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Gene expression in toxicant exposed chironomids

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Gene expression in toxicant-exposed chironomids
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Chapter 1

General introduction
The above quote by Philippus Aureolus Theophrastus Bombastus von Hohenheim, better known as Paracelsus, is perhaps the most renown quote in (eco)toxicology and states in English “All things are poison, and nothing is without poison; only the dose makes that a thing is no poison”. That is to say, it is not only the nature of a compound but also its concentration that determines its effect. This concept of dose-responsiveness has greatly shaped toxicology and ecotoxicology, but later on it became evident that other factors also play pivotal roles in the expression of toxicity. To paraphrase Paracelsus, no compound is a ‘poison’ unless the exposure time is sufficient to induce an effect and there is no such thing as a universal ‘poison’. Instead, the mode of action of a compound determines its toxicity in combination with the intrinsic sensitivity of the exposed species. To complicate matters even more, in realistic ecological settings the sensitivity of individuals and populations to ‘poisons’ depends on many factors, such as combined exposure to other (environmental) stressors and species specific responses.

Historically, toxic effects on biota were primarily investigated on the organism and population level by means of standardized toxicity tests where impacts on life cycle endpoints, such as survival, growth and reproduction were observed in ecologically relevant species (OECD, 2012). During the last decade, however, as molecular biological techniques rapidly advanced, the focus of ecotoxicological research has gradually shifted towards toxicity exerted at lower levels of biological organization (van Straalen and Feder, 2012). This field of ecotoxicology where effects on the genome, transcriptome, proteome and/ or metabolome are studied is commonly referred to as ecotoxicogenomics (Snape et al., 2004) and is generally believed to hold the potential to elucidate the mechanisms of toxicity to an unprecedented detail (e.g. Goetz et al., 2011; van Straalen and Feder, 2012). A report of the U.S. National Research Council entitled ‘Toxicity testing in the 21st century: A vision and a
strategy’ even envisioned a turning point in human health-orientated toxicology, stating that future toxicity testing will rely on detecting and characterizing a compounds ability to initiate cellular perturbations that can ultimately manifest as toxicity, instead of directly measuring effects on health (NRC, 2007). This paradigm shift has also been advocated for ecotoxicology during a SETAC Pellston workshop (Villeneuve and Garcia-Reyero, 2011). While these visions are far stretching and might never be fully realized, it does show that the use of ‘-omics’ technologies in ecotoxicology has raised high expectations and that ecotoxicology might be on the verge of a leap forward. But is this realistic?

Transcriptomics appears to provide the most suitable tools for analysing molecular stress response pathways in toxicant-exposed organisms (Schirmer et al., 2010). Initially, microarray-based large-scale gene expression studies were limited to genomics model 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* (Girardot et al., 2004). Later on, as expressed sequence tags could be generated with subtracted cDNA libraries, microarrays were also developed for species with limited or no genome data and transcriptomics studies could be conducted with ecotoxicologically relevant model organisms such as *Folsomia candida* (Timmermans et al., 2007) and *Lumbricus rubellus* (Owen et al., 2008). However, as this approach was very laborious and time consuming, a real breakthrough in ecotoxicogenomics came with the introduction of the next-generation sequencing technologies (Ekblom and Galindo, 2011). These high-throughput sequencing approaches generate rapidly large quantities of sequence data (Metzker, 2010) thus allowing a cost effective and relatively swift development of microarrays for any species of interest (e.g. Vera et al., 2008; Bellin et al., 2009). Yet the large-scale gene expression studies that were subsequently conducted with toxicant-exposed organisms focused almost exclusively on molecular stress responses that occur shortly after exposure, separately and independently from standardized ecotoxicity tests (e.g. Swain et al., 2010). For ecotoxicogenomics to reach its full potential though, it is necessary to relate the changes detected at the level of the transcriptome to the impacts observed at life cycle endpoints (e.g. Connon et al., 2008).

The discrepancies between measuring early molecular stress responses and assessing long-term life cycle effects may arise from the time dependent uptake of toxicants and the consequent expression of adverse effects (Baas et al., 2010). The tendency to reduce exposure time is not limited to ecotoxicogenomics, and is well known from standard ecotoxicity testing where acute toxicity tests are conducted because of time constraints and costs associated with chronic toxicity tests. Yet, the relevance of such early stress responses for long term toxic effects remain largely unknown. Moreover, chronic toxicity tests that are conducted at lower and thus more environmentally relevant test concentrations are more
informative as they incorporate effects that are exerted in later stages of development or during reproduction (Schulz and Liess, 1995; van Gestel et al., 2001).

Successful efforts have been made to link the effects observed during acute and chronic toxicity testing using the acute-to-chronic ratio, where the acute median lethal concentration (LC50) is divided by a chronic sublethal effect concentration such as the lowest observed effect concentration (LOEC) (Länge et al., 1998; Roex et al., 2000; Ahlers et al., 2006). These studies also showed that compounds with similar modes of action tend to group together, with narcotic compounds having generally lower acute-to-chronic ratios compared to compounds with specific modes of action. The relationships between acute-to-chronic ratios and mode of action are not straightforward though, due to the high variability in acute-to-chronic ratios. Hence, a better grouping of compounds with the same modes of action would reduce the uncertainties in acute-to-chronic ratios improving its reliability as tools in environmental risk assessment.

Chronic ecotoxicity tests that cover an entire generation of the test species, are a great improvement compared to acute ecotoxicity tests (Marinković et al., 2011). However, such chronic tests do not provide information on how species are affected in polluted environments where the exposure spans multiple generations. Under harsh conditions populations can, if they do not migrate, go extinct or eventually become less sensitive to the exposed compounds (Postma et al., 1995b; Sola et al., 2004). These adaptive responses can be of a permanent or reversible nature, the first being caused by heritable changes in the genetic material of the exposed populations (adaptation), and the latter by physiological acclimation of the exposed organisms and subsequent maternal effects (phenotypic plasticity) (Morgan et al., 2007). To unravel the mechanism(s) underlying adaptive responses, multigeneration studies have been conducted where under controlled laboratory conditions, cultures of various species were exposed to toxic compounds and effects on life cycle parameters were monitored over time (Postma and Davids, 1995; Shirley and Sibly, 1999; León Paumen et al., 2008b; Vedamanikam and Shazilli, 2008). Reduced sensitivity was reported in cultures exposed to compounds with specific modes of action and evidence was presented that supported both adaptation and phenotypic plasticity (Postma and Davids, 1995; Shirley and Sibly, 1999; Vedamanikam and Shazilli, 2008). Interestingly, a culture that was exposed for multiple generations to a compound that acts via a non-specific mode of action known as narcosis, showed no changes in sensitivity (León Paumen et al., 2008b). Since the mechanisms underlying adaptive responses, or the lack of adaptive responses, still remain largely unknown transcriptomics holds great potential in elucidating these mechanisms in detail (Schoville et al., 2012).
Aim and objectives

Transcriptomics is suggested to have great potential for elucidating molecular stress response pathways in toxicant-exposed organisms. Yet, to fully exploit transcriptomics it is necessary to study molecular stress responses in ecologically relevant species and to relate these molecular stress responses to the effects observed on life cycle endpoints. Due to advances in molecular biological techniques, large-scale gene expression studies have now become feasible, not only for laboratory test species with fully sequenced genomes, but also for eco(toxico)logical key species. Consequently, this thesis aims to elucidate the relation between life cycle effects and molecular stress responses in the ecotoxicological model species *Chironomus riparius* for compounds with different modes of action.

To this purpose, the following objectives have been set:
- To compare life cycle and multigeneration responses of chironomid larvae to compounds with different modes of action.
- To develop transcriptomics resources for *Chironomus riparius* consisting of an annotated transcriptome and a gene-expression microarray, allowing large-scale gene expression studies with chironomid larvae.
- To compare gene expression and life cycle endpoints in toxicant-exposed chironomid larvae.

Test organism & test compounds

The non-biting midge *Chironomus riparius* (Insecta: Diptera) has been selected for the present study, because of its wide distribution and abundant presence in freshwater ecosystems (Armitage et al., 1995) and because it has a long history in sediment toxicity testing with currently four OECD guidelines being available for acute and chronic toxicity tests (OECD, 2012). This insect species is highly suitable for sediment toxicity tests as it resides predominantly in the sediment, where the larvae settle after hatching and remain till they emerge as adults (Armitage et al., 1995). Its frequent application as ecotoxicological test organism is due to its ease of culturing under laboratory conditions and because its entire life cycle, consisting of an egg stage, four larval stages, a pupa stage, and an adult stage can be completed within three to four weeks (Armitage et al., 1995). For the present study, this species has also been selected because it can be successfully used to study adaptive responses under controlled laboratory conditions (e.g. Postma and Davids, 1995; Vogt et al., 2007). The *C. riparius* larvae used in the present study originated from the University of Amsterdam's in-house laboratory culture.
Four compounds have been selected because they represent different modes of action. Phenanthrene is a polycyclic aromatic compound that acts via a non-specific baseline toxicity known as narcosis (Bleeker et al., 2002) and has been shown to induce the formation of DNA adducts (Scicchitano et al., 2004). The organometal tributyltin is a biocide that has been shown to cause endocrine disruption in *C. riparius* (Hahn and Schulz, 2002). The essential metal copper and the non-essential metal cadmium both cause oxidative stress, however, the processes differ as copper is a redox-active metal, while cadmium is a redox-inactive metal (Ercal et al., 2001; Gaetke and Chow, 2003).

**Outline of the thesis**

Comparing toxicity data generated in different studies and reducing background noise in transcriptomics studies would greatly improve when test conditions would be better standardized. Therefore, in chapter two the focus was on reducing the experimental variability in *C. riparius* sediment toxicity tests by optimizing both the composition of the artificial sediment and the feeding regime. After the best performing test conditions were identified, *C. riparius* larval development was characterized under our specific test conditions.

In chapter three single-generation effects of four compounds with different modes of action were established on *C. riparius* life cycle parameters. To this purpose chronic *C. riparius* sediment toxicity tests were performed. Subsequently, the derived chronic (sub)lethal effect concentrations were used to calculate for each compound a LC50/LOEC ratio that was inspired on the acute-to-chronic ratio, in order to evaluate if ratio’s based
exclusively on chronic data would improve grouping of compounds according to their mode of action.

Long term exposure to toxic compounds can profoundly impact the sensitivity of test species. To understand how the sensitivity of *C. riparius* would be affected by prolonged toxicant exposure, in chapter four a multigeneration experiment was performed where six *C. riparius* cultures were exposed for nine consecutive generations to two exposure scenarios of three of the four compounds that were tested in chapter three. This experiment established the multigeneration effects of the three compounds on *C. riparius* and allowed us to determine if changes in the sensitivity of the exposed cultures had taken place and if so, if the changes were due to adaptation or phenotypic plasticity.

Chapter three and four were based on (sub)lethal effects on life cycle endpoints. To be able to analyse changes in gene expression of toxicant-exposed chironomids, in chapter five it was aimed to develop transcriptomics resources for *C. riparius*. Therefore, a broadly sampled and normalized *C. riparius* transcriptome library was pyrosequenced, and the sequence data was used to assemble and annotate the transcriptome, as well as to develop a *C. riparius* gene expression microarray for large scale gene expression studies in this genomics non-model organism.

In chapter six the microarray developed in chapter five was used to analyse gene expression in individual *C. riparius* larvae that had survived toxicity tests with the four toxicants that differently affected *C. riparius* life cycle parameters in chapter three. This experiment allowed a direct comparison of the sensitivity of gene expression and life cycle endpoints and an analysis of the dose-response profiles of individual differentially expressed transcripts. Moreover, insight was gained in the status of the transcriptome of toxicant-exposed midge larvae at the end of the exposure period when their development has already been affected.

The concluding remarks in chapter seven discuss the main findings of this thesis, focussing on the crucial roles of mode of action and exposure time in assessing the relation between life cycle and molecular stress responses in toxicant-exposed chironomids. Finally, the suggested potential of transcriptomics for ecotoxicity testing and environmental quality and risk assessment is evaluated.
Chapter 2

Development of an easily made artificial sediment that reduces experimental variability

Abstract

_Chironomus riparius_ toxicity tests are commonly performed on natural, thus variable, sediments. The introduction of transcriptomics in ecotoxicology increased the necessity for reduced experimental variability. To this purpose we developed an easily made artificial sediment and monitored larval development. We showed that larval development was synchronized and that the days when specific larval stages are reached can be identified. We conclude that the newly developed artificial sediment will facilitate the use of transcriptomics in ecotoxicology.
Introduction

The first microarrays produced to study gene expression were constructed about fifteen years ago (Schena et al., 1995; Lockhart et al., 1996). Ever since, the field of transcriptomics has been evolving rapidly. Microarrays are nowadays affordable and can be obtained commercially for a growing number of model organisms. This is clearly visible in the field of toxicology, where microarrays have become a standard tool to elucidate the mode of action of toxicants at the molecular level (Andrew et al., 2003), as well as to identify toxicant specific gene expression profiles (Hamadeh et al., 2002). With some delay, transcriptomics also found its way into ecotoxicology. While the first ecotoxicogenomical studies focussed on stress response in model organisms (Momose and Iwahashi, 2001, Seki et al., 2002), lately, gene expression studies are performed on ecologically relevant non-model organisms, such as *Folsomia candida* (Timmermans et al., 2007) and *Daphnia magna* (Poynton et al., 2007). It is expected that with the current technological breakthroughs in the next generation sequencing technologies, the number of gene expression studies performed on non-model organisms will increase rapidly (Vera et al., 2008; Bellin et al., 2009).

One of the organisms for which currently hardly any sequence data is available, but for which a microarray is currently being developed is the non-biting midge *Chironomus riparius*. This chironomid species is ecologically relevant due to its widespread distribution, numerical abundance and importance as prey (Armitage et al., 1995). In addition, *C. riparius* is also widely used in ecotoxicology for acute, chronic and life cycle testing of chemicals according to standardized test protocols (Ristola et al., 1999; Béchard et al., 2008; León Paumen et al., 2008a). One of these protocols is OECD guideline 218, which describes how to perform a sediment-water chironomid toxicity test using spiked sediment (OECD, 2004). To reduce variability within and between experiments, the guideline recommends the use of artificial sediment consisting of 75% quartz sand, 20% kaolin clay and 5% peat. This artificial sediment is a great improvement compared to natural sediments, however, as it contains peat, it still varies inevitably between batches. When studying changes in gene expression upon exposure to toxicants, it is necessary to standardize experimental conditions to such an extent that the observed changes are as little as possible influenced by other variables than the toxicant. Therefore we aimed to develop an improved artificial sediment that would be consistent over time and that could easily be produced with well-defined commercially available components. To test if larval development would be better synchronized on the newly developed artificial sediment, larval growth and development were monitored. Synchronizing larval development will allow to identify the days when specific larval stages are reached.
Material and methods

Test organism and culturing conditions

*C. riparius* larvae used in the experiments originate from the University of Amsterdam’s in-house laboratory culture. This culture has been maintained at 20 ± 1 °C, 65% humidity and a 16: 8 hour light: dark regime. 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.

Experimental design

**Experiment 1: C. riparius growth test with different artificial sediments.** A 14-day growth experiment with *C. riparius* larvae was performed to test the suitability of different artificial sediment compositions. The artificial sediments were made according to the OECD 218 guideline (OECD, 2004), with slight modifications. All sediments consisted of 75% quartz sand (Sibelo NV, Mol, Belgium), 20% Kaolin clay (Keramikos, Haarlem, the Netherlands) and 5% organic matter. As organic matter, grounded peat (tuincentrum het Oosten, Aalsmeer, the Netherlands), cellulose fibbers (homogenized unbleached paper) and α-cellulose powder (Sigma-Aldrich, St. Louis, MO) were used. The pH of the artificial sediments was adjusted with CaCO₃ to 7.0 ± 0.5. Deionised water was added to obtain a moisture content of the artificial sediment of 50%.

Three feeding rates were tested per artificial sediment, 0.25, 0.5 and 1.0 mg food/larva/day. To ensure a homogenous distribution of the food in the sediment, the total amount of food for the entire duration of the test (35, 70 and 140 mg) was added to a glass bottle together with the artificial sediment and placed overnight on a roller bank (20rpm). 60 grams of this homogenized food-sediment mixture was added to each of three replicate 400 ml glass beakers. For the peat containing artificial sediment an additional treatment was included with twice the amount of sediment per beaker (120 grams). These beakers were then carefully topped up with 250 ml of Dutch Standard Water (DSW; deionised water with 200 mg/l CaCl₂*2H₂O, 180 mg/l MgSO₄*H₂O, 100 mg/l NaHCO₃ and 20 mg/l KHCO₃; pH 8.2) and allowed to settle for 4 hours before aeration was turned on. The test beakers were conditioned for one week under the same conditions which prevailed in the subsequent test, after which ten first-instar larvae, less than 24 hours old, were added per beaker. The larvae were allowed to settle on the sediment for 4 hours before aeration was restarted. After 14 days of incubation with constant aeration, the sediment was sieved through a 425 micron sieve. Larvae were collected and photographed. Using analySIS® software (Soft Image System, GmbH) the body length (BL) of the larvae was determined.
Experiment 2: Larval development in the newly developed artificial sediment. A 14-day growth experiment with artificial sediment containing α-cellulose powder as organic matter and a feeding rate of 0.5 mg food/larva/day was conducted as previously described. The experiment was started with 36 replicates. Three days after introduction of the first-instar larvae to the test beakers monitoring of larval development was initiated. Each day three replicates were sieved, the larvae photographed and their body length, head capsule width and head capsule length determined using analySIS® software.

Statistical analysis

Results from the 14-day growth experiment with different artificial sediments were checked for normality using Shapiro-Wilk’s W test and tested for homogeneity of variance using Leven’s test, after which they were compared at a 5% significance level using an one-way analysis of variance (ANOVA) followed by a Tukey posthoc test. The statistical analyses were performed using SPSS® 17 for windows.

Results and discussion

C. riparius growth test with different artificial sediments

Three artificial sediment compositions were tested for larval development, containing either cellulose fibbers, α-cellulose powder or grounded peat as organic matter. For the peat containing sediment an additional treatment was included that had an increased sediment thickness commonly used in our laboratory for natural sediments. Figure 1 shows that feeding level had a much stronger impact on larval development than the composition of the artificial sediment. For the four sediments a significant increase in larval growth was observed with increasing food levels; cellulose fibber (F2.54 = 132,406, p < 0.001), α-cellulose powder (F2.55 = 52,029, p < 0.001), grounded peat (F2.51 = 74,211, p < 0.001) and thicker layer of grounded peat (F2.50 = 122,466, p < 0.001). This finding is in agreement with Ristola et al. (1999) who tested four feeding levels on four natural sediments differing in organic content and particle size distribution. They observed that larval growth was greatly enhanced with increasing food levels in all natural sediments.

No significant differences in larval growth were observed between the three artificial sediment compositions tested. The only sediment where the larvae grew significantly less was the thicker peat containing sediment. This was the case for all three feeding levels; 0.25 mg/larva/day (F3.68 = 6,464, p = 0.001), 0.5 mg/larva/day (F3.71 = 20,842, p < 0.001) and 1.0 mg/larva/day (F3.71 = 12,696 p < 0.001). A possible explanation could be that due to the thickness of the sediment, approximately 1 cm as to the 4 mm of the other treatments, a part of the food was not available for C. riparius larvae that mostly forage on the sediment.
Since larval growth did not differ between the three artificial sediment compositions, it was decided to continue with the most standardized of the three, the $\alpha$-cellulose containing sediment. This artificial sediment has the benefit that $\alpha$-cellulose can be obtained commercially, thus allowing an easy and quick production of artificial sediment, while ensuring reproducibility in time. The suitability of $\alpha$-cellulose had already been advocated by Ribeiro et al. (1999) and Fleming et al. (1998), using other proportions of sand, clay and $\alpha$-cellulose.

**Larval development in the newly developed artificial sediment**

To test if larval development would be better synchronized on the newly developed artificial sediment, a 14-day growth experiment (0.5 mg food/larva/day) was conducted where larval development was monitored on a daily basis. Three parameters were noted for each larva: body length, head capsule length and head capsule width. Previously Watts and Pascoe (2000) compared *C. riparius* and *Chironomus tentans* development and plotted the body length versus the head capsule width. These graphs did not yield distinct groups, so clustering was necessary. In this study we decided to plot the head capsule length versus the head capsule width (Figure 2). This way all larvae fell into one of four distinct groups corresponding to the four larval instars of *C. riparius* (Figure 2) making clustering unnecessary. In future experiments, Figure 2 will allow the determination of the instar stage of larvae that due to toxicant exposure have a delayed development.

![Figure 1: Larval length after 14 days at three feeding regimes, i.e. 0.25, 0.5 and 1.0 mg food/larva/day, in artificial sediment containing 5% organic matter composed of cellulose fibers (light grey; 60g ww), $\alpha$-cellulose powder (dark grey; 60g ww) or peat (light grey lines; 60g ww & dark grey lines; 120g ww). Error bars indicate standard deviation.](image)
With the clear classification of larvae into one of the four instars, we were able to identify the days when certain stages were reached under control conditions (Figure 3). This knowledge will be useful in future ecotoxicogenomics studies that will require larvae of specific stages. We therefore conclude that both the availability of the newly developed standardized sediment as well as the synchronization of larval development growing on it, will be of great assistance in the performance of future ecotoxicogenomics work with *C. riparius*.

Figure 2: Head capsule development. Larvae are distinctly grouped according to the four larval instars when head capsule width is plotted against the length. Experiment was performed in artificial sediment with 5% \( \alpha \)-cellulose at 0.5 mg food/larva/day.

Figure 3: Larval development over time indicated by the percentage of larvae being instar I (light grey), II (dark grey), III (grey horizontal lines) or IV (grey vertical lines). The experiment was performed in artificial sediment with 5% \( \alpha \)-cellulose at 0.5 mg food/larva/day.
Chapter 3

Life cycle responses of the midge *Chironomus riparius* to compounds with different modes of action

Abstract

Compounds with different modes of action may affect life cycles of biota differently. The aim of the present study was therefore to investigate the impact of four chemicals with different modes of action, including the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene, on chronic lethal and sublethal life cycle effect parameters of the non-biting midge *Chironomus riparius*, applying a 28-day sediment toxicity test. Tributyltin and cadmium delayed emergence significantly over a wide range of sublethal concentrations, while this range was narrow for copper and almost absent for phenanthrene. The chronic LC50/LOEC<sub>EmT50</sub> ratio, expressing these differences, amounted to 1.5, 3.5, 12.0 and 18.2 for respectively phenanthrene, copper, cadmium and tributyltin. Thus the more specific the compounds mode of action, the higher the chronic LC50/LOEC<sub>EmT50</sub> ratio, as previously observed for acute-to-chronic ratios (ACRs). Comparison of our results with literature derived LC50/LOEC ratios showed a comparable trend and a lower variability compared to ACRs. We therefore conclude that the presently proposed chronic ratio is indicative for the specificity of a chemicals mode of action and that it is less variable than the ACR.
Introduction

Single species toxicity tests are routinely performed to assess the toxicity of new and existing compounds. Because of costs and time constraints, the majority of these tests focus on acute toxicity, where organisms are exposed to relatively high test concentrations for a short period of time (Bleeker et al., 1998; Hahn and Schulz, 2002; Béchard et al., 2008). Such acute toxicity tests fail to incorporate long-term effects that are exerted in later stages of development or during reproduction (Schulz and Liess, 1995; van Gestel et al., 2002) and hence are only expressed upon chronic exposure. To gain a better understanding of the link between acute and chronic toxicity, the acute-to-chronic ratio (ACR) was introduced, being the acute median lethal concentration (LC50) divided by a chronic sublethal effect concentration. ACRs have been calculated for a multitude of compounds and species (Länge et al., 1998; Roex et al., 2000; Ahlers et al., 2006; Raimondo et al., 2007) and several studies observed a relationship between the ACR and the chemicals mode of action. Länge et al. (1998) reported that compounds with more specific modes of action, such as heavy metals, organometals and pesticides frequently showed very high ACRs, while Roex et al. (2000) and Ahlers et al. (2006) demonstrated that narcotic compounds showed the lowest ACRs, with the smallest variation within and between species. Relationships between ACRs and mode of action are not straight forward though, due to the high variability in ACRs, especially for those calculated for non-narcotic compounds, ranging from less than 1 to greater than 10,000 (Raimondo et al., 2007). We hypothesize that this variability may be reduced by calculating a lethal/sublethal ratio using merely chronic toxicity data, as it has been shown that chronic toxicity is less variable than acute toxicity (Baas et al., 2010). Moreover, although the ACR may be a useful decision making tool, there is not much scientific basis for dividing an acute lethal concentration by a chronic sublethal effect concentration.

The aim of the present study was therefore to compare, under equal experimental conditions, the lethal and sublethal effects of compounds with different modes of actions on life cycle effect parameters of a single species in order to evaluate if a chronic lethal/sublethal effect concentration ratio could be more indicative for the specificity of a compounds mode of action and less variable than ACRs. The non-biting midge *Chironomus riparius* was chosen to perform these chronic studies, as this insect species has a short life cycle with full metamorphosis (Armitage et al., 1995), is easily kept under laboratory conditions and moreover a standardized life cycle toxicity test is available (OECD, 2004). We generated chronic *C. riparius* toxicity data for the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene, using this highly standardized assay (OECD, 2004). Emergence delay was selected as sublethal endpoint as it has previously been shown to be more sensitive than total emergence or growth (Chibunda, 2009). Thus the chronic ratio
was calculated for each compound by dividing the chronic LC50 by the chronic mean emergence time (EmT50) based LOEC (LOEC\textsubscript{EmT50}). Since in recent years, an increasing number of compounds, including (organo)metals (Chibunda, 2009; Roman et al., 2007; Vogt et al., 2007; Nowak et al., 2008), polycyclic aromatic compounds (PACs) (León Paumen et al., 2008a) and agrochemicals (Åkerblom et al., 2008; Agra et al., 2009; Jungmann et al., 2009; Tassou et al., 2009; Egeler et al., 2010; Langer-Jaesrich et al., 2010), have been tested applying the same \textit{C. riparius} life cycle sediment toxicity test, we were able to compare the obtained experimental ratios with ratios derived from literature data to assess the robustness of the chronic LC50/LOEC\textsubscript{EmT50} ratio for a wide variety of compounds and to evaluate if this chronic ratio is indeed less variable than the ACR.

\textbf{Materials and methods}

\textit{Test organism and culturing conditions}

The non-biting midge \textit{Chironomus riparius} (Diptera) is a commonly used test species in chronic sediment toxicity testing (Roman et al., 2007; León Paumen et al., 2008a). This insect species resides predominantly in the sediment, where the larvae settle after hatching and remain till they emerge as adults. The entire life cycle, consisting of an egg stage, four larval stages, a pupa stage and an adult stage can be completed within three to four weeks (Armitage et al., 1995). The \textit{C. riparius} larvae used in the present study originated from the University of Amsterdam’s in-house laboratory culture. This culture was maintained in aquaria containing quartz sand overlaid with Dutch Standard Water (deionised water with 200 mg/l CaCl\textsubscript{2}*2H\textsubscript{2}O, 180 mg/l MgSO\textsubscript{4}*H\textsubscript{2}O, 100 mg/l NaHCO\textsubscript{3} and 20 mg/l KHCO\textsubscript{3}; hardness is 210 mg as CaCO\textsubscript{3}/l and pH 8.2 ± 0.2) at 20 ± 1 °C, 65% humidity and a 16: 8 h light: dark photoperiod (León Paumen et al., 2008a). The culture was fed a mixture of Trouvit\textsuperscript{®} (Trouw, Fontaine-les-Vervins, France) and Tetraphyll\textsuperscript{®} (Tetrawerke, Melle, Germany) in a ratio of 20:1. This mixture was also used as food for all subsequent experiments.

\textit{Test compounds}

The selected compounds included: the essential metal copper (CuCl\textsubscript{2}.2H\textsubscript{2}O, copper standard, Fluka), the non-essential metal cadmium (CdCl\textsubscript{2}, Tritisol\textsuperscript{®}, Merck), the organometal tributyltin (TBT-Cl, 96% purity, Aldrich) and the polycyclic aromatic compound phenanthrene (98% purity, Aldrich). Tributyltin and phenanthrene stock solutions were made in acetone (99.8% purity, Chromasolv\textsuperscript{®}, Sigma-Aldrich).
Sediment preparation and spiking procedures

The toxicity tests were performed using artificial sediment according to OECD guideline 218 (OECD, 2004), with slight modifications. The sediments consisted of 75% quartz sand (Sibelco® M34, Belgium) with a 60 to 250 μm grain size, 20% kaolin clay (WBB vingerling, the Netherlands) and 5% α-cellulose (Sigma). The pH of the artificial sediment was adjusted with CaCO₃ (99% purity, Sigma-Aldrich) to 7.0 ± 0.5. Deionised water was added to obtain a final moisture content of 50%.

The sediment was spiked with the following nominal concentrations of the selected compounds: copper 5, 10, 20, 40, 60, 80 and 100 mg/kg dry weight; cadmium 0.5, 1, 2, 4, 6 and 8 mg/kg dw; tributyltin 0.25, 0.5, 1, 2, 4, 8 16 and 32 mg Sn/kg dw; and phenanthrene 50, 100, 150, 200, 250 and 300 mg/kg dw. Controls were included for all compounds, and additional solvent controls were added for tributyltin and phenanthrene. There were seven replicates per treatment, five replicates for the toxicity tests and two replicates that were sacrificed at the start and half-way through the experiment (day 14) for chemical analysis.

Two different spiking methods were used depending on the water solubility of the compounds. For the readily water soluble copper and cadmium, appropriate amounts of metal stock solution were added to 420 g wet sediment in 1-liter glass bottles. Treatments that required less or no metal stock solution were supplemented with deionised water, so equal volumes were added to all treatments. To this metal-sediment mixture, 980 mg of food was added, corresponding to 0.5 mg food/larvae/day for the entire duration of the test (28 days). The bottle was placed for 24 hours on a roller bank (20 rpm) in order to homogenize the food-metal-sediment mixture, after which it was divided over seven replicate 400 ml glass beakers (60 g/ beaker). These beakers were carefully topped up with 250 ml of Dutch Standard Water and covered with plastic foil to prevent evaporation during the experiments. After settling of the sediment gentle aeration was turned on. The test beakers were conditioned for one week, allowing the compounds to equilibrate with the sediment and a stable sediment layer to be formed.

For the less water soluble compounds tributyltin and phenanthrene, an additional pre-spiking step was introduced according to León Paumen et al. (2008a). The compounds, dissolved in acetone, were added to a 1-liter glass bottle containing 42 g dry sediment, corresponding to 10% of the total amount of dw sediment. Acetone (50 ml) was added to the compound-sediment mixture to allow adequate overnight mixing on a roller bank (20 rpm). The next day, the compound-sediment mixture was dried in a fume cupboard, by allowing the acetone to evaporate. Deionised water was added to the dry compound-sediment mixture to obtain a 50% moisture content, after which the remaining wet sediment was added. From here on the same mixing and equilibration procedure was followed as for the metals.
Toxicity tests

Twenty-eight day life cycle toxicity experiments were performed based on OECD guideline 218 (OECD, 2004). Test beakers were kept under the same conditions as the *C. riparius* culture, i.e. 20 ± 1 °C under a 16:8 h light: dark photoperiod and were constantly aerated. When necessary, deionised water was added to compensate for evaporation losses. The quality of the overlying water was determined at the start, half-way (day 14) and at the end (day 28) of the experiments by measuring dissolved oxygen concentration, conductivity, pH and ammonium concentrations (Supporting Table S1).

The experiments were initiated by introducing ten first instar larvae into each of the test beakers using a stereo microscope and a blunt glass Pasteur pipette. The larvae, less than 24 hours old, were obtained by hatching at least five egg ropes in Dutch Standard Water three days prior to the start of the experiment. To allow settlement of the introduced larvae on the sediment, aeration in the exposure chambers was switched off and restarted after 4 hours. During the experiment two additional feedings of 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, were administered. These feedings were administered 7 and 14 days after the start of the toxicity test. From day 14 on, the test beakers were inspected daily for emerged midges until termination of the experiment on day 28. Emerged midges were removed and sexed. At the end of the experiment, the sediment was sieved through a 350 μm sieve and surviving larvae were counted.

Chemical analyses

Actual toxicant concentrations were determined by sacrificing one replicate per treatment at the start, halfway (day 14) and at the end of the experiments (day 28). For copper and cadmium the actual concentrations were determined in the overlying water, interstitial water and sediment. For the solvent carried toxicants, phenanthrene and tributyltin, actual toxicant concentrations were only determined in the sediment, since previous studies showed that less than 0.02% of the phenanthrene added to the sediment ended up in the interstitial water (León Paumen et al., 2008c). Overlying water samples were collected in 50 ml polypropylene tubes. Sediment was collected in 50 ml polypropylene tubes and centrifuged at 3000 rpm for 15 minutes. The interstitial water was transferred to a new tube. All samples were stored frozen at -20 °C until analysis. The water content of the sediment was determined gravimetrically by oven-drying two subsamples of 2 g for three days at 60°C, and averaged 22 ± 2%.

Copper and cadmium sediment concentrations were determined by digesting duplicate 130 mg oven-dried subsamples in 2 ml of a 4:1 mixture of nitric acid (65% p.a.; Sigma-Aldrich) and hydrochloric acid (37% p.a., Sigma-Aldrich) in tightly closed Teflon® bombs upon heating in an oven at 140 °C for 7 hours. The digested samples were diluted with 8 ml deionised water and allowed to settle overnight at 5 °C. Duplicate 2 ml interstitial and
overlaying water samples were acidified by adding 20 µl nitric acid (69-70% p.a.; Sigma-Aldrich). Copper and cadmium concentrations in the samples were determined by flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 100, Germany). The certified reference material ISE 989 Riverclay (Wageningen Agricultural University, The Netherlands) was used for quality assurance. The measured metal test concentrations were corrected for copper (86%) and cadmium (97%) recovery, and were used to calculate the actual metal concentrations as time-weighted means of the three measurements per test concentration according to OECD guideline 211 (OECD, 1998).

Tributyltin analyses were performed by RWS-Waterdienst, Lelystad, according to their in-house developed method accredited by the Dutch Accreditation Council. The frozen sediment samples were freeze-dried and homogenized. Two 1 g subsamples were extracted by adding 15 ml methanol (J.T. Baker), 1.5 ml acetic acid (99.9%, J.T. Baker) and 7 ml hexane (J.T. Baker). After 5 minutes of mixing, 3 ml of 4M sodium acetate (J.T. Baker) and 4 ml 5% sodium tetraethylborate were added, and the samples were incubated for 22 min. The reaction was stopped with 5 ml 10M NaOH. The ethylated organotins were concentrated in 15 ml hexane using an AlOx column (10% moisture, MP Ecochrom), after which they were transferred into iso-octane using a Kuderna-Danish solvent evaporator and a gentle stream of nitrogen to blow off the hexane. These extracts were analysed with a gas chromatography mass selective detector GC-MSD (GC 6890 Series, Agilent Technologies; MSD: 5973 inert MSD, Agilent Technologies; HP Network Hewlett Packard). The quality control following the RWS-Waterdienst protocol included an internal reference consisting of monopropyltin (111%) and tripropyltin (102%) and reference sediment (Wadden sediment). Tributyltin and the degradation products dibutyltin and monobutyltin were measured in the control at the start of the experiment and in three tributyltin test concentrations, respectively, 0.25, 0.5 and 4 mg Sn/kg dw sediment, at the start and at the end of the experiment. The measured tributyltin test concentrations were corrected for extraction losses of the reference sediment (91% recovery), and ranged between 69 and 80% of the nominal values at the start of the experiment. Using the time-weighted averages of the measured tributyltin test concentrations a correction factor was calculated allowing extrapolation of the remaining actual exposure concentrations.

Actual phenanthrene concentrations were determined by extracting duplicate 1 gram subsamples (León Paumen et al., 2008a). The subsamples were dried with 1 gram of anhydrous sodium sulphate (p.a., Merck) and were Soxhlet-extracted in 25 ml hexane for 5 hours using cellulose extraction thimbles (Whatman). 1 ml of the hexane-extracted samples was transferred into 1 ml acetonitrile by blowing off the hexane using a gentle stream of nitrogen. The samples were then analysed using a Dionex high-performance liquid chromatographic system consisting of a Vydac 201TP reverse-phase column (C18; 5 µm, 4.6 x 250 mm) with a Waters Spherisorb ODS2 Guard Column (C18; 5 µm, 4.6 x 10 mm)
connected to a fluorescence detector (model FP-1520; Jasco, UK) and a diode-array UV detector (model UVD 320, Gynkotek, Germany). Soxhlet extraction efficiency was validated by adding spiking solution to clean sediment and following the same procedure. The measured phenanthrene test concentrations were corrected for recovery (74%) and ranged at the start of the experiment between 74 and 103% of the nominal values. Actual phenanthrene concentrations were calculated as the time-weighted means of the three measurements per test concentration (OECD, 1998).

**Data analyses**

A Student t test (p < 0.05) showed that survival did not differ significantly between the control and solvent control. The solvent controls were used as control treatment for LC50 calculation in the tributyltin and phenanthrene experiments and the controls for copper and cadmium. The LC50, i.e. the actual toxicant concentration in the sediment at which 50% mortality was observed compared to the (solvent) control, was calculated according to the logistic response model adopted from Haanstra et al. (1985). The following equation, \( y = \frac{c}{1 + e^{b (\log(x) - \log(a))}}, \) was fitted through the concentration-response data with \( y \) being the effect parameter (survival), \( x \) the actual exposure concentration, \( a \) the LC50, \( b \) the slope of the logistic curve and \( c \) the average survival in the control. Survival included both emerged midges as well as the larvae recovered from the sediment at the end of the 28 day experiment.

The mean emergence time (EmT50), i.e. the day at which 50% emergence occurred, was calculated for each test concentration at which emergence exceeded 10%, by plotting the cumulative number of emerged midges against time. This was performed separately for males and females, because *C. riparius* has a bimodal emergence pattern, where males emerge prior to females (Watts and Pascoe, 2000). Again the logistic model according to Haanstra et al. (1985) was applied, but in this case \( a \) was the EmT50, \( b \) the slope, \( c \) the average total emergence per replicate and \( x \) the days at which emergence was recorded. To determine at which actual toxicant concentrations emergence was significantly delayed compared to the control for the metals and compared to the solvent control for tributyltin and phenanthrene, EmT50 values for the different test concentrations were compared to the (solvent) control using generalized likelihood ratio tests according to van Gestel and Hensbergen (1997). The lowest actual test concentration that significantly delayed emergence was accordingly termed the LOEC\textsubscript{EmT50}.

The chronic lethal/sublethal effect concentration ratio was calculated for each compound by dividing the LC50 by the LOEC\textsubscript{EmT50}. All statistical analyses were performed in SPSS® 17 for windows.
Results

Chemical analyses

The actual toxicant concentrations in the sediment were for copper: 6.7, 12.0, 17.2, 28.8, 52.0, 65.2, 92.4 and 116.0 mg/kg dw sediment; cadmium: 0.01, 0.5, 1.2, 1.8, 3.9, 5.2 and 5.9 mg/kg dw sediment; tributyltin: <0.001, 0.2, 0.3, 0.7, 1.4, 2.7, 5.5, 10.9 and 21.8 mg Sn/kg dw sediment; and phenanthrene: <0.3, 29.7, 79.9, 106.5, 144.3, 204.4 and 257.8 mg/kg dw sediment (Supporting Table S1). These actual concentrations ranged for copper, cadmium, tributyltin and phenanthrene, respectively, between 98 - 113%, 73 - 112%, 69 - 80% and 59 - 86% of the nominal values. During the experiment 12% of the tributyltin was degraded into dibutyltin and monobutyltin, most probably by biotic processes, which might include tributyltin metabolization by the midge larvae (Stäb et al., 1996; Looser et al., 2000), while phenanthrene degradation amounted on average 32%, probably due to microbial degradation (Yuan et al., 2001; Johnson et al., 2005). The actual concentrations in the overlaying and interstitial water ranged for copper between 0.8 - 341.7 μg/l and for cadmium between 0.0 - 156.7 μg/l (Supporting Table S2).

Quality criteria

All experiments met the OECD guideline 218 validity criteria regarding water quality and emergence (OECD, 2004). The pH was 7.6 ± 0.4, ammonium concentrations remained <0 mg NH₄⁺/l, conductivity was 674 ± 31 μS/cm, dissolved oxygen levels were above 70% air saturation and control emergence ranged between 84 and 98% (Supporting Tables S2 and S3).

Chronic survival

Chronic survival was determined by summing the emerged midges and the larvae that were recovered from the sediment at the end of the experiment. In the (solvent) controls and the lowest test concentrations in each of the four experiments, no larvae were present in the sediment after 28 days, indicating that all surviving midges had emerged. With increasing test concentrations, the number of emerged midges decreased for all compounds. In the copper, cadmium and tributyltin experiments this was accompanied by the recovery of some larvae from the sediment after 28 days, but in the phenanthrene experiment, no larvae were recovered from the sediment after 28 days and, hence, survival equaled emergence. The mean survival data of the four experiments and the associated logistic response models are shown in Figure 1a-d. From these clear concentration-response curves the actual LC50 values with their 95% confidence intervals were calculated for each compound, as shown in Figure 1i-l.
Figure 1: Chronic effects of phenanthrene, copper, cadmium and tributyltin on *C. riparius* survival and mean male emergence time (EmT50). Figure 1a-d: Survival (average % ± stdev.) after 28 days of exposure. Figure 1e-h: EmT50 values with 95% C.I. * EmT50 values significantly different from control value (p<0.05). ▲ Concentrations where no male midges emerged or where male emergence was below 10%. Figure 1i-l: LC50, LOEC/EmT50 and chronic LC50/LOEC/EmT50 ratio for each compound.
Emergence time

A bimodal emergence pattern, with females emerging consistently later than males, was observed in all experiments. Since the four compounds affected the mean emergence time (EmT50) of both genders equally (Supporting Table S3), it was decided to select one of the two genders for subsequent calculations. Because male EmT50 values have been recently reported for several compounds (León Paumen et al., 2008a), we focused on male EmT50 values to facilitate comparison with literature data. As shown in Figure 1e-h, male EmT50 values increased gradually with increasing toxicant concentrations in the sediment for all compounds. Phenanthrene caused a significant delay in male emergence at 79.9 mg/kg dw sediment and higher. This delay was small and did not further increase with increasing phenanthrene concentrations. The lowest test concentration at which copper significantly delayed emergence was 17.2 mg Cu/kg dw sediment. Male EmT50 values for copper exposed midges continued to increase with increasing copper concentrations, till at 65.2 mg Cu/kg dw sediment no midges emerged at all. The range of copper concentrations at which these sublethal effects were observed was narrow in comparison to cadmium and tributyltin, which showed significantly delayed emergence from, respectively, 0.5 till 5.2 mg Cd/kg dw sediment and from 0.2 till 1.4 mg Sn/kg dw sediment. The lowest test concentrations that significantly delayed emergence were accordingly termed the LOEC_{EmT50}, and are shown in Figure 1i-l.

Chronic LC50/LOEC_{EmT50} ratio

The calculated chronic LC50/LOEC_{EmT50} ratios are shown in Figure 1i-l and amounted for phenanthrene, copper, cadmium and tributyltin, respectively, to 1.5, 3.5, 12.0 and 18.2. These chronic ratios increase with increasing specificity of the compounds mode of action.

Discussion

Chronic lethal and sublethal effects

By performing, under equal experimental conditions, four Chironomus riparius life cycle toxicity tests with phenanthrene, copper, cadmium and tributyltin, we were able to compare the chronic sublethal and lethal effects exerted by these compounds. For all four compounds clear concentration-response relationships were observed for emergence time and survival. However, depending on the compounds mode of action, differences between lethal and sublethal effects were observed. Phenanthrene, a polycyclic aromatic compound that acts via a non-specific baseline toxicity known as narcosis (Bleeker et al., 2002), primarily affected survival. All surviving larvae managed to emerge, indicating that larval development was either not or not substantially delayed. This is in agreement with León
Paumen et al. (2008a) who previously reported that emergence equaled survival in phenanthrene exposed *C. riparius* larvae. However, in contrast to that study we did observe sublethal effects. Male emergence was significantly delayed at a concentration of 79.9 mg phenanthrene/kg dw sediment and higher. For the three other compounds that acted via more specific modes of action, i.e. endocrine disruption for the organometal tributyltin (Hahn and Schulz, 2002) and oxidative stress for the redox-active metal copper and the redox-inactive metal cadmium (Gaetke and Chow, 2003; Ercal et al., 2001), the observed sublethal effects were much more profound. These compounds delayed larval development and emergence severely in a concentration dependant manner, with male mean emergence reaching a maximal delay of 8.4, 10.8 and 7.7 days for respectively, copper, cadmium and tributyltin. Our results are in agreement with previously published studies where copper, cadmium and tributyltin exhibited similar effects on *C. riparius* larval development and/or emergence (e.g. Roman et al., 2007; Vogt et al., 2007; Nowak et al., 2008). The concentration range at which emergence was delayed was much narrower for copper in comparison to cadmium and tributyltin. This is most probably because copper, as it is involved in vital biological processes (Gaetke and Chow, 2003), is regulated to a certain point. Regulation of the internal copper concentration, but not of cadmium, has been reported for some species of the genus Chironomus (Krantzberg and Stokes, 1989).

**Chronic LC50/LOEC<sub>EmT50</sub> ratio**

The chronic lethal/sublethal ratio, defined as the chronic median lethal concentration (LC50) divided by the lowest observed effect concentration based on the EmT50 (LOEC<sub>EmT50</sub>), amounted for phenanthrene, copper, cadmium and tributyltin, respectively, to 1.5, 3.5, 12.0 and 18.2. These values clearly indicate that the chronic ratio increases with the specificity of the compounds mode of action. This is in line with previous reports on ACRs, where narcotic compounds showed the lowest ratios (Roex et al., 2000; Ahlers et al., 2006) and compounds with more specific modes of action frequently showed high ACRs (Länge et al., 1998). While for ACRs differences of three to four orders of magnitudes were commonly reported (Länge et al., 1998; Roex et al., 2000; Ahlers et al., 2006; Raimondo et al., 2007) the chronic ratios in our study differed only by one order of magnitude. Given the limitation that we tested only four compounds, additional chronic ratios were derived from *C. riparius* literature toxicity data. To include as many studies as possible, ratios were also calculated for studies that did not report a LOEC based on EmT50. In those cases, the LOEC based on the most sensitive sublethal endpoint, e.g. total emergence or growth, was selected. The LC50/LOEC ratios are shown in table 1. For the narcotic compounds 1,2,3,4-tetrachlorobenzene (Leslie et al., 2004), fluoranthene (Stewart and Thompson, 1995), acridine and phenantridine (León Paumen et al., 2008a) chronic ratios were calculated that were very close to the 1.5 we derived for phenanthrene. The other three narcotic compounds, i.e. anthracene, acridone and phenantridine, gave somewhat higher ratios.
Interestingly, León Paumen et al. (2008a) noted already that during chronic exposure, these three compounds most probably had a more specific effect on emergence time than just narcosis. For the metals mercury (Chibunda et al., 2009) and copper (Roman et al., 2007) ratios were calculated of 3.7 and 3.6. These are in line with the 3.5 we observed for copper. However, since mercury is a non-essential metal it was expected to be more in line with the higher value found for cadmium. Vogt et al. (2007) reported a life cycle toxicity test with cadmium, however, due to the low statistical power (only two replicates) the authors did not obtain significant differences in EmT50’s between treatments, and thus no LC50/LOEC ratio could be calculated. Vogt et al. (2007) also tested the highly toxic biocide tributyltin. Even though male emergence was clearly delayed at low test concentrations, they only obtained a significant delay at higher test concentrations. This resulted in a noticeably lower chronic ratio compared to the ratio calculated in this study. This discrepancy between the two studies is most probably due to differences in statistical analysis. The last two compounds, ivermectin (Egeler et al., 2010) and thiaclopid (Langer-Jaesrich et al., 2010) are both insecticides with specific modes of action. The LC50/LOEC_{EmT50} ratios were therefore expected to be high. Accordingly, ratios slightly above 10 were calculated for both compounds. These ratios might have been even higher if LOECs based on EmT50, instead of respectively, growth and total emergence, had been available. Although several other agrochemicals have been tested with *C. riparius*, these studies could not be included, as they did not report a LC50 (Agra et al., 2009; Jungmann et al., 2009; Tassou and Schulz, 2009) or a LOEC value (Åkerblom). Generally, the ratios obtained from the literature showed the same trend of increasing chronic LC50/LOEC ratios with increasing specificity of the compounds mode of action as the ratios calculated in the present study. The observations indicated a robust relationship between this chronic lethal/sublethal ratio and specificity of the mode of action, but also pointed to variability caused by the methodology used to quantify sublethal effects and variability induced by different test conditions. Nevertheless, the observed variability was several orders of magnitude lower than observed for ACRs. It is concluded that the chronic LC50/LOEC_{EmT50} ratio is indicative for the specificity of a compounds mode of action and that the variation is drastically reduced compared to ACRs.

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*Supporting information available:* Tables S1-S3. This information is available free of charge via the Internet at http://pubs.acs.org.
Chapter 4

Response of the non-biting midge *Chironomus riparius* to multigeneration toxicant exposure

Abstract

The ability of the non-biting midge *Chironomus riparius* to withstand long-term toxicant exposure has been attributed to genetic adaptation. Recently, however, evidence has arisen that supports phenotypic plasticity. Therefore, the present study aimed to investigate if *C. riparius* indeed copes with prolonged toxicant exposure through phenotypic plasticity. To this purpose, we performed a multigeneration experiment in which we exposed *C. riparius* laboratory cultures for nine consecutive generations to two exposure scenarios of, respectively, copper, cadmium and tributyltin. Total emergence and mean emergence time were monitored each generation, while the sensitivity of the cultures was assessed at least every 3rd generation using acute toxicity tests. We observed that the sublethally exposed cultures were hardly affected, while the cultures that were exposed to substantially higher toxicant concentrations after the 6th generation were severely affected in the 8th generation followed by signs of recovery. A marginal lowered sensitivity was only observed for the highly exposed cadmium culture, but this was lost again within one generation. We conclude that *C. riparius* can indeed withstand long-term sublethal toxicant exposure through phenotypic plasticity without genetic adaption.
Introduction

Biodiversity generally decreases with deteriorating environmental conditions (Crunkilton and Duchrow, 1990; Solà et al., 2004; Relyea, 2005). Some species, however, are able to maintain viable populations under conditions that are fatal to others. Non-biting midges belonging to the genus Chironomus (Insecta: Diptera) are such pollution tolerant and persistent species (Armitage et al., 1983; Gabriels et al., 2010). Indeed, larvae of *Chironomus riparius* can cope with low oxygen concentrations (Redecker and Zebe, 1988), increased salinities (Bervoets et al., 1996), wide ranges of pH (Jernelöv et al., 1981; Havas and Hutchinson, 1982) and are, above all, remarkably tolerant to organic (e.g. Gower and Buckland, 1978; Friberg et al., 2010) and heavy-metal pollution (e.g. Winner et al., 1980; Groenendijk et al., 1999). Studies that aimed to unravel the mechanism underlying their ability to withstand long-term pollution have been performed with *C. riparius* populations obtained from heavy-metal polluted environments (Postma et al., 1995a; 1995b), as well as with *C. riparius* laboratory cultures that were exposed for multiple generations to a single toxicant (Postma and Davids, 1995; Miller and Hendricks, 1996; Ristola et al., 2001; Vogt et al., 2007). Except Ristola et al. (2001), these studies reported a decreased sensitivity in the toxicant-exposed midges and suggested, to a greater or lesser extent, that this was due to genetic adaptation. Substantial evidence for genetic adaptation, i.e. heritability of decreased toxicant sensitivity in offspring reared under clean conditions (Morgan et al., 2007), was, however, only provided for historically exposed *C. riparius* field populations (Postma et al., 1995a; 1995b). The other multigeneration studies reported in literature either did not perform this check and could thus not rule out phenotypic plasticity, i.e. physiological acclimation and subsequent maternal effects (Postma and Davids, 1995; Vogt et al., 2007), or found evidence for both mechanisms (Miller and Hendricks, 1996). A recent multigeneration study performed with the closely related species *Chironomus plumosus* (Vedamanikam and Shazilli, 2008) has revitalized this discussion, as they obtained substantial evidence for phenotypic plasticity by showing that decreased metal sensitivity could be induced within six generations, but also lost again after two generations of rearing under clean conditions. Interestingly, the same process of gaining and subsequent losing of decreased toxicant sensitivity was also reported by Vogt et al. (2007) when culturing midges under continuous tributyltin pressure. Triggered by these latter observations, the aim of the present study was to verify if *Chironomus riparius* indeed copes with prolonged toxicant exposure via phenotypic plasticity. To this purpose, we performed a multigeneration experiment where *C. riparius* cultures were exposed for nine consecutive generations to three model toxicants that were previously shown to differently affect *C. riparius* during a single generation sediment toxicity test (Marinković et al., 2011). For each of these three toxicants, i.e. the essential metal copper, the non-essential metal cadmium and the organometal tributyltin, two exposure scenarios were designed. One in
which the sublethal concentration remained constant and one in which after the 6th generation the exposure concentration was increased for three more generations. Total emergence and mean emergence time were monitored during each generation for all cultures. To assess changes in sensitivity, 14-day sediment toxicity tests were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently at least every third generation.

**Materials and methods**

**Test organism and culturing conditions**

The *C. riparius* larvae used in the present study originated from the University of Amsterdam’s in-house laboratory culture. Regular exchange of egg-ropes with other laboratories and the maintenance of a large population size guaranteed a high level of genetic variation as previously reported by Nowak et al. (2007). The culture was maintained in aquaria containing quartz sand overlaid with Dutch Standard Water (deionised water with 200 mg/l CaCl$_2$*2H$_2$O, 180 mg/l MgSO$_4$*H$_2$O, 100 mg/l NaHCO$_3$ and 20 mg/l KHCO$_3$; hardness is 210 mg as CaCO$_3$/l and pH 8.2 ± 0.2) at 20 ± 1 °C, 65% humidity and a 16: 8 h light: dark photoperiod. 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.

**Experimental setup of the multigeneration study**

*Chironomus riparius* test cultures were exposed for nine consecutive generations to artificial sediment spiked with either copper (CuCl$_2$.2H$_2$O, copper standard, Fluka), cadmium (CdCl$_2$, Titrisol®, Merck) or tributyltin (TBT-Cl, 96% purity, Aldrich). The artificial sediment had a pH of 7.0 ± 0.5, a moisture content of 50% and consisted for 75% of quartz sand (Sibelco® M34, Belgium), 20% of kaolin clay (WBB Vingerling, the Netherlands) and 5% of α-cellulose (Sigma). For each toxicant, two exposure scenarios were designed: In the first scenario, the sublethal test concentration remained constant over nine generations (tox cultures). Because the effects on emergence remained limited during the first six generations, a second scenario was designed where the test concentrations were increased from sublethal to partially lethal for three more generations (tox+ cultures). The sublethal test concentrations were based on previously conducted one generation toxicity tests (Marinković et al., 2011) and aimed to reduce total emergence with 15%, while the partially lethal test concentrations were chosen because they caused substantial mortality in the sensitivity testing experiments in the present study. The nominal test concentrations...
were for copper respectively 15 and 30 mg Cu/kg dw; cadmium: 2 and 8 mg Cd/kg dw; and tributyltin: 0.5 and 3 mg Sn/kg dw.

Each generation of the *C. riparius* test cultures consisted of two replicate 22 l aquaria (35*25*25cm) and two additional replicate 400 ml glass beakers that were used for chemical analysis at the start (day 0) and half-way a generation (day 14). A third chemical analysis was conducted at the end of the generation (day 28) by sampling sediment from one of the replicate aquaria. Spiking of the artificial sediment was conducted according to Marinković et al. (2011). In short, appropriate amounts of copper and cadmium stock solution were added to 3.2 kg wet sediment in glass 3 l bottles. To these sediment-metal mixtures 6.0 g of food, which corresponded to 0.25 mg food/larvae/day for the entire duration of a generation (28 days), was added and the bottles were placed for 24 hours on a roller bank (20 rpm). The homogenized food-metal-sediment mixtures were subsequently divided over the test vessels, i.e. 1.5 kg/ 22 l aquarium and 60 g/ 400 ml glass beaker. The test vessels were carefully topped up with Dutch Standard Water (6 l/aquarium; 250 ml/beaker) and covered with plastic foil to prevent evaporation. After settling of the sediment gentle aeration was turned on. The test vessels were conditioned for one week, allowing the compounds to equilibrate with the water and sediment and a stable sediment layer to be formed. For the less water-soluble compound tributyltin, an additional pre-spiking step was performed where the acetone dissolved tributyltin was mixed through 10% of the total amount of dw sediment. The tributyltin-sediment mixture was allowed to dry in a fume board, after which deionized water and the remaining wet sediment were added. From here on the same mixing and equilibration procedures were followed as for copper and cadmium.

All test cultures, including control and solvent control cultures, were started simultaneously from a batch of 50 egg-ropes that originated from our *C. riparius* laboratory culture. Using a stereo microscope and a blunt glass Pasteur pipette, 400 randomly chosen larvae (< 24 hours) were introduced in each of the two replicate 22 l aquaria (35*25*25cm) per treatment. To the two replicate 400 ml glass beakers that were used for chemical analysis, 10 larvae were added from the same batch. To allow settlement of the introduced larvae on the sediment, aeration in the test vessels was temporarily switched off and restarted 4 hours later. Two additional feedings of 700 mg food/aquarium and 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, were administered during each generation at days 7 and 14. On day 14 the plastic cover was replaced by a cage to prevent emerging midges from escaping during exhausting and from this day on, the aquaria were inspected daily for emerged midges until termination of the exposure period (day 28, in case of strongly delayed emergence day 33). Emerged midges were caught with an exhauster, sexed and transferred to a mating cage where they were allowed to swarm and lay egg-ropes. Egg ropes were daily removed from the meeting cage.
and stored in Dutch Standard Water for up to three days at 4 °C. Typically the 30 first laid egg-ropes were hatched at 20° C in Dutch Standard Water and the larvae (< 24 hours) were used to start up the next generation in freshly prepared test vessels. This was possible for all cultures except for the TBT⁺ culture where due to the strongly reduced emergence in the 8th generation only 21 good quality egg ropes could be used to start the next generation.

**Sensitivity testing experiments.**

To determine the sensitivity of the test cultures during the multigeneration experiment, 14-day sediment toxicity experiments were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently every 3rd generation. The control culture was tested with all three compounds. When decreased toxicant sensitivity was observed in a test culture, additional sensitivity testing was conducted with the respective test culture during the following generation to determine the stability of the decreased sensitivity. The sensitivity testing experiments were based on OECD guideline 218 (OECD, 2004) and were performed with the following nominal test concentrations of copper: 10, 20, 30, 40 and 50 mg Cu/kg dw; cadmium: 0.5, 1, 2, 4, and 8 mg Cd/kg dw; and tributyltin: 0.5, 1, 2, 3 and 4 mg Sn/kg dw. Controls and solvent controls were included. Spiking of the artificial sediment was performed the same way as previously described for the multigeneration experiment, with the only difference being that the initial feeding corresponded to 0.5 mg food/larvae/day for a period of two weeks (70 mg food/beaker). Each treatment consisted of seven replicate 400 ml glass beakers of which two were sacrificed for chemical analysis at the start (day 0) and halfway (day 7) the experiment. A third chemical analysis was conducted by sampling sediment from one of the remaining five replicates upon termination of the experiment (day 14). Ten larvae (< 24 hours) that were obtained from a batch of at least five hatched egg ropes per test culture were added to each test beaker. After 7 days an additional feeding of 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, was administered. On day 14 the experiments were terminated, the sediment was sieved through a 350 μm sieve and surviving larvae were counted.

**Quality criteria and chemical analyses**

The quality of the overlying water and the actual toxicant concentrations in the sediment were determined by sacrificing one replicate per treatment at the start, half-way and at the end of both the multigeneration experiment and the sensitivity testing experiments. The actual toxicant concentrations in the sediment were determined as previously described in Marinković et al. (2011). In short, sediment was centrifuged at 3000 rpm for 15 minutes and stored at -20 °C until analysis. For the copper and cadmium samples, the frozen sediment was oven-dried and duplicate 130 mg subsamples were digested for 7 hours at 140 °C in 2 ml of a 4:1 mixture of nitric acid (65% p.a.; Sigma-Aldrich) and hydrochloric
acid (37% p.a., Sigma-Aldrich). The digested samples were diluted with deionised water and analysed by flame atomic absorption spectrophotometry (Perkin Elmer A Analyst 100, Germany). Certified reference material ISE 989 Riverclay (Wageningen Agricultural University, The Netherlands) was included for quality assurance and the measured metal test concentrations were corrected for copper (86%) and cadmium (88%) recovery. The actual metal concentrations in the sediment were calculated as time-weighted means of the three measurements per test concentration according to OECD guideline 211 (1998).

The tributyltin samples were analysed by RWS-Waterdienst, Lelystad, according to their in-house developed method accredited by the Dutch Accreditation Council. Two 1 gram subsamples of freeze-dried sediment were extracted by adding 15 ml methanol (J.T.Baker), 1.5 ml acetic acid (99.9%, J.T.Baker) and 7 ml hexane (J.T.Baker). After 5 minutes of mixing, 3 ml of 4M sodium acetate (J.T.Baker) and 4 ml 5% sodium tetraethylborate were added, and the samples were incubated for 22 min. The reactions were stopped with 5 ml 10M NaOH. The ethylated organotins were concentrated in hexane using an AlOx column (10% moisture, MP Eochrom), after which they were transferred into iso-octane using a Kuderna-Danish solvent evaporator and a gentle stream of nitrogen. These extracts were analyzed with a gas chromatography mass selective detector GC-MSD (GC 6890 Series, Agilent Technologies; MSD: 5973 inert MSD, Agilent Technologies; HP Network Hewlett Packard). The quality control included reference sediment (Wadden sediment) and an internal reference consisting of monopropyltin (111%) and tripropyltin (102%). Tributyltin was measured in generation F1 and F9 of the multigeneration experiment at the start (day 0) and at the end (day 28). The measured tributyltin test concentrations were corrected for extraction losses of the reference sediment (91% recovery), and ranged between 69% and 80% of the nominal values at the start of the experiment. Using the time-weighted averages of the measured tributyltin test concentrations a correction factor was calculated allowing extrapolation of the remaining actual exposure concentrations.

Data analyses

All statistical analyses were performed at a 5% significance level in SPSS® 17 for windows. Emergence data from the multigeneration experiment was used to compare the exposed cultures with the (solvent) control cultures within and over the generations. Total emergence data was arcsine-sqrt transformed and tested for homogeneity of variance with Levene’s test. A t test was used to compare total emergence of the exposed and the control cultures over the generations and per generation. The EmT50, i.e. the day at which 50% emergence occurred during a generation, was calculated for each culture according to Haanstra et al. (1985) by fitting a logistic curve \( y = c / (1 + e^{b(x - \log(a))}) \) through the cumulative number of emerged midges against time. In this equation \( a \) is the EmT50, \( b \) is the slope of the logistic curve, \( c \) is the average total emergence per replicate and \( x \) is the day
at which emergence was recorded. Since *C. riparius* has a bimodal emergence pattern, where males emerge prior to females (Watts and Pascoe, 2000), EmT50 values were calculated for all emerged midges as well as males and females separately. The EmT50 values of the exposed cultures were subsequently compared to the (solvent) control culture EmT50 value using generalised likelihood ratio tests (van Gestel and Hensbergen, 1997).

Larval survival data from the sensitivity testing experiments were used to calculate the concentrations of the test compounds that caused 50% mortality (LC50). The LC50 values were calculated with the actual toxicant concentrations using the same logistic response model (Haanstra et al., 1985) and were compared to the control culture LC50 values using generalised likelihood ratio tests (van Gestel and Hensbergen, 1997).

**Results**

**Quality criteria and actual toxicant concentrations in the sediment**

All experiments met the OECD guideline 218 validity criteria regarding water quality (OECD, 2004). The pH was $7.5 \pm 0.4$, ammonium concentrations remained 0 mg NH$_4^+$/l, conductivity was $665 \pm 46 \mu$S/cm, dissolved oxygen levels were above 70% air saturation and water temperature was $20 \pm 1^\circ$ C. The total emergence of the control culture ranged during the multigeneration experiment between 72% and 92% for all generations except the 6$^{th}$ generation where it amounted to 65% $\pm$ 0.5%. While the control emergence in the 6$^{th}$ generation was just below the required 70%, the multigeneration experiment was considered valid with an average (n=9) control culture emergence of 80 $\pm$ 8.8% over the nine generations.

During the multigeneration experiment, the actual toxicant concentrations of the different treatments hardly varied over the generations (Supporting Table S1), resulting in the following average actual toxicant concentrations for the tox (n=9) and tox$^+$ (n=3) cultures for, respectively, copper: 18.9 and 34.7 mg Cu/kg dw sediment; cadmium: 1.9 and 7.0 mg Cd/kg dw sediment; and tributyltin: 0.3 and 2.1 mg Sn/kg dw sediment. Also the actual toxicant concentrations measured in the sensitivity testing experiments did not show strong deviations from the nominal concentrations (Supporting Table S2) and averaged for, respectively, copper (n=4): 4.7, 14.6, 23.7, 33.2, 43.1 and 54.9 mg Cu/kg dw sediment; cadmium (n=5): <0.01, 0.4, 1.0, 1.9, 3.4 and 7.1 mg Cd/kg dw sediment; and tributyltin (n=4): <0.001, 0.3, 0.7, 1.4, 2.1 and 2.7 mg Sn/kg dw 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.
**Multigeneration exposure of C. riparius**

Average (± st.dev) total emergence of the control culture (n=9) was 80 ± 8.8% during the multigeneration experiment (Figure 1a-b). There were no significant differences between the total emergence of the control culture and the solvent control culture which showed an average (n=9) total emergence of 82 ± 9.1% during the multigeneration experiment (Figure 1c). Exposure of *C. riparius* cultures to sublethal copper, cadmium and tributyltin concentrations resulted for the respective cultures in a significant emergence reduction of 12%, 12% and 33% during the 1st generation (Figure 1a-c). Over the nine

![Figure 1: Effects of the three toxicants on *C. riparius* total emergence and the mean emergence time (EmT50) during nine consecutive generations of exposure. Fig. 1a-c: Total emergence (average % ± st.dev) per generation. Fig. 1d-f: EmT50 (day ± 95% C.I) values per generation. The open circles represent the control culture (for tributyltin the solvent control culture), the black squares the tox culture and the grey triangles the tox+ culture. * indicates values significantly differing from the (solvent) control culture (p<0.05).]
generations only the tributyltin culture had a significantly lower total emergence which averaged (n=8) 65 ± 9.3% (Figure 1c). As shown in Figure 1c, the 5th generation of the tributyltin culture was excluded from the calculations. This was because the concerning aquaria suffered from a two-day power failure that resulted in unreliable emergence data for the tributyltin culture. Nevertheless, sufficient good quality egg ropes were obtained to start the 6th generation. The tox+ cultures, which were exposed to substantially higher toxicant concentrations after the 6th generation, were much stronger impacted (Figure 1a-c). The tributyltin+ culture was immediately significantly impaired with a total emergence of 26% ± 8.5% in the 7th generation, while it took a generation longer to see a comparable significant reduction in the total emergence of the copper+ and cadmium+ cultures. In the 9th generation, total emergence of the three tox+ cultures remained significantly impaired, however, obvious signs of recovery were observed (Figure 1a-c). The average total emergence (n=3) of the cadmium+, copper+ and tributyltin+ cultures was significantly lower than that of the (solvent)control culture and amounted to, respectively, 50 ± 24%, 37 ± 20% and 27 ± 21%.

The mean emergence time (EmT50) of the control culture ranged between 19.0 and 21.1 days and averaged 20.1 ± 0.8 days over the nine generations (Figure 1d-e). A similar pattern was observed for the solvent control culture with an average EmT50 of 20.0 ± 0.8 days and a range of 18.6 to 21.1 days (Figure 1f). The tox cultures showed EmT50’s that deviated in most generations significantly, though limitedly from the (solvent) control culture EmT50’s. While the copper and cadmium cultures had an average emergence delay of 0.7 days over the nine generations and on occasions emerged even faster than the control culture, the midges exposed to the sublethal tributyltin concentration were always delayed compared to the solvent control with an average delay (n=8) of 2.8 days. After the increase of the toxic pressure, all three tox+ cultures were much stronger delayed (Figure 1d-f). The strongest delay was observed in the 8th generation and amounted for the cadmium+, copper+ and tributyltin+ cultures, respectively, 6.8, 6.3 and 9.2 days in comparison to the (solvent) control culture. In the 9th generation a recovery was observed as the emergence delay of the respective tox+ cultures was limited to 3.0, 3.9, and 5.8 days.

To assess if males and females were equally affected we also calculated the EmT50 values for males and females separately (Supporting Figure S1). The observed emergence patterns showed that even though the females emerged consistently later than the males, both sexes were equally affected.

**Sensitivity testing of exposed cultures**

To assess the sensitivity of the test cultures, 14-day toxicity tests were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently every 3rd generation (Figure 2). During each of these sensitivity testing experiments, the control culture was tested with all three toxicants. The LC50 values demonstrate that the
sensitivity of the control culture showed no distinct development over the generations and that the tox cultures remained as sensitive as the control culture throughout the multigeneration experiment. The increase of the toxicant concentration after the 6th generation resulted only for the cadmium+ culture in a significantly higher LC50 value in the 9th generation compared to the control culture. However, one generation later this decreased cadmium sensitivity was not observed anymore.

**Discussion**

In the present study we postulated that phenotypic plasticity, i.e. the capacity of a single genotype to exhibit a range of phenotypes in response to different environmental conditions (Whitman and Agrawal, 2009), underlies *C. riparius’* well documented ability to cope with a wide variety of stressors (e.g. Redecker and Zebe, 1988; Bervoets et al., 1996; Jernelöv et al., 1981; Havas and Hutchinson, 1982; Gower and Buckland, 1978; Friberg et al., 2010; Winner et al., 1980). To verify this postulate we exposed *C. riparius* laboratory cultures for nine consecutive generations to three model compounds that were previously shown to differently affect *C. riparius* during its life-cycle (Marinković et al., 2011). We observed that long-term exposure to sublethal concentrations of cadmium and copper had an impact on the *C. riparius* cultures, yet the total emergence and the mean emergence time (EmT50) of these cultures oscillated around the control culture values over the nine generations. The tributyltin culture was stronger affected, resulting in a significantly lower total emergence.

Figure 2: Sensitivity of the *C. riparius* cultures was assessed at the start of the multigeneration experiment (P) and subsequently after the 3rd, 6th and 9th generation (i.e., F3, F6 and F9) with the appropriate toxicant using 14-day toxicity tests. An additional sensitivity testing experiment was performed with cadmium after the tenth generation (F10). The figures show the LC50 (conc. ± 95% C.I) values obtained for the cultures exposed to, respectively, cadmium, copper and tributyltin. In each figure the open circles represent the control culture (for tributyltin the solvent control culture), the black squares the tox culture and the grey triangles the tox+ culture. * indicates values significantly differing from the (solvent) control culture (p<0.05).
during the nine generations. Interestingly, while previous *C. riparius* multigeneration studies showed that effects worsened with increasing number of exposed generations (Postma and Davids, 1995), we did not observe such a trend. In fact, the sensitivity of the sublethally exposed cultures was not significantly affected during the nine generations of consecutive exposure, as was clearly demonstrated by the sensitivity testing experiments. It might be argued that the chosen sublethal concentrations were too low. However, previous multigeneration studies where *C. riparius* laboratory cultures were exposed to sublethal concentrations of cadmium (Postma and Davids, 1995), zinc (Miller and Hendricks, 1996), and tributyltin (Vogt et al., 2007) showed that the presently tested sublethal concentrations of these compounds can lead to decreased toxicant sensitivity as well as to extinction. Possible explanations for the discrepancies between these studies and the present study might be both the toxic pressure which might be lower in the present study due to the binding capacity of the artificial sediment, as well as the genetic variability of our *C. riparius* laboratory culture that might have been higher and thus effectively prevented extinction.

The three tox+ cultures that were exposed to substantially higher toxicant concentrations after the 6th generation, were, as expected, much stronger impacted and showed a significantly reduced total emergence in the 8th generation. Even though the differences remained significant in the 9th generation, all three tox− cultures showed a marked recovery. The recovery of these cultures was confirmed by the subsequent sensitivity testing experiments where the obtained LC50 values clearly showed that the copper+ and tributyltin− cultures remained equally sensitive as the controls, while a significantly lower sensitivity was only observed for the cadmium+ culture. On first glance, the latter observation seemed to be in good agreement with Postma and Davids (1995), who demonstrated that nine consecutive generations of sublethal cadmium exposure decreased the cadmium sensitivity of *C. riparius*. While these authors did not continue to monitor the cadmium sensitivity of their *C. riparius* culture after the 9th generation, we did and observed that even though the cadmium pressure was retained, the reduced cadmium sensitivity of the cadmium− culture was already lost in the next generation. Similarly, Vogt et al. (2007) observed that a *C. riparius* culture that was exposed for eleven consecutive generations to tributyltin was significantly less sensitive to tributyltin in the 9th and 10th generations, while in the last generation of the multigeneration experiment the sensitivity of the exposed culture was again similar to that of the control culture. This pattern has been observed in other multigeneration studies too (Miller and Hendricks, 1996, Vedamanikam and Shazilli, 2008), however, since in those studies the tolerant midge cultures were reared for two generations under clean conditions, the authors concluded that the lower toxicant sensitivity was lost due to a lack of toxic pressure and/or cost of tolerance which can be the result of between-environment trade-offs (Shirley and Sibly, 1999). Hence, when we consider our results as well as the above discussed observations from other *C. riparius*
multigeneration studies, it appears that response of *C. riparius* to long-term toxicant exposure is rather plastic, i.e. surviving cultures do not always develop a decreased toxicant sensitivity, and, more so, when they do it may be rapidly lost again regardless of the presence/absence of toxic pressure. We therefore conclude that *C. riparius* laboratory cultures can indeed withstand long-term sublethal toxicant exposure without getting genetically adapted due to phenotypic plasticity. This plasticity is apparently responsible for fluctuating responses between successive generations.

With the demonstrated plasticity in mind, we reassessed the studies that were conducted with historically metal exposed *C. riparius* field populations in order to verify if phenotypic plasticity could also explain these field observations. The strongest evidence for genetic adaptation was provided by Postma et al. (1995a; 1995b) who showed that first-generation clean water laboratory reared offspring of historically metal exposed *C. riparius* field populations was less cadmium sensitive than offspring of nearby sampled reference populations. Interestingly, these two studies also showed that one of the tested metal-exposed field populations lost its decreased metal sensitivity in the interval between two successive sampling campaigns. Similarly, Groenendijk et al. (1999) reported that the level of metal sensitivity of several historically metal exposed *C. riparius* populations varied considerably during a five month field sampling campaign. Since these historically metal exposed *C. riparius* field populations were periodically replenished with non-exposed larvae from clean upstream river reaches as well as egg-ropes deposited by non-exposed females, it remains, however, impossible to determine to what extent the observed variability was due to gene flow, to the loss of tolerance or to both. Groenendijk et al. (2002) also showed that crossing midges from polluted and reference sites yielded offspring with intermediate levels of metal sensitivity. They advocated a major genetic component, however, a slight maternal effect was also observable, therefore phenotypic plasticity cannot be ruled out. Finally, it should be noted that although the above discussed field studies showed heritability in first-generation clean water laboratory reared offspring, a prerequisite to postulate genetic adaptation as the responsible mechanism (Morgan et al., 2007), this does not exclude other heritable mechanisms that are not mediated through alterations at the DNA sequence level and that can be reversible, i.e. epigenetic changes (Vandegehuchte et al., 2011) and that are in fact part of the broader term ‘maternal effects’. This is especially worth nothing, since Groenendijk et al. (2002) observed that the decreased metal sensitivity was rapidly lost in the hybrid offspring what might indicate a transgenerational epigenetic effect (Ho and Burggren, 2010). Epigenetic changes, particularly those mediated through DNA methylation, have been suggested to play a key role in the regulation of phenotypic plasticity (Zhang and Meaney, 2010) and insects have been proposed as model organisms for studying this phenomenon (Glastad et al., 2011). Thus, phenotypic plasticity is most likely the mechanism that underlies *C. riparius* ability
to cope with long-term sublethal toxicant exposure, but due to the complexity of field studies it can only be demonstrated under controlled laboratory conditions.

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**Supporting information available:** Tables S1-S2 and Figure S1. This information is available free of charge via the Internet at http://pubs.acs.org.
Combining next-generation sequencing and microarray technology into a transcriptomics approach for the non-model organism

*Chironomus riparius*

Abstract

Whole-transcriptome gene-expression analyses are commonly performed in species that have a sequenced genome and for which microarrays are commercially available. To do such analyses in species with no or limited genome data, i.e. non-model organisms, necessary transcriptomics resources, i.e. an annotated transcriptome and a validated gene-expression microarray, must first be developed. The aim of the present study was to establish an advanced approach for developing transcriptomics resources for non-model organisms by combining next-generation sequencing (NGS) and microarray technology. We applied our approach to the non-biting midge *Chironomus riparius*, an ecologically relevant species that is widely used in sediment ecotoxicity testing.

We sampled extensively covering all *C. riparius* developmental stages as well as toxicant-exposed larvae and obtained from a normalized cDNA library 1.5M NGS reads totalling 501 Mbp. Using the NGS data we developed transcriptomics resources in several steps. First, we designed 844k probes directly on the NGS reads, as well as 76k probes targeting expressed sequence tags of related species. These probes were tested for their affinity to *C. riparius* DNA and mRNA, by performing two biological experiments with a 1M probe-selection microarray that contained the entire probe-library. Subsequently, the 1.5M NGS reads were assembled into 23,709 isotigs and 135,082 singletons, which were associated to ~55k, respectively, ~61k gene ontology terms and which corresponded together to 22,593 unique protein accessions. An algorithm was developed that took the assembly and the probe affinities to DNA and mRNA into account, what resulted in 59k highly-reliable probes that targeted uniquely 95% of the isotigs and 18% of the singletons.

Concluding, our approach allowed the development of high-quality transcriptomics resources for *C. riparius*, and is applicable to any non-model organism. It is expected, that these resources will advance ecotoxicity testing with *C. riparius* as whole-transcriptome gene-expression analysis are now possible with this species.
Introduction

Microarray technology has, 17 years after its introduction (Schena et al., 1995), become a well-established tool for whole-transcriptome gene-expression analyses (MAQC Consortium, 2006). Although under pressure due to the on-going developments in next-generation sequencing (NGS) (Shendure, 2008), this technology is anticipated to have a viable future for the coming decade given its current low cost, relatively limited data-handling burden, as well as accepted pre-processing and data analyses methods. In contrast to NGS, microarray technology allows comprehensive though cost-effective transcriptome analyses, making it thus possible to test various experimental conditions and to use many replicates, the latter being a necessity to account for biological variability (Hanssen et al., 2011). The option of purchasing completely custom-made microarrays containing up to 4.2 million spots each with a different oligonucleotide (http://www.nimblegen.com/products/cgh/custom/4.2m/index.html) allows for flexible experimentation including the design and the use of huge probe libraries. Over the years, many ground-breaking microarray experiments have been performed with respect to unravelling cellular mechanisms, as well as the discovery of predictive/diagnostic biomarkers (van ‘t Veer et al., 2002).

Until recently, microarray studies were mainly restricted to several traditional genome-sequenced model species (Neumann and Galvez, 2002). This meant that in domains that rely on non-model, i.e. non sequenced, organisms, such as ecology and ecotoxicology, microarray technology was only of limited use (Neumann and Galvez, 2002). The introduction of NGS and in particular medium-long (300-500bp) read pyrosequencing (Margulies et al., 2005) changed this (Ekblom and Galindo, 2011), as it became feasible to develop microarrays for any species of interest (Vera et al., 2008; Garcia-Reyero et al., 2008). In general, the approach to develop transcriptomics resources for non-model organisms is as follows. NGS reads are generated from mRNA and, by lack of reference genome, de novo assembled with one or several transcriptome assemblers. Subsequently, microarray probe libraries are designed that target the assembled sequences (contigs/isotigs) and, depending on the microarray format, all or a selection of the un-assembled reads (singletons) (Vera et al., 2008; Garcia-Reyero et al., 2008; Bellin et al., 2009; Gong et al., 2010; Milan et al., 2011; Bass et al., 2012). This approach has several drawbacks, as the microarray design strongly relies on the transcriptome assembly which varies depending on the assembler used (Kumar and Blaxter, 2010; Mundry et al., 2012) and which can result in modified sequences due to the partial assembly of NGS reads (Miller et al., 2011), the insertion of bases to fill gaps and the merging of NGS reads that do not belong to the same transcript. Moreover, as there is no biological confirmation of the obtained NGS reads in this approach, probes can be developed against sequences that do not target the intended organism, as they are the result of sequencing errors (Gilles et al., 2011) and/or contamination (Longo et al., 2011; Schmieder and Edwards, 2011). The eventual
microarray design will therefore include many probes that recognize sequences not present in the target organism. Finally, as this approach relies solely on in-silico methods, a fraction of the probes may also perform badly in actual microarray experiments.

Given the above discussed drawbacks, the aim of the present study was to establish an advanced approach for developing transcriptomics resources for non-model organisms, consisting of an annotated transcriptome and a high-quality microarray. To achieve this, we formulated the following strategy (Figure 1):

- **Generate NGS data:** Perform a NGS experiment on a normalized cDNA library obtained from a broad range of biological samples of the non-model organism.

- **Design probe library:** Design up to one million probes targeting all original NGS reads, as well as, expressed sequence tags (ESTs) of related species.

- **Assemble the NGS reads.** Assemble the NGS reads into a transcriptome for downstream probe selection and functional annotation.

- **Select probes with targets in the genome:** Conduct an array-based comparative genomic hybridization (aCGH) experiment with a probe-selection microarray that contains all the designed probes, to select probes that hybridize well with the genomic DNA (gDNA) of the non-model organism.

- **Select probes for standard mRNA analysis:** Conduct an array-based gene-expression (aGE) experiment with the probe-selection microarray to further select probes according to their 3’ location in mRNA, their signal-intensity and their ability to uniquely interrogate the assembled transcripts. The final probe library targets all non-model organism transcripts that can be uniquely targeted while keeping the number of probes to a minimum.

- **Finalize transcriptomics resources:** Functionally annotate all transcripts using the annotation tool Blast2GO® (Götz et al., 2008) and determine which transcripts are targeted by the high-quality GE microarray.

As microarrays are gaining importance in ecotoxicology (Lettieri, 2006; van Straalen and Feder, 2012), we applied this approach to a non-model organism commonly used in ecotoxicology for which till date no microarray has been developed and for which very limited sequence data can be found in public repositories, even though the transcriptome of this non-model species has recently been published (Nair et al., 2011). The non-biting midge *Chironomus riparius* (Insecta: Diptera) is a member of the Chironomidae family, which are the most widely distributed and often most abundant insects in freshwater ecosystems (Armitage et al., 1995). Consequently, chironomids are routinely used to evaluate and monitor the biological quality of rivers and lakes (Armitage et al., 1983; Gabriels et al., 2010). Their larvae settle in the sediment, where they remain until they emerge as adults. The short life-cycle and ease of rearing have also made *C. riparius* a
commonly used species in sediment ecotoxicology (León Paumen et al., 2008a; Marinković et al., 2011) with currently four standardized OECD guidelines being available for acute and chronic toxicity tests (OECD, 2012). Assessing effects on life cycle endpoints has proven to be an effective method for deriving effect concentrations for environmental risk assessment (Traas and van Leeuwen, 2007). However, life cycle effects are not supported by a mechanistic insight in the toxicants mode of action nor the physiological changes that occur in toxicant-exposed midges. Studies that measure effects at lower levels of biological organization, such as the transcriptome, are therefore required (Steiner et al., 2004; Swain et al., 2010).

Figure 1: Strategy to obtain non-model organism transcriptomics resources. NGS: Next-generation sequencing; ESTs: Expressed Sequence Tags; aCGH: array-based Comparative Genomic Hybridization; GE: Gene Expression; GO: Gene Ontology; EC: Enzyme Commission numbers. * adapted from http://extension.missouri.edu/explorepdf/agguides/pests/g07402.pdf.
Chapter 5

Results and Discussion

Generation of transcriptome next-generation sequencing data

Non-model organisms have no or limited genomics data. Due to the size and complexity of the genome, sequencing the transcriptome is a practical alternative to obtain genomics data for such species (Ekblom and Galindo, 2011). The obvious drawback is that only sequences from genes that are expressed in the sequenced samples will become available. Therefore broad sampling, as well as normalization of the pooled sample, i.e. reducing the frequency of highly abundant transcripts, is highly recommended (Figure 1). For *C. riparius* we included all life cycle stages, i.e. egg ropes, all four larval stages, pupae and male and female adults. Considering *C. riparius* use in sediment ecotoxicology (León Paumen et al., 2008a; Marinković et al., 2011), we also included larvae exposed to different concentrations of several model toxicants (Table 1, extended version in Supporting Table S1). These specimens were used to synthesize a normalised cDNA library that yielded 1,549,146 NGS reads with a total length of 500,673,325 bp which is in line with the specification of the 454 NGS platform and which is more than ten times the amount of reads and base pairs previously reported by Nair et al. (2011) who obtained, respectively, 138,091 NGS reads and 49,774,676 bp by pyrosequencing toxicant-exposed 4th instar larvae.

Table 1: *C. riparius* sample list summary.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Pre-exposed</th>
<th>Exposed</th>
<th>Time/ Dose range</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg ropes</td>
<td>n.a.</td>
<td>n.a.</td>
<td>&lt;1h - 72h post laying</td>
<td>16</td>
</tr>
<tr>
<td>Larvae (instar I-IV)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>&lt;1 day - 14 days post hatching</td>
<td>16</td>
</tr>
<tr>
<td>Pupae</td>
<td>n.a.</td>
<td>n.a.</td>
<td>14-16 days post hatching</td>
<td>4</td>
</tr>
<tr>
<td>Adult males</td>
<td>n.a.</td>
<td>n.a.</td>
<td>&lt;1h-60h post emerging</td>
<td>16</td>
</tr>
<tr>
<td>Adults females</td>
<td>n.a.</td>
<td>n.a.</td>
<td>&lt;1h-60h post emerging</td>
<td>16</td>
</tr>
<tr>
<td>Larvae</td>
<td>n.a.</td>
<td>Cadmium</td>
<td>0.5-4.0 mg Cd/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>n.a.</td>
<td>Copper</td>
<td>10 - 40 mg Cu/ k/ dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>n.a.</td>
<td>Tributyltin</td>
<td>0.5- 3.0 mg Sn/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>n.a.</td>
<td>Phenanthrene</td>
<td>50 - 400 mg Phe/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>Cadmium</td>
<td>Cadmium</td>
<td>0.5 - 4.0 mg Cd/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>Copper</td>
<td>Copper</td>
<td>10 - 40 mg Cu/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>Tributyltin</td>
<td>Tributyltin</td>
<td>0.5- 3.0 mg Sn/ kg dw</td>
<td>4</td>
</tr>
<tr>
<td>Larvae</td>
<td>Phenanthrene</td>
<td>Phenanthrene</td>
<td>50 - 400 mg Phe/ kg dw</td>
<td>4</td>
</tr>
</tbody>
</table>

* n.a.: not applicable.
**Generation of microarray probe library**

In our microarray approach, we started by designing a huge probe-library directly on all adapter trimmed NGS reads that were longer than 60 bp and that did not contain unknown bases in their sequence, using the in-house developed NGS array designer (http://mad1.science.uva.nl/projects/NGSdesigner). Designing probes against NGS reads, instead of the assembled contigs/ isotigs ensured that the designed probes did not target sequences that might have been modified during the assembly process. The probe-library was subsequently extended with probes designed against publically available ESTs of closely related species. These probes could be an enrichment as they might target conserved sequences that were not present in the sequenced sample. For *C. riparius*, this resulted, after testing for cross-hybridization, in 919,821 probes. 843,837 NGS read designed probes targeted almost all NGS reads, while 75,984 probes were designed against ESTs belonging to the genus Chironomus and the closely related dipteran species *Anopheles gambiae, Anopheles darlingi, Anopheles funestus, Aedes aegypti* and *Culex quinquefasciatus*.

**Assembly of transcriptome NGS data**

For the downstream probe selection procedure, as well as the annotation of the non-model species transcriptome, the NGS reads had to be assembled. For *C. riparius* we used Newbler (v2.5.3.) which is a de-facto standard assembler for NGS reads generated by pyrosequencing (Martin and Wang, 2011) and which has been shown to perform best assembling such NGS reads de novo into a transcriptome (Kumar and Blaxter, 2010). An overview of sequencing and assembly statistics is given in Table 2. From the ~1,5 million trimmed NGS reads, 87.2% was fully or partially assembled, 8.8% could not be assembled and was labelled singleton, while the remaining 4.1% was discarded as the NGS reads did not meet the required quality standards. While the large number of singletons undoubtedly contained fragments of rare transcripts (Meyer et al., 2009; Ewen-Campen et al., 2011), we suspected that a substantial portion was the result of NGS errors, artefacts of cDNA library preparation and/or contaminants from other sources. Especially the latter option seems plausible, since entire larvae including their gut flora were used for the cDNA library preparation. To keep track of possible differences between the two transcript sets, we kept the isotigs and singletons separated during our entire study.

Since the Newbler assembler takes alternative splicing into account, the *C. riparius* transcriptome assembly consisted of ~27k contigs (“exons”) that were incorporated into ~23k isotigs (“transcripts”, average length 1,369 bp), which in turn were grouped into ~18k isogroups (“genes”). 66.8% of the isotigs consisted of a single contig, while the others contained up to 13 contigs. Isotigs that shared contigs were grouped together into isogroups, which resulted in 85.8% with one, 10.5% with two, and 3.7% with three or more
isotigs. Correcting for 26 contigs that were not translated by the Newbler assembler into isotigs, we defined the transcriptome as the total of 23,709 isotigs and 135,082 singletons.

Table 2: *C. riparius* transcriptome sequencing and assembly statistics.

<table>
<thead>
<tr>
<th>Category</th>
<th>Sequences</th>
<th>Base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequencing statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw NGS reads</td>
<td>1,549,146</td>
<td>500,673,325</td>
</tr>
<tr>
<td>NGS reads ¹</td>
<td>1,540,849</td>
<td>459,548,838</td>
</tr>
<tr>
<td>NGS reads N50 ²</td>
<td>-</td>
<td>347</td>
</tr>
<tr>
<td><strong>Assembly statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assembled NGS reads</td>
<td>1,342,920</td>
<td>409,774,142</td>
</tr>
<tr>
<td>Discarded NGS reads ³</td>
<td>63,401</td>
<td>10,253,972</td>
</tr>
<tr>
<td>Singletons</td>
<td>135,082</td>
<td>39,520,724</td>
</tr>
<tr>
<td>Singleton N50 ²</td>
<td>-</td>
<td>343</td>
</tr>
<tr>
<td>Contigs (&quot;exons&quot;)</td>
<td>27,334</td>
<td>21,898,252</td>
</tr>
<tr>
<td>Contig N50 ²</td>
<td>-</td>
<td>1,161</td>
</tr>
<tr>
<td>Mean # NGS reads/ contig</td>
<td>59.0</td>
<td>-</td>
</tr>
<tr>
<td>Isotigs (&quot;transcripts&quot;)</td>
<td>23,683</td>
<td>32,429,684</td>
</tr>
<tr>
<td>Isotig N50 ²</td>
<td>-</td>
<td>1,886</td>
</tr>
<tr>
<td>Mean # contigs/ isotig</td>
<td>1.9</td>
<td>-</td>
</tr>
<tr>
<td>Isogroups (&quot;genes&quot;)</td>
<td>18,514</td>
<td>-</td>
</tr>
<tr>
<td>Mean # isotigs/ isogroup</td>
<td>1.3</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ after trimming of adaptor sequences; ² N50 is a weighted median, such that half the bases are contained in sequences equal to or larger than the N50 length; ³ NGS reads that were discarded during the assembly process because they were too short (< 50 bp), contained repeats or were marked as outliers by the Newbler assembler.

**aCGH experiment to select relevant microarray probes using gDNA**

To select the most reliable and biological relevant probes from the previously designed probe-library, a probe-selection microarray was developed that contained the probe-library and ample negative-control probes that did not recognize any sequence in GenBank. Performing an aCGH experiment allowed subsequently the identification of probes that hybridized with the gDNA of the non-model organism. Assuming a limited chance on random homology, the aCGH experiment was expected to considerably clean-up the NGS data, eliminating NGS reads that originated from contamination or technological errors, while simultaneously selecting relevant probes for the final non-model species GE microarray. It is recognized, that his procedure would also select against probes targeting
exon spanning sequences, however, due to the large number of probes this was not considered a problem.

For *C. riparius*, we developed a 1M probe-selection microarray that contained the 919,821 probes from the probe-library and 40,000 negative control probes. This probe-selection microarray was used in an aCGH experiment to analyse the gDNA of *C. riparius* as well as the gDNA of *A. gambiae* that served as a positive control. It turned out that there was a correlation between the GC-content of the negative control probes and their signal-intensity, i.e. above a GC-content of 45% a steady increase in signal intensity was observed (Supporting Figure S1). This prompted us to limit the CG-content for all probes to a maximum of 50%, what resulted in 816,270 *C. riparius* NGS-read probes, 42,636 dipteran-specific probes, and 19,000 negative-control probes.

As expected, the NGS-read probes showed stronger signal intensities after hybridisation with *C. riparius* than *A. gambiae* gDNA (Figure 2a). The opposite was true for the probes designed against *A. gambiae* ESTs. In fact, the *A. gambiae* aCGH signal-intensities of the NGS-read probes were in the range of that of the negative controls, indicating a substantial genomic difference between the malaria mosquito *A. gambiae* and the non-biting midge *C. riparius*. To select probes that hybridized well to the *C. riparius* gDNA, we compared for all probes the log₂-ratio (*C. riparius*/*A. gambiae*) of the aGCH signal intensities with the summed log₂ (*C. riparius* x *A. gambiae*) signal intensities in a MA-plot (Figure 2b). The negative- and positive (*A. gambiae*) control probes behaved as expected, both with low *C. riparius* aGCH signal intensities and only for the positive control probes high signal intensities for the *A. gambiae* gDNA. The distributions of the probes designed against the other dipteran species, with the exception of the Chironomus spp, corresponded to the pattern observed for the *A. gambiae* designed probes (Supporting Figure S2).

Based on the distributions of the control probes (Figure 2b), we defined signal-intensity parameters that allowed a conservative selection of *C. riparius* gDNA specific probes: parameter I was a *C. riparius* log₂ signal of 10, separating probes with a strong signal when hybridized with *C. riparius* gDNA; parameter II was a *C. riparius* log₂ signal of 8, separating probes with an intermediate signal when hybridized with *C. riparius* gDNA; and parameter III was a log₂ *C. riparius*/*A. gambiae* signal ratio of 1, separating probes with a higher signal to *C. riparius* than to *A. gambiae* gDNA. Using these parameters we defined categories to select probes: Category A contained probes above parameter I and III, which are probes that gave a strong and specific *C. riparius* gDNA signal (217,878); Category B contained probes between parameter I and II and above III, which are probes that gave an intermediate and specific *C. riparius* gDNA signal (206,608); and Category C contained probes above I and below III, which are probes that gave a strong and non-specific *C. riparius* gDNA signal (42,379). These categories contained a total of 466,865 reliable probes. The validity of the selection parameters was confirmed by applying the same signal
Figure 2: Array-based comparative genomic hybridization (aCGH) experiment. (A) Box-and-whisker plot summarizing the obtained log2 signal intensity distributions for the indicated probes collections, with the light grey boxes representing the *C. riparius* aCGH signal and the dark grey the aCGH *A. gambiae* signal. (B) MA-plot of the aCGH data. The dots with the different shades of grey represent the entire probe-library. The defined signal-intensity parameters are indicated by the dashed blue line and the captions I, II, III. The categories containing the selected probes are indicated by different shades of grey and the letters A, B and C. The red dots are the neg.control and the green dots the pos.control (*A. gambiae* EST) probes.

Figure 3: Array-based gene expression (aGE) experiment. (A) Schematic representation of the two mRNA linear amplification protocols. The coloured bar represents the mRNA with the 3' polyA tail indicated by the stretch of A's. The arrows represent the amplified cDNA products obtained for the regular and the modified procedure, with the length of the arrows indicating the length of the synthesized cDNA's. (B) MA-plot of the aGE data. The light grey dots represent all aCGH selected probes. The three coloured regions are expected to contain probes targeting transcripts at the 3' side (blue), probes targeting the middle of the transcripts (red) and probes targeting the 5'side as well as probes with no target transcripts (green). (C) Density plot where the relative position of the three probe populations on the isotigs is demonstrated. The colours of the lines correspond to the colours used in panels A and B. The black line represents a random selection of probes that covers, as expected, the isotigs evenly over the entire length.
intensity parameters to the negative and positive control probes, what resulted in the selection of only 2.7% of the negative control probes (Figure 2b) and 86.6% of the positive control probes, with 2,860, 1,428 and 165 positive control probes in the categories A, B, and C, respectively.

Hence, with this approach we were able to reduce our initial pool of probes with 49.6%. However, despite this reduction in probes, still 98.2% isotigs and 60.2% singletons were targeted. This effectively means that the aCGH experiment selected against bad-performing probes, rather than bad NGS reads. A bad-performing probe can be caused by low affinity to the target due to small NGS errors, exon spanning target sequences, reads belonging to other species and sequence-specific microarray technology anomalies.

**aGE experiment to further select 3’ located probes using mRNA**

After the DNA check by the aGCH experiment, we used mRNA derived from the non-model organism in the probe-selection procedure. Given the relative small average size of the isotigs and the many singletons, it was fair to assume that many transcripts in our example represented incomplete mRNA sequences, something that will often be the case in de novo assembled non-model organisms transcriptomes. As the standard aGE protocol uses linearly amplified cDNA, i.e. the mRNA is amplified using oligo-dT primers from the 3’-side, only 3’ located probes will be useful for the final aGE microarray. Identifying probes against the most 3’ located target sequences in the mRNA was possible by combining a standard linear amplification protocol that yielded 3’-end biased labelled material, with a modified linear amplification protocol that yielded highly 3’ restricted material due to the incorporation of dideoxynucleotides during the cDNA synthesizing step (Figure 3a). For this experiment, the same probe-selection microarray was used as in the aCGH experiment.

For *C. riparius*, two aGE samples were synthesized from the same mRNA pool that was pyrosequenced, using the regular and modified linear amplification protocols (Figure 3a). Comparing the two samples on the 1M probe-selection microarray resulted in three populations of probes. First, probes that showed a good intensity signal in both procedures: these probes recognized targets located 3’ in mRNA that was expressed at a high enough level to be detected by microarray technology. Second, probes that performed well in the regular protocol, but not in the 3’ restricted protocol: these probes recognized targets located more 5’ in mRNA that was expressed at a sufficient level. Third, probes that performed badly in both protocols: these probes recognized targets either located too much 5’ in mRNA to be amplified by either protocol, targets from genes that were expressed below the detection level of microarray technology, or targets from genes that were not present in the tested transcriptome, i.e. NGS contamination or erroneous sequences. This approach worked quite well and revealed that the anticipated three probe populations could
be identified (Figure 3b). To demonstrate that these populations indeed represented 5’, middle and 3’ located mRNA target sequences, we determined the relative position of their targets on the assembled isotigs (Figure 3c). Compared to a set of randomly chosen probes, the distributions showed a strong preference for the expected location. Hence, there were likely probes targeting 5’, middle and 3’ located sequences for each mRNA in this NGS data set. Since the different probe populations could not clearly be separated, the aGE data was used to rank the probes based on their signal strengths and thus the target location on the NGS-defined transcripts.

To achieve our goal and design a cost-effective 3’-primed gene-expression microarray, we aimed to keep the number of reliable and biologically relevant probes to a minimum, while making sure that as many as possible transcripts were uniquely targeted. For this, a selection algorithm was defined that was executed for both isotig and singleton sets. This algorithm started by categorizing the probes for isotigs using BLASTN: probes were defined having good matches to isotigs, if the bit score was >80. Probes with no good match to any isotig were discarded. Then, probes were selected only if they were unique to one isogroup. Before the final selection, probes were evaluated to contain only stretches of 7 identical nucleotides or less, as well as 5 subsequent di-nucleotides or less. The selected probes were ranked according to their aGE signal intensity. Starting with the highest-ranked probe, probes were selected for each isogroup until all isotigs within each isogroup were targeted by at least one probe. The same algorithm was then applied to the singleton set.

Applying this algorithm to our *C. riparius* aGE data, we obtained a final probe library that consisted of 59,409 validated probes uniquely targeting 22,507 isotigs, (corresponding to 17,403 isogroups) and 23,782 singletons.

**Finalization of the transcriptomics resources**

To allow down-stream analyses and interpretation of gene-expression studies, functional annotation of the transcriptome was needed. Functional annotation of the isotigs and singletons was performed independently, starting with a BLASTX search of both sets against the GenBank non-redundant (nr) protein database followed by a Blast2GO® analyses identifying relevant Gene Ontology (GO) terms (Ashburner et al., 2000) and unique enzyme commission (EC) numbers.

For *C. riparius*, we used a lenient BLASTX e-value threshold of 1*e⁻³ and found matches to homologous proteins for 71.0% of the isotigs and 17.9% of the singletons (Supporting Table S2). Screening those BLASTX hits for identical protein accessions, showed that 50.0% of all isotigs and 8.9% of the singletons corresponded to unique protein accessions numbers. From the BLASTX results we identified a total of 22,593 unique protein accessions of which only 6% were found in both transcript sets. The fact that the isotigs and singletons almost equally contributed to this total set of unique protein
accessions indicated that the assembly was exhaustive and thus successful, as well as that biologically relevant information was indeed present in the singletons. The number of unique protein accessions obtained in the present study is higher than the 9,512 unique protein accessions previously reported by Nair et al. (2011), most probably because they pyrosequenced less deep and because their biological sample was less diverse as it merely

Figure 4: Taxonomic distribution of the best BLASTX hits matching *C. riparius* transcripts. Distribution of the best BLASTX hits that were matched to the isotigs (black) and the singletons (light grey) according to their taxonomic origin. (A) All transcripts (isotigs n=16,824; singletons n=24,129) that were matched to a BLASTX hit. (B) Transcripts (isotigs n=16,537; singletons n=4,7539) that were matched to a BLASTX hit and that are targeted by the final aGE microarray.
consisted of 4th instar *C. riparius* larvae. Since the genome sequence of *C. riparius* is not elucidated we cannot determine the exact coverage of the transcriptome. However, considering that the sequenced genomes of the closely related mosquitos *A. aegypti*, *C. quinquefasciatus* and *A. gambiae* are predicted to have 16,789, 18,883, respectively, 13,133 transcripts (Severson and Behura, 2012), it seems likely that we covered a substantial part of the *C. riparius* transcriptome.

Distributing all the best BLASTX hits over taxonomic groups (Figure 4a), showed, as expected, that the far majority (92.0%) of isotig-matched proteins belonged to known insect proteins, and especially to the dipterans *A. aegypti* (29.1%), *C. quinquefasciatus* (18.2%) and *A. gambiae* (12.9%). This is in concordance with the distribution reported by Nair et al.

Figure 5: Gene Ontology (GO) terms obtained for *C. riparius* transcripts. The data represents the distribution of the annotated isotigs (black) and the annotated singletons (light grey) over the various level-2 GO terms. Each bar represent the total number of annotated transcripts associated with the specified level-2 GO term as a percentage of the total number of annotated transcripts belonging to the higher-ranked GO category, i.e. cellular component (isotigs n=6,380; singletons n= 9,277), molecular function (isotigs n=10,663; singletons n= 11,359) and biological process (isotigs n=6,249; singletons n= 7,343).
(2011). For the singleton-matched proteins the distribution was different in that 49.8% related to insect proteins and 45.0% to other eukaryotic species. Only small fractions of the isotigs and singletons matched to prokaryote proteins (0.7% and 3.9%), which indicates that the chironomid gut flora did not substantially contaminate the NGS data. Since 0.7% of the isotigs and 21.4% of the singletons matched human proteins, we performed a BLASTN (nucleotide) search against the human genome and transcriptome data available at NCBI, to further estimate the potential human contamination of the NGS data. We detected that 3.5% of NGS reads showed a strong similarity to human sequences over the entire read length and that 94.0% of these ‘human’ NGS reads remained unassembled, i.e. became singletons. As these sequences could also represent conserved genomic sequences, we chose not to remove them from our NGS data.

Assigning functional categories to the C. riparius transcriptome, we were able to annotate 11,895 (50.2%) isotigs and 12,662 (9.4%) singletons with ~55k and ~61k GO terms, respectively (Table 3 and Supporting Table S3). The distribution over the various GO terms was remarkably similar for the isotigs and singletons (Figure 5) and may suggest that the observed patterns at least partially depended on the abundance of certain GO-terms. To visualize the interaction of the annotated transcripts, we assigned with Blast2GO®, 688 unique EC numbers to 2,973 isotigs and 3,611 singletons (Table 3 and Supporting Table S3) and found 126 pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al., 1999).

Table 3: C. riparius transcriptome annotation summary.

<table>
<thead>
<tr>
<th>Category</th>
<th>Isotigs</th>
<th>Singletons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of transcripts</td>
<td>23,709</td>
<td>135,082</td>
</tr>
<tr>
<td>Transcripts with BLASTX match</td>
<td>16,824</td>
<td>24,129</td>
</tr>
<tr>
<td>Transcripts assigned GO terms</td>
<td>14,290</td>
<td>17,698</td>
</tr>
<tr>
<td>Annotated transcripts</td>
<td>11,895</td>
<td>12,662</td>
</tr>
<tr>
<td>• with GO terms for biological processes (# GO terms)</td>
<td>(25,689)</td>
<td>(23,976)</td>
</tr>
<tr>
<td>• with GO terms for molecular functions (# GO terms)</td>
<td>10,663</td>
<td>11,359</td>
</tr>
<tr>
<td>• with GO terms for cellular components (# GO terms)</td>
<td>(19,443)</td>
<td>(23,304)</td>
</tr>
<tr>
<td>Transcripts with Enzyme Codes</td>
<td>2,973</td>
<td>3,611</td>
</tr>
</tbody>
</table>
The final GE microarray (Gene-Expression Omnibus accession numbers GPL15611) targeted 22,507 isotigs and 23,782 singletons. Of these targeted transcripts, 73.5% isotigs and 20.0% singletons had a BLASTX hit, of which 71.4% and 88.3% matched to a unique protein accessions. Importantly, our selected probes targeted 94.9% of the isotigs and 17.6% of the singletons, which covered 98.6% and 34.9% of the unique protein accessions, respectively. The taxonomic distribution of the targeted isotigs was almost the same of that of the entire set of isotigs with 92.7% matching insect proteins. However, for the singletons the percentage that matched insect proteins increased substantially from 49.8% to 82.2% (Figure 4b). As aimed for, the percentage of ‘human contamination’ was substantially reduced in both transcript sets.

Thus by combining NGS and microarray technology we succeeded in designing an annotated high-quality microarray suited for whole-transcriptome gene-expression analysis in a non-model organism. For C. riparius, we selected from a 925k probe library 59k highly-reliable probes that have been proven to perform well in both aCGH and aGE experiments. While we designed probes directly on the NGS reads, our approach is also suitable for validating probe libraries designed against assembled transcripts. Concluding, we now have valuable C. riparius transcriptomics resources, i.e. an annotated transcriptome and a 135K 3’-primed gene-expression microarray, that can advance ecotoxicity testing with C. riparius as whole-transcriptome gene-expression analysis are now possible with this species.

Materials and Methods

Test organism, culturing conditions and sample selection

The Chironomus riparius specimens originated from the University of Amsterdam’s in-house laboratory culture and were maintained on artificial sediment at 20 ± 1 °C, 65% humidity and a 16: 8 h light: dark photoperiod (León Paumen et al., 2008a; Marinković et al., 2011). The genetic fidelity of this C. riparius laboratory culture was previously confirmed by mitochondrial cytochrome oxidase I (COI) gene sequencing (Boonstra et al., 2009) The sample list, including all life cycle stages and toxicant-exposed larvae, is provided in Supporting Table S1. Each sample was immediately snap frozen in liquid nitrogen and stored at -80 °C until processing

RNA isolation, cDNA library construction and next-generation sequencing

The frozen samples were pooled and homogenization in liquid nitrogen. Total RNA was extracted using the RNeasy Mini kit (Qiagen) with an on-column DNase (Qiagen) digestion to remove traces of genomic DNA. RNA integrity was examined using a RNA 6000 Nano
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chip on a 2100 Bioanalyzer (Agilent Technologies), while the RNA yield was determined on a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific). An aliquot of the total RNA sample was sent to GATC Biotech (Konstanz, Germany) where a normalized cDNA library was prepared and sequenced using Titanium chemistry on a GS FLX Instrument (Roche Diagnostics) according to manufacturer’s protocol. Detailed information is presented in Supporting Appendix S1.

De novo transcriptome assembly and functional annotation

NGS reads were, after removal of adapter sequences, assembled with Newbler v2.5.3. (Roche) in the de novo mode using default assembly parameters. The obtained isogroups and isotigs, as well as, the remaining singletons were renamed in the format of “CripIG000001”, “CripIT000001”, “CripSI000001” with “Crip” standing for C. riparius, “IG” for isogroup, “IT” for isotig, “SI” for singleton, and “000001” for an arbitrary assigned number. The C. riparius transcripts, i.e. isotigs and singletons, were functionally annotated using the Blast2GO® suite (Conesa et al., 2005; Götz et al., 2008). Detailed information of the functional annotation procedure is presented in Supporting Appendix S1.

Array-based comparative genomic hybridization (aCGH)

The designed 1x1M probe-selection microarray was obtained from Agilent Technologies and hybridized with C. riparius and A. gambiae genomic DNA (gDNA). gDNA was extracted from 30 pooled fertilized C. riparius egg ropes, respectively, 30 pooled unfed A. gambiae adults. To keep contamination of the C. riparius gDNA sample to an absolute minimum the midges were allowed to deposit the egg ropes in petri dishes filled with clean water. Egg ropes not older than 30 minutes were collected, stringently rinsed with clean water and immediately flash frozen in liquid nitrogen. After pooling, gDNA was extracted using a CTAB DNA extraction method that included a RNAse A (Sigma-Aldrich) digestion step for removal of residual RNA (Brunner et al., 2010). gDNA quality and quantity were determined with gel electrophoresis (0.5% agarose in TAE buffer) and NanoDrop ND-1000 measurements. 200 ng DNA was amplified and labelled by strand displacement amplification. Concentrations of amplified products were measured on the NanoDrop ND-1000 and qualified on the BioAnalyzer with the DNA 1000 Kit (Agilent Technologies). Yield and CyDye incorporation of the final labelled products were measured with the NanoDrop ND-1000 in the Microarray Measurement Mode. The 1M probe-selection microarray was subsequently hybridized with 10 µg of Cy3 labelled C. riparius gDNA and 10 µg Cy5 labelled A. gambiae gDNA according to the Oligonucleotide Array-Based CGH for Genomic DNA Analysis manual (Agilent Technologies version 6.3. The microarray was scanned in an ozone-free room on an Agilent G2505CA scanner at 3 µm resolution and the data was extracted with Feature Extraction version 10.7.3.1 (Protocol CGH_107_Sep09). The log₂ transformed median signals were
analysed in R (www.r-project.org). Detailed information is presented in Supporting Appendix S1.

**Array-based gene-expression (aGE)**

The pooled *C. riparius* RNA sample, of which an aliquot was pyrosequenced, was used to identify probes corresponding to the 3’-end of *C. riparius* transcripts. 200 ng RNA was taken as input for both a regular, as well as, a modified linear RNA amplification. The regular RNA amplification was conducted with the Agilent Low RNA Input Linear Amplification Kit (Agilent Technologies) according to manufacturer’s recommendations. The modified reaction was conducted using the same kit, however, the first step where the mRNA is primed with an oligo (d)T-T7 primer and converted into double-stranded cDNA with the M-MLV reverse transcriptase, was modified by the addition of dideoxynucleotides (ddNTP) in the deoxynucleotide mix. This modification was expected to yield highly 3’-biased cDNA as incorporation of a ddNTP would prematurely terminate cDNA elongation. The modified amplification procedure is described in detail in Supporting Appendix S1. Amplified RNA was checked for quality and quantity using Bioanalyzer and NanoDrop measurements. Yield and CyDye incorporation of the final labelled products were measured with the NanoDrop ND-1000 in the Microarray Measurement Mode. The 1M probe-selection microarray was subsequently hybridized with 2.5 µg Cy3 labelled regularly amplified RNA and 2.5 µg Cy5 labelled alternatively amplified RNA according to the Two-Colour Microarray-Based Gene-Expression Analysis manual (Agilent Technologies version 6.5). The microarray was scanned in an ozone-free room on an Agilent G2505CA scanner at 3 µm resolution. The data was extracted with Feature Extraction version 10.7.3.1 (Protocol GE2_107_Sep09). The log₂ transformed median signals were analysed in R.

**Data deposition**

The *C. riparius* NGS sequence reads were submitted to NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession number SRX147945. The assembled *C. riparius* isotigs have been submitted to NCBI Transcriptome Shotgun Assembly Sequence Database (www.ncbi.nlm.nih.gov/genbank/tsa) and can be accessed through the GenBank accession numbers KA174710-KA198345. Complete raw microarray data and their MIAME compliant metadata have been deposited at NCBI Gene-Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession numbers GPL15610 (1M microarray) and GPL15611 (135K microarray).

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Chapter 6

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

Chapter 6

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 phenantrene, 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$_2$O, copper standard, Fluka): 10, 20, 30, and
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40 mg Cu/kg dw; cadmium (CdCl₂, 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 NH₄⁺/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 tributyltin 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 (Sigele 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
Gene expression patterns and life cycle responses

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.](image-url)
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.

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*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.
Chapter 7

Concluding remarks
In the General Introduction I referred to the daring statement of the US National Research Council that future toxicity testing will evaluate biologically significant perturbations in key molecular pathways instead of measuring toxic effects on life cycle parameters of exposed organisms (NRC, 2007; Villeneuve and Garcia-Reyero, 2011). The present thesis indeed showed that life cycle effects in the model species *Chironomus riparius* are reflected by molecular stress responses. Nevertheless, the results obtained in this thesis also pointed to the importance of other factors that play pivotal roles in the expression of toxicity, i.e. the mode of action of the tested compounds and the exposure time. Furthermore methodical issues need to be reviewed. Below I will discuss the main findings of this thesis in relation to the claim that transcriptomic responses can revolutionize environmental risk assessment.

**Toxicants mode of action**

Organisms exposed to compounds with different modes of action are generally differently affected. The present thesis showed that the three compounds that act via specific modes of action, i.e. the essential metal copper (Gaetke and Chow, 2003), the non-essential metal cadmium (Ercal et al., 2001) and the organometal tributyltin (Hahn and Schulz, 2002), affected both the life cycle and molecular endpoints in a dose-responsive manner. Moreover, genes related to the specific modes of actions of the compounds were only differently expressed upon exposure to the corresponding toxicants. The polycyclic aromatic compound phenanthrene that acts via a non-specific baseline toxicity known as narcosis (Bleeker et al., 2002), on the other hand affected only survival in a dose response manner. The sublethal endpoints larval growth (Chapter 6) and emergence (Chapter 3) were hardly affected with increasing test concentrations, which is in concordance with published accounts (León Paumen et al., 2008a). The present thesis showed for the first time that gene expression in *C. riparius* is also not affected in a dose dependent manner by phenanthrene, since the total number of differentially expressed transcripts did not increase consistently with increasing test concentrations. In fact, a large fraction of the differentially expressed transcripts had already reached their maximal expression at the lowest tested phenanthrene concentration. Thus the response to phenanthrene appeared to be ‘all or nothing’, both on the molecular and on the population level.

Chapter 6 reported compound specific changes in the transcriptome. For the metals copper and cadmium transcripts that encode metallothionein were upregulated, while for the endocrine disruptor tributyltin the expression of transcripts encoding enzymes involved in the endocrine system and insect metamorphosis (e.g. steroid dehydrogenase and juvenile hormone acid methyltransferase) was modified (Shinoda and Itoyama, 2003). For phenanthrene transcripts were identified that are involved in the xenobiotic detoxification (e.g. cytochrome p-450 and epoxide hydrolases). The transcriptomics study (Chapter 6) also showed that for all compounds the expression of transcripts involved in the oxidative
stress response (e.g. glutathione s-transferase) was modified, confirming that all these compounds ultimately cause oxidative stress. Besides the transcripts that could be linked to specific modes of action, we also found transcripts that are part of general stress responses, such as heat shock proteins and various DNA repair systems. It should be noted that a large fraction of the differentially expressed transcripts (ca. 40%) had no known function and could thus not be interpreted, while a substantial fraction of the transcripts that showed homology to known sequences and that could be assigned a hypothetical function were often matched to other hypothetical proteins what makes their annotation doubtful. Indeed, genetically well identified species are less bothered by this issue (e.g. Harris et al., 2010; McQuilton et al., 2012). Nevertheless it is now proven that analysing molecular stress responses even in a ‘non-model species’ is suitable to provide clues to the mode of action of toxicants even though further confirmation of the molecular processes is required.

While this thesis did not focus on compound specific transcriptomic fingerprints, which is certainly a possibility for future research (Yang et al., 2001; Hamadeh et al., 2002), the presently observed compound specific gene expression patterns allowed a modern interpretation of the acute-to-chronic ratio. Chapter 3 allowed grouping of compounds based on the known modes of action conforming earlier studies by Länge et al. (1998), Roex et al. (2000) and Ahlers et al. (2006). However, there was a high variability in acute-to-chronic ratios in the latter studies making the relationships between acute-to-chronic ratios and mode of action less straightforward than required for environmental risk assessment. This thesis showed that the variation could be reduced by calculating a lethal/sublethal ratio that is merely based on chronic data, allowing the grouping of compounds based on life cycle effects to be more reliable (Chapter 3). This systematics is now confirmed by the different gene expression patterns in midge larvae exposed to the same four test compounds (Chapter 6).

It is concluded that mechanistic information can be obtained on compounds modes of action by studying transcripts that encode proteins involved in specific and general stress responses, reflecting life history effects.

**Exposure time**

In order to exert a toxic effect on biota, compounds must first be taken up and transported to a target site. This sets a lower limit to exposure time and is the source of discrepancies between measuring early molecular stress responses and assessing long-term life cycle effects. Large-scale gene expression studies focused almost exclusively on molecular stress responses that occur shortly after exposure, separately and independently from standardized ecotoxicity tests (e.g. Connon et al., 2008). Changes in gene expression shortly after exposure may lead to the identification of transient processes that are involved in the compensation of cellular side-effects rather than being causally linked to the toxicity
of the compounds (van Straalen and Feder, 2012). Short exposure times may therefore over-evaluate primary responses, while long-time gene expression is potentially susceptible to later stage or general stress responses or even to feed back of life cycle changes on gene expression. That is why I argued that it was necessary to relate impacts observed at life cycle endpoints to changes in gene expression in individual *C. riparius* larvae that had survived toxicity tests. This allowed a direct comparison of the sensitivity of gene expression and life cycle endpoints and moreover, insight was gained in the status of the transcriptome of toxicant-exposed midge larvae at the end of the exposure period when their development had already been affected. The results showed that at the lowest test concentrations where the life cycle endpoints of *C. riparius* were hardly affected, already a substantial number of transcripts was significantly differentially expressed and that they corresponded to both general and toxicant specific stress responses. I therefore conclude that the present thesis certainly underpins the power of molecular tools for measuring toxicant effects, but inevitably the expression of molecular stress responses is as time dependent as whole organism effects.

**Multigeneration effects**

The present thesis addressed time as crucial factor in the expression of toxicity, ultimately focusing on multigeneration exposure where the adaptive responses and evolutionary capacities of *C. riparius* were studied. In previous studies *C. riparius* was reported to develop heritable tolerance to metal exposure (Postma et al., 1995b; Postma and Davids, 1995; Groenendijk et al., 1999). In chapter 4 it was shown that for three compounds, including the metals copper and cadmium, such a selection of less sensitive individuals was not evident based on sensitivity tests performed after 3, 6 and 9 generations of exposure. A marginal and temporal decrease in cadmium sensitivity was observed, but also lost in one generation, what led to the conclusion that phenotypic plasticity in *C. riparius* was prominent with maternal effects potentially being important. Previously, Bonduriansky and Day (2009) showed that non-genetic inheritance can play an important role in transgenerational effects, while Coutellec and Barata (2011) stressed that epigenetic effects deserve attention with regard to ecotoxicity testing as they can affect test species sensitivity. The present study indicates that *C. riparius* is a highly plastic species that will allow investigation of the molecular mechanisms that are altered in multigenerational exposed chironomids using the transcriptomics resources developed in Chapter 5.

**Genomic tools**

In the last two chapters of this thesis it was shown that current molecular biological genomics technologies enable the analysis of a large part of the transcriptome (~30,000 genes) of a species for which prior to this thesis hardly any sequence data was available in public repositories. Less than a decade ago this would be hardly possible and incredibly
expensive, because sequencing technology was limiting and commercial microarrays were only available for a few species with sequenced genomes which were not used in ecotoxicity testing (Neumann and Galvez, 2002). Over the last five years however, new sequencing technologies became available and microarray technology matured allowing cost-effective analysis of transcriptomes from ecologically relevant, but genomics non-model species (Ekblom and Galindo, 2011).

One crucial step in the microarray data analysis is transcriptome assembly. Transcriptome assembly is a complex process that can result in wrongly assembled transcripts, due to errors in the sequenced reads (Gilles et al., 2011), the algorithms used in the assembly process (Kumar and Blaxter, 2010; Miller et al., 2011), the alternative splicing of transcripts, the overlap between homolog genes and the possible contamination with sequences from other species that share similar sequences. To minimalize the effects of erroneous assemblies, I developed in chapter five an approach to improve microarray design. To this purpose a million probes were designed directly on the raw sequence reads. Next, each probe was assessed for its affinity to *C. riparius* genomic DNA and RNA, and the best probes were selected using a custom made selection algorithm so that they covered all transcripts that could be uniquely interrogated while reducing the number of probes to a minimum. This process avoided the inclusion of poor quality probes as for instance observed for Affymetrix arrays (Dannemann et al., 2012). The resulting gene-expression microarray was successfully applied in chapter six for a large-scale gene expression study.

The field of genomics is still rapidly developing, as shown by the Oxford Nanopore press release on February the 17th 2012 where the GridION and MinION were announced (http://www.nanoporetech.com/news/press-releases/view/39). These third generation sequencers will change ecotoxicogenomics research as it will become feasible to obtain entire genomes of test species, excluding the difficulties that are associated with transcriptome assemblies. Therefore I expect that within a decade microarray studies will be replaced as the major tools for quantitative transcriptomics studies. The short sequence reads that can cost-effectively be produced with sequencers such as Illumina pose now difficulties for assembly, but with the availability of reference genomes they will be easily matched to genes. In conclusion, the microarray design approach developed in the present project made the first large scale gene expression study with the ecotoxicological test species *C. riparius* possible and has the potential to facilitate transcriptomics research with any species of interest, as in the near future microarrays will still be the preferred transcriptomics tool.
Perspectives on environmental risk assessment

This thesis showed that toxicity of compounds can be assessed with the non-biting midge *C. riparius* using standard ecotoxicity tests (Chapters 3 and 6), extended multigeneration studies (Chapter 4) and transcriptomics studies (Chapters 5 and 6), and that the relation between life cycle effects and molecular stress responses for compounds with different modes of action were elucidated. It was observed for all four compounds that at sublethal test concentrations where growth was hardly affected, large numbers of genes were differentially expressed. Moreover, the expression of a considerable fraction of these genes reached their maximal expression at the lowest tested concentration. These ‘low dose responsive’ genes point towards the higher sensitivity of molecular endpoints in comparison to life cycle endpoints. This supports the claim that transcriptomics can provide sensitive endpoints (e.g. Lobenhofer et al., 2004; Poynton et al., 2008), but as only a small fraction of the differently expressed genes could be linked to specific and general stress responses, the biological relevance of these genes remains to be revealed. The importance of this latter notion is evident when concepts such as the No Observed Transcriptome Effect Level (NOTEL) (Lobenhofer et al., 2004) are proposed for setting environmental hazard thresholds without knowing if the measured transcriptomic response will result in damage at higher levels of biological organization. The present thesis demonstrated that molecular stress responses observed at the end of toxicity tests enable a better understanding of toxicity mechanisms of compounds with different modes of action, albeit a better identification of the patterns of gene expression is still needed. But the question remains how these findings can be valorised for environmental risk assessment and if future environmental risk assessment will indeed be based on biologically significant perturbations in key toxicity pathways instead of measuring toxic effects on life cycle parameters such as envisioned by the U.S. National Research Council (NRC, 2007) and the SETAC Pellston workgroup (Villeneuve and Garcia-Reyero, 2011).

As with most technological developments also in ecotoxicogenomics, expectations initially exceeded reality (van Aggelen, 2010). This has previously happened with physiological and biochemical biomarkers that were also envisioned to serve as early warning indicators that respond before measurable effects can be detected on life cycle parameters. While there have been success stories, e.g. vitellogenin as a biomarker for exposure to endocrine disruptors (e.g. Tyler et al., 1996; Brian et al., 2005), biomarkers in general have not replaced standard toxicity testing in environmental risk assessment (Forbes et al., 2006). Biomarkers with ecotoxicogenomics as a modern and powerful exponent are still mainly used for initial toxicity screenings, hypothesis testing and gaining insight in mechanisms of toxicity. For ecotoxicogenomics to become an integrated part of environmental risk assessment, molecular stress responses will have to show a certain robustness over different testing conditions and preferably different species, as well as be
related to responses measured at the level of organisms and populations. Considerable effort has been made to meet these requirements: transcriptomics studies with the soil ecotoxicological model organism *Folsomia candida* showed that the composition of the soil has a great impact on gene expression patterns measured in toxicity tests (Nota et al., 2010) and that by characterizing different soil types a normal operating range can be defined, thus accounting for gene expression variation that is due to natural conditions (de Boer et al. 2011). This thesis, together with work conducted by others (e.g. Connon et al., 2008; Craig et al., 2009; Beggel et al., 2011) related life cycle and molecular responses in an increasing number of species. Considering the great efforts that are invested in the various disciplines of ecotoxicogenomics, and the ever increasing number of compounds that need to be assessed I believe that, while big hurdles will be on the way, ecotoxicogenomics will become an integrated part of environmental hazard and risk assessment where it will complement standardized ecotoxicity testing without replacing it.

**Conclusions**

This thesis advanced on the understanding of stress responses in toxicant exposed chironomids. I introduced a modified acute-to-chronic ratio where chronic LC50 values are divided by chronic LOEC values, reducing variability and allowing a reliable grouping of compounds according to their modes of action. The sequencing of the *C. riparius* transcriptome yielded transcriptomics resources that included an annotated transcriptome and a microarray. The novel approach led to an improved microarray design which is applicable to any species. These transcriptomics resources allowed the first large-scale gene expression study with the ecotoxicological key species *C. riparius*. By analysing the transcriptomes of chironomids that survived standard ecotoxicity tests, it was possible to relate life cycle effects with molecular stress responses, showing that the latter are compound specific and more sensitive. Therefore this thesis underpins the power of molecular tools for measuring toxicant effects, but also points out that expression of molecular stress responses is as time dependent as whole organism effects. A multigeneration study showed that *C. riparius* ability to cope with long term toxicant exposure is mediated by its plasticity and not always through genetic adaptation such as previously thought. Using the newly developed *C. riparius* transcriptomics resources a better insight might be obtained in the molecular alterations that occur in multigenerational exposed chironomids. Taking into consideration the progress made in the present project and in ecotoxicogenomics in general, it is expected that transcriptomics studies will eventually become an integrated part of environmental hazard and risk assessment where it will complement standardized ecotoxicity testing without replacing the established methods.
References


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Summary

Transcriptomics is generally believed to hold the potential to elucidate the molecular mechanisms of toxicity to an unprecedented detail. Yet, to fully exploit transcriptomics in ecotoxicology it is necessary to relate molecular stress responses to the effects observed on life cycle endpoints. While initially large-scale gene expression studies were only feasible for species with sequenced genomes, advances in molecular biological techniques made it possible to perform transcriptomics studies with non-sequenced eco(toxico)logical key species. Consequently, this thesis aimed to elucidate the relation between life cycle effects and molecular stress responses in the ecotoxicological model species *Chironomus riparius* for compounds with different modes of action. To this purpose, the following objectives were set:

- To compare life cycle and multigeneration responses of chironomid larvae to compounds with different modes of action.
- To develop transcriptomics resources for *Chironomus riparius* consisting of an annotated transcriptome and a gene-expression microarray, allowing large-scale gene expression studies with chironomids.
- To compare gene expression and life cycle endpoints in toxicant-exposed chironomid larvae.

The non-biting midge *Chironomus riparius* (Insecta: Diptera) was selected for the present study, because of its ecological relevance and because of its long history in sediment toxicity testing with currently four OECD guidelines being available for acute and chronic toxicity tests. Four compounds were selected because they represent different modes of action. Phenanthrene is a polycyclic aromatic compound that acts via a non-specific baseline toxicity known as narcosis. The organometal tributyltin is a biocide that has been shown to cause endocrine disruption in *C. riparius*. The essential metal copper and the non-essential metal cadmium cause oxidative stress, however, the processes differ as copper is a redox-active metal, while cadmium is a redox-inactive metal.

The introduction of transcriptomics in ecotoxicology increased the necessity for reduced experimental variability. To this purpose in chapter 2 I developed easily made artificial sediment and monitored larval development. It was shown that larval development was synchronized and that the days when specific larval stages are reached can be identified. It was concluded that the newly developed artificial sediment will facilitate the application of transcriptomics in ecotoxicity testing with *C. riparius*.

Since compounds with different modes of action may affect life cycles of biota differently, the aim of chapter 3 was to investigate the impact of four chemicals with different modes of action on chronic lethal and sublethal life-cycle effect parameters of the
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non-biting midge *C. riparius*. A 28-day sediment toxicity test was performed with the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene. Tributyltin and cadmium delayed emergence significantly over a wide range of sublethal concentrations, while this range was narrow for copper and almost absent for phenanthrene. The chronic LC50/LOEC\textsubscript{EmT50} ratio, expressing these differences, amounted to 1.5, 3.5, 12.0 and 18.2 for respectively phenanthrene, copper, cadmium and tributyltin. Thus the more specific the compounds mode of action, the higher the chronic LC50/LOEC\textsubscript{EmT50} ratio, as previously observed for acute-to-chronic ratios (ACRs). Comparison of our results with literature derived LC50/LOEC ratios showed a comparable trend and a lower variability compared to ACRs. It was therefore concluded that the presently proposed chronic ratio is indicative for the specificity of a chemicals mode of action and that it is less variable than the ACR.

The ability of the non-biting midge *C. riparius* to withstand long-term toxicant exposure has been attributed to genetic adaptation. Recently, however, evidence arose that supports phenotypic plasticity. Therefore, chapter 4 aimed to investigate if *C. riparius* indeed copes with prolonged toxicant exposure through phenotypic plasticity. To this purpose, a multigeneration experiment was performed in which *C. riparius* laboratory cultures were exposed for nine consecutive generations to two exposure scenarios of, respectively, copper, cadmium and tributyltin. Total emergence and mean emergence time were monitored each generation, while the sensitivity of the cultures was assessed at least every 3\textsuperscript{rd} generation using acute toxicity tests. It was observed that the cultures exposed to sublethal concentrations were hardly affected, while the cultures that were exposed to substantially higher toxicant concentrations after the 6\textsuperscript{th} generation were severely affected in the 8\textsuperscript{th} generation followed by signs of recovery. A marginal lowered sensitivity was only observed for the highly exposed cadmium culture, but this was lost again within one generation. It was concluded that *C. riparius* can indeed withstand long-term sublethal toxicant exposure through phenotypic plasticity without genetic adaption.

Whole-transcriptome gene-expression analyses are commonly performed in species that have a sequenced genome and for which microarrays are commercially available. To do such analyses in species with no or limited genome data, i.e. non-model organisms, necessary transcriptomics resources, i.e. an annotated transcriptome and a validated gene-expression microarray, must first be developed. The aim of chapter 5 was therefore to establish an advanced approach for developing transcriptomics resources specifically for non-model organisms by combining next-generation sequencing (NGS) and microarray technology. This approach was applied to the non-biting midge *C. riparius*. I sampled many individuals covering all *C. riparius* developmental stages as well as toxicant-exposed larvae and obtained from a normalized cDNA library 1.5M NGS reads totalling 501 Mbp. Using the NGS data transcriptomics resources were developed in several steps. First, 844k probes
were designed directly on the NGS reads, as well as 76k probes targeting expressed sequence tags of related species. These probes were tested for their affinity to *C. riparius* DNA and mRNA, by performing two biological experiments with a 1M probe-selection microarray that contained the entire probe-library. Subsequently, the 1.5M NGS reads were assembled into 23,709 isotigs and 135,082 singletons, which were associated to ~55k, respectively, ~61k gene ontology terms and which corresponded together to 22,593 unique protein accessions. An algorithm was developed that took the assembly and the probe affinities to DNA and mRNA into account, what resulted in 59k highly-reliable probes that targeted uniquely 95% of the isotigs and 18% of the singletons. It was concluded that this approach allowed the development of high-quality transcriptomics resources for *C. riparius*, and is applicable to any non-model organism. It is expected that these resources will advance ecotoxicity testing with *C. riparius* as whole-transcriptome gene-expression analysis is now possible with this species.

Molecular 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 the aim of chapter 6 was to directly compare the effects on growth, survival and gene expression of the non-biting midge *C. riparius*. To this purpose, I analysed simultaneously life cycle and transcriptomics responses of chironomid larvae exposed to four model toxicants. It was 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.

This thesis advanced on the understanding of stress responses in toxicant-exposed chironomids. [I] The specificity of compounds, or their mode of action, was effectively expressed by a new metric based on life cycle analysis and also by differences in gene expression, albeit the molecular mechanisms need to be further elucidated. [II] Time of exposure and time of effect observation was evaluated as a key factor both in quantifying whole organism response as well as in the identification of gene expression. [III] Multigeneration effects of chemical stress may involve genetic adaptation, but also non-inheritable transgenerational effects. The species *C. riparius* was demonstrated to be highly
plastic and put forward as a suitable test species to verify molecular responses over multigenerational exposure. [IV] The advances in ecotoxicogenomics are rapidly taking place with third generation sequencers on their way to revolutionize the field once again. However, till that time the transcriptomic tools developed in the present study can be applied to any species of interest, and here it allowed the first large-scale gene expression study with *C. riparius*. [V] The claim that ecotoxicogenomics could replace whole organism testing for risk assessment or hazard identification is critically discussed. This thesis showed that investigating transcriptome changes in response to toxic compounds provides a better understanding of the mechanisms of toxicity, and that molecular stress responses are more sensitive than life cycle effects. However, I also pointed out the necessity to relate molecular responses to toxic effect on the whole organism level. Considering these arguments it is expected that transcriptomics studies in ecotoxicology will eventually become an integrated part of environmental hazard and risk assessment where it will complement standardized ecotoxicity testing without fully replacing it.
Samenvatting

Transcriptoom studies hebben – zo neemt men aan - de potentie om de moleculaire mechanismen van toxiciteit tot in het kleinste detail te ontrafelen. Echter, om transcriptoom studies te kunnen toepassen in de ecotoxикologie zullen moleculaire stressresponsen eerst gerelateerd moeten worden aan de levenscycluseffecten van de betreffende testorganismen. De grootschalige genexpressie studies die hiervoor nodig zijn waren aanvankelijk alleen mogelijk met soorten waarvan het genoom volledig gesequenced was. Recente ontwikkelingen in molecuair biologische technieken maken het nu echter ook mogelijk om transcriptoom studies uit te voeren met eco(toxico)logische sleutelsoorten waarvan het genoom niet gesequenced is. Dit proefschrift had daarom als doel om de relatie tussen levenscycluseffecten en moleculaire stress responsen te ontrafelen in het ecotoxicologische modelorganisme *Chironomus riparius* blootgesteld aan toxische stoffen met verschillende werkingssystemen. Hiertoe werden de volgende doelstellingen geformuleerd:

- Het vergelijken van levenscyclus en multi-generatie responsen van chironomidenlarven blootgesteld aan stoffen met verschillende werkingsmechanismen.
- Het ontwikkelen van transcriptomics instrumenten voor *Chironomus riparius* bestaande uit een geannoteerd transcriptoom en een genexpressie microarray, waardoor grootschalige genexpressie studies met chironomiden mogelijk worden.
- Het vergelijken van genexpressie en levenscycluseffecten in chironomidenlarven die zijn blootgesteld aan toxische stoffen.

De dansmug *Chironomus riparius* (Insecta: Diptera) is gekozen als testorganisme voor de huidige studie vanwege zijn ecologische relevantie en vanwege het veelvuldig gebruik van deze soort voor het testen van sedimenttoxiciteit, vastgelegd in vier OESD richtlijnen voor acute en chronische toxiciteitstesten. Vier stoffen met verschillende werkingsmechanismen zijn geselecteerd: Phenanthrene is een polycyclische aromatische verbinding met een niet-specifiek werkingsmechanisme beter bekend als narcose. Het organometaal tributyltin is een biocide die het endocrine systeem van *C. riparius* kan verstoren. Het essentiële metaal koper en het niet-essentiële metaal cadmium veroorzaken beide oxidatieve stress, echter via verschillende mechanismen, omdat koper een redox-actief metaal is, terwijl cadmium redox inactief is.

De integratie van transcriptomics in de ecotoxicologie heeft de behoefte aan het terugdringen van experimentele variatie nog verder versterkt. Daarom heb ik in hoofdstuk 2 een eenvoudig samen te stellen artificieel sediment ontwikkeld en heb ik de larvale ontwikkeling van de dansmuggen in dit sediment gevolgd. Aangetoond werd dat de larven zich synchroon ontwikkelden en dat de dagen waarop specifieke larvale stadia werden bereikt goed geidentificeerd konden worden. Dientengevolge werd de verwachting uitgesproken dat het nieuw ontwikkelde artificiële sediment de toepassing van
transcriptomics in ecotoxiciteitstesten met *C. riparius* zou kunnen faciliteren en is het in alle achtervolgende hoofdstukken toegepast.

Aangezien stoffen met verschillende werkingsmechanismen de levenscyclus van een organisme anders zouden kunnen beïnvloeden, was het doel van hoofdstuk 3 om de effecten van vier chemicaliën met verschillende werkingsmechanismen op chronische letale en subletale levenscyclus-effectparameters van de dansmug *C. riparius* te onderzoeken. Hiertoe werd een 28-daagse sediment toxiciteitstest uitgevoerd met het essentiële metaal koper, het niet-essentiële metaal cadmium, het organometaal tributyltin en de polycyclische aromatische verbinding phenanthrene. Tributyltin en cadmium vertraagden het uitvliegen van de dansmuggen aanzienlijk over een breed scala van subletale concentraties, terwijl dit concentratiebereik smal was voor koper en voor phenanthrene bijna afwezig was. De chronische LC50/LOEC<sub>EmT50</sub> ratio, die deze verschillen tot uitdrukking brengt, bedroeg 1.5, 3.5, 12.0 en 18.2 voor respectievelijk phenanthrene, koper, cadmium en tributyltin. Dus hoe specifieker het werkingsmechanisme van de stof, hoe hoger de chronische LC50/LOEC<sub>EmT50</sub> ratio, zoals eerder waargenomen voor acute-to-chronic ratio’s (ACRs). Vergelijking van onze resultaten met LC50/LOEC ratio’s uit de literatuur liet een vergelijkbare trend zien waarbij onze nieuwe ratio een lagere variabiliteit vertoonde in vergelijking met de ACRs. Daarom werd geconcludeerd dat de hier voorgestelde chronische ratio indicatief is voor de specificiteit van het werkingsmechanisme van een stof en dat deze minder variabel is dan de ACR.

Het vermogen van de dansmug *C. riparius* om langdurige blootstelling aan toxische stoffen te overleven wordt vaak toegeschreven aan genetische adaptatie. Onlangs echter is bewijs geleverd voor fenotypische plasticiteit als onderliggend mechanisme van deze waarnemingen ondersteunt. Daarom werd in hoofdstuk 4 onderzocht of *C. riparius* inderdaad middels fenotypische plasticiteit langdurige blootstelling aan toxische stoffen weerstaat. Hiertoe werd een multi-generatie experiment uitgevoerd waarin laboratoriumculturen van *C. riparius* gedurende negen opeenvolgende generaties werden blootgesteld aan twee blootstellingsscenario’s van respectievelijk, koper, cadmium en tributyltin. Het totale aantal uitgevlogen dansmuggen en hun gemiddelde uitvliegtijd werden elke generatie gemeten, terwijl de gevoeligheid van de culturen tenminste elke derde generatie werd vastgesteld door middel van acute toxiciteitstesten. Er werd waargenomen dat de culturen die aan subletale concentraties waren blootgesteld hier nauwelijks door beïnvloed werden, terwijl de culturen die na de zesde generatie aan aanzienlijk hogere toxische concentraties werden blootgesteld, in de achtste generatie zwaar werden aangetast, waarna voorzichtig herstel volgde. Een marginaal verlaagde gevoeligheid werd alleen waargenomen voor de cultuur die aan hoge concentraties cadmium was blootgesteld, maar dit was de volgende generatie al weer verdwenen. Er werd daarom geconcludeerd dat *C. riparius* langdurige blootstelling aan toxische stoffen inderdaad kan
weerstaan door middel van fenotypische plasticiteit, en niet door genetische adaptatie. *C. riparius* is dus een zeer plastische soort, die geschikt is voor het bestuderen van de moleculaire stressresponsen tijdens multi-generatie blootstelling aan toxische stoffen.

Genexpressie studies die het gehele transcriptoom bestuderen, worden gewoonlijk uitgevoerd met soorten waarvan het genoom volledig gesequenced is en waarvoor microarrays commercieel verkrijgbaar zijn. Om dergelijke analyses te doen met soorten waarvoor geen of hooguit beperkte sequentiedata beschikbaar is moeten eerst de nodige transcriptomics instrumenten worden ontwikkeld, zoals een geannotoerd transcriptoom en een gevalideerde genexpressie microarray. Het doel van hoofdstuk 5 was dan ook om een geavanceerde benaderingswijze te ontwikkelen die het mogelijk maakt om specifiek voor niet gesequencede organismen transcriptomics instrumenten te ontwikkelen middels het combineren van Next-Generation Sequencing (NGS) en microarray technologie. Deze benaderingswijze werd vervolgens toegepast op de dansmug *C. riparius*. Vele individuele dansmuggen werden bemonsterd om een genormaliseerde cDNA bibliotheek te maken die alle *C. riparius* ontwikkelingsstadia bevatte, inclusief larven blootgesteld aan toxische stoffen. Het sequencen resulteerde in 1.5M NGS sequentie fragmenten met een totale lengte van 501 Mbp. De NGS data werd vervolgens gebruikt om in een aantal stappen transcriptomics instrumenten te ontwikkelen. Eerst werden 844K probes direct op de NGS sequentie fragmenten ontworpen, als ook 76k probes op basis van sequenties van verwante soorten. Om de affiniteit van al deze probes voor *C. riparius* DNA en mRNA te bepalen, werd de gehele probe bibliotheek op een 1M probeselectiemicroarray gezet en werden twee microarray experimenten uitgevoerd. Vervolgens werden de 1.5M NGS sequentie fragmenten geassembleerd in 23.709 isotigs en 135.082 singletons, die geassocieerd werden met ~55k, respectievelijk, ~61k genontologie termen, en die gezamenlijk overeenkwamen met 22.593 unieke eiwitten. Een algoritme werd ontwikkeld dat op basis van de transcriptoom assemblage en de probeaffiniteiten voor DNA en mRNA, 59k zeer betrouwbare probes selecteerde die 95% van de isotigs en 18% van de singletons uniek konden bevragen. Geconcludeerd werd dat met deze aanpak de ontwikkeling van hoogwaardige transcriptomicsinstrumenten voor *C. riparius* mogelijk is en dat deze toepasbaar zijn op elk nietgesequencede organisme. De verwachting is dat deze transcriptomicsinstrumenten een bijdrage zullen leveren aan de verdere ontwikkeling en interpretatie van ecotoxiciteitstesten met *C. riparius* aangezien ze genexpressie analyses in deze soort mogelijk maken.

Moleculaire stressresponsen worden vaak gevoeliger geacht dan traditionele ecotoxicologische levenscycluseffecten zoals overleving en groei. De inspanningen om de testduur te verminderen en meer gevoelige effectparameters te genereren heeft ervoor gezorgd dat transcriptomics studies uitgevoerd worden bij lage doses gedurende korte tijd, afzonderlijk en onafhankelijk van traditionele ecotoxiciteitstesten, waardoor de vergelijking
met tussen beiden indirect wordt. Daarom was het doel van hoofdstuk 6 om de effecten op groei, overleving en genexpressie van de dansmug *C. riparius* direct te vergelijken. Om dit doel te bereiken heb ik tegelijkertijd de levenscyclus- en transcriptomicsresponsen geanalyseerd van chironomidenlarven die waren blootgesteld aan vier toxische stoffen. Er werd waargenomen dat al bij de laagste test concentraties veel transcripten significant verschillend tot expressie kwamen, terwijl de levenscyclusparameters van *C. riparius* nog nauwelijks negatief werden beïnvloed. Analyse van deze transcripten liet zien dat de laagste test concentraties aanzienlijke en biologisch relevante cellulare stress induceerden en dat een substantieel deel van deze transcripten al hun maximale respons vertoonde bij deze laagste test concentraties. De directe vergelijking tussen de moleculaire- en levenscyclusresponsen na veertien dagen blootstelling liet zien dat genexpressie gevoeliger is voor toxicanten dan levenscyclus effect parameters, wat het potentieel van transcriptomics voor ecotoxiciteitstesten en milieurisicobeoordeling nogmaals onderstreept.

Dit proefschrift heeft bijgedragen aan een beter begrip van de stressresponse van chironomiden blootgesteld aan toxische stoffen: [I] De specificiteit van stoffen, of anders gezegd hun werkingsmechanisme, werd effectief tot uitdrukking gebracht door een nieuwe ratio die gebaseerd is op levenscyclusanalyse na langdurige blootstelling en ook door verschillen in genexpressie, hoewel het nodig is om deze moleculaire response nog verder uit te werken. [II] De blootstellingsstijl en daarmee de tijd die nodig is om effecten tot uitdrukking te laten komen zijn evenzo belangrijk factoren voor kwantificeren van effecten op de levenscyclus als op de genexpressie. [III] Multi-generatie effecten van chemische stress kunnen genetische adaptatie veroorzaken, maar ook niet-erfelijke transgenerationele effecten. Aangetoond werd dat *C. riparius* een zeer plastische soort is, die geschikt is voor het bestuderen van de moleculaire stressresponse tijdens multi-generatie blootstelling aan toxische stoffen. [IV] De vooruitgang in ecotoxicogenomics vindt snel plaats en het is te verwachten dat met de derde generatie sequencers het onderzoeksveld wederom drastisch zal veranderen. Echter, tot die tijd kan de benaderingswijze die in deze studie ontwikkeld is toegepast worden op elk willekeurig soort en hebben de hier ontwikkelde transcriptomics instrumenten de eerste grootschalige genexpressie studie met *C. riparius* mogelijk gemaakt. [V] Tenslotte is de bewering dat ecotoxicogenomics het testen met organismen voor risicobeoordeling zou kunnen vervangen kritisch besproken. Dit proefschrift liet zien dat het onderzoeken van veranderingen in het transcriptoom als reactie op toxische stoffen een beter begrip van de mechanismen van toxiciteit opleverde, en dat moleculaire stressresponsen gevoeliger zijn dan levenscycluseffecten. Echter, ik heb ook gewezen op de noodzaak om moleculaire responsen te relateren aan toxische effecten op het niveau van het gehele organisme. Rekening houdend met deze argumenten wordt verwacht dat ecotoxicologische transcriptomics studies uiteindelijk een integraal onderdeel zullen worden van milieu risicobeoordeling, waarbij ze gestandaardiseerde ecotoxicologische testen zullen aanvullen in plaats van volledig te vervangen.
Sažetak

Općenito se vjeruje da proučavanje transkriptoma može pomoći u razumijevanju molekularnih mehanizama u pozadini toksičnosti. Ipak, kako bi se u potpunosti iskoristio potencijal takvih istraživanja u ekotoksikologiji potrebno je povezati molekularni odgovor organizma na stres s efektima na razini životnog ciklusa. Međutim, analize transkriptoma, tj. ekspresije svih gena u jednome organizmu, izvorno su bile moguće samo u vrsta s potpuno sekvenciranim genomima. Ipak, napredci u molekularno biološkim tehnikama omogućili su analize transkriptoma u ključnih eko(toksiko)loških vrsta čiji genomi do tada još nisu bili sekvencirani. Cilj ovog doktorata je razjasniti odnos između efekata određenih životnim ciklusa i molekularnih reakcija na stres uzrokovanih spojevima s različitim djelovanjem u vrste Chironomus riparius, koju se koristi kao modelni organizam za ekotoksikološka istraživanja. U tu svrhu, postavljeni su bili slijedeći ciljevi:

- Usporediti multi-generacijske reakcije i one vezane uz životni ciklus u hironomidnim ličinkama nakon izloženosti spojevima sa različitim načinima djelovanja.
- Razviti sredstva koja omogućuju analizu transkriptoma Chironomus riparius koja se sastoje od dokumentiranog (annotated) transkriptoma i ekspresijskog microarray-a.
- Usporediti parametre bazirane na genskoj ekspresiji i životnom ciklusu u hironomidnim ličinkama izloženim toksičnim spojevima.

Dvokrilac Chironomus riparius (Insecta: Diptera) izabran je za ovaj doktorat zbog svoje ekološke važnosti i duge povijesti u ekotoksikološkom testiranju sedimenta, s trenutno četiri OECD smjernice za akutne i kronične toksične testove. Izabrana su četiri spoja s različitim načinima djelovanja: Fenantren je policiklički aromatski spoj nespecifične toksičnosti poznate kao narkoza. Organometal tributiltin je biocid koji može uzrokovati endokrine poremećaje u C. riparius. Iako esencijalan metal bakar i ne-esencijalan metal kadmij oba uzrokuju oksidativni stres, procesi se razlikuju jer je bakar redoks-aktivan metal, dok je kadmij redoks neaktivan.


Budući da spojevi različitog djelovanja mogu drugačije utjecati na životni ciklus organizma, cilj trećeg poglavlja je bio istražiti utjecaj četiri kemikalije različitog djelovanja na kronične letalne i subletalne parametre životnog ciklusa dvokrilca C. riparius. Izvedeni
Su toksikološki testovi koji traju 28 dana koristeći esencijalni metal bakar, ne-esencijalni metal kadmij, organometal tributiltin i policiklički aromatski spoj fenantren. Tributiltin i kadmij su značajno usporavali izlijetanje dvokrilaca nakon korištenja širokog niza subletalnih koncentracija, dok je taj niz bio sužen za bakar, i skoro odsutan za fenantren. Kronični LC50/LOECEmT50 pokazatelj, koji izražava te razlike, iznosio je 1.5, 3.5, 12.0 i 18.2 za fenantren, odnosno, bakar, kadmij i tributiltin. Znači da se u specifičnijem djelovanju spoja odražava veći kronični LC50/LOECEmT50 pokazatelj, što je u skladu s omjerom akutnog i kroničnog (eng. acute-to-chronic ratio’s (ACRs)). Usporedba naših rezultata s LC50/LOEC pokazateljima iz literature potvrdio je sličan trend, s tim da se naš kronični pokazatelj pokazao manje varijabilnim u odnosu na ACRs. Stoga je zaključeno da je ovdje predloženi kronični pokazatelj indikativan za specifičnost načina djelovanja kemikalija i da je manje varijabilan od ACR.


Analiziranje cjelokupnog transkriptoma obično se radi na vrstama čiji je genom sekvenciran i za koji se microarrays komercijalno mogu nabaviti. Kako bi se takve analize moglo provesti na vrstama bez, ili u najboljem slučaju ograničenom količinom, sekvenci, potrebno je razviti načine analiz transkriptoma temeljenje na dokumentiranom transkriptomu i ekspresijskom microarray-u. Cilj petog poglavlja stoga je bio uspostaviti naprednu metodu za razvijanje sredstava koja omogućuju analizu transkriptoma ne-sekvenciranih vrsta kombinirajući Next Generation Sequencing (NGS) i microarray.
tehnologiju. Ova nova metoda zatim je primijenjena na *C. riparius*. Prvo, uzorci svih razvojnih faza *C. riparius*, uključujući lićinke izložene toksičnim spojevima, korištene su za generiranje normalizirane baze DNA sekvenci. Potom, sekvenciranje te baze je rezultiralo s 1.5M NGS sekvenciranih fragmenata ukupne dužine od 501 Mbp. Na temelju NGS fragmenata dizajnirao sam 844k probe, tj. oligonukleotide, potom sam dodatno dizajnirao 76k probe na temelju sekvenci srodnih vrsta. Afinitet svih proba prema *C. riparius* DNA i mRNA testirao sam pomoću dva *microarray* eksperimenata. Nakon toga su 1.5M NGS sekvencirani fragmenti povezani u 23,709 isotig i 135,082 singleton i spojeni pomoću ~55k, odnosno ~61k *Gene Ontology* pojnova, što je odgovaralo 22,593 jedinstvenim bjelančevinama. Zatim je razvijen algoritam koji na bazi spojenih sekvenci i afiniteteta selektirao 59k pouzdanih proba. One su omogućile jedinstveno ispitivanje 95% isotig-a i 18% singleton-a. Zaključeno je da je ovaj pristup omogućio razvoj kvalitetnih parametara za analizu transkriptoma u vrste *C. riparius*, i da se može koristiti za bilo koji organizam koji nema sekvenciran genom. Očekuje se da će ova metodologija dodatno potaknuti ekotoksikološka ispitivanja na *C. riparius* jer omogućuju ispitivanje transkriptoma ove vrste.

Molekularni odgovori na stres često se smatraj osjetljivijima od tradicionalnih ekotoksikoloških parametara kao što su preživljavanje i rast. Nastojanje u ekotoksikologiji da se smanji vrijeme testiranja i da se definiraju osjetljiviji parametri rezultiralo je analizama transkriptoma u kojima se organizme kratkotrajno izlagalo niskim dozama, odvojeno i nezavisno od tradicionalnih ekotoksičnih testova, što komplicira direktno uspoređivanje različitih efekata. Stoga je cilj šestog poglavlja bio izravno usporediti efekte na rast, preživljavanje i ekspresiju gena u dvokrilca *C. riparius*. U tu svrhu sam istodobno analizirao efekte uzrokovane životnim ciklusom i transkriptom u lićinaka izloženih četirima toksikantima. Primijetili smo da se ekspresija puno transkripata značajno promijenila već na najnižim testiranim koncentracijama, a koje nisu porematile životni ciklus *C. riparius*. Analiza transkriptata pokazala je da već najniže testirane koncentracije uzrokuju biološki značajnij stres na staničnom nivou i da se velik dio tih transkriptata modulira kod ovakvih niskih doza. Izravna usporedba molekularnih efekata i onih definiranih životnim ciklusom nakon četrnaest dana izloženosti toksikantima pokazala je da je genska ekspresija osjetljivija od efekata definiranih životnim ciklusom, što podupire potencijal transkriptomike u ekotoksikologiji i procjenivanju rizika za okoliš.

Ovaj doktorat je pridonio razumijevanju reakcija na stres u hironomidima izloženim toksičnim spojevima: [I] Specifičnost spojeva, tj. njihov način djelovanja, učinkovito se uspajela karakterizirati novim pokazateljom baziranim na kronično efektima definiranih životnim ciklusom, kao i s promjenama u genskoj ekspresiji. No, molekularne odgovore na stres potrebno je detaljnije istražiti. [II] Vrijeme izloženosti toksikantima, i sukladno tome vrijeme potrebno da vidljivi efekti nastanu, ocijenjeni su kao važni faktori za kvantificiranje
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Europe (especially Albania and Romania) gave me energy during the phases that the experiments didn’t proceed as I wanted.

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Curriculum vitae

Marino Marinković was born on May 12th 1980 in Rotterdam, the Netherlands. As a kid he spend his summer holidays visiting the exquisite Dalmatian coast, where already at a young age he became fascinated by the Mediterranean flora and fauna. It was therefore no surprise that after graduating from Citycollege St. Franciscus high school he decided to study biology at Leiden University. The potential of molecular biology, combined with his drive to apply scientific knowledge for practical purposes resulted in a specialization in microbiology and biotechnology. However, his attraction to natural systems never really disappeared. Following his MSc in Biology and working for a couple of months at the Molecular Microbiology department at Leiden University, he moved to Ghent where he obtained his second MSc at Ghent University, this time in Aquaculture. Subsequent employment in a Greek marine finfish hatchery proved to be less challenging than anticipated and he returned back to academia where he did his PhD in Ecotoxicology at the University of Amsterdam. The results of his PhD study, where he was able to combine both fields of interest are represented in this dissertation. Extracurricular activities during his PhD, such as the PhD and Postdoc committee, made him realize that besides conducting scientific research he also greatly enjoys organizing and managing projects. Currently, he is searching for a new challenge where he can employ his interest in science, as well as commercial and societal activities.