**PREDICTING EFFECTS OF MULTIPLE STRESSORS ON AQUATIC BIOTA**

**ACADEMISCH PROEFSCHRIFT**

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus Prof. Mr. P.F. van der Heijden ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit op woensdag 17 december 2003, te 10.00 uur

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geboren te Ede

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Predicting Effects of Multiple Stressors on Aquatic Biota
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Proefschrift Universiteit van Amsterdam, FNWI, 2003.
ISBN 90-76894-40-X

This study was financed by the Institute for Inland Water Management and Waste Water Treatment (RIZA), and was conducted at the Department of Chemistry and Ecotoxicology, RIZA, and at the Department of Aquatic Ecology and Ecotoxicology, FNWI, University of Amsterdam.

**Cover:** '*Stressed Out!*'
Inspired by Roy Lichtenstein's '*Peace through Chemistry*', 1970

# Contents

# *Chapter 1*

## General Introduction

The expanding human population and the associated rise in industrial and agricultural activities results in an ongoing pressure on aquatic ecosystems. Anthropogenic activities have changed the global climate and habitats, and increased the input of nutrients and large numbers of chemicals. Water pollution has greatly affected the distribution of aquatic species. Anthropogenic stressors are likely to alter species abundance and persistence, and to reduce biodiversity (Mooney and Godron 1983).

With respect to chemicals, risk assessment procedures have been developed to protect ecosystems against adverse effects. In laboratory toxicity tests, effects of single substances have been tested on few test species (algae, invertebrates, and fish) and the test results have been extrapolated to ecosystems (Van Leeuwen and Hermens 1995). Since levels of several contaminants have declined over the past decade (RIZA and RIKZ, 2001), results of standard toxicity tests suggest that these low levels of substances have no impact on ecosystems. However, the constant and optimal experimental conditions used in toxicity testing lack ecological realism, as environmental conditions in the field are generally variable and suboptimal. Also, in the field many species and substances occur simultaneously, while they are usually investigated separately in experiments. The differences between laboratory and field conditions are likely to result in changes in bioavailability and toxicity of substances, which complicates the extrapolation of results from standard toxicity tests to the field. Therefore, toxicity experiments have been performed under more ecological realistic conditions. For instance, the joint effects of combinations of chemicals were studied in mixture toxicity studies (Greco et al. 1995, Vighi et al. 2003), while interactions between biological species were studied in model ecosystems (Brock et al. 1992), experimental ditches (Van den Brink 1996), and enclosures (Caquet et al. 2000). A relatively new topic is the interaction between environmental factors and chemicals. Although much information is available about the responses of aquatic species to individual environmental factors, little is known about the interaction between, and joint effects of natural factors and chemicals on aquatic biota.

## MULTIPLE STRESS

Many different definitions for stress have been formulated (as reviewed by Grime 1989, Hoffmann and Parsons 1994). Two viewpoints can be distin-

guished: stress is either defined as the *response* of a biological system (ranging from a cell to an ecosystem) to internal or external pressures acting on this system, or as the *agent* causing the response in a biological system, usually called the 'stressor' or 'stress factor' (Hoffmann and Parsons 1994). However, stressors cannot be identified without reference to the biological system on which they act (Koehn and Bayne 1989, Sibly and Calow 1989). The intensity of a stress response may vary from zero to complete inhibition and depends on the biological system, and on the nature, severity, and periodicity of the stressor (Grime 1989).

Environmental conditions in the field are seldom optimal and this would imply that organisms or populations living in natural systems are continually stressed. When deviations from optimal performance due to unfavorable conditions are not too large, compensation can occur. However, stressed organisms or populations are assumed to be more susceptible to additional unfavorable factors, for instance exposure to toxicants. On the other hand, exposure to a toxic substance may narrow the tolerance range for natural factors. In the present thesis, the term 'multiple stress' is used for the combination of natural stressors (abiotic and biotic) and chemicals. Following Grime (1989), the biological effect of multiple stressors is determined as the impairment of the normal functioning of individuals or populations, by reducing resource acquisition, survival, growth or fecundity relative to optimal conditions. Relatively little is known about the interactions between the two types of stressors. Therefore, knowledge of the interactions between multiple stressors, their joint effects, and underlying mechanisms is essential to assess the ecological relevance of multiple stressors, and to predict effects on aquatic species in the field.

## OBJECTIVES

The present thesis addresses the joint effects of substances and environmental factors on aquatic biota and explores the mechanisms of their interaction by combining laboratory experiments and models. The objectives of this thesis are therefore:

- to review the present state of knowledge of joint effects of substances and natural factors on aquatic organisms,
- to assess the influence of multiple stressors at the individual and population level,

- to clarify whether patterns observed in multiple stressor studies are due to changes in exposure to chemicals or to a modified intrinsic sensitivity of the test organisms,
- and to quantify the interaction between compounds and environmental factors.

## STRESSORS AND TEST SPECIES

Although many other natural stressors could have been chosen, this thesis focuses on temperature, food, and salinity. The choice for these three stress factors is extensively discussed in the literature review in Chapter 2. In short, all three factors are ecologically important and are highly variable in the field, which may cause stress responses in organisms. Further, they are likely to interact with substances, as they are major factors influencing the physiology of organisms.

Cadmium was chosen as a model toxicant. It still causes problems in Dutch inland waters, although levels are declining (CIW 2001). For this reason, cadmium is a priority and blacklisted substance in the Netherlands (VROM 2001). It is measurable in low concentrations and it is a nonessential element, which cannot be regulated by most organisms (Rainbow 1998). This makes cadmium an appropriate toxicant for use in accumulation experiments with small animals.

The water flea *Daphnia magna* was used as a model test species. Daphnids have been extensively used for toxicity testing. They are representatives of filter-feeding zooplankton and have a major contribution to food webs in many fresh waters (Lampert 1987). They are easy to culture in the laboratory and due to parthenogenetic reproduction under favorable conditions, populations of one genotype can be obtained. Further, an extensive amount of literature is available about the individual effects of the chosen stressors on *D. magna*.

## OUTLINE

As stated above, this thesis aims to assess the joint effects of substances and environmental factors on aquatic biota, and to analyze the nature of their interaction by studying underlying mechanisms through experiments and

models. The study departs from a literature study searching for studies concerned with the joint effects of multiple stressors in the aquatic environment. The results are given in Chapter 2, where the influences of temperature, nutritional state, and salinity on the responses of several aquatic species to various classes of chemicals are discussed. Quantitative relationships were derived from data presented in literature, which were used to discuss the suitability of uncertainty factors used in risk assessment procedures. From this literature review, several questions arose, which were investigated in subsequent experimental chapters. A stepwise approach was chosen to test successive situations of increasing complexity and ecological relevance. Chapter 3 addresses the question if temperature-dependent toxicity of chemicals is due to acceleration of physiological processes, and thus toxicant uptake rates, or increased sensitivity of the daphnids. With this purpose, data of toxicity and accumulation experiments were analyzed with the DEBtox model. Chapter 4 describes a chronic experiment with *D. magna* in which the influence of temperature and food level on the toxicity of cadmium was investigated at the population level. The DEBtox model was used to link effects on energy budgets of individuals to life history parameters determining population growth and to detect underlying mechanisms. Results of the model analyses are given in Chapter 5. In Chapter 6, the predictability of multiple stressors is discussed, and potential implications for risk assessment are mentioned.

# *Chapter 2*

# A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment

Heugens EHW, Hendriks AJ, Dekker T, Van Straalen NM, Admiraal W. 2001. *Critical Reviews in Toxicology* 31(3): 247-284. With slight modifications.

## ABSTRACT

Risk assessment procedures use toxicity tests in which organisms are subjected to chemicals under otherwise constant and favorable experimental conditions. Because variable and suboptimal environmental conditions are common aspects of natural ecosystems, the hazard of underestimation of risk arises. Therefore, an uncertainty factor is used in the extrapolation of results of standard toxicity tests to field situations. The choice for these uncertainty factors is based on little ecological evidence. This review discusses studies on the toxicity of various chemicals to aquatic organisms, modified by temperature, nutritional state, and salinity, excluding papers on changes in bioavailability of substances. Collected data were analyzed quantitatively to evaluate the validity of toxicity data obtained from standard toxicity tests in the laboratory under field conditions. Generally, organisms living under conditions close to their environmental tolerance limits appeared to be more vulnerable to additional chemical stress. Usually, increasing temperature and decreasing food or nutrient level raised toxicity. The influence of salinity was less clear; metal toxicity increased with decreasing salinity, toxicity of organophosphate insecticides increased with higher salinity, while for other chemicals no clear relationship between toxicity and salinity was observed. The interactions can be explained by several physical and physiological processes, acting on factors such as bioavailability, toxicokinetics, and sensitivity of organisms. Quantitative analysis of data indicated that an uncertainty factor for the laboratory to field extrapolation should be smaller than one for an ecosystem in a temperate region, while a factor greater than one would be appropriate for systems nearby discharge points of cooling water. The factor should be greater than one when varying nutritional state is concerned, but smaller than one with respect to salinity. Dependent on the effect parameter used, the differences in toxicity between laboratory and relevant field situations ranged from a factor of 2.6 to 130 and 1.7 to 15 for the two temperature conditions and 1.2 to 10 for nutritional state. A salinity increase from freshwater to marine water decreased toxicity by a factor of 2.1. However, as less extreme salinity changes are more relevant under field conditions, the change in toxicity is probably much smaller. To obtain uncertainty factors that sufficiently protect natural systems without being overprotective, additional research is required.

## INTRODUCTION

Pollutants enter the aquatic environment in various ways. Risk assessment procedures for substances have been developed to minimize risks for aquatic ecosystems (EPA 1984). In these procedures, a major role is assigned to standard toxicity tests in the laboratory in which sensitivity of organisms to individual substances or field samples is determined. Over the last few years, toxicant levels in Dutch waters have declined (RIZA 1994a, b). Results of standard toxicity tests with these low concentrations of toxic substances sometimes suggest that present pollution levels have no impact on ecosystems. However, the translation of results of standard toxicity tests to field situations is difficult because many factors (for example, bioavailability of substances and adaptation to toxicants), are involved. To reduce the chance of underestimating risk, an uncertainty factor is used to extrapolate these results to field situations. Overestimation of risks is also undesirable, as high costs may be involved to meet quality criteria. As the choice for uncertainty factors is arbitrary and often not based on empirical science, the validity of these factors has been questioned (Chapman et al. 1998). It is unclear whether toxicity in the field is greater or smaller than toxicity found in the laboratory (Van Straalen and Denneman 1989). Arguments for an uncertainty factor greater or smaller than one (toxicity in the field is higher or lower than in the laboratory, respectively) are given in Table 2.1.

**Table 2.1.** Arguments used in establishing a laboratory-field uncertainty factor

| | |
|---|---|
| - | In the field, biological availability of chemicals is often lower than in laboratory tests |
| - | In the field, ecological compensation and regulation mechanisms are operating |
| - | In the field, adaptation to chemical stress may occur |
| + | Adaptation often entails costs in ecological performance |
| + | In the laboratory, organisms are tested under optimal and constant conditions |
| + | In the field, the organisms are exposed to mixtures of many chemicals |

A minus indicates a negative argument for an uncertainty factor greater than one, a plus a positive argument.
From: Van Straalen and Denneman 1989.

The purpose of this review is to analyze interactions between chemicals and other stressors acting on aquatic organisms. Although the number of studies using terrestrial organisms increases rapidly, too few papers are

available at this time to discuss them here. An understanding of interactions is important for the extrapolation of results of toxicity tests conducted in the laboratory to ecosystems and for the design of site-specific quality criteria for substances. Following several authors (Bradshaw and Hardwick 1989, Calow 1989, Koehn and Bayne 1989, Sibly and Calow 1989), stress is defined as an environmental change that affects community structure and ecological functioning of organisms (i.e. growth and reproduction), leading to reduced fitness. Responses to stress are dependent on the level of organization studied, because a stress response at the cellular or molecular level need not become manifest at the level of individuals or populations due to compensatory and regulation mechanisms (Calow 1989, Parker et al. 1999). In this article, the term 'multiple stress' is used for the situation where an organism is exposed both to a toxicant and to stressful environmental conditions, such as suboptimal temperature or food supply.

Toxicants and environmental factors can interact in a variety of ways. Changing environmental conditions may influence the bioavailability of chemicals. For example, sorption of substances to particulate organic matter may indirectly influence toxicity. Toxicokinetics may be altered as well, for example, high temperature or high food levels may induce higher uptake or detoxication rates. Furthermore, the physiological state of an organism may be affected. For instance, food scarcity may change fat, protein, and carbohydrate concentrations in an organism, which may influence the partition of chemicals over the different compartments. Multiple stressors may change an organism's sensitivity in two ways: exposure to a toxic substance may narrow an organism's tolerance range for environmental factors. On the other hand, these factors may influence the toxic effect concentration of pollutants. Papers dealing solely with the first type of interaction (changed bioavailability by pH, water hardness or humic substances) were excluded because this is not the objective of this review.

From the numerous potential environmental stressors, this article considers the influence of temperature, nutritional state, and salinity on the sensitivity of different groups of aquatic organisms to various classes of chemicals. These environmental factors are chosen because they may highly fluctuate in natural systems, causing stress in organisms. Temperature stress in organisms might occur due to climatic extremes or discharges of heated wastewater by power plants (Cairns et al. 1975). Discharges from soda works and potassium and coalmines greatly influence salt

concentrations in freshwaters, resulting in salinity stress (Dieperink 1997). In estuaries, fluctuating temperature and salinity may be natural stressors (Forbes 1991, McLusky et al. 1986). Variable food or nutrient levels are common in natural ecosystems. Food scarcity may even occur in eutrophicated waters, if populations have grown to the carrying capacity (Buikema et al. 1980 [in Klüttgen et al. 1996], Kooijman and Metz 1984).

Three previous reviews have considered the interaction between contaminants and temperature (Cairns et al. 1975), contaminants and salinity (Hall and Anderson 1995), and the combination of these three factors (McLusky et al. 1986). The present article provides information from new literature references published since these reviews. The sections in which this information is given are divided into an introductory section wherein background information on the specific stressor and our expectations concerning possible interactions are given. This is followed by a section with detailed descriptions of individual studies, succeeded by a summary and conclusions section containing general findings and exceptions to the general patterns observed, as well as conclusions regarding our earlier expectations. In addition, the collected data are used to study quantitative relationships that give an indication of the value of uncertainty factors needed to extrapolate results of standard toxicity tests performed in the laboratory to field situations.

## METHODS

### Literature collection

The studies used in this review assessed the toxicity of a chemical to a certain species at a range of values for one or more environmental factors. Only studies with constant experimental conditions, apart from the factors of interest, were included. The databases Current Contents Search and Biological Abstracts were used to acquire relevant literature. References cited in articles were used as well. As the various toxicants have different effects on organisms, chemicals were distinguished into three classes (metals, pesticides, and natural toxicants) and a rest group of infrequently studied chemicals (other toxicants). In each group the studies were arranged according to compounds and then to trophic groups. When toxic effects

increased at rising values of an environmental factor, the factors are positively correlated. A negative correlation indicates decreased toxicity at rising values and an optimum is designated as the situation where toxicity is lowest or highest at intermediate values of the environmental factor. No correlation means that no systematic effect of the factor on toxicity is observed.

### Quantitative relationships

Data were collected from the papers reviewed to study quantitative relationships between toxic effects of contaminants and environmental factors. The data obtained for exposed organisms were scaled to those of the controls. This eliminated the normal effects of temperature, nutritional state, and salinity. By scaling, differences of scale and unit disappeared as well, allowing comparison between experiments. As food quantity and quality varies between studies, food levels in each experiment were scaled to the highest level used in the specific experiment. The relationship between toxicity and temperature is based on the Arrhenius equation and is given by:

$$\log y = \alpha_1 + \beta_T \cdot T^{-1} \tag{2.1}$$

where $y$ is EC50, LC50 or the tissue toxicant concentration causing death (lethal body burden) (mass of toxicant biomass$^{-1}$), $\alpha_1$ is the intercept, $\beta_T$ is a coefficient for temperature, and $T$ is temperature (Kelvin). The relationship between toxicants and nutritional state, salinity, and combinations of environmental factors is given by:

$$\log z = \alpha_2 + \gamma_T \cdot \log T + \gamma_N \cdot \log N + \gamma_S \cdot \log S + \gamma_C \cdot \log C \tag{2.2}$$

where $z$ is EC50, LC50 or a parameter for growth, reproduction, or survival, $\alpha_2$ is the intercept, $\gamma_T$, $\gamma_N$, $\gamma_S$, and $\gamma_c$ are coefficients for temperature, nutritional state, salinity, and toxicant, respectively, $T$ is temperature (Kelvin), $N$ is scaled food or nutrient level (-), $S$ is salinity (‰), and $C$ is toxicant concentration (amount of toxicant volume$^{-1}$). Both linear and multiple regressions were used to fit data to Equations 2.1 and 2.2. As data were scarce,

studies with limited experimental treatments were still used, although this resulted in less reliable regressions. The coefficients of the environmental factors ($\beta$ and $\gamma$) were arranged in groups according to the effect parameters mostly applied: 24-h EC50, 48-h EC50, 24-h LC50, and 48-h LC50 for temperature and growth, reproduction, survival after chronic exposure, 48-h LC50, and 96-h LC50 for nutritional state. Groups were analyzed with a t-test (95% confidence interval). For salinity, too few effect parameters were available to arrange them into groups. Both regressions and statistical analyses were performed with Matlab 5.2 software (The Mathworks, Inc.).

The coefficients of the environmental factors ($\beta$ and $\gamma$) were used to estimate the difference in toxicity under laboratory and relevant field conditions. Standard toxicity tests are usually performed at 20 °C, while a much lower temperature can be found in natural ecosystems situated in temperate regions, for example, the Dutch Lake IJsselmeer during the winter season (RIWA 2000). On the other hand, higher temperatures may occur in systems nearby power plants discharging cooling water (RIZA 1996). Therefore, toxicity at 20 °C was compared with toxicity at 3.6 and 30 °C. Further, the estimated toxicity at an algal concentration of 3 mg C $L^{-1}$ was compared with toxicity at concentrations of 0.109 and 0.375 mg C $L^{-1}$. The first food level is an average food level used for daphnids in standard toxicity tests (OECD 1998), while the others are the average biomasses of green algae in an oligotrophic and an eutrophic Dutch lake during summer: Lake Maarsseveen (Hallegraeff 1976, carbon content calculated from chlorophyll concentration by estimating 2% chlorophyll and 50% carbon on a dry weight basis) and Lake IJsselmeer (Lammens 1999), respectively. For salinity, the change in toxicity was estimated by assuming an extreme salinity increase from 1‰ in the laboratory to 35‰ in the field, which may occur in estuaries (Forbes 1991).

## INFLUENCE OF TEMPERATURE

### Introduction

Most aquatic organisms are ectotherms, which makes temperature an important environmental factor controlling physiological processes. Extreme temperatures may be lethal; the temperature at which death occurs

is determined by developmental, genetic, and environmental factors. Within the genetic limits, acclimatization of an organism to a higher or lower temperature can extend the upper or lower boundaries, respectively (Cairns et al. 1975). As temperature influences all kinds of physiological processes, an interaction between temperature and toxicants can be expected when a chemical acts on these processes. For instance, Prosser (1991 [in Rosas and Ramirez 1993]) proposed that thermal stress acts on the neural membranes, often accompanied by tissue hypoxia. As exposure to toxicants may increase the metabolic oxygen demand, higher temperatures will potentiate the toxic effect. Temperature may also influence toxicokinetics of substances by affecting metabolic rate or locomotory and feeding activity of an organism, thus affecting uptake, elimination, and detoxication rates (Cairns et al. 1975, Donker et al. 1998, Smit and Van Gestel 1997). Also, the physiological condition of an organism may be modified by changing temperature, for example, by the induction of proteins protective against cold or heat, which may influence sensitivity to toxicants. At the thermal tolerance limits, exposure to toxicants can enlarge adverse thermal effects (Cairns et al. 1975, Donker et al. 1998). Considering these statements, we expect increased toxicity at elevated temperatures when both stressors act on metabolic processes, such as energy metabolism or respiration. Further, we expect increased internal toxicant concentrations at high temperatures due to elevated toxicant uptake rates, leading to a rise in adverse effects. On the other hand, detoxication and elimination rates may increase as well. It is not clear if this counteracts the thermal effect on toxicant uptake rates.

To examine the influence of temperature on the toxicity of chemicals, the critical thermal maximum (CTM) is often used as an indicator of the general physiological state of an organism (Rosas and Ramirez 1993). The CTM is a measure for the upper limit of thermal tolerance of aquatic animals, which may be modified as a result of toxicant exposure (Alcaraz et al. 1997). The endpoint criterion is the loss of the animal's control over its swimming performance, when it starts to swim vigorously or just before this, when it starts to lose its sense of direction. Comparison of CTM values from different experiments is only possible when a uniform rate of temperature increase and a similar endpoint criterion is chosen. Further, the organisms need to be acclimated to the same temperature (Becker and Wolford 1980).

## Detailed report of literature

The influence of temperature on toxicity of substances has been discussed in two reviews. Cairns et al. (1975) summarized the effects of temperature on the toxicity of various chemicals to aquatic organisms, mostly fish, while McLusky et al. (1986) described the effects of temperature and salinity on the sensitivity of marine and estuarine invertebrates to metals. Both studies showed that changing environmental conditions may affect sensitivity of organisms to chemicals. In general, toxicity of chemicals increased with rising temperature, and organisms living near to their thermal tolerance limits exhibited greater effect of exposure to toxicants than did organisms living under optimal conditions. Results of studies cited in both reviews are given in Annex 2.1. Studies were only included when mortality was determined, and the number of times that a positive, negative, no correlation, or optimum was found is summed. Annex 2.2 gives a summary of the interactive effects of temperature and toxicants found in recent articles reviewed in the present paper. Parameters most commonly measured are presented; other parameters and details follow in the subsequent section. Figure 2.1 presents the frequency of occurrence of positive, negative, no correlations, and optima for effects of toxicants and temperature on mortality in articles reviewed in Cairns et al. (1975), McLusky et al. (1986), and the present article.

### *Metals*

Rosas and Ramirez (1993) found a decreased thermal tolerance in prawn (*Macrobrachium rosenbergii*) exposed to cadmium and chromium. Compared with control prawns, exposure to 0.05 mg $Cd^{2+}$ $L^{-1}$ in hard water for 24 h reduced the CTM from 35.8 to 33.8 °C. When animals were exposed for 96 h the CTM compared with the control group decreased from 35.4 to 34.6 °C. In prawns exposed to 0.7 mg $Cr^{6+}$ $L^{-1}$ in soft water the CTM decreased from 36.1 °C for controls to 35.3 °C for exposed animals. Exposure time did not significantly influence the decrease in CTM. A reduction in thermal tolerance by cadmium was more prominent in hard than in soft water, while for chromium it was vice versa. A calcium-related uptake of cadmium was assumed; in soft water calcium uptake was reduced, accompanied by a

lower cadmium uptake and toxicity. In contrast, the uptake of chromium is not associated with calcium uptake and bioavailability of chromium in hard water is reduced by complexation. It was stated that the observed decrease in thermal tolerance was caused by changes in neurocyte membranes, accompanied by a decrease in oxygen concentration in the tissues.

Carrier and Beitinger (1984) showed that toxicants do not always influence the thermal tolerance of organisms. The CTM of green sunfish (*Lepomis cyanellus*) exposed to cadmium concentrations of 5.17 mg $L^{-1}$ for 10 d did not significantly differ from that of control fish. This species belongs to the family Centrarchidae, which appears to be resistant to a wide variety of environmental conditions. It was suggested that the lack of response is a result of low cadmium uptake rates, detoxication mechanisms, or high cadmium depuration rates.

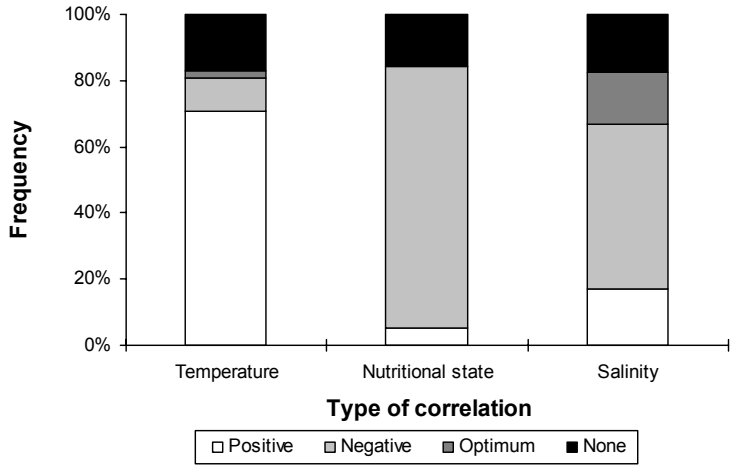


**Figure 2.1.** Frequency of occurrence of four categories of correlations between toxicity of substances and environmental factors (temperature, nutritional state, and salinity) with respect to mortality. The four categories are: *positive correlation*: toxicity increases with increasing values of the environmental factor, *negative correlation*: toxicity decreases with increasing values of the environmental factor, *optimum*: toxicity is lowest or highest at intermediate values of the environmental factor, *no correlation* (indicated as 'none'): the environmental factor does not influence toxicity. Number of considered experiments was 151 for temperature, 19 for nutritional state, and 181 for salinity. Summarized results of Cairns et al. (1975), Hall and Anderson (1995), McLusky et al. (1986), and the present review.

Jacobson et al. (1997) found increased copper sensitivity of glochidia (free-living larvae) of the freshwater mussel *Actinonaias pectorosa* at elevated temperatures. A rise in temperature from 10 to 25 °C resulted in a decrease of the 24-h LC50 from 132 to 42 µg copper $L^{-1}$. A positive linear relationship between temperature and LC50 values was found, indicating greater toxicity of the metal at higher temperatures.

Persoone et al. (1989) observed a similar response to increased temperature in *Daphnia magna* exposed to potassium dichromate for 24 h. Dependent on water hardness, exposure to potassium dichromate combined with a temperature increase from 7 to 28 °C caused a rise in the amount of immobilized animals by a factor 3 to 12. Similar results were reported by Larrain et al. (1998), who exposed the copepod *Tisbe longicornis* to potassium dichromate for 48 h at 13, 16.5, and 20 °C and observed 50% mortality at 25.6, 17.8, and 8.6 mg $L^{-1}$, respectively. The variability between replicate experiments decreased with increasing temperature.

In natural systems, the presence of toxicants and changes in ambient water temperature do not need to coincide. To simulate this, Becker and Wolford (1980) tested toxicant and temperature stress sequentially. The thermal tolerance of juvenile salmonids was tested 24 to 48 h after exposure to sublethal concentrations of nickel by the use of the CTM method. Temperature and time at loss of control over swimming performance and death were determined. Generally, CTM values for exposed fish were lower and more heterogeneous than those from control fish, suggesting that each individual reacts differently to toxicant exposure. Exposure of Coho salmon (*Oncorhynchus kisutch*) to 16 mg $L^{-1}$ nickel for 14 d significantly altered the thermal response. The temperatures at loss of control and death were 28.4 and 29.1 °C for control fish, decreasing to 25.4 and 26.3 °C for exposed fish, respectively. The time at loss of control and death also decreased from 134.1 and 141.9 min (control) to 104.0 and 113.4 min (exposed). Exposure of rainbow trout (*Salmo gairdneri*) to nickel also reduced CTM values compared with those of control fish. For example, an exposure to 1.4 mg $L^{-1}$ nickel for 21 d resulted in a reduction of temperature at loss of control and death of 26.9 and 27.2 °C (control) to 23.2 and 24.7 °C (exposed), respectively. Time at loss of control and death decreased from 188.7 and 193.2 min (control) to 153.7 and 167.2 min (exposed). A two-fold higher nickel burden in exposed fish compared with untreated fish explained their low resistance to elevated temperatures.

### *Pesticides*

Takle et al. (1983) exposed red shiners (*Notropis lutrensis*) to the dipotassium salt of the herbicide endothal (Aquathol-K). Fish were exposed to nominal concentrations of 1 and 30 mg $L^{-1}$ acid equivalents for 48 h, but this did not significantly alter the CTM.

Song et al. (1997) tested the influence of two temperatures on the toxicity of four insecticides on *D. magna* and *Aedes aegypti*. Both species were more tolerant to tebufenozide at 20 than at 27 °C. 48-h LC50 values for the water fleas and mosquitoes were, respectively, 17.37 and 0.92 mg $L^{-1}$ at 27 °C, while at 20 °C less than 50% mortality occurred during the test period. Tebufenozide is a disrupter of the molting process, and its effects are more pronounced at higher temperatures when metabolic rates, and thus growth and development, are enhanced. *D. magna* was more tolerant to aldicarb, a neurotoxin, at 20 than at 27 °C. Corresponding 48-h LC50 values were 0.74 and 0.075 mg $L^{-1}$. The shapes of the mortality curves were similar. When *A. aegypti* was exposed to aldicarb, higher mortality was found at 20 than at 27 °C, although there was no significant difference in LC50 values at the two temperatures (48-h LC50 was 0.27 and 0.29 mg $L^{-1}$, respectively). Temperature did not affect toxicity of dimethoate and imidacloprid in both species. Fisher and Wadleigh (1985) reported that increased temperature significantly lowered the tolerance of midge larvae (*Chironomus riparius*) to the insecticide lindane. 24-h LC50 values were 13.43, 6.63, and 6.77 mg $L^{-1}$ at 15, 25, and 35 °C, respectively. In an additional uptake experiment animals were exposed to $^{14}$C-lindane for 24 h. Internal concentrations of 0.0342, 0.0713, and 0.0436 mg $kg^{-1}$ were found at 15, 25, and 35 °C, respectively. The low accumulation at 15 °C explains the low toxicity at this temperature. At the higher temperatures, however, a similar response was observed, while less lindane was accumulated at 35 than at 25 °C. It was assumed that temperature influences both the ability of lindane to penetrate the nervous system and the sensitivity of the target site.

### *Natural toxicants*

Alcaraz et al. (1997) measured the temperature tolerance of white shrimp *Penaeus setiferus* postlarvae when exposed to ammonia, nitrite, and combi-

nations of these chemicals for 72 h. Ammonia did not affect the temperature response, although the neurotoxic action of ammonia could have masked the temperature effect. Nitrite, however, significantly decreased the temperature tolerance. Concentrations of 50 and 100 mg $L^{-1}$ $NO_2^-$-N caused a decrease in CTM of 5.0 and 8.4%, respectively. The thermal tolerance of shrimp exposed to ammonia and nitrite simultaneously was related to the toxicity ratios of ammonia and nitrite concentrations in the mixtures. The CTM of 38.8 °C in control animals decreased to 36.6 and 35.8 °C in animals exposed to mixtures of $NH_3$ and $NO_2^-$ in toxicity ratios of 1:1 and 1:2, respectively. Supposedly, the reduction in thermal tolerance was caused by the oxidation of haemocyanin by nitrite and a subsequent low capacity to deliver oxygen to tissues. In addition, the increase in water temperature caused an increase in metabolic rate that could enhance ammonia and nitrite accumulation and their toxic effect.

Cyanobacteria may cause adverse effects to daphnids, either by the production of toxic compounds or by the formation of filaments or colonies that disturb the feeding proces (Lampert 1987). Hietala et al. (1997) noted temperature effects on both acute and chronic toxicity of the toxic cyanobacterium *Microcystis aeruginosa* to different clones of *Daphnia pulex*. In the acute test, immobilization was measured at 19 and 24 °C. Thermal effects were seen in nine out of ten clones, where 48-h EC50 values decreased with increasing temperature. The maximal difference in EC50 was 11-fold. Chronic effects at the two temperatures were determined in two clones, by measuring survival, fecundity, and intrinsic rate of natural increase during 20 d. One clone showed a decrease in fecundity when exposed to cyanobacteria and low temperature. The intrinsic rate of natural increase of exposed animals decreased with rising temperature in both clones. As no additional food was given in the acute experiment, the authors suggest that increased toxicant uptake at high temperature was responsible for the observed effects. This temperature effect was of less importance in the chronic test, where non-toxic green algae were provided as a food source. Here, the effects could be explained by an increased metabolic energy demand at high temperatures. When feeding was inhibited by the cyanobacteria, the ingested amount of green algae could not maintain optimal growth and reproduction, leading to a reduced intrinsic rate of natural increase.

Hanazato (1991a) exposed *Daphnia ambigua* to a chemical (a kairomone) released by *Chaoborus* (Diptera) larvae, whereupon the tolerance to water temperatures ranging from 5 to 30 °C was examined. Daphnids developed helmets as a defense mechanism when held in water conditioned by the predator *Chaoborus*. Temperature determined helmet size, which was maximal at 23 °C and became smaller with decreasing temperature. At 5 and 10 °C, no helmets were formed. No interaction between temperature and kairomone was found when growth or reproduction were considered, but for mortality an interaction was found: at high temperatures (28 and 30 °C) survival rates of daphnids exposed to kairomone decreased greatly compared with unexposed animals. For instance, at 30 °C all exposed individuals died before the second day, whereas 42% of the control individuals survived until the seventh day. The high mortality in the exposed group occurred during the juvenile stages in which the daphnids developed helmets. Probably molting to the high-helmeted morph increased the risk of mortality at high temperatures.

### *Other toxicants*

Persoone et al. (1989) studied the influence of temperature on the toxicity of sodium laurylsulfate to *D. magna*. The amount of immobilized daphnids increased with temperature. Dependent on water hardness, a temperature rise of 7 to 28 °C resulted in a 5-fold to 12-fold higher amount of immobilized animals.

Phenol decreased the thermal tolerance of central stoneroller minnow (*Campostoma anomalum*). Chagnon and Hlohowskyj (1989) acclimated fish to 7.5 and 23 °C for at least two weeks, whereupon they were subjected to phenol. The CTM was determined after 48 h and at both acclimation temperatures the thermal tolerance was lowered in fish treated with phenol. Exposure to 12 mg phenol $L^{-1}$ decreased CTM values from 28.8 (control) to 27.1 °C and 35.8 (control) to 32.9 °C for fish acclimated at 7.5 and 23 °C, respectively. As temperature plays a major role in many physiological processes that depend on normal gill function, it is thought that phenol affects the gill tissue, resulting in the observed decrease in thermal tolerance.

Besides physiological factors, physical factors may be influenced by temperature changes as well, which may alter toxicity. This is illustrated by

Van Wezel and Jonker (1998), who used the lethal body burden (LBB) as an effect parameter. The LBB is defined as the molar total body concentration of contaminants causing death. In this article, the influence of temperature on the LBB of 1,2,4-trichlorobenzene in the three-spined stickleback (*Gasterosteus aculeatus*) was determined. At 17.0 and 22.5 °C, the LBB was significantly lower than at a temperature of 5 °C. The differences were 2-fold to 2.5-fold. This was explained by a temperature-dependent partitioning of the narcotic compound over different compartments in the sticklebacks. The compound mainly accumulates in storage lipids and membrane lipids. The toxic effect is thought to be determined by the concentration of the chemical in the membranes. The partitioning over storage lipid and water and over membrane lipid and water changes differently with temperature, implying that at lower temperatures more 1,2,4-trichlorobenzene has to be accumulated in the storage lipids before the critical concentration in the membrane lipids is reached, resulting in a higher LBB.

## Summary and conclusions

The studies reviewed above show that the relationship between toxicants and temperature is mostly investigated with crustaceans and fish as test organisms. In nearly all experiments thermal stress increased the adverse effects of substances, while results of experiments measuring the CTM indicated that exposure to toxicants decreased the ability of organisms to cope with elevated temperatures. Metal toxicity increased at rising temperatures except in one study with fish. This lack of effect was species specific, as the fish used belongs to a family known to be less sensitive to adverse environmental conditions. No clear relationship was observed between pesticide exposure and temperature, as positive, negative, and no correlations between temperature and toxicity were found. The thermal tolerance of fish was not changed by endothal in an acute test. As fish are not the target organisms of this herbicide, the tested concentrations could be too low to cause a response within the exposure period. Temperature did not affect toxicity of the pesticides dimethoate and imidacloprid. The neurotoxic action of these chemicals could mask the thermal effect. Natural substances were more detrimental at high than at low temperature, except for ammonia. Like dimethoate and imidacloprid, ammonia is capable of causing

neurotransmission failure, which may have masked the effects of temperature. The experiments conducted with less frequently studied substances subscribe the mostly reported finding of increased toxicity at elevated temperatures.

Many authors share our hypothesis that temperature potentiates the toxic effects of chemicals acting on metabolism, for example, energy demand and oxygen uptake and distribution. Further, we expected increased internal toxicant concentrations at elevated temperatures due to higher toxicant uptake rates. This idea is often subscribed, but unfortunately in only two studies the internal concentrations were measured. From those papers it is clear that in addition to higher toxicant accumulation due to increased metabolic rate other processes are involved as well, for example accessibility and sensitivity of the target site for chemicals and temperature dependency of the partitioning of narcotic compounds over body compartments. The reviewed literature did not make clear if detoxication and elimination rates increase at higher temperatures. However, the positive correlation between temperature and toxicants mostly observed suggests that these processes are of less importance.

## INFLUENCE OF NUTRITIONAL STATE

### Introduction

Nutritional state has a great impact on life history traits. In relation to toxicity testing, it may influence the sensitivity of organisms to toxic substances, as nutrition has a great impact on the physiological condition of organisms. In addition, sorption of toxicants to food particles may either decrease or increase bioavailability, depending on the route of uptake. Furthermore, metabolic rate is increased by higher food levels, which may influence toxicokinetics. Whether toxicity decreases or increases is determined by the overall effect on uptake, elimination, and detoxication rates. As it is unclear which of the processes described above has the highest impact on the toxicity of chemicals, we hope to detect a general finding in the literature published.

When crustaceans are used as test organisms, algae are added as a food source. In most articles, food availability is reported as number of cells $mL^{-1}$. This density does not contain information on the nutritional value of the algae, as was demonstrated by Naylor et al. (1992), who proposed standardization of food levels using carbon delivered per daphnid $d^{-1}$. Therefore, the results of different experiments can only be compared qualitatively.

### Detailed report of literature

The interaction between toxicants and nutritional state on the parameters mostly used are summarized in Annex 2.3. Figure 2.1 presents the frequency of occurrence of positive, negative, no correlations, and optima for joint effects of toxicants and nutritional state on mortality in the articles reviewed in the following section.

#### *Metals*

Studies investigating the interaction between cadmium toxicity and nutritional state, all used crustaceans as test organisms. Klüttgen and Ratte (1994) exposed *D. magna* chronically to cadmium at different food levels and found higher inhibition of juvenile development at lower food levels, whereas body length and brood size were more affected at high food levels. Compared with controls, exposure to 5 µg Cd $L^{-1}$ repressed juvenile development by 17% at $0.8 \cdot 10^6$ cells *Chlorella* $d^{-1}$, while no significant effect occurred at $16 \cdot 10^6$ cells $d^{-1}$. Body length decreased by 23 and 5% at high and low food levels, respectively. Brood size was inhibited by 69% at high food concentration, but no effect was observed at the low food level. Especially at the high food levels, mortality was lower in treated groups than in controls, which might be due to hormesis, a nonspecific positive reaction to low toxicant concentrations (Stebbing 1982). The intrinsic rate of natural increase was largely independent of algae concentration. Cadmium was thought to interfere with metabolic processes, indirectly affecting food uptake. The effects at high food levels may be explained by higher cadmium accumulation due to increased metabolism. Effects on juvenile

development were only detectable when food was scarce, because juveniles are only stressed by food depletion when algae levels become very low.

Other studies with cadmium were conducted by Chandini (1988a, b, 1989) where *Daphnia carinata* and *Echinisca triserialis* appeared to be more sensitive to cadmium when food levels decreased. At algal concentrations of 0.5, 1.5 and 4.5 · $10^6$ cells *Chlorella* $mL^{-1}$, 96-h LC50 values calculated were 70, 58 and 340 μg Cd $L^{-1}$ for *E. triserialis* and 110, 235 and 480 μg Cd $L^{-1}$ for *D. carinata* (Chandini 1988a). Chronic toxicity also depended on the nutritional state of the animals because cadmium was more damaging at low food concentration. For *E. triserialis*, estimated EC50 values for life history parameters such as longevity, life expectancy at birth, total fecundity, net reproductive rate, generation time, and intrinsic rate of natural increase were in the range of 2 to 13 and 4 to 21 μg Cd $L^{-1}$ at low and medium food doses, respectively (Chandini 1988b). At high food level no EC50 values could be assessed in the concentration range tested, as mortality was too low. EC50 values for the same parameters obtained for *D. carinata* ranged from 27 to 84 μg Cd $L^{-1}$ at low food conditions (Chandini 1989). At intermediate food levels EC50 values rose to a range of 51 to 150 μg Cd $L^{-1}$ and at high food levels values could only be estimated for fecundity and net reproductive rate at 102 and 87 μg Cd $L^{-1}$, respectively. Generation time was not included in this range, as an EC50 could only be estimated at low food level (162 μg Cd $L^{-1}$). Observations of the animals indicated that cadmium caused a disturbance of the feeding process. The decline in survival, growth, and reproduction was thought to be related to increased maintenance costs when daphnids were exposed to cadmium. At high algal densities the total food intake was not as much affected as at low food levels and cadmium effects were mitigated.

Besides the food concentration during experiments, maternal food levels may alter sensitivity of organisms to toxicants as well. Enserink et al. (1990, 1993) conducted acute (48 h) and chronic (21 d) toxicity tests with *D. magna* exposed to cadmium and chromium. After 48 h exposure to cadmium, mortality was three times higher in juveniles descendant from mothers held at high than at poor food conditions, which was related to body size. At low food levels less but large young were born, while mothers at high food density produced lots of small neonates. It was argued that this size-dependent response was either due to the existence of a detoxication mechanism, because costs for detoxication are more easily paid by large individuals

with high energy reserves, or by a more favorable surface to body volume ratio for large animals when the toxic action of the chemical depends on its uptake and distribution. No interactions were found in the other treatments, which makes the authors assume that other factors were of more importance in chronic than in acute experiments. The lack of response in daphnids treated with chromium was associated with a different mode of toxic action of this metal compared with that of cadmium.

Hall et al. (1989a, b) used algae as test organisms. Batch cultures of *Chlamydomonas geitleri* and *Chlorella vulgaris* and chemostat cultures of *C. vulgaris* were exposed to copper under phosphorus- or nitrogen-limited conditions. Although the chemostat cultures were more sensitive, in both kinds of cultures copper exposure induced a decline in cell numbers, which was more pronounced in phosphorus- than in nitrogen-limited cultures. As biomass remained constant, it was suggested that copper caused an uncoupling of cell growth and cell division. Final yield was significantly reduced in phosphorus-limited batch cultures. For *Chlorella*, internal copper concentrations were higher at phosphorus than at nitrogen limitation, probably due to increased cell permeability at low phosphorus concentrations. This was not seen in *Chlamydomonas*. Copper is thought to be detoxicated in intracellular polyphosphate granules, the formation of which depends upon the availability of phosphorus. At low phosphorus levels, the formation of these bodies and thus the detoxication of copper is decreased, resulting in greater sensitivity. Growth could even be further reduced when all available phosphorus is used for detoxication. Nott and Langston (1989) also proposed a role for polyphosphate granules in detoxication of chemicals in *Littorina littorea*, but this hypothesis has not yet been tested. Sunda and Huntsman (1998) found interactive effects of manganese and copper or zinc on *Thalassiosira pseudonana*, a coastal diatom. Both copper and zinc inhibited manganese uptake, resulting in a decreased growth rate at high copper or zinc and low manganese availability.

Koivisto et al. (1992) found an interaction between food level and copper toxicity to five cladoceran species: *Daphnia galeata, D. magna*, *D. pulex, Bosmina longirostris*, and *Chydorus sphaericus*. In all species, effects of copper on survival were amplified by low food concentrations.

Enserink et al. (1995) found interactive effects of lead and food concentration on size and age at first reproduction of *D. magna*. Lowest observed effect concentrations were independent of food levels. Lead

reduced body growth under both food conditions, but this delayed reproduction only at the low food level. Results were explained by increased energy costs for detoxication or repair in daphnids exposed to the metal.

### *Pesticides*

Barry et al. (1995) noted that sensitivity of *D. carinata* exposed to the insecticides endosulfan and esfenvalerate for 9 d depended on algal concentration. Food level did not influence the toxicity of endosulfan to daphnids with respect to mortality and length at maturity. However, age at maturity increased for daphnids exposed to pesticide and low food levels, while fecundity in the first brood was more affected at high than at low food levels. The more pronounced effects at higher food levels were thought to be due to enhanced metabolization of endosulfan by daphnids and algae, resulting in endosulfan sulfate which is a toxic and more persistent product, and increased toxicant uptake rates. Mortality in daphnids exposed to esfenfalerate at high food levels was not significantly different from controls, while there was 100% mortality at low food levels. Fecundity in the first brood and length and age at maturity were only analyzed for the highest food levels. No interaction between insecticide and food level was observed. The higher toxicity at lower food concentrations was explained by a change in bioavailability, as the amount of pesticide adsorbed to algae decreased at low algal density.

### *Natural toxicants*

Reinikainen et al. (1998) studied the effect of food on the tolerance of *D. pulex* to toxic cyanobacteria. When daphnids were exposed to *Microcystis aeruginosa* for 7 d, mortality increased more at low than at high levels of additional, nontoxic food. Egg mortality was also higher in daphnids that were starved before exposure to cyanobacteria than in well-fed daphnids (Reinikainen et al. 1995). Gilbert (1996) exposed rotifers, *Synchaeta pectinata*, and two clones of *Brachionus calyciflorus*, to different food conditions in the presence of cynaobacteria, *Anabaena flosaquae*. In *S. pectinata* exposed to *Anabaena*, both lifespan and fecundity were reduced to a greater extent at

low food concentration, with fecundity as the most sensitive parameter. In two separate experiments, *Anabaena* caused a decrease in fecundity of 29 to 90% at food levels of respectively $2 \cdot 10^4$ and $3 \cdot 10^3$ cells $mL^{-1}$ and of 61 to 95% at $2 \cdot 10^4$ and $5 \cdot 10^3$ cells $mL^{-1}$ of additional food. Only in one of the *Brachionus* clones an interaction between food and *Anabaena* was observed. Lifespan was affected more at low than at high concentrations of additional food. When fecundity was considered, the same clone showed a tendency to be more affected at low food levels, but there was no significant interaction. *Anabaena* contains an alkaloid neurotoxin, anatoxina, which inhibits the feeding process of daphnids. Besides this direct effect, filaments of *Anabaena* may physically interfere with filter feeding. The higher sensitivity to cyanotoxin at lower food levels was explained by the increase in relative concentration of these toxic algae as food levels decrease. In addition, well-fed animals are in a better physiological condition, which may lead to a higher tolerance to cyanobacteria. Walls et al. (1997) found a clear interaction between food concentration and exposure to cyanobacteria. Exposure of *D. pulex* to *Microcystis* for 48 h caused increased mortality at low food levels. No effect on growth was observed. In the same study, daphnids were exposed to a chemical released by *Chaoborus* (Diptera). Growth was not affected, but compared to controls, age at first reproduction decreased at the low food level. A negative effect of the chemical on the total number of offspring was observed under high food conditions, whereas this number slightly increased at the low food level. It was not clear why adverse effects were observed at high food levels and positive or indifferent effects at low food levels. The authors suggested that the increase in offspring number in the low food and *Chaoborus* treatment was caused by the earlier start of reproduction. In a similar experiment, Hanazato (1991b) observed helmet formation in *D. ambigua* as a defense against predation when exposed to a compound excreted by *Chaoborus*. Helmets were even formed when food levels became very low, but the size decreased, as well as growth, reproduction, and survival rates. These effects were probably induced by energy loss associated with helmet formation, resulting in a reduced tolerance to food deficiency.

### *Other toxicants*

Klüttgen et al. (1996) studied effects of 3,4-dichloroaniline on *D. magna* and *Ceriodaphnia quadrangula* under different food conditions. Treated *D. magna* held at low food density exhibited a delay in first reproduction compared with controls. At high food levels, *C. quadrangula* treated with the same substance started reproduction sooner compared with unexposed animals. Toxicant-induced inhibition of the feeding process was only observed in *D. magna*, suggesting that the more pronounced effect at low food concentration was caused by insufficient food intake. In both species, the number of offspring was reduced to a greater extent at low than at high food levels. However, in *C. quadrangula* slightly promoting effects occurred in the treatment with highest food and lowest toxicant concentration. As was already discussed (Enserink et al. 1990, 1993), the feeding condition of daphnids may influence the susceptibility of their young to chemicals. Baird et al. (1989) reported similar findings for *D. magna* exposed to 3,4-dichloroaniline. Offspring from mothers cultured at an algal concentration of 0.5 mg C $L^{-1}$ were about twice as vulnerable as those from mothers cultured at 0.05 mg C $L^{-1}$. 48-h LC50 values of 104 and 195 µg $L^{-1}$ were found for neonates descendant from mothers held at high and low food concentrations, respectively.

## Summary and conclusions

The papers summarized above reveal that food or nutrient levels influence the outcome of toxicity tests. For daphnids, there are also transgenerational effects, as the feeding condition of the mother affects the susceptibility of her young to toxicants. Daphnids from well-fed mothers were more sensitive than young from poor-fed mothers. In most studies, a negative relationship between nutritional state and toxicity was observed; decreasing food or nutrient levels resulted in increased toxicity of chemicals. Almost all researches used crustaceans as test organisms, but studies with algae and rotifers showed the same trend. Some studies found another interaction between toxicity and nutritional state. Adverse effects on growth and reproduction were observed in daphnids treated with cadmium under high food conditions. This was probably related to a higher metabolic rate caused by the elevated food level, resulting in an increased toxicant uptake

rate. The pesticide endosulfan was more toxic at high food levels, what was explained by an enhanced transformation of the pesticide into a more toxic product. It is also suggested that exudates from cyanobacteria have detrimental effects on daphnids, although this effect declined at high levels of nontoxic algae. Further, exposure to kairomone made daphnids reproduce sooner under low food conditions. The underlying mechanism causing this effect is unclear.

In only one study, differences in the bioavailability of the substance at varying food conditions accounted for the observed results. Most authors explained the increased toxicity at low food levels as a decreased ability to pay the energy costs for detoxication or repair mechanisms. On the other hand, insufficient food uptake caused by the inhibition of the feeding process itself was also suggested. In addition to the indirect relation between nutritional state and detoxication of chemicals, it was assumed that polyphosphate granules in algae were directly associated with detoxication of contaminants. Summarizing, we can conclude that generally the impact of food or nutrient level on an organism's physiological state is the most important factor determining the toxicity of chemicals.

## INFLUENCE OF SALINITY

### Introduction

Salinity fluctuations are common in estuaries, but might also occur in freshwater ecosystems as a result of mining activities. Processes for osmoregulation in freshwater differ from those in marine waters. The blood of freshwater organisms is hypertonic relative to their environment. Water flows into the body due to osmotic pressure and there is a passive loss of ions. $Na^+$ and $Cl^-$ ions determine plasma osmolarity and regulation of the ionic balance mainly occurs by active uptake of these ions from the ambient water. In contrast, the blood of marine organisms is hypotonic compared to the ambient water. The osmotic pressure causes inflow of ions and loss of water to the environment. To compensate for these processes, the organisms drink seawater and the excess of ions are excreted by the gills (Vonck 1999).

The pollution of marine environments occurs mainly in coastal areas (Forbes 1991), where organisms may be exposed to contaminants and

simultaneously experience highly fluctuating salinity. This explains why most literature dealing with the toxicant-salinity interaction considers estuarine organisms. This interaction is complex because salinity may act both on physiological processes as well as on the chemical itself; for example, salinity influences the chemical speciation of metals. A rise in salinity increases the degree of metal complexation, decreasing bioavailability (Riedel et al. 1985, Tsuda et al. 1990), and decreasing toxicity because the free ionic form of metals is thought to be most toxic (Bury et al. 1999, Karen et al. 1999). In addition, the increase in ionic strength at higher salt concentrations can decrease the activity of active toxic species. Increased ion concentration, associated with the rise in salinity, may also interfere biochemically with the toxicant (Riedel 1985). However, the decline in toxicity at rising salinity is not equal for all species and is probably caused by different modes of toxic action or pathways of toxicant entry (Bury et al. 1999, Karen et al. 1999). For instance, differences in osmoregulation in fresh and saltwater organisms results in species-specific accumulation and excretion of toxicants. In the light of these considerations, our expectation is that metal toxicity is enlarged at low salinities. When the concentration of the free metal ion is measured, or when other chemicals are used, we predict lowest toxicity at the isosmotic point of organisms, where the exchange of water and ions with the ambient water is least.

### Detailed report of literature

Hall and Anderson (1995) analyzed the effects of salinity on the sensitivity of aquatic organisms, mainly fish and crustaceans, to various classes of chemicals. In 55, 27, and 18% of the 173 articles, a negative, positive, and no correlation was reported, respectively. Metal toxicity was generally negatively correlated with salinity. Bioavailability and toxicity were found to correlate with the free metal ion concentration, which is higher at low salinity. No clear trend was observed for organic chemicals, except for organophosphate insecticides where a positive correlation between toxicity and salinity was found. They concluded that genetic, life history, and ecological factors should be considered when evaluating the influence of salinity on toxicity of chemicals. McLusky et al. (1986) reviewed literature on the interaction between salinity and temperature on the acute toxicity of metals to

marine and estuarine invertebrates. In general, acute toxicity increased as salinity declined and animals living near to their salinity limits were less tolerant to metal stress. Effects of salinity were explained by competition of metals with calcium and magnesium at uptake sites. Results of both reviews are shown in Annex 2.4. Only studies concerned with mortality as effect parameter are included and positive, negative, or no correlations and optima are indicated. Results of the present study are given in Annex 2.5, details and less common parameters are discussed in the following text. Figure 2.1 presents the relative number that a positive, negative, no correlation, and optimum occurs for the effects of toxicants and salinity on mortality in papers summarized in Hall and Anderson (1995), McLusky et al. (1986), and this review.

### *Metals*

Lin and Dunson (1993) reported enhanced cadmium toxicity in the mangrove-dwelling fish *Rivulus marmoratus* at low salinity. 96-h LC50 values for fish held in freshwater and water of 14‰ salinity were 2.96 and 21.12 mg total Cd $L^{-1}$. When the calculated free cadmium concentration was considered instead of total cadmium concentration, the salinity effect disappeared, indicating that changes in free ion concentration were responsible for the differences in mortality. Effect parameters like growth and mortality are most frequently used, but other parameters might be more sensitive. For instance, Vonck (1999) studied the osmoregulation of flounder (*Platichthys flesus*) when exposed to cadmium. Fish were adapted to freshwater, 50% seawater, full strength seawater, and to water fluctuating from freshwater to seawater and back in cycles of 84 h. After adaptation, cadmium was administered intragastrically. Cadmium effects were influenced by the ambient water salinity and the content of the stomach, which is influenced by drinking in seawater fish. At constant salinities, a cadmium concentration of 15 mg Cd $kg^{-1}$ fish caused a doubling of ion-transporting chloride cell density in freshwater fish from 158 to 320 cells $mm^{-2}$, while in 50% and full strength seawater this increase was only 40 and 27%, respectively. Serum osmolarity declined only in freshwater fish from 321 to 304 mOsmol $L^{-1}$. Cadmium exposure combined with fluctuating salinity resulted in a dose-dependent increase of chloride cell density. Cell densities increased

with 82 and 160% when fish were treated with 10 mg Cd $kg^{-1}$ fish during the freshwater and seawater phase of the salinity cycle, respectively. This increase in chloride cell density coincided with lower serum cadmium levels compared with fish exposed to the same cadmium concentration but experiencing constant salinity, which suggests that the chloride cells are involved in the removal of cadmium from the fish. The response to cadmium was thought to be concealed by the effects caused by salinity fluctuation, because no effect of the metal on serum osmolarity was observed. It was assumed that the stronger response in freshwater compared to seawater was due to an increase in free cadmium concentration, which is the most toxic form. Shazili (1995) also found cadmium sensitivity to increase at lower salinity. Seabass, *Lates calcarifer*, was acclimated to 5, 15, and 30‰ salinity for 2 weeks, after which cadmium was added. A concentration of 10 mg Cd $L^{-1}$ resulted in median lethal times (LT50) of 16.7, 163.3, and 458.3 h at the three salinities, respectively. 96-h LC50 values were 1.99, 14.2, and 19.0 mg Cd $L^{-1}$. The author explained the results by an increased fraction of cadmium present in its free anionic and most toxic form at low salinity.

Wildgust and Jones (1998) noted that an estuarine mysid was most tolerant to cadmium at optimal salinity. After acclimation to different salinities for 5 d, *Neomysis integer* was exposed to the free cadmium ion. Mortality was smallest in the isosmotic point of 20‰ salinity, where the 96-h LC50 was 34.4 µg $Cd^{2+}$ $L^{-1}$. At salinities of 12 and 28‰, these values decreased to 5.3 and 3.9 µg $Cd^{2+}$ $L^{-1}$, respectively. Differences in toxicity could not be completely explained by differences in cadmium speciation. Probably, the increase in osmotic stress outside the isosmotic point declined cadmium tolerance.

Forbes (1991) found a different effect of salinity on cadmium toxicity. Cadmium effects on growth of the estuarine gastropod *Hydrobia ventrosa*, cultured at 23‰ salinity, were most prominent at a salinity of 33‰, while at 13‰ no response of cadmium was observed as snails did not grow. At a salinity of 23‰, the growth of the control group followed a logistic growth model, while at the other treatments this model was no longer useful because of the highly size-dependent growth response to cadmium and salinity. The low salinity was thought to give such a severe stress that an additional cadmium stress was completely masked. The high toxicity at 33‰ salinity was explained by increased osmotic stress, making the snails less able to compensate the higher metabolic demand induced by cadmium.

### *Pesticides*

Song and Brown (1998) exposed salt marsh mosquitoes, *Aedes taeniorhynchus*, and brine shrimps (*Artemia* sp.) to four insecticides (aldicarb, dimethoate, imidacloprid, and tebufenozide). To determine salinity effects, the mosquitoes and shrimps were held in isosmotic and hyperosmotic water, which salinity was three and four times the salinity of the isosmotic water, respectively. Mortality in both species was less under isosmotic conditions than under hyperosmotic conditions. 72-h LC50 and 48-h LC50 values were estimated for animals held under isosmotic and hyperosmotic conditions, respectively. For *Aedes* tested at isosmotic and hyperosmotic conditions the following LC50 values were calculated: 0.20 and 0.15 mg aldicarb $L^{-1}$, 0.20 and 0.031 mg dimethoate $L^{-1}$, 0.021 and 0.013 mg imidacloprid $L^{-1}$ and 0.35 and 0.15 mg tebufenozide $L^{-1}$. For *Artemia*, LC50 values were 17.25 and 5.46 mg aldicarb $L^{-1}$ and 10.14 and 15.73 mg dimethoate $L^{-1}$ in isosmotic and hyperosmotic water, respectively. For imidacloprid and tebufenozide, the mortality at isosmotic salinity was too low to estimate LC50 values, but at high salinity LC50 values were 361.23 and 5.53 mg $L^{-1}$. Although the LC50 of dimethoate for *Artemia* was higher in the hyperosmotic environment, the total number of animals that died was less at isosmotic conditions. Identical slopes of the mortality response curves of *Aedes* exposed to aldicarb and tebufenozide and of *Artemia* exposed to imidacloprid at the two salinities indicated a similar response to these contaminants at both salinities. The authors assumed that increased physiological costs for osmoregulation decreased the overall fitness of the animals, reducing the tolerance to toxicants.

### *Other toxicants*

Palawski et al. (1985) exposed 35 day-old striped bass (*Morone saxatilis*) to a mixture of 18 organic and inorganic chemicals. Fish were held i.a. in freshwater with high water hardness and in saline water. 96-h LC50 values at 1 and 5‰ salinity were 1.5 and 3 times higher than in freshwater, respectively. A model was used to calculate the theoretical metal speciation of the inorganic fraction and results indicated that factors like water hardness, alkalinity, salinity, and pH played a major role in metal speciation. The

authors attribute the variation in mortality to this speciation in the test media, resulting in a different bioavailability of the metals.

Toxicity of narcotic compounds is supposed to depend on the toxicant concentration in the membranes. Van Wezel and Jonker (1998) reported that salinity had no influence on the lethal body burden of 1,2,4-trichlorobenzene in three-spined stickleback (*Gasterosteus aculeatus*), suggesting that salinity does not influence the direct toxicity of narcotic compounds.

### Summary and conclusions

Crustaceans and fish are commonly used as test organisms to investigate toxicity-salinity interactions. These interactions were not clear for all types of chemicals. Metal toxicity was often found to increase at low salinities. Severe osmotic stress at low salinities may explain the lack of cadmium effects on gastropods at low salinity, whereas effects were observed at high salinity. No clear relationship was observed for pesticides. An exception were the organophosphate insecticides, where toxicity increased at rising salinity. This could be caused by increased accumulation at high salinity relative to low salinity. Somasundaram and Coats (1991) and Walker (1976) (both in Ordelman et al. 1994) reported a higher degradation half-live of the organophosphate insecticide malathion in seawater (3 to 5 d) compared to freshwater (1 d).

As expected, the general finding was increased metal toxicity at lower salinity. This was associated with increased bioavailability, as competition of the metals with calcium and magnesium at uptake sites decreases and metal speciation declines with lower salinity. Studies using the free metal ion showed no interaction between the chemical and salinity or minimum toxicity at the isosmotic point. Except for the organophosphate insecticides, no clear interaction between other chemicals and salinity were observed. These responses were generally explained by increased physiological costs for osmoregulation, decreasing the overall fitness of organisms and making them less capable to endure toxic stress. This supports our hypothesis that toxicity is lowest in the isosmotic point of organisms.

## INFLUENCE OF COMBINED FACTORS

### Introduction

As illustrated above, few studies considered the effects of environmental factors on toxicity, but even less studies have investigated the combined action of these factors. Papers described in this section are dealing with the interaction between two environmental factors and their effect on toxicity. Regarding the statements made in previous sections, toxicity is expected to be potentiated at high temperature, poor food conditions, and salinities outside the organism's isosmotic point. Further, we expect effects of low food levels and suboptimal salinities to become worse at high temperatures, as metabolic needs increase. The lower energy intake and higher physiological costs for osmoregulation will result into a decline in the organism's fitness, increasing its sensitivity to toxicants.

### Detailed report of literature

#### *Temperature and nutritional state*

Besides food quantity, food quality is also likely to influence sensitivity of organisms to toxicants. Stephenson and Watts (1984) exposed *D. magna* to different temperatures and types of food for 21 d and exposed neonates that were born during the experiment to potassium dichromate. Although both temperature and nutritional value of the food had significant effects on mortality and reproduction of the mothers, neither 24-h nor 48-h EC50 values for immobilization indicated that temperature or food quality influenced the susceptibility of the neonates to potassium dichromate.

#### *Temperature and salinity*

The effects of temperature and salinity on the acute toxicity of metals have been reviewed by McLusky et al. (1986). Most studies were on crustaceans, with some references on mollusks, annelids, and hydrozoans. In general, cadmium and chromium toxicity increased with rising temperatures and

decreasing salinity. One study described effects of copper, lead, mercury, zinc, and nickel on crustaceans. Toxicity increased at high temperature, most notably at high salinity. At constant temperature, toxicity increased at decreasing salinity. The common observation of increased toxicity at high temperature and low salinity was not always seen when organisms were exposed to mercury. However, changes from an organism's optimal condition increased its sensitivity as was reported by several authors. Summarizing, sublethal concentrations at optimal environmental conditions became lethal at suboptimal conditions of temperature, salinity, or both.

McLusky and Hagerman (1987) investigated the survival of the mysid crustacean *Praunus flexuosus* exposed to chromium, nickel, and zinc at temperatures and salinities ranging from 5 to 15 °C and 4.5 to 27‰, respectively. Survival decreased at rising temperatures and salinities above or below the isosmotic point (20 to 24‰). Considering the combined action of the two environmental factors, all metals caused minimum toxicity at the isosmotic point and 5 °C. Maximum toxicity of chromium and nickel was found at 15 °C and 4.5‰ salinity, while zinc caused more adverse effects at the highest salinity. Mortality of animals was linked to a disruption of the osmoregulation process, but metal poisoning and lethality could also indirectly cause this disruption. Acclimation to the experimental salinities for over 3 weeks did not result in different responses to the metals.

Persoone et al. (1989) also investigated the combined action of temperature and salinity on toxicity. The brine shrimp *Artemia salina* and the rotifer *Brachionus plicatilis*, a marine and a brackish water organism, were exposed to potassium dichromate and sodium laurylsulfate, a detergent, at different combinations of temperature and salinity. Both temperature and salinity and their interaction affected toxicity, except for *B. plicatilis* exposed to laurylsulfate, where no significant interaction between the two environmental factors was observed. The effect of salinity on toxicity of dichromate to *A. salina* was temperature dependent; at low temperatures a decrease in salinity resulted in higher toxicity, but at high temperatures the same salinity change caused less effect. Toxicity increased at higher temperatures. Lowest and highest 24-h LC50 values were 8.8 mg $L^{-1}$ at 30 °C en 20‰ salinity and 291.5 mg $L^{-1}$ at 10 °C and 50‰ salinity, while under standard test conditions (25 °C and 35‰ salinity) a 24-h LC50 of 22.2 mg $L^{-1}$ was estimated. The toxicity of laurylsulfate increased at higher salinities and temperatures. Lowest and highest 24-h LC50 values were 7.2 mg $L^{-1}$ at 30 °C

and 50‰ and 154 mg $L^{-1}$ at 10 °C and 5‰, respectively. The LC50 at standard conditions was 21.5 mg $L^{-1}$. Dichromate was more toxic to *B. plicatilis* at lower salinity and higher temperature. Lowest and highest 24-h LC50 values were 130 mg $L^{-1}$ at 31 °C and 5‰ and 690 at 17 °C and 45‰, while under standard conditions a value of 347 mg $L^{-1}$ was found. Effects of laurylsulfate were more pronounced at high salinity and high temperature, but no interactive effect between these factors was found. Lowest LC50 values were estimated at 24 °C and 65‰ (10.9 mg $L^{-1}$) and highest values at 10 °C and 5‰ (29.4 mg $L^{-1}$). In comparison, the LC50 at standard conditions was 15.4 mg $L^{-1}$. In short, toxicity of both compounds was highest at high temperatures. Toxicity of dichromate was highest at low salinity, whereas laurylsulfate was more toxic at high salinity.

### *Nutritional state and salinity*

Riedel (1984) studied the toxicity of hexavalent chromium on the estuarine diatom *Thalassiosira pseudonana* as a function of both salinity and sulfate. At a constant salinity of 3.20‰, concentrations causing 50% reduction of growth rate decreased from 6.56 to 0.40 µM chromium at 2.90 and 0.15 mM sulfate, respectively. A 50% reduction in fluorescence yield, a measure for photosystem efficiency, was obtained at 4.94 to 0.34 µM chromium, respectively. At 0.32‰ salinity and sulfate concentrations between 0.38 and 0.02 mM, EC50 values for growth and fluorescence yield ranged from 0.75 to 0.04 and 0.73 to 0.02 µM chromium, respectively. Inhibition was a function of the chromium to sulfate molar ratio: inhibition occurred when this ratio exceeded 500:1. Adverse effects of chromium were thought to be caused by inhibition of sulfate uptake. To confirm this assumption, the relationship between chromium toxicity and chromium and sulfate uptake in *T. pseudonana* was investigated (Riedel 1985). Chromium affected growth, especially at low sulfate concentrations. Sulfate uptake rates declined at rising chromium concentrations, but it was not clear whether slower uptake reduced growth or if slower growth decreased sulfate uptake. The author speculated that chromium is taken up by sulfate transport systems. The sulfate uptake rate and the ratio of chromium to sulfate would then determine the chromium uptake rate. In the cells, the hexavalent chromium is reduced to trivalent chromium by oxidation of organic molecules. Adverse effects

caused by chromate may be due to the oxidation of essential molecules, or the production of trivalent chromium, that has a high affinity for proteins. When cell damage by oxidization or the trivalent chromium concentration in the cells reached a certain threshold, growth ceased.

### Summary and conclusions

In summary, a small number of studies investigated the influence of combinations of environmental factors on toxicity. Most experiments were conducted with crustaceans. The lack of information on this topic hinders the detection of clear relationships. Maternal exposure to different food sources and temperatures did not influence the sensitivity of neonates. The combined action of temperature and salinity seemed to intensify their separate effects on toxicity. High temperature and low salinity resulted in major toxic effects of chemicals. No clear interaction could be observed between nutritional state and salinity; it seemed that toxic effects were mainly related to nutrition and not to salinity. Despite the lack of data, it appears that toxic effects are more pronounced at suboptimal than optimal conditions.

## QUANTITATIVE RELATIONSHIPS

If available, data were collected from papers reviewed above and fitted to the models described in Equation 2.1 and 2.2 given in the methods section. The calculated coefficients are given in Annex 2.6. In Table 2.2, the ranges and averages of the coefficients are given for the effect parameters mostly used.

The two different coefficients for temperature, $\beta_T$ and $\gamma_T$, are not comparable, as different models were used to fit the data. Nevertheless, the positive values for $\beta_T$ and negative values for $\gamma_T$, indicate a positive relation between toxicity and temperature, as $\beta_T$ is inversely and $\gamma_T$ directly related to temperature. The $\beta_T$ coefficients were divided into four groups: 24-h EC50, 48-h EC50, 24-h LC50, and 48-h LC50. A t-test was performed to reveal which groups were significantly different (95% confidence interval). The results

are given in Figure 2.2A. The thermal effect on toxicity appears to increase with exposure time, although this is not significant for the LC50 values.

The negative relation between nutritional state and toxicity becomes manifest in the positive values of the coefficient ($\gamma_N$) for parameters as growth, reproduction, survival after chronic exposure, 48-h LC50, and 96-h LC50. The variation in coefficient values for reproduction is probably due to the diversity of effect parameters used. Similar to temperature, exposure time determines to which extent nutritional state influences toxicity. This is depicted for the LC50 values in Figure 2.2B. The combined action of nutritional state and salinity was determined with growth as effect parameter. The calculated $\gamma_N$ values are higher compared to those for growth in studies where nutrition is the only environmental variable. Differences in test organisms could account for this variation; in the study with nutrition and salinity, algae were used, while in the others zooplankton was used.

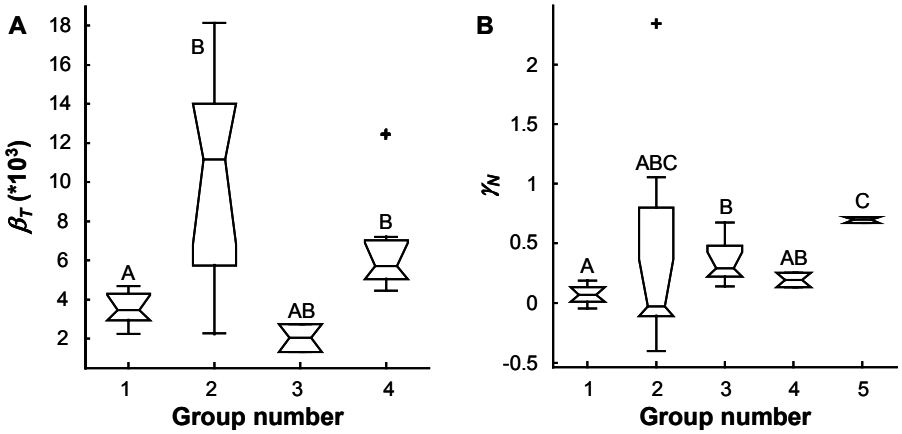


**Figure 2.2.** Distributions of temperature coefficients ($\beta_T$) for 24-h EC50 (group 1), 48-h EC50 (group 2), 24-h LC50 (group 3), and 48-h LC50 (group 4) as effect parameter (A) and of coefficients for nutritional state ($\gamma_N$) with respect to growth (group 1), reproduction (group 2), survival after chronic exposure (group 3), 48-h LC50 (group 4), and 96-h LC50 (group 5) as effect parameter (B). The box has horizontal lines at the lower quartile, median, and upper quartile values. The vertical lines (whiskers) show the extent of the rest of the data. The notches give an estimate of the uncertainty about the means for box-to-box comparison. Values differing more than 1.5 times the interquartile range are outliers, depicted by a plus. Dissimilar letters indicate groups that are significantly different (95% confidence interval).

For salinity, only LC50 values are available. The values for the coefficient ($\gamma_S$) are mostly positive, indicating decreased toxicity at higher salinities.

One exception is a study with the free cadmium ion, where the coefficient has a negative value. Experiments dealing with the combined effects of temperature and salinity on toxicity all used LC50 as effect parameter. Both positive and negative values were found for $\gamma_S$. The negative average value is mainly determined by the negative values obtained from experiments with laurylsulfate.

The coefficients for single factors give an indication of the validity of toxicity data obtained from standard toxicity tests under field situations. The standard tests are usually conducted at 20 °C, while the temperature in natural ecosystems may be much lower or higher, for instance, in waters situated in temperate regions or receiving discharges of cooling water. Considering the average value of the coefficients calculated for temperature, a temperature reduction of 20 to 3.6 °C declines toxicity by a factor of 5.2, 130, 2.6, and 22, while a rise of 20 to 30 °C increases toxicity by a factor of 2.5, 15, 1.7, and 5.6 for 24-h EC50, 48-h EC50, 24-h LC50, and 48-h LC50, respectively. In laboratory tests, food or nutrient availability is ad libitum, while this is less favorable under field conditions. Therefore, a food level of 3 mg C $L^{-1}$, which is used in standard toxicity tests for daphnids (OECD 1998), was compared with toxicity at 0.375 and 0.109 mg C $L^{-1}$, which are average biomasses of green algae in an eutrophic and an oligotrophic Dutch lake during the summer season, respectively (Hallegraeff 1976, Lammens 1999). According to the average value of the calculated coefficients for nutritional state, this means that toxicity in eutrophic and oligotrophic natural systems would be 1.2 to 1.3, 2.1 to 3.3, 2.1 to 3.2, 1.5 to 1.9, and 4.2 to 10 times higher than toxicity determined under standard test conditions, with respect to growth, reproduction, survival after chronic exposure, 48-h LC50, and 96-h LC50, respectively. For salinity, the change in toxicity was estimated by assuming an extreme salinity increase from 1‰ in the laboratory to 35‰ in the field. This results in a 2.1 times lower toxicity under field conditions than in the laboratory.

**Table 2.2.** Ranges and averages of coefficients for temperature ($\beta_T$ and $\gamma_T$), nutritional state ($\gamma_N$) and salinity ($\gamma_S$) (see Equations 2.1 and 2.2), arranged according to effect parameters mostly used[a]

| Factor | Effect parameter | n | Coefficient range | | | Coefficient average ± SD | | |
|---|---|---|---|---|---|---|---|---|
| Temperature | 24-h EC50 | 8 | $2.24 \cdot 10^3$ | - $4.71 \cdot 10^3$ | | $3.55 \cdot 10^3$ | ± $8.7 \cdot 10^2$ | |
| | 48-h EC50 | 9 | $2.20 \cdot 10^3$ | - $1.81 \cdot 10^4$ | | $1.04 \cdot 10^4$ | ± $5.4 \cdot 10^3$ | |
| | 24-h LC50 | 2 | $1.33 \cdot 10^3$ | - $2.75 \cdot 10^3$ | | $2.04 \cdot 10^3$ | ± $1.0 \cdot 10^3$ | |
| | 48-h LC50 | 7 | $4.47 \cdot 10^3$ | - $1.25 \cdot 10^4$ | | $6.66 \cdot 10^3$ | ± $2.7 \cdot 10^3$ | |
| Nutritional state | Growth | 4 | -0.0459 | - 0.188 | | 0.0701 | ± 0.096 | |
| | Reproduction | 13 | -0.402 | - 2.36 | | 0.357 | ± 0.76 | |
| | Survival | 9 | 0.136 | - 0.676 | | 0.347 | ± 0.18 | |
| | 48-h LC50 | 2 | 0.131 | - 0.253 | | 0.192 | ± 0.086 | |
| | 96-h LC50 | 2 | 0.671 | - 0.719 | | 0.695 | ± 0.035 | |
| Salinity | 96-h LC50 | 12 | -0.0339 | - 1.31 | | 0.209 | ± 0.36 | |
| Nutritional state and salinity | EC50[b] | 2 | 0.935 | - 0.973 | (nutrition) | 0.954 | ± 0.27 | (nutrition) |
| | | | 0.982 | - 1.08 | (salinity) | 1.03 | ± 0.069 | (salinity) |
| Temperature and salinity | 24-h LC50 | 4 | -43.3 | - -3.73 | (temperature) | -18.6 | ± 19 | (temperature) |
| | | | -0.582 | - 0.447 | (salinity) | -0.0948 | ± 0.43 | (salinity) |

[a]Symbols indicate the following: n, number of experiments; SD, standard deviation.
[b]Exposure time not reported.

## SYNTHESIS AND DISCUSSION

### Introduction

Although standard toxicity tests are conducted at constant and favorable experimental conditions, results reported in this paper reveal that variable and suboptimal conditions may modify the outcome of toxicity tests. Elevated ambient temperatures were shown to increase toxicity of various classes of chemicals to aquatic organisms. In accordance, exposure to toxicants decreased the thermal tolerance. Further, toxicity of contaminants was usually increased under less favorable nutritional conditions. The influence of salinity on toxicity was less clear, although toxic effects of metals were mostly found to increase in less saline waters, whereas toxicity of organophosphate insecticides increased with higher salinity.

Three previous reviews dealt with multiple stressors. Cairns et al. (1975) studied the interaction between contaminants and temperature and concluded that no generalizations can be made because very little evidence is available. Comparing these conclusions with the findings of the present article, it appears that the amount of literature published has increased considerably since. Hall and Anderson (1995) discussed the interactive effects of contaminants and salinity on aquatic organisms and share our conclusion on the influence of salinity on the speciation of metals and the associated effect on toxicity. Further, they concluded that the different toxicity of chemicals for marine, brackish, and freshwater organisms is determined by physiological characteristics of the test species. This supports our idea of minimal toxicity at the isosmotic point of organisms, which is species specific. McLusky et al. (1986) reviewed literature dealing with the influence of temperature and salinity on acute toxicity of metals to marine and estuarine invertebrates. They found similar correlations as reported in the present article. Their conclusion that toxicity values obtained under fixed temperature and salinity regimes are unsuitable to evaluate the influence of these environmental conditions on metal toxicity agrees with our results, as well as the view that organisms near to their tolerance limits are less capable to withstand toxic stress. Although the newly reviewed articles did not modify the conclusions made in the previous reviews, we think that this new information provides greater

insight into the mechanisms involved in multiple stress interactions. These mechanisms will be discussed in the following sections.

The effects of toxicants on organisms are determined by the bioavailability and toxicokinetics of the substance and the sensitivity of organisms. To understand the interaction between toxicants and environmental conditions, it is important to understand the influence of these conditions on the factors described above. The underlying mechanisms for the observed interactions involve both physical and physiological processes.

### Bioavailability

The studied environmental factors may influence the bioavailability of substances. For aquatic organisms, exposure to toxicants mainly occurs via the ambient water. As the solubility of substances is temperature dependent, varying water temperature influences the bioavailability of toxicants. Food level may determine availability of chemicals as they may adsorb to particulate organic matter, such as algae. The route of exposure determines whether toxicity increases or decreases. Salinity is especially important for the bioavailability of metals. In freshwater, metals mainly exist in the dissociated and most available form, while at higher salinity metals are bound to ligands. Indeed, the few studies that used free metal ion concentration showed either no salinity effect or minimum toxicity at intermediate salinity (Lin and Dunson 1993, Wildgust and Jones 1998). Another interaction between metals and salinity is competition of metals with calcium and magnesium at uptake sites, resulting in decreased bioavailability at high salinity (McLusky et al. 1986). The amount of chemical accumulated by organisms is also determined by the residence time of the chemical. Salinity may influence this process, as the degradation half-life of an organophosphate insecticide was higher in saline water relative to freshwater (Somasundaram and Coats 1991, Walker 1976 [both in Ordelman et al. 1994]).

### Toxicokinetics

Temperature may alter physical factors such as partition coefficients or diffusion rates, in that way affecting toxicokinetics. Modifications in the partitioning of substances over different body parts may result in higher accumulation of the toxicant at the target site, leading to lower effect concentrations (Van Wezel and Jonker 1998). Increased diffusion rates may result into higher toxicant uptake rates. Physiological processes may influence toxicokinetics as well. In ectotherms, an increase of ambient temperature leads to increased metabolic rate. High food or nutrient levels may also raise metabolism in organisms. This may result in higher toxicant uptake rates. Elimination and detoxication mechanisms may counteract the thermal effect on uptake rate, as the rates of these processes may increase as well. The regularly reported increase in toxicity when temperature rises or nutritional condition improves suggests that the temperature effect on uptake rates is of more importance than the effect on elimination and detoxication rates. On the contrary, other processes might be involved as well, and it is unclear to which extent increased uptake rates contribute to the elevated toxicity.

The effects of salinity on uptake rates are complicated because osmoregulation plays a major role. Wright (1995) discussed the influence of salinity on metal uptake in estuarine species. In most studies reviewed, a negative relationship between salinity and uptake rates was observed. Besides salinity effects on metal speciation, metals affected the ionic balance in crustaceans and fish. Gills seem to be the primary site for metal uptake and high concentrations of ion-transporting chloride cells are present in the gills. This and an apparent competition between metals and calcium made Wright suggest a common route of uptake for metals and calcium. The degree to which metal and calcium uptake are coupled may differ between species as well as within species, because calcium needs vary between different developmental stages. Vonck (1999) also reported species-specific effects of salinity on chloride cell density. In addition, Rainbow (1995) reported that some crustaceans could vary metal uptake rates by changing their water permeability. Different ionic selectivity of the gills at low and high salinity, as well as lower apparent water permeability of crabs acclimated to low salinity were observed by Laporte et al. (1997). It was unclear how these factors influence metal uptake. Some studies (in Wright [1995]) mentioned urine as a metal excretory route, especially in freshwater

animals where osmotic inflow of water is compensated by increased urine production. As literature is scarce, the effect of this factor on metal accumulation is unclear.

In contrast to metals, the influence of salinity on uptake of other chemicals was unclear. As salinity does not affect speciation of these chemicals, this subscribes the idea that metal uptake rates are determined by speciation. At the isosmotic point, the uptake of substances other than metals is likely to be decreased, as the inflow and drinking of water and ion exchange is reduced. Thomas and Rice (1986) described the influence of drinking rate on the accumulation of organic chemicals. They measured higher toluene and naphthalene accumulation in the salmonid dolly varden (*Salvelinus malma*) in saline water (30‰) compared with freshwater. This was associated with the increased drinking rate of fish in seawater, resulting in a shorter stomach emptying time and increased uptake of the chemicals from the gut. The elimination of the hydrocarbons by the gills and the urine was also lower in seawater.

### Sensitivity of organisms

Variations in environmental factors may also change toxic effect concentrations by altering the sensitivity of target sites, as was reported for temperature (Fisher and Wadleigh 1985). On the other hand, many organisms are able to regulate the internal toxicant concentration, either by increasing elimination, detoxication, or storage in specific body parts. Because these regulation mechanisms cost energy, less energy is available for other processes (Smit and Van Gestel 1997). It is often assumed in bioenergetics that net assimilated energy is first allocated to maintenance, leaving less energy available for growth or reproduction (Calow 1979, Koehn and Bayne 1989, Kooijman 1993, Kooijman and Metz 1984, Nisbet and Gurney 1989). Geyer et al. (1985, 1990, 1993a, b) observed less negative effects in animals with high fat content relative to animals with low fat content. This indeed indicates that well-fed organisms exposed to toxicants can pay the energy costs for coping with toxic effects more easily than those held at poor food conditions. Maternal food also proved to influence the sensitivity of juvenile daphnids to toxicants (Baird et al. 1989, Enserink et al. 1990, 1993). The maternal lipid reserves are higher in large, less sensitive juveniles (born

from poor-fed mothers) compared with small, sensitive ones (born from well-fed mothers) (Tessier and Consolatti 1989), which may be associated with the available energy to withstand toxic stress. When organisms are exposed to narcotic chemicals for a short time period, adverse effects may also be lower in individuals with a higher fat content because a larger amount of the chemical is stored in the fat compartment, leaving less available to reach the target site. Osmotic stress may also induce lower tolerance to chemicals because of increased energy costs for osmoregulation (i.e. maintenance costs).

### Quantitative relationships

Some of the interactions between chemicals and environmental factors that were described above were also analyzed quantitatively. The calculated coefficients suggest that effects of temperature on toxicity increase with exposure time. This can be understood because toxicant uptake rates are elevated at high temperatures due to increased metabolism. The difference in uptake rates at low and high temperatures results in an increase of toxic effects at the elevated temperature. This difference in toxicity becomes more apparent when the exposure time is extended. The influence of nutritional state on toxicity was determined by exposure time as well. Compared with organisms held at low food levels, well-fed organisms are in a better physiological condition, which makes them more resistant to toxic stress. The difference in toxicity increases when the organisms are exposed to the contaminant for a longer period of time.

The coefficients calculated for the influence of temperature, nutritional state, and salinity on toxicity give an indication of the magnitude of uncertainty factors applicable for laboratory to field extrapolations. Considering temperature, toxicity appears to be smaller than in the laboratory when extrapolating to ecosystems located in temperate regions, which would result in an uncertainty factor smaller than one. When extrapolating to systems nearby discharge points of cooling water, toxicity in the laboratory seems to be underestimated, resulting in an uncertainty factor greater than one. For nutritional state, toxicity under suboptimal conditions appears to be higher than expected from results of laboratory tests; hence the uncer-

tainty factor should be greater than one. An extreme salinity increase from freshwater to saline water results in an uncertainty factor smaller than one.

Chapman et al. (1998) state that uncertainty factors are often overprotective and they recommend a factor that does not exceed the value of 10 and may be much less. Considering the coefficients calculated here, this factor of 10 appears to be sufficient in most situations and it even seems that organisms will be overprotected. However, such an uncertainty factor is not high enough to protect organisms from thermal effects when extrapolating 48-h EC50 values to ecosystems exposed to higher temperatures than in the laboratory, while it is just sufficient for the extrapolation of 96-h LC50 values obtained at favorable food levels to food conditions in an oligotrophic lake. Most lakes in the Netherlands are eutrophic and the food conditions in those lakes will be more favorable. This does not imply that the nutrition-toxicant interaction is unimportant in eutrophicated waters, because food scarcity may even occur in eutrophicated waters, when the population size has reached the carrying capacity of the system (Buikema et al. 1980 [in Klüttgen et al. 1996], Kooijman and Metz 1984). Even if problems with toxicants are absent, they may become manifest when algal concentrations decrease due to declined nutrient inputs. This might be useful to consider before investing effort in reducing nutrient concentrations in surface waters. The factor of 10 seems to be high enough to translate results from standard tests conducted in freshwater to marine systems. However, because freshwater organisms do not occur in marine waters, this extrapolation is irrelevant. When extrapolating toxicity data to freshwaters with elevated salt concentrations, the toxicity change is probably much smaller. Further, as the interaction between salinity and toxicants is not clear, it remains uncertain what a sufficient value of the uncertainty factor will be.

As data were scarce, the coefficients only give an indication of the influence of the environmental factors on toxicity and the outcomes should not be overvalued. Table 2.1 shows that the choice for an uncertainty factor greater or smaller than one is determined by many factors. In this review only one factor was studied: the relation between toxicity of substances and some environmental conditions.

### Final considerations and research needs

It is good to realize that if organisms are exposed to extremely stressful environmental conditions, this may result in such adverse effects that additional stress by chemicals may be overshadowed. Problems may arise when using quantitative endpoints such as growth or reproduction. Further, many studies reviewed used mortality as an endpoint. The lethal toxicant concentrations used in these experiments are often too high to be environmentally realistic. However, the observed interactions were generally the same for studies using lethal and sublethal endpoints, suggesting that the type of correlation between toxicity and environmental factors is independent of the toxicant concentration. In addition, the interaction between environmental factors and toxicants is not constant in natural systems, as environmental conditions, toxicant concentrations, and the physiological state and metabolic needs of organisms may change in time. Nevertheless, the present review shows that environmental factors may modify the toxicity of chemicals to aquatic organisms. Organisms living near their environmental tolerance limits are more susceptible to additional stressors like toxicants and concentrations that are sublethal to organisms living at optimal environmental conditions may become lethal at suboptimal conditions.

In order to obtain uncertainty factors that sufficiently protect natural systems without being overprotective, additional research is necessary. Because elevated temperatures accelerate both toxicokinetics and physiological processses, exposure time should be based on a physiological time scale instead of using fixed test durations prescribed by standard procedures (Donker et al. 1998). This means that exposure times should be adjusted for the different temperatures tested. The influence of acclimating an organism to new experimental conditions before performing the toxicity test should also be considered. For instance, acclimation may decrease the effect of temperature on toxicity, as enzymes are slightly modified by temperature changes. This process takes time, depending on species and body size (Kooijman 1993). Instead of what was usually reported in the reviewed articles, studies performed to investigate the influence of salinity on metal toxicity should include the measurement of the free ion concentration. This would improve the ability to detect mechanisms in the case of changes in toxic effects. Both studies on individuals and populations

are useful, as the first type of studies may provide an understanding of mechanisms involved in multiple stress interactions, while the second type is ecologically more relevant and will help to acquire more accurate uncertainty factors for the extrapolation of laboratory data to field situations. Environmental conditions may alter toxicokinetics, as described above. However, it is still unclear to which extent uptake, elimination and detoxication rates are modified. Measurement of internal toxicant concentrations may help to obtain more insight into these processes and their influence on toxicity.

## CONCLUSIONS

The present review indicates that environmental factors may affect the toxicity of chemicals to aquatic organisms. Organisms living under environmental conditions that are near to their tolerance limits are often less resistant to additional stressors, like exposure to pollution. Generally, toxicity increased with increasing temperature and decreasing food or nutrient supply. Metal toxicity increased with decreasing salinity. To a great extent this was related to chemical speciation. On the other hand, toxicity of organophosphate insecticides increased at higher salinity, possibly due to differences in degradation rates. The influence of salinity on the toxicity of other chemicals was unclear. Both physical and physiological processes influencing factors like bioavailability, toxicokinetics, and sensitivity of organisms should be considered when evaluating these interactions. As standard toxicity tests do not take variable and unfavorable experimental conditions into account, results of these tests are less realistic for toxicity in field situations.

Quantitative analysis of data reveals that an uncertainty factor for laboratory to field extrapolations should be smaller than one when extrapolating to ecosystems in temperate regions, while it should be greater than one for systems receiving discharges of cooling water. When considering nutritional effects, the factor should be greater than one. On the other hand, the factor should be smaller than one when translating results of standard toxicity tests conducted in freshwater to freshwaters with elevated salt concentrations. The toxicity under laboratory and relevant field conditions differed by a factor of 2.6 to 130 and 1.3 to 15 for the two temperature con-

ditions and 1.2 to 10 for nutritional state, dependent on the effect parameter considered. An extreme salinity change from freshwater to marine water decreased toxicity by a factor of 2.1, but as less extreme salinity changes are more relevant under field conditions, the change in toxicity is probably much smaller. Additional research is necessary to obtain uncertainty factors that will sufficiently protect ecosystems without being overly conservative.

## ACKNOWLEDGEMENTS

The study was supported by the Institute for Inland Water Management and Waste Water Treatment. We wish to express our thanks to Gert-Jan de Maagd for valuable comments on a previous version of the manuscript.

**Annex 2.1.** Number of experiments where positive[a], negative[b], no correlations[c] (none), or optima[d] were reported for joint effects of toxicants and temperature on mortality. Summarized from Cairns et al. (1975) and McLusky et al. (1986)

| | Type of correlation | | | |
|---|---|---|---|---|
| Toxicant | Positive | Negative | Optimum | None |
| Metals | | | | |
| Cadmium | 15 | | | 2 |
| Chromium | 3 | | | 2 |
| Copper | 6 | | | 2 |
| Lead | 2 | | | 1 |
| Magnesium | 1 | | | |
| Mercury | 7 | 4 | 1 | 4 |
| Nickel | 3 | | | 2 |
| Zinc | 30 | | 1 | 2 |
| Pesticides | | | | |
| Chlorinated hydrocarbons | 18 | 7 | 1[e] | |
| Herbicides | 2 | | | |
| Organophosphates | 1 | 1 | | |
| Natural toxicants | | | | |
| Ammonia | 2 | | | 2 |
| Other toxicants | | | | |
| Cyanide | 4 | 1 | | 1 |
| Phenols | 1 | 1 | | |
| Synthetic detergents | 3 | 1 | | 2 |

[a]Toxicity increased with increasing temperature.
[b]Toxicity increased with decreasing temperature.
[c]No clear relationship between toxicity and temperature.
[d]Toxicity decreased at intermediate temperatures.
[e]Toxicity increased at intermediate temperatures.

**Annex 2.2.** Overview of positive[a] (+), negative[b] (-), and no correlations[c] (0) between toxicity and temperature with respect to different effect parameters

| Toxicant | Species | Mortality | CTM[4] | Reference |
|---|---|---|---|---|
| Metals | | | | |
| Cadmium | *Lepomis cyanellus* | | 0 | Carrier and Beitinger (1984) |
| Cadmium | *Macrobrachium rosenbergii* | | - | Rosas and Ramirez (1993) |
| Chromium | *Macrobrachium rosenbergii* | | - | |
| Copper | *Actinonaias pectorosa* | + | | Jacobson et al. (1997) |
| Dichromate | *Daphnia magna* | | | Persoone et al. (1989) |
| Dichromate | *Tisbe longicornis* | + | | Larrain et al. (1998) |
| Nickel | *Oncorhynchus kisutch* | | - | Becker and Wolford (1980) |
| Nickel | *Salmo gairdneri* | | - | |
| Pesticides | | | | |
| Aldicarb | *Aedes aegypti* | + | | Song et al. (1997) |
| Aldicarb | *Daphnia magna* | + | | |
| Dimethoate | *Aedes aegypti* | 0 | | |
| Dimethoate | *Daphnia magna* | 0 | | |
| Endothal | *Notropis lutrensis* | | 0 | Takle et al. (1983) |
| Imidacloprid | *Aedes aegypti* | 0 | | Song et al. (1997) |
| Imidacloprid | *Daphnia magna* | 0 | | |
| Lindane | *Chironomus riparius* | + | | Fisher and Wadleigh (1985) |
| Tebufenozide | *Aedes aegypti* | + | | Song et al. (1997) |
| Tebufenozide | *Daphnia magna* | + | | |
| Natural toxicants | | | | |
| Ammonia | *Penaeus setiferus* | | 0 | Alcaraz et al. (1997) |
| Ammonia/nitrite | *Penaeus setiferus* | | - | |
| Cyanobacteria | *Daphnia pulex* (clone 1) | 0 | | Hietala et al. (1997) |
| Cyanobacteria | *Daphnia pulex* (clone 2) | 0 | | |
| Nitrite | *Penaeus setiferus* | | - | Alcaraz et al. (1997) |

**Annex 2.2.** (continued)

| Toxicant | Species | Mortality | CTM[d] | Reference |
|---|---|---|---|---|
| Other toxicants | | | | |
| Phenol | *Campostoma anomalum* | | - | Chagnon and Hlohowskyj (1989) |
| Laurylsulfate | *Daphnia magna* | + | | Persoone et al. (1989) |
| 1,2,4-Trichlorobenzene | *Gasterosteus aculeatus* | + | | Van Wezel and Jonker (1998) |

[a]Toxicity increased with increasing temperature.
[b]Toxicity increased with decreasing temperature.
[c]No clear relationship between toxicity and temperature.
[d]Critical thermal maximum.

**Annex 2.3.** Overview of positive[a] (+), negative[b] (-) and no correlations[c] (0) between toxicity and nutritional state with respect to different effect parameters

| Toxicant | Species | Nutrition | Growth | Reproduction | Mortality | References |
|---|---|---|---|---|---|---|
| Metals | | | | | | |
| Cadmium | *Daphnia carinata* | Algae | | | - | Chandini (1988a) |
| Cadmium | *Daphnia carinata* | Algae | - | - | - | Chandini (1989) |
| Cadmium | *Daphnia magna* | Algae | + (body length)<br>- (juv. developm.) | + (brood size)<br>- (age at mat.) | | Klüttgen and Ratte (1994) |
| Cadmium | *Daphnia magna* | Algae[d] | | | + (acute)<br>0 (chronic) | Enserink et al. (1990) |
| Cadmium | *Echinisca triserialis* | Algae | | | - | Chandini (1988a) |
| Cadmium | *Echinisca triserialis* | Algae | | - | - | Chandini (1988b) |
| Chromium | *Daphnia magna* | Algae[d] | | | 0 | Enserink et al. (1990, 1993) |
| Copper | *Bosmina longirostris* | Algae | | | - | Koivisto et al. (1992) |
| Copper | *Chlamydomonas geitleri* | N/P | 0 | - | | Hall et al. (1989a) |
| Copper | *Chlorella vulgaris* | N/P | 0 | - | | Hall et al. (1989a,b) |
| Copper | *Chydorus sphaericus* | Algae | | | - | Koivisto et al. (1992) |
| Copper | *Daphnia galeata* | Algae | | | - | |
| Copper | *Daphnia magna* | Algae | | | - | |
| Copper | *Daphnia pulex* | Algae | | | - | |
| Copper | *Thalassiosira pseudonana* | Mn | - | | | Sunda and Huntsman (1998) |
| Lead | *Daphnia magna* | Algae | - | - | | Enserink et al. (1995) |
| Zinc | *Thalassiosira pseudonana* | Mn | - | | | Sunda and Huntsman (1998) |
| Pesticides | | | | | | |
| Endosulfan | *Daphnia carinata* | Algae | 0 | + (brood size)<br>- (age at mat.)<br>0 (fecundity) | 0 | Barry et al. (1995) |
| Esfenvalerate | *Daphnia carinata* | Algae | 0 | 0 | - | |

**Annex 2.3.** (continued)

| Toxicant | Species | Nutrition | Growth | Reproduction | Mortality | References |
|---|---|---|---|---|---|---|
| Natural toxicants | | | | | | |
| Cyanobacteria | *Brachionus calyciflorus* (clone 1) | Algae[e] | | 0 | - | Gilbert (1996) |
| Cyanobacteria | *Brachionus calyciflorus* (clone 2) | Algae[e] | | 0 | 0 | |
| Cyanobacteria | *Daphnia pulex* | Algae[e] | | - | | Reinikainen et al. (1995) |
| Cyanobacteria | *Daphnia pulex* | Algae[e] | | | - | Reinikainen et al. (1998) |
| Cyanobacteria | *Daphnia pulex* | Algae[e] | 0 | | - | Walls et al. (1997) |
| Cyanobacteria | *Synchaeta pectinata* | Algae[e] | | - | - | Gilbert (1996) |
| Kairomone | *Daphnia ambigua* | Algae | - | - | - | Hanazato (1991a) |
| Kairomone | *Daphnia pulex* | Algae | 0 | + | | Walls et al. (1997) |
| Other toxicants | | | | | | |
| 3,4-Dichloroaniline | *Ceriodaphnia quadrangula* | Algae | | + (age at mat.)<br>- (brood size) | | Klüttgen et al. (1996) |
| 3,4-Dichloroaniline | *Daphnia magna* | Algae | | - | | |
| 3,4-Dichloroaniline | *Daphnia magna* | Algae[d] | | | + | Baird et al. (1989) |

[a]Toxicity increased with increasing food or nutrient level with respect to effect parameter.
[b]Toxicity increased with decreasing food or nutrient level with respect to effect parameter.
[c]No relationship between toxicity and food or nutrient level.
[d]Algae concentration at which mothers of test animals were cultured.
[e]Additional algae concentration added.

**Annex 2.4.** Number of experiment where positive[a], negative[b], no correlations[c] (none) or optima[d] were reported for joint effects of toxicants and salinity on mortality. Summarized from Hall and Anderson (1995) and McLusky et al. (1986)

| | Type of correlation | | | |
|---|---|---|---|---|
| Toxicant | Positive | Negative | Optimum | None |
| Metals | | | | |
| Arsenic | | | | 3 |
| Cadmium | 2 | 25 | 4 | 7 |
| Chromium | | 6 | | |
| Copper | 2 | 6 | 1 | 2 |
| Lead | | 2 | | 1 |
| Mercury | | 10 | 4 | 2 |
| Nickel | | 7 | | |
| Silver | | | | 1 |
| Zinc | 1 | 7 | 3 | 1 |
| Pesticides | | | | |
| Biocides | 4 | 6 | 1 | |
| Herbicides | 1 | 1 | | |
| Organophosphates | 11 | 1 | | 5 |
| Chlorinated hydrocarbons | 5 | 7 | 1 | 4 |
| Pyrethroids | 1 | 1 | 2 | |
| Carbamates | | 1 | | 1 |
| Other toxicants | | | | |
| Petroleum hydrocarbons | | | | 1 |
| Polycyclic aromatic hydrocarbons | 2 | 1 | 1 | 1 |
| Organic chemicals | 2 | 6 | 2 | 1 |

[a]Toxicity increased with increasing salinity.
[b]Toxicity increased with decreasing salinity.
[c]No relationship between toxicity and salinity.
[d]Minimum toxicity decreased at intermediate salinity.

**Annex 2.5.** Overview of positive[a] (+), negative[b] (-), no correlations[c] (0), or optima[d] (opt.) between toxicity and salinity with respect to mortality

| Toxicant | Species | Mortality | References |
|---|---|---|---|
| Metals | | | |
| Cadmium | *Rivulus marmoratus* | - | Lin and Dunson (1993) |
| Cadmium ($Cd^{2+}$) | *Rivulus marmoratus* | 0 | |
| Cadmium | *Lates calcarifer* | - | Shazili (1995) |
| Cadmium ($Cd^{2+}$) | *Neomysis integer* | opt. | Wildgust and Jones (1998) |
| Pesticides | | | |
| Aldicarb | *Aedes taeniorhynchus* | + | Song and Brown (1998) |
| Aldicarb | *Artemia* sp. | + | |
| Dimethoate | *Aedes taeniorhynchus* | + | |
| Dimethoate | *Artemia* sp. | + | |
| Imidacloprid | *Aedes taeniorhynchus* | + | |
| Imidacloprid | *Artemia* sp. | + | |
| Tebufenozide | *Aedes taeniorhynchus* | + | |
| Tebufenozide | *Artemia* sp. | + | |
| Other toxicants | | | |
| 1,2,4-Trichlorobenzene | *Gasterosteus aculeatus* | 0 | Van Wezel and Jonker (1998) |
| Mixture of organic and inorganic chemicals | *Morone saxatilis* | - | Palawski et al. (1985) |

[a]Toxicity increased with increasing salinity.
[b]Toxicity increased with decreasing salinity.
[c]No relationship between toxicity and salinity.
[d]Minimum toxicity at intermediate salinity.

**Annex 2.6.** Quantitative relations between toxicity of chemicals and temperature, nutritional state, and salinity or combinations of these factors[a]

| Factor | Species | Toxicant | Effect parameter | Exposure time |
|---|---|---|---|---|
| Temperature | *Daphnia magna* | Aldicarb | LC50 | 48 h |
| | *Actinonaias pectorosa* | Copper | LC50 | 24 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia pulex* | Cyanobacteria | EC50 | 48 h |
| | *Daphnia magna* | Dichromate | EC50 | 24 h |
| | *Daphnia magna* | Dichromate | EC50 | 24 h |
| | *Daphnia magna* | Dichromate | EC50 | 24 h |
| | *Daphnia magna* | Dichromate | EC50 | 24 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Tisbe longicornis* | Dichromate | LC50 | 48 h |
| | *Daphnia magna* | Laurylsulfate | EC50 | 24 h |
| | *Daphnia magna* | Laurylsulfate | EC50 | 24 h |
| | *Daphnia magna* | Laurylsulfate | EC50 | 24 h |
| | *Daphnia magna* | Laurylsulfate | EC50 | 24 h |
| | *Chironomus riparius* | Lindane | LC50 | 24 h |
| | *Gasterosteus aculeatus* | 1,2,4-Tetra-chlorobenzene | LBB (wet weight) | |
| Nutritional state | *Daphnia magna* | Cadmium | Body length | Chronic |
| | *Daphnia magna* | Cadmium | Brood size | Chronic |
| | *Daphnia carinata* | Cadmium | LC50 | 48 h |
| | *Daphnia carinata* | Cadmium | LC50 | 96 h |
| | *Echinisca triserialis* | Cadmium | LC50 | 48 h |
| | *Echinisca triserialis* | Cadmium | LC50 | 96 h |
| | *Echinisca triserialis* | Cadmium | Life span | Chronic |
| | *Echinisca triserialis* | Cadmium | Longevity | Chronic |
| | *Echinisca triserialis* | Cadmium | Adult size | Chronic |
| | *Echinisca triserialis* | Cadmium | Size at maturity | Chronic |
| | *Echinisca triserialis* | Cadmium | Neonate size | Chronic |
| | *Echinisca triserialis* | Cadmium | Fecundity | Chronic |
| | *Daphnia carinata* | Cadmium | Life span | Chronic |
| | *Daphnia carinata* | Cadmium | Longevity | Chronic |
| | *Daphnia carinata* | Cadmium | Size at maturity | Chronic |
| | *Daphnia carinata* | Cadmium | Fecundity | Chronic |
| | *Daphnia pulex* | Cyanobacteria | Survival time | Chronic |
| | *Daphnia pulex* | Cyanobacteria | Egg mortality | Chronic |
| | *Brachionus calyciflorus* | Cyanobacteria | Life span | Chronic |

| Number of data points | Intercept ($\alpha_1$ or $\alpha_2$) | Coeff. for environm. factor ($\beta_T$, $\gamma_T$, $\gamma_N$, or $\gamma_S$) | Coeff. for toxicant ($\gamma_c$) | R-square | Reference |
|---|---|---|---|---|---|
| 2 | -42.7 | $1.25 \cdot 10^4$ | | n.s.[b] | Song et al. (1997) |
| 4 | -7.60 | $2.75 \cdot 10^3$ | | 0.991 | Jacobson et al. (1997) |
| 2 | -32.8 | $9.70 \cdot 10^3$ | | n.s. | Hietala et al. (1997) |
| 2 | -45.8 | $1.35 \cdot 10^4$ | | n.s. | Hietala et al. (1997) |
| 2 | -61.7 | $1.81 \cdot 10^4$ | | n.s. | Hietala et al. (1997) |
| 2 | -53.3 | $1.56 \cdot 10^4$ | | n.s. | Hietala et al. (1997) |
| 2 | -45.5 | $1.34 \cdot 10^4$ | | n.s. | Hietala et al. (1997) |
| 2 | -38.4 | $1.12 \cdot 10^4$ | | n.s. | Hietala et al. (1997) |
| 2 | -22.4 | $6.31 \cdot 10^3$ | | n.s. | Hietala et al. (1997) |
| 2 | -14.8 | $4.07 \cdot 10^3$ | | n.s. | Hietala et al. (1997) |
| 2 | -8.62 | $2.20 \cdot 10^3$ | | n.s. | Hietala et al. (1997) |
| 4 | -16.8 | $4.63 \cdot 10^3$ | | 0.963 | Persoone et al. (1989) |
| 4 | -7.55 | $2.24 \cdot 10^3$ | | 0.968 | Persoone et al. (1989) |
| 4 | -10.3 | $3.11 \cdot 10^3$ | | 0.890 | Persoone et al. (1989) |
| 4 | -13.2 | $3.98 \cdot 10^3$ | | 0.956 | Persoone et al. (1989) |
| 3 | -14.3 | $4.47 \cdot 10^3$ | | 0.936 | Larrain et al. (1998) |
| 3 | -21.3 | $6.51 \cdot 10^3$ | | 0.943 | Larrain et al. (1998) |
| 3 | -15.9 | $4.96 \cdot 10^3$ | | 0.975 | Larrain et al. (1998) |
| 3 | -17.0 | $5.28 \cdot 10^3$ | | 0.969 | Larrain et al. (1998) |
| 3 | -23.7 | $7.20 \cdot 10^3$ | | 0.972 | Larrain et al. (1998) |
| 3 | -18.6 | $5.72 \cdot 10^3$ | | 0.962 | Larrain et al. (1998) |
| 4 | -14.9 | $4.71 \cdot 10^3$ | | 0.951 | Persoone et al. (1989) |
| 4 | -8.04 | $2.80 \cdot 10^3$ | | 0.970 | Persoone et al. (1989) |
| 4 | -11.4 | $3.76 \cdot 10^3$ | | 0.921 | Persoone et al. (1989) |
| 4 | -9.43 | $3.21 \cdot 10^3$ | | 0.821 | Persoone et al. (1989) |
| 3 | -3.56 | $1.33 \cdot 10^3$ | | 0.744 | Fisher and Wadleigh (1985) |
| 3 | -4.26 | $1.40 \cdot 10^3$ | | 0.945 | Van Wezel and Jonker (1998) |
| 16 | -0.0610 | -0.0459 | -0.0536 | 0.781 | Klüttgen and Ratte (1994) |
| 16 | 0.581 | -0.402 | -0.442 | 0.865 | Klüttgen and Ratte (1994) |
| 2 | 2.66 | 0.253 | | n.s. | Chandini (1988a) |
| 3 | 2.68 | 0.671 | | 1.00 | Chandini (1988a) |
| 3 | 2.65 | 0.131 | | 0.919 | Chandini (1988a) |
| 3 | 2.39 | 0.719 | | 0.662 | Chandini (1988a) |
| 12 | 0.370 | 0.676 | -0.503 | 0.859 | Chandini (1988b) |
| 12 | 0.0937 | 0.182 | -0.188 | 0.878 | Chandini (1988b) |
| 12 | 0.0333 | 0.0740 | -0.0788 | 0.813 | Chandini (1988b) |
| 12 | 0.0435 | 0.0642 | $-7.80 \cdot 10^{-3}$ | 0.540 | Chandini (1988b) |
| 12 | $-5.30 \cdot 10^{-3}$ | 0.0164 | -0.0275 | 0.280 | Chandini (1988b) |
| 12 | 0.800 | 0.962 | -1.26 | 0.762 | Chandini (1988b) |
| 12 | 0.935 | 0.550 | -0.474 | 0.817 | Chandini (1989) |
| 12 | 0.949 | 0.360 | -0.509 | 0.834 | Chandini (1989) |
| 6 | 1.33 | 0.188 | -0.637 | 0.843 | Chandini (1989) |
| 12 | 8.15 | 2.36 | -4.14 | 0.724 | Chandini (1989) |
| 6 | 1.34 | 0.234 | -0.334 | 0.874 | Reinikainen et al. (1998) |
| 6 | -0.793 | -0.238 | 0.363 | 0.685 | Reinikainen et al. (1995) |
| 3 | -0.179 | 0.455 | | 0.807 | Gilbert (1996) |

**Annex 2.6.** (continued)

| Factor | Species | Toxicant | Effect parameter | Exposure time |
|---|---|---|---|---|
| Nutritional state | *Synchaeta pectinata* | Cyanobacteria | Life span | Chronic |
| | *Synchaeta pectinata* | Cyanobacteria | Fecundity | Chronic |
| | *Synchaeta pectinata* | Cyanobacteria | Life span | Chronic |
| | *Synchaeta pectinata* | Cyanobacteria | Fecundity | Chronic |
| | *Daphnia carinata* | Endosulfan | First brood size | Chronic |
| | *Daphnia carinata* | Endosulfan | Age at maturity | Chronic |
| | *Daphnia pulex* | Kairomone | Age at maturity | Chronic |
| | *Daphnia pulex* | Kairomone | Fecundity | Chronic |
| | *Daphnia magna* | Kairomone | Age at maturity | Chronic |
| | *Daphnia magna* | Kairomone | Fecundity | Chronic |
| | *Ceriodaphnia quadrangula* | Kairomone | Age at maturity | Chronic |
| | *Ceriodaphnia quadrangula* | Kairomone | Fecundity | Chronic |
| Salinity | *Rivulus marmoratus* | Cadmium | LC50 | 96 h |
| | *Lates calcarifer* | Cadmium | LC50 | 96 h |
| | *Lates calcarifer* | Cadmium | LC50 | 12 d |
| | *Lates calcarifer* | Cadmium | LC50 | 21 d |
| | *Morone saxatilis* | Cadmium | LC50 | 96 h |
| | *Neomysis integer* | Cadmium ($Cd^{2+}$) | LC50 | 96 h |
| | *Morone saxatilis* | Carbaryl | LC50 | 96 h |
| | *Morone saxatilis* | Chromium | LC50 | 96 h |
| | *Morone saxatilis* | Copper | LC50 | 96 h |
| | *Morone saxatilis* | Malathion | LC50 | 96 h |
| | *Morone saxatilis* | Nickel | LC50 | 96 h |
| | *Morone saxatilis* | Selenium | LC50 | 96 h |
| | *Morone saxatilis* | Toxaphene | LC50 | 96 h |
| | *Morone saxatilis* | Zinc | LC50 | 96 h |
| Nutritional state and salinity | *Thalassiosira pseudonana* | Chromium | EC50 | Not reported |
| | *Thalassiosira pseudonana* | Chromium | EC50 | Not reported |
| Temperature and salinity | *Praunus flexuosus* | Chromium | LC50 | 48 h |
| | *Praunus flexuosus* | Chromium | LC50 | 96 h |
| | *Praunus flexuosus* | Chromium | LC50 | 192 h |
| | *Artemia salinia* | Dichromate | LC50 | 24 h |
| | *Brachionus plicatilis* | Dichromate | LC50 | 24 h |
| | *Artemia salinia* | Laurylsulfate | LC50 | 24 h |
| | *Brachionus plicatilis* | Laurylsulfate | LC50 | 24 h |

[a]Data were fitted to Equation 2.1 and 2.2 (see text).

[b]n.s., not significant, as only two data points were available.

| Number of data points | Intercept ($\alpha_1$ or $\alpha_2$) | Coeff. for environm. factor ($\beta_T$, $\gamma_T$, $\gamma_N$ or $\gamma_S$) | Coeff. for toxicant ($\gamma_c$) | R-square | Reference |
|---|---|---|---|---|---|
| 3 | $8.33 \cdot 10^{-3}$ | 0.291 | | 0.527 | Gilbert (1996) |
| 3 | 0.0162 | 0.744 | | 0.768 | Gilbert (1996) |
| 3 | -0.144 | 0.136 | | 0.318 | Gilbert (1996) |
| 2 | -0.220 | 1.06 | | n.s. | Gilbert (1996) |
| 5 | -0.188 | -0.162 | | 0.664 | Barry et al. (1995) |
| 5 | -0.0120 | -0.0361 | | 0.452 | Barry et al. (1995) |
| 2 | 0.0325 | 0.0644 | | n.s. | Walls et al. (1997) |
| 2 | -0.123 | -0.200 | | n.s. | Walls et al. (1997) |
| 9 | 0.0569 | -0.0936 | -0.0337 | 0.501 | Klüttgen et al. (1996) |
| 6 | 0.720 | 0.443 | -0.891 | 0.662 | Klüttgen et al. (1996) |
| 9 | -0.0496 | -0.0555 | -0.0298 | 0.477 | Klüttgen et al. (1996) |
| 6 | 0.291 | -0.032 | -1.12 | 0.546 | Klüttgen et al. (1996) |
| 2 | 1.13 | 0.166 | | N.S. | Lin and Dunson (1993) |
| 3 | -0.551 | 1.31 | | 0.926 | Shazili (1995) |
| 3 | -1.24 | 1.698 | | 0.940 | Shazili (1995) |
| 2 | -1.55 | 1.66 | | n.s. | Shazili (1995) |
| 2 | 1.88 | 0.318 | | n.s. | Palawski et al. (1985) |
| 3 | 0.994 | -0.0339 | | $1.50 \cdot 10^{-4}$ | Wildgust and Jones (1998) |
| 2 | 3.36 | 0.120 | | n.s. | Palawski et al. (1985) |
| 2 | 4.76 | 0.0791 | | n.s. | Palawski et al. (1985) |
| 2 | 2.28 | 0.0697 | | n.s. | Palawski et al. (1985) |
| 2 | 1.81 | 0.106 | | n.s. | Palawski et al. (1985) |
| 2 | 4.32 | 0.183 | | n.s. | Palawski et al. (1985) |
| 2 | 3.19 | 0.0170 | | n.s. | Palawski et al. (1985) |
| 2 | 0.881 | 0.0371 | | n.s. | Palawski et al. (1985) |
| 2 | 2.63 | 0.139 | | n.s. | Palawski et al. (1985) |
| 10 | 0.314 | 0.935 (nutrition)<br>0.982 (salinity) | | 0.987 | Riedel (1984) |
| 10 | 0.171 | 0.973 (nutrition)<br>1.08 (salinity) | | 0.966 | Riedel (1984) |
| 12 | 20.3 | -8.15 (temp.)<br>0.824 (salinity) | | 0.892 | McLusky and Hagerman (1987) |
| 12 | 27.2 | -11.1 (temp.)<br>0.901 (salinity) | | 0.857 | McLusky and Hagerman (1987) |
| 10 | 24.9 | -10.0 (temp.)<br>0.422 (salinity) | | 0.562 | McLusky and Hagerman (1987) |
| 20 | 109 | -43.3 (temp.)<br>-0.0104 (salinity) | | 0.841 | Persoone et al. (1989) |
| 16 | 12.3 | -4.22 (temp.)<br>0.447 (salinity) | | 0.796 | Persoone et al. (1989) |
| 20 | 54.5 | -21.1 (temp.)<br>-0.582 (salinity) | | 0.892 | Persoone et al. (1989) |
| 16 | 10.7 | -3.73 (temp.)<br>-0.234 (salinity) | | 0.827 | Persoone et al. (1989) |

# *Chapter 3*

# Temperature-Dependent Effects of Cadmium on *Daphnia magna*: Accumulation versus Sensitivity

Heugens EHW, Jager T, Creyghton R, Kraak MHS, Hendriks AJ, Van Straalen NM, Admiraal W. 2003. *Environmental Science and Technology* 37(10): 2145-2151. With slight modifications.

## ABSTRACT

Standard toxicity tests are performed at one constant, optimal temperature (usually 20 °C), while in the field variable and suboptimal temperatures may occur. Lack of knowledge on the interactions between chemicals and temperature hampers the extrapolation of laboratory toxicity data to ecosystems. Therefore, the aim of this study was to analyze the effects of temperature on cadmium toxicity to the water flea *Daphnia magna* and to address possible processes responsible for temperature-dependent toxicity. This was investigated by performing standard toxicity tests with *D. magna* under a wide temperature range. Thermal effects on accumulation kinetics were determined by estimating uptake and elimination rates from accumulation experiments. To study temperature dependency of the intrinsic sensitivity of the daphnids to cadmium, the DEBtox model was used to estimate internal threshold concentrations (ITCs) and killing rates from the toxicity and accumulation data. The results revealed that increasing temperature lowered the ITC and increased the killing rate and the uptake rate of the metal. Enhanced sensitivity of *D. magna* was shown to be the primary factor for temperature-dependent toxicity. Since temperature has such a major impact on toxicity, a temperature correction may be necessary when translating toxicity data from the laboratory to the field.


## INTRODUCTION

Standard toxicity tests performed in the laboratory are used extensively to predict the effects of chemicals in ecosystems. These toxicity tests are mostly performed at a constant and favorable temperature, usually 20 °C, while in the field temperature is highly variable because of season and climate. As most aquatic organisms are ectotherms, temperature is an important factor having a high impact on the rate of most physiological processes. This may have great effects on the exposure of organisms to toxicants. For instance, differences in the ambient temperature may affect uptake, elimination, and detoxication rates because of changes in metabolic, locomotory, and feeding activity of organisms (Cairns et al. 1975, Smit and Van Gestel 1997, Donker et al. 1998, Fisher et al. 1999). Besides alterations in exposure, the sensitivity of organisms to chemicals may be modified by changes in the physiological

condition, for example, by the induction of cold- or heat-protective proteins. Further, close to the thermal tolerance limits, temperature stress may enlarge adverse effects of toxicants (Cairns et al. 1975, Donker et al. 1998, Heugens et al. 2001).

A number of reviews considered the joint effects of contaminants and temperature (Cairns et al. 1975, McLusky et al. 1986, Heugens et al. 2001) and revealed that temperature is of major importance for the outcome of toxicity tests. Although many authors cited in these reviews proposed underlying mechanisms responsible for the observed interactions between temperature and chemicals, such as altered accumulation kinetics and sensitivity of the organisms, few studies have actually tested these hypotheses. The aim of this study was therefore to analyze the influence of temperature on the acute toxicity of metals and to address the processes responsible for a possible temperature-dependent toxicity. With this purpose, the water flea *Daphnia magna* was exposed to a range of water temperatures, and thermal effects on toxicity and accumulation kinetics of cadmium were determined. The influence of temperature on the intrinsic sensitivity of the daphnids to cadmium was evaluated by relating tissue cadmium concentrations to toxic effects in time, assuming that the tissue concentration determines the effect. It is hypothesized here that, if temperature only affects accumulation kinetics of chemicals, then the intrinsic sensitivity should be the same for all temperature regimes. This hypothesis was investigated with the mathematical model DEBtox (Bedaux and Kooijman 1994). DEBtox is able to describe time-dependent toxicity data, which contains information about the dynamic aspect of the occurrence of effects. The model was adapted to fit the data from the toxicity and accumulation experiments simultaneously to reveal if thermal effects on cadmium toxicity resulted from changes in accumulation kinetics or intrinsic sensitivity of the daphnids or both.

## MATERIALS AND METHODS

### Culture conditions

The *Daphnia magna* population used in the present study was obtained from the Institute for Inland Water Management and Waste Water Treatment (RIZA, Lelystad, the Netherlands), where it was cultured for several years.

The culture consisted of cohorts with a density of 20 daphnids $L^{-1}$. A cohort was kept for 4 to 5 weeks, after which a new cohort was started with at least third-brood neonates (<24 h). Artificial Elendt M7 medium was used for culturing (OECD 1998). The medium was renewed three times a week and juveniles were removed. The culture was maintained under a light-dark regime of 16:8 h and at a temperature of 20 °C. On working days, the daphnids were fed with 2.0 mg C $L^{-1}$ of a concentrated suspension of *Selenastrum capricornutum*. The algae were cultured in a chemostat in Woods Hole medium (Guillard 1975). Every week, algae were harvested and centrifuged at 3000 rpm for 10 min. The supernatant was removed and the algae were resuspended in Elendt M7 medium, after which the total organic carbon concentration of the suspension was measured with a total carbon analyzer (College Station, TX). The suspension was stored at 4 °C in a dark room until used for feeding.

## Experimental design

To study temperature-dependent toxicity and to distinguish between thermal effects on accumulation kinetics and sensitivity of the daphnids, the following experiments were conducted:

*Influence of temperature on toxicity*

To study temperature effects on cadmium toxicity to the daphnids, acute toxicity tests at a temperature range of 10 to 35 °C were performed.

*Influence of temperature on accumulation kinetics*

To examine how temperature affects accumulation kinetics, short-term accumulation experiments were executed at 10 to 26 °C. This temperature range was chosen because at these temperatures no control mortality occurred because of temperature stress during the test period.

*Influence of temperature on sensitivity*

(a) To detect if the sensitivity of the daphnids is altered by temperature, time-dependent toxicity tests at 10 to 26 °C were performed. In these tests, the survival of the daphnids was measured at several exposure times, in contrast to the toxicity experiments at 10 to 35 °C described above.

(b) The results of (a) were combined with the outcomes of the accumulation experiments and analyzed with the DEBtox model. To ensure that the cadmium accumulation pattern was independent of the exposure concentration, a short-term accumulation experiment was performed at 20 °C in which cadmium accumulation was studied at exposure concentrations used in the time-dependent toxicity tests.

The daphnids were not acclimated to the test temperature prior to exposure because acclimation may result in differentiation between individuals. During the acclimation period, the daphnids at the higher temperatures will reach a larger body size than those at the lower temperatures, leading to variation in the initial body size of the daphnids, which hampers the interpretation of the test results. Furthermore, acclimation of animals to temperatures in the higher temperature range may prove to be useless because animals exposed to these stressful temperatures are likely to die within the acclimation period.

## Toxicity tests

### *Acute toxicity tests at 10 to 35 °C*

Acute toxicity tests were performed in accordance with standard protocols (OECD 1984), except where noted. Test animals used were at least third-brood neonates. Groups of five daphnids (<24 h) were assigned to 60-mL polypropylene tubes containing 50 mL of test medium without food. Per temperature, at least six cadmium concentrations were tested, obtained by diluting a solution of cadmium chloride (Titrisol, Merck) in Elendt M7 medium. The test tubes were placed at 10, 13, 16, 20, 23, and 26 °C (two replicates per treatment) and at 29, 32, and 35 °C (three replicates per treatment). A light-dark regime of 16:8 h was applied. After 24 and 48 h, the number of animals not responding to gentle stimulation with a pipet was scored. Those animals were considered to be dead. To determine the actual cadmium concentrations in the water, 1-mL water samples of each treatment were taken in duplicate after 1, 24, and 48 h. The samples were acidified with 20 µL of 65% nitric acid (Merck, p.a.) and analyzed by air-acetylene flame (Perkin-Elmer 1100B) or graphite furnace atomic absorption

spectrometry (AAS) (Perkin-Elmer 5100PC/HGA600/AS60), depending on the metal concentration in the samples. The oxygen concentration in the test solutions during the experiment was at least 86% of the air saturation value at the temperature used.

### *Time-dependent toxicity tests at 10 to 26 °C*

Toxicity tests were performed at 10, 20, and 26 °C following the procedure described above. However, in these experiments, the survival of the daphnids was scored twice a day during 96 h of exposure (three replicates per temperature and cadmium treatment). Each day, water samples were taken in duplicate for measurement of the actual cadmium concentration.

## Accumulation experiments

### *Short-term accumulation experiments at 10 to 26 °C*

The influence of temperature on cadmium accumulation was studied in neonates (<24 h) descending from at least the third brood. Three groups of 60 and three groups of approximately 200 daphnids were randomly transferred to 600-mL glass beakers containing 500 mL of Elendt M7 medium (control) and test medium with an actual concentration of 101 ± 0.24 (SE) µg Cd $L^{-1}$ Elendt M7 medium, respectively. One control and one cadmium-containing beaker were placed at each of the selected temperatures: 10, 20, and 26 °C. After 2, 5, 8, 24, and 45 h, 8 to 15 cadmium-exposed daphnids were collected from each temperature treatment. Control animals were gathered at the start of the experiment and when a treatment was ended. Only daphnids that were not killed or immobilized were used. Therefore, the number of animals gathered at the end of the experiment could be smaller than at the beginning, but enough animals survived the treatment to ensure a reliable analysis of the cadmium concentrations. Depending on the number of daphnids available, the number of replicates per exposure time and temperature was two or three. The collected daphnids were kept in a beaker containing clean, double-distilled water for 10 min, pooled in 2-mL polyethylene tubes, lyophilized, weighed, and digested in 65% nitric

acid (J.T. Baker, Ultrex) and 30% hydrogen peroxide (Fluka, purum p.a.) using the micro-destruction method described in Timmermans et al. (1989). The concentrated samples were diluted with 500 µL of acidified analytical grade water (5 mL of 65% nitric acid $L^{-1}$ [J.T. Baker, Ultrex]) and analyzed by graphite furnace AAS (Perkin-Elmer 5100PC/HGA600/AS60). Water samples (1 mL, in triplicate) were taken after 1, 24, and 45 h. After acidification with 20 µL of 65% nitric acid (Merck, p.a.), the actual cadmium concentration in the samples was analyzed by air-acetylene flame AAS (Perkin-Elmer 1100B).

### *Short-term accumulation experiments at 20 °C*

The accumulation experiments at 10 to 26 °C described above focused on the accumulation pattern of cadmium at only one exposure concentration (101 µg Cd $L^{-1}$). To verify if the uptake and elimination rate constants were independent of the cadmium concentration, a simplified accumulation experiment at 20 °C was performed. The same procedure as was described for the accumulation experiments at 10 to 26 °C was used, with the following exceptions. One group of 90 and three groups of 270 daphnids (<24 h) were allocated to 600-mL glass beakers containing 500 mL of Elendt M7 medium (control) and test media with actual concentrations of 217 ± 0.39, 452 ± 0.59, and 1112 ± 2.2 µg Cd $L^{-1}$ Elendt M7 medium, respectively. All beakers were placed at a temperature of 20 °C. After 0 and 72 h (control); 24, 48, and 72 h (217 µg Cd $L^{-1}$); 5, 24, and 48 h (452 µg Cd $L^{-1}$); and 5, 10, and 24 h (1112 µg Cd $L^{-1}$), 15 mobile animals were collected in triplicate and analyzed for cadmium tissue concentration following the method described above. The choice for these exposure times was based on the outcomes of the toxicity tests: the effects of cadmium on survival of the daphnids started between the first and the last sampling time. Water samples (1 mL) were taken in triplicate daily for measurement of the actual cadmium concentration.

## Data analysis

### *Acute toxicity tests at 10 to 35 °C*

Thermal effects on acute cadmium toxicity were assessed by calculating 24- and 48-h LC50 values for all temperatures by means of nonlinear regression, using the logistic response model (Haanstra et al. 1985):

$$Y = \frac{S_0}{1 + e^{b(X-a)}} \tag{3.1}$$

where $Y$ and $S_0$ (%) represent survival in cadmium and control treatments, respectively, $a$ is the log LC50 (LC50 in µg Cd $L^{-1}$), $b$ is the slope, and $X$ is the log concentration (actual exposure concentration in µg Cd $L^{-1}$).

At temperatures ranging from 10 to 26 °C, there was no mortality in the control treatment and $S_0$ was set at 100%. However, at the higher temperatures control mortality occurred. Therefore, $S_0$ was estimated by use of Equation 3.1. Following Van Gestel and Hensbergen (1997), the LC50 values obtained in the range of temperatures were tested two by two for significant differences by fitting the data simultaneously by the logistic response model, once by using a separate LC50 parameter ($a$ in Equation 3.1) for each temperature treatment and once by using the same LC50 parameter for both treatments. The outcomes of these fits were then compared using a likelihood ratio test (Sokal and Rohlf 1995). The significance level was adjusted for multiple comparisons by the Bonferroni correction (Sokal and Rohlf 1995). This implied that α was lowered from 0.05 to 0.002. Nonlinear regressions and statistical analyses were performed with the computer program SPSS (version 10.0.5, SPSS Inc.).

### *Short-term accumulation experiments at 10 to 26 °C*

After log-transforming the tissue cadmium concentrations, the differences in cadmium accumulation by daphnids exposed at 10, 20, and 26 °C were tested for significance by analysis of variance (ANOVA). When significant treatment effects were revealed ($\alpha = 0.05$), a Student-Newman-Keuls post hoc test ($\alpha = 0.05$) was used to determine which treatments differed from

each other.

The cadmium concentrations in the daphnids were used to estimate uptake and elimination rate constants assuming the simple linear one-compartment model:

$$C_i = \frac{k_1}{k_2} C_e (1 - e^{-k_2 t}) + C_{i,0} e^{-k_2 t} \quad (3.2)$$

where $C_i$ (mg Cd kg$^{-1}$ dw [dry weight]) is the tissue cadmium concentration, $k_1$ (L kg$^{-1}$ dw h$^{-1}$) and $k_2$ (h$^{-1}$) denote the uptake and elimination rate, respectively, $C_e$ (mg Cd L$^{-1}$) is the cadmium concentration in the test medium, $t$ (h) represents time, and $C_{i,0}$ (mg Cd kg$^{-1}$ dw) is the cadmium concentration in the daphnids at the start of the experiment.

The parameters were estimated by nonlinear regression. The differences between uptake and elimination rates at the three temperatures were tested for significance ($\alpha$ = 0.017, Bonferroni correction for multiple comparisons [Sokal and Rohlf 1995]) using the method of Van Gestel and Hensbergen (1997) described above. Nonlinear regressions as well as all statistical analyses were executed with the computer program SPSS (version 10.0.5, SPSS Inc.).

### *Time-dependent toxicity tests at 10 to 26 °C*

The data of the time-dependent toxicity tests were evaluated by the DEBtox model, which was used to reveal whether the influence of temperature on the response to cadmium was due to changes in the intrinsic sensitivity of the daphnids. Since the original model is not able to fit accumulation and toxicity data simultaneously, additional equations were programmed in MatLab 6.1 (The Mathworks, Inc.). A full description of the model is given in Bedaux and Kooijman (1994), but the most important assumptions and equations as well as extensions of the model are summarized here (see also Péry et al. [2002]). The kinetics of the compound is assumed to follow a simple linear one-compartment model as given by Equation 3.2 but assuming that the initial tissue cadmium concentration is an inert fraction that is not eliminated. Instead of estimating $k_1$, DEBtox fits the ratio of $k_1$ and $k_2$, which is also known as the bioconcentration factor (BCF, L kg$^{-1}$), as a model

parameter.

According to DEBtox, the toxicity of a chemical is given by the survival probability of individuals, which is specified via the hazard rate ($h$). The product $h\Delta t$ can be interpreted as the probability to die in the small time interval $\Delta t$, given that the animal has survived up to that moment. The survival probability can be expressed as:

$$q(t, C_e) = \exp(-\int_0^t h(\tau, C_e) d\tau) \tag{3.3}$$

where $q(t,C_e)$ (-) is the probability to survive until time $t$ and $h(\tau,C_e)$ ($h^{-1}$) is the hazard rate at time $\tau$, both a function of the toxicant concentration in the water ($C_e$).

DEBtox assumes the existence of a true no-effect concentration (NEC); a concentration causing no additional mortality of the organisms, even after long exposure. Normally, internal concentrations are not measured in toxicity experiments; therefore DEBtox treats the internal concentration as a hidden variable: the tissue concentration ($C_i$) is scaled with the BCF in order to obtain a quantity that is directly proportional to the tissue concentration but has the dimension of an external concentration. In the present study, however, the elimination rate ($k_2$) was very small, which hampers an accurate estimation of the BCF and, as a result, the NEC. Instead, the tissue cadmium concentrations measured in the accumulation experiments were used, making it possible to estimate an internal threshold concentration (ITC), which is the internal analogue of the NEC. When the ITC is exceeded, the hazard rate is assumed to increase proportionally to the difference between $C_i(t,C_e)$ and the ITC:

$$h(t, C_e) = \begin{cases} k_† \cdot (C_i(t, C_e) - ITC) + h_0(t) & \text{if } C_i(t, C_e) > ITC \\ h_0(t) & \text{if } C_i(t, C_e) \le ITC \end{cases} \tag{3.4}$$

where $k_†$ (kg dw $mg^{-1}$ Cd $h^{-1}$) represents the killing rate, $h_0(t)$ ($h^{-1}$) is the background hazard rate, and $ITC$ (mg Cd $kg^{-1}$ dw) is the internal threshold concentration.

The killing rate is the proportionality factor that describes the relation between the hazard rate and the tissue concentration that exceeds the ITC. It

is a measure for the toxicity of a compound and has the dimension (tissue concentration · time)$^{-1}$. Both the ITC and the killing rate are measures for the intrinsic sensitivity of the daphnids to cadmium.

The blank hazard rate ($h_0$) is assumed to be constant in the standard DEBtox model. In the current study, however, control mortality increased with temperature and time, which was probably caused by starvation. Therefore, when control mortality occurred in a temperature treatment, blank mortality was assumed to follow a Weibull function:

$$h_0(t) = \alpha \cdot t^{\beta} \tag{3.5}$$

where $\alpha$ and $\beta$ are empirical constants.

The DEBtox model fitted the results of the toxicity and accumulation experiments simultaneously, the model parameters were estimated by maximum likelihood methods, and the 95% confidence intervals were determined using the profile likelihood (Meeker and Escobar 1995).

## RESULTS

### Influence of temperature on toxicity

In the acute toxicity tests at 10 to 35 °C, the average cadmium recovery in the water at the end of the experiment (as percentage of the concentration at the start of the experiment) was 103 ± 2.3 %. The effect of temperature alone on survival of *D. magna* can be determined by analyzing control survival for temperatures ranging from 10 to 35 °C, which is given in Figure 3.1.

Up to 26 °C, there was no control mortality during the exposure period, but above this temperature, survival decreased drastically to zero at 35 °C. This decline was more severe after 48 h than after 24 h. Figure 3.1 also shows that the LC50 after 24 and 48 h decreased with increasing temperatures. A longer exposure time generally decreased the LC50 value, but the influence of temperature on the LC50 value showed the same trend for both 24 and 48 h of exposure. LC50 values were not calculated for 35 °C, as no daphnids survived in any of the treatments. The results of the statistical analyses are given in Table 3.1. The cases where statistical differences could not be determined were caused by a poor fit of the dose-

response curve or by high control mortality. Table 3.1 shows that all 24-h LC50 values at the various temperature regimes differed, but that the 48-h LC50 values in the high-temperature range were similar. The 48-h LC50 obtained for the 16 °C treatment did not differ from the other treatments, which was probably due to the large confidence interval.

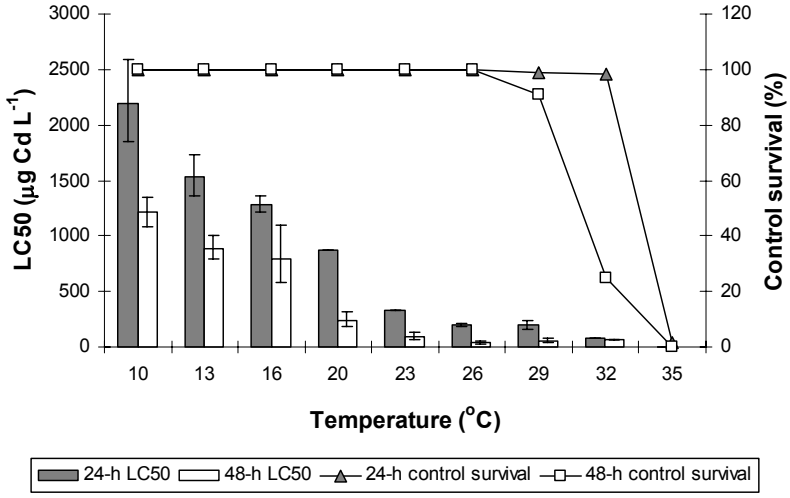


**Figure 3.1.** Control survival of and LC50 values for *D. magna* exposed to cadmium at 10 to 35 °C for 24 and 48 h. Error bars represent 95% confidence intervals.

### Influence of temperature on accumulation kinetics

In the accumulation experiments at 10 to 26 °C, the average cadmium concentration in the water that was recovered at the end of the experiment was 107 ± 0.53 % of the initial concentration. In the 26 °C treatment, a high number of animals was immobilized due to the cadmium exposure; therefore, this treatment was already ended after 24 h. Survival in control and exposed groups was 95 and 92% at 10 °C, 100% and 90% at 20 °C (45-h exposure period) and 98 and 82% at 26 °C (24-h exposure period), respectively. Control animals contained very little cadmium (4.65 ± 0.12 mg Cd $kg^{-1}$ dw). Figure 3.2 shows the cadmium concentrations in daphnids held at the three temperatures after different exposure times. Generally, elevated temperatures resulted in higher tissue concentrations. The cadmium tissue con-

centration could not be determined after 45 h for the highest temperature due to mortality, caused by the relatively high exposure concentration of 101 μg Cd $L^{-1}$. As the results of the acute toxicity test show, this concentration is above the 48-h LC50 obtained at 26 °C (Figure 3.1). Therefore, the accumulation curve for this temperature treatment should be considered with care. From the course of the accumulated cadmium concentration over time, uptake rates were estimated (summarized in Table 3.2). In Figure 3.2 can be seen that the uptake is still in the linear range, which makes the estimation of elimination rates impossible. Statistical analysis revealed a significant difference between the uptake rates at 10 and 20 °C, but no difference between the rates at 10 and 26 °C nor between those at 20 and 26 °C (likelihood ratio test, $\alpha = 0.017$). Again, the high test concentration could have inhibited the normal functioning of the daphnids in the 26 °C treatment, hampering a further increase in the cadmium uptake rate.

**Table 3.1.** Significant differences in 24-h and 48-h LC50 values between temperatures ranging from 10 to 32 °C as tested with a likelihood ratio test ($\alpha = 0.002$)[a]

| 24 h | Temperature (°C) | 10 | 13 | 16 | 20 | 23 | 26 | 29 |
|---|---|---|---|---|---|---|---|---|
| | 13 | nd | | | | | | |
| | 16 | * | - | | | | | |
| | 20 | * | * | * | | | | |
| | 23 | nd | nd | nd | nd | | | |
| | 26 | * | * | * | * | nd | | |
| | 29 | * | * | * | * | * | - | |
| | 32 | * | * | * | nd | nd | * | * |

| 48 h | Temperature (°C) | 10 | 13 | 16 | 20 | 23 | 26 | 29 |
|---|---|---|---|---|---|---|---|---|
| | 13 | * | | | | | | |
| | 16 | * | - | | | | | |
| | 20 | * | * | - | | | | |
| | 23 | * | * | - | nd | | | |
| | 26 | * | * | - | * | nd | | |
| | 29 | * | * | - | nd | nd | - | |
| | 32 | nd | * | nd | nd | nd | - | - |

[a]Symbols indicate the following: *, significant difference; -, no significant difference; nd, not determined. No comparisons were made for 35 °C since there were no surviving daphnids in any of the treatments.

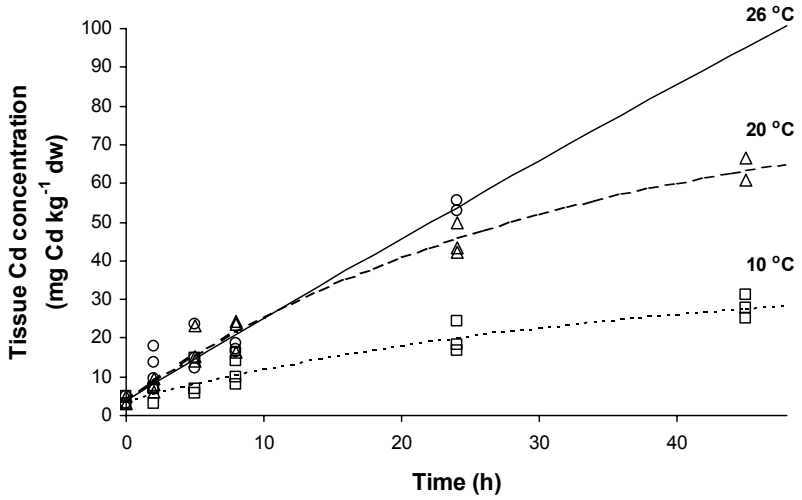


**Figure 3.2.** Tissue cadmium concentrations of *D. magna* exposed to 101 ± 0.24 (SE) µg Cd $L^{-1}$ at 10, 20, and 26 °C for 45 h. Symbols represent data points, while the lines are fitted to the data following Equation 3.2.

**Table 3.2.** Uptake rates with 95% confidence intervals (CI) estimated for *D. magna* exposed to 101 ± 0.24 (SE) µg Cd $L^{-1}$ for 45 h at 10 and 20 °C and for 24 h at 26 °C.

| Temperature (°C) | $K_1$ (L $kg^{-1}$ dw $h^{-1}$) | 95% CI |
|---|---|---|
| 10 | 10.5[a] | 5.5 – 15 |
| 20 | 25.3[b] | 20 – 30 |
| 26 | 22.2[ab] | 11 – 33 |

[a] and [b] indicate treatments that are significantly different as tested with a likelihood ratio test ($\alpha$ = 0.017).

### Influence of temperature on sensitivity

The average cadmium recovery in the water at the end of the time-dependent toxicity tests was 104 ± 0.96 % of the concentration that was initially present. In Figure 3.3, the cadmium concentration in the daphnids (upper panel) and the fraction of surviving daphnids (lower panel) are plotted against time. The simple one-compartment model used by DEBtox provides an adequate fit to the cadmium concentration in the daphnids. The accumulation data at 20 °C showed that the uptake and elimination rate constants were independent of the exposure concentration. The survival of the

daphnids was described well by the estimated tissue concentrations, and the results were comparable with the toxicity tests at 10 to 35 °C, with toxicity increasing with rising temperature. At 20 °C, however, the best fit was obtained when mortality in the lower cadmium treatments was regarded as control mortality, resulting in an overestimation of control mortality. Otherwise, DEBtox was incapable of fitting all cadmium exposures with one ITC. The daphnids at low cadmium concentrations appeared to become more vulnerable to cadmium after a longer exposure time (resulting in a lowering of the ITC), which was presumably due to starvation, as in chronic experiments with food and the same temperature treatments no control mortality was observed (unpublished results). The daphnids at the higher concentrations were not living long enough to experience these adverse effects. For the same reason, the longer exposure period caused high control mortality at a temperature of 26 °C, in contrast with the previous toxicity experiments at 10 to 35 °C. Better fits for 20 and 26 °C could be obtained by assuming that the ITC decreased in time, but since it is highly speculative in which way the ITC changes in time, these model fits were not shown.

In Table 3.3, the estimated model parameters are given. The BCF is highly correlated with the elimination rate. Since the elimination rate is very small, the two parameters could not be accurately estimated and comparisons of the parameters at the three temperatures cannot be made. Nevertheless, the model fits resulted in reliable estimates of the ITCs and killing rates at the three temperatures. Little difference was observed between the ITC at 10 and 20 °C, but the ITC was significantly lower at 26 °C. The killing rates increased at elevated temperature, with significant differences between the rates at 10 and 26 °C, and those at 20 and 26 °C. These findings indicate that cadmium became more toxic at higher temperatures.

## DISCUSSION

### Influence of temperature on cadmium toxicity

The results of the acute toxicity tests showed that the effect of cadmium on the survival of *D. magna* is highly temperature-dependent and that temperature itself may become lethal as well when it exceeds the thermal tolerance limit of the daphnids. Thermal effects on survival of daphnids were

also reported by Work and Gophen (1999), who found that survival time of 2 to 3 day-old *D. lumholtzi* decreased from 29 d at 15 °C to 17 d at 29 °C. The toxicity data are in accordance with the findings of other authors (Lewis and Horning 1991, Stuhlbacher et al. 1993, Wolf et al. 1998), who observed lower cadmium resistance of *D. magna* at elevated temperatures.

Although this study as well as the available literature agrees that temperature increases toxicity, the question remains if the observed temperature-dependent toxicity is due to altered toxicant accumulation, susceptibility of the daphnids, or a combination of these factors. In the following sections, the significance of these factors is discussed.

### Influence of temperature on cadmium accumulation

The present study showed that cadmium uptake rates at 20 °C were significantly higher than the rate at 10 °C. Few studies considered the influence of temperature on cadmium kinetics in *Daphnia*. Stuhlbacher et al. (1993) observed four times higher cadmium accumulation at 30 than at 10 °C in *D. magna* treated with 100 μg Cd $L^{-1}$ for 24 h at 10, 20, and 30 °C. Overall, the cadmium concentrations measured in the daphnids were 3 to 5 times higher than in the present study, probably due to differences in the test medium used. Studies concerning thermal effects on the accumulation of cadmium by other test species showed the same trend as observed in this paper: uptake rates and the amount of cadmium accumulated during a certain period of time was higher at elevated temperatures. For instance, Bervoets et al. (1996) found a 14 times higher cadmium uptake rate at 25 °C compared with 5 °C in the midge *Chironomus riparius*. Higher cadmium body concentration at elevated temperature were also reported for several other species,such as fingerlings of perch (Edgren and Notter 1980), Asiatic clams (Graney et al. 1984), freshwater isopods (Van Hattum et al. 1993), Japanese eel (Yang and Chen 1996), and burrowing mayfly nymphs (Andres et al. 1998). These observations can be explained by an increase in metabolic rate and thus oxygen demand, when ectothermic organisms are exposed to a temperature rise. This causes elevated ventilation rates, which may lead to higher cadmium accumulation at higher temperatures (Cairns et al. 1975, Graney et al. 1984). The increased metabolic rate may also result in higher active transport of cadmium across the membranes, which may

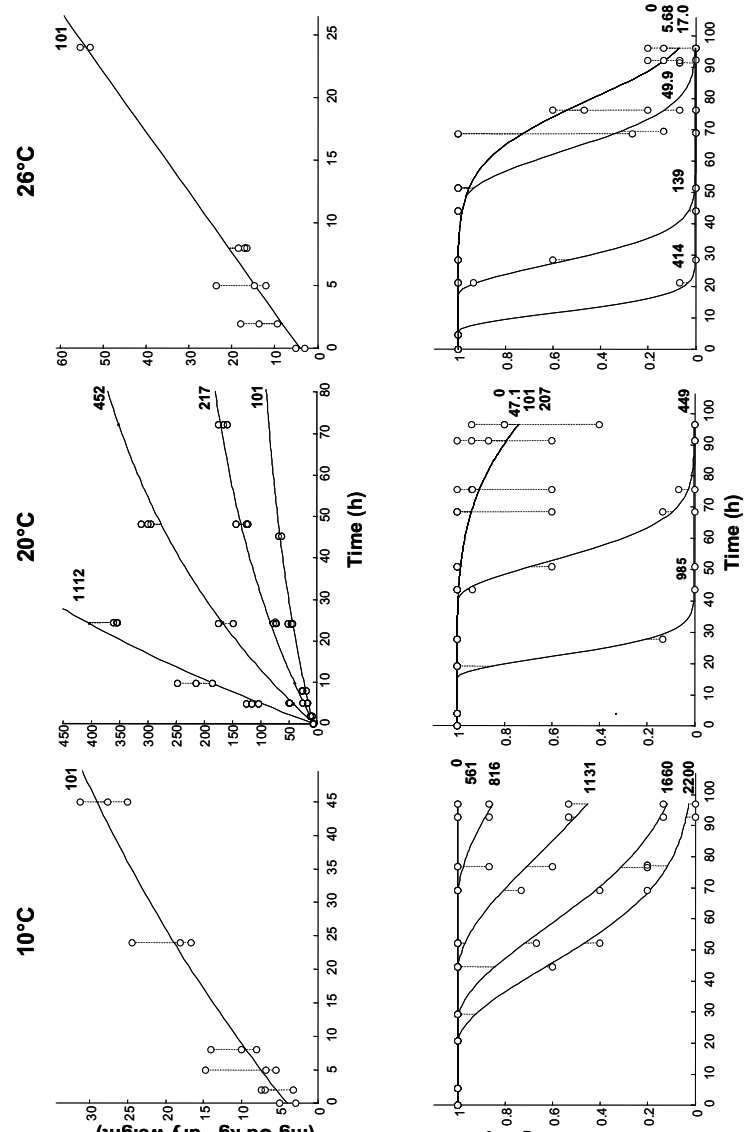


**Figure 3.3.** Tissue cadmium concentrations of *D. magna* (upper panel) and the fraction of surviving *D. magna* at 10, 20, and 26 °C (lower panel). Test duration of accumulation experiments was 45 h (10 °C and 101 µg Cd $L^{-1}$ at 20 °C), 24 h (26 °C), and 0 to 72 h (other concentrations at 20 °C). Test duration of toxicity tests was 96 h. Symbols represent data points, while lines are fitted to the data by the DEBtox model. The numbers on the right side of each line correspond to the actual cadmium concentration in the treatment (µg Cd $L^{-1}$).

**Table 3.3.** Parameters estimated by DEBtox by fitting toxicity[a] and accumulation[b] data simultaneously

| Symbol | Description | Unit | Temperature (°C) | Parameter value | Likelihood based 95% CI[c] |
|---|---|---|---|---|---|
| $\alpha$ | Parameter of Weibull function | | 10 | ne[d] | |
| | | | 20 | $1.02 \cdot 10^{-9}$ | $8.0 \cdot 10^{-15}$ - $3.0 \cdot 10^{-6}$ |
| | | | 26 | $3.47 \cdot 10^{-12}$ | $2.8 \cdot 10^{-15}$ - $1.4 \cdot 10^{-9}$ |
| $\beta$ | Parameter of Weibull function | | 10 | ne | |
| | | | 20 | 3.60 | 1.7 - 6.3 |
| | | | 26 | 5.40 | 4.0 - 7.0 |
| *BCF* | Bioconcentration factor | L $kg^{-1}$ | 10 | 653 | $3.6 \cdot 10^{2}$ - $6.1 \cdot 10^{4}$ |
| | | | 20 | $1.13 \cdot 10^{3}$ | $8.8 \cdot 10^{2}$ - $1.6 \cdot 10^{3}$ |
| | | | 26 | $1.00 \cdot 10^{6}$ | $2.9 \cdot 10^{3}$ - $\infty$ |
| $k_2$ | Elimination rate | $h^{-1}$ | 10 | 0.0109 | $9.5 \cdot 10^{-5}$ - 0.024 |
| | | | 20 | 0.0157 | 0.010 - 0.022 |
| | | | 26 | $2.15 \cdot 10^{-5}$ | $-\infty$ - $8.1 \cdot 10^{-3}$ |
| $k_f$ | Killing rate | kg dw $mg^{-1}$ Cd $h^{-1}$ | 10 | $1.23 \cdot 10^{-4}$ | $7.9 \cdot 10^{-5}$ - $1.8e \cdot 10^{-4}$ |
| | | | 20 | $1.59 \cdot 10^{-3}$ | $8.8 \cdot 10^{-4}$ - $2.8 \cdot 10^{-3}$ |
| | | | 26 | $3.26 \cdot 10^{-3}$ | $1.9 \cdot 10^{-3}$ - $5.4 \cdot 10^{-3}$ |
| *ITC* | Internal threshold concentration for survival | mg Cd $kg^{-1}$ dw | 10 | 270 | $2.2 \cdot 10^{2}$ - $3.3 \cdot 10^{2}$ |
| | | | 20 | 239 | $2.1 \cdot 10^{2}$ - $2.6 \cdot 10^{2}$ |
| | | | 26 | 51.6 | 42 - 61 |

[a] Time-dependent toxicity tests at 10 to 26 °C with a 96-h test duration.

[b] Short-term accumulation experiments at 10 to 26 and 20 °C with a 24- to 45-h and 0- to 72-h test duration, respectively.

[C] Confidence intervals.

[d] ne, not estimated.

increase accumulation rates as well (Cairns et al. 1975).

In contrast to uptake rates, the temperature dependence of cadmium elimination appears to be less clear. The depuration rates of Asiatic clams were not altered by temperature (Inza et al. 1998). In contrast, a small but significant temperature effect on cadmium elimination was found in freshwater isopods since the metal was eliminated at 5 °C but not at 10 and 20 °C (Van Hattum et al. 1993). Burrowing mayfly nymphs eliminated cadmium rapidly, and a small but significant increase of the elimination rate was observed when temperature was elevated (Odin et al. 1997).

Summarizing, in the present study as well as in others, increased cadmium uptake rates and accumulation at elevated temperatures are reported and some small but significant effects of temperature on elimination rates. Many authors suggest therefore that increased cadmium toxicity at elevated temperatures is caused by enhanced cadmium accumulation. Since it is still uncertain if changes in the sensitivity of the daphnids are involved as well, the subsequent section deals with this topic.

### Temperature effects on sensitivity of *D. magna*

When the sensitivity of the daphnids to cadmium is not changed by temperature, the ITC and the killing rate should be equal at all temperature regimes. The temperature dependency of the ITCs and killing rates estimated by DEBtox showed that this hypothesis is invalid. The ITC was smaller at 26 °C compared with 10 and 20 °C, implying that at 26 °C less cadmium needed to be accumulated to induce lethal effects than at lower temperatures. The killing rate was elevated at high temperature relative to low temperature, indicating that cadmium effects were amplified with rising temperature. It is thus concluded that thermal effects on cadmium toxicity cannot be ascribed to accumulation kinetics alone and that altered susceptibility of the daphnids plays an important role as well.

### Accumulation versus sensitivity

The results presented in this study showed that temperature stress alone caused mortality of daphnids when the temperature reached a certain

upper thermal tolerance limit. Furthermore, temperature had a major impact on cadmium toxicity, which was attributable to various mechanisms. In the lower temperature range, temperature rise accelerated uptake kinetics, causing higher cadmium toxicity. In the higher temperature range, increased uptake was less important, while the contribution of increased sensitivity of the daphnids became more significant, as shown by the lowering of the ITC. Also the temperature dependency of the killing rate indicated that the intrinsic sensitivity to cadmium increased sharply with temperature. The present model allowed quantitative comparison of the roles of increased uptake and susceptibility of the daphnids throughout wide temperature ranges. Determination of cadmium accumulation and survival in time proved to be vital parameters. Although accumulation kinetics as well as sensitivity of the daphnids was influenced by temperature and both processes in conjunction determined the wide ranges of cadmium toxicity observed, intrinsic temperature-dependent sensitivity to cadmium was shown to be the primary factor for toxicity in daphnids.

Many previous studies (reviewed in Cairns et al. [1975], McLusky et al. [1986], Heugens et al. [2001]) have considered the influence of temperature on toxicity of chemicals. The generally observed temperature-toxicity relationship was thought to be related to changes in accumulation kinetics or sensitivity of the test organisms. Since these assumptions were never tested, the relative importance of the interacting processes responsible for temperature-dependent toxicity remained elusive. The present study proved that a combined approach of experiments and modeling is essential to disentangle interacting processes and to test hypotheses on the role of mechanisms of multiple stress. The present study is likely to have consequences for the widespread practice of extrapolating laboratory results to ecosystems that so far totally ignores temperature-modified toxicity.

## ACKNOWLEDGEMENTS

The study was supported by the Institute for Inland Water Management and Waste Water Treatment. We wish to express our thanks to Albert Espeldoorn for experimental support.

# *Chapter 4*

# Population Growth of *Daphnia magna* influenced by Multiple Stressors – Joint Effects of Temperature, Food, and Cadmium

Heugens EHW, Tokkie LTB, Kraak MHS, Hendriks AJ, Van Straalen NM, Admiraal W. Submitted.

## ABSTRACT

Aquatic organisms in the field are often exposed to combinations of stress factors of various origin. Little is known of the interaction between different types of stressors and hence the predictability of their joint effects is low. Therefore, this study analyzed the joint effects of temperature, food and cadmium on the population growth rate of the water flea *Daphnia magna*. The results revealed that the interaction between the three factors, in addition to their separate effects, influenced life history parameters and cadmium accumulation, leading to effects on population growth rate. In general, population growth rate increased at high temperature and food level, but decreased when cadmium was present. The positive temperature effect on population growth rate was smallest at limiting food levels. Negative cadmium effects on the growth rate were enhanced at elevated temperatures, while high food levels protected the daphnids from adverse cadmium effects. Population growth rates ranging from 0.6 to -0.04 $d^{-1}$ were found when the three factors were combined. To avoid over- or underestimation of toxicity of substances to field populations, results of standard toxicity tests should be interpreted in a location-specific way.


## INTRODUCTION

Aquatic organisms in natural waters are often subjected to various stress factors of physical, chemical, and biological origin. A substantial amount of information on the interacting effects of chemicals is available from mixture toxicity studies (Greco et al. 1995, Vighi 2003), but it still is relatively unknown how combinations of different types of stressors interact on the population level. This study focused on the joint effects of two major natural stress factors, temperature and food, and a chemical stressor, cadmium, on life cycle parameters (survival, reproduction) of the water flea *Daphnia magna*. These chronic endpoints describe trends in population density or abundance and predict future population sizes (Sibly and Hone 2002), making them relevant tools to assess the fate of field populations under multiple stress conditions. Temperature and food were chosen because these factors may be highly variable in the field and are of major importance for the physiological state of organisms. Cadmium was chosen as a model

toxicant, because it is still causing problems in Dutch inland waters, even though the concentrations are declining (CIW 2001).

Based on previous literature reviewed in Heugens et al. (2001), an interaction between the three factors was expected. A temperature rise increases metabolic rate in ectothermic organisms (Cairns et al. 1975), and thus feeding activity. For cladocerans, this activity depends on food level as well, with feeding rate increasing with food concentration, up to a threshold concentration, the 'incipient limiting level', above which the feeding rate remains relatively constant (Lampert 1987). Increased food uptake results in a higher amount of energy available for growth and reproduction. Hence, the population growth rate of animals living at high temperatures and abundant food is expected to be higher than at low temperatures and poor food conditions. Cadmium on the other hand, affects the population growth rate of cladocerans (Marshall 1978, Bertram and Hart 1979, Wong and Wong 1990). Acute experiments revealed that cadmium is more toxic to daphnids at high than at low temperature (Lewis and Horning 1991, Stuhlbacher et al. 1993, Heugens et al. 2003). At high food levels, toxicity may decrease if the greater amount of energy can be directed to withstand toxic stress. Alternatively, the bioavailability of cadmium may change with food level as the negatively charged surface of algal cells, a common food source, has a high affinity for positively charged metal cations, resulting in a reversible binding of cadmium ions to the cell surface (Allen et al. 1995). The hypotheses on the interaction between the three variables as stated above were tested by exposing *D. magna* to several cadmium concentrations in the water at combinations of three temperatures and three food levels ranging from 10 to 26 °C and 0.50 to 2.0 mg C $L^{-1}$ of *Selenastrum capricornutum*, respectively. Tissue cadmium concentrations were measured to relate toxic effects caused by cadmium to the amount of toxicant accumulated by the daphnids. In this way, potential effects of temperature and algal density on the bioavailability of cadmium were determined as well.

## METHODS

### Culture conditions

*Daphnia magna* cultured in our laboratory descended from a culture kept at the Institute for Inland Water Management and Waste Water Treatment (RIZA, Lelystad, the Netherlands). The culture consisted of cohorts with a density of 20 daphnids per liter of artificial Elendt M7 medium (OECD 1998). A cohort was kept for four to five weeks, after which a new cohort was started with at least third brood neonates (<24 h). Three times per week, the medium was renewed and juveniles were removed. The culture was maintained under a light-dark regime of 16:8 h and at a temperature of 20 °C. On working days, the daphnids were fed with 2.0 mg C $L^{-1}$ of a concentrated suspension of *Selenastrum capricornutum*. The algae were cultured in a chemostat in Woods Hole medium (Guillard 1975). Every week, algae were collected and centrifuged at 3000 rpm for 10 min. After removal of the supernatant, the algae were resuspended in Elendt M7 medium and the total organic carbon concentration of the suspension was measured with a total carbon analyzer (College Station, Texas). The suspension was stored at 4 °C in a dark room until used for feeding.

### Chronic toxicity tests

The standard *Daphnia* reproduction test (OECD 1998) was adjusted to study the influence of temperature, food and cadmium on life history traits of *D. magna*. Each experiment consisted of 12 treatments, composed of combinations of four cadmium concentrations (including one control) and three food levels (0.50, 1.0 and 2.0 mg C $L^{-1}$). The experiments were consecutively performed at 10, 20 and 26 °C. In the 20 °C experiment, the cadmium concentrations in the low-food treatment proved to be too low to exert effects. Therefore, an additional experiment with higher exposure concentrations was performed at 20 °C. The experimental design is summarized in Table 4.1. The temperatures used are within the range of temperatures observed in the field. Food levels ranged from concentrations used in the standard *Daphnia* reproduction test (OECD 1998) to lower food levels that are common in aquatic ecosystems. The lowest food level was derived from pilot

experiments in batch systems and proved to sustain sufficient reproductive output to be able to analyze negative effects due to cadmium exposure. This low food level is close to the average biomass of green algae in the eutrophic Lake IJsselmeer in the Netherlands, for which a food level of 0.375 mg C $L^{-1}$ was reported (Lammens 1999).

**Table 4.1.** Experimental set-up with actual cadmium concentrations (μg Cd $L^{-1}$) measured in the different temperature, food, and cadmium treatments. Values in brackets denote standard errors. *S. capricornutum* was used as a food source.

| | Actual cadmium concentration (μg Cd $L^{-1}$) | | | |
|---|---|---|---|---|
| Food level | Temperature (°C) | | | |
| (mg C $L^{-1}$) | 10 | 20(I)[a] | 20(II)[b] | 26 |
| 0.50 | <dl[c] | <dl | <dl | <dl |
| | 48.6 (7.8) | 14.4 (3.1) | 27.4 (4.3) | 19.7 (2.9) |
| | 78.2 (9.2) | 23.0 (3.6) | 76.8 (5.8) | 29.4 (5.1) |
| | 136 (11) | 37.8 (4.3) | | 49.1 (7.3) |
| 1.0 | <dl | <dl | | <dl |
| | 46.5 (8.7) | 23.4 (4.3) | | 19.5 (3.2) |
| | 78.0 (9.1) | 37.3 (3.8) | | 29.2 (4.1) |
| | 136 (11) | 85.7 (4.9) | | 48.2 (6.5) |
| 2.0 | <dl | <dl | | <dl |
| | 132 (12) | 85.0 (5.2) | | 30.2 (3.8) |
| | 228 (15) | 140 (6.4) | | 50.5 (6.7) |
| | 358 (20) | 234 (9.0) | | 80.8 (7.4) |

[a]First 20 °C experiment.
[b]Additional 20 °C experiment.
[c]<dl, below detection limit (control treatment).

To maintain constant cadmium concentrations in the experiments and to avoid loss of algae from the water column by sedimentation, an intermittent flow-through system was used (Figure 4.1). By the use of this flow-through system, the algae concentration in the water column was kept relatively constant, and distortion of the results by food shortage of the daphnids at elevated temperatures due to higher filtration rates was prevented. Per cadmium and food treatment in each temperature experiment, 10 neonates (<24 h) of at least the third brood were placed individually in poly-propylene tubes, from which the middle part had been replaced by gauze. The 10 tubes were positioned together in a plastic aquarium that was filled beforehand with 3 L of test solution. The test solutions consisted of artificial Elendt M7 medium (OECD 1998) with different cadmium and food levels (Table 4.1) that were prepared in 3-L Erlenmeyer flasks and stirred for

minimal half an hour before usage. This duration was considered to be sufficient to reach equilibrium between cadmium in solution and cadmium adsorbed to algal cell surfaces (Taylor et al. 1998). Eight times a day, 375 mL of the corresponding solution was discharged into each aquarium by peristaltic pumps connected to a timer and the same volume was removed from the aquarium simultaneously. In this way, a volume of test solution equivalent to the volume in the aquarium flowed through the system once a day. A light-dark regime of 16:8 h was used. The temperature was kept constant by placing the aquaria in temperature-controlled water baths.

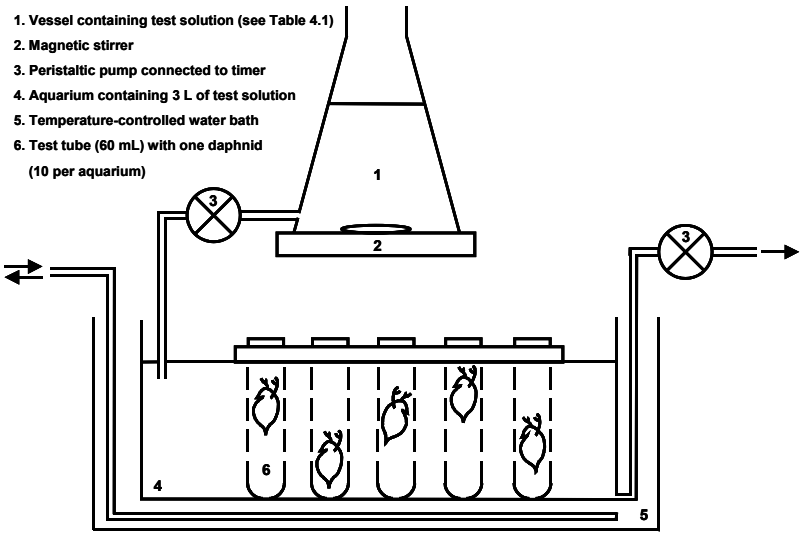


**Figure 4.1.** Intermittent flow-through system.

In order to determine actual cadmium concentrations in the water, 1.5-mL water samples were taken just before and 1 h after the addition of freshly made solution and centrifuged at 3000 rpm for 10 min. 1 mL of supernatant was transferred to a new Eppendorf container and acidified with 20 µL of 65% nitric acid (Merck, p.a.), whereafter they were analyzed by air-acetylene flame atomic absorption spectrometry (AAS) (Perkin-Elmer 1100B). Stability of the algae concentrations was checked by measuring cell numbers and volumes in 5-mL water samples, diluted with 15 mL of salt solution (1.2% $NaNO_3$), with a coulter counter. When the food level in a treatment started to increase due to sedimentation of the algae, the gauze tubes with the daphnids in the remaining water at the bottom of the tube were

removed from the aquarium. The test medium was transferred to an Erlenmeyer flask and the bottom of the aquarium was flushed with double distilled water to remove sedimented algae, after which the test medium and the test tubes were put back in the aquarium. During an experiment, this occurred at most once per treatment. Since temperature accelerates physiological processes, a certain exposure time at high temperature represents a longer period of physiological time than at low temperature. To prevent problems with interpretation, the test duration was based on physiological time: the time in which the daphnids produced three clutches of neonates. This physiological endpoint was chosen because population growth rate (see below) approached its maximum value after the production of the third brood. This resulted in test periods of 41, 15 and 11 d at 10, 20 and 26 °C, respectively.

Every day, the following life history traits of the daphnids were recorded: survival, presence of eggs in the brood pouch and the amount of living neonates produced. Newborn neonates were removed. From these daily-observed life history characteristics, the age at maturity (i.e. the day on which the first clutch of eggs was deposited in the brood pouch) and the age at first reproduction were determined. The life history parameters were integrated by calculating the population growth rate according to the Euler-Lotka equation:

$$\sum_{x=0}^{x=x_m} l_x m_x e^{-rx} = 1 \qquad (4.1)$$

where $l_x$ is the fraction surviving until age $x$, $m_x$ is the number of offspring produced per surviving female between age $x$ and $x+1$, $r$ ($d^{-1}$) is the population growth rate and $x_m$ is the maximum age. The outcome of Equation 4.1 was obtained by iterative calculations, resulting in only one value for r per treatment. Since no uncertainty could be calculated from this single value, the Jackknife method was used to generate a variance, as described by Meyer et al. (1986).

Protocols for standard toxicity testing with *Daphnia* prescribe that control mortality does not exceed 20%, and is preferably absent (OECD 1998). This results in a theoretical population growth rate, which cannot be achieved in the field. When natural mortality rates of field populations are known, control mortality can be included in Equation 4.1 to calculate more realistic

population growth rates. However, because mortality rates of field populations of *Daphnia* under different temperatures and food levels are unknown, the population growth rates presented in this paper are approximations of the actual rates that can be realized.

To determine somatic growth of the daphnids during the test period, body lengths (measured from the middle of the eye to the base of the tail) of neonates (<24 h) descending from the same cohort as the test animals were measured at the start of the experiments using an image analyzer (Leica). This was repeated at the end of the experiment for surviving daphnids. For each treatment, growth was calculated as the difference in average body length at the start and the end of the experiment. After length measurement, the animals were kept in clean, double distilled water for 10 min, after which one to three daphnids were pooled in 2-mL polyethylene tubes. Following the micro-destruction method described in (Timmermans et al. 1989), the animals were lyophilized, weighed and digested in 65% nitric acid (J.T. Baker, Ultrex) and 30% hydrogen peroxide (Fluka, purum p.a.). The concentrated samples were diluted with 500 µL of acidified analytical grade water (5 mL of 65% nitric acid $L^{-1}$ (J.T. Baker, Ultrex)) and analyzed by air-acetylene flame (Perkin-Elmer 1100B) or graphite furnace AAS (Perkin-Elmer 5100PC/HGA600/ AS60), depending on the metal concentration in the samples. To determine the maternal investment per neonate (as average neonate size) and the investment in total reproductive output (as average neonate size · brood size), body lengths of neonates descending from the third brood were measured as well.

### Statistical analyses

Data for all parameters were tested for homogeneity and normality. When these conditions were met, a three-way analysis of variance (ANOVA) was performed. Otherwise, a three-way ANOVA was performed on rank data. Rank transformation was chosen because the variance of rank data is automatically stable (Potvin and Roff 1993). Cadmium treatments were compared with the corresponding control treatment by t-tests with Bonferroni adjustment with an overall error of 0.05.

As was mentioned above, the test period was based on physiological age, resulting in a longer exposure time at low temperature than at high temperature. Because of this difference in test duration, life span was analyzed within temperature groups by performing a two-way ANOVA on rank data, instead of a three-way ANOVA. The life span of animals that died within the first 24 h was set at 0 days, while the life span of an animal that lived until the end of the experiment equals the test duration. This approach may have led to an underestimation of the effects on survival, since the differences in life span between treatments where daphnids died during the experiment and those where no mortality occurred were smaller than if the test duration would have been prolonged.

## RESULTS

### Cadmium accumulation

Tissue cadmium concentrations were determined in daphnids that survived until the end of the experiment (Figure 4.2). Data of only one exposure concentration was available for the 10 °C and high-food treatments, since no daphnids survived the other cadmium treatments. Because the corresponding control treatment was lost during the experiment as well, the body cadmium concentration nor the other effect parameters (see below) could be determined for this treatment. The poor performance of the control animals could not be attributed to a treatment effect and therefore, the experiment was continued without the control group. Sufficient treatments remained to determine the effects of cadmium and food level and their interactions on *D. magna*.

The amount of cadmium accumulated by the daphnids during the experiment significantly increased with increasing cadmium concentrations in the water, higher temperatures and lower food levels. These effects were magnified when a combination of these conditions was applied, as indicated by the significant interactions between temperature and cadmium, and food and cadmium (three-way ANOVA, Table 4.2).

**Table 4.2.** Two and three-way ANOVA for effects of the main factors temperature (T), food level (F), and cadmium (Cd) and their interactions on life span, growth, age at maturity and first reproduction, total number of neonates produced per mother, population growth rate, investment in individual neonates, and investment in total reproductive output of *D. magna*. Except growth data, all data was rank transformed before analysis. Values denote *F*-values, degrees of freedom (between brackets), and significance level: n.s., P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001

| Effect parameter | | T | F | Cd | TxF | TxCd | FxCd | TxFxCd |
|---|---|---|---|---|---|---|---|---|
| Tissue Cd concentration | | 22.862 (2, 34) *** | 13.257 (2, 34) *** | 117.249 (6, 34) *** | 0.406 (3, 34) n.s. | 13.979 (6, 34) *** | 4.026 (8, 34) *** | 2.162 (4, 34) n.s. |
| Life span | 10 °C | | 1.316 (2, 11) n.s. | 6.667 (5, 11) *** | | | 2.501 (3,11) n.s. | |
| | 20 °C | | 5.350 (2, 11) ** | 2.565 (6, 11) * | | | 0.538 (2, 11) n.s. | |
| | 26 °C | | 3.316 (2, 12) * | 0.663 (4, 12) n.s. | | | 1.959 (5, 12) n.s. | |
| Somatic growth | | 245.210 (2, 32) *** | 348.119 (2, 32) *** | 59.546 (5, 32) *** | 4.638 (3, 32) ** | 12.733 (6, 32) *** | 16.802 (8, 32) *** | 11.159 (4, 32) *** |
| Age at maturity | | 1196.099 (2, 37) *** | 47.715 (2, 37) *** | 10.269 (11, 37) *** | 10.466 (3, 37) *** | 16.791 (4, 37) *** | 17.171 (8, 37) *** | 18.072 (3, 37) *** |
| Age at first reproduction | | 861.290 (2, 36) *** | 36.196 (2, 36) *** | 11.925 (7, 36) *** | 0.156 (3, 36) n.s. | 1.893 (7, 36) n.s. | 10.243 (8, 36) *** | 1.091 (4, 36) n.s. |
| Total number of neonates | | 43.316 (2, 36) *** | 124.252 (2, 36) *** | 51.772 (7, 36) *** | 2.540 (3, 36) n.s. | 4.464 (7, 36) *** | 19.557 (8, 36) *** | 3.655 (4, 36) ** |
| Investment per neonate | | 9.089 (2, 31) *** | 9.796 (2, 31) *** | 3.526 (6, 31) ** | 2.749 (3, 31) * | 4.307 (5, 31) *** | 1.596 (6, 31) n.s. | 0.661 (3, 31) n.s. |
| Total investment in reproduction | | 79.466 (2, 31) *** | 152.326 (2, 31) *** | 8.390 (6, 31) *** | 0.606 (3, 31) n.s. | 8.572 (5, 31) *** | 2.045 (6, 31) n.s. | 2.911 (3, 31) * |
| Population growth rate | | 124.093 (2, 36) *** | 77.425 (2, 36) *** | 26.741 (7, 36) *** | 3.356 (3, 36) * | 7.019 (7, 36) *** | 5.651 (8, 36) *** | 1.961 (4, 36) n.s. |

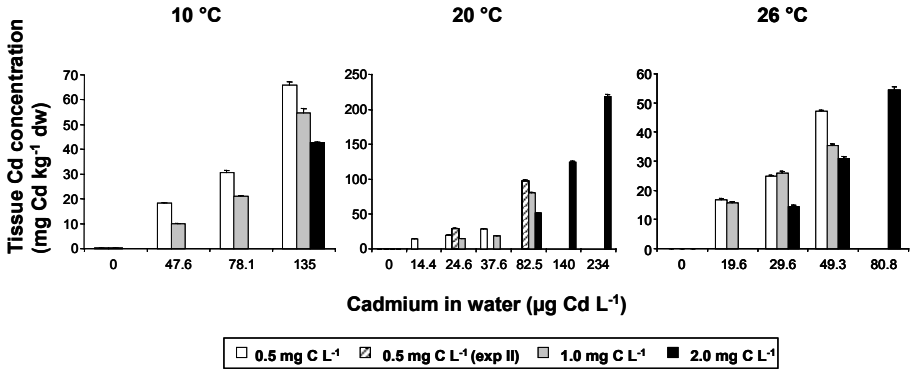


**Figure 4.2.** Tissue cadmium concentrations of surviving *D. magna* at 10, 20, and 26 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum*, as a function of the cadmium concentration in the water. Error bars represent standard errors. The test duration was 41, 15 and 11 d at 10, 20, and 26 °C, respectively. In all treatments, tissue cadmium concentrations were significantly higher relative to the corresponding control (t-tests with Bonferroni correction), except in the 10 °C, medium-food and 47.6 µg of Cd $L^{-1}$ treatment.

## Survival

The survival of the daphnids during the experiments is given in Figure 4.3. From these survivorship curves, average life span was calculated for all exposure conditions (not shown). The effects of cadmium and food level on life span were analyzed within each temperature treatment using two-way ANOVA (Table 4.2). At 10 °C, cadmium significantly decreased life span, while no effect of food was found. At 20 °C, both cadmium and food had a significant effect on survival. Cadmium decreased life span, but the influence of food level on life span was not consistent and no clear trend could be seen. At 26 °C, only the food concentration significantly influenced life span. Again, no clear pattern could be observed. Although the life span of the daphnids at the highest cadmium concentration was significantly shorter than in the control (t-test with Bonferroni correction), the high variation in life span of the daphnids in the cadmium treatment was the reason that cadmium was a non-significant factor with regard to life span. No interactions between cadmium and food were present at any of the tested temperatures.

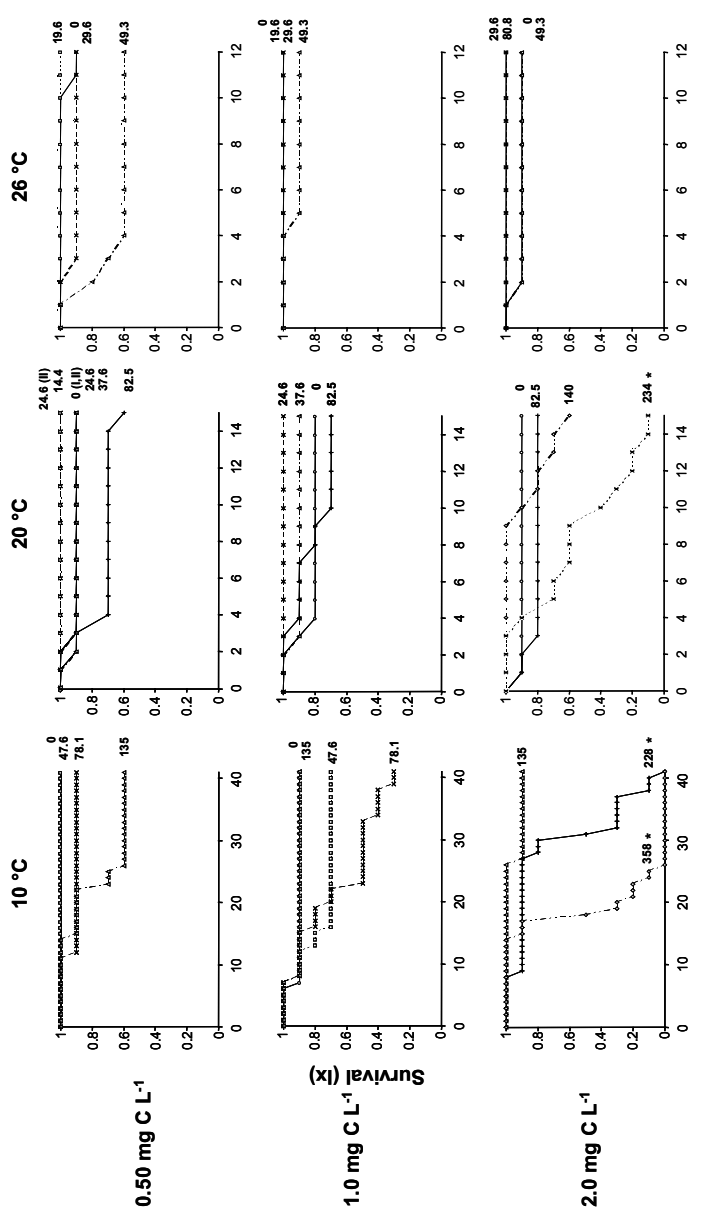


**Figure 4.3.** Survival of *D. magna* exposed to various cadmium concentrations (as indicated by the numbers on the right side of the graphs) at 10, 20, and 26 °C and 0.50, 1.0 and 2.0 mg C $L^{-1}$ of *S. capricornutum* as a function of time. When exposure concentrations were equal, (I) or (II) were used to discriminate between data of the first and the additional 20 °C experiment, respectively. * indicates a significant difference in life span relative to the corresponding control (or lowest cadmium treatment for the 10 °C and high-food treatment) (t-tests with Bonferroni correction).

## Growth

The effects of temperature, food and cadmium, and their two- and three-factor interactions on growth were all significant (three-way ANOVA, Table 4.2). Figure 4.4 shows the increase in body length during the test period for daphnids that were still alive at the end of the experiment. The daphnids reached a larger body size at low temperature and high food level. Exposure to cadmium decreased growth, which was enhanced by the low temperature and high food conditions.

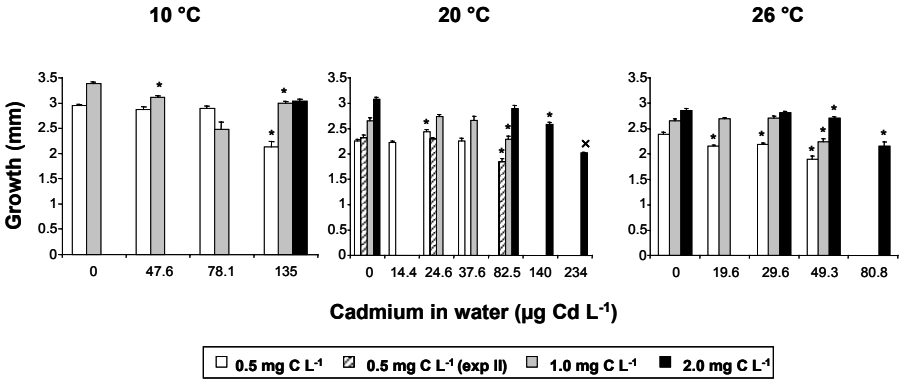


**Figure 4.4.** Growth of surviving *D. magna* at 10, 20, and 26 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum*, as a function of the cadmium concentration in the water. Error bars represent standard errors. The test duration was 41, 15 and 11 d at 10, 20, and 26 °C, respectively. ∗ indicates a significant difference in growth relative to the corresponding control (or lowest cadmium treatment for the 10 °C and high-food treatment) (t-tests with Bonferroni correction), × indicates that no statistical comparison with the corresponding control could be made because too few data was available.

## Reproduction

### *Age at maturity and first reproduction*

In Figure 4.5, wherein the cumulative number of neonates per living female as a function of times is given, the age at first reproduction can be seen as well. Results of a three-way ANOVA (Table 4.2) indicated that elevated temperature and food level significantly reduced the age at maturity and the age at first reproduction of the daphnids, while exposure to cadmium

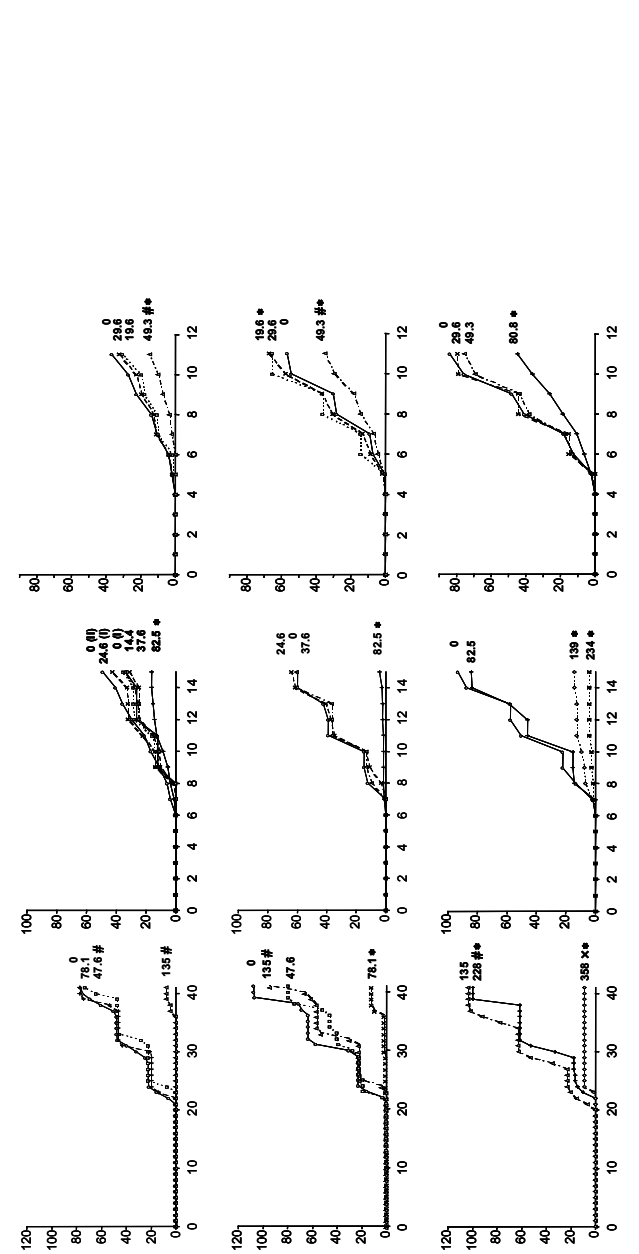


**Figure 4.5.** Cumulative number of neonates produced by *D. magna* exposed to various cadmium concentrations (as indicated by the numbers on the right side of the graphs) at 10, 20, and 26 °C and 0.50, 1.0 and 2.0 mg C $L^{-1}$ of *S. capricornutum* as a function of time. When exposure concentrations were equal, (I) or (II) were used to discriminate between data of first and additional 20 °C experiment, respectively. # and ∗ indicate a significant difference in age at first reproduction and total number of neonates, respectively, relative to the corresponding control (or lowest cadmium treatment for the 10 °C and high-food treatment) (t-tests with Bonferroni correction), × indicates that no statistical comparison with the corresponding control could be made because of too few data.

postponed the onset of reproduction. The significant two- and three-factor interactions point out that the effects of cadmium on the age at maturity were enhanced by high temperature and low food level. With respect to the age at first reproduction, only the interaction between food and cadmium was significant, which implied that a high food level protected the daphnids against adverse effects of cadmium.

### *Total number of neonates*

The total number of neonates produced was significantly affected by temperature, food and cadmium (three-way ANOVA, Table 4.2). Except the interaction between temperature and food, all two- and three-factor interactions were significant as well. Figure 4.5 shows that the number of neonates born during the test period was significantly higher at 10 °C than at 20 and 26 °C, where the numbers were similar. High food levels increased the reproductive output. When comparing cadmium effects at the different food and temperature treatments, it appeared that cadmium affected reproductive output more at low food levels and high temperatures.

### *Maternal investment in reproduction*

Although the effects of temperature, food and cadmium, and the joint action of temperature and food, and temperature and cadmium on maternal investment per neonate (as neonate size) were significant (three-way ANOVA, Table 4.2), no clear relationship between the three factors and neonate size were found. However, distinct relationships were found for the total investment in reproduction (as neonate size · brood size) (Figure 4.6).

The total investment was affected by temperature, food and cadmium. Significant interactions between temperature and cadmium, and between temperature, food and cadmium were present as well (three-way ANOVA, Table 4.2). In general, the overall investment in reproduction increased with low temperature and high food level, and decreased when the animals were exposed to cadmium. The negative effects of cadmium were increased at elevated temperature. Since no effects were observed for any of the three

factors on neonate size, the differences in total investment could entirely be attributed to the differences in brood size.

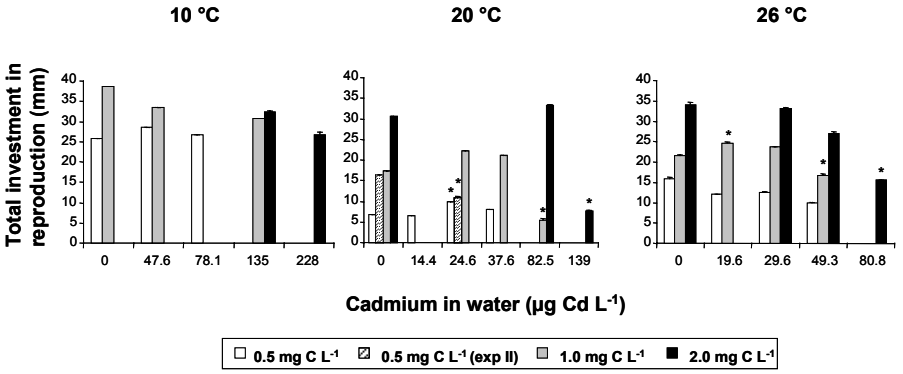


**Figure 4.6.** Total investment in reproduction of *D. magna* at 10, 20, and 26 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum*, as a function of the cadmium concentration in the water. Error bars represent standard errors. * indicates a significant difference in population growth rate relative to the corresponding control (or lowest cadmium treatment for the 10 °C and high-food treatment) (t-tests with Bonferroni correction).

## Population growth rate

Population growth rate was significantly affected by temperature, food and cadmium, as well as their two-factor interactions (three-way ANOVA, Table 4.2). Figure 4.7 shows the population growth rate at the different exposure regimes. Population growth rate increased with elevated temperature and food levels and decreased with increasing concentration of cadmium. However, at 26 °C and medium food level, cadmium concentrations up to 29.6 µg Cd $L^{-1}$ caused an increase in population growth rate relative to the control treatment. At 10 °C, population growth rate was hardly influenced by food level, but at elevated temperatures of 20 and 26 °C, high levels of food were observed to stimulate population growth. In general, cadmium became more damaging at low food levels. The cadmium-induced decrease in growth rate was amplified at elevated temperature because similar exposure concentrations resulted in a larger reduction in growth rate than at low temperature.

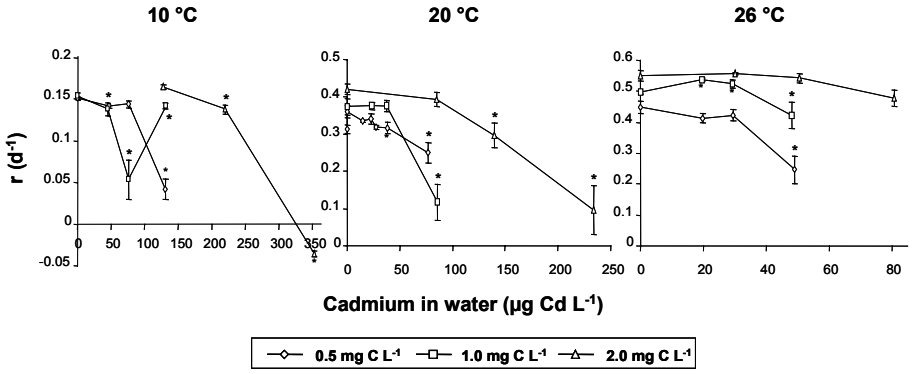


**Figure 4.7.** Population growth rate of *D. magna* at 10, 20, and 26 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum*, as a function of cadmium concentration in the water. Error bars represent 95% confidence intervals. The test duration was 41, 15 and 11 d at 10, 20, and 26 °C, respectively. * indicates a significant difference in population growth rate relative to the corresponding control (or lowest cadmium treatment for the 10 °C and high-food treatment) (t-tests with Bonferroni correction).

## DISCUSSION

The results of the present study revealed that temperature, food and cadmium, as well as their interactions, were important factors that influenced life history parameters and as a consequence, the population growth rate of *D. magna*.

The age at maturity and the age at first reproduction decreased at high temperature, enhancing population growth. This is in accordance with the common observation that higher temperatures accelerate physiological processes in ectotherms, as most aquatic organisms are (Cairns et al. 1975). In contrast, somatic growth of the daphnids, as well as the number of neonates produced, was higher at 10 °C than at 20 and 26 °C. Possibly, increased metabolic costs at elevated temperatures resulted in less energy available for growth. As smaller daphnids produce less offspring (Glazier 1992), the lower reproduction at elevated temperatures could be caused by the smaller body size of the daphnids at these temperatures. However, this decrease in reproduction did not result in a lower population growth rate at 20 and 26 °C compared with 10 °C. Similar outcomes were reported for *D. magna* by Sakwińska (1998). Daphnids raised at lower temperature matured later and at a larger size, resulting in a higher number of offspring in the

first two clutches, but population growth rate was still higher at elevated temperature.

Higher population growth rates were obtained at high food levels. This was due to a decrease in the age at which the daphnids started to reproduce, in combination with an increase in the amount of neonates produced. The latter could be attributed to the larger body size of the daphnids when food was abundant. At high food levels, more energy is available for growth and reproduction (Kooijman 2000), which was reflected by the better performance of well-fed relative to poor-fed daphnids.

Cadmium exposure decreased the population growth rate, since the metal affected all parameters, except life span at 26 °C. This is in accordance with the findings of previous studies (Marshall 1978, Bertram and Hart 1979, Wong and Wong 1990).

The interaction between temperature and food significantly affected the age at maturity, growth of the daphnids, and population growth rate, but none of the other life history parameters studied. The increase in growth rate when temperature rose from 10 to 20 °C was consistent for the three food levels, but at 26 °C, the increase was smaller at low food levels relative to the higher food levels. Apparently, food deficiency in the low-food treatment increased with temperature. Since the algae concentration in the water column was relatively constant (data not shown here), this deficiency was not due to the test design, but more likely to physiological processes, such as a higher metabolic rate. The findings suggest that at the lowest food concentration, food intake of the daphnids could no longer support the higher metabolic energy needs induced by high temperature, which resulted in a lower population growth rate. Many authors indeed state that temperature effects are most pronounced at non-limiting food conditions (Orcutt and Porter 1984, Giebelhausen and Lampert 2001). Clearly, the daphnids can only gain from elevated temperature when enough food is available to cope with increased metabolic costs.

Both the two-factor interactions between temperature and cadmium, and between food and cadmium influenced the population growth rate. This was due to differences in the number of neonates produced under the tested conditions. Temperature and cadmium interacted in a way that temperature enhanced the negative effects of cadmium on the population growth rate. Previous studies also reported increased cadmium toxicity at elevated temperatures in acute experiments (Lewis and Horning 1991, Stuhlbacher et al.

1993, Heugens et al. 2003), which was due to enhanced cadmium uptake rates as well as elevated intrinsic sensitivity of the daphnids (Heugens et al. 2003). Indeed, the tissue cadmium concentrations measured in the present study revealed that daphnids accumulated a higher amount of cadmium at elevated temperatures. Results of other chronic experiments were in accordance with the findings of the present study. For instance, decreased population growth rates at rising temperature were found for *D. pulex* exposed to toxic cyanobacteria (Hietala et al. 1997). A similar temperature response was presented for survival of *D. ambigua* exposed to *Chaoborus* kairomone, but no effects on growth and reproduction were observed (Hanazato 1991). In both studies, the observed interactions between the stress factors were explained by increased metabolic costs associated with high temperatures. When an additional stress factor was present, the daphnids could no longer cope with these increased metabolic needs, either because the stress factor inhibited food intake (cyanobacteria), or because it induced a defense mechanism (kairomone).

The interaction between food level and cadmium became apparent from the lower toxicity of cadmium in well-fed daphnids compared to poor-fed ones. Apparently, high food level protected the daphnids against adverse effects of cadmium. This resulted in an earlier start of reproduction, a larger body size and the production of a higher amount of neonates, which led to higher population growth rates at high food level than at low food level. Studies that focused on the influence of food level on chronic cadmium toxicity to the cladocerans *D. carinata* and *Echinisca triserialis* also found increased effects of cadmium on population parameters with decreased food levels (Chandini 1988, 1989). Cadmium disturbed the feeding processes and it was suggested that food intake by poor-fed daphnids was insufficient to maintain normal functioning, especially when metabolic costs increased due to cadmium stress. Although Klüttgen and Ratte (1994) found a similar relationship between cadmium and food for effects on juvenile development, they observed enhanced adverse effects of cadmium on growth and reproduction in adult daphnids held at high food concentrations. With respect to population growth rate, they reported that this rate was largely independent of food level. The authors suggested that the response of adults could be due to the low calcium concentration in the test medium, which caused an increase in cadmium uptake via calcium-specific carriers, especially at higher food levels due to an elevated metabolic rate.

Although the authors did not specify the calcium content, the total water hardness in their test water was four times lower than the calcium concentration in our test medium. This could explain the different cadmium-food interaction observed in our study.

Another possible mechanism is related to the binding of the metal onto algal cell surfaces. Tissue cadmium concentrations determined in daphnids that survived until the end of the experiment revealed that less cadmium was accumulated by the daphnids held at high than at low food concentration. Apparently, more cadmium ions were adsorbed per algal cell when food was less abundant. Allen et al. (1995) found that cations such as free cadmium induced feeding inhibition at sublethal concentrations, in contrast to anionic toxicants which caused inhibition of feeding only at lethal concentrations. Reduced feeding rates in cadmium-exposed *D. magna* were also observed in other studies (Bodar et al. 1988, Chandini 1988, 1989, Taylor et al. 1998, Barata et al. 2002). These findings indicate that adsorption of cadmium ions to the negatively charged surface of algal cells is a potential exposure route of cadmium to particle-grazing daphnids. When food uptake was also impaired in the present experiment, the effects would be higher at low food level, both because the higher amount of cadmium absorbed to the algae and because the net food intake is affected more at low food levels. The effect on feeding is likely to affect growth and reproduction, finally resulting in reduced population growth rates.

Although a three-factor interaction between temperature, food and cadmium was found for effects on age at maturity, growth and the number of neonates, such an interaction was not observed for population growth rate. We know of only one other study that dealt with chronic experiments with *Daphnia* exposed to three stress factors. Folt et al. (1999) analyzed the joint effects of temperature, food and the detergent sodium decodecyl sulfate (SDS) on cumulative reproduction and survival of *Daphnia*. Cumulative reproduction in multiple stressor treatments was generally less than or equal to reproduction in single stressor treatments. Comparable to our findings, reproduction rose with increasing temperature, until temperature became lethal. At low food level, reproduction started later and was lower than at high food dose. Despite these significant effects of temperature and food, no interaction between the two factors was found. The interaction between food and SDS was opposite to our findings: SDS reduced reproduction to a greater extent at high food level than at low food level. A three-

factor interaction revealed that the influence of food on toxicity was stronger at high temperatures. Similarly, toxicant-induced mortality increased at high temperature, and this effect was more pronounced at high food level. It was suggested that these interactions were related to increased toxicant uptake rates from the water and food at high temperature and food concentration due to elevated feeding rates. The difference in toxicant mode of action (SDS versus cadmium) could account for the contrasting results of Folt et al. (1999) and the current study.

Organisms living under field conditions are subjected to a variety of environmental stressors. Still, relatively little is known about the combined effects of these stress factors. Although a substantial amount of literature is available about the distinct effects of temperature, food, and cadmium, less information is at hand about the joint effects of two factors, and studies about three-factor interactions are exceptional. Moreover, conflicting results have been reported, which is probably due to the large variation in test protocols.

Results of the present study revealed that temperature, food, and cadmium are important factors influencing population growth of *D. magna*. In contrast to standard laboratory conditions (20 °C and a surplus of high-quality food), organisms living under natural conditions are subjected to a variety of environmental stressors. Temperatures below 20 °C may occur for most of the year in aquatic systems located in temperate regions. As illustrated by this study, this may reduce population growth of daphnids compared to standard conditions. However, higher temperatures are likely in small ponds during the summer season or may occur in the future through global climatic warming. This may lead to enhanced population growth, as long as the upper thermal tolerance limit is not exceeded. In contrast to the excessive food quantity often used in the laboratory, food levels in the field are limiting during large parts of the year, especially when *Daphnia* populations have reached carrying capacity (Kooijman and Metz 1984). According to the outcomes of this study, low food availability in the field will decrease population growth rates. With regard to cadmium exposure, negative effects of cadmium on field populations are expected to decrease at low temperature, whereas higher cadmium toxicity will occur at high field temperatures and low food levels. These interactions indicate that results of

standard toxicity tests should be interpreted in a location-specific way to avoid both over- and underestimation of effects on field populations.

## ACKNOWLEDGEMENTS

The study was supported by the Institute for Inland Water Management and Waste Water Treatment. We wish to express our thanks to Clarissa Veenstra for performing range-finding experiments and testing the intermittent flow-through system.

# *Chapter 5*

## Modeling Life Cycle Parameters of *Daphnia magna* influenced by Multiple Stressors

Heugens EHW, Jager T, Kraak MHS, Admiraal W. To be submitted.

## ABSTRACT

Organisms living in the field are commonly exposed to combinations of stress factors. As little information is available about interactive effects between different types of stressors, this study focused on the joint effects of temperature, food, and cadmium on the water flea *Daphnia magna*. Data obtained from a life history experiment were analyzed with a dynamic energy budget model (DEBtox) to disclose underlying processes accounting for temperature- and food-modified effects of cadmium on development and reproduction of *D. magna*. Although more extensive modeling work needs to be done, results of the model analyses suggested that increased cadmium toxicity at high temperature and low food level was due to increased sensitivity of the daphnids to cadmium. The model parameters obtained by fitting the model to the data were used to simulate population growth of *D. magna* under multiple stress conditions. The results indicate that at high temperature and limiting food conditions cadmium causes adverse effects at levels that are not toxic under laboratory conditions.


## INTRODUCTION

Field populations in their natural environment are often subjected to combinations of chemical, physical and biological stressors, but it is still unclear how these stressors interact with each other. Although a number of studies considered the joint effects of chemical and physical stressors (reviewed in Cairns et al. 1975, McLusky et al. 1986, Hall and Anderson 1995, Heugens et al. 2001), an advanced understanding of the underlying processes is lacking. Insight into these processes is essential to understand and predict the dynamics of natural populations living under multiple stress conditions. For this reason, the present study analyzes the joint effects of temperature, food and cadmium on life cycle parameters of the water flea *Daphnia magna* in a model setting. The data were obtained from a previous study (Chapter 4). This study revealed that temperature, food and cadmium alone and in combination had a high impact on the performance of the daphnids. In general, population growth of the daphnids was highest at high temperature and food level. Adverse effects of cadmium were enhanced at higher temperatures and lower food levels. Cadmium accumulation in the daphnids

was elevated under these conditions, but it remained unclear if other factors were important as well, such as altered sensitivity of the daphnids to the metal, as was previously observed in short-term experiments (Heugens et al. 2003).

The mechanistic DEBtox model (Kooijman and Bedaux 1996) was chosen to analyze the data of the study described in Chapter 4. DEBtox is based on the dynamic energy budget theory (Kooijman, 2000) and describes how energy is assimilated by organisms and subsequently allocated to maintenance, growth, development and reproduction. De Coen and Janssen (2003) linked energy budgets to life history characteristics of control and cadmium-exposed *D. magna* by measuring changes in available energy reserves and energy consumption. The use of the DEBtox allows the disentangling of processes responsible for the observed responses of the daphnids to the different temperature, food and cadmium treatments. Instead of fitting the data of each observation time separately which leads to several response curves, all data is fitted simultaneously. This results in a response surface, which contains information on the dynamic expression of toxicant effects. Moreover, DEBtox estimates a true no effect concentration (NEC), which is process-based and independent of exposure time.

Previous studies showed that the DEBtox model was successful in analyzing the effects of jointly acting stressors: acute effects of cadmium and temperature on daphnids (Heugens et al. 2003) and chronic effects of copper and food on chironomids (Péry et al. 2003). The results presented in the current study are preliminary, as the modeling operation is still in progress and the fitting routine is under review. At this time, observations at 10 °C have not been included in the model analyses. Nevertheless, the outcomes of the present study can be considered as an example of the application of the DEBtox model in toxicity testing, the results of which are used to evaluate the influence of multiple stressors on field populations.

## MATERIALS AND METHODS

### Chronic experiments

The joint effects of cadmium, temperature and food on population parameters of *Daphnia magna* cultured in the laboratory were studied in chronic

experiments. An extensive description of these experiments is given in Chapter 4. In short, an experiment consisted of 12 treatments, composed of four cadmium concentrations (including one control) and three food levels (0.50, 1.0 and 2.0 mg C $L^{-1}$ of the algae *Selenastrum capricornutum*), which was consecutively performed at 10, 20 and 26 °C. Extra cadmium concentrations were included in an additional experiment at 20 °C, since the exposure concentration in the first experiment proved to be too low to exert effects. The experimental design is given in Table 4.1.

To maintain constant cadmium and food levels, an intermittent flow-through system was used. Per cadmium and food treatment in each temperature experiment, 10 neonates (<24 h) were exposed. As temperature accelerates physiological processes in ectotherms such as *Daphnia*, the experiment was ended when the daphnids reached a marked developmental endpoint: the production of the third brood. This led to exposure durations of 41, 15 and 11 d at 10, 20 and 26 °C, respectively. Several life history features of the daphnids were determined, from which the amount of living neonates produced over time, and body length and tissue cadmium concentration at the start and the end of the experiment were used in the present study. Data of the 10 °C experiment was not yet analyzed in the present study, because a missing control treatment at high food level complicates the model analysis. The effects of temperature, food, and cadmium on survival were small, and to reduce the amount of model parameters to be fitted, survival was not estimated.

### Model description

DEBtox is a mechanistic model that rests on the Dynamic Energy Budget (DEB) theory (Kooijman 2000). This theory describes the transfer of energy from food to processes involved with maintenance, growth and reproduction. The model is able to describe time-dependent data, wherein information about the dynamic aspect of the occurrence of effects is enclosed. A full description of the DEBtox model is given in Kooijman and Bedaux (1996), but an overview of the most important assumptions, equations and extensions are given in the following paragraphs.

*Growth and reproduction in the absence of cadmium*

When no toxicants are present and food levels are constant, growth of the isomorphic growing daphnids (body shape remains unchanged during growth) can be described by a Von Bertalanffy growth curve:

$$l(t) = l_\infty - (l_\infty - l_b)\exp(-r_B t) \tag{5.1}$$

where *l(t)* is body length as fraction of the maximum length ($l_\infty$) that the daphnids can reach when food is abundant (i.e. scaled length) (-) at time *t* (d), $l_b$ is scaled length at birth (-) (initial length in the present study was 0.853 ± 0.0074 (SE) and 0.874 ± 0.0088 mm for the 20 and 26 °C experiment, respectively), $r_B$ is the Von Bertalanffy growth rate ($d^{-1}$). To include dependency on different food levels, DEBtox uses *f* instead of $l_\infty$, which is the ingestion rate as a fraction of the maximum ingestion rate (i.e. scaled food density).

Both body size and food availability control reproduction. In the offspring number used to estimate the reproduction rate (*R* in number $d^{-1}$) in the present study, the number of living and dead neonates, and the number of aborted eggs $d^{-1}$ were included, since energy costs are involved in all three types of reproductive output. At a constant food level, *R* as a function of scaled length is given by:

$$R(l) = \frac{R_m}{1 - l_r^3}\left(\frac{g + l}{g + f} f l^2 - l_r^3\right) \tag{5.2}$$

with $R_m$ the maximum reproduction rate at maximum size and abundant food (number $d^{-1}$), *g* is the energy investment ratio (-) (see Kooijman and Bedaux [1996]), and $l_r$ is the scaled length at puberty (i.e. length at which for the first time energy is invested in reproduction) (-).

When food is abundant, *f* in Equation 5.1 and 5.2 equals 1, but this value becomes smaller than 1 under limiting food conditions. By use of this parameter, food limitation was included in the model.

### *Growth and reproduction in the presence of cadmium*

Exposure to toxicants may either result in direct or indirect effects on growth and reproduction. Growth is directly affected by toxicants when they increase the costs for growth, but indirectly when they lower the amount of incoming energy by reducing the assimilation rate or efficiency. Similarly, reproduction is directly influenced by toxicants when they decrease the survival probability of eggs or increase the energy costs per egg. When the assimilation is lowered by exposure to chemicals, or when the energy costs for maintenance or growth are increased, reproduction is indirectly affected. Indirect effects of toxicants on reproduction are associated with a delay of the start of the reproductive cycle, whereas this effect is not seen with direct effects. The first step in this study was to select which mode of toxic action was most relevant for cadmium. This was accomplished by fitting the model to the data while assuming different modes of toxic action. The results (not shown here) suggested that cadmium is affecting growth and reproduction of the daphnids indirectly by reducing the assimilation rate. This mode of toxic action was therefore used for all subsequent model analyses.

DEBtox assumes the existence of a no-effect concentration (NEC). Below this concentration, no toxic effects occur, independent of exposure duration. In the present study, cadmium concentrations in the daphnids were determined, which allowed the estimation of the internal analog of the NEC: the internal threshold concentration (ITC) for growth and reproduction. For this purpose, the original model was extended with additional equations, which were programmed in MatLab 6.1 (The Mathworks, Inc.). The effect of cadmium on the assimilation rate was considered to be proportional to the tissue cadmium concentration exceeding the ITC.

The accumulation kinetics of cadmium by the daphnids is assumed to follow a simple linear one-compartment model, provided that the cadmium and food concentrations in the water are constant and the absorption of cadmium to the algae cells, and the partitioning of cadmium over the different body compartments are instantaneous. Instead of estimating the uptake rate constant, $k_1$, DEBtox fits the ratio of $k_1$ and the elimination rate constant, $k_2$, which is also known as the bioconcentration factor (*BCF* in L $kg^{-1}$). For growing animals, the accumulation kinetics of cadmium can then be described by:

$$\frac{d}{dt}C_i = \frac{C_e \cdot BCF \cdot k_2 \cdot f}{l} - C_i \cdot \left( \frac{k_2 \cdot f}{l} + \frac{d}{dt} \ln l^3 \right) \tag{5.3}$$

wherein $C_i$ is the tissue cadmium concentration (mg Cd kg$^{-1}$ dw [dry weight]), $C_e$ is the cadmium concentration in the environment (dissolved fraction and fraction absorbed to food particles) (mg Cd L$^{-1}$) which is assumed to be constant in time, and the term $d/dt\ ln^3$ accounts for dilution by growth. In the present study, the likelihood of the model fits was not influenced when the *BCF* and $k_2$ were considered to be independent of food quantity. Therefore, to reduce the number of model parameters, one *BCF* and one $k_2$ were fitted for all food levels.

The effects on growth and reproduction rates resulting from cadmium-induced decrease in assimilation can be then described by:

$$\frac{d}{dt}l = r_B \left( f\left(1 - s(C_i)\right) - l \right) \tag{5.4}$$

and

$$R = \left(1 - s(C_i)\right)^3 \frac{R_m}{1 - l_r^3} \left( \frac{g + l}{g + f} fl^2 - l_r^3 \right) \tag{5.5}$$

respectively, with $s(C_i)$ as the stress function that is linear in the tissue cadmium concentration above the ITC:

$$s(C_i) = \left( \frac{C_i - ITC}{C_A} \right)_+ \tag{5.6}$$

where $C_A$ is the tolerance concentration for assimilation (mg Cd kg$^{-1}$ dw), which determines the magnitude of the effect caused by the tissue cadmium concentration exceeding the ITC. The '+' indicates that the stress function should be positive. Both the ITC and the tolerance concentration ($C_A$) are measures for the intrinsic sensitivity of the daphnids to cadmium. Estimation of these parameters at the different temperature and food treatments will therefore reflect changes in the sensitivity of the daphnids that may contribute to the observed temperature- and food-dependent cadmium toxicity.

Model parameters were estimated simultaneously (additional equations were programmed in MatLab 6.1 (The Mathworks, Inc.) by use of maximum likelihood methods and 95% confidence intervals were obtained by using the profile likelihood (Meeker and Escobar 1995).

## RESULTS

Figures 5.1 and 5.2 present the data and model fits of the tissue cadmium concentration, body length and cumulative reproduction of *D. magna* exposed to different combinations of cadmium and food levels at 20 and 26 °C, respectively. In general, the model fits that were obtained matched the data well. Parameter estimates with their likelihood-based 95% confidence intervals are given in Table 5.1. Non-overlapping confidence intervals indicate a significant difference between treatments.

Parameter estimates for the scaled food density (Table 5.1) show that the performance of the daphnids at the three food levels used in the experiments differed significantly from each other. Comparison of the scaled food densities at 20 and 26 °C reveals that the effects of food limitation imposed on the daphnids was more severe at low than at high temperature.

The course of the calculated tissue cadmium concentration over time was influenced by growth dilution (Figures 5.1 and 5.2). Once growth ceased (see growth curves), the effect of growth dilution disappeared and the cadmium tissue concentration increased rapidly. Some of the fits for cadmium accumulation (especially at 26 °C, low and medium food level) underestimated the tissue cadmium concentration. This was likely due to the small amount of data as the tissue concentrations and body lengths were only measured at the beginning and the end of the experiments. When comparing the different temperature, food and cadmium treatments, the tissue cadmium concentration of the daphnids turned out to be higher at elevated cadmium concentrations in the water and lower food levels. Corresponding parameter estimates (Table 5.1) show that the bioconcentration factor of cadmium was slightly lower at 26 °C than at 20 °C, but this difference was not significant as the confidence intervals overlapped. For the elimination rate an opposite temperature effect was found, with a significantly higher rate at 26 °C than at 20 °C.

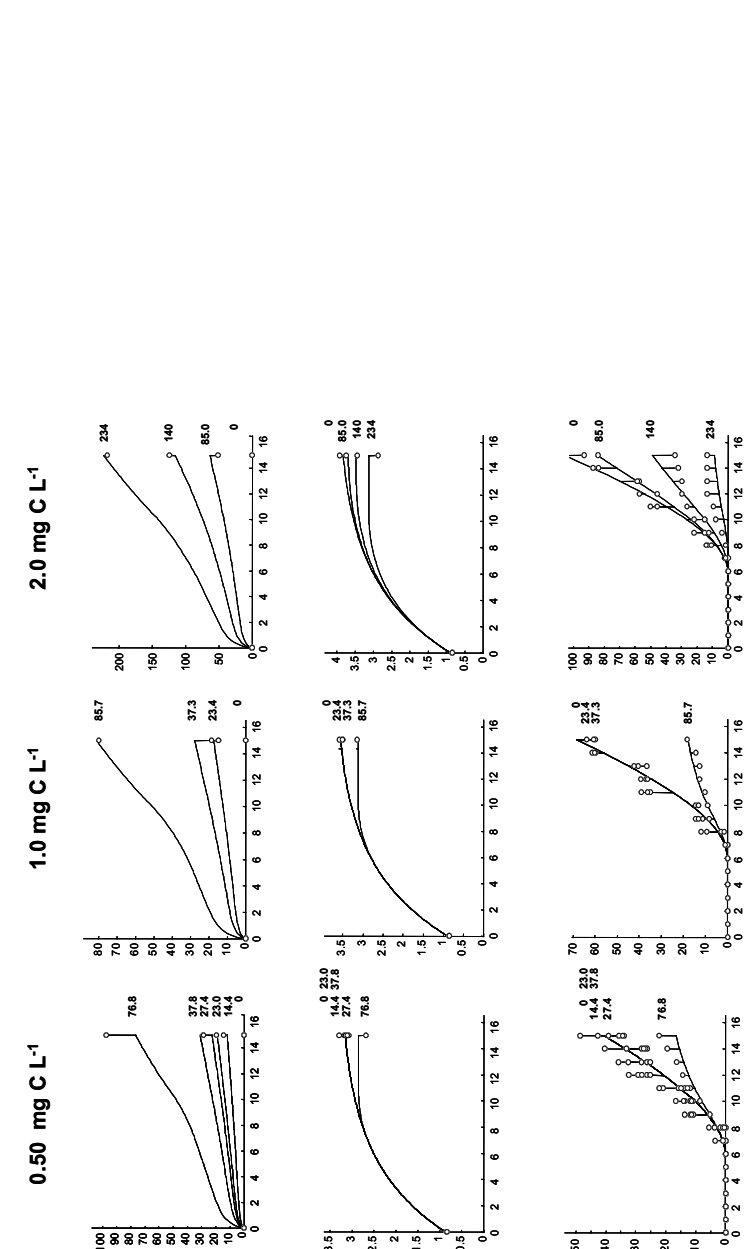


**Figure 5.1.** Cadmium accumulation (left panels), growth (center panels), and reproduction (right panels) of *D. magna* exposed to various cadmium concentrations at 20 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum* as a function of time. The numbers on the right side of each line correspond to the actual cadmium concentration in the water (μg Cd $L^{-1}$). Symbols represent data points, while lines are fitted to the data by the DEBtox model.

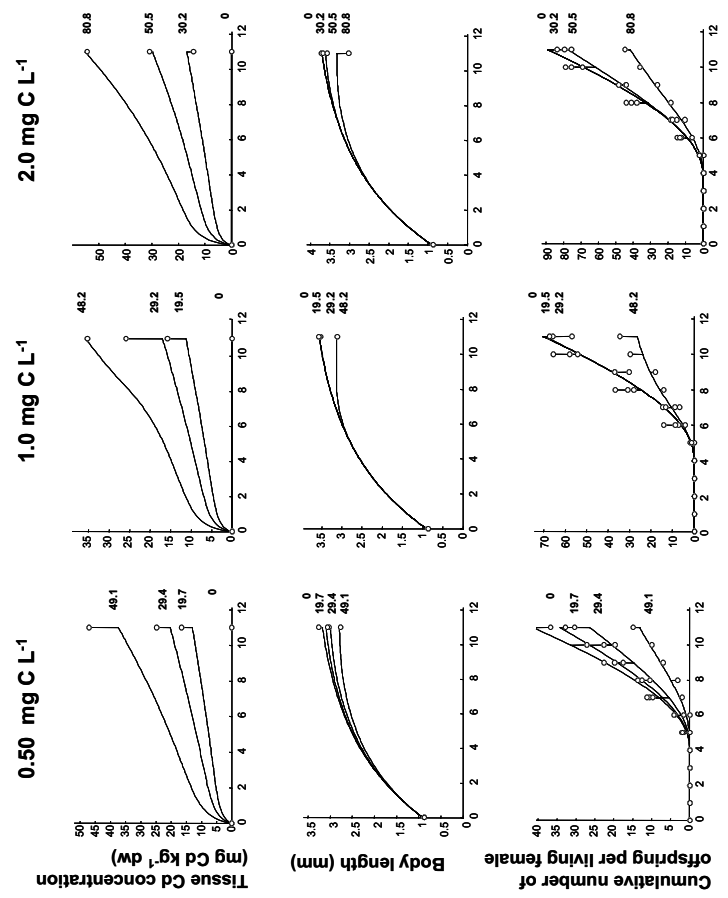


**Figure 5.2.** Cadmium accumulation (left panels), growth (center panels), and reproduction (right panels) of *D. magna* exposed to various cadmium concentrations at 26 °C and 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum* as a function of time. The numbers on the right side of each line correspond to the actual cadmium concentration in the water (µg Cd $L^{-1}$). Symbols represent data points, while lines are fitted to the data by the DEBtox model.

With respect to body length, daphnids grew less at low food level (Figures 5.1 and 5.2). Growth reduction by cadmium was pronounced at high temperature and low food level. Parameter estimates in Table 5.1 show that the maximum body size achieved by the daphnids at the two temperatures did not differ, but the Von Bertalanffy growth rate parameter was significantly higher at 26 °C than at 20 °C. Increasing food level increased this length at both temperatures, although this increase was stronger at 20 °C than at 26 °C.

The onset of reproduction can be seen in the reproduction curves (Figures 5.1 and 5.2), which allow a day-to-day comparison of simulated values and observations. The age at first reproduction increased at low temperature and low food conditions. Cadmium prolonged the juvenile period, and this effect was more pronounced at low food levels. These findings were supported by the length at puberty (Table 5.1), which was significantly lower at 26 °C compared to 20 °C, indicating that reproduction was enhanced at the high temperature. Increasing food level increased this length significantly at both temperatures, although this increase was stronger at 20 °C than at 26 °C. The number of neonates produced during the test period was similar at both temperatures (Figures 5.1 and 5.2). However, the number was lower at limiting food levels, and exposure to cadmium and high temperature enhanced this effect. The maximum reproduction rate was significantly higher at 26 °C than at 20 °C (Table 5.1).

As measures for the intrinsic sensitivity of the daphnids to cadmium, the ITC for growth and reproduction and the tolerance concentration for assimilation ($C_A$) (see Equation 5.6) with likelihood-based 95% confidence intervals were determined at the different temperature and food treatments (Figure 5.3 and 5.4). The overlapping confidence intervals in the 20 °C experiment reveal that there is no significant effect of food level on the ITC (Figure 5.3). At 26 °C however, the ITC at low food level was a factor of three lower than those at medium and high food levels, which were not significantly different. Comparison of the estimates made at the two temperatures points out that the ITC at low and intermediate food levels was significantly lower at 26 than at 20 °C, respectively. The difference is five-fold and two-fold for the low-food and medium-food treatment, respectively. Temperature did not affect the ITC at the high food level. The results indicate that the daphnids became more sensitive to cadmium under the combined conditions of high temperature and low food dose.

**Table 5.1.** Parameters estimated by the DEBtox model for the different temperature and food treatments[a]

| Symbol | Description | Unit | Temperature (°C) | Food level (mg of C $L^{-1}$) | Parameter value | Likelihood-based 95% confidence intervals |
|---|---|---|---|---|---|---|
| f | Scaled food density | - | 20 | 0.50 | 0.821 | 0.81 - 0.82 |
| | | | | 1.0 | 0.926 | 0.91 - 0.93 |
| | | | | 2.0 | 1.00[b] | - |
| | | | 26 | 0.50 | 0.852 | 0.84 - 0.86 |
| | | | | 1.0 | 0.960 | 0.95 - 0.97 |
| | | | | 2.0 | 1.00[b] | - |
| $l_m$ | Maximum length | mm | 20 | 0.50 – 2.0 | 4.02 | 4.0 - 4.3 |
| | | | 26 | 0.50 – 2.0 | 3.93 | 3.9 - 4.2 |
| $r_B$ | Von Bertalanffy growth rate | $d^{-1}$ | 20 | 0.50 – 2.0 | 0.186 | 0.18 - 0.21 |
| | | | 26 | 0.50 – 2.0 | 0.236 | 0.23 - 0.28 |
| $l_r$ | Length at first reproduction | mm | 20 | 0.50 | 2.63 | 2.6 - 2.6 |
| | | | | 1.0 | 2.95 | 2.9 - 3.0 |
| | | | | 2.0 | 3.12 | 3.1 - 3.1 |
| | | | 26 | 0.50 | 2.59 | 2.6 - 2.6 |
| | | | | 1.0 | 2.88 | 2.8 - 2.9 |
| | | | | 2.0 | 2.94 | 2.9 - 3.0 |
| $R_m$ | Maximum reproduction rate | number $d^{-1}$ | 20 | 0.50 – 2.0 | 24.4 | 24 - 27 |
| | | | 26 | 0.50 – 2.0 | 28.2 | 27 - 32 |
| BCF | Bioconcentration factor | L $kg^{-1}$ | 20 | 0.50 – 2.0 | $2.07 \cdot 10^3$ | $2.0 \cdot 10^3$ - $2.4 \cdot 10^3$ |
| | | | 26 | 0.50 – 2.0 | $1.85 \cdot 10^3$ | $1.8 \cdot 10^3$ - $2.0 \cdot 10^3$ |
| $k_2$ | Elimination rate | $d^{-1}$ | 20 | 0.50 – 2.0 | 0.0423 | 0.039 - 0.050 |
| | | | 26 | 0.50 – 2.0 | 0.0676 | 0.061 - 0.082 |

[a]Estimations for *ITC* and $C_A$ are given in Figure 5.3 and 5.4, respectively.
[b]Not fitted.

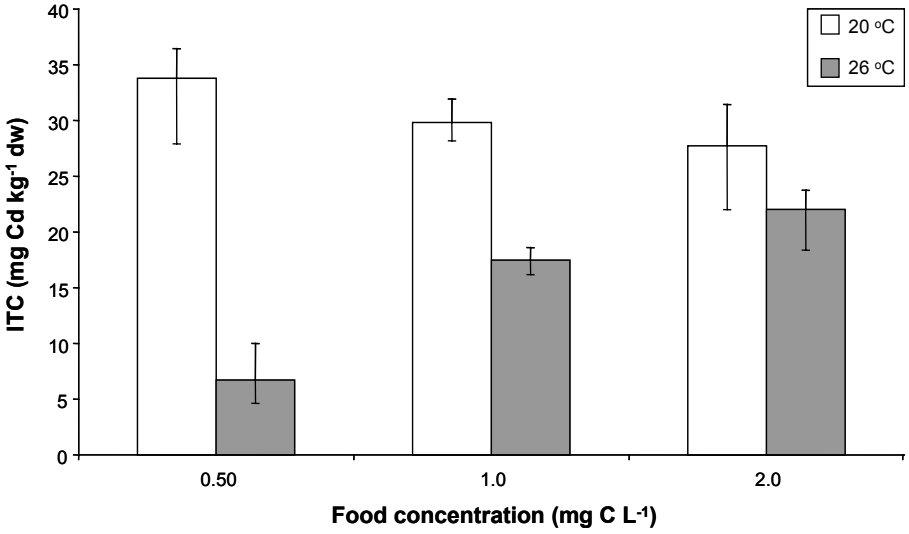


**Figure 5.3.** Estimations for the ITC of *D. magna* at 20 and 26 °C as a function of food level. The test duration was 15 and 11 d at 20 and 26 °C, respectively. Error bars represent likelihood-based 95% confidence intervals. Non-overlapping intervals indicate significant differences between treatments.

Estimations for the tolerance concentration for assimilation ($C_A$) at the different temperature and food combinations are presented in Figure 5.4. At 20 °C, the tolerance concentration was four times higher at the high food level than at the intermediate and low food level, where the tolerance concentrations did not significantly differ from each other. In agreement with the results found for the ITC, this indicates that the daphnids were more vulnerable to cadmium when less food was available. At 26 °C however, ambiguous results were obtained as a three times lower tolerance concentration was found at the medium food level compared to the low and high food levels, where the concentrations were comparable. This suggests that the sensitivity of the daphnids was increased at the intermediate food level. However, these findings may also be the result of experimental variation in this food treatment, since the daphnids at the two lowest cadmium concentrations performed better than the control animals, which may have had consequences for the model fit. When the tolerance concentrations at the two temperatures were compared, significant temperature effects were found at the high food level. At this food quantity, the tolerance concentration was two times lower at 26 °C than at 20 °C, indicating that the daph-

nids were two times less resistant against cadmium at elevated temperature, which is in accordance with the findings for the ITC.

Both the ITC and the tolerance concentration reflect the intrinsic sensitivity of the daphnids. Overall, the effects of temperature and food level on the two parameters were consistent, indicating that the daphnids became more vulnerable to cadmium at high temperature and low food levels.

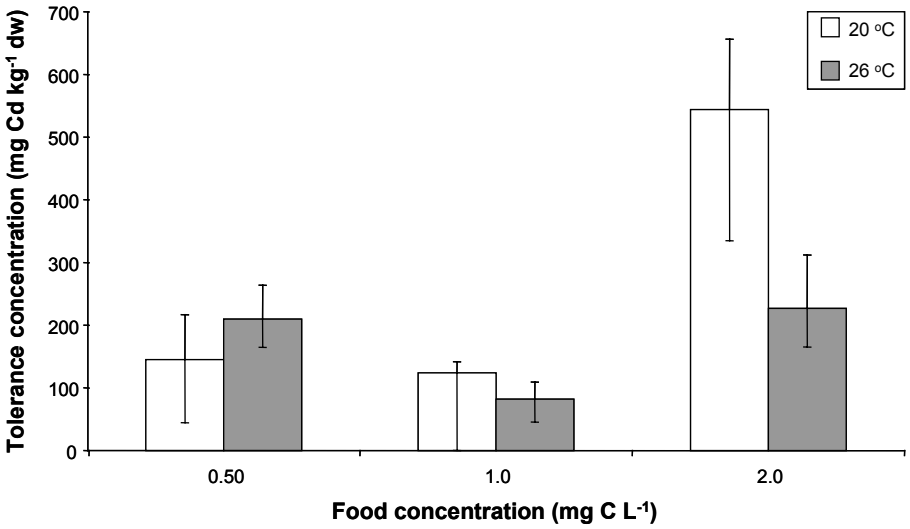


**Figure 5.4.** Estimations for the tolerance concentration for assimilation ($C_A$) of *D. magna* at 20 and 26°C as a function of food level. The test duration was 15 and 11 d at 20 and 26 °C, respectively. Error bars represent likelihood-based 95% confidence intervals. Non-overlapping intervals indicate significant differences between treatments.

## DISCUSSION

The conclusions given here are preliminary and may have to be adapted when the model is run with an improved fitting routine and when the model fits for the data obtained at 10 °C become available.

In this study it was demonstrated that DEBtox was able to describe the effects of temperature, food and cadmium on life history parameters of *D. magna* adequately. The first step of the model analyses was the selection of the most relevant mode of toxic action of cadmium (direct or indirect effects

on growth or reproduction, see materials and methods section). Based on the results (not shown here), it was assumed that cadmium indirectly influenced growth and reproduction by its effect on assimilation. Apparently, the amount of energy assimilated was reduced in cadmium-exposed daphnids, leaving less energy available for growth and reproduction. In other studies, it was indeed observed that cadmium interfered with the feeding process of cladocerans (Chandini 1988, 1989, Taylor et al. 1998, Barata et al. 2002). Bodar et al. (1988) found no effects of cadmium on assimilation efficiency of *D. magna*, but consumption and assimilation rates decreased. It was unclear if assimilation rate was directly influenced by cadmium or indirectly by reduced consumption. However, Gulati et al. (1988) reported that cadmium inhibited assimilation rates of Crustacea, including *Daphnia*, more than consumption rates, which resulted in a strong decline in assimilation efficiency. Reduction of the available energy for population growth has also been measured for *D. magna* exposed to cadmium and other toxicants (De Coen and Janssen 2003).

In Heugens et al. (submitted), the observed increase in cadmium toxicity at high temperature and low food level was partly explained by elevated cadmium accumulation under these conditions. The model analyses presented in the current study revealed that changes in the intrinsic sensitivity of the daphnids to cadmium, as defined by the ITC and the tolerance concentration for assimilation ($C_A$) also contribute to the altered cadmium toxicity. In general, the ITC and tolerance concentration decreased at elevated temperature and low food level, which points at increased vulnerability of the daphnids to cadmium at high temperature and low food levels. Increased sensitivity of *D. magna* to cadmium at elevated temperatures was also shown in short-term experiments (Heugens et al. 2003). In these acute experiments in which no food was given to the daphnids, the results suggested an interaction between starvation and increasing temperature: cadmium-exposed daphnids that survived long enough to experience starvation appeared to become more susceptible to cadmium, resulting in a lowering of the ITC for survival in time (results not published). This effect was more pronounced at high temperature.

The implications of interactive effects of temperature, food, and cadmium on field populations are difficult to predict. Positive effects of elevated tem-

perature and food level on reproduction may be canceled out by the increased sensitivity of the daphnids to cadmium at high temperature and low food quantity. Therefore, the parameters estimated with the DEBtox model were used to calculate population growth rates at different stressor combinations as a function of cadmium concentration in the water (Figure 5.5). For this purpose, survival at the different treatments was estimated, while all other parameters were fixed at the values given in Table 5.1 (for equations, and estimates of the additional parameters for survival see Annex 5.1).

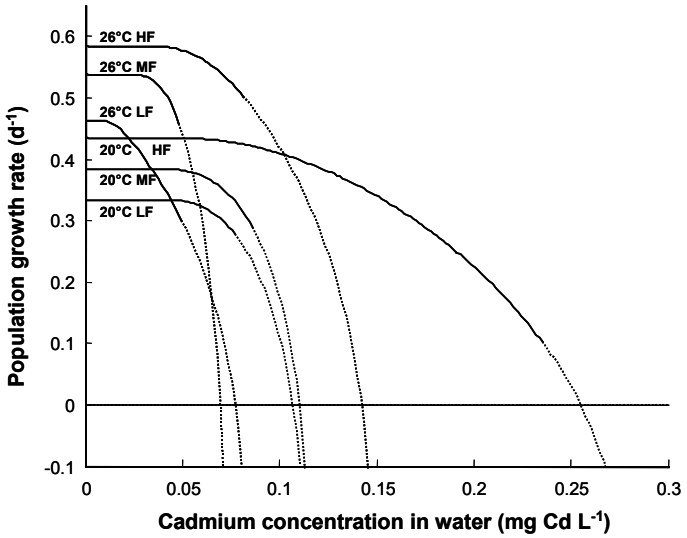


**Figure 5.5.** Population growth rate of *D. magna* at 20 °C and 26 °C, and 0.50 (LF), 1.0 (MF), and 2.0 mg C $L^{-1}$ (HF) of *S. capricornutum* as a function of cadmium concentration in the water. Solid lines represent population growth rates at tested cadmium concentrations, while dotted lines are extrapolated population growth rates at non-tested cadmium concentrations. For equations and parameter estimates see Annex 5.1.

As can be seen in Figure 5.5, the highest population growth rate is achieved at high temperature and high food condition. However, when cadmium is present, the performance of the daphnids declines more rapid in this treatment relative to the other treatments, even below levels attained at low temperature and low food level. In the standard *Daphnia* reproduction tests (OECD, 1998), experimental conditions equal the 20 °C and high-food treatment. Figure 5.5 shows that the daphnids in this treatment are least affected by cadmium. These conditions may not be realistic for natural

waters, where higher water temperatures may occur during the summer season, whereas food levels generally are much lower than those used in standard toxicity tests. Regarding Figure 5.5, elevated temperature would lead to increased population growth rates relative to standard conditions, irrespective of food quantity. Simultaneous exposure to cadmium however, would decrease population growth rates especially at high temperature and low food levels, canceling out the stimulating effect of temperature. These differences in toxicity induced by changes in temperature and food level are within the ranges observed in literature (reviewed in Heugens et al. 2001). This example illustrates how natural factors may influence the outcomes of standard toxicity tests. The current risk assessment for chemicals relies heavily on such toxicity tests, which may introduce a considerable bias and may therefore fail to protect ecosystems when environmental conditions in the field differ from experimental conditions used in the laboratory.

## ACKNOWLEDGEMENTS

The study was supported by the Institute for Inland Water Management and Waste Water Treatment. We wish to express our thanks to Jan Hendriks and Nico van Straalen for valuable comments on previous versions of the manuscript.

## ANNEX 5.1

To understand the implications of the interactive effects of temperature, food, and cadmium on field populations, population growth rates were determined using the data presented in Heugens et al. (submitted). The equations given below are extensively discussed in Kooijman and Bedaux (1996). Population growth rates ($r$) at different combinations of temperature, food, and cadmium were calculated by use of the following equation:

$$1 = \int_{t_0}^{t_{\max}} q(t, C_e) \cdot R(t, C_e) \exp(-r(C_e) \cdot t) \tag{5.8}$$

where $t_0$ and $t_{max}$ (d) represent the times at the start and end of the experiment, respectively, $q(t,C_e)$ (-) is the probability of individuals to survive until time $t$, and $R(t,C_e)$ (number $d^{-1}$) the reproduction rate, both a function of time $t$ and the cadmium concentration in the water $C_e$ (mg Cd $L^{-1}$). $R$ is calculated following Equation 5.2 and 5.5. The survival probability is specified via the hazard rate ($h$). The product $h\Delta t$ can be interpreted as the probability to die in the small time interval $\Delta t$, given that the animal has survived up to that moment. The survival probability can be expressed as:

$$q(t, C_e) = \exp(-\int_0^t h(\tau, C_e) d\tau) \tag{5.9}$$

where $h(\tau,C_e)$ ($d^{-1}$) is the hazard rate at time $\tau$, which is a function of the toxicant concentration in the water ($C_e$). When the ITC for survival is exceeded, the hazard rate is assumed to increase proportionally to the difference between $C_i(t,C_e)$ and the ITC:

$$h(t, C_e) = \begin{cases} k_\dagger \cdot (C_i(t, C_e) - ITC_{surv}) + h_0(t) & \text{if } C_i(t, C_e) > ITC_{surv} \\ h_0(t) & \text{if } C_i(t, C_e) \le ITC_{surv} \end{cases} \tag{5.10}$$

where $k_\dagger$ (kg dw $mg^{-1}$ Cd $d^{-1}$) represents the killing rate, $h_0$ ($d^{-1}$) is the background hazard rate, and $ITC_{surv}$ (mg Cd $kg^{-1}$ dw) is the internal threshold concentration for survival. The killing rate is the proportionality factor that

describes the relation between the hazard rate and the tissue concentration that exceeds the ITC.

The parameters for survival and population growth rate were estimated while all other parameters were fixed at the values given in Table 5.1. For 20 °C, the following estimates were obtained: $h_0$ = 8.92·10$^{-3}$ d$^{-1}$, $ITC_{surv}$ = 54.8 mg Cd kg$^{-1}$ dw, $k_f$ = 2.47·10$^{-3}$ kg dw mg$^{-1}$ Cd d$^{-1}$. For 26 °C, $h_0$ = 7.20·10$^{-3}$ d$^{-1}$. Effects of cadmium on survival were small at 26 °C, and no $ITC_{surv}$ and $k_f$ were fitted for this temperature as the best fit was obtained when mortality due to cadmium was neglected. The calculated population growth rates are presented in Figure 5.5.

# *Chapter 6*

## Predicting Effects of Multiple Stressors

This thesis addresses the joint impact of natural factors and toxicants on aquatic species and studied the nature of the interaction between the two types of stressors. The findings showed that environmental conditions may influence toxicity of chemicals to a great extent. The experimental chapters gave more insight into the mechanisms responsible for the interactive effects of temperature, food level, and cadmium (i.e. changes in accumulation kinetics and sensitivity of the test organisms).

Knowledge of multiple stressor effects is vital for the protection of species living under variable field conditions. Risk assessment for chemicals is used for the regulation of chemical emissions, which aims at protecting ecosystems against adverse effects of man-made substances. One of the elements of the risk assessment process, effects assessment, relies heavily on laboratory toxicity tests in which the toxicity of single chemical is determined for a few species (algae, invertebrates, and fish). When the amount and quality of toxicity data is sufficient, species sensitivity distributions can be used to derive a concentration of the chemical below which adverse effects in the specific environmental compartment are not expected to occur (i.e. predicted no-effect concentration [PNEC]) (Van Straalen 2002). However, when the amount of toxicity data is limited, a PNEC is estimated by applying a constant assessment or uncertainty factor on the data for the most sensitive species to adjust for intra- and interspecies variation. The magnitude of these factors depends on the type (acute, chronic, or field) and quantity of toxicity data available. In the European Union, a constant factor of 10 is applied for each extrapolation step: the assessment factor ranges from 10 when sufficient chronic toxicity data is available to 1000 for acute toxicity data for a few species (Van Leeuwen and Hermens 1996). Similar approaches are used by the U.S. EPA (see Chapman 1998).

Current risk assessment for chemicals does not take the potential effects of multiple stress conditions into account. Therefore, the question remains how to predict the effects of multiple stress conditions in the field. This question is addressed here by discussing the findings of this thesis in the context of the predictability of multiple stress responses. The mechanistic approach that was followed in the previous chapters is extrapolated to the current practice in risk assessment.

## PREDICTING MULTIPLE STRESS RESPONSES

Two approaches will be evaluated to predict the joint effects of temperature, food level, and cadmium on population growth rates of *D. magna* that were presented in Chapter 4. The first approach has previously been used in Chapter 2 in which data obtained from literature studies were analyzed quantitatively. This resulted in coefficients for temperature, nutritional state, salinity, and toxicant concentration (when parameters other than LC50 or EC50 were considered), which specify the change in toxicity in relation to the change in the natural factor. With the second approach, multiple stress responses are calculated using the responses to single stress factors following Folt et al. (1999). The DEBtox model is not discussed as a predictive risk assessment tool here, as the model was fitted to the data of Chapter 4 and no other data set is available to validate the estimated parameters.

### Coefficients for temperature- and food-modified cadmium toxicity

In Chapter 2, coefficients for temperature and food level were derived from literature data using linear regression. With these coefficients, changes in cadmium toxicity due to variation in temperature and food level can be calculated. Here, this predicted change in toxicity is compared with the actual change in chronic cadmium toxicity observed in Chapter 4 for the population growth rate of *D. magna*.

The coefficients obtained in Chapter 2 were used to assess the change in toxicity due to shifts in temperature and food level by applying equation 1, which is based on Equation 2.2:

$$\frac{\log y_1}{\log y_2} = \frac{\beta_T \cdot \log T_1 + \beta_F \cdot \log F_1}{\beta_T \cdot \log T_2 + \beta_F \cdot \log F_2} \qquad (6.1)$$

in which $y_1$ and $y_2$ are the responses under the two treatments to be compared, $\beta_T$ and $\beta_F$ are the coefficients for temperature and food level, -14.5 and 0.357 (see Table 2.2), respectively, $T_1$ and $T_2$, and $F_1$ and $F_2$ are the temperatures (Kelvin) and food levels (mg C $L^{-1}$) of treatment 1 and 2, respectively. The coefficient for temperature (-14.5) was the average of temperature coefficients obtained from studies focusing on combined effects of tem-

perature and salinity (Annex 2.6). This coefficient was based on seven sets of LC50 data for marine and brackish water species, as all other available data were fitted to another model (Equation 2.1). This resulted in disparate coefficients that cannot be compared. For food, a coefficient of 0.357 was used (Table 2.2). This coefficient was based on 13 sets of reproduction data, mainly for crustaceans. As mortality was low in the experiments described in Chapter 4, the use of this coefficient seems appropriate for population growth rate as well. Population growth rates in cadmium treatments presented in Chapter 4 were normalized by dividing the population growth rates by the rates obtained in the corresponding control treatments. By this scaling, the cadmium toxicity-modifying effects of temperature and food were separated from the direct effects of the natural factors on population growth rates. Temperature- and food-induced toxicity changes were determined by calculating the ratios of population growth rates obtained in treatments with different temperatures and food levels, but similar cadmium exposure concentrations. These experimentally derived ratios were compared with the ratios calculated with Equation 6.1.

The ratios of the observed and the predicted change in toxicity due to shifts in temperature and/or food are given in Table 6.1. Values smaller than one indicate that the observed change in toxicity was lower than predicted, while values higher than one denote the opposite. The values were skewed to the right, with a geometric mean of 1.57 (1.0 – 3.5 ($5^{th}$ – $95^{th}$ percentile)), and a maximum of 4.3. This deviation is considered to be acceptable. The results indicate that the coefficients calculated from literature data of multiple stressor experiments were appropriate to predict the temperature- and food-modified effects of cadmium on population growth rates of *D. magna* measured in Chapter 4 (i.e. similar patterns as in literature were observed in Chapter 4).

A major drawback of this method is that scaling of experimental data to the corresponding control treatment eliminates the direct effects of temperature and food. Although this is an advantage for the separation of the influence of natural factors on toxicity and their direct effects on population growth rate, and for the comparability of studies (see Chapter 2), the influence of ambient temperature and food levels may be of greater importance to field populations than the toxicity-modifying nature of the factors. To distinguish between these two aspects, coefficients for temperature, food level, and

cadmium were determined using population growth data of *D. magna* (Chapter 4), but now by using both normalized and non-normalized data. The coefficients were estimated with multiple regression (SPSS version 10.0.5, SPSS Inc.) by use of Equation 6.2 (see also Equation 2.2):

$$\log y = \alpha + \beta_T \cdot \log T + \beta_F \cdot \log F + \beta_{Cd} \cdot \log Cd \qquad (6.2)$$

in which $y$ is either population growth rate normalized to the rate in the corresponding control treatment, or non-normalized population growth rate ($d^{-1}$), $\alpha$ is the intercept, $\beta_T$, $\beta_F$ and $\beta_{Cd}$ are coefficients for temperature, food and cadmium, respectively, $T$ is temperature (Kelvin), $F$ is food level normalized to the highest food level of 2 mg C $L^{-1}$, and $Cd$ is the actual cadmium concentration in the water (μg Cd $L^{-1}$).

The coefficients for temperature, food and cadmium calculated using normalized and non-normalized population growth rates are summarized in Table 6.2. The coefficients for normalized data specify the effects of temperature and food level on cadmium toxicity. The negative temperature and cadmium coefficients imply that a lower population growth rate, i.e. increased cadmium toxicity, is achieved at elevated temperatures and cadmium concentrations. The opposite occurs for food level, where the positive coefficient indicates that population growth is increased, i.e. cadmium toxicity is reduced, at elevated food levels. The overall effects of temperature and food level on population growth rate (i.e. cadmium toxicity-modifying effect *and* enhancing effect on population growth rate) are given by the coefficients for non-normalized data. In contrast to negative temperature coefficient for normalized data, the coefficient for non-normalized data is positive. Apparently, the influence of temperature on cadmium toxicity is of lesser importance than the stimulating effect of temperature on population growth. No differences were observed for the food coefficients for normalized and non-normalized data as the confidence intervals overlap. Apparently, the beneficial effect of food was not large enough to become apparent in the food coefficient for non-normalized data. However, the cadmium coefficient for non-normalized data is smaller than that for normalized data, suggesting that the favorable effect of temperature on population growth rate partly canceled out the adverse effects of cadmium.

**Table 6.1.** Comparing observed and predicted change in toxicity of cadmium to *D. magna* due to shifts in temperature and food density. The comparison is based on the ratio of observed (derived from data presented in Chapter 4) and predicted effects (ratio obs/pred). The predicted change in cadmium toxicity induced by temperature and food density is described in Equation 6.1. If the ratio is greater than unity, cadmium is more toxic than expected, in other words, temperature and food density in combination with cadmium reduces population growth rate more than was expected from literature data. Treatments with similar cadmium concentrations in the water were compared[a]

| Treatments compared | | [Cd] | Ratio obs/pred | Treatments compared | | [Cd] | Ratio obs/pred |
|---|---|---|---|---|---|---|---|
| 26 HF | 26 MF | 31 | 0.749 | 26 LF | 20 LF | 31 | 1.43 |
| | | 52 | 0.907 | | | 52 | 0.733 |
| | 26 LF | 31 | 0.653 | | 10 MF | 52 | 1.74 |
| | | 52 | 1.09 | | 10 LF | 52 | 1.30 |
| | 20 HF | 85 | 1.25 | 20 HF | 20 MF | 85 | 2.33 |
| | 20 MF | 31 | 1.06 | | 20 LF | 85 | 0.815 |
| | | 52 | 1.22 | | 10 HF | 145 | 1.17 |
| | | 85 | 2.91 | | | 246 | 0.450 |
| | 20 LF | 31 | 0.763 | | 10 MF | 85 | 3.48 |
| | | 31 | 0.933 | | | 145 | 0.988 |
| | | 52 | 0.802 | 20 HF | 10 LF | 85 | 0.992 |
| | | 85 | 1.02 | | | 145 | 2.58 |
| | 10 MF | 52 | 1.91 | 20 MF | 20 LF | 31 | 0.723 |
| | | 85 | 4.35 | | | 31 | 0.884 |
| | 10 LF | 52 | 1.43 | | | 52 | 0.780 |
| | | 85 | 1.24 | | | 85 | 0.351 |
| 26 MF | 26 LF | 19 | 1.89 | | 10 MF | 52 | 1.85 |
| | | 31 | 0.872 | | | 85 | 1.50 |
| | | 52 | 1.21 | | 10 LF | 52 | 1.39 |
| | 20 MF | 31 | 1.41 | | | 85 | 0.427 |
| | | 52 | 1.35 | 20 LF | 10 MF | 52 | 1.85 |
| 26 MF | 20 LF | 31 | 1.02 | | | 85 | 4.27 |
| | | 31 | 1.25 | | 10 LF | 52 | 1.78 |
| | | 52 | 0.885 | | | 85 | 1.22 |
| | 10 MF | 52 | 2.10 | 10 HF | 10 MF | 145 | 0.845 |
| | 10 LF | 52 | 1.57 | | 10 LF | 145 | 2.20 |
| 26 LF | 20 MF | 31 | 1.62 | 10 MF | 10 LF | 52 | 0.748 |
| | | 52 | 1.11 | | | 85 | 0.285 |
| | 20 LF | 19 | 3.95 | | | 145 | 2.61 |
| | | 31 | 1.17 | | | | |

[a]Symbols indicate the following: [Cd], cadmium concentration (µg Cd $L^{-1}$), here given as nominal concentrations; 10, 20, 26: 10, 20, and 26 °C; LF, MF, HF: 0.50, 1.0, and 2.0 mg C $L^{-1}$ of *Selenastrum capricornutum*.

Summarizing, the coefficients determined from literature data were successful in predicting the temperature- and food-modified effects of cadmium on population growth rates presented in Chapter 4. Estimation of coefficients for temperature, food and cadmium using normalized and non-normalized population growth data gave insight into the importance of temperature

and food level as factors that modified cadmium toxicity and population growth. The results indicate that the performance of field populations can only be accurately predicted when the effects of all prevalent environmental conditions, consisting of both natural and anthropogenic stressors, are considered.

**Table 6.2.** Coefficients with 95% confidence intervals (in brackets) for temperature ($\beta_T$), food ($\beta_F$), and cadmium ($\beta_{Cd}$), as well as the intercept $\alpha$, and R-square derived from population growth rates of *D. magna* presented in Chapter 4. The coefficients were estimated using Equation 6.2. Both normalized and non-normalized population growth rates were used as effect parameter[a]. The coefficients for normalized data represent temperature- and food-induced changes in cadmium toxicity whereas the coefficients for non-normalized data also denote the change in toxicity, as well as the direct effects of temperature and food density

| | Effect parameter | | | |
|---|---|---|---|---|
| Regression parameter | Normalized population growth rate | | Non-normalized population growth rate | |
| $\beta_T$ | -4.48 | (-13.1 – 4.17) | 24.3 | (18.3 – 30.3) |
| $\beta_F$ | 0.268 | (-0.0528 – 0.589) | 0.0971 | (-0.129 – 0.323) |
| $\beta_{Cd}$ | -0.513 | (-0.787 – -0.239) | -0.0187 | (-0.0377 – $3.63 \cdot 10^{-4}$) |
| $\alpha$ | 11.9 | (-9.78 – 33.6) | -60.4 | (-75.2 –- 45.7) |
| R-square | 0.443 | | 0.704 | |

[a]The negative population growth rate at 10 °C, high food and 370 μg Cd $L^{-1}$ could not be used in Equation 6.2 and was discarded.

## Calculating effects of multiple stressors from effects of individual stress factors

The second approach to predict the joint effects of temperature, food, and cadmium on population growth rates obtained in Chapter 4 explores to which extent combined effects of multiple stressors can be predicted from effects of the individual factors. Following Folt et al. (1999), the interaction between the single factors temperature, food, and cadmium is assumed to be comparative (joint effect equals effect of dominant or single worst stressor (Bruland et al. 1991), additive (joint effect equals sum of individual effects (Hay et al. 1994, Hay 1996), or multiplicative (joint effect equals multiplication of individual effects (Begon 1996, Pennings 1996)).

Population growth rates in all treatments were expressed as ratios of the performance under optimal conditions, where the highest population growth rate was achieved (see also Folt et al. 1999). This was in the treat-

ment with 26 °C, high food, and 30 µg Cd $L^{-1}$ (Figure 4.6). Since the performance in this treatment did not differ significantly from that in the corresponding control treatment, and to facilitate comparisons between different exposures, the 26 °C, high-food, and no-cadmium treatment was considered to be optimal. Effects of individual stressors effects were calculated by comparing population growth rate under optimal conditions to rates obtained in treatments where only a single factor was altered. For instance, the low temperature effect is the population growth rate in the 10 °C, high-food and no-cadmium treatment divided by the population growth rate in the 26 °C, high-food, no-cadmium treatment. As cadmium concentrations could differ between temperature and food treatments, not all combinations of stressors could be analyzed. Responses to the individual stressors were used to calculate multiple stress responses by assuming a comparative (dominant stressor determines multiple stress response), additive (responses to individual stressors are added), and multiplicative interaction (single stress responses are multiplied) between the separate factors. For example, when the population growth rates at low temperature, and at low food level were 0.9 and 0.8 times the population growth rate at optimal conditions, the population growth rate at the combination of low temperature and low food level is 0.8 (comparative), 1 - (1 - 0.9 + 1 - 0.8) = 0.7 (additive), and 0.9 x 0.8 = 0.72 (multiplicative) times the population growth rate at optimal conditions.

Table 6.3 shows the ratios of the observed and predicted multiple stress responses at various treatments for the three methods. While the predictions made with the comparative and multiplicative interaction were generally underestimating the actual responses (20 and 15 times out of 21, respectively), the additive interaction mostly overestimated (13 times out of 21) the effects induced by multiple stressors. The deviations of the predictions from the actual observed values were skewed to the right. When responses were overestimated, the geometric means of the differences between observed and predicted responses were 1.25 (1.0 – 2.5 ($5^{th}$ – $95^{th}$ percentile)) and 1.08 (1.0 – 1.2) and a maximum of 2.6 and 1.2 for the additive and multiplicative interactions, respectively. No geometric mean was determined for the comparative interaction as only one of the predictions overestimated the response (by a factor of 1.06). The geometric means of the differences between observed and predicted multiple stress responses when underestimation occurred were 1.35 (1.1 – 3.1), 1.34 (1.0 – 2.3), and 1.28 (1.0 – 2.6) for the comparative, additive, and multiplicative interaction, respec-

tively. The predicted response differed from the observed response up to a maximum of 3.6, 2.5, and 2.8 for the three interactions. As was argued above, these deviations are regarded as acceptable.

**Table 6.3.** Comparing observed effects of temperature, food, and cadmium on population growth rate of *D. magna* (data presented in Chapter 4) and those predicted by assuming a comparative, additive, and multiplicative interaction between the stressors (ratio obs/pred)[a]

| | | Comparative | | Additive | | Multiplicative | |
|---|---|---|---|---|---|---|---|
| Treatment | [Cd] | Ratio obs/pred | Under- or overestimation | Ratio obs./pred. | Under- or overest | Ratio obs./pred. | Under- or overest. |
| 10 LF | 0 | 1.09 | - | 2.43 | + | 1.13 | + |
| 10 MF | 0 | 1.08 | - | 1.38 | + | 1.03 | + |
| 20 LF | 0 | 1.34 | - | 1.01 | - | 1.09 | - |
| 20 LF | 0 | 1.17 | - | 1.13 | + | 1.05 | + |
| 20 MF | 0 | 1.13 | - | 1.02 | + | 1.02 | - |
| 10 LF | 52 | 1.16 | - | 2.58 | + | 1.08 | + |
| 10 LF | 85 | 1.15 | - | >10 | + | 1.24 | + |
| 10 MF | 52 | 1.20 | - | 1.33 | + | 1.06 | - |
| 10 MF | 85 | 3.09 | - | 1.34 | + | 2.42 | - |
| 20 LF | 31 | 1.23 | - | 1.05 | + | 1.02 | - |
| 20 LF | 31 | 1.32 | - | 1.02 | - | 1.09 | - |
| 20 LF | 52 | 1.33 | - | 1.02 | + | 1.07 | - |
| 20 LF | 85 | 1.68 | - | 1.02 | + | 1.19 | - |
| 20 MF | 31 | 1.12 | - | 1.01 | + | 1.02 | - |
| 20 MF | 52 | 1.12 | - | 1.04 | + | 1.00 | + |
| 20 MF | 85 | 3.59 | - | 2.52 | - | 2.82 | - |
| 20 HF | 85 | 1.07 | - | 1.13 | + | 1.08 | + |
| 26 LF | 31 | 1.06 | - | 1.07 | - | 1.07 | - |
| 26 LF | 52 | 1.82 | - | 1.79 | - | 1.80 | - |
| 26 MF | 31 | 1.06 | + | 1.04 | + | 1.04 | + |

[a]Symbols indicate the following: 10, 20, 26: 10, 20, and 26°C; LF, MF, HF: 0.5, 1.0, and 2.0 mg C $L^{-1}$ of *S. capricornutum*; [Cd]: cadmium concentration in the water (μg Cd $L^{-1}$), here given as nominal concentrations; -, +: under- or overestimation of actual response.

Considering these results, the actual multiple stress responses are worst predicted by the comparative interaction, since this interaction nearly always underestimated responses and the deviations were largest. The additive interaction mostly overestimated the effects of multiple stressors, whereas the multiplicative interaction generally underestimated effects. Although it seems safer to use a method that is overprotective than to have a chance of underestimating risk, the predictions made with the multiplicative interactions differed less from the actual responses than those made with the additive interaction. It is therefore concluded that of the three

methods (comparative, additive, and multiplicative), the multiplicative method predicted the joint effects of temperature, food, and cadmium on population growth rates of *D. magna* presented in Chapter 4 best.

### Comparison of the two approaches used to predict multiple stress responses

Joint effects of temperature, food, and cadmium on population growth of *D. magna* were predicted by use of coefficients that were derived from literature data, and by calculating multiple stress responses from responses induced by individual stress factors relative to optimum conditions. Since the results of the second approach revealed that multiplication of fractional responses to single stressors gave the best predictions, only this method is compared with the first approach using coefficients.

Both methods were able to predict the results of Chapter 4 adequately. Still, the predictions made by multiplying the responses to single stressors differed less from the actual observed effects than the use of literature-derived coefficients. However, to use the methods for a range of values for the natural factors, both methods require many data to obtain accurate coefficients for natural factors or to quantify the responses to single stress factors correctly. However, once appropriate coefficients for the natural stress factors have been determined, they can be used for a range of values for the natural factors to predict multiple stress effects, except values exceeding the tolerance range of species. In contrast, when predicting effects by multiplying responses to separately acting stressors, the response to every new value of the factor that deviates from the optimum conditions needs to be established first, for example by performing new experiments.

Both methods were able to predict the joint effects of temperature, food, and cadmium on population growth of *D. magna* presented in Chapter 4 within acceptable ranges. However, the use of literature-derived coefficients is less laborious because effects of non-tested values of natural factors can also be determined once coefficients are available. Therefore, it is concluded that this approach has the best potential for extrapolating toxicity estimates from one condition to another (for example, from the laboratory to the field).

Although environmental factors may affect both the exposure of organisms to substances and toxicity of these substances to organisms, current risk assessment for chemicals does not take the potential effects of multiple stressors into account. While much progress is made in predicting the influence of natural factors on the bioavailability of chemicals with biotic ligand models (Paquin et al. 2002, Janssen et al. 2003), the assessment of multiple stressor responses is still lacking. A straightforward implementation of multiple stressors in legislation (for example, by multiplying existing quality criteria for temperature, eutrophication, and chemicals) seems problematic because the units of multiple stressors are unlike and risks induced by multiple stressors can only be expressed in units of responses rather than in concentrations. However, the use of responses is not practical for legislation of emissions and translation of altered responses to safe concentrations is therefore crucial.

As was argued above, the uncertainties related to differences between laboratory test conditions and natural conditions in ecosystems can be estimated by the use of literature-derived coefficients. These uncertainties can be accounted for by applying an assessment factor for multiple stressor effects on toxicity data obtained under standard laboratory conditions. In Chapter 2, the magnitude of these factors with respect to effects of temperature, food level, and salinity on toxicity was discussed. It was argued that the factor should be smaller than one when extrapolating to ecosystems with lower temperatures or higher salinities than in the laboratory. On the other hand, the value should be greater than one when field temperatures are higher and food levels are lower than those in the laboratory. Translating the changes in toxicity due to the three natural factors found in literature to relevant field conditions suggests that the magnitude of the factor should fall with the range of 0.01 to 15 for temperature, and 1.2 to 10 for nutritional state. The range for salinity could not be determined. These values only give an indication of the change in toxicity due to environmental factors and should not be overvalued. The use of assessment factors seems valuable in the short term. However, on the long term it would be more effective to replace the use of constant assessment factors with statistical and mechanistic models that also specify the margins of uncertainty for the range of factors that modify the toxicity of chemicals.

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# *Summary*

# *Samenvatting*

Over the last few years, the levels of several toxicants in the aquatic environment have declined. Standard toxicity tests with these low toxicant concentrations often suggest that present pollution levels have no impact on ecosystems. However, toxicity testing is generally performed under optimal and constant experimental conditions (e.g. temperature and food level), while in the field, species are exposed to combinations of biological, physical, and chemical stressors. These suboptimal and variable field conditions are likely to influence the toxicity of chemicals to organisms. Although knowledge of interactions between different types of stressors is vital to predict effects of multiple stressors in the field and to protect ecosystems, relatively little is known about the interactions and effects of jointly acting stressors. Therefore, this thesis addressed the combined effects of toxicants and environmental factors on aquatic biota and explored the mechanisms of their interaction.

The first step in this study was to evaluate the existing literature on multiple stressors. This literature review revealed that temperature, nutritional state and salinity had a major impact on the toxicity of various chemicals to aquatic organisms (Chapter 2). Organisms exposed to conditions close to their environmental tolerance limits were generally more sensitive to additional chemical stress, while exposure to toxicants narrowed the tolerance range for natural factors. Overall, toxicity increased at high temperatures and low levels of food or nutrients. The influence of salinity on toxicity depended on the type of chemical, but the relationship between both factors was not clear for all classes of compounds. Quantitative analyses of litera-

ture data resulted in coefficients for the three natural factors studied, which gave an indication of the magnitude of uncertainty factors that could be used to extrapolate results obtained under laboratory conditions to the field. Although the observed interactions were explained by changes in toxicant bioavailability, accumulation kinetics, and sensitivity of the test organisms due to the natural factors, these processes were have rarely been investigated so far.

The mechanisms responsible for temperature-dependent toxicity of cadmium to *Daphnia magna* were therefore studied in toxicity and accumulation experiments and the data were analyzed with the DEBtox model (Chapter 3). Extreme temperatures caused complete mortality, independent of cadmium exposure. Below this upper thermal tolerance limit, temperature increased cadmium toxicity. This temperature-dependent cadmium toxicity was attributable to various mechanisms. In the lower temperature range, a temperature rise accelerated uptake kinetics, causing higher cadmium toxicity. In the higher temperature range, increased uptake was less important, while the contribution of increased intrinsic sensitivity of the daphnids became more significant.

To increase the ecological relevance of the study, chronic experiments were performed in an intermittent flow-through system and food level was introduced as a second natural factor. In these life history experiments, the joint effects of temperature, food, and cadmium on population growth of *D. magna* were analyzed (Chapter 4). High temperature and food level were found to stimulate population growth rate, whereas negative effects of cadmium were enhanced at elevated temperatures and low food levels. Tissue cadmium concentrations showed that increased cadmium accumulation at high temperature and low food conditions contributed to the observed temperature- and food-modified toxicity.

In Chapter 5, the data of the chronic experiments reported in Chapter 4 were analyzed with the DEBtox model. The findings presented are preliminary and will be adjusted when new parameter estimates become available. Model runs suggested that cadmium indirectly influenced reproduction of the daphnids by reducing the amount of energy assimilated from food in cadmium-exposed daphnids. In addition to the increased cadmium accumulation (Chapter 4), the model fits revealed that the intrinsic sensitivity of the daphnids to cadmium was increased as well under the combined conditions of high temperature and low food levels.

Finally, the predictability of the effects of multiple stressors and the implications for risk assessment are discussed in Chapter 6. Two approaches are worked out and evaluated to predict the population growth data presented in Chapter 4. First, the coefficients for temperature and nutritional state calculated in Chapter 2 were used to predict changes in the toxicity of cadmium at varying temperature and food levels. Second, multiple stress responses were calculated using the responses to the single stress factors observed in Chapter 4 by assuming a comparative, additive, and multiplicative interaction between the factors, of which the multiplicative interaction predicted the responses to the multiple stressors best. When both the predictive potential of the two approaches and the simplicity to use them were taken into account, the use of literature-derived coefficients for temperature and food was considered to be the best approach to predict the joint effects of temperature, food level and cadmium on population growth of *D. magna* presented in Chapter 4.

Current risk assessment for chemicals does not account for the potential effects of multiple stressors. The literature-derived coefficients may help to develop assessment factors to correct for the uncertainties related to differences between laboratory test conditions and natural conditions in ecosystems. Although the application of an assessment factor to correct for multiple stress conditions seems valuable in the short-term, in the long-term the use of constant extrapolation factors should be replaced with statistical and mechanistic models that also specify the margins of uncertainty for the range of factors that modify the toxicity of chemicals.

## SAMENVATTING

De laatste jaren zijn de concentraties van veel verontreinigingen in het aquatische milieu gedaald. Standaard toxiciteitstesten met deze lage concentraties suggereren vaak dat de huidige graad van vervuiling geen effect meer heeft op ecosystemen. Deze toxiciteitstesten worden echter veelal uitgevoerd onder optimale en constante experimentele condities, bijvoorbeeld wat betreft temperatuur en voedselniveau, terwijl in het veld soorten bloot staan aan combinaties van stressfactoren van biologische, fysische en chemische oorsprong. Deze suboptimale en variabele veldcondities hebben waarschijnlijk invloed op de toxiciteit van chemicaliën voor organismen. Relatief

weinig is bekend over de interacties en effecten van combinaties van stressoren. Dit belemmert het maken van goede voorspellingen die gebruikt worden in het overheidsbeleid voor stoffen. Dit proefschrift heeft daarom tot doel om de gecombineerde effecten van chemicaliën en natuurlijke factoren op aquatische biota te beschrijven en de onderliggende mechanismen van hun interactie bloot te leggen.

Deze studie begon met het evalueren van de bestaande literatuur over meervoudige stress. Uit dit literatuuronderzoek bleek dat temperatuur, voedselconditie en saliniteit een grote invloed uitoefenen op de toxiciteit van verschillende stoffen voor aquatische organismen (hoofdstuk 2). Organismen die bloot stonden aan condities die de tolerantiegrens voor de desbetreffende natuurlijke factoren bereikten, waren doorgaans meer gevoelig voor additionele chemische stress, terwijl blootstelling aan toxicanten de mate van tolerantie voor natuurlijke stress reduceerde. Over het algemeen nam de toxiciteit van stoffen toe bij hoge temperaturen en lage voedselconcentraties. De invloed van saliniteit op toxiciteit hing af van het type toxicant, maar de relatie tussen beide factoren was niet altijd eenduidig. Kwantitatieve analyse van data verkregen uit de literatuur resulteerde in coëfficienten voor de drie onderzochte natuurlijke factoren. Deze coëfficienten geven inzicht in de grootte van extrapolatiefactoren die toegepast kunnen worden bij de vertaling van laboratorium resultaten naar de veldsituatie. De waargenomen interacties tussen toxicanten en natuurlijke factoren worden veelal verklaard door veranderingen in de biologische beschikbaarheid van de toxicant, de accumulatie kinetiek en de gevoeligheid van de testorganismen veroorzaakt door de natuurlijke factoren. De onderliggende processen zijn tot nu toe nog nauwlijks bestudeerd.

Vervolgens werd in hoofdstuk 3 de interactie tussen temperatuur en cadmium voor de watervlo *Daphnia magna* experimenteel onderzocht, waarbij onderscheid gemaakt werd tussen de effecten van temperatuur op cadmium accumulatie en toxiciteit. De waarnemingen werden vervolgens geanalyseerd met het zogenaamde DEBtox model, waardoor ook onderzocht kon worden of temperatuur de intrinsieke gevoeligheid van de watervlooien voor cadmium beïnvloedde. Extreem hoge temperaturen veroorzaakten volledige sterfte, onafhankelijk van cadmium blootstelling. Onder deze limiet versterkte toenemende temperatuur de toxiciteit van cadmium. Deze temperatuursafhankelijke effecten van cadmium konden toegeschreven worden

aan verschillende mechanismen. In het lagere temperatuurstraject versnelde een temperatuurssteiging de opname kinetiek van cadmium, wat een hogere toxiciteit tot gevolg had. In het hogere temperatuurstraject was verhoogde cadmium opname van minder belang, maar werd de hogere toxiciteit voornamelijk veroorzaakt door een toename van de intrinsieke gevoeligheid van de watervlooien voor cadmium.

Om de ecologische relevantie van de studie te vergroten werden vervolgens chronische experimenten uitgevoerd in een semi-continu doorstroom-systeem waarbij naast temperatuur ook voedsel als natuurlijke factor werd geïntroduceerd. In deze levenscyclustesten werden de gecombineerde effec-ten van temperatuur, voedsel en cadmium op de populatiegroei van *D. magna* onderzocht (hoofdstuk 4). Hoge temperaturen en voedselconcentra-ties bleken zoals verwacht stimulerend te werken op de populatiegroei-snelheid, terwijl de negatieve effecten veroorzaakt door cadmium versterkt werden door hoge temperaturen en lage voedselniveau's. Uit cadmiumcon-centraties gemeten in de watervlooien kwam naar voren dat de door tempe-ratuur en voedsel veranderde cadmium toxiciteit deels veroorzaakt werd door hogere accumulatie van dit metaal bij hoge temperaturen en lage voedselconcentraties.
In hoofdstuk 5 werden de data verkregen uit de chronische experimenten (hoofdstuk 4) geanalyseerd met het DEBtox model. De modelanalyses suggereerden dat cadmium de reproductie van de watervlooien indirect beïnvloedde door het reduceren van de energie-assimilatie uit voedsel. Naast de hogere accumulatie van cadmium (hoofdstuk 4) wezen de modelanalyses uit dat de intrinsieke gevoeligheid van de watervlooien voor cadmium eveneens toenam bij hoge temperatuur en lage voedselconcentratie.

Tenslotte werd in hoofdstuk 6 de voorspelbaarheid van de effecten van gecombineerde stressoren bediscussieerd. Twee benaderingen om de populatiegroei (gepresenteerd in hoofdstuk 4) te voorspellen werden uitgewerkt en geëvalueerd. Ten eerste werden de coëfficienten voor temperatuur en voedsel zoals berekend in hoofdstuk 2 gebruikt om veranderingen in toxiciteit als gevolg van verschillen in temperatuur en voedselconcentratie te berekenen. Ten tweede werd de respons op meervoudige stress berekend met behulp van de in hoofdstuk 4 waargenomen respons op de afzonderlijke stressfactoren. Hiervoor werd achtereenvolgens een comparatieve, additieve en multiplicatieve interactie tussen de factoren verondersteld, waarvan de multiplicatieve interactie de populatiegroei het beste bleek te

voorspellen. Wanneer de de twee benaderingen werden vergeleken, waarbij zowel de voorspelbaarheid als de toepasbaarheid van de methoden in beschouwing werden genomen, bleken de coëfficienten verkregen door middel van data uit de literatuur de beste methode om de gecombineerde effecten van temperatuur, voedsel en cadmium op de populatiegroei van *D. magna* gepresenteerd in hoofdstuk 4 te voorspellen.

De huidige risicobeoordeling voor stoffen houdt geen rekening met potentiële effecten van combinaties van stressfactoren. Coëfficienten afgeleid uit literatuurdata kunnen helpen bij de ontwikkeling van extrapolatiefactoren waarmee gecorrigeerd kan worden voor de verschillen tussen experimentele condities tijdens laboratoriumtesten en natuurlijke condities in ecosystemen. Op de korte termijn lijkt de toepassing van extrapolatiefactoren om te corrigeren voor de effecten van natuurlijke factoren op de toxiciteit van stoffen waardevol. Echter, op de lange termijn zou het gebruik van een constante extrapolatiefactor vervangen moeten worden door de toepassing van statistische en mechanistische modellen die onzekerheidsmarges aangeven voor een reeks van factoren die de toxiciteit van chemicaliën beïnvloeden.

# *Dankwoord*

De afgelopen jaren is me vaak gevraagd of de term multistress op het onderzoek sloeg of op mij. Ik ben bang dat het laatste meer het geval was naarmate de promotie naderde. Gelukkig heb ik veel steun gekregen van allerlei mensen, waarvan ik een aantal hier wil bedanken.

Speciale dank voor mijn promotoren Wim Admiraal en Nico van Straalen en mijn co-promotoren Jan Hendriks en Michiel Kraak. Wim benaderde me aan het eind van mijn studie met de vraag te solliciteren naar een AIO plek bij zijn vakgroep. Ik liep destijds stage in Tromsø in Noorwegen en was erg verbaasd dat een voor mij onbekende professor mij wist te vinden op zo'n afgelegen plek boven de poolcirkel. Bedankt dat jullie mij deze kans hebben gegeven. Wim, in het begin vond ik de samenwerking nogal verwarrend. Dit kwam door je levendige beeldspraak, met touwtjes om manuscripten, kapstokken met ronde of rechte haakjes, al dan niet kwispelende hondjes, maar ik kan niet anders zeggen dan dat ik het erg prettig vond om met je samen te werken. Ben ik je beter gaan begrijpen of ben je echt concreter geworden? Nico, ik heb jouw uitvoerige commentaar op mijn manuscripten altijd erg op prijs gesteld, dank hiervoor. Jan, je begeleidde me op het RIZA (of in de trein, als dat zo uit kwam). Als rizanees probeerde je me te behoeden voor die academen die altijd maar weer dieper wilden graven en wees je me op de toepasbaarheid van het onderzoek voor het beleid. Bedankt dat ik altijd bij je terecht kon. Michiel, jij was altijd al een luisterend oor, maar je werd dat helemaal toen je mijn begeleider werd. Ik ben je hier erg dankbaar voor, want door de gesprekken met jou kreeg het onderzoek voor mij meer structuur. Bedankt voor alle hulp, discussies en peptalk!

Tineke Dekker begeleidde me in de eerste fase van het onderzoek. Ik wil haar bedanken voor de gezelligheid (waaronder eten met de zoöplanktongroep en picknicken en zwemmen in Maarsseveen), de hulp met het opzetten van de *Daphnia*-kweek en alle discussies over de experimenten. Hannie

Maas wil ik bedanken voor de discussies over ecotoxicologie in het algemeen, *Daphnia* in het bijzonder en de opzet en uitwerking van de experimenten. Hannie, ik vond het altijd fijn om even bij je binnen te wippen en bij te kletsen. Toen ik krap zat in mijn tijd heeft Albert Espeldoorn me erg geholpen door een experiment voor me uit te voeren, veel dank hiervoor. Voor het analyseren van mijn data heb ik contact gezocht met Tjalling Jager. Tjalling, jij hebt me ingewijd in de Debtoxiaanse wetenschap en 'multidimensionale hypervlakken'. Het duizelde me af en toe wel, maar ik vond het erg leuk om de data modelmatig te analyseren. Bedankt voor al je hulp hierbij.

Omdat ik het eerste jaar voornamelijk lezend en schrijvend doorgebracht heb op het RIZA, wist ik in het tweede jaar nog steeds de weg niet op de UvA. Gelukkig was daar Gerdit die me wegwijs maakte op het lab en met de AAS. Bedankt voor alle hulp en gezelligheid tijdens het soms frustrerende meten op de oven-AAS. Wanneer er problemen waren met apparatuur kon ik gelukkig uitwijken naar de VU. Ingrid en Rudo, bedankt dat ik een aantal monsters kon komen meten; door jullie tips kon ik vervolgens zelf aan de slag op de UvA. Harm wordt ook wel de helpdesk van AEE genoemd en voor mij heeft hij die naam zeker waargemaakt. Bedankt voor het veilig maken van het doorstroomsysteem (hier heeft Bas ook geholpen: bedankt voor de broodtrommeltijdschakelaar!), het opvangen van mijn studenten toen ik ziek was en voor je hulp bij het drukklaar maken van dit boekje. Fijn dat ik tot 's avonds laat nog bij jullie terecht kon Harm en Sas! Ik zal nooit meer Palatino als lettertype kiezen! Elske, ik zal je missen als kamergenootje. Met wie moet ik nu uit volle borst '*una paloma blanca*' zingen? Bas en Saskia, bedankt voor jullie hulp met de algenkweek. Verder natuurlijk alle (oud-)collega's van de afdelingen Chemie en Ecotoxicologie van het RIZA en Aquatische Ecologie en Ecotoxicologie van de UvA: bedankt voor de leuke tijd en jullie interesse in de voortgang van het onderzoek. Ik wil ook alle studenten bedanken die me geholpen hebben: Reanne, Clarissa, Roy en Lonneke. Bedankt voor jullie inzet en alle lol die we gehad hebben. Ik vond het super dat Clarissa en Lonneke ook in het weekend naar het lab kwamen om de chronische testen in het doorstroomsystem door te laten draaien, al duurde zo'n test soms wel erg lang.

Mijn paranimfen Helen en Marie-Elène wil ik bedanken dat ze me terzijde willen staan tijdens het uur der waarheid. Helen, ik zal het missen om samen in weer en (tegen!)wind naar huis te fietsen. Bedankt voor al je hulp

en steun de afgelopen jaren. Marie-Elène, ik leerde je kennen als student toen je stage liep bij ons op de afdeling en inmiddels werk je zelf ook aan een promotieonderzoek. Bedankt voor al je pepmailtjes en leuke uitjes, veel succes met het afronden van jouw onderzoek!

De promotiecommissie, bestaande uit Bas Kooijman, Remi Laane, Matthias Liess, Steph Menken, André de Roos en Kees van Gestel, wil ik bedanken voor de tijd die ze in het lezen van het proefschrift hebben gestoken en de adviezen die ze hebben gegeven voor het afronden van het boekje.

Gelukkig was er ook een leven naast het werk, waaronder etentjes met Ingrid en Esther, mijn jaargenootjes uit Wageningen die min of meer tegelijkertijd ook als AIO in Amsterdam begonnen. Bedankt voor alle steun! Verder wil ik nog de kanovrienden, de wijnvrienden, het S.L.A. (Sisi Liberation Army), en alle overige vrienden bedanken die voor de broodnodige afleiding gezorgd hebben.

De meeste dank ben ik verschuldigd aan het thuisfront. Mijn ouders wil ik bedanken dat ze mij altijd aangemoedigd hebben om te studeren. Pappa en mamma, jullie vertrouwen in mijn kunnen is altijd een grote steun in de rug geweest. Marcel en Daniëlle, bedankt voor jullie interesse, al snapten jullie misschien niet hoe iemand vier jaar lang onderzoek kan doen aan watervlooien. Lieve Theo, bedankt voor al je hulp en je nimmer aflatende optimisme. Tegelijkertijd promoveren was soms wel zwaar, maar ik vind dat we het goed gedaan hebben: met zijn tweeën is het leuker dan alleen! Nog even volhouden en daarna gaan we samen beginnen aan ons volgende avontuur. Je bent een kanjer… maar ik heb gewonnen!

# *Curriculum vitae*

Evelyn Heugens werd geboren op 1 april 1975 te Ede. In 1993 behaalde zij haar Atheneum diploma aan het Edese Marnix College. In hetzelfde jaar begon zij de studie milieuhygiëne aan de Landbouwuniversiteit Wageningen. Daar koos zij voor de specialisatie Waterkwaliteit binnen de oriëntatie Waterkwaliteitsbeheer en Aquatische Oecologie. Zij volgde afstudeervakken aan de vakgroep Waterkwaliteitsbeheer en Aquatische Oecologie en via deze vakgroep bij het Laboratorium voor Water- en Drinkwateronderzoek van het RIVM. Ook deed zij een stage bij het onderzoeks- en adviesbureau Akvaplan-Niva te Tromsø, Noorwegen. In 1998 kreeg zij haar diploma. Van december 1998 tot maart 2003 werkte zij aan een promotieonderzoek bij het Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling (RIZA) in Lelystad, de Universiteit van Amsterdam (UvA) en de Vrije Universiteit in Amsterdam. Het onderzoek is uitgevoerd bij de afdeling Chemie en Ecotoxicologie van het RIZA en de afdeling Aquatische Ecologie en Ecotoxicologie van de UvA. De resultaten hiervan zijn beschreven in dit proefschrift. In oktober 2003 werkte zij in opdracht van het RIZA aan een beleidsnotitie over multistress. Thans is zij werkzaam bij de afdeling Toxicological Risk Assessment van TNO Voeding in Zeist.