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 straight forward 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.