A Combined Approach of Experiments and Modelling for the Implementation of Freshwater Copepods in Ecological Risk Assessment

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vorgelegt von

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“Prediction is very difficult, especially if it's about the future.”

Niels Bohr

Erklärung

Ich versichere, dass ich diese Doktorarbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Weiterhin versichere ich, die aus benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht zu haben.

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Zusammenfassung

Standardisierte Testrichtlinien, wie sie in der ökologischen Risikobewertung von Chemikalien verwendet werden, stehen nur für eine relativ kleine Gruppe von Arten zur Verfügung. Zum Beispiel wird der Wasserfloh *Daphnia magna* in den meisten Fällen als einzige repräsentative Art für Süßwasserinvertebraten gefordert. Dies erfolgt unter der Annahme, dass solche Tests mit Standardarten in Kombination mit relativ großen Extrapolationsfaktoren auch die Arten im Freiland ausreichend schützen. Standardtestarten werden in der Regel wegen ihrer intrinsischen Empfindlichkeit, ihrer Verfügbarkeit bzw. Zuchtfähigkeit im Labor und ihre Eignung für die Tests ausgewählt. Die zu schützenden Arten im Freiland können jedoch ganz andere Eigenschaften in Bezug auf Lebensdaten und Verhalten haben, die neben der physiologischen Empfindlichkeit die Vulnerabilität gegenüber Chemikalien bestimmen. Die Variabilität in der physiologischen Empfindlichkeit der verschiedenen Arten kann durch die Prüfung zusätzlicher Arten im Labor und die Ableitung von Empfindlichkeitsverteilungen adressiert werden, während die ökologische Empfindlichkeit mit Populations- oder Lebensgemeinschaftsstudien, z.B. in Mesokosmen, oder mit Hilfe ökologischer Modellierung untersucht werden kann. Ruderfußkrebse (Copepoda) spielen als Fressfeinde von kleinerem Zooplankton und als Futter für Fische eine wichtige Rolle beim Transfer von Stoffen und Energie in der aquatischen Nahrungskette. Aufgrund ihres komplexen Lebenszyklus sind Copepoden potenziell ökologisch vulnerable Organismen und daher als ökologische Indikatoren des Risikos einer chemischen Belastung geeignet. Zwar wird zur Zeit eine OECD Prüfrichtlinie für eine marine Copepodenart entwickelt, in der Risikoabschätzung für Binnengewässer werden Copepoden jedoch bisher, mit Ausnahme von Mesokosmosstudien, nur selten betrachtet. Vor diesem Hintergrund liefert die vorliegende Arbeit die Grundlagen eines kombinierten Ansatzes von Laborexperimenten und Populationsmodellierung, um Süßwassercopepoden besser in der Risikoanalyse von Chemikalien, insbesondere

Pflanzenschutzmitteln, berücksichtigen zu können. Zunächst wurde eine erschöpfende Literaturrecherche durchgeführt, in der *Mesocyclops leuckarti* als eine Copepodenart identifiziert wurde, die einen guten Kompromiss zwischen ökologischer Vulnerabilität aufgrund ihres komplexen Lebenszyklus und Praktikabilität ökotoxikologischer Tests darstellt. Die Eignung der ausgewählten Art für die Haltung im Labor wurde durch die Etablierung eines stabilen Laborkultur gezeigt. Die Durchführung von ökotoxikologischen Tests und die intrinsische Empfindlichkeit wurden in Akuttests mit der Beispielsubstanz Triphenylzinn (TPT) demonstriert. Die Experimente zeigten, dass *Mesocyclops leuckarti* gegenüber TPT empfindlich ist, wobei die Nauplien empfindlicher als die Adulttiere waren. Darüber hinaus wurden verschiedene Experimente durchgeführt, um für *M. leuckarti* Lebensdaten zu Nahrungsaufnahme, Entwicklung, Reproduktion und Überleben für verschiedene Futterarten und Futtermengen zu erhalten. Diese Daten dienten als Grundlage für die Entwicklung eines Individuen-basierten Populationsmodells (IBM), mit dem von Effekten auf Organismusebene auf die für die Risikoabschätzung relevantere Ebene der Population extrapoliert werden kann. Als Beispiel wurden die Toxikokinetik und Toxikodynamik von TPT mit Hilfe des „General Unified Threshold Models of Survival“ (GUTS) beschrieben. Die Kombination von GUTS mit dem Populationsmodell erlaubt die mechanistische Modellierung der Auswirkungen von für Pflanzenschutzmittel typischen zeitvariablen Expositionsmustern auf die Populationsdynamik von *M. leuckarti*. Das kombinierte Modell wurde verwendet, um die Effekte von TPT auf *M. leuckarti*-Populationen unter verschiedenen Futterbedingungen zu analysieren. Modell-Simulationen zeigten, dass der Kannibalismus von Nauplien, die bereits durch TPT Exposition belastet waren, die synergistischen Effekte von abiotischen und biotischen Faktoren verstärkten und zu einer doppelten Belastung und einer höheren Anfälligkeit der Population führten im Vergleich zu Simulationen, in denen kein Kannibalismus stattfand. Weiterhin wurde mit Hilfe von Populationsmodellen die ökologische Empfindlichkeit von *M. leuckarti* gegenüber TPT mit

der des Wasserflohs *Daphnia magna* und der der Büschelmücken *Chaoborus chrystallinus* verglichen. Dabei stellte sich heraus, dass die Populationsebene empfindlicher war als die Organismenebene. Weiterhin war die Empfindlichkeit der drei Arten auf der Populationsebene umgekehrt gereiht wie auf der Organismenebene. Auf der Populationsebene war *C. crystallinus* die empfindlichste Art und *D. magna* die unempfindlichste. Im Gegensatz zur Populationsebene war *M. leuckarti* auf Organismenebene weniger empfindlich als *D. magna.* Diese Arbeit belegt die Relevanz und Eignung von Copepoden für die ökologische Risikoanalyse von Chemikalien sowie die Bedeutung der Modellierung für die Extrapolation von auf Organismenebene gemessenen Effekten auf die Ebene der Population. Der hier verfolgte Ansatz, Laborexperimente und Modellierung für eine repräsentative vulnerable Art zu kombinieren, um mechanistisch auf Populationsebene und zwischen verschiedenen Expositionsmustern zu extrapolieren, kann auch auf andere Taxa angewendet werden, um für verfeinerte Risikoabschätzungen einen Satz an praktikablen Tests und Modellen für verschiedene Arten zu erhalten.

Summary

Standardized test guidelines used in ecological risk assessment (ERA) consider a relatively small set of test species. For instance in most standard risk assessments, *Daphnia magna* is the only required species representing freshwater invertebrates. This is done under the assumption that tests with such standard species in combination with relatively large assessment factors are protective for other species in the field. Standard test species are usually selected based on intrinsic sensitivity as well as practicability i.e. the ease of culturing them and conducting experiments in the laboratory. However, species in the field may employ variable life-history strategies which may have consequences concerning the ecological vulnerability of these species to toxicants. The variability in the intrinsic sensitivity of different species can be assessed by testing additional species and constructing species sensitivity distributions while ecological vulnerability can be addressed using community-level studies e.g. mesocosms and ecological modelling. Copepods are important animals in the aquatic food chain. They are predators of other plankton and act as prey for fish, consequently enabling the transfer of energy and substances though the food chain. Copepods, owing to their complex life-history strategies, are potentially vulnerable organisms and therefore, useful as ecological indicators of risk. For marine risk assessment, copepods are now being considered and an OECD test guideline for bottom-dwelling harpacticoid copepods is under way. However, in freshwater ecotoxicology, copepods are largely ignored except in mesocosm studies. To facilitate the consideration of freshwater copepods in higher-tier ERA there is a need for the development of robust test methods and models to facilitate extrapolation between environmental conditions, exposure patterns and species. Bearing this in mind, this thesis delivers the basics of a combined approach of laboratory experiments and modelling for better consideration of freshwater copepods in ERA. With a particular focus on plant protection products, an exhaustive literature review was carried out to identify a

representative species of freshwater copepods, which could be a good compromise between a potentially vulnerable relevant species, owing to its complex life-history strategies, as well as a good laboratory species based on the ease of rearing it in the laboratory. Consequently, the literature review revealed *Mesocyclops leuckarti* as a good representative species for freshwater copepods for ecotoxicological studies. The suitability of this selected species was demonstrated by establishing a stable laboratory culture. To demonstrate the intrinsic sensitivity of this species, a model chemical, triphenyltin (TPT), was used to conduct acute toxicity tests with this species in the laboratory. Laboratory toxicity experiments confirmed *M. leuckarti* to be sensitive to TPT with the naupliar stages showing a higher sensitivity compared to older stages. Furthermore, various experiments to study the life-cycle processes of this species namely- feeding, development, reproduction, survival, etc. were conducted using different food sources and feeding regimes. To facilitate the extrapolation of individual-level effects to more relevant population-level responses, an individual-based model (IBM) was developed for this selected species. The model was parameterised based on parameters of eco-physiological processes obtained from laboratory experiments. The toxicokinetics and toxicodynamics of TPT were described and modelled using the General Unified Threshold model for Survival (GUTS) based on the aforementioned laboratory toxicity tests. This model allows the mechanistic modelling of the effects of time-variable exposure patterns. The combined model was used to analyse the population-level effects TPT under different feeding regimes. Model simulations showed that cannibalism of nauplii that were already stressed by TPT exposure contributed to synergistic effects of biotic and abiotic factors and led to a two-fold stress being exerted on the nauplii, thereby resulting in a higher population vulnerability compared to the scenario without cannibalism. Furthermore, a case study wherein the ecological sensitivity of *M. leuckarti* was compared to *D. magna* and *Chaoborus crystallinus* by means of population modelling was carried out. It was observed that population-level sensitivities of the three species used in the case study were higher than those on the

individual level. Also, the sensitivity ranking of the three species on the population level was the converse of that on the individual level i.e. the species that was least sensitive at the individual level (*C. crystallinus*) was found to be most sensitive on the population level. Furthermore, *M. leuckarti* was less sensitive than *D. magna* at the individual level and more sensitive than *D. magna* at the population level. This thesis confirmed the relevance and practicability of copepods for ERA as well as the significance of population modelling in predicting population-level responses from individual-level data. This approach of combining laboratory experiments and population modelling of a representative vulnerable species to allow mechanistic extrapolation to the population level and to other exposure patterns can also be applied to other taxa in order to build up a set of test species and models useful for refined and more realistic ERA.

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Contents

Chapter 1

General introduction

1.1 Background and scope of the thesis

The ecological risk assessment (ERA) of chemicals looks at the risk posed by harmful substances to the environment. This is achieved by conducting acute and chronic toxicity experiments following guidelines for a relatively small set of standard species and applying safety factors to protect a wide array of species in the field. The species chosen for these studies are usually those which are easy to rear and have short life cycles (Stark et al 2004b). However, variability in physiological traits might affect intrinsic sensitivity to toxicants (Baird and Van den Brink 2007), while life-history traits (e.g. life span, reproduction, dispersal ability), which drive population dynamics (Stearns 1997; Stark et al 2004a) and recovery from toxic stress (Barnthouse et al 2007; Gergs et al 2011), determine the ecological sensitivity of a species. Species that are easy to rear, usually having short generation times and high intrinsic rates of increase, are less susceptible to population-level stress (Stark et al 2004a). For ERA to be objective, more relevant species need to be considered (Calow and Forbes, 2003).

For risk assessment in freshwater bodies, *Daphnia magna* is the organism of choice and is considered a representative species for freshwater invertebrates (EFSA 2013). Regulatory acceptable concentrations based on *Daphnia* EC_{50} values alone may not always protect sensitive species (Brock et al 2012). Species sensitivity distributions (SSD), which are being widely used in ERA, aim to determine a chemical concentration protective to a multitude of species (Wheeler et al 2002; Solomon et al 1996). Forbes and Calow (2002) suggest that a community sensitivity distribution would provide a better estimate of an ecologically relevant effects threshold. For the effect assessment of plant protection products, an aquatic invertebrate other than *Daphnia* should also be tested depending on the mode of action of the substance (SANCO Technical Guidance Doc 2002). For more realistic ERA, SSDs need to

include a greater number of sensitive species in order to represent a higher sensitivity (Solomon et al 1996).

Therefore, the present thesis focused on freshwater copepods as candidates for being additional test species constituting Tier 2 of the tiered risk ERA scheme (EFSA 2013) for plant protection products. Copepods are important animals in aquatic ecosystems found in almost every aquatic habitat (Reid 2001). They are food for fish and predators of plankton, and therefore, they enable the transfer of energy through the food chain (Raisuddin et al 2007). Planktonic freshwater copepods are susceptible to pesticide exposure via spray drift, drainage or run-off events. When compared with daphnids, copepods are smaller in size, have relatively longer life cycle and complex feeding habits, all of which make them potentially more vulnerable to toxic stress and therefore good candidates to be considered in ecotoxicological studies. Despite the significant need for copepods in ERA, there are no test methods or guidelines available on how to employ copepods for this exercise. Therefore, there is a need to identify a representative species for freshwater copepods useful for ERA and to develop a methodology to employ this species in ERA.

Laboratory studies however, focus on individual-level effects. For aquatic invertebrates, population-level effects are more relevant. Mesocosms and other community-level studies act as experimental tools to focus on population-level effects under semi-field conditions. However, mesocosm studies require relatively greater effort and resources to setup and run. Therefore, a tool to extrapolate from individual-level data to population-level responses as well as across different species and exposure scenarios is of paramount importance to ERA. Recently, mechanistic population models have been suggested as useful tools to extrapolate individual-level observations to population-level effects (Preuss et al 2009, Preuss et al 2011, Forbes et al 2011, etc.). Population models can not only add ecological complexity to ERA

but also reduce uncertainty thereby adding value to ERA (Forbes et al 2011). Population modelling constitutes higher-tier risk assessment and will soon be employed in assisting pesticide registration in the European Union (EFSA 2013). Within this context a Marie Curie Initial Training Network funded by the European Commission within the 7th Framework Programme was launched. This project was called CREAM (Mechanistic Effect Models for the Ecological Risk Assessment of Chemicals) (Grimm et al 2009). The main purpose of this project was to deliver a suite of mechanistic ecological effect models for a range of organisms and ecosystems relevant for chemical risk assessments (Grimm et al 2009).

The present thesis was part of the work package Aquatic Invertebrates, with a particular focus on the employment of freshwater copepods for ERA. This thesis delivers the basics of a combined approach of laboratory experiments and modelling for better consideration of freshwater copepods in ecotoxicology. The objectives of the study are as follows-

- To identify a representative species of freshwater copepods suitable for ERA through an exhaustive literature review. This species should not only be a good laboratory species (easy to culture and work with in the laboratory) but also be a relevant and potentially ecologically vulnerable species.
- To establish a stable culture of the selected test species, as well as the food for this species, in the laboratory.
- To conduct toxicity tests with this species to test for sensitivity to a model toxicant.
- To conduct ecological experiments to study the life-cycle processes of this species namely- feeding, development, reproduction, survival, using different food sources and feeding regimes.
- To develop an individual-based population model (IBM), parameterised with data from ecological and toxicological experiments conducted in the laboratory, for the

selected test species including responses to the selected toxicant to facilitate extrapolation of individual-level effects to the population level.

- Finally, to highlight the applicability of the model through a case study comparing individual and population-level sensitivities to the selected toxicant for the selected test species as well as two other planktonic organisms- *D. magna* and *Chaoborus crystallinus.*

1.2 Structure of the thesis

The thesis is structured into 6 chapters beginning within the ongoing chapter. Each chapter has its own introduction, methods, results and discussion section. Each succeeding chapter is based on the achievements of the preceding chapter. All references cited within the chapters have been jointly listed in a separate section towards the end. All the aforementioned objectives were realised during the study and have been presented in the thesis within chapters 2-5 as follows:

Chapter 2 *A plea for the use of copepods in freshwater ecotoxicology*

This chapter creates a background for the chapters ahead. It mainly covers the general context and the current state of affairs in ecotoxicological research with a particular focus on copepods. It describes how copepods fit into the current chemical risk assessment framework. Following a thorough literature search, a representative species for freshwater copepods is identified. Furthermore, combined approach of laboratory experiments and individual-based modelling is proposed to assess the potential risk of chemicals to copepods in freshwater ecosystems.

Chapter 3 *Life-stage-dependent sensitivity of the cyclopoid copepod Mesocyclops leuckarti to triphenyltin*

This chapter focusses on the setting up of laboratory cultures and toxicity experiments with *Mesocyclops leuckarti*. A model toxicant is chosen and used to perform acute toxicity studies with *M. leuckarti*. The recently proposed toxicokinetic and toxicodynamic model called the General Unified Threshold model for Survival (GUTS) is used to describe the toxicity of triphenyltin to the different stages of *M. leuckarti*. The modelling exercise presented here is used to develop a toxicity submodel linked to the main *M. leuckarti* IBM which is presented in the next chapter.

Chapter 4 *Eco-physiological triggers of population-level sensitivity to chemical stress in Mesocyclops leuckarti*

This chapter describes ecological experiments carried out with *M. leuckarti* in the laboratory on different food sources and feeding regimes. An IBM developed for *M. leuckarti* to simulate population dynamics under toxicant exposure based on data from these laboratory experiments is presented. This chapter aims to determine which life-cycle processes are most significant in determining density dependence and explore how this mechanism of density dependence affects extrapolation from individual-level effects the population level for *M. leuckarti* populations exposed to triphenyltin. There are different density-dependent processes that moderate the dynamics of populations, namely- bottom-up or top-down. Bottom-up processes are triggered by food availability whereas top-down processes are consequences of predation. Additionally, there exist self-regulating mechanisms like cannibalism or reduced reproduction and variation in brood number and size in response to high population size. Therefore, this chapter also investigates how two different mechanisms of density dependence would influence population-level ERA of the cyclopoid copepod *Mesocyclops leuckarti*.

Chapter 5 *Does intrinsic sensitivity imply population vulnerability? A tale of three models*

This chapter presents a case study with three common planktonic species- *D. magna*, *M. leuckarti* and *C. crystallinus.* All three species have distinct life-history strategies. The main issue addressed here is whether there is any difference between individual and population-level responses for these three species. Three distinct individual-based models developed for these three species were used to simulate population level responses to TPT exposure. These responses were compared with the individual-level effects observed for the same organisms in toxicity experiments in order to test whether there were differences between the individual- and population-level sensitivities for the three test species under TPT exposure. The power of population modelling in this exercise is highlighted.

Chapter 6 *General conclusions*

This chapter summarizes the main achievements within each of the preceding chapters as well as the main outcomes of the thesis. This chapter also provides a general idea of how the work presented within this thesis can be beneficial to freshwater ecotoxicology and ERA. Finally, a general outlook on necessary future efforts to build on the work done within this thesis is provided.

Chapter 2

A plea for the use of copepods in ecotoxicology

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2.1 Introduction

Environmental risk assessment (ERA) for plant protection products should be protective such that no long-lasting adverse effects on populations will occur in the environment (European Commission 2009). ERA is predominantly based on the results of laboratory studies testing a range of single compound concentrations in order to derive concentration-response relationships for standard test organisms (Holmstrup et al 2010). In order to account for uncertainties related to extrapolation from well-controlled laboratory conditions using standard test organisms to field conditions, ERA procedures typically consider safety factors to provide conservative estimates of environmental concentrations expected to result in acceptable risks for organisms in the field (Chapman et al 1998). There is a growing interest in estimating environmental risks of chemicals in a way that is more ecologically relevant (e.g. Stark and Banken 1999). As a consequence, the use of exposure scenarios based on climate and soil conditions (FOCUS 2001) or spatially explicit exposure modelling (Hendley et al 2001; Seuntjens et al 2008; Schulz et al 2009) contribute to more current and unambiguous approaches in ERA. Although, scientists have called for more realistic toxicity tests for decades (van Leeuwen et al 1985; van der Hoeven 1990; Seitz and Ratte 1991), this is mostly ignored in the hazard assessment portion of ERA. Regulatory acceptable concentrations based on *Daphnia* EC_{50} values alone may not always protect sensitive species (Brock et al 2012). Species sensitivity distributions (SSDs), which are being widely used in ERA, aim to determine a chemical concentration protective to a multitude of species (Wheeler et al 2002; Solomon et al 1996). Forbes and Calow (2002) suggest that a community sensitivity distribution would provide a better estimate of an ecologically relevant effects threshold. For more realistic ERA, SSDs need to include a greater number of sensitive species in order to represent a higher sensitivity (Solomon et al 1996).

The characteristics of species chosen for toxicological studies have been summarized by Robinson and Thorn (2005) as availability, relevance, ease of handling, sensitivity and database of background information. It has also been suggested that sensitive species should be cultured in the laboratory as potential subjects for toxicity studies (Adams and Rowland 2003). Moreover, surrogate species are required to represent populations of species in potentially exposed ecosystems (Wogram 2009). In most studies however, species chosen are usually those which are easy to culture and maintain in the laboratory (Stark et al 2004b). This is evident in the case of Cladocerans, *Daphnia magna* in particular, which are typical laboratory test species due to their short life cycles and ease of culturing (Wogram 2009). However, ERA is more objective when more relevant species are used (Calow and Forbes 2003). Variability in physiological traits might affect intrinsic sensitivity to toxicants (Baird and Van den Brink 2007), while life-history traits (e.g. life span, reproduction, dispersal ability), which drive population dynamics (Stearns 1997; Stark et al 2004a) and recovery from toxic stress (Barnthouse et al 2007; Gergs et al 2011), determine the ecological sensitivity of a species. Species that are easy to rear, usually having short generation times and high intrinsic rates of increase, are less susceptible to population-level stress (Stark et al 2004a).

For the effect assessment of plant protection products, an aquatic invertebrate other than *Daphnia* should also be tested depending on the mode of action of the substance (SANCO Technical Guidance Doc 2002). Copepods are important animals in aquatic ecosystems found in almost every aquatic habitat (Reid 2001). They are food for fish and predators of plankton, and they enable the transfer of energy through the food chain (Raisuddin et al 2007). Humes (1994) reported that there are 11500 species of copepods known worldwide with a large number still left undescribed.

The present study looks at risk assessment for plant protection products to invertebrates by focussing on freshwater ecosystems. Freshwater ecosystems are more susceptible to exposure with pesticides because of direct entry by way of spray drift or run-off (Leonard et al 1999). The objective of the study was to analyse if copepods should also be considered in more detail in risk assessments for freshwater ecosystems. We focused on the risk assessment for plant protection products because a tiered approach including standard tests, additional species testing and microcosm and mesocosm studies is the most advanced one, and can be compared to frameworks for other chemicals (Hommen et al 2010). We compared literature available on copepods and daphnids, the latter being preferred organisms in freshwater ecotoxicological research, in ecology and ecotoxicology and discussed the extent of focus on these groups. Moreover, the studies found on ecotoxicology of copepods were discussed in detail, the potential of copepods in ecotoxicological research was highlighted and a possible way ahead towards selecting a freshwater copepod species for studying the potential effects of plant protection products was suggested.

2.2 Literature search

2.2.1 Data source

We conducted a literature search for copepods using the online tool ISI Web of Knowledge^{SM} (© 2010 Thomson Reuters) in the fields of ecology and ecotoxicology by means of the keywords copepod [AND] ecol*, copepod [AND] ecotox*, copepod [AND] tox*, and similarly daphnia [AND] ecol*, daphnia [AND] ecotox*, daphnia [AND] tox*, in distinct searches in the month of September 2011. We searched for hits to compare studies done on *Daphnia* and copepods. The operator '*' enables searching for alternative forms of the search terms used e.g. ecotoxicology, ecological, etc. We chose to compare copepods as a group with the genus *Daphnia* because *Daphnia*, which are used in all frameworks related to chemical

risk assessment, are preferred organisms for ecotoxicological studies facilitated by the provision of detailed guidelines. At first it would have seemed fair to compare Copepoda and Cladocera or Branchiopoda with respect to ecological literature. However, publication titles seldom mention *Daphnia* as being cladocerans and assume that readers take this fact for granted. On the contrary for copepods, it is necessary to mention the word 'copepod' in the title and keywords as a descriptor in order to aid comprehension.

2.2.2 Data analysis

To illustrate the relevance of copepods in ecology, we extracted information from the publications obtained on the distribution of copepods across different aquatic habitats, the different life-history variables of copepods, the food choices shown by copepods, reproduction in copepods and the position of copepods in aquatic food webs. Hits obtained using the keywords copepod [AND] ecotox* were scanned in detail and summarized depending on the amount of information provided in the publications based on species information (group, species and stage), study information (scale, level of interest, substance and endpoint) and location. We looked not only for studies which particularly focused on copepods as subjects of ecotoxicological studies but also for studies where copepods were not the main focus of the studies. Mesocosm studies, for example, were carried out at the community level, and although they did not focus on copepods in particular, they revealed the potential vulnerability of copepod populations within these communities.

2.3 Review of literature

The literature search (Table 2.1) revealed that the number of literature hits for copepods and *Daphnia* in ecology were comparable, and which were 10,985 and 9,631 respectively. Yet,

the same was not true for a comparison of ecotoxicological literature on *Daphnia*, with 1,063 hits and copepods, with 149 hits.

Table 2.1- Keywords and the respective number of hits obtained from the literature search carried out in September 2011 using the ISI Web of knowledge.

Keyword	Number of hits for copepod [AND]	Number of hits for daphnia [AND]
ecol*	10,985	9,631
ecotox*	149	1,063
tox*	1070	9,374

This indicated that the publications on copepod ecology were three orders of magnitude higher than those on copepod ecotoxicology. From 1990 to 2010, the publications on copepod ecology amounted to an average of 425 publications per year, while those on copepod ecotoxicology averaged to only six publications per year (Fig 2.1). It is important to note that there is probably no direct correlation between the number of papers published and the importance of the taxa discussed. We assumed within the scope of this chapter that the number of publications on a particular taxon, *Daphnia* for example, indicated that this taxon was drawing attention and gaining importance from the scientific community.

2.3.1 Relevance of copepods in freshwater ecosystems

As reviewed by Boxshall and Defaye (2008), copepods are widely distributed into parasitic and free-living forms in freshwater ecosystems. In the present study, we focused only on the characteristics of copepods found in freshwater ecosystems like ponds and ditches, which are susceptible to pesticide exposure due to spray drift or run-off. Spray drift and run-off are important for the entry of pesticides into surface waters (Schulz 2001). Depending on their

feeding habits, copepods occupy different places in the food chain, feeding on other soft-bodied zooplankton like rotifers, various phytoplankton (Fryer 1957; Vijverberg 1989) and also on mosquito larvae (Marten 1990). On the other hand, they are prey for fish and can transfer toxic substances through the food chain (Raisuddin et al 2007). Order Calanoida, Cyclopoida and Harpacticoida are the three orders of free-living copepods, of which order Cyclopoida contains the largest copepod family called Cyclopidae (Boxshall and Defaye 2008) in freshwater. Copepods form a dominant biomass in planktonic ecosystems (Williams et al 1994) and show a high recolonisation potential within ephemeral water bodies (Frisch et al 2009). Cyclopoid copepods, along with rotifers, are rapid colonizers of experimental tanks (Cohen and Shurin 2003, Cáceres and Soluk 2002, Jenkins and Buikema 1998). With a few exceptions, copepods also dominate subterranean (e.g. groundwater) habitats (Galassi et al 2009) and are also known to show micro-habitat (e.g. hole in a rock or a tree trunk, etc) preferences (Galassi 2001). Copepods also play an important role in the transfer of energy through different trophic levels (Lampert and Sommer 1997). To summarize, copepods, like other zooplankton taxa, are abundant in aquatic ecosystems and important components of aquatic food webs.

2.3.2 Biology of freshwater copepods

Somatic growth in copepods has been observed to be a discontinuous process with respect to body size (Vijverberg 1989). However, with respect to weight it has been observed to be a continuous process, with temperature along with food quality and quantity being the most influential environmental factors (Vijverberg 1989). The life cycle of freshwater copepods is quite complex and is intricately divided, post hatching, from eggs into six naupliar stages (NI-NVI), five copepodid stages (CI-CV) and finally adults (Santer 1998). The life cycle can be interspersed with a period of dormancy to survive harsh environmental conditions (Alekseev et al 2007) like freezing or drying up of the habitat.

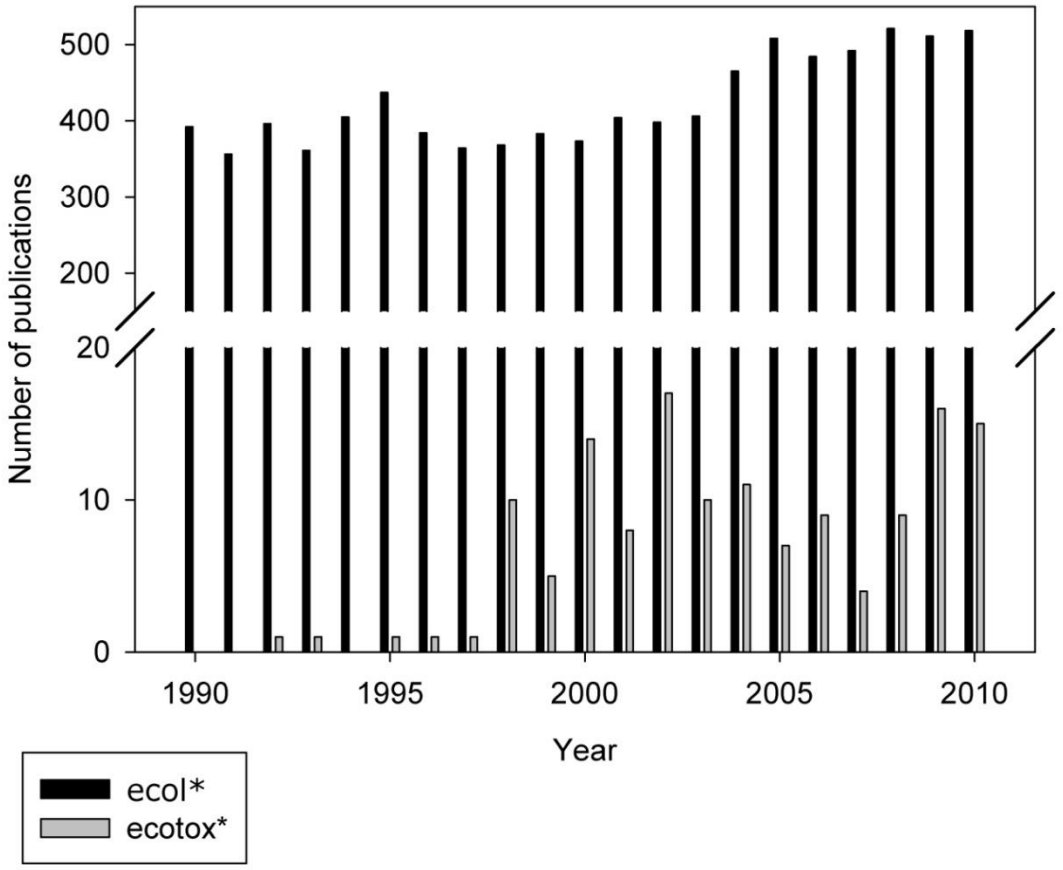

Fig 2.1- Citations on copepods in ecology and ecotoxicology from 1990 to 2010

Santer (1998) summarized the life cycles of the 3 orders of freshwater free-living copepods namely Calanoida, Cyclopoida and Harpacticoida. She reported that, "Calanoid copepods, found in pelagic zones of freshwater bodies possibly produce offspring without resting stages and their life cycle strategies have been found to be flexible. All life stages may coexist to increase chances of survival when under stress due to increased predation or starvation. Cyclopoid copepods on the other hand represent both pelagic as well as bottom dwelling species. While calanoid and harpacticoid copepods produce resting eggs to survive harsh conditions, cyclopoid copepods do not. Development is blocked at a certain stage and in general diapause is observed. Bottom dwelling harpacticoid copepods show both sexual and parthenogenetic reproduction. In oligotrophic environments, females have more food in the

absence of males and hence reproductive success is high. Some species also show continuous reproduction without diapausing or producing resting eggs. Copepods exhibit both grasping and filtering modes of feeding. Cyclopoids grasp food particles and show omnivory in the copepodite stages. Adults essentially show carnivory, while nauplii show herbivory. Harpacticoids employ surface browsing and filtration techniques for feeding" (Santer 1998). Herbivorous copepods feed rather selectively, basing selection on prey size, while calanoid copepods are 'macro-filtrators' and show both 'active and passive capture modes' (Vijverberg 1989). Carnivorous copepods require animal food for production of eggs (Hansen and Santer 1995) and in the absence of adequate animal prey they feed on copepod nauplii. Rotifers with soft carapaces are preferred food for carnivorous copepods (Williamson 1984). What separates copepods from cladocerans, rotifers and ostracods are their complex life-cycle strategies (Table 2.2), which can lead to higher population vulnerability. Stark et al (2004b) explained the importance of complex life-history strategies in influencing the susceptibility of species with complex life-history strategies.

2.3.3 Copepods in ecotoxicology

There was a noticeable increase in the number of published studies on copepods in ecotoxicology (Fig 2.1). In this section, we have discussed the studies we found on copepod ecotoxicology, both in the marine and freshwater environments, because of the following reasons-

- Firstly, most of the ecotoxicological work that was conducted focused on copepods in marine environments and there were very few studies on freshwater copepod ecotoxicology.

- Secondly, our main motive was to elucidate the fact that copepods are as vulnerable to exposure with contaminants, if not more, than other standard invertebrate organisms used in aquatic ecotoxicology.

Recently, the importance of copepods in marine ecotoxicology was realized (Raisuddin et al 2007) and an OECD guideline for marine copepods is now under preparation. After the publication of a full life-cycle test report for harpacticoids *Nitocra spinipes* and *Amphiascus tenuiremis*, and the calanoid *Acartia tonsa*, the species selected for further experimental work was *A. tenuiremis* (OECD 2011). There is still no protocol for acute or chronic toxicity tests with freshwater copepods. Microcosm and mesocosm studies conducted to analyse the responses of freshwater communities to different toxins revealed copepods to be potentially sensitive (Table 2.3). While testing for effects of the insecticide chlorpyrifos in outdoor experimental ditches, copepods were found to be most sensitive (van Wijngaarden et al 1996). Copepods were also found to be more sensitive than *Daphnia* when exposed to lambda cyhalothrin in freshwater mesocosms (Wijngaarden et al 2006), and to a mixture of the herbicide atrazine and the insecticide lindane (Van den Brink et al 2009). The sensitivity of Copepods in these studies were not determined in detail taxonomically. They were identified either as nauplii, copepodites, adults or on most occasions only upto the level of taxonomic order namely Calanoida, Cyclopoida or Harpacticoida. copepods to the fungicide azoxystrobin was also reported by Gustafsson et al (2010) and Zafar et al (2012).

Within the 149 hits obtained for copepod [AND] ecotox*, most studies that were carried out at the species level

Table 2.2- Different life-history strategies of planktonic organisms

Characteristic	Copepods	Cladocerans	Rotifers	Ostracods	Planktonic Insects
Average life span	2-6 months	4-6 weeks	1-2 d	2-6 months	2 months to 2 years
Trophic status	Primary consumers	Primary consumers	Primary consumers	Primary consumers	Secondary consumers
Dispersal	Passive	Passive	Passive	Passive	Active
Reproductive strategies at optimum conditions	Sexual	Mostly Asexual	Sexual or asexual	Asexual	Sexual
Resting stages/eggs	Yes	Yes	Yes	Yes	Mostly no
References	Gophen 1976; Maier 1994, 1998; Hopp and Maier 2005; Santer 1998; Reid 2001; Hansen and Santer 2003	Sarma et al 2005; Lynch 1980; Korovchinsky 1990; Nandini and Sarma 2006;	Duncan 1989; Kirk 1997; Stelzer 2005	Butlin et al 1998; Havel and Talbott 1995; Fergusson 1944	Fedorenko and swift 1972; Kajak and Rybak 1979; Fischer and Moore 1993

were acute or chronic toxicity studies that focused mainly on marine species of calanoid and harpacticoid copepods looking at the effects of various metals, oil, nanoparticles, sediments, organic matter and various mixtures (e.g. Kusk 1997, Hansen et al 2011, Macken et al 2009). The end points that were investigated included alteration of development, physiological changes, mortality, recovery, fecundity inhibition, gene expression, decrease in population abundance, etc. Although some papers that simply mentioned the word ‘toxicity’ or ‘ecotoxicology’ and ‘copepods’ within the text also came up as hits, further reviewing showed that they did not focus on toxicity tests with copepods (e.g. Drillet et al 2011).

Table 2.3- Relative sensitivities of copepods and daphnids in micro/mesocosm pesticide toxicity studies.

Toxicant	Type	Sensitivity			Reference
		Copepods	Daphnids	Highest species weight in principal response curve	
Chlorpyrifos	Insecticide	NOEC < 0.1 µg l^{-1} (Most sensitive)	-	Yes	Van Wijngaarden et al 1996
Cypermethrin	Insecticide	4 h NEC < 0.01 µg l^{-1} (Nauplii most Sensitive)	1 d NEC < 0.03 µg l^{-1}	Yes	Wendt-Rasch et al 2003
Lambda-Cyhalothrin	Insecticide	NOEC 25 ng l^{-1}	NOEC 100 ng l^{-1}	No	Van Wijngaarden et al 2006
Atrazine+ Lindane	Herbicide + Insecticide	Negative effects at 30 µg l^{-1} (More sensitive than *Daphnia*)	Negative effects at 100 µg l^{-1}	No	Van den Brink et al 2009
Azoxystrobin	Fungicide	NOEC for reproduction 10 µg l^{-1}	-	Yes	Zafar et al 2012

Some of the search hits obtained were reviews on potential methods for studying toxic impacts on copepods (e.g. Raisuddin et al 2007). As described in Table 2.4 we found 51 studies on harpacticoid copepods wherein 36 studies provided information at the species level. The most frequent species considered in these studies were *Tisbe battagliai* (e.g. Diz et al 2009, Hutchinson et al 1999) and *Tigriopus japonicus* (e.g. Ki et al 2009, Kang et al 2011). *Nitocra spinipes* was the next most favoured species (e.g. Dahl and Breitholtz 2008). 35 of these total studies on harpacticoids were carried out in the laboratory (e.g. Chandler et al 2004). We found 34 studies in all on calanoid copepods with 21 laboratory studies of which

15 were carried out at the species level (e.g. Gutierrez et al 2010). The calanoid copepod *Acartia tonsa* was the most favoured species within these tests (e.g. Kusk 1997).

Finally, we found only six studies on cyclopoid copepods, mainly mesocosm studies, of which only two were laboratory studies and two species level studies (e.g. Mösslacher 2000). The ecotoxicological carried out with copepods in freshwater habitats were fewer in number than those in marine, brackish and other habitats. Looking at the overall search results, there were five full life-cycle tests while most others were acute and chronic toxicity tests. As for the substances tested, marine sediments and copper were most common followed by effluents and pesticide components.

Table 2.4- Distribution of studies on copepods in ecotoxicology from the literature search carried out.

Group	Calanoida	Cyclopoida	Harpacticoida	Miscellaneous
Laboratory studies	21	2	35	-
Species Level	15	2	36	-
Freshwater	9	6	7	9
Other habitats	25	-	44	49

As many as 58 out of the total publications that appeared as hits for copepods in ecotoxicology contained no specific ecotoxicological data. These studies either reported copepods as potentially useful organisms in ecotoxicological studies, or were on toxicity to zooplankton in general; or as in most cases, simply mentioned the word copepod either in references or within the text (e.g. Drillet et al 2011). However, it was clear from this literature

search that copepods represented potentially vulnerable organisms in freshwater toxicity studies. Therefore, it was important to identify a suitable representative for freshwater copepods which could later be used in higher–tier risk assessment e.g. in population modelling.

2.4 Selecting a freshwater copepod species for ecotoxicological tests

According to the Scientific Opinion of the European Food Safety Authority on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the Guidance Documents on Aquatic and Terrestrial Ecotoxicology (EFSA 2010), it is important to find key drivers (representative taxonomic groups) and to identify vulnerable species for each key driver. These may not necessarily be standard species, but may be species for which toxicological sensitivity as well as life-cycle traits would influence recovery potential (EFSA 2010). Therefore, the selection of a suitable laboratory species requires a compromise between sensitivity and relevance on one hand and ease of rearing on the other.

We used a top-down approach (Fig 2.2) to select a relevant species for toxicity tests in order to assess the risk posed by pesticides to freshwater copepods. We chose to focus on cyclopoid copepods which are becoming increasingly important in food webs post eutrophication (Maier 1998), are important food items for freshwater aquaculture and are of great economic value (Szlauer and Szlauer 1980), important for control of mosquito larvae (Marten 1990) and abundant in edge-of-field water bodies. Being littoral and pelagic in occurrence, they are susceptible to pesticide exposure via spray drift and run-off and have the potential for being good indicators of the health of the ecosystem. Due to the concentration of many substances at the surfaces of water bodies, surface dwelling organisms will be more exposed (van

Straalen 1994) than sub-surface and sediment dwelling forms. The family Cylopidae is the largest freshwater copepod family with more than 800 species (Boxshall and Defaye 2008). As mentioned before, cyclopoid copepods show the phenomenon of diapause, which may occur either in winter or in summer. The species which diapause in winter are called summer species and those that diapause in summer are called winter species. Summer species (e.g. *Mesocyclops leuckarti*) have relatively shorter life cycles in comparison with winter species (e.g. *Cyclops vicinus*) (Maier 1994). Therefore, it seemed ideal to select a species with a relatively shorter life cycle i.e. a summer species in order to facilitate full life-cycle tests in the laboratory. Also, application of pesticides mostly occurs in spring and summer rather than in winter, and therefore a summer species would be ideal to study such exposure. *M. leuckarti* is one of the most well studied freshwater cyclopoid copepod summer species in Germany, in particular and Europe, in general.

A fair amount of ecological research has been done on this species, which has established that *M. leuckarti* is a common planktonic species in European freshwater habitats, shows herbivory at naupliar and early copepodite stages, switches to carnivory at the copepodite IV stage and grows best with a mixed diet of algae and rotifers (Smyly 1961, Gophen 1976, Maier 1993, Maier 1994, Hansen and Santer 1995, Santer 1998, Hopp and Maier 2005). It is a small sized species, 0.9–1.0 and 0.7–0.8 mm for the females and males, respectively (Hopp and Maier 2005). Although life cycles in the field may be long, copepods were shown to have relatively shorter life cycles under laboratory conditions, e.g. *M. leuckarti*, which was found to have an average development time from egg to adult of 70 d in the field, showed an average development time of 18 d under laboratory conditions (Smyly 1961).

However, these development times depend on the quality and quantity of food provided to the copepods and the temperature. This species also shows an increased egg production when fed a mixed diet of algae (*Chlamydomonas* or *Cryptomonas*) and rotifers (*Brachionus*) and this

increased egg production is not due to increased carbon content but rather due to the high quality

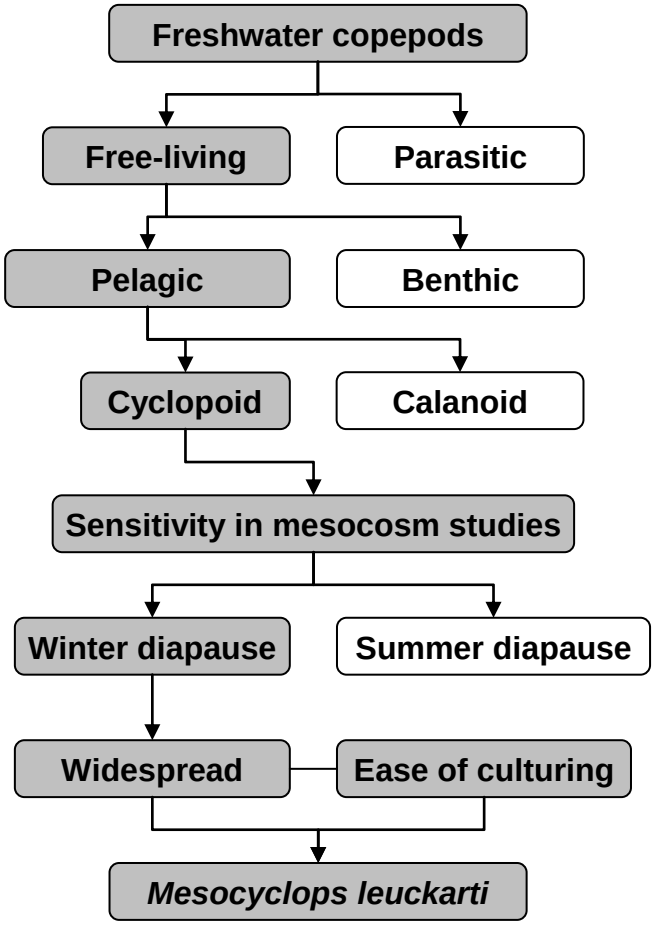

Fig. 2.2- Selecting *M. leuckarti* as a suitable representative for laboratory toxicity studies on cyclopoid copepods.

of food in *Brachionus* (Hansen and Santer 1995). Owing to its relatively shorter life cycle, planktonic distribution, its summer occurrence and ease of culturing in the laboratory, *M. leuckarti* qualifies as a suitable laboratory species to conduct full life-cycle tests.

2.4.1 Laboratory species versus relevant species

Finding a good laboratory species, which is also environmentally relevant, is a good example of an optimisation problem. Although *M. leuckarti* qualifies as a good laboratory species for

pesticide toxicity studies, a more relevant species for thorough risk assessment would be one with a longer life cycle, lower fecundity and higher vulnerability under toxicant exposure. Many other species of freshwater cyclopoid copepods have longer life cycles of six months to one year. *C. vicinus* populations have been shown to diapause for ten months (Hansen and Santer 2003). *M. leuckarti*, being a summer species has a relatively shorter life cycle. For species with longer life cycles, there are chances of repeated exposure within one life cycle due to multiple applications or run-off events. Species with longer life cycles would also require more time to recover post exposure. *M. leuckarti* is a good compromise between environmental relevance and ease of ecotoxicological testing. *M. leuckarti* is a species which is often found in edge of field water bodies (Smyly 1961; Gophen 1976). We can easily conduct full life-cycle tests with *M. leuckarti* (Hopp and Maier 2005; Unpublished data from our own experiments) and use these data to parameterize an individual-based population model. Therefore, it would be beneficial to link studies on a laboratory experimental copepod species (e.g. *M. leuckarti*) to those on a more relevant species found in the field by extrapolating laboratory-level effects on individuals to population-level effects in the field and across species. This objective can be achieved by exploiting the power of an individual-based population modelling approach (e.g. Preuss et al 2009, 2010).

2.4.2 Employment of a modelling approach

To address the complexity in the life-cycle strategies of *M. leuckarti*, we suggest the employment of an individual-based population modelling approach to predict the effects of pesticides on population dynamics of this species. An individual-based model for *M. leuckarti* will help to address the consequences of affected life-cycle traits for population vulnerability (including recovery), food selection based on stage and environmental conditions (algae, soft bodied zooplankton, cannibalism) and extrapolation to cyclopoid copepods with longer life

cycles (e.g. *C. vicinus*). Laboratory experiments on feeding, development, reproduction and survival are good starting points for an ecological model for the simulation of population dynamics under laboratory conditions (Preuss et al 2009). Further, acute and chronic toxicity tests on this species using a selected pesticide will help to calibrate the effect sub-model as demonstrated for *Daphnia* by Preuss et al (2010). The combination of the ecological and effect models will help to calibrate individual-level effects on the species and then to simulate population-level responses (Preuss et al 2010).

2.5 Discussion and outlook

The objective of this literature study was to demonstrate the relevance of copepods in ecology and their potential importance in ecotoxicology. It should be noted that we compared the Subclass Copepoda with the genus *Daphnia* to highlight the under-representation of freshwater copepods in particular and copepods in general, within ecotoxicological research. There is an obvious different between the extent of literature on copepods in ecotoxicology, which is clearly outweighed by that in ecology. Although copepods have been shown to be important components of aquatic ecosystems, there is a great need for including them in ecological risk assessment. As far as surrogate species are concerned, their usage is only appropriate if used at the taxonomic order level, thereby justifying the use of *Daphnia* as a surrogate for Branchiopoda rather than also for Copepoda (Sánchez-Bayo 2006). Copepods are mostly avoided in ecotoxicological research owing to their long generation times in the field and mixed food choices. However with appropriate abiotic conditions (medium quality, temperature, etc.) and good quality food, copepods can be reared in the laboratory without much effort (Vijverberg 1989; Maier 1998; Hopp and Maier 2005; unpublished data from our own experiments).

According to the population vulnerability conceptual framework model by Van Straalen (1994), a vulnerable species is one which runs a high risk of being exposed, is intrinsically sensitive and is not able to recover easily after exposure. It would be interesting to see how copepods fit into this framework:

(i) Exposure: Littoral freshwater copepods are more susceptible to pesticide exposure as a consequence of spray drift or run off to freshwater bodies than other deep water and bottom-dwelling forms. Copepods usually have longer life cycles in the field, compared to *Daphnia,* and therefore face a high risk of multiple exposures within one life span. Copepods have mixed food preferences depending on the life stage and these in turn may influence the amount of toxicant ingested. Toxicants can accumulate in the food chain (Maul et al 2006) and there can be a higher toxicant exposure to predators despite limited exposure within the habitat (Rubach et al 2011).

(ii) Intrinsic sensitivity: Copepods are generally smaller in size when compared with *Daphnia*. *M. leuckarti* nauplii are 0.101 mm (Dahms and Fernando 1993) and adult females are 1 mm in length (Hopp and Maier 2005), while *D. magna* neonates are 0.93 mm in size (Cleuvers 1995) and the adults can grow up to 5 mm (Hammers-Wirtz and Ratte 2000). Different stages show differential sensitivities to toxicants (Green et al 1996). Copepod nauplii are smaller and more sensitive than the later stages as shown in some mesocosm experiments (Wendt-Rasch et al 2003; Gustafsson et al 2010, Zafar et al; 2012). It was shown for rotifers that sexual reproduction is more sensitive to toxicants than asexual reproduction (Snell and Carmona 2009) and as seen with copepods, has been identified as one of the prerequisites for toxicity test organisms (Turesson et al 2007).

(iii) Recovery: Cyclopoid copepods have been known to show high sensitivity and slow recovery in comparison with *Daphnia sp,* e.g. in lufenuron-stressed experimental ditches

(López-Mancisidor et al 2008). Roessink et al (2006) revealed in a microcosm study that cladocerans recovered within 4 weeks while copepods recovered after 12 weeks post exposure to triphenyltin, an organotin fungicide. As reviewed earlier in this chapter, copepods have long life spans and generation times in the field when compared with other zooplankton (Table 2.2). Longer generation times do contribute to slower recovery such that intrinsic recovery by way of population growth is slower (Stark et al 2004b). Therefore, copepods can be considered as zooplankton with a relatively low potential for intrinsic recovery.

Ecotoxicological studies, summarized earlier in this chapter, carried out directly on copepods or on microcosm or mesocosm communities, in general, have shown copepods to be sensitive to a range of insecticides and fungicides. The fact that some studies showed copepod nauplii to be the most sensitive stage, and others showed copepods to be more sensitive than the standard test genus *Daphnia*, implies that copepods should not be overlooked as important indicator species. Mesocosm studies, mentioned earlier in this chapter under section 2.3.3, did not focus on copepods in particular. Instead, they were community level studies carried out to study the effects of toxicants on micro and macroinvertebrate communities in aquatic ecosystems. However, this discovery that copepods are potentially vulnerable organisms, compliments the ecotoxicological work done with marine copepods. This will encourage the realisation of the potential of copepods as important organisms in ecotoxicological research.

There is a great need for well planned, exhaustive studies on copepods in ecotoxicology to bridge the large gap between ecological and ecotoxicological studies on copepods with the goal to identify and consider appropriate representative species for freshwater ecosystems. A modelling approach certainly provides an amicable solution, helping to extrapolate results obtained with a suitable laboratory species, i.e. one with a short life cycle to a more vulnerable species with a longer life cycle and a higher sensitivity. Although this present study primarily focuses on cyclopoid copepods, it can also be extended to calanoids and

harpacticoids using a modelling approach. This would make it convenient to incorporate the life-cycles of other species of copepods and to extrapolate across species.

For the sake of realism in the ERA of pesticides, freshwater copepods need to be considered. SSDs can more effectively be representative of sensitivity when they include more sensitive species (Solomon et al 1996). Along with other sensitive taxa, copepods can also be included in the SSD approach, not only for acute but also for chronic SSDs. To facilitate the consideration of freshwater copepods in ERA there is a need for the development of robust methods and guidelines for studies on important freshwater representative species understandable for risk assessors.

Chapter 3

Life-stage-dependent sensitivity of the cyclopoid copepod *Mesocyclops leuckarti* to triphenyltin

Published in a slightly modified form as

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3.1 Introduction

Ecological risk assessment for plant protection products primarily employs single species toxicity tests in the laboratory (Holmstrup et al 2010). The *Daphnia magna* acute toxicity test is one of the most widely used bioassays for the toxicity screening of chemicals. Within this bioassay, the 48 h EC_{50} value is used (OECD 2004). The same principle is also applied to the risk assessment of fungicides, which have been widely used as plant protection products in the EU (51% of all plant protection products used in the EU-15 in 2003 (EUROSTAT 2007)). The organotin compound triphenyltin hydroxide (TPT), also known as fentin hydroxide, is a non-systemic fungicide and an anti-fouling agent. TPT resists photodegradation and hydrolysis, indicated by half-life values of 93 to 111 d in irradiated water samples and 155 d in dark control samples (Duft et al 2003), and also persists in sediments (Fent et al 1991) with a DT_{50} value of 75 in the field (Footprint, 2011). TPT can enter into the aquatic environment in diverse ways e.g. erosion of contaminated soil particles during events of rain or irrigation (Schulte-Oehlmann et al 2000). Although the use of TPT as a biocide has been banned in the EU (EU, 2003), its widespread use in the past makes it a good model compound for experiments. Several freshwater toxicity studies highlighted both acute toxicity (Dimitriou et al 2003; Stoner, 1966) and long term delayed effects (Roessink et al 2006) of TPT. For the water flea *Daphnia pulex*, the LC_{50} value after a 3 h exposure was 350 µg l^{-1} (Nishiuchi and Hashimoto, 1967). Short-term laboratory single species tests of the effect of TPT on aquatic invertebrates showed the groundwater cyclopoid copepod *Acanthocyclops venustus* to be the most sensitive species with a 96 h LC_{50} value of 0.8 µg l^{-1}, more sensitive than *Gammarus pulex* and *Daphnia galeata* with 96 h LC_{50} values of 12.6 and 16.0 µg l^{-1} respectively (Roessink et al 2006).

Cyclopoid copepods, owing to their complex life-cycle strategies and planktonic occurrence, are potentially vulnerable organisms when exposed to plant protection products, and are therefore good candidates for ecotoxicity tests (Chapter 1). The pelagic freshwater cyclopoid copepod *Mesocyclops leuckarti* is a well-studied copepod in ecology. It has a dominant presence in European freshwaters, is a good representative species for freshwater cyclopoids and can be conveniently reared in the laboratory for ecological and ecotoxicological experiments (Chapter 1). The different life stages of copepods are known to be differentially sensitive to toxicants, with early larval stages usually being more sensitive than the later more mature stages (Brown et al 2005; Verriopoulos and Moraitou-Apostolopoulou, 1982).

Our aim was to study the acute toxicities of TPT to the naupliar, copepodite and adult life stages of *M. leuckarti* after 96 h to investigate whether different life stages of *M. leuckarti* showed differential sensitivity to TPT. We also investigated how the sensitivities of this copepod after 48 h and 96 h differ with respect to TPT exposure.

Recently, the General Unified Threshold model of Survival (GUTS) has been proposed by Jager et al (2011). This model integrates "all previously published toxicokinetic and toxicodynamics models" (Jager et al 2011). The model comprises toxicokinetic dose metrics (external concentration, internal concentration and scaled internal concentration) and toxicodynamic assumptions (stochastic death (SD), individual tolerance (IT)) (Jager et al 2011). We used this model to mechanistically describe the toxic effects of TPT to the different life stages of *M. leuckarti* over time.

3.2 Materials and methods

3.2.1 Test animals

We established three simultaneously running batch cultures of algae, rotifers and copepods using a high quality culture medium, COMBO (Kilham et al 1998), which is suitable for both phytoplankton and zooplankton cultures. Cultures were acclimated for more than six weeks to laboratory conditions i.e. 20±1°C and a light: dark rhythm of 16:8 h. The cultures were shaken or stirred twice every day to ensure sufficient aeration. The algae, *Cryptomonas obovoidea*, were obtained from the Culture Collection of Algae at the University of Cologne (CCAC), Germany. These algae were inoculated into sterile 2 l Erlenmeyer flasks and grown as batch cultures illuminated by cool white fluorescence lights. Cultures of the rotifer *Brachionus calyciflorus* were kindly provided by Dr. Claus-Peter Stelzer, Institute of Limnology, Mondsee, Austria. These rotifers were batch cultured in 5 l beakers. They were fed a combination of the algae *Desmodesmus subspicatus* as used in Klüttgen et al (1994) and *C. obovoidea* in high densities (~15 mg C l^{-1}). Copepods were originally sampled by Ulrich Hopp from a lake in Neu-Ulm, southern Germany as copepodites (CIV-CV instars). These copepodites were transferred to 2 l beakers and fed with a combination of *C. obovoidea* (~15 mg C l^{-1}) and *B. calyciflorus* (~3500 ind l^{-1}) at the Institute for Environmental Research, RWTH Aachen University, Aachen, Germany. Once the animals developed into adults and on mating produced eggs, nauplii that hatched from these eggs were immediately transferred to new 2 l beakers with medium and were fed *C. obovoidea* (~5 mg C l^{-1}). After the batch cultures were established, the naupliar stages and copepodite (CI-CIII instars) were fed only algae (~10 mg C l^{-1}) while mixed food comprising algae and rotifers (~15 mg C l^{-1}) was fed to copepodite (CIV-CV instars) and adults (males and females).

3.2.2 Test substance TPT

The test compound TPT was obtained as solid Triphenyltin hydroxide, also known as fentin hydroxide, from Sigma Aldrich, Germany. 0.5 mg of this odourless white powder was weighed with an Ultra-microbalance (UMX5 Comparator, Mettler Toledo, USA) and subsequently dissolved in approximately 200 ml COMBO medium in a 500 ml volumetric flask. The medium was mixed gently and filled up to the 500 ml mark. The flask was covered in aluminium foil and stirred for 3 h at 750 rpm on a magnetic stirrer. To verify the constancy of exposure conditions over the duration of the different experiments, chemical analysis was conducted for all stock solutions. In accordance with Roessink et al (2006), water samples were first extracted into hexane using HAc + NaAc buffer and 2% sodium tetraehtylborate, and subsequently transferred to GC vials. Chemical analysis was carried out using a GC-MSD (GC: HP Agilent 6890 with auto-injector HP Agilent 7683; MSD: HP Agilent 5973 Network MSD). The analysis revealed that all concentrations were within ±20% of the nominal concentrations. Previous experiments with daphnids and radioactive TPT under the same experimental conditions determined that the concentration of TPT remains constant under these conditions as was measured using liquid scintillation counting and radio-HPLC (Preuss, unpublished). Therefore, all results presented in this chapter are based on the nominal concentrations. A dilution series, between 6.25 and 200 μg l^{-1} with a dilution factor of two, was prepared by pipetting the calculated aliquot of the stock solution (1 mg l^{-1}) for each treatment in 100 ml volumetric flasks, which were subsequently filled with COMBO medium to a final volume of 100 ml.

3.2.3 Acute toxicity tests

Four different acute toxicity tests were conducted to compare the sensitivities of different life stages of *M. leuckarti* to TPT. 40 individual nauplii (24 h old), copepodites (CII-CIV), and adults (males and females) respectively, were used in each treatment. Six different TPT

concentrations 6.25, 12.5, 25, 50, 100 and 200 µg l^{-1} were selected. The naupliar dataset was not tested with 100 and 200 µg l^{-1} because pre-tests showed 100% naupliar mortality at 50 µg l^{-1}. The different life stages of *M. leuckarti* were exposed to TPT in 96-well plates with a volume of 250 µl per test vessel for each organism.

None of the animals were fed during the test. Nauplii were collected prior to the test by separating egg sacs from individual gravid females and placing them in 24 well plates with approximately 2 ml medium in each well. As soon as the eggs hatched, the volume of the medium was slowly and gently reduced in order to concentrate the nauplii in the well. Individual nauplii were subsequently caught in 10 µl medium with a 100 µl Eppendorf pipette under a binocular microscope (Nikon SMZ 1500; Nikon Instruments Europe, England). Copepodite and adult test organisms were isolated with a 45 µm sieve, picked up carefully with 10 µl medium using a 100 µl Eppendorf pipette. The pointed section of the pipette tip was cut off to enlarge the tip opening and minimize the risk of damaging the copepods. The individuals were picked up with 10 µl medium and added to 240 µl test medium in the intended well of the 96-well plate to achieve the calculated TPT test concentration. The test organisms were placed in separate wells during the test to avoid cannibalism and intraspecific stress.

The endpoint immobility (correspondingly survival) of the copepods was checked at nine different time points during the tests (1, 17, 24, 41, 48, 65, 72, 89 and 96 h). At each time point, the test organisms were observed under a stereomicroscope (Nikon SMZ 1500) in an air-conditioned laboratory at a constant room temperature of 20°C to avoid possible impacts of temperature fluctuations on the organisms. LC_{50} values after 48 and 96 h were calculated using the biotest statistics software ToxRat® Professional version 2.10 (ToxRat solutions GmbH, Germany).

3.2.4 The GUTS application

Due to the lack of toxicokinetic data (uptake and elimination rates) to estimate internal TPT concentrations in *M. leuckarti*, the scaled internal concentration was selected as dose metric (*M*) which is given by

$$dC_i^*(t)/dt = k_e\,(C_w(t) - C_i^*(t))$$

where, C_i^* [µg l^{-1}] (internal concentration), C_w [µg l^{-1}] (external concentration) will be equal at equilibrium and k_e [h^{-1}] is the dominant rate constant (Jager et al 2011). We ran simulations with different assumptions of the model to compare the mechanisms of lethality: SD and IT approach. The tolerance distribution of the organisms for the latter was based on a log-logistic function which requires two parameters- α, the median of the distribution and β, the slope of the distribution. The k_e will reflect the slowest compensating process (elimination or damage repair) dominating the overall dynamics of toxicity (Jager et al 2011). The SD model requires the threshold for effects *z* [µg l^{-1}] and a killing rate k_k [l μg^{-1} h^{-1}] in addition to the k_e. When the scaled dose metric exceeds this threshold value, the increased probability of an individual to die in comparison with the control is referred to as the hazard rate (for threshold *z*) which is assumed to increase linearly with the dose metric:

$$h_z(t) = k_k \max(0, M(t) - z) + h_b(t)$$

where, h_b [h^{-1}] is the background hazard rate (Jager et al 2011). The mortality in the control is assumed to be a stochastic event independent of toxic effects and the background hazard for short-term experiments can be assumed to be constant (Jager et al 2011).

The survival of different life stages of *M. leuckarti* over 96 h of exposure was simulated using a Delphi® (Embarcadero Technologies, USA) implementation of the GUTS (Preuss, unpublished). To estimate appropriate parameter values, the GUTS was fitted to the survival data over time for all test concentrations. The calibrations were performed by maximising the likelihood function (Jager et al 2011) using the Simplex-Algorithm (Dantzig, 1963).

Calibrations were run with random start values and those with the best likelihood were selected for parameter estimation. To generate 95% confidence intervals of survival rates over time, the concept of profiling the likelihood function (Meeker and Escobar, 1995) was applied.

3.2.5 Statistical comparison of 96 h LC_{50} values

To test whether there were significant differences between the 96 h LC_{50} values of the different life stages, their confidence intervals were compared as a first step. If the confidence intervals did not overlap then the two quantal endpoints were assumed to be significantly different (Environment Canada, 2005). However, when confidence intervals overlapped, the following equation was used wherein a chi-squared test was performed to test whether or not a significant difference existed between the LC_{50} values of more than two groups,

$$\chi^2 = \Sigma^k_{i=1} w_i (logLC50_i (\Sigma^k_{i=1} w_i logLC50_i / \Sigma^k_{i=1} w_i))^2 \quad \text{(Eq 1)}$$

where k is the number of LC_{50} values and w is the weight (Environment Canada, 2005). The weight w is calculated as

$$w = (1/SE_{logLC50})^2$$

where, SE is the standard error (Environment Canada, 2005).

3.3 Results

3.3.1 Acute toxicity of TPT

Control mortality in all experiments did not exceed 10%. Naupliar control mortality was only seen after 89 h. No control mortality was observed for the copepodites and the males. At the lowest concentration of 6.25 µg l^{-1}, 33% naupliar mortality was seen, while at 12.5 µg l^{-1}, 51% nauplii died after 96 h. At both these lowest concentrations, there were no significant effects observed on the survival of copepodites, adult males or adult females. The adult females showed no significant mortality also at 25 µg l^{-1}. 100% naupliar mortality was seen at

50 µg l^{-1} after 72 h. After 96 h at 100 µg l^{-1}, the mortality of copepodites, adult males and females was 87%, 90% and 85% respectively. At 200 µg l^{-1}, 100% mortality was observed for all stages: after 65 h for the copepodites, and after 48 h for the adult males and females. The LC_{50} values of TPT to different life stages of *M. leuckarti* after 48 h and 96 h are presented in Table 3.1.

LC_{50} values after 48 h for nauplii were lower by a factor of more than 2.5 compared to the other stages. Also, 96 h LC_{50} values for nauplii were lower by more than a factor of 4.5 compared to the other stages. The 48 and 96 h LC_{50} values for nauplii and the other stages differed by a factor of 2 and a factor of 3 respectively. Statistical analysis (section 2.5) revealed that after 96 h, nauplii were significantly more sensitive than all other life stages and that there were no significant differences between the 96 h LC_{50} values for the copepodites, adult males and adult females.

3.3.2 The GUTS calibration

The calibration results for the different stages are shown (Fig 3.1-3.4). At 50 µg l^{-1} for the nauplii and 6.25, 100 and 200 µg l^{-1} for the other stages, both approaches managed to describe the survival trend well. At intermediate concentrations, both the SD and the IT approaches described the trend differently with the SD approach mostly under-predicting survival. The calibrated GUTS parameters are shown in Table 3.2. For the SD approach, the dominant rate constant k_e was the smallest for the nauplii dataset. For copepodites and adults, the k_e values were in a similar range. The k_k values on the other hand for this model were higher for the nauplii compared to the other three stages. For the IT approach, the k_e values did not show significant differences between the nauplii and the other stages. Despite a low k_e for all stages, α for the naupliar stage was lower than the other stages by a factor of 2, with a 95 % confidence interval of 4.74×10^{-7} – 6.21×10^{-7}. However, β values for all the stages were similar.

Table 3.1- Control mortality, LC_{50}, 95% confidence intervals (CI) and slope of the Probit regression curves after 48h and 96 h exposure of different life stages of *M. leuckarti* to TPT.

	Survival rate in control [%] after	48 h		96 h	
	96 h	LC_{50} µg l^{-1} (± 95% CI)	Slope value	LC_{50} µg l^{-1} (± 95% CI)	Slope value
Nauplii	90	33.9 (n.d.)	3.09	10.3 (8.2-12.5)	2.85*
Copepodites	100	96.8 (n.d.)	2.80	51.0 (18.4-143.1)	3.01*
Male	100	81.0 (51.3-144.9)	3.68*	44.8 (21.4-96.6)	2.92*
Female	90	86.1 (73.4-102.6)	3.68*	49.8 (19.8-145.9)	2.85*

Slope values with an asterisk * are significantly different from zero (tested by F-test, p (F) < 0.05). In this case, curves show a significant increase indicating a relationship between concentration and mortality.

n.d. means not determined due to mathematical reasons (according to Fieller's theorem, (Fieller, 1944))

3.4 Discussion

3.4.1 *M. leuckarti* as a test organism

The mortality rate after 96 h in the control did not exceed 10% in any of the tests. No significant differences in the survival of the different life stages in the control could be measured.

This confirms that none of the copepods were stressed during the test by starvation or any other environmental factor that could compromise the toxicity test. *M. leuckarti* in this study showed similar sensitivities to TPT, as was also reported for *D. magna* and *G. pulex* (Roessink et al 2006). Sensitivity, low control mortality and ease of laboratory culture indicate that *M. leuckarti* is a good laboratory species for ecotoxicity tests as suggested before (Chapter 1). The LC_{50} values for all the stages continued to decrease over the test duration. The average 48 h LC_{50} to 96 h LC_{50} ratio for all stages was 2.2, indicating that frequently used time intervals of 24 and 48 h for acute toxicity tests may not be appropriate for tests with

Table 3.2- Calibrated GUTS parameters (with 95% CI) with the mechanisms of SD and IT (coupled with the log-logistic distribution). Calibrations were conducted with the scaled internal concentration as dose metric. R-squared values were calculated for predicted vs measured plots (not shown here).

Parameters for stochastic death					
Life stage	**k_e [h^{-1}]**	**k_k [l μg^{-1} h^{-1}]**	***z* [μg l^{-1}]**	r^2	Likelihood
Nauplii	0.07 (0.05-0.12)	$8.59x10^{-4}$ ($6.6x10^{-4}$-$1.05x10^{-3}$)	1.55 (0-2.74)	0.91	380.78
Copepodite	0.45 (0.43-1)	$1.8x10^{-4}$ ($1.5x10^{-4}$-$2.2\ x10^{-4}$)	8.59 (0-8.95)	0.88	285.25
Male	0.33 (0.12-6.72)	$2.1x10^{-4}$ ($1.7x10^{-4}$- $2.5x10^{-4}$)	5.29 (2.15-6.20)	0.93	245.55
Female	0.47 (0.4-1)	$2x10^{-4}$ ($1.06x10^{-4}$-$2.5\ x10^{-4}$)	10.43 (1-11.66)	0.95	337.57

Parameters for individual tolerance (log-logistic distribution)					
	k_e [h^{-1}]	**α**	**β**	r^2	Likelihood
Nauplii	$3.97x10^{-10}$ ($3.49x10^{-10}$ – $4.57x10^{-10}$)	$5.41x10^{-7}$ ($4.74x10^{-7}$ – $6.21x10^{-7}$)	2.28 (1.91-2.72)	0.93	379.56
Copepodite	$2.38x10^{-10}$ ($1.98x10^{-10}$ – $2.91x10^{-10}$)	$1.18x10^{-6}$ ($9.84x10^{-7}$ – $1.45x10^{-6}$)	1.54 (1.28-1.84)	0.89	276.41
Male	$2.30x10^{-10}$ ($1.99x10^{-10}$ – $2.67x10^{-10}$)	$9.03x10^{-7}$ ($7.85x10^{-7}$ – $1.05x10^{-6}$)	2.09 (1.74-2.50)	0.95	239.78
Female	$2.38x10^{-10}$ ($1.99x10^{-10}$ – $2.83x10^{-10}$)	$1.11x10^{-6}$ ($9.37x10^{-7}$ – $1.32x10^{-6}$)	1.67 (1.38-1.98)	0.95	325.40

copepods using substances like TPT, which have a slow mechanism of action as was also observed before (Roessink et al 2006).

3.4.2 Life-stage-dependent sensitivity of *M. leuckarti*

The R-squared as well as the likelihood values for both the approaches were similar but the difference lay in the meaning of the parameters (Table 3.2). According to the SD approach, the naupliar dataset had the least favourable parameters for survival, with a low k_e, a high k_k and a low *z*. On the other hand, the IT approach showed a slightly higher k_e for nauplii and a slightly lower α. Low individual variability throughout the entire dataset was confirmed by

similar values for β. Overall, the nauplii showed significantly higher sensitivity compared to the other stages (Table 3.1). The lower sensitivity of smaller and earlier life stages of organisms has been demonstrated in literature (Hutchinson et al 1998). The relatively higher sensitivities of nauplii to chemicals other than TPT were also observed in mesocosm experiments (Wendt-Rasch et al 2003, Zafar et al 2012). In general, one assumption is that the lower sensitivity of earlier life stages is due to a higher surface-to-volume ratio leading to faster uptake (Gerritsen et al 1998; Hendricks et al 2001; Preuss et al 2008; Roessink et al 2006). Translating this assumption to the GUTS (with scaled internal concentration as dose metric) would lead to k_e explaining the differences in sensitivity between the different life stages, with a higher k_e implying faster uptake and elimination (Gerritsen et al 1998). It is clear from the parameter values for both the SD and the IT assumptions that this does not necessarily hold true for copepods (Table 3.2). For the SD approach, the nauplii showed a significantly lower k_e, a significantly higher k_k and a lower z compared to the other stages. For the IT approach, a significantly higher k_e and a significantly lower α were observed for the nauplii. Therefore, the surface-to-volume ratio and subsequently the k_e, and α together explain the differences in sensitivity for the IT approach. For the SD approach, the surface-to-volume ratio alone cannot explain the differences in sensitivity. Instead, the k_k and the k_e explain the differences.

Since surface-to-volume ratio alone cannot explain the differences in sensitivity between the life stages, there must be some other process involved with the toxicodynamics of TPT in the nauplii leading to a lower threshold. At the cellular level, TPT targets oxidative photophosphorylation of the production of energy in mitochondria (Chandra et al 1989) and blocks cellular glutathione S-transferases, a detoxification-enzyme of the Phase II metabolism (Al-Ghais and Ali, 1999).

We assume that the mode of action of TPT in the mitochondria of nauplii will be same as the interaction in the mitochondria of adults. Nonetheless, the log K_{OW} of TPT is 3.53 indicating lipophilicity (Arnold et al 1997). Thus, the TPT that is taken up by the older stages may move into the body lipids (Klosterhaus et al 2003) and not produce as rapid an effect in older stages as in the nauplii. The higher tolerance of late copepodites and adults to TPT exposure may be a consequence of their higher lipid contents as was observed for other toxicants (Klosterhaus et al 2003). Additionally, the nauplii expend energy on both development and maintenance while the adults require energy for maintenance and reproduction (Kooijman, 2010). Under adverse conditions, it may be more economical for the adult animals not to lay eggs, thereby saving energy and keeping their energy budget balanced (Kooijman, 2010), which is not an option for the nauplii. This may lead to the effect of TPT being comparatively more pronounced on nauplii than on the older stages. All these factors may collectively contribute to a higher threshold in the late copepodites and adults compared to the nauplii.

In comparison with the case studies presented in the original GUTS paper (Jager et al 2011), the k_e values we found using the IT approach were significantly lower than those found using the SD approach. The k_e values can be interpreted meaningfully only in conjunction with the z or the β values and not by direct comparison between the two approaches.

We presume that the different k_e values obtained using the SD and the IT approaches are properties of the respective models. As was indicated by Jager et al (2011), it is still not possible at this stage to say which of the two approaches, SD or IT, is more realistic in an absolute sense. This can be ascertained by laboratory experiments. Further research on different calibration routines and calibrations using several different datasets for copepods and other organisms will provide new insights into this issue.

This study however, shows the potential of using the GUTS to understand how toxic compounds interact with different life-stages. To further elucidate this, it is necessary to segregate the toxicokinetics and the toxicodynamics of TPT and to measure the toxicokinetics explicitly, taking into account the distribution of TPT between the lipid fraction and the sites of action.

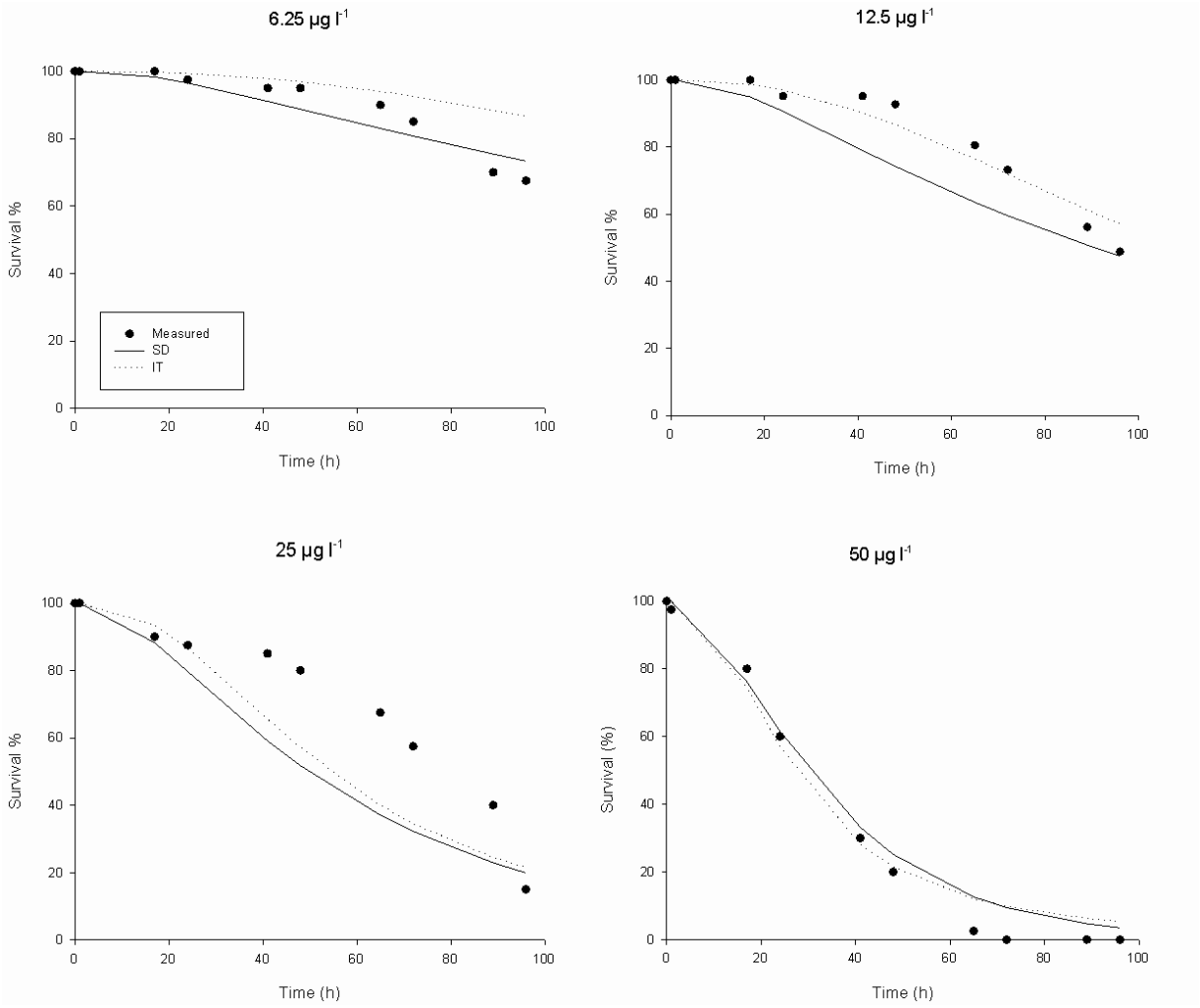


Fig 3.1- Survival rates of nauplii of *M. leuckarti* at different TPT concentrations as simulated by the two mechanisms of lethality- SD and IT. The lines are the GUTS simulations and the points are measured data.

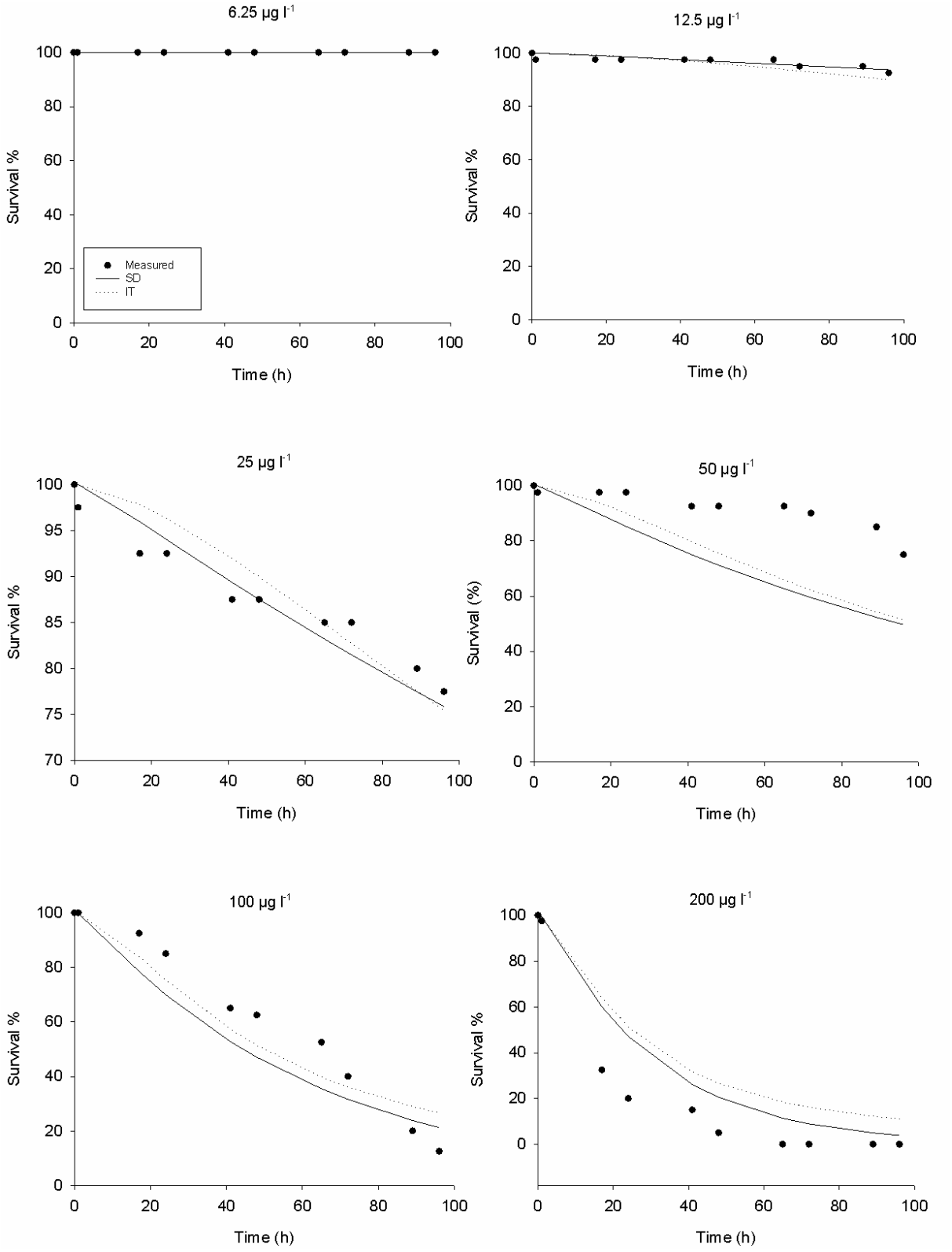


Fig 3.2- Survival rates of copepodites of *M. leuckarti* at different TPT concentrations as simulated by the two mechanisms of lethality- SD and IT. The lines are the GUTS simulations and the points are measured data.

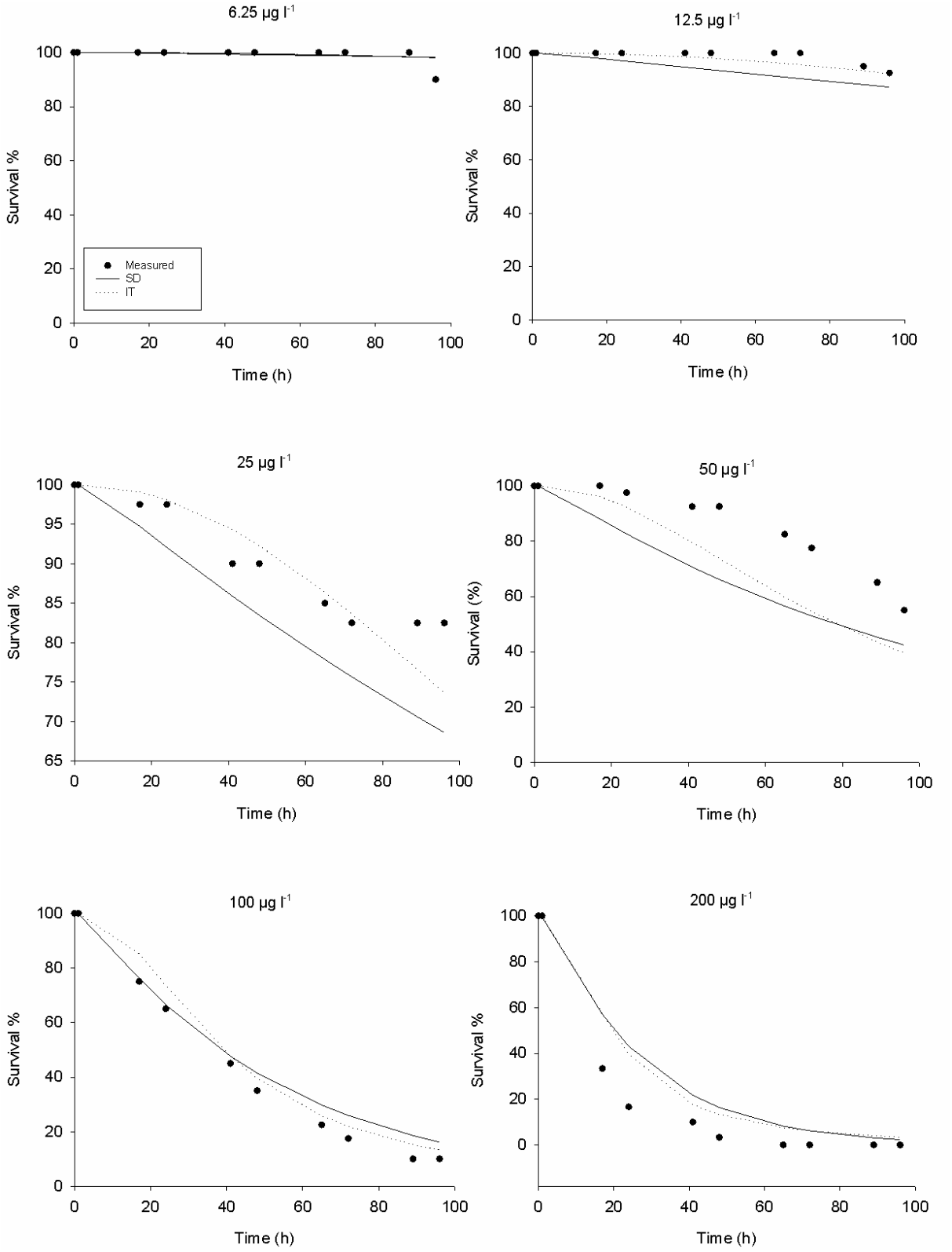


Fig 3.3- Survival rates of adult males of *M. leuckarti* at different TPT concentrations as simulated by the two mechanisms of lethality- SD and IT. The lines are the GUTS simulations and the points are measured data.

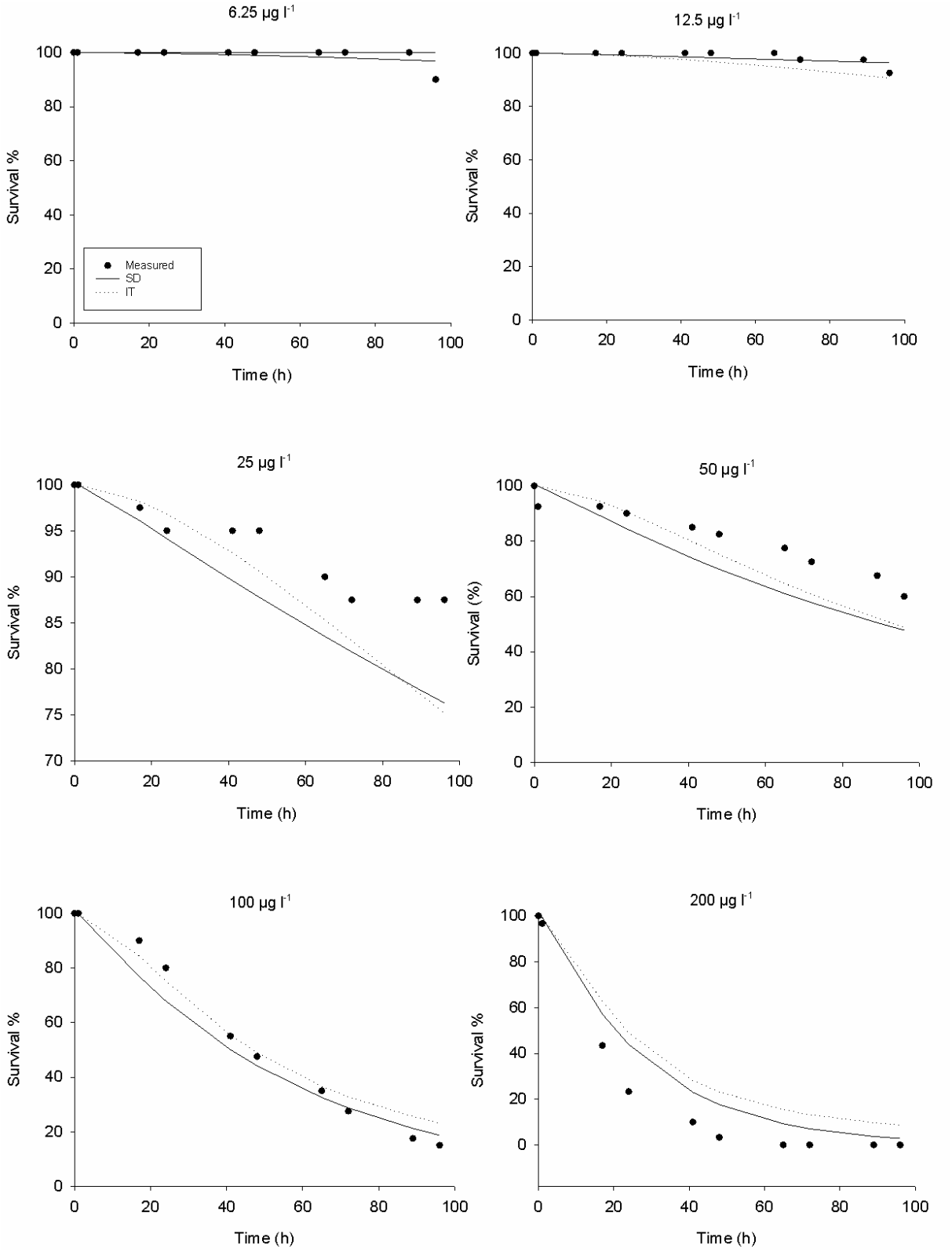


Fig- 3.4 Survival rates of adult females of *M. leuckarti* at different TPT concentrations as simulated by the two mechanisms of lethality- SD and IT. The lines are the GUTS simulations and the points are measured data.

3.5 Conclusions

M. leuckarti is a good laboratory species for ecotoxicity testing. The naupliar stages of *M. leuckarti* are significantly more sensitive to TPT than the later stages. For the IT approach, the k_e and α together explain the differences in sensitivity, whereas for the SD approach, the k_k and the k_e explain the differences in sensitivity for the different life stages of *M. leuckarti*.

The GUTS application demonstrated that the higher sensitivity of nauplii may be due to a lower intrinsic threshold compared to the later stages. Toxic effects of TPT on copepods after 96 h were considerably higher than those after 48 h and therefore, for tests with substances with a slow mechanism of action like TPT on copepods, 24 or 48 h quantal endpoints may not be appropriate. Further experiments to provide a more detailed understanding of toxicokinetics of TPT in copepods could be useful.

Chapter 4

Eco-physiological triggers of population-level sensitivity to chemical stress in *Mesocyclops leuckarti*

4.1 Introduction

Ecological risk assessment purposes to quantify risks posed by substances released into the environment (EEA 1998). Current schemes for ecological risk assessment (ERA) are based on organism-level endpoints which are vastly different from the ecosystems they want to protect (Forbes et al 2011). Therefore, within the higher tiers of ERA, population-level endpoints are being considered (EFSA 2013). At the population level, the phenomenon of density dependence is one of the most important ecological processes that influence population dynamics (Fowler 1981; Preuss et al 2009; Hazlerigg et al 2013; Sibly et al 2000). While this process has been widely explored in ecology, it has yet to be explored in detail from an ecotoxicological perspective. There are different density-dependent processes that moderate the dynamics of populations, namely- bottom-up or top-down. Bottom-up processes are triggered by food availability (e.g. Rublee 1992) whereas top-down processes are consequences of predation (Estes et al 2001). Additionally, there exist self-regulating mechanisms like cannibalism (e.g. in copepods (Lazaretto and Salvato 1992)) or reduced reproduction and variation in brood number and size in response to high population size (e.g 'crowding effects' in daphnids (Cleuvers et al 1997)).

In this study, we investigated how two different mechanisms of density dependence would influence population-level ERA of the cyclopoid copepod *Mesocyclops leuckarti*. *M. leuckarti*, owing to its intrinsic sensitivity as well as its population-level vulnerability, was suggested as a good representative species of freshwater cyclopoid copepods for ecotoxicity tests in the laboratory (Chapter 2, Chapter 3). The life cycle of copepods is complex, characterized by different developmental stages, sexual reproduction, and diapause under unfavourable environmental conditions (Santer 1998). Copepod feeding habits are also complex (Price 1988) and the life-history strategies of copepods are influenced by the quality

and quantity of food available (Hansen and Santer 1995; Hopp et al 1997; Maier 1994; Vijverberg 1989). There are two important aspects of copepod feeding biology that have profound implications for population dynamics- food switching during the copepodite stages and cannibalism- (1) Food switching is a complex strategy employed by many copepods. Many copepod species have been known to be herbivorous in the earlier stages and to switch to omnivory or carnivory in the later stages (Adrian and Frost 1993; Santer 1998, Vijverberg 1989). Adult stages of some copepods require animal food, more than others, to produce eggs (Hopp et al 1997; Hansen and Santer 1995). (2) Cannibalism is an important characteristic of many copepod species (e.g. Lazaretto and Salvato 1992; Havel 1980; Daan 1988). In the presence of low densities of animal prey, cyclopoid copepod species have been known to show cannibalism to obtain animal proteins needed for egg production (Hansen and Santer 1995).

How do these two mechanisms of density dependence affect extrapolation from individual-level effects of toxicants to the population level? To answer this question we used a combination of laboratory experiments and individual-based modelling. Ecological models are increasingly being used to highlight ecological risk and to extrapolate from individual-level laboratory-based scenarios to realistic population-level conditions in the field (Forbes et al 2011). Individual-based modelling is a powerful tool to mechanistically model individual-level processes and extrapolate them to the population level (Grimm and Railsback 2005). Within this approach, individuals are attributed different properties with respect to their life-history traits and population dynamics emerge as consequences of individual-level variability (DeAngelis and Mooij 2005).

Here we present an IBM developed for *M. leuckarti* to simulate population dynamics under toxicant exposure based on data from laboratory experiments. We used laboratory

experiments to parameterise the IBM. Parameters for modelling eco-physiological processes like feeding, development, reproduction and survival were determined under different feeding regimes for the three different life stages of *M. leuckarti*. Data from toxicity experiments with triphenyltin (TPT) (Chapter 3) were used to calibrate the toxicity submodel. The main aims of this chapter are to determine which life-cycle processes based on feeding strategies are most influential in determining density dependence in *M. leuckarti* under triphenyltin (TPT) exposure and to explore how this mechanism affects extrapolation from individual-level effects to the population level.

4.2 Materials and methods

4.2.1 Laboratory experiments

Feeding, development, reproduction and survival experiments were conducted for the nauplii, copepodite (early copepodite stages I to III and late copepodite stages IV and V) and adult stages. Two different feeding regimes were tested, a pure algal diet and a mixed diet of algae and rotifers. The mixed diet was only tested with late copepodite stages and adults because pre-tests showed that lower copepodite stages and nauplii did not feed on rotifers. Therefore, a similar procedure was also used for cannibalism experiments with nauplii. Laboratory cultures (alga-*Cryptomonas obovoidea*, rotifer-*Brachionus calyciflorus* and copepod-*Mesocyclops leuckarti*) were already established and acclimated for more than six weeks to laboratory conditions i.e. 20±1°C and a light: dark rhythm of 16:8 h. Details of the establishment and maintenance of laboratory cultures can be found in (Chapter 3). These cultures were used for all the following experiments. Algal concentrations in the medium were estimated by measuring fluorescence using the Tecan Infinite® M 200 PRO series microplate reader and correspondingly plotting a calibration curve against cell number counted using the Bürker-Türk counting chamber. The total organic carbon content of *C.*

obovoidea was measured as a difference between total carbon and total inorganic carbon using the Ströhlein C-MAT 5500 carbon analyzer at the Fraunhofer IME, Schmallenberg, Germany. Toxicity was implemented into the model using the General Unified Threshold model of Survival (Jager et al 2011) using results from previous laboratory toxicity experiments, as has been described in Chapter 3.

4.2.1.1. Feeding experiments

Feeding experiments on a pure algal diet were performed for all stages

Feeding on algae

Algal concentrations in the series 1, 2.5, 5, 10, 15, 20, 25, 30 mg C l^{-1} were used for all copepod feeding experiments with algae. The experiments were carried out in 20 ml glass bottles. Each bottle was filled with 10 ml medium with the respective algal concentration. 20 nauplii, 5 copepodites and 5 adults were separately introduced into each test bottle. There were three replicates and one control per food concentration. The animals were allowed to feed over a 24 h period and fluorescence was measured. The ingestion rates of the copepods were first calculated as:

$$Ingestion\ rate = \frac{C_c - C_t \;\; V}{N \; t}$$

where C_c and C_t are the algal concentrations in the test and the control, *V* is the volume of the sample, *N* is the number of rotifers, and *t* is the time in hours.

These calculated ingestion rates were plotted against the food concentration and the different Holling equations (Holling 1959a, 1959b, 1966) were used to simulate the functional response

of *M. leuckarti* using SigmaPlot 12. This has been expanded upon in the section Submodels (4.2.2.6).

Feeding on rotifers

Feeding experiments with *B. calyciflorus* were carried out in similar glass bottles but without algae. Five early copepodites (CI-CIII instars), five late copepodites (CIV-CV instars) and five adults were separately placed in glass bottles containing 10 ml medium and different numbers of *B. calyciflorus*- 1, 3, 5, 7, 9, 10. The bottles were observed after 24 h for the presence of living rotifers. If no living rotifers were observed, they were considered ingested. Cannibalism experiments were carried out for the late copepodites and adults with 5 replicates of 10 nauplii each and one set of controls. Cannibalism experiments were not carried out with early copepodites and nauplii because pre-tests showed no cannibalistic behaviour in these stages.

4.2.1.2. Development experiments

Development experiments were carried out in transparent 24-well plates (Fischer Scientific, Germany) with 2 ml COMBO medium and one animal in each well. Ten animals per food concentration were used. The medium was renewed every day and food in the desired test concentrations was provided. Two different food regimes were tested- a pure algal diet and a mixed diet of algae and rotifers. Five different algal concentrations were tested- 1, 2.5, 5, 10, 15 mg C l^{-1}. For the experiments on a mixed diet, seven rotifers per food concentration were provided. Development times were measured for nauplii (NI-NVI), early copepodites (CI-CIII), and late copepodites (CIII-CVI).

4.2.1.3. Reproduction experiments

Egg production by *M. leuckarti* on the algal and mixed regimes was also tested in transparent 24-well plates. For the pure algal diet, the concentrations tested were similar to the

development experiments. However, for the mixed diet, 12 different food concentrations were tested- one set of experiments had 0.5 mg C l^{-1} with 1, 3, 5, 7 and 9 rotifers, and the other set had seven rotifers with the five different algae concentrations as in the development experiments. 170 copepodites (CV instars) were isolated and acclimated to the different experimental food regimes until they developed into adult females. Once these developed into adult females, they were introduced into new wells containing adult males to mate. The females were introduced into the test wells after mating. There were 10 replicates per food concentration for both food regimes. Egg production was observed up to four broods. The medium and corresponding food concentrations were renewed daily.

4.2.1.4. Survival experiments

Survival times for nauplii (NI-NVI), early copepodites (CI-CIII), late copepodites (CIII-CVI) and adults were measured on a pure algal diet using the same algae concentrations as in the development experiments. For the mixed diet regime, survival times of late copepodites and adults were measured with seven rotifers and the five algae concentrations as in the development experiments.

4.2.2 Model description

The following model description follows the ODD (Objects, Design concepts, and Details) protocol (Grimm et al 2006; Grimm et al 2010).

4.2.2.1 Purpose

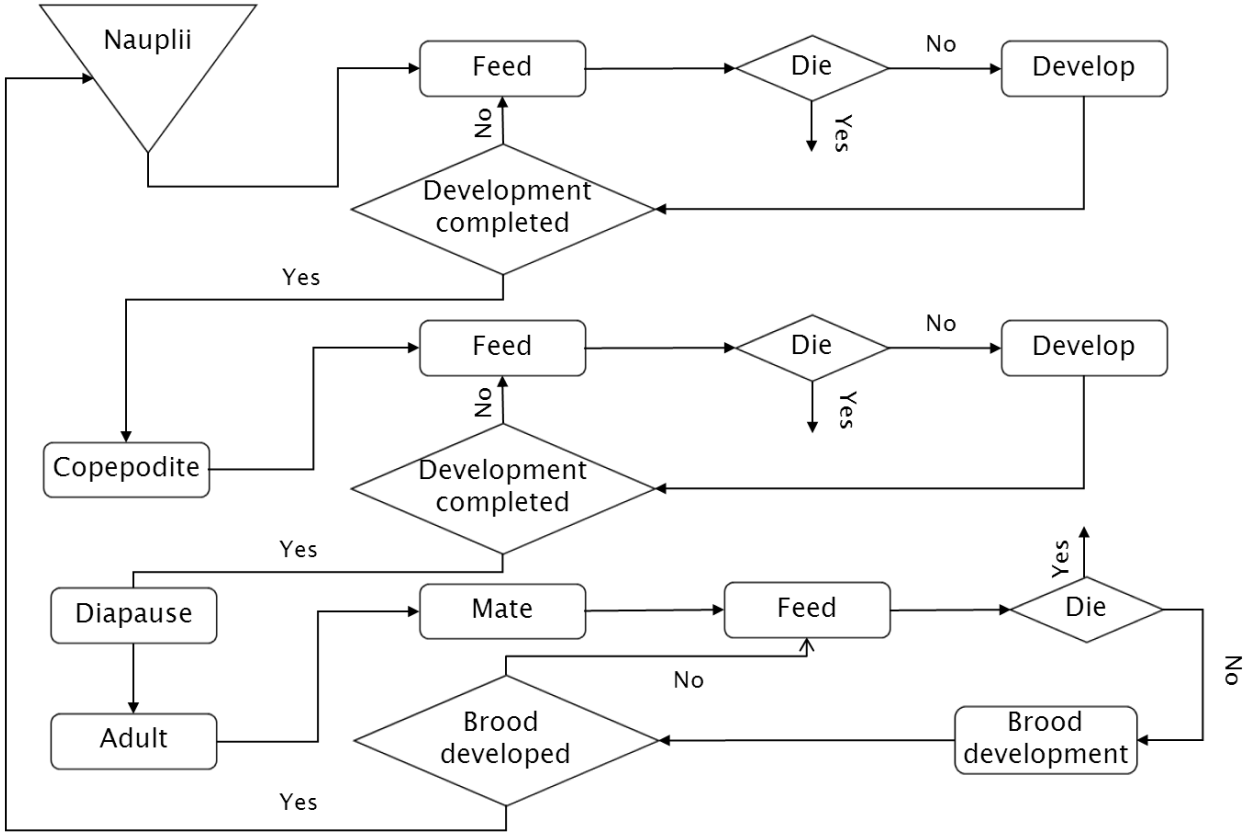
The purpose of the model is to simulate the population dynamics of *M. leuckarti* under TPT exposure and to compare the influence of different density-dependent processes on the risk assessment at the population level.

4.2.2.2 Entities, state variables and scales

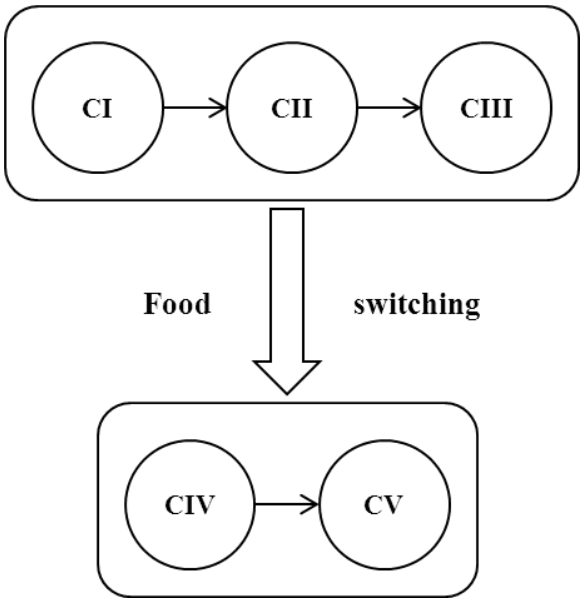
The model includes two types of entities, the environment and the individuals. The environment is characterised by food concentration (algae or number of rotifers), the volume of the test vessel and feeding regime. The life cycle of each individual (Fig 4.1a and 4.1b) is modelled to describe the changes to the state variables (Table 4.1) dependent on food (Table 4.2). The state variables vary with respective individual properties and therefore, the model output is stochastic. The vessel volume (and correspondingly the volume of the medium) is 10 l with no inflow or outflow of medium or organisms. The algae, rotifers and copepods are homogenously distributed within the medium. The model proceeds in discrete daily time steps, with the overall simulation time being 365 days. Space has not been represented in the model.

4.2.2.3 Process overview and scheduling

Within one day the following processes are executed in subsequent order: update of the environment (algae and/or rotifers are added to the environment, change in algae concentration as a result of feeding is calculated, as well as the remaining food from the previous day is carried over; temperature dependency is implemented contingent on the day of the year), counting of the number of individuals in each stage, feeding (algae (ingestion rates are calculated), rotifers (rotifer growth rate is calculated), cannibalism on nauplii), development (creation and development of individual copepods in different life stages based on food concentration and food regime, diapause at CIV stage depending on temperature), survival (depending on temperature, food concentration and food regime), reproduction (update latency, brood size, brood number depending on temperature, food concentration and food regime), initiate toxicity (run GUTS simulations for different stages), discard dead copepods. Algae and the rotifers remaining in the environment at the end of the simulation day are added to the total food in the vessel at the beginning of the next day.



(a)



(b)

Fig 4.1- (a) Conceptual diagram of the sexual life cycle of *M. leuckarti*. The rectangles indicate individual-level processes and queries are indicated in rhombi (b) Segregation of the copepodite stages of *M. leuckarti* in the model based on feeding habits.

4.2.2.4 Design concepts

Emergence

The model does not include any emergent behaviour of individuals; rather, individual variability and the response of individuals to changing food conditions and the toxicant are imposed by empirical rules derived from laboratory experiments described in this chapter as well as other publications (Preuss et al 2011, Chapter 3).

Interaction

Individuals interact indirectly via competition for food. The only direct interaction in the model is the cannibalising of nauplii by the late copepodites and the adults. Thus, cannibalism is the only direct interaction in the model.

Stochasticity

To represent individual variability, all individual-level life-cycle traits have been modelled in a stochastic way (Table 4.1). The corresponding distributions and their parameters were derived from experimental data.

Observation

Food concentration, population structure and size are determined at the beginning of each time step. For each female, the total number of newborns over four broods is observed. The arithmetic mean, minimum and maximum values per day are calculated for the population abundance from the population properties saved for each run for Monte–Carlo simulations. The mean of 100 Monte-Carlo simulations is calculated.

4.2.2.5 Initialization

The initial state for the control simulations is- vessel volume 1 l, population structure 25 nauplii and 5 adults, 1000 rotifers, algae concentration of 0.5 mg C l^{-1}, growth rate of rotifers 0.52 d^{-1} and 0 µg l^{-1} TPT. When other initial conditions were used, they are mentioned in the corresponding figure captions.

Table 4.1- State variables and parameters of the model for *M. leuckarti*

State variables	Description	Parameter/ variable	Unit	Mean	SD
Individual					
N_{dev}	Naupliar development	pN_{dev}	d^{-1}	0.111	nND
$CI\text{-}CIII_{dev}$	Early copepodite development	$pCI\text{-}CIII_{dev}$	d^{-1}	Eqn	nND
$CIII\text{-}CVI_{dev}$	Late copepodite development	$pCIII\text{-}CVI_{dev}$	d^{-1}	Eqn	nND
$mixCIII\text{-}CVI_{dev}$	Late copepodite development on mixed food	$pmixCIII\text{-}CVI_{dev}$	d^{-1}	Eqn	nND
L	Latency	pL	d^{-1}	0.25	0.06
E_{dev}	Embryo development	pE_{dev}	d^{-1}	0.29	0.14
BS	Brood size	pBS	-	Eqn	
	Number of broods per female	pNB	-		
S	Survival probability	pS_{Nau}	d^{-1}	0.05	-
		$pS_{CI\text{-}CIII}$	d^{-1}	Eqn	-
		$pS_{CIII\text{-}CVI}$	d^{-1}	Eqn	-
		$pSmix_{CIII\text{-}CVI}$	d^{-1}	Eqn	-
		pS_{Adu}	d^{-1}	Eqn	-
		$pSmix_{Adu}$	d^{-1}	Eqn	-
Gender	Male/female		-		
Environment					
vol	Volume of the vessel		ml		-
algae_conc	Algae concentration		mg C l^{-1}		-
total_ingrot	Total number of rotifers in the vessel		-		-
Cw	Concentration of TPT in water		µg l^{-1}		-

nND: Parameters were not normally distributed; distributions have been given under submodels.

All parameters were calibrated on experimental data.

dev: development; SD: Standard deviation; -: not applicable; Eqn: parameter values have been described as equations depending on the food regime.

Mixed feeding and cannibalism can be selected by checking the respective checkboxes during initialization. Pure algae feeding can be selected by leaving the checkboxes for mixed feeding and cannibalism unchecked as well as changing initial number of rotifers, rotifer growth rate and rotifer threshold to zero.

Table 4.2- Equations for individual variables of *M. leuckarti*

Variable	Equation	r^2
$pCI\text{-}CIII_{dev}$	$\mathbf{0.3566(1 - exp(-81.5578C))}$	0.91
$pCIII\text{-}CVI_{dev}$	$\mathbf{0.1908C\ \ (0.003 + C)}$	0.86
$pmixCIII\text{-}CVI_{dev}$	$\mathbf{0.2574(1 - exp(-128.0446C_1))}$	0.94
pBS	$\mathbf{41.5158C_B\ \ (0.0044 + C_B)}$	0.86
$pS_{CI\text{-}CIII}$	$\mathbf{0.001 + 0.0722(-exp(-81.5578C))}$	0.99
$pS_{CIII\text{-}CVI}$	$\mathbf{0.089 * 0.0087\ \ (0.0087 + C)}$	0.80
$pSmix_{CIII\text{-}CVI}$	$\mathbf{0.0395 * 0.0013/(0.0013 + C_1))}$	0.95
pS_{Adu}	$\mathbf{0.0934 * 0.0056\ \ (0.0056 + C_A)}$	0.83
$pSmix_{Adu}$	$\mathbf{0.0103 + 0.4081exp(-915.068C_B)}$	0.99

C, C_A: ingestion rates for the respective stages on a pure algal diet; C_1, C_B: ingestion rates on a mixed diet

4.2.2.6 Submodels

Update environment

Algae and rotifers are added to the vessel at the beginning of the simulations. From the second day onwards the algae and rotifers remaining from the previous day are carried over. The change in algae concentration as a result of feeding is calculated by Euler integration in steps of 1 min and then summed up for the entire day as follows:

$$\frac{dC_a}{dt} = \frac{C_i - I.R.}{vol}$$

where, C_a is the algae concentration in the test vessel and C_i is the initial algae concentration provided and *I.R.* is the ingestion rate. The change in the number of rotifers is brought about by a combined effect of feeding as well as growth of the rotifers. The growth rate of the rotifers (d^{-1}), as recalculated from Stemberger (1990) is given by

$$brachi\_gr = -1.1145 + 1.6172(1 - 0.1126^{\frac{A}{V}})$$

where, *A* is the total algae in the medium and *V* is the volume of the vessel, and has been implemented in the model. However, a fixed growth rate of 0.54 d^{-1} was assumed for the present study. The remaining rotifers and algae from the previous day are added to the total food on the next day. The upper threshold value for the number of rotifers in the vessel has been set to 3000 ind l^{-1}. An annual temperature (ºC) dependency was implemented in the model using the following equation by Straškraba and Gnauck (1983)

$$T = 11.1 + 10.165 \sin(day + 240)$$

This equation considers the variation in annual temperature in European freshwater bodies. The empirical relationship between copepod development and survival, and temperature was recalculated from Hart (1990), and a Bělehrádek function was fitted to it.

$$dt = \frac{250(t + 7.2)^{-1.86}}{0.5365891}$$

This was implemented in the model by dividing the development, survival and reproduction parameters by by the change in temperature given by the Bělehrádek function.

Feeding

The calculated ingestion rates were plotted against the food concentration in SigmaPlot 12 to simulate a Holling type functional response (Holling 1959a, 1959b, 1966). *M. leuckarti* exhibited a Holling type III functional response (Fig 4.2):

$$I.R. = a * (\frac{C^2}{C^2 + b^2})$$

where, *C* is the algal concentration and *a* and *b* are parameters specific to each stage. No differences between tests and controls were detected after 24 h in the naupliar feeding experiments.

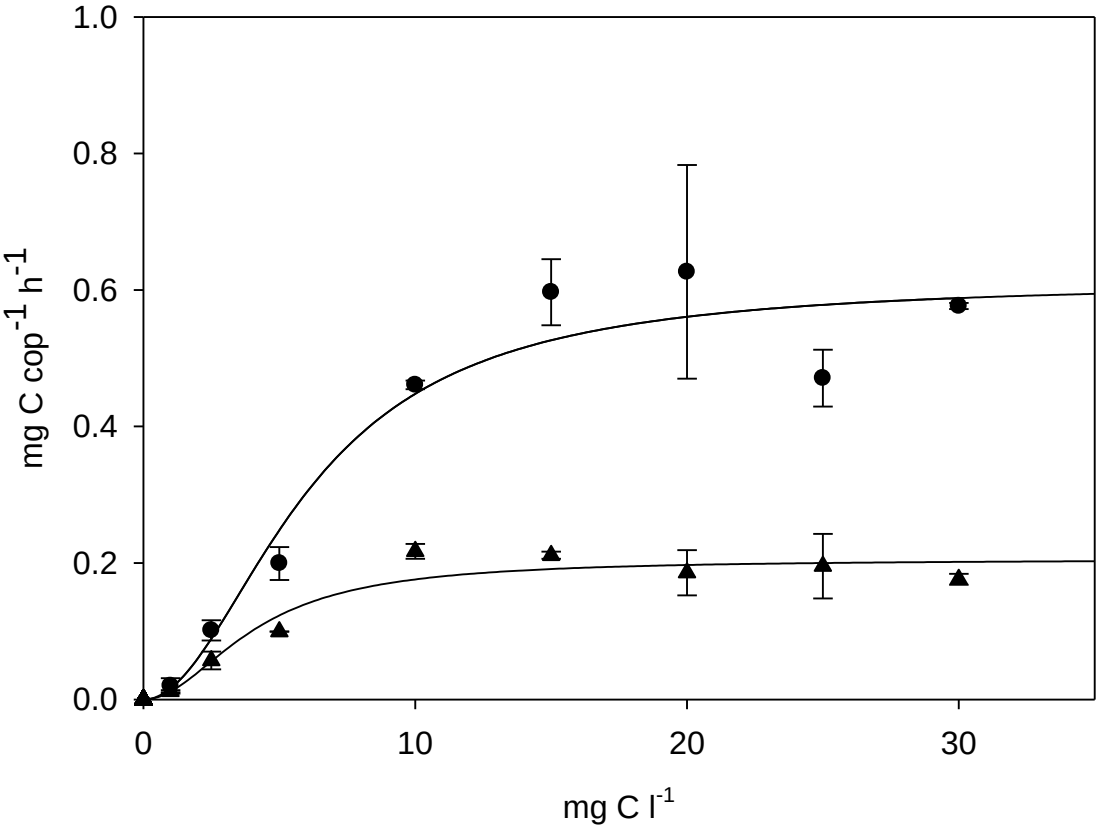


Fig 4.2- Feeding rates of copepodites (Triangles) and adults (Circles) on a pure algal diet of *C. obovoidea*.

Therefore, it is assumed in the model that the naupliar stages do not feed. The parameters for the Holling type III functional response curve as observed for the different stages of *M. leuckarti* are shown (Table 4.3).

Table 4.3- Holling type III functional-response parameters for *M. leuckarti* copepodite and adult life stages.

Parameter	Copepodites	Adults
a	0.2053	0.6121
b	4.0868	6.0602

In the feeding experiments on rotifers, all rotifers in the bottles with adults and late copepodite instars were consumed. Therefore, a maximum of ten rotifers or nauplii are consumed per late copepodite and adult. However, in the bottles with early copepodite instars, less than 5% rotifer mortality was seen and therefore, we assumed that these stages did not feed on rotifers. Cannibalism experiments showed that all nauplii were consumed after 24 h. 0% control mortality was seen in these experiments. From feeding experiments on rotifers and cannibalism experiments on nauplii, it was observed that the late copepodites and adults consumed all of the ten rotifers or nauplii added. Therefore, a maximum of 10 nauplii are cannibalised per copepod in the model. For the sake of convenience, the carbon content of rotifers and nauplii has been assumed to be the same i.e. 0.00029 mg C (Dumont 1975). In the model, the total carbon from animal food is added to the carbon obtained from the ingestion rates on the available algae and these values are used to calculate development variables. Each late copepodite and adult consumes a maximum of 10 animals (rotifers or nauplii). Cannibalism of the nauplii by the late copepodites and adults has been implemented in the model. This probability of encountering rotifers and nauplii is calculated based on their densities in the vessel. The late copepodites and adults feed on either rotifers or nauplii whose numbers are effectively reduced from the vessel.

Development

Development parameters (Fig 4.3) were calculated as described in Preuss et al (2011). Separate development rates were calculated for nauplii (NI-CI), early copepodites (CI-CIII), late copepodites (CIII-CVI) (Table 4.2). The daily development rates were calculated as follows:

$$\frac{\Delta DEV_N}{\Delta t} = pN_{dev*rdm\_Dev_N}$$

$$\frac{\Delta DEV_{CICIII}}{\Delta t} = pCICIII_{dev*rdm\_Dev_{CICIII}}$$

$$\frac{\Delta DEV_{CIIICVI}}{\Delta t} = pCIIICVI_{dev*rdm\_Dev_{CIIICVI}}$$

where, rdm_Dev is a number given to the individual at birth (Preuss et al 2011). Values for indices of development (ID), as proposed by Soussi and Ban (2001), were calculated for each stage over the entire range of the food concentrations tested.

$$ID = 1 + \frac{D - M(D)}{M(D)}$$

where *M(D)* is the median duration of a particular development stage and *D* is the duration of an individual in this development stage. The values generated have a median of 1 (Soussi and Ban, 2011) and are multiplied, later on within the IBM, with the development rate.

Gamma distribution parameters (which cannot be negative) were estimated (Table 4.4) because the distribution of ID values failed the normality test as stated by (Preuss et al 2011). The gamma distribution was implemented in the model to deal with individual variability as stated by (Preuss et al 2011). Diapause is initiated in the copepods when the temperature falls below 7ºC. Diapause has not been tested in laboratory experiments. The assumption of diapause has been made based on cues from literature (Santer and Lampert, 1995; Hopp and Maier 2005). Diapause is dependent on the annual change in temperature that has been incorporated into the model. During diapause, the copepods do not show any feeding or development and are in a state of inactivity. Diapause occurs only at the copepodite IV stage.

Naupliar development was independent of the range of food concentrations tested. Furthermore, naupliar development data, similar to the other stages, were not normally distributed. Therefore, Dixon's Q test (Dean and Dixon 1951) was performed.

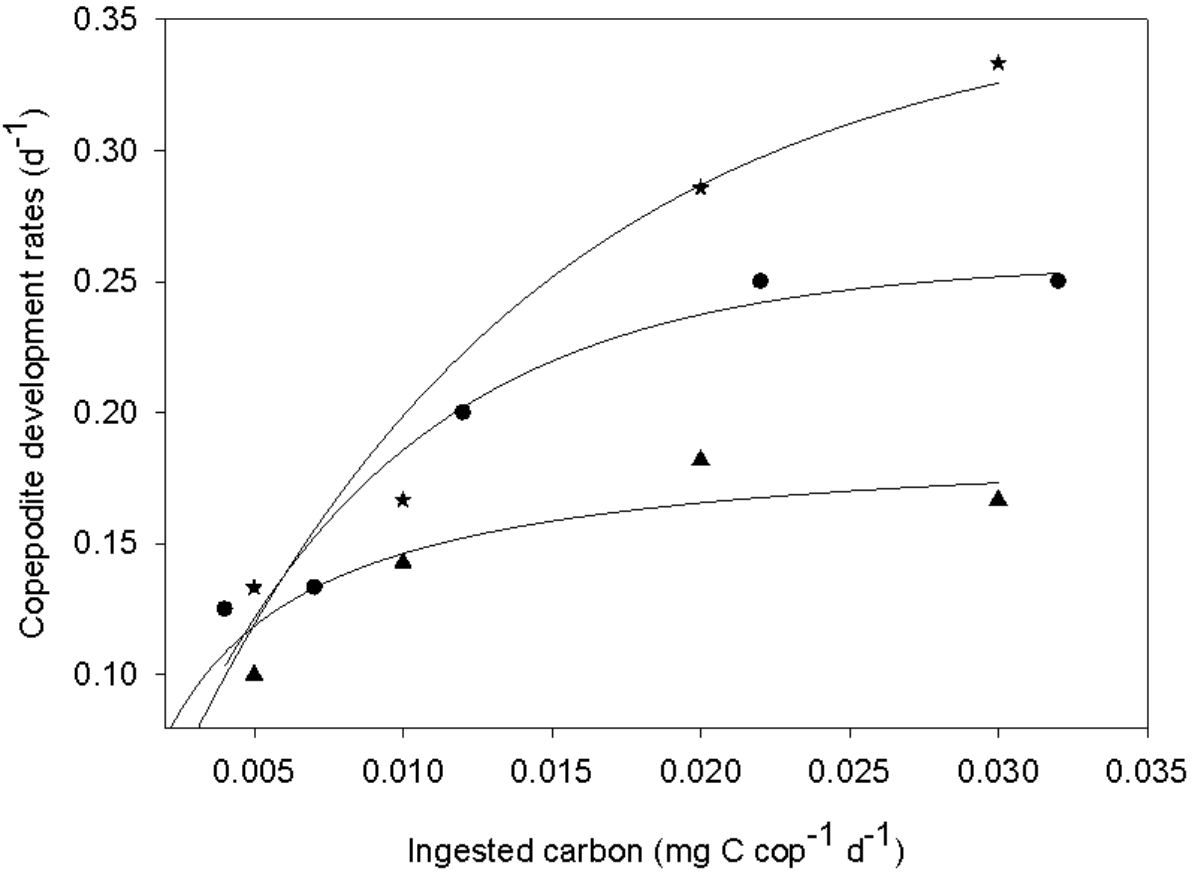


Fig 4.3- Early and late copepodite development parameters on algal and mixed diets. Early copepodite pure algae (star) late copepodite mixed (circle) late copepodite pure algae (triangle).

Table 4.2- Distributions and the respective parameters to simulate individual variability for development rates of *M. leuckarti*

		Gamma distribution		Normal distribution		
	Median	α	β	Mean	SD	N
NI-CI	*Table 4.2	102.12	0.0097	0.99	0.1003	44
CI-CIII	*Table 4.2	10.16	0.0932	0.95	0.2894	43
CIII-CVI	*Table 4.2	5.72	0.2581	1.48	0.6288	39
mixCIII-CVI	*Table 4.2	10.06	0.0952	0.96	0.3112	46

*Table 4.2- Equations given in Table 4.2.

The outlier value for development rate 0.117 d^{-1} was identified and rejected, and the development rate was set to 0.111 (Table 4.1) and implemented in the model. All development descriptors are divided by the change in temperature given by the Bělehrádek function.

Reproduction

Reproduction in adult female copepods is influenced by latency (time from mating up to fertilisation), embryo development, brood size and the number of broods in the model.

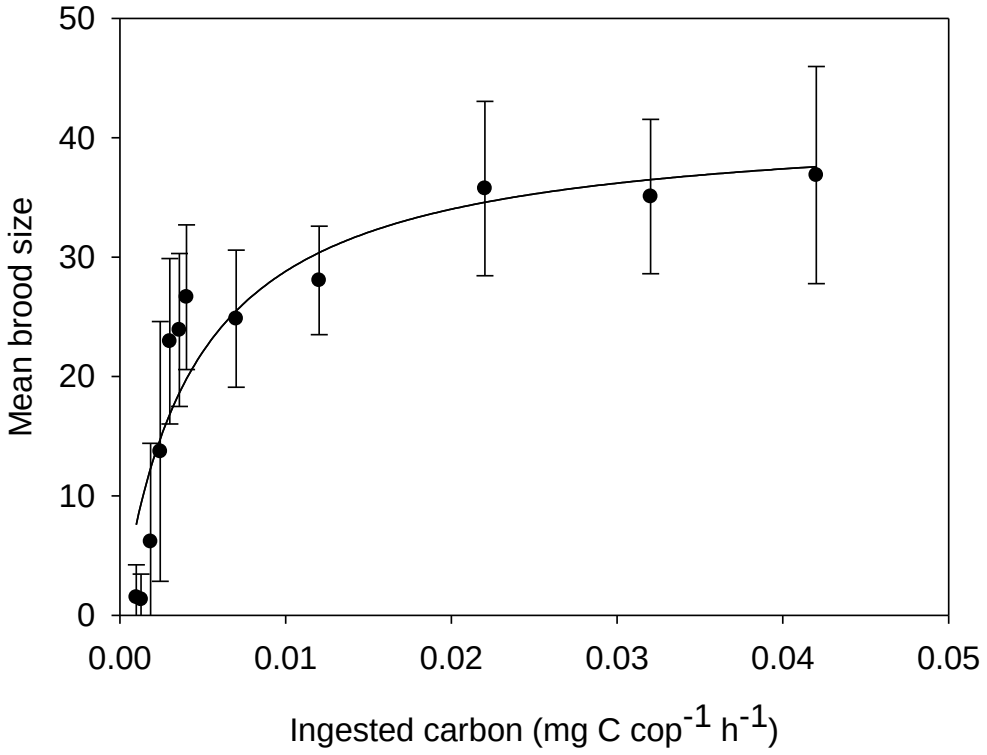


Fig 4.4- Mean brood size of *M. leuckarti* on a mixed diet.

Latency and embryo development were calculated and normal distributions were fitted to the data according to Preuss et al (2011). Brood number was restricted to four broods because a previous study on the reproductive parameters of *M. leuckarti* has shown a mean of 3.7 clutches per female under mixed feeding conditions (Hopp et al 1997). Mean brood size over

four broods (Fig 4.4) was calculated and used to parameterize the equation for brood size at different food concentrations (Table 4.2). A sex ratio of 1:1 is assumed for the male and the female copepodites. All reproduction descriptors are divided by the change in temperature given by the Bělehrádek function.

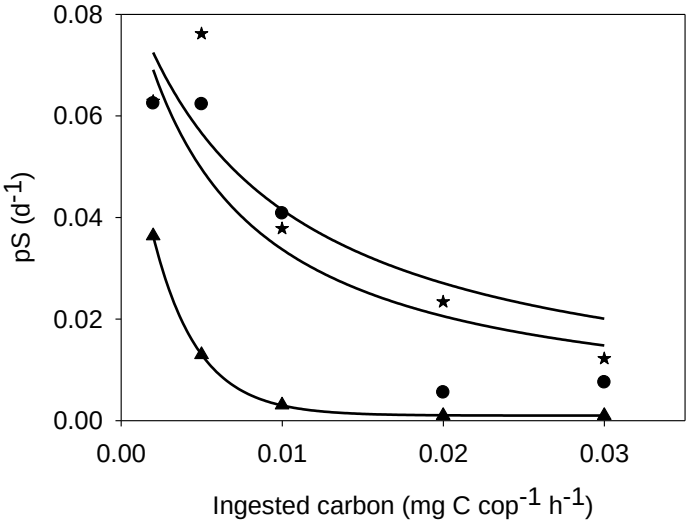
Survival

A daily mortality rate and consequently survival rate was calculated (Table 4.2) on a pure algal diet for the early copepodite stages, the late copepodite stages and the adults (Fig 4.5a), and on a mixed diet for the late copepodites and the adults (Fig 4.5b), and is added to the relative lifetime values from 0 to 1 according to Preuss et al (2011). Copepods die when the relative lifetime values are greater than 1. Naupliar survival was independent of food concentration for the range of food concentrations tested. Naupliar survival data were also not normally distributed. The outlier was identified using Dixon's Q test (Dean and Dixon 1951) and the survival rate obtained was 0.0016 d^{-1}. However, it is highly improbable for nauplii to survive for 625 d. Therefore, a more realistic survival rate of 0.05 d^{-1} was assumed for the nauplii in the model based on the maximum survival of nauplii under laboratory conditions from our experiments. Similarly for adults, a lower limit of survival was placed at 0.01 d^{-1}. All survival descriptors are divided by the change in temperature given by the Bělehrádek function.

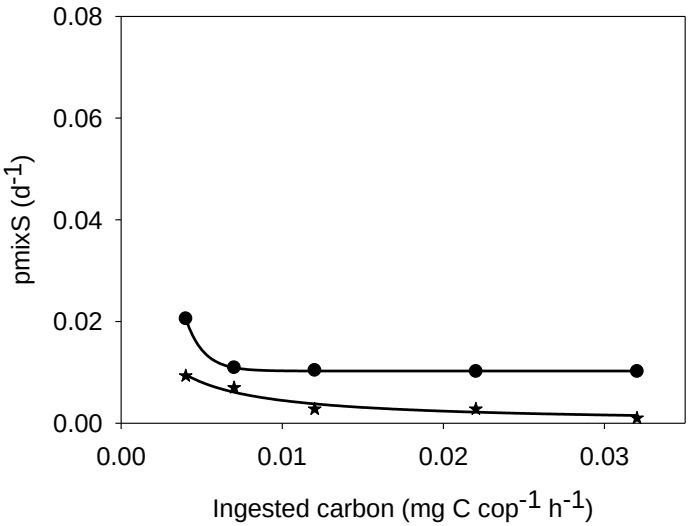
Toxicity

The toxicity submodel was implemented using the GUTS (Jager et al 2011). Survival data of different copepod stages over time from acute toxicity experiments were used to calibrate the parameters for the model. The toxicokinetics of TPT were described by using the scaled-internal concentration due to lack of uptake and elimination data, while the toxicodynamic assumption was selected to be stochastic death. The concentration of TPT in water is added as

an input variable (Table 4.1). The GUTS application has been described in Kulkarni et al (2013b) and was directly implemented into the IBM. Within the model, toxic effects occur



(a)



(b)

Fig 4.5- Survival parameters of the different stages of *M. leuckarti* on (a) a pure algal diet, early copepodite (triangle), late copepodite (star), adult (circle) and (b) a mixed diet, late copepodite (triangle) and adult (circle).

only on the different copepod stages and no toxic effects have been assumed for the rotifers or the algae.

4.2.3. Model implementation

The programming platform used was Delphi® 2009 (Embarcadero Technologies, USA).

4.2.4 Model simulations

To test for the influence food limitation on *M. leuckarti* populations, simulations were run for two different feeding conditions- (i) Constant algae (0.5 mg l^{-1}) concentration and different rotifer numbers (100, 250, 500, 750, 1000) (ii) Constant rotifer number (1000) and different algae concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 mg C l^{-1}). Under both conditions rotifer growth rate and threshold values were set to zero, and cannibalism was switched on. Furthermore, in order to observe long-term population dynamics, simulations over 10 years were run and the trends in population dynamics were observed.

To test the effect of TPT exposure on the different mechanisms of population regulation, test and control simulations were run for the same initial conditions (365d; initial population of 25 nauplii and 5 adults; 10 l vessel volume) under a two peak exposure (peaks administered at day 65 and day 100) scenario (at 10, 25 and 50 µg l^{-1} TPT) for each of the following three food regimes-

Case 1- Pure algae feeding

In this scenario, the copepods were given a pure algal diet of 0.5 mg l^{-1}.

Case 2- Mixed feeding

Here, a mixed diet of algae (0.5 mg l^{-1}) and 1000 rotifers was provided.

Case 3-Cannibalism

Cannibalism was initiated in this case and copepods fed on algae (0.5 mg l^{-1}), and the probability of encountering rotifers and nauplii is calculated based on their densities in the vessel. Cases 1 and 2 were designed to explain the influence of food availability on density dependence whereas case 3 was used to explain the impact of cannibalism on density dependence under toxicant exposure.

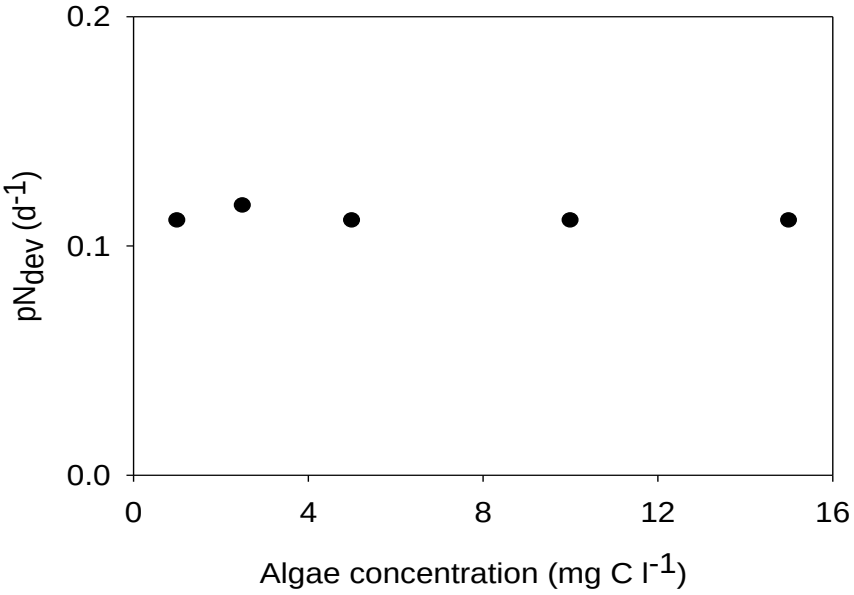
4.3 Results

The results of laboratory experiments were used to calibrate the model as reported under the Model Description section. The results of survival and development experiments with nauplii (Fig 4.6) are reported first. The most notable of the results was the independence of naupliar survival and development on food. Therefore for the food concentrations tested, no direct relationship between the food concentration and naupliar survival and development could be established.

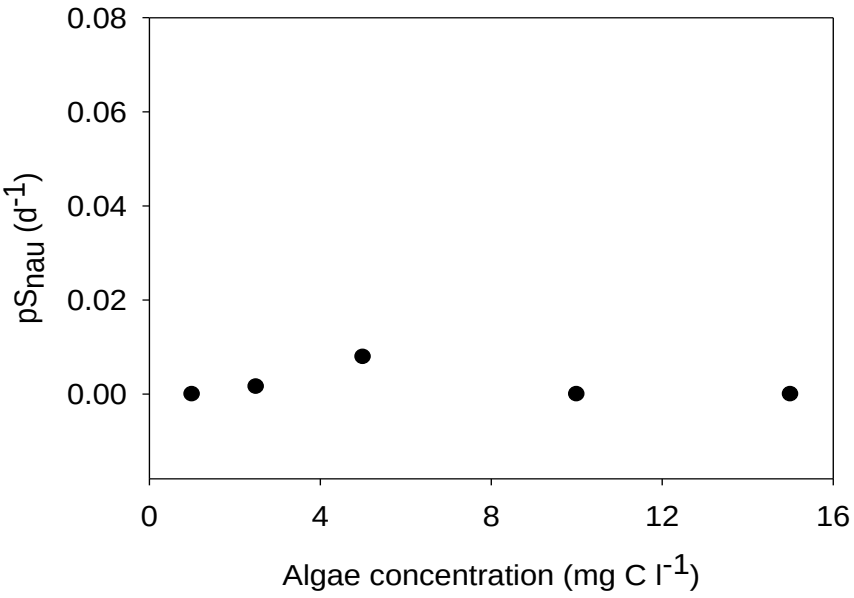
The results of model simulations, wherein different scenarios were tested (Fig 4.6-4.8), are described below.

4.3.1 Food limitation

At a constant algae concentration (0.5 mg C l^{-1}), the mean of the population dynamics (mean of 100 Monte Carlo simulation runs) over 365 d increased with the number of rotifers (Fig 4.7a). The rise was exponential initially and reached maximum after 2000 rotifers. At a constant rotifer concentration (1000 per 10 l), the mean of the population dynamics reached a plateau at algae concentrations above 0.3 mg C l^{-1} (Fig 4.7b).

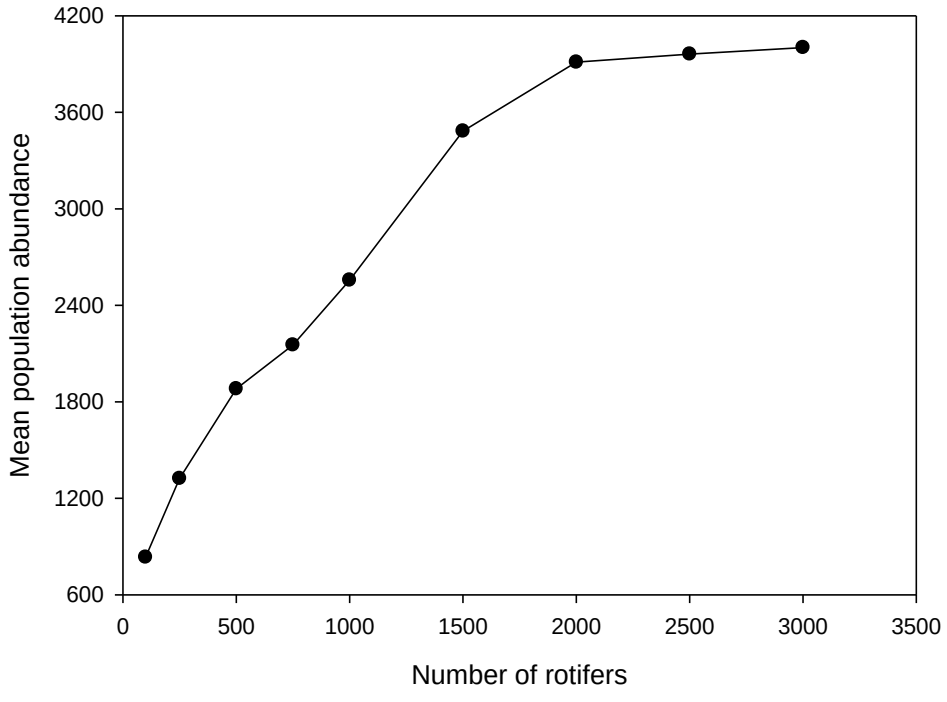


(a)

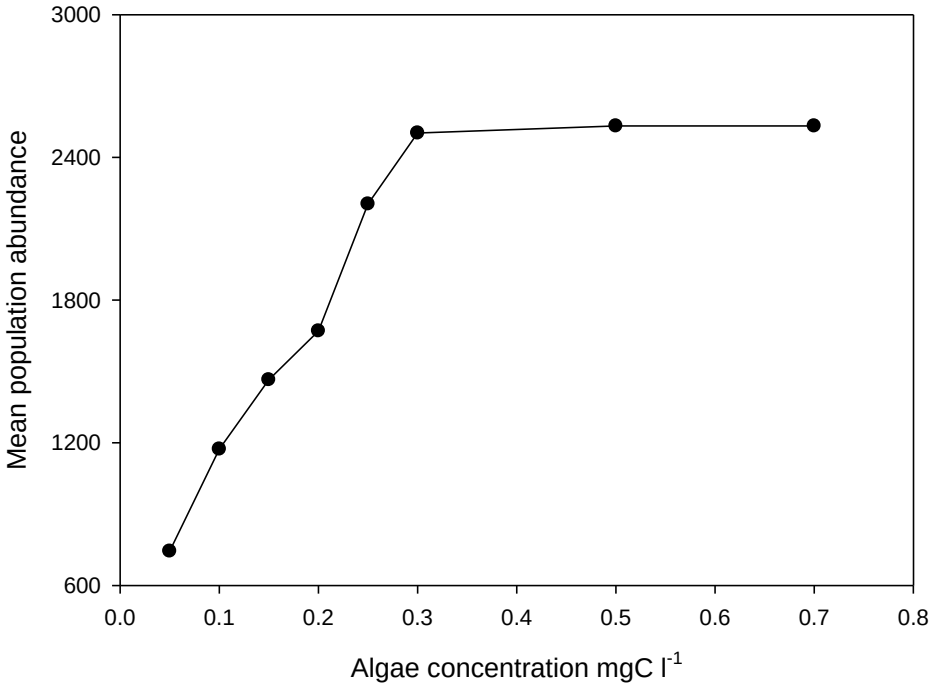


(b)

Fig 4.6- (a) Development rates and (b) Survival rates for the naupliar stages of *M. leuckarti* at different food concentrations.



(a)



(b)

Fig 4.7- Mean population abundance of *M. leuckarti* under different feeding regimes - (a) Constant algae concentration (0.5 mg l^{-1}) and varying rotifer concentrations (100, 250, 500, 750 and 1000) (b) Constant rotifer concentration (1000) and varying algae concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 mg C l^{-1}). Under both conditions rotifer growth rate and threshold values were set to zero, and cannibalism was switched on.

4.3.2 Control simulations

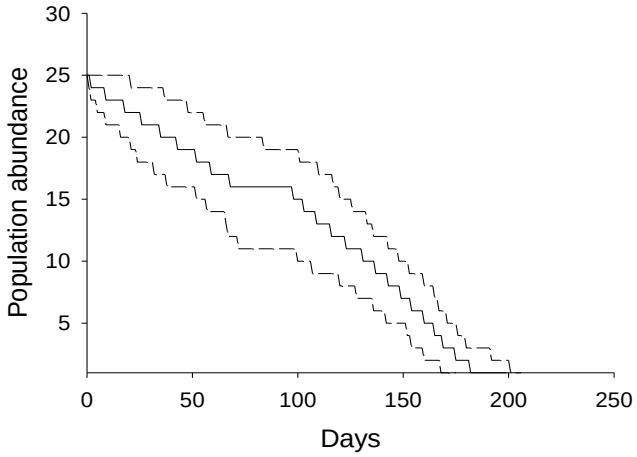
The copepod populations did not reproduce on a pure algal diet and went extinct around day 180 (Fig 4.8). Population dynamics in the controls (i.e. excluding TPT) for both cases 2 and 3 are shown (Fig 4.8). The population showed a steady increase up to day 110. At this point, a large number of adults were present in the population, which later reproduced under favourable food conditions. This resulted in the boost of nauplii which led to a sudden increase in population abundance around day 150. The population then showed a sharp decrease in abundance followed by two steady peaks around day 210 and 270. There were no significant differences between the population dynamics of the control scenarios in case 2 and 3. For long-term population dynamics, a characteristic yearly trend was seen (Fig 4.10). This consisted of a first large peak around April and a second peak in population abundance around September, with two smaller peaks in between.

4.3.3 Test simulations

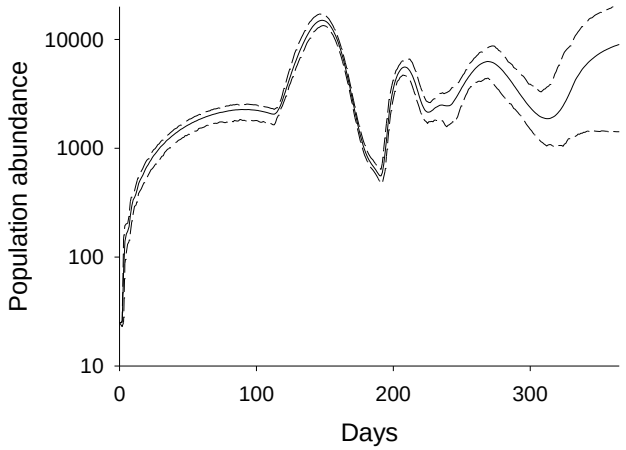
As population dynamics could not be sustained on a pure algal diet, test results for this case have not been reported. Marked differences were seen between test population dynamics for cases 2 and 3 (Fig 4.9). As seen in the control simulations, the populations showed a steady increase upto day 65, where the TPT dose was administered. However, lethal effects were seen after the administration of the TPT dose.

Case 2- Mixed feeding

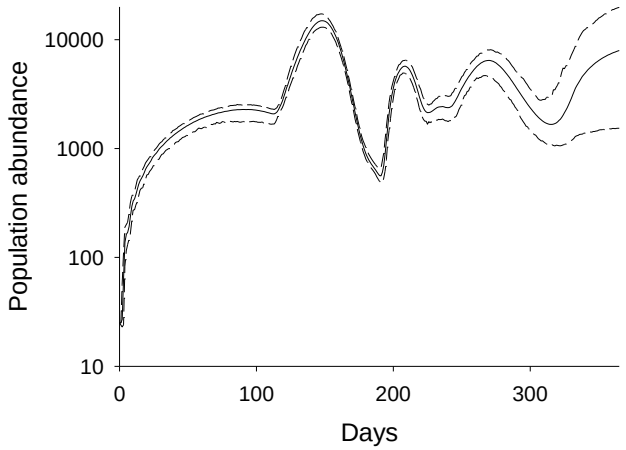
At 10 µg l^{-1} after the administration of the first TPT peak, the affected population showed a steady recovery just before the second TPT peak was administered. Approximately 20 days after the administration of the second peak, the population again showed a steady recovery.



Case 1



Case 2



Case 3

Fig 4.8- *M. leuckarti* population abundance with 95 % confidence intervals on different feeding regimes, Case 1 (Pure algae feeding), Case 2 (mixed feeding), Case 3 (Cannibalism).

A sudden dramatic overshooting in population abundance was seen around day 180 followed by a steep decline after 200 d (Fig 4.9). A more pronounced toxic effect of TPT was seen at 25 µg l^{-1}. Comparatively higher mortality was seen after the second peak was administered at this concentration. At 50 µg l^{-1} a sharp population decline after the first peak that continued after the second peak was seen. Absolute values of mean population abundance (not shown here) were 1873 individuals at 65d and 52 individuals at 100d. Recovery began only around day 150 and population abundances rose to 102 individuals after 150d. Complete recovery of the population was only seen around day 190. At 75 µgl^{-1}, there were 1976 individuals at 65d which fell to 33 individuals at 100d. Abundances dropped to 10 individuals on day 102. The population began to recover around day 150 with mean population abundances rising to 38 individuals after 160d. Complete recovery was seen on day 190, 90 d after the administration of the second peak.

Case 3- Cannibalism

Population abundances in case 3 were similar to those observed in case 2 at 10 and 25 µg l^{-1}. At 50 µg l^{-1}, mean population abundances went from 1970 on day 65 to 49 on day 100 and subsequently 14 on day 108. However, the lower percentile of population abundance suggested that 5% of the simulation runs went down to zero. At 75 µgl^{-1}, the population went extinct at the immediate administration of the second peak. 1999 individuals at 65 d were reduced to 29 on day 100 and zero on day 111.

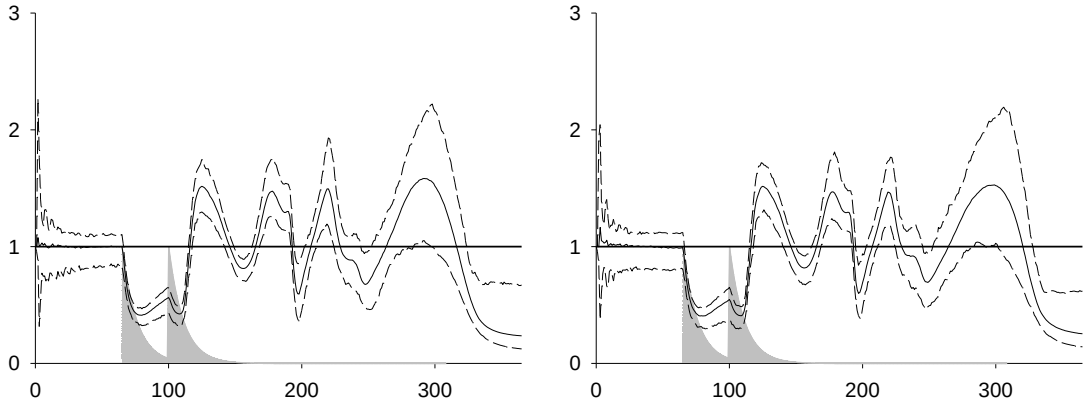
4.4 Discussion

4.4.1 Naupliar survival and development

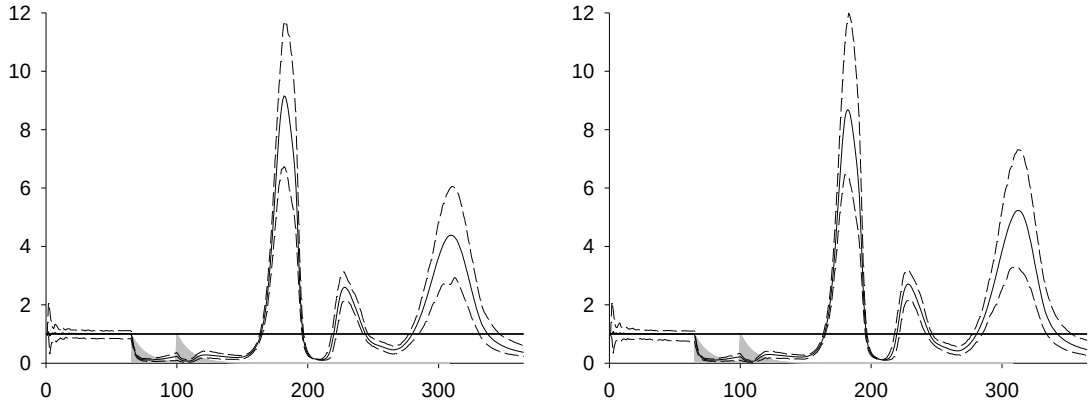
The independence of naupliar development and survival on the food concentrations used in our experiments was an interesting observation. At 16.5 ºC, Hansen and Santer (1995)

reported that the threshold food concentration for the successful naupliar development of *M. leuckarti* was 0.3 $mgCl^{-1}$. The lowest food concentration tested in our experiments was 1 $mgCl^{-1}$.

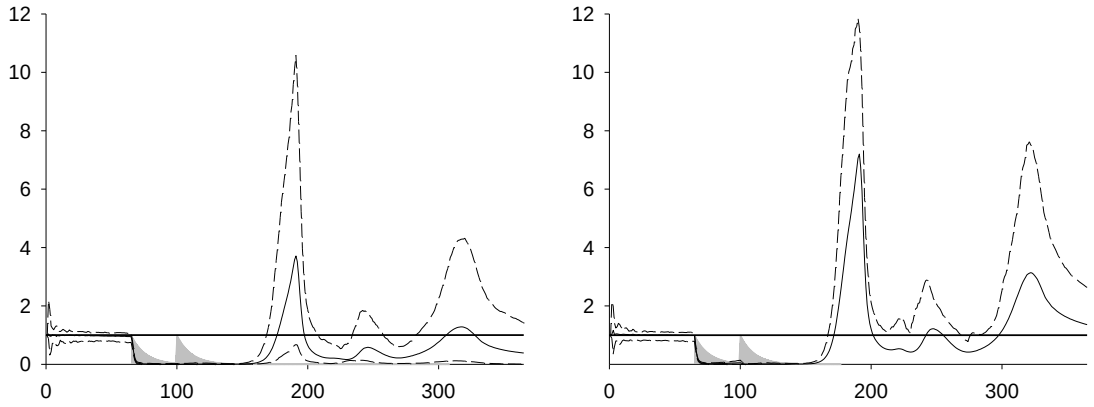
Case 2 (Mixed feeding) **Case 3 (Cannibalism)**



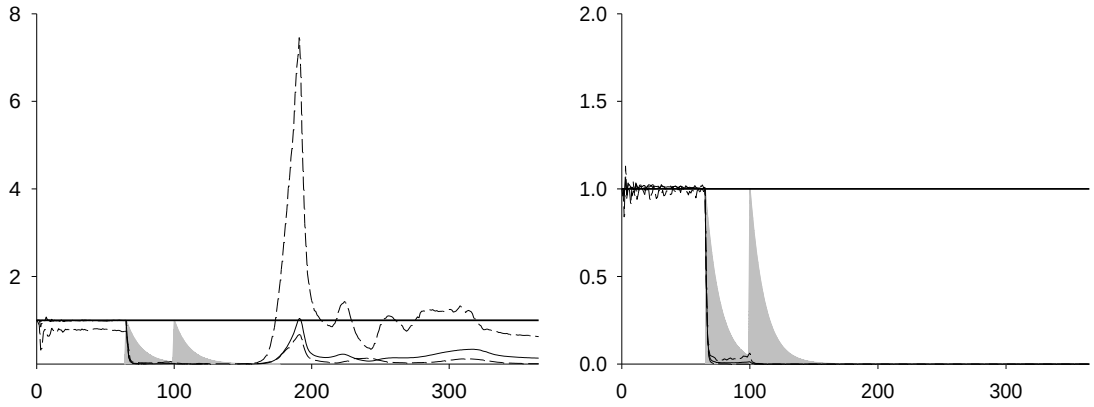
10 µg l^{-1}



25 µg l^{-1}



50 µg l^{-1}



75 µg l^{-1}

Fig 4.9- Population abundances of *M. leuckarti* on a logarithmic scale (y-axis) relative to control with 95 % confidence intervals over 365 days (x-axis) under two peak TPT exposure.

This concentration may already have been high enough to facilitate successful survival and development of nauplii in our experiments. However, naupliar resistance to starvation was evident in our previously reported toxicity experiments where during the test the nauplii in the control survived for 96h without food (Chapter 3). Naupliar feeding is unclear and a highly debated area. There are varying views on naupliar feeding. Nauplii, being "inefficient herbivores", are considered bottlenecks in copepod development and therefore, some

copepods have to undergo diapause to avoid this limitation (Hopp and Maier 2005, Santer and Lampert 1995). Nauplii have been shown to be sometimes feeders on 378% of their body weights (Böttjer et al., 2009) and sometimes lecithotrophic, living off the fat reserves obtained from the egg (Chullasorn et al., 2012). NI and NII instars in some harpacticoids e.g. *Tegastes falcatus* have shown the absence of anal openings leading to the presumption that they do not feed (Ivanenko et al., 2008). Some studies have reported nauplii to feed on flagellates (Santer and Lampert, 1995) and some on bacterioplankton (Turner, 2004). The fact that nauplii can live on a range of food types, not limited to plankton, may explain food independence at the range of algal concentrations tested here. To better understand food-dependence of *M. leuckarti* nauplii, more experiments, which not only look at food limitation using alternative food sources (e.g. bacteria) but also take into account the dependence of nauplii of nauplii on fat reserves obtained from the eggs, are necessary. Within the model, naupliar population dynamics were independent of food concentration for the range tested. The only limiting factors for nauplii in the model were the greater and more significant stressors- cannibalism and toxicity.

4.4.2 Food limitation

It is evident from the first set of simulation results that it is not algae but rotifers that limit *M. leuckarti* population abundance (Fig 4.7). The threshold concentration of algae for stable population abundance was observed to be 0.3 mg C l^{-1} which suggests that algae are required to sustain the population. However, equilibrium was observed at all concentrations of algae higher than the threshold concentration. Therefore, algae concentration seemingly does not drive the population dynamics of *M. leuckarti*. Conversely, increasing mean population abundance at a constant algae concentration and increasing rotifer concentration confirmed that *M. leuckarti* population dynamics are driven by the presence and amount of animal food.

Overall, these findings are similar to other studies in the past which reported this copepod species to favour omnivory (Hansen and Santer, 1995, Hopp et al., 1997, Hopp and Maier 2005, etc.). Hansen and Santer (1995) reported, and we concur, that although there wasn't any significant difference between the carbon content of *Cryptomonas* sp. and *Brachionus* sp., the addition of the latter proved significantly beneficial to *M. leuckarti*. Therefore, we also conclude that it is not the quantity of food that matters but rather the quality and for studies with freshwater as well as marine copepods, omnivory needs to be carefully investigated.

4.4.3 Model results

Population dynamics of copepods emerged from individual properties. In the control dynamics (Fig 4.8), after the first boost around day 150, the population peaks were smaller. Long-term simulations (more than 5 years) showed similar annual patterns fluctuating with the temperature dependence incorporated into the model (Fig 4.10). The first large peak observed around April when spring temperatures favour population growth. The second large peak was observed around September. This is followed by diapause through winter. These observations are consistent with Vijverberg and Richter (1982) who described population abundances of *M. leuckarti* in a lake. Although our simulations show population dynamics under small-scaled laboratory conditions (Fig 4.10), the annual trend seen in the field (by Vijverberg and Richter 1982) is replicated. The effect of cannibalism on population dynamics was clearly highlighted by the model results and has been described as follows:

4.4.3.1 Comparison of Cases 1, 2 and 3

Case 1 proved to be most unfavourable for the copepod population. The population could not reproduce and therefore, could not be sustained on a pure algal diet. Contrary to our

expectations, no significant differences were seen between control population dynamics in cases 2 and 3 (Fig 4.8).

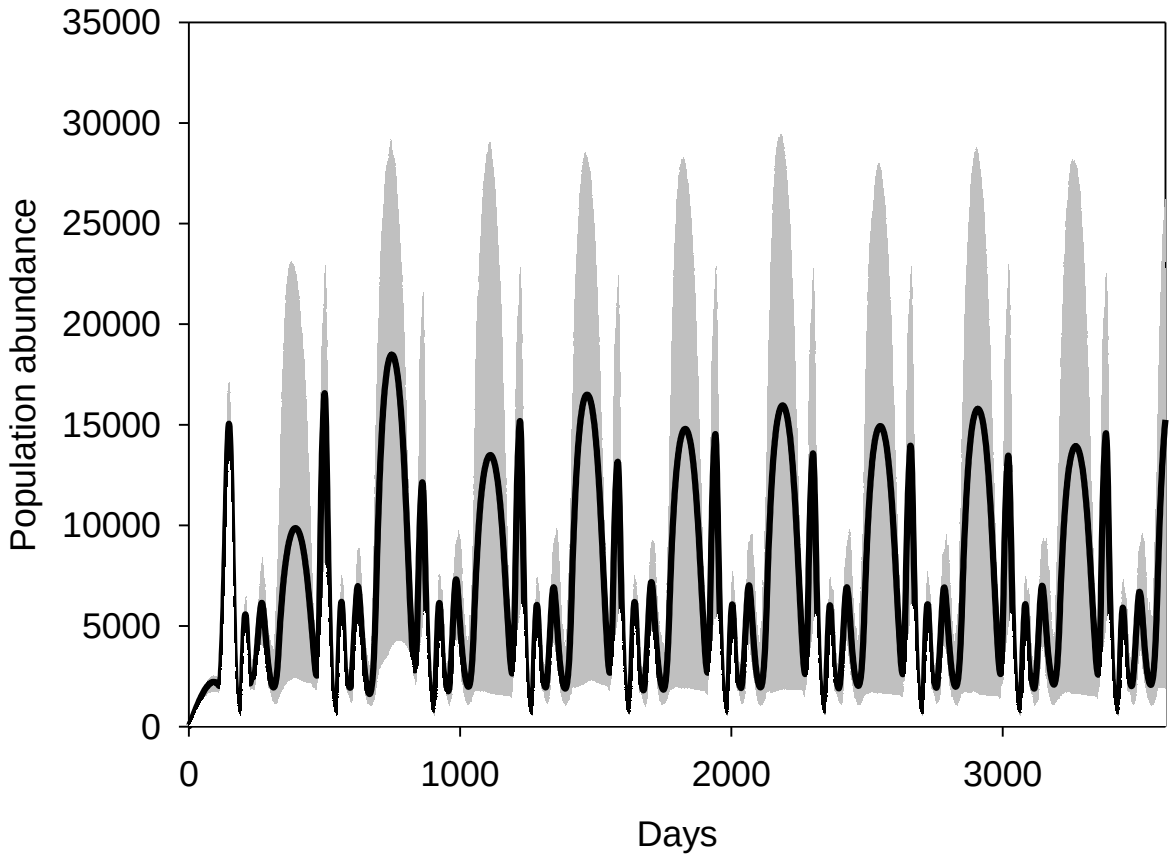


Fig 4.10- Simulation of the population abundance of *M. leuckarti* over 10 years. The shaded area shows the 95% confidence interval.

For the test simulations, however, the differences between these two cases become more prominent particularly at the highest concentration of 75 µg l^{-1}. Studies (Gabriel 1985, Van den Bosch and Gabriel 1991 and Gabriel and Lampert 1985) have proposed that cannibalism in cyclopoid copepods serves a two-fold purpose- it stabilises population boosts (sudden and high increase in population abundance) and provides an alternative food source when animal food is scarce. Although food selection does occur in copepods, it may generally be driven by the size of the prey (Schultz and Kiorboe 2009). The rotifers and nauplii in our study were

both soft-bodied and small enough to be preyed upon by the copepods. In this study however, cannibalism seems to have had an adverse effect on the nauplii in the population. Despite the presence of an alternative prey in the form of rotifers, the naupliar population was severely affected. The nauplii therefore, were exposed not only to toxic stress but also to cannibalistic predation.

4.4.3.2 Toxic effects

TPT is known to be a slow-acting compound (Roessink et al 2006). Therefore, a delay in recovery was observed in the exposed copepod populations. Owing to the delayed effects of TPT and subsequently longer recovery times, the copepod populations did not fully recover before the second peak was administered (Fig 4.9). The duration between the two peaks was 35 d which is higher than the life-cycle duration of *M. leuckarti* under optimal conditions (i.e. approximately 21 days (Gophen 1976)). Population abundances for both feeding scenarios (cases 2 and 3) were similar at 10 and 25 µg l^{-1}. There is a difference between the effects of stressors at lower and higher population densities (Sibly et al., 2000). At lower population densities, less cannibalistic stress was added to the already intoxicated nauplii, and a steady reproductive output was maintained. At higher population densities, the probability of cannibalism on nauplii was higher. The dramatic overshooting of population densities observed for cases 2 and 3 at the 25 and 50 µgL^{-1} concentrations (Fig 4.9) can be attributed to a trade-off between food availability, metabolism of toxicant and reproductive strategy in the copepod populations. Studies with *Daphnia magna* (Agatz et al., 2013) and marine copepods (Guisande et al., 1996) have shown that both these species are able to adopt modified reproductive strategies influenced by food availability. When food is abundant they are known to produce numerous but less fit offspring whereas at low food concentrations they direct more energy towards offspring size rather than number (Guisande et al., 1996). An

analysis of population structure (not shown here) showed that the population was mainly constituted by nauplii at these concentrations. Following TPT exposure in our study, the individuals in the population had to expend energy on metabolising the toxicant as well as reproduction. An increase in performance of one trait must therefore be balanced by a decrease in the performance of another (Jager et al., 2013). Consequently, population abundances having decreased following TPT exposure resulted in higher food availability. This adaptive strategy may have led to a higher reproductive output with the production of a high number of nauplii resulting in higher population densities after the second TPT peak at these concentrations. The presence of competitors and predators in the field usually serves to control population boosts and reduce fluctuation (e.g. Olenick, 1983) but this interaction is absent in single species tests.

Above 50 μgl^{-1}, the nauplii were drastically affected by the cannibalism scenario (case 3). 5% of the simulation runs went down to zero immediately after the administration of the second peak implying that there was at least a 5% chance of population extinction at this concentration. The highest effect was seen at 75 μgl^{-1} where the population reached 100% extinction immediately after the second peak. Studies (Gabriel, 1985, Van den Bosch and Gabriel, 1991, Gabriel and Lampert, 1985) have proposed that cannibalism in cyclopoid copepods serves a two-fold purpose- it stabilises population boosts (sudden and high increase in population abundance) and provides an alternative food source when animal food is scarce. Although food selection does occur in copepods, it may generally be driven by the size of the prey (Schultz and Kiorboe, 2009). The rotifers and nauplii in our study were both soft-bodied and small enough to be preyed upon by the copepods. Both the rotifers and the nauplii were not distinguished as different prey items in the model (relative density determined probability of encounter and predation by copepods). Despite these facts, cannibalism seems to have had

an adverse effect on the nauplii in the population at 75 μgl^{-1} (Fig 4.9) which is supported by the absence of peaks leading to the conclusion that the nauplii produced at this concentration were severely impacted. Despite the presence of an alternative prey in the form of rotifers, the naupliar population was severely affected. The nauplii of *M. leuckarti* are more sensitive to TPT compared to the adult stages (Kulkarni et al., 2013b). The nauplii, therefore, were exposed not only to toxic stress but also to cannibalistic predation. A synergistic effect of cannibalism as well as toxicity to TPT seems to have resulted in a higher vulnerability of copepod populations compared to the scenario without cannibalism.

4.4.3.3 Significance of omnivory

As was expected (e.g. Hopp et al 2005), the simulated copepod populations could not be sustained using a purely algal diet. Animal food, as mentioned before, is vital for egg production especially for a species with a strong predatory instinct like *M. leuckarti.* A high concentration of algal food alone is not enough to sustain the population. In the absence of animal food, cannibalism is observed (Hansen and Santer, 1995). Although adult copepods are able to cannibalise each other too, studies have shown that the cannibalism of nauplii and smaller copepodites is a more common phenomenon (Vijverberg 1989). *M. leuckarti* has a high reproductive output under favourable feeding conditions. Therefore, as seen in the control simulations (Fig 4.8), high boosts in population abundances, dominated by nauplii, were seen. Due to high fecundity as a consequence of an optimum animal food concentration, a relatively smaller number of adults could produce a high number of nauplii.

4.4.3.4 Implications for risk assessment

For ecotoxicology and the ERA of chemicals, these findings may have profound implications. During the standardisation of experimental conditions for ecotoxicological tests (e.g. within OECD guidelines), the focus lies on common abiotic factors like temperature, food quality

and quantity, type of medium, etc. It may be argued that on one hand, setting a ration too high may lead to interactions between limited food availability (leading to cannibalism) and toxicants may be missed while on the other hand, setting a ration too low may lead to increased cannibalism making it difficult to distinguish between biotic (cannibalism) and toxic stress. However, in mesocosm studies the effects of toxic stress are assessed as differences to control (e.g. Roessink et al. 2006). Owing to similar ecological interactions occurring in the control, the difference between biotic and abiotic stressors can be clearly established. However, the most important phenomenon that may be missed is that of biomagnification. It may be the case that nauplii are exposed to the toxicant before being cannibalised. In such cases, the adults may face direct exposure to the toxicant as well as magnified exposure through food. This could lead to the overestimation of toxic effects and the misinterpretation of experimental observations. Furthermore in the absence of population-level density regulation in the form of cannibalism, the effects of the toxicant could be underestimated.

4.5 Conclusions and outlook

Model simulations showed that cannibalism of nauplii that were already stressed by TPT exposure contributed to synergistic effects of biotic and abiotic factors and led to a two-fold stress being exerted on the nauplii, thereby resulting in a higher population vulnerability compared to the scenario without cannibalism. Our results suggest that in population-level risk assessment, it is easy to underestimate toxicity unless underlying ecological interactions including mechanisms of population-level density regulation are considered. More experiments are warranted to provide independent datasets to validate the model. Moreover, multifactorial experiments (e.g. combining toxicant exposures, different feeding regimes and temperature regimes) which take into consideration the fluctuation of rotifer dynamics based

on food and abiotic factors as well as those that focus on toxic impacts on rotifers and algae are necessary to make the model more realistic. This model provides a fair insight into the population-level responses of *M. leuckarti* under TPT exposure and has the potential to be further extended to different copepod species and toxicants.

Chapter 5

Does intrinsic sensitivity imply population vulnerability? A tale of three models

5.1 Introduction

Current ecological risk assessment (ERA) procedures deal with the estimation of hazards wherein in the adverse effects caused by a particular substance to the environment are addressed. This is mainly done by measuring lethal and sub-lethal individual-level endpoints like growth, development and reproduction and applying safety factors to cover a wide range of species.

However, individual-level variables change with species and chemicals, and therefore, individual-level responses can be vastly different from population-level responses (Forbes et al 2011). For instance, Roessink et al (2006) showed that for similar acute toxicity values to triphenyltin (TPT) in the laboratory, population-level responses were vastly different. NOEC values for chaoborids, cladocerans and copepods in mesocosms were 10 µg l^{-1} (week 8 to 42), 30 µg l^{-1} (week 0.4 to 12) and 1 µg l^{-1} (week 2 to 8) respectively. Studies like this confirm the fact that different species may employ varied life-history strategies which could influence their sensitivities to toxicants. Consequently, life-history strategies may influence toxic stress, population dynamics and time to recovery, and therefore current ERA approaches may lead to overestimation or underestimation of risk (Stark et al 2004, Forbes et al 2008). Although the use of uncertainty factors may reduce the underestimation of toxicity using simplistic measures, the variations for species with contrasting life-history strategies may be vastly different (Stark et al 2004). Therefore, to fulfil EFSA protection goals, population-level effects need to be better understood (EFSA 2010).

Recently, mechanistic population models have been suggested as useful tools to extrapolate individual-level observations to population-level effects (Preuss et al 2009, Preuss et al 2011, Forbes et al 2011, Kulkarni et al 2013a, amongst others). Population models can not only add ecological complexity into risk assessment but also reduce uncertainty thereby adding value

to ERA (Forbes et al 2011). Population modelling constitutes higher-tier risk assessment and will soon be employed in assisting pesticide registration in the European Union (EFSA 2013).

Table 5.1- Different life-history strategies of *D. magna, M. leuckarti and C. crystallinus* under optimal conditions (Data from laboratory experiments as well as from the literature reviewed in Chapter 2).

Characteristic	***D. magna***	***M. leuckarti***	***C. crystallinus***
Life stages	Neonate, Juvenile, adult	Nauplii, copepodite, adult	Larva, pupa, adult
Generation time	~2 weeks	~4-6 weeks	~5-7 weeks
Reproductive strategy	Iteroparous	Iteroparous	Semelparous
Juvenile development time	7-10 days	15-20 days	30-50 days
Feeding habits	Herbivory	Omnivory	Carnivory

This chapter presents a case study with three common planktonic species- the cladoceran *Daphnia magna*, the freshwater cyclopoid copepod *Mesocyclops leuckarti* and the multi-voltine phantom midge *Chaoborus crystallinus.* All three species have distinct life-history strategies (Table 5.1). *D. magna* and *M. leuckarti* are both true zooplankton species, where the former has a relatively shorter life cycle and simple feeding habits while the latter has a relatively longer life cycle and complex feeding habits. *C. crystallinus*, also a zooplankton species, is an aquatic insect that spends its larval stages in water before emerging as adult and migrating (Rudstam 2009). The main issue addressed here is whether there is any difference between individual and population-level responses for these three species. Three distinct individual-based models (IBMs) developed for these three species were used (Preuss et al 2009, Kulkarni et al Strauss et al) to simulate population level responses to TPT exposure. These responses were compared with the individual-level effects observed for the same

organisms in toxicity experiments. Our aim was to test whether there were differences between the individual- and population-level sensitivities for the three test species under TPT exposure. A combined approach of laboratory experiments and mechanistic modelling has been employed. Simulation results have been compared and potential implications for risk assessment have been discussed.

5.2 Materials and methods

5.2.1 Toxicity experiments and GUTS calibration

Acute immobilisation tests under TPT exposure were conducted for *D. magna* and *C. crystallinus* similar to those conducted with *M. leuckarti* (Chapter 3). The Aachener Daphnien Medium (ADaM) was used for experiments with *D. magna* and *C. crystallinus* while the COMBO medium was used for *M. leuckarti* experiments as mentioned before (Chapter 3). All experiments were conducted at constant temperature (20 ± 1 °C) with a light-dark rhythm of 16:8 h. None of the animals were fed during the experiments. The TPT stock solution was prepared and chemical analysis was performed identical to Kulkarni et al (2013b). The tests were conducted using a static approach because it was determined previously that under these conditions TPT was stable over 96 h (Chapter 3).

For all species, concentration response curves were generated and LC_{50} values after 48 h were calculated using a Probit analysis (conducted using ToxRat® Professional, ToxRat solutions GmbH, Germany) followed by the application of the toxicokinetic and toxicodynamic approach using the GUTS (Jager et al 2011). The toxicokinetic dose metric scaled internal concentration and the toxicodynamic assumption stochastic death were used to describe the toxicity of TPT to these organisms (Refer to Chapter 3 section 3.2.4). Parameter values for the GUTS were estimated by fitting the GUTS to survival data over time for all three species.

According to Kulkarni et al (2013b), calibrations were run with random start values, and by maximising the likelihood function using the simplex algorithm (Dantzig 1963), values with the best likelihood were selected for parameter estimation. 95% confidence intervals for the survival rates were generated by profiling the likelihood function according to Meeker and Escobar (1995).

5.2.2 Population-level model simulations

Three existing IBMs for *D. magna* (Preuss et al 2009), *M. leuckarti* (Chapter 4) and *C. crystallinus* (Preuss and Strauss 2012) were used. These models, which were parameterised using laboratory experimental data, comprehensively describe the detailed life-history strategies of the respective species and are able to simulate population dynamics based on individual-level properties. All three models are implemented in the programming platform Delphi® (Embarcadero Technologies, USA). The toxicokinetics and toxicodynamics (TKTD) of TPT were implemented in all three population models using the GUTS (Jager et al 2011).

Two different exposure scenarios, one peak and two peaks of TPT, were tested for the three models (Fig 5.1). The simulation conditions for both scenarios were as follows-

- Temperature 20°C
- Spiking was done at equilibrium
- Greater than 90% food saturation

For the two-peak scenario, the first peak was administered on day 65 while the second peak was administered on day 100. The maximum number of individuals during the simulations was comparable for all three organisms.

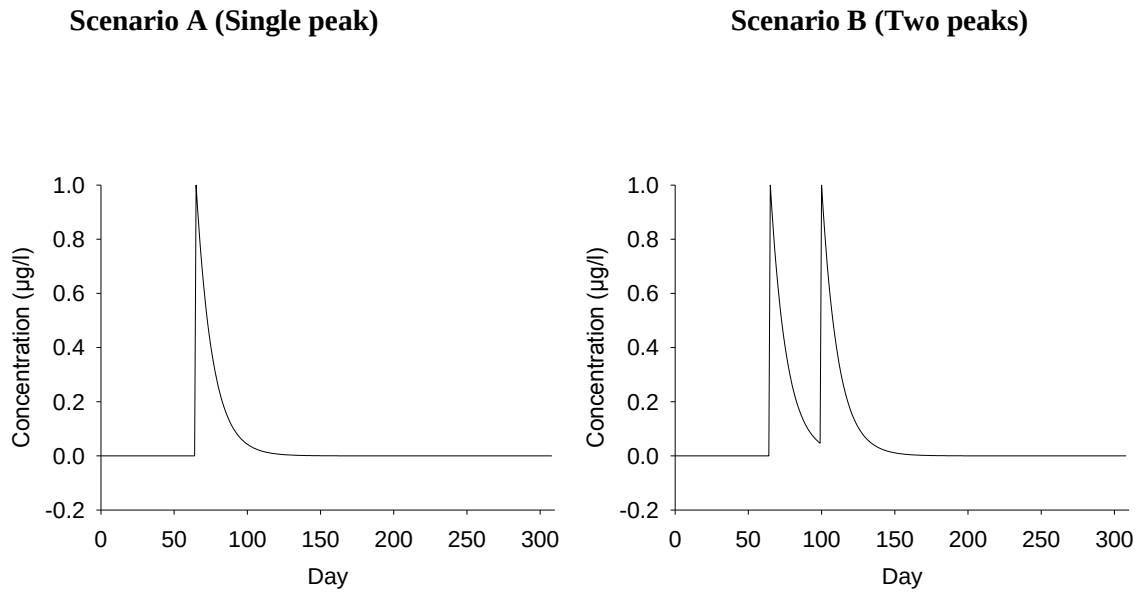


Fig 5.1- The two exposure scenarios (single-peak and two-peak) to TPT which were tested with all three models (Temperature 20°C, *ad libitum* food concentration, spiking at equilibrium). The time interval between the peaks for Scenario B was 35 days.

Extinction probabilities for the populations of the three test species were calculated under both exposure scenarios and population-level responses were compared with individual-level responses.

5.3 Results

5.3.1 Individual-level sensitivity

48 h LC_{50} values for the three test species and concentration response curves are shown (Table 5.2 and Fig 5.2 respectively). *D. magna* neonates were seen to be most sensitive while *C. crystallinus* larvae were found to be least sensitive to TPT at the individual level. A common observation for *D. magna* and *M. leuckarti* was that the neonate larval stages were more sensitive than the adult stages (Table 5.2).

Table 5.2- 48 h acute toxicity data from laboratory experiments with TPT for *D. magna, M. leuckarti* and *C. crystallinus*.

Organism	LC_{50} µg l^{-1}	Lower 95% CI	Upper 95% CI
D. magna neonates	25.9	n.d.	n.d.
D. magna adults	41.9	32.8	53.5
M. leuckarti nauplii	33.9	n.d.	n.d.
M. leuckarti adults	86.1	73.4	102.6
C. crystallinus	135.1	61	643379.8

n.d. means not determined due to mathematical reasons (according to Fieller's theorem, Fieller (1944))

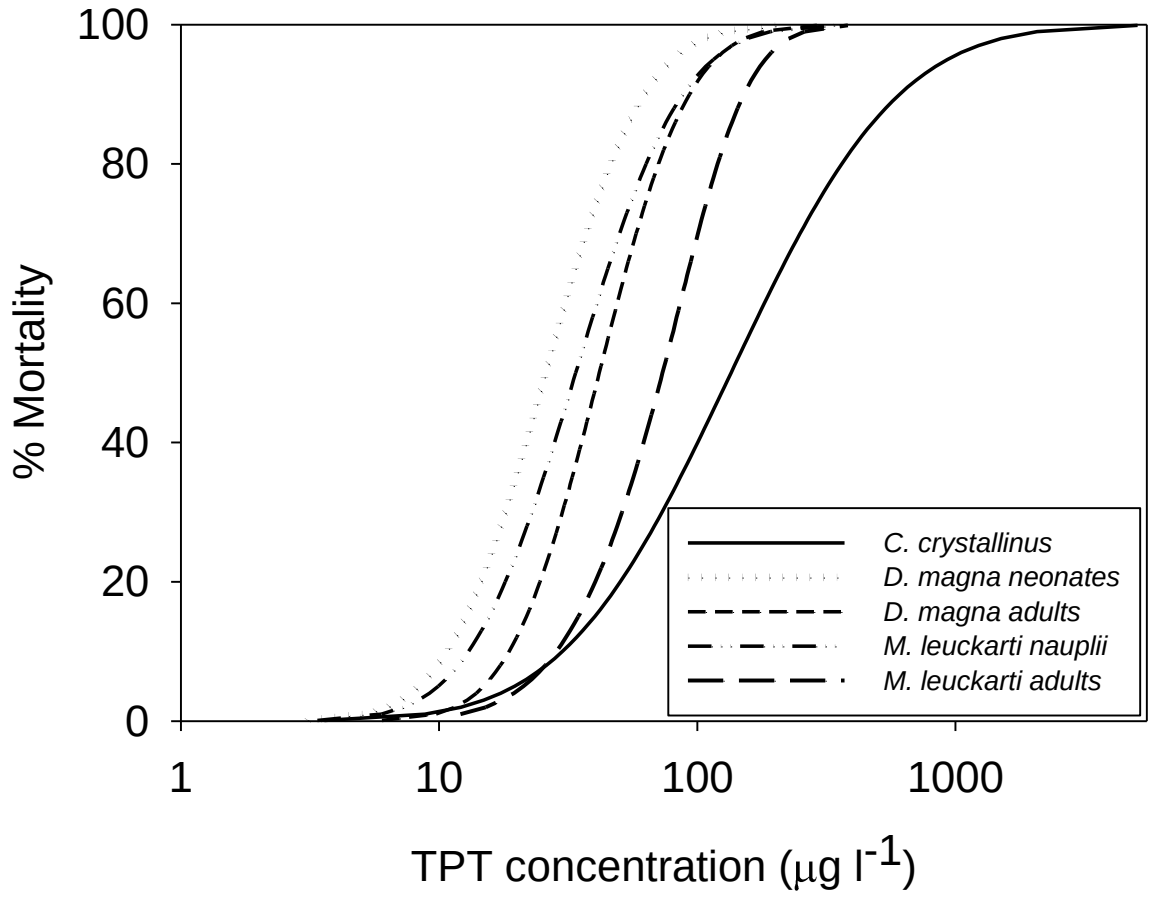


Fig 5.2- Concentration response curves over 48 h for all three test species under TPT exposure.

Table 5.3-GUTS calibration results for the larval stages of the three test species (Parameters for *M. leuckarti* are from Chapter 3).

Parameter	k_k (ml $\mu g^{-1} h^{-1}$)	k_e (h^{-1})	z ($\mu g\ l^{-1}$)
D. magna	2.19	0.11	0.02
	(1.66-2.59)	(0.07-0.17)	(0-0.27)
M. leuckarti	0.86	0.07	1.55
	(0.66-1.05)	(0.05-0.12)	(0-2.74)
C. crystallinus	1.35	0.01	0.47
	(0.95-1.85)	(0-0.01)	(0-1.06)

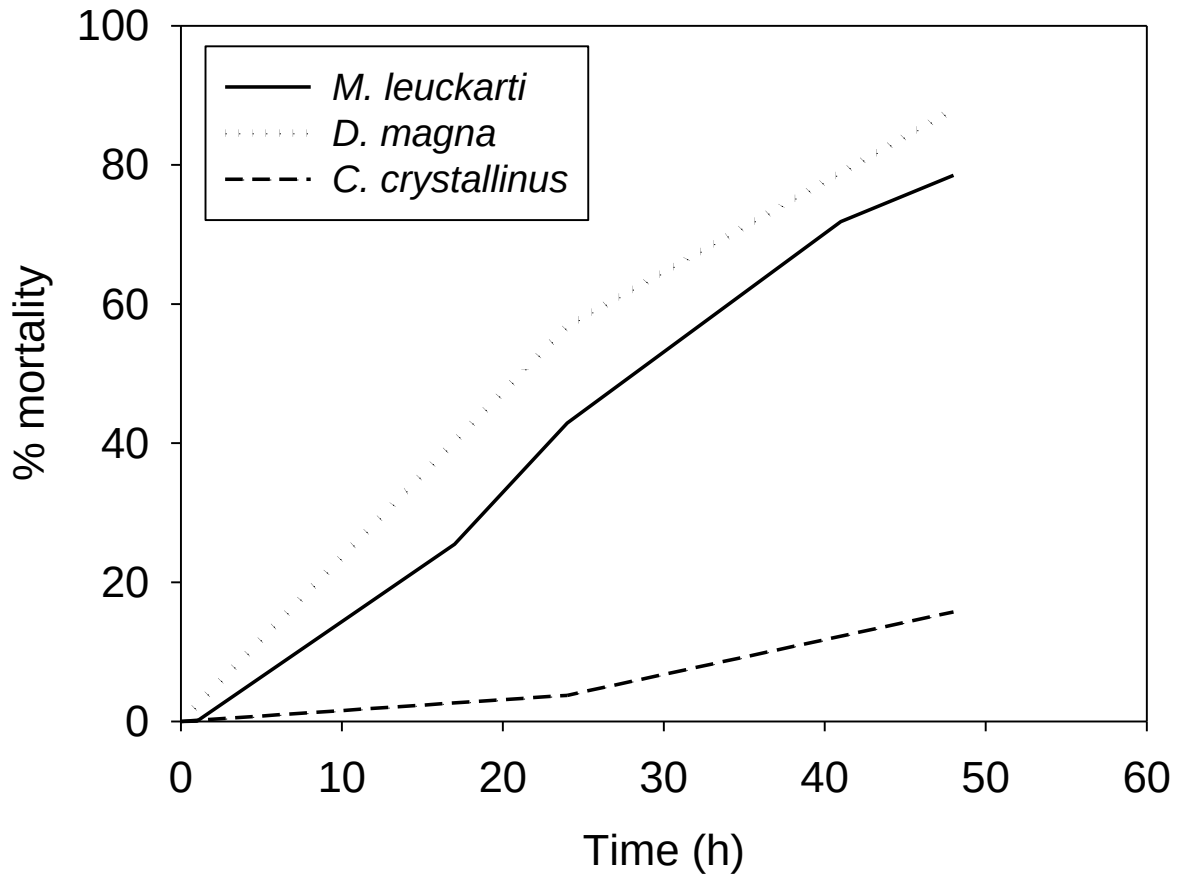


Fig 5.3- Comparison of GUTS parameter simulations for the three species after 48h at 50 $\mu g\ l^{-1}$

A factor of 3.6 was observed between the sensitivity of *D. magna* neonates and the larvae of *C. crystallinus* while a factor of 2.7 was seen between *M. leuckarti* nauplii and the larvae of *C. crystallinus*. *D. magna* adults were twice as sensitive as *M. leuckarti* adults.

Parameter estimates using the GUTS for the larval stages of the three species- the killing rate (k_k), the elimination rate (k_e) and the threshold for survival (z), are shown (Table 5.3). A comparison between the GUTS simulations for the three species at 50 µg l^{-1} described the differences in sensitivities at the individual-level (Fig 5.3). The parameter set for *D. magna* was the most unfavourable with the highest k_k and the lowest z (Table 5.3). Therefore, *D. magna* was the most sensitive organism at the individual level (Fig 5.3).

The ranking in sensitivity at the individual level for the three species was-

D. magna > *M. leuckarti* > *C. crystallinus*

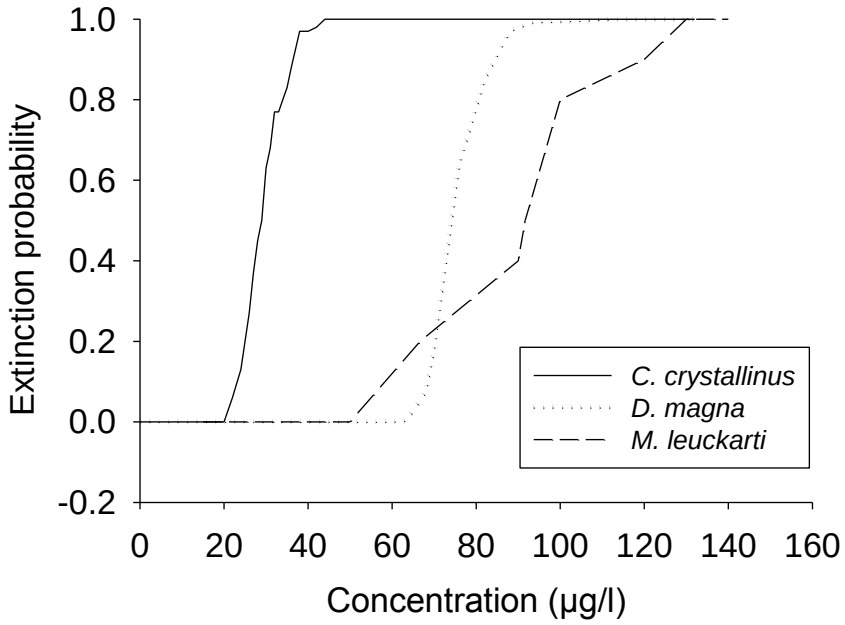
5.3.2 Population-level sensitivity

Extinction probabilities for the three test species under TPT exposure are shown (Fig 5.4). Converse responses were observed on the population level compared to individual-level responses.

- Scenario A (Single peak)

  The most interesting observation at the population level was the sensitivity of the *C. crystallinus* population (Fig 5.4). 100% population extinction was reached by the *C. crystallinus* population at 44 µg l^{-1} TPT.

Scenario A (Single peak)



Scenario B (Two peak)

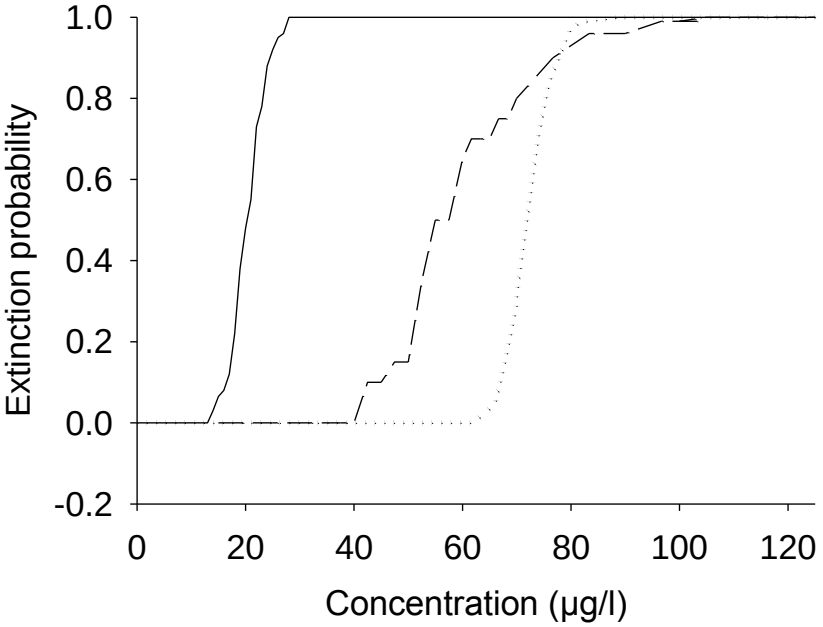


Fig 5.4- Extinction probabilities calculated by the models for the three organisms under TPT exposure.

Extinction for this species began at 22 µg l^{-1}. Conversely, the *D. magna* population, beginning after 60 µg l^{-1}, was driven to complete extinction at 114 µg l^{-1}. *M. leuckarti*, showed sensitivity similar to *D. magna* reaching 100% extinction risk at 130 µg l^{-1}.

- Scenario B (Two peak)

  Extinction probabilities in this scenario were especially unique for the *M. leuckarti* population (Fig 5.4). Population extinction for *M. leuckarti* began after 40 µg l^{-1}. Total population extinction was seen at 105 µg l^{-1}. This overlaps with the *D. magna* population curve which begins much later (after 62 µg l^{-1}) and ends at 90 µg l^{-1}. *C. crystallinus* was again observed to be most sensitive and was completely extinct at 28 µg l^{-1}, much before the other two species showed any signs of mortality (Fig 5.4). Overall, *C. crystallinus* was observed to be most sensitive on the population level and *D. magna* was found to be least sensitive (Fig 5.4). The *D. magna* population recovered completely before the second peak. The *C. crystallinus* population was 1.6 times more sensitive under Scenario B than under Scenario A.

  The sensitivity ranking on the population level was-

  C. crystallinus > *M. leuckarti* > *D. magna*

5.4 Discussion

5.4.1 Differences in sensitivities related to life histories

5.4.1.1 Individual level

At the individual level, the response of an organism to a toxicant is purely a product a combination of its own physiological processes as well as the physical and chemical properties of the toxicant. The solubility of the substance in water, the uptake of the substance

by the organism, the assimilation of the substance within the organism and the harmful effects of the substance by interfering with cellular processes are what determine the ultimate effect (Hodgson 2004). From the GUTS calibration (Table 5.3), it was clear that the sensitivities of the three species were results of a combination of different factors, as has also been observed before (Chapter 3). All three test species showed similar k_k values which highlights the fact that TPT has a similar mode of action in all the three species. The *z* was lowest for the daphnids and explains the high sensitivity of *D. magna* at the individual level.

Overall, it has been suggested that in short-term laboratory experiments with TPT that long term effects that are seen in cosm experiments, such as longer exposure via food and water, are ignored (Roessink et al 2006). Therefore, for organisms with a relatively longer life cycle like *C. crystallinus*, short-term laboratory experiments may underestimate sensitivity. In such cases, sensitivity needs to be observed over a longer time.

5.4.1.2 Population level

At the population level, all complex interactions, interference with food, as well as bioaccumulation and bio-magnification, and a wide range of limiting processes e.g. density dependence, cannibalism, predation, etc are present (Forbes et al 2008, Preuss et al 2009, Nicolle et al 2011). Therefore, the responses seen at the population are drastically different compared to individual-level responses. Each of the three test species showed population-level responses uniquely determined by its own life history:

D. magna

Population dynamics in the *D. magna* model are regulated by crowding and food availability (Preuss et al 2009). The regulation of offspring size and fitness related to maternal environment in *D. magna* seems to be contingent on variables such as food or environmental

stress. Crowding affects survival of neonates and juveniles as well as reproduction and brood size (Preuss et al 2009). Food dependence influences all the different life-cycle processes (Preuss et al 2009). The neonates are more sensitive to TPT compared to the adults (Table 5.2) at the individual level. Adverse effects on the individual level may not be the same on the population level. TPT may also cause long-term delayed effects (Roessink et al 2006) which may be manifested in subsequent generations. Although it may not seem too far-fetched to consider that R-strategists like *D. magna* may be able to reproduce their way out of an event of toxic exposure, toxicodynamics can be far more complex than we realise. Under exposure to Dispersogen A, *D. magna* produced numerous but less fit neonates were produced at the individual level and this led to an overall reduction in population size (Hammers-Wirtz and Ratte 2000). It was also shown that reduction in population size under toxic stress may, in case of toxicants like Dispersogen A, not be due to effects on survival but rather due to the reduction in offspring fitness (Gabsi et al 2013). Nevertheless, it is clear that the life-history strategies of *D. magna* play a significant role in determining population-level effects.

M. leuckarti

It has been shown for marine copepods that the effects of a stressor at high population density may be different from those at low population density (Sibly et al 2000). The most important life-cycle processes driving the population dynamics of *M. leuckarti* are mixed feeding and cannibalism (Chapter 4).

Therefore, density dependence in *M. leuckarti* populations is determined predominantly by cannibalism and food concentration. In this study, food was sufficient and therefore, only cannibalism determined population susceptibility. Furthermore, the nauplii of *M. leuckarti* are more sensitive to TPT compared to the adults (Table 5.2). Therefore, although cannibalism can, in some cases e.g. in the absence of prey, be a stabilising mechanism for population

dynamics (Gabriel W 1985), in case of *M. leuckarti*, cannibalism exerted additional stress on the population and therefore, the susceptibility to TPT was increased as was also shown before (Chapter 4). Moreover, the comparatively longer life cycle and higher adult longevity of *M. leuckarti* make it susceptible to multiples exposures within a single lifetime (Chapter 2).

C. crystallinus

In the *C. crystallinus* model, density-dependent mortality determines the carrying-capacity of the model. At high population densities, the adverse effect of TPT is compensated by the decrease in cannibalism. At lower population densities, there is lower cannibalism and a higher susceptibility to TPT. Population density, food availability as well as toxic effects together determine population resilience in this species. Most importantly, the reproductive strategy and development time (Table 5.1) are vastly different from the other two species. Immigration and emigration also play a part in regulating population densities of this species in open systems. However, migration processes have been excluded from the model used in this study. This species best exemplifies the fact that intrinsic sensitivity may not always imply population vulnerability. It showed lowest intrinsic sensitivity at the individual level but as a consequence of a myriad of population-level effects, it was found to be most sensitive at the population level in this study.

5.4.2 Inter-species differences in population-level sensitivity

At the individual level, all three test species have different life-history strategies (Table 5.1). One of the most significant differences, which may have important consequences for recovery, seems to be the juvenile development. *D. magna* matures within two weeks and reproduces comparatively faster than the other two species. The long life cycles of these two species make them more vulnerable to toxic exposure and to the possibility of delayed effects

(Roessink et al 2006). Besides having a longer generation time, cannibalism exerts additional stress on the nauplii in combination with the toxic effects of TPT and therefore, population resilience is reduced. Some life-history strategies of *M. leuckarti* are shared by either of the other two test species. *M. leuckarti*, like *D. magna* is iteroparous, and therefore produces more offspring within one life time than *C. crystallinus.* However, like *C. crystallinus, M. leuckarti* has a long generation time owing to which it recovers slower than *D. magna*. The *C. crystallinus* population was 1.6 times more sensitive under Scenario B than under Scenario A. This can again be attributed to the fact that a longer life-cycle may imply multiple exposures within the lifetime. The *C. crystallinus* population was extinct at concentrations when the probability of extinction for the populations of the other two species was still negligible (Fig 5.4). Under Scenario B, the *M. leuckarti* population had already reached 80% extinction risk at 70 µg l^{-1} whereas the *D. magna* population had only reached 30% extinct risk. Although both populations reached extinction at similar concentrations, the copepod population was at risk much earlier. This may also probably be due to exposure over longer time.

5.4.3 Significance of models

Our model simulations agree with a similar outdoor mesocosm study with TPT (Roessink et al 2006). The contrasting sensitivities at the individual and population level observed in this study were in accordance with Roessink's mesocosm study. In case of toxicants with a multiple effects, standard individual-level toxicity tests may underestimate toxic effects (Gabsi et al 2013), and although cosm experiments are closer to reality compared to laboratory experiments, they are expensive, and cumbersome to setup and monitor. Protection goals for aquatic invertebrates need to be defined at the population level and this is where mechanistic population models can help. Models are cheap virtual laboratories and can be used repeatedly to simulate various scenarios. In case of individual-based models, population

dynamics emerge from individual-level processes (DeAngelis). Even with the absence of uptake and elimination data, models like GUTS can describe TKTD using survival data from simple laboratory experiments. This study highlights the power of population models in simulating and predicting population-level effects from individual-level data. A combination of experimental effects at individual level and population models can assist extrapolation across species and exposure scenarios and reduce uncertainty in ERA.

5.5 Conclusions

Life-history strategies can have implications for population dynamics. Population-level sensitivity to a toxicant may be considerably higher than the individual-level sensitivity. Population models are powerful tools to predict population-level effects from individual-level data. For more realistic ERA, protection goals for aquatic invertebrates need to be developed at the population level and a combined approach of laboratory experiments and mechanistic population modelling needs to be employed.

Chapter 6

General conclusions

As reviewed in Chapter 2 although a few recent studies show growing awareness towards employing copepods in ecotoxicological studies, copepods are largely ignored within freshwater ecotoxicology. The work presented within this thesis primarily highlights the importance of copepods in freshwater ecosystems, advocates the significance of copepods for freshwater ecotoxicology and proposes a way forward to consider copepods in higher-tier risk assessments. To fulfil the need for a freshwater invertebrate species in addition to *Daphnia magna* useful in higher-tier ecological risk assessment (ERA), a representative species for freshwater copepods, *Mesocyclops leuckarti,* was identified in Chapter 2. This species was selected through an exhaustive literature review and is a good compromise between a potentially vulnerable species as well as a good laboratory species. In order to advocate the use of this species, the construction of an appropriate framework was essential. Therefore, a combination of laboratory experiments and mechanistic modelling was proposed in Chapter 2. Generally copepods are mostly avoided in ecotoxicological studies due to their long life cycles and complex culturing requirements. This problem was circumvented by selecting *M. leuckarti* in Chapter 3. Chapter 3 also highlighted the fact that *M. leuckarti* is not a difficult laboratory species to work with and can be conveniently cultured under controlled conditions. The results of the toxicity experiments described in Chapter 3 established *M. leuckarti* as an intrinsically sensitive species. Furthermore, the study also revealed that the naupliar stages of this species were more sensitive than the older stages. When the toxicokinetics and toxicodynamics were investigated in greater detail, it was revealed that the threshold of effects was the most important factor in determining the sensitivity of the naupliar stages of this species and not the killing rate. Considering the fact that the prediction of population-level responses from individual-level data requires population models, an individual-based model was developed in Chapter 4. The main outcome of model simulations was the identification of the synergistic effect of cannibalism as well as TPT stress on the nauplii as

the cause of increased susceptibility of *M. leuckarti* populations under toxic exposure. Chapter 5 showed that adverse effects seen at the individual level may not necessarily be the same at the population level. Different species may respond differently to toxic stress at the individual and the population level as a consequence of their life-history strategies. Nevertheless, it was confirmed that population models are valuable tools in predicting population-level responses from individual-level data.

Although the parameterisation of the toxicity submodel within the model presented here is specific to a single species and toxicant combination, it is not rigid. Different submodels for different toxicants can be integrated into the model. The General Unified Threshold model for Survival is able to describe toxic effects based on relatively basic survival data from laboratory tests. Furthermore, the model can be re-parameterised to describe the life cycles of different copepod species to enable extrapolation across other species and exposure scenarios. The model is veritably intended to support and improve the interpretation of results of toxicity tests. This approach which includes the employment of more relevant species for short and long-term laboratory tests combined with mechanistic modelling should be useful tool for the ERA of chemicals. There are no data on population experiments dealing with toxic effects on *M. leuckarti*. Therefore, future tasks would include conducting population experiments as well as validating the model with independent datasets from population experiments to make the model more reliable.

The experimental work and the modelling within this study fit well within the tiered risk assessment scheme proposed by the European commission (EFSA 2013). Data from laboratory experiments with copepods constitute Tier 2 and be can easily implemented into a model which constitutes Tier 3. The population models should be validated on data from

population experiments and mesocosm studies. After successful testing, the model could be used as a tool to predict the population-level effects on copepods when exposed to toxicants.

To facilitate the consideration of freshwater copepods in ERA there is a need for the development of robust methods and guidelines for studies on important freshwater representative species understandable for risk assessors. This study is one such example which confirms that a combined approach of laboratory experiments and population modelling can prove to be powerful resource for the ERA of plant protection products in freshwater ecosystems.

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Publications

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Kulkarni D, Daniels B, Preuss TG (2013) Life-stage-dependent sensitivity of the cyclopoid copepod Mesocyclops leuckarti to triphenyltin. Chemosphere 92:1145-1153. DOI: 10.1016/j.chemosphere.2013.01.076

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Conference contributions

Kulkarni D, Daniels B, Preuss TG (2013) The influence of food-dependent eco-physiological processes on the response of Mesocyclops leuckarti to triphenyltin. Poster spotlight. 23rd SETAC Europe Annual Meeting, Glasgow, United Kingdom.

Kulkarni D (2013) The influence of food-dependent eco-physiological processes on the response of Mesocyclops leuckarti to triphenyltin. Platform Presentation. CREAM Open Conference "Mechanistic Effect Models for Ecological Risk Assessment of Chemicals", Leipzig, Germany.

Daniels B, Kulkarni D, Preuss TG (2013) Applying mechanistic TKTD models to evaluate acute-toxicity tests: An approach to phase out the NOECs and regression-based LCx. Poster corner. 23rd SETAC Europe Annual Meeting, Glasgow, United Kingdom.

Kulkarni D, Daniels B, Strauss T, Preuss TG (2013) Comparing individual and population level sensitivities to triphenyltin- A tale of three models. Poster presentation, 3rd SETAC Young Environmental Scientists meeting, Krakow, Poland.

Daniels B, Kulkarni D, Ratte HT, Preuss TG (2012) Applying the general unified threshold model of survival (GUTS) to describe toxic effects of Triphenyltin

Hydroxide on the cyclopoid copepod *Mesocyclops leuckarti*. Poster presentation, 6th Setac World Congress, Berlin, Germany.

Kulkarni D, Strauss T, Hommen U, Gergs A, Ratte HT, Preuss TG (2012) Using a modelling approach to compare sensitivities to Triphenyltin at the individual and population levels for three planktonic organisms. Platform presentation, 6th Setac World Congress, Berlin, Germany.

Kulkarni D, Hommen U, Gergs A and Preuss TG (2011) Why a population model of the cyclopoid copepod *Mesocyclops leuckarti* for the ecological risk assessment of chemicals is necessary. Poster presentation, 21st Setac Europe Annual Meeting, Milan, Italy.

Kulkarni D and Preuss TG (2011) How copepods are useful in studying the effects of pesticides in water: A Ph.D. project overview. Poster presentation, Marie Curie Researchers Symposium "SCIENCE – Passion, Mission, Responsibilities", Warsaw, Poland.

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