Chapter 6

Gene expression patterns and life cycle responses of toxicant-exposed chironomids

Abstract

Cellular stress responses are frequently presumed to be more sensitive than traditional ecotoxicological life cycle endpoints such as survival and growth. Yet, the focus to reduce test duration and to generate more sensitive endpoints has caused transcriptomics studies to be performed at low doses during short exposures, separately and independently from traditional ecotoxicity tests, making comparisons with life cycle endpoints indirect. Therefore we aimed to directly compare the effects on growth, survival and gene expression of the non-biting midge *Chironomus riparius*. To this purpose, we analysed simultaneously life cycle and transcriptomics responses of chironomid larvae exposed to four model toxicants. We observed that already at the lowest test concentrations many transcripts were significantly differentially expressed, while the life cycle endpoints of *C. riparius* were hardly affected. Analysis of the differentially expressed transcripts showed that at the lowest test concentrations substantial and biologically relevant cellular stress was induced and that many transcripts responded already maximally at these lowest test concentrations. The direct comparison between molecular en life cycle responses after fourteen days of exposure revealed that gene expression is more sensitive to toxicant exposure than life cycle endpoints, underlining the potential of transcriptomics for ecotoxicity testing and environmental risk assessment.

Introduction

Toxicity of compounds to organisms is routinely assessed by observing effects on life cycle endpoints such as survival, development and reproduction (OECD, 2012). However, prior to the onset of sublethal effects on life cycle endpoints, toxicant-exposed organisms are already impacted at the cellular level (Schrirmer et al., 2010). The impact on the cellular level varies depending on the type, magnitude and duration of the toxicant exposure, which is reflected by the activation of different stress response pathways (Simmons et al., 2009). Analysing molecular stress responses in toxicant-exposed organisms can therefore yield information regarding the toxicants mode of action and the mechanisms underlying the sublethal effects observed at higher levels of biological organization (Vandegehuchte et al., 2010). Since these molecular stress responses are reflected by changes in gene expression, transcriptome responses to a wide variety of toxicants have been investigated in genomics models species such as the yeast *Saccharomyces cerevisiae* (Momose and Iwahashi, 2001), the zebrafish *Danio rerio* (Yang et al., 2007) and the fruit fly *Drosophila melanogaster* (Yepiskoposyan et al., 2006). Technological improvements in sequencing technologies (Vera et al., 2008) and the lower cost of microarrays have now made it possible to perform large-scale gene expression studies with species that previously had limited sequencing information, but that are eco(toxico)logically relevant, e.g. the water flea *Daphnia pulex* (Shaw et al., 2007) and the springtail *Folsomia candida* (Nota et al., 2009).

It is generally recognized that transcriptomics holds great potential for ecotoxicity testing and environmental risk assessment (van Straalen and Feder, 2012), especially as it is frequently presumed to be more sensitive than traditional ecotoxicity testing, where effects are assessed on life-cycle endpoints. Yet, the focus on reducing test duration and the generation of more sensitive endpoints, e.g. the No Observed Transcription Effect Level (Lobenhofer et al., 2004), caused transcriptomics studies to be performed at low doses during short exposures, separately and independently from traditional ecotoxicity tests (e.g. Poynton et al., 2008; Connon et al., 2008), making a comparison between traditional endpoints and transcriptomics endpoints indirect. Hence, a reliable comparison between life cycle and transcriptomics endpoints requires to measure these simultaneously, preferably in the same individuals at the end of the life cycle exposure. Since to our knowledge this has rarely been done, our aim was to assess the effects of toxicants which differently affected the life cycle of the non-biting midge *Chironomus riparius* (Marinković et al., 2011) on *C. riparius* gene-expression. Analysing the transcriptome in individual larvae that survived the ecotoxicity tests allowed us to directly compare the sensitivity of gene-expression endpoints with traditional endpoints. An additional advantage of this approach is that we will gain insight on the status of the transcriptome of toxicant-exposed midge larvae at the end of the exposure period, when their development has already been affected. To this purpose we present here the first large-scale gene expression study with the ecotoxicologically relevant

test species *Chironomus riparius*. This insect species has a long history in sediment toxicity testing (e.g. Marinković et al., 2011; León Paumen et al., 2008a), with currently four OECD guidelines being available for acute and chronic toxicity tests (OECD, 2012). We performed 14-day sediment toxicity tests with four model toxicants, i.e. the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene, and measured the effects on survival and growth. We simultaneously analysed changes in gene-expression in individual larvae that survived the 14-day sediment toxicity tests, using a recently developed *C. riparius* gene-expression microarray (Marinković et al., 2012b). Our test setup enabled us 1) to determine at which concentrations general and specific cellular stress responses are observed and compare these to traditional (sub)lethal effect concentrations and 2) to examine the dose-response profiles of individual differentially expressed transcripts.

Materials and methods

Experimental design

Four 14-day sediment toxicity tests were conducted, one with each toxicant, with each experiment designed to measure both the effects of exposure on life cycle responses, i.e. survival and growth, and to analyse in individual surviving larvae the changes in global gene expression. The transcriptome analyses were performed using *Chironomus riparius* microarrays, which were designed based on the sequencing results of a *C. riparius* mRNA library that included mRNA from toxicant-exposed larvae from the current study (Marinković et al., 2012a).

The experiments were conducted with the University of Amsterdam's *C. riparius* culture that was maintained in aquaria containing quartz sand overlaid with Dutch Standard Water at 20 ± 1 ˚C, 65% humidity and a 16: 8 h light: dark photoperiod (Marinković et al., 2011). The culture was fed a mixture of Trouvit® (Trouw, Fontaine-les-Vervins, France) and Tetraphyll® (Tetrawerke, Melle, Germany) in a weight ratio of 20:1. This mixture was also used as food for all subsequent experiments.

Toxicity tests

The 14-day sediment toxicity tests were conducted based on OECD guideline 218 (OECD, 2004) and have been described in detail in (Marinković et al., 2012a). Therefore, for details concerning the preparation of the artificial sediment, the sediment spiking procedures and the methods used for determining the actual toxicant concentrations in the sediment we refer to Marinković et al. (2011; 2012a). In short, the following nominal test concentrations were used for copper ($CuCl_2.2H_2O$, copper standard, Fluka): 10, 20, 30, and

40 mg Cu/kg dw; cadmium (CdCl$_2$, Titrisol$^®$, Merck): 0.5, 1, 2, and 4 mg Cd/kg dw; tributyltin (TBT-Cl, 96% purity, Aldrich): 0.5, 1, 2 and 4 mg Sn/kg dw: and phenanthrene (98% purity, Aldrich): 25, 50, 100 and 200 mg phe/kg dw. Controls and solvent controls were included. Each treatment consisted of seven replicate 400 ml glass test beakers of which two were used for chemical analysis at the start and the end of the experiment. Sediment spiking was performed by adding copper and cadmium stock solutions directly to wet sediment, while for the acetone dissolved compounds tributyltin and phenanthrene a pre-spiking step was included where the acetone was allowed to evaporate. During the spiking 490 mg of food, corresponding to 0.5 mg food/larvae/day for the entire duration of the test, was added to the 420 g wet sediment-toxicant mixtures. The sediment-toxicant-food mixtures were subsequently divided over the seven test beakers. The beakers were topped up with 250 ml of Dutch Standard Water and allowed to equilibrate for a week, , during which pH, conductivity and dissolved oxygen levels were monitored. Subsequently, ten 1st instar (< 24 hours) larvae were added to each test beaker. At day 7 an additional feeding of 17.5 mg food/ beaker was added. At day 14 the test beakers were sieved and the surviving larvae were counted, their length was measured and they were immediately individually flash frozen in liquid nitrogen and stored at -80˚C until processing.

The sediments were extracted and the actual toxicant concentrations were measured using methods described in detail in (Marinković et al., 2011; 2012a) and amounted for copper: 4.3, 14.7, 23.2, 32.8 and 41.4 mg Cu/kg dw sediment; cadmium: <0.01, 0.5; 0.9, 1.9 and 3.1 mg Cd/kg dw sediment; tributyltin: <.0.001, 0.3, 0.7, 1.4 and 2.7 mg Sn/kg dw sediment; phenanthrene: <0.3, 18, 41, 79 and 182 mg phe/kg dw sediment. It should be noted that the first concentration for each toxicant relates to the control sediment. For the actual concentrations in the water we refer to Marinković et al. (2011) who performed toxicity tests with the same toxicants and under identical experimental conditions, and reported the actual toxicant concentrations in the sediment, overlaying water and pore water. The experiments met the OECD guideline 218 (OECD, 2004) quality criteria, i.e. pH was 7.5 ± 0.4, ammonium concentrations remained 0 mg NH$_4^+$/l, conductivity was 665 ± 46 µS/cm, dissolved oxygen levels were above 70% air saturation and water temperature was 20 ± 1° C.

Microarray experiment

For the gene expression experiment we selected the controls, solvent controls and three test concentrations per toxicant, i.e. copper: 10, 20, and 40 mg Cu/kg dw; cadmium: 0.5, 1, and 2 mg Cd/kg dw; tributyltin: 0.5, 1, and 2 mg Sn/kg dw: and phenanthrene: 25, 50, and 100 mg phe/kg dw. RNA was isolated from individual flash frozen *C. riparius* larvae according to the single embryo RNA isolation protocol of de Jong et al. (2010) that uses Qiazol (Qiagen) for RNA extraction and RNeasy MinElute columns (Qiagen) for RNA purification. RNA yield was determined on a NanoDrop ND-1000 UV-VIS

spectrophotometer (Thermo Fisher Scientific), while RNA integrity was examined using a RNA 6000 Nano chip on a 2100 Bioanalyzer (Agilent Technologies). Per RNA sample, 500 ng total RNA was amplified according to the Agilent QuickAmp kit manual (Agilent technologies). The synthesized amplified RNA (aRNA) was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The labelling of test samples with Cy3 and a reference sample (made by pooling equimolar amounts of RNA from test samples) with Cy5, was conducted according to Bruning et al. (2011) using the Amersham Mono-Reactive CyDye Packs (GE Healthcare). After purification of the labelled aRNA with the E.Z.N.A. MicroElute RNA Clean Up Kit, the efficiency of the labelling was determined by measuring the yields of aRNA and CyDye incorporation on the NanoDrop ND-1000. Hybridization samples, containing 1.1 µg test (Cy3) and 1.1 µg reference (Cy5) sample, were made according to the manufacturer's instructions (NimbleGen Arrays User's Guide for Gene Expression Arrays Version 5.0, Roche/NimbleGen). Hybridization of the samples to the custom made 12*135K *C. riparius* microarrays (Marinković et al., 2012b) was carried out for 18 hours at 42°C using NimbleGen Hybridization System 4 (Roche/NimbleGen). After stringent washes, microarrays were subsequently scanned in an ozone-free room with a Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.5 (Roche/NimbleGen).

Data analyses

The life cycle parameters, survival and larval growth, were used to calculate effect concentrations. First the survival data was arc-sqrt transformed. A student t test (p < 0.05) showed subsequently that the control and the solvent control treatments did not differ significantly regarding their survival or larval growth. Therefore the solvent controls were used as control treatment for the LC50/EC50 calculations in the tributyltin and phenanthrene experiments and the controls in the copper and cadmium experiments. The LC50, respectively, EC50, is the actual toxicant concentration in the sediment at which 50% mortality, respectively, 50% reduction in larval length was observed compared to the control. These effect concentrations were calculated according to Haanstra et al. (1985) by fitting a logistic curve ($y = c / (1 + e^{b*(\log(x) - \log(a))})$) through the concentration-response data, with x being the actual exposure concentration, a the LC50/ EC50, b the slope of the logistic curve and c the average survival/ larval length in the (solvent) control.

Gene expression in individual larvae was analysed with a 12*135K *C. riparius* microarray that targeted 37,368 transcripts with 67,281 probes that were printed in duplicate and whose sequences were taken from an earlier design (Marinković et al., 2012b). The raw microarray data were subjected to a set of quality control checks, i.e. visual inspection of the scans, examining the consistency among the replicated samples by principal components analysis, checking for spatial effects through pseudo-colour plots,

inspecting signal to noise ratios, and visual inspection of pre- and post-normalized data with box plots and RI plots (see Supporting Figure S1). The data was normalized using the vsn algorithm that performs intensity-based variance stabilization (Huber et al, 2002). After collapsing of the duplicate probes per transcript, the normalized data were statistically analysed for probes indicating differential gene expression using a linear model with group means parameterization (Smyth, 2004) for each toxicant separately. A contrast analysis was applied to compare each test concentration with the control. For hypothesis testing a permutation based Fs test was used (Cui et al., 2005) and the resulting P-values were corrected for false discoveries according to Storey and Tibshirani (2003). As most transcripts were interrogated by multiple probes, only the probe indicating the most significant differential expression per transcript was selected.

We also determined which transcripts were differentially expressed at all three test concentrations. If multiple probes per transcript were involved, we selected the probe indicating the most significant differential expression among all three test concentrations. These transcripts were subjected to k-means clustering using correlation as similarity measure in order to analyse their dose response relationships. A two-step clustering procedure, where the transcripts were first separated into two clusters (typically up- and down-regulated), and subsequently further separated into four to six clusters, showed many different gene expression dose response profiles. We quantified the number of transcripts that belonged to two contrasting types of dose response profiles, i.e. transcripts that showed a gradual increase, respectively, decrease as a function of toxicant concentration and that were therefore classified as dose range responsive transcripts, and transcripts that were up- or downregulated in response to the lowest test concentration, but were not increasing or decreasing any further at higher test concentrations and that were therefore classified as low dose responsive transcripts. Transcripts following these profiles were identified as follows. A representative transcript following one of the dose response profiles was selected, and this profile served as centroid for finding other transcripts with this profile with the criterion $abs(cor) > 0.98$.

Data deposition

The microarray data and other MAIME compliant meta data have been deposited in NCBI's Gene Expression Omnibus and are accessible under accession number GPL15611.

Results and discussion

Life cycle effects and differentially expressed genes

Larval survival and the length of the surviving larvae were determined after 14 days of exposure in order to assess the (sub)lethal effects of the four tested toxicants on *C. riparius* life cycle endpoints. For all toxicants clear dose-response relationships were obtained for survival, as indicated by the mean survival data and the associated logistic response models shown in Figure 1a-d. The calculated LC50 values (Figure 1a-d), i.e. the actual concentrations that resulted in 50% mortality, are in concordance with the LC50 values previously reported in a 28-day *C. riparius* life cycle toxicity study (Marinković et al., 2011). As expected, sublethal effects were, in comparison to lethal effects, detected at lower test concentrations for all four toxicants (Figure 1e-h). For the organometal tributytlin and the metals copper and cadmium growth was impaired in a dose dependent manner and consequently EC50 values could be calculated (Figure 1e-h). For the polycyclic aromatic compound phenanthrene that acts via a nonspecific baseline toxicity known as narcosis (Bleeker et al., 2002), the EC50 could not be calculated as there was no dose-responsiveness. This observation is in agreement with previous studies that showed that phenanthrene primarily affects *C. riparius* survival, while development is only slightly delayed (Marinković et al., 2011; León Paumen et al., 2008a).

Prior to the identification of the differentially expressed transcripts (referred to as DEGs) per exposure level, a thorough quality assessment of the microarrays was performed which identified two outliers and revealed that the high cadmium treatment consisted of two subgroups (Supporting Figure S1). The complete list of DEGs identified for the different treatments is shown in Supporting Table S1, while Figure 1i-l shows the number of DEGs as a function of the actual toxicant concentration. Except for phenanthrene, the number of DEGs increased with increasing test concentrations indicating dose-responsiveness for copper, cadmium and tributyltin. Phenanthrene that showed no dose-responsiveness for the life cycle endpoint growth (Figure 1h), also did not show a consistent increase in the number of DEGs, with the lowest number of DEGs identified at the highest exposure concentration. We observed for all four toxicants that the lowest test concentrations, which did not affect *C. riparius* survival and only moderately impaired growth (Figure 1a-h), profoundly impacted gene expression in the exposed larvae, with 830, 2,113, 2,974 and 4,367 transcripts being up- or downregulated in response to cadmium, respectively, copper, phenanthrene and tributyltin (Figure 1i-l).

Interpretation of the gene expression responses

The previous section clearly showed that gene expression in *C. riparius* larvae was already substantially affected at the lowest test concentrations. To determine if these lowest test concentrations already caused measurable stress in the exposed larvae, we analysed the
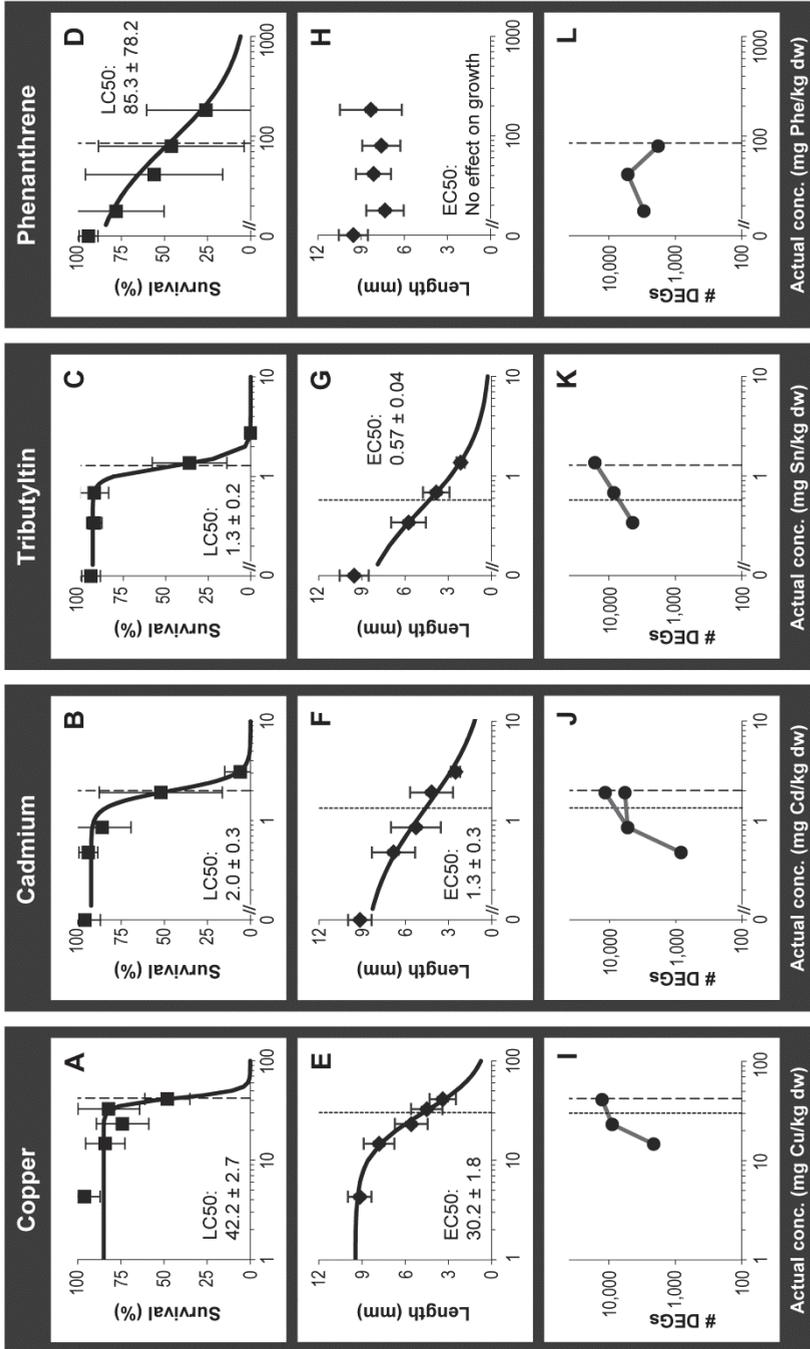
Figure 1: Effects of copper, cadmium, tributyltin, and phenanthrene on *C. riparius* survival, growth and gene expression after 14 days of exposure. Panels A-D: Survival (average ± stdev.) with the fitted log-logistic model. Panels E-H: Larval length (average ± stdev.) with the fitted log-logistic model. Panels I-J: Number of differentially expressed genes (# DEGs) connected with a grey line that does not represent a model fit. The dashed and long dashed vertical lines represent the EC50, respectively, LC50 concentrations calculated in the present study.

DEGs for transcripts indicative of general stress responses (Simmons et al., 2009), and biological effects that are specifically associated with the respective toxicants. Performing functional transcriptome analyses in genomics non-model species such as *C. riparius* is rather challenging, as they have at best a partially annotated transcriptome where the putative functions are based on sequence homologies (e.g., BLASTX, InterPro). Nevertheless, as cellular stress response pathways are conserved among taxa (Kültz, 2005), we were able to interpret our DEGs. Interestingly, our results indicated that relevant stress response pathways were already affected at the lowest test concentrations, which is illustrated by Figure 2a-h. Figure 2a-d shows the significance and fold-change of the DEGs in response to the lowest concentration. In Figure 2e-h we show for each toxicant the gene expression signals of six biologically relevant DEGs both under control and low toxicant conditions. The relevance of these transcripts in the context of stress responses will be discussed in the following sections.

Heat shock response. The heat shock response is a major stress response pathway triggered by a wide range of environmental stressors (e.g. hyperthermia, hypoxia, starvation), as well as toxicants (e.g. metals, pesticides) (Feder and Hofmann, 1999; Gupta et al., 2010). Additionally, it has been reported that some heat shock genes are induced in the absence of stress during embryogenesis and metamorphosis in insects (e.g. Michaud et al., 1997; Gu et al., 2012). For all four toxicants we observed an induction in transcripts encoding members of the heat shock protein family, i.e. small hsp and hsp70 for copper and cadmium (Figure 2e-f), small hsp and hsp67b2 for phenanthrene (Figure 2h) and small hsp, hsp67b2, hsp70 and hsp90 for tributyltin (Figure 2g). Upregulation of hsp70 was previously reported in chironomids exposed to copper and cadmium (Karaouna-Renier and Rao, 2009; Morales et al., 2011). Interestingly, Morales et al. (2011) did not observe upregulation of hsp70 in tributyltin exposed *C. riparius* larvae. This discrepancy might be due to the differences in experimental setup, i.e. the larvae in the present study were exposed to a higher tributyltin concentration for a much longer period of time what most probably resulted in higher stress levels.

Oxidative stress response. All four toxicants have been reported to inflict oxidative stress through the generation of reactive oxygen species (ROS) (Ercal et al., 2001; Liu et al., 2006; Xue and Warshawsky, 2005). For ROS to be produced by phenanthrene, redox-reactive metabolites must first be formed. Indeed, we detected in the phenanthrene exposed larvae an upregulation of transcripts encoding cytochrome P-450 monooxygenases and epoxide hydrolases (Figure 2h), both enzymes that generate redox-reactive metabolites during phase I of xenobiotic detoxification (Nota et al., 2009; Xue and Warshawsky, 2005). Glutathione s-transferases (GSTs) play a key role in antioxidant defense, as they inactivate reactive molecules by glutathione conjugation (Felton and Summers, 1995; Hayes et al., 2005). We observed in all larvae up- and downregulation of GSTs (Figure 2f,h), what is in
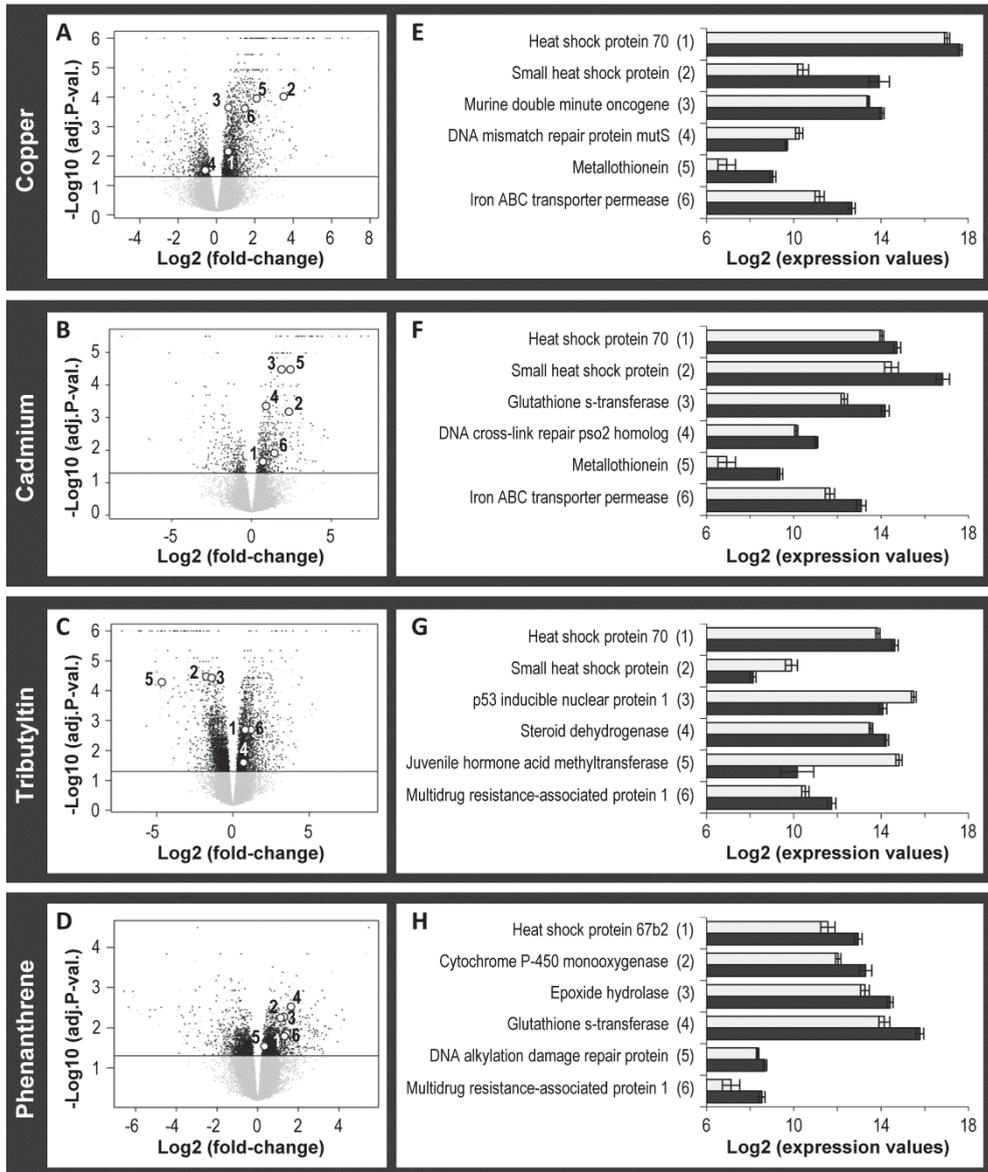
Figure 2: Gene expression responses after 14 days of exposure to low test concentrations of copper, cadmium, tributyltin and phenanthrene. Panels A-D: Volcano plots showing the gene expression responses at the lowest test concentrations. The red dots represent the most significant probe per differentially expressed transcript. The light grey dots below the p=0.05 line are not significantly altered, while the light grey dots above the line represent the less significant probes per transcript. Panels E-H: Six selected differentially expressed transcript that are of biological relevance. The light grey bar represent the expression in the control and the dark grey bar the expression at the lowest test concentrations (average with standard error).

concordance with Nair and Choi (2010) who reported a similar GSTs expression pattern in cadmium exposed *C. riparius* larvae. Considering that GSTs comprise a large family of multi-functional enzymes, e.g. 13 GST genes were recently identified in *C. riparius* (Nair and Choi, 2010), a better characterization of the GSTs transcripts needs to be conducted to interpret these rather complex results. In addition, we also observed for all toxicants an induction of transcripts encoding multidrug resistance-associated proteins (Figure 2g-h) which are ABC transporters that are responsible for eliminating glutathione conjugates from the cells (Borst et al., 2000).

DNA damage response. DNA damage is inflicted by all four toxicants through oxidative stress (Ercal et al., 2001; Liu et al., 2006; Xue and Warshawsky, 2005). Additionally, phenanthrene induces the formation of DNA adducts through reactive metabolites (Scicchitano et al., 2004), while cadmium has been shown to inhibit several DNA repair pathways (Bertin and Averbeck, 2006). We detected altered gene expression of transcripts involved in the p53 signaling pathway, e.g. the oncogene mdm2 in the copper (Figure 2e) and cadmium exposed larvae and the tumor protein p53-inducible nuclear protein 1 (TP53INP1) in the tributyltin (Figure 2g) and phenanthrene exposed larvae. The p53 signaling pathway activates DNA repair proteins or, if the DNA is irreparable, initiates apoptosis (Zou and Elledge, 2000). For all four toxicants we detected upregulation of one or more DNA repair proteins, e.g. DNA mismatch repair protein MutS (Figure 2e), DNA cross-link repair protein pso2 (Figure 2f) and DNA alkylation damage repair protein (Figure 2h). For the complete list per toxicant we refer to Supporting Table S1.

Metal response. In the copper and cadmium exposed larvae we detected an upregulation of transcripts encoding metallothioneins (Figure 2e-f). Metallothionein expression is often induced upon metal exposure, as these cysteine-rich proteins have the capacity to bind a wide range of metals and subsequently protect cells against intoxication (Sigel et al., 2009). This is in concordance with Gillis et al. (2002) who previously reported the production of metallothionein-like proteins in cadmium exposed *C. riparius* larvae. We also measured an upregulation of transcript encoding iron ABC transporter permease in larvae exposed to copper, cadmium and tributyltin, but not in those exposed to phenanthrene (Figure 2e-f). This seems to indicate increased transport of metal ions, possibly out of the cells.

Endocrine disrupting effects. Tributyltin affects molting-hormone biosynthesis and imaginal disc development in *C. riparius* and is therefore considered an endocrine disruptor (Hahn and Schulz, 2002). In the tributyltin exposed larvae we indeed detected altered expression of transcripts encoding steroid dehydrogenase, steroid receptor-interacting snf2 protein and juvenile hormone acid methyltransferase (Figure 2g). The latter enzyme plays an important role in the activation of juvenile hormone and is thus an key regulatory enzyme for insect metamorphosis (Shinoda and Itoyama, 2003). These three transcripts

were not differentially expressed in larvae exposed to the other toxicants, thus our results confirm the endocrine disrupting properties of tributyltin in *C. riparius*.

A more elaborate mechanistic interpretation, such as gene set enrichment analysis (Subramanian et al., 2005), was hampered by the incomplete annotation of this genomics non-model species where ~40% of the DEGs had no putative functions (Supporting Table S1), and where the mapping of gene sets was difficult. Nevertheless, the above sections clearly showed that biologically relevant stress responses already occurred at the lowest test concentrations. To quantify the significance of this finding, we analysed the dose-responsiveness of individual transcripts that were differentially expressed at all three test concentrations (Supporting Figure S2).

Dose response profiles of individual transcripts

A qualitative assessment of the clusters of the individual transcripts (Supporting Figure S3) led to three observations. Firstly, transcripts generally responded unidirectionally, i.e., we found hardly any transcripts that were upregulated in response to one test concentration and downregulated in response to another. Secondly, only a limited number of transcripts showed a gradual increase, respectively, decrease in response to increasing test concentrations. We labelled these genes as dose range responsive transcripts as their changing expression covered the chosen test concentration range. Thirdly and most
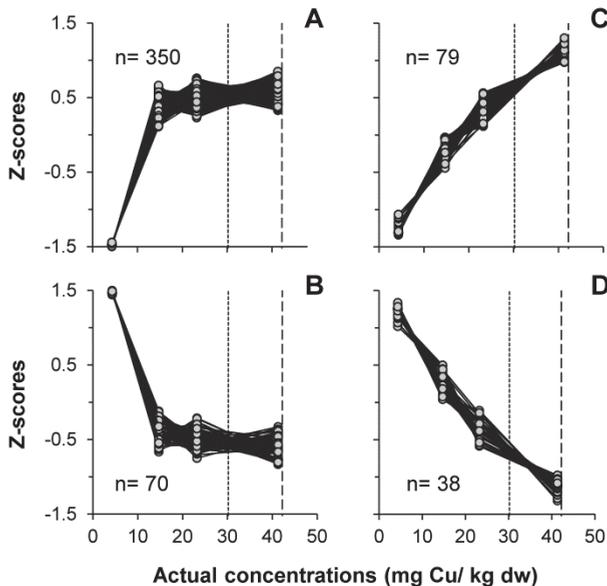

Figure 3: Gene expression profiles obtained for copper. The transcripts that have a low dose responsive profile are shown in panels A and B, while the transcripts matching a dose range responsive profile are shown in panels C and D. In each panel the number of transcripts belonging to the respective group is indicated.

importantly, a substantial number of transcripts had reached their maximal, respectively, minimal expression levels already at the lowest test concentrations. These transcripts were labelled low dose responsive transcripts, since their expression profiles showed that already at the lowest test concentrations transcriptional (dys)regulation had occurred. The consistent differential expression of these low dose responsive transcripts with increasing test concentrations was indicative for their relevance for the toxic response. To further quantify these observations we compared the number of low dose responsive transcripts with the number of dose range responsive transcripts. The gene expression profiles obtained for copper are shown in Figure 3, while Supporting Figure S3 shows the profiles obtained for the three other toxicants. We found 420 low dose responsive transcripts for copper, 111 for cadmium, 382 for tributyltin and 555 for phenanthrene, which was three to twelve times higher than the number of dose range responsive transcripts (Figure 3 and Supporting Figure S3). Thus for all four toxicants a relatively large fraction of the differentially expressed transcripts was already maximally impacted at the lowest test concentrations, indicating more sensitive cellular stress responses.

Comparing life cycle responses and gene expression patterns

This first microarray exposure study with the non-biting midge *Chironomus riparius* demonstrated the power of the simultaneous analysis of gene expression and life cycle responses. Our results showed that already at the lowest test concentrations a substantial number of transcripts was significantly differentially expressed, while the life cycle endpoints of *C. riparius* were hardly affected. Moreover, the low test concentrations were shown to inflict biologically relevant cellular stress in the exposed larvae, as transcripts indicative of both general and toxicant specific stress responses were differentially expressed. Moreover, we were able to identify a large number of differentially expressed transcripts that responded already maximally at the lowest test concentrations. These low dose responsive transcripts may reflect sensitive stress responses and identifying their function may provide a wide range of sensitive transcriptomics biomarkers. The direct comparison between molecular and life cycle responses after fourteen days of exposure, revealed that gene expression is more sensitive to toxicant exposure than life cycle endpoints, underlining the potential of transcriptomics for ecotoxicity testing and environmental risk assessment.

Supporting information available: Supportive Figures S1-S4 and Table S1. This information is available free of charge via the Internet at http://pubs.acs.org.