# Consequences of short-term feeding inhibition from exposure to pesticides for individuals and populations of aquatic invertebrates

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# Abstract

Recently, several scientific committees of the European Commission have identified research needs to enhance the risk assessment of plant protection products (PPPs). This PhD explicitly focuses on contributing to the research needs of assessing effects under highly time-variable exposure, increasing the ecological realism in effect assessment approaches, considering effect assessment of combined stressors (natural and anthropogenic) and improving ecological modelling.

The presented work focuses on the observation of potential impacts of PPPs (imidacloprid and carbaryl) on feeding of aquatic invertebrates (*Gammarus pulex* and *Daphnia magna*) under more environmentally-realistic exposures. Isolated feeding depression and its combination with additional stress is explored. Investigations include the determination of consequences of alterations in feeding for further behavioural traits of individuals and its transposition to the population level. An ecological model is used as a virtual laboratory to allow the interpretation of complex impacts observed which in turn helps to evaluate the model used.

A key finding is that feeding assays with *G. pulex* are able to reveal impacts of PPPs at environmentally-relevant concentrations and that the measurement of recovery potential is important. However, the method used requires further improvement in order to extrapolate impacts to the population and ecosystem level. The possibility of short-term impacts on feeding causing severe impacts at the individual and population level is shown for *D. magna.* Direct extrapolation from the feeding assay with imidacloprid to other individual traits is not possible. Impacts are found to depend on food availability and the individual's reproductive strategy, which is found to be more flexible under multiple stresses than has been reported in the literature. Further research is required in order to generalise these findings.

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# Author's declaration

All research chapters were written in the form of research papers for publication in international peer-reviewed journals. Table 1 gives the latest status of the publication process. The format of published and submitted articles has been changed according to the requirements for this thesis, but only minor alteration to the content of articles was undertaken. Copyright rests with the publishers for those articles published and in the case of acceptance for those submitted for publication.

The presented work has been undertaken as a full-time PhD student enrolled at the University of York and was supervised by Prof. Colin Brown and co-supervised by Dr. Roman Ashauer.

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Parts of the results presented in Chapters 5 and 6 and in Appendix A derive from the work of Tabatha Cole (former BSc student of the University of York) and Wiebke Giessler (visiting student at Fera from the Institute of Environmental and Sustainable Chemistry, TU-Braunschweig, Braunschweig, Germany). Both students were co-supervised by me.

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All research chapters are subject to joint authorship, reflecting the role of co-authorship. Thus, research articles have been subject to improvement by suggestion, advice and editing by co-authors, and in the case of complete publication by two or more anonymous referees throughout the publishing process. However, all publications have been written by myself as main author.

*Table 1: Status of publications for chapters presented in this thesis.*

| **Title** | **Authors** | **Journal** | **Article history** | **Chapter** |
|---|---|---|---|---|
| Natural variability in feeding rate of *Gammarus pulex* | Annika Agatz<br>Colin D. Brown | To be decided | To be submitted | 2 |
| Imidacloprid perturbs feeding of *Gammarus pulex* at environmentally-relevant concentrations | Annika Agatz<br>Roman Ashauer<br>Colin D. Brown | Environmental Toxicology and Chemistry | Submitted 17-09-13<br>Revised 18-11-13<br>Accepted 21-11-13 | 3 |
| Feeding inhibition explains effects of imidacloprid on the growth, maturation, reproduction and survival of *Daphnia magna* | Annika Agatz<br>Tabatha A. Cole<br>Thomas G. Preuss<br>Elke Zimmer<br>Colin D. Brown | Environmental Science and Technology | Submitted 25-11-12<br>Revised 11-2-13<br>Accepted 20-02-13<br>Published 19-03-13 | 5 |
| Evidence for linking feeding inhibition, population characteristics and sensitivity to acute toxicity for *Daphnia magna* | Annika Agatz<br>Colin D. Brown | Environmental Science and Technology | Submitted 02-04-13<br>Revised 09-07-13<br>Accepted 09-07-13<br>Published 20-08-13 | 6 |
| Body size and nutritional status dependent acute toxicity of carbaryl on *Daphnia magna* | Annika Agatz<br>Wiebke Giessler<br>Colin D. Brown | To be decided* | To be submitted | Appendix E |

**Short communication*

# 1. General introduction

Plant protection products (PPPs) are intended for use in the environment to protect crops against pests and diseases and from competition from weeds. Products are used to ensure large scale plant production to meet the increasing demand for food. However, PPPs are designed to be toxic and are produced with the intention to be released into the environment. These chemicals, in particular the newer compounds, have specific mechanisms and modes of action but their use in the field may not only affect target organisms. Non-target organisms can also be directly or indirectly affected by PPPs. The range of possible effects is unclear due to the complexity of our environment which we do not fully understand at present. Nevertheless, great effort is made by different stakeholders (industry, regulators, academia and decision makers) to predict, understand and evaluate the fate and effects of PPPs before they are approved for use in the field. This procedure is called environmental risk assessment (ERA). Within the European Union the risk assessment of PPPs (more precisely of active substances, safeners and synergists in PPPs) is regulated under legislation EC No. 1107/2009 (European Union, 2009). Annex II of this legislation provides a full list of procedures and criteria for the approval of a PPP (European Union, 2009). Overall, a PPP has to be assessed prior to the potential approval regarding its efficacy, methods of analysis, fate and behaviour in the environment (including the relevance of metabolites and its fate and behaviour concerning groundwater) and its impact on human health and the environment. The aim is to protect the environment against impacts with adverse consequences for environmental and human health (European Union, 2009). Several guidance

documents exist and are frequently updated and new guidance is developed to ensure that the risk assessment takes account of the latest knowledge. For example, the guidance on tiered risk assessment for PPPs for aquatic organisms in edge-of-field surface waters has been updated in 2013 by the European Food Safety Authority (EFSA) panel on Plant Protection Products and their Residues (PPR) (EFSA, 2013).

Despite efforts in environmental risk assessment, several examples of PPPs exist where unacceptable impacts have been observed after the regulated use of those substances in the environment (Stockholm Convention, 2009). Currently, 14 pesticides are fully or partially banned under the Stockholm convention (Stockholm Convention, 2009; 2011). Cases such as these, and the fact that PPPs are still proposed to be banned under this convention, indicate the need to improve the risk assessment procedures to prevent false positive assessments. The improvement of the risk assessment to prevent false negative assessments is equally important. Global demand for cereals will double by 2030 (United Nations, 2002). This and the restricted area available for additional agriculture (United Nations, 2002) lead to the need to improve crop production and/or reduce the loss of production due to pests and diseases. The development and registration of a new generation of PPPs and the improvement of PPP use may be a promising way to fulfil this task. However, to do so the ERA of PPPs needs to be improved to be protective in all cases but not over-protective.

This PhD focuses on aspects of the risk assessment of PPPs on the effect assessment for the aquatic environment especially focusing on the evaluation of risk for aquatic invertebrates living in the water column in edge-of-field surface waters (e.g. ponds,

streams and ditches). The ERA-specific protection goal for invertebrates in edge-of-field surface waters is at the population level (EFSA, 2013). Generally, the aquatic risk assessment consists of a combination of exposure and effect assessment (EFSA, 2013); the assessment is undertaken in different steps, the so called tiers. Overall a step is passed when there is a very low risk of unacceptable impacts due to the use of this PPP in the environment and therefore the assessment is completed. If a PPP under assessment fails (e.g. shows potential for a risk to the environment) it can either fail the assessment, meaning it cannot be used in the environment, or enter the next assessment step. In each step the type of data used increases in detail, quality, reflection of environmental reality and specification in order to meet the requirements of the underlying questions which also increase in their specificity with each step (EFSA, 2013).

Recently, a list of relevant research needs to be developed, considering the improvement of risk assessment of PPPs, was highlighted within the framework of several scientific committees of the European commission (SCHER, SCENHIR, SCCS) (Vighi, 2012). A selection of these research needs (European Union, 2013; Vighi, 2012) are provided below and are briefly described in the context of risk assessment of PPPs in the area of aquatic invertebrates.

*"Assessing the effects of highly time-variable exposure."* Degradation, metabolism, frequency of use, application of different volumes depending upon weather (wind force, wind direction, intensity and frequency of rainfall), the distance of the water body from the application site, the soil type and many other factors influence the concentration of PPPs in ground and surface water (Reinert *et al.*, 2002). All these

influences produce a very complex situation and result in a variable occurrence of PPPs in water bodies (Calevro *et al.*, 1999; Wittmer *et al.*, 2010). Despite this knowledge, risk assessment relies usually on effect assessment under constant exposure, especially in the earlier steps (EFSA, 2013).

*"Increasing the ecological realism of effect assessment approaches."* The concentration of the PPP used in effect assessment, especially in the first steps of the risk assessment, is also normally ecologically unrealistic. Concentrations tested in ecotoxicological tests are usually rather high and generally do not reflect concentrations potentially occurring in the habitat of the test species. Mortality of individuals and their reproductive outcome are endpoints which were judged as important when looking at the survival and robustness of a population (OECD, 2004). A combination of these endpoints not being very sensitive and the need to generate standard measures for risk assessment procedures has led to the use of concentrations that are generally ecologically irrelevant. The use of other (sub-lethal) endpoints) is needed to measure potential influences of more environmentally-realistic concentrations (Chapman, 2002). Behaviour may provide a sensitive indication of sub-lethal toxicant effects as it represents an integration of complex biochemical and physiological processes (Rand, 1995) which is generally affected at lower concentrations than mortality. Focusing on feeding as an endpoint in ecotoxicological studies is a useful and sensitive tool to detect sub-lethal impacts on individual organisms with relevance to the population and/or ecosystem level. Energy availability depends on feeding (Sancho *et al.*, 2009; Villarroel *et al.*, 2009) and the energy budget can be considered an indicator of the overall condition of an organism (Calow and Sibly, 1990) which ultimately determines health of the

population. Other changes like altered growth and reproduction, which are also used as endpoints in experiments, are often instigated by an effect on feeding (Guisande and Gliwicz, 1992; Porter *et al.,* 1982; Preuss *et al.,* 2009).

*"Assessing the interactions between combined stressors and environmental factors."* A further mismatch between the risk assessment procedure and the reality in the environment is the lack of consideration of multiple stresses. Multiple stresses may occur from a combination of different PPPs being present at the same time or following each other or due to a combination of anthropogenic (e.g., PPPs) and natural stress. Examples for natural stress are competition for space and food, or the presence of predators. Organisms within standard tests for ERA are usually tested at the individual level under optimal conditions and under constant exposure to one PPP (OECD, 2004). This is mainly to increase the power of the test to generate reliable values for the standard measurement endpoints in the current risk assessment. However, in reality organisms interact with each other (intra- and inter-specific interaction) and more than one compound may enter the habitat of the test species at a time. Concurrent appearance of environmental and anthropogenic stress exposes individuals in the environment to enormously different circumstances than tested in the effect assessment for the ERA. Microcosm and mesocosm studies do consider intra- and inter-specific interaction, but mixture toxicity is totally ignored, at least for risk assessment purposes (EFSA, 2013).

*"Improving ecological modelling."* Ecological and ecotoxicological modelling is a promising tool for ERA (European Union, 2013). It has been suggested frequently that such models can be a powerful means to overcome some of the limitations the

ERA faces (Grimm *et al.*, 2009). One limitation is the contrasting nature of effect assessment and protection aims within the ERA of PPPs. Influences of static exposure to individuals of a particular age class are measured for individuals kept under optimal environmental conditions (OECD 2004 and references therein to the different test protocols). In the environment, however, populations (the protection level) are composed of individuals of all ages which face limiting environmental conditions and dynamic exposures. It is challenging to extrapolate from the individual to the population level because a population consists of a variety of dissimilar individuals. Individuals in a population generally act in response to internal and external factors, both of dynamic nature. Individuals interact with each other and with their environment according to their individual properties which differ between individuals and within one individual over time (Grimm and Railsback, 2005). Thus, a perpetual, variable and sensitive interplay of action and reaction from individuals exists in a population. Individual based population models (IBMs) are developed to address this limitation in extrapolation and can subsequently be expanded with one or more toxicological sub-models as a tool to predict impacts of xenobiotic exposure on the dynamics in populations; this makes these models a useful tool for ERA of pesticides.

This PhD explicitly focuses on contributing to the research needs described above through i) improvement of experimental methods of effect measurements to measure influences of time-variable exposure (Chapter 2); ii) assessment of effects from short-term exposure with a subsequent phase of observation beyond exposure (Chapter 3,5 and 6); iii) investigation of sub-lethal influences of PPPs to aquatic invertebrates (Chapters 2-6) focusing mainly on influences on feeding behaviour

(Chapters 2-4); iv) investigation of the potential transposition of influences on feeding to other behavioural traits of individual aquatic invertebrates (Chapter 5) and to the population level (Chapter 6); v) assessing effects under multiple stress (Chapters 4-6); and vi) improvement of ecological and ecotoxicological modelling by application of existing models (Chapter 5) and by generating data sets needed for model development (Chapters 2-6).

Specific aims and objectives of this project are given below.

Aims:

1) Assessing the feasibility of long-term feeding assays with two aquatic invertebrates to investigate the impact of time-variable exposure to two pesticides on feeding behaviour.
2) Assessing the consequences of short-term feeding inhibition from exposure to pesticides under various degrees of natural stress to individual traits of aquatic invertebrates.
3) Assessing the impact of short-term feeding inhibition from pesticide exposure to populations of aquatic invertebrates under various degrees of additional stress for the population.

Objectives:

1) Development of feeding assays with *Gammarus pulex* and *Daphnia magna* to measure the impact of pesticides on feeding rate at a daily resolution in order to test effects of time-variable exposures.
2) Measure the concentration-dependent impact of imidacloprid and carbaryl on the feeding rate of *Gammarus pulex* and *Daphnia magna*.

3) Assess the feasibility of measuring consequences of pesticide-induced feeding inhibition on growth and reproduction of *Gammarus pulex* and *Daphnia magna* when using the experimental method developed in objective 1.
4) Investigate the transposition of short-term feeding inhibition from pesticide exposure on growth and reproduction for *Gammarus pulex* and/or *Daphnia magna* under low and high food availability for individuals.
5) Explore whether impacts on growth and/or reproduction observed in objective 4 are direct effects of the pesticide or whether changes observed are driven by acclimation to feeding inhibition. Already existing models may be used and/or enhanced.
6) Conducting a population experiment with *Daphnia magna* to investigate the impact of short-term feeding inhibition on the population abundance and population structure when feeding inhibition accurse within the phase of population development or when populations are in their environmental equilibrium.
7) Assess whether impacts of short-term feeding inhibition on populations (result of objective 6) can be predicted from effect assessment at the individual level (result of objective 4).
8) Explore whether short-term feeding inhibition of *Daphnia magna* populations changes the sensitivity of the population to subsequent exposure to an acute toxin.

All work undertaken in this thesis focuses on two aquatic invertebrate species. A standard test species always used for risk assessment (*Daphnia magna*) which lives in non-flowing surface waters and a widely distributed freshwater detritivorous

shrimp (*Gammarus pulex*) living mainly in flowing surface waters. An overview is provided below to explain why these species were chosen, what natural factors are known or thought to influence the sub-lethal endpoints observed, and which PPPs were used within the presented work.

*G. pulex* is one of the aquatic detritus feeders that are present all over the world and play an important role for leaf litter breakdown in the environment (Maltby and Naylor, 1990). Aquatic detritivorous macro-invertebrates, called shredders, have a key role in litter breakdown through fragmentation of leaf material (Gessner *et al.*, 1999; Petersen and Cummins, 1974). This necessitates the study of shredding behaviour under anthropogenic influences. One way of measuring shredding behaviour is the measurement of feeding rate. Experiments measuring the impact of xenobiotics on the feeding rate of *Gammarus* are mostly undertaken with individuals caught in the field throughout the year and a huge variety of materials and methods are used (no standard procedure is available); this makes it hard to compare results from different experiments with each other.

Generally, two different *ex situ* methods were used in the past to detect changes in the feeding activity due to xenobiotic exposure: time-response feeding experiments and mass feeding assays. Time-response feeding experiments measure the time until a fixed amount of food is eaten (Blockwell *et al.*, 1998; Pascoe *et al.*, 1995; Taylor *et al.*, 1991; Taylor *et al.*, 1993). By contrast, in mass feeding assays the mass of eaten food within a fixed time point is measured (Brown and Pascoe, 1989; McCahon and Poulton, 1991). Both methods may be suitable to detect the time-dependent impact of different exposure scenarios. Time-response feeding experiments have the advantage

that the experimental design is straightforward. After exposure the organisms will be fed with a determined amount of food and the time until 50% of the food is ingested is measured. However, this method is limiting when one wants to extend the experiment to investigate the transposition of feeding inhibition to growth and/or reproduction. Bundschuh *et al.* (2009) and Graca *et al.* (1993 and 1994) showed that variability exists in food preference between individuals and for one individual over time. Changing the food source, as is done in time-response feeding experiments, can alter the feeding rate itself. Performing a mass feeding assay seems to be a more promising alternative. There, the food source stays the same for the whole experiment, so multiple measurements over a long time period are possible without an additional impact caused by the change in food source.

Several natural factors influence the feeding rate of gammarids, such as food source, food quality, body size, temperature, reproductive status, parasite infection and water quality (Kunz *et al.*, 2010). Many of these influences have been reported in the past (for detailed information see Chapter 2). However, the quantitative influence on feeding was either not reported or only given for the food source used in the particular test, making comparisons of the quantitative influences impossible.

*Daphnia magna* is an important organism in the food chain of non-flowing surface water because of its crucial role as a primary consumer and as food for predators like small fish and different insect larvae (Brett, 1992; Gliwicz, 1994; Murdoch and Scott, 1984; Stibor, 1992). Daphnids have a fairly short lifecycle whilst reproducing parthenogenetically and are therefore a suitable test organism for whole life cycle testing. This makes it possible to test the transposition of effects on feeding to other

endpoints like growth and reproduction and even allows to empirically investigate transposition of impacts on feeding to the population level. Furthermore, daphnids are known to adapt rapidly and efficiently to a wide range of changes in their environment allowing the investigation of effects from dynamic exposure. In particular they are known to be dynamic in their reproductive outcome. Changes in reproduction has, for example, been found to be influenced by changes in the food source (Boersma, 1997; Cleuvers *et al.*, 1997; Coors, 1999; Enserink *et al.*, 1995; Glazier, 1998; Sokull-Klüttgen, 1998), by presence of predators ((Brett, 1992; Coors, 1999; Gliwicz, 1994; Hammers, 1996; Murdoch and Scott, 1984; Repka *et al.*, 1994; Sakwinska, 1998; Stibor, 1992), or by presence of conspecific pressure (Dodson and Havel, 1988; Goser and Ratte, 1994; Luning, 1992; Machacek, 1993; Weider and Pijanowska, 1993).

The PPPs used for the presented work are the insecticides imidacloprid and carbaryl. Details on why those compounds have been used are given in the separate chapters as reasons differ with the aim of each study undertaken. Both compounds have in common that they act on the nervous system. Their target site within the nervous system differs but the result of their action is the same - an overstimulation of nervous cells disabling the normal function of a nerve cell. Imidacloprid is a neonicotinoid insecticide which binds onto the acetylcholine receptors (EFSA, 2008). Carbaryl inhibits acetylcholinesterase (United Nations 2007), a protein responsible for removing acetylcholine from the receptor. Details on the physico-chemical properties, the fate of the compound in water and its ecotoxicity to aquatic organisms are given in Table 1-1.

*Table 1-1: Key data for the study compounds used in this thesis (PPDB, 2013)*

| | Carbaryl | Imidacloprid |
|---|---|---|
| Pesticide type | Insecticide, Plant growth regulator | Insecticide, Veterinary treatment |
| Substance group | Carbamate | Neonicotinoid |
| Mode of action | Stomach and contact activity with slight systemic properties. Acetylcholinesterase (AChE) inhibitor. | Systemic with contact and stomach action. Acetylcholine receptor (nAChR) agonist. |
| CAS RN | 63-25-2 | 138261-41-3 |
| Chemical formula | $C_{12}H_{11}NO_2$ | $C_9H_{10}ClN_5O_2$ |
| Molecular mass (g $mol^{-1}$) | 201.22 | 255.66 |
| IUPAC name | 1-naphthyl methylcarbamate | (*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine |
| Formulation and application details | Often formulated as a suspension concentrate that is mixed with water and applied using an orchard sprayer. | Often supplied as granules that are mixed with water and applied as a spray, used as a seed treatment or applied directly to compost. For animal use it is usually supplied as a spot-on solution. |
| Solubility - In water at 20$^oC$ (mg $l^{-1}$) | 9.1 | 610 |
| Octanol-water partition coefficient at pH 7, 20$^oC$ | 2.29 X $10^{02}$ | 3.72 X $10^{00}$ |
| Water phase only DT50 (days) | 3.1 | 30 |
| Bio-concentration factor | 44 | 0.61 |
| Acute 48 hour $EC_{50}$ (mg $l^{-1}$) | 0.0064 *Daphnia pulex* | 85 *Daphnia magna* |
| Acute 96 hour $LC_{50}$ (mg $l^{-1}$) | 0.0057 *Americamysis bahia* | 0.046 *Americamysis bahia* |
| Chronic 21 day NOEC (mg $l^{-1}$) | 0.25 *Daphnia magna* | 1.8 *Daphnia magna* |
| Mesocosm study data (NOEAEC (mg l-1)) | 0.02 Aquatic community, inappropriate test conditions | 0.0006 Chironomids, Batidae |

This thesis comprises seven main chapters: *Chapter 1* provides a general introduction to the environmental risk assessment of plant protection products and some research needs provided by different scientific committees of the European commission to improve the risk assessment procedures.

*Chapter 2* summarises work undertaken to quantify the variability in individual feeding rate of *G. pulex* under various individual-based and food source-based influences. The purpose of this work was to improve methods to measure the influence of PPPs on the individual feeding rate of *G. pulex* at a fine temporal resolution enabling the test to be used with more environmentally-realistic exposure patterns.

*Chapter 3* demonstrates empirical measurement of the influence of imidacloprid (main chapter) and carbaryl (Appendix A) on the individual feeding rate of *G. pulex* and the organism's recovery from such influences using the method improvements obtained in Chapter 2.

*Chapter 4* reports empirical evidence that natural stress (here limitation in food quality) prior to anthropogenic stress (here short-term exposure to imidacloprid) can change the results of the effect assessment. Further research needs are discussed to allow feeding assays with gammarids to be considered as a standard test for environmental risk assessment.

*Chapter 5* reports a series of experiments undertaken to measure the impact of short-term imidacloprid exposure on the individual performance of *D. magna* e.g. feeding,

growth, maturation, reproduction and survival. This chapter also reports on the use of an ecological model (individual based population model) as a virtual laboratory to interpret the multiple effects measured during the series of experiments conducted. This chapter was published in Environmental Science and Technology early in 2013 and contains two appendixes. Appendix B provides the supporting information as published and Appendix C contains additional experimental material investigating whether effects on mothers due to short-term imidacloprid exposure are transposed to the next generation.

*Chapter 6* reports on a *D. magna* population experiment undertaken to investigate whether short-term exposure to imidacloprid causes impacts to populations and how these vary with the level of intra-specific competition present while exposure occurs. Additionally, it was investigated whether short-term exposure to imidacloprid alters the sensitivity of the population to subsequent short-term exposure to carbaryl and whether the level of intra-specific competition interferes with those alterations. This chapter was published in Environmental Science and Technology (2013) and contains an appendix providing the supporting information as published (Appendix D).

*Chapter 7* summarises the main findings of the presented work and discusses these in the context of current procedures in environmental risk assessment and the previously mentioned research needs for the risk assessment of PPPs.

# 2. Natural variability in feeding rate of *Gammarus pulex*

Focusing on feeding as an endpoint in ecotoxicological studies is a useful and sensitive tool to detect sub-lethal impacts on individual organisms at more environmentally-realistic exposure concentrations with relevance to higher levels of organisation. Energy availability depends on feeding and can be considered an indicator of the overall condition of individuals because growth and reproduction are instigated by feeding. Several natural factors are known to influence the feeding rate of gammarids, such as food source, food quality, body size, temperature, reproductive status, parasite infection and water quality (Kunz *et al.,* 2010). Many of these influences have been reported in the past (summarised by Kunz *et al.,* 2010) but the quantitative influence on feeding was either not reported or only given for the food source used in the particular test.

This chapter presents several experiments undertaken to identify and quantify the influence of parasite infection and food source, food quality, body size and acclimation time on the feeding rate of *G. pulex*. All these experiments were undertaken to identify a more standardised way to measure the influence of xenobiotics on the individual feeding rate of gammarids at a daily resolution to allow the investigation of effects from short-term exposures. Measurement at the individual level was chosen for three reasons; i) reduction of the number of test organisms to use; ii) reduction of data variability due to cannibalism; and iii) generation of data for the development and/or validation of individual based ecological models that predict leaf litter decomposition due to shredding.

## *2.1. Introduction*

Intra-specific variability is an important influence in ecotoxicology, particularly when studying sub-lethal effects. There may be differences in an endpoint of interest among individuals which are so large that it may be more appropriate to focus on a sub-group of the test species in order to detect changes caused by the tested stressor. To identify which sub-group of *Gammarus pulex* to use in toxicological feeding assays the intra-specific variability in the feeding rate caused by natural factors needs to be quantified.

The food preferences for freshwater detritivores like *Gammarus* are influenced by leaf type (Barloche and Kendrick, 1973a; Irons *et al.*, 1988), fungal species (Barloche and Kendrick, 1973b; Rossi, 1985; Suberkropp and Arsuffi, 1984) and incubation time (Bueler, 1984; Suberkropp and Arsuffi, 1984). Shredders usually prefer leaf material colonised by microorganisms because the bacteria and especially fungi enhance leaf litter palatability to shredders and increase the nutritive value of the leaf litter by modification of the leaf material (Gessner *et al.*, 1999). Enzymatic and mechanical activity from the microorganisms causes a decomposition of the leaf material which is proportional to the incubation time (Graca *et al.*, 1993a). This process of microbial decomposition is called conditioning (Gessner *et al.*, 1999) and represents an important step within the leaf litter breakdown in the environment. *G. pulex* ignore pure fungal mycelia but consume more conditioned than unconditioned leaf material (Graca *et al.*, 1993a). These results confirm previous observations of the role of fungi as modifiers of leaf material (Kostalos and Seymour, 1976; Sutcliffe *et al.*, 1981; Willoughby and Sutcliffe, 1976).

In addition to the impact of food quality, natural environmental changes, the presence of pollutants and infection with parasites can all cause a change in feeding of *G. pulex* (Bollache *et al.,* 2002; Brown and Pascoe, 1989; McCahon *et al.*, 1991). A population of *Gammarus* in the field can be highly infected with acanthocephalan parasites (Brown and Pascoe 1989). Brown and Pascoe (1989) found in a river in the UK an infection rate of 52%. McCahon (1991) and Brown and Pascoe (1989) observed a reduced feeding rate (only 17 - 21% compared to the control), an alteration in sensitivity to chemicals and an increased natural mortality for infected *Gammarus*. Acanthocephalan parasites are known to alter the reproductive biology and physiology of their hosts in various ways (Bollache *et al.,* 2002). In most cases, the prevalence and the intensity of infection with parasites are significantly associated with a decrease in developmental stability (Moller, 1996). *Pomphorynchus laevis* produce a strongly positive phototaxis to *Gammarus* (Brown and Thompson, 1986; Cezilly *et al.,* 2000), reduce the glucose and oxygen consumption (Rumpus and Kennedy, 1974), increase the hemocyanin concentration (Bentley and Hurd, 1993; Bentley and Hurd, 1996) and cause a reduction in fecundity of females (Poulton and Thompson, 1987). *Polymorphus minutes*, another acanthocephalan parasite that uses *G. pulex* as intermediate host, reverses the geotaxis from positive to negative (Brown and Thompson, 1986; Cezilly *et al.,* 2000) and stops the production of eggs in females (Ward, 1986). In addition to the described larval effects of the parasite the cystacanths affect the behaviour of *G. pulex*. The respiration (Rumpus and Kennedy, 1974) and the mating decision (Brown and Thompson, 1986; Poulton and Thompson, 1987) are influenced.

## *2.2. Materials and methods*

### *2.2.1. Test organisms*

*G. pulex* were collected from a small stream in Bishop Wilton UK (Grid reference: SE7963; latitude: 53.985; longitude: -0.787). Organisms were classified by visual observation immediately after collection into those infected (I) or uninfected (nI) with acanthocephalan parasites. The two parasites *Pomphorynchus laevis* and *Polymorphus minutes* were not distinguished.

The organisms were maintained under continuous ventilation at 13±1°C and with a photoperiod of 12:12h at 750-900 Lux in artificial pond water (APW; 294 mg/L $CaCl_2 2H_2O$, 123.3 mg/L $MgSO_3 7H_2O$, 64.8 mg/L $NaHCO_3$ and 5.8 mg/L KCl in deionised water) (Naylor *et al.*, 1989) prior to and throughout experimentation.

### *2.2.2. Food sources*

Food sources used were horse chestnut and alder leafs. Horse chestnut leaves (*Aesculus hippocastanum* (L.)) were collected in November and stored at 20±2°C after drying (air dry) under dry, dark conditions and at room temperature until use. Whole leaves were used as food to maintain the organisms in the laboratory. These leaves were stored in tap water at room temperature and were conditioned with *Cladosporium* sp. for at least three months prior to use. Leaf discs with a diameter of 1.6 cm were prepared for use in the experiments. Leaf types used differed in their decomposition state. These were: decomposed by inoculation with *Cladosporium* sp. (DC), decomposed in tap water (D), and non-decomposed (nD). Culture media for

the leaf type DC was enriched water (66.04 mg/L $(NH_4)_2HPO_4$, 68.05 mg/L $KH_2PO_4$, 87.09 mg/L $K_2HPO_4$, 1.84 mg/L $CaCl_22H_2O$ and 2.54 mg/L $MgCl_26H_2O$ in deionised water) (Naylor *et al.*, 1989) and tap water for the leaf type D. Preparation of the leaf discs DC was by decomposing 150 horse chestnut leaf discs with a diameter of 1.6 cm in 300 mL enriched water inoculated with *Cladosporium* sp. from a culture on malt-extract. The leaf discs nD were prepared two days before the start of the experiment by storing them in tap water in the dark.

Leaf discs prepared from alder leafs (*Alnus glutinosa*) (DS) decomposed with in stream water decomposed alder leafs were used as an additional food source. This food source was obtained from the University of Landau, Germany. Detailed information on the preparation of this food source can be found in Zubrod *et al.*, (2010). In short, leaf discs with a diameter of 2.0 cm were conditioned for 10 days in a nutrient medium. Inoculation of the leaf discs with a river-like microbial community was by addition of alder leaves previously exposed in the Rodenbach, Germany (491330N, 81020E). The discs were dried at 60 C to constant weight and rewetted in APW two days prior the use in an experiment.

### *2.2.3. Experimental design*

APW was replaced every second or third day during all experiments; the oxygen content and pH in the old and new medium were measured. Mortality and moulting status was recorded daily. The feeding rate of moulting organisms was discounted from analysis during the period when the carapax was changed because the impact of moulting on the feeding rate is so far unknown.

### *2.2.4. Impact of food source and parasite infection*

The first experiment consisted of five treatments, each with three replicates. Each replicate comprised four gammarids (body size 0.6 - 1.2 mm), six leaf discs at day one and three leaf discs during subsequent days in 250 mL APW. Three treatments contained uninfected gammarids and one of the three leaf discs D, DC or nD, respectively. Two treatments contained infected gammarids and either the food DC or D. The experiment lasted 96h. Data for the first feeding period ($t_{0h}$-$t_{24h}$) were excluded from further analysis because of a significant difference in feeding rate relative to subsequent periods.

### *2.2.5. Measurement of C-N ratios*

The carbon and nitrogen content of *G. pulex, Cladosporium* sp. and all leaf types was measured using a vario MACRO CN elementar analyser. Within the food type DC three groups of leaves were analysed which were visually classified by their colour (light-, middle- and dark-brown). The leaves and veins were analysed separately for calculating the C-N ratio of the leaf material consumed, because gammarids do not eat the main veins. Prior to analysis, the samples (duplicates) were dried (96h at 105°C), milled and weighed. Sample weight ranged between 6.0 and 28.4 mg.

### *2.2.6. Impact of body mass*

75 organisms with a body mass between 0.48 and 14.6 mg dw were kept individually in 90 mL APW and fed daily with three leaf discs of the type DC. Only organisms

without visible acanthocephalan parasite infection were used. The experimental period was nine days.

### *2.2.7. Individual feeding rate as a function of time for two food sources*

15 organisms with a body mass between 4.64 and 11.96 mg dw were kept individually in 90 mL APW. All organisms were fed daily with three leaf discs. Ten individuals were fed with the food source DC and the remaining organisms were fed with leaf discs type DS. Only organisms without visible acanthocephalan parasite infection were used. The feeding rate of each individual was measured on a daily basis throughout the experimental period of 15 days.

### *2.2.8. Measurement of feeding rate*

The individual feeding rate $F_R$ [mg(food)/(mg(gammarid)*d)] was calculated at a daily resolution (t = exactly 24h) by dividing the amount of eaten food within the observed period $F_{E(t)}$ [mg/d] ($F_{E(t)} = F_{(t-1)} - F_{(t)}$) by the body mass of the individual *G* [mg] ($F_R = F_{E(t)}/G$). To prevent over-estimation of the feeding rate associated with weight loss of the leaf discs caused by leaching and/or decomposition, the measured food at the end of the period $F_{(t)}$ was corrected with a leaching-decomposing-factor *ld*; $F_{E(t)} = F_{(t-1)} - (F_{(t)}/ld)$. *ld* was obtained by dividing the weight of the control leaves at the end of the measuring period by the initial weight. All measurements of weights refer to dry weight.

Body mass of the organisms was measured after the experiment by drying the organisms for at least 24h at 90°C. When the amount of food eaten was observed in

wet weight (ww), the dry weight (dw) of the food material was calculated using the experimentally-derived linear regression of dw = 0.186 * ww ($R^2$ = 0.865; data not shown). Weighing was carried out with a Mettler Toledo XS205 Dual Range balance weighing to a precision of 0.01 mg.

### *2.2.9. Statistical analysis*

One- and two-way ANOVA was performed with the feeding rate [mg(food)/(mg(gammarid)*d)] of replicates. The Shapiro-Wilk test for normal distribution and the Levene-Mediane test for equal variance were performed prior to ANOVA. Multiple comparisons of resulting p-values were by application of the Holm-Sidak test when normal distribution and equal variance was given. Otherwise a Kruskal-Wallis test was used. Statistical analysis of feeding rates was undertaken with SigmaPlot 11.

## *2.3. Results and discussion*

For all experiments the pH ranged between 7.4 and 7.9, the oxygen content was always higher than 75% saturation and the temperature ranged between 12.2 and 14.0°C. The measured pH lies within the optimum (7.2-7.8) for the organism given by Schellenberg (Schellenberg, 1942). Oxygen content and temperature of the test medium fulfilled the conditions preferred by *G. pulex* (Gledhill, 1993).

### *2.3.1. General findings*

The overall feeding rate of experiment one (described in section 2.2.3.1.) was 0.21±0.14 mg(food)/(mg(gammarid)*d) without distinguishing the influence of food source and parasite infection (Figure 2-1, column 2). This overall feeding rate ranged between 0.17±0.13 and 0.37±0.20 mg(food)/(mg(gammarid)*d) when calculated on a daily basis. A reduction of the standard deviation of the test results by 1.6% was observed when discarding the first feeding period from the data analysis (Figure 2-1, column 1 and 2). Data for the first feeding period ($t_{0h}$-$t_{24h}$) were excluded from further analysis because of a significant difference in feeding rate relative to subsequent periods.

The overall feeding rate in the next experiment (described in section 2.2.3.3) without distinguishing in body mass was 0.37±0.28 mg(food)/(mg(gammarid)*d) (Figure 2-1, column 8) and ranged between 0.23±0.25 and 0.51±0.54 mg(food)/(mg(gammarid)*d) when calculated on a daily basis.

Overall the intra-specific variability in feeding rate was very high. The standard deviations of the measured feeding rates were 66 and 75% of the average values for experiments described in section 2.2.3.1 and 2.2.3.3, respectively when no differentiation in the three tested factors was made (Figure 1, column 1, 8). The large variability indicated a low statistical power in any tests of individual feeding rate of *G. pulex* at a daily resolution and thus the need for further work to understand and reduce intra-specific variability.

### *2.3.2. Food source*

Feeding rate of non-infected gammarids was significantly influenced ($p<0.05$) by leaf type (Figure 2-1, column 3-5) for experiments where gammarids were fed with different sources of horse chestnut. The food source nD gave the lowest feeding rate (0.07±0.05 mg(food)/(mg(gammarid)*d), the feeding rate of organisms fed with source DC was intermediate (0.22±0.09 mg(food)/(mg(gammarid)*d)), and food source D resulted in the highest feeding rate (0.36±0.10 mg(food)/(mg(gammarid)*d)). Dangles and Guerold (2001) found the same relationship for the freshwater amphipod *G. fossarum,* and Graça *et al.* (1993b) observed that *G. pulex* ate twice as much when leaf material was conditioned. Other references show that food preferences for freshwater detritivores are related to the time of inoculation with microorganisms (Arsuffi and Suberkropp, 1988; Bueler, 1984).

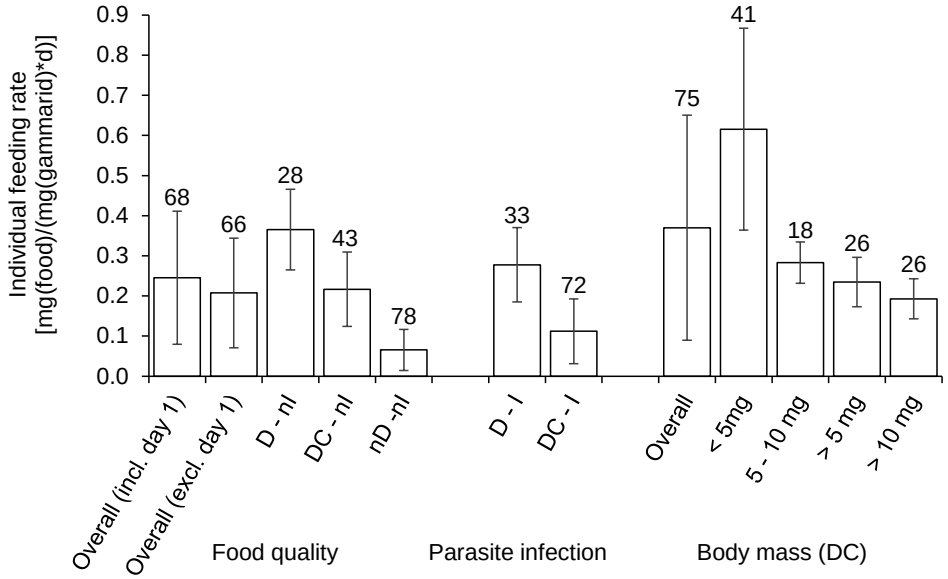


*Figure 2-1: Variation in feeding rate of individual* Gammarus pulex *with type of food, presence (I) or absence (nI) of infection with acanthocephalan parasites; and body mass. The food source is indicated with DC for horse chestnut leaf discs decomposed with* Cladosporium sp.*; D for leaf discs decomposed in water and nD for non-decomposed leaf discs. The numbers represent the standard deviation as a percentage of the average.*

Results demonstrated that standardised food preparation and storage can reduce the variability of the feeding rate of *G. pulex* in laboratory studies (Figure 2-1, food quality) which would increase the potential for detecting stressor-related effects *ex situ*. Within this experiment a maximal reduction of the variability in feeding rate by 38% was found when data were distinguished by food quality. The higher the food quality and thus the feeding rate, the lower the variability (Figure 2-1, food quality).

### *2.3.3. Parasite infection*

Infection with acanthocephalan parasites had a significant influence ($p<0.1$) on feeding rates for both food types tested, and the intensity of the influence was related to the food source (Figure 2-1). The feeding rate decreased with parasite infection from 0.36±0.10 to 0.28±0.09 mg(food)/ (mg(gammarid)*d) when fed with food of source D (Figure 2-1, column 3 vs. 6); and from 0.22±0.09 to 0.11±0.08 (mg(food)/(mg(gammarid)*d)) by feeding with leaves of source DC (Figure 2-1, column 4 vs. 7). This demonstrates a reduction in feeding rate caused by parasite infection of 22 and 50% when fed with the leave types D and DC, respectively. In the present study results show that excluding infected organisms from laboratory studies reduced the variability of the test results by up to 33% (Figure 2-1, column 2 vs. 6).

The results of the present study combined with those of Brown and Pascoe (1989) show that a separation of the organisms according to whether or not they are infected with acanthocephalan parasites will reduce the variability of the test results and thus increase the power in a toxicity study to detect any effects caused by a stressor. Standardisation of either parasite infection or food quality might be suitable to reduce the intra-specific variability in the individual feeding rate for successful toxicity studies at a daily resolution. However, it might be advisable to standardise both

because significant differences in feeding rate were observed for the tested food sources and infection status.

### *2.3.4. Body size*

A strong relationship ($R^2$ 0.79) between feeding rate and body mass (given in dw) could be observed for all observation periods. Figure 2-2 shows the average of the feeding rate as a function of body mass for the whole experimental duration. Feeding rate was consistently higher for smaller organisms. As mentioned by several authors, the growth rate of *G. pulex* is discontinuous (Delong *et al.*, 1993; Gee, 1988; Glazier *et al.*, 1992; Hargeby, 1990; Soderstrom, 1988) and correlated to the feeding rate (Maltby and Naylor, 1990) with a general pattern of faster growth for juvenile organisms (Gee, 1988; Sutcliffe *et al.*, 1981).

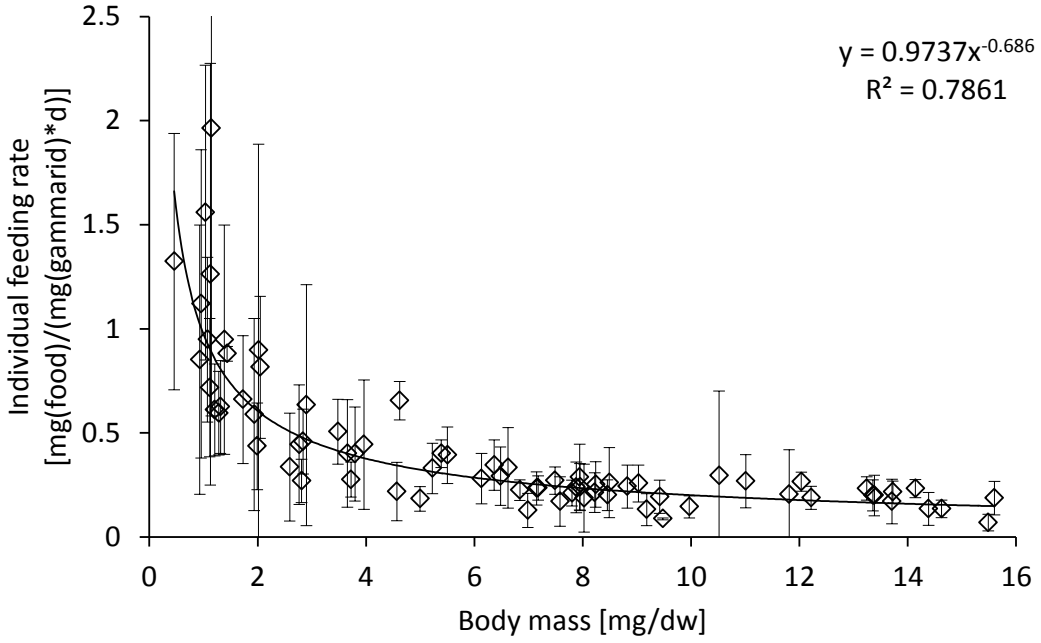


*Figure 2-2: Feeding rate of individual* Gammarus pulex *as a function of body mass. Average ± standard deviation for a measurement period of nine days after changing the food source from non-inoculated (nD) to inoculated food (DC).*

Figure 2-3 shows the individual feeding rate over time for three sizes of organism. The feeding rate of each group was compared over time and it was found that the variability was the greatest and significant for small organisms (<5 mg), whereas for larger organisms no significant differences in feeding rate over time were observed. Smaller organisms (body mass <5 mg) had two to three times higher feeding rate than organisms with a body mass of >5 mg when calculated over the whole experimental duration (Figure 2-1, columns 8-12). Specification, in terms of body mass reduced the variability in feeding rate compared to the mixed groups by 35, 57 and 49% for the groups <5, 5 to 10 and >10 mg, respectively.

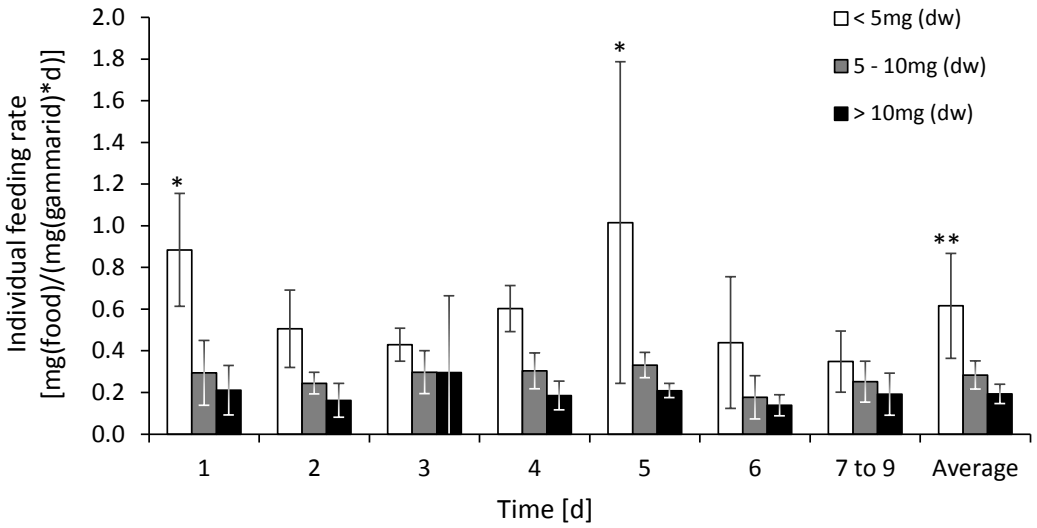


*Figure 2-3: Feeding rate of three different size classes of* Gammarus pulex *over time after changing the food source from non-inoculated (nD) to inoculated food (DC). Average values (n>18 for organisms <5 mg; n>29 for organisms of 5 to 10 mg; n>15 for organisms <10 mg). *Significant difference compared to the same group over time (p<0.05); **Significant difference between large and small organisms (p<0.01); Mann-Whitney rank sum test.*

When making feeding activity assays with *G. pulex* it is advisable to use organisms of a very specific body mass (for example 2.0 to 2.5 mg) to reduce the variability of

the test results and thus increase the possibility of a successful toxicity study. However, restricting the size range of organisms reduces the relevance of test results for the mixed populations found in the environment. A further option would be to use organisms of a higher body mass because of the decreasing strength of the relationship between feeding rate and body mass with increasing body weight. It was shown that organisms >5 mg had a smaller and insignificant variability in feeding rate over time when compared to smaller organisms; however, this was offset by the disadvantage that feeding rate was lower than that for smaller organisms.

It was observed that the feeding rate of organisms <5 mg fluctuated greatly over time, yielding significant differences between different observation periods. Such fluctuations must be excluded for toxicity studies. Thus the results would suggest the use of organisms >5 mg for toxicity tests. Use of larger organisms reduces uncertainties from weighing and such organisms can be collected throughout the year (Demyanov *et al.*, 2006).

### *2.3.5. Food quality (C-N ratio)*

Large variability in the C-N ratio of the food type DC was observed, and this was related to the colour of the leaf discs (Figure 2-4). The relatively high variability in the C-N ratio for the food type DC explained the high variability in feeding rate for gammarids fed with this food type. A further separation of the food source within one preparation procedure by nutrient content (here the C-N ratio) might reduce the variability of the test results even more than the 38% observed in this study, because the whole set of type DC leaf discs was used. Furthermore, it was observed that *G. pulex* had the smallest C-N ratio tested (5.55±0.02) followed by *Cladosporium* sp. (10.32±0.04). These C-N ratios are clearly smaller than those of all horse chestnut

leaf discs tested. Benthic consumers often contain higher amounts of nitrogen and thus have a lower C-N ratio than their food sources (Cross *et al.*, 2005). There seem to be exceptions for this observation as the C-N ratio for the food source DS was close to that of the gammarids themselves (Figure 2-4).

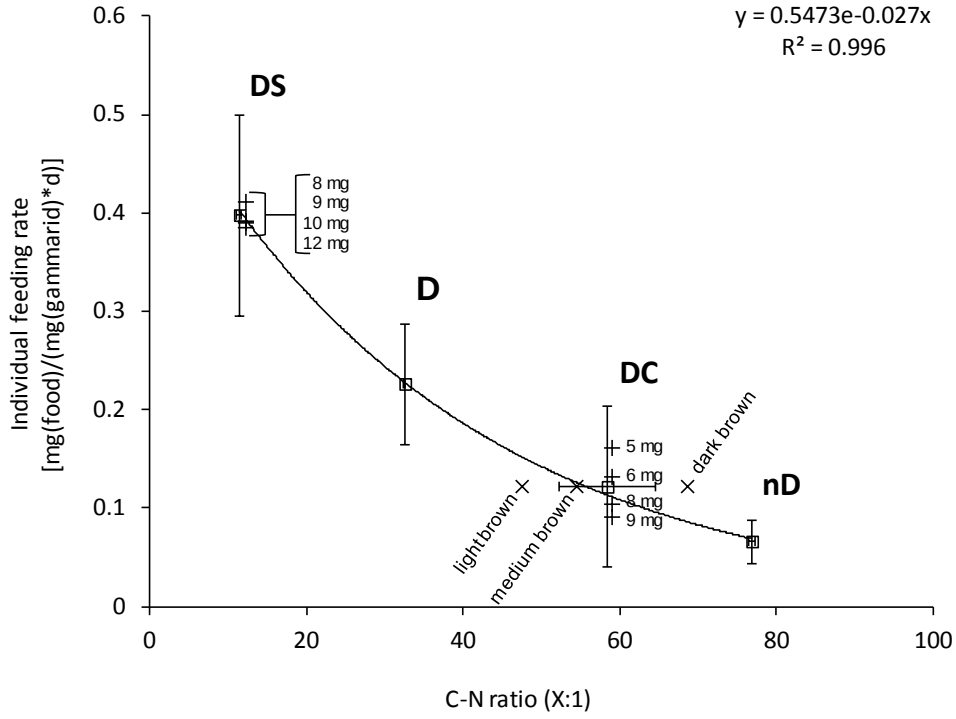


*Figure 2-4: Variation in feeding rate of individual* Gammarus pulex *with the C-N ratio of food eaten. Average values (± standard deviation) are plotted for the four food sources tested □ (D, DC, DS and nD) and for the food sources DS and DC the feeding rate is additionally plotted in dependence of the body weight (dw) of the test organisms +. The C-N ratio for the food source DC was also determined after classification into three groups of leaf colour* X.

Figure 2-4 also shows a relationship ($R^2$ = 0.99) between C-N ratio of food eaten (leaf discs excluding the veins) and feeding rate. The feeding rate decreased with increasing C-N ratio. The decline in the C-N ratio with decomposition time of the

leaf discs is caused by microbial activity and was also observed for alder and beech leaves in the field (Groom and Hildrew, 1989). This microbial activity, called conditioning, is another important part of leaf litter processing in aquatic ecosystems which increases the palatability of detritus for shredding organisms (Gessner *et al.*, 1999). The literature suggests that aquatic shredders prefer food of lower C-N ratios because the quality is higher resulting in better nutritional status (original reference in Leberfinger and Bohman, 2010). However, whether food preference really depends on C-N ratio is unclear, as information in the literature range from no relationship between those two factors at all (Friberg and Jacobsen, 1994; Leberfinger and Bohman, 2010) to a strong relationship (Irons *et al.*, 1988). Nutritional composition is a determining factor of food quality (Frost and Elser, 2002) and adaptation in feeding activity provides a compensation for sub-optimal composition of available food (Albarino and Balseiro, 2001; Cross *et al.*, 2005).

Under the assumption that *G. pulex* only eats the amount of food needed to sustain the energy budget and nitrogen is the limiting factor, food consumption would increase as nitrogen content of the food decreased. However, the opposite relationship was observed. An explanation could be that the content of other important nutrients (e.g. phosphorus) might have been decreased during the decomposition by microbial activity and was than limiting. The compensation for this limitation then forced the gammarids to increase their feeding rate. For *Gammarus fossarum*, another aquatic shredder, it has recently been shown that their growth is negatively correlated to the C-P ratio of the food source (Danger *et al.*, 2012). Our results show that a comparison of feeding activity data generated in experiments using differing leaf species decomposed using different methods might be possible when the C-N ratio of the provided food is measured. However, it is

advisable to include phosphorus into the testing of nutritional status of food sources, because phosphorus is an essential component of food quality alongside carbon and nitrogen (Frost and Elser, 2002).

### *2.4. General discussion and conclusion*

Decreasing intra-specific variability in feeding rate was observed when focusing on the measurement of feeding rate of a sub-group of gammarids. Focusing on organisms of a sub-group in terms of parasite infection and body mass resulted in a reduction in intra-specific variability of up to 50 and 57% respectively. Using a food source of particular quality reduced the variability by up to 38%. Acclimation to test conditions only reduced the variability in test results by 1.6%. Certainly, taking into account each of these options to reduce the intra-specific variability for a feeding assay will maximise the reduction in intra-specific variability, but the result will not be of additive nature.

There are contrasting strategies which can be followed for a feeding assay depending on the objective of the conducted study. The results suggest letting the organisms acclimate to the test conditions for at least one day and to use organisms that are either all infected or all uninfected. The optimum should be the use of non-infected individuals as the infection reduces the feeding rate (present study and Brown and Pascoe, 1989), thus reducing the chance of measuring negative impacts due to the tested stressor. Furthermore, it is not known whether both parasites influence feeding in the same manner as here these parasites were not distinguished. The proportion of infection and the intensity of the infection with the parasites, which may have a different impact on feeding, will depend on geographical location and season. Therefore, the use of uninfected organisms is recommended. Further research could

include the investigation of xenobiotic impacts on feeding of infected organisms as those were shown to be more sensitive than uninfected organisms (Brown and Pascoe, 1989).

The results show that when conducting feeding assays with gammarids attention has to be given to the selection of the test organisms in terms of their body size. In order to increase the chance of measuring influences of the treatment, the results suggest conducting experiments with organisms of different sizes depending on the length of the conducted study. Short-term experiments may be conducted with juvenile organisms of a very particular size because their feeding rate is in general higher than that for adults. Additionally, juveniles have been shown to have a higher sensitivity to toxicants (Adam *et al.*, 2010; Blockwell *et al.*, 1996). However, as the feeding rate of juveniles seems to be unstable over time they may only be suitable for short-term experiments. A further reason to select juvenile individuals may be the increased representativeness of the test for the field situation. The density of organisms within the larger size class in the environment is lower than that of smaller organisms (Allan and Malmqvist, 1989). Experiments with juveniles may be conducted with an increased number of replicates because the total amount of food consumed within a day is rather low which increases the measuring uncertainty. Long-term experiments are particularly important to observe recovery potential following a treatment and for investigation of effects from pulsed exposure. Such experiments should be conducted with adult gammarids to stabilize the control feeding rate over time. A further reason to select adults is their importance for the population sustainability as these individuals reproduce.

Some attention should be drawn to the food source to be used in a feeding assay. The results suggest using conditioned food prepared in a single batch and the C, N and P content of the used food should be measured. Furthermore, more than one leaf disc should be provided per organism in order to reduce the variability of the feeding rate caused by the variability in the food quality. Generally, the longer the leaf material is inoculated with microorganisms the higher is the feeding rate which, again, increases the chance of measuring negative impacts of the treatment. However, one might want to consider that there is likely a maximum feeding rate which is determined by the mechanical shredding behaviour of gammarids. Conducting a feeding assay at such a shredding rate eliminates the possibility to measure treatment-related increases.

For this thesis feeding assays were conducted to measure the impact of imidacloprid and carbaryl (Chapter 3 and Appendix A). The overall aim was to investigate whether feeding assays conducted at the individual level and a daily resolution are sensitive enough to determine a dose-response for feeding inhibition and to determine any recovery from such an impact. It was decided to conduct the studies with non-infected adult individuals with a body mass >5 mg. Three leaf discs of horse chestnut leaves decomposed by inoculation with *Cladosporium* sp. were used and completely exchanged for each feeding period. Organisms had three days for acclimation to test conditions prior exposure.

# 3. Imidacloprid perturbs feeding of *Gammarus pulex* at environmentally-relevant concentrations

The present study aimed to investigate whether imidacloprid affects feeding of *G. pulex* at environmentally-relevant concentrations and whether a feeding assay at the individual level, taking into account the methods to reduce intraspecific variability described in Chapter 2, is sufficiently sensitive to allow testing at a daily resolution. Furthermore, it was explored whether extending the feeding assay to include a recovery phase benefits the interpretation of the results in terms of effects being environmentally relevant.

## *3.1. Introduction*

Studies on food uptake and the energy budgets of detritivores show that food uptake is affected by xenobiotics at much lower concentrations than those causing mortality (Bundschuh *et al.*, 2011; Crane and Maltby, 1991; Maltby and Crane, 1994; Naylor *et al.*, 1989; Zubrod *et al.*, 2010). Food uptake by detritivorous organisms has been measured *in situ* and *ex situ* for several decades (Bundschuh *et al.*, 2011; Kunz *et al.*, 2010; Zubrod *et al.*, 2010), and it was demonstrated that laboratory feeding assays are representative of leaf decomposition in the field (Bloor and Banks, 2006).

Nevertheless, studies observing impacts of xenobiotics on food uptake of gammarids need to be improved. Both, exposure and effects may vary over short timescales. Thus, detection of impacts from realistic exposure patterns requires an appropriate temporal resolution of measurement. Furthermore, the observation of recovery to normal feeding and/or the potential increase of feeding as a compensation for a

decrease in feeding during exposure should be investigated for a more realistic assessment of effects on decomposition of leaf litter in the field.

Generally, ecotoxicological studies have been carried out measuring the composite feeding rate over periods from four to seven days (Kunz *et al.*, 2010) and recovery potential was not included (for an exception see Nyman *et al.*, 2013). The only studies we are aware of where feeding of gammarids was measured at a resolution of one (Graca *et al.*, 1993a; 1993b) or two days (Hargeby, 1990; Hargeby and Petersen, 1988) were carried out in ecological studies without chemical stressors.

Imidacloprid is a neonicotinoid insecticide that is generating concern regarding potential impacts on ecosystems (Pestana *et al.*, 2010). The compound has potential to reach surface waters due to its chemical and physical properties; it has been estimated to potentially reach such waters in concentrations up to 36 µg/L and has been detected in surface waters at concentrations up to 14 µg/L (Jamec *et al*, 2007 and references therein). For daphnids, the most commonly used aquatic invertebrate test species, such concentrations were not relevant for any observed effects measured in standard toxicity tests as concentrations causing effects were in the range of several mg/L (OECD, 2007). *Gammarus* is known to be more sensitive to imidacloprid than *Daphnia* with respect to mortality (Ashauer *et al.*, 2011). As feeding behaviour is a particularly sensitive endpoint, and prolonged starvation contributes to mortality caused by imidacloprid in gammarids (Nyman *et al*, 2013), effects are more likely to occur at field-relevant concentrations of imidacloprid.

## *3.2. Materials and methods*

*G. pulex* were collected in August from a small stream in Bishop Wilton UK (grid reference: SE7963; latitude: 53.985; longitude: -0.787) and were fed prior to experimentation with horse chestnut leaves (*Aesculus hippocastanum* (L.)) that had been stored in tap water for at least three months. These leaves were conditioned with *Cladosporium* sp. at room temperature. The organisms were maintained prior to and within experimentation under continuous ventilation at 13±1°C and with a photoperiod of 12:12h at 750-900 lux in artificial pond water (APW) (Naylor *et al.*, 1989). Organisms were left to acclimatise to those conditions for three days before the start of the experiments.

### *3.2.1. Food source*

Leaf discs with a diameter of 1.6 cm were prepared for the experiment using horse chestnut leaves collected in November. Leaves were stored after drying in the dark and at room temperature (20±2°C) until further preparation. Leaf discs were conditioned with *Cladosporium* sp. for two weeks following the description of Naylor *et al.* (1989) and subsequently stored as leaves previously collected. This food corresponds with the food source DC in Chapter 2 sources however from another batch. All leaf discs were rewetted in APW for two days prior to use.

### *3.2.2. Experimental design*

Gammarids with a dry body mass between 3.8 and 15.0 mg and without visible infection with acanthocephalan parasites were kept individually in 90 mL APW and each individual was provided with three leaf discs at all times. All food was exchanged every 24h and the APW was replaced every 48h. Oxygen content and pH

in the old and new medium were measured and mortality and moulting status was recorded daily. The feeding rate of organisms that moulted during the experiment was discounted from analysis because the exact impact of moulting on the feeding rate is unreported; previous observations show that organisms might stop eating during the period before changing the carapax (Hargeby and Petersen, 1988). Body mass of the organisms was measured after the experiment by drying the organisms for 48h at 65°C and weighing to a precision of 0.01 mg.

The actual experiment was divided into two phases, which were a four-day exposure phase (2*2 d), and a three-day recovery phase. Prior to the experiment and after the acclimatisation to the laboratory conditions (three days), organisms were further acclimatised to test conditions for two days (i.e. food source and separation). Five test concentrations of imidacloprid (0.81, 2.7, 9.0, 30 and 100 µg/L) were selected to range between ca. 0.2 and 20% of the lethal concentration for 50% of the test organisms ($LC_{50}$) determined after 96h of exposure (Beketov and Liess, 2008b; Roessink *et al.*, 2013). The largest concentration tested was similar to the median $LC_{10}$ for *G. pulex* after 96h (99.5 µg/L (Roessink *et al.*, 2013)). No formulations or solvents were used (analytical standard: 99.0% purity, PESTANAL®; Sigma Aldrich). Samples of the stock solution and the media were taken at the beginning and end of both parts of the exposure phase and frozen at -22°C until preparation and chemical analysis using high performance liquid chromatography (HPLC).

Ten individuals were used for each test concentration and the control. Additionally, the experiments included a leaf disc control (three replicates) on each day. These controls detect differences in the weight associated with the drying and weighing procedure and furthermore prevent an over-estimation of the feeding rate associated

with weight loss of the leaf discs caused by leaching and/or decomposition during the experiment. All measurements of weights refer to dry weight.

### *3.2.3. Measurements of feeding rate*

The individual feeding rate $F_R$ [mg(food)/(mg(gammarid)*d)] was calculated at a daily resolution (t = exactly 24h) by dividing the amount of food eaten within the observed period $F_{E(t)}$ [mg/d] ($F_{E(t)} = F_{(t-1)} - F_{(t)}$) by the body mass of the individual $G$ [mg]; $F_R = F_{E(t)}/G$. The measured food at the end of the period $F_{(t)}$ was corrected with the leaching decomposition factor $ld$; $F_{E(t)} = F_{(t-1)} - (F_{(t)}/ld)$. $ld$ was obtained by dividing the weight of the control leaves at the end of the measuring period by the initial weight. The initial weight of the leaf discs was determined by weighing the leaf discs for each replicate prior the two-day rewetting phase in APW.

### *3.2.4. Chemical analysis*

The three smallest test concentrations of imidacloprid (0.81; 2.7 and 9 µg/L) and four standards (range 0.35 to 17.65 µg/L) were pre-concentrated on $C_{18}$-cartridges (Strata; 8B-S001-EBJ; C18-E; 55 µm; 70A) prior to chemical analysis. The cartridges had a bed mass of 100 mg and a column volume of 3 mL. Cartridges were activated with 3 mL methanol, loaded with 10 mL sample at 1 mL/min, and then eluted with 3 mL methanol. The eluted sample was evaporated to dryness under nitrogen and then redissolved in 0.25 mL methanol:water (50:50 v/v).

Analysis of imidacloprid was by injection of 75 µL sample onto HPLC (Agilent 1100 Series, Agilent Technologies, UK Ltd.) equipped with a UV detector (254 nm) and a Discovery® $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco) maintained at 25°C. The mobile phase was methanol/water (45:55; v/v) with a flow rate of 0.5 mL/min.

The limit of detection for imidacloprid (retention time: 5.6 min) was ≤14 µg/L (equivalent to ≤0.35 µg/L in the original samples subjected to pre-concentration) and the recovery through the pre-concentration step was 109±9%.

### *3.2.5. Statistics*

A one-way ANOVA was performed with the control data over time. The Shapiro-Wilk test for normal distribution and the Levene-Mediane test for equal variance were performed prior to ANOVA. The feeding rate [mg(food)/(mg(gammarid)*d)] of each replicate was used as input for this test. Similarly, but with a two-way ANOVA the relative feeding rate of treatments was tested against the control. A modified probit analysis was performed to generate the $EC_{50}$ values. Statistical analysis of feeding rates was undertaken with SigmaPlot 11. The $EC_{50}$ values were determined using ToxRat Professional 2.10 (ToxRat Solutions GmbH, Germany).

## *3.3. Results and discussion*

### *3.3.1. Environmental conditions*

The pH ranged between 7.4 and 7.9, the oxygen content was always higher than 75% saturation and the temperature ranged between 12.2 and 14.0°C. The measured pH lies within the optimum (7.2-7.8) for the organism given by Schellenberg (1942). Oxygen content and temperature of the test medium fulfilled the conditions preferred by *G. pulex* (Gledhill, 1993).

The maximal difference between measured and nominal concentrations of imidacloprid was 11%, whereas the difference for samples that were not pre-concentrated was <5%. A decrease in imidacloprid concentrations by 5.7±0.4%

during both exposure pulses was detected. For further analysis imidacloprid was assumed to be constantly present during the exposure phase at the nominal concentrations tested.

### *3.3.2. Feeding rate over time*

A two-way ANOVA on relative feeding rates during the whole experiment revealed an overall significant effect of treatment ($p=0.025$). In addition, feeding rate in the control and the three smallest concentrations of imidacloprid differed from the highest concentration (100 µg/L) throughout ($p<0.032$). The relative feeding rates during and after exposure to imidacloprid as a function of time are presented in Figure 3-1. There was a clear trend of concentration-dependent influences on the feeding rate in both exposure and recovery phases. Feeding inhibition in the exposure phase increased with increasing concentration (Figure 3-2) and at least for the two highest concentrations tested with exposure time (Figure 3-1). In both cases the impact on feeding was significant within one or more feeding periods at concentrations ≥30 µg/L. Those concentrations were similar or higher than those reported to cause drift (Beketov and Liess, 2008b; Berghahn *et al.*, 2012) and immobility of *G. pulex* (Roessink *et al.*, 2013).

It could be argued that reduced feeding was caused by the inability of organisms to reach food due to loss of movement. However, the food that was provided in this experiment covered the whole base of the test vessel meaning that active movement to reach food was unnecessary. Furthermore, it was observed that organisms were always holding a leaf disc at the time when food was exchanged. Hence, effects observed were due to reduced feeding and not driven by loss of ability of reaching

the food. Lost ability to coordinate the feeding apparatus could be the driving factor for reduced feeding.

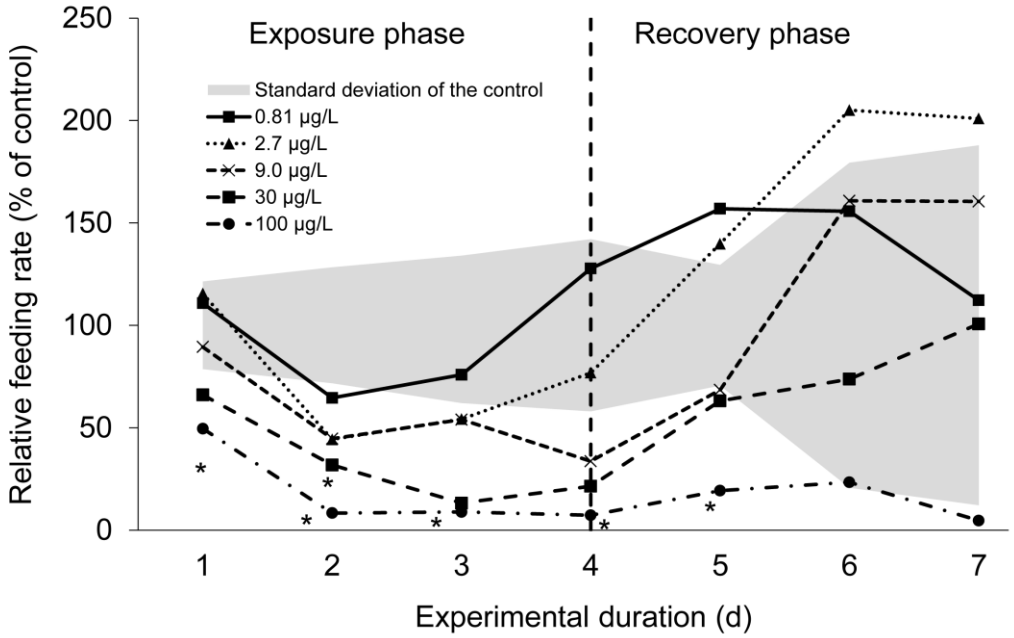


*Figure 3-1: Relative individual feeding rate of* Gammarus pulex *as a function of time within and after exposure to five nominal imidacloprid concentrations. Values shown are the average of treatments (n=10) and the standard deviation of the control (n=10) where the control is set at 100%. * Significant difference to the control (p<0.05).*

Another pattern was observed in the recovery phase. It seems that organisms not showing any (0.81 µg/L) or non-significant (2.7 and 9.0 µg/L) effects on feeding during exposure were nevertheless affected by imidacloprid (Figure 3-1 and Figure 3-2; recovery phase). When the compound was removed from the test vessel those organisms ate significantly more food than in the exposure phase and more than the

control organisms (Figure 3-2). However, this recovery was restricted to those concentrations which did not alter feeding significantly during exposure (Figure 3-2).

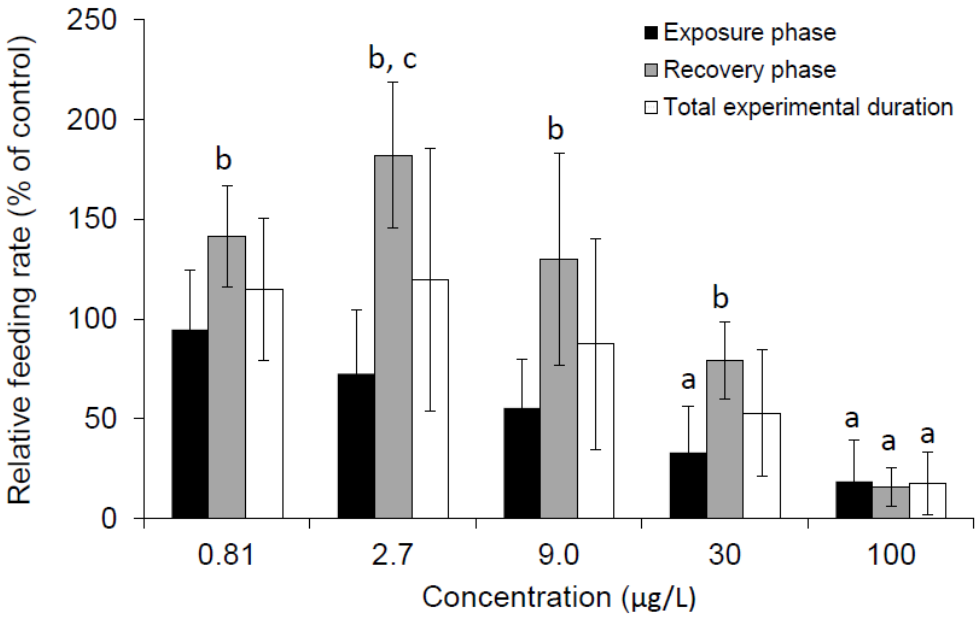


*Figure 3-2: Relative individual feeding rate of* Gammarus pulex *as a function of nominal imidacloprid concentrations. Average ± standard deviation (n=10) calculated for different phases of the experiment. a = significantly reduced compared to control; b = significantly increased compared to the exposure phase; c = significantly increased compared to the control (all at P<0.05).*

Furthermore, recovery was not directly related to exposure concentration in terms of the feeding rate itself, but rather in terms of the time when the feeding rate reached (recovery) or exceeded (compensational feeding) that of the control (Figure 3-1). In the smallest concentration tested (0.81 µg/L) organisms recovered to the control level even before exposure ended (day 4) and feeding exceeded that of the control in the following days (Figure 3-1) leading to a significantly increased overall feeding in the recovery phase compared to the exposure phase (Figure 3-2). Organisms in the next highest concentration (2.7 µg/L) recovered one day later (within one day after

exposure) and exceeded the feeding rate of the control in the following days more intensively compared to organisms exposed to 0.81 µg/L (Figure 3-1). The highest two test concentrations did not allow the organisms to recover within three days after exposure. The highest test concentration did not allow the organisms to recover (e.g. reaching or exceeding the feeding rate of the control on the same day) within three days after exposure.

We cannot distinguish whether the compensational feeding after exposure is a direct consequence of impacts on feeding or acts as compensation for other influences requiring energy, such as detoxification or increased energy demand of any other kind. Nevertheless, we show that low concentrations which do not cause significant effects on feeding (present study), drift (Beketov and Liess, 2008b; Berghahn *et al.*, 2012), immobility (Roessink *et al.*, 2013) or survival (Ashauer *et al.*, 2011; Beketov and Liess, 2008b; Roessink *et al.*, 2013) during exposure can alter feeding subsequent to exposure; this may indicate potential vulnerability to additional and subsequent stress. Roessink *et al.* (2013) reported a 10% effect concentration ($EC_{10}$) for immobilisation of *G. pulex* after exposure to imidacloprid for 96h of 3.6 µg/L. This concentration is four times larger than the smallest concentration tested in the present study for which increased feeding after exposure was observed.

Concluding the test with a recovery phase not only revealed influences at low concentrations, but also led to better insights about the effects. *G. pulex* did not recover within three days from a four-day exposure to imidacloprid at concentrations ≥30 µg/L (Figure 3-2). This result conforms with observations that imidacloprid blocks the post-synaptic nicotinic acetyl-choline receptors (nAChRs) virtually irreversibly (e.g. in insects (Tennekes, 2010) and references therein). Our results also

show that recovery at lower concentrations is possible, suggesting that some degradation of the compound or regeneration of nicotinoid receptors is possible. In fact, it was shown that imidacloprid is eliminated by 95% after 11.2 days in clean water (Ashauer *et al.*, 2010) and that this elimination does not involve formation of metabolites at detectable concentrations (Ashauer *et al.*, 2012).

### *3.3.3. Effect concentrations*

Effect concentrations causing 10 and 50% reduction in feeding rate after one, two, three and four days of exposure are presented in Table 3-1. Effects on feeding rate were observed at concentrations two orders of magnitude lower than those causing mortality (Ashauer *et al.*, 2011; Beketov and Liess, 2008b; Roessink *et al.*, 2013) (when comparing the average LC50 (96h) of 270 µg/L (Ashauer *et al.*, 2011) and the EC50 (96h) of 5.3 µg/L (Table 1)), emphasizing previous findings that feeding rate is a sensitive endpoint for *G. pulex* (Crane and Maltby, 1991; Maltby and Crane, 1994; Naylor *et al.*, 1989). Furthermore, reduction in feeding rate by 50% was observed at a concentration of 5.3 µg/L (Table 3-1), a concentration that is within the range of measured and estimated environmental concentrations (Table 3-2). Hence, our results from the exposure phase indicate that effects on the feeding rate of *G. pulex* caused by imidacloprid might already occur in the environment. Impacts of imidacloprid at concentrations of around 10 to 30 µg/L have already been reported.

Nyman *et al.* (2013) observed that gammarids were able to recover feeding between pulses of exposure to 15 µg/L and survive repeated pulsed exposures. The present study demonstrates such recovery of feeding. *Gammarus pulex* exposed to 30 µg/L were observed to show extra, pesticide induced, drifting activity on top of their natural drifting activity (Beketov and Liess, 2008b) and it was recently demonstrated

that organisms drifted downstream after exposure to 12 µg/L (Berghahn *et al.*, 2012). We show that strong impacts on feeding ($EC_{50}$) occur at slightly lower concentrations. A critical issue would be to determine whether patterns of exposure in the field match those in experiments as it is frequently found that exposure concentrations vary markedly over fairly short periods of time.

*Table 3-1: Concentrations giving 10 and 50% inhibition ($EC_{10}$, $EC_{50}$ plus 95% confidence intervals) of individual feeding rate [mg(food)/(mg(gammarid)*d)] measured in dry weight for different time points from the start of exposure of Gammarus pulex to imidacloprid.*

| Exposure time [h] | $EC_{10}$ [µg/L] | 95% confidence limit | $EC_{50}$ [µg/L] | 95% confidence limit |
|---|---|---|---|---|
| 24 | 9.05 | 5.15 – 12.10 | 18.96 | 14.93 – 23.05 |
| 48 | 3.28 | 0.005 – 8.81 | 20.59 | 6.48 – 72.01 |
| 72 | 2.03 | n.a. | 10.50 | n.a. |
| 96 | 2.05 | n.a. | 5.34 | n.a. |

*Table 3-2: Measured (m) and estimated (e) imidacloprid concentrations in surface and ground water.*

| Location | Type of determination | Concentration [µg/L] | Reference |
|---|---|---|---|
| Sea Wilapa Bay, USA | m | 1.6 | (Jemec *et al.*, 2007)[a] |
| Surface water, Florida, USA | m | 1.0 | (Jemec *et al.*, 2007)[a] |
| Lake Wales Ridge, USA | m | 14 | (Jemec *et al.*, 2007)[a] |
| Ground water, New York, USA | m | 6.7 | (Jemec *et al.*, 2007)[a] |
| Surface water, USA | m | 3.29 | (Starner and Goh, 2012) |
| Surface water (river), USA | m | 0.56 | (Starner and Goh, 2012) |
| Surface water, USA | e (acute exposure) | 36.0 | (Jemec *et al.*, 2007)[a] |
| Surface water, USA | e (chronic exposure) | 17.2 | (Jemec *et al.*, 2007)[a] |
| Ground water, USA | e (acute exposure) | 2.09 | (Jemec *et al.*, 2007)[a] |
| Ground water, USA | e (chronic exposure) | 2.09 | (Jemec *et al.*, 2007)[a] |
| EU [b] | e (acute exposure) | <6.1[c] | (EFSA, 2008) |

[a] *Original reference given in this reference.* [b] *Endpoint identified by the EU-Commission as relevant for Member States when applying the Uniform Principles.* [c] *Depending on the treated monoculture.*

### *3.3.4. Limitations of the test design*

Measurement for individual organisms showed that the feeding rate is not stable over one week. There was a significant difference in the control feeding rate between the first and the last three days ($p<0.008$; data not shown). The feeding rate decreased from 0.17±0.04 to 0.05±0.04 mg(food)/(mg(gammarid)∗d), accompanied by an increase in variability (average divided by standard deviation) as the standard deviation remained stable. Thus, improvement of the test design is needed for longer experiments. Such experiments would be desirable for testing multiple pulses and also to determine effects on growth and reproduction, endpoints important for extrapolating effects to the population level. Furthermore, there was a high variability in the feeding rate measured at a daily resolution which is most likely driven by a range of natural factors including age or size, food source and water quality (Boettger *et al.*, 2012). Hence, measurement at a daily resolution for individuals requires a large number of replicates to determine impacts at low effect intensity. A statistical power analysis for the data generated on day one of the experiment revealed that 20 replicates per treatment would be needed to detect a reduction in feeding rates by 20% at a significance level of 95%. However, such a study would not be guaranteed to succeed because data obtained from moulting individuals should be removed from the analysis and because feeding rate decreases over time.

### *3.3.5. Mortality*

No organisms died in the control or the three smallest test concentrations. After four days of exposure to imidacloprid, one organism died in the largest test concentration of 100 µg/L and on the last day of the experiment two organisms died in the second

largest concentration of 30 µg/L. Lethal concentrations for half of the test organisms ($LC_{50}$) of imidacloprid to *G. pulex* were investigated in three studies where similar $LC_{50}$-values after 96h of exposure were found. Beketov and Liess (2008b) reported a $LC_{50}$ of 270 µg/L (95% confidence limit: 170-450 µg/L). Three years later Ashauer *et al.* (2011) and five years later Roessink *et al.* (2013) found an almost identical $LC_{50}$. Having one individual out of 10 dying after four days of exposure to 100 µg/L matches the $LC_{10}$ of 99.5 µg/L (32.2-307 µg/L) reported by Roessink *et al.* (2013). Nevertheless, it seems that another mechanism also occurred causing the mortality observed at the end of the experiment when individuals were exposed to 30 µg/L. The literature gives evidence which might explain the delayed mortality; which certainly complicates the extrapolation of effects measured in acute toxicity tests to the field. Hervant *et al.* (1997) reported death of *G. fossarum* after 7 days of starvation; Chapter 5 will demonstrate that feeding inhibition due to imidacloprid exposure is the only cause of effects on growth, maturation, reproduction and survival of another crustacean, *D. magna*. A further study shows that imidacloprid has the potential to indirectly cause lethality due to interference with feeding (Nyman et al, 2013). Their multiple stress model explained the mortality by a combination of direct chemical stress and starvation. Apparently, mortality can occur from even lower concentrations when feeding behaviour is affected for longer durations (Nyman et al, 2013). Tennekes and Sánchez-Bayo (2011) reviewed the temporal aspects of imidacloprid exposure assessment. The incorporation of time as a dose factor is especially important for neonicotinoids and other xenobiotic groups which can bind irreversibly to receptors because repeated exposure at low doses has clear potential to cause adverse effects. Additionally, additive effects of different compounds with the same mode of action are possible.

### *3.3.6. General discussion*

We showed a temporary increased feeding rate following exposure to low test concentrations which may have an impact on overall leaf litter breakdown in the field, and thus could lead to changes at the ecosystem level. Diverse literature shows that food uptake of detritivores is directly related to the critical ecosystem-level process of leaf litter breakdown (Bundschuh *et al.*, 2011; Crane and Maltby, 1991; Gessner *et al.*, 1999; Maltby and Crane, 1994; Petersen and Cummins, 1974; Zubrod *et al.*, 2010). Furthermore, changed feeding could lead to effects at the population level. Maltby and Naylor (1989) showed that influences on the scope for growth, i.e. food uptake as the most sensitive part of the scope-for-growth concept, can be related to reproduction of *G. pulex* (Maltby and Naylor, 1990). Alterations in population abundance and population structure caused by short-term feeding inhibition due to imidacloprid exposure and its consequences for population vulnerability to subsequent stress is demonstrated in Chapter 6 for *D. magna*. It is unlikely that for gammarids a single short-term inhibition of feeding leads to similar influences at the population level because the life cycle of daphnids and gammarids is very different in terms of time scale. Nevertheless, instability in population development cannot be ruled out. Multiple pulses of the same compound or of compounds acting in the same manner are likely due to the long life span of *G. pulex*. The combination of multiple exposure and slow elimination of imidacloprid from *G. pulex* (Ashauer *et al.*, 2010) increases the likelihood of additive adverse effects. Compounds that have been shown to affect feeding of aquatic invertebrates include, but are not restricted to, the following pesticides or pesticide metabolites: fenvalerate (Day and Kaushik, 1987; Reynaldi *et al.*, 2006), endosulfan (Fernandez-Casalderrey *et al.*, 1994), diazinon (Fernandez-Casalderrey *et al.*, 1994), pentachlorophenol (Juchelka and Snell, 1995),

chlorpyrifos (Juchelka and Snell, 1995), naphthol (Juchelka and Snell, 1995), tebuconazole (Sancho *et al.,* 2009), molinate (Sancho *et al.,* 2003), carbendazim (Slijkerman *et al.,* 2004) and propanil (Villarroel *et al.,* 2003). Hence, there is a potential risk to aquatic non-target organisms due to the possibility of additive effects on feeding following temporal and spatial co-occurrence of substances influencing feeding in surface waters of agricultural areas.

Appendix A demonstrates the results of a similar experiment undertaken with carbaryl. This appendix shows that another pesticide can be added to the given list of pesticides acting on the feeding behaviour of aquatic invertebrates.

Whether short-term feeding depression of Gammarids, as shown in the present study, is a matter of concern at the population and ecosystem level could be investigated in different ways. A possible technique would be the extrapolation of temporal and spatially explicit measures of reduced feeding to the ecosystem process leaf litter breakdown and assess impacts on the nutrient cycle via changes in shredding activity. Including the extrapolation of individual feeding to shredding activity at the population scale might be a potential or even vital addition for understanding ecosystem level effects of pesticide exposure. Generally, exposure models (European Commission 2001) and ecological models (Galić, 2012; Kupisch *et al.,* 2012) are developed and their combination could address those questions. The incorporation of compensational feeding would be vital for assessing effects of environmentally-realistic exposure, but the actual mechanism of compensational feeding has yet to be determined. To understand the physiological basis of compensational feeding we need to understand how impaired feeding affects an organism’s energy requirements and energy budgets. Individual based, time resolved feeding rate measurements

following different starvation intervals are needed to achieve such understanding. However, the measurement of feeding of individuals needs to be improved in order to conduct the necessary experiments.

When considering acute toxicity, *G. pulex* are two orders of magnitude more sensitive to neonicotinoids than the commonly used standard test species *D. magna* (Ashauer *et al.*, 2011). However insects are even more sensitive than *G. pulex* by about a factor of 35 (Beketov and Liess, 2008a) which suggests that feeding rates of insects may also be affected by imidacloprid at much lower concentrations than those studied here.

### *3.4. Conclusion*

A toxicity study determining sub-lethal effects with an observation period beyond exposure is time- and resource- intensive. However, the extension yielded information on recovery potential and indicated that a low concentration of a stressor can have subsequent effects even though no effects were observable during exposure. Imidacloprid reduced feeding at low concentrations (30 µg/L) that are at the upper end of those likely to occur in the environment. Feeding rates were increased after exposure at even lower concentrations (0.81 µg/L) to compensate for earlier impacts, but recovery did not occur at the higher concentrations due to slow elimination of imidacloprid. To what extent the effects on feeding have a potential to evoke effects at the population level or disturb leaf litter breakdown in the environment needs further investigation. There is a need to improve methods for laboratory experiments with gammarids, to enhance culturing of these organisms, and to develop models that

can help to quantify the propagation of effects along the various levels of organisation from the individual (e.g. feeding, growth, reproduction) to the ecosystem (e.g. leaf litter decomposition, food web).

# 4. Individual lipid content changes the occurrence and intensity of impacts of imidacloprid on the feeding rate of *Gammarus pulex*

Chapter 3 revealed a decrease in the individual feeding rate of the control organisms over time. Further experiments also focusing on measuring the feeding rate of gammarids (Chapter 2, Appendix A and data not presented) show similar reductions of the feeding rate of untreated individuals over time. A clear pattern of reduced feeding rate with time from collection in the field was observed when combining feeding rates measured over time of all control treatments (Figure 4.1).

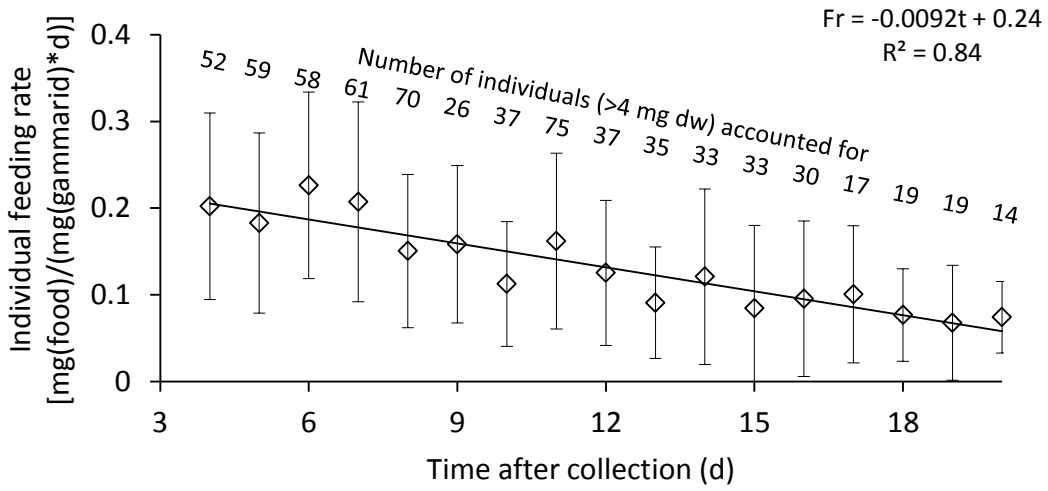


*Figure 4-1: Feeding rate over time for individual* Gammarus pulex *with a body mass >4 mg (dw). Organisms were caught in the field at day 0 and subsequently fed with horse chestnut leaf discs decomposed with* Cladosporium sp. *for two weeks. Average values ± standard deviation of a given number of individuals observed.*

In general, a reduction in feeding rate to half the initial rate was found within three weeks after collecting the organisms from the field (Figure 4-1). It was hypothesised that the quality of food provided drives this inconsistency in feeding rate due to changes in the lipid content of the organisms. Therefore, a study was undertaken to

investigate the lipid content of *G. pulex* over time when fed with two food sources differing in quality. A further aim of this study was to investigate whether a difference in lipid content of the test organisms influences the toxic effect within a feeding assay.

### *4.1. Introduction*

*G. pulex* is frequently used in ecological and ecotoxicological research as a representative species for shredders in floating waters which can be responsible for up to 75% of the fragmentation of detritus (Piscart *et al.*, 2009). Despite attempts at culturing this species in the laboratory, there has been no success in maintaining the culture over several generations. Therefore, organisms are caught in the environment for most experiments undertaken, meaning that there can be large variability between test organisms. One factor which varies in individuals in the field and is considered as important for toxicity of xenobiotics is the lipid content of the organisms. Lipid content of gammarids varies not only with parasite infection (Medoc *et al.*, 2011; Plaistow *et al.*, 2001), gender and reproductive stage (Arts *et al.*, 1995; Clarke *et al.*, 1985); it also changes with season (Clarke *et al.*, 1985). Thus, the lipid content of gammarids is different from experiment to experiment indicating that a comparison of effects observed in tests undertaken at different times and/or with organisms caught at different sites is challenging if not impossible. Lipids are an energy store that reflects the history of ingested food. Thus the food provided prior to and during an ecotoxicological study should be able to keep the lipid content stable, especially when the time between collection in the field and start of the study or the duration of the study is rather long. Changes in lipid content might provoke changes in the

toxicity by changes in uptake and elimination of the xenobiotic; changed intensity and/or occurrence of an effect might result.

## *4.2. Materials and methods*

### *4.2.1. Environmental conditions and food sources*

*G. pulex* with a body mass >5 mg dry weight (dw) were collected in early June from a small stream in Bishop Wilton UK (Grid reference: SE7963; latitude: 53.985; longitude: -0.787). Organisms not infected with acanthocephalan parasites (visually observed) were maintained in two groups under continuous ventilation at 13±1 °C and with a photoperiod of 12:12h at 750-900 Lux in 10 L artificial pond water (APW) (Naylor *et al.*, 1989). Groups differed in the decomposition stage of food provided. Food sources consisted of leaf discs (diameter 1.6 cm) from horse chestnut leaves (*Aesculus hippocastanum* (L.)) collected in November. Leaf discs were either stored in tap water for three months (indirectly decomposed; food source D as used in Chapter 2) or decomposed by inoculation with *Cladosporium* sp. for 10 days subsequent to sterilisation of the discs (directly decomposed; food source DC as used in Chapter 2). Direct decomposition was undertaken following the description of Naylor *et al.* (1989). Leaves and leaf discs were dried and stored under dry, dark conditions and at room temperature (20±2 °C) after collection and food source preparation, respectively. Leaf discs were soaked in APW two days before use in both treatments.

### *4.2.2. Lipid content over time*

Gravimetric determination of lipid content was performed for the food sources used and for gammarids when maintained under the laboratory conditions described and

fed with either food source. The lipid content of gammarids was determined over time, taking samples on days 0, 7, 14 and 19 after collection in the field. Samples (triplicates) with a dry weight of 103.4 - 184.7 mg and 377.2 - 586.3 mg for gammarids and food sources, respectively, were freeze dried (2h), manually milled, and weighed to a resolution of 0.01 mg prior to lipid extraction using cyclohexane (2 mL), de-ionised water (2.2 mL) and propan-2-ol (1.8 mL) as solvents (Smedes, 1999). Lipid extraction was performed twice for each sample. After mixing the sample with the solvents (vortex 30 sec., sonication 5 min), the samples were centrifuged for 5 min at 2000 rpm followed by separation of the cyclohexane-lipid phase and the evaporation of the solvent under nitrogen flow. A liposome solution consisting of 3 mg/mL DSPC (1-2-distearoyl-*sn*-glycero-3-phosphocholine) was used as reference sample.

### *4.2.3. Toxicity of imidacloprid*

A feeding activity assay was conducted subsequent to the observation of the lipid content over time. Organisms of both groups were maintained individually in 90 mL APW and fed with three leaf discs of the same food as for the preceding 19 days, i.e. decomposed by inoculation with *Cladosporium* sp. (directly decomposed; DC) or decomposed in water for at least three months (indirectly decomposed; D). The entire food source was fully replaced each day. Organisms of both groups were divided in three sub-groups of ten organisms each. Two of these sub-groups from both groups were exposed to imidacloprid (analytical standard: 99.0%; Sigma Aldrich; Batch-no.: SZE9112X) at concentrations of 30.0 and 100 µg/L. The third sub-groups of both groups were used as surrogate controls. The experiment included a leaf disc control to prevent feeding rates being over-estimated due to weight loss of the leaf discs

caused by leaching and/or decomposition. The individual feeding rate was measured and calculated as described in section 3.2.3.

### *4.2.4. Chemical analysis*

Analysis of imidacloprid was by injection of a 75-µL sample onto HPLC (Agilent 1100 Series, Agilent Technologies, UK Ltd.) equipped with a UV detector (254 nm) and a Discovery $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco) maintained at 25°C. The mobile phase was methanol/water (45:55; v/v) with a flow rate of 0.5 mL/min. The limit of detection for imidacloprid (retention time: 5.6 min) was ≤14 µg/L. The chemical analysis showed a maximal difference between measured and nominal initial imidacloprid concentration of 5.0% and a daily reduction of the concentration by 6.8±0.5%.

### *4.2.5. Statistics*

A multiple comparison one-way ANOVA was used to test the effect of treatment on average feeding rate [mg(food)/(mg(Gammarid)*d)]. A multiple comparison two-way ANOVA was performed for the lipid contents (% body mass (dw)) over time, setting time as variable and food source as fixed factor. A similar test was used to test the relative feeding rate (% of the surrogate control) of the treatments over time, having time as variable and treatment as fixed variable. The Shapiro-Wilk test for normal distribution and the Levene-Mediane test for equal variance were performed prior to ANOVA. All tests were performed using the program Sigma Plot 12.

***Results***

*4.2.6. Lipid content over time*

The lipid content of the food sources was significantly different. Directly decomposed horse chestnut leaf discs contained 3.61±0.03% lipid per dry mass whereas indirectly decomposed leaf discs had 1.80±0.04% lipid per dry mass.

The lipid content of gammarids caught in the field was 15.06±1.79% of the dry body mass. A linear decrease in lipid content was observed during the maintenance of the organisms under laboratory conditions (Figure 4-2).

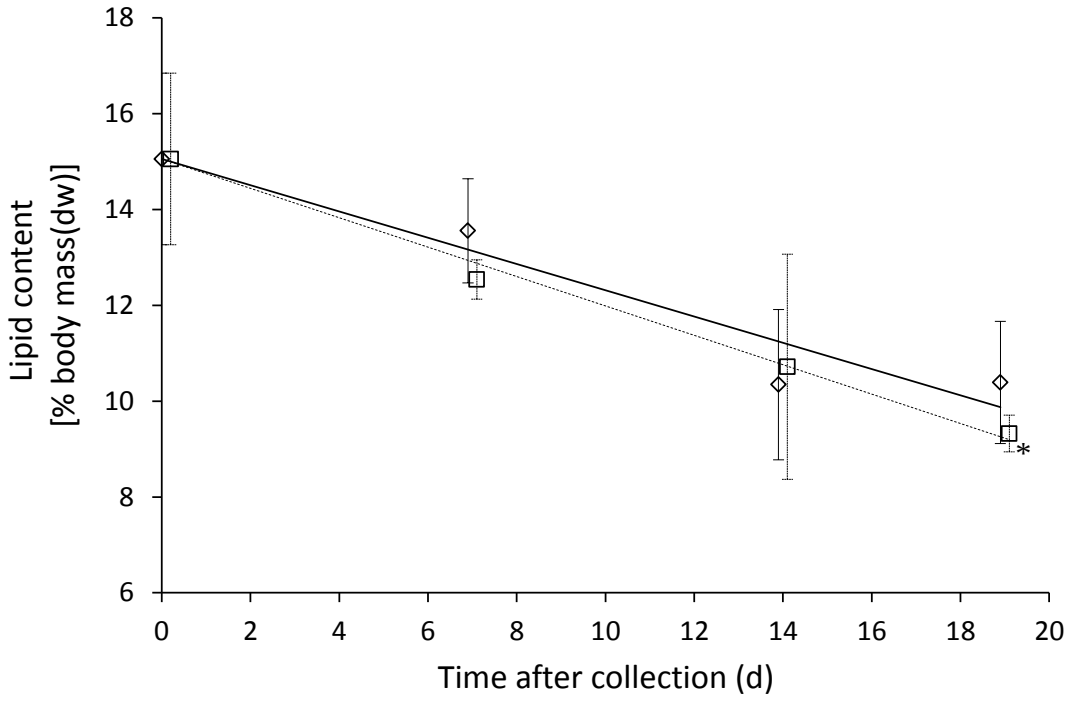


*Figure 4-2: Lipid content over time of* Gammarus pulex *with a body mass <5 mg (dw). Organisms were caught in the field at day 0 and either fed with horse chestnut leafs inoculated in tap water for 3 months (◊, solid line, $R^2 = 0.926$) or with* Cladosporium sp. *for 10 days (dashed line, $R^2 = 0.993$). Average values ± standard deviation (n = 3). * Difference in treatments on the same day with p = 0.053.*

Lipid contents were significantly less ($p<0.05$) than the initial values after one week. Within 19 days a decrease from 15.06±1.79% to 9.32±0.39 and 10.39±0.72% of the dry body mass was observed when the organisms were fed with directly or indirectly decomposed food, respectively. This leads to an overall daily reduction of the lipid content by 1.64±0.22% when organisms were fed with indirectly decomposed food and 2.03±0.27% when organisms were fed with directly decomposed food. Differences in lipid contents depending on the food sources provided were not significant when tested with a two-sided test. Nevertheless, differences increase over time and were comparatively high and almost significant on day 19 ($p=0.053$). A one-sided statistical test suggested a significant difference between the groups of differing food sources ($p=0.027$) on day 19.

### *4.2.7. Feeding rate at different lipid contents*

Within the control treatments the feeding rate of organisms fed with food sources D and DC differed significantly ($p<0.001$). Organisms fed with directly decomposed food (DC; lipid content of 3.6%) ate 0.104±0.049 mg(food)/(mg(gammarid)$_*$d) and organisms fed with indirectly decomposed food (D; lipid content of 1.8%) ate 0.199±0.032 mg(food)/(mg(gammarid)$_*$d) (Figure 4-3). The difference in the feeding rate was 52±25%. Time had no influence on the feeding rate of either control treatment ($p>0.1$), thus the feeding rate was stable over three days.

### *4.2.8. Toxicity of imidacloprid*

Organisms of differing lipid content and fed with different food sources reacted differently when exposed to the same concentrations of imidacloprid (Figure 4-3). The feeding rate of gammarids with a lipid content of 10.4% was significantly

affected within one day of exposure ($p<0.006$), and the effect was continuous over time for both concentrations tested (Figure 4-4). An exposure of organisms with a lipid content of 9.3% to the lowest concentration tested (30.0 µg/L) only had a significant influence on feeding rate after two days of exposure. Additionally, differences in effect intensity were observed. The intensity of the effect did not differ between organisms of differing lipid content when exposed to 30.0 µg/L, but was significantly ($p=0.025$) stronger for organisms with a lipid content of 10.4% when exposed to imidacloprid at a concentration of 100.0 µg/L.

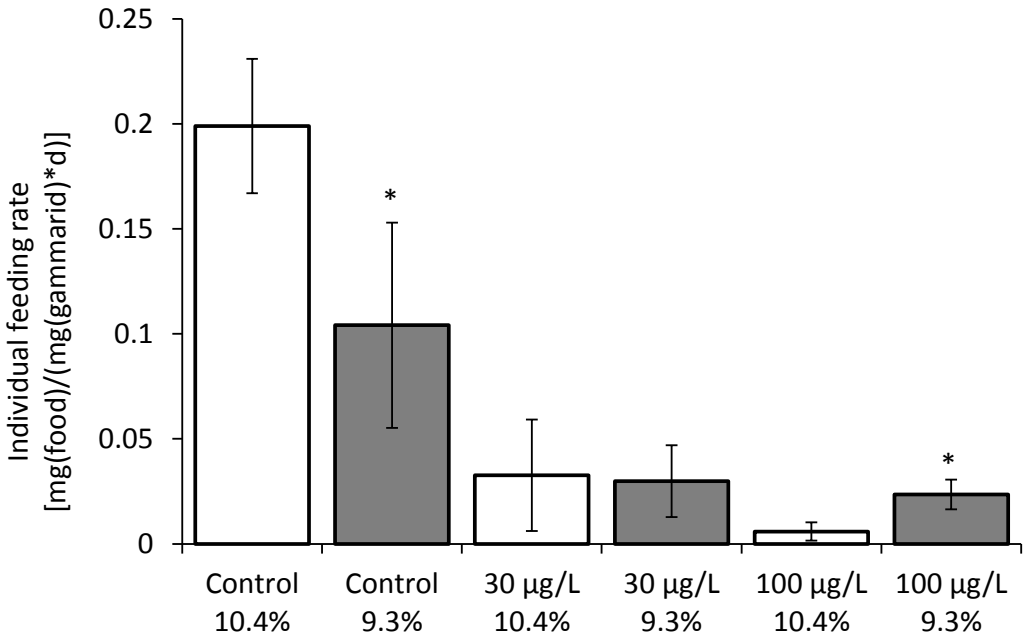


*Figure 4-3: Feeding rate of individual* Gammarus pulex *exposed to two concentrations of imidacloprid. Organisms tested differed in their lipid content (white = 10.4% of the dry body mass; grey = 9.3% of the dry body mass). Data shown are the mean ± standard deviation after three days of exposure. * Significant difference to the surrogate control treatment.*

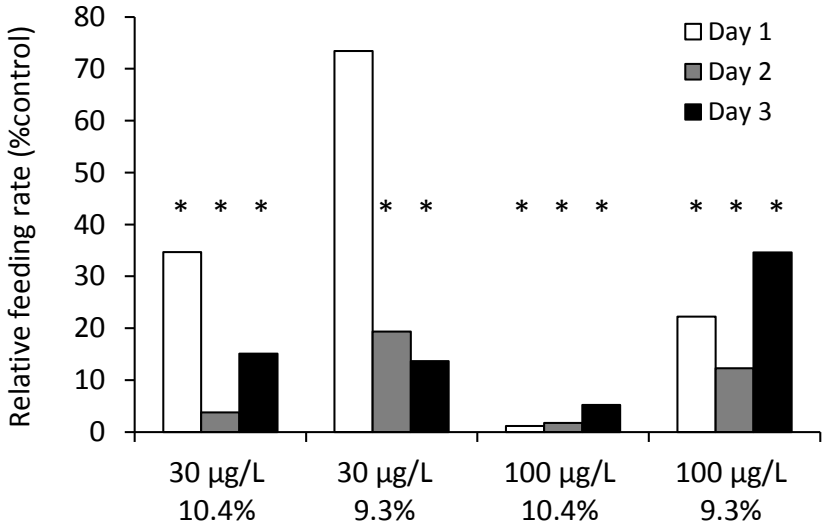


*Figure 4-4: Daily relative feeding rate as a % of the control at different combinations of internal lipid content (9.3% and 10.4% of the dry body mass) and external imidacloprid concentration from a three-day feeding assay. * Significant difference to the surrogate control treatment.*

## *4.3. Discussion*

### *4.3.1. Lipid content over time*

A reduction in the lipid content of the organisms over time while keeping them in the laboratory is a clear sign that the food provided is not sufficient to maintain the organisms in a healthy/stable condition. Stable health however is crucial for comparison of results obtained from different ecotoxicological studies undertaken with these organisms. It is known that toxicity of xenobiotics is often influenced by the lipid content of the test organism (US EPA, 2000). Not only is the comparison of results from one experiment to the next almost impossible, but toxicity can also change over time within one experiment, especially for experiments lasting longer than a few days. The interpretation of toxicant-induced effects will always be

overlaid by an additional time dependency which is species- and laboratory-dependent. Thus, the measurement of the lipid content of the test organisms needs to be done prior to each experiment to allow at least for a restricted comparability of test results. It could be argued that observation of the body weight of organisms prior to the experiment would be sufficient (e.g. less time- and resource- consuming) to determine whether the organisms are in a stable condition. However, this may not be the case because it was shown that body mass (measured in wet weight) was constant over time, whilst a loss in lipid content was counterbalanced by increased water content in the organisms (Hervant *et al.*, 1999).

Comparison of the decrease in lipid over time with that observed by Hervant *et al.* (1999) indicates how limiting the provided food was. Harvant *et al.* (1999) found a reduction in total lipid content of 39% within four weeks of complete starvation (= 1.4% per day). This reduction is similar or even smaller than that observed here (1.64±0.22 and 2.03±0.27%) while feeding the organisms with conditioned horse chestnut leafs.

It was observed previously that organisms that died due to starvation had a lipid content of 5.1% (Gee 1988). Under the assumption of lipid content being the driving factor for death through starvation and continuous linear reduction in the lipid content, the test organisms in the present study would have a life expectancy of 32 to 36 days. The time for 50 % lethality ($LT_{50}$) for *Gammarus fossarum* due to starvation has been reported to be 35.2 days (Hervant *et al.*, 1997). Their experiment revealed that no mortality occurred during the first week and 100% of the population was dead after 70 days (Hervant *et al.* 1997).

### *4.3.2. Feeding rate at different lipid contents*

Whether the differences in feeding rates arise from the differences in lipid content of the food or of the organisms cannot be determined by the data presented here. However, under consideration of data presented in Chapter 2 it can be concluded that the lipid content of the organisms cannot be the driving factor for changes in feeding rate. Here and in Chapter 2 the same food sources were used and the feeding rates measured in both experiments differed similarly depending on the food source. Thus the combination of both investigations indicates that the difference in feeding rate is caused by a characteristic of the food source. Whether it is the difference in lipid content of the food, the differences in the C-N ratio of the food (Chapter 2) or some other difference which has not been investigated (for example the phosphorus concentration) cannot be clarified. It might be just a coincidence that double the amount of food with half the lipid content (i.e. a similar amount of lipid) was eaten when comparing the two control treatments in the present experiment.

### *4.3.3. Toxicity of imidacloprid*

It could be argued that the difference in the toxicity observed derives from changed uptake of imidacloprid via the food source. This is however unlikely to be the case. First of all, the food source within the experiment was changed every 24h, reducing the risk of compound accumulation in the food source. Furthermore, with an octanol-water partition coefficient ($K_{ow}$) of 0.57 (OECD 2007) the main uptake of imidacloprid is via water. Gross-Sorokin *et al.* (2003) showed that this is the main uptake route for a chemical (4-nonylphenol) with a much higher $K_{ow}$. Similarly, Ashauer *et al*. (2010) showed strong evidence that dietary uptake is negligible for compounds with a low log $K_{ow}$.

A difference in effect-occurrence and effect-intensity at a total difference in lipid content of only 1.1% of the organisms body mass shows that this parameter might have an even stronger impact on variability in ecotoxicological testing than thought. This is important in the context of measuring environmentally-realistic exposure patterns (short-term exposure). There are implications for data- comparison and -interpretation, and finally for environmental risk assessment.

### *4.4. Conclusion*

There is no doubt that studying shredding behaviour under anthropogenic stress would benefit the environmental risk assessment of xenobiotics because there are direct links to important ecosystem processes (the protection goal for environmental risk assessment). However, the culture procedures for those organisms in the laboratory (in particular the food sources provided) need to be improved in order to produce comparable results.

# 5. Feeding inhibition explains effects of imidacloprid on the growth, maturation, reproduction and survival of *Daphnia magna*

Chapter 3 showed that imidacloprid has an impact on feeding of *Gammarus pulex* and it was hypothesised that this impact may result in other impacts at the individual level (e.g. alteration in growth and reproduction) and thus might have the potential to provoke changes at the population level. This implies that effects on growth and reproduction might not be caused directly by the compound, but rather arise from acclimation of the organism to stress invoked by feeding inhibition. Chapter 4 suggests that feeding history (reflected in the lipid content of the organism) has the potential to alter the effect of imidacloprid. A consequence may be that the transposition of feeding inhibition on growth and reproduction also depends on the feeding history.

The work on *G. pulex* (Chapters 2, 3, 4 and Appendix A) revealed that extensive improvements in the culture procedures (food sources) are needed to undertake experiments long enough to investigate whether toxicant-induced feeding depression alters individual performance of this species. Therefore, the test species was changed to the water flea *Daphnia magna* because for this aquatic invertebrate test procedures exist which enable the investigation of individual performance over the whole life cycle of individuals. Experiments were conducted to identify effects of short-term exposure to imidacloprid on individual performance (feeding, growth, maturation, reproduction and survival) of *D. magna* under surplus and reduced food availability.

## *5.1. Introduction*

All endpoints of the ecological cascade of feeding, growing, maturing, reproducing and surviving depend on population dynamics and are known to be influenced under inter- and intra-specific competition (e.g. competition for food and space). Xenobiotic impacts on the dynamics of this cascade for *D. magna* are often studied at the population or community level (with the latter defined as a cohort of daphnids in combination with organisms of other species), as neither food nor space is dynamic or limited in the standard tests used to identify effects to individuals. It has been shown that reduced food availability due to intra- specific (Barry, 1996; Knillmann *et al.*, 2012; Pieters and Liess, 2006a; Stampfli *et al.*, 2011; Takahashi and Hanazato, 2007) or inter-specific (Foit *et al.*, 2012; Knillmann *et al.*, 2012; Stampfli *et al.*, 2011) competition can cause differences in the intensity and/or duration of xenobiotic effects. Tests at the individual level can have advantages in determining causal mechanisms for effects; manipulation of feeding density is essential in this context because food is a limiting factor for population density (Antunes *et al.*, 2004 and references therein) and sensitivity to toxicants can depend on nutritional status (Heugens *et al*., 2001).

The effect-intensity of toxicants can be negatively or positively correlated with food availability. Lower bioavailability of the toxicant for daphnids due to accumulation of the compound in alga (Barry *et al.*, 1995) can lead to reduced effect-intensity when combined with negligible toxin uptake via the gut. The reallocation of energy reserves available for reproduction towards survival of offspring, producing less but fitter offspring, also leads to a negative correlation (Barry, 1996; Coors *et al.*, 2004; Pereira and Goncalves, 2007; Pereira *et al.*, 2007). Metabolism of the toxin by the

food (algae) to a more toxic compound can cause increased toxicity with increasing food density (Barry *et al.*, 1995). Additionally, it was observed that adsorption of the compound to the algae can change the feeding rate and thus reduce individual performance in terms of growth and reproduction (Taylor *et al.*, 1998).

Influences of a toxin on feeding could invoke various behavioural changes because energy availability depends on feeding (Sancho *et al.*, 2009; Villarroel *et al.*, 2009) and the energy budget can be considered an indicator of the overall condition of an organism (Calow and Sibly, 1990). Effects of pesticides on the feeding rate of daphnids have been observed within hours of exposure (Sancho *et al.*, 2009); were found for low concentrations of a number of xenobiotics (Allen *et al.*, 1995; Duquesne and Kuester, 2010; Fernandezcasalderrey *et al.*, 1994; McWilliam and Baird, 2002; Pestana *et al.*, 2010; Sancho *et al.*, 2009); and have been observed to be reversible within 30 minutes of removing the stressor (McMahon and Rigler, 1965). Additionally, feeding rate of daphnids has been found to be the most sensitive endpoint among the measurement of feeding rate and content of glycogen, lipid, protein and calories (Sancho *et al.*, 2009).

We hypothesise that various effects of xenobiotics on Daphnid behaviour are not caused directly by the compound, but rather result indirectly from acclimation of the organism to its environment in response to a stress. To test this hypothesis, we carried out experiments at the individual level to quantify effects on all endpoints of the effect-cascade. Imidacloprid was selected as a model substance because it is known to cause feeding inhibition (Pestana *et al.*, 2010). The experiments carried out were a feeding assay, an acute toxicity test and two chronic reproduction tests. We performed the chronic tests under differing food density and included the

measurement of recovery. Additionally, we tested whether feeding inhibition on its own can explain the dynamic effects found further up the cascade using the individual *Daphnia magna* population model "IDamP" (Preuss *et al.*, 2009) as a virtual laboratory.

## *5.2. Materials & Methods*

### *5.2.1. Experimental designs*

Table ***5-1*** gives detailed information on all experiments undertaken to investigate the impact of imidacloprid on the endpoints feeding, growing, maturing, reproducing and surviving. Environmental conditions for all experiments were maintained at those of the *D. magna* culture (see the following section). Four experiments were conducted, namely an acute toxicity test, two reproduction tests and one feeding assay. In the acute toxicity test mortality of imidacloprid to neonate daphnids at concentrations up to 100 mg/L was observed for a period of one week (four replicates; ten individuals per replicate). At the end of this experiment the body length of the remaining individuals was measured.

The two reproduction experiments were undertaken to observe the effects of imidacloprid on growth, maturation (here defined as the day of releasing the first offspring) and reproduction. Therefore, neonates were exposed individually (ten replicates per treatment) for one week and their body length and reproductive status was observed frequently over a period of four weeks. Those tests deviated from the standard OECD test protocol (OECD, 1998) in terms of exposure scenario, food quantity (lowest level recommended by OECD test protocol (OECD, 1998) (Test 1) and 33% lower (Test 2)) and experimental duration. Exposure lasted only one week

*Table 5-1: Experimental details for the observation of effects from a short-term exposure of Daphnia magna to imidacloprid.*

| | **Acute toxicity test** | **Reproduction tests** | | **Feeding assay** |
|---|---|---|---|---|
| | | **Test 1** | **Test 2** | |
| Test duration [d] | 7 | 41 | 34 | 1 |
| Test approach | Quasi-static | Quasi-static | Quasi-static | Static |
| Volume [mL] of test solution (1st week / rest) | 50 | 50 / 80 | 50 / 80 | 2.15 |
| Number of treatments (incl. control) | 7 | 3 | 6 | 7 |
| Number of replicates per treatment | 4 | 10 | 10 | 10 |
| Number of individuals per replicate | 10 | 1 | 1 | 1 |
| Imidacloprid concentration tested [mg/L] | 0.40, 1.20, 3.70, 11.1, 33.3, 100 | 0.15, 12.0 | 0.15, 0.40, 1.3, 4.0, 12.0 | 0.078, 1.56, 7.8, 31.2, 156 |
| Exposure duration [d] | 7 | 7 | 7 | 1 |
| Start of exposure [d] | 1 | 1 | 1 | 1 |
| Number of media changes during exposure | 1 | 1 | 1 | 0 |
| Frequency of media change after exposure [d] | - | 2 – 3 | 2 – 3 | - |
| Chemical analysis performed | Yes | Yes | Yes | No |
| Frequency of feeding [d] | 2 – 3 | 1 | 2 – 3 | Start of test |
| Food density provided [mg TOC/d](1st week /rest)* | 0.035 | 0.050 / 0.100 | 0.035 / 0.070 | 0.073±0.002 |
| Endpoints measured | Mortality, Body size | Mortality, Body size, Reproduction, Maturation | Mortality, Body size, Reproduction, Maturation | Mortality, Feeding activity |
| Frequency of observation of mortality [d] | 1-3 | 1 | 2 – 3 | End of test |
| Frequency of body size determination [d] | End of test | 2 – 3 | 2 – 3 | Start of test |
| Type of body size determination | Microscopy | Image analysis | Microscopy | Image analysis |
| Number of individuals measured each sampling | 10 | 10 | 5-6 (same set) | 10 |
| Frequency for observation of reproduction [d] | - | 1 | 2 – 3 | - |

** In the first week of both reproduction tests the organisms were fed with half of the mentioned food, whereas those food densities ensure excessive food availability for neonate and juvenile daphnids; __Starting conditions for model predictions using the individual* Daphnia magna *population model "IDamP"*

*TOC = Total organic carbon*

to account for a more realistic exposure scenario; organisms were fed with less food to simulate competition for food, as occurs in the environment; and the experiments were extended to gather data on recovery potential after short-term exposure. The cumulative number of organisms produced per mother and the number of offspring produced per brood was calculated. The former was measured over a period of 28 d, because this is the longest recommended test duration according to the standard test procedure, and no mortality was observed within this time frame. The latter was determined until brood number 6, as this was the last brood released by all individuals within the test duration.

The reproduction test under high food availability was prolonged for detection of individual development of the first (F1-1) and second (F1-2) offspring produced. Detailed information can be found in the second appendix of this chapter (Appendix C). Those results are neither presented nor discussed within this chapter.

The fourth experiment was conducted to measure the influence of imidacloprid on the feeding rate of daphnids. Therefore, the amount of food given at the beginning and the end of the feeding period (24h) was measured by detecting the absorption of light by the food source *Desmodesmus subspicatus* at a wavelength of 720 nm. The feeding assay was conducted in 1 cm cuvettes and exposure to imidacloprid began simultaneously with the start of the feeding period. An algal control without *Daphnia* was used to correct feeding for algal growth during the test. The calculation of feeding inhibition was conducted using a simple one-compartment model taking into account the food concentration, the algal growth rate, the filtration rate and the ingestion rate. Detailed information on the calculation of feeding inhibition can be found in the supporting information (Appendix B).

The maximal fluctuation in pH was 0.8 units due to frequent changes of the test solution (Table 5-1). The lowest and highest pH measured were 7.4 and 8.2, representing the measurement of fresh and old media, respectively.

### *5.2.2. Test organisms*

Neonate daphnids (age <24h, body size 0.94±0.05 mm) derived from four-week old daphnids from our own culture were used for all experiments and were fed with the green algae *D. subspicatus*. Cultures of both species were maintained as follows. *D. magna* Straus were cultivated quasi-statically at a group level in M4-media (Elendt, 1990) under constant temperature (20±1°C), a photoperiod of 16h light and 8h dark, and a light intensity of 15-19 μE/$m^2$s. The culture was maintained over time using the offspring of four-week old daphnids. Twenty individuals of the same age (±1d) were cultivated in 1.5 L medium which was changed weekly. Mothers and offspring were separated on the day of feeding. Organisms were fed with *D. subspicatus* three times a week (Monday, Wednesday and Friday) with 0.14 mg TOC per Daphnid. Algae used for feeding were in the logarithmic growth phase obtained by cultivating them under continuous aeration, constant temperature (20±1°C) and constant light with an intensity of 45 μE/$m^2$s. The algae were cultured statically for 2 weeks in 1-L media bottles containing 750 ml Kuhl-medium (Kuhl and Lorenzen, 1964).

### *5.2.3. Test item and chemical analysis*

An analytical standard of imidacloprid (PESTANAL®; 99.0%) obtained from Sigma Aldrich (Batch-no. SZE9112X) was used for the preparation of stock solutions without the use of a carrier solvent. Samples of both prepared solutions and the test media were taken prior to and following each pulse and frozen at -18°C for chemical

analysis. For analysis 75 µL of each sample was injected onto HPLC (Agilent 1100 Series, Agilent Technologies, UK Ltd.). Samples with a nominal concentration >10 mg/L were diluted tenfold prior to injection using M4-media. The HPLC was equipped with a UV detector (254 nm) and a Discovery® $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco) maintained at 25°C. Analysis was performed with a mobile phase of methanol/water (45:55; v/v) and with a flow rate of 0.5 mL/min. Further details of the chemical analyses can be found in the supporting information (Appendix B).

All measured imidacloprid concentrations were within 6.3% of nominal concentrations apart from the concentration >10 mg/L which deviated from nominal by up to 26.4%. The larger difference was attributed to the need to dilute these samples prior to analysis. No influence of food density was found on either the deviation from the nominal concentration or the degradation of imidacloprid. All results are discussed subsequently in relation to the nominal concentrations.

### *5.2.4. Body length determination*

Body length of the daphnids was measured either using a microscope or by image analysis, and was defined as length measured from the top of the eye to the base of the apical spine (ventral). When body size was determined by image analysis, scans of each individual were produced using an incident light scanner (Canon, CanoScan 9000F) at a resolution of 1200 dpi. This procedure was shown to be harmless to daphnids in relation to all endpoints observed in the present studies (Chapter 4). For both procedures, organisms were transferred to a Petri dish and temporarily immobilized by removal of media.

### *5.2.5. Modelling the effects*

The individual *Daphnia magna* population model "IDamP" (Preuss *et al.*, 2009) was used to predict effects on growth, maturation, reproduction and mortality when changing the individual feeding rate. IDamP is an individual based population model for *D. magna*, which explicitly simulates the life-history of individual daphnids and their plasticity at various environmental conditions, including various feeding scenarios. Since it is an individual based model, single individuals, batches of individuals, and populations can be simulated. No model calibration was undertaken for the simulation, and all experimental results were unknown prior to simulation. Input parameters represented the starting conditions of the experiments described above (underlined values in Table 5-1 plus initial body length of 0.94±0.06 mm). The measured mean inhibition in feeding rate (expressed as % of control) was used to modify the feeding rate within the model. Detailed information on the feeding inhibition used for modelling is given in the supporting information (Appendix B; Table A-2). Feeding inhibition was set constant for the exposure phase and reset to zero inhibition immediately after exposure ceased. Thus, underlying assumptions for the effect modelling were time-independence of the feeding inhibition and a spontaneous recovery to the control level.

### *5.2.6. Statistics*

Impacts of exposure on growth over time were evaluated using a two-way ANOVA (reproduction tests). A one-way ANOVA was used to compare body length at the end of the acute toxicity test. Comparison of growth between the reproduction tests was by one-way ANOVA at each sampling time. Linear growth was assumed between successive sampling points and the body length of individuals was calculated by

interpolation when sampling times of the reproduction tests did not match. All endpoints shown in Table 5-2 and Table 5-3 were analysed by one-way ANOVA within experiments and with a two-way ANOVA between experiments. SigmaPlot 12 (Systat Software, Inc.) was used for statistical analysis and each ANOVA followed the Holm-Sidak method. Assumptions on normal distribution and equal variance were tested prior to ANOVA using the Shapiro-Wilk and Levene-Mediane tests, respectively.

## *5.3. Results and discussion*

### *5.3.1. General results*

Several endpoints were significantly affected ($p<0.05$) by imidacloprid. Under surplus food supply the concentration causing an effect increased along the effect-cascade. Food density influenced the intensity and duration of effects from imidacloprid on the endpoints observed, and some effects only became detectable when food density was reduced.

### *5.3.2. Feeding rate*

The feeding assay showed a concentration-dependent influence of imidacloprid (Figure 5-1). The lowest concentration tested (0.078 mg/L) did not affect feeding, whereas the two highest concentrations (31.2 and 156.0 mg/L) caused a reduction in food intake by 100% (Table A-3). A feeding rate of 0.019±0.0003 mg TOC/d was calculated for non-exposed neonates when accounting for algal growth. Concentrations causing 5, 50 and 95% feeding inhibition after one day of exposure were 0.19, 1.83 and 8.70 mg/L, respectively (upper to lower limits for the 95% confidence interval were 0.09-0.32, 1.4-2.2 and 7.3-17.5 mg/L, respectively); these

values were in the same range as those reported by Pestana *et al.* (2010). Feeding of daphnids depend on appendage movement and therefore the coordination of the nervous system (Villarroel *et al.*, 1998). Thus xenobiotic impacts on the function of the nervous system as caused by imidacloprid disturbs or obviates the filtration (Villarroel *et al.*, 1999) and leads to changed feeding.

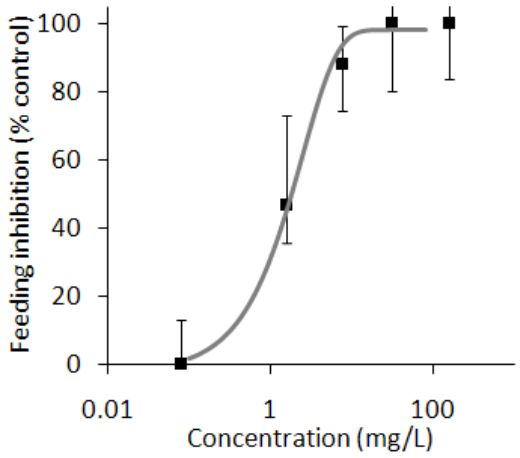


*Figure 5-1: Relative feeding inhibition of* Daphnia magna *when exposed to imidacloprid for 24h (feeding assay). The symbols represent the results calculated from measured data (± relative optimization error; see section 12.2.1.1. in the appendix of this chapter on calculation of feeding inhibition for details), and the lines represent the four-parameter sigmoidal dose-response curve.*

### *5.3.3. Growth*

A significant influence of imidacloprid exposure on growth was found in the acute toxicity and both reproduction tests. The body length of daphnids was reduced in a concentration-dependent fashion by up to 53±11% within seven days of exposure. For further information see Figure A-2 and Table A-4 in the supporting information (Appendix D). Intensity of effect during exposure was independent of food density as there was no difference ($p>0.05$) in body length during exposure at differing food

densities (Figure 5-2). However, food density was a determining parameter for recovery in growth following imidacloprid exposure. Whereas organisms maintained at high food density recovered to the control level within three weeks, no recovery occurred when organisms were fed less (Figure 5-2). Organisms fed less stopped growing nine to eleven days after exposure, remained at the same body size for at least nine days, and then started growing again. The appearance of this discontinuation in growth was concentration-dependent (Figure A-3) and occurred around the time when the organisms released their first brood (Figure 5-2). Reduced food densities resulted in a loss of recovery potential so that a significantly reduced overall rate in growth occurred in all concentrations tested (Table 5-2).

Growth-discontinuation with a loss of recovery potential occurred for concentrations where there was no effect on growth during exposure or the week thereafter (Figure 5-2), indicating that a delayed effect had occurred. Under food limitation, the lowest concentration tested (0.15 mg/L) showed the described growth-discontinuation, whereas organisms at high food density showed no growth-discontinuation even when exposed to 12 mg/L (Figure 5-2). The feeding assay showed that a concentration of 12 mg/L reduced the feeding rate by 97% whereas a concentration of 0.15 mg/L reduced the amount of food eaten by only 3.7%; hence delayed effects on growth occurred under food limitation after short-term feeding inhibition of less than 5%. Differences in the energy reserves caused by the difference in food density might have evoked this behaviour. Reduced individual growth has the potential to cause a change in population structure and this in turn can change sensitivity to additional stress ((Preuss *et al.*, 2010), Chapter 4) and/or the recovery potential of a population (Liess and Foit, 2010). Further research is required to determine whether such effects occur in practice.

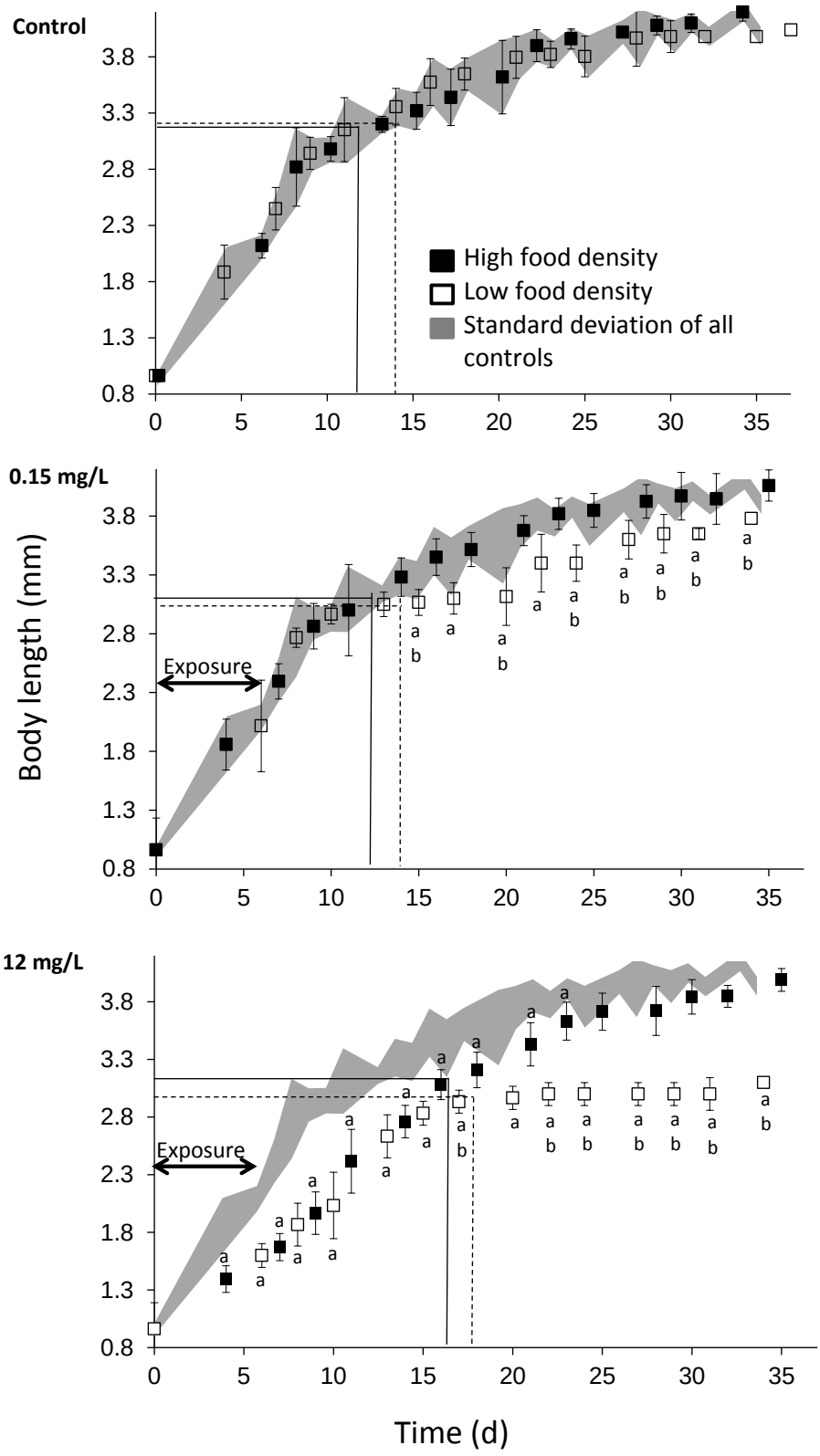


*Figure 5-2: Measured average body length (± standard deviation (SD)) of* Daphnia magna *over time when fed with Desmodesmus subspicatus at differing densities*

*(Reproduction tests 1 and 2). The arrows indicate the presence of imidacloprid, and the grey areas indicate the measurements of the control from both reproduction tests combined. Lines show the time and body length of releasing the firstbroad for organisms with high (----) and low (- - -) food density. a Significantly different from the control treatments, indicating effects of imidacloprid exposure (two-way ANOVA; Holm-Sidak method; p<0.05). b Significantly different from the same treatment of the other experiment, indicating effects of the food density (one-way ANOVA; Holm-Sidak method; p<0.05).*

*Table 5-2: Growth [mm/d] of* Daphnia magna *calculated over a period of 34 days when exposed to different concentrations of imidacloprid for one week. Organisms (<24h) were fed at two different food densities (Reproduction tests 1 and 2). The average (± standard deviation) for measured data, and the range for the predicted data are shown. Data were predicted using the individual* Daphnia magna *population model "IDamP".*

| **Concentration** | **Measured** | | **Predicted** | |
|---|---|---|---|---|
| **[mg/L]** | **0.1 mg TOC/d** | **0.07 mg TOC/d** | **0.1 mg TOC/d** | **0.07 mg TOC/d** |
| 0 | 0.114 ± 0.007 | 0.113 ± 0.002 | 0.060 - 0.129 | 0.054 - 0.123 |
| 0.15 | 0.115 ± 0.006 | 0.098 ± 0.006 [ab] | 0.055 - 0.129 | 0.058 - 0.121 |
| 0.4 | - | 0.081 ± 0.003 [a] | - | 0.046 - 0.125 |
| 1.3 | - | 0.087 ± 0.006 [a] | - | 0.044 - 0.116 |
| 4 | - | 0.077 ± 0.004 [a] | - | 0.038 - 0.101 |
| 12 | 0.110 ± 0.006 | 0.077 ± 0.003 [ab] | 0.040 - 0.093 [c] | 0.043 - 0.092 |

*a Significantly different from the control of the same experiment (p<0.05; one-way ANOVA, Holm-Sidak method); b Significantly different from the same concentration at different food density (p<0.05; two-way ANOVA, Holm-Sidak method); c The range of the prediction does not overlap with the measurement (→ mismatch between measurement and prediction); TOC = Total organic carbon*

### *5.3.4. Maturation*

The time taken to reach the reproductive stage was influenced by food density and imidacloprid exposure (Table 5-3). Reduced food density provoked significantly

delayed reproduction ($p<0.001$) when comparing the control treatments from the two food densities tested. Increasing delay of reproduction was found with increasing imidacloprid concentration when organisms were fed with 0.07 mg TOC/d, whereas a concentration of 12 mg/L delayed reproduction significantly ($p<0.001$). Figure 5-2 shows a relationship between the body length of daphnids and the time when they released the first brood. All organisms released offspring for the first time when reaching a body length between 2.9 and 3.2 mm. whereas body length at first reproduction varied by 10%, a variation of 30% was found for time taken to reach maturity. A delayed maturation time has been demonstrated previously to be influenced by food density (Guisande and Gliwicz, 1992; Porter *et al.*, 1982). At the population level a prolonged maturation time influenced by food density is normal because food density is a limiting factor for population density (Antunes *et al.*, 2004).

### *5.3.5. Reproduction*

Neither reduced food availability nor imidacloprid exposure at high food density caused changes in reproduction (Table 5-3). The combination of both however provoked effects on the cumulative number of offspring by up to 57% when imidacloprid concentration exceeded 1.3 mg/L (Table 5-3). Cumulative number of offspring depends on maturation that is in turn influenced by pesticide exposure and food density. Therefore, the identification of effects on reproduction makes it necessary to look at the number of offspring per brood. A significant change in clutch size in both directions was found (Table 5-3). Clutch size was increased following exposure to small concentrations of imidacloprid (0.15 and 0.4 mg/L); conversely, there was a reduced clutch size at large imidacloprid concentrations, but this was not

*Table 5-3: Key data for sub-lethal endpoints for* Daphnia magna *after a one-week exposure to imidacloprid and when fed at differing food density (reproduction tests 1 and 2). The average (± standard deviation) for measured data, and the 95$^{th}$ percentile for the predicted data are shown. Data were predicted using the individual* Daphnia magna *population model "IDamP".*

| Endpoint | Concentration [mg/L] | Measured | | Predicted | |
|---|---|---|---|---|---|
| | | 0.1 mg TOC/d | 0.07 mg TOC/d | 0.1 mg TOC/d | 0.07 mg TOC/d |
| Time taken until maturation [d] | 0 | 11.7 ± 1.4 | 13.8 ± 0.7 [b] | 10 – 14 | 9 – 15 |
| | 0.15 | 12.2 ± 1.5 | 13.8 ± 1.8 [b] | 9 – 14 | 10 – 15 |
| | 0.4 | - | 13.8 ± 1.5 | - | 10 – 15 |
| | 1.3 | - | 14.3± 1.9 | - | 10 – 15 |
| | 4 | - | 16.3 ± 3.4 | - | 11 – 19 |
| | 12 | 16.6 ± 2.1 [a] | 18.0 ± 2.3 [a] | 14 – 24 | 14 – 20 |
| Cumulative no of offspring / mother (for 28 days) | 0 | 67.7 ± 13.0 | 55.8 ± 15.6 | 14 – 124 | 12 – 90 |
| | 0.15 | 67.5 ± 10.7 | 59.8 ± 16.6 | 13 – 138 | 7 – 132 |
| | 0.4 | - | 63.9 ± 12.1 | - | 8 – 92 |
| | 1.3 | - | 54.9 ± 14.8 | - | 9 – 89 |
| | 4 | - | 31.7 ± 11.1 [a] | - | 4 – 101 |
| | 12 | 58.1 ± 19.0 | 32.1 ± 12.6 [ab] | 8 – 105 | 10 – 78 |
| No of offspring / mother and brood (brood 1-6) | 0 | 14.4 ± 7.2 | 11.8 ± 5.3 | 11.0 ± 2.8 | 8.7 ± 2.3 |
| | 0.15 | 13.6 ± 7.5 | 13.7 ± 5.7 [a] | 11.2 ± 2.7 | 8.7 ± 2.3 |
| | 0.4 | - | 14.3 ± 5.2 [a] | | 9.2 ± 1.9 |
| | 1.3 | - | 12.0 ± 6.3 | | 8.8 ± 2.5 |
| | 4 | - | 9.5 ± 5.0 [c] | | 9.3 ± 1.9 |
| | 12 | 13.8 ± 7.4 | 10.5 ± 6.4 [c] | 12.4 ± 1.9 | 8.8 ± 0.4 |

*a Significantly different from the control of the same experiment ($p<0.05$; one-way ANOVA, Holm-Sidak method); b Significantly different from the same concentration at different food density $p<0.05$; two-way ANOVA, Holm-Sidak method); c Significantly different from the treatments showing a positive influence of the compound within the same experiment ($p<0.05$; two-way ANOVA, Holm-Sidak method); TOC = Total Organic Carbon*

significant relative to the control. Increased reproduction is known to be associated with a reduction in body length (Guisande and Gliwicz, 1992; Hammers-Wirtz, 2002) and/or lipid content of the offspring (Guisande and Gliwicz, 1992) because available energy for reproduction is redistributed towards delivering more but less fit offspring (Hammers-Wirtz and Ratte, 2000). Acclimation of daphnids to their environment is known to be influenced by several environmental factors including food (Boersma, 1997), predation (Gliwicz, 1994; Stibor, 1992) and presence of conspecifics (Cleuvers *et al.*, 1997; Goser and Ratte, 1994). The observed growth-discontinuation in combination with the induced reproduction after exposure to imidacloprid gives evidence that daphnids additionally have the ability of redistributing energy towards reproduction rather than growth when food availability is limited. However, this redistribution relies on organisms reaching maturity which in turn depends on reaching a minimum body length.

### *5.3.6. Mortality*

No significant treatment-related mortality occurred in either the feeding assay or the reproduction tests. Only the highest concentration tested in the acute toxicity test (100 mg/L) caused mortality of daphnids; here, no significant ($p<0.05$) differences from all other treatments were found within the first three days of exposure, whereas all daphnids were dead on the next day of observation (day six) (Figure A-4). This result combined with findings from the feeding assay indicates that feeding inhibition by 97% (12 mg/L) did not result in mortality. Thus, organisms were able to cope with a food intake of 3% of the control in combination with the energy reserves given at birth and the food taken up in the period prior to exposure. However, a longer exposure might lead to death by starvation when the energy needed to sustain

metabolism exceeds the remaining feeding rate and energy reserves are depleted. Neonates caught in the field survived 5-6 days under total starvation (Elendt, 1989; Tessier *et al.*, 1983); this suggests that imidacloprid in concentrations up to 100 mg/L does not have a direct mortal effect on daphnids, but rather causes mortality due to starvation. A further comparison of starvation times is not possible without comparing the status of the mothers and the neonates in terms of lipid content (Tessier *et al.*, 1983) and body mass, because starvation is dependent on both (Perrin *et al.*, 1992). This might be the reason why our results in terms of mortality do not match with those given in the Canadian Water Quality Guideline for imidacloprid (OECD, 2007). There, an effect concentration of 85 mg/L after 48h of exposure to newly hatched daphnids is given, showing mortality occurring within less time than can be explained by starvation.

### *5.3.7. Effect modelling*

In general the blind model simulations fitted the observations for all endpoints (Figure 5-3, Figure A-2, Figure A-4) even though feeding inhibition was the only effect included within the model. This suggests that all effects observed within the experiments were secondary effects provoked by feeding inhibition; a good match to observations would not have been expected if imidacloprid also exerted independent effects on one or more of the other endpoints.

A mismatch between model and experiment occurred for growth at high food density after strong feeding inhibition (Figure 5-3). In the experiment a complete recovery in body length to the control level was observed, whereas the model underestimated the recovery and the final body length was lower than that of the control (Table 5-3).

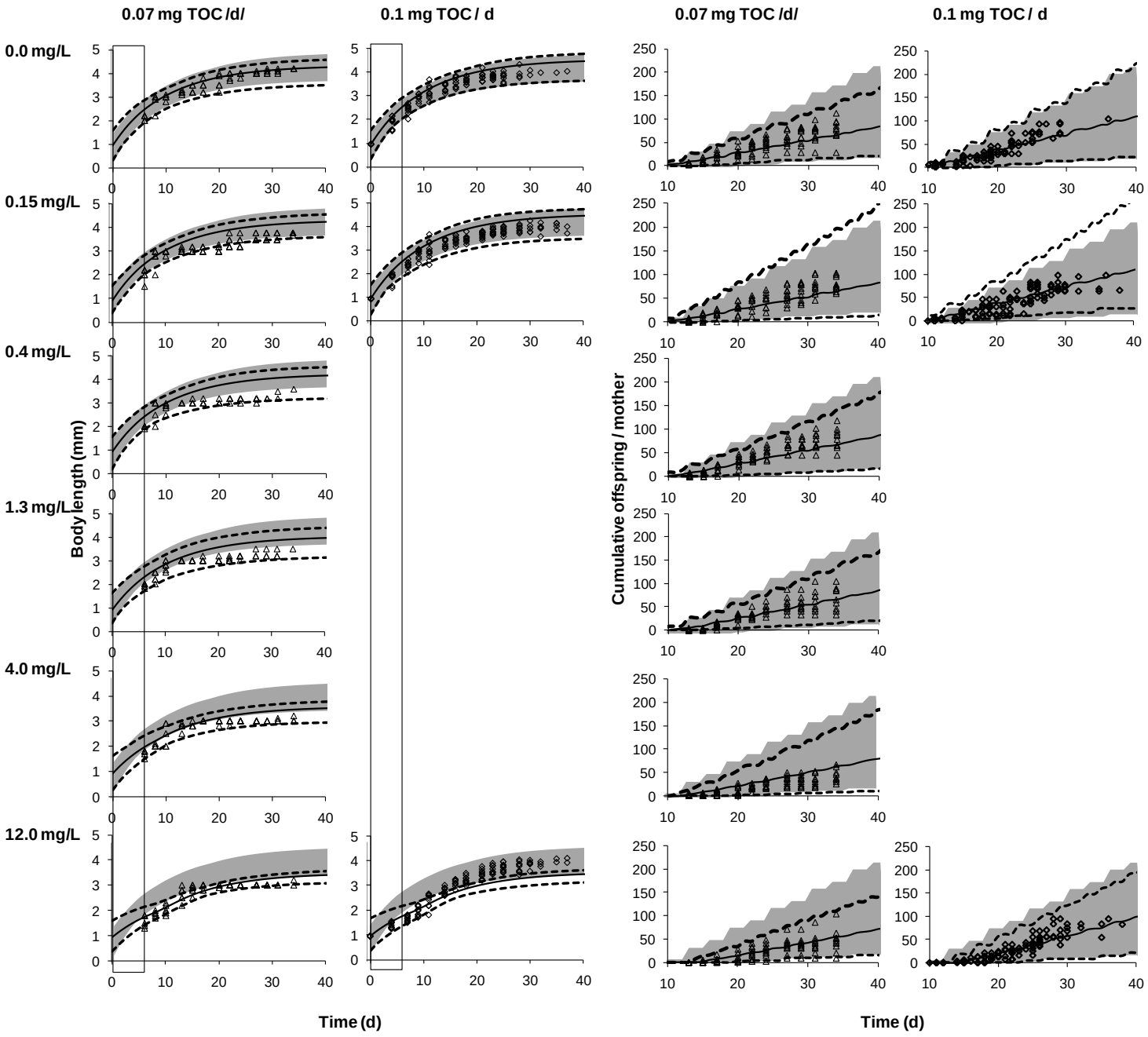


*Figure 5-3: Measured (symbols) and predicted (solid line = average, dotted line = 95$^{th}$ percentile) of body length and reproduction of* Daphnia magna *over time when fed with* Desmodesmus subspicatus *at different densities [mg TOC/d]; TOC = total organic carbon. Daphnids (<24h) were exposed to different imidacloprid concentrations [mg/L]. The rectangles indicate the presence of imidacloprid, and the grey areas indicate the 95$^{th}$ percentile of the control fed with 0.1 mg TOC/d. Data were predicted using the individual* Daphnia magna *population model "IDamP".*

This mismatch results from the assumption of instantaneous recovery from feeding inhibition without compensational food uptake after removal of imidacloprid. Higher feeding rates after removal of the stressor were observed previously (McMahon and Rigler, 1965), and missing this behaviour in the model explains lower prediction of growth at high food levels.

The experiments helped to identify a process that may be missing from the model. There was a consistent mismatch between simulation and observation around the time where the described growth-discontinuation occurred. This mismatch does not affect either the final body length or the reproductive outcome. Having all discussed effects caused by changes in feeding and subsequently by changes in energy reserves might suggest the use of a model following the dynamic energy budget theory (Kooijman, 2010).

### *5.3.8. General discussion*

The combined interpretation of the experiments admits the evidence that the organism's energy reserve is most likely the stimulating factor for all effects observed, even though we did not measure the energy reserves. The energy availability dependes on feeding (Sancho *et al.*, 2009; Villarroel *et al.*, 2009) and the energy budget can be considered as an indicator of the overall condition of an organism (Calow and Sibly, 1990). Feeding rate has been found to be the most sensitive endpoint among the measurement of feeding rate, glycogen-, lipid-, protein- and caloric content (Sancho *et al.*, 2009), and therefore is most likely the driving factor of changes in energy budget. Low food availability resulted in a loss of ability to replenish these reserves after removal of imidacloprid, because energy was redistributed to attaining the minimum body length for reproductive maturity. Low

energy reserves combined with low food availability beyond the stage of maturity resulted in limited energy to sustain reproduction without redistributing the reserves towards reproduction resulting in stopped growth (growth-discontinuation). At low concentrations of imidacloprid the decrease in energy reserves was so low that the growth-discontinuation resulted in increased reproduction; conversely, the impact of high concentrations on energy reserves provoked reduced reproduction, thus the impact was too high to be balanced by redistribution towards reproduction (growth-discontinuation). The results of the presented work might be transferable to other compounds and other species even though only imidacloprid and daphnids were used as model substance and model species, respectively. Impacts of a wide range of xenobiotics on feeding of several non-target species have been observed in the past.

Nevertheless, surely an extrapolation to other compounds acting on the nervous system is possible. Feeding of daphnids depend on appendage movement and therefore the coordination of the nervous system; therefore, xenobiotic impacts on the function of the nervous system disturbs or obviates the filtration (Villarroel *et al.*, 1999) and leads to changed feeding.

We conclude that the interpretation of experimental results should take the effect-cascade, and thus the ecology of the test species into account. Consideration of each experiment on its own would have led to a different interpretation of the effects.

Delayed mortality could have been interpreted as a result of a slow uptake and elimination of the compound by the organisms; induced reproduction at low concentrations might have been interpreted as resulting from increased metabolism due to stress (hormesis); and reduced reproduction on its own might have been considered as symptomatic of a direct effect. However, the consideration of reduced

food availability (as occurring in populations) within experiments at the individual level, the addition of observing recovery potential and the use of an additional tool (IDamP) disclosed delayed, long-lasting secondary impacts on daphnids with potential repercussions for population sustainability.

It is known that food is a limiting factor for population density (Antunes *et al.*, 2004) and changes in food levels may change the energy allocation of organisms (Peeters *et al.*, 2010). Furthermore, the sensitivity to toxicants is usually increased with decreasing food density (Heugens *et al.*, 2001). Food supply is chosen to be unlimited in the standard tests for daphnids, but effects of xenobiotics can vary dramatically depending on food supply under both constant (Heugens *et al.*, 2001; Peeters *et al.*, 2010) and periodic exposure (present study). Therefore, approaches are needed to extrapolate test results to conditions where food supply may be limited. Possibilities might be the model used here and/or the use of a model based on the dynamic energy budget theory. However, both model types in their current stage do not allow the consideration of the described reallocation of energy reserves towards reproduction rather than their own growth, what might implement uncertainties in the model outcome, especially for modelling time variable combinations of stressors.

# 6. Evidence for links between feeding inhibition, population characteristics and sensitivity to acute toxicity for *Daphnia magna*

Chapter 5 revealed that imidacloprid inhibits feeding of daphnids and that this alters the individual performance of the organisms. Both, the occurrence of the effect and the intensity of the effect were shown to depend on food availability. It was hypothesised that short-term feeding inhibition may provoke a shift in population structure that will vary with conspecific pressure. Here, populations of *Daphnia* at different stages of conspecific pressure were exposed to imidacloprid in a manner similar to that in Chapter 5 to test this hypothesis. It was additionally hypothesised that the shift in population structure increases population sensitivity to a subsequent xenobiotic exposure due to size-dependent toxicity. To test this hypothesis within the same experiment, populations were exposed to carbaryl subsequent to the imidacloprid exposure.

## *6.1. Introduction*

*Daphnia magna* is a model organism for ecology and ecotoxicology. It is known that food availability (e.g. concentration in the environment) influences the performance of daphnids in terms of growth (Preuss *et al.*, 2009), maturation (Guisande and Gliwicz, 1992; Porter *et al.*, 1982), reproduction (Boersma, 1997; Bradley *et al.*, 1991; Cleuvers *et al.*, 1997) and survival (Elendt, 1989), and ultimately determines population density (Antunes *et al.*, 2004). It is expected that restricting the intake of food due to xenobiotics daphnids (Day and Kaushik, 1987; Fernandez-Casalderrey *et al.*, 1994; Sancho *et al.*, 2009; Villarroel *et al.*, 1999; Villarroel *et al.*, 2003) will

affect individual performance similarly as both cases lead to less food being taken up by the individual. Empirical studies with different xenobiotics undertaken with surplus food availability provide evidence that toxicant-induced feeding depression leads to similar changes in individual performance as natural acclimation; xenobiotics can alter the feeding behaviour of daphnids (Day and Kaushik, 1987; Fernandez-Casalderrey *et al.*, 1994; Sancho *et al.*, 2009; Villarroel *et al.*, 1999; Villarroel *et al.*, 2003) and this can affect one (Baird *et al.*, 1990; Enserink *et al.*, 1995) or more (Pestana *et al.*, 2010; Pieters *et al.*, 2005; Reynaldi *et al.*, 2006) endpoints associated with individual performance (e.g. growth and reproduction). This raises the question of whether such changes are a concern for populations; dynamic and limiting food availability is a frequent occurrence at this level of organisation and organisms constantly acclimate by altering their individual performance. Studies are needed to investigate effects of toxicant-induced feeding depression and its consequences at different food availabilities. Chapter 5 showed that short-term (one-week) feeding inhibition by only 3.7% (caused by imidacloprid exposure at 0.15 mg/L) within the juvenile development of daphnids can alter the individual performance in terms of growth, maturation and reproduction when food availability is limited. Still, it remains uncertain whether this is a concern for populations as factors other than food availability influence population dynamics; presence of organisms of the same kind (conspecifics) and presence of predators are examples. One study where *D. magna* populations were exposed to fenvalerate, which was later shown to inhibit feeding (Reynaldi *et al.*, 2006), demonstrated impacts at the population level (Pieters and Liess, 2006a). There, the potential to recover from changes in abundance and structure of the population after exposure was observed to depend on the phase of population development (differing intensity

of conspecific pressure) during which exposure occurred. However, for fenvalerate it has not been demonstrated whether the influence on feeding is the only driving factor for observed changes in population abundance and structure, or whether other direct effects of the compound exist. In contrast, the study with imidacloprid (Chapter 5) demonstrated that the compound acts on feeding of daphnids without any further direct effects on growth, maturation, reproduction or mortality for concentrations ≤ 100 mg/L (Chapter 5); these individual-level processes determine population abundance and structure.

The present study investigated whether a short-term sub-lethal impact (feeding depression) can lead to alterations in population abundance and/or structure and whether this leads to changed sensitivity to a subsequent stress (acute toxicity). To test this, we exposed populations of *D. magna* to the feeding-inhibitor imidacloprid for one week, maintained the populations in pesticide-free medium for a further week and then exposed them to carbaryl for one day. The population experiment also compared the impacts of identical exposures to the two pesticides under low and high conspecific pressure.

## *6.2. Materials & Methods*

### *6.2.1. Test organisms and food*

Daphnids from our own culture were used and fed with the green alga *Desmodesmus subspicatus*. Detailed information on cultural maintenance of both species can be found in the supporting information (Appendix D). Briefly, *D. magna* Straus were cultivated quasi-statically at a group level in M4-media (Elendt, 1990) and *D.*

*subspicatus* maintained in the logarithmic growth phase for 2 weeks in Kuhl-medium (Kuhl and Lorenzen, 1964) was used as food source three times a week.

### *6.2.2. Test items*

The compounds were model substances that were selected based on their mode of action rather than their appearance and fate in the environment. It was demonstrated previously that imidacloprid only affects feeding of *D. magna* at concentrations between 0.15 and 100 mg/L, but that this caused secondary effects on several traits of individual performance (e.g. alterations in growth, maturation and reproduction) (Chapter 5). Carbaryl is known to be very toxic to daphnids and short-term exposure reduces survival in a manner that depends on body length (Takahashi and Hanazato, 2007). In line with the experimental hypothesis, there was no intention to match exposure concentrations, exposure durations or exposure sequences measured in the field.

### *6.2.3. Experimental design*

The population test was performed as a batch approach for 46d at 20±1°C and a photoperiod of 16h light and 8h darkness as proposed previously (Hammers-Wirtz, 2002). Populations were started with ten juveniles (five <3d; five <9d) and seven adults (three <16d; three <23d; one <30d) and kept in 900 ml M4-medium (Elendt, 1990). Each population was fed with 0.5 mg TOC (total organic carbon) on a daily basis irrespective of population abundance. Frequently (at least every fifth day, 16 sampling intervals in total) images of each population were taken using an incident light scanner and subsequently populations were transferred to fresh medium.

The experiment was set up with six treatments, each consisting of three replicates. The treatments were split into two groups, representing exposure to imidacloprid under either "spring" or "summer" conditions. These terms were chosen only based on food availability and for clarity of presentation of results; further environmental conditions (e.g. temperature, light intensity, photoperiod, and water quality) remained identical throughout the experiment. Food availability was high for organisms in the spring treatments because the abundance of individuals was rather low, resulting in population growth. Food was limiting for organisms in the summer treatments because the abundance of individuals was high, resulting in declining population abundance. The only difference between these two groups was the time when exposure occurred. Concentration, duration and the time between exposure to imidacloprid and carbaryl were identical but delayed by two weeks for the summer treatments. For the spring treatment, imidacloprid exposure was from day 2 to 9 and carbaryl exposure started on day 16, whereas for the summer treatment imidacloprid exposure was from day 16 to 23 and carbaryl exposure started on day 30. Imidacloprid exposure was for one week at nominal concentrations of 0.15 or 12.0 mg/L. These exposures inhibited feeding of juvenile daphnids by 3.7 and 97%, respectively (Chapter 5). These values for feeding depression were measured on individual daphnids as it was not technically possible to measure feeding rates directly within the population experiments.

One week after the exposure to imidacloprid ended, all treatments were exposed to carbaryl for one day using a nominal concentration of 0.02 mg/L; this is approximately the 50% effect concentration ($EC_{50}$) after two days of exposure to carbaryl for daphnids with a body length of 3 mm and represents twice the $EC_{50}$ for neonates (0.9 mm) (Takahashi and Hanazato, 2007). Detailed information on effect

concentrations after two days of exposure in dependence of body size and food availability has been given by Takahashi and Hanazato 2007. Control treatments had no exposure to imidacloprid, but were exposed to carbaryl (0.02 mg/L) to compare effects of this exposure in the absence of preceding feeding inhibition.

No formulations or solvents were used for either imidacloprid or carbaryl (PESTANAL® analytical standards: 99.0%; Sigma Aldrich). Fresh stock solutions were prepared on the day of usage by dissolving the compound in M4 media. Water samples for chemical analysis of imidacloprid were taken at the beginning and end of each exposure period. Samples were only taken at the beginning of exposure for carbaryl. Samples were frozen at -18°C until analysis (<3 months).

### *6.2.4. Chemical analysis*

Detailed information on chemical analysis and associated results can be found in the supporting information (Appendix D). In short, analysis was performed by injection of a 25-75 µL sample onto high-performance liquid chromatography (HPLC) equipped with a UV or a fluorescence detector. Reduction of interference by algal pigment was unnecessary for carbaryl analysis as samples were taken prior to feeding of populations. The same applied for imidacloprid analysis for samples taken at the beginning of exposure. Samples for the measurement of the imidacloprid concentration at the end of the exposure period were taken from the top third of the test vessel because *D. subspicatus* tends to sink to the bottom of the test vessel when cultivated as for this study.

Throughout the manuscript the nominal concentrations for imidacloprid and the measured concentration for carbaryl (0.0096±0.0008 mg/L) are used for presentation of results and comparison with the literature.

### *6.2.5. Observations and endpoints*

Scans of each population were made on each sampling day, on each day when the exposure regime changed, and at least every fifth day otherwise. Scans were generated by transferring the whole population to a Petri dish and subsequently fixing them by removing the media until a minimal movement of the organisms was observed. The Petri dish was then scanned using an incident light scanner (Canon, CanoScan 9000F) at a resolution of 1200 dpi. The images were used to count and measure the length of each individual. Body length of the daphnids was measured from the top of the eye to the base of the spine (posterior).

For evaluation, the daphnids were divided into four size classes differing in body length. Populations were classified into organisms <1 mm (neonate); 1-2 mm (juvenile); 2-3 mm (primiparae; organisms about to reproduce for the first time) and >3 mm (adult).

### *6.2.6. Statistical analysis*

The statistical analysis was conducted using SigmaPlot 12. A two-way ANOVA with a significance level of 0.05 was performed, keeping time as repeated and treatment as variable factor. Treatments were always tested against their surrogate control population. Additionally, one-way ANOVA's following the Holm-Sidak method were performed for comparison of the endpoints given in Table 6-1. Normal distribution

and equal variance were tested prior to ANOVA using the Shapiro-Wilk and Levene-Mediane tests, respectively.

## *6.3. Results and discussion*

### *6.3.1. Effects of weak feeding inhibition*

Exposure to imidacloprid at 0.15 mg/L inhibited feeding of daphnids by 3.7% within one day (Chapter 5). Such an inhibition did not cause any significant changes in the total population abundance (Figure 6-1a), abundance of different size classes (Figure 6-2a-d left) or population structure (Figure 6-3b left) when exposure occurred while populations were growing (spring exposure); no changes were observed either during exposure or for the week thereafter. In contrast, toxicant-induced inhibition of feeding by 3.7% resulted in significant changes in population abundance when exposure occurred to a declining population (summer exposure; Figure 6-1b). Those populations started growing following imidacloprid exposure whereas the control populations continued to decline, resulting in significantly increased population abundance within ten days of the start of exposure (Figure 6-1b; 0.15 mg/L). This would not normally be considered as an impact of pesticide exposure because the outcome is positive; however, the reversal in population growth dynamics caused by anthropogenic influences should not be disregarded without investigating the possibility that this may lead to additional stress on other traits. It has been argued that adherence to the laws of conservation of mass and energy means that increased performance for one trait will be accompanied by a decrease in performance in another (Jager *et al.*, 2012). Such a shift in use of available energy has been observed for individual daphnids exposed to the same concentration of imidacloprid (0.15 mg/L) for the same duration (seven days) (Chapter 5). There, the mechanism of

growth-discontinuation resulted in increased reproduction accompanied by reduced individual growth after feeding inhibition due to imidacloprid under food limitation (Chapter 5). This mechanism explains why an increase in population abundance was observed even though feeding was inhibited. Here, the increased population abundance is caused by an increase in the number of juveniles (Figure 6-2b right) resulting in a significant reduction in proportion of primiparae in the population structure after exposure to a feeding-inhibitor affecting feeding by 3.7% (Figure 6-3b right). An increase in neonate abundance was not observed because of the frequency of sampling and size classification used; any neonates produced left this size class before being assessed as new members of the population as their initial body length was close to that required to access the next size class (juveniles) of >1 mm (Figure 6-4a; right).

Acclimation of the reproductive effort of daphnids is known to be influenced by food availability (Boersma, 1997; Cleuvers *et al.*, 1997). Under high food availability daphnids follow the reproductive strategy of producing many but small neonates (Enserink *et al.*, 1993; Guisande and Gliwicz, 1992; Pieters and Liess, 2006b; Hammers-Wirtz, 2002) which are less fit (Pieters and Liess, 2006b), whereas under limiting food availability fewer but bigger neonates are produced (Guisande and Gliwicz, 1992). In the latter case, offspring are better able to overcome a phase of starvation as their lipid content is increased compared to neonates produced under food surplus (Tessier *et al.*, 1983). This ability of daphnids to acclimate via reproductive effort is believed to increase the survival of a population living in an environment with highly variable food supply (Pieters and Liess, 2006b). The increase in population abundance following imidacloprid exposure at 0.15 mg/L during population decline was attributable not to the energy taken up by the organism

(which decreased due to feeding inhibition), but rather to the food concentration in the environment (which increased as less was eaten) as previously proposed by Glazier (1998). A decrease in reproductive outcome and thus population abundance would be expected if it would have been the energy taken up that determined the reproductive strategy; this is because a shift towards the production of less but bigger offspring would be expected when less food is taken up. The reallocation of energy towards reproduction causing growth-discontinuation (Chapter 5) would exacerbate the effect.

The observed changes in abundance and structure of the population due to a one-week feeding inhibition of 3% (0.15 mg/L) were not long lasting and did not alter the sensitivity of the population to subsequent carbaryl exposure (Table 6-1).

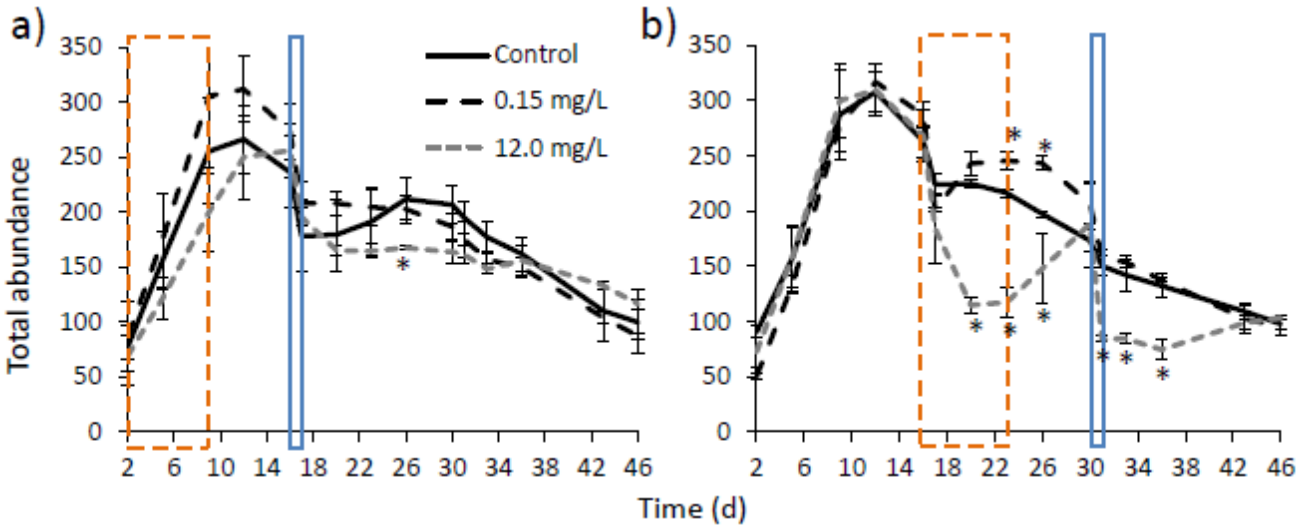


*Figure 6-1: Total average population abundance of* Daphnia magna *(n=3; ±SD) when exposed to either 0.15 or 12.0 mg/L imidacloprid, then subsequently to carbaryl (0.0098 mg/L). Exposure to imidacloprid commenced either a) at the beginning of the population development (spring), or b) delayed by two weeks (summer). Dashed boxes indicate the presence of imidacloprid. Boxes indicate the presence of carbaryl.* Significant differences to the control population (spring or summer) ($p<0.05$; two-way ANOVA; Holm-Sidak method).*

*Table 6-1: Decrease in Daphnia magna population abundance (% of abundance prior to exposure) due to a one-day carbaryl exposure (0.0096 mg/L). Populations were previously exposed to imidacloprid for one week.*

| **Period of imidacloprid exposure** | **Size class** | **Imidacloprid concentration [mg/L]** | | |
|---|---|---|---|---|
| | | **Control** | **0.15** | **12.0** |
| Spring | Neonate & juvenile | 28.8 ± 10.1[a] | 27.7 ± 6.0[a] | 30.6 ± 4.7[a] |
| | Primipara & adult | 12.5 ± 5.7 | 10.9 ± 6.2 | 2.6 ± 14.7 |
| | Total | 25.0 ± 6.8[a] | 23.9 ± 3.6 [a] | 23.3 ± 5.8[a] |
| Summer | Neonate & juvenile | 26.6 ± 8.8 | 27.5 ± 7.5 | 80.8 ± 6.4[b] |
| | Primipara & adult | -14.0 ± 14.5 | 13.0 ± 26.8 | 16.6 ± 13.4 |
| | Total | 13.3 ± 6.7 | 23.1 ± 12.3 | 53.1 ± 13.8[b] |

***a*** *= significant different to the natural population decline (summer treatment) (p>0.05; One-way ANOVA; Holm-Sidak method);* ***b*** *= significant difference to the surrogate control treatment (p<0.05; One-way ANOVA; Holm-Sidak method)*

### *6.3.2. Effects of strong feeding inhibition*

Strong feeding inhibition caused by the higher concentration of imidacloprid provoked a different effect to that when feeding was inhibited by 3%. Exposure at 12.0 mg/L inhibited feeding by 97% (Chapter 5). Here, this was associated with an overall reduction in population abundance (Figure 6-1) and small changes in population structure (Figure 6-3c), both within the exposure period.

Inhibition of feeding by 97% for one week caused mortality of individuals within the population resulting in either reduced population growth or significant population decline depending on the stage of growth that populations were in when exposure occurred (Figure 6-1). The same concentration of imidacloprid was found to have no effect on survival within 7 days when tested at an individual level, even when those individuals were additionally stressed by low food availability (Chapter 5). There it

was shown that mortality due to exposure to imidacloprid at concentrations up to 100 mg/L resulted from starvation and only occurred when the sum of food taken up and existing energy reserves was insufficient for organism viability. It follows that individuals in our populations must have a lower energy reserve than individuals held separately at low food density. In turn, results undermine the direct extrapolation of toxicity data generated at an individual level to assess impacts on populations.

#### *6.3.2.1. Effects of strong feeding inhibition on growing populations*

Mortality during exposure of growing populations to imidacloprid at 12.0 mg/L can only be seen indirectly as reduced population growth (Figure 6-1a) because the rate of mortality was exceeded by the rate of population growth. Reduced total abundance is caused by a decrease in number of primiparae within the population (Figure 6-2a-d left), resulting in a shift of the population structure towards smaller individuals (Figure 6-3c left). Primiparae are likely the most sensitive organisms for starvation due to feeding inhibition because their energy investment within the same time is greater than that of younger and older organisms. Neonates and juveniles expend energy in growing towards the minimum body length for maturation, whilst adults expend energy mainly for reproduction. In contrast, primiparae have to maintain both growth and reproduction and thus their energy reserve are less likely to be sufficient to overcome a period of starvation. This theory has been partly proven in an additional experiment which is described in detail in the supporting information. There, three groups of daphnids in differing age classes, maintained for 2, 8 and 11 days under surplus food availability were subsequently starved for 5 days. Within

those 5 days of complete starvation (no food supply) 0±0, 40±28 and 50±20% of organisms from the 2-, 8- and 11-day old groups died, respectively.

After exposure, the number of neonates continued to increase relative to the control (Figure 6-3a left) and the number of primiparae increased steadily such that the effect during exposure was reversed with significantly more primiparae (Figure 6-3c left). At the same time, the number of juveniles did not increase as in the control, resulting in a shift of the population structure towards fewer juveniles but more neonates (Figure 6-3c). Not only was the abundance of neonates significantly greater following exposure to imidacloprid, but the body length of the offspring when released from the brood pouch was also significantly smaller than that of the control ($p<0.05$; Figure 6-4a left) meaning that offspring in exposed populations spent more time as neonates. Close inspection of the data shows that the minimal body size of the control increased whereas that in populations exposed to imidacloprid remained more or less stable (Figure 6-4a left). This difference occurred first at the last day of exposure and was driven by different reproductive strategies accompanied by reduced individual growth during exposure as shown previously at the individual level (Chapter 5). For the control populations, the reproductive strategy shifted from producing many small offspring to producing fewer but bigger offspring because the food available per organism in the environment reduced continuously due to population growth. Such a shift in reproductive strategy was unnecessary for populations exposed to imidacloprid because mortality resulted in a net increase in food available per organism amplified by the reduced growth of each individual (smaller organisms eat less than bigger ones). As food availability was relatively high, there was no growth-discontinuation for this population and changes in population structure were rather small (shifts were confined to the group of immature

daphnids). As a consequence, feeding inhibition within the phase of population growth did not alter the sensitivity of that population to carbaryl exposure one week later (Table 6-1). However, carbaryl exposure caused a further change in population structure (Figure 6-3c), with significantly fewer juveniles and more primiparae for two weeks thereafter.

#### *6.3.2.2. Effects of strong feeding inhibition on declining populations*

Feeding inhibition of 97% induced by imidacloprid exposure to a declining population altered the population in terms of both structure and abundance to a greater extent than when the exposure occurred during population growth (Figure 6-2 and Figure 6-3). The growing and declining populations had different availability of food and it could be argued that extent of feeding inhibition might have varied accordingly for the two populations. This hypothesis is contradicted by measurements at an individual level that showed the same inhibition of feeding by imidacloprid under differing food intensities (Chapter 5).

There was an accelerated reduction in population abundance (Figure 6-1b) for the first four days after the start of exposure to imidacloprid, resulting in a reduction by 56±7% when compared to the initial abundance prior to exposure. Mortality during exposure occurred in all size classes, resulting in no alteration in population structure (Figure 6-3c; right). Mortality reduced the population abundance such that increased food availability and space shifted the reproductive strategy towards producing more (Figure 6-2a) but smaller (Figure 6-4a) neonates. These neonates were even smaller than those produced by populations exposed to imidacloprid within the phase of population growth (Figure 6-4a).

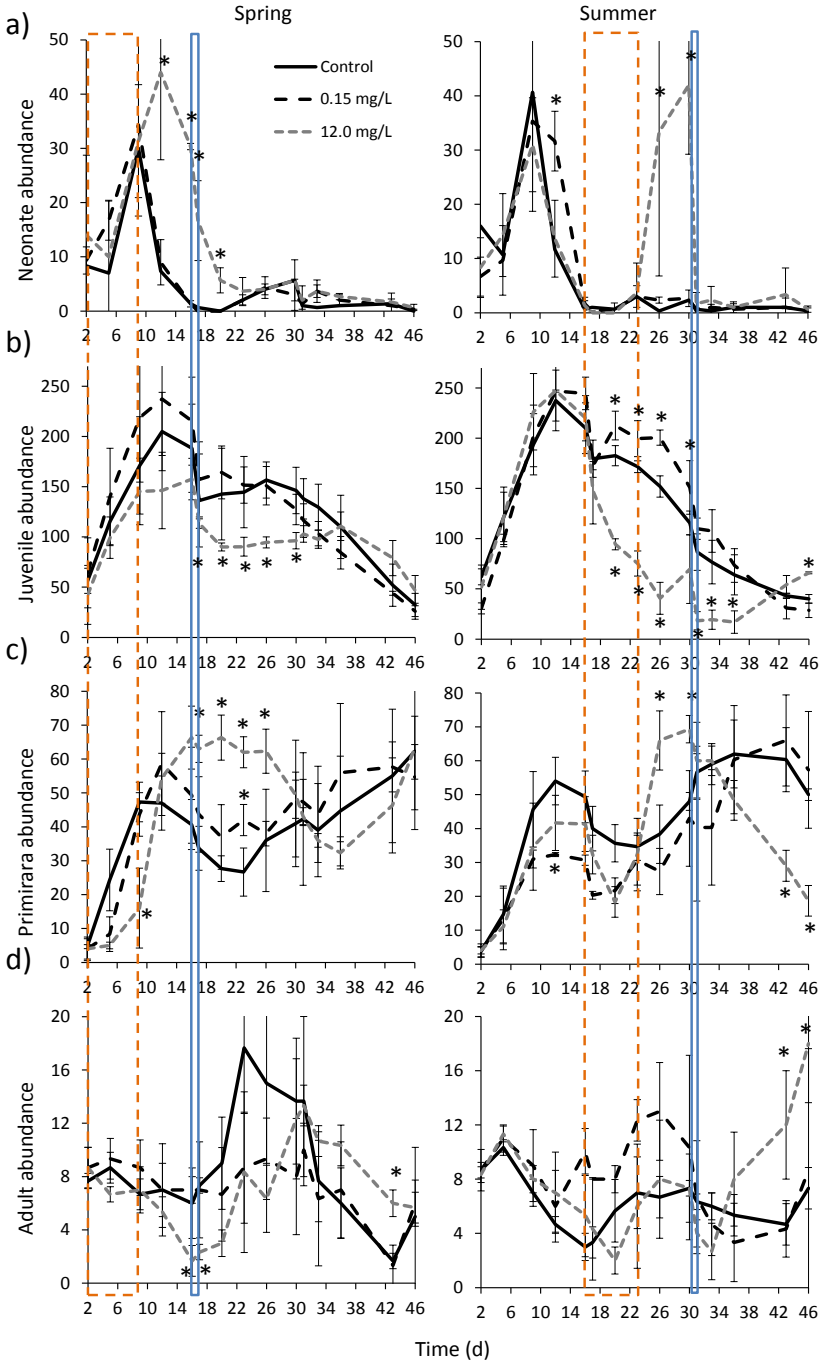


*Figure 6-2: Average population abundance of* Daphnia magna *(n=3; ±SD) when exposed to either 0.15 or 12.0 mg/L imidacloprid, then subsequently to carbaryl (0.0098 mg/L). Exposure to imidacloprid commenced either at the beginning of the population development (spring) or delayed by two weeks (summer). Organisms were grouped according to their body length: a) neonates, b) juveniles, c); primiparae, and d) adults. Dashed boxes indicate the presence of imidacloprid. Boxes indicate the presence of carbaryl.* Significant differences to the control population (spring or summer) (p<0.05; two-way ANOVA; Holm-Sidak method).*

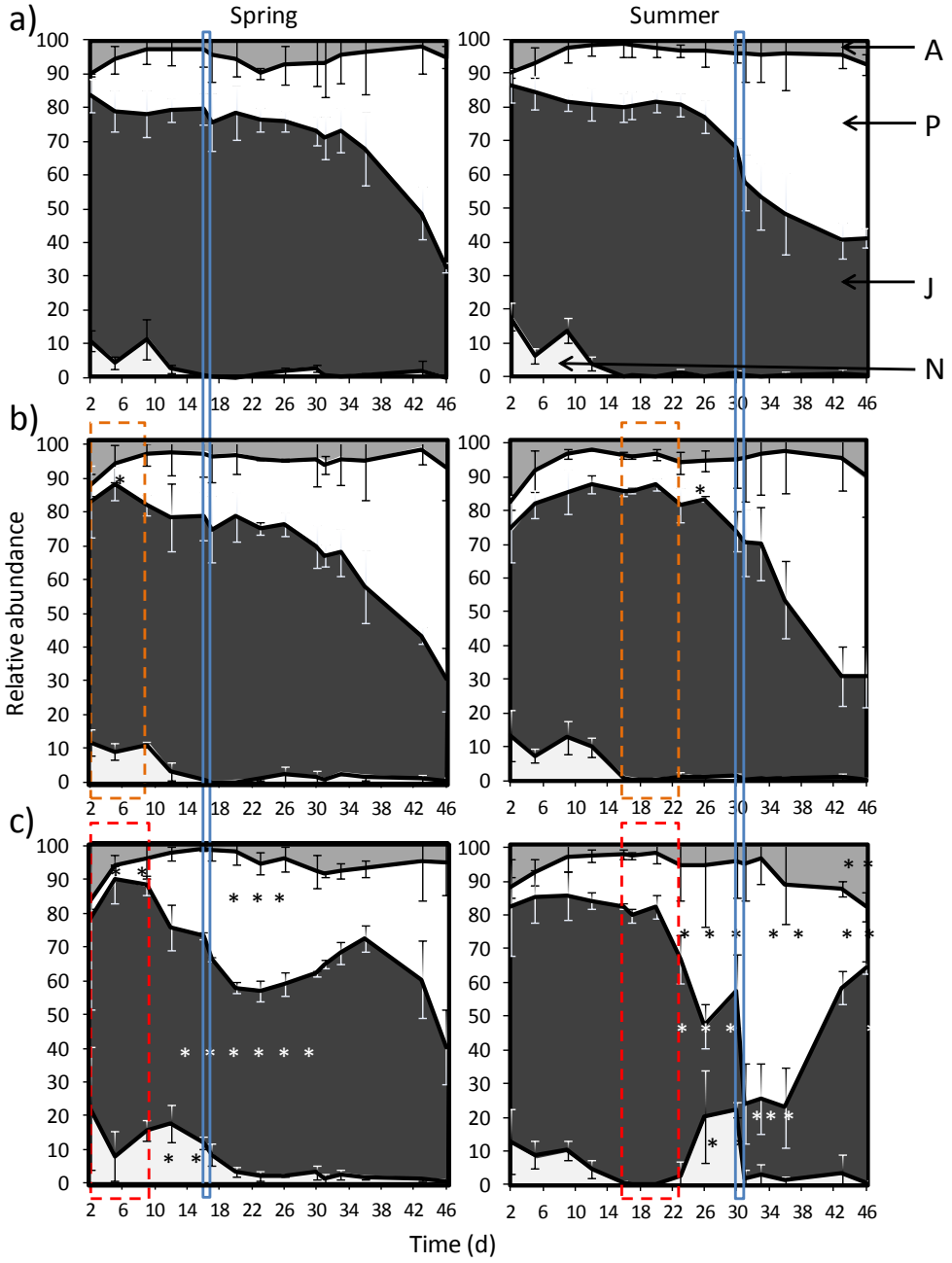


*Figure 6-3: Average population structure of* Daphnia magna *(n=3; ±SD) when exposed to a) control, b) 0.15 mg/L imidacloprid, or c) 12.0 mg/L imidacloprid, and then subsequently to carbaryl (0.0098 mg/L). Exposure to imidacloprid commenced either at the beginning of the population development (spring) or delayed by two weeks (summer). Organisms were grouped according to their body length:* ***A*** *= adults;* ***P*** *= primiparae;* ***J*** *= juvenile;* ***N*** *= neonate. Dashed boxes indicate the presence of imidacloprid. Boxes indicate the presence of carbaryl.* Significant differences to the control population (spring or summer) (p<0.05; two-way ANOVA; Holm-Sidak method).*

This indicates additivity of reduction in offspring size due to body length of the mother and food availability. Whilst both influences generate smaller offspring, the literature suggests that offspring produced under influence of both factors will be less fit than when only the size of the mother is the determining factor (Hammers-Wirtz and Ratte, 2000). This is confirmed in the current experiments when considering the effect of subsequent exposure to carbaryl. Populations pre-exposed to imidacloprid during their growth phase suffered 23±6% mortality when exposed to carbaryl for one day at 0.0096 mg/L. In contrast, populations where imidacloprid exposure occurred during the decline phase had 53±14% mortality in response to carbaryl exposure (Table 6-1); this mortality was primarily associated with neonates and juveniles (81±6% loss of abundance).

Our results show that differences in reproductive strategy and consequences for neonate fitness changed the sensitivity of neonates to carbaryl (Figure 6-2a and Table 6-1). Previous work has demonstrated that the effect of a one-day exposure to carbaryl on *Daphnia* varies with the stage of population development (Hanazato and Hirokawa, 2004; Takahashi and Hanazato, 2007); differences in effects were correlated with food availability within the stages of population development and the differing population structure within these phases (Takahashi and Hanazato, 2007). It has also been demonstrated that the effect of carbaryl on *D. magna* is size-dependent (Pieters *et al.*, 2005) with the lethal concentration for 50 % of the test organisms ($EC_{50}$) decreasing with reduced body length. The slope of this relationship was shown to be influenced by food availability, but only for organisms that had reached the minimal body length required for reproduction. Reducing food availability increased the sensitivity of larger individuals to carbaryl, but had no effect on the sensitivity of neonates (Takahashi and Hanazato, 2007). Our results only partially

agree with those from Takahashi and Hanazato (2007) and they add important additional knowledge. Our results confirm the finding that growing populations are more sensitive to carbaryl than declining ones. Relative to the control, carbaryl exposure killed more individuals within the population previously exposed to imidacloprid during the phase of population decline. Imidacloprid exposure markedly reduced population abundance and transferred the populations into a subsequent phase of growth. The enhanced sensitivity to carbaryl occurred even though population abundance was not significantly different from the control ($p>0.05$) immediately before carbaryl exposure. The new finding here is that we also found that all size classes were sensitive to carbaryl when the population was growing (Figure 6-2 right) as there was a reduction in abundance of all size classes; this eliminates the possibility that the reduction in one size class was an artefact whereby apparent mortality arose because individuals change size classes between observations due to growth. This contrasts with Takahashi and Hanazato (2007) who only observed increased mortality for adult organisms; the deviation might be caused by differences in the separation into size classes or different power of experiments to detect change in mortality.

It could be argued that the increased mortality after carbaryl exposure in populations exposed to imidacloprid during population decline is not a result of changed reproductive strategy but rather results from direct interaction of the compounds. If this was the case, we would have expected to see the interaction independent of when the exposure started as the pattern of exposure was identical. However, only the treatments where imidacloprid exposure started during the phase of population decline showed increased mortality due to carbaryl.

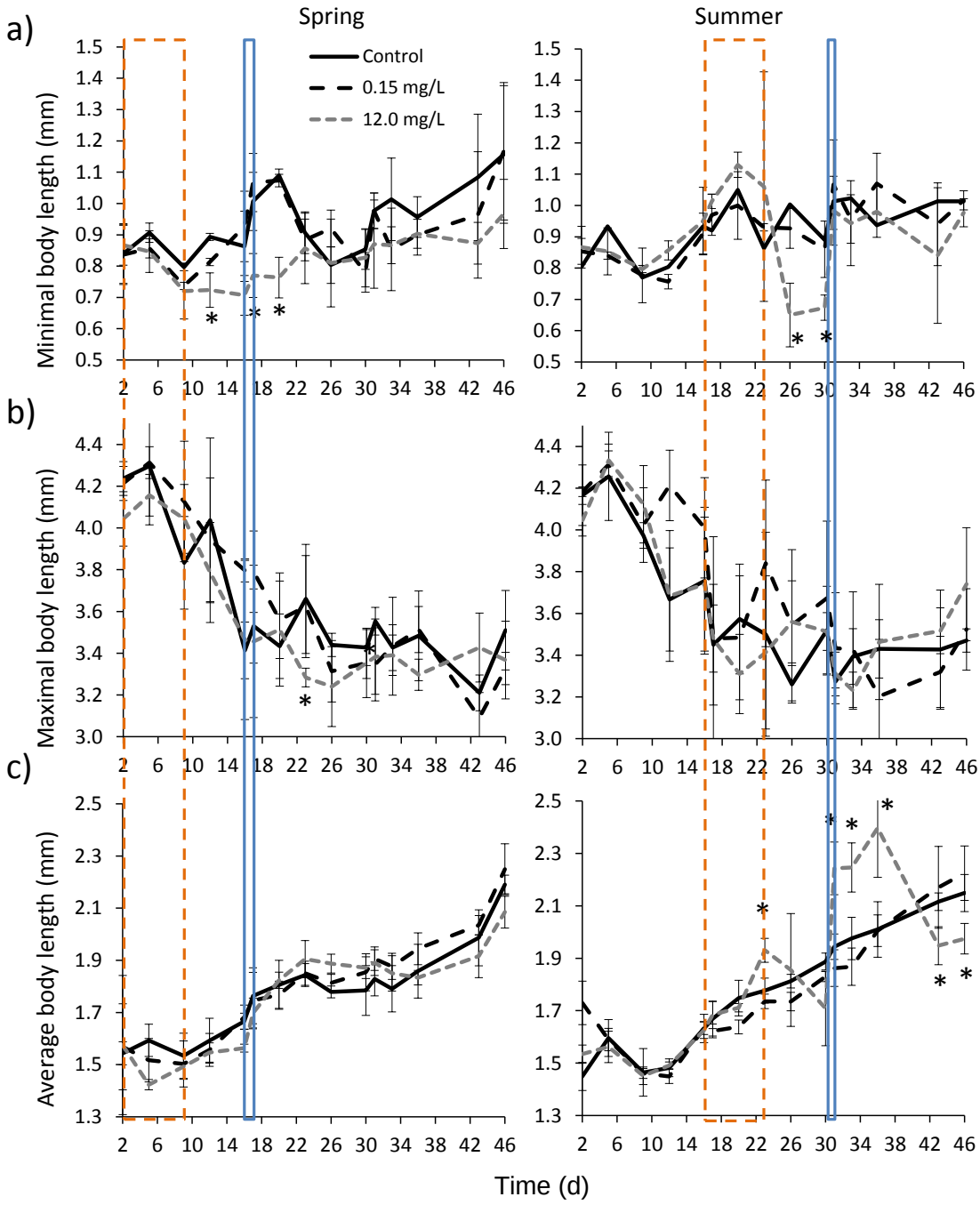


*Figure 6-4: Average body length of Daphnia magna individuals within a population (n=3; ±SD) when exposed to either 0.15 or 12.0 mg/L imidacloprid, then subsequently to carbaryl (0.0098 mg/L). Exposure to imidacloprid commenced either at the beginning of the population development (spring) or delayed by two weeks (summer). a) Minimum body length, b) maximum body length, and c) average body length. Dashed boxes indicate the presence of imidacloprid. Boxes indicate the presence of carbaryl.* Significant differences to the control population (spring or summer) (p<0.05; two-way ANOVA; Holm-Sidak method).*

As discussed there are several questions arising from the observed effect pattern accompanied with acute toxicity of carbaryl to *D. magna* populations. Several experiments were conducted to identify the effects on individuals and clarify whether food availability plays a role in its toxicity pattern to organisms of differing age. The description and discussion of these experiments can be found in the appendix of this chapter (Appendix E).

### *6.3.3. General discussion*

The study confirms that short-term exposure of populations with a feeding inhibitor alters the population structure, and that this effect varies depending on the natural stress occurring from conspecifics. We found that feeding inhibition can lead to mortality within populations even when mortality is not predicted from tests at the individual level. This suggests that sub-lethal effects measured at the individual level might not extrapolate directly to predict effects on populations, but further research is required before the finding can be generalised. The nature of acclimation of daphnids to their environment and the possible absence of a signal for toxicant-induced feeding inhibition might be factors preventing direct extrapolation. It might be expected that changes in individual performance would be identical whether they were a response to reduced food availability (e.g. lower concentration of food in the environment) or toxicant-induced feeding depression. However, there is an important difference that may mean this is not the case. Natural acclimation and adaptation to changes in food availability (Boersma, 1997; Cleuvers *et al.*, 1997; Enserink *et al.*, 1995; Glazier, 1998; Gliwicz and Guisande, 1992) or to presence of conspecifics (Dodson and Havel, 1988; Goser and Ratte, 1994; Luning, 1992; Machacek, 1993) is believed to be driven by external information arising from natural chemicals; the so

called info-chemicals are rarely studied and mostly unknown (Machacek, 1993). In contrast, toxicant-induced feeding inhibition is unlikely to be signalled in the same way as this is a process initiated inside the organism. This would mean that the need to acclimate in terms of growth and reproduction might remain undetected, leading to hidden imbalance in the energy budget, loss of normal individual performance and eventually death through starvation. Hence, for measurements made at the individual and population level, results for mortality due to starvation differ under identical feeding inhibition because the external information and the resulting reproductive strategy differs.

The study also shows that short-term feeding inhibition can cause a population to be more sensitive to a subsequent stress. However, the hypothesis that the change in population sensitivity is solely attributable to size-dependent mortality is not supported. Here, the mortality resulting from feeding inhibition transfers the populations into the phase of population growth; this increases population vulnerability to following stress due to a shift in the reproductive strategy. The strategy deployed involves a high reproduction rate accompanied with low body length of the offspring and is likely to match that of individuals studied in tests at the individual level. Thus direct lethal effects measured at the individual level are more likely to be useful to predict effects for such a population.

Nevertheless, feeding inhibition did alter the population structure towards smaller individuals so still has the potential to increase population vulnerability as hypothesised because smaller individuals are more sensitive than bigger ones. Such an interaction between a shift of population structure towards smaller individuals and increased population sensitivity to a size-dependent acute toxin has been found for *D.*

*magna* previously (Agatz *et al.*, 2012). Results from the current study are inconclusive on the relative contribution of this effect to changed population sensitivity. Studies with a wider range of feeding inhibition (>3% and <97%) will be required to quantify this influence.

The failure to predict mortality due to feeding inhibition shows that methods are needed to extrapolate standard ecotoxicological tests not only to conditions of different food availability (as shown in the study at the individual level (Chapter 5)), but also to the population level. It is known that food is a limiting factor for population density (Antunes *et al.*, 2004) and that changes in food levels change the reproductive strategy of organisms (Enserink *et al.*, 1993; Guisande and Gliwicz, 1992; Pieters and Liess, 2006b; Hammers-Wirtz, 2002). Furthermore, the sensitivity to toxicants is usually increased with decreasing food density (Heugens *et al.*, 2001). Food supply is chosen to be unlimited in the standard tests for daphnids, but effects of xenobiotics can vary dramatically depending on food supply under both constant (Heugens *et al.*, 2001; Peeters *et al.*, 2010) and periodic exposure (present study). Approaches are needed to extrapolate test results to conditions where food supply may be limited or even change over time. Possibilities might be the use of the individual *Daphnia magna* population model "IDamP" (Preuss *et al.*, 2009), the use of a model based on the dynamic energy budget theory (Kooijman, 2010) or a combination of both such as the DEB-IBM network (Martin *et al.*, 2012). At present, none of these modelling frameworks allow for the reallocation of energy from growth to reproduction after feeding inhibition that was reported in Chapter 5. Hence, model development is required before application to this or similar datasets. Such development should be considered a priority given that results indicate a failure to predict mortality in populations from study of the impacts of feeding inhibition at

the individual level. The differences in population sensitivity to a further short-term stress occurring one week after feeding inhibition adds a further challenge to understanding the potential impacts of multiple stressors in the field. Again, this points to the need to develop models able to incorporate such effects.

# 7. General discussion

This PhD explicitly focused on contributing to four research needs stressed by several scientific committees of the European Commission (European Union, 2013; Vighi, 2012) to improve the environmental risk assessment (ERA) of plant protection products (PPPs). Studies considered the aquatic invertebrates *Daphnia magna* and *Gammarus pulex* as model species and imidacloprid and carbaryl as model substances. The research needs addressed are given below and were described in detail in Chapter 1:

i) assessing the effects of highly time-variable exposure
ii) increasing the ecological realism of effect assessment approaches
iii) assessing the interactions between combined stressors and environmental factors; and
iv) improving ecological modelling.

This PhD focused on the observation of potential impacts of PPPs on feeding of aquatic invertebrates under more realistic exposure scenarios and the transposition of such impacts to further behavioural traits of individuals and to the population level. Feeding was chosen as the main endpoint of interest because previous research shows that feeding inhibition is a crucial endpoint to be considered for ERA. Alteration in feeding can directly and indirectly disturb ecosystem function via effects onto the population level and ecosystem processes. Energy availability depends on feeding (Sancho *et al.*, 2009; Villarroel *et al.*, 2009) and the energy budget can be considered an indicator of the overall condition of an organism (Calow and Sibly, 1990). Furthermore, other behavioural changes such as changes in growth

and reproduction are often instigated by an effect on feeding (Guisande and Gliwicz, 1992; Porter *et al.*, 1982; Preuss *et al.*, 2009).

Prior to this PhD, feeding assays for the shredder *G. pulex* had not been set up to allow the consideration of environmentally-realistic exposure patterns, nor to allow investigations of what happens after exposure. Both of these factors are important to consider for the extrapolation of effects to the population and ecosystem level in a more environmentally-relevant manner. Here, influences on feeding were measured at a finer temporal resolution than usually undertaken (Chapter 3 and 4, Appendix A) and feeding behaviour after exposure was investigated. Natural influences on feeding have been studied extensively and reported in the literature (Chapter 2). Thus, the current work contributes to method development for effect assessment. Additionally, all experiments were carried out at the individual level to generate data useful for the development and/or evaluation of individual based ecological and ecotoxicological models. It was observed that feeding of *G. pulex* is disturbed at environmentally-realistic exposure concentrations of imidacloprid and carbaryl (Chapter 3 and Appendix A). Hence, this work gives two examples of potential impacts of PPPs known to be present in the environment. However, the results only show a potential for impacts in the field in terms of concentrations. Whether impacts really do occur in the environment can only be clarified when the feeding assay is improved. *G. pulex* lives in flowing waters where exposure can change rather quickly (Kreuger, 1998; Leu *et al.*, 2004; Reinert *et al.*, 2002; Wittmer *et al.*, 2010). Thus, the exposure duration is a vital criterion for environmentally-realistic effect measurement. It was demonstrated that method improvement should at least involve seeking a more appropriate food source to be used (Chapter 2 and 4).

A further research need is to investigate whether short-term feeding inhibition of individual gammarids provokes influences at the protection level for ERA of PPPs stated in the legislation of the European Union (2009). The question of whether or not short-term feeding depression of gammarids is a matter of concern at the population and ecosystem level could be investigated using individual based population modelling. Ecological models are developed to address such questions (see section 3.3.6. for details) but need to be improved to better match reality. Chapter 3 shows that not only recovery but also compensational feeding occurs which might accelerate leaf litter breakdown in the field. Individual-based, time-resolved feeding rate measurements following different starvation intervals are needed to implement such behaviour into the model. Chapters 2, 3 and 4 show the need to improving the measurement of feeding of individuals in order to generate such data.

The extrapolation of effects to the population level seems easier for daphnids. Several tools to support this are more developed and manageable within a reasonable time scale. The experimental investigation of the transposition of effects on feeding to individual traits like reproduction is possible. Even the empirical investigation of exposures to populations is manageable within a reasonable time scale. Chapter 5 provided novel information on effects of imidacloprid on *D. magna* along the effect-cascade of feeding, growing, maturing, reproducing and surviving. It was identified that feeding is the only endpoint measured which is directly affected by the compound with all other effects being secondary. This work demonstrates how multiple lines of evidence linked by understanding the ecology of the organism are necessary to elucidate xenobiotic impacts along the effect-cascade. Consideration of endpoints individually would have led to a different interpretation of the single

effects observed. This finding poses the question whether several effects of xenobiotics on growth and reproduction reported in the literature may also be secondary effects caused by feeding inhibition rather than a direct effect on growth and/or reproduction.

Chapter 5 also reveals that even for *D. magna*, the most common test organism for aquatic ecotoxicology, the behaviour of this organism is not fully understood. A newly identified mechanism of adaptation of daphnids to stress caused by food limitation has been identified (growth-discontinuation). It is known that food is a limiting factor for population density (Antunes *et al.*, 2004) and changes in food levels may change the energy allocation of organisms (Peeters *et al.*, 2010). Additionally, sensitivity to toxicants is usually greater with decreasing food density (Heugens *et al.*, 2001). Food supply is chosen to be non-limiting in the standard tests for daphnids, but effects of xenobiotics can vary dramatically depending on food supply under both constant (Heugens *et al.*, 2001; Peeters *et al.*, 2010) and periodic exposure (Chapter 5). Approaches are needed to extrapolate test results from standard toxicity studies, where food availability is non-limiting, to conditions where food supply may be limited or fluctuates. Models to do so already exist (Martin *et al.*, 2012; Preuss *et al.*, 2009) and they seem to be able to predict empirical population dynamics with and without anthropogenic stress under differing food conditions (Gabsi *et al.*, 2013; Martin *et al.*, 2013a; Martin *et al.*, 2013b; Preuss *et al.*, 2010). However, currently-available models do not allow the consideration of the reallocation of energy reserves towards reproduction rather than growth of daphnids (growth-discontinuation) that was described in Chapter 5. This is a source of uncertainty in the model outcome, especially for modelling time-variable combinations of stressors.

In Chapter 6 an impact of short-term feeding depression on whole populations was observed. There the potential interplay of anthropogenic (multiple pesticide exposure) and natural stress (intra-specific interaction) for populations was also investigated. It has been observed frequently that toxicity changes with intra- and inter-specific competition (Knillmann *et al.*, 2012; Stampfli *et al.*, 2011) and recent evidence shows that these effects intensify over time when multiple exposures occur (Liess *et al.*, 2013). Here, it was postulated that changes in population structure due to one stressor will alter sensitivity to a subsequent xenobiotic due to size-dependent toxicity (Chapter 6). The postulated change in sensitivity to a subsequent xenobiotic was shown to occur, but it was also shown that this may arise, at least in part, from a change in reproductive strategy and consequent change in the energy budget of the organisms. Hence, it is shown that sensitivity to subsequent stress can occur even when there is little or no change in population structure after the initial stress. Thus, novel information is presented on combined effects of short-term exposure of two pesticides applied consecutively to *D. magna* populations. This work shows that direct extrapolation of toxicity data from individuals to populations is not possible because indirect effects at the population level change the intensity and duration of effects.

A further research need is to investigate whether one or all available individual based population models for *D. magna* are able to predict empirical observations presented in Chapter 6. This data set is suitable not only to evaluate available models (completely independent data set) but is also useful to compare models with each other. Having measured each individual in the population at each sampling point allows the comparison of measurements and model outcome in a very detailed manner without having to change the output of the model. Evaluation of one or more

models would also enable to identify whether the new mechanism of growth-discontinuation (Chapter 5) might need to be considered for population modelling. Chapter 6 indicates that this mechanism did occur within the experiment at the population level and caused a structural alteration of the population.

Furthermore, the data set on *Daphnia* populations (Chapter 6) shows that feeding inhibition can lead to mortality within populations even when mortality is not predicted from tests at the individual level. This suggests that sub-lethal effects measured at the individual level might not extrapolate directly to predict effects on populations. Recently, Newman *et al.* (2013) showed that indirect mortality through toxicant-induced starvation can add substantially to the direct mortality of the toxicant. However, further research is required before the finding can be generalised. Both, the presented data and the study from Newman *et al.* (2013) used the same compound (imidacloprid). The nature of acclimation of daphnids to their environment and the possible absence of a signal for toxicant-induced feeding inhibition might be factors preventing direct extrapolation. It might be expected that changes in individual performance would be identical whether they were a response to reduced food availability or toxicant-induced feeding depression. However, there might be an important difference that is explained in detail in Chapter 6. The difference might cause hidden imbalance in the energy budget which depends on conspecific pressure. Hence, for measurements made at the individual and population level, results for mortality due to starvation differ under identical feeding inhibition because the external information and the resulting reproductive strategy differs. It would be useful to investigate whether these findings can be generalised because a broad range of pesticides are known to affect feeding behaviour of non-target species. The following pesticides or pesticide metabolites have been shown to affect

feeding behaviour: imidacloprid and carbaryl (presented work), fenvalerate (Day and Kaushik, 1987; Reynaldi *et al.*, 2006), endosulfan (Fernandez-Casalderrey *et al.*, 1994), diazinon (Fernandez-Casalderrey *et al.*, 1994), pentachlorophenol (Juchelka and Snell, 1995), clorpyrifos (Juchelka and Snell, 1995), naphthol (Juchelka and Snell, 1995), tebuconazole (Sancho *et al.*, 2009), molinate (Sancho *et al.*, 2003), carbendazim (Slijkerman *et al.*, 2004) and propanil (Villarroel *et al.*, 2003). Application of this research may not be restricted to pesticides. Human and veterinary pharmaceuticals, which are present in the environment, share the characteristic of being designed to have a specific mechanism of action (e.g. acetylcholine antagonism and acetylcholinesterase inhibition).

Overall this PhD was conducted to assess the feasibility of long-term feeding assays with aquatic invertebrates to investigate the impact of time-variable exposure to pesticides on feeding behaviour, to assess the consequences of short-term feeding inhibition for further individual traits and populations considering single and multiple stress for the test organisms. Method improvement allowed testing of some important hypotheses aimed at better understanding sub-lethal effects (e.g. feeding inhibition causes secondary effects on individual behaviour) and the relative importance of such impacts at the level of interest for environmental risk assessment (e.g. feeding inhibition changes sensitivity of populations to following stress). This PhD identified some rather subtle behaviours that were previously unknown (e.g. growth-discontinuation), improved the knowledge on some key questions and opened some additional uncertainties for environmental risk assessment.

# Appendix A: The influence of carbaryl exposure on the feeding rate of *Gammarus pulex*

Chapter 3 showed that imidacloprid affects feeding of gammarids at environmentally-relevant concentrations. The present study aimed to investigate whether another pesticide, which also acts at the nervous system of aquatic invertebrates, affects feeding of *G. pulex* in the same way as observed for imidacloprid. An identical experiment as performed in Chapter 3 was conducted using environmentally-relevant concentrations of carbaryl. This experiment was conducted prior to the analysis of the experiment with imidacloprid; this means that the suggested improvements of the experimental method could not be considered.

*Material and methods*

Food sources used, experimental design, measurement of feeding inhibition and statistical analysis were the same as those of the experiment with imidacloprid (Chapter 3). The influence of carbaryl on the individual feeding rate of *G. pulex* was measured at concentrations one order of magnitude lower than those of imidacloprid to correct for differences between lethal concentrations of the two compounds. Lethal concentrations for 50% of the test organisms after three days of exposure to carbaryl were reported to be 25 µg/L (Bluzat and Seuge, 1979) and 44 µg/L (Ashauer *et al.*, 2007). Concentrations tested were 0.081, 0.27, 0.9, 3.0 and 10 µg/L.

Analysis of carbaryl was by injection of 75 µL sample onto Liquid chromatography-mass spectrometry (LC-MS, Agilent 1100 Series, Agilent Technologies, UK Ltd.)

equipped with a fluorescence detector (Ext. = 274; Em. = 335) and a Discovery® $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco) maintained at 25°C. The mobile phase was methanol/water (70:30; v/v) with a flow rate of 0.8 $mL_{*}min^{-1}$. The lowest concentration tested (0.081 µg/L) was higher than the limit of detection (0.05 µg/L) via LC-MS.

*Results and discussion*

The pH, temperature and oxygen content were not significantly different from those measured in the experiment with imidacloprid and thus also fulfilled the requirements for *G. pulex* at all times as described in section 3.4.1.

The maximal difference between measured and nominal concentrations of carbaryl was 18%. A decrease in carbaryl concentrations by 10±13% was detected during both exposure pulses. For experimental analysis, carbaryl was assumed to be constantly present during the exposure phase at the nominal concentrations tested.

Within 24 hours of exposure, one organism died in the lowest concentration of 0.081 µg/L, in the middle of the experiment one organism of the control treatment died and on the last day of the experiment one organism died in the highest concentration of 10 µg/L. Thus a concentration dependent mortality was not observed. In contrast to the experiment with imidacloprid, here no evidence for mortality due to starvation was found. However, the results indicate that similar mortality could occur at higher concentrations. Figure A-1 shows that carbaryl seems to reduce feeding in a concentration-dependent manner and that organisms are capable of recovering from

this, just as was found for imidacloprid. The highest concentration tested reduced the overall feeding rate during exposure by 60±16%; thus higher concentrations should have been tested for a complete dose-response curve and to determine whether recovery at higher concentrations follows the same pattern as was observed for imidacloprid.

Due to the high variability in the data, 10 and 50% effect concentrations ($EC_{10}$, $EC_{50}$) and their 95% confidence limits could only be obtained after 24h of exposure. The 10 and 50% effect concentrations after 24h of exposure were 0.66 (0.08-1.40) and 7.24 (4.45-17.44) µg/L, respectively. The $EC_{10}$ is within the range of concentrations measured in a stream in Spain (Table A - *1*).

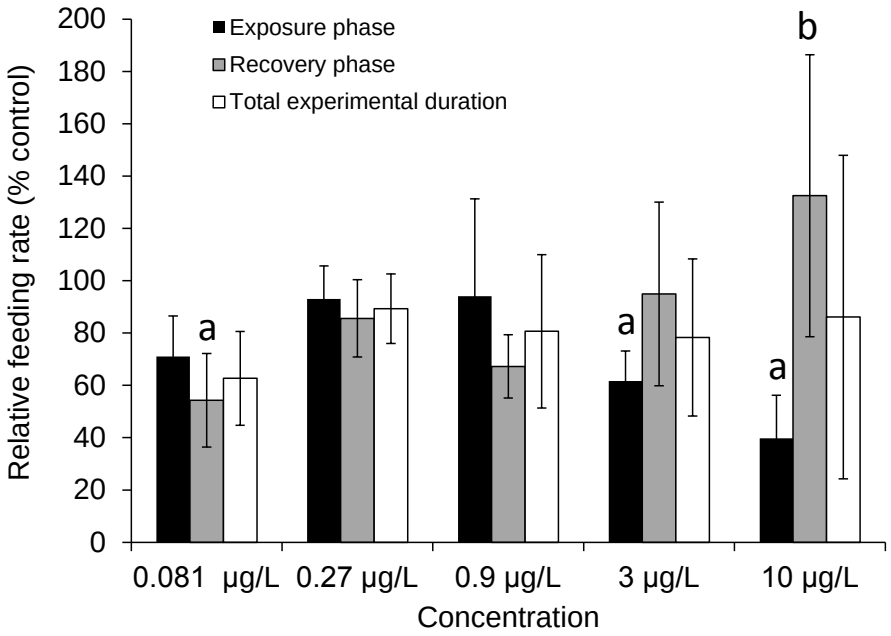


*Figure A-1: Relative individual feeding rate of* Gammarus pulex *as a function of nominal carbaryl concentrations. Average ± standard deviation calculated for different phases of the experiment. a = significantly reduced compared to control; b = significantly increased compared to the control*

It appears that carbaryl provokes similar effects on individual gammarids as imidacloprid. However, there was one deviation from the findings for imidacloprid. At the lowest concentration tested (0.081 µg/L), the organisms ate significantly less after the compound was removed from the test system (Figure A-1). The measurement of feeding inhibition at this concentration has been repeated with the same experimental setup as described previously, as this anomaly was only observed at this concentration and was only just significant (p=0.048). The repetition revealed a similar influence on feeding after exposure (data not shown). However, to verify those findings it would be recommended to repeat this experiment with a range of concentrations around that of 0.081 µg/L and a larger number of replicates/treatment.

*Table A - 1: Measured (m) and estimated (e) carbaryl concentrations in surface and ground water.*

| Location | Type of determination | Concentration [µg/L] | Reference |
|---|---|---|---|
| Campo de Nijar, Almeria (Spain); upstream | m | 0.04 – 1.37 | (Marco *et al.*, 1995) |
| Campo de Nijar, Almeria (Spain); downstream | m | 0.05 – 0.89 | (Marco *et al.*, 1995) |
| EU surface water | e | <47.19 | (European Union, 2007) |
| EU ground water | e | <0.001 | (European Union, 2007) |

# Appendix B: Supporting Information for Chapter 5 as published

*Material and methods: Calculation of feeding inhibition*

Relative feeding inhibition was calculated with a simple one-compartment model developed in ModelMaker 4.0 (Sherwell Scientific Ltd; UK). Two steps were undertaken to account for algal growth and the dependency of ingestion rate on food availability during the experiment before the inhibition in filtration rate for each concentration tested was calculated.

The following steps were carried out to calculate the relative inhibition in feeding:

1) Modelling the algal growth rate *Ag* [/h] by optimising *Ag* of the following model against the measured amount of algae *Ca* [cells/ml] from the algal control treatment at the beginning and end of the experiment.

$dCa/dt = Ca*Ag$

2) Modelling *FR* [ml/Daphnid*h] from the control treatment by optimising *FR* of the following model against the algal growth rate *Ag* obtained in step 1) and the measured amount of algae *Ca* [cells/ml] from the control treatment at the beginning and end of the experiment.

$dCa/dt = Ca*Ag-IR$

$IR= FR*Ca$

$\rightarrow dCa/dt = Ca*Ag-(FR*Ca)$

3) Modelling inhibition in filtration rate $FR_{inhib}$ [ ] by optimising $FR_{inhib}$ of the following model against the algal growth rate *Ag* obtained in step 1), the filtration rate without inhibition *FR* obtained in step 2) and the measured amount of algae *Ca* [cells/ml] from the treatment (imidacloprid concentration) at the beginning and end of the experiment. This procedure was undertaken for each concentration.

$dCa/dt = Ca*Ag-IR$

$IR= FR*(1-FR_{inhib})*Ca$

→ $dCa/dt = Ca*Ag-(FR*(1-FR_{inhib})*Ca)$

The resulting values for the relative inhibition in filtration rate (and their optimisation errors) given as % in Table A-3 and plotted in Figure 5-1. SigmaPlot 12 (Systat Software, Inc. SigmaPlot for Windows) was used to derive a dynamic curve fit in order to estimate the effect of concentrations not tested to use as input for modelling of the other effects using IDamP (Table A-2). A Sigmoid curve with 4 parameters (Y = y0+a/(1+exp(-(x-x0)/b)) was used. The following parameter values were obtained from SigmaPlot:

| Parameter | Value | StdErr | CV [%] | Dependencies |
|---|---|---|---|---|
| A | -2.27E+09 | 9.50E+15 | 4.19E+08 | 1 |
| B | -2.48E+00 | 7.43E-01 | 3.00E+01 | 0.999867 |
| x0 | -4.20E+01 | 1.04E+07 | 2.47E+07 | 1 |
| y0 | 9.80E+01 | 4.44E+00 | 4.53E+00 | 0.562324 |

*Material and methods: Test item and chemical analysis*

Further details of chemical analyses:

HPLC: (Agilent 1100 Series, Agilent Technologies, UK Ltd.

Column: Discovery® $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco)

Column temperature: 25°C

Detector: UV detector

Detection wave length: 254 nm

Mobile phase: methanol/water (45:55; v/v)

Flow rate (mobile phase): 0.5 mL/min

Chemical: Imidacloprid (PESTANAL®; 99.0%) from Sigma Aldrich (Batch-no. SZE9112X)

Sample storage: frozen at -18°C

Injection volume: 75 µL

Sample preparation: samples with a nominal concentration >10 mg/L were diluted tenfold prior to injection using M4-media.

Concentration of standards tested: 0.01, 0.1, 1.0, 5.0 and 10.0 mg/L

Retention time imidacloprid [min]: 5.7

Slope of the regression line [area/mg]: 348.17

$R^2$ of regression line: 0.9999

LOD (limit of detection = 3.3 (SD/S)): 0.024 mg/L

LOQ (limit of quantification = 10 (SD/S)): 0.074 mg/L

Elimination of interference with algae pigment: Samples for the detection of concentrations at the start of exposure were taken prior to feeding, so no interference was possible. Samples for the measurement of the concentration at the end of exposure were taken from the top one-third of the test vessel. *Desmodesmus subspicatus* sinks to the bottom of the test vessel when cultivated as for this study.

*Material and methods: Modelling parameter*

*Table A-2: Values for feeding inhibition of* Daphnia *magna caused by imidacloprid that were used for modelling effects onto growth, maturation, reproduction and mortality.*

| Experiment | Concentration [mg/L] | Feeding inhibition [%] |
|---|---|---|
| Reproduction tests | 0.15 | 3.7 |
| | 0.4 | 12.7 |
| | 1.3 | 38.7 |
| | 4 | 78 |
| | 12 | 97.2 |
| Acute toxicity | 0.4 | 12.7 |
| | 1.2 | 36.7 |
| | 3.7 | 75.5 |
| | 11.1 | 96.8 |
| | 33.3 | 100 |
| | 100 | 100 |

*Results and discussion; Feeding rate*

*Table A-3: Feeding inhibition of* Daphnia magna *during imidacloprid exposure for one day.*

| Concentration [mg/L] | Feeding inhibition [% control] | Optimisation error of feeding inhibition [%] |
|---|---|---|
| 155 | 100 | 20 |
| 31.2 | 100 | 14 |
| 7.8 | 88.30191 | 11 |
| 1.56 | 46.88141 | 26 |
| 0.078 | 0 | 13 |

*Results and discussion; Growth*

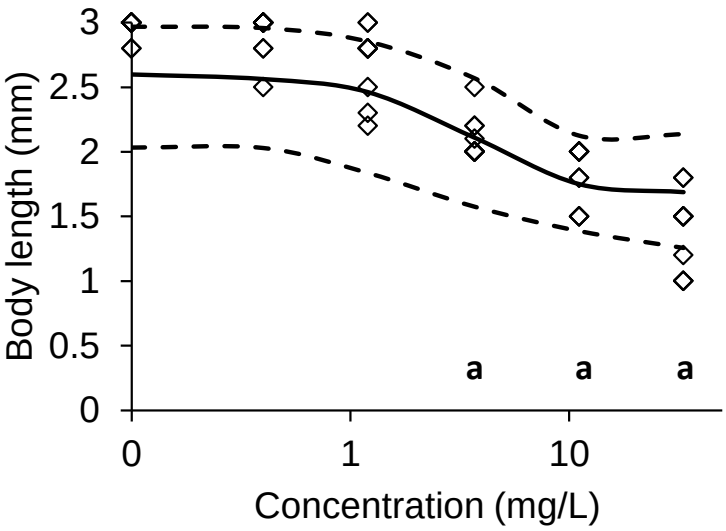


*Figure A-2: Measured (symbols) and simulated (solid line = average, dotted line = 95$^{th}$ percentile) body length of* Daphnia magna *when exposed to different imidacloprid concentrations for one week. Organisms (<24h) were fed with 0.035 mg TOC (total organic carbon) of the green algae* Desmodesmus subspicatus. *a Significant differences to the control treatments (one-way ANOVA; Holm-Sidak method; p<0.05). Data were simulated using the individual* Daphnia magna *population model "IDamP".*

*Table A-4: Relative decrease in body length of* Daphnia magna *after exposure to imidacloprid for seven days (acute toxicity test).*

| Concentration [mg/L] | 0.4 | 1.2 | 3.7 | 11.1 | 33.3 |
|---|---|---|---|---|---|
| Average | 1.4 | 8.5 | 28.8 [a] | 38.6 [a] | 53.2 [a] |
| Standard deviation | 5.6 | 9.3 | 5.5 | 7.7 | 10.6 |

[a] *Significant differences to the control treatments (one-way ANOVA; Holm-Sidak method; $p<0.05$).*

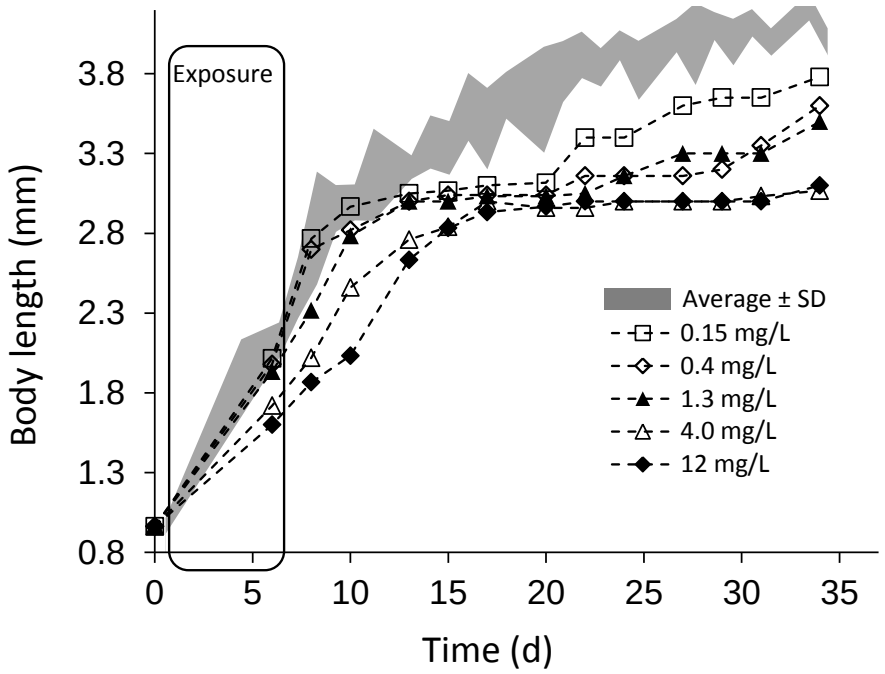


*Figure A-3: Measured average body length of* Daphnia magna *over time when fed with Desmodesmus subspicatus at low density (Reproduction tests 2). The rectangle indicates the presence of imidacloprid. The symbols are means for treated individuals, and the grey area indicates ± one standard deviation (SD) around the mean of measurements of the controls from both reproduction tests combined.*

*Results and discussion; Mortality*

*Explanation for Figure A-4:* A further mismatch between measured and simulated results occurred at an imidacloprid concentration of 33.3 mg/L in the acute toxicity test; in the experiment no significant mortality was observed, whereas the model predicted the same mortality as for the concentration of 100 mg/L. This overestimated mortality is related to an overestimation of the feeding inhibition from the data fit in the feeding assay around this concentration (Figure 5-1). For modelling the mortality following exposure to 33.3 mg/L, a feeding inhibition of 100% was used. More accurately, a reduction of >97% but <100% should have been used. Whereas concentrations causing 97% feeding inhibition do not result in mortality due to starvation within seven days, a complete feeding inhibition results in death within four to six days. We decided not to remodel the results with a more accurate feeding inhibition, because we wanted to keep the approach of blind modelling throughout the manuscript to demonstrate what we would be able to predict without having further information on the feeding inhibition.

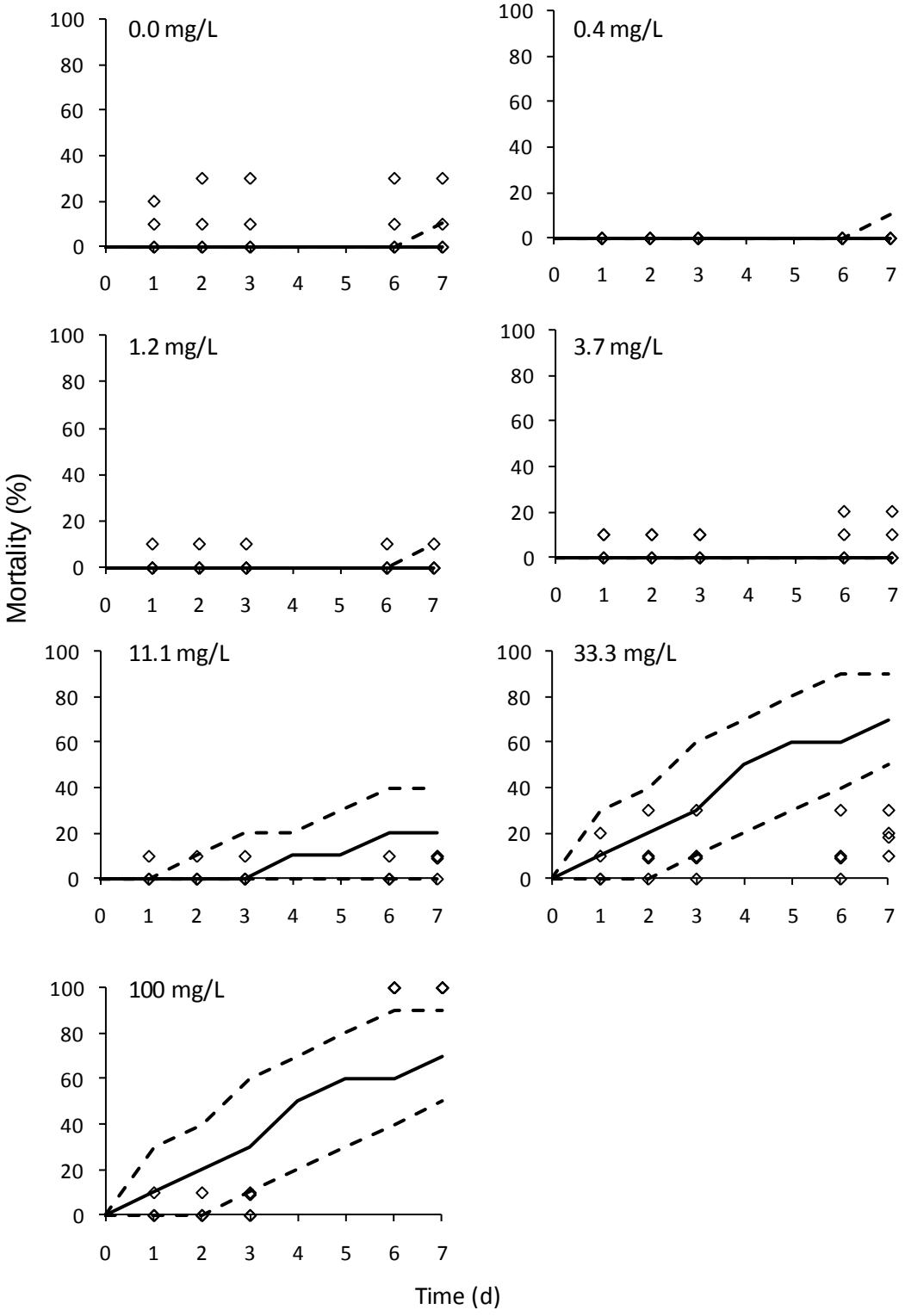


*Figure A-4: Measured (symbols) and simulated (solid line = average, dotted line = 95$^{th}$ percentile) mortality of newly hatched* Daphnia magna *over time when exposed to different concentrations of imidacloprid. Organisms were fed with Desmodesmus subspicatus.*

# Appendix C: Individual performance of *Daphnia magna* offspring after short-term feeding inhibition of mothers

In Chapter 5 it was investigated what effects occur from a short-term exposure of daphnids to imidacloprid. It was hypothesised that the effects derive from acclimation to feeding inhibition. Within this work it was additionally investigated whether such exposure provokes a changed individual performance of the offspring produced following short-term exposure. To do so, growth and reproduction of the first and second brood produced from the test organisms in the reproduction test 1 described in Chapter 5 was measured until these individuals produced at least their third brood.

*Materials and methods*

The continuation of the experiment (Reproduction Test 1) was undertaken following the same experimental setup (ten individuals per treatment, change of media every 2-3 days), environmental conditions (100ml M4 media/individual, 0.05/ 0.1 mg TOC ($1^{st}$ week/ rest of experiment), temperature, light-dark-rhythm) and methods for endpoint observation as described in the surrogate chapter (section 5.3.). Individual performance of the first (F1-1) and second (F1-2) brood from individuals exposed for one week within their development (F0) was observed over time. Offspring from each treatment at one day (when more than 10 neonates were produced) were mixed and subsequently ten per treatment were randomly separated for further observation.

*Results and discussion*

Maturation (Figure A-5), embryo-development (Figure A-6), growth (Figure A-7) and reproduction (Figure A-8) were investigated frequently over 25 and 22 days for the F1-1 and F1-2 generations, respectively. Individual performance of the offspring was compared within the generation to that of the control and between generations within the same treatment.

Monitoring the individual performance of the offspring from mothers exposed to imidacloprid during their juvenile development revealed that there was no adaptation of daphnids to imidacloprid. Neither maturation (Figure A-5), embryo-development (Figure A-6), growth (Figure A-7) or reproduction (Figure A-8) of offspring (F1) was significantly ($p>0.05$) affected by the exposure of mothers to imidacloprid.

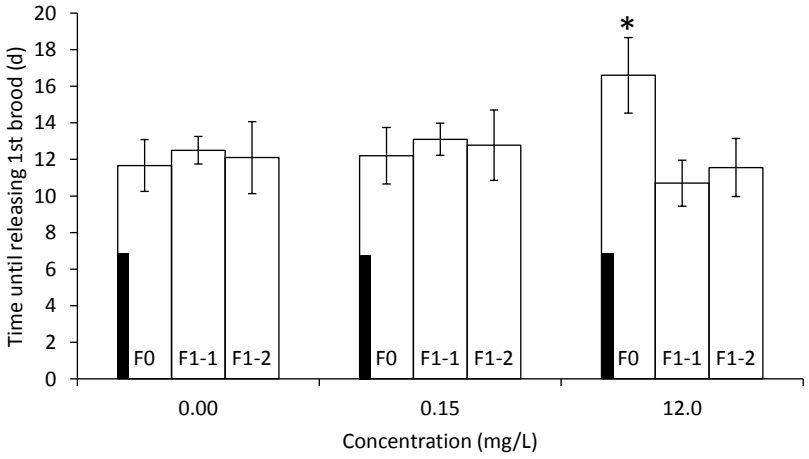


*Figure A-5: Maturation (Average ± SD) of* Daphnia magna *when exposed to imidacloprid at three concentrations for seven days during their development (F0) and of their first (F1-1) and second (F1-2) brood. The black box indicates the presence of imidacloprid.*Significant difference to all other treatments (two-way ANOVA; Holm-Sidak method; $p<0.05$).*

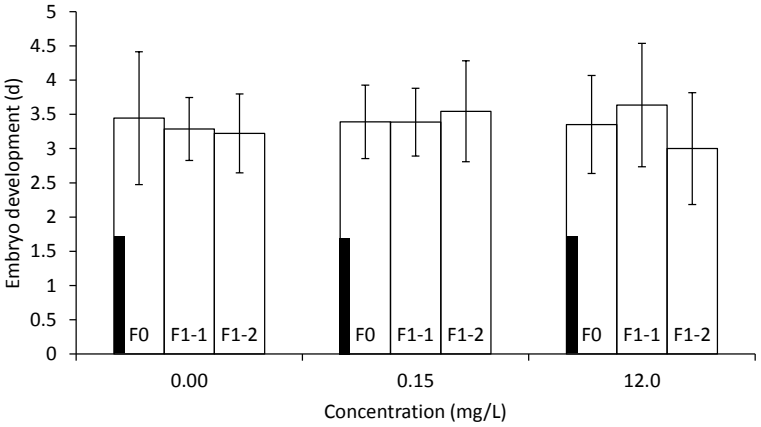


*Figure A-6: Embryo development (Average ± SD) of* Daphnia magna *when exposed to imidacloprid at three concentrations for seven days during their development (F0) and of their first (F1-1) and second (F1-2) brood. The black box indicates the presence of imidacloprid. There is no significant difference between treatments (two-way ANOVA; Holm-Sidak method; $p<0.05$).*

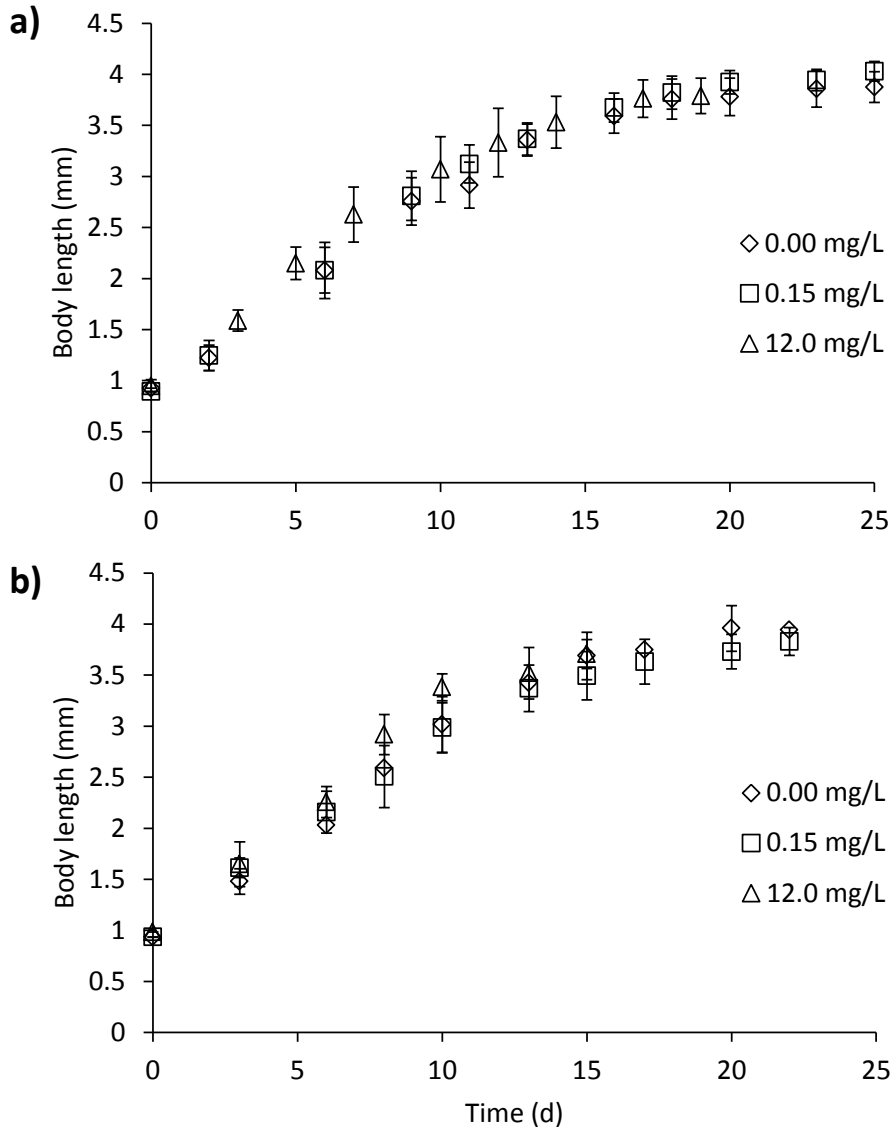


*Figure A-7: Body length (average ± SD) of* Daphnia magna *over time when mothers were exposed to imidacloprid at three concentrations for seven days during their development. Data show the body length of their first (F1-1) and second (F1-2) brood in a) and b), respectively. There is no significant difference between treatments (two-way ANOVA; Holm-Sidak method; $p<0.05$).*

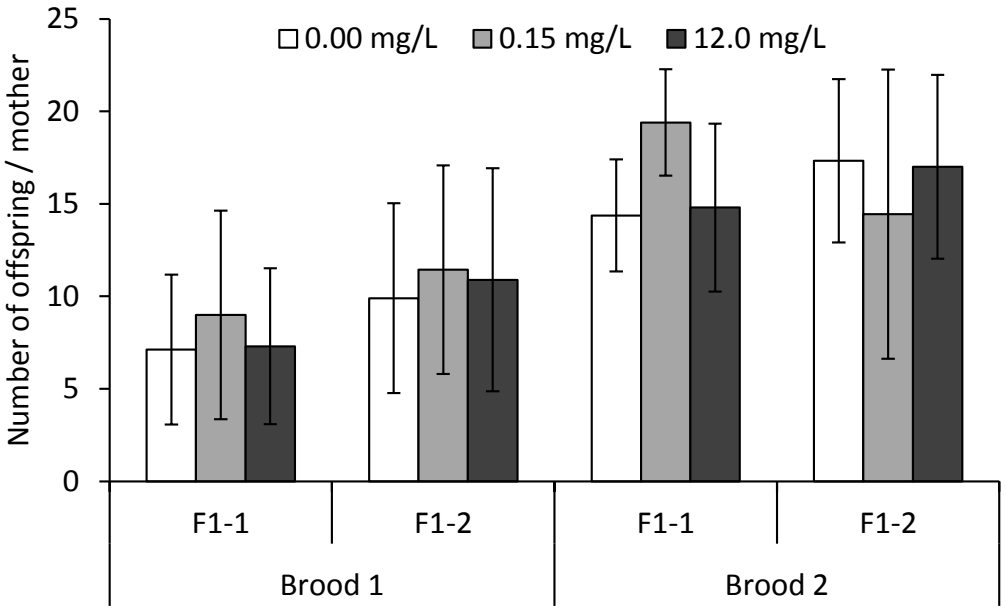


*Figure A-8: Reproduction of* Daphnia magna *when exposed to imidacloprid at three concentrations for seven days during their development. Data show the brood size (average ± SD) for their first (F1-1) and second (F1-2) offspring. There is no significant difference between treatments (two-way ANOVA; Holm-Sidak method; $p<0.05$).*

# Appendix D: Supporting Information for Chapter 6 as published

*Material and methods: Chemical analysis*

Analysis was performed by injection of a 25-75 µL sample (25 µL for all samples with a nominal concentration >1 mg/L) onto HPLC (Agilent 1100 Series, Agilent Technologies, UK Ltd.) equipped with a UV detector (254.1 nm) or a fluorescence detector (extinction=274; emission=335) for quantification of imidacloprid and carbaryl, respectively. A Discovery® $C_{18}$ column (15 cm x 4.6 mm; 5 µm; Supelco) was used and maintained at 25°C. The mobile phase had a flow rate of 0.5 mL/min and comprised methanol and water in a ratio of 45:55 and 70:30 (v/v) for imidacloprid and carbaryl, respectively. Analytical parameters for imidacloprid and carbaryl were retention time 5.3 and 5.6 min respectively, limit of detection 0.038 and 0.0028 mg/L respectively, and limit of quantification 0.116 and 0.0084 mg/L respectively. Reduction of interference by algal pigment was unnecessary for carbaryl analysis as samples were taken prior to feeding of populations. The same applied for imidacloprid analysis for samples taken at the beginning of exposure. Samples for the measurement of the imidacloprid concentration at the end of the exposure period were taken from the top third of the test vessel as *D. subspicatus* cultivated as for this study tends to sink to the bottom of the test vessel.

Measured concentrations of imidacloprid for the smallest concentration used were consistently higher than the nominal concentration (0.15 mg/L) with a deviation of

19±7%. A deviation of 2±2% was found for the nominal concentration of 12.0 mg/L. The measured concentrations for imidacloprid were 0.178±0.005 mg/L and 12.23±0.04 mg/L. There was no significant difference ($p>0.34$) in the measured concentrations between spring and summer exposure. Concentrations decreased within one week by 8.8±3.4% and 1.2±0.5% for nominal initial concentrations of 0.15 and 12.0 mg/L, respectively.

The measured concentrations for carbaryl were consistently lower than the nominal concentration of 0.02 mg/L. Measured concentrations were 52±4% lower than the nominal concentration resulting in an applied concentration of 0.0096±0.0008 mg/L. There was no significant difference ($p>0.5$) in the measured concentrations across all populations; thus the carbaryl pulses were comparable between spring and summer treatments.

*Material and methods: Test organisms and food*

Daphnids from our own culture were used and fed with the green alga *Desmodesmus subspicatus*. Cultures of both species were maintained as follows. *Daphnia magna* Straus were cultivated quasi-statically at a group level in M4-media (Elendt 1990) under constant temperature (20±1°C), a photoperiod of 16h light and 8h dark, and a light intensity of 15-19 $\mu E/m^2s$. The culture was maintained over time using the offspring of four-week old daphnids. Twenty individuals of the same age (±1d) were cultivated in 1.5 L medium which was changed weekly. Mothers and offspring were separated on the day of feeding. Organisms were fed with *D. subspicatus* three times a week (Monday, Wednesday and Friday) with 0.14 mg TOC (=total organic carbon)

per Daphnid. Algae used for feeding were in the logarithmic growth phase obtained by cultivating them under continuous aeration, constant temperature (20±1°C) and constant light with an intensity of 45 µE/$m^2$s. The algae were cultured statically for 2 weeks in 1-L media bottles containing 750 ml Kuhl medium (Kuhl and Lorenzen 1964).

*Results & discussion: Experiment for clarification of primipara being more sensitive to starvation in terms of mortality*

Daphnids were cultured according to the culture procedures described above until they reached an age of 2, 8 and 11 days. Each age class was split into two groups, transferred into clean media and either fed as usual or starved for four days. After four days the body length of the daphnids now being 6, 12 and 15 days old was measured according to the described method of body length determination. Subsequently, the organisms were placed into fresh media without food (four times five individuals in 100 ml M4-media). Mortality was recorded after 3.5, 8 and 24 h.

*Table A-5: Mortality of* Daphnia magna *due to starvation.*

| | **Age [d]** | **Body length [mm]** | **Size class according to population experiment** | **Mortality [%] after x h of starvation** | | |
|---|---|---|---|---|---|---|
| | | | | **3.5** | **8.5** | **24** |
| Starved | 6 | 1.5±0.2 | Juvenile | 0±0 | 0±0 | 0±0 |
| for 4 d | 12 | 2.5±0.4 | Primipara | 5±10 | 10±12 | 40±28 |
| | 15 | 2.9±0.2 | Primipara | 5±10 | 15±19 | 50±20 |
| Continuously fed as in culture | 6 | 1.9±0.2 | Juvenile-primipara | 0±0 | 0±0 | 0±0 |
| | 12 | 2.8±0.4 | Primipara-adult | 0±0 | 0±0 | 0±0 |
| | 15 | 3.1±0.2 | Primipara-adult | 0±0 | 0±0 | 0±0 |

# Appendix E: Body size and nutritional status dependent acute toxicity of carbaryl on *Daphnia magna*

*Introduction*

Carbaryl is very toxic to daphnids and sensitivity correlates with body length (Takahashi and Hanazato, 2007). Takahashi and Hanazato (2007) demonstrated that the effect concentration for 50% of the test organisms ($EC_{50}$) increases with increasing body length (i.e. smaller organisms are more sensitive). Their data also show that this dependency of effect concentrations on body length can be influenced by food availability prior to exposure. However, food availability prior to exposure was found to only influence the $EC_{50}$ when individual daphnids reached the reproductive stage which corresponds with just less than 3 mm body length (Figure A-9).

Recently we have shown that short-term feeding inhibition (i.e. a decrease in nutritional status of individuals) caused by imidacloprid exposure resulted in increased vulnerability of a *Daphnia* population to carbaryl exposure of one day (Chapter 6). There, increased population vulnerability was observed even though only marginal changes in population structure occurred. It was also found that all size classes of daphnids within the population were more strongly affected by carbaryl subsequent to feeding inhibition (Chapter 6). Thus, it can be hypothesised that the effect of carbaryl is not only size-dependent and changes with nutritional status of adult individuals as shown by Takahashi and Hanazato (2007), but rather depends to

a large extent on the nutritional state of individuals of every size. Nutritional status (e.g. energy availability) depends on feeding (Sancho *et al.*, 2009; Villarroel *et al.*, 2009) and the energy budget of the organism (e.g. use of available energy for different traits) which is dynamic (Kooijman, 2010) and changes for example with the growth and reproductive strategy an individual follows. The nutritional status can be considered an indicator of the overall condition of an organism (Calow and Sibly, 1990).

*Materials and methods*

Here, organisms of differing age (1, 6, 12 and 15 days old) were exposed for 48h to a measured carbaryl concentration of 12±0.3 µg/L. Organisms were either provided with no food or given food *ad libitum* for four days prior to exposure to produce a difference in their nutritional status. Environmental conditions were: constant temperature (20±1°C), a photoperiod of 16h light and 8h dark, and a light intensity of 15-19 $\mu E/m^2s$. Daphnids were taken from our own culture and were fed with the green alga *Desmodesmus subspicatus ad libitum* (0.2 mg total organic carbon per Daphnid per day). No organisms were fed during exposure to carbaryl to ensure comparability with the study from Takahashi and Hanazato (2007). The experiment was conducted with four replicates each comprising five individuals in 100 ml M4-media for each age group and nutritional status. There was no starvation prior to exposure for organisms that were less than one day old. There were controls without exposure for all treatments. Body length of the organisms was observed prior to exposure by image analysis as described previously (Agatz *et al.*, 2012). Mortality was recorded after 3.5, 8.5, 24 and 48h. Organisms that were not swimming but

moved their antenna were counted as survivors. Data were tested for statistical analysis by two-way ANOVA using the Holm-Sidak test with a significance level of $p \leq 0.05$. Assumptions on normal distribution and equal variance were tested prior to ANOVA using the Shapiro-Wilk and Levene-Mediane tests, respectively. Carbaryl was analysed by high performance liquid chromatography as described previously (Chapter 6).

*Results and discussion*

Table A-6 summarises the results of the experiment. Almost all individuals of all size classes tested (except new-borns) survived exposure to carbaryl for two day when not previously stressed by starvation. The observed mortality for neonates conforms to the effect concentration after two days of exposure ($EC_{50(48h)}$) for this size class given by Takahashi and Hanazato (2007) because we found 47±21% mortality after two days when using a concentration (12 µg/L) very close to the stated $EC_{50(48h)}$ (Figure A-9).

Individuals of all size classes were more sensitive to carbaryl when previously stressed by starvation; the sensitivity increased with decreasing energy reserves. In fact, up to 100±0% of starved juveniles, primipara and adults died within two days of exposure whereas without previous starvation no mortality was observed in all those size-classes (Table A-6). Food availability *per se*, and a resulting change in toxicity due to changed uptake of the compound and or metabolism due to higher concentration of food in solution, can be neglected as a driving factor for observed changes in sensitivity because food was absent during exposure. Nutritional status had a stronger influence than body size. Body size between fed and starved

individuals at the beginning of exposure was marginal but sensitivity to identical exposure varied dramatically (Table A-6).

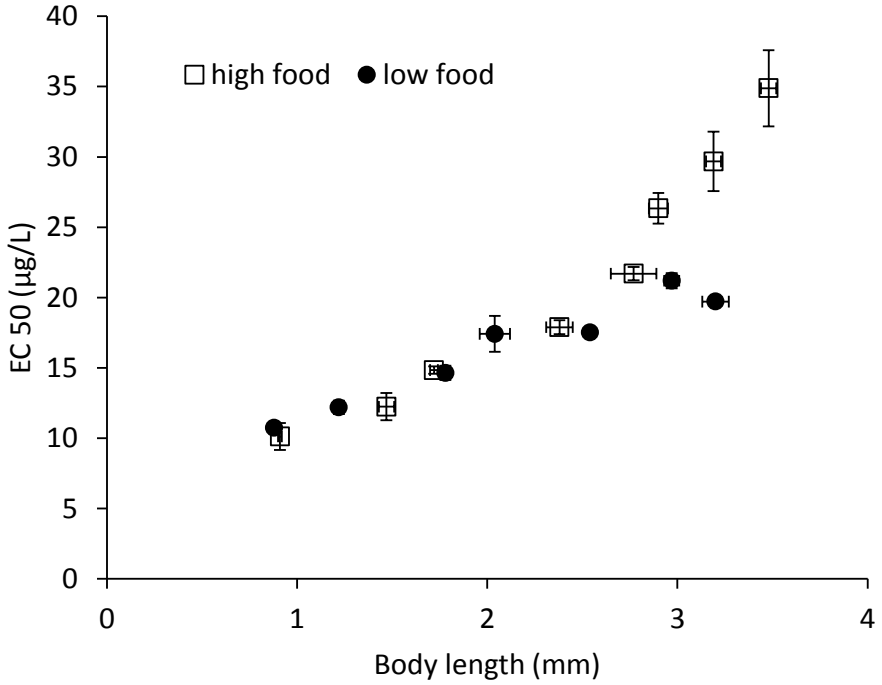


*Figure A-9: Effect concentration for immobility [µg/L] of* Daphnia magna *as a function of body length [mm] after exposure for 48h (data from Takahashi and Hanazato (2007)). Shown are average values and their standard errors. The food availability refers to the food provided while daphnids where raised for the test; high food: 5*10$^5$ cells/L, low food: 5*10$^4$ cells/L (*Chlorella*). During exposure organisms were not fed.*

The observed change in sensitivity to carbaryl for juveniles seems to conflict with observations from Takahashi and Hanazato (2007) who did not find an influence of nutritional status on the sensitivity of juveniles (Figure A-9). They probably did not find an influence of nutritional status for juveniles because of the method used to produce organisms with differing nutritional status. Takahashi and Hanazato (2007) provided two different concentrations of food to organisms, rather than providing or

not providing food (as conducted here). Both food concentrations provided were not limiting for juveniles and therefore there was no difference in the nutritional status of juveniles when carbaryl exposure occurred.

Qualitative determination of the relative importance of each factor (body size and nutritional status) in determining sensitivity to carbaryl cannot be derived from these data. Possibly this cannot be determined by any experiment because nutritional need depends not only on body size but also on the growth and reproductive strategy, what is in fact highly variable. It has been observed in the present study that differences in growth and reproductive strategy can influence the sensitivity. Individuals categorised into the size class of primipara were found to be the most sensitive when considering exposure duration. More than 50% of primipara died within 3.5h after exposure started; within 24h almost all organisms were dead; and after 48h there were no survivors (Table A-6). Similarly, juveniles and adults were more strongly affected over time, but were significantly less sensitive than primipara within the first day of exposure. Only after 48h of exposure there was no difference in the sensitivity depending on the life stage of the daphnids.

Primiparae were likely the most sensitive organisms because the sensitivity increases with decreasing energy reserves and primipara have a greater energy investment per unit time than younger and older organisms. Juveniles expend energy in growing towards the minimum body length for maturation, whilst adults expend energy mainly for reproduction. Primiparae have to maintain growth and reproduction and therefore their energy reserves are depleted quicker than those of juveniles and adults making them more sensitive to starvation (as has been demonstrated in Chapter 6).

*Conclusion*

This study demonstrates that it is important for effect assessment to not only know the organism's feeding history but also their reproductive status. Here, effects varied between no effect and 100% mortality. A difference in toxicity as observed is especially of concern for environmental risk assessment when the tested concentration of the compound is close to that occurring in the environment.

*Table A-6: Mortality of* Daphnia magna *due to carbaryl exposure (0.012±0.003 mg/L) in dependence of body size and nutritional status *.*

| | Age [d] | Body length [mm] | Size class | Mortality [%] after x h of exposure | | | |
|---|---|---|---|---|---|---|---|
| | | | | 3.5 | 8.5 | 24 | 48 |
| Starved | 6 | 1.5±0.2 | Juvenile | 25±10[a] | 40±16[a] | 65±10[a] | 83±33 |
| for 4d | 12 | 2.5±0.4 | Primipara | 63±11[a, b] | 89±22[a, b] | 92±17[a, b] | 100±0[a] |
| | 15 | 2.9±0.2 | Primipara-adult | 32±20[a] | 24±23[a] | 80±40[a] | 50±100[a] |
| Continuously | 1 | 0.9±0.1 | Neonate | 0±0 | 0±0 | 25±10[b] | 47±21[b] |
| fed as in | 6 | 1.9±0.2 | juvenile-primipara | 0±0 | 0±0 | 0±0 | 0±0 |
| culture | 12 | 2.8±0.4 | primipara-adult | 0±0 | 0±0 | 0±0 | 5±0 |
| | 15 | 3.1±0.2 | primipara-adult | 0±0 | 0±0 | 0±0 | 0±0 |

** Given mortality is corrected for mortality due to starvation (control mortality); a: significant difference to surrogate control (same age of continuously fed as in culture) (Two-way ANOVA; Holm-Sidak test; $p<0.05$); b: significant difference between age classes within starved and fed, respectively (Two-way ANOVA; Holm-Sidak test; $p<0.05$).*

# Appendix F: Definitions

***Ag*** [/h] = Algal growth rate

**APW** = Artificial Pond Water

***Ca*** [cells/ml] = Algal concentration

**D** = Indirectly decomposed horse chestnut leaves; decomposition by storing leaves in tab water for at least 3 month

**DC** = Directly decomposed horse chestnut leaves; decomposition by inoculation with *Cladosporium sp.* for two weeks

**DEB** = Dynamic energy budget

**DS =** Indirectly decomposed elder leaves; decomposition by in stream inoculation

**dw =** dry weight

$\mathbf{EC_x}$ = Effect concentration for x% of test organisms (other endpoints than mortality)

**EFSA** = European Food Safety Authority

**ERA** = environmental risk assessment

**F0** = First generation (mothers)

**F1-1** = Second generation produced from the first brood of mothers

**F1-2** = Second generation produced from the second brood of mothers

$\boldsymbol{F_{E(t)}}$ [mg/d] = amount of eaten food within the observed period (t)

**FOCUS =** FOrum for Co-ordination of pesticide fate models and their USe

$\boldsymbol{F_R}$ [mg(food)/mg(gammarid)*d] = individual feeding rate of *Gammarus*

***FR*** [ml/Daphnid*h] = Feeding rate of *Daphnia*

$\boldsymbol{FR_{inhib}}$ [] = Inhibition in filtration rate

***G*** [mg] = body mass of the individual

**IBM** = Individual-based population model

**IDamP** = Individual *Daphnia magna* population model

**$K_{ow}$** = octanol-water partition coefficient

**LC-MS** = Liquid chromatography-mass spectrometry

**$LC_x$** = Lethal concentration for x% of test organisms

***ld*** = leaching-decomposing-factor*; ld* was obtained by dividing the weight of the control leaves at the end of the measuring period by the initial weight.

**LOEC =** Lowest Observed Effect Concentration

**LOD** = limit of detection

**LOQ** = limit of quantification

**$LT_{50}$** = The time for 50 % lethality

**nAChRs** = nicotinic acetyl-choline receptors

**nD** = Not decomposed horse chestnut leaves

**NOEC =** No observed effect concentration

**NP** = nonylphenol

**PPP =** Plant protection product

**SCCS** = Scientific Committee on Consumer Safety

**SCENHIR** = Scientific Committee on Emerging and Newly Identified Health Risks

**SCHER** = Scientific Committee on Health and Environmental Risks

**SD** = standard deviation

**TKTD modelling =** Toxicokinetic-toxicodynamic modelling

**TOC** = Total Organic Carbon

**ww =** wet weight

# Appendix G: Glossary

**Conspecific** = Individuals of the same species

**Growth-discontinuation** = Mechanism whereby daphnids acclimatise to environmental conditions in terms of their individual performance in growth and reproduction. The energy allocation in growth is redirected towards reproduction under limiting food availability probably for maintenance of the population.

**Info-chemicals** = Molecular cues regulating the adjustment of species behaviour towards increased survival.

**Neonate** = New born daphnids; in standard toxicity tests used to appoint individuals younger than 24h; here, in population tests also used to appoint individuals with a body length less than 1 mm.

**Primipara** = Daphnids about to reproduce for the first time

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