Chapter 1

General introduction

> *"Alle Ding' sind Gift und nichts ohn' Gift; allein die Dosis*
> *macht, das ein Ding' kein Gift ist."*
> Paracelsus (1493-1541)

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.

Figure 1: Paracelsus. After an engraving by Hirschvogel from a portrait taken in 1540 at Laibach or Vienna when he was forty-seven years old (adapted from Stoddart, 1911).

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 end 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 straight forward 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 test (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 sensitivity to the exposed compounds (Postma et al., 1995b; Solà 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.

Figure 2: Adult *Chironomus riparius* (Jan van Arkel/ IBED[©]).

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.