5. Mixture exposure advances the onset of biotransformation genes during early exposure

Muriel E. de Boer, Jacintha Ellers, Kora Montagne-Wayer, Cornelis A.M. van Gestel, Nico M. van Straalen, Dick Roelofs

Abstract
Environmental pollution often consists of complex mixtures of chemicals that, upon exposure, can cause interactions in organisms that may differ from the expected effects based on the toxicity of each of the individual compounds. Application of transcriptional profiling is a method that could enhance the mechanistic understanding of toxicological effects by focusing on the molecular responses. Moreover, expression profiling is sensitive enough for testing within the sub-lethal exposure range of exposures, which may provide insight into the possible risks of mixtures with individual compound concentrations below intervention or limit values.

We here report on the dynamics in gene expressions over the first two weeks of exposure of *Folsomia candida* to a mixture of cadmium and phenanthrene, and to cadmium as a single compound. Expression profiles consisted of a set of 86 quantitative high throughput RT-PCR assays designed for indication of molecular stress responses caused by chemical contamination of soil. Time-series analysis with multivariate Principal Response Curves showed a mixture treatment related advance in the onset of transcripts related to biotransformation and a delay in transcripts related to cadmium deposition. These temporal transcriptional differences were associated with antagonistic effects found for reproduction. Activated biotransformation might promote chelation of cadmium to glutathione and thus delay progression of oxidative stress in the early phase of the exposure. This may be related to the antagonistic toxicity effects on reproduction that became manifest over longer term. Temporal dynamics of stress response mechanisms underlying mixture toxicity can serve risk assessment by defining a framework of relevant time intervals for the use of early warning biomarker methods for measuring toxicity pathways of adverse population level effects.
5. Mixture exposure advances onset of biotranformation gene expression

5.1 Introduction

The majority of polluted soil sites consists of a mixed pollution in which different chemicals simultaneously affect biological systems present in the area. Since the ‘omics revolution’ mixture toxicity assessment has increasingly taken a systems biology approach. This new approach entails a mechanistic understanding of mixture effects, in which cellular processes and stress responses play an important role (Bundy et al. 2008).

Transcriptional profiling focuses on the molecular state of mRNA within the cells at a particular moment, in order to describe the cellular processes underlying phenotypic toxicological effects. The advantage of gene expression studies is that they capture the early molecular responses after short exposure times. A transcriptional profile may give insight in the nature of the exposure (e.g. Chapter two of this thesis, Panti et al. 2011) or serve as ‘early warning’ system for phenotypic effects on the organismal or population level after longer term exposures (Robbens et al. 2007; Owen et al. 2008).

For these reasons, the application of molecular biomarkers could become particularly useful in the sublethal concentration ranges that often occur in chemical mixtures present in the environment (see for instance Roelofs et al. 2012). Assessing the mechanisms of mixture toxicity at the transcriptomic level is particularly challenging, because uptake and elimination rates differ between chemicals (Baas et al. 2007). Internally, this causes a variable mixture ratio in the early phase of exposure. This phase often coincides with the exposure time of molecular experiments. These influences may intervene with fundamental transcriptional dynamics and thus, affect the transcriptional effects observed in mixtures (Beggel et al. 2012). Also, cellular stress and detoxification responses can interfere with internal physiological processes such as molting cycle or circadian systems (Schibler 2005, Rinaldi et al. 2007, Posthuma et al.1992, Goodwin 1965) which can add to the dynamic structure of gene expression. Moreover, considering the fact that genes expressed in an early stage of exposure might not be the same as those causally associated with phenotypic effects on a longer time scale, it is essential to understand the sequence of molecular dynamics in order to establish a link between the molecular level and higher levels of effect (Van Straalen and Feder 2012).

The springtail *Folsomia candida* is a common test species in ecotoxicology (Fountain and Hopkin 2005) and was recently introduced as a transcriptomic model in soil ecotoxicology (Timmermans et al. 2007). In a previous ecotoxicogenomics study on *F. candida*, we studied toxic effects of binary mixtures of cadmium and phenanthrene (Chapter four). We found an antagonistic interaction on reproduction between cadmium and phenanthrene after 28 days of exposure. In addition, transcriptional effects were studied after four day exposure to concentrations of single compounds and mixtures that caused 50% effect in the 28-day reproduction test. Measurements
indicated biotransformation, inflammatory and immune system-related transcripts were differentially induced in a treatment-specific manner. This led to the view that oxidative stress levels may have been lower in samples from the mixture treatments than in those from the single compound treatments (Chapter four).

Here, we report on the temporal dynamics of transcriptional responses in *F. candida*. This study is part of the larger experiment on which we reported (Chapter four). The current study compares transcriptional responses to cadmium as a single compound with transcripts of exposure to a binary mixture of cadmium with a background level of phenanthrene at different exposure times up to fourteen days of exposure. Treatment concentrations were selected on the basis of the estimated effect levels found in the before-mentioned study (Chapter four). A set of high throughput RT-qPCR assays was used in a Canonical Redundancy Analysis (RDA) and Principal Response Curve analysis (PRC).

We hypothesized that differences in toxicity on reproduction would be related to differences in uptake and/or elimination kinetics between the treatments, which would be reflected in the transcription profiles. Internal metal burden and effects on reproduction of *F. candida* were therefore measured and related to the results on transcriptional response. Our results show differences in the timing between treatments of induction of different groups of related assays, including biotransformation. This may potentially protect the cells against increasing levels of oxidative stress on the longer term.

### 5.2 Material and Methods

**Animals**

*Folsomia candida* (Collembola, Isotomidae, Berlin strain, cultured in our lab) was reared in plastic containers with a water-saturated bottom of plaster of Paris containing 10% charcoal. During rearing as well as toxicity testing, animals were kept at 20°C in a 12:12 light-dark photoperiod with dried baker's yeast (Dr. Oetker) as food. All animals used in the experiments originated from a single *F. candida* culture strain. Since *F. candida* reproduces parthenogenetically, all individuals have identical genotypes.

**Test design**

To assess the dynamics of mixture toxicity on different environmental toxicological levels, effects on reproduction after 28 days (ISO 1999; guideline 11267) were
compared to internal cadmium concentrations and gene expression dynamics over the first two weeks of exposure.

The current experiment was conducted jointly with the gene expression study described earlier (Chapter four). The treatments used in the current experiment were selected on the basis of the findings of Chapter four of this thesis, for the estimated EC_{50} level (28-days; reproduction) for cadmium, which was 36 mg Cd/kg dry soil. In a binary mixture of cadmium and phenanthrene, an antagonistic interaction was found such that 36 mg/kg cadmium + 13 mg/kg of phenanthrene gave an estimated effect level of only around 20%.

We exposed *F. candida* for 2, 4, 7 or 14 days to test soils treated with cadmium (36 mg/kg cadmium) or a binary mixture treatment of 36 mg/kg cadmium + 13 mg/kg phenanthrene, or solvent control soils.

**Treatments**

Lufa 2.2 reference soil (characteristics: soil pH-CaCl₂ 5.5, total organic carbon content 2.1%, water-holding capacity 46.5%; LUFA-Speyer, Speyer, Germany) was spiked following the procedure of ISO (1999). For the mixture treatment, phenanthrene (purity 96%; Sigma-Aldrich Chemie, Germany) was dissolved in acetone (Riedel-de Haën, Seelze, Germany) and poured over the soil which was then completely submerged. For the control and cadmium treatments, soils were submerged in using the same volume of pure acetone as in the mixture treatment. Acetone was evaporated overnight under the fume hood. The next day the soils were moistened to 50% of the water holding capacity using water in the control treatments, and water in addition of the required amount of cadmium solution (CdCl₂·H₂O, purity 99%; J.T. Baker, The Netherlands) in the cadmium and mixture treatments. The soils were thoroughly mixed, transferred to the test containers and left overnight to stabilize before test animals were introduced.

Each treatment×time combination was replicated five times. Each biological replicate consisted of an exposure jar with ten intermediate-sized adult *F. candida* of approximately one month of age, on top of a compressed layer of ca 10 g moist freshly prepared test soil. After exposure periods of 0, 2, 4, 7 and 14 days, animals were snap-frozen in N₂(l) and stored at -80°C. One randomly selected individual was used for internal cadmium measurement while the other nine animals were pooled for RNA isolation.

**28-day test for effects on reproduction**

Reproduction was assessed after 28 days of exposure following the ISO guideline 11267, for inhibition of reproduction of *F. candida* by soil pollutants (ISO 1999). In
short, ten 10-12 day old age-synchronized animals were exposed in each exposure jar in 30 g moist uncompressed soil. Animals were extracted after four weeks, by submerging the soil in water and gentle and careful stirring, after which the animals would float to the surface. Automated counting of the number of juveniles was done via a digital photograph (taken with an Olympus C-5060 camera) and Cell Imaging software (Olympus, Zoeterwoude, Netherlands). See for further details Chapter four of this thesis.

**Internal cadmium accumulation**

Only alive animals were sampled; due to poor survival in some treatment×time combinations, the number of animals analyzed varied between 2 and 5. Each sample was weighed and transferred to a small thoroughly cleaned Pyrex tube for digestion using a block heater and HNO$_3$ + HNO$_4$ (Ultrex 2 (71%) and Ultrex; 7:1). After evaporation to dryness, residues were dissolved in 300 μl 0.1 M HNO$_3$ and analyzed by graphite-furnace atomic-absorption spectrometry (Perkin Elmer 5,100). The accuracy of the analytical procedure, measured using certified dogfish liver material (Dolt-2) as reference, was within 10% of the certified value.

**Gene expression analysis**

Total RNA was isolated from the snap-frozen pools of nine individuals per replicate using the SV Total RNA isolation system (Promega) according to the manufacturer's instructions and described in detail in Chapter four. cDNA was synthesized using the reverse-transcriptase system of Promega with the M-MLV reverse transcriptase enzyme and an oligo-dT primer according to manufacturer’s instructions. RNA input amounts were equalized to approximately 0.3 μg per reaction. All reverse transcriptions were performed in a single run in a single 96-wells plate. cDNAs were diluted four times prior to qPCR. The Biomark high throughput qPCR platform, in combination with the 48 x 48 Dynamic Array chips (Fluidigm), was used to run a set of 88 qPCR assays; 86 genes of interest and two validated reference genes (Chapter three). The method has been described in detail in Chapter two.

**Data analysis**

Univariate analysis of variance (ANOVA) was used to test for differences in effect level among treatments from the 28-day test for reproduction. Differences in internal cadmium accumulation were tested with a two-way ANOVA with treatment and time.
5. Mixture exposure advances onset of biotransformation gene expression. Analyses were performed in GraphPad Prism v5 (GraphPad Software, San Diego, USA).

For calculation of the uptake and elimination rate constants for cadmium accumulation in the animals, a first-order one-compartment kinetics model was used (Newman 1998):

\[ C_t = C_0 + \frac{k_1}{k_2} \cdot C_{\text{exp}} \cdot \left(1 - e^{-k_2 \cdot t}\right) \]

where \( C_t \) is the concentration in the organism at time \( t \) (μg/g dry body weight), \( C_0 \) is the concentration in the organism at time 0 (μg/g dry body weight), \( k_1 \) is the uptake rate constant (g dry soil/g dry body weight per day) and \( k_2 \) the elimination rate constant (per day), and \( C_{\text{exp}} \) is the cadmium exposure concentration in the test soil (mg/kg dry soil), \( t \) is time (d).

We used the mean cadmium concentration measured in the control animals as \( C_0 \) (0.340 μg/g dry body weight) and the nominal cadmium concentration as \( C_{\text{exp}} \) (36 mg/kg dry soil). The values of \( k_1 \) and \( k_2 \) and the corresponding 95% confidence interval were calculated by non-linear regression analysis. To compare the uptake and elimination rate constants for cadmium in the animals exposed to cadmium only or to the mixture of cadmium and phenanthrene, a likelihood ratio test was performed. All these analyses were done in SPSS 18.0 for Windows.

The qPCR data were preprocessed in Genex Light v4.3.5 (MultiD Analyses AB 2008). Four technical qPCR replications were inspected and averaged. Average Ct-values were normalized for differences in input using the geometric mean of the expression levels of two reference genes (SDHA and YWHAZ, Chapter three of this thesis) and log2 transformed.

Multivariate analyses were performed using CANOCO for Windows 4.5 (Ter Braak and Šmilauer 2002). Prior to analysis, autoscaling and maximum normalization transformation were performed, in order to eliminate influences of differences in amplitudes and basal expression levels between the assays.

The choice between linear or unimodal response modeling was based on the gradient length present in the data set. The gradient length was determined using Detrended Correspondence Analysis (DCA), and was around 1 standard deviation. In this case the data can best be described using linear models; see Van den Brink et al. (2003) for details on the method.

Redundancy Analysis (RDA) and Principal Response Curve analysis (PRC) were applied to examine gene expression profiles in relation to the treatments in time (Van den Brink and Ter Braak 1999). We followed the step-by-step directions provided by Lepš and Šmilauer (2003; chapter 15). For statistical significance testing of the relationships between the samples and the gene expression and time interactions, Monte Carlo testing was performed with 1000 permutations and the full model settings, with different designs for restricted permutation for each hypothesis tested.
5. Mixture exposure advances onset of biotranformation gene expression

First an RDA was run with restricted Monte Carlo permutations within treatment blocks and the interaction between time and treatment, in order to determine if gene expression changes in time were different between the treatments. Next, PRC analysis was performed to study the temporal expression dynamics between the two treatments in detail. PRC is originally developed to improve the interpretation of treatment related effects in time in research related to community responses (Van den Brink and Ter Braak 1999). It is an applied RDA where the different exposure times are used as covariables and the interactions of treatment × time as explanatory variables. A precondition of a balanced dataset applies for this analysis. Due to mortality, we lost some replicates in particular in the 14-day exposures. We therefore decided to do an initial PRC using 2 replicates of each treatment×time combination, by randomly leaving out excess replicates (The diagram of the first PRC of this analysis is added as Additional file 1). Subsequently, a second PRC was run using four replicates, by omitting the data of the last (14 days) time point. For further analyses we restricted ourselves to the second PRC analysis.

5.3 Results

Ecotoxicological effects on reproduction
The concentrations of cadmium and phenanthrene used were expected to cause 50% reduction of reproduction for the cadmium treatment and 20% for the mixture treatment compared to control (Chapter four). Although the reproduction data from the 28-day toxicity test showed differences between the mean values of the treatments reflecting this trend (mean number of juveniles per pot ± SD: control = 637 ± 235; cadmium = 271 ± 227, 57% effect; mixture = 352 ± 192, 45% effect), the differences could not be statistically confirmed (ANOVA, F_{2,11} = 0.39, p = 0.7).

Cadmium bioaccumulation
Internal cadmium concentrations increased over time (two-way ANOVA, F_{4,30} = 37.4, p < 0.0001; Figure 1), up to 80.2 ±5.0 and 77.2 ±18.4 μg Cd/g dry body weight (mean ±SD) respectively, after 14 days of exposure in the cadmium and the mixture treatment. No significant differences were found between the treatments (two-way ANOVA, F_{4,30} = 0.85, p = 0.5). The uptake rate was relatively slow; after the initial uptake internal cadmium concentrations did not significantly differ between 2 and 4 days (Tukey’s post hoc test; p > 0.05). Also, no significant differences in internal cadmium concentrations were found between 7 and 14 days of exposure (Tukey’s post
5. Mixture exposure advances onset of biotranformation gene expression (Figure 1). Therefore, uptake ($k_1$) and elimination ($k_2$) rate constants for cadmium in the animals (mean ± 95% confidence intervals) were estimated using the one-compartment, first-order kinetic model. Results were for cadmium only exposure: $k_1 = 0.361$ (0.277-0.444) g dry soil/g dry body weight/day; $k_2 = 0.129$ (0.065-0.193) per day and for the mixture $k_1 = 0.344 ± (0.236-0.453)$ g dry soil/g dry body weight/day; $k_2 = 0.138$ (0.053-0.222) per day. Differences between treatments were not significant with $\chi^2_{df=1} < 3.84$ for both $k_1$ and $k_2$.

**Figure 1.** Curve fit of internal cadmium concentrations in Folsomia candida during two weeks of exposure to 36 mg/kg dry soil of cadmium or a mixture of 36 mg/kg dry soil cadmium + 13 mg/kg dry soil phenanthrene. Internal cadmium concentrations increased over time, but did not significantly differ between 2 and 4 days and between 7 and 14 days of exposure (Two-way ANOVA, $F_{2,30} = 37.4$, $p < 0.0001$, followed by Tukey’s multiple comparisons post hoc test; $p < 0.05$). Different letters above each measurement indicate significant differences between exposure times. Differences between treatments were not significant with $\chi^2_{df=1} < 3.84$ for both $k_1$ and $k_2$. 
5. Mixture exposure advances onset of biotransformation gene expression

Figure 2. Heatmap view of RT-qPCR assays average normalized relative gene expression values for each treatment (control = acetone control; cadmium = 36 mg/kg dry soil cadmium; mixture = 36 mg/kg dry soil cadmium + 13 mg/kg dry soil phenanthrene). Folsomia candida were exposed to the treatments for different exposure times (0; 2; 4; 7; 14 days). Darker color intensities represent increased expression values. Details of the assays can be found in Table 3 and the Additional file 1 of Chapter four.)
Transcriptional profiling in time

Figure 2 shows a heatmap of the normalized relative expression values for 86 RT-qPCR assays in each treatment. To investigate if the temporal gene expression changes were treatment-specific, multivariate Principal Response Curve analysis was used. Details of the assays can be found in Table 3 and the Additional file 1 of Chapter four. First, we ran an RDA with an interaction between time and treatment followed by Monte Carlo permutation testing. Significant differences in gene expression dynamics were found between the two treatments over time (RDA, F-ratio = 1.76, p = 0.0020). The percentage of variation in the gene expression profiles’ explained by the first ordination axis (a measure of the explanatory power of the variables), was 3.8% (R 1st axis = 0.67). The low value is explained by the fact that for both treatments (Cd; Cd-Phe mixture) the general cadmium response profile was dominant. Most of the assays did not show differences between the treatments.

We were interesting in finding out which of the assays did deviate and in what way, between treatments in time, therefore we used Principal Response Curve analysis (PRC) to investigate the temporal dynamics of gene expression over time. In a PRC analysis, the controls are used as references and deviations from the controls over time could be observed in the cadmium and the mixture treatment.

Figure 3 shows the first three PRCs in a separate diagram each. Monte Carlo permutation tests confirmed the significance of the first two PRCs. Together, they contributed 29.9% of the treatment variance (PRC1: 23.1%, F-ratio = 10.814, p = 0.0010; PRC2: 6.8%, F-ratio = 3.802, p = 0.0130). PRC1 captures the difference in gene expression between the reference and both treatments, while the main difference in gene expression between the two treatments was characterized by PRC2. Both these responses were present from the initial exposure time of 2 days onward (Figure 3). The third PRC showed an additional response after 7 days exposure which occurred in the cadmium treated samples.
5. Mixture exposure advances onset of biotransformation gene expression

Figure 3. Principal response curves (PRCs) showing treatment effects for various exposure times (0-7d) in F. candida exposed to 36 mg/kg d.s. cadmium (red line) or 36 mg/kg d.s. cadmium + 13 mg/kg d.s. phenanthrene (blue line). The acetone controls are depicted as a horizontal line in time. PRC 1 (left) accounts for 23.1% of the total variation; PRC 2 (middle) for 6.8%. The assay correlation weights (bk) shown on right hand side of the graph can be interpreted as the affinity of the particular assay with the PRC. For clarity of presentation, not all assays are shown.
Gene assays correlating highly with each of the three PRCs are depicted in the associated plot of assays weight in Figure 3. It should be noted that in the first PRC, the negative values for assays weight reflect a positive correlation with the treatment, as the values of the canonical coefficients are negative.

PRC1 includes genes highly induced by both treatments in time, as compared to the control. These include genes related to binding of cadmium (WAP, GLRA1 and MT2), and to inflammation and immune function (IRG6, ACVS, IPNS, SID1). The exact function of the highly correlated MOXD1_2 gene is not known, only that it encodes for an ER localized DBH-like monooxygenase enzyme MOXD1 (Xin et al. 2004). Assays associated with PRC1 reflected the global response to cadmium, and were largely in agreement with our findings from the 4-day period expression profiling experiment (Chapter four of this thesis).

In the second PRC, the cadmium exposed animals showed correlations (negative assay coefficient values) with the lysosomal cathepsin-L like cysteine proteinase (CtSSL), two extracellular matrix glycoproteins; laminin-beta-2 and laminin-a (LAMB2, LAM4), monooxygenase DBH-like 1 (MOXD1_1), dipterincin-D (DPTD) and HAD 1 and 2, which are found in P type ATPases that include cation/copper and heavy metal transporters and proton pumps (Interpro; www.ebi.ac.uk/interpro). This pattern seems to reflect the important pathway of metal binding by metallothionein (correlated in the first PRC) and deposition in lysosomal vesicles. The mixture treatment pattern showed correlations with the induction of phase I biotransformation enzymes (positive assay weight values; different cytochrome P450 (CYP) enzymes) starting from day 2 onwards. Also, phase II (GSTO) and two ABC transporters were correlated with this pattern. Both patterns converged after 7 days. Expression of the assays on the lower end of the diagram, seen in the cadmium treatment after two days of exposure, emerged in the mixture treated samples after 7 days of exposure (see the relative expression levels in Figure 2). Conversely, induction of CYPs occurred in the cadmium treatment. The 7-day exposures were more alike than the short-time exposures.

PRC3 shows that after 2 days, the alcohol dehydrogenase assay (ADHA) and two ABC transporter assays (ABCC1 and ABC/MOAT-D) were induced in both treatments. Also other genes, involved in cell signaling (LIP, SDR9) transcription/translation (DDX18, DNK, CTCFL, HISTHA, IARS2) and transportation (CFL, SCRIB) correlated with this early response after 2 days, but less strongly. Furthermore, PRC3 showed a response of the cadmium treatment after 7 days, which included the induction of cytochrome P450 assays and heat shock proteins.

A biplot of the correlations of all assays with each treatment×time interaction of the first and second PRC is depicted in Figure 4. It reflects the most dominant gene expression changes in the treatments over time. PRC1 (horizontally) separates the controls from the treatments, and can be interpreted as a pattern of severity of stress.
PRC2 (vertically) shows more the nature of the stress, by separating both treatments from each other. The treatment×time arrows of the 7-day exposures point in a more similar direction than the shorter exposures, thus indicating that treatment-specific transcription profiles were most different in the first days of exposure, and treatment profiles were more alike after 7 days of exposure.

**Figure 4.** Biplot of the first two principal response curves (PRCs) showing the assays scores to the different treatment×time interactions of Folsomia candida exposed to 36 mg/kg dry soil cadmium (cadm) or 36 mg/kg dry soil cadmium + 13 mg/kg dry soil phenanthrene (mixt) for various exposure times (T0-T7). PRC 1 (horizontally) accounts for 23.1% of the total variation; PRC2 (vertically) for 6.6%.

**5.4 Discussion**

The aim of this study was to investigate the dynamics of early response transcriptional effects of a binary mixture of sublethal concentrations of cadmium and phenanthrene. In previous experiments, binary mixtures of cadmium and phenanthrene were found to have an antagonistic interaction effect on springtail reproduction (Chapter four). The effect levels of 57% (cadmium) and 45% (mixture) for cadmium and the mixture, respectively exceeded the effect levels estimated by MixTox modeling, which were...
5. Mixture exposure advances onset of biotranformation gene expression

50% and 20% respectively, but still supported the antagonistic interaction by in the fact that addition of phenanthrene reduced the effects on reproduction. No treatment-specific differences in cadmium accumulation were found over time. Therefore, the expectation that the antagonism in the mixture treatment would be associated with a changed cadmium kinetics, was not confirmed.

Due to intrinsic variation between biological replicates (Crouau and Cazes 2003, Van Gestel and Mol 2003) it is difficult to demonstrate significant differences between treatments in 28-day toxicity tests, in particular in the lower effect level ranges. Notwithstanding, the data of the current study clearly demonstrated an antagonistic interaction between cadmium and phenanthrene in their effects on reproduction. We observed no treatment-specific differences in cadmium accumulation over time. Accumulation slowed down over the second week of exposure. Values of 80 μg Cd/g dry body weight after two weeks were in agreement with the literature. Van Gestel and Hensbergen (1997) reported equilibrium of cadmium uptake in *F. candida* to be reached within 2 to 3 weeks. They found internal cadmium concentrations in *F. candida* between 12 and 30 μg Cd/g dry body weight after two weeks of exposure to 50 mg Cd/kg dry soil, in an artificial soil. These lower concentrations could be due to differences in soil type.

Microbial degradation causes the concentration of phenanthrene in the test soil to decrease in the course of the experiment. The half-life for phenanthrene degradation was estimated around 44 days in a study on enchytraeids exposed in Lufa 2.2 soil (Amorim et al. 2011). Although degradation rates may vary depending on the soil and organisms present, based on the nominal concentration of 13 mg/kg dry soil at the beginning of the experiment approximately 10 mg/kg phenanthrene would still have been present by the end of the 14-day expression profiling exposure and approximately 8 mg/kg phenanthrene by the end of the 28-day reproduction toxicity test. According to earlier MixTox modeling of binary mixtures of cadmium and phenanthrene (Chapter four) the addition of 13 or 8 mg/kg phenanthrene to 36 mg/kg cadmium was estimated to have the same effect on reproduction after 28 days. We therefore assume that this binary mixture exposure can be held representative for a low level exposure range of phenanthrene in simultaneous exposure with the EC$_{50}$ level of cadmium.

The transcriptional response to cadmium that we observed was in agreement with our earlier findings (Chapter four): induction of transcripts for sequestration as well as inflammation and immunity. This response increased in time. Between days 2 to 4 the pattern deviated between the treatments (Figure 3). Important differences were present in the early response between the treatments.

The treatment specific pattern revealed in the early response of the cadmium samples (PRC2) indicates for induction of transcripts involved in a well-described cadmium sequestration system (see for instance Van Straalen and Roelofs 2005) of
metallothionein-binding and uptake of the MT-Cd complex in lysosomal vesicles (HAD 1 and 2 induction, cathepsin-L like protease induction, induction of laminins, which are extracellular glycoproteins), and some genes without any annotational genbank hits (NO HITS 1 and 9). Although MT2 expression is induced early on, induction of the other assays of this vesicular/granular excretion route was postponed in the mixture samples until 7 days of exposure. The instant induction of biotransformation, observed in the mixture samples, maybe related to conjugation of free cadmium to glutathione (GSH) as an alternative to metallothionenin binding to Cd. GSH is one of the most versatile and pervasive binding ligands and forms complexes with metals via non-enzymatic reactions (Wang and Ballatori 1998). Cd-glutathione complexes were identified in yeast (Li et al. 1997a, 1997b) and in bivalve molluscs (Zaroogian and Jackim 2000). This last study showed that GSH functioned in acute protection against Cd\(^{2+}\) toxicity prior to MT induction. In F. candida, conjugation of free cadmium to GSH could instigate a rapid Cd\(^{2+}\) detoxification in the mixture exposed samples. Tentatively, the 7-day response seen in the cadmium samples may be related to induction of the oxidative stress defense system triggered by elevated ROS levels caused by cadmium. This is supported by the co-induction of HSP70s.

An arrest in cadmium accumulation was observed between 2 and 4 days of exposure. A similar multi-phasic pattern of cadmium accumulation was present in data of Tubifex tubifex (Steen Redeker and Blust 2004). Reinecke et al. (1999) described a pathological phenotype of Eisenia fetida having a bloated translucent appearance, possibly indicating a disturbed water balance, and speculated that this may be related to disturbed kidney functioning, concurring with the fact that cadmium can have detrimental effects on the kidneys in a number of animal groups. It may be argued that the early response arrest in cadmium accumulation in F. candida is caused by cadmium elimination through renal excretion of Cd-glutathione conjugates.

After 7 days of exposure (Figure 4) responses of the treatments converged to a more general stress response pattern. Consequently, treatment-specific early effects diminished. Common features of the 7-day exposures were induction of cadmium-binding (e.g. MT2, MOXD1), heat shock proteins and the immune response. These common assays represent a progression in oxidative stress levels. Our previous study (Chapter four) indicated that oxidative stress levels may have been higher in the single cadmium treatment than the mixtures. This study extends on this view. The oxidative stress response is under mild stress conditions characterized by the antioxidant defense system plus the induction of the inflammation response when stress intensifies to intermediately levels (Zhang et al. 2008). The suggestion that oxidative stress levels are attenuated in mixtures over the initial exposure phase is supported by the current study: In the biplot, (Figure 4), the location of the inflammatory and immune assays at the lower end, correlated with the cadmium treatment. The direction of the mixture
exposures changed the direction of the cadmium exposures in time, indicating that stress levels in the mixture were increasing.
Our results showed for both treatments that the system is highly dynamic in the early stage of exposure. Transcription profiles in the cadmium and the mixture treatments were more alike after 7 days than after shorter exposure times. Hence, in case of single time point analysis, conclusions about the transcriptional effects of the treatments would depend on the time point considered. Setting an optimal exposure time for the use of stress response profiles as ‘early warning system’ to biomarkers of phenotypic effect (reproduction after 28 days) is therefore not straightforward.

5.5 Conclusions

Multiple time point testing is essential for a mechanistic understanding of transcriptional dynamics of mixture toxicity. The current study illustrates that selection of different time points could easily have led to different conclusions on the effects of the treatments at the transcriptional level, for instance with regards to the induction of biotransformation. Baas et al. (2007) stressed the need to incorporate the factor time in mixture toxicity modeling: Speaking of ‘the’ interaction in the mixture for certain phenotypic effects is impossible, since it depends on the time point considered. We showed that this conclusion also holds for transcriptional profiling.
For risk assessment of mixture toxicity, there is a need to understand in what ways early-phase cellular dynamics of responses affect higher-level toxicity effects. To create a system of sensitive gene expression biomarkers linked to ecotoxicological endpoints, investigation over longer, multiple, exposure times is needed. This is the only way to find those time intervals that avoid complex transcriptional dynamics, but at the same time ensure adequate indication of features such as the nature and degree of toxicity, or the most dominant stress occurring from mixed pollutions.
**Additional file 1.** First principal response curve showing treatment effects for various exposure times (0-14d) in Folsomia candida exposed to 36 mg/kg dry soil cadmium (red line) or 36 mg/kg dry soil cadmium + 13 mg/kg dry soil phenanthrene (blue line). The acetone controls are depicted as the horizontal line in time. The assay correlation weights ($b_i$) shown on right hand side of the graph can be interpreted as the affinity of the particular assay with the PRC. For clarity of presentation, not all assays are shown.