools include profiling of gene expression responses (using microarrays or RNA sequencing – RNSseq), that represent a signature of the internal physiological condition of an organism at a particular moment in time. Comparing gene expression profiles of individuals exposed to stressors to individuals in unstressed condition can elucidate the underlying mechanisms/mode of action of a toxin or stress. Moreover, ecotoxicogenomics can be used as an early warning system, preceding effects on organismal end points usually assessed in ecotoxicology (van Straalen and Roelofs, 2006). Analyses at the level of communities investigate potential detrimental effects on a more ecologically realistic scale. The decision matrix is therefore complemented with microcosm studies using plant material (instead of pure compounds) and mul-

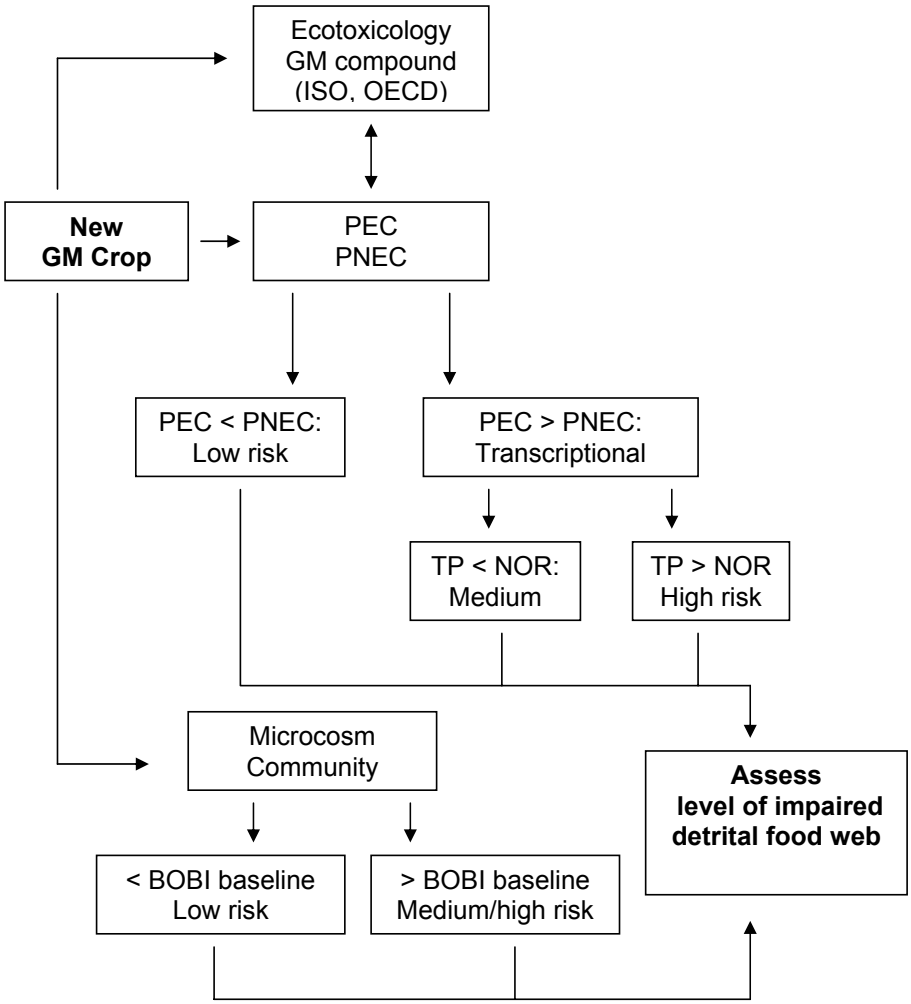

Figure 1.4: Design of the decision matrix for the assessment of potential risks of novel (GM) crops to the soil ecosystem. PEC: predicted environmental concentration, PNEC: predicted no–effect concentration, NOR: normal operation range, TP: transcriptional profile of stressed soil invertebrates, BOBI: baseline field data (Bodembiologische Indicator).

tiple species in different community structures. This approach allows for the study of species interactions and soil ecosystem processes in a simplified and controlled context (Birch et al., 2007; Romeis et al., 2011). Comparison of laboratory and microcosm results to baseline and field data is necessary to put the obtained information into the proper perspective for risk assessment (Birch et al., 2007; Romeis et al., 2008, 2011).

1.6 Outline of the thesis

Using the decision matrix, can we identify the potential risks of *Brassica oleracea* (GM and non–GM) on non–target organisms before allowing novel GM crops to be grown on a commercial scale? This general question fuelled the following research questions that accompany this thesis:

1. What are the effects of ITC and GSL–containing plant material on survival and reproduction of soil invertebrates?
2. What is the underlying mechanism of the toxic effects, based on gene expression analyses? And can we identify indicator genes that serve as early warning signals for the detrimental effects of the toxins?
3. What is the effect of GM compared to non–GM plant material on soil invertebrates?
4. What are the effects of GSL containing plant material on soil community interactions and soil ecosystem functioning?
5. How do the results compare to field baseline data?

In this thesis, four soil invertebrate species are used, which were chosen as representatives of key functional groups within the soil ecosystem; the earthworm *Eisenia andrei* (ecosystem engineer), the isopod *Porcellio scaber* (litter fragmenter) and two collembolans (springtails) *Folsomia candida* (fungivorous grazer) and *Protaphorura fimata* (facultative herbivore). *Eisenia andrei*, *P. scaber* and *F. candida* are further well–known ecological relevant model organisms for ecotoxicological studies (Hornung et al., 1998; OECD, 2004; Fountain and Hopkin, 2005) and gene expression analyses (Nota et al., 2008; Gong et al., 2010). The collembolan *P. fimata* (Heckmann et al., 2006) was chosen to investigate if GSL and their hydrolysis products have similar effects on herbivores (target) and beneficial (non–target) soil organisms.

Chapter 2 describes an ecotoxicological study investigating the effects of two different hydrolysis products of 2–phenylethyl GSL on the beneficial isopod *P. scaber*. Toxic effects on survival over time were investigated using both a dose–response curve and a toxicokinetic–toxicodynamic (TKTD) approach. This study tests the hypothesis that ITC are more toxic than other GSL hydrolysis products, such as nitriles.

To investigate if ITC have similar effects on herbivores and beneficial soil invertebrates, chapter 3 is a study with the collembolan species *F. candida* and *P. fimata*. Using an ecotoxicological approach, the toxic effects of 2–phenylethyl ITC on survival and reproduction were tested for both species. Moreover, a gene expression (transcriptome) analyses was performed for *F. candida*, to elucidate the underlying mode of action of this natural toxin.

In chapter 4 toxic effects of 2–phenylethyl ITC on the earthworm *E. andrei* are reported. It includes toxicity and gene expression data for *E. andrei* to investigate the susceptibility of earthworms to ITC and the underlying mechanisms of such toxic effects. To this end, cross–species hybridisation with *E. andrei* DNA and mRNA was validated and performed on an *E. fetida* microarray platform.

The GSL composition and total concentration varies within *B. oleracea* cultivars. Chapter 5 describes a study measuring GSL concentration and composition differences for leaf material of three *B. oleracea* cultivars. Simultaneously, the consequences of variation in GSL concentration and composition for survival and reproduction of beneficial soil invertebrates and the soil microbial community is investigated. Furthermore, a genetic analysis of the plant cultivars was carried out to elucidate the physiological and gene expression background of the difference in GSL concentration and composition.

To investigate possible effects of plant material containing toxic compounds (e.g. GSL) in a more ecologically relevant setting, a microcosm study with different combinations of soil invertebrates was performed, as described in chapter 6. The aim was to study if toxic stress, induced via plant material, could affect species interactions and soil processes. This study was performed with two plant species; *B. oleracea* plants differing in GSL concentration and *Noccaea cearulescens* differing in cadmium concentration.

During this project a GM was created for two *B. oleracea* cultivars: Winspit and AG1012. Chapter 7 examines how GM changes the GSL concentration and composition of the plants, compared to a non–GM, and if this in turn affects the survival and reproduction of two beneficial soil invertebrates. Two GM

lines were used per plant cultivar, one with increased gene expression of genes involved with 2–propenyl GSL production, and one without this increased gene expression, compared to a non–GM. The purpose was to tease out if a 'GM–effect' could be found, i.e. an effect of manipulation of the plant genome without an actual phenotypic effect.

To put the obtained results into an ecological risk assessment (ERA) perspective, chapter 8 provides a summarizing discussion of the data in this thesis, including a comparison of the obtained data to known baseline and field data. Finally an evaluation of the decision matrix is made, to evaluate the applicability of our tool for ERA in order to assess GM crop effects on soil ecosystems that can aid in more informed decision making by governmental institutions.

CHAPTER 2

Time–related survival effects of two gluconasturtiin hydrolysis products on the terrestrial isopod *Porcellio scaber*

A.E. Elaine van Ommen Kloeke, Tjalling Jager, Cornelis A.M. van Gestel, Jacintha Ellers, Marinda van Pomeren, Thibault Krommenhoek, Bjarne Styrishave, Martin Hansen, Dick Roelofs
Chemosphere 89, 2012, 1084–1090

2.1 Abstract

Glucosinolates are compounds produced by commercial crops which can hydrolyse in a range of natural toxins that may exert detrimental effects on beneficial soil organisms. This study examined the effects of 2–phenylethyl

isothiocyanate and 3–phenylpropionitrile on the survival and growth of the woodlouse *Porcellio scaber* exposed for 28 d. 2–Phenylethyl isothiocyanate dissipated from the soil with half–lives ranging from 19 to 96 h. Exposure through soil showed toxic effects only on survival. The LC_{50}s after 28 d were significantly different at 65.3 mg/kg for 2–phenylethyl isothiocyanate and 155 mg/kg for 3–phenylpropionitrile. A toxicokinetic–toxicodynamic (TKTD) approach, however, revealed that both compounds in fact have very similar effect patterns. The TKTD model was better suited to interpret the survival data than descriptive dose–response analysis (LC_x), accounting for the fast dissipation of the compounds in the soil. Found effects were within environmentally relevant concentrations. Care should therefore be taken before allowing these natural toxins to enter soil ecosystems in large quantities.

2.2 Introduction

Litter decomposition is one of the most important processes for proper soil ecosystem functioning and depends on the physiochemical soil environment, litter quality and detritivore community composition. Litter–feeding soil fauna, e.g. millipedes and isopods, have extensive impact on decomposition by performing essential 'ecological services' such as litter fragmentation (Wardle, 2002; Hättenschwiler et al., 2005). Certain toxic compounds, such as abamectin and doramectin, are known for their adverse effects on litter decomposers (Hornung et al., 1998; Kolar et al., 2008). Most ecotoxicological studies focus on anthropogenic contaminants. Toxins are, however, also synthesized by organisms, so–called natural toxins. Examples can be found in various organisms such as mycotoxins originating from fungi (Bennett and Klich, 2003) or plant derived phytoanticipins or phytoalexins (Morant et al., 2008).

Glucosinolates (GSLs) are sulphated aldoximes (aldoxime–N–sulphates), derived from amino acids and are compounds which can hydrolyse in a range of natural toxins. They are found in many *Brassica* species, including agriculturally important plants such as broccoli, oilseed rape and the scientifically important model *Arabidopsis thaliana* (Wittstock and Halkier, 2002; Traka and Mithen, 2009). Evolved as anti–herbivore defence mechanism, glucosinolates are hydrolysed by the enzyme myrosinase (β–thioglucoside glucohydrolases) upon tissue disruption, such as chewing by herbivores (Wittstock and Halkier, 2002; Morant et al., 2008; Bednarek and Osbourn, 2009). After forming unstable aglucones, a spontaneous rearrangement occurs into various metabolites,

such as isothiocyanates (ITCs) (Wittstock et al., 2003; Bones and Rossiter, 2006; Morant et al., 2008). Although ITCs are the predominantly formed metabolites, various *Brassica* species are also able to produce simple nitriles, epithionitriles or organic thiocyanates instead of ITC, especially when protein factors such as nitrile–specifier proteins (NSPs) and epithiospecifier proteins (ESPs) are present (Wittstock et al., 2003). Simple nitriles can also be formed in the absence of ESP under conditions of low pH values ($<$ pH 5) or high concentrations of iron ions (Wittstock and Burow, 2007).

ITCs are the most common products following hydrolysis of GSL and their toxic effects have been extensively studied. These compounds have detrimental effects on a wide range of organisms, including bacteria, fungi and invertebrates (reviewed in: Wittstock et al. (2003)). For example, 11 μM 2–phenylethyl ITC caused 50% mortality of the population of the root–knot nematode *Meloidogyne incognita* (Lazzeri et al., 2004). In like manner, species that do not directly interact with the plants (non–target species) can be affected. For example, 65 nmol benzyl ITC per gram soil caused a 50% reduction in reproduction of the non–target soil arthropod, *Folsomia fimetaria* (Jensen et al., 2010). However, less is known about effects of simple nitriles on invertebrates and even fewer studies have directly compared the effects of different hydrolysis products derived from the same glucosinolate (Wittstock et al., 2003; Wittstock and Burow, 2010). In general, nitriles are considered to be less toxic than ITC (Wittstock et al., 2003) Table 5.4 as was for instance shown for *Caenorhabditis elegans*, where the ITC was almost 100 times more toxic than the nitrile (Donkin et al., 1995).

The aim of this study was to investigate the effects of two hydrolysis products of the GSL gluconasturtiin, 2–phenylethyl GSL, on the survival and growth of the terrestrial isopod *Porcellio scaber*. This root GSL is studied especially for its ITC hydrolysis product which is a potential anti–cancer agent (Jeffery and Araya, 2009; Traka and Mithen, 2009). Isopods are macro–detritrivores and occur abundantly in various ecosystems and habitats (Drobne, 1997). Their litter fragmentation activity causes an increased moisture retention ability of litter, which favours microbial growth and results in enhanced nutrient mineralisation (Bradford et al., 2002; Wardle, 2002). Moreover, isopods such as *P. scaber* are recognised as useful test organisms for the characterisation of chemical toxicity (Drobne, 1997; Hornung et al., 1998; Kolar et al., 2010). 2–Phenylethyl isothiocyanate and 3–phenylpropionitrile were investigated individually. Lethal (mortality) and sub–lethal (growth) effects were measured

through time and analysed using dose–response curves and a toxicokinetic–toxicodynamic (TKTD) model derived from the general unified threshold model for survival (GUTS, (Jager et al., 2011)). To investigate which route of exposure was most important, exposure was investigated using both contaminated soil and contaminated food. As ITCs are reported to be readily biodegradable (Brown and Morra, 1997; Jensen et al., 2010) concentrations of 2–phenylethyl ITC in the soil were measured over time. This is the first study comparing effects of different hydrolysis products derived from the same glucosinolate on a beneficial non–target soil invertebrate.

2.3 Materials and methods

2.3.1 Animals

The common litter fragmenter, the woodlouse *P. scaber* (Hopkin, 1991), was used in the present study. Lab–cultured first generation of *P. scaber* specimens were used, originating from two field locations (courtesy of C.A.M. van Gestel): parental individuals for the soil exposure experiments were sampled at an allotment garden nearby Utrecht in the Netherlands and parental individuals for the food exposure experiments were retrieved from a compost heap in a garden near Utrecht. Lab–cultured animals were kept in climate chambers at 20°C, 75% relative humidity and a 12:12 h light: dark regime. Housing was provided in glass aquaria with a layer of plaster of Paris, moistened potting soil, with frequently replenished leaf litter (mainly *Populus sp.* and *Acer sp.* leaves) and dry cat food as food source. Test animals had a body length $\geq$ 15 mm and a body weight of 20–30 mg. The sex ratio of female : male was 6:4 for all experiments. Pregnant females were excluded from tests.

2.3.2 Experimental soil, compounds and spiking

LUFA 2.2 soil (Speyer, Germany) was used for all experiments, which has a pH–value ($\pm$ SD) of 5.5 $\pm$ 0.1 and an organic C content (in %) of 2.09 $\pm$ 0.40. The soil was dried at 60°C for 24 h before usage. 2–Phenylethyl ITC [CAS: 2257–09–2] and 3–phenylpropionitrile [CAS: 645–59–0] were obtained from Sigma Aldrich (www.sigmaaldrich.com) as a liquid solution ($\geq$ 99% pure). The compounds were spiked into the soil or food using acetone as a solvent using 1 mL acetone for each g dry weight (DW) soil (Brinch et al., 2002). Ten

percent of the total amount of soil or food needed for each treatment was spiked with the desired concentration, shaken thoroughly and incubated for 24 h in preservation jars. Afterwards, jars were left open overnight under a fume hood to facilitate evaporation of the acetone, thereafter the remaining 90% of the total soil or food was added, mixed together thoroughly. Finally, the soil was moistened to 50% of the water holding capacity (WHC) of 45.2%, corresponding to 22% water of the soil DW. Food was moistened to 50% moisture relative to DW. For the soil exposure tests the soil was spiked only once, at the start of the experiment. For the food experiment, at the start of every week, freshly spiked (or non–spiked) food was made and used during one week, as the food was prone to fungal growth very quickly. Spiked food was kept in dry condition and was only moistened when needed for feeding.

2.3.3 Measuring dissipation in soil

In a separate experiment, without the presence of *P. scaber*, the rate of dissipation of 2–phenylethyl ITC from the soil was measured for three concentrations falling within the ecotoxicological test range: 25, 100 and 400 mg/kg soil. From the spiked soils 5 g samples were taken at time 0 (hydrated soil) and 1, 3, 5, 24, 48, 72, 168 h after initial spiking. The samples were extracted by adding 5 mL ethyl acetate and 100 μL benzyl ITC solution (500 μmol/L in ethyl acetate) as analytical internal standard (IS) to the samples which were then stored at –18°C in darkness. Prior to analysis, samples were vortexed and the ethyl acetate phase was filtered and dried using Pasteur pipettes packed with quartz wool (inactivated, silica treated) and 2.0 g anhydrous Na_2SO_4. This procedure was repeated with an additional 5 mL of ethyl acetate added to the initial soil. The eluate was then evaporated with nitrogen to nearly dryness (less than 200 μL), transferred to GC–vials, and later analysed by gas chromatography tandem mass spectrometry as described in van Ommen Kloeke et al. (2012b). Calibration curves of both benzyl and 2–phenylethyl ITC were used to calculate the factual concentrations of 2–phenylethyl ITC present in the soil samples. First–order degradation kinetics was assumed to estimate the dissipation half–lives, using SPSS 15.0. Dissipation data for the concentration 3.28 mg/kg (20.1 nmol/kg), measured in a former GC–MS experiment (van Ommen Kloeke et al., 2012b), was added to complement the dataset needed for an integrated model analysis with the general unified threshold model for survival (GUTS, see section 2.3.6).

2.3.4 Ecotoxicological experiments

Two tests were performed for each compound: exposure through soil and exposure through food. The method for ecotoxicological testing on *P. scaber* of Hornung et al. (1998) was used to determine effects on the survival and growth. Treatments for both exposure tests were 3.9, 15.6, 62.5, 250 and 1000 mg/kg DW complemented by a normal control (C) using only LUFA 2.2 soil or food and an acetone control (AC) with LUFA 2.2 soil or food spiked with only acetone. All treatments comprised five biological replicates. Each replicate, consisting of 150 g moist soil and ten isopods, was kept in 600 mL glass jars and incubated at 20°C, 75% relative humidity in a 12:12 h light: dark regime. Test food consisted of *Populus sp.* leaves, commercial rabbit food and potato powder ground together in a 50–40–10% ratio (Hornung et al., 1998). Before usage, leaf material was dried overnight at 60°C, after which it was frozen for a day at –20°C. Food was presented *ad libitum* in small plastic dishes (18 mm diameter) and refreshed three times a week. To maintain 50% WHC of the soil, moisture content of the soil was checked once a week and replenished when needed. For shelter three moistened pieces of roof tile were put on top of the soil surface. Survival was checked three times a week and dead individuals were removed from the jar. The mass of all living individuals was measured per replicate once a week on a microbalance.

2.3.5 Dose response curves

Median and 10% lethal effect concentration (LC_{50} and LC_{10}, respectively) values were calculated using the log–logistic response model after Haanstra et al. (1985):

$$Y(c) = \frac{Y_{max}}{1 + \frac{x}{(100-x)}\left(\frac{c}{LC_x}\right)^b}$$

In which Y is the percentage of survival as a function of the concentration c. Y_{max} is the estimated survival in the untreated control, LC_x is the estimated concentration for the selected percentage of effect x (here either 10 or 50) and b the slope parameter of the dose–response curve. SPSS 15.0 was used to fit the model by least–squares analysis. To investigate if the LC_x values differed significantly between the two compounds, a generalised likelihood ratio test was performed (Sokal and Rohlf, 1995).

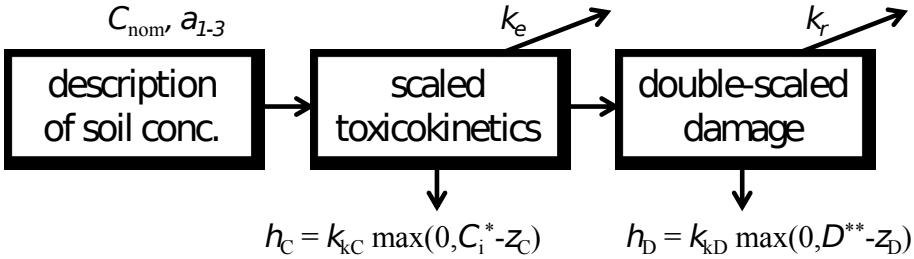

Figure 2.1: Schematic representation of the model used to analyse the survival data for *Porcellio scaber* exposed to two gluconasturtiin hydrolysis products in LUFA 2.2 soil. The soil concentration over time is described empirically with three coefficients(a_1–a_3) and the nominal concentration (C_{nom}). This concentration is used to calculate the scaled internal concentration C_i^*, which links to a hazard rate (h_c). The scaled internal concentration is subsequently used to calculate a double–scaled damage (D^{**}), also linking to a hazard rate (h_D). The parameters k_e and k_r describe the two rate constants, z_C and z_D two thresholds and k_{kC} and k_{kD} two killing rates. Model parameters are explained in Table 2.

2.3.6 Time course modelling – GUTS

To analyse the time course of the toxic effects on the organisms, the survival data were further analysed using the GUTS model (Jager et al., 2011) with the assumption of stochastic death. This toxicokinetic–toxicodynamic (TKTD) framework makes use of all data for survival over time and also allows accounting for the dissipation of the test compounds (see Figure 2.1). The simplest model in which hazard is linked to a single mechanism based on scaled internal concentration, was not able to fit the data satisfactorily, i.e. the single mechanism model was not able to capture the individual treatments. The slowly building up of the effects at low concentrations could not be reconciled with the rapid and strong effects at the highest doses (see Supporting information, Figure S1). Therefore, a second mechanism was added using a stage of damage. The scaled damage is calculated from the scaled internal concentration, making the damage double scaled, i.e. it obtained the dimensions of an external concentration (Jager et al., 2011).

Consequently, GUTS was adapted to account for two hazard rates for toxi-

cant effects; one calculated from the scaled internal concentration and one from the damage stage. Two mechanisms of toxicity were thus assumed: one related to the body residues and, linked to that, another related to the damage caused by the body residue. Apart from the fact that the body residues determine the damage levels, the two causes of death are assumed to be independent, so their associated hazard rates can be added.

This resulted in two rate constants (k_e and k_r), two thresholds (z_C and z_D) and two killing rates (k_{kC} and k_{kD}). Adding a Weibull function for background mortality (S_b), which increased slightly in time, improved the fit to the data. An accurate description of the background mortality can help to identify deviations from the control:

$$S_b = exp(-(h_b t)^F)$$

In which h_b is the hazard rate in the control, t is time and F is the shape coefficient.

Overall survival was calculated by multiplying S_b with the survival fractions calculated from the sum of the hazards due to the chemical. Finally, the model was fit to the survival data for both compounds simultaneously using maximum likelihood estimation (Jager et al., 2011), and confidence intervals were calculated using the profile likelihood (Meeker and Escobar, 1995).

As the model accounts for dissipation of the test compounds, the analysis of the survival data required an adequate description of the actual exposure concentration over time. The measured concentrations of 2–phenylethyl ITC in LUFA 2.2 soil over time did, however, not match the nominal test concentrations. Therefore, the actual soil concentration at each time point was estimated using a model fit on the measured dissipation data. As a consequence, the dissipation rate constant (k_d) decreased with increasing initial concentration in soil (C_0), which could be described with a log–linear function:

$$k_d = a_1 - a_2 log C_0$$

The initial concentration is assumed to be a fixed fraction of the nominal concentration (C_{nom}):

$$C_0 = a_3 C_{nom}$$

The three coefficients a_1–a_3 were estimated from the complete set of measured dissipation data, four concentrations combined, using maximum likelihood estimation and assuming independent and normally distributed errors

after log transformation. Confidence intervals were calculated by profiling the likelihood function (Meeker and Escobar, 1995). The best estimates were used in the survival analysis for both compounds, as there is no dissipation data available for 3–phenylproprionitrile. The complete set of equations of the model can be found in the supporting information.

Alternatively to stochastic death, the GUTS framework can use individual tolerance as a limit case (Supporting information, Figure S2). The current data set is, however, not strong enough to distinguish between stochastic death and individual tolerance.

2.4 Results and discussion

2.4.1 Dissipation of 2–phenylethyl ITC in soil

At the start of the experiment (t = 0), recovery rates of 2–phenylethyl ITC varied between 69.7% and 100%. 2–Phenylethyl ITC dissipated rapidly in natural LUFA 2.2 soil, showing an exponential decrease over time (Figure 2.2). Half–lives were 19.4 (± std error (SE): 1.86) h for 3.28 mg/kg: 32.5 (± 2.33) h for 25 mg/kg: 60.9 (± 4.73) h for 100 mg/kg and 95.8 (± 12.5) h for 400 mg/kg soil. Rate constants decreased with increasing initial concentration, giving rise to four different dissipation models. To be able to use the dissipation data for the GUTS model (at different initial concentrations) the dissipation data was fitted into a single descriptive model (see section 2.3.6). The resulting fit is shown in Figure 2.2, and the parameter estimates (with corresponding 95% confidence intervals) were $a_1 = 0.932$ (0.868–0.997), $a_2 = 0.144$ (0.129–0.159), and $a_3 = 0.729$ (0.663–0.803).

These dissipation patterns of 2–phenylethyl ITC in natural soil can also be found for lower concentration ranges. For instance, concentrations ranging between 0.5 and 3.28 mg/kg soil showed half–lives of around 16 h, depending on the initial concentration (van Ommen Kloeke et al., 2012b). Additionally, benzyl ITC, which has a chemical structure similar to 2–phenylethyl ITC, showed similar dissipation patterns in non–sterile or natural soil (Gimsing et al., 2009; Jensen et al., 2010), while in sterile soils the dissipation of benzyl ITC was much slower. Microbial degradation is therefore likely the main driver responsible for the natural dissipation process of ITCs (Gimsing et al., 2009).

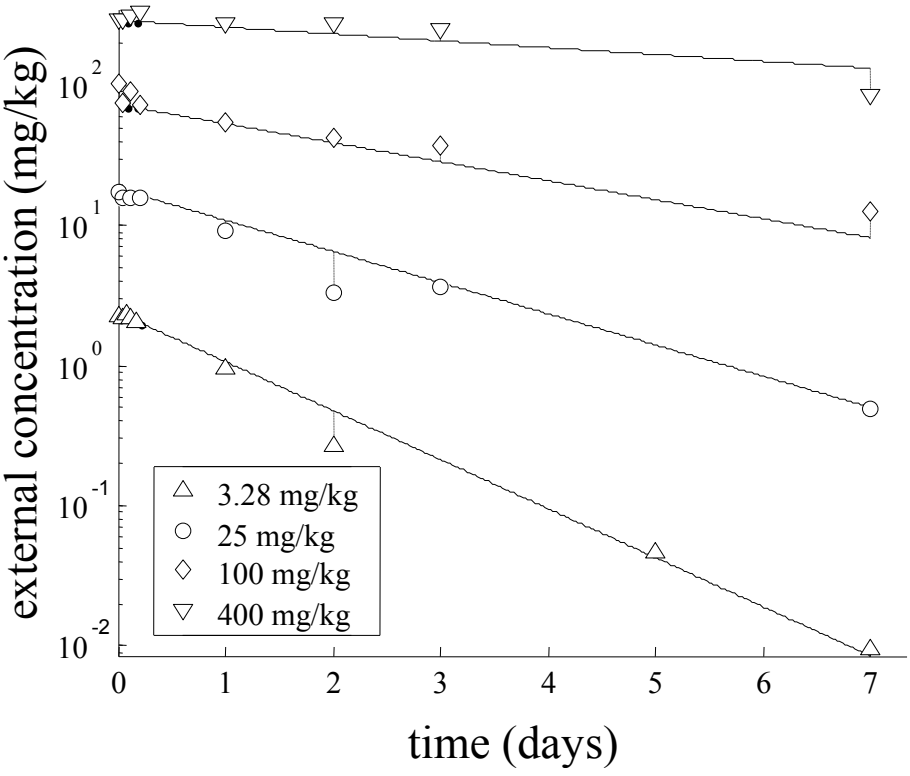

Figure 2.2: Dissipation of 2–phenylethyl ITC (mg/kg soil) as a function of time (days) in moist LUFA 2.2 soil at 20°C for four different starting concentrations. Model curves represent the simultaneous fit of an empirical model (see main text).

2.4.2 Toxic effects through food exposure

Exposure via food did not affect survival or growth of *P. scaber*. The highest death rate was found for 3–phenylproprionitrile at the lowest concentration of 3.9 mg/kg soil with 58% (± SE 15.9) survival. Glucosinolate hydrolysis products are known for their particular odour and taste (Fahey et al., 2001; Halkier and Gershenzon, 2006). It is therefore very likely that *P. scaber* was able to detect the compounds and avoided the spiked food at higher concentrations.

2.4.3 Toxic effects through soil exposure

Growth was measured throughout all the experiments, but did not show any coherent pattern and was therefore excluded from further analyses.

Log–logistic response modelling

Dose–response curves of the effects of 2–phenylethyl ITC and 3–phenylpropionitrile on survival of *P. scaber* after 7 d and 28 d exposure are presented in Figure 2.3. On average, adult survival of *P. scaber* for the control (C) in the 2–phenylethyl ITC test was (± SE) 88 ± 3.7% and 80 ± 5.5% for the acetone control (AC) group, after 28 d of exposure. Adult survival of *P. scaber* for C in the 3–phenylpropionitrile test was on average 80 ± 4.5% and 88 ± 3.7% for AC, after 28 d of exposure. There were no significant differences between the C and AC group for each compound; $p = 0.262$ for to 2–phenylethyl ITC and $p = 0.207$ for 3–phenylpropionitrile. Moreover, C and AC did not differ between the two compounds.

Both 2–phenylethyl ITC and 3–phenylpropionitrile proved to be toxic for *P. scaber* when exposed through the soil (Figure 2.3), with a mortality of 100% at 1000 mg/kg soil. Lethal concentrations (based on nominal concentrations) were calculated after 7 and 28 d and are shown in Table 2.1. For 2–phenylethyl ITC, the LC_x estimates differed substantially when calculated after 7 or 28 d, with higher LC_x estimates after 7 d. To be able to understand this difference in the LC_x estimates, the data was further analysed using time–course modelling (see section 2.4.3).

Overall, *P. scaber* seems relatively resilient to both hydrolysis products compared to other beneficial soil invertebrates. For instance, the collembolan *Folsomia candida* and *Protaphorura fimata* showed an LC_{50} estimate of 2.51–2.48 mg/kg soil, more than 25 times lower, when exposed to 2–phenylethyl ITC

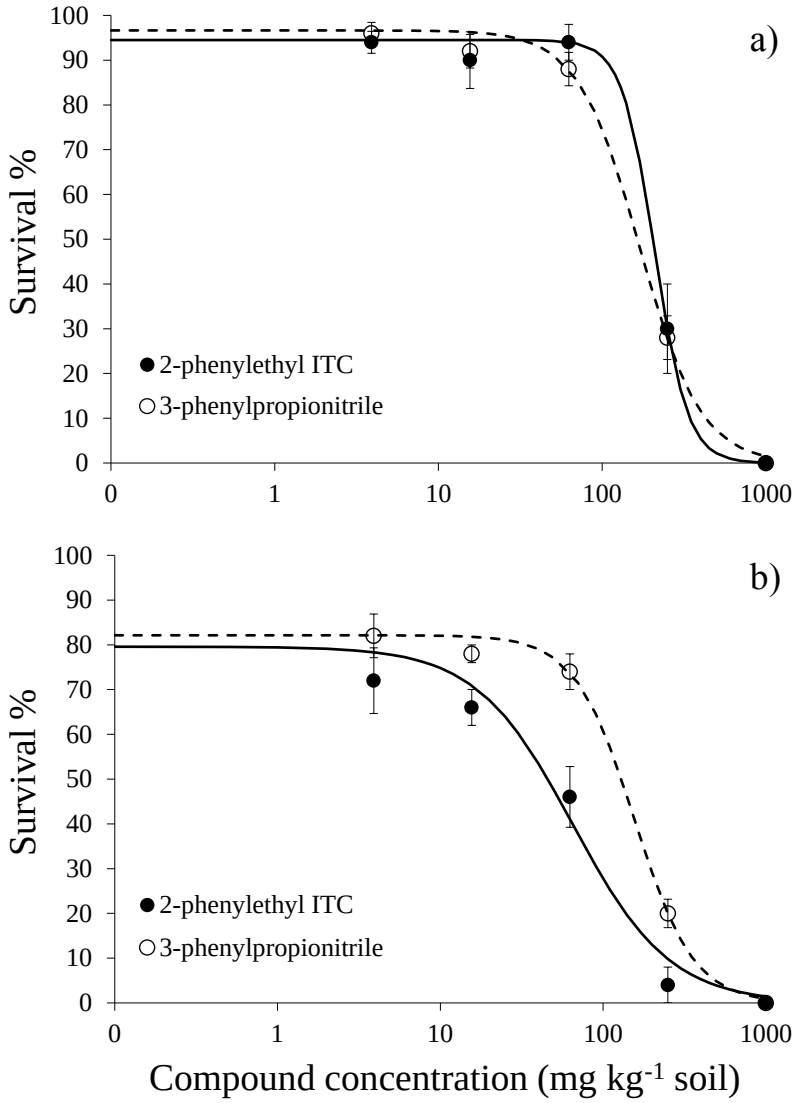

Figure 2.3: Effects of 2–phenylethyl isothiocyanate and 3–phenylpropionitrile (nominal concentrations) on the survival of *Porcellio scaber* after: (a) 7 d and (b) 28 d exposure in LUFA 2.2 soil. Lines show the fit of the log–logistic dose response model to the data with continuous line: fit to 2–phenylethyl ITC and dashed line: fit to 3–proprionitrile. Error bars are standard errors (n = 5).

Table 2.1: LC–values for the effects of to 2–phenylethyl isothiocyanate (2–PEITC) and 3–phenylpropionitrile (3–PEN)on survival of *Porcellio scaber* after 28 d exposure in LUFA 2.2 soil. Values are in μg compound per gram DW soil (nominal concentrations).

		2–PEITC	CI	3–PEN	CI	χ^2
7 days	LC_{10}	126	(0–431)	65.5	(42.7–88.3)	1.85
	LC_{50}	210	(75.8–343)	169	(145–194)	1.79
	LC_{10}	14.6	(2.16–27.0)	62.0	(34.1–89.9)	5.00
28 days	LC_{50}	65.3	(42.0–88.7)	155	(123–188)	18.9

CI = 95% confidence interval. LC_x differences between 2–phenylethyl isothiocyanate and 3–phenylpropionitrile are deemed significant ($p < 0.05$) if $\chi^2 > 3.84$.

after 28 d of exposure (van Ommen Kloeke et al., 2012b). The toxicity of 2–phenylethyl ITC to *P. scaber* is comparable to that of other natural toxins. For instance abamectin, a natural fermentation product of soil bacteria and known antiparasitic veterinary medicine, showed a LC_{50} of 71 mg/kg soil for *P. scaber* after 21 d of exposure (Kolar et al., 2010). Abamectin also rapidly degrades in soil partly due to photodegradation (Wislocki et al., 1989). Furthermore, both 2–phenylethyl ITC and 3–proprionitrile are known to cause inhibition of soil nitrifying bacteria communities with 2–phenylethyl ITC being most toxic (Bending and Lincoln, 2000). For 3–phenylpropionitrile no other toxicity data with regard to invertebrates or other soil organisms is known.

Comparing the toxicity of 2–phenylethyl ITC versus 3–phenylpropionitrile at the two different time points gave very different results. LC_x estimates calculated after 7 d of exposure did not significantly differ between 2–phenylethyl ITC and the 3–phenylpropionitrile while there was a significant difference in toxicity of the two compounds after 28 d, with the ITC being more toxic (Table 2.1). The majority of other studies did show a difference in toxicity between corresponding ITCs and nitriles (same precursor glucosinolate) with ITC being more toxic than the nitrile (reviewed in Wittstock et al. (2003)). The results presented here are therefore only partly consistent with previous studies. The deviating effects of the different hydrolysis products depend, however, on species and exposure method (Wittstock et al., 2003).

Time course modelling – GUTS

LC_x estimates may be less relevant as measures of toxicity in studies experiencing rapid loss of the compound from the soil, as observed in this study (Figure 2.2). Descriptive dose–response models are of limited use in handling time–varying exposure concentrations. The resulting LC_x is only a description for this particular situation (single dose, this dissipation pattern, and this exposure duration); extrapolation to other scenarios or comparison to other compounds (with different dissipation kinetics) is therefore impossible. The GUTS–based survival model is a TKTD approach and thus allows for the analysis of the survival data, accounting for the fact that the external concentrations were not constant (Figure 2.4). The complete set of survival data for the two chemicals was estimated with a single parameter set (model parameters are given in Table 2.2 with their confidence intervals) as the survival patterns were actually very similar. Toxic effects of both compounds were especially rapid at the highest test concentration, resulting in death to all individuals in less than 2 d.

It is likely that two mechanisms are responsible for the toxicity of the compounds, i.e. the large effect of the two highest concentrations could not be reconciled with smaller effects at lower doses. To stay close to the GUTS model, a stage of damage was added with effects being linked both to the internal concentration and to the damage level (Figure 2.1). However, the damage dynamics were very fast in this case, which implies that damage closely follows the internal concentration, or perhaps that the internal concentration itself affects two target sites. A possible justification for the existence of two mechanisms might thus be that there are two receptors for the compound, each producing a different pattern of effect. Alternatively, the parent compound might be metabolised into a transformation product that increases the probability of death through a different mechanism. The current data set is insufficient to explore these possibilities further. The most appropriate choice of mechanism requires a more dedicated study. The possible existence of a two–mechanism effect is not uncommon and was for instance also proposed for *F. candida* exposed to chlorpyrifos via food (Jager et al., 2007).

Figure 2.4: Fit of the hazard model from GUTS (Figure 2.1) to the observed data of the survival of *Porcellio scaber* over time upon exposure to: (a) 2–phenylethyl ITC and (b) 3–proprionitrile in LUFA 2.2 soil. The model is simultaneously fit to the data for both compounds. Parameter estimates are provided in Table 2.2. The legend provides nominal concentrations; calculations were performed using the estimated soil concentrations based on the fit in Figure 2.2.

Table 2.2: Estimated model parameters of the fit (with 95% confidence intervals) to the data of the effect of 2–phenylethyl ITC and 3–proprionitrile on the survival of *Porcellio scaber* in LUFA 2.2 soil. See Figure 2.4. for the corresponding model fits.

Parameter	Estimate (95% confidence)	Unit
Blank hazard rate (h_b)	0.0161 (0.0132–0.0189)	d^{-1}
Weibull factor (F)	1.84 (1.50–2.26)	$(-)$
Elimination rate body residue (k_e)	0.212 (0.184–0.250)	d^{-1}
Threshold for body residue (z_C)	70.0 (65.5–75.2)	$mgkg_{soil}^{-1}$
Killing rate for body residues (k_{kC})	0.136 (0.0892–0.200)	$kg_{soil}mg^{-1}d^{-1}$
Repair rate for damage (k_r)	9.76 (1.06–10)[a]	d^{-1}
Threshold for damage (z_D)	0 (0–4.50)	$mgkg_{soil}^{-1}$
Killing rate for damage (k_{kD})	0.00143 (0.00101–0.00195)	$kg_{soil}mg^{-1}d^{-1}$

[a] The repair rate of 10 per day is the maximum allowed in the analysis for numerical reasons. This value indicates very rapid equilibration of damage (damage kinetics thus follow the kinetics of the body residue).

The same model parameters were used to fit the data for both compounds, assuming that the two compounds have similar effects on survival. This is in accordance with the logistic response modelling when using data for 7 d of exposure. As can be seen from Figure 2.4, any possible difference in the effects on survival between the two compounds is largely driven by the difference in response at 62.5 mg/kg in the last week of exposure. The same diverting data points are also responsible for the different LC_x estimates found for 2–phenylethyl ITC calculated after 7 and 28 d.

At the moment it is unclear whether the two compounds really exert a comparable effect. The current data set does not allow a clear distinction, since soil concentrations or dissipation rates for 3–phenylproprionitrile were not available. However, the GUTS modelling does show the shortcomings of the descriptive dose–response modelling that is generally used in ecotoxicological studies. When the exposure concentration decreases during the test, LC_x estimates based on nominal concentrations will underestimate the toxicity of the compound. Therefore, a TKTD approach should be preferred in these situations. However, even when exposure is constant, there are compelling

reasons to prefer TKTD modelling (Ashauer and Escher, 2010; Jager et al., 2011). TKTK modelling is limited to situations where survival is followed over time (and preferably also the exposure concentrations), which limits its applicability. Even though the number of parameters is quite large (Table 2.2), the model fit describes all of the effects data over time, where the descriptive dose response would require three parameters per observation time. Furthermore, the GUTS parameters in principle allow extrapolation to other exposure scenarios such as pulsed exposures (Ashauer and Escher, 2010). However, in this particular case, the complexity of the mechanism (Figure 2.1), and the number of parameters needed (Table 2.2, and a_1–a_3), does not seem to provide a solid basis for extrapolation.

In any case, ecotoxicological research should include environmental dissipation data in order to get a more realistic understanding of the acute or chronic effects of highly degradable compounds.

2.5 General discussion

The aim of this study was to demonstrate the toxic effects of two hydrolysis products of gluconasturtiin on the beneficial litter fragmenter *P. scaber*. These natural toxins clearly showed lethal effects on this isopod after only a few days of exposure to contaminated soil. ITC toxicity is expected to be due to irreversible and nonspecific reactions of the compounds with proteins and amino acids, which result in inactivation of enzymes (Brown and Morra, 1997). Toxic effects of nitriles are likely related to the cyano group, inactivating especially enzyme systems involved in cellular respiration such as cytochrome oxidase (Brown and Morra, 1997). Survival patterns for both compounds looked very similar for *P. scaber*. GUTS modelling gave more insight in the toxic effects through time and accounted for the fast dissipation rates of the compounds. These patterns suggest the presence of two effect mechanisms, operating in different concentration ranges. Thus, care has to be taken when extrapolating effects from short–term, high–exposure tests to long–term, low–exposure situations, and vice versa.

Recent interest in GSL containing crops surged due to the discovery of their potential as anti–cancer agent (Jeffery and Araya, 2009; Traka and Mithen, 2009), their natural ability for crop protection (Halkier and Gershenzon, 2006) and use as a natural pesticide in agriculture in the form of biofumigation (Morra and Kirkegaard, 2002). At present, concentrations up to 100 nmol/g for ITCs

are found in laboratory experiments and in the field after using effective biofumigation strategies, which is equivalent to 16.3 mg/kg 2–phenylethyl ITC (Gimsing and Kirkegaard, 2009). The toxic ranges investigated in this study are therefore likely to be found, and even exceeded, in the field. The detrimental effects on beneficial soil invertebrates such as isopods can have serious repercussions on soil functioning. Care should therefore be taken before allowing natural toxins to enter the soil ecosystem at these levels.

Acknowledgments

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Appendix A. Supplementary material

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CHAPTER 3

Molecular and life–history effects of a natural toxin on herbivorous and non–target soil arthropods

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3.1 Abstract

Natural toxins, such as isothiocyanate (ITC), are harmful secondary metabolites produced by plants. Many natural toxins occur in commercial crops, yet their possible negative repercussions on especially non–target soil organisms are largely unknown. This study examined life–history and gene transcriptional responses to 2–phenylethyl ITC on two soil arthropod species: *Folsomia*

candida and *Protaphorura fimata*. To that end the standardized ISO guideline for ecotoxicological tests and a microarray for *F. candida* were used. The dissipation of 2–phenylethyl ITC in natural soil was investigated using GC–MS/MS for quantification. Half–lives, tested at four concentration levels in natural soil, were on average 16 h with biodegradation as the plausible main removal process. Regardless, toxic effects on reproduction were shown for *F. candida* and *P. fimata*, with EC_{50} values of around 11.5 nmol/g soil illustrating the toxic character of this compound. Gene expression profiles revealed the importance of fatty acid metabolism at low exposure concentrations (EC_{10}), which is associated with the lipophilic nature of 2–phenylethyl ITC. At higher concentrations (EC_{50}) gene expression became more ubiquitous with over–expression of especially stress–related genes and sugar metabolism. The regulation of a gene encoding a precursor of follistatin, furthermore, implied the inhibition of reproduction and may be an important molecular target that can be linked to the observed adverse effect of life–history traits.

3.2 Introduction

The soil ecosystem is extremely complex and comprises many different organisms, each with their own function, niche and interactions. Soil ecosystem functioning is challenged by many anthropogenic toxins such as metals (He et al., 2005), pesticides (Edwards and Bohlen, 1992) and polycyclic aromatic hydrocarbons (Leon Paumen et al., 2009). These compounds may disrupt the soil ecosystem through mortality or reduced reproduction of soil organisms, as was shown by a large number of studies. Natural toxins, on the other hand, are rarely considered a threat to the environment yet can be lethal at low dosages. Natural toxins are organic compounds that are produced as secondary metabolites in fungi, bacteria, algae, plants or animals. Plant toxins (phytotoxins) are used by many edible plants as part of their natural defence system against pathogens and herbivory (van Egmond, 2004; Hoerger et al., 2009).

Natural toxins can also be found in the plant family *Brassicaceae*, including broccoli, cabbage and mustard, which all produce glucosinolates (GSL) as secondary metabolites. Tissue damage, due to, for instance, herbivory, causes the enzyme myrosinase to hydrolyse GSL into several possible products of which the most common and most toxic is isothiocyanate (ITC) (Brown and Morra, 1997; Halkier and Gershenzon, 2006). ITCs have well–known negative effects on several invertebrate species such as fruit flies, wireworms, symphilids and

nematodes (Brown and Morra, 1997). For instance, 11 μM 2–phenylethyl ITC caused 50% mortality of populations of the root–knot nematode *Meloidogyne incognita* (Lazzeri et al., 2004). Toxic effects are mainly due to irreversible and nonspecific reactions of ITCs with proteins and amino acids, which result in inactivation of enzymes (Brown and Morra, 1997).

Most ecological studies involving ITC have focussed on GSL–containing plants and their effects on specific plant pests such as insect herbivores (Hopkins et al., 2009). In contrast, little is known about effects on non–target species; beneficial or neutral species which are unintentionally affected by the toxicant via indirect contact (non–herbivory). Non–target soil organisms can be exposed to ITCs through the decomposition of crop litter, biofumigation or root exudates of GSL–containing plants (Wardle et al., 2004). ITCs do not seem to be target–specific (Brown and Morra, 1997) and may, therefore, also affect non–target species. For instance, Jensen et al. (2010) showed a 50% reduction in reproduction of the non–target soil arthropod, *Folsomia fimetaria* when exposed to 65 nmol benzyl ITC per gram soil. Such effects on non–target, but beneficial soil organisms, can have severe negative repercussions on soil functioning.

The present study examined the effect of 2–phenylethyl ITC (the hydrolysis product of 2–phenylethyl GSL) on the survival and reproduction of the non–target fungivorous springtail *Folsomia candida* and the herbivorous springtail *Protaphorura fimata*. Springtails (Collembola) are one of the most abundant soil organisms, providing a significant contribution to nutrient mineralisation and decomposition (Berg et al., 2001; Filser, 2002). Moreover, *F. candida* is an important model species for soil quality assessments (Fountain and Hopkin, 2005). By complementing measurements on key life–history traits (survival and reproduction) with gene expression profiling (microarray techniques), we also aimed to assess the underlying modes of action of 2–phenylethyl ITC (van Straalen and Roelofs, 2008). A microarray for *F. candida* was, therefore, applied to provide insight in the molecular response pathways invoked and the biological processes affected by 2–phenylethyl ITC. Concentrations of 2–phenylethyl ITC in the soil were measured over time, as ITCs are reported to be readily biodegradable (Brown and Morra, 1997; Jensen et al., 2010). This is the first report on effects on soil organisms caused by a natural toxin in which measures of survival and reproduction are associated with molecular mechanistic information.

3.3 Materials and methods

3.3.1 Animals

Folsomia candida, a parthenogenetic collembolan (Fountain and Hopkin, 2005), was used for both ecotoxicological and microarray experiments. A synchronized culture of either 10–12 days old (ecotoxicological experiments) or 23–25 days old (microarray) animals was prepared from the laboratory stock culture ('Berlin strain', VU University Amsterdam). This difference in age is chosen due to standardization and aims (reproduction and gene expression effects, respectively) of the existing protocols (ISO, 1999; Nota et al., 2009) and the size of the animals at the time of harvesting; microarrays after 2 days and ecotoxicity tests after 28 days. Standardized methods for synchronization and stock maintenance followed the International Standardization Organization (ISO) guideline 11267 (ISO, 1999). In short, springtails were kept on moistened plaster of Paris mixed with charcoal, at 20°C, 75% relative humidity and a 12 h light/dark cycle. *Protaphorura fimata*, a herbivorous collembolan (Endlweber et al., 2009), also reproduces parthenogenetically and was cultured in a similar fashion as *F. candida*. This species is, however, difficult to synchronize (Heckmann et al., 2006). Therefore, a method was developed to synchronize animals with a maximum of 1 week difference in age. In short, a large quantity of 'almost ready to hatch' eggs (yellow/ brown coloured, oval shaped) were carefully transferred from the stock culture to a fresh Petri dish (with a bottom of moist plaster of Paris mixed with charcoal), using a dissecting needle. In order to keep the eggs clean (avoid microbial growth), $\sim$ 10 adult females were included in this 'breeding dish'. After 1 week all juveniles were transferred to a fresh Petri dish using a glass Pasteur pipette. Each culture weekly received a fresh quantity of baker's yeast. To ensure that animals were reproductively active, they were only used for experiments if large quantities of eggs were present in the breeding dish. *P. fimata* used in the toxicity tests were 5–6 weeks old.

3.3.2 Experimental soil, compound and spiking

For all experiments natural LUFA 2.2 soil (Speyer, Germany) was used, which is a loamy sand soil with a pH of ($\pm$SD) 5.5 ± 0.1 and an organic C content of $2.09 \pm 0.40\%$. Before usage the soil was dried at 60°C for 24 h. 2–Phenylethyl ITC [2257–09–2] was obtained from Sigma Aldrich (www.sigmaaldrich.com) as a liquid solution (99% purity). Ten percent of the total amount of soil needed

for each treatment was spiked with the desired concentration of 2–phenylethyl ITC, ranging from 3.06 to 20.1 nmol per g dry weight soil, by using stock solutions with acetone as solvent (1:1 ratio, i.e. ml acetone: g dry weight soil, DW) (Brinch et al. 2002). The spiked soils were thoroughly shaken and stored for 24 h in preservation jars to allow the soils to absorb the test chemical. Thereafter, to facilitate complete evaporation of all acetone, jars were left open overnight under a fume hood. Next, the remaining 90% of the total amount of soil needed for each treatment was mixed thoroughly in with the 10% spiked soil. Finally, the soil was moistened to 50% of the water holding capacity of 45.2%, corresponding to 22% water of the soil DW.

3.3.3 Measuring dissipation in soil

To measure the soil dissipation rate of 2–phenylethyl ITC 5 g samples were taken from the soils spiked with 3.06–4.90–7.84–12.56–20.09 nmol/g soil, representing several concentrations within the test ranges used for the toxicity tests. Time intervals were: dry start (no water added to dry soil), wet start (hydrated soil, time 0), 1, 2, 3, 4, 24, 48, 120, 168 h after initial spiking. The samples were extracted by adding 5 ml ethyl acetate and 100 ll benzyl ITC (500 μmol/l in ethyl acetate) as analytical internal standard (IS) to the samples which were then stored at –18°C in darkness. Prior to analysis, samples were thawed, shaken on a vortex and the ethyl acetate phase was transferred on top of Pasteur pipettes packed with quartz wool inactivated, silica treated) in the bottom and 2.0 g anhydrous Na_2SO_4 above to filter and dry the samples. The purified sample eluate was collected in a 10 ml test tube. This procedure was repeated with an additional 5 ml of ethyl acetate added to the initial soil and again transferred to the same Pasteur pipette and eluate into the same test tube. The eluate was then evaporated with nitrogen to nearly dryness (less than 200 μl) and transferred to GC vials. Samples were analysed by gas chromatography tandem mass spectrometry (GC–MS/MS, Varian CP–3800 and Varian 1200 triple–quadruple) with electron ionization (70 eV). The MS was operated in selected reaction ion monitoring mode acquiring data on the ion transitions 163.0 > 105.0 for 2–phenylethyl ITC and 149.0 > 91.0 for benzyl ITC. The GC–column was a 30 m x 0.25 mm, 0.25 μm Factor Four VF–5 MS (Varian). The injection volume was 1 μl in splitless mode achieved by a CTC CombiPAL autosampler and a PTV–injector kept at 200°C at all times. The initial column oven temperature was 40°C and the final temperature was

285°C (15°C/min) with a total analysis time of 18.3 min. Helium was used as carrier gas at 1.0 ml/min. Calibration curves of both benzyl and 2–phenylethyl ITC were used to calculate the amount of 2–phenylethyl ITC present in the soil samples. First order degradation kinetics was used to estimate the degradation half–lives, which was performed in SPSS 15.0.

3.3.4 Ecotoxicological experiments

Ecotoxicological experiments to determine effects on the survival and reproduction for *F. candida* and *P. fimata* were carried out following the ISO guideline 11267 (ISO, 1999). Concentrations of 2–phenylethyl ITC were for the *F. candida* experiment: 3.06–4.90–7.84–12.56–20.09 nmol/g soil and for the *P. fimata* experiment: 3.06–5.51–9.92–17.89–32.16 nmol/g soil. All experiments included a normal control (C) using only LUFA 2.2 soil and an acetone control (AC) with LUFA 2.2 soil spiked with only acetone. Samples were kept in 100 ml jars and consisted of 30 g moist soil, ten animals, a few grains of baker's yeast and were incubated at 20°C, 70% relative humidity and a 12 h light/dark cycle. Once a week jars were aerated, moisture content was checked and fresh food added. After 28 days 100 ml of water was added to test containers, stirred gently and completely poured out into a glass beaker. For each sample several digital photographs were taken to register all living springtails that came floating to the surface. To establish survival and total number of juveniles, photos were analyzed using the life science microscopy imaging software CellD (Olympus) (Broerse and van Gestel, 2010). Dose–response curves were calculated to establish lethal concentrations (LC_x) and effect concentrations (EC_x, a specific percentage of reduction in juvenile production), using the logistic response model after Haanstra et al. (1985):

$$Y(c) = \frac{Y_{max}}{1 + \frac{x}{(100-x)}\left(\frac{c}{EC_x}\right)^b}$$

In which Y is the percentage of survival (LC_x) or the number of juveniles for (EC_x), with Y_{max} the estimated maximum number in the untreated control, x is the percentage of effect (here either 10 or 50), c is the concentration and b the slope of the dose–response curve. To investigate if the LC_x and EC_x values differed significantly between the two species a generalized likelihood ratio test was performed (Sokal and Rohlf, 1995).

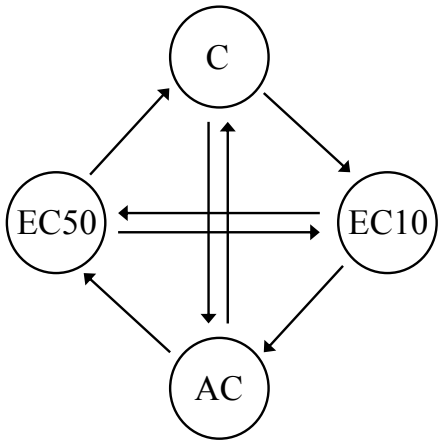

Figure 3.1: Microarray hybridisation loop design for the four exposure treatments of *Folsomia candida* after 48 h exposure to 2–phenylethyl isothiocyanate in LUFA 2.2 soil. Each treatment had four biological replicates. C control, AC Aceton Control, EC_{10} concentration corresponding to a 10% reduction of reproduction and EC_{50} concentration corresponding to a 50% reduction of reproduction. Arrows indicate which samples were hybridized together on one two–coloured microarray. Arrow direction shows if a sample was labelled with CY3 (green) or CY5 (red): start of arrow = CY3 and end of arrow = CY5.

3.3.5 Microarrays experiments

Gene expression analysis was performed for *F. candida* exposed to four treatments including two toxic treatments and the water and acetone control. The two toxic treatments consisted of sub–lethal concentrations of 2–phenylethyl ITC close to EC_{10} and EC_{50} values for effects on reproduction (6.21 and 10.7 nmol/g; being the average of the EC_{10} and EC_{50} values obtained in the present experiments and in an unpublished pilot experiment). Each treatment had four biological replicates, each consisting of 30 synchronized animals of 23–25 days old. After 2 days exposure, the flotation technique (100 ml demineralised water) was used to harvest animals from the soil. After scooping animals from the water surface with a spoon, they were placed on plaster of Paris to remove excess water. Quickly thereafter animals were placed in a micro–centrifuge tube

and snap frozen in liquid nitrogen. RNA preparation, labelling and hybridisation were performed as described by Nota et al. (2009). For hybridisation a loop design was used (Figure 3.1). Dyes were swapped at the biological replication level; for each treatment two samples were labelled with the fluorescent dye Cy3 and two with Cy5. A custom made 60–mer, 8 x 9 15k oligo microarray (Gene Expression Omnibus accession number: GPL7150) based on Agilent technologies was used for this experiment. This microarray contains 5,069 unique probes representing 5,069 different gene clusters from Collembase (www.collembase.org). All probes were spotted randomly in triplicate (Nota et al., 2009).

After feature extraction (Agilent FE software version 9.5.1.1) microarray data was further analysed using the package 'Limma' (version 2.18.3 Smyth (2004)) in the statistical environment R (version 2.9.0). In short, normexp background correction (Ritchie et al., 2007), LOESS normalization (Smyth and Speed, 2003) and aquantile normalization (Hahne et al., 2008) were performed for the whole dataset. As each microarray contained three replicates of the same probe, the consensus correlation, which is a robust average, was taken so that only one data point per microarray remained for each gene (Smyth et al., 2005). Each gene was thus represented by four data points (biological replicates/microarrays). Differential expression was then assessed by means of linear models and empirical Bayes methods. Finally the Benjamini–Hochberg's false discovery rate method (Benjamini and Hochberg, 1995) was used for multiple testing corrections (adjusted $p < 0.05$ was considered significant). MA–plots and boxplots were used for quality control of the data for each array. The expected and observed log ratios of the Agilent spike–in control probes showed a $R^2 \geq 0.95$ for all arrays. Differential expression analysis was performed for several contrasts between the four treatments resulting in a mean log_2 expression ratio (treated/untreated) and a p–value for each probe on the array. Using Blast2GO (Conesa et al., 2005), part of the differentially expressed genes were annotated to known genes, assigned to a gene ontology (GO)–term or referenced to an InterPro number, setting the hit threshold at e–value $< 1.0e^{-6}$. The raw and processed microarray data are available from the NCBI Gene Expression Omnibus (GEO) under accession number GSE29239.

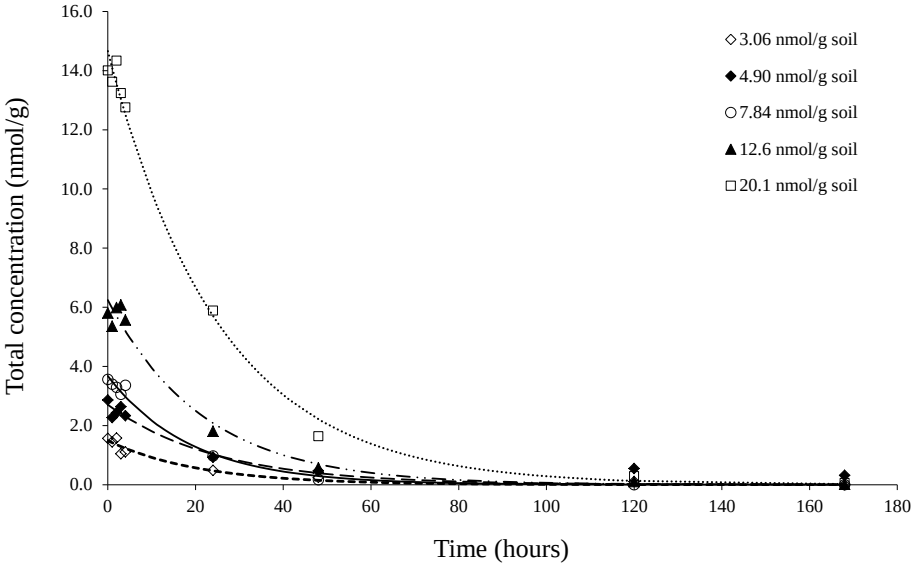

Figure 3.2: Dissipation of 2–phenylethyl ITC (nmol/g soil) as a function of time (h) in moist LUFA 2.2 soil at 20°C for five different starting concentrations. Lines show fits of first–order degradation kinetics.

3.4 Results and discussion

3.4.1 Dissipation of 2–phenylethyl ITC in natural soil

Recovery rates of 2–phenylethyl ITC varied between 52.8 and 78.6% based on the dry start concentrations. Adding water to the soil (dry vs. wet start) caused a significant decrease in the total concentration of 2–phenylethyl ITC in the soil ($p = 0.03$, Student's t–test between measured concentrations of the dry and wet start). A similar effect of water addition was shown by Gimsing et al. (2009) for benzyl ITC, i.e. an increase in water content of the soil increased the degradation rate of benzyl ITC (Gimsing et al., 2009).

2–Phenylethyl ITC could only be detected in the LUFA soil samples for the first week (Figure 3.2), indicating that this compound dissipates rapidly in natural soil. Half–lives were dependent on the starting concentrations and

were 14.4 (95% confidence interval: 6.50–22.04) h for 3.06 nmol/g, 17.2 (9.53–24.7) h for 4.90 nmol/g, 13.1 (9.67–16.6) h for 7.84 nmol/g, 15.2 (9.44–20.9) h for 12.6 nmol/g, and 17.6 (14.4–20.8) h for 20.1 nmol/g soil. For the 4.90 nmol/g data set the two last data points (120 and 168 h) were excluded in the half–live model, as these values (still shown in Figure 3.2), if included, would yield a very high and different half–life (55.9 h). Jensen et al. (2010) and Gimsing et al. (2009) showed similar dissipation patterns in nonsterile or natural soil for benzyl ITC, a compound with a chemical structure similar to 2–phenylethyl ITC. In sterile soils the dissipation of benzyl ITC was much slower, thus, indicating that microbial degradation is the chief driver responsible for the natural dissipation process of ITCs (Gimsing et al., 2009). We note that the stability of 2–phenylethyl ITC may also be influenced by the presence of test organisms and their associated food source, which was not investigated in this study.

Currently, data on ITC metabolites and their potential toxicity are unknown. Most likely 2–phenylethyl ITC is degraded to benzene, phenol and benzoic acid, which are deemed less toxic than the parent compound.
However, as long as the toxicophore (NCS), the ITC part of the molecule, remains intact in any formed metabolite, the molecule is toxic. The potential effects of ITC metabolites, therefore, remain an important issue, which requires attention in future studies.

3.4.2 Toxic effects on life–history traits

Toxic effects of 2–phenylethyl ITC on reproduction of both Collembola species are presented in Figure 3.3. On average, adult survival of *F. candida* was (± SE) 84 ± 5.1% for the control (C) and 90 ± 0.0% for acetone control (AC) group. Adult survival of *P. fimata* was on average 88 ± 2.0% for C and 90 ± 3.2% for AC. On average *F. candida* produced 275 ± 33.5 juveniles in C and 307 ± 22.9 juveniles in AC. For *P. fimata* the average number of juveniles was 162 ± 22.5 in C and 190 ± 22.6 juveniles in AC. There was no significant difference in survival or reproduction between the two controls for both *F. candida* (survival: $p = 0.273$ and reproduction: $p = 0.448$, Student's t–test) and *P. fimata* (survival: $p = 0.608$ and reproduction: $p = 0.412$, Student's t–test). Although *P. fimata* is not a common test species for ecotoxicological tests, the species met the validity criteria described by the *F. candida* ISO guideline (1999) with regard to adult survival (mortality in controls $\leq$ 20%)

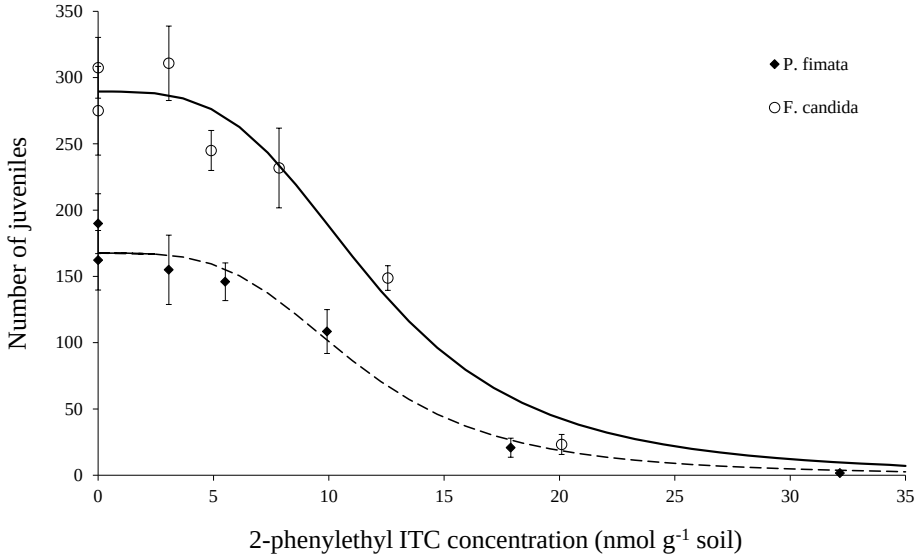

Figure 3.3: Effects of 2–phenylethyl isothiocyanate (nominal concentrations) on the reproduction of *Folsomia candida* and *Protaphorura fimata* after 28 days exposure in LUFA 2.2 soil. Lines show the fit of the logistic dose response model to the data. Error bars are standard errors (n = 5).

and reproduction (per control vessel $\geq$ 100 juveniles).

2–Phenylethyl ITC proved to be toxic for both collembolan species. At exposure concentrations around 30 nmol/ g soil reproduction was completely impaired (Figure 3.3). Estimated LC_{10}, LC_{50}, EC_{10} and EC_{50} values for both species (based on initial concentrations) are shown in Table 3.1. It should be noted that the *LC*– and *EC*–values do not reflect the rapid loss of the compound from the soil. *LC*– and *EC*–values did not significantly differ between *F. candida* and *P. fimata* (Table 3.1), suggesting that 2–phenylethyl ITC exerts comparable effects on life–history traits of a non–target species as well as an herbivorous pest species. This supports the common assumption that ITCs are not target–specific (Brown and Morra, 1997).

Furthermore, 2–phenylethyl ITC is approximately five times more toxic than benzyl ITC of which the EC_{50} is 65 nmol/g for *F. fimetaria* (Jensen

Table 3.1: *LC*– and *EC*–values for the effects of 2–phenylethyl isothiocyanate on survival and reproduction, respectively of *Folsomia candida* and *Protaphorura fimata* after 28 days exposure in LUFA 2.2 soil. Values are in nmol ITC per gram soil (nominal concentrations).

	F. candida	CI	*P. fimata*	CI	χ^2
LC_{10}	10.4	(8.67–12.1)	9.32	(6.81–11.8)	0.44
LC_{50}	15.4	(14.1–16.7)	15.2	(13.5–17.0)	0.03
EC_{10}	6.29	(3.73–8.84)	6.08	(2.92–9.24)	0.01
EC_{50}	12.0	(9.94–14.0)	11.2	(8.63–13.8)	0.18

CI = 95% confidence interval. Life–history trait differences between *F. candida* and *P. fimata* are deemed significant ($p < 0.05$) if $\chi^2 > 3.84$.

et al., 2010). Apparently, a slight difference in chemical structure (one CH_2 less) changes the toxicity of ITCs. Many studies show that 2–phenylethyl ITC is among the most toxic ITCs causing adverse effects on a wide range of soil organisms (van Dam et al., 2009). In comparison to anthropogenic toxicants this natural toxin is much more harmful. Fountain and Hopkin (2005) compiled a list of several metals and organic chemicals which were tested with *F. candida* using the ISO guideline (1999). Half of the listed organic chemicals have higher EC_{50} values than 2–phenylethyl ITC (1.96 mg/kg = 12.0 nmol/g) indicating lower toxicity (Fountain and Hopkin, 2005).

The chemical structure of the compound gives insight as to why soil organisms can still come into contact with the toxin; the hydrophobic phenyl group binds firmly to the organic matter of the soil, while the ethyl tail (isothiocyanate), positioned outside soil particles, remains accessible to soil dwelling organisms (Potter et al., 1998). Furthermore, if bioavailable, lipophilic compounds easily bind to lipids, such as the hydrophobic tail region in phospholipid bilayer of cell membranes, which increases the permeability of membranes and contributes in turn to higher contact toxicity (van Dam et al., 2009).

Apparently, even though 2–phenylethyl ITC dissipates rapidly in natural soil and is only present in the soil for a few days, it is able to exert toxic effects on soil arthropods after 28 days. Adult death could be an explanation for reduction in reproduction. In that case the LC_x and corresponding EC_x should be overlapping. However, confidence intervals only slightly overlapped

Table 3.2: Total numbers of significantly differentially expressed genes in *Folsomia candida* exposed for 48 h to 2–phenylethyl ITC at concentrations similar to the 28–day EC_{10} and EC_{50} for effects on reproduction compared to the acetone control

	EC_{10}	EC_{50}
Up	65	64
	4994	*4962*
Down	10	43

Non–differentially expressed genes are given in italics.

(Table 3.1). Reproduction thus also seems impaired in surviving adults on a physiological level, for which gene expression analysis can give more insight.

3.4.3 Gene expression

Differential expression in *F. candida* was analysed among 5,069 unique genes between control (C), acetone control (AC), versus EC_{10} and EC_{50}. At both life–history levels, survival and reproduction, the two control groups C and AC did not significantly differ, even though animals in AC seemed to perform better. At the gene expression level, however, a total of 130 genes were significantly differentially expressed between C and AC (for a complete list we refer to Appendix A of the Supporting Information, Table A1). As 2–phenylethyl ITC was spiked into the soil with acetone as solvent, AC was considered the most appropriate control and thus used for further statistical analysis to identify significant transcriptional responses to EC_{10} and EC_{50} treatments.

The complete list of significantly expressed genes for the comparisons EC_{10} versus AC and EC_{50} versus AC can be found in the Supporting Information (Appendix A, Table A2). In the EC_{10} versus AC comparison, 75 genes were differentially expressed, of which 65 were up–regulated and 10 down–regulated (Table 3.2). In the EC_{50} versus AC comparison, 107 genes were differentially expressed, of which 64 were up–regulated and 43 down–regulated. Moreover, 46 genes showed similar differential expression at the EC_{10} and EC_{50} level (Figure 3.4), so that 29 genes were differentially regulated only at the EC_{10} and 61 were unique to the EC_{50}. Approximately 30% of all significant genes

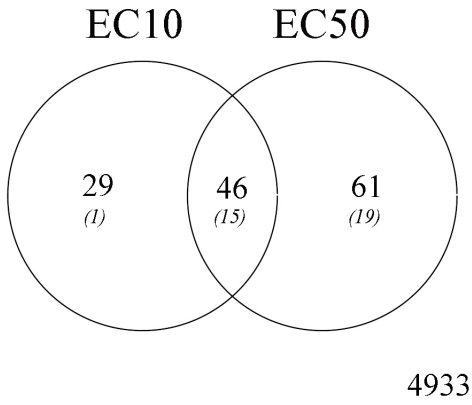

Figure 3.4: Venn–diagram showing overlap in the total number of significantly differentially expressed genes in *Folsomia candida* after 48 h exposure to 2–phenylethyl isothiocyanate in LUFA 2.2 soil at concentrations corresponding to the EC_{10} and EC_{50} for effects on reproduction. Number of annotated genes are represented in brackets.

were annotated using Blast2GO (Conesa et al., 2005). Thirty of the annotated and not fully annotated differentially expressed genes were placed in the context of biological processes (Table 3.3) and are discussed in the following sections.

Transcriptional response at EC_{10} level

At the EC_{10} level, 2–phenylethyl ITC mainly induced metabolic processes (Table 3.3), especially lipid metabolism, which was indicated by the up–regulation of, for instance, acetyl–coenzyme A carboxylase alpha (Fcc00050), fatty acid delta–6 desaturase (Fcc01007), fatty acid synthase (Fcc02959) and genes encoding for the lipid transport proteins SEC14–like 1 and 2 (Fcc03602 and Fcc06054) (Saito et al., 2007). On the other hand, genes involved in lipid synthesis (diacylglycerol *o*–acyltransferase: Fcc03980) and the binding of small lipophilic molecules (cral–trio domain: Fcc04835), such as 2–phenylethyl ITC, were down–regulated. The cral–trio domain (sec–14 domain) also

encompasses SEC 14–like proteins; this domain was thus represented in both the up–regulated and down–regulated genes. It is, however, likely that SEC14–only proteins act as lipid transporters, while the multidomain SEC14–containing proteins (indicated by cral–trio) have more complex functions, integrating lipid metabolism with other biochemical processes (Saito et al., 2007).

Alkaline ceramidase (Fcc03847) was also up–regulated, which codes for an enzyme that is part of a complex bioactive lipid system that mediates cell proliferation, differentiation, apoptosis, adhesion, and migration. It hydrolyses ceramide to sphingosine, a mediator of cellgrowth arrest and apoptosis (Mao and Obeid, 2008).

Moreover, Fcc00558 was up–regulated. Although not fully annotated by Blast2GO, this sequence had high similarity with a gene encoding for chitin binding peritrophin, which functions in the formation of the extracellular envelope from chitin (peritrophic matrix). This envelope protects the mid–gut of arthropods from physical damage by food particles and from attacks of plant toxins such as 2–phenylethyl ITC (Merzendorfer and Zimoch, 2003). Activity of such a process was supported by the up–regulation of genes involved with chitinase (endochitinase: Fcc02522 and acidic mammalian chitinase–like: Fcc00881), an enzyme involved in the moulting process. Taken together, gene expression at the EC_{10} level revolved around fatty acid metabolism and moulting processes, which can be associated with the lipophilic nature of 2–phenylethyl ITC.

Table 3.3: Functional grouping of up–regulated or down–regulated genes in *Folsomia candida* after 48 hours exposure to 2–phenylethyl ITC in LUFA 2.2 at concentrations corresponding to the EC_{10} (6.21 nmol/g) and/or EC_{50} (10.7 nmol/g) for effects on reproduction compared to the acetone control.

Process	Gene name	Blast2GO description	E–value	E–code	InterPro	EC_{10} logFC	EC_{50} logFC
Fatty acid metabolism							
GO:0006633	Fcc00050[a]	acetyl–coenzyme a carboxylase alpha	3.32E–82	EC:6.4.1.2		**0.961**	**1.086**
GO:0006629	Fcc01007[a]	delta–5 fatty acid desaturase	7.10E–34		IPR005804	**0.989**	
GO:0006633	Fcc02959[a]	fatty acid synthase	4.94E–23	EC:1.3.1.10	IPR000794	**1.082**	**1.266**
GO:0080090	Fcc00142	sterol regulatory element–binding protein 1	1.43E–15				**0.458**
GO:0045540	Fcc06054[a]	sec14–like 2 (cerevisiae)	9.79E–09		IPR001251	**0.693**	**0.594**
	Fcc03602	sec14–like 1 (cerevisiae)	1.85E–15		IPR001251	**0.681**	
	Fcc04835	sec14–like protein 4 (cral trio domain–containing protein)	1.44E–17		IPR001251	*–1.184*	
	Fcc03980	diacylglycerol o–acyltransferase	2.44E–09			*–1.554*	
	Fcc03847	alkaline ceramidase	2.66E–16			**0.643**	
Oxidative stress							
GO:0005515	Fcc00494[a]	glutathione s–transferase	7.64E–39		IPR004045	**0.703**	**0.830**
GO:0005515	Fcc05973[a]	glutathione s–transferase	1.48E–42		IPR004045		**1.123**
GO:0055114	Fcc02155[a]	cytochrome p450	2.84E–32		IPR001128		**1.002**
GO:0005215	Fcc04663[a]	alpha–tocopherol transfer	4.36E–42		IPR001071		*–0.746*
GO:0006979	Fcc00022[a]	rec8	1.54E–20		IPR002007		*–1.150*

Process	Gene name	Blast2GO description	E–value	E–code	InterPro	EC_{10} logFC	EC_{50} logFC
Immune response							
GO:0009058	Fcc00057[a]	isopenicillin n synthetase	2.16E–29				**0.856**
GO:0006950	Fcc03623[a]	advillin	2.71E–47		IPR003128	**0.604**	**0.596**
Sugar metabolism							
GO:0003824	Fcc05611[a]	trehalase	2.51E–46		IPR001661		**0.868**
GO:0050660	Fcc04789[a]	glucose dehydrogenase	1.54E–15		IPR007867		**0.827**
GO:0006565	Fcc02499[a]	cystathionine beta–synthase	5.25E–61	EC:4.2.1.22			**0.788**
GO:0009744	Fcc00529[a]	maltase– intestinal–like	8.26E–30	EC:3.2.1.26	IPR000322	**1.188**	**1.309**
GO:0015986	Fcc03280[a]	vacuolar atpase subunit d	5.11E–71	EC:3.6.3.6	IPR002699		*–0.582*
Growth and development							
GO:0005214	Fcc03188[a]	adult cuticle	3.21E–17		IPR000618		*–0.801*
GO:0005576	Fcc04617[b]	follistatin precursor	6.66E–29		IPR001239		*–1.351*
	Fcc00558	chitin binding peritrophin–	4.14E–07			**0.595**	
GO:0006032	Fcc02522[a]	endochitinase	1.33E–29		IPR000726	**1.110**	**1.080**
GO:0006032	Fcc00881[a]	acidic mammalian chitinase–like	1.40E–51	EC:3.2.1.0	IPR001223	**1.124**	**0.889**
GO:0008152	Fcc03673[a]	juvenile hormone acid methyltransferase	2.34E–23	EC:2.1.1.0	IPR013216	**0.794**	**1.034**
Transcription							
GO:0043967	Fcc03584[a]	dna methyltransferase 1 associated protein 1	9.69E–71				*–0.518*
GO:0003899	Fcc04065[a]	dna–directed rna polymerase ii subunit rpb4	1.61E–51	EC:2.7.7.6	IPR005574		*–0.716*

Process	Gene name	Blast2GO description	E–value	E–code	InterPro	EC_{10} logFC	EC_{50} logFC

Bold indicates up–regulation of genes while italics indicate down–regulation of genes. Empty cells indicate the gene was not differentially expressed compared to the acetone control.
Process: Biological process category and related GO–code of gene (if available)
Gene name: Collembase accession number
Blast description: Best Blast2GO hit
E–value: Blast2GO E–value
E–code: Enzyme commission number
InterPro: First code of hits found in InterPro database
logFC: The log2 transformed ratio (treated/untreated)
[a] Sequence was fully annotaded by Blast2GO
[b] Second best annotated Blast2GO description for this sequence

Transcriptional response at EC_{50} level

The effects of 2–phenylethyl ITC on gene expression at the EC_{50} level were more diverse than the EC_{10} response (Table 3.2). Up–regulation of a single cytochrome P450 gene (CYP450: Fcc02155) and two glutathione s–transferases (GST: Fcc00494 and Fcc05973) indicated detoxification by phase I and II biotransformation of 2–phenylethyl ITC to be processed for elimination through the aqueous phase. It is well known that genes coding for CYP450 and GST contribute to the biotransformation of xenobiotics and are oxidative stress responsive as described by their associated GO–term (Hayes and Pulford, 1995). Interestingly, this process is also activated in humans exposed to 2–phenylethyl ITC and is supposed to be associated with chemoprevention (Cheung and Kong, 2010), suggesting that 2–phenylethyl ITC is a xenobiotic compound. Genes associated with vitamin E transport (alpha–tocopherol transfer: Fcc004663), known for its anti–oxidant properties (Dutta-Roy, 1999), and oxidation reduction (rec8: Fcc00022) were, however, down–regulated. In addition, isopenicillin N synthase (Fcc00057) and advillin (Fcc03623) were up–regulated. These genes are involved in microbial defence and immune response, suggesting a more general stress response.

Furthermore, 2–phenylethyl ITC affects transcription of genes involved in sugar metabolism in *F. candida*. This is illustrated by the up–regulation of, for instance, trehalase (Fcc05611), glucose dehydrogenase (Fcc04789), cystathionine beta–synthase (Fcc02499) and maltase–intestinal–like (Fcc00529). These transcriptional responses probably serve to increase energy production, which was supported by the down–regulation of vacuolar ATPase subunit d (Fcc03280), an enzyme that converts ATP into ADP. Up–regulation of trehalase was also found as a response of *F. candida* to desiccation stress (Timmermans et al., 2009). Changes in sugar metabolism indicate that organisms invest in many energyconsuming processes, in order to survive stressful conditions.

In addition, a gene encoding the formation of adult cuticle (Fcc03188), important for development and growth, was exclusively down–regulated at the EC_{50} level. For sequence Fcc04617, the second best Blast2GO hit with a similarity of 79%, coded for a precursor of follistatin. Genes involved with follistatin have been shown to modulate activity of members of the TGF–β super family (bone morphogenic proteins 2 and 4) in vertebrates, which in turn are important for e.g. axis formation, development of the nerve system and embryonic development. In *Drosophila*, follistatin is expressed throughout development

where it is, for instance, important for morphogenesis in pupae (Bickel et al., 2008; Pentek et al., 2009). Perturbation of this gene may, therefore, result in failure of early embryonic development and as such overall reduction in reproduction. However, care should be taken to directly extrapolate gene expression to life history effects due to the differences in exposure conditions between ecotoxicological and microarray experiments.

Finally, other gene expression patterns involved the down–regulation of DNA methyltransferase (Fcc03584), mediating DNA methylation, and DNA directed RNA polymerase ii subunit rpb4 (Fcc04065), involved in RNA synthesis. This suggests that specific parts of DNA, e.g. stress response genes, were more accessible while overall mRNA production was decreased.

Overlap in genes between EC_{10} and EC_{50}

A large overlap was found for gene expression at the EC_{10} and EC_{50} treatments (Table 3.3), which also showed similar direction of regulation (up or down) for both treatments. Three particular genes, up–regulated in both treatments, are worth mentioning. The first two are genes encoding for endochitinase (Fcc02522) and acidic mammalian chitinase (Fcc00881). Chitinase is an enzyme which is a key constituent of the moulting process of the cuticle and the peritrophic matrix of arthropods (Merzendorfer and Zimoch, 2003). Apparently, the moulting process was stimulated as a reaction to the toxin. We speculate that this points towards the lipophilic nature of 2–phenylethyl ITC, resulting in stimulated excretion of epithelium to which the compound is bound. Second, a gene coding for juvenile hormone acid methyltransferase (Fcc03673) was up–regulated in both treatments. Juvenile hormone acid methyltransferase is an enzyme involved in the final step of juvenile hormone synthesis. This group of acyclic sesquiterpenoids regulate many physiological processes such as development, growth, reproduction and diapause (Shinoda and Itoyama, 2003). Up–regulation of this gene indicates that these crucial life–cycle processes of *F. candida* are affected by 2–phenylethyl ITC.

3.5 Conclusion

2–Phenylethyl ITC had a noticeable effect on survival and reproduction of both springtail species and the gene expression of *F. candida*. These gene profiles represent hypotheses for explaining the underlying modes of action

and acute effects of the toxin on survival and reproduction. For instance, due to the lipophilic nature of the ITC, we observed genes involved in fatty acid metabolism to be severely affected and overall gene activity to be altered. This in turn activated genes encoding biotransformation enzymes, to counteract toxic activity and facilitate excretion of cyclic compounds such as ITC. The regulation of a gene encoding follistatin, furthermore, implied the inhibition of reproduction and may be an important molecular target that can be linked to the observed adverse effect of life history traits. This study clearly shows the detrimental effect of this natural toxin on beneficial non–target and herbivorous springtails. Loss of especially beneficial, functionally important soil invertebrates can result in serious negative repercussions on soil ecosystem functioning (Berg et al., 2001). Natural toxins are present in many agricultural systems and probably at increased levels in the near future. Current interests in ITCs focus on the toxic characteristics that have, for instance, been exploited for alternative pest management methods, so–called biofumigation (Matthiessen and Kirkegaard, 2006). Several field and laboratory studies have shown that ITC concentrations can rise up to 100 nmol/g soil after using effective biofumigation strategies (Gimsing and Kirkegaard, 2009). Other interests concern the possible chemopreventive nature of ITCs involved with cancer (Traka and Mithen, 2009). Such social–economic uses of ITC call for novel commercial health crop varieties with enhanced GSL levels (Traka and Mithen, 2009). By definition, as indicated by their secondary metabolite nature, they exert toxicological effects (Hoerger et al., 2009). Potential risks to the environment of these natural toxins, readily available in every–day crops, should therefore be carefully assessed. This study is the first step towards an understanding of these potential risks by studying the effects of a natural toxin at a life–history trait and molecular level.

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Electronic supplementary material

The online version of this article (doi:10.1007/s10646–012–0861–z) contains supplementary material, which is available to authorized users.

CHAPTER 4

Effects of a natural toxin on life–history and gene expression of *Eisenia andrei*

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Submitted

4.1 Abstract

Earthworms perform key functions for a healthy soil ecosystem, such as bioturbation. The soil ecosystem can be challenged by natural toxins such as isothiocyanates (ITC), produced by many commercial crops. Therefore, the effects of 2–phenylethyl ITC were investigated on the earthworm *Eisenia andrei* using an ecotoxicogenomics approach. Exposure to 2–phenylethyl ITC reduced both survival and reproduction of *E. andrei* in a dose–dependent manner (EC_{50} = 556 nmol/g). Cross–species comparative genomic hybridisation

validated the applicability of an existing 4x44k *E. fetida* microarray to *E. andrei*. Gene expression profiles revealed the importance of metallothionein (*mt*) as an early warning signal when *E. andrei* was exposed to low concentrations of 2–phenylethyl ITC. Alignment of these *mt* genes with the *mt*–2 gene of *Lumbricus rubellus* showed that at least two *mt* gene clusters are present in the *Eisenia sp.* genome. At high exposure concentration gene expression was mainly affected by inhibiting chitinase activity, inducing an oxidative stress response, and stimulating energy metabolism. KEGG pathway analysis, furthermore, implied that the high concentration may have caused impaired light sensitivity, angiogenesis, olfactory perception, learning and memory. Increased levels of ITC may be found in the field in the near future. The presented results call for a careful investigation to quantify the risk of such compounds before allowing them to enter the soil on a large scale.

4.2 Introduction

A healthy soil is essential for fertile, sustainable agricultural practices. Key to that is a soil ecosystem comprising a diversity of organisms with different functions (Wardle et al., 2004). An important function is bioturbation, which increases moisture and aridity of the soil and provides favourable conditions for microbes (Wardle et al., 2004). Bioturbation is mainly carried out by ecosystem engineers such as earthworms (Kula and Larink, 1998; Satchell, 1983). Earthworms are therefore essential for a healthy soil ecosystem.

Anthropogenic introduction of pesticides, heavy metals, and other compounds, are well–known threats to soil ecosystem functioning and diversity (Edwards and Bohlen, 1992), but only recently have toxicity studies focused on the effects of natural toxins. Natural toxins are organic compounds produced as secondary metabolites, for instance by fungi or by plants, including many commercially important crops. For example, phytotoxins known as isothiocyanates (ITC) are hydrolysis products of glucosinolates (GSL) synthesized in almost all plants of the order *Brassicales* including broccoli, kale, and Brussels sprouts produce (Halkier and Gershenzon, 2006). When the plant tissue is damaged, GSL is hydrolyzed by the enzyme myrosinase into various compounds (Wittstock and Halkier, 2002; Bednarek and Osbourn, 2009), of which ITCs are the most common and toxic (Halkier and Gershenzon, 2006; Brown and Morra, 1997).

Isothiocyanates are used by plants as a defense against herbivores, but also act on species that do not directly interact with these plants, i.e. non–target species. It has recently been shown that beneficial soil arthropods exposed to ITC experience severely reduced reproduction (Jensen et al., 2010; van Ommen Kloeke et al., 2012b). Negative effects on beneficial soil organisms can critically affect soil quality especially when essential ecosystem functions, such as bioturbation, are compromised.

To investigate such effects of natural toxins, a combination of standardised ecotoxicological experiments and gene expression analyses (ecotoxicogenomics) were performed with the earthworm *Eisenia andrei* exposed to 2–phenylethyl ITC as a model system. *Eisenia andrei* is one of the standard test species used in soil ecotoxicology to assess survival, growth and reproduction (OECD, 2004). Gene expression analyses, using microarrays, may give insight in the mechanisms underlying toxic exposure, as was shown for *E. fetida*, a species closely related to *E. andrei* (Gong et al., 2010). In this study, the effect of the natural toxin 2–phenylethyl isothiocyanate on the ecotoxicological endpoints survival and reproduction of *E. andrei* was assessed according to the international guideline. Simultaneously, we studied transcriptional responses at sub–lethal exposure levels to investigate mechanistic consequences of this compound. To the best of our knowledge, this is the first study to investigate the effects of an isothiocyanate on an earthworm at both the ecotoxicological and the genomic levels.

4.3 Materials and methods

4.3.1 Animals

Eisenia andrei (*Lumbricidae*) originated from synchronized laboratory stock cultures, was cultivated at the VU University in Amsterdam. Maintained in large plastic containers filled with a mixture of horse dung and pot soil, stocks were given a fresh batch of horse dung (without antibiotics) every week. The containers were kept under constant conditions in a climate chamber with a temperature of 20°C, a 12:12 hours light: dark cycle and 75% relative humidity. Test organisms had a maximum age difference of one month, and they were all sexually mature and reproductively active, i.e. each worm bearing a visible clitellum. Prior to the experiment, test organisms were isolated and acclimatized to the control LUFA 2.2 soil (Speyer, Germany) for 24 hours.

4.3.2 Experimental soil, test chemical and spiking

LUFA 2.2 soil had a pH–value of 5.5 ± 0.1 (± Std. Deviation, SD) and an organic C content of 2.09 ± 0.40%. Prior to usage, the soil was dried at 60°C for 24 hours. 2–Phenylethyl ITC [CAS: 2257–09–2] was purchased from Sigma Aldrich (www.sigmaaldrich.com) as a liquid solution (≥ 99% pure). Due to its low water solubility, the compound was spiked into the soil using acetone. Spiking was executed with only ten percent of the total amount of soil needed for each treatment, in a 1:1 ratio (i.e. adding 1 ml of acetone to 1 g dry weight soil) (Brinch et al., 2002). The appropriate concentration of 2–phenylethyl ITC for each treatment (calculated for the total amount of soil) was added to the acetone. The amended soil was shaken thoroughly and incubated for 24 hours in preservation jars. To facilitate evaporation of the acetone, jars were left open for another 36 hours under a fume hood. Subsequently, the remaining 90% of the total soil was added in batches, manually mixed by hand and kitchen blender, and moistened to 22% water content (equivalent to 50% of the soil's water holding capacity).

4.3.3 Ecotoxicological experiments

Exposures were performed in accordance with the standardized OECD Guideline 222 (OECD, 2004). The nominal concentrations of 2–phenylethyl ITC were 0 (acetone only solvent control or AC), 242, 363, 545, 817, 1225 and 1838 nmol/g dry weight of soil. Every treatment comprised 5 replicates, with each replicate consisting of 500 g of moist soil held in a 600 ml glass jar under the same ambient environmental conditions as described above. At the start of the experiment 11 worms per replicate were rinsed with water, dried by blotting, weighed per group and added to the soil. For the first 24 hours after exposure a piece of gauze and a rubber band was used to close off the test jar, preventing worms from escaping and stimulating submerging of worms into the soil. Afterwards, jars were capped with loosely closing lids to facilitate air exchange. Four days after exposure, one worm was removed from each replicate, snap frozen in liquid nitrogen and stored at –80°C for gene expression analyses. The remaining adult worms and soil were placed back in to their respective original jars. Soil moisture content and food (horse dung) availability were checked once a week and replenished as needed. After 28 days all surviving adult worms were removed by hand, and mortality and biomass were determined. The remaining

soil with juveniles and cocoons was returned to the test jars and incubated for another 28 days, after which reproduction was measured. Reproduction was assessed manually by counting the number of juveniles that migrated to the soil surface after placing the jars in a 60°C water bath.

4.3.4 Dose response curves

The log–logistic response model after Haanstra et al. (1985) was used to calculate the 50% and 10% lethal concentrations (LC_{50} and LC_{10}, respectively) and effective concentrations (EC_{50} and EC_{10}, respectively):

$$Y(c) = \frac{Y_{max}}{1 + \frac{x}{(100-x)}(\frac{c}{EC_x})^b}$$

Where Y is the percentage of survival (LC_x) or the number of juveniles for (EC_x),as a function of the concentration c; Y_{max} is the estimated maximum number in the untreated control; x is the selected percentage of effect (here either 10 or 50) and b the slope parameter of the dose–response curve. Least squares regression implemented in SPSS v.15.0 was employed to fit our experimental data to the model.

4.3.5 Microarray validation and annotation

Gong et al. (2010) developed a custom–designed Agilent microarray (Gene Expression Omnibus accession number: GPL16366, Agilent amadid no.: 022725) for *Eisenia fetida*, a species closely related to *E. andrei*. This microarray contains 43,803 60–mer probes representing unique transcripts of *E. fetida* spotted on the 4 x 44k Agilent platform. In order to validate the applicability of this array platform to *E. andrei*, a two–colour, comparative genomic hybridisation (CGH) experiment (see Figure S1) was carried out using four arrays and three genomic DNA samples from both species (one pooled sample from *E. fetida* and two samples from individual *E. andrei*). Prior to genomic DNA extraction, individual worms were ground with mortar and pestle in liquid nitrogen. No more than 25 mg of ground tissue was used for gDNA extraction, using the Qiagen QIAamp DNA Mini Kit with the tissue protocol. After quality control on a Nanodrop (OD260:OD280 and OD260:OD230), gDNA was labelled with both Cy3 and Cy5 dye using the CGH Labeling Kit for Oligo Arrays (Enzo Life Sciences) according to manufacturer's instructions. Hybridisation

and scanning were performed according to Agilent's protocol for array CGH. A dye–swap was carried out as a quality control step. Both cross–species hybridisation and within species hybridisation (two different individuals from the same stock) were performed. After hybridisation, scanning and Agilent Feature Extraction, data was analysed in R 2.15.1 using software packages CGH Base (version 1.14), SyMisc (version 0.9–65) and CGH–call (version 2.16). Data was normalized by 'median' normalization and differential hybridization of the genomic DNA was investigated by making contrasts between the two species and the two individuals of *E. andrei*. MA plots of the CGH results were generated in R.

In order to retrieve most information on gene function, the transcripts represented by the microarray probes were re–annotated using Blast2GO (Conesa et al., 2005) (Blast2GO.com). Default settings were used with a BlastX Expect value of $1.0e^{-3}$ for Blast results and an E–value Hit filter of $1.0e^{-6}$ for annotation.

4.3.6 Gene expression analysis

Global gene expression was analysed for *E. andrei* exposed to 363 nmol and 545 nmol 2–phenylethyl ITC per gram soil, in addition to the solvent (acetone) control, which served as reference. These two concentrations were chosen as they closely resembled the derived EC_{10} and EC_{50} values (see Table 4.1). Four individual worms per treatment (each worm representing one biological replicate) were ground in liquid nitrogen with mortar and pestle. RNA was isolated from approximately 25 mg of ground tissue using the SV Total RNA Isolation System (Promega, Madison, WI, USA) with minor adaptations to compensate for excessive amounts of tissue: 500 μl instead of 350 μl Dilution buffer and 285 μl instead of 200 μl ethanol were applied to circumvent clogging of the nucleic acid binding matrix. Furthermore, 800 μl instead of 600 μl RNA Wash Solution (RWA) was applied to increase release of cellular debris from the matrix during the washing steps. Only one RNA isolate was prepared per worm and considered as one biological replicate, resulting in four biological replicates per treatment. RNA quality and quantity were determined using Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and Nanodrop (Thermo Fischer Scientific, Wilmington, DE, USA), respectively. Subsequently, 150 ng of total RNA was amplified by reverse transcription followed by cRNA synthesis during which either CY3 or CY5 dye was incorporated, using the Agilent Low–Input

Quick Amp Labeling Kit according to the manufacturer's instructions. RNeasy columns (Qiagen, Valencia, CA, USA) were used to purify the cRNA. Using Agilent Gene Expression Hybridization Kits, hybridisation was conducted at 65°C for 17 h with array hybridization chambers rotating at 4 rpm in an incubator. The biological replicates were dye–swapped for each treatment. Two samples with contrasting dyes were hybridised per array according to a loop design (Figure 4.1). Two technical replicates (a and b) per biological replicate were hybrizided, resulting in 12 microarray hybridisations for 12 samples. After washing with the Agilent Gene Expression Wash, arrays were scanned in an Agilent DNA microarray scanner G2505B and signal intensity data was obtained using Agilent Feature Extraction software (version 10.5.1.1 and 10.7.3.1). The acquired data passed quality assurance and quality control, as the expected and observed log ratios of the Agilent spike–in control probes on the array showed a $R^2 \geq 0.98$ for all arrays.

The two–colour microarray dataset was analysed using the software package Limma (version 2.18.3) (Smyth, 2004) in the statistical environment R (version 2.15.0). Prior to statistical analysis, LOESS normalization (Smyth and Speed, 2003), normexp background correction (Ritchie et al., 2007) and aquantile normalization (Hahne et al., 2008) were performed to correct for the technical biases caused by differential dye intensities within and between arrays. Significance of differential gene expression was assessed using a modified t–test based on an empirical Bayes method implemented in Limma. A multiple testing correction method developed by Benjamini and Hochberg (1995) was applied to control the false discovery rate, resulting in an adjusted p–value of < 0.01 as the threshold value for differentially expressed genes. The raw and processed microarray data are available from the NCBI Gene Expression Omnibus (GEO) under accession number GSE43600.

A gene function enrichment analysis was performed on the significantly regulated transcripts using Blast2GO. Briefly, a 2–tailed Fisher's Exact Test was conducted in Blast2GO to determine if any particular groups of Gene Ontology (GO)–terms were over– or under–represented in any treatment as compared to the complete set of GO–terms associated with the array (False Discovery Rate corrected p–value < 0.05). Pathway analysis was performed to map significantly altered genes to KEGG pathways using RefNetBuilder with default settings (Li et al., 2011), and to identify the pathways enriched with the highest percentage of differentially expressed genes.

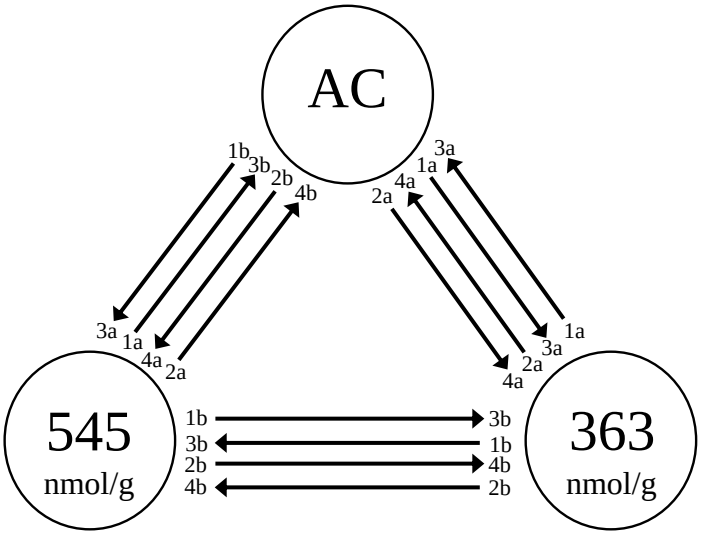

Figure 4.1: Microarray hybridisation loop design for three treatments of *Eisenia andrei* after 96 hours of exposure to 2–phenylethyl isothiocyanate in LUFA 2.2. soil. Each treatment had four biological replicates (1–4) and two technical replicates (a–b). Arrows indicate which pair of samples were hybridized together on one array, with one sample at arrowhead labelled with CY5 and the other at arrow end labelled with CY3.

4.3.7 DNA sequence alignment and phylogenetic analyses

The 15 contigs that were annotated as metallothioneins (*mt*) (Table S1, S2d) were aligned to two isoforms of *mt* sequence 2 (*wMT*–2) derived from *Lumbricus rubellus* using the molecular evolution and genetic analysis package MEGA version 5.0 (Tamura et al., 2011). Sequences were aligned using the ClustalW tool with Gap opening penalty set at 15 and gap extention penalty set at 6.6. The alignment was used for phylogenetic analysis performed with the Maximum likelihood method using a gamma substitution model. Confidence values for branching clades were generated by 1000 bootstrap replications of the ML calculation.

Table 4.1: The 28 day–LC and 56 day–EC derived for the effects of 2–phenylethyl ITC on survival and reproduction of *Eisenia andrei* in LUFA 2.2 soil.

	estimate	std. error	CI
LC_{10}	358	46.9	(262–453)
LC_{50}	533	27.5	(477–589)
EC_{10}	386	40.8	(303–469)
EC_{50}	556	22.8	(510–603)

Values in nmol ITC per gram soil, nominal concentrations. LC = lethal concentration; EC = effective concentration; CI = 95% confidence interval.

4.4 Results and discussion

4.4.1 Toxic effects on life–history traits

Exposure to 2–phenylethyl ITC reduced both survival and reproduction of the earthworm *Eisenia andrei* in a dose–dependent manner (Figure 4.2). Survival of adult worms in the acetone controls (AC) was 100% with an average of 90.2 juveniles (± 5.10 standard error). At concentrations around 800 nmol/g soil on average less than one adult (0.8 average, 0.37 ± standard error) survived, and some juveniles were observed even when only one adult survived. A summary of the lethal (LC_x) and effective (EC_x) concentrations can be found in Table 4.1. It should be noted that the LC– and EC–values, do not reflect the rapid dissipation of the compound from the soil (half lives ranging from 32 to 96 hours), as was shown in an earlier study (van Ommen Kloeke et al., 2012a). Effects on survival and reproduction showed partially overlapping confidence intervals (Table1). This indicates that the impairment of reproduction may be partially explained by adult death. However, reproduction may also be impaired in surviving adults on a physiological level. Gene expression analysis can give more insight into the underlying mechanisms (see section 4.4.4).

2–Phenylethyl ITC is one of the most toxic ITCs causing adverse effects on a wide range of soil organisms (van Dam et al., 2009). The current data clearly show that *E. andrei* is much less sensitive to 2–phenylethyl ITC than other soil invertebrates. We previously showed that 2–phenylethyl ITC reduced reproduction by 50% at exposure concentrations around 12 nmol/g soil

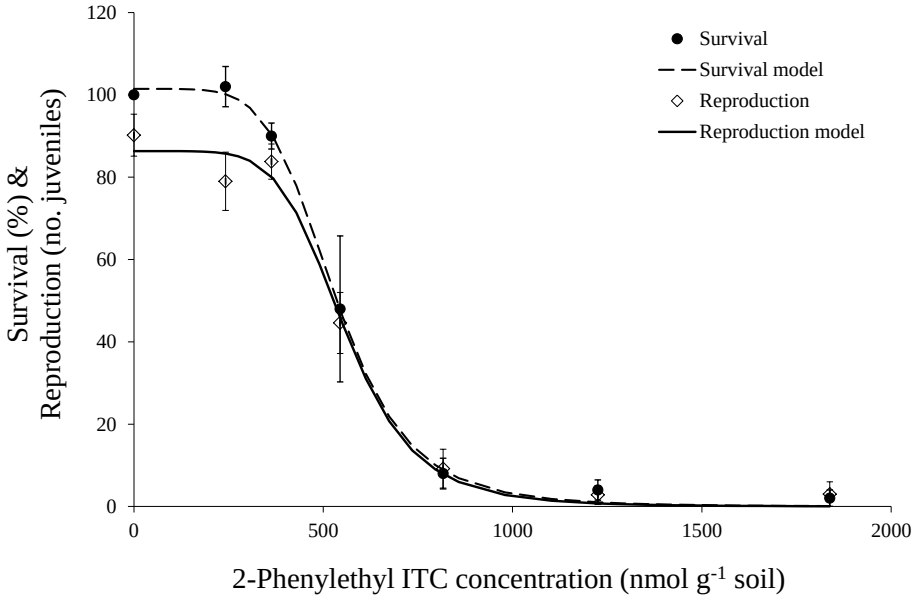

Figure 4.2: Dose–response curve of *Eisenia andrei* survival and reproduction after 28 and 56 days of exposure, respectively, to different concentrations of 2–phenylethyl ITC in LUFA 2.2 soil. Nominal concentration range = 242–1838 nmol/g soil. Error bars are standard errors (n = 5).

(ca. 2 mg/kg) and caused 50% lethality at 15 nmol/g soil (ca. 2.5 mg/kg) for two collembolan species, *Folsomia candida* and *Protaphorura fimata* (van Ommen Kloeke et al., 2012b). Other studies also show lower sensitivity of *E. andrei* compared to *F. candida*. For instance, a study with the insecticide carbofuran, widely used in agricultural practises against e.g. aphids, found that collembolans were the most sensitive species while *E. andrei* had an LC_{50} at least 8 times higher than did *F. candida* (Chelinho et al., 2012). The higher tolerance of earthworms to natural toxins might be attributed to their evolutionary adaptation as they frequently burrow through the top organic layer of soil enriched with both humus (food) and natural toxins–containing leaf litter (Morgan et al., 2007).

4.4.2 Comparative genomic hybridization using *E. fetida* and *E. andrei* gDNA

A comparative genomic hybridisation (CGH) experiment was conducted to validate cross–species hybridisation of *E. andrei* with the existing *E. fetida* microarray (Gong et al., 2010). A pool of *E. andrei* gDNA was labelled with Cy5 or Cy3 and hybridised against *E. fetida* labelled gDNA to the 43,803 unique probes (two–colour hybridization, see Supplementary information Figure S1). Probes were considered species–specific if log_2 (signal intensity ratio Cy5/Cy3) exceeded 1. Only 126 probes (or 0.3% of the total array probes) showed a log_2 ratio between 1 and 3.8 in the comparison *E. fetida* versus *E. andrei*. On the other hand, variation in gDNA between two *E. andrei* individuals was also observed, where 87 probes had a log_2 ratio between 1 and 2.5. These 213 probes were flagged and excluded for further statistical data analysis in the gene expression experiment. These results clearly demonstrate that more than 99.5% of the 44K *E. fetida* probes showed no preferential hybridization to either of the two species. Therefore, the CGH experiment validated the applicability of the *E. fetida* microarray to *E. andrei* gene expression studies. Array CGH has been used frequently for ecological relevant species, i.e. making use of a microarray developed for a model species to investigate another more ecological relevant species. For instance, an *Arabidopsis thaliana* microarray was used for gene expression analyses of a metal hyperaccumulator *Thlaspi caerulescens*, with only 220 genes of the 13,500 not feasible for usage ($>$ 3–fold change) (van de Mortel et al., 2006).

4.4.3 *Eisenia* array re–annotation

The *Eisenia* microarray contains 43,803 non–redundant probes, each targeting a unique transcript, selected from a total of 63.5K validated probes (Gong et al., 2010). To update the putative functions of these transcripts, we re–annotated this array using Blast2GO (Conesa et al., 2005) (Blast2GO.com). Out of the 43,803 target transcript sequences, 7,423 (17%) had significant BLASTX hits ($E \leq 1.0e^{-3}$), whereas the remaining 36,380 (83%) did not show enough homology to sequences in all of the searched databases (Gene onthology (GO), ENZYME, InterPro and KEGG). The homology hits of 5,293 transcript sequences (12%) met a higher filter stringency ($E \leq 1.0e^{-6}$) and these sequences were defined as 'fully annotated' as they had GO–term, Enzyme Commission

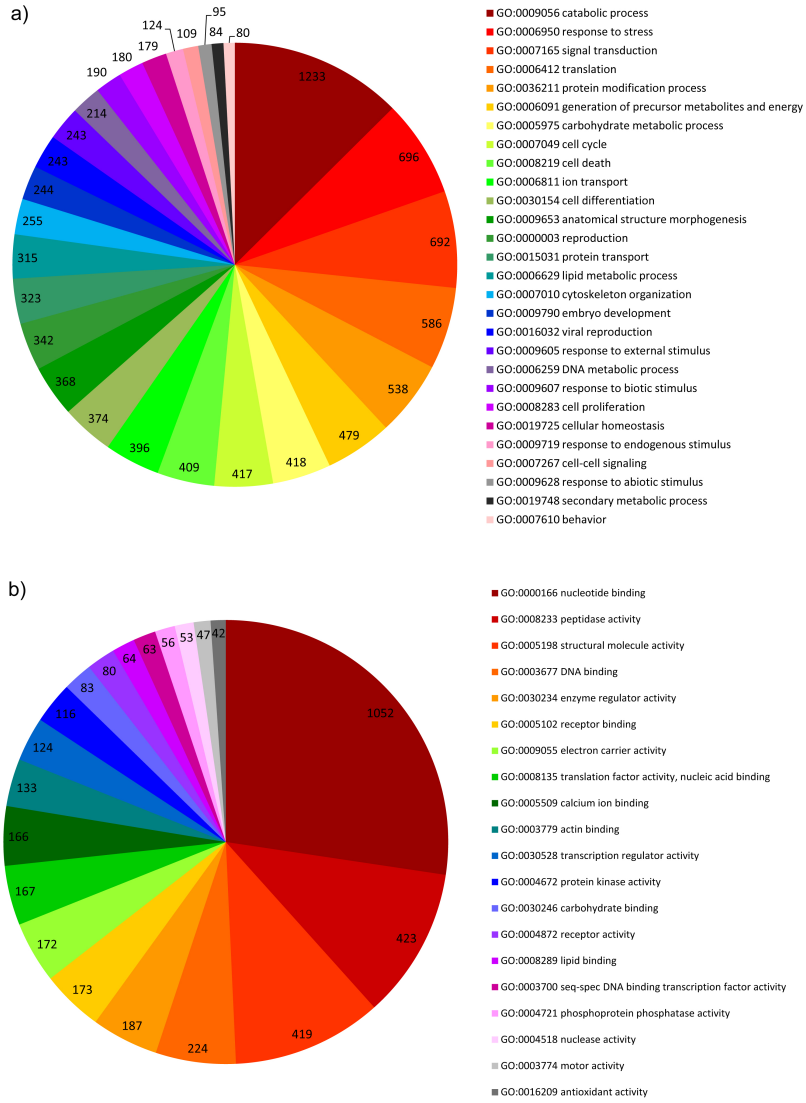

Figure 4.3: Blast2GO multilevel pie charts giving a general overview of the most represented specific Gene Ontology (GO)–terms on the *Eisenia* microarray that belong to a) biological processes and b) molecular functions. Numbers indicate the number of genes assigned to the GO. The multilevel Pie chart was made after using GO–slim, node level 5 and a sequence cut–off of 10 sequences.

(EC) number and InterPro entry. The complete list of probes represented on the *Eisenia* microarray and their target sequence annotation is available (see Supplementary information Table S1).

In total 41,733 Gene Ontology (GO)–terms could be assigned, of which 19,937 are categorized into biological processes, 11,566 into molecular functions, 9,988 into cellular components, and 242 do not belong to any of the three categories. Figure 4.3 shows a general overview of the most represented specific GO–terms, belonging to two categories: (a) biological process and (b) molecular function; using a Blast2GO multilevel Pie chart with GO–slim, node level 5 and a sequence cut–off of 10 sequences. For instance, in the biological processes category, the largest group of genes (1233 genes) were assigned GO:0009056 'catabolic process', while the second largest group of gene (696 genes) were assigned the GO–term 'response to stress' (GO:0006950). Two smaller sets of genes were assigned GO–terms 'reproduction' (GO:0000003) (342 genes) and 'secondary metabolic process' (GO:0019748 – 84 genes), respectively. Although a substantial number of genes could not be annotated, important processes are represented so that underlying mechanisms in response to stressful conditions could be studied. There are two possible explanations for the low level of annotation. First, phylogenetic divergence of earthworms from the current genomic models may have introduced substantial genetic variation preventing reliable similarity searches in public databases. Second, the EST read length may be insufficient for similarity searches. The median target transcript sequence length on this particular microarray is 135 base pairs and their mean length is 200 base pairs (Gong et al., 2010). We believe these two factors in combination have led to the low level of annotation.

4.4.4 Gene expression

Gene expression analysis was used to investigate the underlying mechanisms of 2–phenylethyl ITC toxicity on survival and reproduction of *E. andrei*. Gene expression was compared between the acetone control (AC) and two effective concentrations, 363 nmol/g and 545 nmol/g, used in the ecotoxicological experiments (Figure 4.2). The complete list of significantly differentially expressed genes for the comparisons between the three treatments can be found in the Supplementary information Table S2.

Gene expression altered by exposure to a low concentration of 2–phenylethyl ITC

Only 24 transcripts were identified as differentially expressed between the AC and 363 nmol/g treatments (Table 4.2 and Table S2–a). Interestingly, 13 of these transcripts putatively coding for metallothionein (*mt*), were consistently up–regulated, with an average log_2 fold change of 1.76 ($\pm$ 0.20, standard deviation) (Table S2–d). Gene enrichment analysis (Fisher Exact, adjusted p–value < 0.05) confirmed that the GO–terms corresponding with metallothionein, 'metal ion binding' (GO:0046872), 'cation binding' (GO:0043169) and 'ion binding' (GO:0043167) were over–represented in this treatment (see Supplementary information Table S3–a). This result is not surprising as a total of 15 target transcripts on the *Eisenia* microarray had a blast description of either 'metallothionein', 'metallothionein–2' or 'metallothionein–2c', of which 13 were affected by the 363 nmol 2–phenylethyl ITC/g treatment. (Table S1).

Metallothionein (*mt*) is a protein that detoxifies free metals such as cadmium (Hamer, 1986). It enables organisms to tolerate higher levels of metals in their environment as was demonstrated in a study with the springtail *Orchesella cincta*. This species not only exhibited up–regulation of *mt* genes in response to metals exposure, but exerted higher basal *mt* expression in metal–tolerant populations (Timmermans et al., 2005; Roelofs et al., 2009) suggesting that the tolerant phenotype has undergone transcriptional regulatory evolution. Similar results were observed for the earthworm species *E. fetida* and *Lumbricus rubellus* in response to cadmium (Gruber et al., 2000; Sturzenbaum et al., 2004), although the increased *mt* transcriptional level was shown to be a result of acclimation and not genetic adaptation.

Clearly, *mt* is involved in stress responses to heavy metal contamination. However, other studies have shown that *mt* is also induced in response to oxidative stress, and is triggered by chemical or physical stress to induce formation of oxygen free radicals (Sato and Bremner, 1993). The current study suggests that low concentrations of 2–phenylethyl ITC induced oxidative stress in *E. andrei*, which in turn activated *mt* production.

Gene expression altered by exposure to a high concentration of 2–phenylethyl ITC

A total of 1,142 genes were significantly differentially expressed in *E. andrei* by 96 hour–exposure to 545 nmol/g 2–phenylethyl ITC (Table 4.2), of which

Table 4.2: Total numbers of significant differentially expressed genes in *Eisenia andrei* exposed for 96 hours to 2–phenylethyl ITC at two concentrations (nmol/g soil)

	363 nmol/g vs. AC	545 nmol/g vs. AC	546 vs. 363 nmol/g
Up	22	464	405
	43779	*42661*	*42946*
Down	2	678	452

Concentrations are close to the 56–day EC_{10} and EC_{50} for effects on reproduction compared to the acetone control (AC). Non–differentially expressed genes are given in italics.

271 showed mapping results (23.8%) and 17.8% could be fully annotated (204 genes). This high annotation percentage allowed us to perform a gene enrichment analysis, which shows that 126 GO–terms are either over– or underrepresented (Fisher Exact Test, adjusted p–value < 0.05). The complete list of results is available (see Supplementary information Table S3–b). Of the 126 enriched GO–terms, 21 could be related to a biological meaning in regard to the present study (Table 4.3) and will be discussed in the following sections.

The most overrepresented biological process was associated with chitin related processes (GO:0004568; GO:0006030; GO:0006032; GO:0008061; GO:0008843), representing a total of 41 unique sequences. Chitin is a key component of the exoskeleton of several invertebrates, including earthworms and arthropods. In contrast to arthropods, however, chitin only occurs in the chaetae (bristles used for locomotion) and part of the digestive system of earthworms (Peters and Walldorf, 1986). It occurs in the peritrophic membrane secreted by the mid–gut and parts of the crop and gizzard, as was found for several *Lumbricidae* including *E. fetida* (Peters and Walldorf, 1986).

Table 4.3: Select list of overrepresented GO–terms determined by gene enrichment analysis (Fisher Exact Test adjusted $p < 0.05$). Differentially expressed genes in *Eisenia andrei* after 96 h–exposure to 535 nmol/g 2–phenylethyl ITC in LUFA 2.2 soil compared to the acetone control were chosen as the test set, whereas the whole array as the reference set.

GO–ID	Term	Category	Adjusted p–value	no.Test	no.Ref
GO:0000272	polysaccharide catabolic process	P	5.92E–31	36	32
GO:0003824	catalytic activity	F	3.96E–03	139	2641
GO:0004568	chitinase activity	F	1.87E–46	36	2
GO:0005975	carbohydrate metabolic process	P	7.95E–26	71	350
GO:0005976	polysaccharide metabolic process	P	8.11E–30	43	70
GO:0006004	fucose metabolic process	P	3.53E–02	3	2
GO:0006022	aminoglycan metabolic process	P	4.32E–35	40	34
GO:0006026	aminoglycan catabolic process	P	4.69E–36	36	18
GO:0006030	chitin metabolic process	P	1.37E–48	40	5
GO:0006032	chitin catabolic process	P	1.87E–46	36	2
GO:0008061	chitin binding	F	4.34E–44	35	3
GO:0008843	endochitinase activity	F	6.90E–45	34	1
GO:0009045	xylose isomerase activity	F	2.90E–04	4	0
GO:0009308	amine metabolic process	P	2.09E–12	61	484
GO:0009607	response to biotic stimulus	P	2.40E–17	41	153
GO:0019321	pentose metabolic process	P	2.75E–02	4	6
GO:0030247	polysaccharide binding	F	9.78E–39	35	10
GO:0042732	D–xylose metabolic process	P	2.90E–04	4	0
GO:0042806	fucose binding	F	1.72E–02	3	1
GO:0046496	nicotinamide nucleotide metabolic process	P	2.11E–02	9	47
GO:0050896	response to stimulus	P	7.11E–03	81	1322

GO–ID = Gene ontology code
Term = GO–description
Category = GO–category with P = biological process and F = molecular function
Adjusted p–value = FDR corrected p–value of Fischer Exact Test
no. Test = number of genes with the GO–term in the treatment gene set
no. Ref = number of genes with the GO–term in microarray minus the ones also found in the treatment gene set.

The peritrophic membrane serves several functions, including improvement of digestion, protection against mechanical and chemical damage and serving as a barrier to infection by pathogens (Lehane, 1997; Merzendorfer and Zimoch, 2003). In addition, worm phagocytes can produce and release the highly conserved chitinase as a component of innate immunity against chitin–containing pathogens because chitinase is involved in cell–wall chitin metabolism and catabolism (van Eijk et al., 2005). Genes involved in chitin regulation were found to be up–regulated in the soil arthropod *Folsomia candida* in response to 2–phenylethyl ITC, indicating an increased moulting process (van Ommen Kloeke et al., 2012b). Consistent with previous observations in *E. fetida* exposed to 2,4,6–trinitrotoluene (an oxidative stress inducer) (Gong et al., 2007), these genes were down–regulated in *E. andrei*, suggesting impaired innate immunity and decreased protection against physical, chemical and pathogenic damages.

Oxidative stress response was also one of the overrepresented processes, although their corresponding GO–terms are less descriptive. For instance, 'catalytic activity' (GO:0003824) was represented by five up–regulated transcripts encoding glutathione s–transferase (GST) (TA1–115917; TA1–165734; TA2–04556; TA2–152184; TA2–213208, see Table S2–b). Activation of this gene indicates that a detoxification process was initiated in response to 2–phenylethyl ITC, a xenobiotic compound (Hayes and Pulford, 1995). An oxidative stress response to 2–phenylethyl ITC was also identified for the springtail *F. candida* (van Ommen Kloeke et al., 2012b) and humans. In humans, phase II enzymes such as GSTs are known to contribute to the biotransformation of xenobiotic compounds, and therefore induced expression of GSTs aids in chemoprevention (Cheung and Kong, 2010). The same GO–term also included transcripts coding for heat shock protein (HSP90: TA2–056944), quinone oxidoreductase (TA2–004419) and dehydrogenase reductase (TA2–004470). Moreover, the overrepresented GO–terms 'amine metabolic process' (GO:0009308), 'response to biotic stimulus' (GO:0009607) and 'response to stimulus' (GO:0050896) encompassed multiple up–regulated genes encoding oxidative stress related proteins (e.g. HSP90: TA1–014841, 10 kda heat shock mitochondrial: TA1–145406 and chaperonin containing subunit 5: TA1–080073). Heat shock proteins are highly conserved chaperone proteins that are usually up regulated in response to heat stress. Heat shock proteins assist among others in signal transduction, protein folding, and stabilization of various other proteins, e.g., tumor growth proteins, proteins transport and degradation

(Chen et al., 2006). Up–regulation of all these genes underlines the general pattern of oxidative stress response triggered by 2–phenylethyl ITC.

Stress responses require re–allocation of energy, as indicated by the differential regulation of a great number of energy and sugar metabolic processes. A substantial number of GO–terms (GO:0000272; GO:0005975; GO:0005976; GO:0006004; GO:0006022; GO:0006026; GO:0009045; GO:0019321; GO:0030247; GO:0042732; GO:0042806; GO:0046496) including polysaccharide catabolic and metabolic processes were overrepresented in the *E. andrei* transcriptome responding to 2–phenylethyl TC. Again, this response was comparable to *F. candida*, which also showed up regulation of several sugar metabolic genes at higher concentrations of 2–phenylethyl ITC (van Ommen Kloeke et al., 2012b).

In addition to the 13 putative *mt*–coding transcripts induced at the low concentration, one more putative *mt*–coding transcript and one *mt* precursor gene were also up–regulated at the high concentration (Table S2–b). Interestingly, the high concentration induced a slightly (5.3%) but significantly (paired t–test, $p = 0.018$) higher expression of all 13 putative *mt* genes (mean log_2 fold change = 1.85; Table S2–d). Taken together, the results of both low and high concentrations demonstrate that *mt* expression can be induced by an oxidative stress producer like 2–phenylethyl ITC, and it may serve as a biomarker of oxidative stress if its dependence on exposure concentration can be further demonstrated.

In summary, 2–phenylethyl ITC at the high exposure concentration affected *E. andrei* gene expression mainly by inhibiting chitinase activities, inducing an oxidative stress response, and stimulating energy metabolism.

Pathways altered by exposure to a high concentration of 2–phenylethyl ITC

The 1,143 genes significantly altered by the high concentration of 2–phenylethyl ITC were mapped to KEGG pathways using RefNetBuilder (Li et al., 2011). Out of the 225 KEGG reference pathways, 189 were mapped with at least one differentially expressed gene (Table S4). We sorted these pathways according to the proportion of differentially expressed genes to the genes in an entire pathway. The four most differentially expressed genes–enriched pathways are phototransduction (ko04745), VEGF signalling (ko04370), olfactory transduction (ko04740) and long–term potentiation (ko04720). The percentage of differentially expressed genes in the four pathways was 44%, 34%, 33% and 30%, respectively. A further functional analysis of the differentially expressed genes reveals that calcium signalling represented by calcium/calmodulin–dependent protein kinase II (CAMK2) and classical protein kinase C (CPKC) is involved in all four pathways. The 5–fold inhibition of CAMK2 putatively led to decreased CPKC and increased transient–receptor–potential–like

(TRPL) protein activities in the phototransduction pathway, and an enhanced olfactory receptor activity via activated type III adenylyl cyclase in the olfactory transduction pathway. The altered calcium signalling pathway within the VEGF signalling pathway was signified by decreased CPKC and regulatory subunit of protein phosphatase 3 (PPP3, CNB), In addition to the calcium signalling pathway, the MAPK signalling pathway was also inhibited in the long–term potentiation pathway, ultimately leading to elevated p90 ribosomal S6 kinase (RSK2) and E1A/CREB–binding protein (CREBBP). These results imply that exposure to the high concentration of 2–phenylethyl ITC may cause impaired light sensitivity, angiogenesis (e.g. wound healing), olfactory perception (olfaction), learning and memory.

Differential gene expression between high and low concentrations of 2–phenylethyl ITC

A total of 857 genes were differentially expressed in *E. andrei* between the high and low 2–phenylethyl ITC treatments (Table 4.2), of which 84 showed mapping results but only 7.4% could be fully annotated (53 genes). Due to this low percentage, gene enrichment analysis resulted in only 5 overrepresented GO–terms (Fisher Exact adjusted p–value < 0.05, see Supplementary information Table S3–c). An interesting result was the differential expression of two genes encoding ferritin (TA1–103650, TA1–183928, down–regulated) and a gene encoding a ferritin–like protein (TA2–068791, up–regulated). Ferritin is well–known as the iron storage protein and plays a role in the internal iron homeostasis in various species including for bacteria, plants and animals (Harrison and Arosio, 1996; Romney et al., 2011). Less investigated functions include reduction of iron toxicity, which suggest an anti–oxidant function, although pro–oxidant activities have also been reported (Harrison and Arosio, 1996).

Sequence alignment and phylogeny of *mt* transcripts

In the earthworm *Lumbricus rubellus* 22 full–length *mt* cDNA sequence were identified in a previous study, which phylogenetically clustered in 3 *mt* gene subgroups (*wMT*–1,2 and 3) (Sturzenbaum et al., 2004). The isoform *wMT*–2 is supposed to be involved in cadmium sequestration. In contrast, so far, only one *mt* gene has been identified for *E. fetida* (Gruber et al., 2000). Alignment of the current 15 *E. fetida mt* transcripts with all three *mt* genes of *L. rubellus*, showed that all *E.fetida mt* contigs are most similar to *wMT*–2 (data not shown). The resulting phylogeny of these contigs with the two isoforms of *wMT*–2 (Figure 4.4) suggests that at least two *mt* gene clusters are present in *Eisenia sp.* supported by bootstrap values of at least 80%. Within these clusters substantial genetic variation can be observed, especially within the cluster

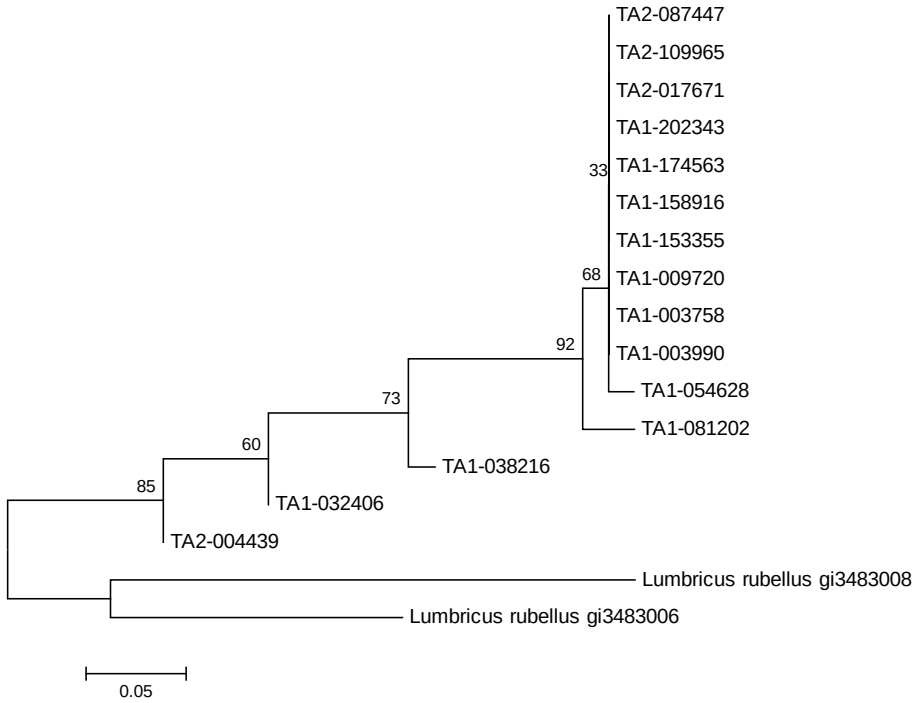

Figure 4.4: Phylogeny of *Eisenia* metallothionein transcripts with two isoforms of *Lumbricus rubellus wMT*–2 as outgroup (gI3483008 and gI3483006). Numbers above branches indicate percentage bootstrap support (1000 replicates).

that contains TA1–04439, TA1–032406 and TA1–038216, suggesting the presence of different isoforms.

4.5 Conclusion

The natural toxin 2–Phenylethyl ITC had a noticeable effect on survival, reproduction and gene expression of the earthworm *Eisenia andrei*. This species was less sensitive to 2–phenylethyl ITC than the soil arthropod *Folsomia candida* exposed under the same conditions. We can only speculate on the cause of this low sensitivity to 2–phenylethyl ITC, but a likely explanation may be the natural ability of earthworms

to actively bioaccumulate, sequester and excrete compounds such as heavy metals (Morgan and Morgan, 1990). This ability is due to the presence of the chloragosome matrix, tissue found especially in the intestinal region of earthworms (Sturzenbaum et al., 2004). This mechanism is hypothesized to minimize damage by cytotoxins and is related to gene expression of metallothionein genes (Morgan and Morgan, 1990) which are known detoxification agents (Hamer, 1986). This study showed the annotation of multiple *mt* transcripts present on the *Eisenia* array which played an important role in the stress response of *E. andrei* when exposed to 2–phenylethyl ITC. The gene expression results presented in this study are a first step for explaining the underlying modes of action and acute effects of the toxin on survival and reproduction.

Isothiocyanates receive increasing attention due to their chemopreventive nature and potential anti–cancer activity in human beings (Traka and Mithen, 2009). Furthermore, their natural ability as anti–herbivorous agent makes them interesting for alternative agricultural pest manage methods, such as biofumigation (Morra and Borek, 2009). These natural toxins already occur in the field with ITC concentrations rising up to 100 nmol/g soil after biofumigation (Matthiessen and Kirkegaard, 2006). Their socio–economic benefits call for engineered crops with enhanced levels of GSL, inevitably leading to even higher levels of ITCs in the soil ecosystem and detrimental effects on the performance of soil invertebrates, as described here. Losing beneficial soil invertebrates such as earthworms could have serious repercussions for the soil ecosystem (Satchell, 1983), as earthworms perform essential functions in the soil. Such risks should, therefore, be carefully investigated before allowing these natural toxins to enter the soil on a large scale.

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Supplementary information

Can be obtained digitally upon request

Figure S1 – Two–color comparative genomic hybridization results showing log_2 (Ratio Cy5/Cy3) of four hybridizations on the *Eisenia* 44K–probe array: F vs. E1, E1 vs. F, E1 vs. E3, and E3 vs. E1. Three used gDNA samples are F = *E. fetida*, E1 = *E. andrei* sample 1 and E3 = *E. andrei* sample 3.
Table S1 – Re–annotation of *Eisenia* microarray (Agilent amadid no.: 022725; 43803 probes) using Blast2GO with default threshold settings (BlastX Expect value $\leq 1.0e^{-3}$) and an E–value Hit filter of $1.0e^{-6}$.
Table S2 – List of differentially expressed genes in *Eisenia andrei* significantly altered by 96 hour exposure to a) 363 or b) 545 nmol 2–phenylethyl ITC per gram LUFA 2.2 soil in comparison with the acetone control (AC), c) treatments compared to each other and d) a comparison of metallothionein gene expression effect between the low and the high exposure concentration of 2–phenylethly ITC.
Table S3 – Results of gene enrichment analysis (Fisher Exact test, adjusted p–value < 0.05) of differentially expressed genes in *Eisenia andrei* after 96 h–exposure to 363 or 545 nmol/g 2–phenylethyl ITC in LUFA 2.2 soil compared to the acteone control.
Table S4 – Mapping of 1143 significantly altered genes by high ITC to KEGG pathways using RefNetBuilder and detailed functional analysis of the top four differentially expressed genes–enriched KEGG pathways.

CHAPTER 5

Biofumigation using a high–sinigrin wild *Brassica oleracea* accession affects beneficial soil invertebrates

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5.1 Abstract

Several *Brassicaceae* species are known for their production of aliphatic glucosinolates (AGSLs), the degradation of which leads to toxic compounds, that affect harmful and beneficial, non–target soil organisms when *Brassicas* are used for biofumigation. We

explored the biofumigation effects on non–target soil organisms and aimed to relate those effects to differences in glucosinolate (GSL) profiles and the relevant biosynthesis genes. Leaf material of two *B. oleracea* cultivars and the wild *B. oleracea* accession Winspit was analysed for AGSL production and whole genome gene expression, and used for biofumigation experiments on beneficial soil organisms. Total AGSL levels varied between genotypes, with particularly sinigrin and gluconapin being abundantly or exclusively present in Winspit. The genes *BoGSL–ELONG*, *BoMYB29* and *BoAOP2* were higher expressed in Winspit than in the cultivars. When incorporated into soil, especially the Winspit plant material exerted toxic effects to non–target soil invertebrates such as springtails.The high expression of *BoGSL–ELONG* and *BoAOP2* correlates with the high production of sinigrin and gluconapin in Winspit and is probably caused by high expression of *BoMYB29*. The high sinigrin content explains the toxicity of Winspit for soil invertebrates. These results can be used to optimise *B. oleracea* crops for biofumigation.

5.2 Introduction

Plant species of the family *Brassicaceae*, including commercially important crops such as oilseed rape, many vegetables such as cabbages, turnips and broccoli, and the scientifically important model *Arabidopsis thaliana*, are rich in sulphur–containing secondary metabolites called glucosinolates (GSLs) (Wittstock and Halkier, 2002). More than 120 different GSLs have been identified, of which approximately 50% are aliphatic glucosinolates (AGSLs), methionine derived GSLs that mainly occur in the leaves and flowers of the plant (Halkier and Gershenzon, 2006; Hopkins et al., 2009). Glucosinolate composition and content can vary widely within and between species. For instance, in *Brassica oleracea* 'Winspit', a natural, wild accession originating from the coast of Dorset (England), total GSL concentrations are high compared to other *B. oleracea* wild populations and cultivated genotypes (Gols et al., 2008). Glucosinolate composition also varies between *B. oleracea* populations and genotypes, although the AGSL 2–propenyl GSL (sinigrin) is almost universally present (Mitchell and Richards, 1979; Gols et al., 2008). Biosynthesis of GSLs is regulated by several genes (Halkier and Gershenzon, 2006).

An explanation for the observed variation in GSL total content and composition between genotypes and species may therefore be found in the differences in the expression of the genes encoding for enzymes and regulators of the GSL biosynthetic pathways. For instance, although *B. rapa* and *A. thaliana* belong to the same family, they differ in GLS composition and content, with the GSL content of *B. rapa* vegetative tissues up to seven times higher than that of *A. thaliana*. Glucoraphanin and glucobrassicanapin are the most abundant GSLs in *B. rapa*, while glucoraphanin is the most abundant GSL in *A. thaliana* (Larsen Petersen et al., 2002; Padilla et al.,

2007). Comparative genomics studies show that *A. thaliana* GSL pathways overlap only to a limited extent with *B. rapa* GSL pathways (Zang et al., 2009; Wang et al., 2011). For *B. oleracea* these biosynthetic pathways are also expected to overlap, but they have not yet been investigated in detail.

The current interest in GSL is mainly focused on the toxic characteristics of their hydrolysis products, of which isothiocyanates (ITCs) are the best–known (Brown and Morra, 1997; Halkier and Gershenzon, 2006). The release of ITCs is mediated by the enzyme myrosinase in response to tissue disruption, caused, for instance, by chewing of herbivores (Wittstock and Halkier, 2002; Bednarek and Osbourn, 2009). Isothiocyanates have been exploited for alternative pest management methods, a procedure called biofumigation. Biofumigation aims at using different *Brassica* species and the related *Brassicaceae* species *Sinapis alba*, for the release of high levels of ITCs, upon mixing of high–GSL containing plant material into the soil, to suppress soil–borne pests (Matthiessen and Kirkegaard, 2006). For instance, a reduction in the occurrence of nematode cysts in potato plants, can be observed due to the release of especially the aliphatic 2–propenyl GSL (sinigrin) and the aromatic 2–phenylethyl GSL (Aires et al., 2009). The toxic nature of ITCs can, however, also affect beneficial soil organisms, which are essential for proper soil functioning. For example, the beneficial soil arthropod *Folsomia candida* has a reduced survival and reproduction, even at very low concentrations of pure 2–phenylethyl ITC spiked into the soil (van Ommen Kloeke et al., 2012b). The effects of biofumigation on beneficial soil invertebrates using plant material, has remained unstudied so far. Such knowledge is essential for developing sustainable biofumigation strategies.

The objective of this study was to explore the effects of biofumigation on non–target soil organisms using different *B. oleracea* genotypes. Furthermore, we wanted to relate the observed biofumigation effects to differences in GSL content and composition and to identify genes that are likely to be responsible for these differences. These data will aid in the development of proper biofumigation strategies, by predicting the consequences of biofumigation practises on beneficial soil invertebrates. To that end, we studied the phenotypic variation for AGSL production and analysed the expression of genes of the AGSL pathway in three *B. oleracea* genotypes, which were chosen for their expected difference in AGSL content and composition. We then investigated the biofumigation effects of these genotypes on the bacterial soil community and the ecologically important soil invertebrate model species *Eisenia andrei*, an ecosystem engineer (Sheppard et al., 1998) and *F. candida*, a fungivorous collembolan (Filser, 2002). Finally, the fate of GSLs and ITCs was traced after incorporation of the plant material into the soil, revealing the biofumigation potential of *B. oleracea* and the duration of a potential effect.

5.3 Materials and methods

5.3.1 Plant material

Three *B. oleracea* genotypes were used: Winspit (WIN), a natural wild accession originating from the coast of Dorset, England, and two cultivars: the purple sprouting broccoli 'Santee' (PSB) and the Savoy cabbage 'Wintessa' (SAV). The two cultivated genotypes were chosen for their expected elevated AGSL content, based on information provided by the breeding company Bejo Seeds BV, the Netherlands (personal communication Dr. H. Huits). Seeds of Winspit were provided by Dr. Rieta Gols (Laboratory of Entomology, Wageningen University, the Netherlands). F1 hybrid seeds of PSB and SAV were obtained from Bejo Seeds BV (Warmenhuizen, Netherlands).

All the seeds were sown in 17∅ cm pots containing a peat–based commercial potting mixture ('Lentse potgrond nr. 4'; 85% peat, 15% clay). After approximately eight weeks, three mature fully expanded, but not old, leaves were harvested, stored at –80°C and used for GSL and gene expression analysis. Since Winspit seeds were collected in the field and are likely to be genetically heterogeneous, material from four individual Winspit plants (WIN5, WIN7, WIN9 and WIN14) was used for GSL analysis and gene expression experiments. Twelve plants of both F1 cultivars, which were assumed to be genetically similar, were distributed in four groups of three plants, which were analysed separately. A different batch of plants of the same genotypes was grown under the same conditions. Plant material was freeze–dried and stored at room temperature (RT) until used for the biofumigation experiments. For all genotypes, material of several plants was combined in order to obtain enough for the amounts needed for the experiments.

5.3.2 Glucosinolate analysis by HPLC

GSLs from fresh or freeze–dried plant material (200 mg respectively 50 mg) were extracted and subsequently analysed for GSL content and composition using high performance liquid chromatography (HPLC) analysis as described by Hennig et al. (2012). Benzyl GSL (glucotropaeolin, 25 μL, 3mM) was used as internal standard in the analysis.

5.3.3 Analysis of Brassica microarray data

The Affymetrix Brassica Exon 1.0 ST Array (Love et al., 2010) was used for microarray analysis. Hybridizations were carried out at the Nottingham Arabidopsis Stock Centre Affymetrix service (NASC, University of Nottingham, UK). Total RNA

samples were labelled, hybridised, and scanned following the standard protocol from the manufacturer (GeneChip Expression Analysis, Affymetrix, www.affymetrix.com). The GeneChip Command Console Software (AGCC; Affymetrix) was used to generate '.cel' files for each of the hybridisations. These have been deposited in NCBI Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE39951 (www.ncbi.nlm.nih.gov/geo/). The raw chip data were normalised using the Robust Multichip Average (RMA) pre–normalisation algorithm (Irizarry et al., 2003) in the GeneSpring GX (version 11.5; Agilent Technologies) analysis software package. Following RMA pre–normalisation the signals were further normalized by standardizing the signal value of each probe–set to the median of that probe–set across all hybridisations. All further analysis was carried out using different functions in GeneSpring GX software. Differentially expressed genes were identified when the WIN plants were analysed together using a two–step process; (i) an unpaired t–test using a Benjamini and Hochberg false discovery rate correction ($p < 0.05$) and (ii) a fold–change > 1.5. When the WIN plants were analysed separately a probe–set was identified as differentially expressed if the fold–change was > 2. Gene Ontology (GO) analysis was performed using the GO analysis function in Genespring GX, with the p–value calculated using a hypergeometric test with Benjamini–Yekutieli correction (Benjamini and Yekutieli, 2001). The GO analysis was performed on the *A. thaliana* paralogue of the *Brassica* gene, identified as the top hit from a BLAST (using NCBI BLASTn algorithm) analysis of the probe sequences against the *A. thaliana* genome.

5.3.4 Real–time Reverse Transcriptase PCR

Real–time quantitative reverse transcriptase (RT) PCR (Q–RT–PCR) was used to confirm the microarray results. The expression of *B. oleracea* AGSL biosynthetic genes was analysed using the SYBR Green dye and a CFX96^{TM} Real–Time PCR Detection System (Bio–Rad Laboratories, Richmond, CA). Expression levels were calculated relative to the expression of the *BoGAPDH* housekeeping gene. Primers for *B. oleracea* GSL gene expression analysis were designed based on *B. oleracea* sequences if available, otherwise on *B. rapa* sequence (Table S1). *Brassica* sequences were found in BRAD (brassicadb.org/brad/) and NCBI (www.ncbi.nlm.nih.gov) databases. Most of the evaluated genes were chosen according to the gene inventory of the GSL pathway and *B. rapa* orthologues (Wang et al., 2011). *BoMAM* genes were evaluated on the bases of the *B. oleracea* information already reported (Li and Quiros, 2002; Gao et al., 2006). Special effort was put into primer design in order to distinguish the different isoforms/paralogues of each gene. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA, using the Script cDNA Synthesis Kit (Bio–Rad Laboratories, Richmond, CA) according

to the manufacturer's instructions. Real–time Q–RT–PCR experiments were carried out using 50 ng of cDNA and the iQ SYBR Green Supermix (Bio–Rad Laboratories, Richmond, CA) following the manufacturer's protocol. Relative quantification of each single gene expression was performed using the comparative C_T method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin no.2 (Applied Biosystems). The relative expression levels were represented as heat maps, using BAR Heatmapper Plus software (http://bbc.botany.utoronto.ca/ntools/).

5.3.5 Biofumigation experiments

For the biofumigation experiment the springtail *Folsomia candida* (Fountain and Hopkin, 2005) and the bioturbator *Eisenia andrei* were used. *F. candida* came from a synchronized culture of 10–12 days old animals which was prepared from a laboratory stock culture ('Berlin strain', VU University Amsterdam). The International Standardization Organization (ISO) guideline 11267 (ISO, 1999) was used for synchronization and stock maintenance. Springtails cultures were kept on moistened plaster of Paris mixed with charcoal and *ad libitum* baker's yeast as food source. *E. andrei* originated from a synchronized laboratory stock culture (VU University Amsterdam). Worm cultures were cultivated in a substrate of potting soil and peat, fed abundantly with horse manure (devoid of pharmaceuticals). For the experiments, adult worms were used with a fully developed clitellum and with a maximum age difference of one month. Both cultures were kept under constant conditions in a climate chamber with a temperature of 20°C, a 12/12 hours light/dark cycle and 75% relative humidity.

Biofumigation was performed using freeze–dried leaves of the three different *B. oleracea* genotypes SAV, PSB and Winspit, as described earlier. Leaves of approximately 8 weeks old were harvested from the plant, snap frozen in liquid nitrogen and thereafter directly freeze–dried in order to preserve the GSL content for a longer period of time and for easy storage. One percent of freeze–dried leaf material relative to total soil (on a dry weight (DW)/DW base) was used for biofumigation. Leaf material was completely fragmented using a laboratory blender (IKA, A11 basic) to ensure maximum tissue rupture and, in turn, GSL release efficiency and hydrolysis potential (Gimsing and Kirkegaard, 2009). Fragmented plant material was mixed thoroughly in the soil by hand and kitchen blender. Finally, the soil was moistened to 50% of the water holding capacity of 45.2%, corresponding to 22% water of the soil DW. The natural soil LUFA 2.2 (Speyer, Germany) was used, which is a loamy sand soil with a pH of 5.5 ± 0.1 (± SD) and an organic C content of 2.09 ± 0.40%. Before usage the soil was dried at 60°C for 24 hours. Test jars were incubated in climate rooms at 20°C, 70% relative humidity and a 12 hour light/dark cycle for 28 or 56 days. Once a week jars were aerated and moisture content was adjusted if needed.

The *F. candida* test consisted of 6 replicates per plant genotype, kept in 100–ml jars and consisted of 15 g moist soil and 10 animals. After 28 days 100 ml of water was added to test containers, stirred gently and completely poured out into a glass beaker. For each sample several digital photographs were taken to register all living springtails that came floating to the surface. To establish survival and reproduction, photos were used for hand counting the number of adult survivors and juveniles on a computer screen. The *E. andrei* test consisted of 5 replicates per plant genotype, kept in 600–ml jars and consisted of 250 g moist soil and 5 animals. After 28 days, survival was checked by hand sorting and removing the surviving adults from the soil. The remaining soil was incubated for another 28 days after which the number of hatched juveniles were extracted and counted using a water bath at 60°C.

One–way ANOVA and post–hoc tests were performed with SPSS 15.0 to investigate significant differences between treatments for each species. For the survival data of *F. candida* a Welch adjustment and Games–Howell post–hoc test (Quinn and Keough, 2002) had to be used as the assumption of homogeneity of variances was not met for this dataset.

5.3.6 Microbial community analysis

Changes in microbial community structure after biofumigation were determined by cultivation–independent analysis of PCR–amplified 16S rRNA gene fragments using denaturing gradient gel electrophoresis (DGGE). Samples were taken at several time points: 24 hours, 48 hours, 1 week, 2 weeks and 4 weeks after incorporation of the plant material. Samples consisted of 250 mg of moist soil with plant material and free of animals. Samples were frozen at –18°C until further analysis. DNA was extracted using the PowerSoil DNA kit (MoBio Laboratories, Solana Beach, CA) following instructions of the manufacturer. PCR DGGE was performed as described by de Boer et al. (2012), using GC–clamped primers to amplify the V3 region of bacterial 16S rRNA genes (Muyzer et al., 1993). The software GelCompar II (Applied Maths, Kortrijk, Belgium) was used to analyse the DGGE profiles by using a band assignment–independent method (Pearson product–moment correlation coefficient and unweighted pair–group clustering method using arithmetic averages (UPGMA). The binary matrix with Pearson correlation coefficients was analysed by nonmetric multidimensional scaling (NMDS) in PAST (PAleontological STatistics), version 2.15 (Hammer et al., 2001), which is an unsupervised statistical technique to explore similarities or distances between data points. The algorithm (Taguchi and Oono, 2005) places the data in a two– or three dimensional coordinate system to visualise the data while preserving the ranked differences and minimizing the stress (Hammer et al., 2001). For this data set the 'user similarity' was used and visualised in 2D. A stress measure smaller than 0.3 was deemed a good fit of the data.

5.3.7 Determining the fate of GSL and ITC in soil

To investigate the fate of GSLs, GSL hydrolysis into ITC and ITC dissipation, soil samples were taken from the biofumigated soil (without effect of animals) at several time points: 1 hour, 24 hours, 48 hours and 1 week after incorporation of the plant material. Samples consisted of approximately 5 g of moist biofumigated soil and were frozen at the particular time point at –18°C (in darkness) until further analyses.

GSL analysis using HPLC

GSL content and composition of the soil samples was measured using a HPLC procedure similar as the one used for the plant material, but for larger samples. Samples were first extracted in 12 ml of boiling 100% methanol for 20 min, while vortexing the samples regularly. The supernatant was collected after centrifugation (10 min at 25 000 x g), stored on ice and the pellet was re–extracted twice in the same way, using 10 ml of 70% methanol and putting the samples in a water bath at 75°C during the 20 min incubation. Glucotropaeolin (1 ml, 3 mM) was added during extraction as internal standard. DEAE–columns were prepared using plastic 2–ml syringes packed with 1 cm glass wool and 3 ml DEAE Sephadex ion exchanger, and placed in 10–ml glass collection tubes. Columns were washed twice with 1 ml milli–Q water, after which 2 ml of the extracted GSL supernatant was added. Columns were washed twice with 1 ml 0.02 M $NaAC$ solution and after placing the columns in new collection tubes, 75 μl of freshly prepared sulphates was added. After the on–column desulphation overnight at RT (collection tubes were covered with parafilm), desulphated GSL samples were eluated using milli–Q water, 3 times 0.5 ml, and samples were filtered using a 0.45–μM 13–mm syringe filter.

The desulpho–GSLs were subsequently analysed using HPLC analysis and quantified as described by Verkerk et al. (2001).

ITC analysis using GC–MS

Gas chromatography–mass spectrometry (GC–MS) analysis was used to investigate the presence of ITCs in the soil after biofumigation. The samples were extracted by adding 5 ml ethyl acetate and 100 μl benzyl ITC (500 μmol/L in ethyl acetate) as analytical internal standard (IS) to the samples. Prior to analysis, samples were thawed, shaken on a vortex and the ethyl acetate phase was transferred on top of Pasteur pipettes packed with quartz wool (inactivated, silica treated) in the bottom and 2.0 g anhydrous Na_2SO_4 above to filter and dry the samples. The purified sample eluate was collected in a 10–ml test tube. This procedure was repeated with an additional 5 ml of ethyl acetate added to the initial soil and again transferred to the same Pasteur pipette and eluated into the same test tube.

The eluate was then evaporated in a water bath at around 80°C (> boiling point ethyl acetate) to nearly complete dryness (less than 2 ml) and transferred to GC vials. Samples were analysed by gas chromatography tandem mass spectrometry (GC–MS/MS, Varian CP–3800 and Varian 1200 triple–quadruple) with electron ionization (70 eV). The MS was operated in selected reaction ion monitoring mode acquiring data on the ion transitions 163.0 > 105.0 for 2–phenylethyl ITC and 149.0 > 91.0 for benzyl ITC. The GC–column was a 30 m x 0.25 mm, 0.25 μm Factor Four VF–5 MS (Varian). The injection volume was 1 μl in splitless mode achieved by a CTC CombiPAL autosampler and a PTV–injector kept at 200°C at all times. The initial column oven temperature was 40°C and the final temperature was 285°C (15°C/min) with a total analysis time of 18.3 min. Helium was used as carrier gas at 1.0 ml/min. Quantification of the ITC proved difficult due to a lack of quantifiable external standards, therefore only qualitative and relative analysis could be done.

5.4 Results

5.4.1 GSL analysis in a wild accession of *Brassica oleracea* and two cultivars

Three *Brassica oleracea* genotypes were used for GSL analysis in leaves and roots: Winspit (WIN), a wild accession collected from the south coast of Dorset, and two cultivated F1 hybrid varieties: the Savoy cabbage 'Wintessa' (SAV) and the purple sprouting broccoli 'Santee' (PSB). The leaves of the tested genotypes showed large differences in aliphatic and indolic GSL composition and content (Figure 5.1). On the other hand, the roots of these plants showed several indolic GSLs but no aliphatic GSL (data not shown). While the two F1 hybrid varieties are genetically homogenous, the wild accession Winspit was expected to be heterogeneous due to its out–crossing nature, which is why four individual plants were examined separately. The highest total GSL content was found in the Winspit plants WIN5, WIN9 and WIN7 (respectively 1.07, 1.89 and 4.59 μmol/g fresh weight (FW)) (Figure 5.1). 3–Butenyl GSL (gluconapin) and 4–pentenyl GSL (glucobrassicanapin) were present in all four Winspit plants. 2(R)–2–hydroxy–3–butenyl GSL (progoitrin) was present just in WIN14 and 2–propenyl GSL (sinigrin) was present only in WIN5 (0.20 μmol/g FW) and WIN7 (0.71 μmol/g FW). None of the three genotypes selected for GSL analysis contained AGSLs in the roots. Only in Winspit indolic GSLs were present in the roots (data not shown).

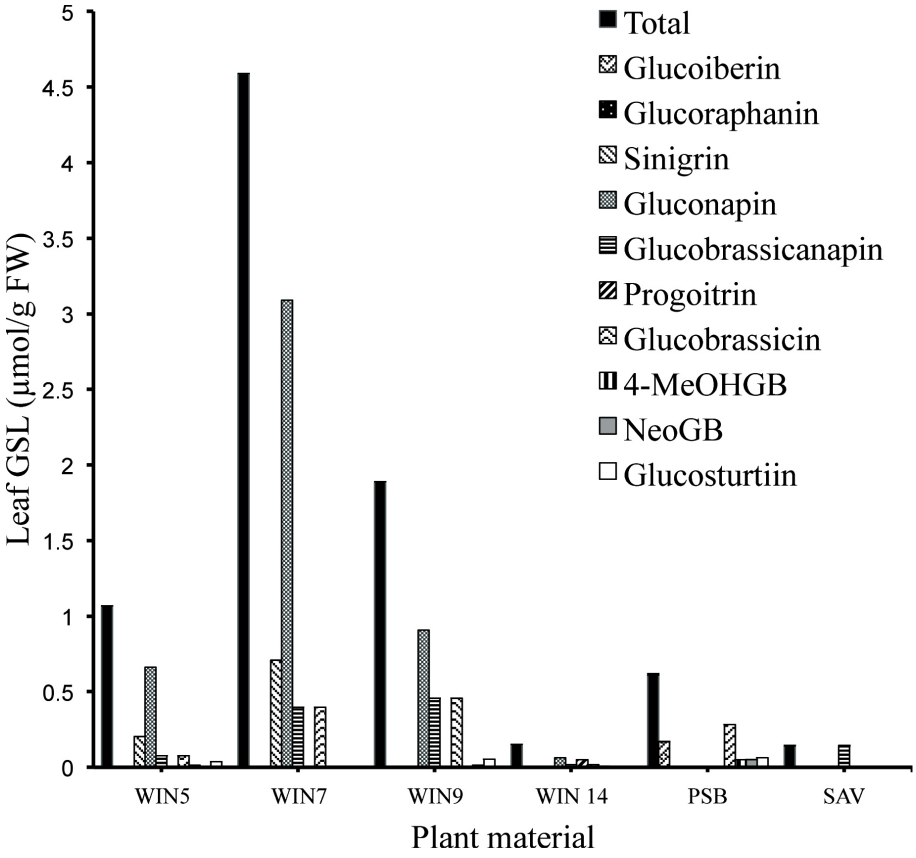

Figure 5.1: Aliphatic GSL content and composition determined by HPLC analysis of the leaves of four *B. oleracea* Winspit (WIN) plants (WIN5, WIN7, WIN9, WIN14) and the F1 hybrid cultivars Purple Sprouting Broccoli 'Santee' (PSB) and Savoy cabbage 'Wintessa' (SAV).

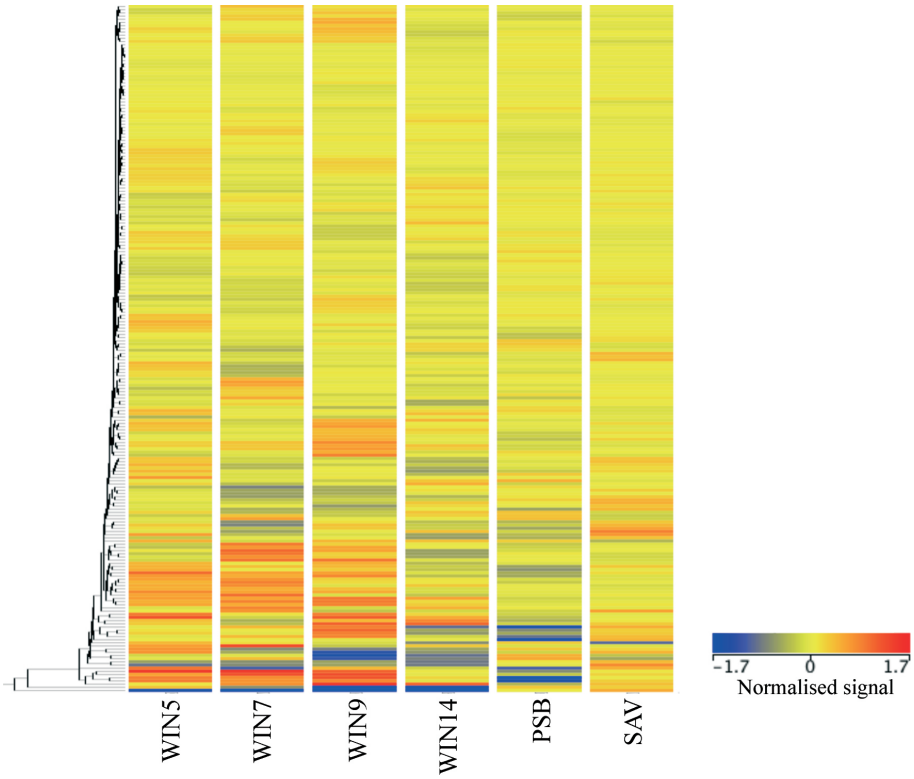

Figure 5.2: Hierarchical clustering of GSL genes according to gene expression profiles as determined by microarray analysis of leaves of four Winspit (WIN) plants (WIN5, 7, 9 and 14) and the F1 hybrid cultivars Purple Sprouting Broccoli 'Santee' (PSB) and Savoy cabbage 'Wintessa' (SAV). Colouration is based on normalized expression values.

5.4.2 Expression of GSL biosynthesis related genes in Winspit and two *Brassica oleracea* cultivars using the Affymetrix *Brassica* Exon 1.0 ST array

The striking differences in AGSL levels between the examined genotypes are likely to be caused by differences in either expression or function of genes involved in GSL

biosynthesis. To examine this, the same material that was used for GSL analysis was used for gene expression analysis, using the Affymetrix *Brassica* Exon 1.0 ST array (Love et al., 2010). A principle component analysis of the data (Figure S1), showed that the three genotypes are different from each other, with the WIN plants being more similar to SAV than to PSB plants and, as expected, in general more heterogeneous than both F1 hybrids. When the individual Winspit plants were analysed together against the two cultivars, 656 probe sets were significantly differentially expressed ($p < 0.05$, fold change > 1.5) between WIN and PSB and 699 probe sets between WIN and SAV. In total, 170 probe sets were consistently significantly differentially expressed ($p < 0.05$, fold change > 1.5) between WIN and both PSB and SAV (Table S2). Gene Ontology analysis of these differentially expressed genes revealed that GO terms related to mucilage metabolism and extrusion, seed coat development and seedling development were significantly over represented (Table S3).

To analyse GSL biosynthetic gene expression analysis, the list of relevant genes as identified by Wang et al. (2011) was used. In total 216 probe sets were identified from this list and the number of genes differentially expressed were identified between Winspit plants and the two cultivars (Table S4). A hierarchical clustering of these genes' expression is presented in Figure 5.2, which demonstrates the variation in GSL biosynthetic gene expression between the genotypes. When the four WIN plants were analysed together, five probe sets, representing four genes, were significantly differentially expressed ($p < 0.05$, fold change > 1.5) between WIN and PSB. These are Bra021670 (*2–Oxoacid dependent dioxygenase; BrGSL–OH*) and Bra004744 (*Isopropylmalate dehydratase; BrIPMI SSU2*), which are both down–regulated in WIN, while Bra022815 (GSTF) and Bra008132 (*Desulfoglucosinolate sulfotransferase 5a; BrST5a*) are both up–regulated in WIN. Of these, only Bra022815 (GSTF) was not differentially expressed between WIN and SAV.

5.4.3 Gene expression analysis of *B. oleracea* the GSL pathway by real–time Q–RT–PCR assays

Based on the microarray analysis, several genes involved in the biosynthesis of AGSLs were found to be differentially expressed in wild and cultivated *B. oleracea* genotypes. To confirm the microarray results, and especially to accurately determine the specific expression of different paralogues of these genes, which may not have been distinguished by microarray analysis, real–time quantitative (Q–)RT–PCR assays were performed on the same material as used for the microarrays. Samples from fully expanded leaves of Winspit, SAV and PSB were analysed for relative mRNA levels of 42 different genes involved in the AGSL pathway, including the paralogues of the genes found to be significantly differentially expressed.

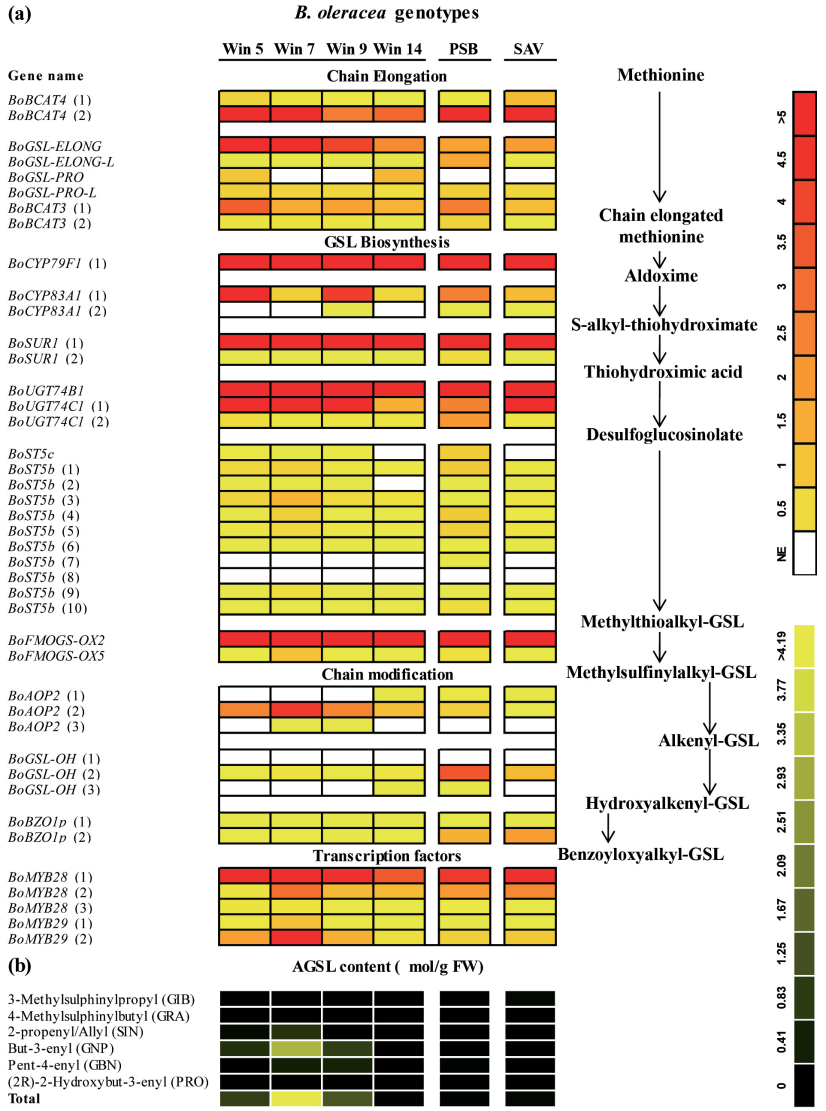

Figure 5.3: Relative expression levels of AGSL biosynthesis genes in *B. oleracea*. (a) Real–time quantitative RT–PCR analyses of the aliphatic GSL biosynthesis genes in four Winspit plants (WIN5, 7, 9 and 14), F1 hybrid cultivars Purple Sprouting Broccoli 'Santee' (PSB) and Savoy cabbage 'Wintessa' (SAV). Colours indicate expression level compared to the expression of the housekeeping gene *BoGAPDH*. (b) Aliphatic GSL content in the same material. NE = not expressed. On the right side of the figure, the aliphatic GSL pathway is displayed and genes are arranged according to their role in the pathway. Full gene names and primers are listed in Table S1.

Q–RT–PCR experiments showed high expression of *BoCYP79F1* and *BoFMOGS–OX2* genes in the three *B. oleracea* genotypes. Only for *BoST5b–8* and *BoGSL–OH–1* no expression was observed in any of the investigated genotypes (Figure 5.3). The difference in the AGSL production in the plants was correlated with the expression of *BoGSL–ELONG*, *BoAOP2* and *BoMYB29* genes (Figure 5.3). The high AGSL containing genotype WIN7 showed the highest relative expression of these genes. In contrast, the cultivated *B. oleracea* plants and WIN14, which displayed low AGSL production, also showed low expression of the above–mentioned genes.

5.4.4 Biofumigation using Winspit plant material on soil invertebrates and bacterial community

The effects of the three different *B. oleracea* genotypes on the survival and reproduction of the springtail *Folsomia candida*, after incorporation into LUFA 2.2. soil, were substantially different from effects on the earthworm *Eisenia andrei* (Figure 5.4). *E. andrei* survival was unaffected by any of the three genotypes. Reproduction, on the other hand, showed significant differences between the three genotypes ($F = 6.06$, $p = 0.015$). Biofumigation with SAV resulted in significantly higher reproduction (38 $\pm$ 2.33 SE hatched juveniles after 56 days of exposure) than treatment with Winspit (Bonferroni, $p = 0.016$), but not compared to PSB. Reproduction was lowest with Winspit, although this was not significantly different from PSB (Bonferroni, $p = 0.109$). In contrast, *F. candida* showed significant differences in both survival (Welch's statistics = 69.20, $p = 0.000$) and reproduction ($F = 200.5$, $p = 0.000$) among *B. oleracea* genotypes (Figure 5.4, c–d). The highest rate of survival and reproduction for *F. candida* was found for organisms exposed to SAV showing a survival of 98% ($\pm$ 1.67) with a corresponding reproduction of 692 ($\pm$ 25.3) juveniles. Survival and reproduction showed intermediate levels for biofumigation with PSB and was lowest for Winspit. For reproduction all genotypes differed significantly from each other (Bonferroni, all $p = 0.000$), whereas for survival, PSB did not differ from Winspit (Games–Howell, $p = 0.071$). Both *E. andrei* reproduction and *F. candida* survival and reproduction almost completely corresponded to the total GSL content levels of plant material used from the three genotypes (Figure 5.4, e).

Nonmetric multidimensional scaling of soil microbial DNA revealed that effects of the *B. oleracea* plant material on the bacterial community structures did not differ between the genotypes, i.e. data points did not cluster together according to the three different genotypes on either axis (Figure 5.5). Only a temporal pattern could be discovered, as the bacterial community structures were highly similar for all three genotypes across three of the four time intervals (Figure 5.5). The largest difference in bacterial community structure between the genotypes was one week after biofumigation, however, after two weeks all genotypes clustered together again.

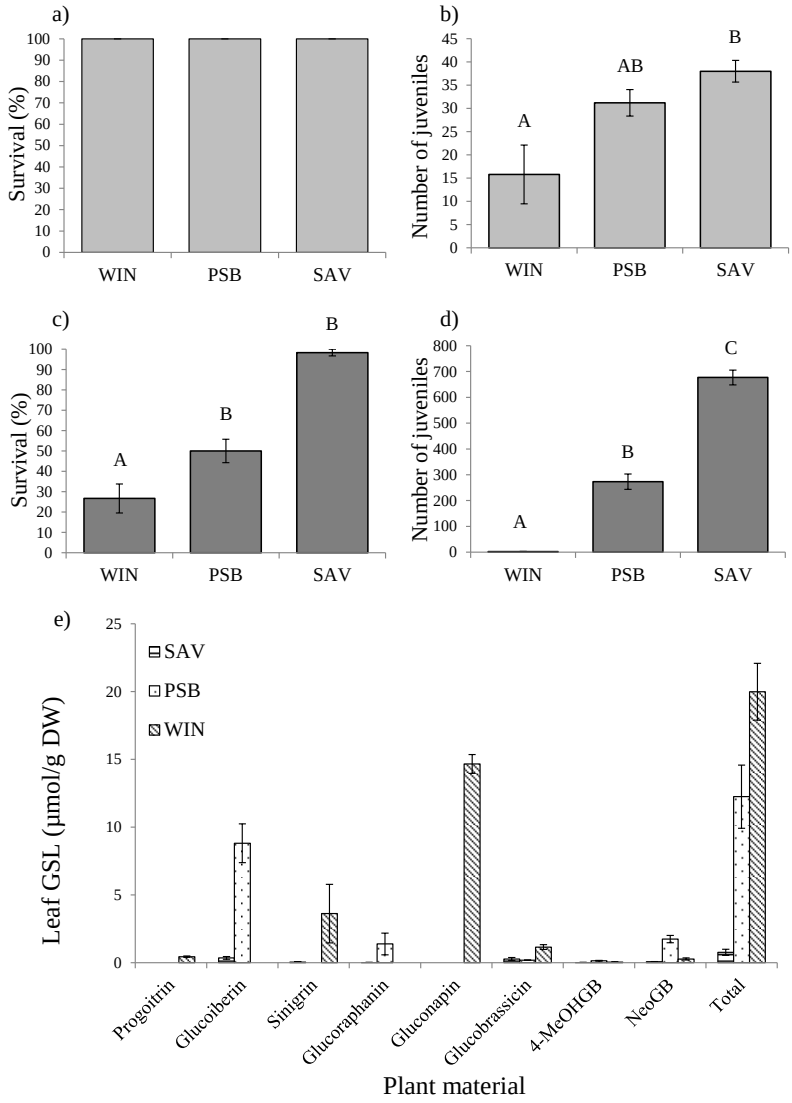

Figure 5.4: Biofumigation effects of three *Brassica oleracea* genotypes on the survival and reproduction of indicator species and GSL content and composition of used leaf material. a–b) *Eisenia andrei* and c–d) *Folsomia candida* after 28 days or 56 days exposure in LUFA 2.2 soil. Exposures were done using 1% freeze–dried and fragmented plant material compared to dry weight of LUFA 2.2 soil. SAV = Savoy, PSB = Purple Sprouting Broccoli and WIN = Winspit. Error bars indicate standard errors (n = 5 for E. andrei and n = 6 for F. candida). Letters indicate significant differences between treatments (ANOVA, $p < 0.05$). e) GSL content and composition of WIN, SAV and PSB plant material used for biofumigation. Error bars indicate standard errors (n = 4 for WIN and n = 3 for SAV and PSB).

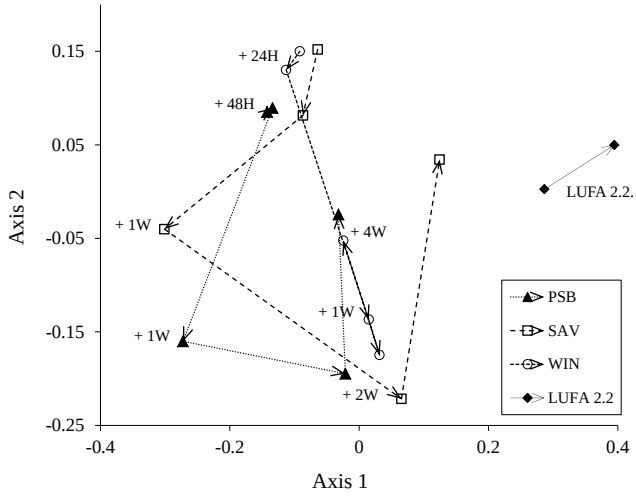

Figure 5.5: Non–metric multi dimensional scaling (NMDS) data of biofumigation effects of three *Brassica oleracea* genotypes on the bacterial community structures at time intervals; 24 hours (+24h), 48 hours (+48h), 1 week (+1W), 2 weeks (+2W) and 4 weeks (+4W) exposure in LUFA 2.2 soil mixed with plant material. Exposures were done using 1% freeze–dried fragmented plant material compared to dry weight of LUFA 2.2 soil. SAV = Savoy, PSB = Purple Sprouting Broccoli and WIN = Winspit. Arrows indicate the sequence in time, i.e. temporal changes in bacterial community structure, exposed to a specific genotype. NMDS is an unsupervised statistical technique to visualise a the distance between data points (dissimilarity matrix of Pearson correlation coefficients) (Hammer et al., 2001). This data set used 'user similarity' and 2D visualisation, resulting stress 2D = 0.188 (a stress measure smaller than 0.3 is deemed a good fit of the data).

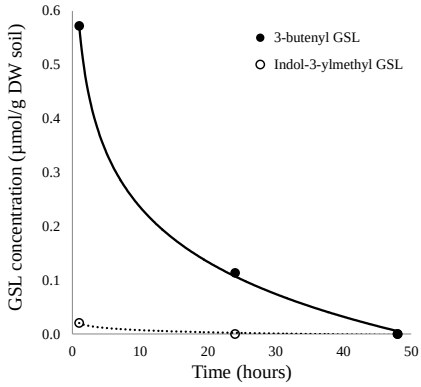

Figure 5.6: Fate of GSLs originating from Winspit after incorporation of plant material in LUFA 2.2 soil. Presence of detectable GLS after incorporation of Winspit plant material in LUFA 2.2 soil. Exposures were done using 1% freeze–dried fragmented plant material compared to soil dry weight.

5.4.5 GSL analysis of the soil after biofumigation

The fate of GSLs was evaluated after incorporation of fragmented plant material into LUFA 2.2 natural soil. Glucosinolates could only be detected in soils treated with Winspit. Already one hour after biofumigation 95% of the potential amount of 3–butenyl GSL (gluconapin) and 43% of potential indole–3–ylmethyl GSL (glucobrassicin) could be detected in the soil (Figure 5.6), based on the total GSL content contained by the Winspit plant material incorporated into the soil. These two GSLs together comprised 72% of potential total GSLs. However, both 3–butenyl GSL (gluconapin) and indole–3–ylmethyl GSL (glucobrassicin) disappeared completely after 48 hours (Figure 5.6). 2–Propenyl GSL (sinigrin), the second most abundant GSL in the Winspit material, was completely undetectable. Also 3–methylsulfinylpropyl GSL (glucoiberin), the main GSL of PSB, could not be detected at all, regardless of the substantial amount present in the original plant material.

Of all the potential ITCs that could be detected in the soil after biofumigation only 3–butenyl ITC, the hydrolysis product of 3–butenyl GSL (gluconapin) was detected. The highest quantity of 3–butenyl ITC was determined one hour after biofumigation, of which approximately 90% disappeared after 24 hours. Small quantities could, however, be detected in the soil after one week. Further specific quantification of the ITC proved difficult, therefore only qualitative and relative analyses could be done.

5.5 Discussion

5.5.1 A high AGSL content in the wild accession Winspit

In general, very low AGSL contents were found in the leaves of the two cultivated genotypes and much higher contents in the plants from the wild accession. The higher contents of AGSLs in Winspit plants was also described by Gols et al. (2008), when comparing Winspit to the Brussels sprouts cultivar 'Cyrus' or to other wild *B. oleracea* populations. The general absence of 2–propenyl GSL (sinigrin) in the cultivated genotypes, was also previously found by Gols et al. (2008), but seems to be typical for wild populations of *B. oleracea* (Mitchell and Richards, 1979).

Although Savoy cabbage in general has been reported to contain high GSL levels, including 2–propenyl GSL, compared to other cabbage varieties (Sones et al., 1984), this was not the case for the cultivar 'Wintessa', most likely reflecting a relatively large standing variation in GSL profiles and quantities among different Savoy cabbage varieties. The absence of 2–propenyl GSL sinigrin in PSB was expected, based on earlier analyses by (Lewis et al., 1991). It is also in line with the claimed high levels of glucoiberin in the purple curds of 'Santee' (www.freshplaza.com).

5.5.2 High expression of GSL biosynthesis related genes in Winspit

In the two WIN plants producing sinigrin, the expression of the *BoAOP2* gene encoding *2–oxoglutarate–dependent dioxygenase* was up–regulated when compared to PSB and SAV. The *BoAOP2* gene is responsible for the conversion of methylsulfinylalkyl–GSLs into alkenyl–GSLs, and thus has an essential role in the production of 2–propenyl GSL (sinigrin). Therefore, the wild *B. oleracea* Winspit is very likely to contain a functional copy of *BoAOP2*, which ensures the conversion of 3–methylsulphinyl propyl GSL (glucoiberin) in 2–propenyl (sinigrin). The expression of *BoAOP2* can strongly affect the properties of *Brassica* genotypes regarding GSL composition. For instance, *BoAOP2* is non–functional in *B. oleracea* L. var. italica (broccoli) (Gao et al., 2004), which is the reason for the high 4–methylsulphinylbutyl GSL (glucoraphanin) content and which substantiates the health claims for consumption of high glucoraphanin containing broccoli to protect against cancers (Juge et al., 2007).

5.5.3 *GSL–ELONG*, *BoMYB29* and *BoAOP2* expression contributes to the high AGSL content in Winspit

The correlation between AGSL content and the relative expression level of the *GSL–ELONG*, *and 2–oxoglutarate–dependent dioxygenase (BoAOP2)* genes with that of the

BoMYB29 transcription factor gene in the studied *B. oleracea* plants, suggests that *BoMYB29* regulates the expression of *BoGSL–ELONG* and *BoAOP2* in *B. oleracea*. Such would be very similar to what has been found for *A. thaliana*, where overexpression of *AtMYB29* contributes to expression of *AtMAM1*, the *A. thaliana* orthologue of *BoGSL–ELONG* (Gigolashvili et al., 2008), *AtAOP2* and several other genes in the AGSL pathway (Sønderby et al., 2007). Unfortunately the analysis of potential knock–out mutants of *AtMYB29* was not conclusive as to its direct transcriptional targets in *A. thaliana* (Sønderby et al., 2010).

The high AGSL concentration in WIN7 was mainly due to the high production of 3–butenyl GSL (gluconapin). This alkenyl–GSL is the direct product of the conversion of 4–methylsulphinylbutyl GSL (glucoraphanin), which reaction is catalysed by the 2–460 oxoglutarate–dependent dioxygenase encoded by the *AOP2* gene. However, in order to have high levels of the end product, also high levels of the substrate need to be provided, which is taken care of by the upstream steps in the GSL biosynthesis pathway. In *B. oleracea*, two steps involved in the amino acid chain elongation part of the AGSL biosynthesis pathway have been identified; one involving the *GSL–PRO* genes, controlling the production of 3–carbon (C3) side–chain AGSLs and the other is encoded by *GSL–ELONG* which is responsible for the production of 4–carbon (C4) side–chain AGSLs (Li and Quiros, 2002; Li et al., 2003). In *B. oleracea* there are two genes for each of these steps known (Figure 5.3). The high expression of *BoGSL–ELONG* contributes to the increased production of 3–butenyl GSL (gluconapin) at the expense of 2–propenyl GSL (sinigrin), which in WIN7 appears to be mainly dependent on expression of the *BoGSL–PRO–L* gene rather than the *BoGSL–PRO* gene, which is not expressed. Thus, the relatively high production of gluconapin in three of the four Winspit plants appears to be mainly due to the higher expression of *BoGSL–ELONG* compared to the other genotypes. This assumption is supported by experiments performed in *B. napus*, where silencing of the *GSL–ELONG* gene significantly induced the production of 2–propenyl GSL (sinigrin). Sinigrin had not been detected before in the original *B. napus* line, thus illustrating the importance of this gene for GSL composition (Liu et al., 2011). Our results indicate that a similar pathway is present in *B. oleracea*, since *BoMYB29* up–regulates *GSL–Elong* expression in *B. oleracea*, favouring the production of but–3–enyl GSL instead of the 2–propenyl GSL. Therefore, a breeding program aiming at producing a more efficient biofumigant *Brassica* plant should take into account the overexpression of the *MYB29* transcription factor (or any of its paralogues *MYB28* and *MYB76* (Sønderby et al., 2010) the up–regulation of *GSL–PRO* gene and silencing of *GSL–ELONG*.

Zang et al. (2009) and Wang et al. (2011) described the AGSL pathway in *B. rapa*, thus creating a comprehensive map of the involved genes. In contrast, knowledge about the AGSL pathway in *B. oleracea* is limited. Our expression analysis results contribute to the elucidation of the AGSL pathway in *B. oleracea*. The expression of

most homologues of *B. rapa* genes was found for the aliphatic GSL genes analysed in the studied *B. oleracea* genotypes. However, for the *BoST5b* and *BoGSL–OH* genes, one paralogue identified in *B. rapa*, was not found in *B. oleracea*. Therefore, these two genes may be not present or not expressed in *B. oleracea*. These findings, together with the GSL content and composition analyses (in the corresponding plants), suggest that there is a high potential to modulate the AGSL pathway towards the development of improved genotypes for biofumigation.

5.5.4 Biofumigation using Winspit plant material caused negative effects on soil invertebrates but not on the bacterial community

Springtail survival and reproduction and earthworm reproduction almost completely corresponded to the total GSL content levels of plant material used from the three genotypes (Figure 5.4), making the GSL content the most likely cause of the effect, rather than the GSL composition. For springtails, however, experiments with single pure compounds have shown that there can be an extensive difference between the effects of different ITCs on survival and reproduction of soil invertebrates. For instance, 2–phenylethyl ITC is approximately five times more toxic than benzyl ITC, when regarding the reproduction of *F. candida* and *F. fimentaria* respectively, even though these compounds differ in just one CH_2–group (van Ommen Kloeke et al., 2012b). In this study, the most predominant GSL in PSB was glucoiberin, while the predominant GSLs in WIN were sinigrin and gluconapin. The toxic effects of the pure ITCs corresponding to glucoiberein, sinigrin and gluconapin on soil invertebrates are currently unknown. In this respect, we cannot fully acertain if total GSL content or the presence of a particular GSL in the leaf material was responsible for the observed toxic effects on the invertebrates in the data.

Surprisingly, earthworm survival was not affected by the *B. oleracea* plant material. In studies with single compounds, earthworms are regularly found to be less sensitive than springtails, as for instance was found for the insecticide carbofuran (Chelinho et al., 2012) and 2–phenylethyl ITC (unpublished data). Moreover, earthworms are known to have a high tolerance towards heavy metals. This tolerance is usually attributed to their evolutionary adaptation as they frequently burrow through the top organic layer of soil enriched with both humus (food) and natural toxins–containing leaf litter (Morgan et al., 2007). These characteristics may also be responsible for the higher tolerance of adult earthworms towards the *B. oleracea* plant material.

Overall, the bacterial community was quite resilient to biofumigation with a peak effect only after one week, and restoration to original values the week thereafter. In contrast, Rumberger and Marschner (2003) observed a change in soil microbial community structures even at 3970 pmol of 2–phenylethyl ITC per gram soil. However,

they analysed community structure only once, after five days of exposure. This exposure time is close to when we found the largest, but transient, differences in our study. (Bending and Lincoln, 2000) showed that both aliphatic and aromatic ITCs had an inhibitory effect on soil nitrifying bacteria. Our study, however, focussed on changes in the overall bacterial community and as such no conclusions can be made considering specific groups of micro–organisms.

5.5.5 GSLs disappear quickly from soil after biofumigation

The data in this study show that the GSLs disappear very quickly from the soil after incorporation of the plant material, which is in line with previous studies that showed GSLs to disappear very rapidly from soils, mainly due to hydrolysis into ITC. For instance, Gimsing and Kirkegaard (2009) concluded that the rate of disappearance depended on the method of incorporation and release efficiency. It seems that the methods we used, freeze–drying and complete fragmentation with a laboratory blender, was effective in rapidly converting the GSLs.

During optimal biofumigation practises up to 100 nmol/g ITC can be released in the soil and an efficiency of conversion measured under field conditions is around 60% Gimsing and Kirkegaard (2009). In our study, the potential amounts released were estimated to reach 88 nmol 3–butenyl ITC (gluconapin) per gram soil (1% DW plant material compared to DW soil, with an average of 14.65 μmol 3–butenyl GSL per gram DW plant material), if a comparable conversion efficiency is assumed. The amounts of GSL–containing plant material applied in this study are thus comparable to those found in the field.

In conclusion, our results demonstrate that *B. oleracea* plant material, enriched in AGSL, is toxic for non–target soil invertebrates, despite the fact that their hydrolysis products are quickly broken down in natural soil. Especially springtails seem to suffer from elevated AGSL containing plant material when incorporated in the soil. The presented data can be useful in the optimization of biofumigation practises, using the gene expression pathways important for AGSL production that are shown in this study.

Future studies should, however, focus on effects of biofumigation under more realistic conditions of agricultural practise. For instance the effects of root material instead of leaf material should be further investigated. Roots are known to have different GSL content and composition compared to leaf material, including some GSLs that can hydrolyse into the more toxic aromatic ITCs (van Dam and Raaijmakers, 2006). Moreover, our study made use of freeze–dried and completely fragmented plant material, in order to ensure maximal GSL hydrolysis (Gimsing and Kirkegaard, 2009). In the field, however, it will be more likely that fragments are larger, fresh instead of freeze–dried or complete plants parts left behind after harvesting. This

will surely decrease the release of GSLs and subsequent hydrolysis into ITCs and thus moderate the biofumigation potential and effects on soil invertebrates.

The results presented in this paper are part of the design of a decision matrix to investigate potential risks of novel crops, such as future genetically modified *Brassica* with elevated GSL levels, on the soil ecosystem. Such a decision matrix is crucial in order to have a full understanding of these risks before high levels of natural toxins, such as ITC, are allowed in the field. The identification of *B. oleracea* plants rich in toxic AGSL and testing their effects on beneficial soil invertebrates represents the first step to establish this decision matrix.

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Supplementary information

Can be obtained digitally upon request

Table S1: List of PCR primers
Table S2: Gene Ontology analysis of differentially expressed genes
Table S3: Differentially expressed GSL genes between WIN, PSB and SAV
Table S4: Differentially expressed genes between WIN, PSB and SAV
Figure S1: Principle component analysis of the array samples.

CHAPTER 6

Exposure of soil ecosystem engineers through toxin–containing litter does not affect soil processes

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6.1 Abstract

Soil processes, such as decomposition, are dependent on the identity and interactions between soil invertebrates. Interactions between species can have non–additive effects on soil processes compared to the sum of single species effects (additive effects). Toxic stress may affect single species differently within a species assemblage and in turn may alter these non–additive or additive effects on soil processes. This study investigated if plant material containing toxic compounds could induce chemical stress on soil invertebrates, alter faunal survival and affect soil processes in single and multiple species treatments. Toxins included glucosinolates (GSL) and cadmium (Cd) which

were added to the natural LUFA 2.2. soil via mixing of fragmented leaf material containing toxic compounds. Overall, exposure of soils via plant material containing toxic compounds induced chemical stress on invertebrate species (springtails) performance, but did not influence CO_2 production. This was mainly because earthworms were not affected by the imposed chemical stress and were the sole species responsible for observed changes in CO_2 production. Non–additive effects of multiple species were not observed, regardless of toxic stress, even though earthworm presence positively affected springtail survival. Results confirm the important role of earthworms as ecosystem engineers and that effects of species assemblages on soil processes depend on the functional identity. The current study shows that exposure of soil ecosystem engineers through litter containing toxic compounds, does not affect soil processes

6.2 Introduction

Soil invertebrate species perform many essential functions, which contribute to the maintenance of a healthy soil ecosystem. For instance, litter decomposition, nutrient cycling and soil physical structure all benefit from the belowground activities of soil invertebrates (Bardgett, 2005; Bradford et al., 2002; Lavelle et al., 2006). The detritivorous community is extremely diverse with many species performing a specific function within a complex interactive food web, e.g. leaf litter fragmentation, bioturbation and fungal grazing (Wardle, 2002; Hättenschwiler et al., 2005). Recent biodiversity–ecosystem function experiments have shown that soil processes depend especially on the identity (function) of soil invertebrates, and not species richness per se (Hättenschwiler et al., 2005; Heemsbergen et al., 2004; Hooper et al., 2005; Loreau and Hector, 2001). In addition, interactions between species may result in additive or non–additive (facilitation or inhibition) effects on soil processes compared to expected performance based on individual species effects, depending on functional identity of the species (Heemsbergen et al., 2004; Loreau and Hector, 2001; Zimmer et al., 2005). For instance, combined activities of isopods (litter fragmenters) and earthworms (bioturbation), have shown positive and negative non–additive effects on decomposition processes depending on the food source (Zimmer et al., 2005). Both species facilitate the soil microbial community by altering e.g. soil organic matter quality and structure and soil properties (Edwards and Bohlen, 1996; Hättenschwiler et al., 2005). Additive effects on soil processes take place if co–occurring species perform similar functions and predict that loss of one species does not necessarily result in changes in soil processes, known as functional redundancy (Laakso and Setälä, 1999; Setälä et al., 2005). In short, soil processes, such as decomposition, are dependent on functional identity and interaction of co–occurring species within the soil food web.

Studies investigating soil processes and food webs commonly describe effects observed in unstressed conditions. Effects of species assemblages on soil processes under stressed conditions, due to for instance the presence of toxins in litter, lacks attention. Species can react differently to chemical stress, which may potentially affect soil processes through changes in the relative abundance of functionally different species or their interactions (Salminen et al., 1995). For instance, decomposition rate (CO_2 production) of spruce needle litter from heavy metal contaminated sites in Sweden significantly declined with increasing heavy metal concentration in the leaf litter due to a reduction in fungal activity (Ruhling and Tyler, 1973). Chemical stress for decomposers can also result from litter containing toxic compounds via, for instance, agricultural practices such as biofumigation, which involves incorporation of glucosinolate (GSL)–containing plant material in the soil (Matthiessen and Kirkegaard, 2006). Another example is the re–location of contaminated plant debris from phytoremediated soils, which may expose soil ecosystems to heavy metals such as cadmium (Meagher, 2000). Cadmium and GLS are both known to have detrimental effects on soil invertebrate survival and reproduction at concentrations encountered in field sites (Crommentuijn et al., 1995; van Ommen Kloeke et al., 2012a,b). Although direct effects of such chemical stress on individual species are usually known, the consequences of changes in the species performance under multiple species assemblages on soil processes are hardly investigated.

Possible effects of chemical stress on species performance and soil processes could be modified by the presence of particular species, depending on their functional identity. For instance, earthworm bioturbation increases microbial activity, which can indirectly increase the availability and mobility of metals (Sizmur and Hodson, 2009) and possibly other chemical compounds. As a result, earthworms may increase toxicity levels and, in turn, negatively affect survival of other species with consequences for soil processes (negative non–additive effect). On the other hand, increased microbial activity can enhance biodegradation of organic compounds (Gimsing and Kirkegaard, 2009), neutralizing the toxic effects (positive non–additive effect). Moreover, in case earthworms are the most sensitive species in a species assemblage under chemical stress conditions, the above mentioned non–additive effects may reverse of disappear.

Here we test such hypotheses by investigating the effect of plant material containing toxic compounds on soil fauna performance (e.g. survival) and soil processes (e.g. CO_2 production), using microcosms with control and toxic stress conditions. Two types of toxic compounds were tested: glucosinolates (GSL) and cadmium (Cd). Glucosinolates are natural phytotoxins found in varying concentrations in a large number of *Brassicaceae* (van Dam et al., 2009). The heavy metal Cd is found in a group of plants known as hyperaccumulators, in which foliar concentrations of Cd can reach up to 100 times the concentration in non–accumulating plants (Richau and Schat, 2009). In this study three soil invertebrate species were chosen as representatives

for three different feeding groups: *Protaphorura fimata*, a herbo–fungivorous collembolan, the earthworm *Eisenia andrei*, a detritivorous ecosystem engineer, and the isopod *Porcellio scaber*, a leaf litter fragmenter. All three species indirectly enhance soil processes such as CO_2 production, but have a different effect on soil respiration due to their differences in feeding strategy (Bardgett, 2005). Species were tested in monocultures and different combinations of multiple species, as detrimental effects of toxins co–occurring species in the community may alter the effect of toxins on soil processes. In the current study, earthworms are hypothesized to enhance toxic effects of Cd and reduce toxic effects of GSL on CO_2 production and faunal survival. This is the first study to investigate effects of plant material containing toxic compounds on soil processes via soil invertebrate performance and interactions.

6.3 Materials and methods

6.3.1 Experimental design

To investigate the effect of leaf material containing toxic compounds on soil invertebrate species survival and soil processes, two experiments were conducted; one with GSL containing leaf material and one with Cd containing leaf material (see section 6.3.3). Both experiments were performed in open–top non–transparent poly–ethylene microcosms (12.5 ∅, 8.5 cm deep), with a mesh (250 μm) covering the bottom and a transparent Perspex lid with mesh (500 μm) in the centre. This system allows air circulation and entry of moisture. Each microcosm contained 250 g moist prepared soil (see section 6.3.4) and a combination of soil invertebrates according to the design shown in Table 6.1. Invertebrate species combinations consisted of a monoculture containing only a single species, or a mixed assemblage consisting of multiple species, which resulted in a total of 7 invertebrate species treatments per plant species. Monoculture treatments had seven replicates and mixed assemblage treatments 5 replicates. In addition, microcosms without any invertebrates served as control treatment for animal addition (Table 6.1) and replicated seven times. Total animal DW biomass was kept similar over all treatments and across species to control for any biomass effect on the data (Table 6.1). This also entailed pre–selecting individuals from stock cultures that would fall into particular weight and/or size classes (see section 6.3.2). For the earthworms and isopods this could be done by taking equal total weight of animal biomass. In case of springtails this was impossible, as this would have resulted in overcrowding. Springtail densities in the field can range up to 8500 per m^2 in sandy soils (Wallwork, 1970), which would result in having over 3000 springtails per monoculture. Instead 1000 springtails were used in the monoculture and this number was adjusted accordingly when springtails were part of mixed communities.

Table 6.1: Overview of soil fauna assemblage compositions in the microcosm experiments. Assemblages were exposed to LUFA 2.2. soil mixed with either glucosinolate or cadmium containing leaf material (or their corresponding control treatment soils) for 21 days.

code	**N**	**species composition**	**No. of individuals**		
			P. fimata	*P. scaber*	*E. andrei*
No	7	None animal	–	–	–
Pr	7	*Protaphorura fimata*	1000	–	–
Po	7	*Porcellio scaber*	–	12	–
Ei	7	*Eisenia andrei*	–	–	8
Pr&Po	5	*P. fimata* & *P. scaber*	500	6	–
Pr&Ei	5	*P. fimata* & *E. andrei*	500	–	4
Po&Ei	5	*P. scaber* & *E. andrei*	–	6	4
Pr&Po&Ei	5	*P. fimata* & *P. scaber* & *E. andrei*	333	4	3

code short code used to describe the species composition, *N* number of replicates

Small pieces of ceramic roof tile (ca. 2–4 cm) were added to microcosms containing *P. scaber* (isopod), as this is a non–burrowing species which has to hides during the day under substrate. The experiments were performed in a climate–controlled chamber of 20°C, 75% relative humidity and 12:12 hour light:dark cycle (no direct light source) and lasted for 21 days. Eight microcosms were placed in a randomized block design on plastic trays, one of each treatment per tray. Twice per week moisture content was checked by measuring loss in total weight on a balance and replenished to keep moisture conditions constant by adding demineralized water.

At the end of the experiment, surviving animals were retrieved per species and counted to investigate species survival. Hand sorting was used to collect isopods and earthworms. Springtails were collected by using flotation with tap water, gentle stirring to assist trapped individuals and scooping all surviving individuals from the water surface with a small filter. Dry weight was determined of all surviving animals after freeze–drying on a Mettler Toledo micro balance (till the nearest 0.01 mg).

6.3.2 Animals

All animal species used in the experiments originated from laboratory stock cultures maintained at the VU University, Amsterdam. Each species has been maintained

under standard conditions as described below and housed in climate chambers with a 12:12 hours light:dark cycle and 75% relative humidity (RH).

The stock culture of *Protaphorura fimata*, a herbivorous collembolan (Endlweber et al., 2009) was maintained at 20°C on moistened plaster of Paris mixed with charcoal (360 g plaster: 400 ml tap water: 30 g charcoal). Cultures received *ad libitum* quantities of baker's yeast refreshed on a weekly basis. For the experiments, individuals were chosen based on size and selected using a sieve (mesh 3.0 mm).

Eisenia andrei, an epigeic compostworm was maintained at 20°C in large plastic containers filled with a mixture of horse dung and pot soil. Stocks were given a fresh batch of horse dung (without antibiotics) every week. Test organisms were all sexually reproductive, a clitellum was present, and had a maximum age difference of one month. Before the experiments, individuals were weighed to check if their body mass corresponded to the selected weight class necessary for the experiments (see section 6.3.1). All individuals used in the experiments thus had a body weight between 204–337 mg fresh weight (FW) corresponding to 30.1–45.3 mg dry weight (DW).

The isopod *Porcellio scaber* was kept at 16°C in glass aquarium filled with a layer of moistened pot–soil topped with a mixture of *ad libitum* dry leaf litter from various deciduous trees (e.g. *Acer campestre*, *Quercus petraea* and *Corylus avellana*). Individuals were selected based on their size (body length), as their size is correlated with body weight (Supplementary information Figure 6.4). All individuals used in the experiments had a body length of 10–12 mm corresponding to 16.9–28.5 mg DW. Gravid females were avoided to prevent reproduction in the experiments.

6.3.3 Plant material

For the experiment with glucosinolate (GSL)–containing plant material, two different genotypes of *B. oleracea* were used; Winspit, a wild *B. oleracea* originating from the coast of Dorset (England) and the cultivated Savoy cabbage. Winspit is known for its high GSL concentration whereas for Savoy low quantities of GSL in the leaf tissue have been shown (Zuluaga et al., *unpublished*, thesis chapter 5). Winspit seeds were kindly provided by Dr. Rieta Gols (Entomology Department, Wageningen University, the Netherlands) and seeds of F1 Savoy cabbage (*B. oleracea* L. var. *wintessa*) were provided by Bejo Zaden B.V. (www.bejo.nl). Plants were grown from seeds in a greenhouse, using 17 cm ∅ pots containing a peat–based commercial potting compost (Lentse potgrond nr. 4; 85% peat, 15% clay). Climate conditions were a 16:8 hours light:dark cycle, temperatures 23/19°C day/night and RH 75%. Plants were watered once a day. After approximately eight weeks, healthy, fully expanded leaves were harvested, snap frozen in liquid nitrogen, freeze–dried at –40°C (Ly ph–Lock 6,

Labconco or Modulyo, Edwards) and stored at room temperature until the start of the experiment.

For the experiment with Cd–containing plant material, the heavy metal hyperaccumulator *Noccaea caerulescens* (Ganges ecotype, southern France) was used. Five weeks after sowing, individual seedlings were transferred to a hydroponics system containing Hoagland's nutrient solution (Richau and Schat, 2009). The solution was changed twice a week. Growth conditions were kept standard in a climate chamber with a 14:10 hours light:dark cycle, temperatures 20/15°C day/night, photon flux density 250 μmoles/m^2/s at plant level, and 75% RH. Seven weeks hereafter, 25μM Cd was added to the Hoagland solutions of half of the grown plants whereas the other half received only the Hoagland solutions. The Cd treatment lasted for two weeks after which all plants were harvested, roots were separated from leaf parts with a scissor, snap frozen, freeze–dried at –40°C (Ly ph–Lock 6, Labconco or Modulyo, Edwards) and stored at room temperature until further use. Only leaf material was used for experiments.

6.3.4 Soil preparation

For all experiments, the natural soil LUFA 2.2 (Speyer, Germany) was used, which is a loamy sand soil with a pH of (± standard deviation, SD) 5.5 ± 0.1 and an organic C content of 2.09 ± 0.40%. Before usage the soil was dried at 60°C for 24 hours. The soil was mixed with freeze–dried, fragmented leaf material of either *B. oleracea* for the GSL experiment or *N. caerulescens* for the Cd experiment. The DW of freeze–dried leaf material relative to total soil DW was 1%. Leaf material was completely fragmented using first a kitchen blender (Tomado, maximum speed) to fragment leafs into large fragments and hereafter using a laboratory blender (IKA, A11 basic, one speed) to fragment the material into powder. The fragmenting process was used to ensure maximum tissue rupture and release of toxic compounds (Gimsing and Kirkegaard, 2009). Fragmented leaf material was mixed through the soil by hand and kitchen blender to ensure a homogeneous distribution of the leaf material. Finally, the soil was moistened (at once) with demineralised water to 50% of the maximum water holding capacity, corresponding to a water content of 22% of soil DW. For both the original leaf materials and the mixed soil, total nitrogen, total carbon and total organic matter were determined (Supplementary information Table 6.5). Total carbon and nitrogen were determined by dry combustion with a Flash EA1112 elemental analyzer (Thermo Scientific, Rodana, Italy). Organic matter content of the soil was determined as loss on ignition at 500°C for 6 hours.

6.3.5 GSL analyses

Total GSL concentration and composition in the *Brassica* leaf material was determined, using 10 mg of freeze–dried and fragmented material. Glucosinolates were extracted with 1 ml of 80% methanol solution containing 0.05 mM intact 4–hydroxybenzyl GSL as internal standard, desulfatased with arylsulfatase (Sigma–Aldrich) on DEAE Sephadex A 25 columns overnight. The resulting desulfoglucosinolates were eluted with 0.5 ml demineralized water and were separated using high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies, Waldbronn, Germany) on a reversed phase C18 column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5 μm, Macherey–Nagel, Düren, Germany) with an water–acetonitrile gradient (1.5% acetonitrile from 0–1 min, 1.5–5% acetonitrile from 1–6min, 5–7% acetonitrile from 6–8min, 7–21% acetonitrile from 8–18min, 21–29% acetonitrile from 18–23min, followed by a washing cycle; flow 1 ml min–1). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. We used the following response factors: aliphatic GSL 2.0, indole GSL 0.5 for quantification of individual GSL (Burow et al., 2006).

6.3.6 Cadmium analyses

Total Cd concentration was measured in leaf material, in soil after mixing with the leaf material and in the earthworms, isopods and springtails from the monoculture treatments. Three samples of ± 100 mg were taken per treatment, freeze–dried or dried for 24 h at 60°C and digested in a mixture of Milli–Q, concentrated HCl and concentrated HNO_3 (1:1:4 by vol.) using an oven (CEM MDS 81–D) for 7 h at 140°C. Digested samples were analysed for total Cd concentration by flame atomic absorption spectrometry (AAS: Perkin–Elmer 1100B).

Total Cd concentration in springtails was measured via mini–destruction. Five samples of individual, freeze–dried springtails per monoculture treatment were put in a block heater with HNO_3 and HNO_4 solution (Ultrex 2 (71%) and Ultrex; 7:1). After evaporation to dryness, residues were taken up in 300 μl 0.1 M HNO_3 and analysed by graphite furnace AAS (Perkin Elmer 5,100). Dolt–2 was used a reference material (Broerse et al., 2012).

Pore water concentration of Cd was measured to analyse the water available fraction of Cd. Three samples of ± 50 g wet soil were randomly taken from the batches of biofumigated soil, pooled per plant treatment. After saturation with demineralised water (100% WHC) and 24 h equilibration, pore water was collected by centrifugation (Centrifuge Falcon 6/300 series, CFC Free). Centrifugation was done over two round filter (S&S 11 μm) and a membrane filter (Whatman 0.45 μm) for 50 min at 2,000 g relative force (Hobbelen et al., 2004)). Approximately 10 ml pore water per sample

was collected for analysis by flame AAS (Perkin–Elmer 1100B).

6.3.7 Soil variables

To investigate soil ecosystem processes, CO_2 production, total NH_4^+ and NO_3^- content were determined. CO_2 production (ppm) was measured for all microcosms one day after the start and hereafter every week during the experiment, using an EGM–4 infrared gas analyzer (IRGA: PP Systems, Hertfordshire UK). Measurements were taken at t = 0 and t = 20 minutes after closing the microcosms with an air–tight, poly–ethylene bottom and lid, the latter including a hole with a silicon plug. Just before closing the microcosms, the inner space was rinsed with compressed air to eradicate any previous build up in CO_2. One sample of 5 ml of head space was taken per measurement with a syringe by penetrating the silicon plug, after which the sample was directly inserted into the IRGA to measure CO_2 content. CO_2 production was calculated by subtracting the t = 0 CO_2 content from t = 20. Measurements were corrected for background soil respiration by subtracting the average CO_2 production measured in the 'None animal' (without any invertebrates) treatment (Table 6.1).

NH_4^+ (mg/L) and NO_3^- (mg/L) were measured at the start and end of the experiments, by taking 10 g FW soil to be extracted with 1M KCl solution. Samples were shaken for 1 h at 200 rpm with 25 ml KCl solution, centrifugated and filtered over a 1.2 μm Whatman GF/C glass fiber filter. NH_4^+ and NO_3^- were measured photometrically by means of the indophenolblue method and the sulfanylamide/naphtyl–ethylene–diamine method, respectively (Skalar SA–40 continuous flow analyzer, Skalar, Breda, The Netherlands). These measurements were taken on the combined soil from all replicates per invertebrate species treatment, due to insufficient soil.

6.3.8 Data analyses

Survival data (percentage) was investigated to determine whether or not a species would perform better in the presence of other species both in control and stressful conditions. Separate analyses were carried out for the two experiments and for each invertebrate species using a 2–way ANOVA with the factors: 'exposure' (leaf material treatment) and two of the three factors 'presence of *P. fimata*', 'presence of *E. andrei*' and 'presence of *P. scaber*', depending on the species investigated. A possible block effect was investigated, including it as a random factor in the design. As block effect was not significant for both experiments, it was omitted from the model. The ANOVA assumptions of normality and homogeneity of variances were examined. In case they were not met, only highly significant p–values ($p < 0.01$) were considered.

Furthermore, the effect of soil fauna assemblage on CO_2 production, through time, was analyzed with a 'split–plot repeated measures' design which uses the same

linear model as used in regular ANOVA analyses (Quinn and Keough, 2002). As within factor 'Time' was used, which encompasses the CO_2 measurements for all time points. The between factors were 'exposure' and 'species presence', as described before. A possible block effect was investigated, including it as a random factor in the design. For data from the GSL experiment block was not significant and therefore omitted from the model. However, data from the Cd experiment did show a significant effect for block, which had an interaction with time (Greenhouse–Geisser correction, $F= 57.868$, $p < 0.001$) and was therefore kept as a factor in the model. A smaller model, with fewer factors and interactions, was also tested. Two data points in the Cd experiment (control treatment) and one data point in the GSL experiment (Winspit treatment) showed values that deviated more than 2.5 times the standard deviation of all the measurements within that treatment (including the data points). These data point were therefore marked as outliers and replaced by the average of their treatment. All residuals were checked and had a normal distribution (one sample Kolmogorov–Smirnov test, $p > 0.1$). For most analyses, sphericity (ε) could not be assumed, in which case the Greenhouse–Geisser adjustment was used. Moreover, we calculated the net diversity effect (Heemsbergen et al., 2004) on CO_2 production. The net diversity effect ($\triangle Y$) is the difference between the observed soil function value (O) to the expected value (E), based on the average performance of the corresponding monocultures:

$$\triangle Y = O - E$$

If the net diversity effect deviates from zero, it suggest that the species involved either facilitate each other ($\triangle Y > 0$) or have an inhibitory effect ($\triangle Y < 0$) on each other.

To compensate for changes in biomass during the experiment, CO_2 data was divided by the total biomass (DW) of the corresponding microcosm. This entailed that 'start CO_2' values were divided by total biomass measured at the start of the experiment and 'end CO_2' values by the total biomass measured at the end of the experiment. Start biomass DW was calculated from a dataset on DW to FW correlations for the species used in the experiments (Supplementary information Figure 6.4). Net diversity effects was also analyzed with a 'split–plot repeated measures' design with 'Time' as within factor (start and end time point) and 'exposure' and 'community' as between factors, describing the four different community compositions used in the experiment. A possible 'block' effect was investigated, including it as a random factor in the design. For data from the GSL experiment 'block' was not significant and therefore omitted from the model. However, data from the Cd experiment did show a significant effect for 'block' and was therefore kept as a factor in the model, although it did not change the final results. Except for the net diversity effect data of week 2, all residuals had a normal distribution (one sample Kolmogorov–Smirnov test, $p >$

0.1), which could not be solved by transforming the data. For most analyses sphericity (ε) could not be assumed, in which case the Greenhouse–Geisser adjustment was used. All statistical analyses were performed in SPSS 20.0.

6.4 Results

6.4.1 Glucosinolate concentration and composition

Total GSL concentration and composition was measured for the *Brassica oleracea* leaf material to confirm differences in GSL between Winspit and Savoy varieties. As expected, Winspit showed a higher GSL total concentration (Figure 6.1), which was mainly due to the high concentrations in 3–butenyl GSL.

6.4.2 Cadmium concentration

Cadmium (Cd) was detected in all components of the exposure route: in leaf material, soil mixed with leaf material, soil porewater, and invertebrates (Table 6.2). For leaf material, soil with leaf material, and soil porewater Cd–exposed material had a higher concentration than control material (all three comparisons, Student's t–test, $p < 0.001$).

Furthermore, presence of *E. andrei* or *P. scaber* did not affect the Cd concentration in the soil pore water compared to Cd–exposed soil pore water without any invertebrates. Cd concentration was also significantly higher in two of the three exposed invertebrates species; *E. andrei* had Cd levels more than 20 times higher then control animals (Student's t–test, $p = 0.001$) and *P. scaber* showed more than 2.5 times higher Cd levels compared to control animals (Student's t–test, $p = 0.003$). Between *E. andrei* and *P. scaber* a significant difference was found (Student's t–test, $p = 0.002$), with *E. andrei* having on average more than 4.5 times higher Cd levels than *P. scaber*. For *P. fimata* no significant difference was found between Cd–exposed and control animals.

6.4.3 Species survival

Glucosinolate experiment

Significant differences in the survival of *P. fimata* were found between treatments, which were explained by three main effects (Table 6.3), plant treatment, presence of *E. andrei* and presence of *P. scaber*. Survival decreased in presence of Winspit (high GSL levels) leaf material compared to Savoy leaf material (exposure effect, $F = 179.315$, $p < 0.001$). Furthermore, survival of *P. fimata* increased in the presence

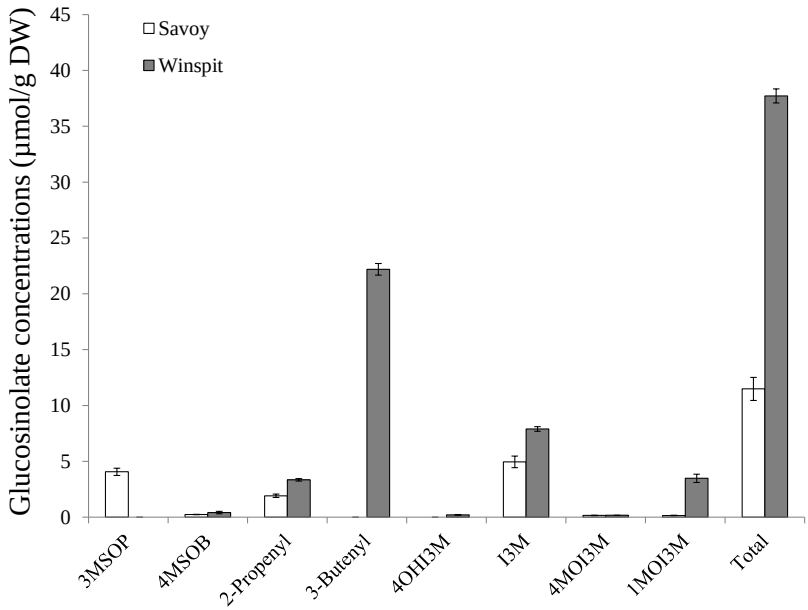

Figure 6.1: Glucosinolate (GSL) concentration and composition present in the leaves of *Brassica oleracea* types Savoy and Winspit. 3MSOP = 3–methylsulfinylpropyl GSL, 4MSOB = 4–methylsulfinylbutyl GSL, 2–propenyl = sinigrin/allyl GSL, 3–butenyl = 3–butenyl GSL, 4OHI3M = 4–hydroxy–indol–3–ylmethyl GSL, I3M = indol–3–ylmethyl GSL, 4MOI3M = 4–methoxy–indol–3–ylmethyl GSL, 1MOI3M = 1–methoxy–indol–3–ylmethyl GSL. Error bars represent standard deviation (n = 4).

Table 6.2: Concentrations of cadmium (ug/g) measured in *Noccaea caerulescens* leaf material, in soil treated with leaf material, soil pore water, and invertebrates exposed to the treated soil.

	control		**cadmium**	
leaf material	0.51	*0.04*	288	*7.63*
–				
soil	0.34	*0.04*	2.74	*0.04*
–				
pore water	0.33	*1.53*	21.0	*1.00*
& *E. andrei*			24.3	*3.21*
& *P. scaber*			21.7	*0.58*
–				
E. andrei	1.71	*0.30*	35.5	*6.55*
P. scaber	2.93	*0.92*	7.72	*0.95*
P. fimata	0.16	*0.29*	2.55	*2.87*

Italics display standard deviations. N=3 except for *P.fimata* for which N=5.

of *E. andrei* (F = 12.957, p = 0.001) but decreased in the presence of *P. scaber* (F = 4.838, p = 0.034), irrespective of the plant treatment.

Survival of *E. andrei* was significantly affected by plant treatment, being higher under conditions with Winspit leaf material compared to Savoy leaf material (exposure effect, F = 40.991, p < 0.001). Moreover, survival of *E. andrei* was affected by an interaction between plant treatment and the presence of *P. scaber* (F = 8.444 , p = 0.006). Overall survival of *E. andrei* was increased in the presence of *P. scaber* under conditions with Savoy leaf material (low GSL levels), but the presence of *P. scaber* had no effect on survival of *E. andrei* when exposed to Winspit leaf material. However, the *E. andrei* survival dataset did not meet the necessary assumption needed to perform an ANOVA test (no normal distribution, Levene's test F = 4.305, p = 0.002). Transformation (natural logarithm) only slightly improved the homogeneity of variances (F = 3.451, p = 0.007), but did reduce the p–value for the interaction between leaf material and the presence of *P. scaber* (F = 10.079, p = 0.003). Unfortunately, interactions cannot be tested by non–parametric tests. Despite these shortcomings of the analysis, the interaction is highly significant, suggesting that this effect does have a significant impact on survival of *E. andrei*.

P. scaber was unaffected by the plant treatment and presence of other species.

Table 6.3: Survival (%) of *Protaphorura fimata*, *Eisenia andrei* and *Porcellio scaber* after 21 days in a microcosm as single species or in the presence of other species, exposed to low (Savoy) or high (Winspit) glucosinolates containing leaf material mixed with LUFA 2.2. soil.

	Savoy			Winspit		
	P. fimata	*E. andrei*	*P. scaber*	*P. fimata*	*E. andrei*	*P. scaber*
single	47.4	44.6	89.3	30.2	100	90.5
	5.93	*17.5*	*4.07*	*5.54*	*0.00*	*5.75*
& *P. fimata*		40.0	90.0		100	90.0
		28.5	*9.13*		*0.00*	*14.9*
& *E. andrei*	53.6		90.0	34.4		86.7
	2.08		*14.9*	*4.60*		*13.9*
& *P. scaber*	44.5	65.0		28.0	95.0	
	2.88	*37.9*		*3.79*	*11.2*	
3 species	50.2	86.7	90.0	31.3	100	95.0
	2.49	*29.8*	*13.7*	*4.61*	*0.00*	*11.2*

Italics indicate standard deviation for each treatment. N=7 for single species and N=5 for combination of species.

Cadmium experiment

Leaf material containing Cd significantly affected survival of only one of the three species (Table 6.4). *Protaphorura fimata* had a slight, but consistently higher survival when exposed to Cd–containing leaf material (exposure effect, $F = 12.563$, $p = 0.001$). Furthermore, there was a significant interaction effect between the presence of *E. andrei* and *P. scaber* ($F = 5.597$, $p = 0.023$), showing a combined negative effect on survival of *P. fimata* compared to when just one of these species is present.

Survival of neither *E. andrei* nor *P. scaber* was significantly affected by either Cd–containing leaf material or the presence of other species in this experiment.

6.4.4 CO_2 production and net–diversity effects

Net–diversity effects

Overall, no significant effects of plant treatment or faunal species assemblage were found for the net–diversity effect in both experiments (Supplementary information

Table 6.4: Survival (%) of *Protaphorura fimata*, *Eisenia andrei* and *Porcellio scaber* after 21 days in a microcosm as single species or in the presence of other species, exposed to control or cadmium contaminated *Noccaea caerulescens* leaf material mixed with LUFA 2.2. soil.

	Savoy			Winspit		
	P. fimata	*E. andrei*	*P. scaber*	*P. fimata*	*E. andrei*	*P. scaber*
single	34.7	100	85.7	39.5	98.2	75.0
	2.95	*0.00*	*11.5*	*3.79*	*4.72*	*9.62*
& *P. fimata*		100	80.0		100	86.7
		0.00	*7.45*		*0.00*	*13.9*
& *E. andrei*	39.2		80.0	45.6		83.3
	5.28		*13.9*	*3.85*		*20.4*
& *P. scaber*	36.0	100		40.6	100	
	4.59	*0.00*		*7.25*	*0.00*	
3 species	33.3	100	95.0	39.2	100	85.0
	7.03	*0.00*	*11.2*	*5.24*	*0.00*	*13.7*

Italics indicate standard deviation for each treatment. N=7 for single species and N=5 for combination of species.

Figure 6.5 and 6.6), probably due to the large variation in the data.

CO_2 production glucosinolate experiment

CO_2 production in the microcosms changed significantly over time (Greenhouse–Geisser, $F = 19.770$, $p < 0.001$). In general, CO_2 production was highest at the start of the experiment and decreased over time (Figure 6.2). Time also showed a significant interaction with presence of *P. fimata* (Greenhouse–Geisser, $F = 3.795$, $p = 0.045$) and a trend for the interaction with presence of *P. scaber* (Greenhouse–Geisser, $F = 3.288$, $p = 0.063$), both species initially had a positive effect on CO_2 production, which changed during the course of the experiment into a negative effect. Furthermore, an overall significant positive effect of the presence of *E. andrei* on CO_2 production was found ($F = 34.001$, $p < 0.001$), which also had a significant interaction with leaf material ($F = 4.390$, $p = 0.040$). As a result, the positive effect of *E. andrei* on CO_2 production was higher under conditions of Winspit (high GSL) leaf material.

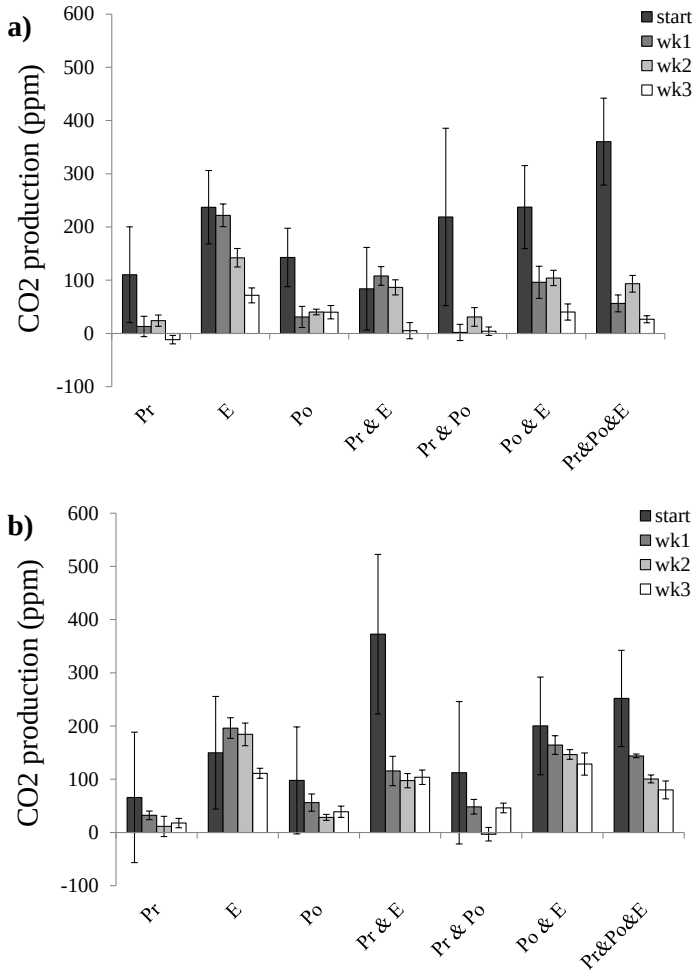

Figure 6.2: CO_2 production (corrected for background production) in microcosms containing LUFA 2.2. soil mixed with 1% *Brassica oleracea* leaf material with a) low or b) high glucosinolate content and different invertebrate species assemblages. Pr = *Protaphorura fimata*, Po = *Porcellio scaber*, E = *Eisenia andrei*. Error bars are standard error (N = 7 for single species treatments and N = 5 for multispecies treatments).

CO_2 production cadmium experiment

For CO_2 production a significant effect was found for time (Greenhouse–Geisser correction, F= 50.372, p < 0.001) and a significant interaction with plant material (Greenhouse–Geisser correction, F= 51.212, p < 0.001). In other words, CO_2 production was higher for Cd–containing leaf material initially, but at the end of the experiment CO_2 production was almost equal for both plant materials (Figure 6.3). Furthermore, a significant interaction between time, leaf material and the presence of *P. scaber* was found (Greenhouse–Geisser correction, F= 4.237, p = 0.040). This entailed that CO_2 production with Cd–containing leaf material and in the presence of *P. scaber* was higher than for control plant material, but only at the start of the experiment. This effect reversed in one week and disappeared completely after two weeks. Finally, an overall significant effect of the presence of *E. andrei* was found (Greenhouse–Geisser correction, F= 6.922, p = 0.011) with higher CO_2 production in the presence of *E. andrei*.

6.4.5 Other plant and soil parameters

Other soil processes and parameters (NH_4^+, NO_3^-, % N, % C, total organic matter) measured for both experiments, did not show any patterns related to plant treatment or faunal species assemblage (Supplementary information Tables 6.5 and 6.6).

6.5 Discussion

Our results demonstrate that exposure of soil ecosystems via plant material containing toxic compounds can induce chemical stress conditions and affect individual invertebrate species performance, but does not influence overall soil processes. Chemical stress conditions for GSL were obtained via Winspit leaf material, which had over 3 times higher total GSL concentrations compared to Savoy. Cadmium–containing leaf material increased cadmium (Cd) levels throughout the exposure route, from plant to invertebrate. Contrary to expectations, presence of earthworms did not modify effects of chemical stress of either Cd or GSL on CO_2 production or other soil invertebrates.

Earthworm activity can have a significant impact on metal availability in soil (see review of Sizmur and Hodson (2009)). In the present study, however, the presence of earthworms did not enhance Cd levels as was hypothesized, i.e. the presence of earthworms did not increase the bioavailable fraction of Cd (porewater concentrations). Therefore, only low concentrations of Cd were observed which logically did not induce adverse effects towards faunal survival or CO_2 production. Detrimental effects of Cd on faunal survival have been found in toxicological studies at much higher concentrations than the 2.74 mg/kg soil observed in this study. For instance, for *E. andrei* LC_{50}

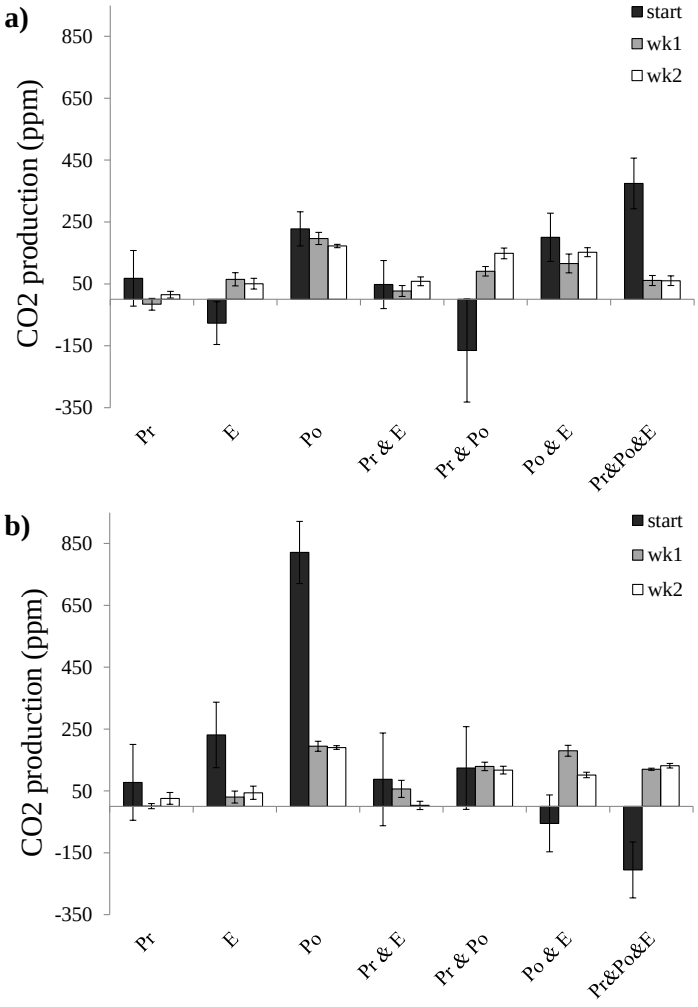

Figure 6.3: CO_2 production (corrected for background production) in microcosms containing LUFA 2.2. soil mixed with 1% *Noccaea caerulescens* leaf material of a) control plants or b) contaminated with cadmium and different invertebrate species assemblages. Pr = *Protaphorura fimata*, Po = *Porcellio scaber*, E = *Eisenia andrei*. Error bars are standard error (N = 7 for single species treatments and N = 5 for multispecies treatments).

(reducing 50% of survival) is well over 1000 mg/kg soil (van Gestel et al., 1992), and the EC_{50} (reducing 50% of reproduction) for the springtail *Folsomia candida* was estimated to be 51–780 mg/kg soil (Fountain and Hopkin, 2001). Bioavailable Cd levels in the soil thus have to be much higher to affect the soil ecosystem and its inhabitants.

In this study, presence of earthworms was hypothesized to reduce toxic effects of GSL on soil processes and faunal survival by enhancing biodegradation of GSL via, for instance, their bioturbation activity. Overall CO_2 production was not affected by GSL–containing leaf material and neither by the presence of earthworms under chemical stress compared to unstressed conditions. Moreover, effects of earthworms on survival of other species, or vice versa, were not affected by GSL–containing plant material. In contrast, a laboratory study using the pure chemical organic compound pentachlorophenol have found such effects, i.e. exposed nematode populations were more strongly affected by the toxin in the presence of springtails than by just the toxin (Salminen et al., 1995). Our results indicate that toxic effects on CO_2 production and faunal survival are not reduced by earthworm presence.

High GSL conditions did affect single species, significantly reducing springtail survival, whereas isopods and earthworms were not affected by enhanced GSL levels. Glucosinolates are hydrolysed into isothiocyanates (ITC) upon tissue damage. Depending on their chemical structure, these compounds are known to be toxic to springtails. For instance, 2–phenylethyl ITC reduced survival of *P. fimata* population by 50% at concentrations of 15.2 nmol/g soil (van Ommen Kloeke et al., 2012b). ITC concentrations in our experiment were estimated to be much higher, with 133 nmol/g soil for 3–butenyl ITC alone (assumed conversion efficiency 60%), the most abundant GSL in Winspit plant material. This is comparable to ITC concentrations found in fields after biofumigation practices, for which concentrations can range up to 100nmol/g soil (Gimsing and Kirkegaard, 2009). The effects of natural toxins observed in our study are therefore relevant for field situations.

Although effects of chemical stress on CO_2 production were not observed, interactions within species assemblages are able to cause non–additive effects on soil processes, depending on the functional identity of the species (Heemsbergen et al., 2004; Loreau and Hector, 2001; Zimmer et al., 2005). Regardless of chemical stress, non–additive effects on soil processes were thus expected in this study due to the different feeding strategies of the chosen invertebrate species. For instance, earthworms could increase collembolan survival by enhancing microbial growth (Lavelle et al., 1995), which in turn could stimulate CO_2 production via fungal grazing (Bardgett et al., 1993). A facilitation effect of earthworms was indeed found for the overall springtails survival (all plant treatments), but this did not result in increased CO_2 production. Isopods, on the other hand, had an overall negative effect on springtail survival, which was most likely due to competition for food. In this study, we

made use of completely fragmented plant material, while isopods are known as litter fragmenters, foraging on larger parts of litter (Drobne, 1997). Such an approach was chosen to create a worst–case scenario, by releasing the maximum amount of toxins stored in the plant material (Gimsing and Kirkegaard, 2009). The absence of litter and consequently a reduction in springtail densities in presence of isopods, did however not alter CO_2 production. Effects of fungal grazing by springtails on soil processes is highly dependent on species densities (Bardgett et al., 1993; Hanlon and Anderson, 1979). Apparently, the springtail densities in this study were not optimal to significantly alter CO_2 production. Overall non–additive effects of multiple species, regardless of toxic stress were, therefore not observed,.

CO_2 production was, however, affected by presence of single species. In both experiments, the presence of *E. andrei* as a single species did have significant positive effects on CO_2 production. For exposure to GSL–containing leaf material this was mainly because earthworms performed well under high GSL conditions. Observations of earlier studies with GSL–containing plant material showed no difference in survival of *E. andrei* between Savoy (low GSL) and Winspit (high GSL) (Zuluaga et al., *unpublished*, thesis chapter 5). Current results showing decreased survival under low GSL conditions were therefore unexpected and may be due to the presence of other compounds in Savoy, such as kaempferol–glucoside–rhamnoside and ascorbigen. Positive effects of earthworms on CO_2 production are in concurrence with other food web studies using multiple invertebrate species and underlined the irreplaceable function of analid species, such as *Cognettia sphagnetorum* and *Lumbricus rubellus* (Heemsbergen et al., 2004; Laakso and Setälä, 1999; Zimmer et al., 2005). Positive effects on CO_2 production can be explained by the bioturbation activity of earthworms which is known to increases microbial activity and hence decomposition processes (Lavelle et al., 1995). In our study isopods and collembolans did not have any effects on CO_2 production (independent of GSL conditions), regardless of their known ability to affect the microbial community and thereby soil functioning (Berg et al., 2001; Hättenschwiler et al., 2005). Our results thus confirm that identity of species rather than number of species drives soil ecosystem processes.

In conclusion, our findings show that toxin containing plant material may cause sufficient stress to affect individual species performance and soil processes, which has been little appreciated in food web studies so far. Especially earthworms were shown to be key drivers of CO_2 production and thus soil ecosystem health may depend on their performance, also under stress conditions. The current study shows that exposure of soil ecosystem engineers through toxin–containing litter does not affect soil processes.

Acknowledgments

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Supplementary information

6.5.1 Species biomass calculations

In order to control for any biomass effect on the data, total faunal biomass (dry weight, DW) was kept similar across species for all faunal treatments. To that end, individuals of *Eisenia andrei* and *Porcellio scaber* were pre–selected from stock cultures based on their fresh weight (FW) or size (body length), which were correlated to DW. Correlations for body mass DW and body mass FW or size were obtained from earlier obtained unpublished datasets (Figure 6.4). For *E. andrei* this entailed the use of individuals with a body weight between 204–337 mg FW corresponding to 30.1–45.3 mg DW. Individuals of *P. scaber* had a body length of 10–12 mm corresponding to 16.9–28.5 mg DW.

6.5.2 Net–diversity effects on CO_2 production

Glucosinolate experiment

No significant effects were found for the net–diversity effect in the glucosinolate experiment (Figure 6.5), probably due to the large variation in the data.

Cadmium experiment

The dissimilarity effect of CO_2 production showed an interaction effect between time and exposure in the cadmium experiment (Greenhouse–Geisser, $F = 8.783$, $p = 0.006$). We found overall inhibition in both plant treatments, but the magnitude of the inhibitory effect weakened over time in the mesocosms with cadmium–containing leaf material (Figure 6.6). Species composition was not a significant factor for the net–diversity effect.

6.5.3 Other plant and soil parameters

Both for *B. oleracea* and *N. caerulescens*, leaf material and soil mixed with the leaf material no differences were observed regarding total nitrogen, carbon and organic

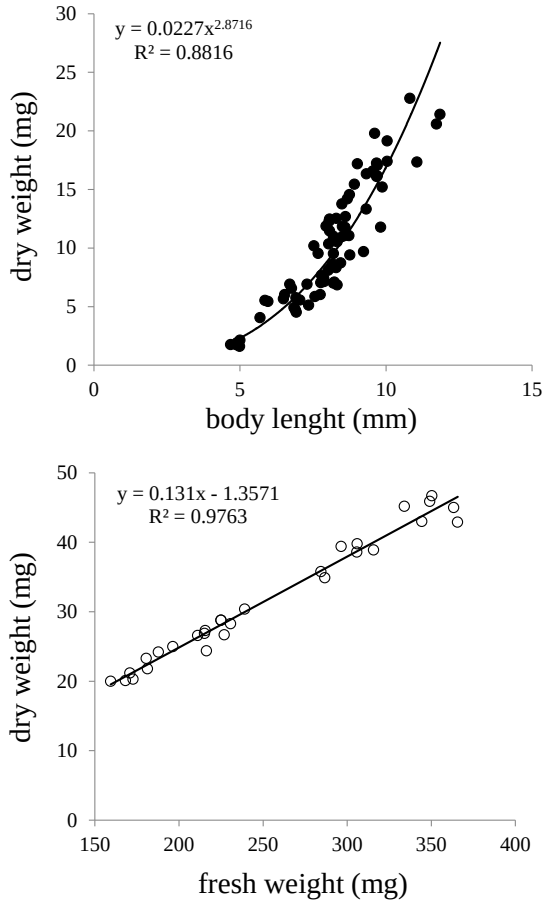

Figure 6.4: Correlations between a) *Porcellio scaber* body length versus dry weight body mass and b) *Eisenia andrei* body mass fresh weight versus dry weight. For *P. scaber* N = 74 and for *E. andrei* N = 29.

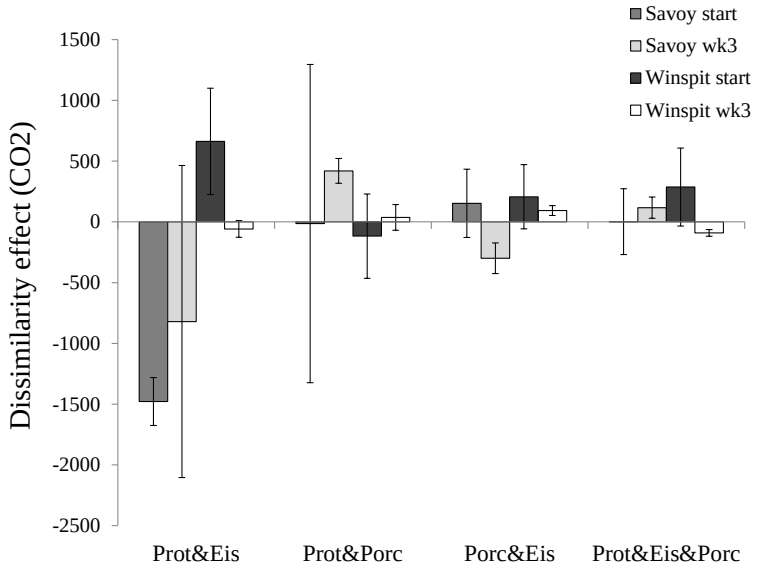

Figure 6.5: Dissimilarity effects on CO_2 production in mesocosms containing LUFA 2.2. soil mixed with 1% *Brassica oleracea* leaf material with low (Savoy) or high (Winspit) glucosinolate content and different invertebrate species assemblages. Pr = *Protaphorura fimata*, Po = *Porcellio scaber*, E = *Eisenia andrei*. Error bars are standard error (N = 5).

matter content (Table 6.5). Only Winspit showed a slightly lower nitrogen content for the leaf material compared to Savoy (Student's t–test, $p < 0.001$).

In the GLS experiment, NO_3^- production was higher for nearly all invertebrate treatments with Savoy (low GSL) leaf material, compared to Winspit (high GSL) leaf material, with *P. fimata* as single species showing the highest average production (Table 6.6). In the Cd experiment, NH_4^+ content was higher in microcosms with Cd–containing leaf material, especially when *P. scaber* was present.

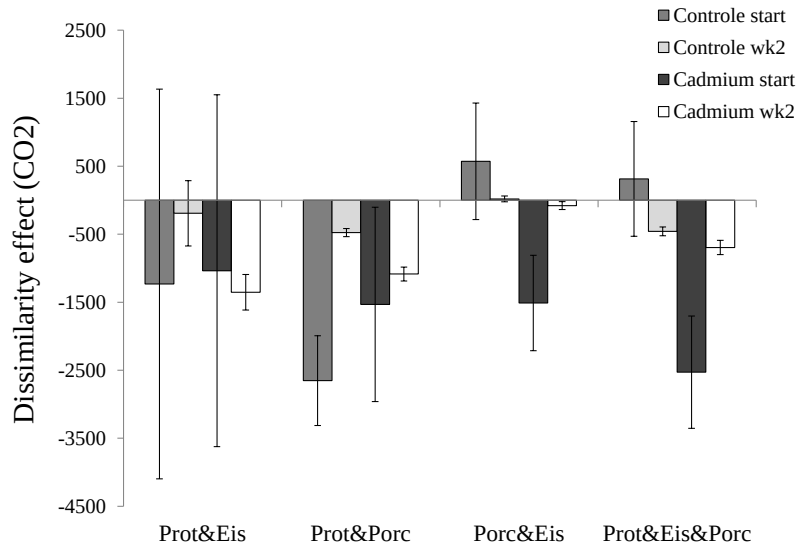

Figure 6.6: Dissimilarity effects on CO_2 production in mesocosms containing LUFA 2.2. soil mixed with 1% *Noccaea caerulescens* leaf material of control plant or plants contaminated with cadmium and different invertebrate species assemblages. Pr = *Protaphorura fimata*, Po = *Porcellio scaber*, E = *Eisenia andrei*. Error bars are standard error (N = 5).

Table 6.5: Total nitrogen, carbon and organic matter content in leaf material and soil at the start of experiments after incorporation of 1% leaf material compared to DW LUFA 2.2. soil

	% N		**% C**		**organic matter (%)**
	leaf	soil	leaf	soil	soil
Winspit	2.09	0.188	41.0	2.40	4.45
	0.04	0.01	0.23	0.11	0.20
Savoy	3.52	0.192	40.9	2.12	4.46
	0.01	0.02	0.03	0.22	0.14
CdNoc	3.30	0.189	41.2	2.13	4.54
	0.03	0.01	0.11	0.08	0.06
CoNoc	3.04	0.173	41.9	1.96	4.43
	0.06	0.01	0.09	0.13	0.05

Winspit = *Brassica oleracea* leaf material with high glucosinolates (GSL), Savoy = *B. oleracea* leaf material with low GSL, CdNoc = cadmium treated *Noccaea caerulescens*, CoNoc = control *N. caerulescens*. Italics indicate standard deviation.

Table 6.6: NO_3^- and NH_4^+ production (mg/L) in microcosms with different soil fauna assemblages exposed to different leaf materials containing toxic compounda mixed through LUFA 2.2 soil for 21 days. Corrected for background production from 'None animal' treatment

	Savoy		**Winspit**		**Control**		**Cadmium**	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
P. fimata	53.9	15.7	5.62	1.66	–1.96	0.80	6.97	0.10
E. andrei	20.6	15.5	7.88	15.9	9.54	2.52	8.24	4.73
P. scaber	2.82	3.26	0.93	0.06	–0.78	8.6	–4.02	31.5
P. fimata & *E. andrei*	7.12	7.81	0.68	7.67	19.6	3.36	14.5	3.40
P. fimata & *P. scaber*	3.79	3.51	5.25	1.21	8.29	8.82	0.48	15.8
P. scaber & *E. andrei*	14.6	9.56	1.23	7.68	14.8	14.0	–9.06	23.0
3 species	10.9	6.48	–1.81	6.02	5.44	0.53	–17.4	11.3

Glucosinolate containing leaf material came from Savoy (low) and Winspit (high) genotypes of *Brassica oleracea*. Cadmium containing leaf material and corresponding control came from *Noccaea caerulescens*.

CHAPTER 7

Soil ecotoxicology of *Brassica oleracea* genetically modified in glucosinolate concentrations

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7.1 Abstract

Brassica species contain secondary metabolites, glucosinolates (GSL), of which especially the hydrolysis products are known to have several socio–economic benefits. To optimize these benefits, genetically modified (GM) *B. oleracea* plants with altered GSL concentrations were recently developed. In previous studies effects of non–GM *B. oleracea* plants, differing in GSL concentrations and composition, on the soil ecosystem were investigated. Here, we study the impact of GM enhanced

GSL in *B. oleracea* compared to non–GM plants. To that end, effects of GM and non–GM *B. oleracea* plant material on the survival and reproduction of three soil invertebrate species were tested via mixing of freeze–dried, fragmented leaf material into natural LUFA 2.2 soil. Two *B. oleracea* strains, genetically modified to enhance GSL content, were compared to each of their conventional strains. Only one of the four GM plants showed negative effects for all invertebrate species, which could be ascribed to increased 2–propenyl GSL concentrations, while total GSL concentration was unchanged. The remaining GM plants did not show any changes in total GSL concentrations and also did not exert negative effects on soil invertebrates when compared to their non–GM counterparts. A specific GM effect could therefore not be observed.

7.2 Introduction

Agricultural areas containing genetically modified (GM) crops, currently occupy over 160 million hectare agricultural soil globally, of which over forty per cent is located in the U.S.A. (James, 2011). A well–known example is Bt maize, expressing the insect–specific toxin 'Cry1Ab gene' in order to become resistant to e.g. the European corn borer *Ostrinia nubilalis* (Cortet et al., 2007). A positive side effect of Bt is the reduction of mycotoxin levels (fungal produced natural toxins that are harmful to humans and livestock) within maize by decreasing *Fusarium spp.* fungal infections (Ostry et al., 2010).

Brassica species contain secondary metabolites, called glucosinolates (GSL), which also serve several valuable socio–economic functions. Tissue damage of the plant material (for instance due to herbivory) hydrolyses GSL into several bioactive compounds, such as isothiocyanates (ITC), are well–studied (Wittstock and Halkier, 2002). Several reports demonstrate a causal relation between ITC activity and anti–carcinogenic effects in humans (Traka and Mithen, 2009; Zhang, 2010). Furthermore, ITCs can act as natural pesticides in the alternative agricultural practise, commonly known as biofumigation (Morra and Kirkegaard, 2002). Such benefits have raised strong interest in the modification of *Brassica* genotypes with elevated GSL levels (Gigolashvili et al., 2007a; Mithen et al., 2003; Wittstock and Halkier, 2002). Natural *Brassica oleracea* genotypes and populations differ in GSL concentration and composition (Gols et al., 2008). However, health benefits and/or agricultural use of *B. oleracea* could be maximized by increasing expression of desirable GSL by means of genetic modification. Recently, the Winspit *MYB29* gene (*WinMYB29*), encoding a transcription factor for the synthesis of aliphatic GSL in *Brassicaceae*, was over-expressed in Winspit and the double haploid (DH) *B. oleracea* type AG1012. The overexpression of this gene increases the synthesis of specific aliphatic GSL in the

transgenic lines (Zuluaga et al., *unpublished*). As a result, levels of particular GSL in the GM plants exceed the highest levels found in natural *B. oleracea*e genotypes.

Novel (GM) crops can, however, unintentionally affect beneficial soil organisms, jeopardizing soil health and quality (Conner et al., 2003; Snow et al., 2005). Important soil ecosystem processes, such as decomposition of dead organic matter, are dependent on a complex community of e.g. microbes and beneficial soil invertebrates (Hättenschwiler et al., 2005; Wardle et al., 2004). Activity of microbes and their faunal grazers, such as collembolans, are essential for decomposition and mineralisation processes, and indirectly primary productivity (Berg et al., 2001; Wardle et al., 2004). Soil organisms can be exposed to detrimental effects of GM crops via root exudates, via litter, or via input of plant remnants after harvesting (Mulder and Lotz, 2009). Moreover, biofumigation agricultural practise entails the deliberate incorporation of GSL–containing plant material (e.g. green manure or seed meals) into the soil to reduce plant pests and pathogens (Gimsing and Kirkegaard, 2009; Björkman et al., 2011). However, ITC toxicity is non–specific and irreversible (Brown and Morra, 1997) and several studies have shown negative effects on survival and reproduction of soil invertebrates in response to ITC exposure (Jensen et al., 2010; van Ommen Kloeke et al., 2012a,b). In addition, ITCs differ in level of toxicity depending on their chemical side–chain (Gimsing and Kirkegaard, 2009), toxic effects of GM *B. oleracea* are thus also most likely dependent on concentration and composition of particular GSL.

Currently, there is no comprehensive system available to investigate GM effects on soil ecosystems, due to the fact that insufficient scientific data are at hand (Snow et al., 2005). This calls for studies that address a better quantification of the impact of GM crops and which investigate such effects with regard to baseline variation. One scientific view point is that only studies at an ecologically relevant scale (e.g. field trials) are suitable predictors for adverse effects caused by these compounds. An alternative approach is to test 'no harm' of a GM crop compared to its conventional crop, in an ecotoxicological context under lab–controlled condictions (Birch et al., 2007; Mulder and Lotz, 2009; **?**; Romeis et al., 2008; Snow et al., 2005; Rombke et al., 2010). The latter approach has been applied in this study. Previously, we investigated effects of *B. oleracea* varying in GSL concentration and composition on survival and reproduction of beneficial soil ecosystem model species (Zuluaga et al., *unpublished*, thesis chapter 5). Here, we investigate if GM induced changes in GSL concentration and composition affect soil invertebrates compared to their untransformed accession. The use of several GM lines and laboratory controlled studies can give insight in potential worst–case scenario for effects on soil ecosystems compared to baseline variation (Romeis et al., 2011). The soil invertebrate species *Eisenia andrei* (earthworm, ecosystem engineer) and *Folsomia candida* (fungivorous collembolan) were chosen as representatives of important soil ecosystem functions such as decomposition (Edwards and Bohlen, 1992; Fountain and Hopkin, 2005).

Protaphorura fimata, a herbo–fungivorous collembolan (Endlweber et al., 2009), was included to deduce if effects are similar between beneficial and pest species. The presented data are essential in understanding the potential impact of GMs on soil ecosystems.

7.3 Materials and methods

7.3.1 Animals

The collembolan species *Folsomia candida* is a fungivorous collembolan (Fountain and Hopkin, 2005), while the (facultative) herbivorous *Protaphorura fimata* is known to feed on plant roots (Endlweber et al., 2009). Both species reproduce parthenogenetically and originated from stock cultures (VU University Amsterdam), maintained on moistened plaster of Paris mixed with charcoal. Stock culture conditions were kept standard in climate chambers at 20°C with a 12:12 hours light:dark cycle and 75% relative humidity (RH). Cultures received a fresh quantity of baker's yeast on a weekly basis. For the experiments, a synchronised batch of 10–12 days old individuals was prepared following the International Standardization Organization (ISO) guideline 11267 (ISO, 1999).

Eisenia andrei, an epigeic compostworm, was maintained in synchronized (max. one month age difference) stock cultures (VU University Amsterdam) maintained in large plastic containers filled with a mixture of horse dung (without antibiotics) and pot soil. Stocks were kept under constant conditions in a climate chamber with a temperature of 20°C, a 12:12 hours light:dark cycle and 75% relative humidity. Animals were selected to contain a visible clitellum, which is indicative for the sexual reproductive state. Prior to the experiment, test organisms were isolated and acclimatized to the control LUFA 2.2 soil (Speyer, Germany) overnight.

7.3.2 Plant material

Two *B. oleracea* types were selected: Winspit, a natural wild accession originating from the coast of Dorset (England) and the double haploid genotype AG1012 (DH AG1012) (Sparrow et al., 2004), derived from a cross between *B. oleracea* var. *alboglabra* (A12DHd) and *B. oleracea* var. *italica* (Green Duke GDDH33). Winspit is known for its high GSL concentrations whereas DH AG1012 has shown lower quantities of GSL in leaf tissue (Zuluaga et al., *unpublished*). Original Winspit seeds were kindly provided by Dr. Rieta Gols (Entomology Department, Wageningen University, the Netherlands) and seeds of the original DH AG1012 by Dr. Penelope Sparrow (John Innes Centre, Norwich, UK). Genetically modified (GM) lines were available (see below) for both Winspit and AG2012, which can be compared to their non–GM

counterpart genotype. For convenience these non–GM plants were indicated as 'wild type' (WT).

Genetic modification of both *B. oleracea* genotypes was performed as described by Zuluaga et al. (*unpublished*). In short, *Brassica oleracea* double haploid (DH) genotype AG1012 and the wild cabbage Winspit were transformed using the coding sequence (CDS) of the *BoMYB29* gene, isolated from a Winspit plant. The GM Winspit (GM1 and GM2) and DH AG1012 (GM5 and GM6) plants, were obtained after the insertion of the 12.148 kb *Pro35S::WinMYB29::Ter35S* genetic cassette, using the Agrobacterium tumefaciens method (Birch, 1997). Transformation of the DH AG1012 and Winspit was carried out following the method of Sparrow et al. (2004) with minor modifications. GM plants came from in vitro regeneration of *B. oleracea* cotyledons and every GM line used in this study is derived from an independent transgenic event. After transformation, GM *Brassica* plants were maintained in in vitro conditions. Multiple clones were obtained through propagation by cutting original plants and allowing regeneration of leaves and roots. When needed, GM plants were transferred to soil pots in greenhouse conditions. The Winspit primary transgenic and DH AG1012, T2 generation have been used for this study. The T2 generation of DH AG1012 resulted from self–pollination of the regenerated transformants.

Based on previous data on the relative gene expression of the inserted *MYB29* (Zuluaga et al., *unpublished*) we selected two GM lines per genotype with potentially different GSL concentration compared to the non–GM, based (Table 7.1). We experimentally verified the GSL concentrations of all genotypes via HPLC and the relative gene expression of WinMYB29 was checked for DH AG1012 GM plants (see section below).

Non–GM WT plants of Winspit and DH AG1012 and the T2 generation GM AG1012 plants were grown from seeds in a greenhouse, using 17 ∅ cm pots containing a peat–based commercial potting compost (Lentse potgrond nr. 4; % peat, 15% clay). GM Winspit plants originated directly from in vitro regeneration of *B. oleracea* cotyledons and thus clones were transferred to soil pots in the greenhouse. Climate greenhouse conditions for both, GM and non–GM plants, were a 16:8 hours light:dark cycle, temperatures 23/19°C day/night and RH 75%. Plants were watered once a day. After approximately eight weeks, healthy, fully expanded leaves were harvested, snap frozen in liquid nitrogen, freeze–dried and stored at RT until soil preparation for the ecotoxicological experiments.

7.3.3 Soil preparation

The natural soil LUFA 2.2 (Speyer, Germany) was used for all experiments, which is a loamy sand soil with a pH of (± SD) 5.5 ± 0.1 and an organic C content of 2.09 ± 0.40%. Before usage the soil was dried at 60°C for 24 hours. A batch of

Table 7.1: Plant genotypes and lines used for exposure of three invertebrate species to LUFA 2.2. soil mixed with the plant material. GM lines were chosen based on their relative gene expression of the *MYB29* gene compared to the WT.

code	**plant description**	**FC *MYB29***
WIN WT	Winspit genotype wildtype	
WIN GM1	Winspit genotype GM line 1	6
WIN GM2	Winspit genotype GM line 2	8
AG WT	DH AG1012 genotype wildtype	
AG GM5	DH AG1012 genotype GM line 1	>18
AG GM6	DH AG1012 genotype GM line 2	>18

code short code used to describe the plant genotype and line, *description* full description of plant genotype and line, *FC MYB29* is fold change in gene expression of *MYB29* gene compared to the wild type.

approximately 4 plants was used per plant line and genotype (Table 7.1) in order to obtain 0.5% of freeze–dried leaf material relative to total soil dry weight (DW). This resulted in 6 plant treatments which was complemented by a LUFA control; soil without any plant material, but a few grains of baker's yeast or horse dung as food source. To ensure maximum release of isothiocyanates, leaf material was completely fragmented (Gimsing and Kirkegaard, 2009) using a laboratory blender (IKA, A11 basic). Subsequently, fragmented plant material was mixed through the soil by hand to ensure a homogeneous distribution. Finally, the soil was moistened with demineralised water to 22% water content (equivalent to 50% of the soil's water holding capacity). Soil was used the same day for the experiments with the soil invertebrates.

7.3.4 Experimental design

Each invertebrate species was exposed to soil with each of the 6 plant treatments (Table 7.1) together with the LUFA control. Each treatment was represented by 5 biological replicates. Ecotoxicological tests with the springtail species were conducted with 10 animals in 100–ml jars consisting of 30 g moist soil. After 28 days 100 ml of water was added to test containers, stirred gently and completely poured into a glass beaker. For each sample several digital photographs were taken to register all living springtails that came floating to the surface. The images were used for visual

counting of the number of adult survivors (survival) and juveniles (reproduction) on a computer screen.

The effects of GSL material on *E. andrei* was tested in 600–ml jars, which consisted of 300 g moist soil and 5 individuals. Survival was examined after 28 days of exposure by hand sorting and removing the surviving adults from the soil. The remaining soil, including juveniles and cocoons, was returned to the test jars and incubated for another 28 days. Reproduction was subsequently assessed by manually counting the number of juveniles that migrated to the soil surface after placing the jars in a 60°C water bath.

All test jars were kept in a climate room at 20°C, 70% relative humidity and a 12 hour light/dark cycle for 28 or 56 days. Once a week jars were aerated and moisture content was adjusted if needed.

One–way ANOVA tests were performed to investigate significant differences between plant treatments per invertebrate species. Except for *P. fimata* survival under exposure of DH AG1012 plant material and *F. candida* survival and *P. fimata* reproduction under exposure of Winspit plant material, the assumption of homogeneity of variances was not met. For those datasets a Welch adjustment (Robust Tests of equality of means) and Games–Howell post–hoc were used. SPSS 20.0 was used for all statistical analyses. Student's t–tests were used to investigate significant differences between the two springtail species regarding affected survival and reproduction per plant. Finally Pearson correlations were conducted to investigate if GSL concentration could be linked to either survival or reproduction of an invertebrate species.

7.3.5 GSL analyses

Glucosinolate concentrations and composition in all the plant material were measured by high performance liquid chromatography (HPLC) analyses at the Max Planck Institute for chemical ecology in Jena, Germany. Using 10 mg of freeze–dried fragmented plant material, GSL were extracted overnight with 1 ml of 80% methanol solution containing 0.05 mM intact 4–hydroxybenzyl GSL as internal standard, desulfatased with arylsulfatase (Sigma–Aldrich) on DEAE Sephadex A 25 columns. The resulting desulfoglucosinolates were eluted with 0.5 ml water and were separated using HPLC (Agilent 1100 HPLC system, Agilent Technologies, Waldbronn, Germany) on a reversed phase C18 column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5 μm, Macherey–Nagel, Düren, Germany) with an water–acetonitrile gradient (1.5% acetonitrile from 0–1 min, 1.5–5% acetonitrile from 1–6 min, 5–7% acetonitrile from 6–8 min, 7–21% acetonitrile from 8–18 min, 21–29% acetonitrile from 18–23 min, followed by a washing cycle; flow 1 ml min–1). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Response factors were: aliphatic GSL 2.0, indole GSL 0.5 for quantification of individual GSL (Burow et al., 2006).

7.3.6 Quantitative polymerase chain reaction (Q–PCR)

As described earlier, the DH AG1012 GM plants are T2 generations of the original GM plants, which showed over 18 times elevated relative gene expression of the *MYB29* gene compared to the non–GM (Zuluaga et al., *unpublished*). To verify the presence of the transgene in plants used in the current experiments (T2 generation), gene expression of coding for *MYB29* was measured through Quantitative Polymerase Chain Reaction (Q–PCR). Leaf tissue (five discs ∅ 60 mm), was taken while the plants were approximately seven weeks old, snap frozen in liquid nitrogen and stored in –80°C until further analyses. Total RNA was isolated from two to three leaf discs, with the Qiagen RNeasy mini kit for plant tissue, following the manufacturer's protocol. Integrity of the RNA was checked on a 1% agarose gel. Next, cDNA was synthesized using the Promega reverse–transcriptase system with 5 μl RNA template, M–MLV reverse transcriptase enzyme and oligo–dT primers, according to the manufacturer's protocol. The DNA engine Opticon1 (Biorad) was used to perform Q–PCR, with samples consisting of 2 μl cDNA template, 1 μl of 1 μM R– and F–primers, 7 μl H_2O and 10 μl SybrGreen mastermix (Bioline no–rox mix). Primer sequences for the GM gene *MYB29* (*WinMYB29*) and a *B. oleracea* housekeeping gene (*BoGAPDH*) (Zuluaga et al., *unpublished*), and were ordered via MWG eurofins. Both genes were simultaneously amplified for each sample, in triplicate. A two step amplification protocol was performed (10 sec. 95°C followed by 30 sec. 60°C) for 40 cycles. Melting curve analysis was performed between 60–90°C by recording fluorescence every 0.05°C increase per second. Cycle threshold (C_t) values of both genes were obtained by the Opticon Monitor 3 software (Biorad) provided with the machine, using standard settings. Primer efficiency was tested for both primers by a dilution range and was 2.3 for *MYB29* and 2.2 for *BoGAPDH*. Relative gene expression of the *WinMYB29* gene compared to WT was calculated for all samples with the Q–Gene module (Muller et al., 2002) as previously described by (Roelofs et al., 2006).

7.4 Results

7.4.1 Gene expression of *WinMYB29* gene in DH AG1012 *B. oleracea* plants

Expression of the *MYB29* gene was measured in DH AG1012 GM lines and DH AG1012 WT. Delta C_t value for WT plants was on average 5.73 (± standard deviation 0.82). DH AG1012 GM 5 plants showed the highest gene expression of *MYB29*, whereas GM6 plants had around equal gene expression values compared to the WT (Table 7.2).

Table 7.2: Relative gene expression of the inserted *WinMYB29* gene in genetically modified AG1012 *B. oleracea* plants compared to non–GM (WT) plants

plant	relative gene expression	standard deviation
AG GM 5.2	8.95	1.91
AG GM 5.9	4.30	0.64
AG GM 5.21	13.4	2.21
AG GM 6.16	1.27	0.36
AG GM 6.19	0.99	0.20
AG GM 6.35	1.37	0.12
AG GM 6.39	1.33	0.42

plant code to describe the genotype, line and plant number

7.4.2 Glucosinolate concentrations and composition in *B. oleracea* plants

Total GSL concentrations and composition were determined in leaf material for each plant (Figure 7.1). Overall, Winspit contained significantly higher GSL levels when compared to DH AG1012 (Student's t–test, $p < 0.001$), with 3–butenyl GSL concentration being especially elevated in Winspit. Both GM lines of Winspit had lower GSL total concentrations when compared to WT (37.7 μmol/g). However, specific GSLs were more abundant in GM plants.

For instance, Winspit GM1 synthesized twice the amount of 2–propenyl GSL (sinigrin – allyl GSL) compared to WT, while indol–3–ylmethyl GSL (I3M) concentration decreased two fold as compared to WT. Winspit GM2 produced only half of the total GSL concentrations (14.8 μmol/g) observed in WT, especially due to a low concentration of 3–butenyl GSL.

The most common GSL present in DH AG1012 was 4–methylsulfinylbutyl GSL (4MSOB – sulforaphane). AG1012 GM lines also showed comparable or lower total GSL concentrations when compared to the AG1012 WT. AG1012 GM5 GSL concentrations and composition were similar to the WT, while AG2012 GM 6 contained much lower total GSL concentrations, especially due to low amounts of 4–methylsulfinylbutyl (4MSOB) GSL.

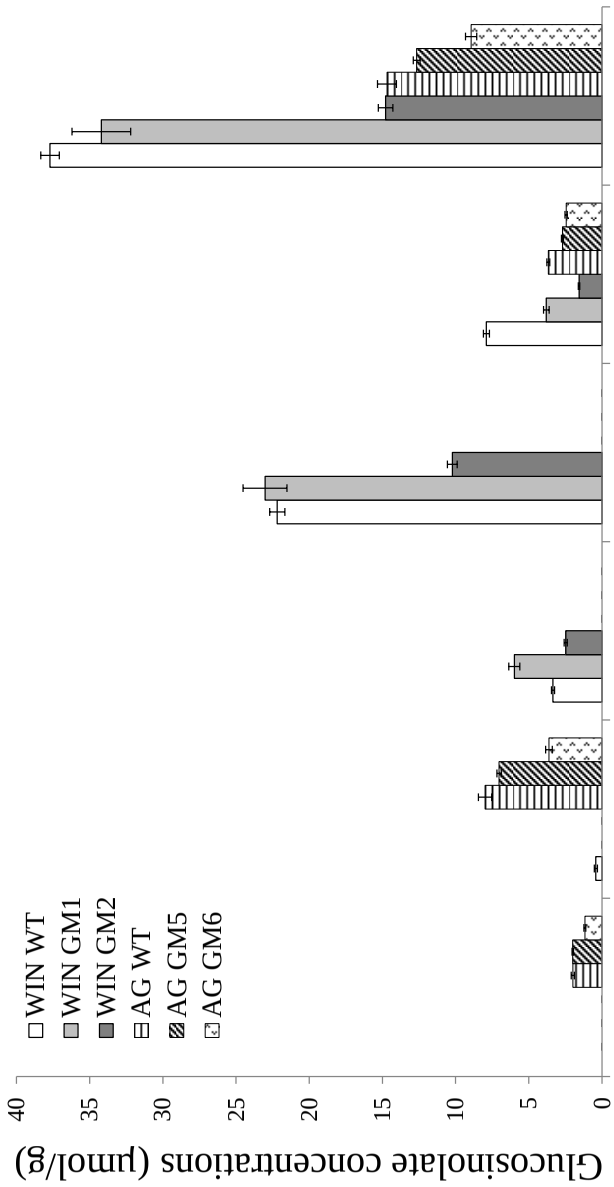

Figure 7.1: Total glucosinolate (GSL) concentration and composition in leaf material of *B. oleracea* plants used for experiments with soil invertebrates, measured by high performance liquid chromatography (HPLC) analyses. 3MSOP = 3–methylsulfinylpropyl, 4MSOB = 4–methylsulfinylbutyl, 2–Propenyl = 2–propenyl GSL (sinigrin), 3–Butenyl = 3–butenyl GSL, I3M = indol–3–ylmethyl GSL. For all plants N = 4.

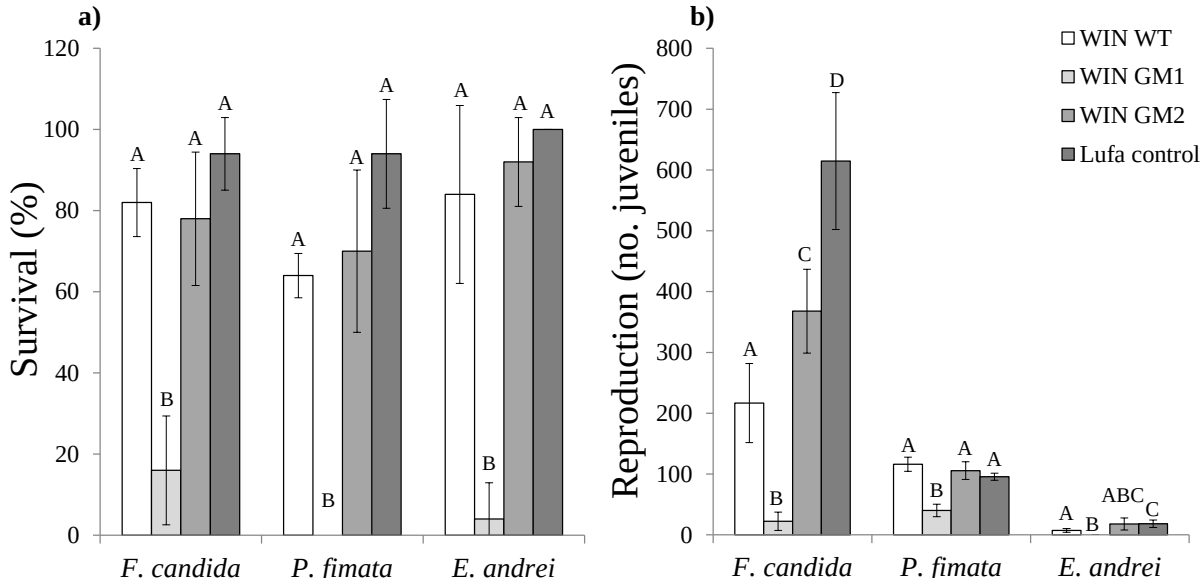

Figure 7.2: Effects of exposure to *B. oleracea* genotype Winspit plant material of wild type (WT) and genetically modified (GM) lines on a) survival and b) reproduction of 3 invertebrate species after 28 or 56 days of exposure in LUFA 2.2. soil. Plant material treatments were complemented by a control treatment without any plant material. Exposures were done using 0.5% freeze–dried and fragmented plant material compared to dry weight soil. Error bars indicate standard deviation. Letters indicate which treatments are significantly different from each other within each species.

7.4.3 Survival and reproduction of soil invertebrates

Winspit leaf material effects on soil invertebrates

Significant differences for all invertebrate species on survival and reproduction were found only between treatments with Winspit GM1 and all other Winspit lines and the LUFA control (Figure 7.2). Winspit GM1 significantly reduced survival for all three invertebrate species compared to the other treatments and the LUFA control (Games–Howell, $p < 0.001$). Moreover, this pattern was complemented by reproduction data (Figure 7.2 b) with a few exceptions; e.g. for *F. candida* reproduction, all treatments were significantly different from each other, with GM2 showing the lowest and the LUFA control the highest number of juveniles. *E. andrei* reproduction much lower than springtail reproduction and only just significantly different between Winspit WT and the control treatment ($p = 0.050$), but just not between GM1 and GM2 ($p = 0.052$).

Pearson correlations between GSL concentration and survival or reproduction of the invertebrate species revealed that increased concentrations of 2–propenyl GSL caused significant declines in both survival and reproduction of all species (Figure 7.3).

Moreover, no significant differences were found for the toxic effects of Winspit GM1 between the two collembolans species regarding survival (Student's t–test, $p = 0.056$) and reproduction (Student's t–test, $p = 0.066$).

AG1012 leaf material effects on soil invertebrates

Treatments with AG1012 plant material showed no effect on survival of either of the three invertebrate species compared to the control, except for a decrease in P.fimata survival in the AG GM5 treatment compared to the WT ($F = 3.67$, $p = 0.035$) (Figure 7.4 a).

Reproduction was significantly decreased in *F. candida*, between the LUFA control and all other AG treatments (Welch statistics = 54.19, $p < 0.001$) (Figure 7.4 b), but no effect of AG1012 plant material was found for *P. fimata* or *E. andrei* reproduction. Reproduction of *E. andrei* was completely impaired by all the plant treatments and therefore showed a significant difference between the LUFA control and the plant material treatments (Welch statistics = 6.708, $p = 0.012$).

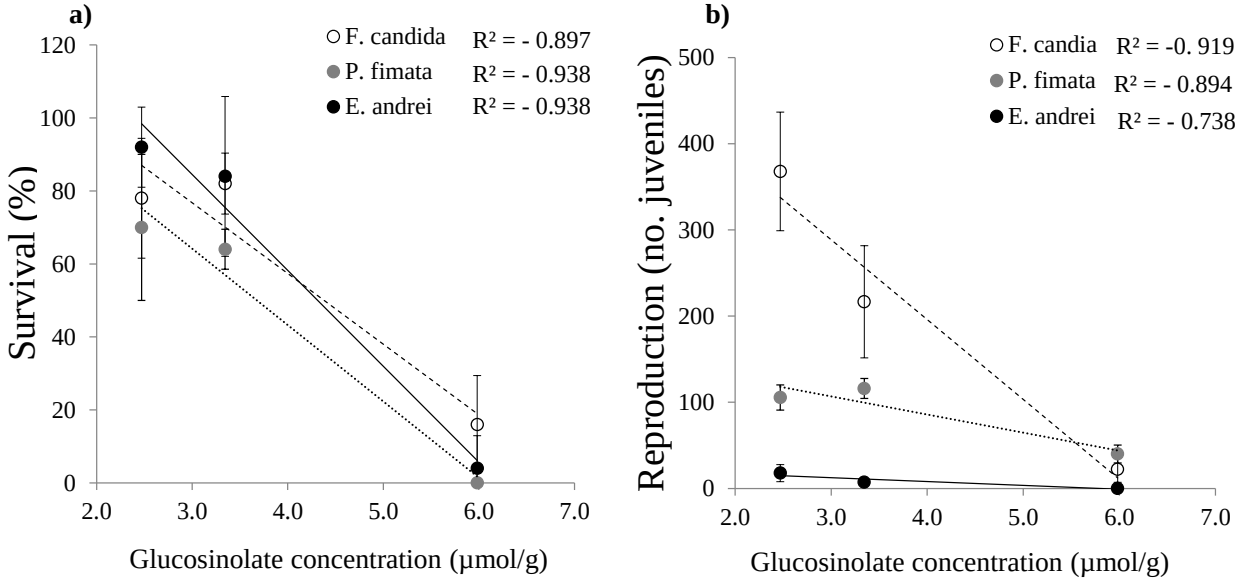

Figure 7.3: Figure 3. Correlations between 2–propenyl glucosinolate concentration and a) survival or b) reproduction of the soil invertebrates *Folsomia candida*, *Protaphorura fimata* and *Eisenia andrei* after 28 or 56 days of exposure in LUFA 2.2. soil. Concentrations of 2–propenyl GSL were measured via HPLC analysis (Figure 7.1). Error bars indicate standard deviation with N = 5 per GSL concentration. PC = Pearson Correlation coefficient.

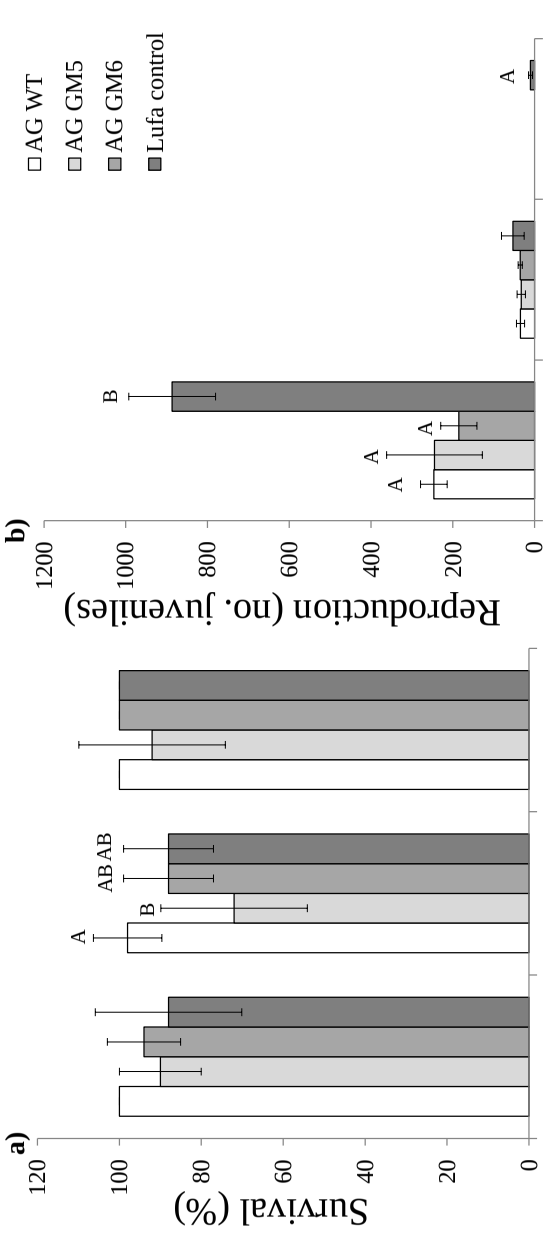

Figure 7.4: Effects of exposure to *B. oleracea* genotype AG1012 plant material of wild type (WT) and genetically modified (GM) lines on a) survival and b) reproduction of 3 invertebrate species after 28 or 56 days of exposure in LUFA 2.2. soil. Plant material treatments were complemented by a control treatment without any plant material. Exposures were done using 0.5% freeze–dried and fragmented plant material compared to dry weight soil. Error bars indicate standard deviation. Letters indicate which treatments are significantly different from each other within each species.

7.5 Discussion

In this study we investigated how alteration in GSL concentrations and composition, induced by GM, affects soil invertebrates as compared to conventional non–GM counterparts, representing baseline GSL variations. Our approach allowed for controlled testing of worst–case scenario of effects on soil ecosystems using GM with enhanced GSL possibly exceeding natural variation. Moreover completely fragmented plant material ensured maximum release of bioactive hydrolysis products of GSL, ITC (Gimsing and Kirkegaard, 2009). Overall, decreases in soil invertebrate survival and reproduction were found for only one GM and correlated with an increase in a specific GSL, 2–propenyl GSL (sinigrin – allyl GSL), rather than the total amount of GSL.

None of the GM lines showed enhanced total concentrations of GSL compared to the conventional non–GM *B. oleracea* genotypes (WT) plants, but only increases in specific GSL as was earlier described by Zuluaga et al. (*unpublished*). For instance, even though AG1012 GM5 plants showed high relative gene expression of the GM gene *WinMYB29*, this did not increase any of the GSL concentrations in the plant material. Moreover, Winspit GM2 even had less than half the total GSL concentrations compared to non–GM and comparable results were found for AG1012 GM6. We could not determine a significant correlation between GM plants and reduction in survival or reproduction for any of the three soil invertebrates tested in this study. This is in concurrence with studies on existing GM crops, such as BT maize that is modified by the introduction of a bacterial gene coding for an insect–specific toxin. In this case, detrimental effects were attributed to natural variation between maize varieties and changes in agricultural practises (Krogh and Griffiths, 2007; Cortet et al., 2007).

The only GM that did show altered GSL concentrations was Winspit GM1, having increased levels of 2–propenyl GSL compared to the non–GM, even though relative total GSL concentrations were not higher. These concentrations could be correlated to decreases in survival and reproduction of both non–target and herbivorous soil invertebrates (Figure 7.3). Earthworms and collembolans alike, experienced substantial decreases in survival and reproduction compared to the non–GM and the other GM line. Moreover, there was no significant difference between *F. candida* and *P. fimata* survival and reproduction when exposed to Winspit GM2, indicating that GSL–containing material has similar effects on non–target and herbivorous invertebrate species. This is most likely due to the non–specific mode of action of the GSL hydrolysis products (Brown and Morra, 1997), as was reported earlier (van Ommen Kloeke et al., 2012b). The higher production of 2–propenyl GSL in Winspit could be due to the *MYB29* regulation of specific genes such as *AOP2*, which is directly involved in the synthesis of alkenyl–GSLs as it was shown by Zuluaga et al. (*unpublished*). Additionally, *MYB29* which positively regulates the aliphatic GSL

production could be a negative regulator of the indolic GSL pathway in *Brassica* as it happens in Arabidopsis thaliana (Gigolashvili et al., 2008). Therefore, this can explain the lower production of I3M observed in the Winspit overexpressing plants compared to the WT plants. The observed concentrations of 2–propenyl GLS in Winspit GM1 are, however, not exceptional compared to natural baseline variations found for Winspit (Gols et al., 2008), underlining that this GM is not more hazardous than conventional non–GM plants.

Slight differences in chemical structure have been reported to cause substantial differences in toxicity (Gimsing and Kirkegaard, 2009). From our results it appears that 2–propenyl ITC, the main hydrolysis product of 2–propenyl GSL, was more toxic compared to, e.g. 3–butenyl and indol–3–ylmethyl ITC, which occurred at higher concentrations in the non–GM. 3–Butenyl ITC is very similar to 2–propenyl ITC, however, previous studies show that 2–phenylethyl ITC was five times more toxic than benzyl ITC for collembolan species, while this compound only differed in one extra CH_2 (Jensen et al., 2010; van Ommen Kloeke et al., 2012b). Still, 2–propenyl ITC seems to have a relatively weak toxic effect compared to e.g. 2–phenylethyl ITC. This was, for instance, found for ITC effects on the human pathogenic bacteria *Helicobacter pylori*, where 2–phenylethyl ITC was up to twenty times more toxic than 2–propenyl ITC (Shin et al., 2004). Such inferior toxic effects were also found for *E. andrei* and *F. candida* (van Ommen Kloeke et al., *unpublished*), and are probably the result of the volatile nature of 2–propenyl ITC (logKow = 1.58), causing fast dissipation from the environment and hence limited exposure of soil invertebrates.

Isothiocyanates, such as 2–propenyl ITC are known to be effective chemopreventive compounds against e.g. cancer, inflammation and cardio diseases (Traka and Mithen, 2009; Zhang, 2010) and are also widely used as alternative pest management with biofumigation practises (Gimsing and Kirkegaard, 2009). GM *Brassica* varieties with enhanced GSL concentrations or enriched specific GSL, such as the *B. oleracea* tested here may thus provide these socio–economic benefits. This study showed, that a plant can be more toxic due to changes in specific GSL, even without an increase of total GSL concentrations. However, uncertainty remains about effects of possible changes in other plant compounds which were not investigated in this study. Moreover, we aimed at worst case scenario. To come to a more comprehensive assessment of potential impact on soil communities we propose future studies in a more ecologically relevant setting.

Acknowledgments

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CHAPTER 8

General discussion

In 2011 the human population reached 7 billion souls. Feeding every individual while safeguarding the protection of natural areas, remains one of the biggest challenges of this century. At the heart of a solution to this challenge is improved agriculture (Da Silva, 2012). Genetically modified (GM) crops with enhanced traits, such as resistance to pests and herbicides or enhanced nutritional value, provide promising opportunities to feed the world with more efficient land use (James, 2011). GM crops with enhanced traits, for instance producing toxic compounds, may also exert potential detrimental effects on the soil ecosystem. This thesis developed a decision matrix to assess potential risks on non–target organisms before allowing novel GM crops to be grown on a commercial scale. Using *Brassica oleracea* (GM and non–GM) as model crop, the decision matrix used a tiered approach (Romeis et al., 2011) to investigate effects of glucosinolates (GSL) and their hydrolysis products, e.g. isothiocyanates (ITC), on the soil ecosystem. This approach provides comprehensive information by integrating multiple levels of biological organisation ranging from; molecular to the community and soil process level.

8.1 Impacts of pure compounds on single soil invertebrate species

Glucosinolates and their hydrolysis products have been intensively studied, with most research focussing on GSL production in plants, GSL effects on herbivores or inter-

actions with the aboveground food web (Gigolashvili et al., 2007a; Halkier and Gershenzon, 2006; Soler et al., 2007; van Dam et al., 2009). Knowledge about effects on belowground non–target species, however, has been lacking so far. Isothiocyanates are known for their non–specific and irreversible mode of action (Brown and Morra, 1997) and can thus form a potential hazard for beneficial soil invertebrates. The first step to investigate possible detrimental effects on beneficial soil invertebrates was therefore to conduct laboratory studies under worst–case–scenarios with pure compounds (Romeis et al., 2011). The pure compound 2–phenylethyl ITC spiked in LUFA 2.2 natural soil was tested on isopods, collembolans and earthworms, including estimations of lethal– (LC_x) and effect concentrations (EC_x) (chapters 2, 3, 4). 2–Phenylethyl ITC proved to be toxic for all tested invertebrates, with the collembolan *Folsomia candida* as the most sensitive species, reducing reproduction with 10 percent, even at concentrations of 1 mg/kg soil (chapter 3). Several other studies have also described the toxic effects of 2–phenylethyl ITC, both for beneficial and herbivorous invertebrates (Bjorkman et al., 2011; Hopkins et al., 2009; Van Dam et al., 2009). Effects have, for instance, been found for bacterial and eukaryote communities (Bending and Lincoln, 2000; Rumberger and Marschner, 2004, 2003), root–knot nematodes (Lazzeri et al., 2004; Serra et al., 2002), whitefringed weevil larvae (Matthiessen and Shackleton, 2000, 2005)). This confirms that ITC are not target–specific (Brown and Morra, 1997), which is in accordance with the overlapping toxic effect concentrations found for *F. candida* and the facultative herbivore *Protaphorura fimata* (chapter 3).

Ideally a species sensitivity distribution (SSD) curve is made with ecotoxicological data, in order to assess which fraction of an ecosystem is at risk at particular concentrations of a compound. This widely used statistical risk assessment tool, makes use of no–observed–effect (NOEC) or effect concentrations (LC_x or EC_x) to describe variation among a set of species in toxicity of a certain compound (Posthuma et al., 2002). However, in the case of 2–phenylethyl ITC, a SSD analysis is not feasible due to the lack of consistent exposure methods found for the above mentioned studies. Methods ranged, for instance, from exposure via fumigation, diet, direct contact or addition to soil. Exposure method can substantially affect toxicity results, e.g. some ITC are more toxic than others via fumigation, while opposing results are found when exposure is done via other methods (Omirou et al., 2011). For a statistically sound SSD analysis a minimum of eight species within an ecosystem is necessary, exposed via the same method and with the same compound (Posthuma et al., 2002).

One reason for choosing 2–phenylethyl ITC was its occurrence in the root tissue of plants (van Dam et al., 2009), making it a likely compound to be encountered by soil invertebrates. Furthermore, some studies regard this ITC as one of the most toxic (van Dam et al., 2009), which is crucial when testing a worst–case scenario. For instance, sulforaphane (4–methylsulfinylbutyl ITC), a leaf tissue ITC, was

tested for *F. candida* up to 80 mg/kg soil with no observed toxic effects (*unpublished data*). 2–Propenyl ITC (allyl ITC) was also tested for springtails and earthworms, showing overall lower toxicity than 2–phenylethyl ITC (*unpublished data*). However, 2–propenyl ITC is very volatile (Hopkins et al., 2009), which affects exposure conditions and makes it difficult to obtain consistent results that can be compared between species and ITCs.

In short, specific ITCs were toxic to beneficial soil invertebrates, even at very low concentrations, a result that was unknown at the start of this thesis. Such knowledge is a first step in the risk assessment process of potentially hazardous compounds.

8.2 Impacts of GSL–containing plant material on single soil invertebrate species

Brassica species contain multiple GSL in their tissues (Agerbirk and Olsen, 2012; van Dam et al., 2009). In the field, invertebrates are therefore more likely to encounter multiple ITC at the same time than just a single ITC. Invertebrates were, therefore, exposed to plant material of three different *B. oleracea* cultivars in order to create a more ecologically relevant exposure (chapter 5). Furthermore, *B. oleracea* plants naturally differ in their GSL concentrations and composition between genotypes and populations (Gols et al., 2008). Therefore, this set–up simultaneously obtains toxicological data for effects of natural (baseline) variation within *B. oleracea* on soil invertebrates. The genotype Winspit, rich in 3–butenyl and 2–propenyl GSL (sinigrin – allyl GSL), had the highest GSL concentration and most severe toxic effects on springtail survival and reproduction, and on earthworm reproduction, while the soil microbial community remained largely unaffected. These result mainly showed that naturally occurring variation in GSL total concentration within *B. oleracea* determines the extent of detrimental effects on soil invertebrates. At the moment it remains unclear if these differences in toxicity are indeed due to the total GSL concentration or the (combined) presence of specific GSL (composition). In order to answer this question, toxicity of the pure ITC corresponding to the specific GSL has to be tested per soil invertebrates, data which is currently lacking.

A worst–case–scenario with plant material was tested by using a method, described by Gimsing and Kirkegaard (2009), which ensures maximum GSL release and hydrolysis. In short, plant material was snap–frozen in liquid nitrogen, freeze–dried, fragmented to powder and thawed within moist soil. This method proved very efficient in GSL hydrolysis as GSL levels measured in the soil quickly disappeared after mixing the plant material into the soil, to be replaced by ITC (chapter 5). Pilot experiments with less fragmented plant material, or material that was not freeze–dried, did not result in effective test environments (van Ommen Kloeke *unpublished data*).

Studies with plant material form an essential part within the decision matrix, as they represent more ecologically relevant conditions of exposure and assess potential risks of a GM plant as a whole instead of the individual compounds.

8.3 Underlying mechanism of toxicity – gene expression under ITC stress

Gene expression data can reveal the underlying mechanisms of toxic effects of compounds (van Straalen and Roelofs, 2008), giving insight into whether a compound has a specific mode of action, or cause a more general response, such as narcosis (Janssens et al., 2011). To elucidate the underlying modes of action of 2–phenylethyl ITC, gene expression profiles of the springtail *F. candida* and earthworm *Eisenia andrei* were studied using microarrays, comparing toxic to control conditions. For *F. candida*, the lipophilic nature of 2–phenylethyl ITC dictated gene expression at low effect concentrations (chapter 3). At high effect concentrations the perturbation of a gene encoding follistatin was revealed, a gene directly involved with reproduction (Bickel et al., 2008). Metallothionein, known for its role in metal toxicity stress (Hamer, 1986) was identified as a key player in the molecular response to low and high effect concentrations of 2–phenylethyl ITC (chapter 4). Whether this response is species–specific or can be generalized towards soil invertebrates remains to be elucidated

Gene expression analysis is regarded as a fast and sensitive tool that can complement the more classical ecotoxicological approach (Ankley et al., 2006), especially when combined with quantitative Polymerase Chain Reaction (qPCR). The regulation of specific genes can serve as an early warning signal of hazardous field conditions. Taking 'the field into the lab', the gene expression profiles (or regulation of specific indicator genes) of laboratory–bred invertebrates exposed to potentially toxic soils can indicate potential risks within a couple of days. For instance, in the case of *F. candida* and *E. andrei* the above mentioned indicator genes (follistatin and metallothionein, respectively) can serve as early warning signals to ITC stress. Of course, in order to fully benefit from this technique, studies should be expanded taking into account other ITC, lower lethal or effect concentrations, time and possibly life stage of the invertebrate species.

To fully understand gene expression profiles, transcriptome data should also be compared to baseline fluctuations in gene regulation caused by natural variation found in field soil, the so–called Normal Operating Range (NOR). For *F. candida* this was tested for 26 field soils, varying in physiochemical soil properties, such as pH and fertility, which can greatly influence availability of toxic compounds (de Boer et al., 2011). de Boer et al. (2011) found that a total of 936 genes were differentially ex-

pressed between clay and sandy soils, indicating that soil type is very important factor for NOR. This is, for instance, much higher than the 107 differentially expressed genes found for *F. candida* in response to 2–phenylethyl ITC for EC_{50} concentrations (chapter 3). Clay may cause differences in habitat for springtails and the microbial community on which they feed, e.g. *F. candida* prefers soils with high organic matter content (de Boer et al., 2011). The main insight from the NOR experiments is that when field soil is tested, a proper control should be taken along as reference.

One pitfall of the currently available microarrays is the low annotation, i.e. around 30% of *F. candida* genes and only 12% of *E. andrei* genes on the microarray are fully annotated. Techniques to sequence long RNA fragments and full genomes (e.g. next generation sequencing and RNAseq) are, however, becoming cheaper by the day (Cahais et al., 2012). Together with the increasing attention for bioinformatic data mining, this should solve a large part of the annotation problems.

Taken together, genomic tools are promising assets to reveal early signals of specific stresses and should have a more prominent role in future risk assessment analyses.

8.4 GM versus non–GM plant material

Risk assessment research has often led to a multitude of investigations in order to understand all possible interactions of the hazard to the ecosystem. In this case, the hazard is a novel GM crop. For decision makers, more specific, and often theoretical ecological knowledge, can be irrelevant or confusing (Johnson et al., 2007; Raybould, 2007). Clear and simple hypotheses testing the effects of a GM versus a non–GM is, therefore, usually more informative for decision makers (Raybould, 2007; Romeis et al., 2011). To that end, chapter 7 describes a study which directly compares effects of GM vs. non–GM *B. oleracea* leaf material on survival and reproduction of springtails and earthworms. Per *B. oleracea* genotype (Winspit and DH AG1012) two GM lines and the corresponding non–GM were tested. Three out of the four GM lines did not show enhanced GSL concentration compared to non–GM plants and also did not have negative effects on soil invertebrates. These results suggest that GM per se does not have any detrimental effects. One GM line did show increased levels of 2–propenyl GSL (sinigrin – allyl GSL) and affected survival and reproduction of soil invertebrates, even though total GSL concentration was not increased compared to the non–GM (chapter 7). These results clearly show that possible risks are dependent on GSL identity, rather than total GSL concentrations within plant material. Slight differences in chemical structure can change the toxicity. This was also shown for the closely related species *F. candida* (chapter 3) and *F. fimentaria* (Jensen et al., 2010), exposed under the same conditions to 2–phenylethyl ITC and benzyl ITC, respectively. Although these compounds differ only by one methylene (CH_2), 2–phenylethyl was ca. five times more toxic than benzyl ITC (chapter 3). The main

conclusion of these results is thus that the total concentration of compounds does not always predict the level of toxicity, but is dependent on the identity of the compounds present or even the combination of (interacting) compounds.

8.5 GSL–containing plant material effects on soil processes and invertebrate interactions

In order to investigate potential risks of GM crops within a more ecological context, laboratory tests systems have to be up–scaled to include more realistic scenarios, such as the inclusion of multiple invertebrate species, in which indirect effects are measured via interactions or whole plant approaches (Conner et al., 2003). The second tier of risks assessment specifically aims at increasing ecological relevance, in which typically either microcosms or semi–field tests, such as controlled greenhouse are conducted (Romeis et al., 2011). Chapter 6 describes a microcosm study with three soil invertebrate species that are exposed to GSL–containing plant material mixed into the soil, either as single species or an assemblage of multiple species. Survival and CO_2 production were measured to investigate if these were affected by enhanced GSL levels and presence of multiple species. Overall, higher GSL levels did not affect CO_2 production because earthworms performed well under high GSL levels, which even stimulated springtail survival. Isopods and springtails did not have any effects on CO_2 production, independent of GSL conditions. This is in agreement with the study of Heemsbergen et al. (2004) which showed that identity of species rather than number of species is important for soil ecosystem processes. The same study also concluded that the earthworm *Lumbricus rubellus* had the largest impact on most soil processes, as a single species, and facilitation effects in multiple treatments using various combinations of species (Heemsbergen et al., 2004). These results and the results of chapter 6, confirm the important role that earthworms play in soil ecosystems, which can be attributed to their function as ecosystem engineer (Edwards and Bohlen, 1996). Loss of this functional group can therefore have serious consequences for the soil ecosystem, whereas springtails and isopods seem less important, at least under the microcosm test conditions, used in chapter 6. As the experimental scale becomes more complex, while moving towards more ecologically relevant conditions, so do interpretations and predictions made from the obtained results (Birch et al., 2007). Extrapolating laboratory test results to large scale field studies, on the other hand, also creates difficulties. Microcosm studies therefore form a practical intermediary to test risks under more ecologically relevant, but still controlled, experimental conditions (Romeis et al., 2011).

8.6 Ecological risk assessment – realistic hazard scenarios

The data obtained for the decision matrix within this thesis, mainly gives information on potential hazard of GSL and their hydrolysis products. Ecological risk assessment (ERA), however, is a combination of hazard impact and the likelihood of exposure (Conner et al., 2003; Johnson et al., 2007; Romeis et al., 2011). To that end, laboratory data should be compared with field experimental data in combination with baseline information on field conditions. Likelihood of exposure depends on multiple aspects, such as routes of exposure, persistence of the compound in the soil and agricultural practises involved with the use of GM crops. In the case of GSL, exposure can occur via root systems of growing plants, litter or dead organic matter after harvest, but also via intentional biofumigation practises.

Roots of *B. oleracea* show high levels of several GSL, with 2–phenylethyl GSL being one of the most dominant (Kabouw et al., 2010a; van Dam et al., 2009). Root exudation of GSL or ITC into the rhizosphere is reported by few studies. For instance, 2–phenylethyl ITC was found in the rhizosphere of *B. napus*, with concentrations ranging up to ca. 12 nmol per gram soil. Concentrations changed over the growing season, but peak ITC concentrations were sufficiently high to slightly alter soil bacterial communities (Rumberger and Marschner, 2004). Another study showed variations in rhizosphere conditions of different *B. oleracea* cultivars, with one cultivar having a positive effect on root–feeding nematodes, due to its lack of 2–phenylethyl GSL. Earthworms and springtails were, however, unaffected by the different growing *B. oleracea* cultivars (Kabouw et al., 2010b). Moreover, a semi–field study investigating the habitat preference of the earthworms *Lumbricus terrestris* under growing crops, also did not observe any deterrent effects of growing *Brassica* crops (Valckx et al., 2011). Based on these studies, root exudation of GSL or ITC seems a harmless route of exposure for soil invertebrates. It must be noted, however, that of these discussed studies only Kabouw et al. (2010a) measured GSL concentrations of the crops (Kabouw et al., 2010a), Table 2) and observed GSL concentrations in root tissue (average = 11.15 μmol/g $\pm$ 2.49 standard deviation) and shoot tissue (average = 3.14 μmol/g $\pm$ 0.79 SD). These values are much lower than the GSL concentrations found for Winspit leaf material (chapter 7), which could reach up to 37.7 μmol/g $\pm$ 0.63 SD in the wild type alone. This potential route of exposure, therefore, deserves more attention and field tests with GM and non–GM *B. oleracea* crops differing in GSL concentration should be conducted.

Information on the concentration and composition of *Brassica* litter is currently very limited. Only one study was found in the literature, in which air dried residues of several crops, including two *Brassicaceae*, were tested via litter bags in a field with the earthworm *L. terrestris* (Valckx et al., 2011). Valckx et al. (2011) demonstrated

that there was no preference between *Brassicaceae* litter or other crop litter, based on litter disappearance under field conditions. However, it is difficult to draw conclusions about the potential exposure via litter based on a single study using crops with unknown GSL concentrations. Litter bag tests do, however, seem a practical method for testing such effects.

A more deliberate route of exposure is via biofumigation, an agricultural practise which incorporates GSL–containing tissue into the soil in order to control for soil–borne pests (Kirkegaard and Sarwar, 1998; Gimsing and Kirkegaard, 2009). This technique was developed as a more natural alternative for synthetic pesticides and soil fumigants, such as metam sodium and methyl bromide (Björkman et al., 2011; De Nicola et al., 2013). Level of tissue disruption, presence of water, and high soil temperature all positively affect release efficiencies of ITC from GSL–containing plant material, which can potentially induce concentrations up to 100 nmol ITC per gram soil (Gimsing and Kirkegaard, 2006; Matthiessen and Kirkegaard, 2006; Gimsing and Kirkegaard, 2009). Only very few field studies report effects of biofumigation on beneficial soil invertebrates. One study that incorporated commercially available *B. carinata* seed meal (seeds ground to powder) into the soil (2500 kg per hectare) showed suppressive effects on the entomopathogenic nematode *Steinernema feltiae* (Henderson et al., 2009). Moreover, biofumigation strategies have developed substantially over the years. Methods to measure air emissions of ITC (volatile fraction of ITC) in the field after biofumigation have been developed, showing concentrations of 188, 6.1, and 0.7 μg per m^3 for 2–propenyl, benzyl, and phenethyl ITC, respectively (Trott et al., 2012). The effects of these ITC emissions after biofumigation on the soil ecosystem are however currently unknown. The most recent development in biofumigation practises introduces an irrigation system with liquid products of *B. carinata* containing high levels of 2–propenyl GSL with conversion efficiencies to 2–propenyl ITC 86% (De Nicola et al., 2013). The liquid, however, also showed lethal effects for *F. candida* with LC_{50} values already at 1.7 g per litre defatted seed meal, corresponding to 3.3 mg 2–propenyl ITC per kg soil (De Nicola et al., 2013). Taken together, biofumigation practises could increase field conditions to levels that have proven to be toxic for beneficial soil invertebrates, especially when techniques become even more refined. Earthworms are the most important species in agricultural fields (Edwards and Bohlen, 1996) and although they seem less affected by ITC with respect to survival (chapters 4, 5, 6), sufficiently high concentrations can affect reproduction (chapter 5) and even survival (chapter 7). Agricultural systems making use of biofumigation should thus take the observed effect concentrations of beneficial soil invertebrates into careful consideration when choosing their levels of exposure.

8.7 Compound stability

From an environmental point of view, one major advantage of ITC, compared to conventional pesticides, is the high biodegradability, which minimizes the risk of persistence in the environment or leaching (Brown and Morra, 1997; Gimsing and Kirkegaard, 2006). For instance, the dissipation of 2–phenylethyl ITC in natural soil showed in half–lives ranging between 13 and 96 hours depending on initial concentrations (chapters 2 and 3). Benzyl ITC showed similar dissipation patterns in natural and non–sterile soil (Gimsing and Kirkegaard, 2009; Jensen et al., 2010). Both are aromatic ITC, which characteristically have a very lipophilic nature and thus easily sorb to soil particles (van Dam et al., 2009). Regardless, they are lost from the soil within days, which is ascribed to microbial degradation, based on a study comparing dissipation rates between sterile and non–sterile soils (Gimsing and Kirkegaard, 2009). Moreover, water content of the soil affects degradability (chapter 3), Gimsing and Kirkegaard (2009)). Aliphatic ITC have other chemical characteristics and are usually more volatile (less lipophylic) than aromatic ITC, such as benzyl ITC (Gimsing and Kirkegaard, 2009). For instance, 2–propenyl ITC was measured after spiking into LUFA 2.2. soil, but showed losses of over fifty percent of nominal concentration at time zero, even at concentrations of 600 mg ITC per g soil (*unpublished data*). This means that during the preparation of the soil for ecotoxicological experiments more than half of the intended concentration is already lost due to the volatile nature of 2–propenyl ITC. Dissipation rates of ITC are rarely studied, possibly due to their fast disappearance, making them hard to detect, especially when time intervals are not chosen carefully. A disadvantage of the fast degradation of ITC is that most classical ecotoxicological models, such as dose response curves, do not take this into account, which limits their usability (chapter 2, Jager et al. (2011)). Degradation of GSL and ITC have rarely been studied directly in the field (Gimsing and Kirkegaard, 2009), and could be affected by temperature, moisture and activity of soil biota. What is certain is that application of GSL–containing material for biofumigation will result in a pulse dosage, after which GSL, and subsequently ITC, will disappear rapidly. From an environmental point of view this is highly desirable, but for pest management this could mean that the fumigating effect will not last long enough.

8.8 Agricultural practise

Finally, a comparison should be made to current agricultural practises, as indirect impacts of GM crops may arise from changes in managing practises compared to conventional crops (Graef et al., 2012; Hails, 2002). In principle, any kind of soil manipulation will affect the soil ecosystem (Mulder and Lotz, 2009). For instance, novel crops can change agricultural practises that can actually be beneficial to the

environment, e.g. less use of pesticide or no–tillage systems when GM crops are grown (Raybould, 2007). This was, for instance, shown for Bt maize, where observed effects on the soil ecosystem were most likely the results of changes in agricultural practises rather than GM (Cortet et al., 2007; **?**). At the moment GM *B. oleracea* is not grown on large field sites or commercial scales. Assessing effects of altered agricultural practises is therefore not possible at this moment.

Field studies using GM *B. oleracea* are currently (to my knowledge) non–existent. This is mainly because such crops were not available for large scale field experiments, and because strict legislations surrounding GM crops make initiation of field studies difficult and time–consuming. EU legislation does, however, demand field trials for every GM crop before they can be used on a commercial scale. Moreover, field trials are essential for higher tier evaluation of GM crops, evaluating for instance long–term effects or changes in agricultural practises, as discussed above (Romeis et al., 2011). To that end, Graef et al. (2012) recently proposed the establishment of an environmental monitoring framework, ENSyGMO. This framework entails long term field studies within a network of various field testing sites in Europe, including the application of harmonized experimental designs and procedures. Such a network can form the basis of scientifically sound baseline data, much needed for GM crop research on higher–tier scales.

8.9 Perspectives

In conclusion, all aspects of the decision matrix designed for the risk assessment of potential detrimental effects of novel GM crops have been addressed using *B. oleracea* varying in GSL and several soil invertebrates as ecological models. The combined results and the choice for a tiered approach (Romeis et al., 2011) give a comprehensive overview of potential adverse effects at multiple organizational scales ranging from molecular to soil process effects. The decision matrix can thus be useful for risk assessment of novel GM crops.

Moreover, its applicability should not be limited to research on novel traits, but can be extended to other plant traits and environmental conditions that are possibly hazardous to the soil ecosystem. A first attempt in this direction was made in chapter 6, using cadmium–containing plant material (mimicking phytoremediation of contaminated sites), investigating its effects on soil processes, survival of invertebrate species and their interactions in a microcosm study. Cadmium exposure levels, obtained via the plant material, were not high enough to exert detrimental effects on soil invertebrate survival or species interactions, nor did it have long–term negative effects on CO_2 production. Still, these results prove that such experiments with different plant species and traits, potentially inducing stress to the soil ecosystem, are feasible within the decision matrix.

Field tests and baseline data are essential to put the obtained results of the decision matrix into perspective, but remain a challenge due to complicated legislations surrounding GM. Laboratory and semi–field tests, such as described within the decision matrix, are therefore a more practical and feasible approach which can answer most hypotheses regarding GM hazards towards the environment (Romeis et al., 2011; Raybould, 2007). In some cases this may even make higher–tier test (field) less necessary (Romeis et al., 2011).

The social, economic and sometimes even environmental advantages of GM crops call for production on commercial scales, rapidly increasing their proportion of total agricultural land use in the near future. Novel GM crops are developed every day, not only with single novel traits, but also multiple GM traits stacked within one crop (James, 2011). With every novel crop, the decision matrix presented in this thesis provides essential information needed to make an informed decision on potential risks towards the soil ecosystem.

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Summary

Agriculture is essential to feed our ever increasing human population and depends largely on healthy and fertile soil. Aboveground production of crops, or plants in general, is inextricably linked to a complex web of an astonishing number of organisms, chemical reactions, processes and interactions; the soil ecosystem. Especially the decomposition of dead organic matter is one of the most important soil processes which returns essential nutrients to the soil ecosystem, ready to be taken up by plants. Decomposition is, amongst other things, dependent on microbes and soil invertebrates, including earthworms, isopods and many other species, each performing a particular function. The identity of species and functional diversity within the soil community, rather than the number and biodiversity of species, drive soil ecosystem processes. The loss of a single species that represents an essential soil function may, therefore, have serious consequences for soil ecosystem functioning, quality and plant production.

Soil ecosystems can be challenged by many factors, including the implementation of novel crops on an agricultural field. Novel crops can, for instance, arise via genetic modification (GM). As with any new technology, GM raised many concerns about potential undesired ecological impacts, such as effects on essential soil invertebrates. This thesis therefore, focused on how novel (GM) crops can affect the soil ecosystem and how potential risks can be assessed.

As a model for novel GM crops, *Brassica oleracea* was chosen. Many of our daily vegetables belong to this species, such as broccoli, cabbage and Brussels sprouts. *Brassica* species are well–known for to their anti–herbivore defence strategy involving the production of glucosinolates (GSL). Tissue damage of these crops, due to for instance chewing by herbivores, converts the GSL into several toxic compounds, of which the isothiocyanates (ITC) are the most well–known. ITCs have shown indistinct toxicity towards insect herbivores and could thus potentially also have detrimental effects on beneficial soil invertebrates. Recent studies discovered that ITCs are anti–oxidants and, for instance, show anti–carcinogenic activity in humans. Furthermore, plants containing GSL are used as an alternative to synthetic pesticides for

pest management on agricultural fields, making use of the natural toxicity of these plants. This practise is better known as biofumigation. These socio–economic benefits have raised the demand for crop with elevated GSL levels, which can be obtained via GM. However, before such novel GM crops are allowed in the field, potential risks towards the environment have to be investigated.

The aim of my PhD was therefore to develop a practical tool, based on scientific studies, which should aid governmental decision makers in the assessment of potential risks of novel (GM) crops towards soil ecosystems. To that end, a decision matrix was developed, representing a tiered approach to investigate potential adverse effects of GSL and their hydrolysis products at different organizational levels (from molecular to community). This approach combined the research fields of toxicology, genomics and community ecology. Five key questions formed the backbone of this thesis: i) What are the effects of ITC and GSL–containing plant material on survival and reproduction of soil invertebrates? ii) What is the underlying mechanism of the toxic effects on a gene expression level? iii) What is the effect of GM compared to non–GM plant material on soil invertebrates? iv) What are the effects of GSL containing plant material on soil invertebrate communities? v) How do results found in laboratory studies compare to field baseline data?

To answer the first question, effects of 2–phenylethyl GSL hydrolysis products were investigated for four soil invertebrates; the springtails *Folsomia candida* (fungivore), *Protaphorura fimata* (facultative herbivore), the earthworm *Eisenia andrei* and the isopod *Porcellio scaber*. Using internationally standardized guidelines, experiments with 2–phenylethyl ITC demonstrated that these natural toxins can have serious impacts on survival and reproduction of soil invertebrates. These effects were within environmentally relevant concentrations found in agricultural soils. Concentrations much lower than reported in soils after biofumigation, for instance, easily reduced 50% of the reproduction and survival of the invertebrate species investigated. This illustrates the toxic character of these compounds on soil invertebrates. Results, furthermore, showed that springtail species were the most sensitive to ITC compared to earthworms and isopods. Moreover these natural toxins are not target specific, affecting both herbivore (pest species) and non–herbivore (non–target) species to a similar degree.

Besides tests with specific ITC, experiments were conducted using material from plants which varied naturally in GSL concentration and composition. In this setting, soil invertebrates were exposed to a wide range of ITCs simultaneously via fragmented plant material mixed through soil (biofumigation). These experiments confirmed that increased total concentration of GSL (and thus ITC), decreased survival and reproduction of soil invertebrates, although earthworm survival was not affected. Moreover, the soil microbial community was investigated over time following exposure. A peak effect on microbial communities was found after one week, with

subsequent restoration to original communities taking place. Overall, the microbial community therefore seems quite resilient to GSL stress conditions.

In order to study the underlying modes of action of 2–phenylethyl ITC, gene expression profiles of the springtail *F. candida* and earthworm *E. andrei* were investigated. Comparing such profiles obtained under control and stressed conditions, illustrate changes in the physiological status of an organism at a particular time. For springtails, the importance of changes in fatty acid metabolism was revealed, reflecting the lipophilic character of the compound. Furthermore, a direct link to reproductive impairment was found among the affected genes of springtails, while this was not apparent in earthworms. In worms, ITC mainly affected chitin production and induced several general and specific oxidative stress related responses. Gene expression profiles found in these studies yield valuable leads for early warning signals of ITC toxic stress in soil invertebrates.

High GSL levels in *B. oleracea* plants occur naturally, but can be further enhanced via genetic modification (GM). To understand if GM per se could affect soil invertebrates, species were exposed to GM *B. oleracea* plant material of several GM plants developed during the project. Effects of GM plant material were tested via biofumigation (mixing through soil) and directly compared to non–GM material. Most of these GM plants did not show any additional negative effects on soil invertebrates compared to naturally occurring *B. oleracea* plants. Only one GM plant was more toxic due to the increase in concentration of one specific GSL, whereas total GSL concentration was the same as non–GM natural varieties. These results show that it is not GM per se, but GSL specific concentrations that could potentially form a risk towards the environment.

In order to better reflect the complexity of soil ecosystems, experiments with *B. oleracea* plant material were conducted using various ensembles of soil invertebrates in microcosms. Using either plant material with high or low GSL concentration, effects on invertebrate survival and soil processes (CO_2 production) were measured. This set up would reveal if particular species and soil processes would perform better or worse in the presence of other species and under stress of GSL. Exposure to high GSL plant material, reduced springtails survival, but at the same time, springtail survival was positively affected by the presence of earthworms. Soil processes remained largely unaffected mainly because earthworms performed well under high GSL conditions. These results confirm the important role of earthworms within the soil ecosystem. Their well–being is therefore of utmost importance when considering the introduction of new crops.

This thesis ends with a general discussion to put the obtained results into perspective of what could potentially be found in the field. This is because ecological risk assessment (ERA) is a combination of hazard impact (toxic potential) and the likelihood of exposure to the hazard. Thus comparisons of laboratory obtained data

to field conditions including natural variations found in the field, so–called baseline data, were made. In the case of GSL (in general), exposure can occur via root systems of growing plants, decomposition of dead plant material, but also via intentional mixing of material into the soil (biofumigation). Field data on GSL are currently very limited, hence the assessment of potential risks is hard to predict and deserves more attention. Nonetheless, exposure via roots and decomposition of dead plant material seem harmless. Biofumigation practises however, have shown to reach concentrations that are toxic to the soil invertebrates investigated within this thesis. Especially as techniques to increase the efficiency of GSL hydrolysis in soil, and thus toxicity, are improving rapidly, care should be taken in order to maintain a healthy soil ecosystem.

An important issue to consider is the stability of GSL and ITC in soil. It is generally known that both compounds disappear rapidly from the soil via evaporation or degradation into harmless compounds. This was also studied within this thesis, showing that concentrations of 2–phenylethyl ITC were halved within, on average, 18 hours. This suggests that soil ecosystem communities are only exposed for a very short time to toxic conditions, created by GSL containing plant material, and may be able to recover quickly after GSL release.

Field experimental data on GM *B. oleracea* plants is, to my knowledge, currently non–existent, primarily due to the very strict legislation surrounding GM crops. Such data is essential to study, for instance, long–term effects and indirect impacts of GM crops which may arise from changes in agricultural managing practises compared to conventional crops. What should be noted is that soil ecosystems could also actually benefit from GM crops due to changes in agricultural management. For instance, with some GM crops the use of synthetic pesticides is decreased, providing more advantageous conditions for beneficial soil invertebrates and thus a healthier soil ecosystem.

In conclusion, the decision matrix developed within this thesis offers a comprehensive overview of potential adverse effects of novel (GM) crops on multiple organizational scales ranging from molecular to soil process effects. The decision matrix can thus be a useful tool for risk assessment. Moreover, its applicability can be extended to other plant traits and environmental conditions that are potentially hazardous to the soil ecosystem.

Samenvatting

Leven in een broccoli wereld: Ontwerp van een beslis matrix ter beoordeling van de impact van nieuwe (GM) gewassen op het bodemecosysteem

Landbouw is essentieel voor de voedselvoorziening van mensen en sterk afhankelijk van een gezonde vruchtbare bodem. Bovengrondse productie van gewassen, of planten in het algemeen, is onlosmakelijk verbonden met een complex web van een verbazingwekkend aantal organismen, chemische reacties, processen en interacties, kortom het bodemecosysteem. Vooral de afbraak van dood organisch materiaal (decompositie) is een van de meest belangrijke bodemprocessen dat zorgt voor de terugkeer van essentiële nutriënten aan het bodemecosysteem. Deze nutriënten worden vervolgens weer door planten opgenomen. Decompositie is onder andere afhankelijk van microben en bodem invertebraten, zoals wormen, pissebedden en vele andere organismen, elk met een eigen functie. Vooral de identiteit van soorten en functionele diversiteit van een bodemgemeenschap, in plaats van totaal aantal en biodiversiteit van een soort, sturen bodem ecosysteem processen. Verlies van een enkele soort die een bepaalde functie vertegenwoordigd kan daardoor serieuze consequenties hebben voor bodemecosysteem–functionaliteit, kwaliteit en plant productie.

Bodemecosystemen kunnen onder druk komen te staan door vele factoren waaronder de implementatie van nieuwe gewassen op landbouwgrond. Nieuwe gewassen kunnen onder andere gecreëerd worden via genetische modificatie (GM). Zoals met elke nieuwe technologie, heeft GM ook de nodig bezorgdheid opgeroepen over potentiële ongewenste ecologische impact, zoals effecten op essentiële bodem invertebraten. Dit proefschrift richt zich vanuit die context op hoe nieuwe (GM) gewassen het bodemecosysteem kunnen beïnvloeden en hoe potentiële risico's beoordeeld kunnen worden.

Als model voor een nieuw gewas is gekozen voor *Brassica oleracea*. Veel van onze dagelijkse groenten behoren tot deze plantensoort zoals broccoli, kool en spruitjes. *Brassica* soorten staan bekend om hun anti–herbivoor verdedigingsstrategie die te maken heeft met de productie van glucosinolaten (GSL). Weefsel beschadiging, bijvoorbeeld veroorzaakt door grazende herbivoren, converteert (hydrolyse) in deze

gewassen de GSL in verschillen toxische stoffen, waarvan isothiocyanaten (ITC) het meest bekend zijn. ITCs staan bekend om een niet onderscheidend toxisch vermogen richting herbivore insecten en kunnen daardoor potentieel ook schadelijke effecten hebben op waardetoevoegende bodem invertebraten. Recente studies hebben ontdekt dat ITCs anti–oxidanten zijn en, onder andere, een anti–kankerverwekkende werking hebben in mensen. Daarnaast worden planten met GSL gebruikt als een alternatief voor synthetische pesticides bij gewassenbescherming in de landbouw, gebruikmakend van de natuurlijke toxische eigenschappen van deze planten. Een methode die bekend staat als biofumigatie. Deze sociaal–economische voordelen hebben de vraag naar gewassen met verhoogde GSL gehaltes aangewakkerd, die verkregen kunnen worden via GM. Voordat zulke nieuwe gewassen echter toegestaan worden op het veld, moeten potentiële risico's op het milieu daarvan worden onderzocht.

Het doel van mijn PhD was, vanuit bovenstaande, om een praktische instrument, gebaseerd op wetenschappelijke studies, te ontwikkelen. Deze tool dient beleidsbepalers binnen de overheid bij te staan in de beoordeling van potentiële risico's van nieuwe gewassen op het bodemecosysteem. Daartoe is een beslis matrix ontwikkeld met een gelaagde aanpak, om zo potentieel negatieve effecten van GSL en de daarbij behorende hydrolyse producten te onderzoeken op verschillende organisatorische niveaus (van moleculair tot gemeenschap). Met deze aanpak zijn de verschillende onderzoeksgebieden van toxicologie, genomics en gemeenschapsecologie gecombineerd. Vijf kernvragen vormden de ruggengraat van dit proefschrift: i) Wat is het effect van ITC en GSL bevattend plantmateriaal op de overleving en voortplanting van bodem invertebraten? ii) Wat zijn de onderliggende mechanismen van de toxische effecten op het niveau van gen expressie? iii) Wat is het effect van GM versus niet–GM plantenmateriaal op bodem invertebraten? iv) Wat zijn de effecten van GSL bevattend plantenmateriaal op gemeenschappen van bodem invertebraten? v) Hoe verhouden resultaten van studies uitgevoerd in het laboratorium zich met waarden gevonden in het veld inclusief de natuurlijke variatie daarbinnen.

Om de eerste vraag te beantwoorden zijn de effecten van 2–phenylethyl GSL hydrolyse producten onderzocht voor vier bodem invertebraten; de springstaarten *Folsomia candida* (schimmel eter), *Protaphorura fimata* (facultatieve herbivoor), de worm *Eisenia andrei* en de pissebed *Porcellio scaber*. Gebruikmakend van internationale gestandaardiseerde testrichtlijnen, toonden experimenten met 2–phenylethyl ITC aan dat deze natuurlijke toxische stoffen zeer schadelijke gevolgen kunnen hebben op de overleving en voortplanting van bodem invertebraten. Deze effecten zijn gevonden binnen milieurelevante concentraties, gevonden in landbouwgronden. Concentraties die veel lager dan zijn gevonden in bodems na biofumigatie konden bijvoorbeeld gemakkelijk overleving en voortplanting van de onderzochte invertebrate soorten halveren. Dit illustreert het toxisch karakter van deze stoffen. Resultaten lieten verder zien dat springstaart soorten het meest gevoelig waren voor ITC in verhouding tot

wormen en pissebedden. Bovendien bleken deze stoffen niet doel–specifiek maar hadden hetzelfde effect op herbivoren (pest soorten) als op niet–herbivore (niet–doel) soorten.

Naast de testen met specifieke ITC, werden er experimenten uitgevoerd met materiaal van planten die van nature variëren in GSL concentratie en compositie. In deze samenhang werden bodem invertebraten, via het mixen van gefragmenteerd plantenmateriaal door de bodem (biofumigatie), tegelijkertijd blootgesteld aan meerdere ITCs. Deze experimenten bevestigden dat een toename in GSL concentratie (en dus ITC), de overleving en voortplanting van bodem invertebraten reduceert. Uitzondering hierop waren wormen, die niet geaffecteerd werden in overleving. Daarnaast werd ook de bodem microbiële gemeenschap na blootstelling door de tijd bestudeerd. Hieruit bleek dat alleen een week na blootstelling een piek effect te meten was, waarna de gemeenschap zich herstelde naar oorspronkelijke waarden. Microbiële gemeenschappen zijn daarmee veerkrachtig onder GSL stres condities.

Om de onderliggende werkingsmechanisme van 2–phenylethyl ITC te onderzoeken werden genexpressie profielen van de springstaart *F. candida* en worm *E. andrei* bestudeerd. Vergelijking van dergelijke profielen verkregen onder controle en gestreste omstandigheden, illustreren veranderingen in de fysiologische toestand van een organisme op een bepaald tijdstip. Voor springstaarten werd het belang van veranderingen in de vetzuurmetabolisme geopenbaard, waarin de vet minnende aard van ITC gereflecteerd wordt. Verder werd een directe link naar voortplantingsschade gevonden onder de geaffecteerde genen van springstaarten, terwijl dit niet zichtbaar was in wormen. ITC beïnvloedde in wormen vooral chitine productie en veroorzaakte een aantal algemene en specifieke oxidatieve stress gerelateerde reacties. Genexpressie profielen die in deze studies gevonden zijn leveren waardevolle aanwijzingen voor vroegtijdige signalen van ITC gerelateerd toxische stress in bodem invertebraten.

Hoge niveaus van GSL in *B. oleracea* komen van nature voor, maar kunnen verder verhoogd worden via genetische modificatie (GM). Om te begrijpen of GM van zichzelf bodem invertebraten beïnvloed, zijn soorten blootgesteld aan GM *B. oleracea* plantenmateriaal van verschillende GM planten die zijn ontwikkeld tijdens dit project. Effecten van GM plantenmateriaal werden getest via biofumigatie (mixen door bodem) en direct vergeleken met niet–GM plantenmateriaal. De meesten van deze GM planten hadden geen additionele negatieve effecten op bodem invertebraten anders dan in het wild groeiende *B. oleracea* planten. Slechts een GM plant was meer toxisch door een verhoogde concentratie van een specifieke GSL, terwijl de total concentratie GSL hetzelfde was als niet–GM natuurlijke variaties. Deze resultaten laten zien dat GM niet per se een potentieel risico vormt voor het milieu, maar GSL specifieke concentraties wel.

Om de complexiteit van het bodemecosysteem beter weer te geven, werden experimenten uitgevoerd met *B. oleracea* plantenmateriaal en verschillende samenstellingen

van bodem invertebraten in microkosmossen. Gebruikmakend van plantenmateriaal met hoge of lage GSL, werden effecten op overleving van bodem invertebraten en bodemprocessen (CO_2 productie) gemeten. Deze setup diende aan te tonen of bepaalde soorten het beter of minder goed doen in de aanwezigheid van andere soorten en onder GSL stres. Blootstelling aan plantmateriaal met hoge GSL reduceerde overleving van springstaarten, maar tegelijkertijd werd de overleving van springstaarten positief beïnvloed door de aanwezigheid van wormen. Bodemprocessen werden voor het merendeel niet beïnvloed vooral omdat wormen het goed deden onder condities met hoge GSL. Deze resultaten bevestigen de belangrijke rol die wormen hebben binnen het bodemecosysteem. Hun welzijn is daarom zeer belangrijk wanneer de introductie van nieuwe gewassen overwogen wordt.

Dit proefschrift eindigt met een algemene discussie om gevonden resultaten in het perspectief te zetten van wat mogelijk gevonden kan worden in het veld. Dit is nodig omdat ecologische risicobeoordeling (ERA) de combinatie is van gevaar invloed (toxisch potentieel) en de kans op blootstelling aan het gevaar. Daarom werden in het laboratorium verkregen resultaten vergelijken met veldomstandigheden, inclusief natuurlijke variatie daarbinnen. In het geval van GSL (in het algemeen), kan blootstelling optreden via het wortelstelsel van groeiende planten, afbraak van dood plantenmateriaal, maar ook via opzettelijke vermenging van materiaal in de bodem (biofumigatie). Veldgegevens over GSL zijn op dit moment zeer beperkt, vandaar dat de beoordeling van mogelijke risico's moeilijk te voorspellen is en meer aandacht verdient. Toch lijkt blootstelling via wortels en afbraak van dood plantenmateriaal onschuldig. Biofumigatie technieken kunnen echter concentraties bereiken die toxisch zijn voor de bodem invertebraten die in dit proefschrift onderzocht zijn. Vooral nu technieken om de efficiëntie van GSL hydrolyse in grond, en dus toxiciteit, snel verbeteren, moet gelet worden op handhaving van een gezond bodemecosysteem.

Een belangrijk punt dat meespeelt in overweging is de stabiliteit van GSL en ITC in de bodem. Het is algemeen bekend dat beide stoffen snel uit de bodem verdwijnen via verdamping of degradatie in onschadelijke verbindingen. Dit werd ook bestudeerd in dit proefschrift, waaruit bleek dat de concentraties van 2–phenylethyl ITC binnen gemiddeld 18 uur werden gehalveerd. Dit suggereert dat bodemecosysteem gemeenschappen alleen gedurende een zeer korte tijd aan toxische omstandigheden, gecreëerd door GSL bevattend plantmateriaal, blootgesteld worden en in staat zijn om snel te herstellen na GSL vrijlating.

Experimentele data over GM *B. oleracea* planten uit het veld bestaat tot op het moment van dit schrijven nog niet, wat onder andere te wijten is aan de zeer strenge wetgeving rond GM gewassen. Dergelijke data is essentieel om, bijvoorbeeld, lange termijn effecten en indirecte effecten van GM gewassen te bestuderen die kunnen ontstaan door veranderingen in de landbouw managementmethoden in vergelijking met conventionele gewassen. Opgemerkt dient te worden dat de bodemecosystemen

ook daadwerkelijk kunnen profiteren van GM gewassen als gevolg van veranderingen in de landbouw managementmethoden. Bijvoorbeeld, bij een aantal GM gewassen kan het gebruik van synthetische pesticiden worden verlaagd, waardoor gunstigere omstandigheden voor waardetoevoegende bodem invertebraten ontstaan en daarmee een gezonder bodemecosysteem.

Concluderend, de beslis matrix die ontwikkeld is binnen dit proefschrift biedt een uitgebreid overzicht van de mogelijke schadelijke effecten van nieuwe (GM) gewassen op verschillende organisatorische niveaus, variërend van moleculaire tot de bodemproces effecten. De beslis matrix is daarmee een nuttig instrument voor risicobeoordeling. Bovendien kan de toepasbaarheid worden uitgebreid tot andere planteigenschappen en milieufactoren die mogelijk schadelijk zijn voor het bodemecosystem

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Finally I would like to take this opportunity to express my deepest gratitude to the love of my life: Raymond. You are my soulmate, you complete me, challenge me, charm me, tease me when I become to serious (which is often), laugh at my craziness or make me laugh when a bit down, but most of all you make me want to be a better person. For ten years already we have grown up together and I wish for nothing more than to continue to reach for the stars with you!

Curriculum vitae

As a child I already ran around the garden with worms, spiders or whatever creepy crawly I could find. As early as I can remember, nature fascinated me! In early life, this interest resulted in many a watercolour painting of some kind of animal and critical investigations of anything living out there.

At high school following pre–university secondary education (vwo), I chose a wide range of subjects ; biology (of course!), mathematics, but also art and even economics. After high school, I had to choose between art and science. Since both alternatives didn't promise great career opportunities, I decided to pick my brain a bit more and to leave art for my spare time (although the cover of this thesis suggests otherwise...).

With my BSc Biology at Utrecht University and MSc Ecology at the VU University, science became an endless quest to unravel the many mysteries of nature. The decision to accept the PhD position offered to me, was therefore an easy one. The interdisciplinary and applied character of the PhD subject really triggered my enthusiasm. My PhD time was fantastic and a great opportunity to develop myself as an independent researcher. I learned so many awesome techniques and skills, even some that I never expected: how to handle obnoxious students, deal with disappointments (the many experiments that failed!) or fun; setting up international collaborations.

Although I am really passionate about science, I always had so many other interests which were not directly linked to academia, such as organizing many workshops or fun activities. The energy this gave me, made me realize that perhaps a career in science would not be the best option after my PhD.

Hence, I choose to try something outside academia after my PhD. So when the opportunity came to start as Publisher for Elsevier, I took it! In this new environment I found the link between several of my interest, combining science with business, organising, travelling, and even some art. It is the start of an exciting time, with many possibilities, opportunities and challenges opening up to me.

Nobody knows what the future holds. What I do know is...
I will always surpass the one I was yesterday!

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C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Ann Elisabeth Elaine van Ommen Kloeke

born on 26 February 1984 in Elburg, The Netherlands

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Amsterdam, 2 July 2013

the Chairman of the SENSE board — Prof. dr. Rik Leemans

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AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Mrs. Elaine van Ommen Kloeke** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 60 ECTS, including the following activities:

SENSE PhD Courses
- Environmental Research in Context
- Research Context Activity: Initiating and organizing reflection meeting with relevant role models in science and beyond, entitled: Words of Wisdom, 2010-2012
- Principles of Ecological Genomics
- Environmental Risk Assessment of Micropollutants

Other PhD Courses
- Bioinformatics-II: Analysis of genome-wide expression data
- Reading Series in Community Ecology
- International Course in Automated Functional Annotation and Data Mining
- Career orientation course

Management and Didactic Skills Training
- Organising the *Phd & Post-doc weekend*, Department of Animal Ecology, VU Amsterdam
- Supervision of four BSc theses
- Practical supervision for the BSc course *Evolutionaire ecologie & gedrag*

External training at a foreign research institute
- GC-MS analysis of compounds, University of Copenhagen, Denmark
- HPLC-MS analysis of compounds, Max Planck Institute for Chemical Ecology, Germany

Oral Presentations
- *Effects of elevated Glucosinolate levels in crops on function and diversity of non-target soil invertebrates*. Ecogenomics day 2010, KNAW, 21 April 2010, Amsterdam, the Netherlands
- *Natural toxins and their molecular and life-history effects on non-target soil invertebrates*. Netherlands Annual Ecology Meeting (NAEM) , 8-9 February 2012, Lunteren, the Netherlands
- *Genetically modified crops: risks towards soil ecosystem services.* SENSE Science Market: Towards a Biobased economy, 25 October 2012, The Hague, the Netherlands
- *Effects of glucosinolate products on non-target organisms.* 2nd Conference on Glucosinolates, 24-27 May 2009, Elsinore, Denmark
- *Effects of elevated Glucosinolate levels in crops on function and diversity of non-target soil invertebrates*. 20th SETAC Europe annual meeting, 23-27 May 2010, Seville, Spain

SENSE Coordinator PhD Education

Drs. Serge Stalpers