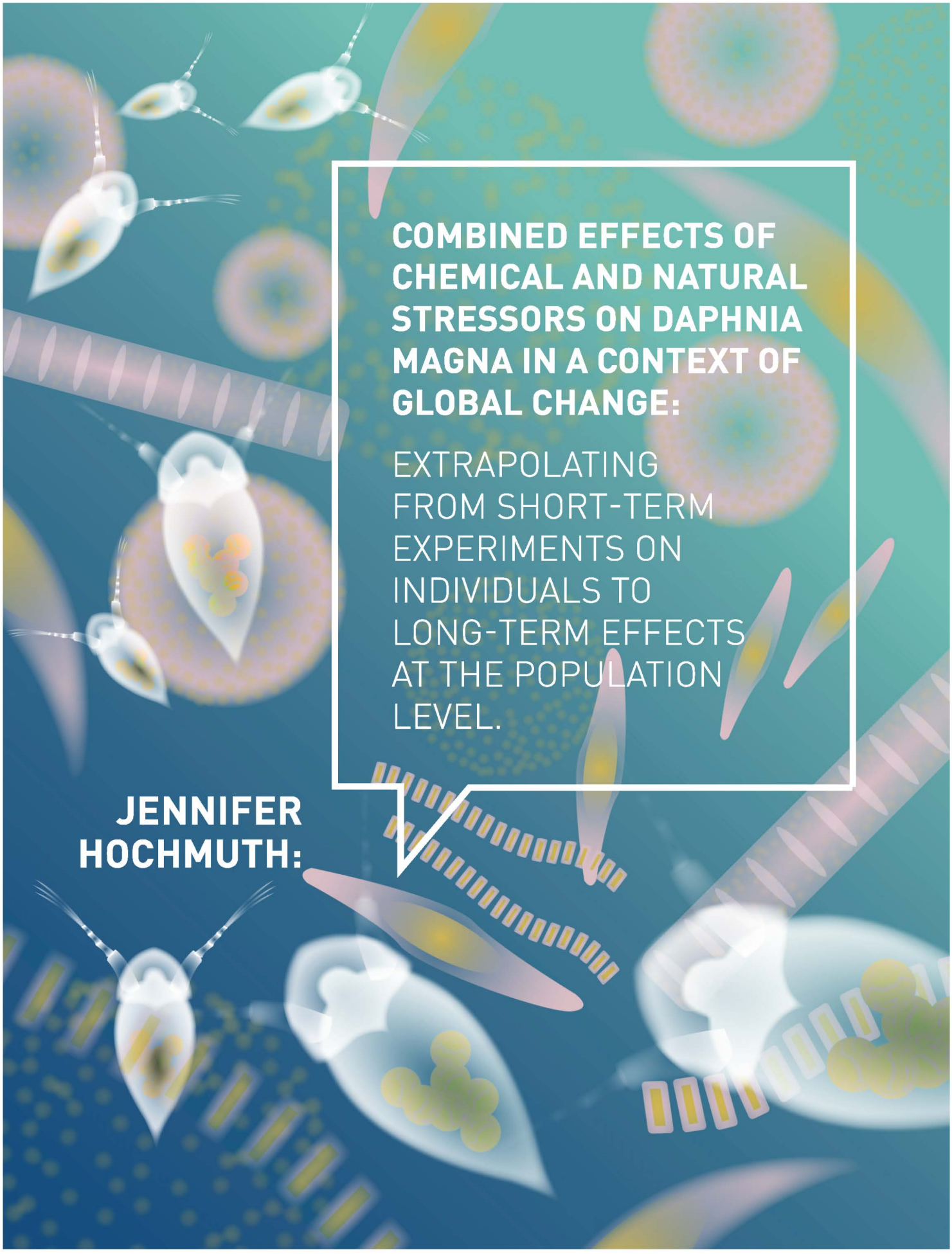

COMBINED EFFECTS OF CHEMICAL AND NATURAL STRESSORS ON DAPHNIA MAGNA IN A CONTEXT OF GLOBAL CHANGE:

EXTRAPOLATING FROM SHORT-TERM EXPERIMENTS ON INDIVIDUALS TO LONG-TERM EFFECTS AT THE POPULATION LEVEL.

JENNIFER HOCHMUTH:

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

“No scientific theory is worth anything unless it enables us to predict something which is actually going on. Until that is done, theories are a mere game of words, and not such a good game as poetry.”

J.B.S. Haldane (polymath, 1892-1964)

Promoters: Prof. dr. ir. Karel De Schamphelaere

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Combined effects of chemical and natural stressors on *Daphnia magna* in a context of global change: Extrapolating from short-term experiments on individuals to long-term effects at the population level.

Thesis submitted in fulfilment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad

Doctor in de Bio-ingenieurwetenschappen

Dutch translation of the title:

Gecombineerde effecten van chemische stoffen en natuurlijke stressfactoren op de watervlo *Daphnia magna* in een context van globale klimaatsverandering: Extrapolatie van kortetermijn experimenten op individueel niveau naar langetermijn effecten op populatie niveau.

Refer to this work as:

Hochmuth, J.D., 2016: Combined effects of chemical and natural stressors on *Daphnia magna* in a context of global change: Extrapolating from short-term experiments on individuals to long-term effects at the population level. Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences, Ghent University, Ghent, Belgium.

ISBN-number:

978-90-5989-930-8

Cover artwork by Timmy Segers (2016)

Jennifer Hochmuth was the recipient of a personal AFR-PhD grant supported by the Fonds National de la Recherche, Luxembourg (AFR-PhD Grant Agreement PhD 2011-1, Project Reference 1330121).

aides à la formation recherche

Fonds National de la Recherche Luxembourg

The research conducted during this PhD dissertation was finically supported with funding from the UGent Special Research Fund (BOF project 01N01211), BELSPO IAP projects AquaStress (P7/31) and SPEEDY (P7/04), the KU Leuven Research Fund project PF/2010/07, the Copper Alliance and the International Zinc Association.

GHENT UNIVERSITY

Belgian Science Policy Office
belspo

KU LEUVEN

Cu Copper Alliance™

international zinc association

Acknowledgement

The quote “There is scarcely any passion without struggle.” by one of my favourite authors, Albert Camus, taken from his philosophical essay on the myth of Sisyphus, perfectly sums up an important insight that my past 5 years in Ghent provided me with. Many a time, when I was confronted with yet another hurdle along the steep hill, I could identify myself with Sisyphus, condemned to repeat forever the same seemingly absurd task of pushing a rock up a mountain, only to see it roll down yet again… However I have come to appreciate, similarly to Camus that “Each atom of that stone, each mineral flake of that night filled mountain, in itself forms a world. The struggle itself toward the heights is enough to fill a man's heart. One must imagine Sisyphus happy.”

That is because the PhD, as so many other aspects in life, is about overcoming challenges, and all challenges come with both successes and failures. Knowledge doesn’t have to be gained on the basis of one success after another. Failure has to be accepted as an inherent part of success, as it provides us with the essential perspective to keep learning and growing. As in the myth of Sisyphus, this is a process that never truly ends. As we roll our rock uphill, it’ll get harder and harder to keep pushing it higher. In fact there’s no top to that hill, even if it appears there is, and sometimes it feels as though everything comes tumbling down, taking us back to square one. But each struggle is valuable as it keeps challenging us and drives our dediction and perseverance to keep pushing and hence keep learning.

Fortunately, I was not left alone to push the heavy rock, that was my PhD, and along this steep hill I was given help by a great number of people that gave me the necessary strength to succeed. First I want to express my deepest gratitude to my promoters Prof. dr. ir. Karel De Schamphelaere and Prof dr. Colin Janssen. Thank you both for your continuous stimulating input and for teaching me the most import skill for any scientist: Challenging me to develop my critical thinking. Karel, thank you for putting your faith in me and always steering me in the right direction. Our long discussions were a crucial component and

gave me strength to stay motivated to keep pushing my PhD-rock! I would also like to extend my appreciation to the members of my doctoral examination committee. Prof. dr. Luc De Meester, Prof. dr. ir. Frederik De Laender, Prof. dr. ir. Lander Baeten, Prof. dr. Marleen De Troch, Dr. Katrien Delbeke and Prof. dr. ir. Guy Smagghe, your critical examination and insightful suggestions allowed me to substantially improve the quality of the final dissertation.

Next I want to thank everyone at the GhEnToxLab, I feel very lucky to have landed in this research group. Marianne, I am especially indebted to you for your assistance, which greatly surpassed administrative issues. You provided me with limitless caring support on all personal matters too. For instance when I first arrived in Ghent, we went fridge shopping and you made sure that I didn't have to push the fridge up the 3 flights of stairs in my apartment! Nancy, I can't thank you enough for your continuous enthusiasm and patience on all lab matters. You are the indispensable glue that holds everything together! Emmy and Jolien, without your joined efforts with my large experiments and the culturing of my (nearly 500!!!) daphnid clones, I would probably still be busy in the lab or just starting to write up my results…. Gisèle, thanks for teaching me the ins and outs of the beautiful *Daphnia magna* and for making sure that my little waterfleas always had plenty of the best algal food! Marc, thanks for turning my experimental designs into reality, you are such a creative mastermind! I still have fond memories of our field sampling trip, thanks again for pushing me over that fence! Jana and Dieter, I have learned such a great deal from the both of you, especially at that start of my PhD. Karel, you were my best (DEB-)buddy at the lab and always made time for me to fix yet another bug, even while you were finishing your own dissertation and planning your own defense. Chapter 6 and the Dutch summary are all thanks to you! The 3 ladies: Jana, Charlotte and Cecilia were my longest office companions: I deeply value the mutual support we gave one another on all professional and personal matters. Emmanuel and Jozef, my last (but not least) office-buddies, I much appreciated the laid-back atmosphere in the office, which helped reducing the stress during the final push, after I returned from almost a year at the European Commission. David, my smoking buddy, thanks for providing me with the many much needed cigarette breaks, spent reflecting together on scientific issues or just simply to distract me from them… Jan, always willing to help, you taught me a lot about birds and statistics and I even got a guinea pig from you! Hopefully I won't have to miss out on our enjoyable chats after leaving the lab. To all the other colleagues, new and old, that I haven't mentioned and to everyone from the AECO research group, I

will value the fond memories of the coffee and lunch breaks, special lab events, as well as the casual after work drinks to decompress together after long working hours. I will miss all of you!

“In order to understand the world, one has to turn away from it on occasion." Fortunately, the difference between us and Sisyphus is that we are free to walk away from time to time, to let the rock make its way back downhill and pick it up again a later time, or to get someone else to help us support it. Sometimes you’ll have to try again right away, keep improving, while other times what you really need is time with someone elso to reflect and refine your approach before you tackle it again. Therefore my final thank you goes out to my family and friends who did exactlly that and provided me with indispensable support along the way.

Daddy, thank you for always giving me the freedom of choice and for never stopping to believe in me! Denise, your love and emotional support have been the warm protective blanket throughout my life! Mimi, it was your generous nature that made all my studies possible, I hope you would be proud of me! Samira, my longest friend, you have always been on my side and our bond is the living proof that true friends are never apart, perhaps by distance but not by heart! “We will always be best friends…” Kathleen, I feel so lucky to have met you! You have been the ultimate support along my journey in Ghent and without you I might have not been able to write this acknowledgement and therefore this PhD!

I don’t know yet what my next struggles will entail but what I do know is that I won’t have to face them alone and that I can look back proudly at having accomplished this milestone with the help and support from all the amazing people around me.

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List of abbreviations

AHA	Aldrich Humic Acid
AIC	Aikake Information Criterion
ANOVA	ANalysis Of VAriance
Ana	*Anabaena*
Aph	*Aphanizomenon*
CA	Concentration Addition
Cd	Cadmium
CASA	Concentration Addition Synergism/Antagonism deviation
Ch	Chapter
CI	Confidence Interval
CICCM	Cawthron Institute Culture Collection of Micro-algae
Cu	copper
Cyano	Cyanobacteria
Cyl	*Cylindrospermopsis*
DIC	Dissolved Inorganic Carbon
DNA	DeoxyriboNucleic Acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EC	European Commission
ECx	Effect Concentration for *x*% of the organisms
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DDT	DichloroDiphenylTrichloroethane
DRC	Dose Response Curve
DEB	Dynamic Energy Budget
DEB-IBM	Dynamic Energy Budget Individual Based Model
EDTA	EthyleneDiamineTetraacetic Acid
USEPA	US Environmental Protection Agency

EQS	Environmental Quality Standard
ERA	Ecological Risk Assessment
EU	European Union
HC5	5% Hazardous Concentration
IA	Independent Action
IASA	Independent Action Synergism/Antagonism deviation
IC	Inorganic Carbon
IBM	Individual Based Model
IPCC	Intergovernmental Panel on Climate Change
LCx	Lethal Concentration for x% of the organisms
MC (*Mc*)	*Microcystis aruginosa*
MoA	Mode of Action
Na	sodium
NOEC	No Observed Effect Concentration
Nod	*Nodularia*
OCEE	Optimal Concentration range for Essential Elements
OECD	Organisation for Economic Co-operation and Development
Osc	*Oscillatoria*
PCB	PolyChlorinated Biphenyl
PCC	Pasteur Culture Collection
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollution
ppt	parts per trillion
Pseudo	*Pseudokirchneriella subcapitata*
PUFA	Poly-Unsaturated Fatty Acid
PwC	Pairwise Comparison
QTL	Quantitative Trait Loci
RCR	Risk Characterization Ratio
R0	Total Reproduction
r_m	Intrinsic rate of natural increase

ROS	Reactive Oxygen Species
SCCAP	Scandinavian Culture Collection of Algae and Protozoa
SCHER	Scientific Committee on Health and Environmental Risks
SETAC	Society of Environmental Toxicology and Chemistry
SSE	Sum of Squared Errors
TU	Toxic Unit
USEPA	US Environmental Protection Agency
UTEX	University of Texas, Austin, USA
WFD	Water Framework Directive
Zn	zinc

1

General introduction and conceptual framework

The research conducted in the context of this PhD dissertation is situated in the field of ecotoxicology, which is concerned with investigating how individuals, populations, communities, and ecosystems respond to chemicals. The exponentially increasing human population and the associated rise in chemical and nutrient input through ongoing urban, industrial and agricultural activity have exerted substantial pressure on aquatic ecosystems. As such, ecotoxicology underpins many important legal frameworks related to environmental protection, such as ecological risk assessment (ERA) and the setting of environmental quality standards (EQS) for chemicals. Typically, the effects of single substances have been tested by means of laboratory toxicity tests, on a few (more or less relevant) model test species (algae, zooplankton, fish, see Figure 1.1) (Van Leeuwen and Hermens, 1996).

Figure 1.1. Model phytoplankton (*Pseudokircherniella supcapitata*), zooplankton (*Daphnia magna*) and fish (*Oncorhynchus mykiss*) species commonly used in freshwater ecotoxicity testing.

1.1 Standard ecotoxicity testing vs. ecological realism

The main protection goal of ERA is ensuring the sustainability of populations and higher levels of organization. Conventional risk assessment is based on ecotoxicity tests with individual chemical stressors, measured at the level of the individual (e.g. survival or reproduction). This contrasts with ecological reality for two major reasons. Firstly, natural populations are often exposed to a combination of chemical and non-chemical stressors, rather than single substances, over many generations (long-term) under time-variable, non-optimal conditions (Eggen et al., 2004, Altenburger et al., 1996).

Box 1.1. Terminology used in ecotoxicology.

Ecological risk assessment (**ERA**) is the process for evaluating how likely it is that the environment may be impacted as a result of exposure to one or more environmental stressors such as chemicals, land change, disease, invasive species and global climate change.

Environmental Quality Standards (EQS) Water quality standards are legal provisions that describe the desired condition of a waterbody or the level of protection or mandate how the desired condition will be expressed or established for such waters in the future.

In this PhD dissertation **stress** is defined as a condition evoked in an organism by one or more environmental factors that bring the organism near to or over the edges of its fundamental ecological niche (Van Straalen, 2003).

Optimal conditions are defined as the environmental conditions, which produce maximum fitness in an organism. **Fitness** is defined as an organism's capacity to survive and reproduce, i.e. its contribution to the next generation.

In the literature **stress** is used to define (i) the factor causing it (i.e. the stressor) or (ii) the effects caused by it (i.e. the resulting physiological state), which can lead to misinterpretation. For instance Sibly and Calow (1989) defined stress as "an environmental condition that, when first applied, impairs **Darwinian fitness**" (Sibly and Calow, 1989). Grime (1989) defined stress as "external constraints limiting the rates of resource acquisition, growth or reproduction of organisms" (Grime, 1989, Ward and Robinson, 2005). Throughout the PhD dissertation, to avoid confusion, **stress** will be defined as a change in the physiological state of an organism, while the term **stressor** will be used to describe any factor (chemical or non-chemical) causing stress (i.e. a change in the physiological state of an organism).

Changes in the environmental factors can cause stress and may affect the performance (i.e. fitness) of a species, population or individual in an ecosystem. Non-chemical stressors typically increase sensitivity to chemicals and vice versa (Coors and De Meester 2008 ; Heugens et al. 2001). Figure 1.2 illustrates that is important to bear in mind that the concept of stress is not absolute, in other words, it

can only be defined with reference to the ecological niche of the species (i.e. the normal range of ecological function) (Van Straalen 2003).

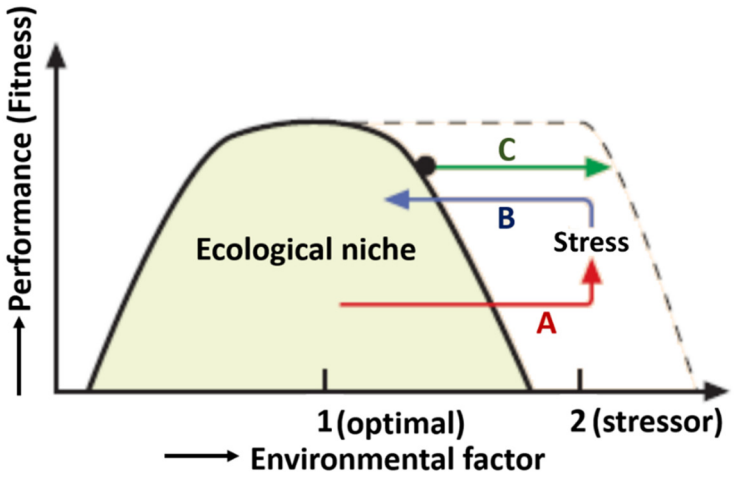


Figure 1.2. Graph illustrating the definition of stress based upon the ecological niche of a species, population or individual. Ecological stress arises when the intensity of an environmental factor increases from 1 (optimal conditions) to 2 (stressful conditions, i.e. becomes a stressor) in such a way that in position 2 the organism is placed outside its ecological niche (A). This will evoke stress and stress-response reactions, until the environmental factor relaxes and the organism returns to its ecological niche (B). Another type of response is to move the border of the niche (C) by genetic adaptation in such a way that moving the environmental factor from 1 (optimal conditions) to 2 (i.e. 2 is no longer a stressor) is not experienced as stress anymore (indicated by the dashed lines). Adapted from Van Straalen (2003).

Secondly, natural populations are generally characterized by genetic variability, which provides the potential for multi-generational microevolutionary responses, allowing populations to genetically adapt to chemical stressors (see response C in Figure 1.2), but potentially at the expense of associated costs (Van Straalen and Timmermans, 2002, Medina et al., 2007). This mismatch not only complicates the extrapolation of results from standard laboratory toxicity tests to realistic field conditions but may, moreover, result in misrepresentations of the actual effects on aquatic ecosystems.

There is however a pressing need to evaluate the combined effects of stressors, as research suggests that mixtures at No-Observed-Effect-Concentration (NOEC) levels of individual substances may cause adverse effects when they are combined in mixtures (Breitholtz et al., 2008, Silva et al., 2002, Versieren et al., 2016). Ecotoxicology has dealt with mixture toxicity for several decades to overcome some of the limitations to environmental realism of simple toxicity testing and enable more accurate predictions of the effects to the natural environment (Hermens et al., 1984, Silva et al., 2002, Backhaus et al., 2003, Norgaard and Cedergreen, 2010, Altenburger et al., 1996).

1.2 Reference models to study the combined effects of stressors

A variety of statistical methods have been employed to study mixture toxicity, of which two reference models are the most widely used and recognized. These are Bliss independence or Independent Action (IA) (Bliss, 1939) and Loewe additivity or Concentration Addition (CA) (Loewe, 1926). Their general null hypothesis is that the relative toxicity of the mixture equals the relative toxicity of the individual components. However, these two reference models differ both conceptually, as well as mathematically.

Independent action (IA, also known as response addition or effects addition) assumes that the components of a mixture act independently from each other, usually through dissimilar modes of action that do not influence one another. The null-hypothesis of this reference model states the response of one component in the mixture is independent of the response of the other component(s) in the mixture (Jonker et al., 2005).

Therefore, the effects of each of the components of the mixture are independent of each other from a probabilistic point of view:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \qquad \textbf{(Equation 1.1)}$$

where the effect of the mixture *E(Cmix)* is calculated from the product of the effects of (*n*) components $E(c_i)$. Effects are expressed as fractions of a maximum possible effect (0% ≤ E ≤ 100%).

Box 1.2. Terminology used in modelling of the combined effects of stressors.

Combined effects are defined as the overall **observed effects** of all stressors combined. Combined effects can be (i) equal to the combined toxicities of the individual components of the mixture (in the case of **non-interaction**), (ii) more toxic than the combined toxicities of the individual components of the mixture (in the case of **synergism**)), (iii) less toxic than the combined toxicities of the individual components in the mixture (in the case of **antagonism**).

Interactive effects of stressors, conversely, can only be inferred by a model if the predicted toxicity of the mixture differs in a statistically significant manner from the actual observed toxicity of the mixture. Interactive effects can be (i) **additive** (if the predictions from the reference model do not significantly deviate from the observations), **synergistic** (if the reference model predicts significantly lower toxicity of the mixture than the observed combined effects), or antagonistic (if the reference model predicts significantly higher toxicity of the mixture than the observed combined effects).

The terms **mixture toxicity** and **interactive effects** are often used synonymously in the literature, despite the fact that a mixture can be toxic without interactive effects of the individual components (or stressors). In this PhD dissertation, to avoid confusion, the term **mixture toxicity** is avoided and **combined effects** of stressors is used throughout this PhD dissertation.

Concentration Addition (CA) on the other hand assumes that the mixture components only differ in the concentrations needed to elicit a toxic effect (i.e. their relative potency). That is, when corrected for their relative potency, each component can be replaced by equivalent concentrations (i.e. dilutions) of another chemical without changing the overall toxicity of the mixture. Due to this conceptual idea, CA is thought to describe the joint action of components that have a similar mode or mechanism of action. If the CA model holds, then the sum of the toxic units equals 1 in a mixture resulting in x% effect. It looks at mixture effects of chemicals in terms of a ‘dilution” principle. It therefore assumes that a component in a mixture can be exchanged partly or completely for another component with an equi-effective concentration without changing the overall toxicity of the mixture, as long as the sum of the toxic units remains the same.

It can be expressed as:

$$\sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad \textbf{(Equation 1.2)}$$

Where *n* is the number of components of the mixture, *TU* is the toxic unit of the i^{th} component of the mixture defined as the ratio between c_i, the concentration of the i^{th} component of the mixture, and ECx_i, the x% effective concentration of the i^{th} component of the mixture (as under a single exposure).

1.2.1 Deviations from additivity (i.e. non-interaction)

Any theoretical model of additivity (i.e. non-interaction) assumes that neither of the mixture components influences another component's action. Interaction is inferred if the level of response produced by any combination of different substances differs from the response expected on the basis of a theoretical model of additivity (i.e. non-interaction) (McCarty and Borgert, 2006). CA and IA are two reference models that can be used to calculate the theoretical combined additive effects. The difference between the calculated expected effects is defined in quantitative terms in relation to the observed combined effects of the mixture components (van Gestel et al., 2010). Synergism is inferred if the observed combined effects of the mixture components are greater than those predicted by either IA, CA or any other reference model. Antagonism is inferred if the components in the mixture produce smaller observed combined effects than predicted by any reference model.

1.2.2 Comparison of the reference models and their limitations

From section 1.2.1 it is obvious that the CA and the IA reference models differ both conceptually and mathematically. Both reference models also require different input to accurately estimate the EC_{xi}. The IA reference model requires a minimum of a response of the individual components tested at the same concentrations as those concentrations present in the mixture. The CA reference model requires a full dose response curve for each individual component, making it more data intensive.

However it is also important to stress the fact that both reference models assess the effects of mixtures at low (more environmentally relevant) concentrations differently. The IA model assumes no effect of a mixture, if each of the components are present at a concentration that would not cause any effect

individually. In other words only components present at concentration higher than the NOEC contribute to the toxicity of the mixture. On the contrary, according to the CA reference model all components (even those present at concentrations below the NOEC) contribute to the combined effects proportionally to the toxic units of the mixture.

Theoretically both concepts seem equally valid, but the question arises which reference model to select, especially if the models produce contrasting predictions of the mixture effects. A mechanistic approach would be to base the choice of the model on the known modes of toxic action of the mixture components. CA is recommended for mixture components with similar toxic action, while IA tends to be applied for mixture components with different modes of toxic action (Loewe, 1926, Cedergreen et al., 2008, Backhaus et al., 2000, Bliss, 1939). Aside from limited knowledge on the known toxic modes of action of many chemicals, a key issue lies in the exact definition of a “similar mode of action”, as two similar phenomenological effects could arise from two very dissimilar molecular mechanisms (van Gestel et al., 2010). Strictly pharmacologically speaking, similar modes of toxic action can be defined as interacting with the same molecular target site. However more broadly speaking similar mechanisms of toxic action can also be defined as leading to a common toxicological response and it has therefore been suggested that the CA model is universally applicable (Berenbaum, 1989). In the majority of studies to date CA has yielded more conservative predictions of the combined effects for chemicals with dissimilar modes of toxic action, even if the predictions yielded with the IA have been more accurate (Backhaus et al., 2000, Faust et al., 2003, Cedergreen et al., 2008).

Regardless of the choice of the model, there are also some general limitations in terms of applicability. A central goal in the study of mixture toxicity is to predict the effects of a mixture based on the toxicity of its individual components. Currently the use of the CA and IA reference models can only infer interaction by comparing the model predictions to the empirical observations (Belden and Lydy, 2006). This is a major drawback for risk assessment as interactive effects can only be pragmatically resolved through experimental validation and quantitatively comparing the effects predicted by both models with observed effects. Another limitation of these models is that the mixture toxicity of mixture treatments should be investigated simultaneously with those of the single components, as the conclusions drawn about the mixture effects may otherwise be erroneous (De Laender et al., 2009).

1.3 From individuals over one generation to populations over multiple generations

A major shortcoming of conventional ecotoxicology is that it still primarily focuses on determining short-term (typically less than one generation) toxicological responses of organisms to chemical exposure, mostly under invariable and (near-)optimal conditions, and using laboratory populations with limited genetic variability (De Schamphelaere et al., 2011, Heugens et al., 2001 , Coors and De Meester, 2008 , Barata et al., 1998, Van Straalen and Timmermans, 2002, Medina et al., 2007 , Van den Brink, 2008 , Van Straalen, 2003). This contrasts, mentioned in **section 1.1**, with the ecological reality and the protection goals of ERA.

A population level study of 46 days illustrated the potential risk of drawing erroneous conclusions from standard ecotoxicity tests with the cladocerans *Daphnia magna* (Agatz et al., 2012). While the authors identified that dispersogen A stimulates the reproduction of juveniles in such a standard test (considered as a positive effect), they found that continuous exposure to dispersogen A not only altered the daphnid population structure by shifting towards smaller individuals, but also increased the sensitivity to the pulse exposure to another chemical, *p*-353-nonylphenol. (Agatz et al., 2012). This highlights a potential mismatch between protection aim and risk assessment practice. The authors showed that a positive effect observed at the individual level can be translated to a negative effect at the population level and that mixtures of stressors can also have considerable impacts on the structure of populations, which again cannot be assessed using standard ecotoxicity tests.

1.3.1 Mechanistic modelling approach using DEB-IBM

A major challenge in ecotoxicology is to develop predictive models that can take into account the ecological complexity displayed in real ecosystems. Ecological and ecotoxicological modelling is a promising tool for ERA (European Union, 2013). It has been suggested frequently that such models can be a powerful means to overcome some of the limitations that ERA faces (Grimm et al., 2009). As stressed in section 1.1, ecotoxicology is faced with a mismatch between the level of interest or the protection goals, which are populations or higher levels of organizations, while the effect assessment of stressors is based on individuals. The ideal type of model can overcome this mismatch by extrapolating effects on individuals to effects on populations (Bradbury et al., 2004, Forbes and Calow,

2012). Mechanistic models that use inherent properties from individuals to derive effects at higher organization levels make for ideal models. It is an established fact that patterns observed at one scale are often the emergent result of processes occurring at smaller scales (Chave, 2013, Levin, 1992, Grimm and Railsback, 2005). Such models also offer a pragmatic advantage because shifting from higher to lower levels of biological organisation decreases the time and costs required to collect the data required. It is also much more practical to collect data on individuals than populations. Ideally, the large existing amount of historical ecotoxicology data could be used for modelling purposes and the resulting models can reduce the need for additional ecotoxicological testing and the number of test animals needed. The missing link is the development of theory and methods to use lower-level data to understand and predict higher-level patterns.

Individual Based Models (IBMs) seem particularly suited for use in ERA because they consider processes occurring at the individual level such as feeding, growth and reproduction, the focal level of standard ecotoxicity tests (Figure 1.3) (Martin et al. 2013b; Gabsi et al. 2014b). This contrasts with other population models, such as classical differential equation models or matrix models, which use state variables at the level of the populations (e.g. population density). Incorporating chemical effects on individuals in IBMs allows to explore how these effects extrapolate to the population level. In recent years, IBMs have been applied successfully to predict the population dynamics of *Daphnia magna* (Preuss et al., 2009, Preuss et al., 2010).

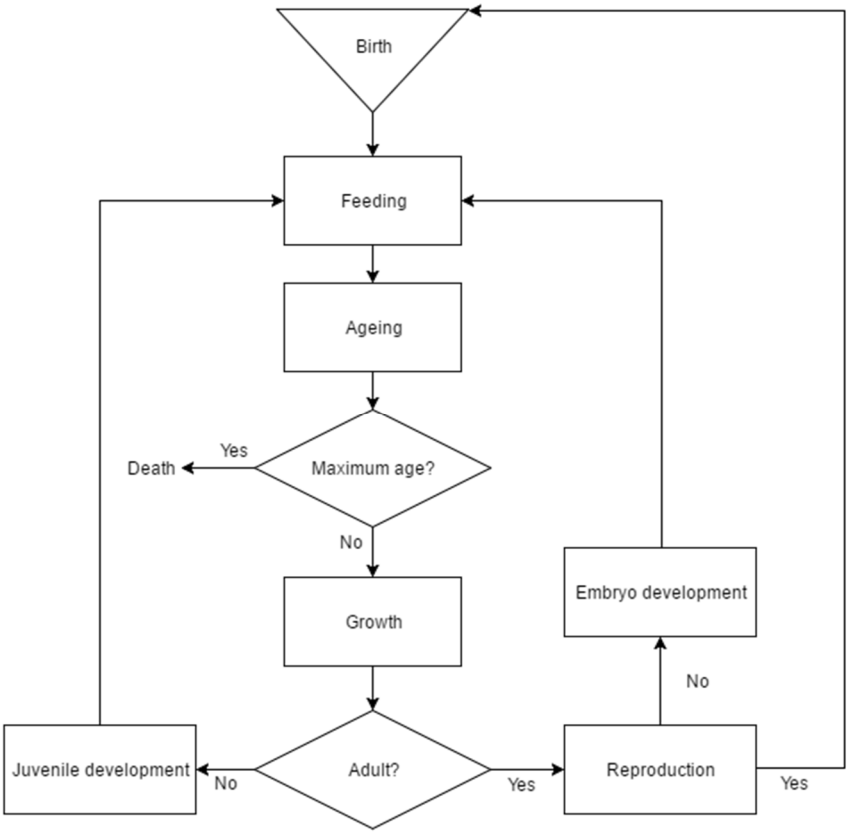


Figure 1.3. Conceptual diagram of an IBM model, which keeps track of each individual in a population, from birth (indicated by the inverted triangle) to death. Rectangles indicate processes occurring at the individual level and queries are expressed as rhombs.

A drawback of IBMs, however, is that they are usually developed to answer very specific research questions, and the structure and parameterization of models defining the life-history of organisms tends to differ widely. This reduces the suitability of IBMs as generic models, as modellers often have to start from scratch when modelling a new species. To make the IBMs as widely applicable as possible, they should be based on a generic theory. Dynamic Energy Budget (DEB) theory is such a theory (Nisbet et al., 2000, Kooijman and Metz, 1984). The underlying goal of the DEB approach is to understand the dynamics of biological systems, from cells to ecosystems, via a balance approach for mass and energy (Figure 1.4). Similarly to IBMs, DEB theory consider individuals as the key unit of interest for understanding dynamic systems at higher levels of organisation. An extensive overview of DEB theory and its applications can be found in key papers (Nisbet et al., 2000, van der Meer, 2006, Sousa et al., 2010, Kooijman and Metz, 1984).

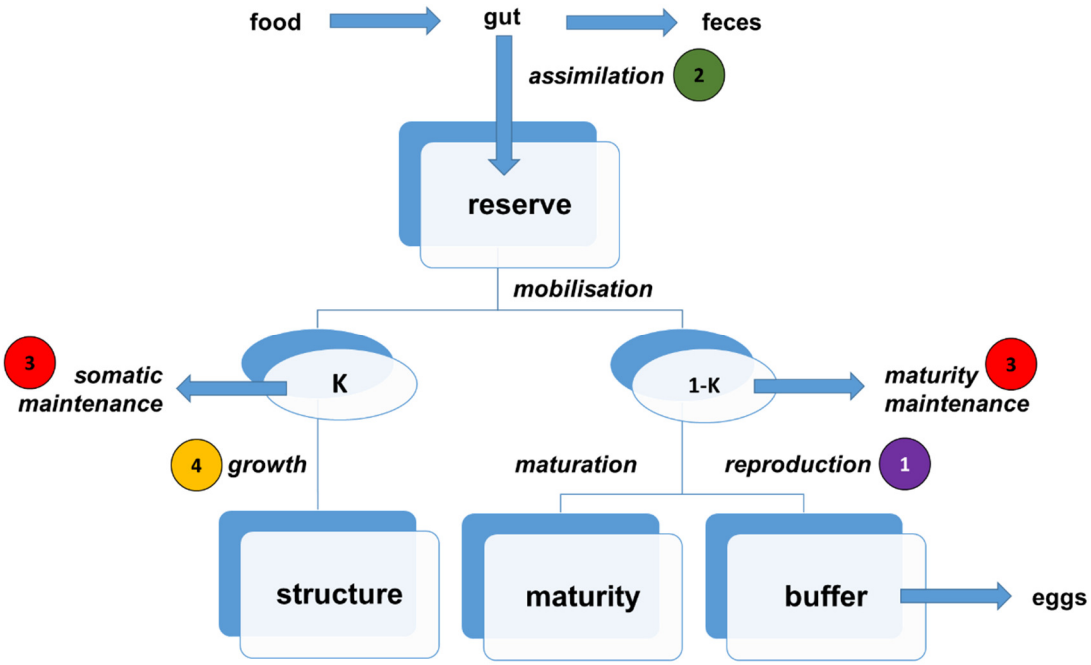


Figure 1.4. Schematic diagram of the standard DEB model adapted from Martin et al. 2013b. The primary states variables (reserve, structure, maturity, and the reproduction buffer) are depicted as rectangular boxes and fluxes are shown in italic. The oval boxes represent the energy assimilated and mobilized as a fixed fraction (*K*) to somatic maintenance and growth, with remainder of the mobilized energy (*1-K*) being allocated to maturity maintenance and the reproduction buffer. The different Physiological Modes of Action (PMoAs) are indicated by the small numbered circles: 1 = increase in cost per egg, 2 = decrease in assimilation efficiency, 3 = increase in maintenance costs, 4 = increase in overhead costs of growth.

The dynamic energy budget (DEB) theory was originally developed in the 1980's (Kooijman and Metz, 1984). In DEB theory, all processes and states of an individual are expressed as energy (or mass). Next, an energy (or mass) balance for the individual is created (Kooijman, 2010). DEB models describe processes at the level of the individual because, compared to sub- and supra-individual levels of biological organization, it is relatively easy to make energy and mass balances at the individual level (Kooijman, 2010). DEB translates environmental conditions to individual performance (growth, survival and reproduction), which is important because the trade-offs in life history traits that DEB specifies (growth vs reproduction, time and size to maturation) turn out to strongly influence population dynamics (Denney et al., 2002). Besides, DEB is a generic theory, as its key assumption is that the mechanisms

governing metabolic organization are similar among species. Therefore, IBMs can benefit from the generality of DEB, while IBMs enable extrapolating from the individual DEB-model to populations.

Only recently a generic implementation of DEB theory in an individual-based model was developed (Martin et al., 2013a). Using this DEB-IBM framework they were successfully able to predict population growth rates and peak densities of experimental populations in multiple experimental settings from the properties of individuals using *Daphnia magna* as a model species. They went further to use his DEB-IBM framework to extrapolate chemical stress from the individual to the population level, using information at the individual level on the effect of 3,4-dichloroanailine on *D. magna* (Martin et al., 2013b). Stressors were modelled as changes in the value of one or more parameters in the DEB sub-model, thereby altering one or more of the energetic fluxes leading to different patterns in growth and or reproduction. The pattern of the stressed life history output depends on the physiological mode of action (PMoA), of which they identified 4 potential PMoAs: Reproduction, Feeding/assimilation, Maintenance and growth cost (Figure 1.4). The individual data suggested a direct effect on reproduction as previous individual level data sets indicated no significant effects on growth. Assuming direct effects on reproduction, the model was able to accurately predict the population response to increasing concentrations of 3,4-dichloroaniline. The model predictions suggest that the combination of DEB theory with IBMs is a promising tool for ERA.

1.3.2 Microevolutionary effects of chemical and natural stressors

Conventional ecological risk assessment of chemicals is usually based on ecotoxicity tests using laboratory populations with limited genetic variation, often even a single isolate/ genotype, with exposure times rarely exceeding one generation (Baird, 1992 , Forbes and Depledge, 1992, De Schamphelaere et al., 2011). Although this guarantees low variability and high reproducibility of ecotoxicity test results, it is not a realistic reflection of the long-term effects of chemical exposure in the field (Barata et al., 1998, Messiaen et al., 2010). However, natural populations are typically characterized by genetically distinct individuals, which may give rise to considerable genetic variability in tolerance to chemical stress within populations (Van Straalen and Timmermans, 2002, Medina et al., 2007). This genetic variability can also be as described as a Genotype x Environment interaction (G x

E interaction), i.e. defined as a change in the relative performance of two or more genotypes measured in two or more environments (Bowman 1972). G x E interactions may therefore involve changes in rank order of genotypes (i.e. genotypes with the highest fitness) between environments and changes in the absolute and relative magnitude of the genetic, environmental and phenotypic variances between environments.

Genetic variation is one of the three pillars of biodiversity recognized at the Rio Convention (1993). The genetic variability in life-history traits under stress within a population sets the scope for microevolutionary responses under exposure to that stress (Klerks et al., 2011 , Messiaen et al., 2013). This may have very important implications for the ultimate effects (on multigeneration, or microevolutionary time scales) of chemicals on natural populations). For instance, exposure of a natural population to a chemical may result in directional selection favouring those genotypes that are more tolerant to the chemical, i.e., those genotypes that can maintain higher fitness under exposure to the chemical, and this process may allow the population to genetically adapt to chemical pollution (De Schamphelaere et al., 2011).

One potential consequence of such acquired genetic adaption to chemical stressors is that short-term toxicity testing (conventional ecotoxicology) would actually overestimate chemical toxicity in field populations, because by definitions individuals in single-generation ecotoxicity tests can't. Conversely, an alternative hypothesis is that every adaptation to chemical exposure bears a ''cost of tolerance'' or ''cost of adaptation'' (Van Straalen and Timmermans 2002; Medina et al. 2007), implying that conventional ecotoxicology approaches may be underestimating the adverse effects of chemical stressors. Consequently, genetic adaptation to stressors can be envisaged as a double-edged sword, as current practices in ecotoxicology may be, depending on the exposure scenario, too conservative or not conservative enough with respect to environment protection.

Box 1.3. Terminology used in evolutionary ecology.

Microevolution can be defined as the change in allele frequencies that occurs over time within a population. Microevolution can be a result of four different processes: natural selection, mutation, gene flow, and genetic drift (Hartl and Clark, 1980). In this thesis only microevolution as a result of natural selection will be considered. Through the process of **natural selection,** also known as "selection of the fittest" (i.e. more tolerant individuals are favoured over less tolerant ones), populations can genetically adapt to chemical stress, which may protect them from local extinction (Van Straalen and Timmermans, 2002, Medina et al., 2007 , Agra et al., 2010 , Agra et al., 2011).

In this thesis we define **genetic adaptation** as result of a population increasing in fitness following the selective pressures exerted by environmental stressors (Bock, 1980). In contrast **acclimation** is a property of phenotypic features of an individual adjusting to a change in their environment (e.g. chemical stress) due to phenotypic plasticity, thus the capacity for morphological, physiological, or life-history modifications on time-scales less than one generation (Ensminger et al., 2005).

The **genotype** is the set of genes responsible for a particular trait, while the **phenotype** is the physical expression, or characteristics, of the same trait. A **Genotype – Environment interaction** (or G × E interaction) is inferred when two different genotypes respond to environmental variation in different ways.

Genetic adaptation to a stressor may come with an altered allelic constitution of the population, which is more suitable to deal with the chemical stressor, but not necessarily to deal with future, novel stressors. This process is commonly termed **cost of adaptation** or **cost of tolerance** (De Schamphelaere et al., 2011, Van Straalen and Timmermans, 2002, Medina et al., 2007 , Agra et al., 2010). Conversely, increased tolerance to novel stressors may also arise, referred to as cross-tolerance or co-tolerance (Lopes et al., 2005, Ward and Robinson, 2005).

Even before the term ecotoxicology was first coined, two case studies from the 1950's serve as text book examples to illustrate that anthropogenic pollution can act as a selective force, causing microevolution in wild populations (Busvine and Nawab, 1955, Kettlewell, 1955). Kettlewell (1955) first demonstrated that the melanic form of the peppered moth *Biston betularia* was increasing in frequency relative to the black-and-white speckled wild type, due to a camouflage advantage on darkened tree barks, covered with soot from air pollution, as a consequence of the industrial revolution. At about the same time, Busvine (1954) demonstrated that houseflies could genetically adapt to and become resistant to DDT (dichlorodiphenyltrichloroethane). There reviews on increased resistance after adaptation to chemicals in the field (Medina et al., 2007 , Morgan et al., 2007, Agra et al., 2010). Recently there has also been an increase in experimental evolution studies in ecotoxicology (Ward and Robinson, 2005 , Lopes et al., 2009 , Jansen et al., 2010).

One experimental evolution study addressed the question of cost of metal adaptation in a multi-generation context with a *D. magna* population composed of 8 genotypes varying in their Cd 48h-LC50 (26 to >120 mg/L) (Ward and Robinson, 2005). The authors observed a 3-fold increase of the mean 48h-LC50 of the population after 8 generations but they didn't observe any difference in tolerance to temperature stress between the cadmium adapted and control populations. A 3-month selection experiment in *D. magna* under carbaryl exposure resulted in increased tolerance to carbaryl at the expense of higher susceptibility to parasite infection (an illustration of a cost of adaptation or cost of tolerance), possibly due to a reduced efficiency of the early immune response (upregulation of phenol-oxidase) in carbaryl- selected populations (Jansen et al., 2011 -a). Busvine (1954) discovered that the houseflies were also resistant to a whole group of organochlorine compounds that they hadn't previously been exposed to. 'Cross-tolerance' has been reported before for metals in *D. magna*: for cadmium and lead (Ward and Robinson, 2005) and for copper and zinc (Lopes et al., 2005). A recent selection experiment not only demonstrated genetic change in the capacity of the *D. magna* to tolerate higher temperatures but also demonstrates that existing natural populations have evolved increased tolerance to higher temperatures using a layered dormant egg bank to reconstruction the evolution over a forty year period (Geerts et al., 2015).

Overall, the knowledge in this microevolutionary context is still fragmentary and more systematic understanding is needed, especially in the light of global change, to unravel the combined effects of chemical and natural stressors on microevolutionary time-scales.

1.4 Impact of global change on the combined effects of chemical and natural stressors

Through our increasing population growth, resource consumption, disturbance of natural systems, and technological advancement, we have been changing the global climate and environment in a manner that is unique over Earth's history. Global change is the term used to describe the effects of human activities on Earth (see Box 1.4).

Box 1.4. Definition of global change.

> The Ecosystems Panel defines **global change** as the interactions between natural changes in the Earth's physical and biological structure and the broader effects of human activity, meaning that it has both natural and anthropogenic components. Global change connected with human activities first came to broad public attention through forecasts of climate change occurring primarily as a result of human-induced enrichment of the atmosphere with greenhouse gases.

With global change projections, co-occurences of chemical and natural (both abiotic and biotic) stressors are only predicted to increase (Moe et al., 2013). Considerable information already exists on interactive effects between chemicals and abiotic variables, whereas information on the effect of biotic stressors on the toxicity of chemicals is sparse (Holmstrup et al., 2010, Laskowski et al., 2010, Fischer et al., 2013, Couillard et al., 2008). The claim has even been made that we presently know more about how future climates are likely to shift across the globe than about how species will respond to those environmental changes (Fordham, 2015).

The analysis of the effect of biotic stressors (such as parasites or harmful cyanobacteria) on organisms is complicated by the fact that biotic stressors (as opposed to chemical stressors) have the ability to

adapt to their environment as well. Such Genotype-Genotype Interactions (G x G interactions) give rise to a geographic mosaic of coevolution. Geographic mosaic theory is a view of coevolution that implies that coevolution is a genetic and ecological process that relentlessly reshapes interactions among species (Thompson 2005). In studies of coevolution, Genotype-Genotype-Environment Interactions (G x G x E interactions) can then be viewed at the level of how natural selection acts on two or more interacting species across many contrasting environments. Geographic mosaic theory implies that the structure of selection, the intensity of reciprocal selection, and the distribution of genetically based traits available to natural selection continually change over time, as the environment changes (Thompson 2005). In the light of global change it will become increasingly important to understanding how these components of the coevolutionary processes interact.

1.5 Model organism: *Daphnia magna*

In a first approach to investigate the combined effects of chemical and natural stressors, there is a clear need for model species that are ecologically relevant, geographically widely distributed and easy to manipulate experimentally in the laboratory. The cladoceran *Daphnia* qualifies as such a model organism as it plays a pivotal role in the food web of freshwater ecosystems, affecting both phytoplankton communities in terms of biomass as well as species composition (as a primary grazer) and fish production (as a major food source) (Lampert, 2006, Dodson and Hanazato, 1995). Additionally its physiology (including resource allocation processes) is well-documented, and well-calibrated mechanistic models (DEB) and physiologically structured individual-based models (DEB-IBM) are available (Baas et al., 2010, Martin et al., 2013a). Its short life cycle under asexual reproduction (parthenogenesis) is very convenient for investigating the whole life cycle and populations dynamics, as well as genetic variation and microevolutionary effects (van Doorslaer et al., 2009 -b, Colbourne et al., 2011). More specifically, because of their cyclical parthenogenetic reproduction, within- and between-clone comparisons can demonstrate genetic variation for various traits within and between populations, enhancing our understanding of evolutionary ecology (Ebert, 2005).

We used *Daphnia magna* (Strauss, 1820), as it is widely used as an invertebrate model for setting water quality standards and is a recommend species according to the guidelines of the Organisation for

Economic Co-operation and Development (OECD, 1998), and considerable information is already available for *D. magna*. (Figure 1.5).

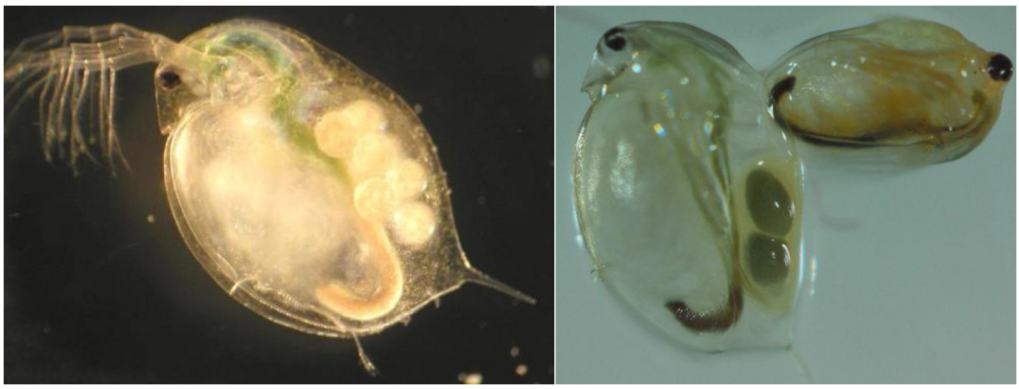


Figure 1.5. Female *Daphnia magna* carrying parthenogenic (asexual) eggs (left), female *Daphnia magna* carrying a sexually produced ephippium (right), as well as an asexually produced male *D. magna* (top right).

D. magna is a cyclic parthenogen that reproduces asexually (clonal reproduction) when conditions are favorable (Figure 1.6) (Lampert, 2006). Once environmental conditions deteriorate (e.g. short day length, food depletion, and high population density, but also chemical stress) males are produced asexually and females produce two haploid eggs which are fertilized by the males (Kleiven et al., 1992, Hobaek and Larsson, 1990, Oda et al., 2005). This sexual reproduction leads to the production of dormant or resting eggs called “ephippia”. Ephippia can remain dormant for several decades and tend to hatch once environmental conditions become more favorable again (Lampert, 2006).

Having alternative life-history strategies allows cladocerans such as *Daphnia* to maximize reproductive rates asexually when conditions are favorable and to ensure long term survival of the population by producing offspring sexually when the environment becomes unsuitable. Ephippia production is fundamental to the maintenance of many *Daphnia* populations in the wild. At the start of the growing season populations are reestablished from banks of resting eggs. In ephemeral habitats, such as temporary ponds, total annual production of ephippia is the most important measure of the fitness of a clone because all adult animals in the population die at the end of each growing season (Shurin and

Dodson, 1997). Even in overwintering populations, hatchlings derived from ephippia can make up a significant proportion of the juveniles born in the spring (Wolf and Carvalho, 1989).

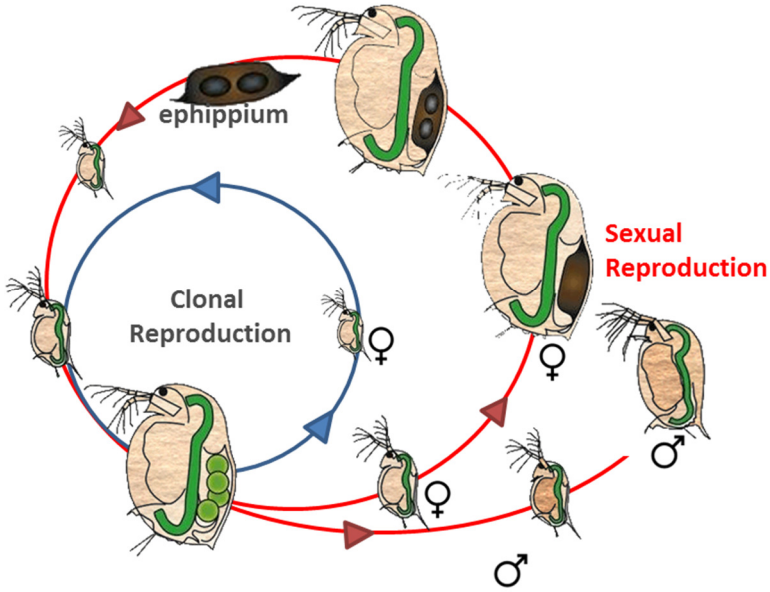


Figure 1.6. Schematic representation of the life cycle of *Daphnia* (adapted from (Ebert, 2005).

1.6 Model stressors

In order to improve ERA and EQS derivation, there is a need to generate systematic, mechanistic and quantitative knowledge about the responses of individuals and natural populations to chemical stress in a context of multiple, simultaneous and time-variable non-chemical stressors. In this PhD thesis copper and zinc are used as model chemical stressors, while the focus in terms of non-chemical stressors lies on cyanobacteria and temperature (in a context of global change). Copper and zinc were chosen as both classify as by the European Commission under list II substances, which are considered less dangerous substances than those under list I, but which nevertheless can have a deleterious effect on the aquatic environment (EC, 2001). Further, the issue of copper and zinc emissions into the environment is pertinent in the context of global change, as both metals are linked to many anthropogenic activities, such as power stations (copper and zinc), automobile vehicles (copper is used in brake pads, tyre wear releases zinc) and other applications (zinc is used in galvanized products, copper in building construction and electronical products). The application of copper-based algaecides is still one of the most common measures to eradicate freshwater phytoplankton, including cyanobacterial blooms (Jancula and Marsalek, 2011, Garcia-Villada et al., 2004).

1.6.1 Chemical stressors

Contrary to man-made organic chemicals, metals are naturally occurring substances and life has evolved in the presence of these elements. Some of these, the essential metals (like copper and zinc), are crucial to survival, growth and reproduction of organisms (Marx, 1987; Linder, 1991; Keen et al., 1993; O'Halloran, 1993). For each essential element, there is a species-specific optimal concentration range for which metabolic requirements and development occur in an optimal way (Hopkin, 1989). This range, over which homeostatic regulation occurs, has been termed the optimal concentration range of essential elements (OCEE) (Van Assche et al., 1997) (Figure 1.7).

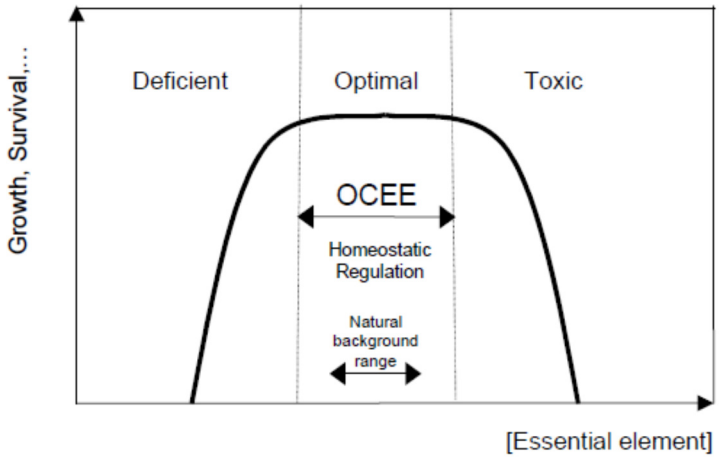


Figure 1.7. The Optimal Concentration range for Essential Elements (OCEE) for a species in a given habitat-type (adapted from Van Assche et al., 1997).

When the external concentration of the essential element becomes too low or too high, homeostatic regulation will not be sufficient and deficiency or toxicity can occur, respectively. Therefore essential metals have a "double" toxicity threshold: deficiency at too low concentrations and toxicity at too high concentrations (Chapman et al., 1996) (Figure 1.7). Organisms regulate their internal concentrations of essential metals to counter potential toxicity with three main strategies are used: reduced intake, enhanced excretion, or storage/detoxification or a combination thereof (see Chapman et al. 1996 and references therein). The focus of this study is on the chronic toxicity of the essential elements copper and zinc, i.e. on copper or zinc concentrations at which the homeostatic regulation capacity of organisms is exceeded.

Copper

Copper (Cu) is found in the Earth´s crust in a variety of forms (sulphide, carbonate, and silicate deposits, as well as pure “native” Cu) and naturally occurs in surface waters. It is an essential element and therefore indispensable for humans, animals and plant alike. The global demand for Cu continues to grow: world refined usage has more than tripled in the last 50 years, as a result of expanding sectors such as electrical and electronic products, building construction, industrial machinery and equipment, transportation equipment, and consumer and general products (International Copper Study Group, 2014). The demand for Cu is expected to be met by the discovery of new deposits, technological improvements, efficient design, and by taking advantage of the renewable nature of Cu, as virtually all products made from Cu can be recycled and recycled copper loses none of its chemical or physical properties. An overview of the primary uses of Cu and the global production figures for 2014 is given in Figure 1.8.

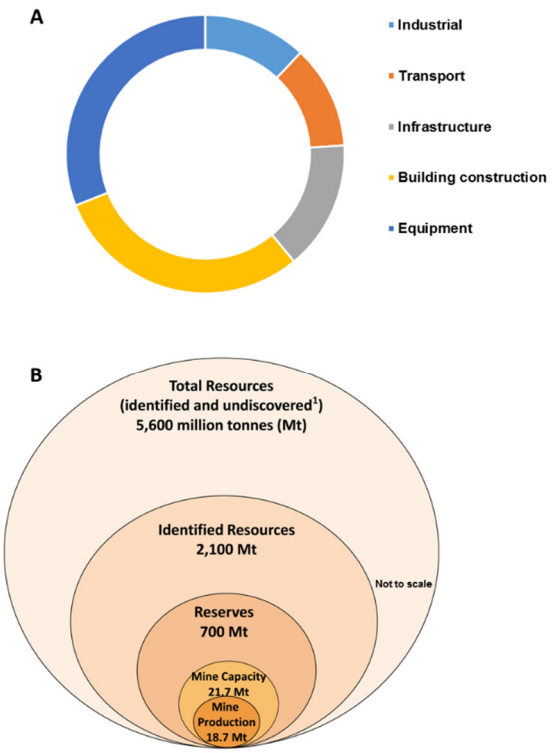


Figure 1.8. Primary end uses (**A**) and production figures (**B**) in 2014 of Cu.

Mt = million tons. (International Copper Study Group, 2014)

Natural background dissolved concentrations of Cu across Europe in 2006 are shown in Figure 1.9 and have been obtained from the FOREGS Database (Salminen et al., 2005). The FOREGS-data set is considered to be of high quality. A detailed description of sampling methodology, sampling preparation and analysis is given by Salminen et al. (2005). Human induced inputs of Cu into the aquatic environment are copper mining and smelting, electrical industry, agriculture, sewage treatment, fabrication of metal products, as well as the combustion of fossil fuel, municipal waste waters, manure, fertilizers and antifouling measures (USEPA, 2007, Salminen et al., 2005).

The most toxic form of copper is the ionic Cu^{2+}. Copper can lead to the formation of reactive oxygen species when levels of the metal are high. Copper toxicity decreases with increasing dissolved organic matter (DOM), usually referred to as dissolved organic carbon (DOC), because copper binds to DOC with high affinity forming a complex that reduces copper binding and uptake, therefore reducing the bioavailable copper and hence its toxicity (Wood et al., 2011, De Schamphelaere et al., 2004). Copper pollution in surface water can locally reach levels that may cause toxicity to *Daphnia* sp., for instance in waters affected by surface run-off from vineyards, where copper is still used as a biocide against fungus diseases (Banas et al., 2010) Additionally, copper is found in biocides, and the application of copper containing algaecides typically results in dissolved copper concentrations in the range of 10-100 µg/L (Jancula and Marsalek, 2011). Copper toxicity has been indirectly linked to the inhibition of active sodium uptake in *Daphnia magna* (De Schamphelaere et al., 2007), inhibition of neuronal signal transmission and acetylcholinesterase (AChE) activity (Untersteiner et al., 2003), and oxidative stress (Barata et al., 2005, Xie et al., 2006). Genetic adaptation to copper has already been shown in field populations of *Daphnia longispina*, with initial evidence of costs of adaptation (Agra et al., 2010).

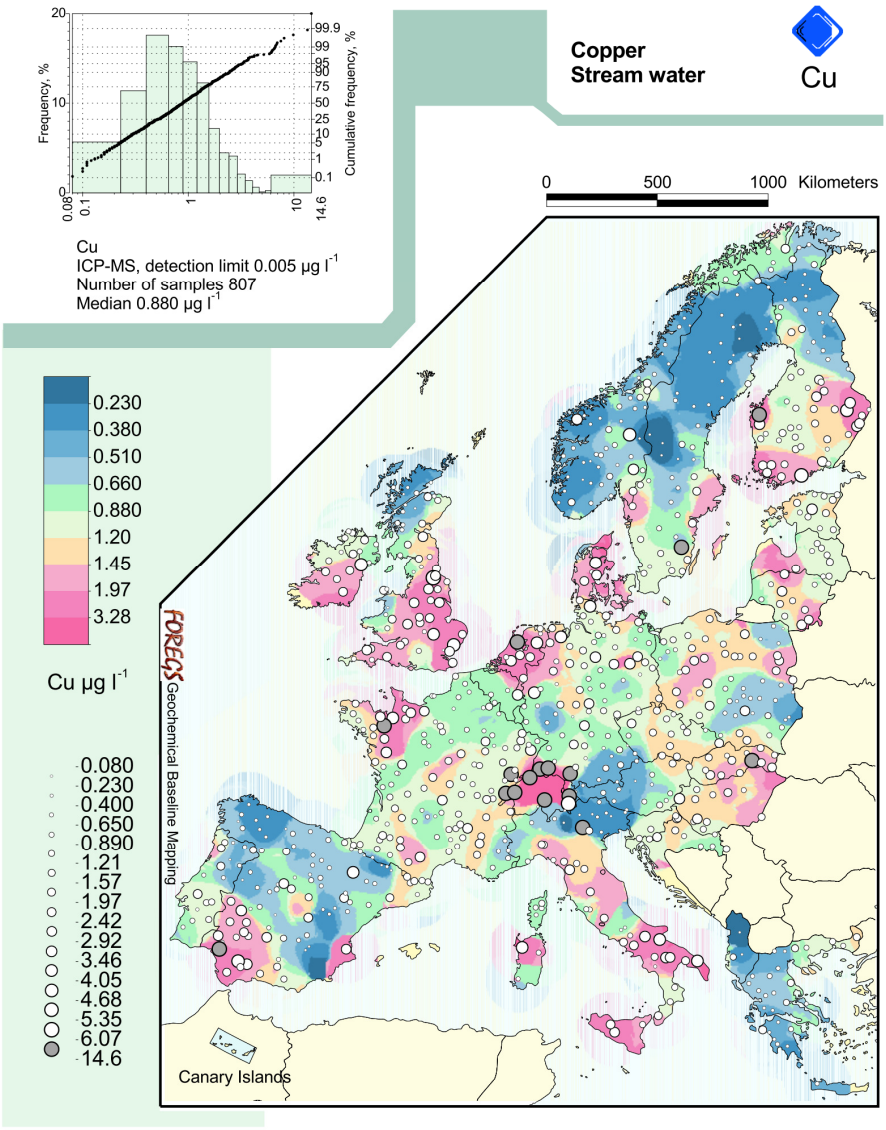


Figure 1.9. Dissolved background Cu concentrations in European surface waters in 2006 (taken from FOREGS Geochemical Baseline Programme)

Zinc

Like copper (Cu), zinc (Zn) is a natural component of the earth's crust, (present in rock, soil, air, and water), and it is also essential for plant, animal and human life. Zn occurs as ore deposits or mineral form (ILZSG, 2015). Natural sources of Zn may include the weathering of zinc-containing bedrocks which give rise to Zn^{2+} in solution. Anthropogenic sources of Zn are significant, arising mainly from industrial activities, such as mining, coal and waste combustion and steel processing. It had also important markets in the brass and construction industries and in chemicals and constitutes an essential nutritional element. The world's Zn production is still on the rise and industrial applications tend to disperse Zn widely in the natural environment, leading to levels above pre-industrial concentrations in air, soil and water. An overview of the primary uses of Zn and the global production figures for 2014 is given in Figure 1.10.

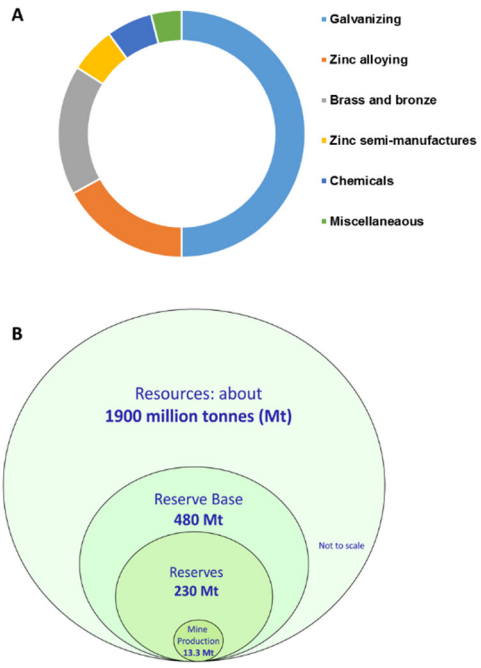


Figure 1.10. Primary end uses (**A**) and production figures (**B**) in 2014 of Zn.

Mt = million tons. (ILZSG, 2015)

Natural background dissolved concentrations of Zn across Europe in 2006 are shown in Figure 1.11 and have been obtained from the FOREGS Database (Salminen et al., 2005). In some areas, particularly at discharges from local mining sites or where industrial activities are carried out concentrations can be much higher (Luomo and Rainbow, 2008). Based on evidence with freshwater fish and daphnids, an important mode of action of zinc is believed to be an impaired calcium uptake, which can lead to hypocalcaemia (Spry and Wood, 1985, De Schamphelaere et al., 2008 , Muyssen et al., 2009).

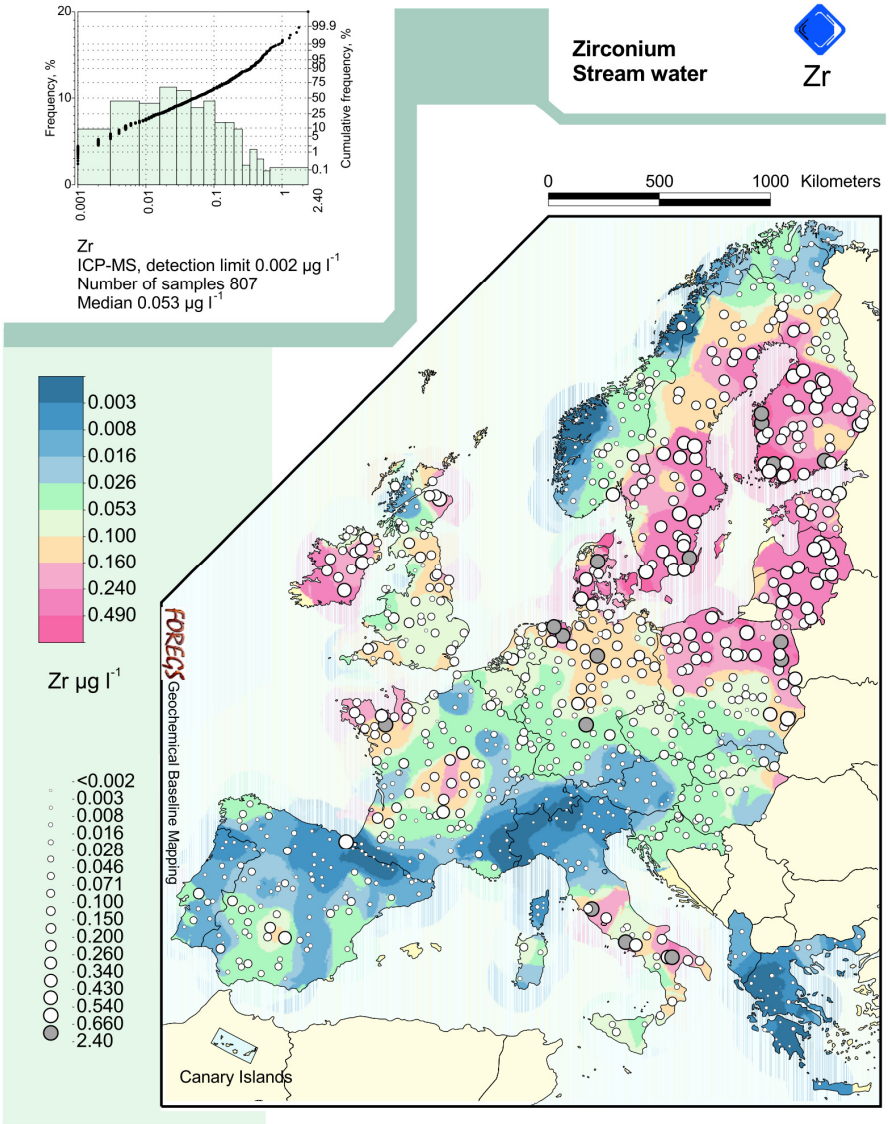


Figure 1. 11. Dissolved background Zn concentrations in European surface waters in 2006 (taken from FOREGS Geochemical Baseline Programme).

1.6.2 Natural stressors

Non-chemical stressors that are currently considered most urgent are those that are linked to global climate change (Noyes et al., 2009) (Wenning et al., 2010). In addition, the Society of Environmental Toxicology and Chemistry (SETAC) has recently launched an urgent call to investigate how global climate change may combine and interact with chemical pollution and how to incorporate this into ERA and chemicals management (Wenning et al., 2010). Global change will undoubtedly bring about important challenges to freshwater organisms, and this PhD thesis will focus on temperature (as a abiotic natural stressor) and toxic cyanobacterial blooms (also termed harmful algal blooms, as a biotic natural stressor). While the effects of the individual stressors on the fitness of freshwater zooplankton are well-studied, the combined effects of increased temperature and cyanobacteria on chemical toxicity have only rarely been documented (Noyes et al., 2009, Luerling, 2003). Further the combined effects of abiotic and chemical stressors have received more attention than the combined effects of biotic stressors on chemicals (Heugens et al., 2001 , Sokolova and Lannig, 2008).

Cyanobacteria

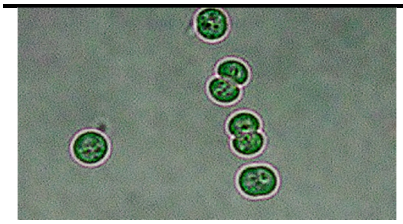
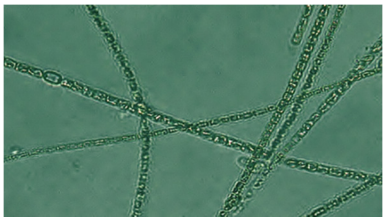
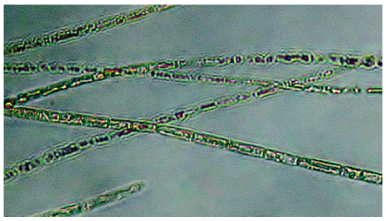
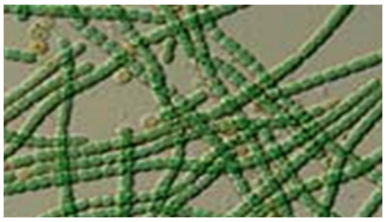
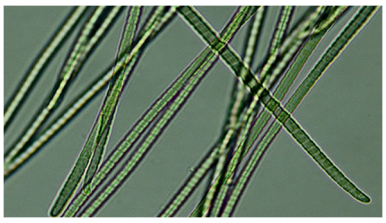
Cyanobacteria are a perfect illustration of non-chemical biotic stress, as they are considered as an emerging threat to freshwater environments. Cyanobacteria pose serious risks to environmental and human health, and large scale ecosystem wide effects have been attributed to their extensive bloom formation (also termed harmful algal blooms) and toxin production (Falconer, 2001, Johnk et al., 2008, Downing et al., 2001, Davis et al., 2009). These effects are only expected to worsen under global change as a number of factors, including rising nutrient loading due to anthropogenic pollution and water temperatures, duration of summer stratification, are predicted to increase prevalence and severity of these cyanobacterial blooms (Paerl and Huisman, 2008 , Kosten et al., 2012, Paul, 2008, O'Neil et al., 2012). The proliferation of cyanobacteria will increased the likelihood of the combined exposure with other stressors.

Cyanobacteria play an important part in the functioning of aquatic ecosystems, as they are a major constituent of phytoplankton communities. Cyanobacteria have a significant impact on freshwater

zooplankton in particular, as they can become a food source to these invertebrates due to their bloom formation in freshwater lakes and ponds (Paerl et al., 2001). It has already been demonstrated that *Daphnia* cannot distinguish between toxic and non-toxic *Microcystis* strains as food source (Demott, 1999, Rohrlack et al., 1999). Several studies have already demonstrated the harmful effects associated with cyanobacteria serving as a food source for freshwater invertebrates such as *Daphnia* (Haney et al., 1995, Demott et al., 1991, Dao et al., 2010). Yet, little agreement can be found in these studies as to the cause of these adverse effects (Rohrlack et al., 1999, Lurling and van der Grinten, 2003). Effects have mainly been associated with four factors or a combination thereof: cyanobacterial toxins (e.g. microcystins, cylindrospermopsins) (Rohrlack et al., 1999, Nogueira et al., 2004, Dao et al., 2010, Demott et al., 1991), feeding inhibition (Lurling, 2003 , Demott et al., 1991), morphology (Wilson et al., 2006, DeMott et al., 2001) and the lack of essential nutrients (Martin-Creuzburg and von Elert, 2009). Although cyanotoxins exhibit high toxicity to vertebrates, including mammals (Wiegand and Pflugmacher, 2005), several studies have reported no significant differences between the effects of cyanotoxin producing and non-toxin producing cyanobacteria on zooplankton, albeit such studies have mainly focused on *Microcystis aeruginosa* (Tillmanns et al., 2008, Wilson et al., 2006). In *Daphnia* the the mechanistic basis of the harmful effects of cyanobacteria remain to be tested.

While it is well-known that cyanobacteria reduce the fitness of *Daphnia* sp. (Cerbin et al., 2010a, Lurling, 2003a) their combined effects with chemicals have hardly been documented, with a few exceptions. In one study the pesticide carbaryl and the microcystin LR producing *Microcystis aeruginosa* caused a synergistic effect in the response of *Daphnia pulicaria* (Cerbin et al., 2010a). Adverse effects of the filamentous cyanobacterium *Cylindrospermopsis raciborskii* on the growth of *Daphnia longispina* were magnified by the presence of a xenobiotc (PCB) (Bernatowicz and Pijanowska, 2011). On the other hand a lack of interactive effects of *Microcystis aeruginosa* with cadmium has been described in *Daphnia magna* (De Coninck et al., 2013), while antagonistic effects have been reported for mixtures of *Microcystis aeruginosa* and different pesticides in *Daphnia pulex* (Asselman et al., 2013). The primary focus of the thesis is on the most ubiquitous cyanobacterium *Microcystis aeruginosa*. Additionally five other cynanobacteria will also be investigated to some extent (Table 1.1)

Table 1.1. Overview of the cyanobacterial genera used in this PhD dissertation. Only the toxin produced by the strain used in the dissertation is mentioned. The known modes of toxic action were obtained from Wiegand and Pflugmacher (2005).

	Cyanobacterium	**Known toxin**	**Mode of toxic action**
	*Microcystis aeruginosa*	Microcystin	Inhibition of protein phosphatases (PP1 and PP2A)
	*Aphanizomenon flos-aquae*	Saxitoxin	Na channel blocking in neurons
	*Anabaena lemmermannii*	Anatoxin-a(s)	Inhibition of activity of acetylcholine esterase
	*Cylindrospermopsis raciborskii*	Cylindro-spermopsin	Protein synthesis inhibitor
	*Nodularia harveyana*	Nodularin	Inhibition of protein phosphatases (PP1 and PP2A)
	*Oscillatoria sp.*	Anatoxin-a	inhibition of acetylcholine receptor

Temperature

Different climate scenarios predict the increase of global average air temperature between about 1.1 ℃ and 6.4 ℃, with average rise of 3.9 ºC, by the end of this century (IPCC, 2007). Global average surface temperature is predicted to increase on average by 1.8 – 4℃ by the end of this century (Caldwell et al., 2015, Meehl, 2007). Most aquatic organisms are ectotherms, which makes temperature a crucial environmental factor controlling physiological processes. While extreme temperatures may be lethal, temperatures outside the optimal range but within the genetic tolerance limits, can lead to acclimatization of individuals to a higher or lower temperature and can extend the upper or lower boundaries, respectively (Cairns et al., 1975, Donker et al., 1998)

Although a range of factors contribute to the proliferation of cyanobacteria, global warming alone is probably the most significant factor to contribute to the predicted increase in occurrence and prevalence of cyanobacteria (Kosten et al., 2012, Abrantes et al., 2006). If cyanobacterial abundance increases in response to global climate change, this could lead to increased exposure in zooplanktonton and other organisms higher up the food chain. The mechanisms by which temperature increases, or decreases, the sensitivity of *D. magna* to different cyanobacteria are not known. Temperature, even if not stressful in itself, can affect both the mechanisms by which stressors produce cellular effects (toxicodynamics), as well as the bioavailable amount of a stressor available for uptake or elimination reaching the target site (toxicokinetics) (Fischer et al., 2013). Harmful effects on *D. magna* reproduction increasing with temperature in some cyanobacteria, while decreasing with others, may therefore be the result of temperature affecting the balance of uptake, internal distribution and elimination (toxicokinetics), or the balance between damage and repair processes (toxicodynamics) differently after exposure to the cyanobacteria.

Even temperatures within the tolerance range affect the balance of uptake, internal distribution, biotransformation, and elimination (toxicokinetics), as well as the balance between damage and repair processes (toxicodynamics) differently after exposure to stressors because of changes in metabolic and behavioral activity of organisms (Fischer et al., 2013, Heugens et al., 2003). This has been shown in the freshwater bivalve *Unio douglasiae*, in which microcystin is eliminated at a higher rate at 25 ℃ than at 15 ℃ (Yokoyama and Park, 2003). For most metal pollutants studied, toxicity increases with

temperature (Heugens et al., 2001). However a recent study on the annelid worm *Enchytraeus crypticus* also reported lower reproduction with decreasing temperature for copper and cadmium (Cedergreen et al., 2013). Our results support the authors' conclusion that no consistent relationship between metal toxicity and temperature across species can be made.

1.7 Conceptual framework

This PhD dissertation focused on several aspects of ecological risk assessment (ERA) of metals on the effect assessment for the aquatic environment, especially focusing on the evaluation of the risk for aquatic invertebrates. Yet, as mentioned in sections 1.1 and 1.3, until now ERA focuses almost exclusively on the effect characterization of single chemical stressors on individuals in short term experiments. This contrasts with ecological reality, where natural populations adapt by means of natural selection to continuously changing conditions of multiple stressors under non-optimal conditions (e.g., food shortage, non-optimal temperature, and predation). With predictions of global change (as mentioned in section 1.4), occurrences of the combined exposure of organisms to chemical and natural (both abiotic and biotic) stressors are only predicted to increase. Therefore ERA may not be protective across different environments, such as conditions predicted under global change, as it doesn't account for multiple stressors nor genetic adaptation. Below, the rationale behind the research carried out in each chapter, as well as the research questions are formulated. The conceptual framework and outline of this PhD dissertation is described in Figure 1.12.

Non-chemical stress typically increases the sensitivity to chemicals and vice versa (Heugens et al., 2001, Coors and De Meester, 2008). An important concern is the combined effects of cyanobacteria, sometimes referred to as harmful algal blooms, with chemical stressors. As discussed in section 1.6, copper remains the most commonly applied chemical algaecide and is also often detected in eutrophic run-offs that promote harmful algal blooms. As highlighted in section 1.6.2, *M. aeruginosa* is the most studied genus, but as other genera may also relevant in the context of global change, the response of two *D. magna* clones to the combined effects of copper and five cyanobacterial genera were evaluated in chapter 2 using two reference models.

Research question 1: Can the combined effects of copper and cyanobacteria be predicted using CA/IA reference models, based on the known effects of individual exposures?

Research question 2: Can the combined effects between copper and cyanobacteria be generalized across different cyanobacterial and daphnid genera?

As cyanobacterial blooms are likely to become more prevalent with global change, the effects of six different cyanobacterial genera were investigated in chapter 3 at three different temperatures (15°C, 19°C and 23°C) on multiple endpoints of *D. magna* using standard ecotoxicity experiments.

Research question 3: Does *D. magna* become more sensitive to the harmful effects of cyanobacteria as temperature increases?

Research question 4: Are the different cyanobacterial genera more harmful to *D. magna* than starvation alone?

As was emphasized in section 1.1, standard ecotoxicity tests are conducted for single chemicals under constant and favorable experimental conditions. In natural communities, however, the toxicity of chemicals may be influenced by abiotic and biotic environmental factors. In chapter 4 the influence of temperature and total food concentration (both related to global change) on the nature of the combined effects of copper and *Microcystis aeruginosa* on *D. magna* was examined (i.e. whether the combined effects deviated from non-interaction).

Research question 5: Are 21-day Cu NOEC concentrations derived under optimal conditions protective under non-optimal conditions?

Natural populations are faced with variable non-optimal conditions, characterized by the presence of multiple stressors, as mentioned in section 1.1. In chapter 5 a population experiment was carried out to compare the total density of a *D. magna* population exposed to regulatory and environmentally relevant copper concentrations under different environmental conditions, equivalent to a seasonal increase of temperature and the proportion of the total diet consisting of *M. aeruginosa* under current temperature conditions, as well as a 4°C temperature increase, predicted under global climate change.

Research question 6: Are 21-day Cu NOEC concentrations derived under optimal conditions protective under time-variable non-optimal conditions at the population level?

As stressed in section 1.3.1, ecotoxicology is faced with the challenge to develop more ecologically realistic predictive models. In chapter 6 a DEB-IBM model was calibrated based on total reproduction after 21 days measured in chapter 4. The independent results from the population level experiment in chapter 5, under combined exposure to copper and *M. aruginosa*, under realistic (global change) time-variable conditions, were used to validate the predictions resulting from this DEB-IBM model.

Research question 7: Can a mechanistic model (DEB-IBM) extrapolate the effects at the individual level to more ecologically relevant effects at the population level?

As mentioned in sections 1.1 and 1.3.2, natural populations are generally characterized by genetic variability, which offers the potential for multi-generational micro-evolutionary responses, allowing populations to genetically adapt to chemical stressors (and become more tolerant to them), but potentially at the expense of a cost-of-tolerance (Van Straalen and Timmermans, 2002, Medina et al., 2007 , Agra et al., 2010 , Agra et al., 2011). Previous studies have shown the ability of individual *D. magna* clones to physiologically acclimate to a range of copper and zinc concentrations (Muyssen and Janssen, 2001, Muyssen et al., 2002 , Bossuyt and Janssen, 2003 , Bossuyt and Janssen, 2004 , Muyssen and Janssen, 2005), but as yet, no research has been dedicated to the potential for genetic adaptation to these metals. In Chapter 7, a 10 week microevolution experiment was conducted with a genetically diverse *D. magna* population at different copper and zinc exposures.

Research question 8: Can a genetically diverse populations adapt to lethal metal concentrations (equivalent to the 8-day LC50)?

Research question 9: Do populations display lower genetic diversity after adaptation than prior to selection?

In a follow up study with the Zn-adapted populations, the effect of Zn-adaptation on the tolerance was observed to the absence of the stressor, as well as to additional stressors relevant for global change (temperature, *M. aeruginosa,* and cadmuim).

Research question 10: Is adaptation to chemical stressors associated with costs of adaptation?

In the final chapter 8, the findings of the PhD dissertation are critically assessed, pointing to methodological strengths and weaknesses, and providing some perspectives for future research.

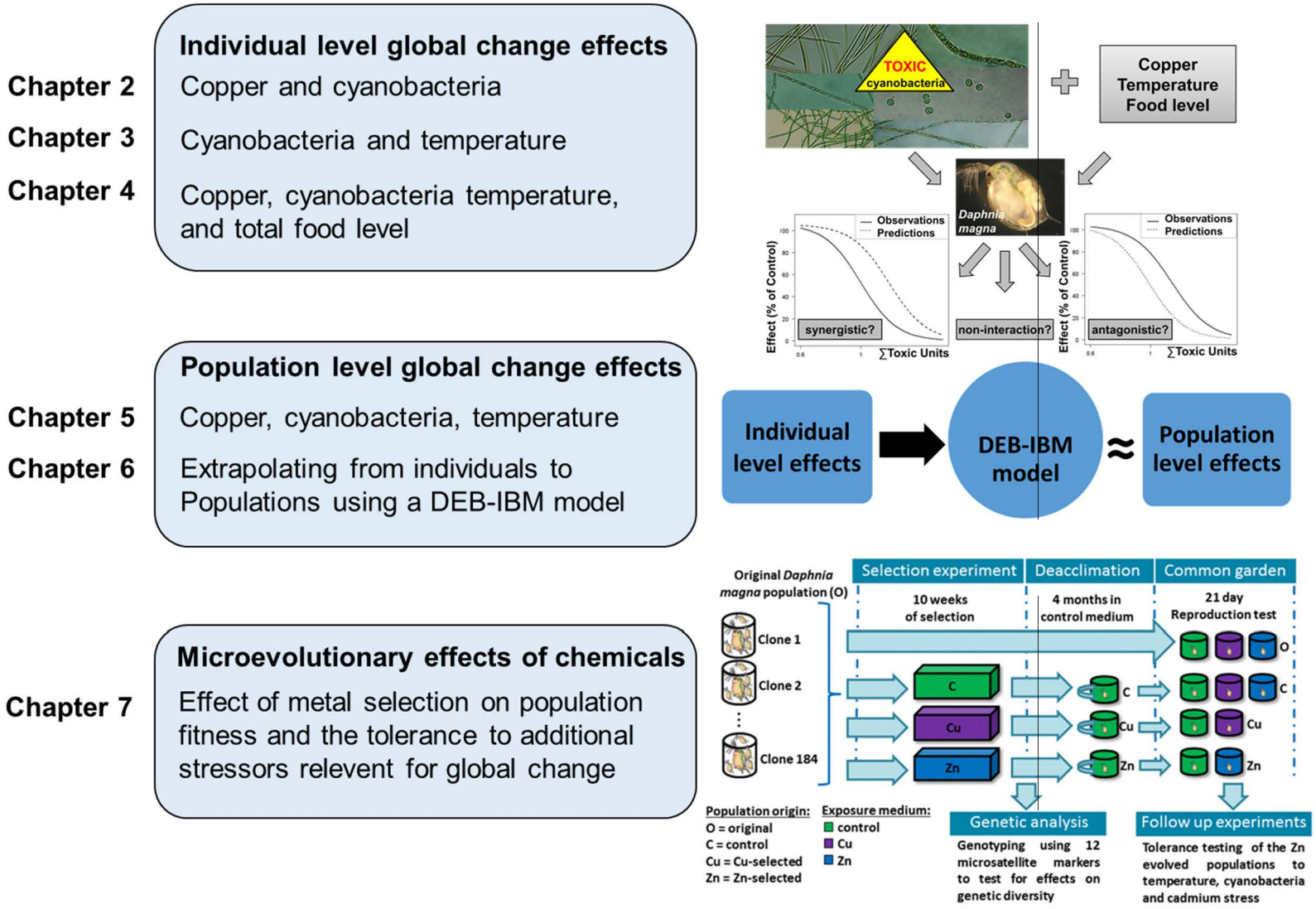


Figure 1.12. Conceptual framework and outline of the PhD dissertation.

2

Testing for interactive effects of copper and different cyanobacteria in *Daphnia magna*

Redrafted after:

Jennifer D Hochmuth, Jana Asselman, Karel AC De Schamphelaere. 2014. Are interactive effects of harmful algal blooms and copper pollution a concern for water quality management? Water Research 60: 41-53.

2.1 Introduction

Harmful algal blooms pose a serious risk to environmental and human health, and the management and restoration of water quality following such a bloom can be challenging. Large scale ecosystem wide effects have been attributed to their extensive proliferation and toxin production (Falconer, 2001, Johnk et al., 2008, Downing et al., 2001, Davis et al., 2009). The application of copper-based algaecides is still one of the most common measures to eradicate freshwater phytoplankton, including cyanobacterial blooms (Jancula and Marsalek, 2011, Garcia-Villada et al., 2004). Furthermore, copper itself is listed as priority pollutant by the U.S. Environmental Protection Agency (McKnight et al., 1983), and many EU countries are developing Environmental Quality Standards (EQS) for copper in surface waters under the EU water framework directive (Comber et al., 2008). Copper pollution in surface waters can locally reach levels that may cause toxicity to aquatic species, for instance in waters affected by surface run-off from vineyards and citrus farms, where copper is still used as a biocide against fungus diseases (Banas et al., 2010, Graves et al., 2004).

As a consequence, it can be anticipated that cyanobacteria and copper pollution often co-occur in freshwater systems, either in situations where surface run-off is enriched with both copper and nutrients or where copper is actively used as a major component in chemical applications to eradicate cyanobacteria blooms. Furthermore, anthropogenic copper pollution may also act interactively with cyanobacterial stressors on aquatic biota. Interaction (e.g. synergism or antagonism) is said to occur if the level of response produced by any combination of different stressors differs from the response expected on the basis of a theoretical reference model of non-interaction (McCarty and Borgert, 2006). Considerable information already exists on interactive effects between chemical and non-chemical stressors, whereas information on the interactive effects between chemical stressors and cyanobacterial stressors is sparse (Holmstrup et al., 2010, Laskowski et al., 2010, Fischer et al., 2013, Couillard et al., 2008). Indirect “smaller than expected” effects of copper on non-target organisms could occur as copper actively eradicates the cyanobacteria, but indirect “larger than expected” effects of copper are also possible, as copper may induce cyanobacterial cell lysis, which increases external cyanobacterial toxin concentrations (Jones and Orr, 1994, Kenefick et al., 1993). Consequently an important concern is that conventional risk assessment may not be conservative enough, as it currently excludes combined and

potentially interactive effects of mixtures of stressors that cannot be predicted form individual toxicities alone.

Furthermore the interactive effects of cyanobacteria with chemicals have rarely been investigated, with few exceptions. In one study the insecticide carbaryl and *Microcystis aeruginosa* caused a synergistic toxicity response in *Daphnia pulicaria* (Cerbin et al., 2010a). More recently, antagonistic effects were reported between carbaryl and four cyanobacterial genera in *Daphnia pulex* (Asselman et al., 2013). Despite the lack of any direct influence of two polychlorinated biphenyls (PCB52 and PCB153) on the fecundity, growth and depth selection of *Daphnia longispina*, adverse effects of the filamentous cyanobacteria *Cylindrospermopsis raciborskii* on fecundity (but not on growth and on depth selection) were magnified by PCB52 in 25.8% of the clones tested and reduced in 33% of the clones, while no significant interactions were observed with PCB153 for any endpoint (Bernatowicz and Pijanowska, 2011). Previous studies have confirmed the existence of a genetic basis for the response to single chemicals or stressors between different *Daphnia* clones within the same species complex (Wilson and Hay, 2007, Barata et al., 1998, Soares et al., 1992, Barata et al., 2002a, Bednarska et al., 2011). These studies highlight that there is a need to investigate interactive effects between cyanobacteria and chemicals, and that the type of interaction is potentially influenced by the chemical, the cyanobacteria species, and the *Daphnia* genotype considered.

In this study the combined effects of five different cyanobacterial genera and copper on reproduction of two *Daphnia magna* clones in 21-day exposure experiments. We chose 5 different cyanobacterial genera, all commonly reported in blooms, and known to differ in their toxin production (Wiegand and Pflugmacher, 2005), as well as their morphology (Araoz et al., 2010, Komarek and Mares, 2012, Watanabe, 1995), in order to cover a broad range of possible cyanobacterial effect profiles (**Table 2.1**). The overall aim was to obtain a descriptive overview of the effects on *Daphnia magna* upon combined exposure to copper and live cyanobacteria cells, accounting for all major factors which could affect fitness (cyanotoxins, feeding inhibition, morphology and lack of essential nutrients) by using a wide range of cyanobacteria species differing in those factors. Because the modes of action of copper and cyanobacteria are various and not fully understood, and because some modes of action may be (partly) similar, we decided a priori to use both the reference model for similarly acting chemicals, i.e. the

Concentration Addition (CA) model (or Loewe Additivity, first introduced by Loewe and Muischnek, 1926) and the reference model for dissimilarly acting chemicals, i.e. the Independent Action Model (IA) (first introduced by Bliss 1939) for the data analysis.

2.2 Material and Methods

2.2.1 *Daphnia* culture and test medium

The *Daphnia magna* clones (Iinb1 and Xinb3) used in all exposures were obtained from the Ebert group (Zoological Institute, Evolutionary Biology, University of Basel, Rheinsprung 9, 4051 Basel, Switzerland, http://evolution.unibas.ch/ebert/). Both clones were derived from the same clonal isolates used in the first-generation *Daphnia magna* genetic linkage map (Routtu et al., 2010). A modified M4 medium was used for both culturing and actual exposures. This medium differs from the original composition (Elendt and Bias, 1990) as follows: hardness was reduced to 180 mg $CaCO_3$/L (Ca and Mg concentration were reduced by 30%), background Cu and Zn concentrations were modified to 5 and 28 µg/L respectively and Na_2EDTA was omitted and replaced with Aldrich humic acid (AHA, Sigma Aldrich, Bornem, Belgium) at a nominal concentration of 5 mg dissolved organic carbon (DOC) per litre.

All stock solutions for the medium were made from analytical grade products (Sigma-Aldrich, Bornem, Belgium) by dissolving them in deionized water, with the exception of AHA, which was dissolved in a solution of 0.04g NaOH per litre of deionized water and filtered to 0.45µm (Acrodisc, PALL Life Sciences, Port Washington, NY, USA). The modified M4 medium was aerated for 24-48h at 20°C +/- 1°C in 25L or 50L polyethylene vessels. After this time the required volume for each treatment per change out was spiked with Cu stock solutions and transferred into sealed polyethylene vessels. After vigorous shaking the test solutions were allowed to equilibrate for 48 hours at 20°C before being used in the toxicity tests. For each medium renewal fresh medium was prepared in the same manner and the spiked medium was always obtained from the same batch as the control medium.

2.2.2 Algae and cyanobacteria cultures

Algal and cyanobacterial species used in this study were grown from continuous cultures that have been maintained successfully in our lab for many years and that have originated from certified culture institutions (Table 2.1). Green algae were cultured at 20°C ± 1°C under continuous light (240 µmol photon m^{-2} s^{-1}) with continuous aeration in carbon filtered aerated city tap water (Ghent, Belgium), to which modified Provasoli's ES enrichment (Bold, 1978) at 1/2 strength and, additionally, 1.4 mg/L $FeSO_4.7H_2O$, 15 mg/L $NaH_2PO_4.2H_2O$, 150 mg/L $NaNO_3$ and 2.35 mg/L $MnCl_2.4H_2O$ were added (Appendix A Table A.1). Cyanobacteria were cultured in modified reference culture media (Allen, 1968, Kotai, 1972a) (Appendix A Tables A.2 - A.4) at 20 ± 1°C under constant light intensity (14 µmol photon m^{-2} s-1) with gentle aeration. At the mid to late log phase (+/- 10 days after culture initiation) both algae and cyanobacteria cultures were concentrated by centrifugation and re-suspended in clean culture medium. Because of the large variation in cell sizes and morphologies among the different algae and cyanobacteria used in this study, both algae and cyanobacteria concentrations were measured as mg dry weightt/mL by drying and weighing a known a subsample (2 mL) of the concentrated culture at 60°C for 24 hours and subsequently deriving the amount of carbon per liter from the dry weight using a conversion factor of 0.4mg C/mg dry weight (1mg C/L 2.5mg dry weight/L) (Evens et al., 2009 , De Schamphelaere and Janssen, 2004). Feeding ratios (algae/cyanobacteria) in all treatments were based on the dry wt per volume unit ratio of the concentrated culture suspensions.

2.2.3 Experimental Design

Chronic toxicity experiments were performed according to a modified central composite design. This design has been advocated as one of the superior experimental designs for evaluating the combined effects data with both the IA and CA models (Lock and Janssen, 2002, Jonker et al., 2005). The standard central composite design was further modified by including four additional points in the mixture treatments (Appendix A Figure A.1). In addition, six points for each of the two single stressor treatments were added, to simultaneously evaluate the effects of the single stressor treatments. This was based on the evidence that non-simultaneous testing of single and mixture treatments leads to incorrect conclusions of the combined effects (De Laender et al., 2009).

Table 2.1. List of algal (A) and cyanobacterial (C) species obtained from different culture collections with their respective strain number, culture medium, as well as a description of the known toxin production, associated mode of action and basic morphology of the cyanobacteria used.

Species	Culture Institution[a]	Strain	Culture Medium[b]	Known toxin (Mode of toxic Action) [c]	Morphology[d]
Chlamydomonas reinhardtii (A)					
	CCAP	CCAP 11/32B	modified Provasoli's ES	NA	NA
Pseudokirchneriella subcapitata (A)					
	CCAP	CCAP 278/4	modified Provasoli's ES	NA	NA
Anabaena lemmermannii (C)					
	SCCAP	K-0599	Z8	Anatoxin-a(s) (inhibition of activity of acetylcholine esterase)	solitary straight filaments heterocysts and akinetes present
Aphanizonmenon sp. (C)					
	CICCM	CAWBG01	$BG11_0$	Saxitoxin (Na channel blocking in neurons)	solitary cylindrical filaments with tendency to form colonies heterocysts and akinetes present
Cylindrospermopsis raciborskii (C)					
	UTEX	LB 2879	Z8	Cylindrospermopsin (protein synthesis inhibitor)	solitary free-floating filaments heterocysts and akinetes present
Microcystis aeruginosa (C)					
	PCC	PCC7806	$BG11_0$	Microcystin (protein phosphatase inhibitor)	unicellular or large colonies no heterocysts and akinetes
Oscillatoria sp. (C)					
	PCC	PCC6412	BG11	Anatoxin-a (inhibition of acetylcholine receptor)	cylindrical filaments no heterocysts and akinetes

[a] Culture Collection of Algae and Protozoa (CCAP), SAMS Research Services Ltd. Scandinavian Culture Collection for Algae and Protozoa (SSCAP), Cawthorn Institute Culture Collection of Microalgae (CICCM), Pasteur Culture Collection (PCC), University of Texas (UTEX). [b] Full composition of culture media (Appendix A Tables A.1 – A.4). [c] Modes of action of toxins were obtained in Wiegand and Pflugmacher (2005). NA = not applicable. [d] Information on morphology was obtained in Komarek and Mares (2012) for heterocystous cyanobacteria, in Araoz (2010) for *Oscillatoria* and in Watanabe et al. (1995) for *Microcystis aeruginosa*

The entire study was split into 5 separate sub-experiments over time, one for each of the 5 cyanobacteria tested. For each cyanobacteria tested, exposures with both clones were carried out simultaneously. In each of these sub-experiments single stressor treatments (cyanobacteria and copper) and mixture treatments were tested simultaneously. As a consequence, single dose response curves of copper could be estimated for each separate sub-experiment, resulting in 5 replicate estimates of the 21 day EC_{50} and slope parameters of copper for both clones (Figure 2.1). For each cyanobacteria sub-experiment one control exposure was carried out simultaneously with the single dose exposures of Cu (6 concentrations), cyanobacteria (6 concentrations) and the mixture treatments (13 combinations of Cu and cyanobacteria concentrations), resulting in a total of 26 treatments per sub-experiment (Table 2.2).

At the start of each test, juvenile animals from the third clutch (< 24h old) of isoclonal females, which had already been grown for a minimum of two generations under experimental control conditions, were transferred individually to polyethylene cups containing 50 ml of the test medium (4 replicates per treatment). Individual reproductive output (measured as number of juveniles) was monitored daily for a period of 21 days. Exposures took place under controlled light cycles (16 h of light: 8 h of dark) and constant temperature (20℃ ± 1℃) according to OECD guideline No. 211 (OECD, 1998). All animals were fed daily with a total food density of 5 mg of dry weight/L. In control treatments, animals were fed with 100% green algae consisting of a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*, which has been shown to result in sufficient dietary need fulfilment under control condition and has been used in our lab for over a decade (Muyssen and Janssen, 2001, Asselman et al., 2013). In the treatments where the diet was contaminated with cyanobacteria, a set percentage of the total diet concentration consisted of cyanobacteria, while the remainder was composed of the green algae mixture. For example, the 20% cyanobacteria treatment had 1 mg of dry weight/L of cyanobacteria and 4 mg of dry weight/L of the green algae mixture. The test medium was renewed 3 times a week (Monday, Wednesday, and Friday).

Table 2.2. Summary of the treatments in the experimental design. ± indicates the standard error (SE) around the mean of the different sub-experiments (measured as time weighted averages according to OECD guidelines, OECD 1998). For a scheme of the experimental design see Appendix A Figure A.1.

Treatment type	nominal Cu (µg Cu/L)	dissolved Cu (µg Cu/L)	total Cu (µg Cu/L)	Cyanobacteria (% in the total diet)
Cu single dose treatments [a]				
	5	3 ± 0.25	4.5 ± 0.34	0
	66	43.6 ± 5.8	67.0 ± 4.2	0
	100	72.2 ± 4.7	106.1 ± 3.1	0
	142	93.5 ± 6.0	138.8 ± 2.9	0
	200	116.1 ± 4.4	188.4 ± 7.2	0
	300	138.2 ± 8.8	219.7 ± 38.5	0
	400	172.7 ± 12.4	297.1 ± 36.8	0
Cyanobacteria single dose treatments [b]				
	5	3 ± 0.25	4.5 ± 0.34	20
	5	3 ± 0.25	4.5 ± 0.34	40
	5	3 ± 0.25	4.5 ± 0.34	50
	5	3 ± 0.25	4.5 ± 0.34	60
	5	3 ± 0.25	4.5 ± 0.34	80
	5	3 ± 0.25	4.5 ± 0.34	100
Mixture treatments [b]				
	50	33.2 ± 4.7	52.7 ± 3.7	10
	50	33.2 ± 4.7	52.7 ± 3.7	25
	50	33.2 ± 4.7	52.7 ± 3.7	40
	66	43.6 ± 5.8	67.0 ± 4.2	15
	66	43.6 ± 5.8	67.0 ± 4.2	30
	100	72.2 ± 4.7	106.1 ± 3.1	10
	100	72.2 ± 4.7	106.1 ± 3.1	25
	100	72.2 ± 4.7	106.1 ± 3.1	40
	133	82.5 ± 3.9	127.8 ±4.1	15
	133	82.5 ± 3.9	127.8 ±4.1	30
	142	93.5 ± 6.0	138.8 ± 2.9	10
	142	93.5 ± 6.0	138.8 ± 2.9	25
	142	93.5 ± 6.0	138.8 ± 2.9	40

[a] Cu single dose treatments were repeated 5 times for each cyanobacteria sub-experiment. [b] Cyanobacteria single dose and mixture treatments were carried out once for each cyanobacteria. All separate sub-experiments were carried out simultaneously for both *Daphnia magna* clones.

2.2.4 Chemical analysis

Concentrations of Cu and dissolved organic carbon (DOC), as well as pH were measured twice a week, once in the fresh medium (prior to addition of algae or cyanobacteria) and once in the old medium (after transferring the daphnids to the fresh medium). Samples were taken as both total (unfiltered), and dissolved (filtered through a 0.45 mm filter, Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). Samples for metal analysis were acidified to a final concentration of 0.14 $molL^{-1}$ of HNO_3 (Normaton Ultrapure 69% HNO_3, Prolabo) prior to storage and subsequently analysed by Atomic Absorption Sepectroscopy (AAS) using a flame-atomic absorption spectrophotometer (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). All data analyses were performed based on the time-weighted mean dissolved Cu concentrations (OECD, 1998). Samples for DOC analysis were measured with a TOC analyser (TOC5000, Shimadzu, Duisburg, Germany) as non-purgeable organic carbon (NPOC). This analysis involves the removal of inorganic carbon by acidification and subsequent purging with N_2 gas prior to analysis. The average dissolved DOC concentration was 3.10 ± 0.16 mg/L and the total DOC concentration was 3.78 ± 0.17 mg/L (average values ± standard deviation). The pH measurements were performed with a pH meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use by using a pH 7 buffer (Merck, Darmstadt, Germany). Average pH in the new clean medium (before daphnids, cyanobacteria and algae were added) was 7.62 ± 0.18 and average pH in the old medium (after daphnids were transferred to a new clean vessel) was 7.83 ± 0.24 (average values ± standard deviation).

2.2.5 Data analysis

For Cu and cyanobacteria, single dose concentration response curves were fitted to each dataset with the log logistic function (Equation 2.1, Figure 2.1 and Figure 2.2). Each single stressor concentration response curve was expressed as % of control and characterized by two parameters, the median effect concentration and the slope:

$$y = \frac{100}{1 + \left(\frac{x}{EC_{50}}\right)^{s}}$$

(**Equation 2.1**)

where *y* is the response of the measured endpoint (total reproduction as % of control), *x* is the concentration of the stressor, 100 is the response (% of control) of the measured endpoint (total

reproduction) at $x = 0$, s is the slope parameter and EC_{50} is the median effective concentration, resulting in a decline of 50% of the response variable relative to control treatment.

As a first step we visualized (Figure 2.3) the interactive effects of copper (Cu) and cyanobacteria (Cyano) by plotting the observed response to the mixtures together with the predicted responses according to both the IA (Equation 2.2) and CA (Equation 2.3) models, which assume non-interaction between the two stressors, using the EC_{50} and slope parameters from the fitted single stressor concentration response curves (Equation 2.1) as input. Both observed and predicted responses were plotted against the sum of toxic units (∑TU) of Cu and cyanobacteria (Equation 2.4):

$$\mathrm{y} = 100 \cdot \frac{1}{1 + \left(\frac{\mathrm{x}_{Cu}}{\mathrm{EC}_{50\mathrm{Cu}}}\right)^{s_{Cu}}} \cdot \frac{1}{1 + \left(\frac{\mathrm{x}_{Cyano}}{\mathrm{EC}_{50\mathrm{Cyano}}}\right)^{s_{Cyano}}}$$

(**Equation 2.2**

$$\frac{\mathrm{x}_{Cu}}{\mathrm{EC}_{50\mathrm{Cu}} \cdot \left(\frac{100 - y}{y}\right)^{\frac{1}{s_{Cu}}}} + \frac{\mathrm{x}_{Cyano}}{\mathrm{EC}_{50\mathrm{Cyano}} \cdot \left(\frac{100 - y}{y}\right)^{\frac{1}{s_{Cyano}}}} = 1$$

(**Equation 2.3**)

$$\sum TU = TU_{Cu} + TU_{Cyano} = \frac{\mathrm{x}_{Cu}}{\mathrm{EC}_{50\mathrm{Cu}}} + \frac{\mathrm{x}_{Cyano}}{\mathrm{EC}_{50\mathrm{Cyano}}}$$

(**Equation 2.4**)

We then tested for an improved model fit by extending the reference models IA (Equation 2.5) and CA (Equation 2.6) with one additional deviation parameter a (to account for synergistic or antagonistic deviations from the reference models) according to Jonker et al. (2005):

$$\mathrm{y} = 100 \cdot \phi\left(\phi^{-1}\left(\frac{1}{1 + \left(\frac{\mathrm{x}_{Cu}}{\mathrm{EC}_{50\mathrm{Cu}}}\right)^{s_{Cu}}} \cdot \frac{1}{1 + \left(\frac{\mathrm{x}_{Cyano}}{\mathrm{EC}_{50\mathrm{Cyano}}}\right)^{s_{Cyano}}}\right) + \frac{a \cdot TU_{Cu} \cdot TU_{Cyano}}{\left(TU_{Cu} + TU_{Cyano}\right)^2}\right)$$

(**Equation 2.5**)

$$\frac{\mathrm{x}_{Cu}}{\mathrm{EC}_{50\mathrm{Cu}} \cdot \left(\frac{100-y}{y}\right)^{\frac{1}{s_{Cu}}}} + \frac{\mathrm{x}_{Cyano}}{\mathrm{EC}_{50\mathrm{Cyano}} \cdot \left(\frac{100-y}{y}\right)^{\frac{1}{s_{Cyano}}}} = \exp\left(\frac{a \cdot TU_{Cu} \cdot TU_{Cyano}}{\left(TU_{Cu} + TU_{Cyano}\right)^2}\right)$$

(**Equation 2.6**)

where ϕ is the standard cumulative normal distribution function. Because roots are notoriously difficult to solve for, we solved the implicit function of CA (Equations 2.3 and 2.6) using (100-y)/y as a single unknown, solving the root and then subsequently determining y.

The statistical analysis was performed in the statistical environment R (R Development Core Team, 2011). The EC_{50} concentrations of the single concentration response curves (Equation 2.1) were compared between the two clones with a paired *T*-test. In order to test whether the EC_{50} concentrations of each cyanobacteria species differed significantly between both clones the Wheeler ratio test was applied (Wheeler et al., 2006). In order to fit the reference models (Equations 2.2 and 2.3) we sampled 5000 sets of parameter values simultaneously that would solve the equations (i.e. EC_{50}, slope and deviation parameter values were estimated simultaneously in one sample run). Each parameter value was taken from a normal distribution with the mean and standard deviation from the parameter values originating from the single concentration response curves. Then the best set of parameter values was selected based on the lowest sum of squared errors (SSE). For the deviation models (Equations 2.5 and 2.6) we used the best parameter estimate from the reference model as starting values and a mean of 0 for the deviation parameter *a*, which is analogous to the generally accepted approach of Jonker et al. (2005).

We statistically tested the presence of a hormesis effect in the single-stressor concentration-response data of Cu and cyanobacteria using the method described by Van Ewijk and Hoekstra (Van Ewijk and Hoekstra, 1993). After verifying assumptions of normality and homoscedasticity of the residuals of the models using a Shapiro-Wilk test and a Levene test respectively, the nested models (Equation 2.3 vs. Equation 2.6, Equation 2.4 vs. Equation 2.7) were compared using an *F*-test, to test whether including the deviation parameter *a* resulted in a statistically improved fit compared to the reference model, which translates in other words into synergistic or antagonistic deviations from the non-interaction assumption

of the reference model (Asselman et al., 2013, Jonker et al., 2005). As the two reference models (Equations 2.2 and 2.3) are mathematically different and not nested, they cannot be compared through formal statistical tests. We therefore used the Akaike Information Criterion (AIC) to provide quantitative measures about model quality as recommend previously (Jonker et al., 2005).

2.3 Results

The EC_{50} of copper (as estimated from the single stressor concentration response data, Equation 2.1 was repeatable between the different experiments within each clone, but we noted consistent differences between the two clones (Table 2.3, Figure 2.1). Xinb3 was more tolerant to Cu (EC_{50} ranging between 86.4 and 106 µg Cu/L) than Iinb1 (EC_{50} ranging between 64.5 and 79.9 µg Cu/L) (p = 1.748e-04, n = 5, df = 4, paired T-test). Based on the estimated EC_{50} for each cyanobacteria (Table 2.3, Figure 2.2), *Microcystis* was the most toxic for both clones (EC_{50} of 23.5% of the total diet for Xinb3 and of 28% of the total diet for Iinb1). The overall sensitivity to the cyanobacteria did not differ considerably between both clones (p = 0.1537, n = 5, df = 4, paired T-test). However Iinb1 was significantly more sensitive to *Aphanizomenon* (EC_{50} 37.1% of diet) compared to Xinb3 (EC_{50} 72.7 % of the diet) ($p < 0.0001$, Wheeler ratio). No significant hormesis effect was noted with respect to exposure to any of the cyanobacteria in both *D. magna* clones. We observed a general hormesis trend for the Xinb3 clone at the lowest Cu concentration, which was however not consistent over the 5 replicate sub-experiments, as the hormesis parameter in the Van Ewijk and Hoeksrat model (1993) was statistically significant in only one of the 5 sub-experiments (i.e. the *Cylindrospermopsis* sub-experiment, p = 0.003). For the Iinb1 clone the hormesis parameter was also significant in only one sub-experiment (*Anabaena* sub-experiment, p = 7.79e-10). As the hormesis effect was not consistently repeatable across sub-experiments we did not consider it further.

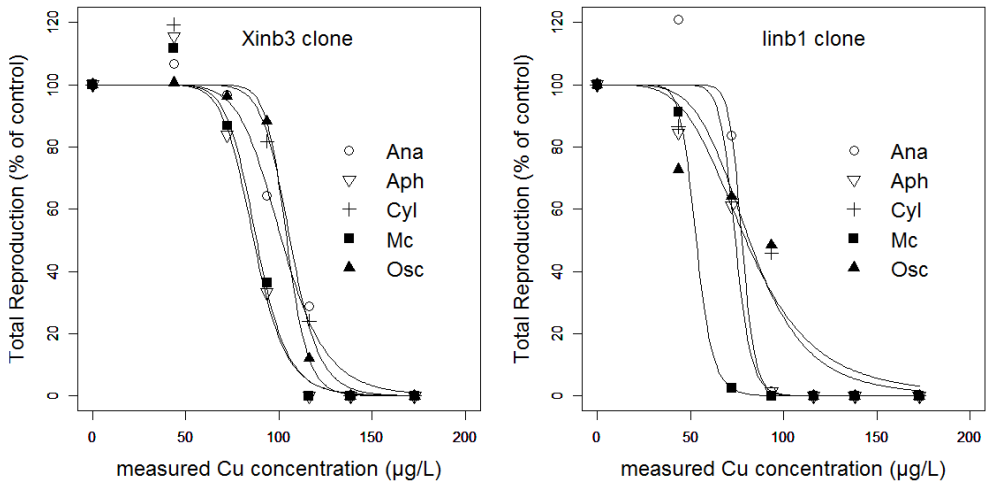


Figure 2.1. Single stressor concentration response curves for Cu (Equation 2.1) for the five different experiments for the Xinb3 and linb1 clones. *Ana* = *Anabaena* (sub-experiment 1), *Aph* = *Aphanizomenon* (sub-experiment 2), *Cyl* = *Cylindrospermopsis* (sub-experiment 3), *Mc* = *Microcystis* (sub-experiment 4), *Osc* = *Oscillatoria* (sub-experiment 5).

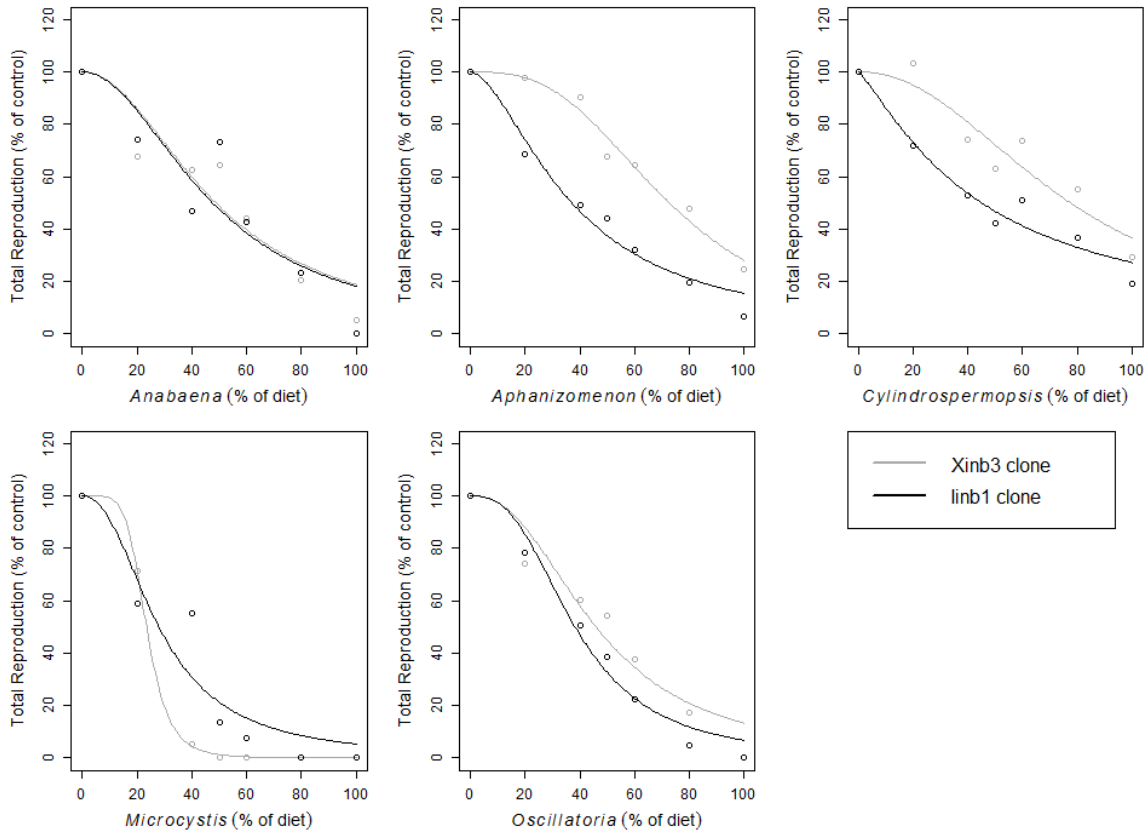


Figure 2.2. Single stressor concentration response curves the five different cyanobacteria (**Equation 2.1**) for the Xinb3 (grey line) and linb1 (black line) clones.

Table 2.3. Summary table of the single stressor concentration response parameters slope and EC_{50} (Equation 2.1) of copper (Cu) and Cyanobacteria (Cyano) in each of the 5 sub-experiments for both *Daphnia* clones (± standard error).

	$slope_{Cu}$	$slope_{Cyano}$	*Cu* EC_{50} (µg/L)	*Cyano* EC_{50} (% of diet)
Anabaena **(sub-experiment 1)**				
Xinb3 clone	8.581 ± 1.399	2.038 ± 0.770	101.490 ± 2.221	48.872 ± 7.103
Iinb1 clone	22.342 ± 26.471	2.028 ± 0.949	77.659 ± 6.929	47.637 ± 8.756
Aphanizomenon **(sub-experiment 2)**				
Xinb3 clone	10.063 ± 2.742	2.945 ± 0.413	86.418 ± 2.914	72.653 ± 3.009
Iinb1 clone	17.521 ± 18.211	1.714 ± 0.289	74.089 ± 2.338	37.142 ± 3.179
Cylindrospermopsis **(sub-experiment 3)**				
Xinb3 clone	13.647 ± 7.338	2.173 ± 0.640	105.996 ± 6.201	77.529 ± 8.063
Iinb1 clone	5.376 ± 1.711	1.234 ± 0.269	81.666 ± 5.178	45.066 ± 4.785
Microcystis **(sub-experiment 4)**				
Xinb3 clone	10.656 ± 2.519	5.724 ± 0.237	87.963 ± 2.62	23.483 ± 0.193
Iinb1 clone	11.931 ± 0.0499	2.26 ± 0.851	53.096 ± 0.047	28.023 ± 5.589
Oscillatoria **(sub-experiment 5)**				
Xinb3 clone	18.424 ± 1.188	2.401 ± 0.681	104.217 ± 0.731	45.928 ± 4.731
Iinb1 clone	4.423 ± 1.884	2.731 ± 0.492	79.845 ± 8.259	38.357 ± 2.677

Interactive effects were detected in the plots of the observed responses and the predicted responses of the reference models against the sum of the toxic units of Cu and the cyanobacteria (Figure 2.3). We observed that the predictions of the mixture effects based on the IA reference model matched the observed response data more closely than those predictions made with the CA reference model in 4 out of the 5 cyanobacterial genera (Figure 2.3). The CA model appeared to overestimate the combined effects in those cases, i.e. an antagonistic interaction is identified when CA is used as the reference model. An exception was noted with mixtures of *Microcystis* and copper, which were considerably better predicted by the CA model compared to the IA model. For *Microcystis* the observed toxicity of the mixture with copper was underestimated by IA predictions, i.e. a synergistic interaction is identified when IA is used as the reference model for copper and *Microcystis* mixtures. These observations were statistically confirmed and are summarized in Table 2.4, showing the interaction type and the *p*-values for the *F*-test comparing the extended model against the reference model.

The statistical comparison of the reference model (Equation 2.2 and Equation 2.3) against the extended model with the deviation parameter (Equation 2.5 and Equation 2.6) revealed four clear trends. First, exactly the same conclusion about the interaction type of the combined effects of Cu and cyanobacteria could be drawn for both clones (Table 2.4). Second, for 4 out of the 5 cyanobacteria species non-interaction was identified with IA as a reference model and antagonism with the CA model (Table 2.4, Figure 2.3). Only for *Microcystis* we noted synergism according to the IA model and non-interaction according to the CA model (Table 2.4, Figure 2.3). Third, overall we observed a lower AIC under the IA concept (thus higher model accuracy), compared to the CA concept, with the exception of *Microcystis*, where the AIC was lower under the CA concept (Appendix A Tables A.5 and A.6). Fourth, the CA reference model (Equation 2.3) consistently overestimated the observed the combined effects compared to the IA model (Equation 2.2) (Figure 2.3). Thus the CA model was either conservative compared to the observed response data (predicting antagonism) in 4 out of 5 cases, or it matched the observed response data quite well (predicting non-interaction in the case of *Microcystis*). In contrast, the IA model on the other hand was under protective compared to the observed response data in one case as it underestimated the observed toxicity of *Microcystis* and Cu (predicting synergism), while it was in line with the observed response in the other cases (predicting non-interaction).

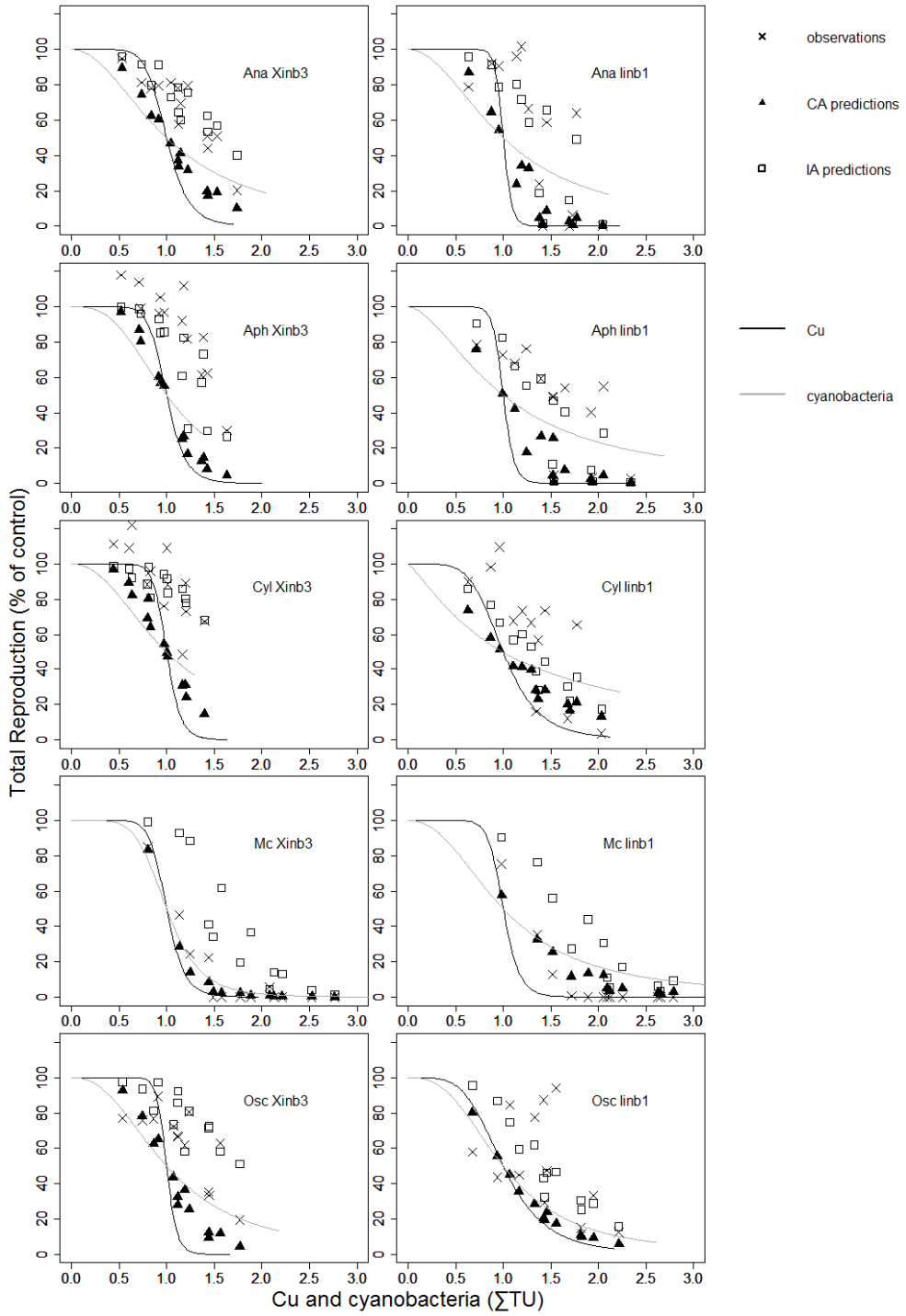


Figure 2.3. Comparison of the predicted interactive effects against the observed combined effects. Predictions were made according to IA and CA using the slope (s) and EC_{50} parameter values derived from the single stressor concentration response curves (Table 2.2). Both observations and predictions were plotted as per cent reproduction of the control against the sum toxic units (∑TU) based on the EC_{50} values and slopes of the single stressor concentration response curves. Additionally, the single stressor concentration responses curves of Cu (black line) and cyanobacteria (grey line) were plotted. *Ana* = *Anabaena, Aph* = *Aphanizomenon, Cyl* = *Cylindrospermopsis, Mc* = *Microcystis, Osc* = *Oscillatoria*

Table 2.4. Summary of the identified interaction types of the Cu and cyanobacteria mixtures. For both *Daphnia magna* clones and for each of the 5 cyanobacterial genera the reference models of Independent Action (IA) (Equation 2.2) and Concentration Addition (CA) (Equation 2.3) were compared against their respective synergism/antagonism deviation model (Equation 2.5 and Equation 2.6) with an *F*-test. A p-value < 0.05 (depicted by *) indicates a significant improvement of the model fit by including the deviation parameter and hence an interactive effect (antagonism or synergism). For the values of all parameter estimates in this statistical analysis we refer to Appendix A Tables A.5 and A.6. The plots of the observed against the fitted response for the reference and extended models including the deviation parameter can also be consulted in the Appendix A Figures A.2-A.6. *Ana* = *Anabaena, Aph* = *Aphanizomenon, Cyl* = *Cylindrospermopsis, Mc* = *Microcystis, Osc* = *Oscillatoria*.

Daphnia clone	**Xinb3**		**Iinb1**	
Cyanobacteria	**IA**	**CA**	**IA**	**CA**
Ana	Non-interaction	Antagonism	Non-interaction	Antagonism
p-value	0.777	6.515e-06*	0.140	9.844e-03*
Aph	Non-interaction	Antagonism	Non-interaction	Antagonism
p-value	0.357	6.922e-07 *	0.137	6.158e-05*
Cyl	Non-interaction	Antagonism	Non-interaction	Antagonism
p-value	0.520	3.421e-04*	0.411	6.817e-03*
Mc	Synergism	Non-interaction	Synergism	Non-interaction
p-value	3.13e-10*	0.521	2.292e-05*	0.428
Osc	Non-interaction	Antagonism	Non-interaction	Antagonism
p-value	0.505	5.693e-05*	0.678	1.885e-02*

2.4 Discussion

As interactive effects of natural stressors and chemical pollutants could potentially complicate chemical risk assessment and management of water quality, investigations of such interactive effects are increasingly being carried out (Holmstrup et al., 2010, Laskowski et al., 2010). Here, we evaluated combined effects of five different freshwater harmful bloom-forming cyanobacterial genera with copper, which is commonly used as an algaecide to eradicate cyanobacterial blooms, and which frequently occurs in eutrophic run-off (e.g. vineyards and citrus farms). In this chapter 4 major findings are highlighted. First, the conclusions drawn on the combined exposures of copper and the five cyanobacterial genera were the same for both clones. In other words, there was no genotype effect in the combined or interactive response of *Daphnia magna* to copper and cyanobacteria exposure. We observed that the Iinb1 clone was more sensitive to Cu (Figure 2.1) and to *Aphanizomenon* (Figure 2.2) than Xinb3. There were however no differences in the type of interactive effects when the clones were simultaneously exposed to *Aphanizomenon* and copper. There are currently only a few studies published that provided concrete evidence of clonal (genotype based) differences in interactive effects between stressors, i.e. mixtures of cadmium + zinc (Barata et al., 2002b), and mixtures of cyanobacteria (*Cylindrospermopsis raciborskii*) + polychlorinated biphenyl (PCB52) (Bernatowicz and Pijanowska, 2011). In the light of risk assessment the primary goal is to protect populations rather than individuals and therefore attempts to generalize the effects of exposure to mixtures should preferably be conducted on multiple genotypes. A recent study also didn't report statistically significant differences in interactive effects of *Microcystis* and cadmium between 20 different *Daphnia magna* clones (De Coninck et al., 2013). Nevertheless, they cautioned against the use of a limited number of clones in order to make generalizations at the species level, as they observed significant antagonism for 1 clone only, while non-interaction occurred for all the 19 other clones tested.

Second, we noted significant differences in the interactive effects of the two stressors, depending on the cyanobacteria species considered. Synergism was only detected in mixtures of copper with *Microcystis* (but only if the IA was used as the reference model), while antagonism was detected in mixtures of copper with the four other cyanobacteria (but only if CA was used as the reference model). It could be argued that this difference might be explained based on consideration of known modes of action (MoA) of both copper and the cyanobacteria. For instance, copper is known to cause redox cycling and has

been shown to synergize oxidative stress responses to other oxidative stress agents (Xie et al., 2006). As *Microcystis aeruginosa* is a well-known oxidative stress agent in *Daphnia* (Wiegand and Pflugmacher, 2005, Dao et al., 2013), it could explain its synergistic action in combination with copper. Yet, although less-well studied, the capacities of the four other cyanobacteria to induce oxidative stress cannot be excluded, as exemplified by the fact that oxidative stress has been reported in *Daphnia magna* exposed to extracts of a wide variety of cyanobacteria strains, including *Anabaena* and *Cylindrospermopsis* (Dao et al., 2013). Therefore oxidative stress is certainly not a definitive mechanistic explanation for the differences in interactive effects with copper between *M. aerugionosa* and the four other cyanobacteria. A similar line of reasoning develops when considering the review of (Deneer, 2000), who reported that, in general, synergistic effects are most common for chemicals acting via similar MoA (albeit most data were on combinations of insecticides acting on AChE system and neuronal signal transmission). It could be argued that this would predict synergistic action between copper and two cyanobacterial genera (*Anabaena lemmermannii* and *Oscillaloria sp.*), as all of these have been demonstrated to act on the AChE system (Wiegand and Pflugmacher, 2005, Untersteiner et al., 2003). Similarly, the fact that both copper and *Aphanizomenon* are known to inhibit Na channels, could predict synergistic action between those two stressors. However, the reality is that neither *Anabaena*, *Oscillatoria* or *Aphanizomenon* were observed to act synergistically in combination with copper at the level of reproductive fitness. Thus, taken all together, the known modes of action of the different cyanobacteria are not able to satisfactorily explain as to why synergistic joint action with copper is only observed for *Microcystis* and not for at least three other cyanobacteria. When considering potential explanations for antagonistic interactions (relative to the CA reference model), a similar conclusion is reached. One possible explanation for antagonism between copper and the cyanobacteria could be related to feeding inhibition. Copper has been implicated in reduced feeding activity of *D. magna* (Ferrando and Andreu, 1993), and thus exposure to copper could diminish ingestion of cyanobacteria and thus reduce exposure to and resulting effects of cell-bound cyanotoxins. Yet, on the other hand, because cyanobacteria are also implicated in reduced feeding (Rohrlack et al. 2001), it could be argued – based on what is mentioned before regarding joint action of stressors with a similar MoA (Deneer, 2000) that this mechanism could also predict synergism (for instance when both copper and cyanobacteria exposure is sufficiently high to each cause food intake inhibition on their own). Thus, without detailed mechanistic studies, it seems very premature to pinpoint the feeding inhibition MoA as

an important explanatory factor for the differences in interactive effects with copper between *M. aeruginosa* and the four other cyanobacteria. Finally, it is worth mentioning that even more complex explanations may be put forward, inherent to the complexity of the *Daphnia*-cyanobacteria-copper-cyanotoxin system, including changing cell-bound to solution cyanotoxin ratios due to copper-induced cell-lysis (Demott et al., 1991, Gilbert, 1990, Zhou et al., 2013)(Demott et al. 1991, Gilbert 1990, Zhou et al. 2013) or reduced copper bioavailability due to copper complexation by cyanobacterial exudates (Choueri et al., 2009, Nogueira et al., 2004). However, it should be clear that, in order to identify the mechanistic basis of the difference of the interactive effects with copper between *M. aeruginosa* and the four other cyanobacteria, an in-depth study would be needed in which several of the possibly involved MoA (mentioned above) should be measured in parallel across the entire array of the five cyanobacteria.

Third, we observed slightly lower AIC values for the IA reference model in almost all models compared to the CA reference model (except for *Microcystis*), suggesting a better model fit under IA than under CA, i.e. that the predicted response correlates better with the observed data (Altenburger et al., 1996, Cedergreen et al., 2008). It has been advocated that model accuracy in itself is less important in risk assessment than in pharmacokinetic research, and that differences between observed and predicted mixture toxicities, regardless of the reference model, are generally within a factor 2 (Altenburger et al., 1996). Backhaus and Faust (2013) went further to suggest that mode of action (MoA) driven analyses should only be applied if error estimations indicate the possibility for substantial differences between CA- and IA-based assessments, rather than as a first priority. As our results indicate relatively small differences between the CA and IA reference models, and given the complex nature of the study system, the in depth MoA analysis (as the one proposed in the previous paragraph) is less of a priority and not strictly needed for use in water quality management.

Fourth, we noted differences between the two reference models of IA and CA across all reference model comparisons. It has previously been noted that (i) the mathematical relationship between IA and CA is a function of the concentration response model function (here log-logistic), the slope parameters of these curves and the tested mixture concentrations and, (ii) that the response predicted by CA usually differs from the response predicted by IA (Drescher and Boedeker, 1995). This can also be further underlined by the dissimilar mathematical formulae related with both concepts (Jonker et al., 2005). With exception

of *Microcystis*, CA overestimated the toxicity of all the binary mixtures, while IA predicted the observed responses reasonably well. For *Microcystis* however, the observed toxicity of the mixtures was predicted well by CA and underestimated by IA. In other words, IA predicted lower combined effects compared to CA, as IA identified the interaction type of copper an cyanobacteria mixtures as non-interaction (or synergism with *Microcystis*) and, while CA identified the interaction type of copper an cyanobacteria mixtures as antagonism (or non-interaction with *Microcystis*). As a consequence the CA model delivers consistently across all cases more conservative estimates of the ombined effects for risk assessment than the IA model, which is in general agreement with the literature (Bellas, 2008, Asselman et al., 2013, Cedergreen et al., 2008, Altenburger et al., 1996).

As it has been advocated that CA is equally useful for predicting toxicities of dissimilarly acting chemicals than IA, it has been suggested to apply CA as a precautious first step worst case scenario, regardless of the modes of action of the chemicals considering that mixture toxicities higher than those predicted by CA are rare findings (Cedergreen et al., 2008, Rodney et al., 2013, Backhaus and Faust, 2013). In terms of ecological risk assessment protection of aquatic ecosystems is an important goal and therefore the use of a conservative model could be the choice for implementation. The data collected in this chapter identified the CA model to be suitable in delivering such conservative predictions of the combined effects of cyanobacteria and copper. Thus in water quality management decisions, the concentration addition (CA) reference model could form a rational basis to account for the combined effects of cyanobacteria and copper.

The results are also consistent with previous studies on interactive effects of cyanobacteria and other chemical stressors (De Coninck et al., 2013, Asselman et al., 2013), as we did not observe strong synergisms (no single case of synergism with the CA model) between cyanobacteria and copper in *Daphnia*. Investigations of additional environmental variables such as nutrient status, resulting in more or less eutrophic conditions (and hence a higher total food concentration) or other model organisms, especially vertebrates, would be necessary to confirm whether the results can be generalised on an ecosystem wide scale.

3

A comparison of the sensitivity of *Daphnia magna* to different cyanobacteria under different temperatures

Redrafted after:

Jennifer D Hochmuth, Karel AC De Schamphelaere. 2014. The effect of temperature on the sensitivity of *Daphnia magna* to cyanobacteria is genus dependent. Environmental Toxicology and Chemistry 33 (10): 2333–2343.

3.1 Introduction

Temperature is one of the main drivers affecting biodiversity and exerts considerable effects on ectothermic freshwater organisms, as their metabolic rates are directly controlled by ambient temperature (Lampert, 2006). The impact of increasing temperature on *D. magna* may in itself be positive (provided that dietary requirements are fulfilled) because maturation time and developmental time are shortened (Orcutt and Porter, 1984), but it could also be negative, as higher temperatures lead to higher energy demands (leading to starvation if energy demands are not fulfilled) (Paul et al., 2004). The effects of increasing temperatures on *D. magna* dynamics have extensively been reviewed elsewhere (Wojtal-Frankiewicz, 2012).

It is well-known that cyanobacteria reduce the fitness of *Daphnia* sp., either by the presence of toxic compounds such as cyanotoxins (e.g. microcystins, cylindrospermopsins) (Dao et al., 2010, Demott et al., 1991, Nogueira et al., 2004, Rohrlack et al., 1999), by mechanistically interfering with the ability of *Daphnia* to feed on algae (Demott et al., 1991, DeMott et al., 2001, Lurling, 2003b, Wilson et al., 2006), or by their lack of essential sterols and fatty acids (Martin-Creuzburg et al., 2008, Volkman, 2003). Furthermore cyanobacteria are predicted to benefit from the consequences of increasing temperatures (Paerl and Huisman, 2008 , Kosten et al., 2012, Paul, 2008, Paerl and Paul, 2012, Elliott, 2012, Abrantes et al., 2006). This may pose considerable risks to environmental and human health, and large scale ecosystem wide effects have already been attributed to their extensive bloom formation (also termed harmful algal blooms) and toxin production (Falconer, 2001, Johnk et al., 2008, Downing et al., 2001, Davis et al., 2009, Abrantes et al., 2006). A survey on small eutrophic lakes in Canada highlighted that warmer spring and summer temperatures in 2006 (compared to 2005) were responsible for a significant increase in cyanobacterial biomass, as well as a shift in the dominant taxa, and that this in turn was correlated with a decline in daphnid abundance (Dupuis and Hann, 2009).

Comparatively little effort has been directed at investigating how temperature influences the response of *D. magna* to cyanobacteria. Cyanobacteria are deficient in phytosterols (Volkman, 2003), which are essential for *Daphnia* to form the membrane component cholesterol (Martin-Creuzburg and Von Elert, 2004). It has been shown that daphnids require more cholesterol at higher temperatures, which suggests that potential dietary sterol limitation of *D. magna* feeding on cyanobacteria could be further intensified

at higher water temperatures (Sperfeld and Wacker, 2009). Prolonged population-level exposure to low dosage microcystin has shown to mask potential responses to changes in temperatures, as warmer temperatures and cyanotoxins were both shown to result in reduced growth and smaller clutch sizes in *D. magna* (Domis et al., 2013). An experiment with a presumably non-cylindrospermopsin-producing strain of the filamentous cyanobacterium *Cylindrospermopsis* found that the number of aborted eggs in *D. magna* increased from 16°C to 24°C (Bednarska and Slusarczyk, 2013). Separate studies on two different genera of cyanobacteria have reported opposite trends of the effect of temperature. In a chronic ecotoxicity test with *Daphnia pulex* the intrinsic rate of natural increase was slightly less reduced by *Microcystis* exposure at 24°C compared to 19°C (Hietala et al., 1997). Similarly higher temperature (30°C compared to 20°C) was observed to mitigate the adverse effects of *Microcystis* across different species of *Daphnia* (Nandini, 1998). Another study compared the harmfulness of two different species of the same genus at different temperatures (*Anabaena affinis* and *Anabaena flos-aquae*, at 12°C, 19°C and 25°C) and noticed that the inhibitory effects of both on *D. magna* reproduction increased with increasing temperature (Claska and Gilbert, 1998). To our knowledge, no studies so far have attempted to simultaneously compare the harmful effects of more than one genus of cyanobacteria to *Daphnia* across a range of temperatures.

Temperature may not only affect the direct response of *D. magna* to cyanobacteria as a food source but also exert indirect effects on *D. magna* due to cyanobacteria-temperature interactions. Yet these interactions appear to be of a more complex nature. For instance microcystin concentrations have been reported to positively correlate with water temperature in one study (Wicks and Thiel, 1990), while another study found no correlation of microcystins with water temperature (Rapala and Sivonen, 1998). It has been hypothesized that toxin production is linked to conditions that are most favorable for cyanobacterial growth rather than temperature per se (Orr and Jones, 1998). However experimental results do not support this hypothesis as higher temperatures resulted in higher growth rates and lower peptide contents in *Microcystis*, and in lower growth rates but higher peptide contents in *Anabaena* (Tonk et al., 2009). Temperature is also known to alter morphology and may therefore affect the dietary uptake by daphnids. The trichome length of *Cylindrospermopsis* was observed to decrease with temperature (Soares et al., 2013). Changes in temperature can also affect sterol content of cyanobacteria (see (Volkman, 2003) and references therein).

The goal of this chapter was to contribute to unraveling effects of temperature on *Daphnia*-cyanobacteria interactions. We chose to only account for direct effects of temperature on the harmful effects of cyanobacteria on *D. magna*, while indirect effects on *D. magna*, via effects of temperature on the cyanobacteria themselves were excluded. Therefore, cyanobacterial genera were cultured at a single temperature. Experiments were carried out with six cyanobacteria spanning a wide range of characteristics potentially affecting *D. magna* life history, each belonging to a separate genus and differing in their known toxin production, morphology, and geographic distribution (Onodera et al., 1997, Merel et al., 2013, Mahmood and Carmichael, 1986, Humpage et al., 1994, Yilmaz and Phlips, 2011, Hawkins et al., 1985, Fastner et al., 2003, Wood and Stirling, 2003, Beattie et al., 2000, Edwards et al., 1992, James et al., 1997) (Table 1) We use the term “harmfulness” throughout instead of “toxicity” to emphasize that we consider the entire arsenal of harmful effects of live cyanobacteria cells rather than limiting ourselves to their toxin production. This is because harmful effects of cyanobacteria have been ascribed to more than their cyanotoxins produced, and because cyanobacteria may produce several toxins simultaneously, some of which still remain unidentified (Sivonen, 1999). We investigated the sensitivity of *Daphnia magna* to these six different cyanobacterial genera as a food source on multiple endpoints at three different temperatures in a 21-day life table experiment. The temperatures tested are comparable to current late spring and early summer temperatures (15°C and 19°C) across Western Europe or to late spring and early summer temperatures that are predicted to be 4°C higher due to climate warming (19°C and 23°C) (Christensen, 2007).

Two specific research questions were addressed in this chapter:

(1) Does *D. magna* becomes more sensitive to the harmful effects of cyanobacteria as temperature increases?

(2) Are the different cyanobacterial genera more harmful to *D. magna* than starvation?

3.2 Material and Methods

3.2.1 Organism cultures and tests media

The *Daphnia magna* clone used in this chapter was the Xinb3 clone. The experimental organisms originated from cultures described in sections 2.1.1 and 2.1.2 in chapter 2 (Appendix A Tables A1-A4). The modified M4 medium was prepared and aerated 72 h before each medium renewal at 20 ℃ in 25 L or 50 L polyethylene vessels. The aerated medium was subdivided and stored at the specific exposure temperature (15 ℃, 19 ℃, and 23 ℃) 24 h prior to medium renewal.

3.2.2 Experimental Design

Chronic 21 day life table experiments were carried out with Daphnia magna at 3 different temperatures (15℃, 19℃, 23℃), exposed to six different cyanobacteria (*Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia, Oscillatoria*) and with a diet varying in the fraction of cyanobacteria (0%, 20%, 40%, 50%, 60%, 80%, 100% of the total diet on a dry wt basis). In addition we added a starvation control treatment (no food, i.e. neither green algae nor cyanobacteria) to assess whether the effect of the cyanobacteria was greater than the effect of starvation alone. We used a full factorial design for each factor combination resulting in a total of 144 treatments (3 temperature levels, 6 cyanobacterial genera and 8 diet treatments).The complete experiment was split into two sub-experiments, which were carried out sequentially. In the first sub-experiment all exposures with *Microcystis, Nodularia* and *Oscillatoria* were carried out, while in the second sub-experiment all exposures with *Anabaena, Aphanizomenon* and *Cylindrospermopsis* were conducted. Within each sub-experiment all treatments were run simultaneously. Daphnids were acclimated for two generations to their respective exposure temperatures prior to the start of the test. At the start of each test, juvenile animals from the third clutch (< 24h old) of the 2nd generation females were transferred in individual polyethylene cups containing 50 ml of the test medium. For each treatment 6 replicate juveniles were set up individually. Exposures were carried out under controlled light cycles (16 h of light: 8 h of dark, with an average light intensity of 14 µmol photon $m^{-2}s^{-1}$ according to OECD guideline No. 211 (OECD, 1998).

Table 3.1. List of algal (A) and cyanobacterial (C) genera obtained from different culture collections with their respective strain number, culture medium, including a description of the known toxin production and the location of isolation of the cyanobacteria strains used in this study. NA = not applicable.

Species	**Strain number (Culture Institute)$_a$**	**Culture medium$_b$**	**Known toxin production**	**Location of Isolation**
Chlamydomonas reinhardtii (A)	CCAP11/32B (CCAP)	modified Provasoli's ES	NA	Amherst, Massachusetts, USA
Pseudokirchneriella subcapitata (A)	CCAP278/4 (CCAP)	modified Provasoli's ES	NA	River Nitelva, Akershus, Norway
Anabaena lemmermannii (C)	K-0599 (SCCAP)	Z8	Anatoxin a(s)	Jutland, Denmark
Aphanizonmenon sp. (C)	CAWBG01 (CICCM)	$BG11_0$	Saxitoxin	Durham, New Hampshire, USA
Cylindrospermopsis raciborskii (C)	LB 2879 (UTEX)	Z8	none known (Hawkins et al., 1985)	Indiana, USA
Microcystis aeruginosa (C)	PCC 7806 (PCC)	$BG11_0$	Microcystin	Braakman, the Netherlands
Nodularia harveyana (C)	PCC 7804 (PCC)	$BG11_0$	Nodularin	Dax, France
Oscillatoria sp. (C)	PCC 6412 (PCC)	BG11	Anatoxin-a	California, USA

$_a$ Culture Collection of Algae and Protozoa (CCAP), SAMS Research Services Ltd. Scandinavian Culture Collection for Algae and Protozoa (SSCAP), Cawthorn Institute Culture Collection of Microalgae (CICCM), Pasteur Culture Collection (PCC), University of Texas (UTEX).$_b$ The culture media compositions can be found in Appendix A Tables A.-A.4.

During the entire test period daily records of survival and reproduction were made and at the end of the test individual length was measured of the surviving adults. Measured endpoints were: total reproduction (number of juveniles per female), intrinsic rate of natural increase (r_m), age at first brood, first brood size and length on day 21. The intrinsic rate of natural increase (r_m) was calculated for each individual replicate based on age-specific fecundity, solving the implicit function (Equation 3.1) (Caswell, 2001, Messiaen et al., 2013):

$$\sum_{x=1}^{21} F_x e^{-r_m x} = 1$$

(**Equation 3.1**)

where x is the age of the individual, F_x is the age specific fecundity (number of alive offspring born on day x) and r_m is the intrinsic rate of natural increase.

All test animals were fed daily with a total food density of 5 mg of dry weight/L (≈ 2.5mg C/L (Geller, 1975)). In control treatments, animals were fed with 100% green algae mixture consisting of a 3:1 ratio (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*, respectively. In the treatments where the diet was contaminated with cyanobacteria, a set percentage (20%, 40%, 50%, 60%, 80%, or 100%) of the total diet concentration consisted of cyanobacteria, while the remainder was composed of the green algae mixture. For example, the 40% cyanobacteria treatment had 2 mg of dry weight/L of cyanobacteria and 3 mg of dry weight/L of the green algae mixture. The test medium was renewed 3 times a week (Monday, Wednesday, and Friday).

3.2.3 Chemical analysis

Dissolved organic carbon (DOC) and pH were measured twice a week, once in the fresh medium (before any algae or cyanobacteria were added) and once in the old medium (after transferring the daphnids to new fresh medium). Samples were taken as both total (unfiltered), and dissolved (filtered through a 0.45 mm filter, Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). Samples for DOC analysis were measured with a TOC analyser (TOC5000, Shimadzu, Duisburg, Germany) as non-purgeable organic carbon (NPOC). The pH measurements were performed with a pH meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use by using a pH 7 buffer (Merck, Darmstadt, Germany).

3.2.4 Data analysis

Concentration response curves for total reproduction (R_0) relative to the control level were fitted in Statistica 7.0 software (Statsoft, Tulsa, OK) using a log logistic model (Equation 3.2):

$$y = \frac{100}{1 + \left(\frac{x}{\exp(a)}\right)^s}$$

(**Equation 3.2**)

where *y* is the response of the measured endpoint (total reproduction as % of control), *x* is the concentration of the cyanobacteria (in % of the total diet), *100* is the response (% of control) of the measured endpoint (total reproduction) at x = 0, *s* is the slope parameter, a is the ln(EC_{50}) and EC_{50} is the median effective concentration resulting in a decline of 50% of the response variable relative to the control treatment. Parameter estimation and calculation of the 95% confidence limits was carried out using the Levenberg-Marquardt method (Levenberg, 1944, Marquardt, 1963). The Wheeler ratio was used to test for significant pairwise differences in the EC_{50} and slope parameter values, which is based on the delta-method to estimate approximate confidence intervals from standard errors of the two estimated parameter values (Wheeler et al., 2006).

We further investigated the effect of increasing cyanobacteria concentrations under different temperatures on the additional endpoints of length (after 21 days), the intrinsic rate of natural increase (r_m), age at first brood and first brood size. As a log-logistic concentration response curve could not fit the response of these endpoints we opted for a non-parametric regression model based on the Theil-Sen estimator to conduct pairwise comparisons of the slopes and intercepts of the regression lines of the endpoints against the cyanobacteria concentration to test for an interaction between temperature and cyanobacteria concentration (Wilcox, 2012). For pairwise comparisons we excluded concentrations where no data were available (i.e. cases where individuals died before the onset of reproduction occurred and without taking length measurements). Significantly different slopes indicate an interaction between temperature and cyanobacteria concentration. Additionally significantly different intercepts can be interpreted as different effects of the different temperatures on the endpoint without the addition of cyanobacteria, while a significant correlation between the endpoint and the cyanobacteria concentration (using the Spearman's *Rho*) would confirm a significant effect of an increasing concentration of the cyanobacteria on the endpoint at a fixed temperature. Additionally to test whether the order of

harmfulness (from most to least harmful cyanobacteria) remained constant over the temperature range we conducted pairwise correlations (using the Spearman's *Rho*) of the EC_{50} and slope parameters at all temperature pairwise combinations. The Bonferroni-Holm correction method was used to adjust all the p-values for multiple comparisons. The above analysis was performed in the statistical environment R (R Development Core Team, 2011) using the WRS package (Wilcox, 2012).

In order to evaluate whether the effects of the cyanobacteria could be attributed to more than a lack of nutritional value we compared the age specific survivorship (l_x) of daphnids fed on a cyanobacteria diet only (100% cyanobacteria and 0% green algae in the diet) against that of starved individuals (0% cyanobacteria and 0% green algae in the diet) at the 3 different temperatures (15°C, 19°C and 23°C) using a Cox proportional hazards regression model (Equation 3.3) together with the Wald test statistic (Cox, 1972, Therneau, 2000):

$$h(t, x_i) = e^{B} h_0(t)$$

(**Equation 3.3**)

where $h(t,x_i)$ is the hazard at time t, for an individual characterized with value x_i for the covariate x, $h_0(t)$ is the baseline hazard and e^B is a function making $h(t,x_i)$ proportional to the baseline hazard at all exposure times. The hazard rate is a ratio of the probability of an event (i.e. death) occurring in an exposed group (i.e. cyanobacteria diet) versus a reference group (i.e. starvation treatment).The hazard rate indicates the likelihood of death for each point of increase in a predictor and is calculated by raising the log odds parameter estimate (B) to the base of natural logarithms. This model was chosen as the data was right censored, which means that the event of interest (death in this case) did not occur before the end of the test period, i.e. several individuals were still alive after 21 days. The analyses were performed with the survival package (Therneau, 2000) in R (R Development Core Team, 2011).

3.3 Results

The pH remained stable over the exposure duration at 7.9 (± 0.1) in the new medium and 7.8 (± 0.1) in the old medium, mean (± SE). Average Total Organic Carbon (TOC) was 4.4 (± 0.1) mg/L in the new medium and 3.8 (± 0.1) mg/L in the old medium, while average Dissolved Organic Carbon (DOC) was 3.5 (± 0.2) mg/L in the new medium and 3.6 (± 0.9) mg/L in the old medium (mean ± SE). No significant

differences in TOC and DOC among the different temperatures or among the different cyanobacteria treatments were observed (one-way ANOVA, *p*-value < 0.05).

Table 3.2. Summary of the *p*-values of the pairwise comparisons (PwC) of the slopes of the non-parametric Theil-Sen regression models[a].

Endpoint	PwC	*Ana*	*Aph*	*Cyl*	*Mc*	*Nod*	*Osc*
length (µm)	15℃-19℃	0.778	0.2136	0.852	0.2571	0.4408	1
	15℃-23℃	0.621	0.0801	0.852	<0.0001*	0.03*	1
	19℃-23℃	0.621	0.26	0.852	0.0334*	0.4408	1
r_m **(day^{-1})**	15℃-19℃	<0.0001*	0.04*	0.1	0.5944	1	0.0301*
	15℃-23℃	0.0066*	0.0734	0.9215	0.2805	1	<0.0001*
	19℃-23℃	0.1836	0.7613	0.09	0.8515	0.187	0.0134*
age 1st brood	15℃-19℃	0.075	1	1	1	0.928	0.1336
(days)	15℃-23℃	0.548	1	1	1	0.989	0.05*
	19℃-23℃	0.548	1	1	1	0.2754	0.0934
1st brood size	15℃-19℃	<0.0001*	<0.0001*	0.7329	0.9799	1	0.7212
(# of juveniles)	15℃-23℃	0.0067*	<0.0001*	0.566	0.3172	1	0.1202
	19℃-23℃	0.7596	0.0534	0.1953	0.3038	1	0.2805

The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A complete overview of the pairwise comparisons (PwC) of the slopes and intercepts and the 95% confidence intervals around each PwC of the endpoints against the cyanobacteria concentration as well as the Spearman's correlation coefficient (*Rho*) can be consulted in ***Appendix B Table B3 and B4***). *Ana=Anabaena, Aph=Aphanizomenon, Cyl=Cylindrospermopsis, Mc=Microcystis, Nod=Nodularia, Osc=Oscillatoria*. * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria concentration) at $p < 0.05$.

Visual inspection of the concentration response curves suggested that harmful effects on reproduction decreased with increasing temperature in 3 cyanobacteria (*Aphanizomenon, Microcystis* and *Nodularia*) and that harmful effects on reproduction increased with increasing temperature in 2 cyanobacteria (*Anabaena* and *Oscillatoria*), while no clear trend was noted for the remaining cyanobacteria (*Cylindrospermopsis*) (Figure 3.1).

Significant differences in both 21-day EC_{50} of total reproduction and slopes of the concentration response curves were noted for comparisons between temperatures for each cyanobacteria individually (depicted by different upper case letters in Figure 3.2, Appendix B Table B.2). For daphnids exposed to *Anabaena* the 21-day EC_{50} of total reproduction decreased with increasing temperature (harmful effects at 23°C > 19°C > 15°C; p <0.0001, Appendix B Table B.2). For *Oscillatoria* the EC_{50} was significantly lower at 23°C compared to 19°C (p = 0.0002, Appendix B Table B.2) and 15°C (p < 0.0001, Appendix B Table B.2) (harmful effects at 23°C > 19°C = 15°C).

On the contrary for *Aphanizomenon* and *Nodularia* the EC_{50} significantly decreased with increasing temperature (harmful effects at 15°C > 19°C > 23°C) (p =< 0.003 for *Aphanizomenon*, p-value =< 0.009 for *Nodularia*, Appendix B Table B.2). Similarly for *Microcystis* the EC_{50} was significantly lower at 15°C and at 19°C compared to 23°C (harmful effects at 15°C > 19°C = 23°C) (p < 0.0001, Appendix B Table B.2).

For *Cylindrospermopsis* the EC_{50} did not change significantly with increasing temperature (harmful effects at 15°C = 19°C = 23°C, Appendix B Table B.2).

For *Anabaena* the slope was significantly steeper at 15°C compared to 19°C (p = 0.002, Appendix B Table B.2) and 23°C (p = 0.0005, Appendix B Table B.2). The same trend was observed for *Cylindrospermopsis* (p = 0.001 and p =0.03, Appendix B Table B.2), while for *Microcystis* the slope was significantly steeper at 19°C compared to 15°C (p = 0.003, Appendix B Table B.2) and 23°C (p = 0.03, Appendix B Table B.2). For *Oscillatoria* the slope of the concentration response curve was significantly steeper at 15°C than at 23°C (p = 0.05, Appendix B Table B.2). For *Nodularia* and *Aphanizomenon* no differences between the slopes were observed at the different temperatures.

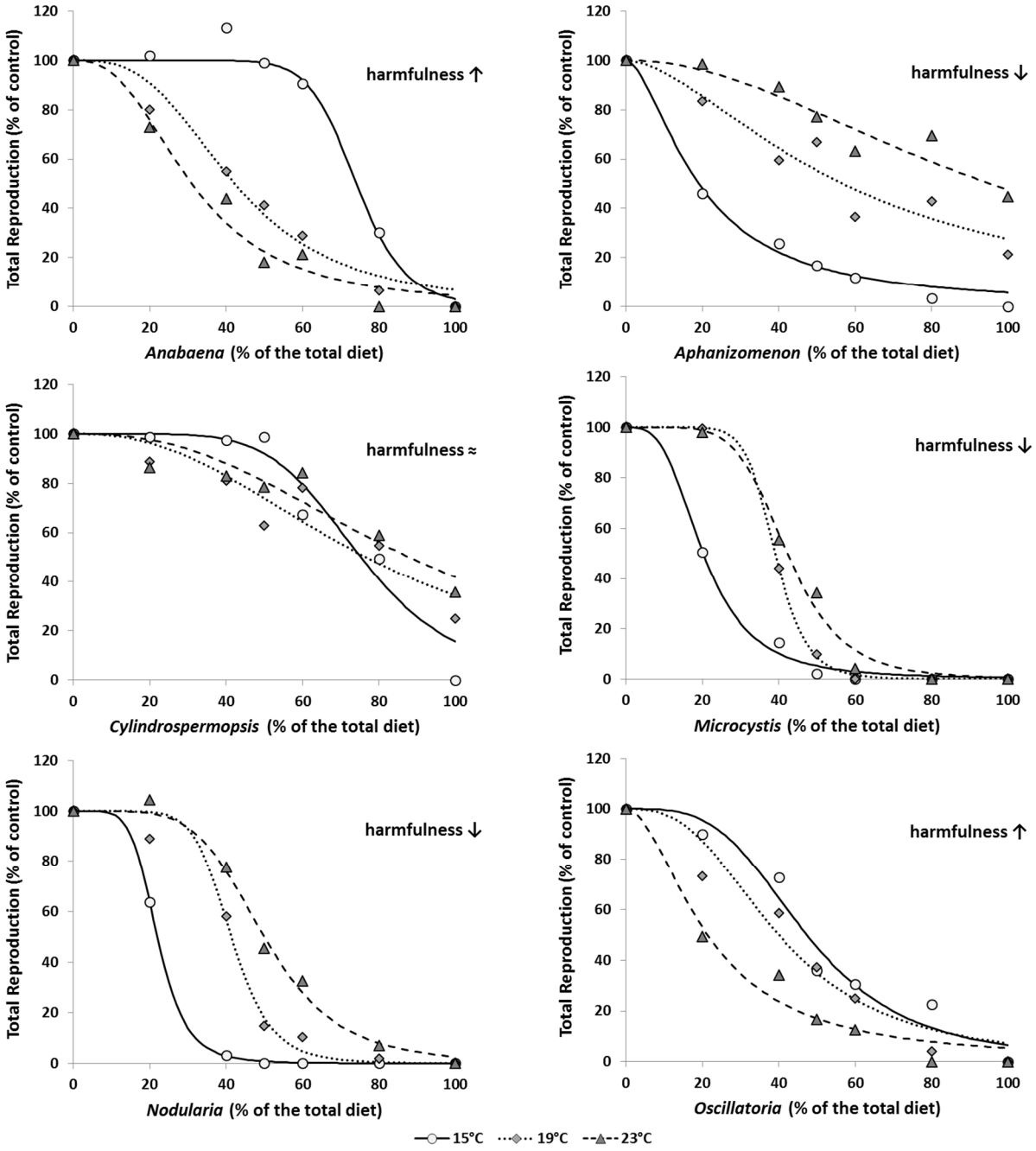


Figure 3.1. Concentration response curves of *D. magna* total reproduction after 21 d for 6 cyanobacterial genera (*Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia,* and *Oscillatoria*) at 3 different constant temperatures (15℃, 19℃, and 23℃). Averaged data (relative to the control) are depicted by marker points and fitted values are depicted by lines. The concentration response parameters (-95%/+95% confidence interval) can be consulted in Appendix B Table B.1. An arrow pointing up indicates that toxicity increases with increasing temperature, arrow pointing down indicates that toxicity decreases with increasing temperature, and the symbol ≈ indicates no significant change in toxicity with increasing temperature.

Additionally significant differences in both 21-day EC_{50} of total reproduction and slopes were also noted for comparisons between the different cyanobacteria within each temperature (depicted by different lower case letters in Figure 3.2, Appendix B Table B.3). At 15°C the order from most to least harmful cyanobacteria was as follows: *Microcystis* = *Nodularia* = *Aphanizomenon* > *Oscillatoria* > *Anabaena* = *Cylindrospermopsis* (p = < 0.0002, Appendix B Table B.3). At 19°C the order from most to least harmful was as follows: *Nodularia* = *Anabaena* = *Microcystis* = *Oscillatoria* > *Aphanizomenon* > *Cylindrospermopsis* (p = < 0.03, Appendix B Table B.3). At 23°C the order from most to least harmful was as follows: *Oscillatoria* > *Anabaena* > *Microcystis* > *Nodularia* > *Aphanizomenon* = *Cylindrospermopsis* (p- =< 0.002, Appendix B Table B.3). At 15°C the order from the steepest to the shallowest slope was: *Anabaena* > *Cylindrospermopsis* > *Nodularia* > *Oscillatoria* > *Microcystis* > *Aphanizomenon*. At 19°C the order was: *Microcystis* > *Nodularia* > *Anabaena* > *Oscillatoria* > *Cylindrospermopsis* > *Aphanizomenon*. At 23°C the order was: *Microcystis* > *Nodularia* > *Anabaena* > *Cylindrospermopsis* > *Aphanizomenon* > *Oscillatoria*. There were no significant correlations between neither EC_{50} values nor slopes at different temperatures (Spearman's *Rho* p > 0.1), which suggests that the order of the most to least harmful cyanobacteria to *D. magna* reproduction changed over the temperature range studied.

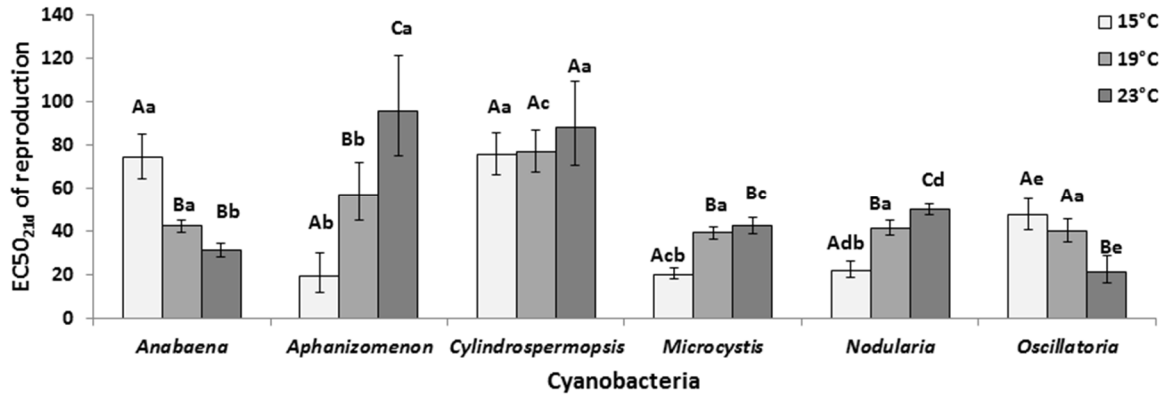


Figure 3.2. Median effective concentrations (EC_{50}) of total reproduction at 21 d for daphnids exposed to 6 cyanobacterial genera (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia, Oscillatoria*) under 3 different constant temperatures (15°C, 19°C, 23°C). Lowercase letters indicate significant differences of the EC_{50} values between different cyanobacteria species within the same temperature. Uppercase letters indicate significant differences of the EC_{50} values between different temperatures within a particular cyanobacterium.

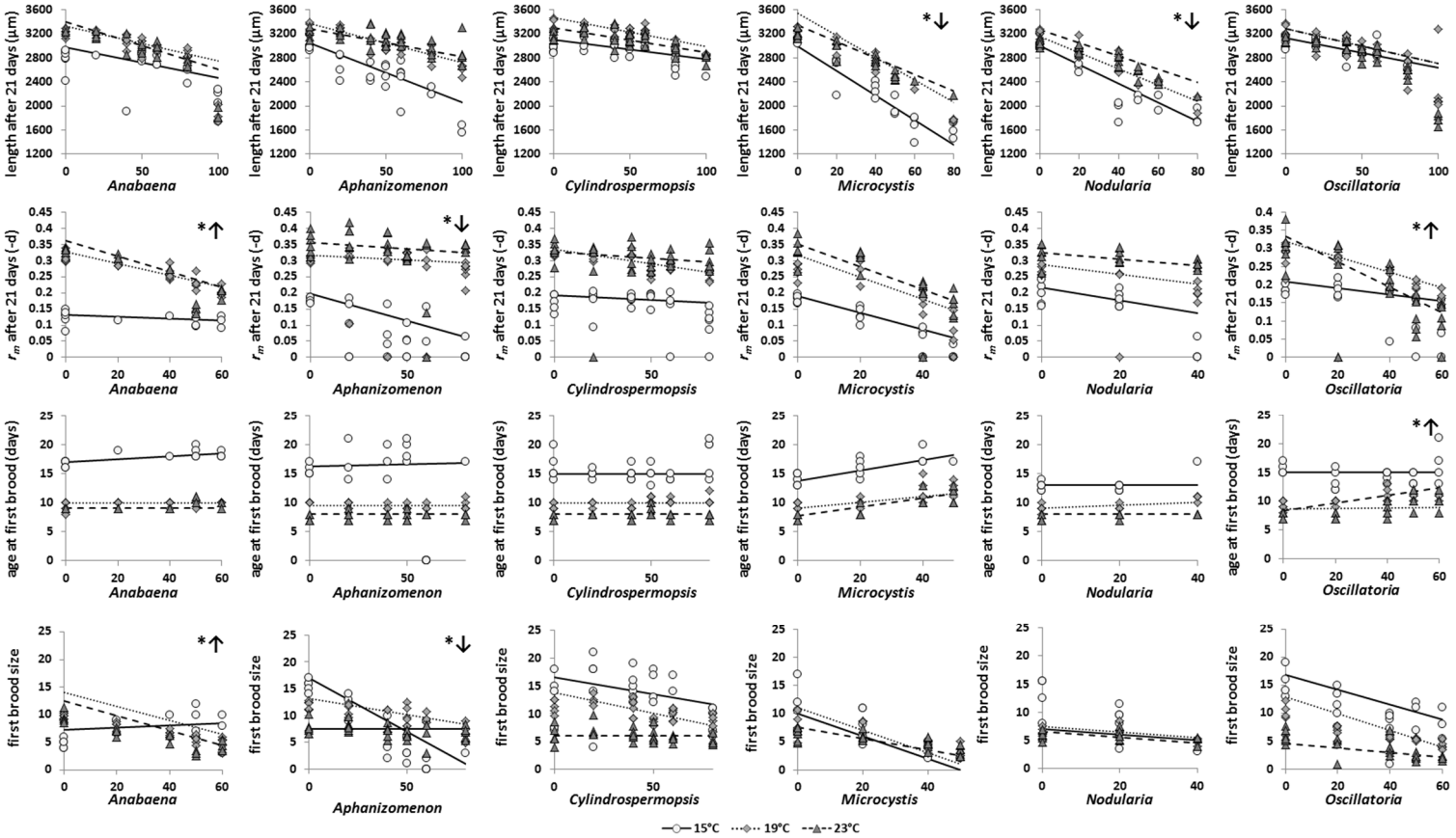


Figure 3.3. Effect of an increasing concentration of 6 different cyanobacterial genera (% cyanobacteria in total diet) on length after 21 d, intrinsic rate of natural increase (r_m), age at first brood, and first brood size under 3 different temperatures. The raw data are depicted by marker points, and regression lines are fitted using the nonparametric Theil–Sen regression model. The asterisk (*) indicates at least one case of interactive effect between temperature and cyanobacteria concentration on the endpoint considered. An arrow pointing up indicates that toxicity increased with increasing temperature, and an arrow pointing down indicates that toxicity decreased with increasing temperature.

Next we investigated the effect of the different cyanobacteria across temperatures on additional endpoints that followed a linear rather than a sigmoidal trend in response to increasing cyanobacteria concentration in the diet: length at the end of the test (after 21 days), intrinsic rate of natural increase (r_m), age at first brood and first brood size (Table 3.2, Figure 3.3). For daphnids exposed to *Anabaena* we noticed interactive effects of cyanobacteria concentration and temperature (i.e. significantly different slopes of the regression lines). r_m and first brood size decreased significantly at 19° and 23°C, while at 15°C r_m remained constant (non-significant *Rho*, Appendix B Table B.5) and first brood size actually increased with increasing cyanobacteria concentration. For exposures with *Aphanizomenon,* the opposite trend was noted: a greater decrease in r_m and first brood size at lower temperatures. These results are in line with the 21-day EC_{50} of total reproduction decreasing with increasing temperatures in *Anabaena* exposures and increasing with increasing temperatures in exposures with *Aphanizomenon*.

No interactive effects of temperature on length and on age at first brood were observed in exposures with either of the two cyanobacteria. In *Cylindrospermopsis* treatments no interactive effects were observed for any of the studies endpoints, which is line with the absence of significant differences in 21-day EC_{50} of total reproduction (Figure 3.1, Appendix B Table B.2). In daphnids exposed to *Microcystis* length and first brood size decreased significantly less at 23°C than at lower temperatures. However no interactive effects were noted for the other endpoints. Similarly in *Nodularia* treatments length decreased at a significantly faster rate at 15°C compared to 23°C. For exposures with *Oscillatoria* we observed r_m to decline significantly faster as well as a later onset of first reproduction with increasing temperature. We did however notice that for any given temperature, the first brood size differed considerably between replicates (e.g. 5 juveniles in the *Anabaena* exposure vs. 15 juveniles in the *Aphanizomenon* exposure under the control treatment, Figure 3.3), which indicates a high degree of variability among replicates.

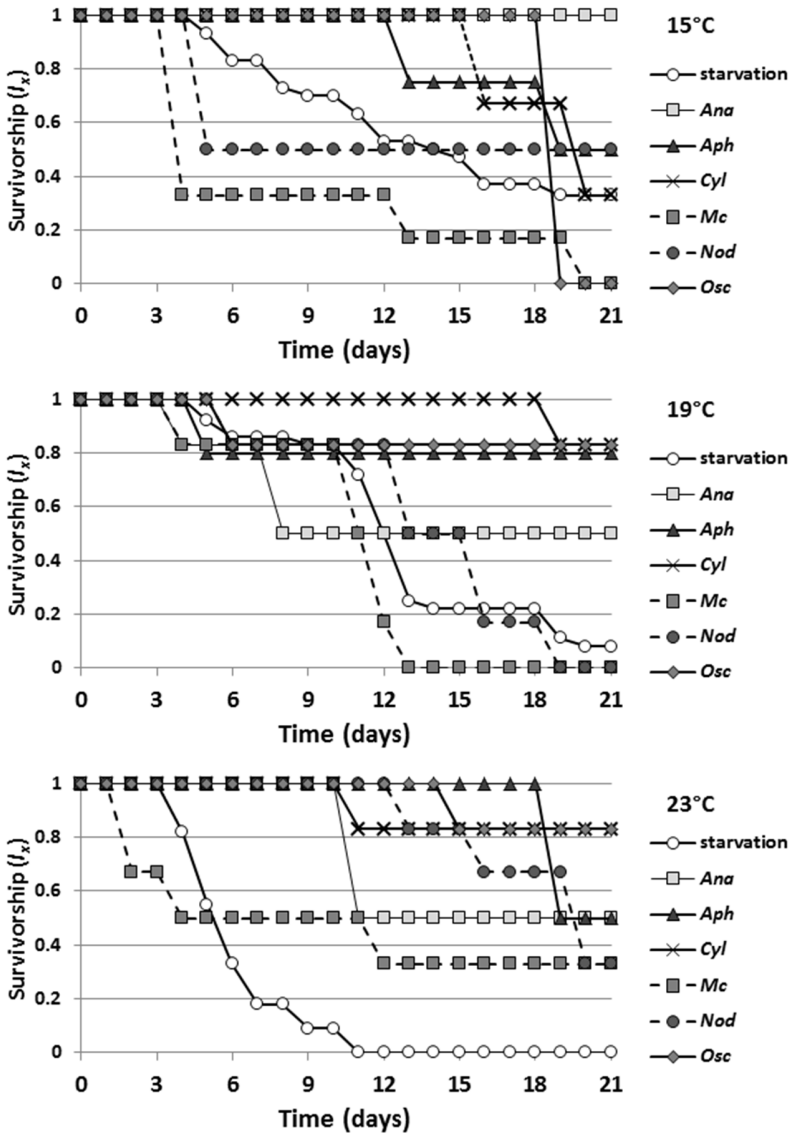


Figure 3.4. Age specific survivorship (l_x) of *D. magna* over the entire exposure period grown under different cyanobacterial diets (100% cyanobacteria and 0% green algae) compared to starvation (0% cyanobacteria and 0% green algae) under 3 different temperatures (15℃, 19℃ and 23℃). *Ana = Anabaena; Aph = Aphanizomenon; Cyl = Cylindrospermopsis; Mc = Microcystis; Nod = Nodularia; Osc = Oscillatoria.*

The age specific survivorship (l_x) under different diets and temperatures can be consulted in Figure 3.4. We found evidence for overall significant differences between survival curves at 19°C (Wald test, χ^2=16.9; d.f.=6; p =0.0097) and at 23°C (Wald test, χ^2=38.54; d.f.=6; p =8.791e-07) but not at 15°C (Wald test, χ^2=7.84; d.f.=6; p =0.2504). At 15°C there was no significant difference between starved daphnids compared to those fed on 100% cyanobacteria, with the exception of the 100% *Microcystis* diet, which increased the daily hazard rate of survival by a factor of 3 (Cox proportional-hazards regression model, exp (B) = 3.2110, p = 0.0128, Table 3.3). At 19°C diets consisting of 100% *Aphanizomenon* and *Cylindrospermopsis* both decreased the daily hazard rate of survival by approximately a factor of 10 (Cox proportional-hazards regression model, *Aphanizomenon*: exp (B) = 0.1050, p = 0.0273, *Cylindrospermopsis*: exp (B) = 0.0732, p = 0.0103, Table 3.3) compared to starved animals. A diet of 100% *Microcystis* increased the hazard rate 2-fold, however this trend was only marginally significant (Cox proportional-hazards regression model, exp (B) = 2.282, p = 0.0718, Table 3.3). At 23°C all cyanobacteria significantly decreased the daily hazard rate of survival compared to starved animals by more than 10-fold, apart for *Microcystis* exposure for which the hazard rate was reduced by roughly a factor of 7 (Table 3.3).

3.4 Discussion

The aim of this chapter was to examine the harmful effects of six different cyanobacterial genera on multiple endpoints of *D. magna* under at three temperatures (15°C, 19°C and 23°C). At present there is little evidence whether *D. magna* become more or less sensitive to cyanobacteria as a food source as temperature increases. Here we show that the direct harmful effects on total reproduction (R_0) of five out of the six cyanobacteria are temperature dependent (Figure 3.1). We observed the 21-day EC_{50} of total reproduction to increase (i.e. decrease in harmful effects) with increasing temperature for three cyanobacteria (*Microcystis, Nodularia and Aphanizomenon*), while the EC_{50} was noted to decrease (i.e. increase in harmful effects) with increasing temperature in two cyanobacteria (*Anabaena* and *Oscillatoria*) (Figures 3.1 and 3.2). Our results further confirm results by Hietala and colleagues (1997) who noted *Daphnia pulex* intrinsic rate of natural increase under *Microcystis* exposure to be lower at 19°C compared to 24°C and those of Claska and Gilbert (1998) that the toxic effects on reproduction increase with temperature for *Anabaena*.

Table 3.3. Results of the Cox proportional-hazards regression model (Equation 3.3) examining the hazard rate of 6 cyanobacteria compared to a starvation reference at 3 different temperatures[a].

	***B* (SE)**	**exp (*B*)**	**95% CI for exp (*B*)**	***p*-value**
15℃				
Starvation	NA	1	NA	NA
Ana	-18.5 (4810)	0.0000	(0-Inf)	0.9969
Aph	-0.0688 (0.549)	0.9336	(0.3186-2.735)	0.9002
Cyl	-0.4 (0.743)	0.6706	(0.1563-2.876)	0.5906
Mc	1.17 (0.469)	3.2110	(1.2818-8.044)	0.0128 *
Nod	-0.256 (0.621)	0.7745	(0.2292-2.616)	0.6807
Osc	0.0844 (0.552)	1.0880	(0.3686-3.212)	0.8786
19℃				
Starvation	NA	1	NA	NA
Ana	-0.916 (0.611)	0.4001	(0.1208-1.327)	0.1337
Aph	-2.25 (1.02)	0.1050	(0.0142-0.7767)	0.0273*
Cyl	-2.62 (1.02)	0.0732	(0.0099-0.54)	0.0103*
Mc	0.825 (0.458)	2.2820	(0.9296-5.6018)	0.0718
Nod	-0.132 (0.447)	0.8767	(0.3649-2.1067)	0.7686
Osc	-19.0 (3610)	0.0000	(0-Inf)	0.9958
23℃				
starvation	NA	1	NA	NA
Ana	-2.7 (0.81)	0.0634	(0.0130-0.3091)	0.000644*
Aph	-3.10 (0.72)	0.0452	(0.0110-0.1867)	1.87E-05*
Cyl	-4.20 (1.09)	0.0150	(0.0018-0.1279)	0.000122*
Mc	-2.03 (0.65)	0.1307	(0.0365-0.4679)	0.001764*
Nod	-2.75 (0.66)	0.0637	(0.0176-0.2311)	2.80E-05*
Osc	-4.25 (1.09)	0.0143	(0.0017-0.1218)	0.000102*

[a]The coefficient *B* measures the effect of each cyanobacteria compared to the starvation treatment (reference). The exp (*B*) gives the relative hazard rate (relative to the reference), therefore the reference has a set hazard rate of 1. NA: information not available for reference.* indicates significantly different hazard rates at $p < 0.05$. *Ana=Anabaena, Aph=Aphanizomenon, Cyl=Cylindrospermopsis, Mc=Microcystis, Nod=Nodularia, Osc=Oscillatoria*, NA = not applicable. * indicates significantly different hazard rates at $p < 0.05$.

Additionally the rank order of the most to the least harmful cyanobacteria (based on the EC_{50} of total reproduction) changed considerably at the three temperatures. The data suggest that there is no major change in the most harmful cyanobacteria (measured as 21-day EC_{50} of total reproduction) at 15°C and 19°C, as *Microcystis* and *Nodularia* appear to be the most harmful under both temperatures. The only exception could be *Aphanizomenon* as it is according to our results as harmful as the most harmful (*Microcystis* and *Nodularia*) at 15°C but the least harmful (together with *Cylindrospermopsis*) at 23°C. However at 23°C *Anabaena* and *Oscillatoria* appear to be more harmful to *Daphnia magna* than *Microcystis* and *Nodularia. Cylindrospermopsis* is overall the least harmful at all temperatures. The results indicate that *D.* magna is more sensitive to *Anabaena* and *Oscillatoria* at warmer temperatures, while the harmful effects on *D. magna* fed a diet that includes *Microcystis*, *Nodularia* and *Aphanizomenon* are more severe at colder temperatures.

The examination of other endpoints in addition to total reproduction (R_0) served two main purposes. Firstly to confirm the trends observed in the dose response analysis of the 21-day EC_{50} of total reproduction and secondly to provide a more in depth investigation of the harmful effects of different cyanobacteria under different temperatures on the life history of *D. magna*. We observed interactive effects (i.e. a greater or lesser harmful effect with changing temperature) under all cyanobacteria exposures, with the exception of *Cylindrospermopsis*, which matches the results from the Wheeler ratio (Appendix B Table B.2). Although significant changes were noticed in total reproduction in 5 out of the 6 cyanobacteria exposures, in only 3 of these 5 exposures were they matched by a significant change in r_m. The r_m endpoint therefore seems to be less sensitive than total reproduction. In *Anabaena* and *Aphanizomenon* treatments a greater decline of r_m at higher and lower temperatures respectively appears to be related to a steeper decline in first brood size and not a significantly later onset of reproduction. For exposure with *Oscillatoria* treatments on the other hand a greater decline of r_m at higher temperatures appears to be related to a significantly later onset of reproduction rather than a smaller first brood size. However as the first brood size varied considerably under control conditions at a given temperature across simultaneously performed tests, first brood size may be a less reliable indicator of fitness than the other endpoints assessed in the present study and, consequently, extrapolations based on the first brood size metric should be made with great caution. In *Microcystis* and *Nodularia* the significantly lower total reproduction at 15°C seems to be related to an interactive

effect of increasing cyanobacteria concentration and temperature on length rather than r_m or the related age at first brood or first brood size. As fecundity is directly correlated to body size in *Daphnia* (Lampert, 1993), it seems likely that the harmful effects on length are a main driver in the response of *Daphnia magna* to *Microcystis* and *Nodularia* exposures. In general the findings suggest that the cyanobacteria affect *Daphnia magna* life history traits adversely in slightly different ways. These findings are supported by the fact that all 6 cyanobacteria differ considerably in their morphology and their known toxins produced (Table 3.1).

Furthermore, we observed significantly different slopes of the concentration response curves across different temperatures for all cyanobacteria (Appendix B Table B.2-B.4). Differences between slopes at different temperatures are an indication for differences in the mode of action (Loewe, 1926).The mechanisms by which temperature increases, or decreases, the sensitivity of *Daphnia* to the different cyanobacteria are not known. Temperature, even if is not stressful in itself, can affect both the mechanisms by which stressors produce cellular effects (toxicodynamicss), as well as the bioavailable amount of a stressor reaching the target site (toxicokinetics) (Fischer et al., 2013). Harmful effects on *Daphnia* reproduction increasing with temperature in some cyanobacteria, while decreasing with others, may therefore be the result of temperature affecting the balance of uptake, internal distribution, biotransformation and elimination (toxicokinetics), or the balance between damage and repair processes (toxicodynamics) differently after exposure to the cyanobacteria. For instance microcystin is eliminated more rapidly in the freshwater bivalve *Unio douglasiae* at 25℃ than at 15℃ if (Yokoyama and Park, 2003). If harmful effects of *Microcystis*, *Nodularia* and *Aphanizomenon* are primarily related to their toxin content, lower detoxification rates at lower temperatures may explain the observed increase in harmfulness with decreasing temperatures. It also may be that sensitivity to the toxins is temperature dependent. *Daphnia pulex* became more sensitive to anatoxin-a derived of *Anabaena flosaque* at higher temperatures (Claska and Gilbert, 1998). Moreover temperature may also affect the uptake rates of the cyanobacteria cells, as viscosity of the water decreases with increasing temperature. Higher edibility of the filamentous cyanobacteria *Cylindrospermopsis raciborskii* by *Daphnia galeata* was observed at higher water viscosity (thus colder temperatures), which is most probably caused by lack of interference with filtering combs (Abrusan, 2004). Therefore especially for filamentous cyanobacteria, feeding inhibition could be higher at higher temperatures. Also, sterol limitation of *Daphnia* fed with

cyanobacteria can be intensified by elevated temperatures, due to increase in *Daphnia* demands for sterols with rising temperatures (Sperfeld and Wacker, 2009).

The results presented in this chapter also suggest that at 23°C daphnids survived significantly longer in all cyanobacteria species treatments on a diet with 100% cyanobacteria (i.e. no green algae added) compared to the starvation control (no added food at all) (Table 3.2, Figure 3.3). However the age specific survival at 23°C was actually lower for animals fed 100% *Microcystis* compared to starved animals for the first 5 days (Figure 3.4). Furthermore at 15°C and 19°C daphnids fed with 100% *Microcystis* died significantly faster than under starvation. This suggests that the harmful effect of *Microcystis* outweighs any nutritional value of the cyanobacteria and that death does indeed occur due to an additional factor than feeding inhibition or poor nutritional value alone (potentially toxin production). Although some other cyanobacteria diets resulted in lower survivorship in the first few days of the exposure (i.e. *Nodularia* at 15°C and *Anabaena* at 19°C) than starvation, survivorship was lower at all temperatures under starvation than under any of the other 5 cyanobacteria. These results can most likely be linked to a combination of increased metabolism and energy needs of *D. magna* at higher temperatures and that survival depends on the balance between potential cyanobacteria toxicity and nutritional value. This could be linked to a higher metabolic rate of *D. magna* at increasing temperatures and the fact that the cyanobacteria still contain some nutritional value (Paul et al., 2004, Martin-Creuzburg et al., 2008). As we used whole cyanobacteria cells, it can be difficult to disentangle effects due feeding inhibition from those due to toxicity. However for those comparisons where we noticed survival to be significantly lower at 100% cyanobacteria than in the starvation treatment lethal effects can be at least partly be attributed to toxin production. These results also suggest that *Microcystis* is the only cyanobacteria that caused a significantly greater mortality to the daphnids than starvation did. The mortality caused by the five other cyanobacteria is less or (at most) similar to the mortality in the starvation control.

An important reason as to why temperature-dependent differences in harmful effects of cyanobacteria should be considered is in relation to projected climate warming. Under realistic conditions in the field indirect effects on *Daphnia* fitness may also occur, due to a direct effect of temperature on characteristics of the cyanobacteria themselves (e.g. toxin content, morphology and sterol content). A

limitation of our study, which therefore does not warrant a simple extrapolation to a realistic climate warming scenario, is that we did not account for such effects of temperature on cyanobacteria themselves, as they were cultured at a single temperature. Further research should consider growing the cyanobacteria at temperatures that are identical to the temperatures used in the *Daphnia* experiments. Such studies would preferably also need to measure toxin production and morphological parameters, as well as sterol content to quantify the relationship with temperature. Furthermore more realistic climate warming projections may want to consider mixtures of more than one cyanobacteria as field research suggests that the competitive abilities of different cyanobacteria differ at different temperatures (Dupuis and Hann, 2009). Therefore shifts in dominant cyanobacteria species are likely to occur under global change and could have major implications when replacement taxa are more harmful to zooplankton. Future studies may also want to consider multiple clones as an experiment with four *Daphnia magna* clones found that the interactive effects of a non-toxic *Cylindrospermopsis* strain and temperature were highly genotype-dependent, which could possibly trigger microevolutionary changes at the population level (Bednarska et al., 2011).

The findings from this chapter do nonetheless highlight that both the sensitivity of *Daphnia magna* to cyanobacteria and the order of harmfulness of the cyanobacteria studied are temperature dependent. Overall there appears to be no universal increase or decrease in the harmful effects of cyanobacteria to *Daphnia magna* with temperature, but rather an intricate combination of mechanisms causing certain cyanobacteria to increase their harmful effect on *Daphnia* at higher temperatures while others may be more harmful at lower temperatures.

4

Combined effects of copper and *Microcystis aeruginosa* on *Daphnia magna* under constant conditions at the individual level

Redrafted after:

Jennifer D Hochmuth, Colin R Janssen, Karel AC De Schamphelaere. 2016. Temperature and food concentration have limited influence on the mixture toxicity of copper and *Microcystis aeruginosa* to *Daphnia magna*. Environmental Toxicology and Chemistry 35 (3): 742–749.

4.1 Introduction

Aquatic communities are typically exposed to mixtures of stressors rather than to single substances and/or other stressors (Eggen et al., 2004). Nevertheless most regulatory assessments focus almost exclusively on the effect characterization of individual substances on a chemical by chemical basis (van Gestel, 2010). Previous research suggests that mixtures at No-Observed-Effect-Concentration (NOEC) levels of individual substances may already cause adverse effects (Breitholtz et al., 2008, Silva et al., 2002). Consequently, a major concern is that conventional risk assessment approaches may not be conservative enough if they do not account for the combined and the potentially interactive effects of toxicant mixtures.

Anthropogenic pollutants may also interact with natural stressors and the knowledge of interactions is important for the extrapolation of results of laboratory toxicity to field situations and for the design of site-specific Environmental Quality Standards (Heugens et al., 2001). Under global change, the exposure to combinations of natural and chemical stressors are predicted to increase, which may substantially complicate ecological risk assessment (Moe et al., 2013, Christensen, 2007). Two extensive reviews provided solid evidence that synergistic effects are commonly observed between chemicals and abiotic stressors (Holmstrup et al., 2010, Laskowski et al., 2010). However, no studies related to the interaction of chemical mixtures and biotic stressors (with the exception of parasites) were discussed by these authors. Cyanobacteria are a perfect illustration of such biotic stress, as they are considered as an emerging threat to freshwater environments. Large-scale ecosystem effects have been attributed to cyanobacterial bloom formation and their toxicity to aquatic organisms (Falconer, 2001, Johnk et al., 2008, Downing et al., 2001, Davis et al., 2009). It is well established that cyanobacteria reduce the fitness of zooplankton taxa like *Daphnia sp.* (Dao et al., 2010, Demott et al., 1991, Rohrlack et al., 2004, Rohrlack et al., 2005, Sarnelle et al., 2010, Shurin and Dodson, 1997). Freshwater communities can be compromised if cyanobacteria outcompete green algae, i.e. if cyanobacteria serve as primary food source for zooplankton under cyanobacterial bloom conditions (Moe et al., 2013). The adverse effects on zooplankton fitness may be enhanced if the proliferation of cyanobacteria interacts with both chemical stressors and/or abiotic stressors.

Copper (Cu) is commonly used as an algaecide to eradicate freshwater phytoplankton, including cyanobacterial blooms (Jancula and Marsalek, 2011). It tends to be applied in concentrations ranging from tens to hundreds of micrograms per liter (Jancula and Marsalek, 2011). It has been reported, however, that it can take up to two months to return to background levels, which in some cases coincides with the same time frame that cyanobacteria can reappear (Van Hullebusch et al., 2002), especially in warmer weather. Increasing temperatures, nutrient loads and extended summer stratification are predicted to increase the prevalence and severity of cyanobacterial blooms (Paerl and Huisman, 2008 , Kosten et al., 2012, Paul, 2008, O'Neil et al., 2012).

The nature of the interactive effects of cyanobacteria and other stressors depends on in the mixture composition. Synergistic effects between cyanobacteria have already been described for the combined exposure of *Microcystis aeruginosa* and the pesticide carbaryl to *Daphnia pulicaria* (Cerbin et al., 2010b) and for *Daphnia longispina exposed to Cylindrospermopsis raciborskii* and pentachlorophenol mixtures (Bernatowicz and Pijanowska, 2011). Conversely, a lack of interactive effects of *Microcystis aeruginosa* with cadmium was observed in experiments with *Daphnia magna* (De Coninck et al., 2013), while antagonistic effects in *Daphnia pulex* were reported for mixtures *of Microcystis aeruginosa* and different pesticides (Asselman et al., 2013). The results from chapter 2 have shown, that the type of interaction of the combined exposure to different cyanobacteria and copper (non-interaction, synergism or antagonism) was dependent on which reference model was used. For mixtures of *Microcystis aeruginosa* and copper synergism was observed relative to the Independent Action reference model (IA) and non-interaction relative to the Concentration Addition reference model (CA) (Hochmuth et al., 2014).

The results from chapter 2 also showed that, based on standard ecotoxicity tests with *D. magna* performed under standard environmental conditions, interactive effects of harmful algal blooms and copper pollution appear to be of limited concern for water quality management. However, in chapter 3, I observed and reported an increasingly harmful effect of *M. aeruginosa* on *D. magna* fitness at lower temperatures (Hochmuth and De Schamphelaere, 2014). More generally, it is well known that both temperature and food concentration can influence the toxicity of metals and other chemicals to *D. magna* (Holmstrup et al., 2010, Heugens et al., 2001). Based on all this, it could be expected that interactive

effects between Cu and harmful algae blooms might be dependent on factors such as temperature and total food concentration, which can be very different in the field compared with standard test conditions.

The main aim of this chapter was to evaluate if under-estimation of copper toxicity can occur when results obtained in standard toxicity tests under optimal conditions are extrapolated to variable and sub-optimal field situations. Building on the findings from chapters 2 and 3, we investigated whether the combined effects of copper and *M. aeruginosa* were affected by less optimal environmental conditions. We selected two important factors with a known effect on copper toxicity: temperature, which generally increases Cu toxicity, and total food concentration which reduces copper toxicity with increasing concentrations (for details consult the extensive review by Heugens et al 2001). An additional aim was to assess to what extent the three factors (total food concentration, % of *M. aeruginosa* in the diet and temperature) combined to influence Cu toxicity. We addressed this aim by evaluating how much of the variance in the observed chronic copper median effective concentrations was explained by *M. aeruginosa*, temperature and total food concentration.

4.2. Material and Methods

4.2.1 Organism cultures and test media

The *Daphnia magna* clone used in this chapter was the Xinb3 clone. The experimental organisms originated from cultures described in sections 2.1.1 and 2.1.2 in chapter 2 (Appendix A Tables A1-A4). The preparation of culturing and test media has previously been described in chapter 3 Section 2.1.1. Test animals were taken from the third clutch (< 24h old) and acclimated for 2 generations to their respective exposure temperature prior to the start of the experiments. The modified M4 medium was prepared and aerated for 72h before each medium renewal at 19°C in 25L or 50L polyethylene vessels. 24h prior to medium renewal the aerated medium was divided and stored at the specific exposure temperatures (19°C, 15°C and 23°C).

4.2.2 Experimental Design

A chronic 21 day toxicity experiment was carried out using a full factorial design composed of 8 Cu concentrations (dissolved concentrations: control 2.2 µg Cu/L, 28 µg Cu/L, 39 µg Cu/L 55 µg Cu/L, 71 µg Cu/L, 103 µg Cu/L, 132 µg Cu/L and 175 µg Cu/L), and 4 concentrations of *M. aeruginosa* (control: 0%, 10%, 20%, and 40% of the total diet) at 3 different constant temperatures (control 19°C, 15°C, 23°C) and 2 total food concentrations (high: 2 mg C/L and low: 0.8 mg C/L). The first level mentioned in parentheses for each factor indicates the control level and follows the OECD test guideline (OECD, 1998). This full factorial design was extended with one higher concentration of *M. aeruginosa* (80%) in the single dose concentration response curves (Cu only at control with a measured dissolved concentration of 2.2 µg/L), resulting in a total of 198 treatments.

For each treatment, 4 replicates with one juvenile each were set up in 50 mL of the test medium. All test animals were fed daily with the total food density based on dry weight of either 0.8 or 2 mg C/L. In control treatments, animals were fed with 100% *Pseudokirchneriella subcapitata*. In the treatments where the diet was contaminated with a set fraction of cyanobacteria, a fixed percentage in terms of milligrams of carbon (10%, 20%, 40% and 80%) of the total food concentration consisted of *M. aeruginosa*, while the remainder was composed of *P. subcapitata*. exposures were carried out under controlled light cycles (16 h of light: 8 h of dark) according to OECD guideline No. 211 (OECD, 1998). The test medium was renewed 3 times a week (Monday, Wednesday, and Friday). Reproduction and survival were scored daily.

4.2.3 Chemical analysis

Dissolved organic carbon (DOC), total organic carbon (TOC) and pH were measured twice a week, once in the fresh/new medium (before any algae or cyanobacteria were added) and once in the old medium (after transferring the daphnids to new fresh medium). Samples for metal and organic carbon analysis were taken as both total (unfiltered), and dissolved (filtered through a 0.45 mm filter, Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). All samples for Cu analysis were acidified to a final concentration of 0.14 mol/L of HNO_3 (Normaton Ultrapure 69% HNO_3, Prolabo). Total and dissolved Cu concentrations of the control medium were measured using graphite furnace atomic absorption

spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA), while all exposure media with added copper (nominal Cu concentration >45 µg/L were measured using flame atomic absorption spectrophotometry (SpectrAA100, Varian, Mulgrave, Australia). The measurements for dissolved and total copper concentration can be consulted in the Supplemental Data (Supplemental Data: Table S3). Samples for organic carbon analysis were measured with a TOC analyser (TOC5000, Shimadzu, Duisburg, Germany) as non-purgeable organic carbon (NPOC). The pH measurements were performed with a pH meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use by using a pH 7 buffer (Merck, Darmstadt, Germany). The pH remained stable over the exposure duration at 7.87 (± 0.02) in the new medium and 7.87 (± 0.03) in the old medium across treatments, mean (± SE, n = 9). Average Total Organic Carbon (TOC) was 4.1 mg/L (± 0.1) in the new medium and 3.1 mg/L (± 0.2) in the old medium, while average Dissolved Organic Carbon (DOC) was 2.6 m/L (± 0.1) in the new medium and 2.9 mg/L (± 0.4) in the old medium, mean (± SE, n = 9).

4.2.4 Data analysis

Single dose response curves fitted to Cu and *M. aeruginosa* for each temperature and total food concentration and the statistical mixture analysis were performed as described in chapter 2 section 2.1.5 (Hochmuth et al., 2014).

Additionally an ANOVA analysis was carried out to predict the Cu EC_{50} using % of *M. aeruginosa*, temperature, total food concentration and their two-way interactions as explanatory categorical factors. As there were no replicate observations of the EC_{50} parameter values for any factor combination the ANOVA model could not include a 3-way interaction term. In addition, to further investigate the relationship between *M. aeruginosa* and Cu toxicity we used a linear model to predict the Cu EC_{50} as a function of the amount (in mg C/L) of *P. subcapitata* and *M. aeruginosa* expressed as continuous factors. To assess the non-linear correlation between Cu EC_{50} and % of *M. aeruginosa* an approximate Spearman correlation test with 9999 Monte Carlo resamplings was carried out. Assumptions of normality and homoscedasticity of the residuals of the models were verified using a Shapiro-Wilk test and a Levene test, respectively. Reference models were compared against the extended models using an *F*-

test, to test whether including the deviation parameter *a* resulted in a statistically improved fit compared to the reference model. A p value <0.05 associated with this F-test translates into a significant synergistic or antagonistic deviation from the non-interaction null-hypothesis of the reference model (Asselman et al., 2013, Jonker et al., 2005). As the two reference models (IA and CA) are not nested models, they cannot be compared through formal statistical tests. Instead we used the Akaike Information Criterion (AIC) to compare model fit as recommend previously (Jonker et al., 2005, Asselman et al., 2013).

An ANOVA analysis was carried out to predict the Cu EC_{50} using % of *M. aeruginosa*, temperature, total food concentration and their two-way interactions as explanatory categorical factors. As there were no replicate observations of the EC_{50} parameter values for any factor combination the ANOVA model could not include a 3-way interaction term. In addition, to further investigate the relationship between *M. aeruginosa* and Cu toxicity we used a linear model to predict the Cu EC_{50} as a function of the amount (in mg C/L) of *P. subcapitata* and *M. aeruginosa* expressed as continuous factors. To assess the non-linear correlation between Cu EC_{50} and % of *M. aeruginosa* an approximate Spearman correlation test with 9999 Monte Carlo resamplings was carried out. All analyses were conducted in R (Team, 2011).

4.3 Results

The response of *D. magna* to mixtures of Cu and *M. aeruginosa* was more accurately predicted with the CA reference model (Equation 2.3) than with the IA reference model (Equation 2.2) (Figure 4.1). This is supported by a consistently lower AIC regardless of temperature or total food concentration (CA: 217-255, IA: 258-295, Table 4.1). Overall, non-interaction was identified according to CA (*a* not significantly different from 0, *F*-test, $p > 0.05$, Table 4.1) while synergism between Cu and *M. aeruginosa* was identified according to IA (*a* significantly smaller than 0, *F*-test, $p > 0.05$, Table 4.1), again regardless of temperature and total food concentration (Table 4.1). The *a* parameter (measure of deviation from non-interaction) had the same magnitude across all combinations of temperature and total food concentration.

Overall, the observed Cu 21d-EC_{50} values (based on reproduction) varied between 20 and 100 µg Cu/L (Figure 4.2). We observed a significant main effect of *M. aeruginosa*, temperature and total food

concentration on the EC_{50} of Cu, as well as significant interactions between temperature and total food concentration and between temperature and *M. aeruginosa* (Table 4.2). There was a clear effect of increasing Cu toxicity with increasing *M. aeruginosa* concentration in the total diet (Figure 4.2). Furthermore the adverse effect of *M. aeruginosa* was more pronounced at colder temperatures, as illustrated by the fact that no reproduction occurred at 15°C with a diet containing 40% *M. aeruginosa*, regardless of the total food concentration. The main effects of temperature and total food concentration were less significant than the effect of *M. aeruginosa*. However, there was a trend of decreasing Cu toxicity with increasing total food concentration. Cu toxicity was lowest at the reference temperature of 19°C, while it increased at 15°C and 23°C (Figure 4.2). Without any addition of *M. aeruginosa*, Cu toxicity was not affected by total food concentration at 15°C (black line in Figure 4.2A), while it decreased with a higher total food concentration as temperature increased to 19°C and 23°C (black lines in Figures 4.2B and C). The main effect of *M. aeruginosa* had the lowest p-value and when considered alone the % of *M. aeruginosa* already explained 76% of the variance in the Cu EC_{50} (Table 4.2, Figure 4.3A). Addition of the other two main effects (temperature and total food concentration) explained a further 11% of the variation (Figure 4.3B) and the full model with all two-way interactions increased the explained variance to 99% (Figure 4.3C).

Table 4.1. Summary of the identified interaction types of the Cu and cyanobacteria mixtures. For each temperature and food concentration the reference models of IA and CA reference models were compared against their respective synergism/antagonism deviation model with an F-test. For the values of all parameter estimates in this statistical analysis we refer to the Appendix C Table C.3.

0.8 mg C/L	***15°C***	***19°C***	***23°C***
IA	**Synergism**	**Synergism**	**Synergism**
a	-4.07	-3.353	-4.95
p-value	3.87e-06*	1.13e-05*	3.03e-07*
AIC	262.3	294.0	279.0
CA	**Non-interaction**	**Non-interaction**	**Non-interaction**
a	0.723	-0.223	-0.199
p-value	0.2220	0.5128	0.9836
AIC	243.1	254.9	249.7
2 mg C/L	***15°C***	***19°C***	***23°C***
IA	**Synergism**	**Synergism**	**Synergism**
a	-4.472	-4.867	-3.2346
p-value	4.54e-07*	4.45e-9*	0.0003*
AIC	257.6	295.2	290.4
CA	**Non-interaction**	**Non-interaction**	**Non-interaction**
a	-0.420	-0.3814	-0.1072
p-value	0.8090	0.2604	1
AIC	217.0	240.5	254.3

* indicates a p-value < 0.05, i.e. a significant improvement of the model fit by including the deviation parameter and hence an interactive effect (antagonism or synergism). P-values were corrected for multiple testing using the Benjamini-Hochberg correction method.

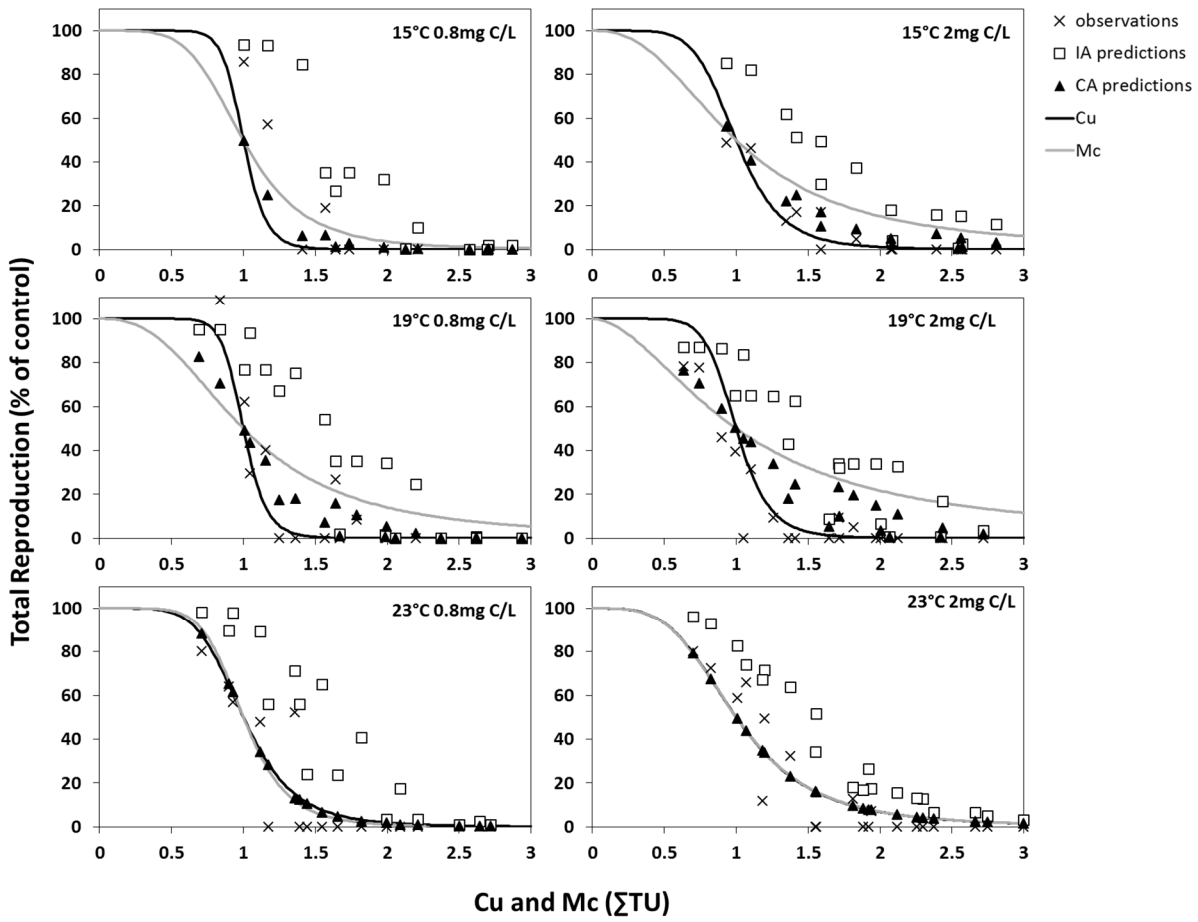


Figure 4.1. Comparison of the predicted responses to the mixture exposure relative to the observed response data. Predictions were made according to the independent action and concentration addition reference models using the slope and median effective concentration (EC_{50}) parameter values derived from the single-stressor concentration–response curves. Both observations and predictions were plotted as a percentage reproduction of the control against the sum toxic units based on the EC_{50} and slope values of the single-stressor concentration–response curves (Equation 2.1). In a mixture, under the concentration addition reference model, the sum of the toxic units is assumed to equal 1 in case of no interaction. The single-stressor concentration–responses curves of Cu (black line) and cyanobacteria (gray line) are also plotted. A summary table of the single-stressor concentration–response parameter values of Cu and *M. aeruginosa* as well as the single-dose response curves can be accessed in Appendix C Tables C.3 and C.4, Figures C.1 and C.2. CA = concentration addition; IA = independent action; Mc = M. aeruginosa; ∑TU = sum toxic units.

Table 4.2. ANOVA summary of the effect of *M. aeruginosa*, temperature and total food concentration on the Cu EC_{50}.

Source of variation	DF	SS	MS	F	p	R^2
Mc	3	7009	2336.4	120.653	4.39e-05*	0.76
Temp	2	759	379.5	19.598	0.0043*	0.08
Tf	1	293	15.117	292.7	0.0115*	0.03
Temp x Tf	2	284	142.0	7.334	0.0326*	0.03
Mc x Tf	3	413	137.7	7.112	0.0297*	0.05
Mc x Temp	5	386	77.2	3.987	0.0777	0.04
Residuals	5	97	19.4			0.01

* indicates significant p-values at the 95% significance level. DF= degrees of freedom, SS= sum of squares, MS= mean sum of squares, F= F statistic, p= p-value, Mc=*Microcystis aeruginosa* (% in total diet), Temp=temperature (°C), Tf= total food concentration (mg C/L), R^2= variance in Cu EC_{50} explained

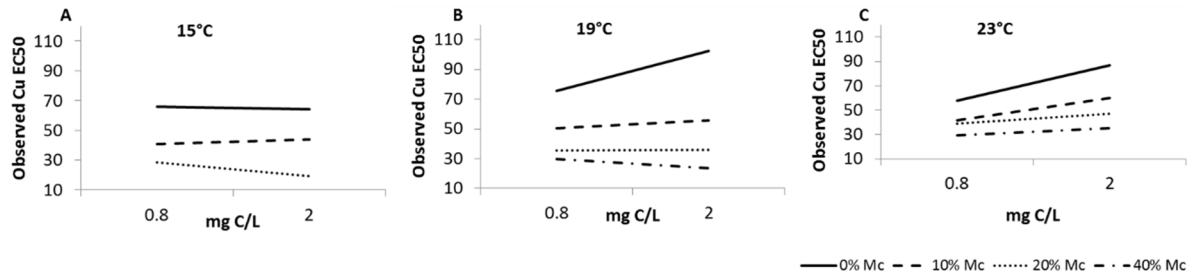


Figure 4.2. Interaction plots of the effects of percentage *M. aeruginosa*, temperature and total food concentration on the observed Cu EC_{50}. The plots aid visual interpretation of the main effects as well as interactive effects quantified in the ANOVA analysis (Table 4.2).

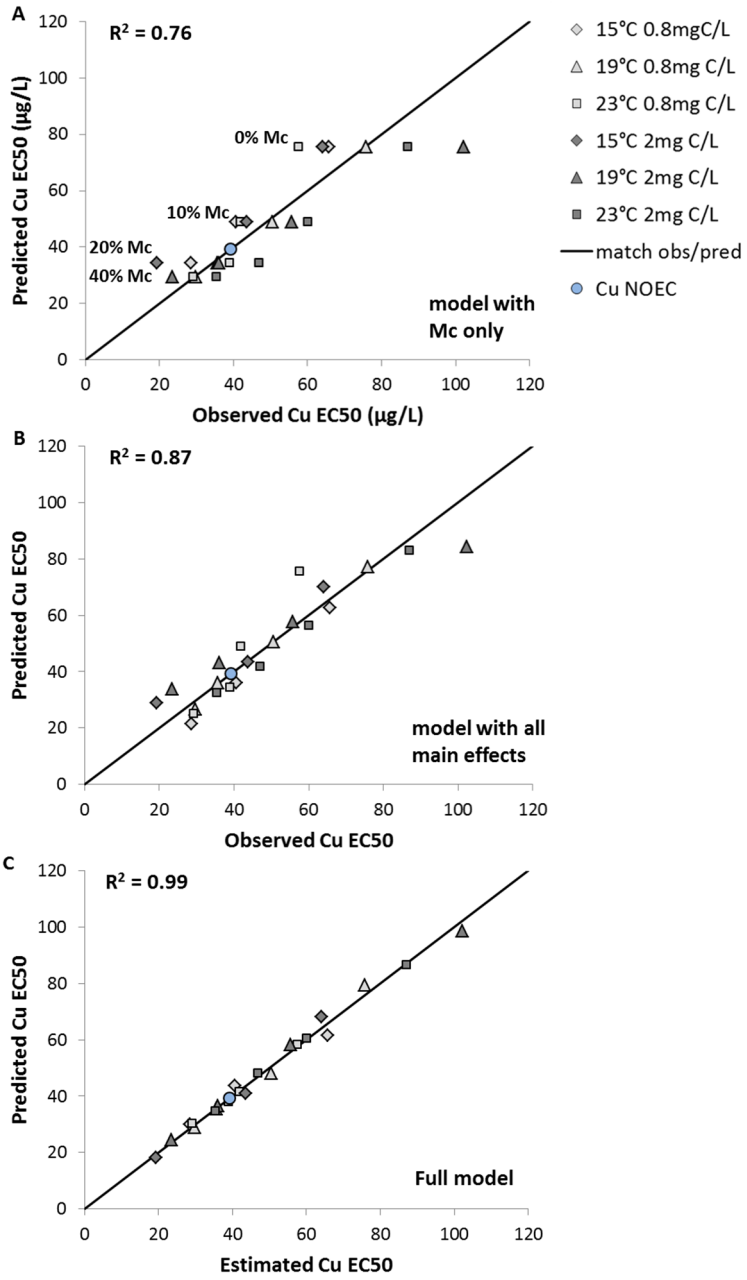


Figure 4.3. Regression of the predicted vs. observed Cu EC_{50} values using only the percentage of *M. aeruginosa* (A), percentage of *M. aeruginosa* as well as temperature and total food concentration without interactions (main effects (B), or all main effects as well as all 2 two-way interactions (C). The observed Cu NOEC under standard conditions (19°C, 2 mg C/L, 0% *M. aeruginosa*) is also depicted (see the Supplemental Data Figure S1 for the dose response curves). Note that the predictions are made using categorical factors (ANOVA). To visualize the goodness of fit a (1:1) line was added. R^2 = variance explained by the factor(s).

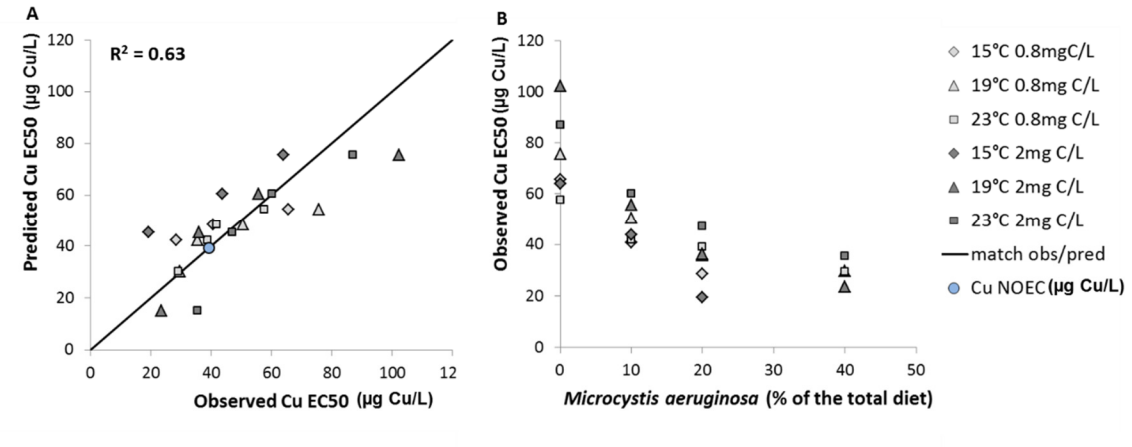


Figure 4.4. Regression of the predicted vs. observed Cu EC_{50} values using the amount (in mg C/L) of *P. subcapitata* and *M. aeruginosa* as continuous factors (A) and correlation of the observed Cu EC_{50} values with the percentage of *M. aeruginosa* in the diet (B). The observed Cu NOEC under standard conditions (19°C, 2 mg C/L, 0% *M. aeruginosa*) is also depicted (see the Supplemental Data Figure S1 for the dose response curves). To visualize the goodness of fit a (1:1) line was added. R^2 = variance explained by the sum of the predictors used in the model (see Table 4.3).

To place our results in regulatory context we compared the observed Cu EC_{50} values under different factor combinations against a "reference" value, i.e. the copper EC_{50} concentration obtained under standard OECD test guideline conditions (19°C, 2mg C/L, 0% *M. aeruginosa*) for the clone in this study (EC_{50} = 102 µg/L, Appendix C Figure C.1). All other Cu EC_{50} values (i.e. in all other conditions investigated here) were lower than this reference value. We further noted that the Cu EC_{50} values at 20% and 40% *M. aeruginosa* were lower than the copper NOEC obtained under OECD reference conditions in this study (0% Mc, 19°C, 2mg C/L, Figure 4.3A, see Appendix C Figure C.1).

A linear model with the concentration of *P. subcapitata* and *M. aeruginosa* (in mg C/L) as continuous factors explained 63% of the variance in the observed Cu EC_{50} (Figure 4.4A). The amount of *M. aeruginosa* explained nearly twice as much of the variance as the amount of *P. subcapitata* (40% vs. 23%, Table 4.3). We observed a significant correlation between the observed Cu EC_{50} and the % of *M. aeruginosa* in the total diet, irrespective of the temperature and total food concentration (approximate Spearman correlation: $Z = -4.115$, $p < 0.0001$, Figure 4.4B).

Table 4.3. Summary of the linear model using the amount mg C/L of *P. subcapitata* (Pseudo) and *M. aeruginosa* (Mc) as continuous factors. The table includes the estimates of the coefficients, of the predictor variables, the standard error (SE), significance (p-value) and explained variance (R^2).

Predictor	coefficient	SE	p	R^2
intercept	40.08	7.32	<0.0001*	NA
mg C/L of Mc	-57.70	12.61	0.0002*	0.40
mg C/L of Pseudo	17.74	5.26	0.0032*	0.23

* indicates significant p-values at the 95% significance level.

4.4 Discussion

Without considering potentially interactive effects, predicted effects of mixtures based on single concentration response curves may either under- or over-estimate the actual effects observed in the environment (van Gestel, 2010, Moe et al., 2013). Here we provide two important insights regarding predictions of the combined effects of stressors. Firstly, we found that, regardless of the reference model used, the combined and interactive effects of copper and *M. aeruginosa* to *D. magna* reproduction are not affected by the temperature and the total food concentration. Secondly, we found that at any given food concentration or temperature the median effective concentration of copper is lower in the presence than in the absence of *M. aeruginosa*, while the effects of temperature and total food concentration were less important. The second insight is actually a logical mathematical consequence of the observed synergisms between copper and *M. aeruginosa* and equal *a* parameter estimates across all temperature and total food concentrations relative to the IA model described in the first insight. Under the assumptions of the IA reference model the fractional effects of the individual mixture constituents (e.g. 50% effect level) are expected to be independent from each other in a probabilistic sense (Faust et al., 2003). By analyzing the shift of the concentration-response curve of one component in the mixture under fixed concentrations of a second component it can be assessed whether their combined effect is in agreement with IA assumptions. We observed a significant effect of *M. aeruginosa* on the Cu EC_{50}, which is underlined by the fact that the EC_{50} of Cu decreases with increasing *M. aeruginosa* concentration (Table 4.2, Figure 4.4B). This has important implications for all studies applying the IA reference model to combined effects of stressors. Below our findings are discussed in more detail.

Irrespective of temperature or total food concentration, synergism was consistently identified with the IA model (i.e. the observed combined effects were consistently underestimated), while non-interaction was identified with the CA model (i.e. the observed combined effects were accurately predicted) (Table 4.1, clearly visible in Figure 4.1). Given that the CA reference model provides both more accurate and conservative (i.e. protective) predictions than the IA reference model, the former represents a sensible choice for risk assessment purposes. Our results add to the accumulating evidence in the literature that the CA reference model typically delivers more conservative estimates of the combined effects (Hochmuth et al., 2014, Asselman et al., 2013, Altenburger et al., 1996, Bellas, 2008) and performs well as a first tier approach even without in depth knowledge of the mode of action of the mixture components (Backhaus and Faust, 2013).

The percentage of *M. aeruginosa* in the diet alone explained most (72%) of the variance in the median effective concentration of copper and when it was used as the only explanatory variable all predicted Cu EC_{50} values were within a two-fold range of the observed EC_{50} values (Figure 4.3A). This suggests that the contribution of temperature and total food concentration (together only explaining and additional 11% of the variance in Cu EC_{50} values) is much less important. The latter is supported by the fact that the *a* parameter (which is based on the whole dose response data and not just on the EC_{50}) is very similar across all temperature and total food combinations. When the percentage of *M. aeruginosa* exceeded 20% the combined effects of copper and *M. aeruginosa* were much greater at 15°C than at higher temperatures. Higher total food concentration generally reduced the harmful effects of *M. aeruginosa* on Cu toxicity at higher temperatures, while the opposite was observed at lower temperatures (higher total food concentration increasing the harmful effects of *M. aeruginosa* on Cu toxicity). These observations suggest that the effect of *M. aeruginosa* in mixtures with copper is more harmful at lower temperatures, as is also demonstrated in the single dose responses curves (Appendix C Figure C.2) and in chapter 2 (Hochmuth and De Schamphelaere, 2014). Currently the mechanism by which increasing temperatures decrease the sensitivity of *D. magna* to *M. aeruginosa* remains unknown, but increased detoxification rates at higher temperatures have been suggested as a possible mechanism (Yokoyama and Park, 2003).

The results presented in this chapter suggest that the adverse effects of *M. aeruginosa* may be a consequence of more than just a decrease in overall food quality as the amount of *M. aeruginosa* explained 40% of variance in the Cu EC_{50} values, while the amount of *P. subcapitata* explained this variance to a lesser extent (23% of variance, Figure 4.4A, Table 4.3). This is further supported by the strong negative correlation between the percentage of *M. aeruginosa* and Cu EC_{50} values (Figure 4B). It is also in line with our previous reports, in which we showed that the adverse effects of *M. aeruginosa* can be attributed to more than just starvation-like effects in *D. magna* (Hochmuth and De Schamphelaere, 2014, Asselman et al., 2014).

Without addition of *M. aeruginosa*, Cu toxicity increased with lower total food concentration and was least toxic at the intermediate temperature (19°C) increasing at both the higher (23°C) and lower (15°C) temperatures (Figure 4.3A). These observations are in accordance with the existing literature, as lower food concentrations have been reported to enhance copper toxicity in five cladoceran species including *D. magna* (Koivisto et al., 1992), with Cu toxicity being lowest at 20°C, and higher at 10 and 30°C (Boeckman and Bidwell, 2006). Our findings therefore indicate that copper toxicity does change with temperature and total food concentration, which is in contrast with standardized experiments at one temperature and an *ad libitum* food supply. It has been postulated that organisms living in environments close to their tolerance limits experience an increased vulnerability to additional stress (Heugens et al., 2001). However, the temperatures and total food concentrations that we selected and tested here are still well within the tolerance range of the *D. magna* clone investigated, which may be one explanation as to why these two factors do not have a major influence on the combined effects of copper and *M. aeruginosa*.

Here we highlight that the EC_{50} of copper decreases 5-fold with increasing *M. aeruginosa* percentage in the total diet, while the difference in EC_{50} is much less pronounced at different temperatures or total food concentrations. Importantly, all Cu EC_{50} values obtained under non-standard conditions in our multivariate experiment(s) are lower than the reference value obtained under standard OECD guideline conditions (see Appendix C table C.3). The decrease of EC_{50} values (i.e. increase of copper toxicity) at increasing *M. aeruginosa* concentrations questions the use of copper-based algaecides in combating harmful algae blooms. Further, a diet composed of only 10% *M. aeruginosa* (= NOEC, see Appendix C

Figure C.2) results in copper EC_{50} values close to the copper NOEC under standard conditions (Figure 4.3A), which confirms that mixtures at NOEC levels of individual substances can result in adverse effects (Breitholtz et al., 2008, Silva et al., 2002). It may also become necessary to derive separate Environmental Quality Standards for eutrophic systems. These may – as, with global change temperature and cyanobacteria are predicted to increase - be part of climate management strategy (Moe et al., 2013). In conditions with a *M. aeruginosa* percentage of 20% or higher the Cu EC_{50} is lower than the copper NOEC obtained under standard (test) conditions (19°C, 2mg C/L, 0% *M. aeruginosa*) for the clone used in this research (=39.3 µg/L, Appendix C Figure C.1). This observation has regulatory significance as, when *M. aeruginosa* was added we observed more than 50% decline in daphnid reproduction at copper levels around the NOEC obtained under standard conditions. The results therefore suggest that predicted no effect concentrations (PNECs) or Environmental Quality Standards (EQSs) based on copper NOECs derived from standard toxicity tests may not be protective in systems that experience *M. aeruginosa* blooms.

5

Combined effects of copper and *Microcystis aeruginosa* on *Daphnia magna* under time-variable conditions at the population level

Redrafted after:

Jennifer D Hochmuth, Colin R Janssen, Karel AC De Schamphelaere. Can synergistic effects of copper and *Microcystis aeruginosa* in *Daphnia magna* be extrapolated from the individual to the population level on individual to the population level? *Accepted with revisions in* Environmental Toxicology and Chemistry.

5.1 Introduction

Daphnia magna is widely used as model organism in both ecotoxicology and ecology because of its short generation time, high sensitivity to many chemicals and keystone position in the aquatic food web (Wogram and Liess, 2001, Mark and Solbe, 1998, Lampert, 2006, Koivisto, 1995). An important limitation of conventional risk assessment is that it primarily focuses on determining short-term (typically less than one generation) toxicological responses of individual organisms to exposures of single chemicals, mostly under constant and (near-)optimal conditions (Van den Brink, 2008 , Calow and Forbes, 2003 , OECD, 1998). This contradicts the main goal of ecological risk assessment, which is to protect of populations and ecosystems rather than single individuals (Hommen, 2010). Indeed, standard tests in ecotoxicology are in sharp contrast with ecological reality, as natural populations are often exposed to a combination of chemical and non-chemical stressors and seek to persist over many generations (long-term) under time-variable, non-optimal conditions. Non-chemical stressors can be both abiotic (e.g. sub-optimal temperature, hypoxia) and biotic (e.g., low food quantity or quality, predation, pathogens) and typically increase the sensitivity to chemicals and vice versa (Coors and De Meester, 2008 , Heugens et al., 2001, Agatz and Brown, 2013).

Current knowledge is still insufficient and too fragmentary to integrate these ecologically relevant aspects into ecological risk assessment (ERA) in a scientifically robust manner (Van den Brink, 2008). Research is needed to investigate the influence of chemical stressors under more realistic exposure scenarios and at higher organisational levels. A recent study found that the combined exposure to imidacloprid and carbaryl can result in a significant decrease in population density, even when no mortality was observed at the individual level (Agatz and Brown, 2013). Another study demonstrated that the combined stressors *p*-353-nonylphenol and predation drove daphnid populations to the brink of extinction, although the effects of both stressors were cryptic (i.e. hard to detect statistically) in single stressor treatments (Gergs et al., 2013). Together these two studies highlight the potential for unexpectedly strong combined stressor effects and the need to assess their consequences at the population level.

Copper contamination in surface water can locally reach levels that may cause toxicity to *Daphnia sp.*, for instance in waters affected by surface run-off from vineyards, where copper is still used as a biocide against fungus diseases (Banas et al., 2010). Copper is also an active ingredient in some algaecides used to combat harmful algal blooms, the application of which typically results in dissolved copper concentrations in the range of 10-100 µg/L (Jancula and Marsalek, 2011). This suggests that ecological risk assessment of copper should consider specific situations where harmful *M. aeruginosa* blooms can co-occur with elevated copper exposure. By the end of this century, global surface water temperatures are expected to increase on average by 1.8 – 4°C (Christensen, 2007, IPCC, 2007), which will enhance the frequency of thermal stratification periods in lakes and thereby increase the prevalence of cyanobacteria blooms (Wagner and Adrian, 2009).

We have previously observed in chapter 4 that mixtures at 21d NOEC levels of individual stressors can result in adverse effects, as a diet composed of only 10% *M. aeruginosa* (= 21d NOEC, see Appendix C Figure C.2) resulted in copper 21d EC_{50} on reproduction values lower than the copper 21d NOEC under standard conditions. The aim of this chapter was to assess the response of more ecologically relevant endpoints (population-level density) to the 21-day Cu NOEC for reproduction derived at the individual level under realistic time-variable conditions of global change. Here we compared the population density of *D. magna* over a seasonal increase of temperature representative of late spring/early summer in Western Europe under current conditions (15°C-19°C) with that of a population experiencing a 4°C increase (19°C-23°C) under global change projections. This temperature change was coupled with a realistic temperature-dependent percentage of *M. aeruginosa* in the total food source (Table 5.1) (Davis et al., 2009, Wagner and Adrian, 2009, Lurling et al., 2013, IPCC, 2007), and at copper levels equivalent to or lower than the 21-day copper reproduction NOEC (NO Effect Concentration) concentration for the studied clone (Hochmuth et al., 2014, Hochmuth et al., 2016).

5.2 Material and methods

5.2.1 Organism cultures and test media

The *Daphnia magna* clone used in this chapter was the Xinb3 clone. The experimental organisms originated from cultures described in sections 2.1.1 and 2.1.2 in Chapter 2 (Appendix A Table A1-A4).

The preparation of culturing and test media has previously been described in Chapter 3 section 2.1.1 Test animals were acclimated for 2 generations to their respective exposure temperature prior to the start of the experiments. The modified M4 medium was prepared and aerated for 72h before each medium renewal. 24h prior to the medium renewal the aerated new medium was divided and stored in the same water bath as the test jars (to acquire the same temperature as the experimental vessels) at 19℃ in 25L or 50L polyethylene vessels.

Dissolved organic carbon (DOC), total organic carbon (TOC), and pH were measured twice a week, once in the fresh medium (before any algae or cyanobacteria were added) and once in the old medium (after transferring the daphnids to new fresh medium). Samples were taken as both total (unfiltered), and filtered (through a 0.45 mm filter, Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). Samples for organic carbon analysis were measured with a TOC analyser (TOC5000, Shimadzu, Duisburg, Germany) as non-purgeable organic carbon (NPOC). The pH measurements were performed with a pH meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use by using a pH 7 buffer (Merck, Darmstadt, Germany).

Measured dissolved Cu concentrations were 2.4 µg/L (± 0.6, control), 25 µg/L (± 8.9), and 44 µg/L (± 8.6), mean +/- standard deviation. Measured total Cu concentrations were 4.4 µg/L (± 0.9, control), 43 µg/L (± 12), and 81 µg/L (± 8.8). The pH remained stable during the entire exposure duration at 7.6 (± 0.8) in the new medium and at 7.5 (± 0.1) in the old medium across treatments. Average Total Organic Carbon (TOC) was 4 mg/L (± 0.1) in the new medium and 3.2 mg/L (± 0.1) in the old medium, while average Dissolved Organic Carbon (DOC) was 3.0 mg/L (± 0.1) in the new medium and 2.7mg/L (± 0.1) in the old medium.

5.2.2 Experimental Design

We conducted a population level experiment for a total duration of 68 days. The experimental setup consisted of a 3 x 4 design, with one constant factor (3 Cu concentrations) and one time-variable factor (4 natural stressor levels). As copper is an essential element, it follows and optimal concentration range (as illustrated in Chapter 1 Figure 1.5) The control and lower Cu concentrations were representative of

the low and high end of the optimal Cu concentration range for *D. magna* and based on results derived in the same medium (3µg Cu/L = control, 25µg Cu/L = lower Cu concentration) (Bossuyt and Janssen, 2004). The higher Cu concentration (dissolved Cu concentration of 45µg Cu/L) was comparable to the NOEC level derived in the same test medium (43.6µg Cu/L = higher Cu concentration) (Hochmuth et al., 2014, Hochmuth et al., 2016). The time-variable factor consisted of natural stressor 4 levels mimicking a realistic seasonal increase in temperature and cyanobacteria concentration under projected global change (IPCC, 2007, Davis et al., 2009, Wagner and Adrian, 2009, Lurling et al., 2013): (i) control, (ii) a diet change of 10-20% of the diet consisting of *Microcystis aeruginosa* under control temperature conditions, (iii) a temperature rise of 4°C relative to the control without a diet change, and (iv) temperature rise of 4°C relative to the control with a diet change consisting of 20-40% *M. aeruginosa* (see Table 5.1 for details).

Table 5.1. Summary table of the experimental design used for the time-variable natural stressor levels. The different treatment factors are: [a] time-variable control conditions under the control temperature 15°C-19°C), [b] time-variable *M. aeruginosa* concentrations and temperatures under the control temperature (15°C-19°C), [c] time-variable temperature conditions under the projected global change regime (temperature = control + 4°C, i.e. 19°C-23°C), [d] twice the amount of time-variable *M. aeruginosa* concentrations as in [b] and temperature conditions under projected global change (temperature = control + 4°C).

	Control conditions		**Global change conditions**	
Period	**Control[a]**	**MC[b]**	**+4°C[c]**	**2xMC+4°C[d]**
1 (Day 0-14)	0% MC-15°C	10% MC-15°C	0% MC-19°C	20% MC-19°C
2 (Day 14-28)	0% MC-16°C	12.5% MC-16°C	0% MC-20°C	25% MC-20°C
3 (Day 28-42)	0% MC-17°C	15% MC-17°C	0% MC-21°C	30% MC-21°C
4 (Day 42-56)	0% MC-18°C	17.5% MC-18°C	0% MC-22°C	35% MC-22°C
5 (Day 56-68)	0% MC-19°C	20% MC-19°C	0% MC-23°C	40% MC-23°C

Each factor combination was replicated 4 times resulting in a total of 48 experimental units. Prior to the start of the test, populations were acclimated for 2 generations to their respective start exposure temperatures (15°C or 19°C) and fed *ad libitum*. At the start of the test, jars containing 1L of modified M4 medium (= experimental unit) were inoculated with 3 egg-carrying females of the same age (14 days, +/- 1 day) and 5 neonates (< 24 hrs). To each experimental unit a total food density of 2 mg dry weight/L (≈0.8 mgC/L) was added daily (Evens et al., 2009 , De Schamphelaere and Janssen, 2004). In treatment combinations with green algae only this consisted of 100% *Pseudokirchneriella subcapitata*, while in treatment combinations with cyanobacteria this consisted of a mixture of *P. subcapitata* and *M. aeruginosa*. Every two weeks (on day 14, 28, 42, 56) the experimental conditions were changed: temperature was increased by 1°C (control conditions: 15°C at the start to 19°C at the end; global change conditions: 19°C at the start to 23°C at the end), % *M. aeruginosa* was increased by 2.5% (on dry weight basis) under control conditions (10% at start to 20% at end) and by 5% under global change conditions (20% at start to 40% at end). The medium was completely renewed 3 times per week and population abundance was recorded by gently pouring the contents of the jars over 3 sieves with consecutively smaller mesh sizes. This allowed us to determine 3 population size classes: 'adults' (>2mm, retained on a 800µm sieve), 'juveniles' (non-egg carrying, 1.2-2mm, retained on a 500µm sieve) and 'neonates' (typically < 48h old, <1.2mm, retained on a 200µm sieve). Individuals present on each sieve were counted and carefully transferred with a pipette to the fresh medium. To simulate predation, every two weeks (simultaneously with the changes in the other time-variable experimental conditions) 50% of each of the 3 different size classes (adults, juveniles, neonates) were removed at random. In line with negative frequency dependent selection, a threshold abundance of 50 daphnids per litre was above which predation events were carried out (Hampton et al., 2006, Visser, 1982).

5.2.3 Data analysis

For the statistical analysis we split the exposure duration into 5 time periods reflecting the 5 changes in the time-variable conditions and artificial predation event (Table 5.1). A mixed linear model with period (5 levels), Cu concentration (3 levels) and natural stressor factor (4 levels) as fixed effects and jar (experimental unit) as random effect was applied. We included different weights for the fixed effects to consider for unequal variances as well as a correlation structure correlating abundance at day t+2 (or

t+3, as abundance was recorded on Monday, Wednesday and Friday) per jar with the abundance at day t. The final model was selected based on the lowest AIC, by comparing models with different or no weights or correlation structures. We tested for effects of the single factors and specifically for statistically significant two-way interactions between Cu concentration and the natural stressor level (i.e combined exposure) for each time period using a Wald Chisquare test wit Holm adjustment for multiple testing. Treatments were always compared against the control populations. The statistical analysis was conducted in R using the package *phia* (Rosario-Martinez, 2013). To test for interactive effects between the copper and the natural stressor levels at each time point, interaction effect sizes were calculated for each observation of population density using Hedges *d*, an estimate of the standardized mean difference not biased by small sample size (Jackson et al., 2016, Gurevitch and Hedges, 2001). Hedges *d* (also termed 'interaction effect size') for each observation was calculated as the difference between the observed effect of both stressors and the predicted additive effect based on the sum of their single independent effects (see (Jackson et al., 2016) and the Supplementary material therein for equation and model details). A significant interactive effect (i.e. different from non-interaction) was assessed using 95% confidence intervals calculated around each effect size. If the 95% confidence interval includes zero non-interaction was concluded. Negative interaction effect sizes (less than zero) represent antagonism (i.e. observed total population density is larger than the density predicted from single additive effects of the individuals stressors) while positive effect sizes (greater than zero) represent synergism (i.e. observed total population density is smaller than the predicted density from single additive effects of the individuals stressors).

5.3 Results

5.3.1 Individual and interactive effects of copper and the natural stressor levels at different time periods

The effects of the individual factors were limited (Figure 5.1A). Under an exposure of 44µg Cu/L population density was significantly lower compared to the control in period 2 ($p < 0.001$) but higher in period 4 ($p = 0.035$). Population density was higher under the control in period 2 ($p = 0.0315$) and higher under the +4℃ stressor level in period 4 ($p = 0.0263$). Under the MC stressor level (2.4 µg Cu/L) total density was lower than under the control in periods 2 ($p = 0.004$) and 5 ($p = 0.0334$) but higher in period

4 (p = 0.0135). Under the 2xMC+4℃ stress level total density was higher than the control at period 1 (p = 0.0126) but lower at period 2 (p = 0.0056). Interactive effects between copper and natural stressor levels were consistently observed only when *M. aeruginosa* was present (MC and the 2xMC+4℃ stress level), regardless of the temperature (Figure 5.1B and Figure 5.1C). Under an exposure of 25µg Cu/L total population density was much lower in the MC stressor level in period 4 (p < 0.001) and in the 2xMC+4℃ stress level in period 5 (p = 0.001) compared to the control (Figure 5.1B). Under an exposure of 45µg Cu/L total population density was significantly lower in the MC stressor level in periods 3 (p = 0.016) and 4 (p < 0.001) and at the end of the experiment this population was one the verge of extinction, with 2 out of the 4 replicates already extinct and a total of only 3 non-egg carrying adults remaining in the other two experimental units (Figure 5.1C). The interactive effects between copper and natural stressors were more pronounced under the 2xMC+4℃ stressor level than under the MC level. At the end of the experiment, total population density declined significantly under the combined exposure to 25µg Cu/L and the 2xMC+4℃ stressor level (period 5: p < 0.001, Figure 5.1B). Under the combined exposure to 45µg Cu/L in the 2xMC+4℃ stressor level total abundance was significantly lower compared to the control (p < 0.002) at all time periods exempt for period 2 (p = 0.1559, Figure 5.1C). Further, under this exposure the first replicate population went extinct on day 23 and by day 54 the last replicate population reached complete extinction.

5.3.2 Interactive effects of copper and the natural stressor levels at each time point

Significant interaction effect sizes (Hedge's *d*) (i.e. 95% confidence interval not including zero) were observed for all stressor combinations at various time points. However, the occurrence and magnitude of interactive effects differed between factor combinations. The combination of copper with the MC stressor level resulted in consistent positive effect sizes, i.e. synergisms, which were greater (larger Hedge's d) at 45µg Cu/L (Figure 5.2E) than at 25µg Cu/L (Figure 5.2B). The combination of copper with the 2xMC+4℃ stressor level increased the magnitude of the significant synergisms (i.e. positive effects sizes, Figure 5.2C and Figure 5.2F). Consistently negative interaction effects sizes, i.e. antagonisms, were only observed when no *M. aeruginosa* was added, regardless of the copper level or temperature (Figure 5.2A and Figure 5.2D). This indicates that copper and temperature together had a stimulating effect on total population abundance, if no *M. aeruginosa* was present.

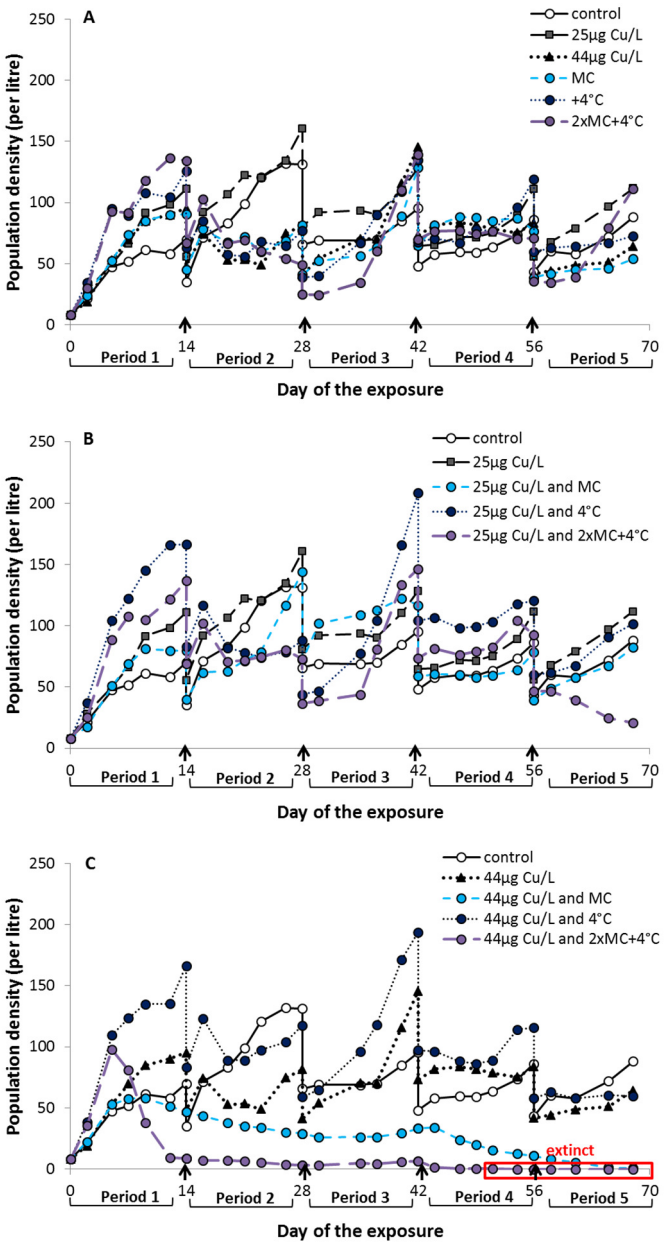


Figure 5.1. Average (n = 4) population density of *Daphnia magna* over the course of the exposure. Upward pointing arrows (↑) indicate the specific days where half of the population was removed at random to mimic predation. A: The effect of the individual stressors on population density. B: The combined effect of 25µg Cu/L and climate stressors. C: The combined effect of 44µg Cu/L and natural stressors. See Table 5.1 for a description of the natural stressor levels. Control: time-variable control conditions under the control temperature regime (15℃-19℃), MC: *M. aeruginosa* concentrations and temperatures under control temperature (15℃-19℃). +4℃: time-variable temperature conditions under the projected global change (temperature = control + 4℃, i.e. 19℃-23℃). 2xMC+4℃: twice the amount of time-variable *M. aeruginosa* concentrations as in MC and temperature conditions under projected global change (temperature = control + 4℃, i.e. 19℃-23℃).

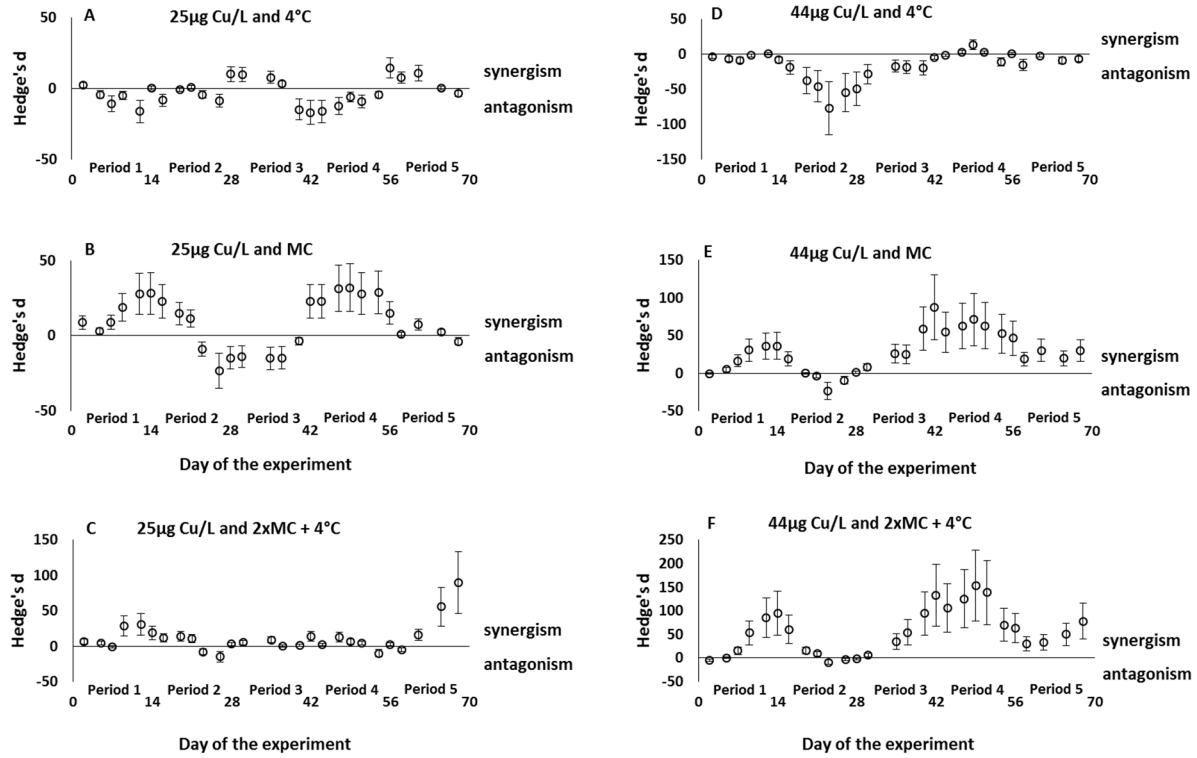


Figure 5.2. The observed combined effects of copper and natural stressor levels expressed as individual effect sizes (Hedge's d), relative to the predicted additive effect based on the sum of their single independent effects (= 0). If the 95% confidence interval includes zero non-interaction was concluded (i.e. no significant difference between measured and predicted response variable under the combined exposure). Negative interaction effect sizes (Hedge's *d* less than zero) represent antagonism (i.e. observed total population density is larger than the density predicted from single additive effects of the individuals stressors) while positive effect sizes (Hedge's *d* greater than zero) represent synergism (i.e. observed total population density is smaller than the predicted density from single additive effects of the individuals stressors). Figures 5.2A-C show the combined effects from an exposure to 25µg Cu/L and Figures 5.2D-F show the combined effects from an exposure to 44µg Cu/L. Please note a difference in the scale of the y-axis across the (Figures 5.2A-F).

5.4 Discussion

In the present study the changes in population density over time were monitored in a *D. magna* population exposed to environmentally relevant copper concentrations under realistic global change conditions (notably the addition of *M. aeruginosa* to the diet and a temperature rise of 4℃). The aim of this chapter was to assess the response of more ecologically relevant endpoints (population-level density) to the 21-day Cu NOEC for reproduction derived at the individual level under realistic time-variable conditions of global change.

Firstly, the results suggest that interactive effects predicted from standard ecotoxicity tests can be translated to more complex realistic situations. Here we observed predominantly synergistic interactions between copper and global change conditions (almost exclusively in the presence of *M. aeruginosa*), when analysing the population abundance at each time point (Figure 5.2). Our results suggest limited effects on total population density of Cu, *M. aeruginosa* and temperature, when considered individually (Figure 5.1A) Synergistic interactions are especially a concern for population and community-level ecological risk assessment, because they complicate extrapolations and predictions from standard individual ecotoxicity tests (Moe et al., 2013, Van den Brink, 2008). Standard ecotoxicity tests typically only cover one generation and may therefore not accurately capture the long term effects over several generations at the population level (Heugens et al., 2001). At the individual level we previously reported that the synergistic effects of copper and *M. aeruginosa* to *D. magna* reproduction were not affected by the temperature (Hochmuth et al., 2016). Similar results were reported in another recent study, where short-term (< 14 days) exposure to low dosages of toxic *M. aeruginosa* resulted in higher population density compared to a control without *M. aeruginosa*, while prolonged exposure (>14 days) to low-dosage *M. aeruginosa* resulted in a strong decrease in population densities, regardless of the temperature (Domis et al., 2013). We have previously documented that the Cu 21 day EC_{50} of the same clone as used in this study decreases 5-fold with increasing MC in diet (Hochmuth et al., 2016). Here we have provided evidence that future global change can cause extinction even at the copper 21-day NOEC concentration of the freshwater ectotherm *D. magna*. Our results obtained at the population level confirm previous findings obtained at the individual level (Hochmuth et al., 2016) and suggest that copper NOECs derived from standard toxicity tests may not be protective in systems that experience *M. aeruginosa* blooms.

Secondly, we highlight the need to consider combined effects of stressors in ERA. We observed that under global change related stressors the daphnid population was clearly more sensitive to the Cu 21 day NOEC for the *D. magna* clone in our study (44µg Cu/L) and to some extent already to a concentration representative of the higher end of the optimal copper concentration (25µg Cu/L, see (Bossuyt and Janssen, 2004)) compared to the Control (2.4µg Cu/L). While the effects of all copper, *M. aeruginosa* and temperature levels were limited when evaluated in single exposures, the combination of all 3 resulted in the extinction of the population under an exposure to 44µg Cu/L with added *M. aeruginosa* and a temperature increase of 4°C (Figure 5.1C). Hence, without the data on mixture effects at the individual level, we could not have predicted the population level extinction, based on single stress exposures to copper and *M. aeruginosa* alone. This is an example of an ecological surprise, i.e. multiple stressors having combined effects that are not expected from the effects of the single stressors (Paine et al., 1998). Evidence from the literature raises concern over the future occurrence of such ecological surprises, given the extent to which the freshwater ecosystems are impacted by a range of simultaneous stressors (Hecky et al., 2010, Jackson et al., 2016, Segner et al., 2014, Darling and Cote, 2008). The prevalence of ecological surprises may increase in the light of global change and multiple, interacting environmental stressors. Contaminants, for which the effects are getting more toxic in the wake of global change, may be require stronger environmental quality standards (Moe et al., 2013).

Further, we highlight the need to overcome the mismatch between the level of interest of ecological risk assessment, which is populations or higher levels of organizations and the currently used endpoints in ecotoxicology, which are at the level of individuals. The standard ecotoxicological concepts used in ecological risk assessment to protect ecosystems are an oversimplification, as the so-called “predicted no effect concentrations” (PNEC) and “no effect concentrations” (NOEC) are typically derived from effect threshold concentrations determined in laboratory toxicity tests using single species. It is an ongoing challenge for ecotoxicologists to extrapolate the combined effects of toxicants and global change, measured at the individual level (e.g., reduced survival and reproduction) to the population level (e.g., population growth rate, extinction risk) and community level (e.g. species richness, food-web structure) (Moe et al., 2013).

The difficulty to extrapolate from laboratory to field conditions has been raised repeatedly (Moe et al., 2013, Van den Brink, 2008). We have demonstrated that the exposure to copper around the geometric mean 21 day Cu NOEC for *D. magna* reproduction can already affect total abundance under global change projections to such an extent that daphnid populations may go extinct within two months. The question arises whether we could have predicted the outcome of our experiment with standard ecotoxicity data. We have previously documented that the concentration addition model accurately predicted the synergistic effects between copper and *M. aeruginosa* on *D. magna* at the individual level (Hochmuth et al., 2014, Hochmuth et al., 2016). The fact that the model predicts that the combined effects are greater than zero even if the individual effects are both zero, suggests that population level effects may be predicted from individual level effects. Recent developments in modelling frameworks, such as mechanistic effect models, combining individual level reproduction and survival models with an individual-based population model (IBM) (e.g. DEB-IBM) offer a possibility to not only understand the individuals' responses but further to extrapolate them to the population level (Martin et al., 2013b). Extending such a modelling framework to account for changing global conditions may be an important step towards a more realistic risk assessment of chemicals.

6

Extrapolating the combined effects of copper and *M. aeruginosa* from the individual to the population level using a DEB-IBM

6.1 Introduction

Risk assessment is faced with a mismatch between the level of interest, which is the protection of populations or higher levels of organizations, and the effect assessment of stressors in practice, which is based on standard ecotoxicity tests with individuals. Natural populations are often exposed to multiple contaminants under non-optimal and time-variable conditions. Non-chemical stressors, both biotic and abiotic (e.g., food shortage, non-optimal temperature, and predation), typically increase the sensitivity of organisms to chemicals and vice versa (Heugens et al., 2001, Coors and De Meester, 2008). Standard ecotoxicity tests derive short-term (typically less than one generation) toxicological endpoints (typically survival or reproduction) of individuals under chemical exposure for individual substances, mostly under invariable and (near-)optimal conditions (De Schamphelaere et al., 2011, Heugens et al., 2001 , Coors and De Meester, 2008 , Van den Brink, 2008).

ERA is faced with the challenge to develop predictive models that can take into account the ecological complexity displayed in real populations and ecosystems (European Union, 2013) (Grimm et al., 2009). Mechanistic models, that use inherent properties from individuals to derive effects at higher organization levels, could address some of the current limitations in ERA, as patterns observed at higher scales tend to emerge of processes occurring at lower scales (Bradbury et al., 2004, Forbes and Calow, 2012, Chave, 2013, Grimm and Railsback, 2005). Recently a generic implementation of Dynamic Energy Budget (DEB) theory in an individual-based model (IBM) was developed (Martin et al., 2013a). The combination of DEB and IBM has many advantages over other mechanistic models. The IBM component of the model allows for population level responses to emerge from the properties of individuals (Figure 6.1). IBMs in particular seem particularly suited for ERA because they consider processes occurring at the individual level such as growth and reproduction, the focal level of standard ecotoxicity tests (Jager et al., 2014, Martin et al., 2013b, Gabsi and Preuss, 2014) (Figure 6.1). Incorporating chemical effects on individuals in IBMs therefore allows to explore how these effects propagate to the population level. In recent years, IBMs have been applied successfully to predict the population dynamics of *Daphnia magna*, a test species used in ecotoxicology (Preuss et al., 2009, Preuss et al., 2010, Gabsi and Preuss, 2014, Gabsi et al., 2014).

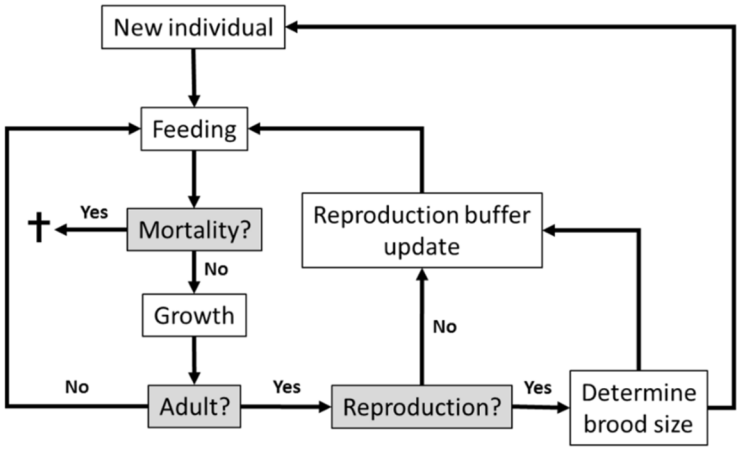


Figure 6.1. Schematic diagram of the organisation in an IBM. An IBM follows the entire life cycle of each individual at discrete time steps. Each individual follows the steps in the same order as illustrated in the diagram. The steps that change the outcome of the state of the individual are shaded in grey.

The underlying goal of the DEB approach is to understand the dynamics of biological systems, from cells to ecosystems, via a balance approach for mass and energy (Nisbet et al., 2000, Kooijman and Metz, 1984, Kooijman, 2010). (Figure 6.2). DEB is therefore a generic theory, theoretically universal in its application, as its key assumption is that the mechanisms governing metabolic organization are similar among species (Kooijman, 2010) (Figure 6.2). Similarly to IBMs, DEB theory considers that individuals are the key unit of interest for understanding dynamic systems at higher levels of organisation. DEB translates environmental conditions to individual performance (growth, survival and reproduction), which is important because the trade-offs in life history traits that DEB specifies (growth vs reproduction, time and size to maturation) turn out to strongly influence population dynamics (Denney et al., 2002). Therefore, IBMs can benefit from the generality of DEB, while IBMs enable scaling from the individual DEB-model to populations. An extensive overview of DEB theory and its applications can be found in several key papers (Nisbet et al., 2000, van der Meer, 2006, Sousa et al., 2010, Kooijman and Metz, 1984).

Using this DEB-IBM framework, Martin and colleagues were successfully able to predict population growth rates and peak densities of experimental *D. magna* populations in multiple experimental settings from the properties of individuals (Martin et al., 2013a, Martin et al., 2012). The same DEB-IBM

framework was used to extrapolate chemical stress from the individual to the population level, using information at the individual level on the effect of 3,4-dichloroanailine on *D. magna* (Martin et al., 2013b). Stressors were modelled as changes in the value of one or more parameters in the DEB sub-model, thereby altering one or more of the energetic fluxes leading to different patterns in growth or reproduction. The pattern of the stressed life history output depends on the physiological mode of action (PMoA). In their paper they identified 4 potential PMoAs: feeding/assimilation, maintenance, growth costs, reproduction costs, and embryonic hazard (Figure 6.2 and Table 6.1) (Martin et al., 2013b). The individual level data sets indicated no significant effects on growth, excluding all PMoAs that have an effect on growth (all except the reproductions costs and embryonic hazard PMoAs). Assuming direct effects on reproduction, the model was able to accurately predict the population response to increasing concentrations of 3,4-dichloroaniline. This suggests that the combination of DEB theory with IBMs is a promising tool for ERA.

Table 6.1. DEB parameters affected by stress depending on the Physiological Mode of Action (PMoA). Parameter values in combination with environmental conditions determine the magnitude of energy fluxes as governed by a set of coupled differential equations (More details on the DEB parameters affected by stress can be found in Appendix D Table D.2).

PMoA	Description	Affected parameter	Stressed value
Feeding/assimilation	Decrease in feeding ability	f	$f_s = f(1-s)$
Maintnenace	Increase in maintenance costs	$\dot{k}_m, \dot{k}_j$	$\dot{k}_{M,s} = \dot{k}_M(1+s)$ * $\dot{k}_{J,s} = \dot{k}_J(1+s)$
Growth costs	Increase in overhead costs of growth	$\dot{k}_m, g$	$\dot{k}_{M,s} = \dot{k}_M/(1+s)$ † $g_s = g(1+s)$
Reproduction costs	Increase in cost per egg	κ_R	$\kappa_{R,s} = \kappa_R/(1+s)$
Embryonic hazard	Decrease in survival during embryonic period	κ_R	$\kappa_{R,s} = \kappa_R \exp(-s)$

* We used the same assumption as Martin et al. (2013), making the assumption that both maturity and somatic maintenance costs are both equally affected, however effects on each parameter independently are also possible.

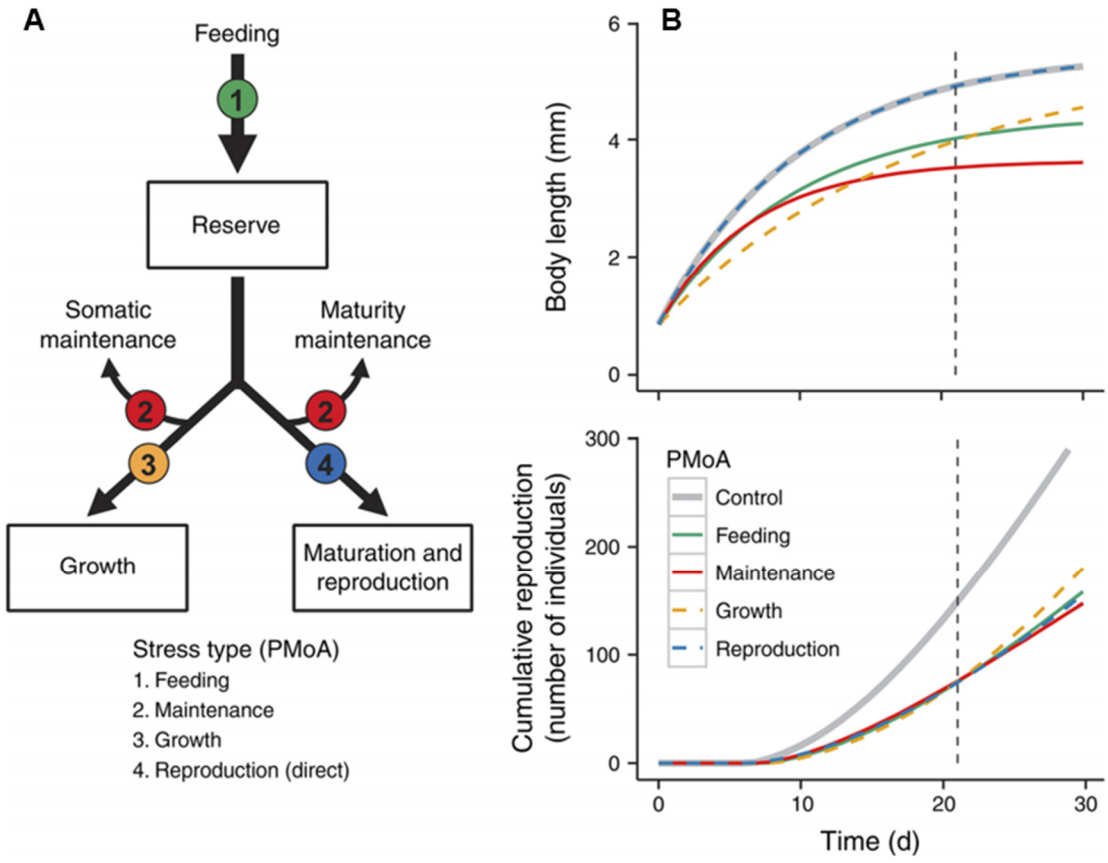


Figure 6.2. Diagrams illustrating energy flow according to DEB theory and the implementation of toxic stress on physiological endpoints (reproduction and growth) via the DEB framework. A: Scheme of the standard DEB-theory and physiological mode of actions (PMoAs). The state of the environment (characterized here by food density) determine the magnitude of the energy fluxes which in turn affect the life history of the organism, including growth and reproductive output. Stress is modelled in DEB as changes in the value of one or more parameters, resulting in changes in one or more of the energetic fluxes, hence leading to different patterns in growth and or reproduction. The pattern of the physiological response under stress (here reproduction and length are shown) depends on the PMoA. Each number in the left diagram represents the point where toxicity via a specific PMoA disrupts energy flow. In Table 6.3, the parameters affected by the different PMoAs are listed. B: Length and reproduction associated with stress via each PMoA are shown. The growth and reproductive predictions for each PMoA correspond to the stress level required to result in a reduction in reproduction of 50% relative to control after 21 days (indicated by the vertical dashed line) in a standardized ecotoxicity test (diagrams taken from Martin et al. 2014).

The aim of this chapter was to test whether DEB-IBM is a suitable modelling approach to predict population level effects of chemical and natural stressors using standard toxicity data collected at the individual level. This aim was addressed in 2 steps:

(1) A DEB-IBM developed by Martin et al. (2013) was calibrated using data on total reproduction after 21 days (i.e. a standard endpoint in ecotoxicology) collected in chapter 4.
(2) The predictions made by the DEB-IBM were validated with the independent population level data collected in chapter 5.

6.2 Material and Methods

6.2.1 Individual and population level experiments

Below follows a short description of the experimental design. See the methods sections in chapter 4 (individual level experiment) and chapter 5 (population level experiment) for a more detailed description. In chapter 4, a standard 21-day ecotoxicity experiment was carried out with a full factorial design using 8 copper and 4 concentrations of *Microcystis aeruginosa* at 3 different constant temperatures (15°C, 19°C, 23°C) and 2 total food concentrations (low: 0.8 mg C/L and high: 2 mg C/L). In chapter 5, a population level experiment was conducted for a total duration of 68 days. The experimental setup consisted of 3 x 4 design, with one constant factor (3 copper concentrations) and one time-variable factor (4 natural stressor levels). Every two weeks (on days 14, 28, 42, 56) the experimental environmental conditions changed: temperature was increased by 1°C (control: 15°C at the start and 19°C at the end, global change: 19°C at the start and 23°C at the end), % *M. aeruginosa* increased by 2.5% in the control climate regime (10% at start and 20% at end) and by 5% in global change regime (20% at start and 40% at end). Each experimental population was fed daily with a total food density of 0.8 mg C/L based on dry weight of (2 mg dry weight/L). To simulate predation, every two weeks (simultaneously with the changes in the other time-variable experimental conditions) 50% of each of the 3 different size classes (adults, juveniles, neonates) were removed at random. In line with negative frequency dependent selection, a threshold abundance of 50 daphnids per litre was above which predation events were carried out (Hampton et al., 2006; Visser, 1982). The comparison of the experimental conditions between the experiment by Preuss et al. (2009) (used for the development of

the original DEB-IBM) and the experimental conditions in chapter 4 and 5 (used to calibrate the original DEB-IBM) can be consulted in Table 6.2.

Table 6.2. Comparison of the experimental conditions in chapter 4 and chapter 5 to the experimental conditions by Preuss et al. (2009) used for the development of the original DEB-IBM model. Please note that the differences in the experimental conditions were accounted for in the model calibration (see section 6.2.3).

Experimental condition	**Preuss et al. (input for DEB-IBM)**	**Chapter 4**	**Chapter 5**
D. magna clone	Clone B	Xinb3	Xinb3
Volume	900mL	40mL	1000mL
Initial density	5 neonates < 24hrs old, or 3 adults and 5 neonates	1 neonate < 24hrs old	3 adults (14 days old +/- 1 day) 5 neonates < 24hrs old
Food source	*Desmodesmus subspicatus*	*Pseudokirchneriella subcapitata*	*Pseudokirchneriella subcapitata*
Feeding	cells were added each day Monday – Thursday, and 3x the normal food level on Friday	cells were added each day	cells were added each day
Medium change	Medium renewal 3 times a week	idem	idem
Endpoints	3 times a week the population was counted in 3 size classes, representative for different life stages separated by sieving with different mesh sizes (adults: ≥2.2mm, juveniles: ≥1.4mm and <2mm, neonates: <1.1mm).	Daily record of survival of adults and number of neonates produced	3 times a week the population was counted in 3 size classes, representative for different life stages separated by sieving with different mesh sizes (adults: ≥2mm, juveniles: ≥1.1mm and <2mm, neonates: <1.1mm).
Duration	42 days	21 days	68 days
Temperature	20°C	15°C, 19°C, 23°C	Weekly increase of 1°C under control conditions (15-19°C) and global change predictions (19-23°C)
Artificial predation	none	Juveniles removed 3 times a week (at medium renewal)	50% removal across each of the 3 size classes every two weeks

6.2.2 Description of the original DEB-IBM model

In this chapter the same DEB-IBM published by Martin and colleagues was used (Martin et al., 2013a). It has eight 'scaled' parameters, with two additional parameters for the aging sub-model (ageing parameters), two parameters for the feeding sub-model (prey dynamics parameters) and three *D. magna* specific parameters (Appendix D Tables D.1 and D.2) (Martin et al., 2013a). A full description of the DEB-IBM implemented by Martin, following the ODD protocol for describing IBMs (Grimm et al., 2006), the user manual, and the NetLogo file of DEB-IBM can be accessed online (https://popecology.wordpress.com/deb-ibm/). In the DEB framework each individual is characterized by four primary state variables (referred to as "DEB state variables") that describe the energy content of four different compartments: "structure", which determines the actual size, feeding rates, and maintenance costs; "reserves", which serve as a buffer between energy flow from feeding to metabolic processes; "maturity", which is a continuous state variable governing transitions between three development stages (embryo, neonate, adult) at fixed maturity levels, and a "reproduction buffer", into which mature individuals (i.e. adults) direct energy for reproduction. A schematic design of the energy flow at the level of the individual can be consulted Figure 6.2.

DEB theory use differential equations to describe how the energy from food is used at the individual level for physiological processes such as maintenance, growth and reproduction (Nisbet et al., 2000). For in-depth reviews of DEB the following papers are a good starting point (Jager et al., 2014, van der Meer, 2006, Ananthasubramaniam et al., 2015, Sousa et al., 2010). In summary, food is ingested by the individual (via the feeding flux J_X) and then assimilated (via the assimilation flux J_A) to fuel different metabolic processes. Embryos do not assimilate food but consume their egg buffer W_B until birth. A fraction K of the assimilation flux J_A is used for maintenance (J_M) and for somatic growth (J_V) of the structural biomass W_V. A fraction $1 - K$ of the assimilation flux J_A is used by juveniles for maturation. After puberty, this $1 - K$ fraction of the assimilation flux J_A describes the mass flux J_R towards the reproduction buffer W_R. General DEB theory makes no assumptions on how the reproduction buffer is converted into offspring, as too many reproductive strategies exist. In the specific case of *D. magna*, the energy in the reproduction buffer is converted into embryos during discrete reproductive events as daphnids reproduce in clutches (i.e. broods). Energy allocated to the reproduction buffer is accumulated over one molt and the energy content per egg is assumed to be fixed throughout the life cycle. The

number of eggs is determined by the size of the reproduction buffer and by the mass per egg. The embryos develop during the next molt, and hatch at the end of that molting period (Martin et al., 2013a). At reproduction events, the available reproduction buffer is converted to eggs.

We started off by using the same DEB parameters as Martin et al. (2013a) (without modifications for our *D. magna* clone) and calibrated the DEB-IBM model, using the standard ecotoxicity data set obtained in chapter 4. We then use this calibrated model to make *a priori* simulations of population abundance under time-variable conditions. The population experiment data from chapter 5 was compared to the model predictions to validate DEB-IBM. What follows is a brief overview of the DEB-IBM and how it was adjusted and modified to the purpose of this research.

6.2.3 Calibration of the original DEB-IBM model

The original DEB-IBM (Martin et al., 2013a) model was calibrated using the control data on total reproduction after 21 days (no Cu or MC added) from chapter 4, which was available for two food concentrations (0.8 and 2mg C/L) and three temperatures (15, 19 and 23°C). It was calibrated on the basis of differences in the experimental design (see Table 6.2). 3 specific adjustments were made: (1) the adjustment of temperature to include the effect of the different time-variable temperatures, (2) the adjustment of feeding related parameters to account for the different algal species and *D. magna* clone (3) the estimation of the stress level for two potential PMoAs (feeding and maintenance). See Appendix D Tables D.1 and D.2 for a complete list of the DEB-IBM model parameters and which parameters are affected by the correction factors.

6.2.4 Arrhenius factor as temperature adjustment

All metabolic rates depend on the body temperature. As an ectotherm, the body temperature of daphnids matches that of its environment closely. As all rates in the DEB-IBM, as well as the time between molts depend on temperature, a temperature correction was used. A well-established method to account for temperature within the homeostatic range for *D. magna* is the Arrhenius relationship (Kooijman, 2010, Rinke and Vijverberg, 2005). The Arrhenius temperature is species-specific and has

been determined for *D. magna* using the von Bertelanffy growth (1957) (= 6400 °K) (Kooijman, 2010, Kooijman, 1988). The Arrhenius law predicts that the natural logarithm of mass-corrected metabolic rates is a linear function of the inverse absolute temperature. To implement the effect of temperature in the model, all the rates that are temperature dependent were multiplied by a temperature adjustment factor (Arrhenius factor, *kT*), based on the Arrhenius relationship (Kooijman, 2010, Kooijman, 1988) to calibrate:

$$k(T) = k_1 \exp\{\frac{T_A}{T_1} - \frac{T_A}{T}\} \qquad \text{(Equation 6.1)}$$
$$T_A = 6400 \text{ K}; \quad T_1 = 293 \text{ K}$$

Where $k(T)$ is the Arrhenius factor at temperature T (°K), k_1 is the Arrhenius factor at the standard temperature and is set to 1 (no temperature correction at 20°C), T_A is the Arrhenius temperature (in Kelvin), T is the absolute temperature (in Kelvin, = 293°K), T_1 is the chosen reference temperature (in Kelvin).

6.2.5 Adjustment of feeding related parameters

In the original DEB-IBM, algae were administered as number of *Desmodesmus subspicatus* cells per volume and the cell number was equated to mg C, assuming that *D. subspicatus* has an average carbon content of 1.95×10^{-8} mgC cell^{-1} (Sokull-Kluettgen, 1998; unpublished recent results from the laboratory of the Institute of Environmental Research, RWTH Aachen University in Martin et al. 2013). Although we used a different food source (*Pseudokircheriella subcapitata*) than Preuss and colleagues, we initially tried to fit the model by solely expressing it as the equivalent quantity of *D. subspicatus*, as the amount of carbon administered in the test. We fed 2.5 or 5 mg/L *P. supcapitata* based on dry weight (≈ 0.8 or 2mg C/L, knowing that the carbon/dry weight ratio is ~0.4, (Evens et al., 2009 , De Schamphelaere and Janssen, 2004). However the simulated individuals reproduced much more than in our observations (2-10-fold, depending on the treatment). We hypothesized that this divergence was a consequence of using both a different food source and a different clone. Different feeding related parameters have an effect on the food quality and size, as well as handling time by the daphnid. Assimilation is the remaining energy fixed into reserves, after removal of the energy lost during

digestion. By assuming that assimilation efficiency is independent of feeding rate (as it depends on a diet specific parameter), assimilation rate becomes proportional to ingestion rate, which has been experimentally demonstrated (Kooijman, 2010). The half-saturation constant can be understood in terms of species-specific physiological characteristics of the consumers in response to their specific food source (e.g. handling and digestion time). Differences in the half-saturation constant have been reported within one order of magnitude for *D. magna* clones of the same size, while even higher difference can be expected among *D. magna* clones differing in body size (Mulder and Hendricks, 2014). We therefore re-calibrated the model by optimizing the maximum ingestion rate (J_{XAm}) (to the same extent as the maximum assimilation rate (J_{Am}), which is scaled out of the model) and the half-saturation constant *K*. As feeding rates vary with temperature and total food concentration, the complete data set (three temperatures and two total food concentrations) was used for the optimization procedure (Yurista, 1999). Optimization was done by randomized sampling (10,000 iterations) and selection of the best parameter combination using the simulation with lowest SSE compared to the control observations.

6.2.6 Stress level determination

At the individual level only sub-lethal effects were considered. Toxicants, once taken up by an individual, are assumed to affect one or more physiological processes. According to DEB theory, stress will affect one or more individual parameters (dependent on the mode of action of the stress), which in turn alters life-history output over time. Thus the type of sub-lethal effect invoked by a toxicant depends both on the physiological process (which parameter is affected), and the magnitude of the effect on that parameter. Martin et al. (2013) referred to effects on different parameters as different ‘‘Physiological Modes of Action’’ (PMoA). The 5 different PMoAs identified are depicted in Figure 6.2 and how they affect the parameters is illustrated in Table 6.2. The stress levels fitted to the individual data were used for the simulations at the population level. Given that we observed negative effects of both copper and *M. aeruginosa* on daphnid length in the life table data (see Appendix D Figures D.1 and D.2), we could *a priori* exclude reproduction as a PMoA, as this PMoA doesn’t simulate a change in growth in response to stress (see illustration of the effect on length over time in Figure 6.2) Further, growth was excluded as a potential PMoA *a posteriori,* on the basis of the simulation results (shown in Appendix D Figure

D.3 and not further discussed in this chapter). Simulations using growth as PMoA significantly overestimated the combined effects compared to the other 2 PMoAs (growth PMoA predicted the populations to already go extinct in the 25µg Cu/L+MC treatment and die out instantly in all other mixture treatments).

Given that the results of chapter 2 and chapter 4 suggest that the Concentration Addition (CA) reference model delivers the most accurate predictions of the combined toxicity of copper and *M. aeruginosa*, we decided to convert the concentrations of the two stressors to their toxic units relative to their EC_{50} values obtained in chapter 4. In line with the CA model, we used the sum of the Toxic Units (TU) of copper and *M. aeruginosa* (obtained from the single dose response curves) to fit the stress level to each TU combination using a hockey stick regression:

$$Stress = \begin{cases} a \cdot (\sum TU) + b, & \text{if } \sum TU > -b/a \\ 0, & \text{if } \sum TU \leq -b/a \end{cases} \quad \textbf{(Equation 6.2)}$$

Where *a* is the slope and *b* the intercept of the hockey stick relationship between the stress level and the sum of the toxic units (∑TU). The complete data set from chapter 4 was used in order to account for a temperature and food density effect. Based on trial simulations and the knowledge of the range of the (∑TU) in the life table experiment, we allowed both parameters to be sampled from the following ranges: $0 < a < 10$ and $-2 < b < 0$.

Optimization of the stress levels per PMoA was done by randomized sampling (1000 itineration) and selection of the best parameter combination using the simulation with the lowest SSE compared to the control observations. The conversion of the sum of the toxic units to stress level can be accessed in the Appendix D (Tables D.3-D.6).The DEB-IBM framework was implemented in NetLogo (Wilensky, 1999) which was specifically designed for IBMs and has been used before to implement DEB IBMs (Martin et al., 2013a, Martin et al., 2012).

6.3 Results

6.3.1 Model calibration

The assimilation (*assim_optim* = 0.957) and the half-saturation (*K_optim* = 10.58) parameters were optimized for the individual-level DEB-IBM based on the lowest SSE out of 100000 simulations under control conditions for 3 temperatures and 2 total food concentrations (Figure 6.3A). The 10-times higher half-saturation constant is in the realistic range, as differences in the half-saturation constant within one order of magnitude for *D. magna* clones of the same size have been reported (as well as higher difference among *D. magna* clones differing in body size) (Mulder and Hendricks 2014).

The stress level parameter was derived via equation 6.1 using the entire data set by converting the concentrations of copper and *M. aeruginosa* to their toxic units (as a common denominator). Using the CA reference model, the assumption was made that the toxic units of the components of the mixture treatments could be summed up. Figure 6.3B and Figure 6.3C show the predicted and the observed reproductions using the best parameter set (lowest SSE) for the feeding and maintenance PMoAs, respectively. Figure 6.4A and Figure 6.4B show the hockey stick regression for the feeding and maintenance PMoAs, respectively. The stress levels corresponding to the single and mixture treatment toxic units per PMoA can be accessed in the Appendix D (Tables D.3-D.6).

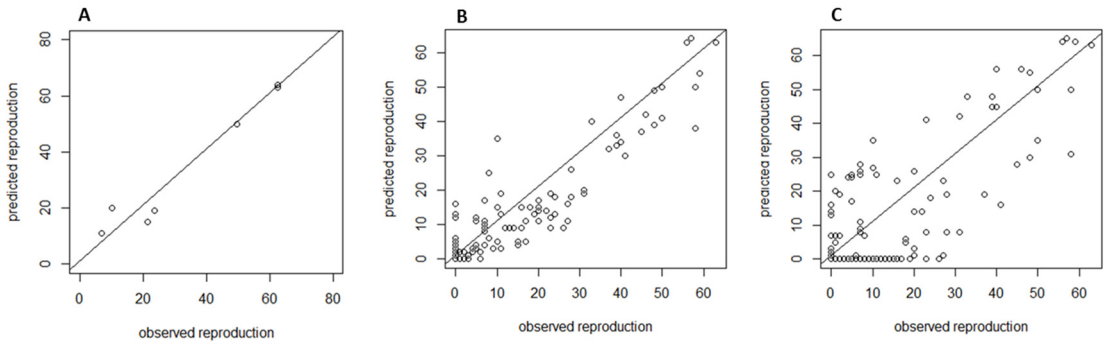


Figure 6.3. Parameter optimization results. A: The best parameter set (*assim_cor* = 0.957*, K_cor* = 10.58), based on the lowest SSE out of 10000 simulations to correct for a different food source, was used to plot the predicted against the observed reproduction is plotted. B: The best parameter set for the feeding PMoA (*a* = 0.516, *b* = -0.195) based on the lowest SSE out of 1000 simulations was used to plot the predicted against the observed reproductions. Best parameter set (0.95702, 10.5789) is plotted. **C**: The best parameter set for the feeding PMoA (*a*= 4.633, *b*= -1.377) based on the lowest SSE out of 1000 simulations was use to plot the predicted against the observed reproductions.

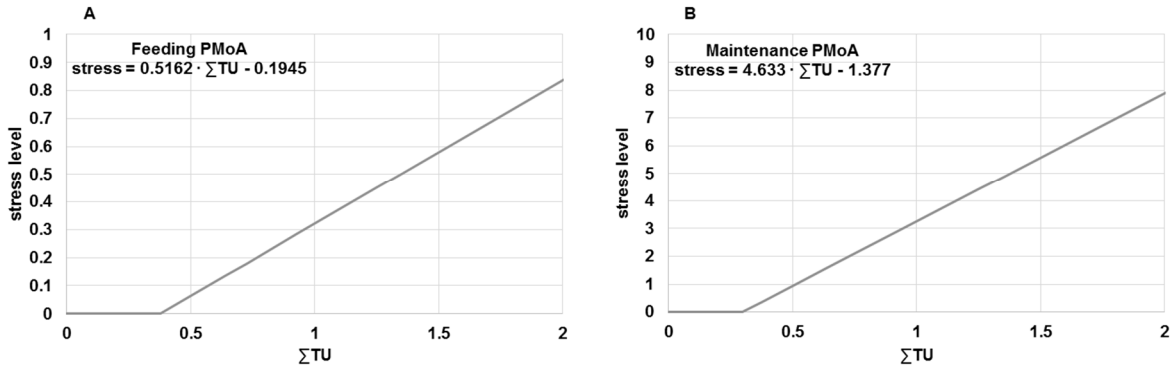


Figure 6.4. Hockey stick regression of the stress level in function of the sum of the toxic units using Equation 6.2. A: feeding as PMoA. B: maintenance as PMoA.

6.3.2 Individual level predictions

The predicted and the observed endpoints (total reproduction after 21 days, age at first reproduction, and length after 21 days) under control conditions are shown in Figure 6.5. For both total food concentrations (0.8 and 2mg C/L), the temperature trend is correctly captured and the predictions match the observations closely: At the lower food concentration (0.8mg C/L), reproduction increases from 15℃ to 19℃ and then decreases at 23℃ (Figure 6.5A1). At the higher total food concentration (2mg C/L) reproductions increases linearly from 15℃ to 23℃. For the other endpoints the predictions are also in line with the observations: Age at first reproduction decreases linearly with temperature (Figure 6.5A2 and Figure 6.5B2), while length after 21 days remains very similar across temperatures (Figure 6.5A3 and Figure 6.5B3).

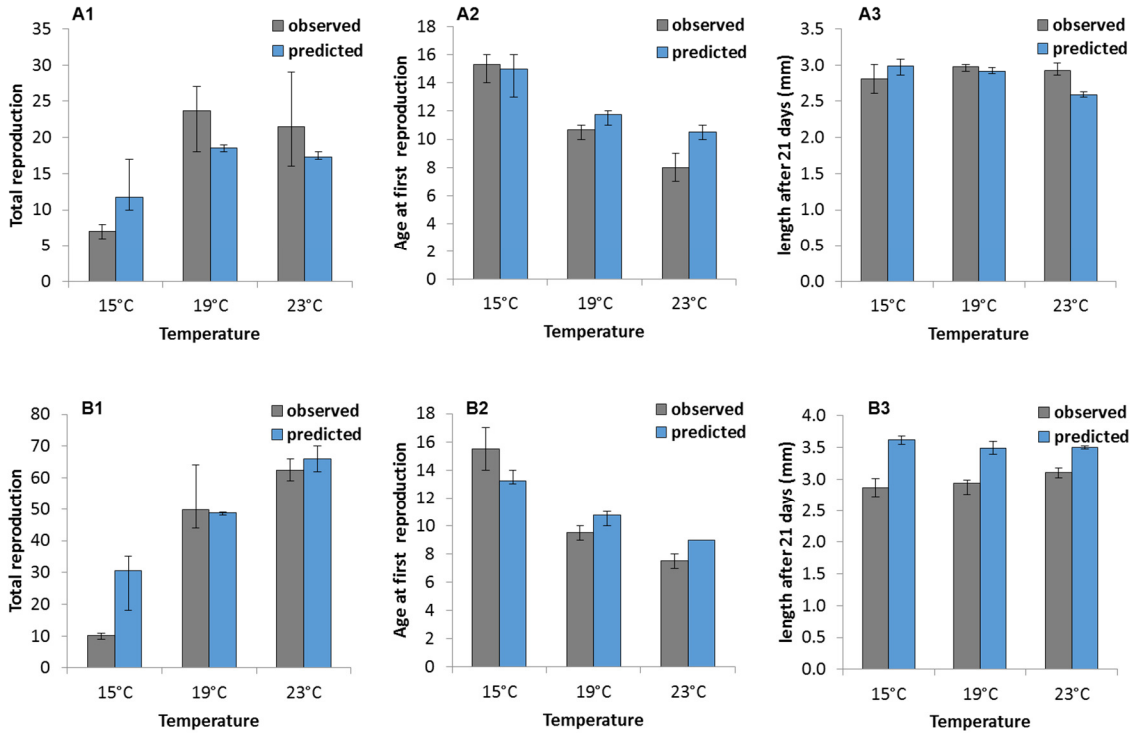


Figure 6.5. Observed (grey bars) and predicted (blue bars) endpoints for the standard ecotoxicity test from chapter 4: 1 = total reproduction, 2 = age at first reproduction, 3 = length after 21 days); at two different total food concentrations: A = 0.8mg C/L and B = 2mg C/L). Observations and predictions are for control conditions only (at 2µg Cu/L = Control, without any *M. aeruginosa* added) and expressed as the mean value. Errors bars indicate the minimum and maximum values (n = 4).

6.3.3 Population level predictions of control conditions

As is shown in Figures 6.6A and C and Figures 6.7A and C, the simulations under control conditions (without added copper or *M. aeruginosa* stress) follow the trend of the observed total population abundance very closely. In both temperature regimes, the peak abundances (+/-100 individuals/L), as well as the total density at the end of the simulations overlapped with the observations. One difference, compared to the observations, is that under the control temperature regime total population density initially increases much faster in the simulations (Figure 6.6A and 6.7A)

6.3.4 Population level predictions under the PMoA feeding

In Figure 6.6 the simulations using feeding as the PMoA for copper and *M. aeruginosa* stress are plotted against the observations. The simulations follow the observed trend, in terms of total abundance over time and total density at the end, for copper stress under both temperature regimes (control: Figures 6.6E and Figure 6.6I, global Change: Figures 6.6G and Figure 6.6K). Under the global change temperature regime (Figure 6.6D) control copper and *M. aeruginosa* stress, population density at the end of the simulations also matches that of the observations, while the total density at the end of the test is over predicted by a factor 2 (100 vs. 50 individuals/L) by the simulations under the control temperature regime (Figure 6.6B). At the lower copper concentration (25µg Cu/L), the simulations of the combined effects of copper and *M. aeruginosa* are in line with the observations (Figures 6.6F and Figure 6.6H). The only exception is that under the global change temperature regime, total density at the end of the experiment was overpredicted by a factor 4 (100 vs. 25 individuals/L) by the simulations (Figure 6.6H). The greatest discrepancy between predictions and observations is noted for combined effects at the highest copper concentration (44µg Cu/L) under the current climate regime (Figure 6.6J). While near extinction was observed in the experiment, simulations predict a stable population density fluctuating between 50 and 100 individuals/L. Under the global change regime (Figure 6.6L), the total abundance was 3 or less individuals and populations went extinct after 54 days, while in the simulations the total density only declines toward extinction after 64 days.

6.3.5 Population level predictions under the PMoA maintenance

In Figure 6.7 the simulations using maintenance as the PMoA for copper and *M. aeruginosa* stress are plotted against the observations. Under single exposures with copper, the simulations also followed the observed trend, similarly to the simulations using feeding as PMoA (Control: Figures 6.7E and Figure 6.7I, Global Change: Figures 6.7G and Figure 6.7K). Under single exposures with *M. aeruginosa* the trend at the end of the experiment was reversed when maintenance was implemented as PMoA. Under the control temperature regime (Figure 6.7B) control copper and *M. aeruginosa* stress, population density at the end of the simulations also matches that of the observations, while the total density at the end of the test was underpredicted by a factor 3 (30 vs. 100 individuals/L) by the simulations under the global change temperature regime (Figure 6.7D). At the lower copper concentration (25µg Cu/L), the

simulations of the combined effects of copper and are *M. aeruginosa* are over-estimating the observed effects (Figures 6.7F and Figure 6.7H). Under the control temperature regime, total density at the end of the experiment is underpredicted by a factor 3.5 (25 vs. 85 individuals/L) by the simulations (Figure 6.7F). Under the global change temperature regime, the population is predicted to go extinct after 28 days, while the observed total density only declines towards the end of the experiment (Figure 6.7H). At the higher copper concentration (44µg Cu/L), the predictions of the combined effects of both stressors match the observations better than the feeding PMoA (Figure 6.7J and Figure 6.7L). For both temperature regimes, not only is the initial increase captured well but also the decline phase and the (near-)extinction.

As is shown in Figure 6.8, the predictions follow the trend of the observed total population density very closely. In both temperature regimes and PMoAs, the difference is mostly less than 50%, with a few exceptions. Using feeding as the PMoA, predictions are much higher (+100%) than observations in mixtures with high copper (44µg Cu/L) under both temperature regimes. (Figures 6.8A and 6.8B). Using maintenance as the PMoA, predictions are much lower (-150%) than observations in mixtures with high copper (44µg Cu/L) under both temperature regimes. (Figures 6.8C and 6.8D).

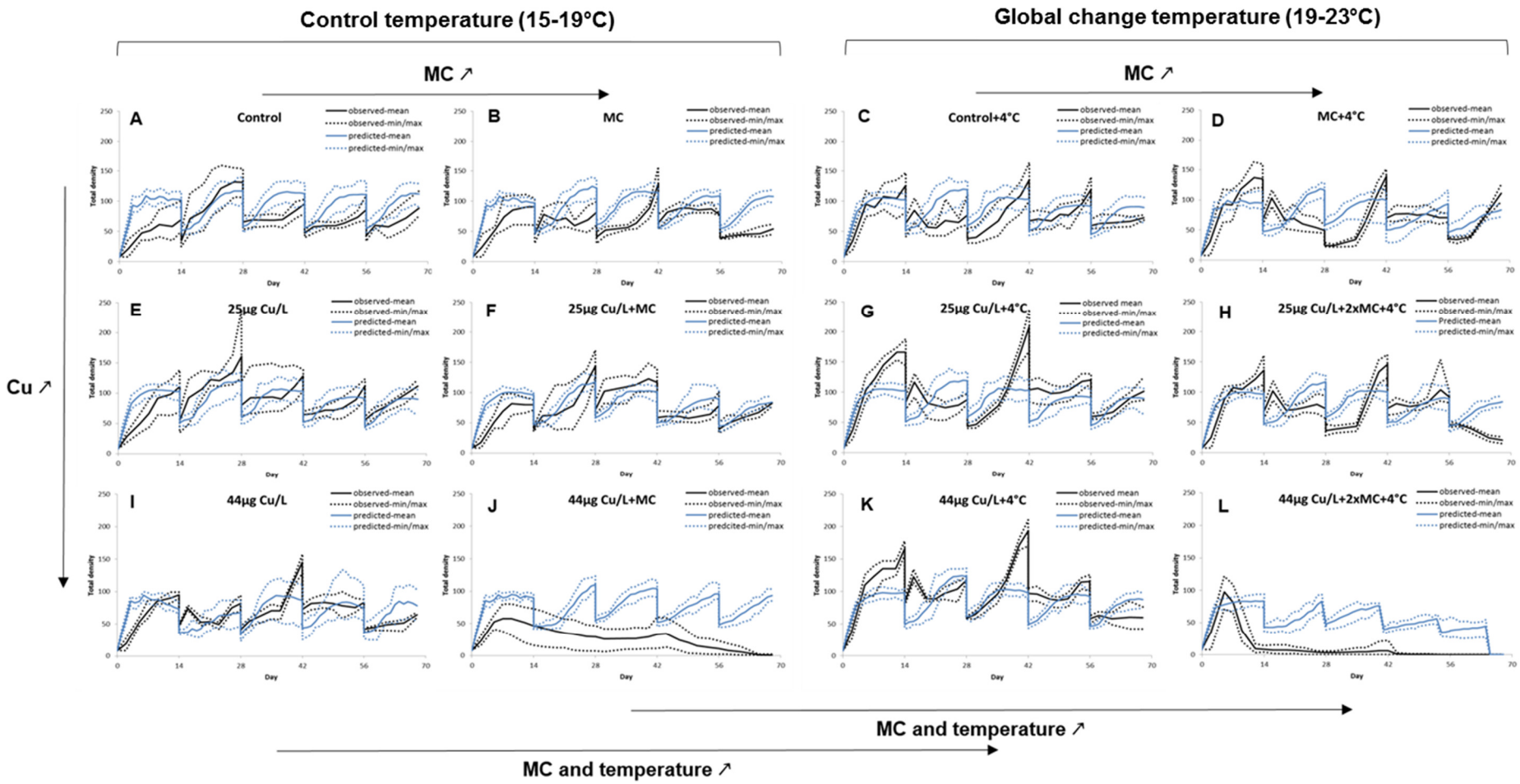


Figure 6.6. Observed and predicted population dynamics for *D. magna* under copper (Cu) and *M. aeruginosa* (MC) stress using the feeding PMoA. Full lines indicate mean total abundance (observations: = black, predictions = blue), while the dotted lines indicate the minimum and maximum observations (n = 4) and predictions (n = 10). Arrows indicate increasing levels of stress. Please note that Figures 6.6A and 6.7A are identical, as well as Figures 6.6C and 6.7D.

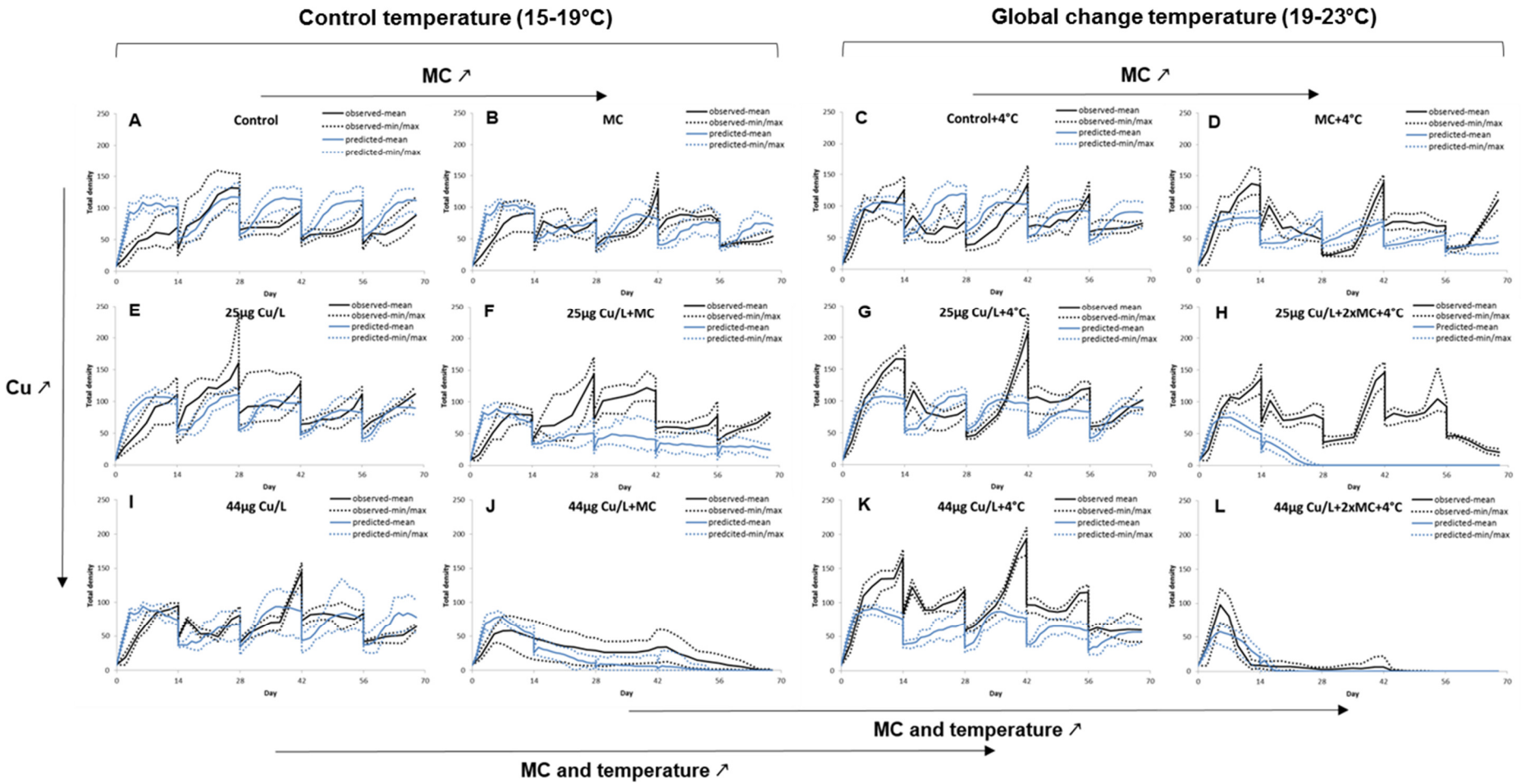


Figure 6.7. Observed and predicted population dynamics for *D. magna* under copper (Cu) and *M. aeruginosa* (MC) stress using the maintenance PMoA. Full lines indicate mean total abundance (observations: = black, predictions = blue), while the dotted lines indicate the minimum and maximum observations (n = 4,) and predictions (n = 10). Arrows indicate increasing levels of stress. Please note that Figures 6.6A and 6.7A are identical, as well as Figures 6.6C and 6.7D.

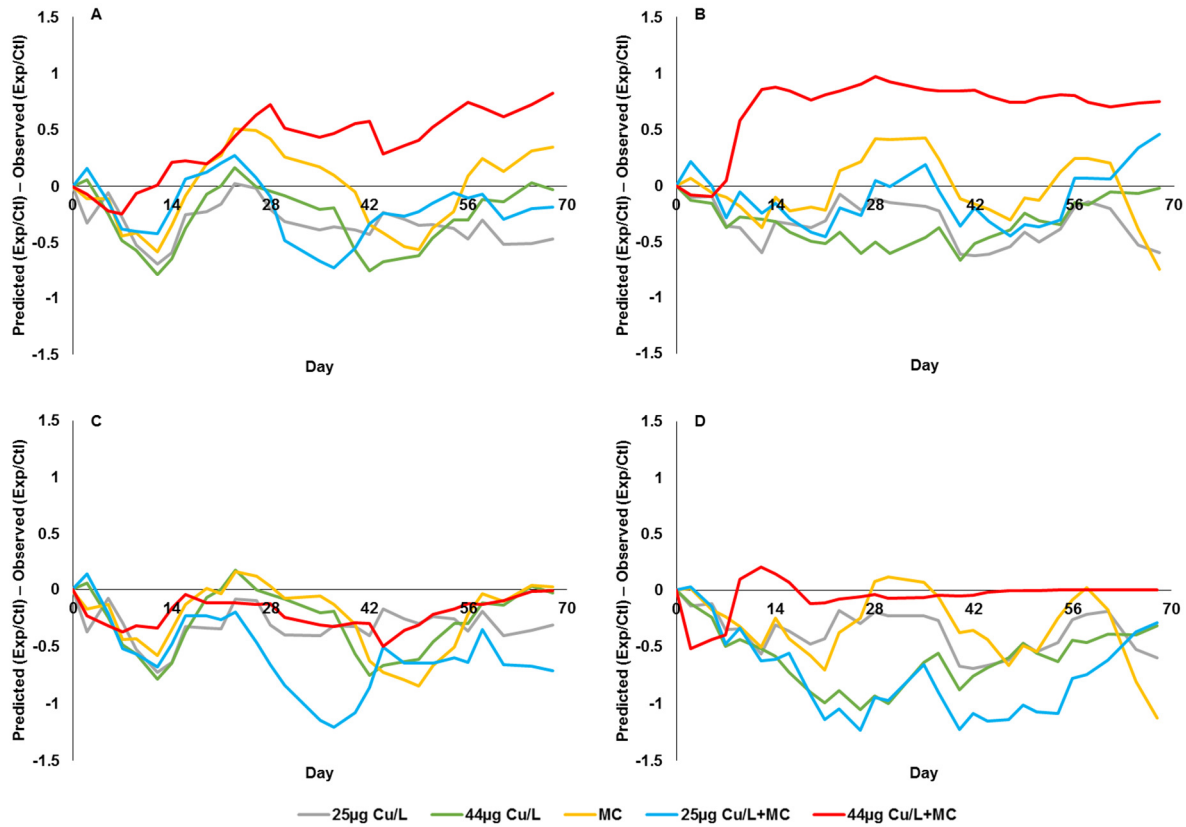


Figure 6.8. Difference between the predicted and the observed values of total population density over the duration of the experiment. For both, the predicted and the observed values of total abundance, the relative abundance was used as a ratio of the exposed density over the control density (Exp/Ctl). **A**: Control temperature (15-19℃) using feeding as the PMoA. **B**: Global change temperature (19-23℃) using feeding as the PMoA. **C**: Control temperature (15-19℃) using maintenance as the PMoA. **D**: Global change temperature (19-23℃) using maintenance as the PMoA

6.4 Discussion

DEB models are often criticized for their complexity and their inherent requirements for additional experiments (beyond the data acquired in standard toxicity tests) to obtain species (or clone) – specific DEB parameters. Our aim was to test the hypothesis that reproduction measured at the individual level under constant conditions could be extrapolated to total density under more realistic scenarios at the population level. The results from this chapter have shown that the combination of standard ecotoxicity endpoints and an existing DEB-IBM model with minimal calibration is able to deliver reasonable predictions for the combined effects of copper and *M. aeruginosa* on population abundance under global

change conditions. In this chapter a different *Daphnia magna* clone, as well as a different algal species as food source were used. Despite this difference, both the individual and the population level predictions matched our observed data closely, using an existing model with minimal calibration. The modelling predictions from chapter 6 also illustrate that the DEB-IBM is applicable for mixtures that follow the CA. In chapters 2 and 4 it was previously demonstrated that the Concentration Addition (CA) reference model delivers accurate predictions for the combined effects were copper and *M. aeruginosa*.

There were a few inconsistencies in the model predictions, which beyond model uncertainty, can also be ascribed to the quality of the data set used for fitting the DEB-IBM. For instance total reproduction at 15℃ was overpredicted by the model for both total food concentrations (Figure 6.4), which is also reflected in the overpredictions of the model of the intial population density peak at the control temperature (15℃ at the start, Figures 6.6A and 6.7A). In chapters 4 and 5 we reported that individuals were acclimated for 2 generations to the test tempertaures before the start of the expsoures. However, it is possible that acclimation time before the start of the experiment was too short. Further, recovery after artificial predation events was considerably faster in simulations compared to the observed total population density. This could potentially by a result of the handling stress inflicted on the individuals during these events, which is not captured in the model (Rousseaux et al., 2010).

DEB is a natural choice for the extrapolation of toxic effects from the individual to the population level, as it is based on the mechanistic understanding of growth and reproduction of individual. Toxicity was described as the impairment of one of different physiological modes of action (PMoA), which affect survival, assimilation, growth, maintenance and reproduction (Kooijman, 2010, Jager et al., 2014, Jager and Zimmer, 2012). Both PMoAs deliver accurate predictions of population density for the single stressor effects. For the mixture treatments, different outcomes are predicted depending on the PMoA. When feeding is used as PMoA, mixtures with the lower copper concentration (25µg Cu/L) and *M. aeruginosa* are accurately predicted under both temperature regimes. However, the observed toxicity is underpredicted in mixtures with the higher copper concentration (44µg cu/L) and *M. aeruginosa* under both temperature regimes. Conversely, when maintenance is used as PMoA, the combined effects were overpredicted at the higher copper concentration (44µg cu/L), while simulations of the mixtures with the lower copper concentration (25µg Cu/L) and *M. aeruginosa* are accurately predicted under both

temperature regimes. This is an interesting finding, as it suggests a switch of dominant copper toxicity mechanism between 25µg Cu/L (maintenance) and 44µg Cu/L (feeding) that contributes most to the mixture effect.

Based on the knowledge of the potential modes of action for copper and *M. aerugiosa* both PMoAs are possible. In *Daphnia*, copper toxicity has been linked to inhibition of active sodium uptake (De Schamphelaere et al., 2007), inhibition of neuronal signal transmission and acetylcholinesterase (AChE) activity (Untersteiner et al., 2003), and oxidative stress (Barata et al., 2005, Xie et al., 2006). While it is well-known that cyanobacteria reduce the fitness of *Daphnia* sp., there appears to be no general concensus in the literature about the main mechanisms underlying this effect. Effects have mainly been associated with four factors or a combination thereof: cyanobacterial toxins (e.g. microcystins, cylindrospermopsins) (Rohrlack et al., 1999, Nogueira et al., 2004, Dao et al., 2010, Demott et al., 1991), feeding inhibition (Lurling, 2003 , Demott et al., 1991), morphology (Wilson et al., 2006, DeMott et al., 2001) and the lack of essential nutrients (Martin-Creuzburg and von Elert, 2009). Although cyanotoxins exhibit high toxicity to vertebrates, including mammals (Wiegand and Pflugmacher, 2005), several studies have reported no significant differences between the effects of cyanotoxin producing and non-toxin producing cyanobacteria on zooplankton, albeit such studies have mainly focused on *Microcystis* (Tillmanns et al., 2008, Wilson et al., 2006).

From what is known about the modes of action of copper and *M. aeruginosa*, both PMOAs are equally likely, or even a combination of the two. In this DEB-IBM the same assumption that both maturity and somatic maintenance costs are both equally affected was used, however effects on each parameter independently are also possible. Stressors can thus have a wide range of effects on individuals and their physiology, and it is hence difficult to discern which modes of action are relevant for a specific chemical The simulations depicted in Figure 6.2 illustrate that it is very difficult to discern the effects of the different PMoAs on growth and even more so on reproduction (even for stress levels equivalent to the 21day EC_{50}). It may therefore also be necessary to include additional endpoints (e.g. feeding and metabolic rates). To identify the actual physiological mode of action of a chemical, ideally full life-cycle studies are used (Jager and Zimmer, 2012). Ecotoxicology urgently needs robust tools that identify the mechanistic basis of measured endpoints to (i) interpret them in an ecological way and (ii) translate

them to ESQs in ERA. In support of mode of action based risk assessment, the concept of adverse outcome pathways (AOPs) has been proposed as a framework to organize and communicate the existing knowledge on the toxicity mechanisms and outcomes across levels of biological organization (Ankley et al., 2010). An AOP is a sequence of events from the first critical molecular event (known as the molecular initiating event or MIE) to an in vivo adverse outcome (AO) (Ankley et al., 2008).

Although both PMoAs investigated in this chapter delivered reasonably good fits, the maintenance PMoA provided the most protective predictions in terms of extinction probability (Figures 6.6 and 6.7). Without any in depth knowledge of the mechanistic basis of the combined effects of copper and *M. aeruginosa*, two approaches can be followed. Firstly the feeding PMoA could be applied when the known copper contamination is equal to or lower than 25µg Cu/L, while the maintenance PMoA could be applied when the known copper contamination is higher than 25µg Cu/L. Alternatively, as a worst case scenario approach, the maintenance PMoA should be selected, as it either delivers accurate or overprotective predictions, while the feeding PMoA is underprotective in mixtures of copper at the 21 day NOEC and *M. aeruginosa*.

If multiple stressors have interactive effects that are not predictable from single stressor impacts (e.g. ecological surprises, *sensu* Paine et al. 1998), a major source of uncertainty is added to projections of biodiversity (Sala et al., 2000) and ecosystem resilience (Folke et al., 2004). Furthermore, if drivers of global change interact synergistically, predictions based on additive expectations may underestimate the ecological impacts of global environmental change (Hoffman et al., 2003, Przeslawski, 2005). The results from this chapter demonstrate that, if maintenance was selected as PMoA, the extinction probability of the population could have accurately been predicted by the DEB-IBM. In other words, an ecological surprise could have been forecasted based on the calibrated model presented here.

7

Microevolutionary effects of copper and zinc in a natural *Daphnia magna* population

Redrafted after:

Jennifer D Hochmuth, Luc De Meester, Cec ília M Pereira, Colin R Janssen, Karel AC De Schamphelaere. 2015. Rapid adaptation of a *Daphnia magna* population to metal stress is associated with heterozygote excess. Environmental Science & Technology 49: 9298-9307.

7.1 Introduction

Conventional risk assessment of chemicals is usually based on ecotoxicity tests using laboratory populations with limited genetic variation, often even monoclonal populations, with exposure times rarely exceeding one generation (Baird, 1992 , Forbes and Depledge, 1992). However, natural populations are typically characterized by genetically distinct individuals, which may give rise to considerable genetic variability in tolerance to chemical stress within populations. The genetic variability in life-history traits under stress within a population sets the scope for microevolutionary responses under exposure to that stress (Klerks et al., 2011 , Messiaen et al., 2013). Microevolution can be defined as the change in allele frequencies that occurs over time within a population. This change can be due to four different processes: natural selection, mutation, gene flow, and genetic drift (Hartl and Clark, 1980). Through the process of directional selection (i.e. more tolerant individuals are favoured over less tolerant ones), populations can genetically adapt to chemical stress, which may protect them from local extinction (Van Straalen and Timmermans, 2002, Medina et al., 2007 , Agra et al., 2010 , Agra et al., 2011).

Because selection by the presence of chemicals eliminates out less tolerant genetic combinations, it is expected to result in the loss of genetic variation. In cyclical parthenogens like the water flea *Daphnia,* a key model species in ecotoxicological testing, this translates into a reduction in clonal diversity in the population, as less tolerant clonal lines (genotypes) become less frequent. Selection may also induce the loss of allelic diversity, which has been termed 'genetic erosion' (Van Straalen and Timmermans, 2002). Conversely the opposite may be possible too, referred to as 'balancing selection', as an increase of genetic diversity was observed under unfavourable conditions (Hoffmann and Merila, 1999).An additional cost of selection may involve that populations adapted to a given chemical stressor are less tolerant to novel stressors or have lower fitness in the absence of the stressor compared to non-adapted populations (Van Straalen and Timmermans, 2002, Medina et al., 2007 , Agra et al., 2010). For instance, a 3-month selection under carbaryl exposure lead to reduced sensitivity to the pesticide but in an increased susceptibility to parasite infection in *Daphnia magna* populations (Jansen et al., 2011 -a). Conversely, increased tolerance to novel stressors may also arise, particularly to a related stressor with similar modes of action. This 'cross-tolerance' has been reported before for metals in *Daphnia*: for cadmium and lead (Ward and Robinson, 2005) and for copper and zinc (Lopes et al., 2005).

Experimental evolution allows to study the genetic responses of populations to selection pressures under standardized conditions and thus to investigate whether populations are able to adapt to a given stressor (Conner, 2003). Such experiments are, however, rarely used in ecotoxicology. One study observed a 3-fold increase of the mean 48h-LC50 for cadmium over 8 generations in a laboratory population of *Daphnia magna* initially composed of 8 clones (Ward and Robinson, 2005). A limitation of this study was that the design did not include several generations of removing maternal effects so as to establish genetic adaptation as the sole reason for the observed increased tolerance to cadmium. To discriminate genetic adaptation from physiological acclimation and maternal effects, any difference in population level tolerance to a stressor (comparison of tolerance to a same stressor before and after selection) needs to remain significant after several generations under conditions without that stressor present (Klerks and Levinton, 1989, Walker, 2001).

The main aim of this chapter was to determine whether microevolutionary responses (genetic adaptation) and complete recovery of population densities are possible in a natural *D. magna* population under copper and zinc stress and whether this genetic adaptation has potential consequences for the tolerance to novel stressors (i.e. cost of adaption vs. cross-tolerance). Our study consisted of two phases (Conner, 2003 , van Doorslaer et al., 2009 -b) (Jansen et al., 2011 -b). First a 10 week selection experiment was carried out using the standing genetic variation present in a natural *D. magna* population exposed to 70 µg Cu/L and 630 µg Zn/L (dissolved concentrations equivalent to the 8-day LC50) as a selection pressure. The focus of our study was on the clonal selection phase, under otherwise favourable conditions (i.e. the spring to fall growth cycle). A common garden experiment was then carried to evaluate the degree of genetic differentiation between the non-selected 'original' population and the experimentally selected populations under 'control' and metal conditions ('Cu' and 'Zn'). We further investigated the tolerance to novel stressors (temperature, cyanobacteria and cadmium) in a follow-up study with the Zn selected populations and used micro-satellite genotyping to determine the potential consequences of selection on clonal and allelic diversity.

In this chapter, 3 research questions with potential implications for ecological risk assessment were assessed:

(1) Can a genetically diverse natural *D. magna* population adapt to lethal metal concentrations (equivalent to the 8-day LC50)?

(2) Do populations display lower genetic diversity after adaptation than prior to selection?

(3) Is adaptation to chemical stressors associated with costs of adaptation?

7.2 Materials and Methods

7.2.1 Establishment and maintenance of the experimental population

For the microevolution experiment we used a natural *D. magna* population established from ephippia collected from a temporal pond in the vicinity of Knokke (West Flanders, Belgium, 51° 21' 01.97"N, 03° 19' 49.58"E). The upper 5cm layer of sediment was sampled in several locations of the pond using a Van Veen grab sampler. In the laboratory the pooled sediment was sieved over 500 µm. More than 200 ephippia of *D. magna* were identified (Vandekerkhove and J.M., 2004) and placed individually in polyethylene vessel containing 50mL of tap water at either 20℃ or 23℃ under a 16 h light : 8 h dark photoperiod. Immediately upon hatching a single hatchling from each ephippium (in case both eggs of an ephippium hatched one hatchling was selected at random) was used to establish a clonal lineage. As ephippial eggs of *D. magna* are produced by sexual reproduction (Ebert et al., 1993), each clonal lineage can be considered genetically distinct (Barata et al., 2000). Juvenile hatchlings were placed individually in a 50 mL polyethylene cup filled with modified M4 medium (Hochmuth, 2014) and kept at 20℃ and under a 16h:8h light:dark cycle. Daphnids were fed daily 5 mg dry weight of *Pseudokirchnerialla subcapitata* per litre and the culture medium was refreshed 3 times a week. A total of 7 offspring of the 3rd brood of the hatchlings were transferred to 1L glass jars and culture medium was refreshed once a week. With every medium renewal, the next generation of each clone was established by randomly picking 4 juveniles and 3 adult daphnids (daphnids carrying eggs in the brood pouch) of the previous generation. As ephippial eggs of *D. magna* are produced by sexual reproduction (Ebert et al., 1993), each clone can be as considered genetically distinct (Barata et al., 2000). The microevolution experiment consisted of 3 discrete test phases (selection experiment, de-acclimation phase and common garden experiments).

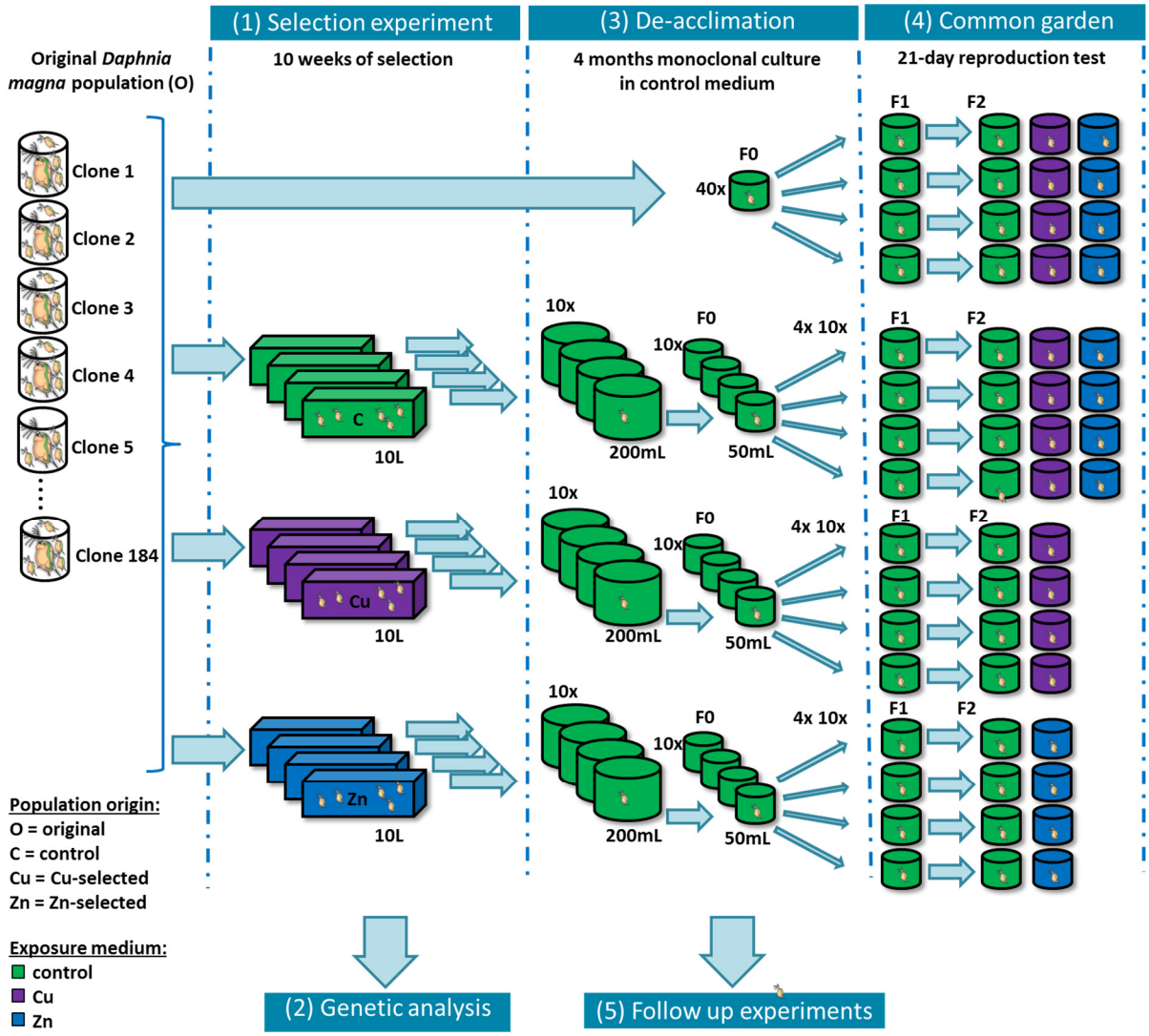


Figure 7.1 Overview of experimental design in this chapter. (1) Selection experiment: 184 clonal lines (< 48 hours-old) were inocculated in 10L aquaria for 10 weeks under either control, Cu or Zn selection (2) Genetic analysis: At the end of the selection experiment 20 clones per aquarium 40 clonal lines from the original population were genotyped using 12 microsatellite markers to test for effects on genetic diversity. (3) De-acclimation: 10 clones per aquarium (i.e. 40 per selection treatment) were picked at random and cultured under monclonal conditions in 200mL vessels in control medium for 4 months. Thereafter one juvenile (< 24 hours-old) per clone was placed in a 50mL vessel (F0) and 4 juveniles from the third clutch were used from each F0 clone to initiate the F1 generation. (4) Common garden: For the common garden experiment, 1 juvenile (F2-generation, < 24 hours-old) from the third clutch of each F1 mother was assigned to control, Cu or Zn expsoure medium, while per exposure medium (control, Cu or Zn) each replicate originated from a different mother. (5) Follow up experiments: Additionally tolerance to temperature, cyanobacteria and cadmium stress was assessed.

7.2.2 Selection experiment

We conducted a selection experiment with 3 treatments: control, copper (Cu) and zinc (Zn). The concentrations used for the Cu and Zn treatments were based on pilot assessments and correspond to the 8-day LC50 concentrations (70 µg Cu/L and 630 µg Zn/L) (Figure 7.1). Each treatment was replicated 4 times (i.e. 4 aquaria per treatment), resulting in a total of 12 aquaria. A set of 184 different clones (one individual/per clone) were inoculated in each 10L aquaria and the exposure duration was 10 weeks. Every day the concentration of *Pseudokirchnerialla subcapitata* in each aquarium was adjusted for each aquarium separately to maintain 2mg dry weight per day per L (≈0.8mg C/L) (Evens et al., 2009 , De Schamphelaere and Janssen, 2004), using a Coulter particle counter (Beckman-Analis, Namur, Belgium). Once per week half the medium was renewed with fresh medium. To avoid population collapses due to overcrowding , starting from the second week, 50% of the population was randomly removed randomly removed in each experimental unit every 2 weeks (as in Van Doorslaer et al. 2009 a). Random culling was achieved by gently mixing the medium in the aquarium with a beaker to distribute the animals evenly before removing 50% of the volume (i.e. 5L). We adopted this culling regime as it avoids population crashes but at the same time results in strong fluctuations in population densities, thereby allowing for episodic exponential growth, which can promote faster replacement of genotypes through time and has previously been shown to result in repeatable microevolutionary responses in a similar experimental evolution trial (Van Doorslaer et al., 2009 -a). Population density (total abundance per 10L aquaria) was recorded after 1, 2, 3, 4, 6 and 8 weeks. Ephippia were removed weekly to ensure that no sexually produced eggs could hatch and contribute to the population

7.2.3 De-acclimation phase

After 10 weeks 10 parthenogenic egg carrying females were randomly collected from each experimental unit and placed individually in 50mL vessels of control modified M4 medium to initiate clonal lines (Figure 7.1). It is important to note that there may be replicate (i.e. identical) clones among these randomly initiated clonal lines. The medium was renewed 3 times a week. Each vessel received a suspension of *P. subcapitata* (equivalent to 5 mg/L dry weight). To eliminate any effects due to acclimation (phenotypic plasticity of individuals) or maternal effects and thus allow a direct test for genetic adaptation (altered allele frequencies in the population) all clonal isolates of the original, control and metal selected

populations were cultured for 4 months (≈14 generations as generation time ≈ 8.5 days) under common garden conditions (control medium) before we conducted the common garden experiments under Cu and Zn exposure (Stoks et al., 2014, Klerks and Levinton, 1989, Walker, 2001). As the individuals were cultured as clonal lines there was no interference from selection among clonal lines in this phase.

7.2.4 Common garden experiments

After the de-acclimation phase of 4 months we conducted a 21-day life-table experiment under common garden conditions to investigate if the populations selected under Cu and Zn had a higher tolerance to Cu and Zn, respectively, than the control populations and the original population (Figure 7.1). We thus investigated whether genetic adaptation to Cu or Zn, measured as an increase in mean population fitness, had occurred. We exposed the original and control populations as well as the Cu-selected or Zn-selected population to the same 8-day LC50 Cu or Zn concentrations, respectively. A clear effect of natural selection is only inferred if the selected populations differ significantly in their response to the selective force compared to both the original and control populations. This type of design allows to separate significant differences in populations due to natural selection from other un-accounted factors (e.g. random genetic drift or lab-selection). In total 10 clonal lines from each of the 4 replicate aquaria, thus a total of 40 clonal lines from the control and 40 clonal lines from each of the selected populations, and a subset of 40 clones from the original population were used. Per clone or clonal line 4 replicates, each from a different mother, were (Figure 7.1). Individuals (< 24 hours) were exposed in 50mL cups and the medium was renewed 3 times a week (Monday, Wednesday and Friday). We also compared the fitness of all populations under control conditions in the absence of elevated Cu or Zn concentrations to test for a cost of adaptation upon return to uncontaminated medium, by comparing total reproduction after 21 days of the Cu- and Zn selected populations against that of the control and original populations.

7.2.5 Microsatellite genotyping

At the end of the selection experiment 20 adult females were randomly picked from each aquarium (i.e. a total of 80 individuals from each of the control, Cu and Zn-selected population types) and tissue samples were preserved individually in 100% ethanol (molecular biology grade, Sigma Aldrich) and

stored at -20℃ until DNA extraction. These samples were genotyped together with 40 individuals of the original population for a total of 13 microsatellite markers divided over two multiplexes (M01 and M03, see Appendix E Table E.2) (Jansen et al., 2011). Genomic DNA was extracted with the column NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions with a few modifications: incubation time was overnight (12 to 14 hours), centrifuging time was increased from 1 to 3 minutes, an additional third washing step of the silica membrane in a new collection tube was performed, and 40µL of Buffer BE was added instead of 100µL to elute the DNA. PCR amplification was performed with the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) on a Thermocycler PCR machine (Biometra, Westburg, Germany) following the manufacturer's instructions. PCR cycling conditions were as follows: an initial denaturation step of 15 min at 95℃ was followed by 25 cycles of 94 ℃ for 0.5 min, 1.5 min at the annealing temperature (Tm) (56℃ for M01 , and 54℃ for M03) and 1.5 min at 72 ℃. A final extension step of 30 min at 60℃ completed the cycling. The forward primers were obtained from Qiagen and the reverse primers from Eurogentec (Maastricht, Netherlands). Microsatellite genotyping was performed by capillary electrophoresis using an ABI PRISM 3031 automated sequencer (Applied Biosystems, Foster City, USA). In each sample well 1µL of the PCR product was combined with 8.8 µL Hi-Di formamide and the 0.2 LIZ size standard. Primers were labelled using the standard DS-33 set (G5) of four dyes (6FAM, NED, VIC, PET) and GeneScan-500 LIZ as size standard (35 and 250 bp peaks were omitted from the analysis). DNA Microsatellite alleles were scored with the Gene Mapper (Version 4.0) software (Applied Biosystems). One microsatellite (B030) was removed from further analysis as accurate scoring of the alleles was prevented due to excessive stuttering and amplification failure across the majority of DNA sample

7.2.6 Tolerance to novel stressors of Zn adapted populations

To test for an effect of adaptation on the tolerance to 3 novel stressors, we carried out a follow-up experiment using the Zn-selected, original and control populations by exposing them to temperature stress as a general abiotic stressor, to a cyanobacteria strain known for its toxin production and considered to be a low quality food source (*Microcystis aeruginosa* strain PCC 7806), as a non-related natural stressor to zinc, and to cadmium (Cd) as chemical stressor related to Zn. To evaluate whether Zn selection had an effect on the tolerance to temperature stress, reproduction at 28℃ was compared

among selection treatments. Tolerance to cyanobacteria stress was evaluated by comparing reproduction under a diet containing *M. aeruginosa* (equivalent to 40% of the total dry weight) against a control diet free of *M. aeruginosa* (see (Hochmuth and De Schamphelaere, 2014) for more details on cyanobacteria culturing). Tolerance to Cd was assessed in a full dose response with Cd (0, 1, 3, 6, 12 and 28 µg Cd/L, measured as dissolved Cd concentration). All tolerance tests were conducted as standard 21-day life table tests under the same conditions as the common garden experiments, with the only difference that 1 rather than 4 replicates per clone or clonal line was used and replication was at the level of the aquaria (i.e. 4 replicates per treatment), as our focus was at the population rather than at the clonal level.

7.2.7 Chemical analysis

Concentrations of Cu, Zn, Cd and organic carbon, as well as pH were measured once a week, once in the fresh medium (prior to addition of algae or cyanobacteria) and once in the old medium (after transferring the daphnids to the fresh medium). The chemical analysis was carried out as described in section 2.2.4. The results of the chemical analysis can be consulted in the supportive info (Appendix E Tables E.3 and E.4).

7.2.8 Population density during the selection experiment data analysis

To measure the effect of exposure to metals in the selection experiment on population densities a mixed linear model with time point of the experiment and selection treatment as fixed effects and aquaria as random effect was applied. We tested whether total abundance differed between selection treatments at each time point using a Wald Chisquare test with Holm adjustment for multiple testing with the R package *phia* (Team, 2011).

7.2.9 Common garden experiments data analysis

To test for a microevolutionary response in the Cu and Zn populations in the selection experiments under Cu and Zn exposure, respectively, we compared total number of juveniles produced per female in 21 days in common garden environments. As mortality was very high (resulting in a lack of reproduction for many individuals) under the Cu and Zn exposures, the distribution of the reproduction data was zero-inflated. We solved this by applying a hurdle model to the reproduction data. The hurdle model is a modified count model in which the two processes generating the zeros and the positives are not constrained to be the same. The first part is a binary outcome model, and the second part is a truncated count model. The binary part models the probability that the threshold is crossed (non-zero or zero), in our case whether reproduction occurred (probability of reproduction), while the second part counts the number of outcomes crossing the threshold, in our case quantifying the number of juveniles produced by reproducing females. The probability of reproduction was measured as the fraction of reproducing females in a population. We tested for an effect of selection treatment on reproduction using selection treatment as a fixed factor, aquaria as a random factor nested in selection treatment and clone or clonal line as a random factor nested in aquaria. Modelling was performed with the package *glmmADMB* (Fournier et al., 2012) in the statistical platform R. We tested for a cost of adaption of the Cu and Zn selected populations upon return to control conditions by comparing the total reproduction after 21 days using a mixed linear model with selection as fixed factor, aquaria nested in selection and clone or clonal line nested in aquaria as random factors using the R package *phia*. Differences in tolerance to temperature and cyanobacteria stress were assessed using a Hurdle model as described above. To assess if cadmium tolerance differed between populations the 21-day EC_{50} values of total reproduction and survival were compared using the Wheeler ratio (Wheeler et al., 2006).

7.2.10 Microsatellite marker analysis data analysis

We used Micro-Checker to check for scoring errors due to stuttering, the presence of null alleles, or large allele drop out, which could lead to a false increase in homozygosity (Van Oosterhout et al., 2004). Micro-Checker generates expected homozygote and heterozygote allele size difference frequencies via bootstrapping (Monte Carlo simulation) and uses the Hardy-Weinberg theory of equilibrium to calculate expected allele frequencies and the frequency of any null alleles detected.

Clonal richness (CR) was defined as the number of multilocus genotypes detected in each population sample and the fraction of maximum clonal richness was calculated by the ratio of clonal richness to the total number of individuals in the sample (CR_{fmax} = CR/N). Clonal diversity was quantified as Simpson's index of clonal diversity (D), which is generally not very sensitive to sample size. We statistically compared estimates of the Simpson Index of clonal diversity between the populations using a one-tailed bootstrap test as implemented in the software GENODIVE with 10,000 permutations (vers. 1.1,(Meirmans P.G., 2004)).

Whereas clonal richness and the Simpson index of clonal diversity refer to multilocus genotype diversity in the population, we also quantified allelic (i.e. genetic) diversity. Allelic richness was estimated using rarefaction for each combination of locus and selection treatment (ElMousadik and Petit, 1996) and mean allelic richness was obtained by averaging the locus specific allelic richness estimates over all microsatellite loci in a population using the R package *PopGenReport* (Adamack and Gruber, 2014). As our smallest sample size was 19 individuals per population, we chose to compare allelic richness after rarefaction to a common sample size of 38 (2 alleles per locus). The population inbreeding coefficient (F_{IS}) measures the excess or deficit of heterozygotes within populations relative to Hardy–Weinberg expectations. It is thus a measure of significant difference between expected and observed heterozygosities (H_e and H_o).

Genetic variation was partioned among selection treatments, aquaria nested within selection treatment and individuals nested within aquaria using a three-level hierarchical analysis of molecular variance (AMOVA) in Genalex (Peakall and Smouse, 2006, Peakall and Smouse, 2012). The relative magnitude of differences in genetic variation among levels can be interpreted in function of the relative importance of deterministic and stochastic microevolution acting on the populations. It is expected that drift would cause significant differences to accumulate among replicates for any selection treatment, whereas selection would cause significant differences among selection treatments. Additionally we used G'_{ST} estimates as a standardized measure of genetic differentiation to statistically measure genetic differentiation through pairwise comparisons among all populations (Hedrick, 2005). Expected and

observed heterozygosities, and global F_{IS} and pairwise G'_{ST} estimates with bootstrapped 95% confidence intervals were estimated using the *DiveRsity* package in R (Keenan, 2013).

To assess the amount of genetic differentiation at the treatment level (i.e. by combining aquaria replicates) we used Discriminant Analysis of Principal Components (DAPC) in the *adegenet* 1.3-4 package in R (Jombart et al., 2010). DAPC is a multivariate method designed to identify and describe clusters of genetically related individuals using a few synthetic variables (called discriminant functions). This method was favoured over more traditional methods such as PCA (Principal Component Analysis) or PCoA (Principal Coordinates Analysis), which focus on the entire genetic variation, because DAPC seeks linear combinations of the original variables (alleles) that maximize differences among populations while minimizing variation within populations. Furthermore the DAPC method does not assume Hardy–Weinberg equilibrium or linkage disequilibrium and is more appropriate for situations where such assumptions are not met than conventional approaches such as Structure (Pritchard, 2000).

7.3 Results

7.3.1 Population density during the selection experiment

After one week of metal exposure we already observed a reduction in population density in the metal-selected populations, while population density was much less reduced in the control populations (Figure 7.2B, Appendix E Table E.5). As no reproduction had occurred in the first week, any loss of individuals in that period translates into the loss of a clone. Under Cu selection a total of 48% of all clones were lost (88 clones of the initial 184 clones). In the Zn selection treatment survival after one week was even lower, with 74% loss of clonal richness (137 clones of the initial 184 clones, Figure 7.2B). In the control populations mortality was low (8 %, 15 clones of the initial 184 clones). Populations under Cu selection displayed significantly lower population densities compared to the control populations throughout the 10 week selection experiment (Figure 7.2A , Wald Chisquare test: p-values < 0.05, see Appendix E Table E.5). Population densities in the Zn-selected populations were significantly lower than densities in the control populations until week 4 (Figure 7.2A , Wald Chisquare test: p-values < 0.05, see Appendix E Table E.5). In weeks 6 and 8 there was no longer a significant difference between the population

densities of the Zn-selected and control populations (Figure 7.2A , Wald Chisquare test: p-values > 0.2, see Appendix E Table E.5).

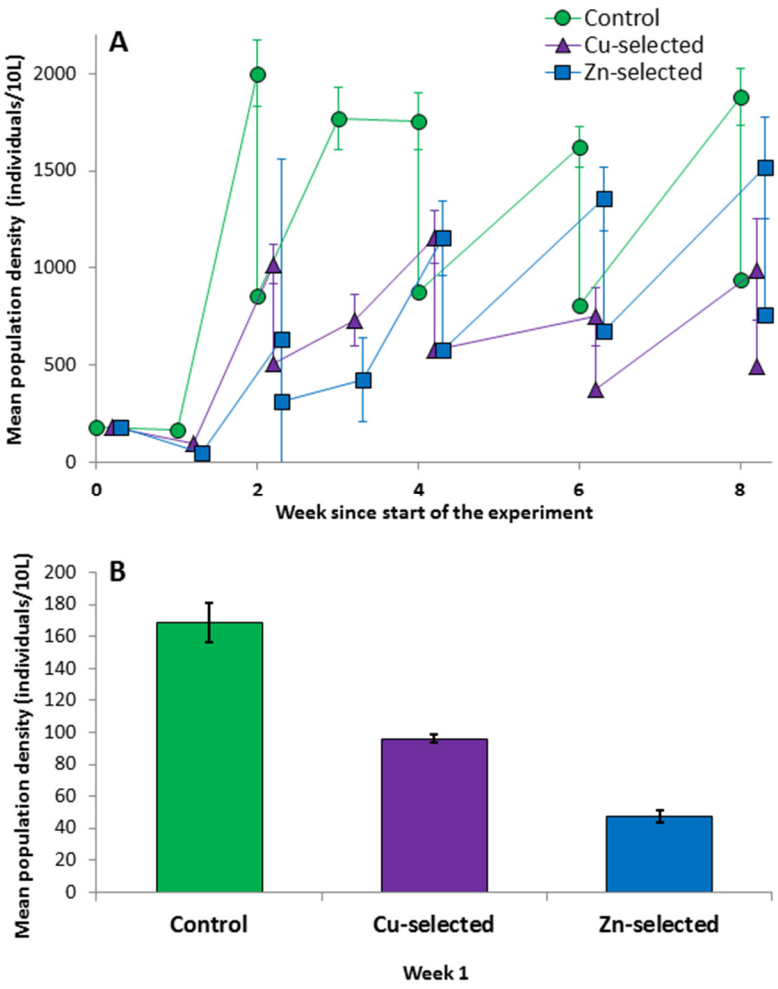


Figure 7.2. (**A**) Population density (+/- standard error) during the selection experiment. Note that the density was reduced by 50% in weeks 2, 4, 6, and 8 as part of the experimental procedure. For visibility symbols for different treatments are slightly drifted relative to each other. No density measurements were available for week 10. 1. (**B**) Box-plot of population densities (+/- standard error) after one week during the selection experiment. Mean (+/- standard deviation) density estimates as well as p-values of the one-way ANOVA and Tukey HSD post-hoc tests can be found in **Appendix E Table E.5**.

7.3.2 Common garden experiments testing for metal adaptation

We observed a clear increase in the probability of reproduction in both Cu- and Zn-selected populations compared to the original and control populations under Cu and Zn common garden exposure,

respectively. While only 20% of the females of control and original populations reproduced when exposed to Cu, reproduction occurred in 45% of the individuals of the Cu-selected populations (Figure 7.3A). This difference was significant (hurdle model followed by Tukey-HSD: Cu-selected vs. original: p = 0.0084, Cu-selected vs. control: p = 0.0058), while the probability of reproduction did not differ between the original and control populations (p = 0.9949). Under Zn common garden we also observed a significant increase in the probability of reproduction from 20% or less in the control and original populations to 70% in the Zn-selected populations (Figure 7.3A, hurdle model followed by Tukey-HSD: Zn-selected vs. original: p <1e-04, Zn-selected vs. control: p = <1e-04; no difference between control and original populations: p = 0.83). There was no significant difference in the total number of offspring that were produced by females that did reproduce under Cu exposure (Figure 7.3B, hurdle model followed by Tukey-HSD: Cu-selected vs. original: p = 0.570, Cu-selected vs. control: p = 0.501, original vs. control: p =0.989) and Zn exposure (Figure 7.3B, hurdle model followed by Tukey-HSD: Zn-selected vs. original: p = 0.774, Zn-selected vs. control: p = 0.157, original vs. control: p = 0.166).

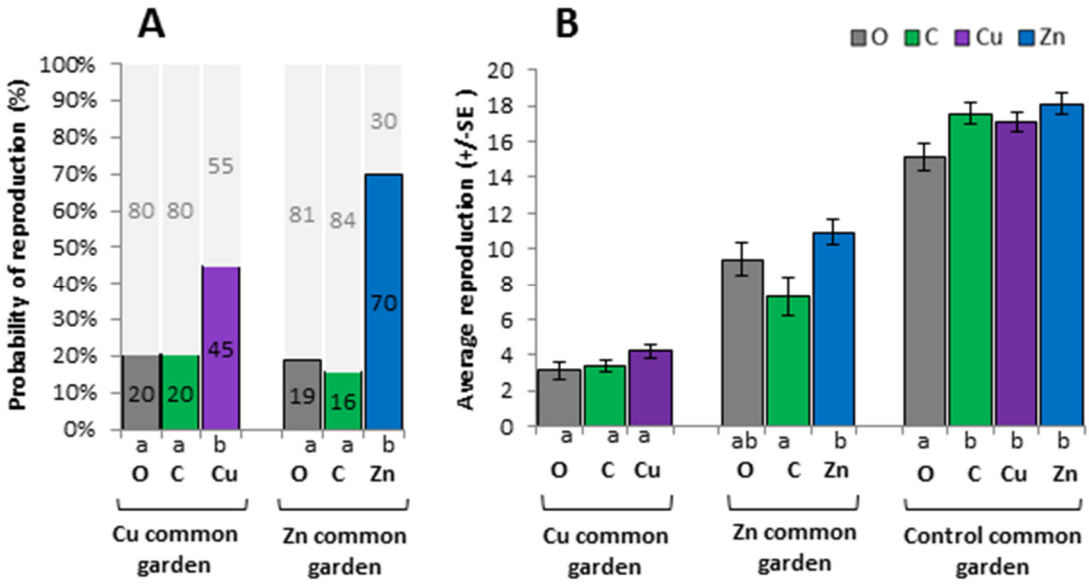


Figure 7.3. Probability of reproduction (**A**) and average number of juveniles produced per female in 21 days of females that reproduced (**B**) in the common garden experiment upon exposure to 8-day LC50 of Cu and Zn and in the absence of such exposure (control condition) of clonal isolates from the metal selected (Cu and Zn), original (O) and control (C) populations. Different lower case letters indicate a significant difference between selection treatments (significance level p = 0.05). Error bars represent one standard error.

7.3.3 Follow-up experiments testing for a cost of adaptation and cross-tolerance to novel stressors

We did not observe any significant difference in total reproduction between metal-selected, control and original populations under control conditions (Figure 7.3B, linear mixed model: $p = 0.053$). At 28℃ the probability of reproduction in the Zn-selected populations was significantly lower than in the control populations but did not differ significantly from the original population (Figure 7.4A, hurdle model followed by Tukey-HSD: Zn-selected vs. original: $p = 0.5681$, Zn-selected vs. control: $p = 0.0371$, original vs. control: $p = 0.1010$), and no difference in average reproduction of reproducing individuals was observed (Figure 3B, hurdle model: followed by Tukey-HSD: Zn-selected vs. original $p = 0.945$, Zn-selected vs. control $p = 0.702$, original vs. control $p = 0.746$). There were no differences in either probability of reproduction (Figure 3A, hurdle model: followed by Tukey-HSD: Zn-selected vs. original $p = 0.705$, Zn-selected vs. control $p = 1$, original vs. control $p = 0.470$) or average number of offspring of reproducing females between populations derived from different selection treatments when fed a diet in which 40% of the dry weight consisted of the cyanobacterium *Microcystis aeruginosa* (Figure 3B, hurdle model: followed by Tukey-HSD: Zn-selected vs. original $p = 0.999$, Zn-selected vs. control $p = 0.669$, original vs. control $p = 0.475$).

The effect of Zn selection on cadmium (Cd) tolerance was more complex and depended on the effect concentration used as a criterion. When total reproduction was used as an endpoint, the Zn-selected populations showed a 20% reduction at a low Cd concentration (21-day reproduction EC_{20}= 2.0µg Cd/L) than both the control (21-day reproduction EC_{20}= 1.6µg Cd/L) and original populations (21-day reproduction EC_{20}= 1.5µg Cd/L), reflecting that they became less sensitive, although the actual difference in EC_{20} was less than 25% (Figure 7.4C, Wheeler ratio: $p < 0.05$, Appendix E Tables E.6 and E.7). With respect to the 21-day reproduction EC_{50}, Zn-selected populations were less sensitive than the control populations but equally sensitive as the original population. For the 21-day reproduction EC_{80}, the Zn-selected populations proved more sensitive to Cd than the original population but equally sensitive as the control populations (Figure 7.4C, Appendix E Tables E.6 and E.7). When survival over the test period was considered as an endpoint the only difference in Cd tolerance was that Zn-selected populations had a significantly lower 21-day survival LC80 than the original population but that their 21-day survival LC80 did not differ from that of the control populations (Figure 7.4D, Appendix E Tables E.6

and E.7). The slopes of the concentration response curves for both reproduction and survival were significantly steeper in the Zn-selected populations than those of the original population but did not differ from the slopes of the control population (Wheeler ratio: $p < 0.05$, Appendix E Tables E.6 and E.7).

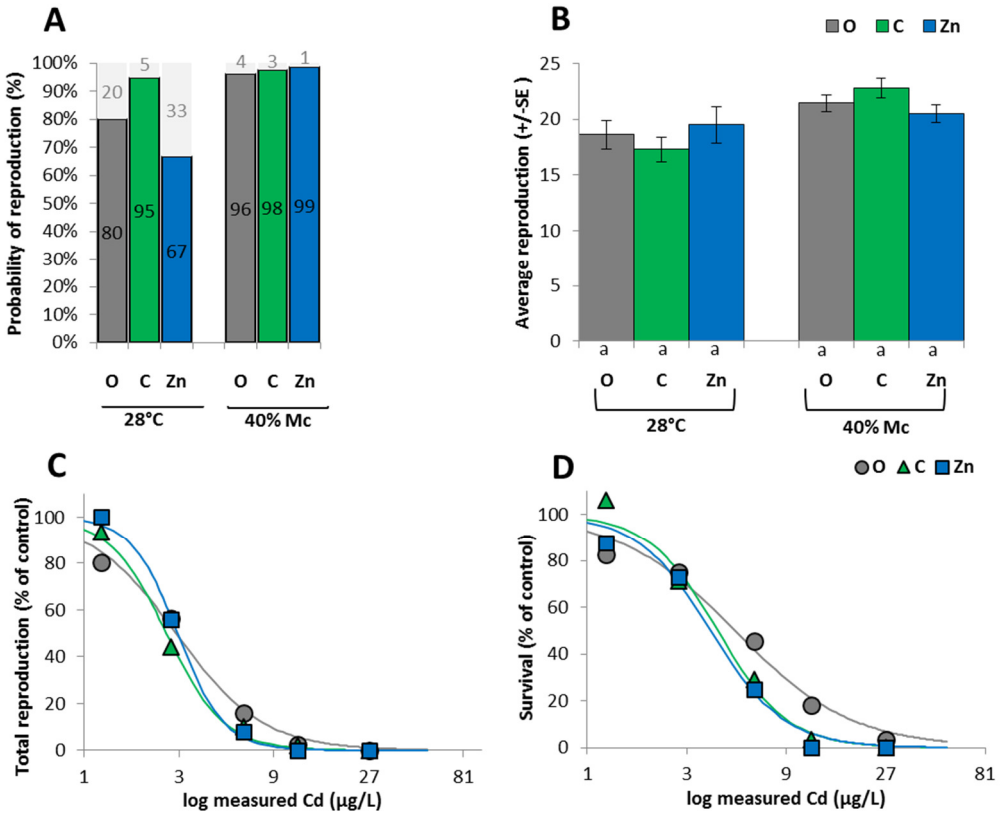


Figure 7.4.. (A) Tolerance to novel stressors of the metal selected (Cu and Zn), original (O) and control (C) populations. Probabilities of reproduction under high temperature conditions (28℃) and when fed cyanobacteria (40% *Microcystis aeruginosa* in the total diet). (B) Average number of offspring of clones that did reproduce. Different lower case letters indicate a significant difference between selection treatments (significance level $p = 0.05$). (C) Concentration response curve under cadmium stress for total reproduction. (D) Concentration response curve under cadmium stress for survival.

7.3.4 Testing for effects of metal selection on clonal and allelic diversity

Both clonal richness and the Simpson index of clonal diversity were lower in the Cu- and Zn-selected populations than in the original and control populations (Table 7.1). The one-tailed bootstrap analysis of the Simpson Index confirmed that both the original and control populations had considerably higher clonal diversity than both the Cu- and Zn-selected populations ($p < 0.05$, 10000 permutations), while no difference in clonal diversity was observed between the Cu and Zn populations ($p > 0.05$, 10000 permutations) and between the original and control populations ($p > 0.05$, 10000 permutations, exact p-values can be consulted in the supportive info: Table S8).

Average allelic richness ranged from 2 to 3 alleles per locus (Table 7.1, see Appendix E Figures E.2 and E3 for locus specific allele frequencies). When averaged over all loci, the observed heterozygosity was consistently higher than the expected heterozygosity (i.e. gene diversity) in the Cu- and Zn-selected populations, which is indicative of heterozygote excess in the latter populations (Table 7.1). The inbreeding coefficient (F_{IS}) provides further evidence of a significant heterozygote excess in the Cu- and Zn-selected populations, while there was no excess of heterozygotes in the original and control populations (bootstrapped 95% C.I. includes 0).

The Discriminant Analysis of Principal Components (DAPC) on the allele frequencies shows that the Cu- and Zn-selected populations form two mostly non-overlapping clusters, while the original and control populations largely overlap with each other (Figure 7.5). The 1st principal component mainly separated the Zn- and Cd-selected populations from each other and to a lesser extent also from the original and control populations, while the 2nd principal component) mainly separated the metal-selected from the control and original populations and, to a lesser extent, also the control from the original populations. Pairwise G'_{ST} values confirm that there is no significant differentiation between the original and control treatments, while the Cu and Zn-selected populations are significantly differentiated from the original and control and from one another (as indicated by the bootstrapped 95% C.I., supportive info: table S9). The hierarchical AMOVA revealed that most allelic variation is found within populations, but that treatment also resulted in significant genetic differences (Table 7.2).

Table 7.1. Estimates of clonal and genetic diversity of the original *D. magna* population and the experimental populations (control, Cu- and Zn-selected).[a]

			clonal diversity			allelic diversity			
population	**aq.**	**N**	**CR**	**CR_{fmax}**	**D**	**AR**	**H_o**	**H_e**	**F_{IS} (95% CI)**
original	NA	40	36	0.90	1	3.36	0.45	0.48	0.05 (-0.01 to 0.1)
control	1	19	14	0.74	0.95	2.92	0.45	0.44	-0.03 (-0.19 to 0.09)
	2	19	11	0.58	0.92	2.83	0.42	0.38	-0.12 (-0.25 to 0.01)
	3	20	16	0.80	0.97	3.49	0.47	0.51	0.07 (-0.01 to 0.14)
	4	19	15	0.79	0.97	3.17	0.41	0.41	-0.001 (-0.09 to 0.07)
Cu-selected	1	20	12	0.60	0.85	3.00	0.56	0.44	-0.28 (-0.48 to -0.13)*
	2	20	9	0.45	0.82	2.81	0.54	0.43	-0.26 (-0.44 to -0.10) *
	3	20	10	0.50	0.80	2.66	0.59	0.44	-0.33 (-0.53 to -0.19) *
	4	20	4	0.20	0.55	1.91	0.51	0.31	-0.62 (-0.77 to -0.53) *
Zn-selected	1	20	8	0.40	0.70	2.82	0.64	0.44	-0.45 (-0.72 to -0.24) *
	2	19	9	0.47	0.81	2.33	0.59	0.39	-0.52 (-0.72 to -0.34) *
	3	19	5	0.26	0.64	2.17	0.52	0.37	-0.39 (-0.64 to -0.17) *
	4	19	13	0.68	0.95	3.17	0.55	0.47	-0.18 (-0.32 to -0.06) *

[a] aq.: replicate (aquarium); *N*: number of genotyped individuals; CR: clonal richness; CR_{fmax}, clonal richness expressed as a fraction of maximum clonal richness; D: clonal diversity measured as the Simpson's index; AR: mean allelic richness of the studied loci measured as the average number of alleles detected per locus, based on rarefaction to a common sample of 38 alleles using the method of El Mousadik and Petit (1996); Ho, He: observed and expected heterozygosity, respectively, averaged over loci; FIS: inbreeding coefficient with bootstrapped 95% confidence intervals as a measure of deviations from Hardy-Weinberg equilibrium within populations (positive values indicate homozygote excess, negative values heterozygote excess). * indicates significant deviations from the Hardy-Weinberg equilibrium.

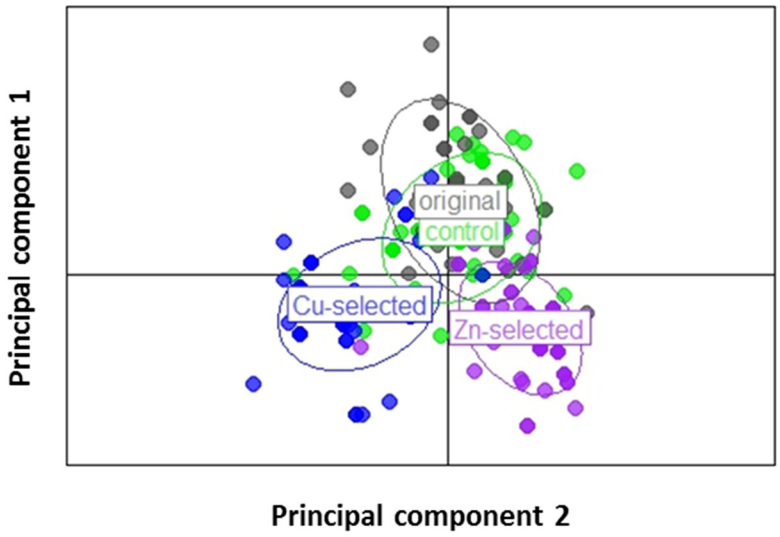


Figure 7.5. Discriminant analysis of principal components (DAPC) on the multi-locus genotype data for all populations. Individual multilocus genotypes appear as circles. Horizontal and vertical axes are the first and second principal component, respectively. A visual representation of the membership probability of the different multilocus genotypes to the different populations can be found in Appendix E Figure E.4).

Table 7.2. Hierarchical analysis of molecular variance for the microsatellite data partitioning variance among and within 4 treatments and within 4 aquaria replicates.

Hierarchical level	df	SS	MS	variance	% variation	*p-value*
among treatments	3	64.8	21.6	0.052	2%	0.018*
among aquaria within treatments	9	106	11.8	0.236	8%	0.084
within aquaria	535	1383	2.58	2.58	90%	0.001*

* indicates a significant difference at the 0.05 significance level.

7.4 Discussion

Conventional risk assessment based on tests on monoclonal populations may be over-conservative because of the artificial reduction of evolutionary potential compared to the natural situation. On the other hand genetic adaptation or acquired resistance - brought about by natural selection - may have costs and these too may interfere with conventional risk assessment practice. In this study we showed that natural populations of *Daphnia magna* have the ability to adapt to both copper and zinc even when exposed to concentrations that strongly impact population densities. We have further demonstrated that these populations can recover fully in the case of zinc but not for copper. We obtained no strong evidence for either costs or gains associated with zinc adaptation in terms of the populations' tolerance to novel stressors or in the absence of the initial stressor. While metal selection clearly leads to a reduction in clonal diversity, allelic richness of the metal-selected populations remained unaffected compared to the control and original populations due to a selection for heterozygous individuals. Below each of these findings is discussed in more detail.

7.4.1 Microevolutionary response to Cu and Zn selection

Using a selection experiment followed by a common garden approach, we have shown that a natural *D. magna* population harbours sufficient evolutionary potential to support rapid adaptation to Cu and Zn stress. We observed a significant increase in Cu and Zn tolerance in a period of only 10 weeks, which is approximately 8 generations of parthenogenetic reproduction (generation time ≈ 8.5 days). The response was strong for the ability to reproduce under Cu and Zn stress but not for the number of eggs produced by individuals that could reproduce. This may be related to the fact that we chose rather high and near-lethal copper and zinc concentrations (8-day LC50) causing a more acute than chronic effect.

Although both Cu and Zn selection caused a significant increase in tolerance to the respective metal, the dynamics of the resulting populations differed considerably. Under the conditions we established in our experiment, the control populations maintained a stable population growth reaching densities between 150 and 200 individuals per L following each culling event. The Zn-selected populations matched the population densities observed in the control populations from week six onwards, while the Cu-selected populations never completely recovered to reach densities similar to the control

populations. Although significantly higher than in the original and control populations, Cu tolerance and average reproduction of the Cu-selected populations in the common garden experiment overall remained rather low. The number of offspring produced by reproducing individuals of the Cu-selected populations when exposed to Cu in the common garden experiment was only 15% of that of these same populations under control conditions (Figure 7.3B).

Although the initial selection on survival was less severe in the Cu-selected compared to the Zn-selected populations (50% mortality during the first week in the Cu-exposed aquaria populations compared to 75% mortality under the Zn-exposed aquaria populations), our results suggest that the natural *D. magna* population used in this study has a higher capacity to adapt to zinc than to copper. Previous studies have established that the microevolutionary potential in response to exposure to another trace metal, cadmium, may differ considerably among populations (Messiaen et al., 2013, Barata et al., 2002a). This warns against generalizations, as it suggests that observations of the evolutionary consequences of long-term exposures to lethal effect concentrations (8-day LC50s in our study) of a given pollutant cannot be extrapolated to other substances.

7.4.2 Costs of adaptation and cross-tolerance

No evidence was observed for a cost of metal adaptation of the Cu- or Zn-selected populations when returned to control conditions. This finding is somewhat in contrast with previous work on *Daphnia longispina* adapted to copper in the wild, where a cost of adaptation upon return to unpolluted conditions was observed (Agra et al., 2011 , Agra et al., 2010). This can, however, be related to the fact that copper is an essential element and Agra and colleagues did not include any copper at all in their control medium, which may have resulted in Cu deficiency in animals that had been selected under higher Cu concentrations. Indeed, the essential metal deficiency hypothesis postulates that a potential cost of metal adaptation is associated with less efficient metal uptake (Van Straalen, 2000, Harper et al., 1997). Our control medium still included 5µg Cu/L and 28µg Zn/L (nominal concentrations), which lies within the optimal concentration range of both metals for *Daphnia* (Bossuyt and Janssen, 2004 , Muyssen and Janssen, 2005).

We obtained only weak evidence for a cost of selection in the follow-up experiments in which we exposed Zn-selected, control and original populations to two additional stressors, a high temperature and a suboptimal food. Exposed to a temperature of 28°C, the Zn-selected populations showed a lower probability to reproduce than the control populations. Although this suggests a cost of adaptation in the Zn-evolved populations, this evidence is incomplete given the lack of a significant difference compared to the original population. Ward and Robinson (2005), in a selection experiment involving 8 clones, also did not observe a difference in temperature tolerance associated with metal adaptation (Cd in their study). Similarly Zn-selection had no influence on the performance of the *Daphnia* when fed a diet composed of 40% *Microcystis aeruginosa.*

We observed cross-tolerance of the Zn-selected populations to cadmium for the 21-day EC_{20} of total reproduction. Yet, this effect was not very strong given that the actual difference in EC_{20} was less than 25%. Moreover at the median and high effect levels (EC_{50} and EC_{80}), rather than observing an effect of Zn-selection, we observed what was most likely an effect of laboratory selection as both the Zn-selected and control populations displayed a significantly lower tolerance to cadmium than their original population. Therefore, despite similar survival at lower cadmium concentrations, control and Zn-selected populations appear to have lower survival tolerance at higher concentrations ($>EC_{50}$) than the original population. Although this study is limited to the asexual part of the life cycle, it is important to bear in mind that under field conditions the selection to one stressor may be separated from the exposure to novel stressors by genetic recombination by means of sexual reproduction. One on narrow sense heritability suggests that there may be long-term adaptive potential of *D. magna* populations to cadmium, but only under one of two temperature conditions investigated (Messiaen et al., 2012) which suggests that the longterm (selection) effects of one stressor can be context-dependent and that extrapolations to true field conditions may be difficult.

7.4.3 Interplay between genetic drift and selection in the selection experiment

The among-population genetic differences we observe among aquaria can in principle be a consequence of genetic drift and differential natural selection (Hartl and Clark, 1980). In addition to our observation of an adaptive increase in Zn and Cu tolerance in the Zn- and Cu-selection experiment, respectively, also our data on microsatellite markers provide multiple lines of evidence that in our experiment, selection was a determining factor. Genetic drift would result in a large amount of variation among aquaria irrespective of treatment, whereas natural selection would result in differences that are related to the treatments. First, the discriminant analysis shows repeatable differences among treatments. Second, the pair-wise G_{ST} values between the original and control populations were non-significant, whereas they were significantly different between the control, Cu- and Zn-selected populations. Thirdly, the AMOVA shows that allelic variation for the 12 studied microsatellite markers among treatments is significant whereas variation among replicate aquaria within treatments is not.

Selection was strong in our experiment, given that high mortality of clones was observed already during the first week. After one week of exposure to either copper or zinc, already 50 to 75 % decline in population density was observed in the metal-selected populations (Figure 7.2B, Appendix Table E.5), which translated in an equally strong reduction in the number of clones. The response was very similar across the four replicate aquaria in both metal-selection treatments (cf. small error bars in Figure 7.2B). This strong decline in the metal-selection treatments reflects that experimental populations were exposed to a strong selection pressure, corresponding to the eight-day lethal concentration for each metal. Given this strong selection pressure, it is striking that the Zn-selected populations entirely recovered in terms of their population densities during the experiment, as they reached similar densities as the control populations by the sixth week of the experiment. This may represent a case of evolutionary rescue (Gomulkiewicz and Shaw, 2013).

7.4.4 Selection for heterozygotes

A significant excess of heterozygosity was observed in the Cu- and Zn-selected populations but not in the original and control populations (significant F_{IS}; Table 7.2 and Figure 7.4A). This suggests that the exposure to quite high levels of metals we imposed on the populations selected for heterozygotes. Heterozygote excess has been reported before in field *Daphnia* populations that experience prolonged periods of clonal reproduction (Hebert, 1974b, Hebert, 1974a, Young, 1979a, Young, 1979b, Hamrova et al., 2011) and has also been reported in experimental mesocosm studies (Haag and Ebert, 2007), but not as a response to chemical stress. We are only aware of two other studies (Peles et al., 2003, Jordaens et al., 2006), cited in a recent review on the effect of metal pollution on the genetic diversity (Mussali-Galante et al., 2014) that also observed an increase in heterozygosity following metal-induced selection. However, both of these studies were performed with sexually reproducing terrestrial species (earthworms and land snails) and with mixtures of metals. To our knowledge, our study is the first to report that multigenerational exposure of a natural freshwater population to metal stress resulted in a reduction of clonal diversity while allelic diversity was maintained, with heterozygote excess being the outcome of chemically induced natural selection in favor of heterozygotes

The increase in heterozygosity observed in the selection experiment has important consequences, as it contributes to the maintenance of a higher allelic diversity. Although we observed a strong decline in clonal richness in the metal-selected populations, there is no decline in allelic richness. This is important as allelic diversity determines a population's ability to respond to long-term selection across generations (Allendorf, 1986).This might reduce one of the potential costs of adaptation, which is reduced genetic variation. The fact that allelic richness was not significantly reduced in the Cu- and Zn-selected populations suggests that the heterozygote excess observed in the metal-selected populations may act as a mechanism that contributes to the maintenance of allelic richness under metal stress. Future research needs to determine to what extent this mechanism could help to maintain the adaptive potential of metal-selected populations to novel stressors and to what extent increased heterozygosity is preserved after genetic recombination following periods of sexual reproduction. This study demonstrates that the exposure of a natural population to levels equivalent to the 8 day LC50 does not exhaust the genetic diversity and that a natural population can quickly recover from chemical stress (at least in the case of zinc) at such high metal concentrations in terms of population density.

8

General conclusions and future research perspectives

8.1 Introduction

Conventional ecotoxicology primarily focuses on determining short-term (typically ≤ one generation) toxicological responses of individual organisms (typically clonal) exposed to single chemicals, mostly under invariable and (near-)optimal conditions. This contrasts with ecological reality, where natural populations adapt by means of natural selection to continuously changing conditions of multiple stressors under non-optimal conditions (e.g., food shortage, non-optimal temperature, and predation). Therefore ERA may not be protective across different environments, such as conditions predicted under global change, as it doesn't account for multiple stressors nor genetic adaptation.

The aim of this PhD dissertation was to investigate the combined effects of metals with natural stressors at different organization levels (individual vs. population), and time-scales (short term vs. long term) on the freshwater cladoceran *Daphnia magna*. First, this final chapter recapitulates the key findings linked to the specific research questions listed in in the conceptual framework (Box 8.1). Secondly, both the methods and results followed to address the specific research questions are critically assessed in the context of ecological risk assessment, pointing to methodological strengths and weaknesses, and providing perspectives for future research. At the end of this chapter the overall contribution of the PhD dissertation to ecological risk assessment is highlighted (Box 8.2).

Box 8.1. Summary of the key findings linked to the research questions formulated in chapter 1.

1: Can the combined effects of copper and cyanobacteria be predicted using models?

YES, CA is more accurate for *M. aeruginosa* and IA is more accurate for the other cyanobacteria.

2: Can the combined effects of copper and cyanobacteria be generalized?

Unlikely, future research is needed to investigate G x G x E interactions.

3: Do rising temperatures increase the harmful effects of cyanobacteria to *D. magna*?

Harmful effects **increase for *Anabaena* and *Oscillatoria***, **decrease for *Microcystis*, *Nodularia* and *Aphanizomenon***, and remain **unchanged for *Cylindrospermopsis***.

4: Are the different cyanobacterial genera more harmful to *D. magna* than starvation?

Only *Microcystis* was observed to be more harmful than starvation alone.

5: Is the Cu NOEC protective for combined effect of copper and *M. aeruginosa*?

NO, as the 21d Cu EC_{50} on *D. magna* reproduction values were lower than the 21d NOEC in treatment where *M. aeruginosa* was added to the diet.

6: Is the Cu NOEC protective for the combined effects of copper and *M. aeruginosa* at the population level?

NO, population extinction was observed under the combined exposure, despite the effect of *M. aeruginosa* on the population density of *D. magna* being negligible under the copper control.

7: Can a mechanistic model (DEB-IBM) extrapolate the effects observed at the individual level to more ecologically relevant effects at the population level?

YES, the model could extrapolate the effects of the individual stressors on the individual level to the combined effects of both stressors at the population level (i.e. predict population extinction).

8: Can populations adapt to lethal metal concentrations (equivalent to the 8-day LC50)?

YES, rapid adaptation was observed to copper and zinc after only 10 weeks.

9: Do populations display lower genetic diversity after adaptation than prior to selection?

NO, clonal diversity was reduced but allelic (i.e. gene) diversity was not affected by selection.

10: Is adaptation to chemical stressors associated with costs of adaptation?

Not necessarily, future research is needed to identify the imprint of adaptation on populations.

8.2 Combined effects of copper and cyanobacteria

A central aim of the PhD dissertation was to investigate the combined effects of cyanobacteria and copper (commonly applied as algaecide to combat cyanobacteria) on *D. magna* under more realistic conditions than those used in standard ecotoxicity experiments, as the exposure to multiple stressors is predicted to increase under global change.

Research question 2: Can the combined effects between copper and cyanobacteria be generalized across different cyanobacterial and daphnid genera?

The results from chapter 2 suggest non-interaction between *M. aeruginosa* and copper according to the CA reference model and non-interaction according to the IA reference model. The opposite was observed for the other 4 cyanobacteria: antagonism according to the CA reference model and non-interaction according to the IA reference model. We have highlighted that combined effects of copper and cyanobacteria can't be generalized across different cyanobacteria genera. The results show that *M. aeruginosa* is the most harmful of the studied cyanobacteria in combined exposure with copper and suggest differences in the mode of action of *M. aeruginosa* and the other cyanobacteria. The results in chapter 2 were also the same for the two *D. magna* clones investigated.

Only two *D. magna* clones and one strain per cyanobacterial genus and known toxin

In ERA the primary goal is to protect populations rather than individuals and therefore attempts to generalize the effects of exposure to combined effects should preferably be conducted on multiple genotypes. The two *D. magna* clones are not sufficient two make generalizations, as it is important to cover the genetic variation present in the natural population. In contract in chapter 7, a more representative sample of the genetic variation of a natural population was used. The two specific *D. magna* clones were used because they were the chosen clonal isolates used for the first-generation *D. magna* genetic linkage map (Routtu et al., 2010). However the Xinb3 clone actually offers little ecological relevance, as it was isolated from a small temporary rock pool in Tvärminne (Finland). Although the Iinb1 clone, which was isolated in Münich (Germany), has a higher ecological relevance than the Xinb3, the latter was used in chapters 3-6 as the Iinb1 produces close to 50% male offspring. As only one strain (each with a different known toxin production) was used per cyanobacterial genus in the experimental design, we can't identify the mode of action of the cyanobacteria. As mentioned in chapter 1,

disentangling the effect of cyanobacteria on *D. magna* is further complicated by the fact that the cyanobacteria have the ability to adapt to their environment as well. For instance, an analysis of the genome sequence of the *M. aeruginosa* strain used in this PhD dissertation highlights that it has a particularly high genome plasticity compared to other cyanobacterial strains (Frangeul et al., 2008). Such Genotype-Genotype-Environment Interactions (G x G x E interactions) give rise to a geographic mosaic of coevolution (Thompson, 2005).

Importance of Genotype-Genotype-Environment Interactions

As the main goal of ERA is to protect natural populations or ecosystems, future studies should focus on clonal genotypes with ecological relevance. Genotype-Genotype-Environment Interactions (G x G x E interactions) offer a possible explanation for the seemingly inconsistent literature reports on zooplankton–cyanobacteria interactions and may even provide insight on how that zooplankton can contribute to the suppression of cyanobacteria blooms. Moreover, *Daphnia* can increase its tolerance to *Microcystis* through maternal effects (Gustafsson et al., 2005) and microevolution (Hairston et al., 1999). The presence or absence of a cyanobacteria bloom may therefore reflect the outcome of interactions between defences and counterdefences (cyanobacteria that protect themselves against grazing and their grazers that protect themselves against toxicity) in predator-prey dynamics.

This geographic mosaic of coevolution can be described as how natural selection acts on two or more interacting species across many contrasting environments. One study showed that genotype x genotype (G x G) interactions are an important factor in explaining the mortality in short-time exposures of *D. magna* to *M. aeruginosa* (Lemaire et al., 2012). This suggests that *D. magna* may develop specific responses rather than generalized responses to adapt to local assemblages of cyanobacteria strains (genotype x strain dependent), and vice versa. Another recent study revealed strong intraspecific differences in the tolerance of *D. galeata* clones to MC/non-MC-producing cyanobacteria in their diet, suggesting microevolutionary effects (Druga et al., 2016)(Druga et al. 2016). This confirms previous findings of food (nutrient) quality playing a role in microevolutionary responses to changing stoichiometric conditions in natural populations of *D. pulex* (Weider et al., 2005). Future studies should focus on the confirmation of such genotype x genotype interactions under a geographic mosaic of coevolution between *D. magna* and cyanobacteria. Genotype x Genotype x Environment Interactions

(G x G x E interactions) offer a possible explanation for the seemingly inconsistent literature reports on zooplankton–cyanobacteria interactions and may even provide insight on how zooplankton can contribute to the control or suppression of cyanobacteria blooms.

Research question 3: Does *D. magna* becomes more sensitive to the harmful effects of cyanobacteria as temperature increases?

A decrease in harmful effects on reproduction with increasing temperature (from 15℃ to 23℃) was observed for *Microcystis*, *Nodularia* and *Aphanizomenon*, while an increase in harmful effects with increasing temperature was noted for *Anabaena* and *Oscillatoria* and no effect of temperature on *D. magna* sensitivity to *Cylindrospermopsis* was observed.

Limited applicability of results as the cyanobacteria and algae were grown at one temperature

While the findings in chapter 3, 4 and 5 are limited to the direct effects of temperature on *D. magna* sensitivity to cyanobacteria (as cyanobacteria were cultured at a single temperature), cyanobacteria and algae are themselves also affected by temperature. While this limitation enabled the focus on only the direct effects of Cu, *M. aeruginosa*, temperature and total diet concentration on the reproduction of *D. magna* (by removing the indirect effects of temperature on the harmfulness of *M. aeruginosa*), extrapolations to realistic exposure scenarios under global change should be interpreted carefully.

Culturing of cyanobacteria and algae at exposure temperature

The present findings could be place in a more ecological relevant context if the effect of temperature on cyanobacteria would be taken into account by culturing the cyanobacteria at the experimental temperatures or at temperatures within the range of where they were isolated. As shown in Table 3.1 of chapter 3, the cyanobacteria and algal strains were isolated in different geographical regions. Moreover, the findings presented in chapter 3 could suggest that *M. aeruginosa* blooms could potentially become less of a concern in the future with temperature predicted to increase. Several additional physical (e.g. vertical mixing) and chemical (e.g. nutrient input) factors influencing the prevalence and intensity of *M. aeruginosa* blooms would have to be included in future experiments to draw more reliable conclusions.

Research question 4: Are the different cyanobacterial genera more harmful to *D. magna* than starvation alone?

In chapter 3, some adverse effects of cyanobacteria on *D. magna* fitness in terms of food quality have been highlighted, as the effects of the cyanobacteria were comparable to (or less than) starvation-like effects in most treatments. These results corroborate those from another study in our laboratory, in which adverse effects on reproduction could be explained largely by starvation effects in the 6 cyanobacterial genera studied in *D. pulex* and in 3 out of the 6 cyanobacterial genera (*Microcystis*, *Nodularia* and *Anabaena*) in *D. magna* (Asselman et al., 2014). The results from chapter 3 also confirm those from chapter 2 and suggest that *Microcystis* is the most harmful cyanobacterium to *D. magna*, as it was the only cyanobacterium that caused a significantly greater mortality in *D. magna* than starvation, while the mortality caused by the five other cyanobacteria was less than or (at most) similar to the mortality in the starvation control.These results advocate against the central focus on cyanobacterial toxins in regulations to protect zooplankton (and potentially other aquatic species), as cyanobacterial toxins do not seem to be the sole driving force behind the adverse effects on zooplankton species (contrary to effects on human health or livestock). Even *M. aeruginosa*, the most harmful cyanobacterium tested, appears to provide at least some nutritional properties to *D. magna*, as the results in chapter 3 demonstrate that at 23°C individuals die faster under starvation then when they were fed with *M. aeruginosa* alone. Hence, the use of cyanobacterial cells rather than toxin concentrations appears more appropriate in ERA.

Analysis of nutritional properties vs. toxin content

Future research could focus on the analysis of the nutritional quality of cyanobacteria relative to green algae and toxin analysis. This follows observations also made by Von Elert and Wolfram (2001) and others (Martin-Creuzburg et al., 2008, Martin-Creuzburg and von Elert, 2009), who observed limited to no effects of cyanobacteria on *Daphnia* when supplemented with nutritional factors such as poly unsaturated fatty acids (PUFAs) and sterols. Additionally, a molecular analysis of the mechanisms affected by cyanobacteria in *D. magna* would be essential to investigate the relative importance of both factors (nutritional quality and toxin effect) in determining overall cyanobacterial toxicity. Future research could include feeding experiments to determine the parameters of the functional response for both algae

and cyanobacteria, which could then also serve as input for feeding-related parameters in the DEB-IBM described in chapter 6.

Research question 5: Are 21-day Cu NOEC concentrations derived under optimal conditions protective under non-optimal conditions?

The findings in chapter 4 are a testimony that mixtures at 21d NOEC levels of individual stressors can result in adverse effects, as a diet composed of only 10% *M. aeruginosa* (= 21d NOEC, see Appendix C Figure C.2) resulted in copper 21d EC_{50} on reproduction values lower than the copper 21d NOEC under standard conditions. Moreover, the results highlight that the interactive effects between copper and *M. aeruginosa*, were not significantly affected by temperature and total food concentration. The 21-day EC_{50} for copper based on reproduction (EC_{50}) varied between 20 and 100 µg/L and the results indicate that the percentage of *M. aeruginosa* explained 76% of the variance in the copper EC_{50} across all conditions studied (with higher *M. aeruginosa* correlating with higher Cu toxicity), while the effects of the temperature and the total food concentration were limited (together explaining 11% of the variance).

The study design doesn't allow for extrapolations to temperatures and food conditions beyond the ecological niche

The temperature and total food condition levels didn't qualify as stressors themselves. Consequently the statement made above, of temperature and total food content having limited influence on the combined effects of copper and *M. aeruginosa* should be taken with caution. Future experiments should place different *D. magna* clones outside their ecological niche (as illustrated in Chapter 1, Figure 1.2), by including the genotype-specific upper- and lower critical temperatures, as well as a starvation treatment.

Limitations of the ecological relevance of the results in chapters 2, 4 and 5

The results presented in chapters 2, 4, and 5 are limited in their applicability to natural systems, as the experimental design doesn't account for effects of copper on algae and cyanobacteria. Indirect effects on *D. magna* can result, as both algae and cyanobacteria serve as a food source. Cyanobacteria are expected to be most sensitive to copper as copper-based algaecides serve the specific purpose to target

cyanobacterial blooms. While *D. magna* is more sensitive than both green algae and fish at low pH levels, green algae have been shown to be most sensitive test species to copper at high pH levels, similar to the medium used in this dissertation (De Schamphelaere et al. 2003). This suggests additional indirect effects of green algae and resulting food shortage under copper exposure on *D. magna*.

Future research using controlled flow-through system to increasing ecological realism

A useful study design to disentangle the Genotype-Genotype-Environment interactions (G x G x E interactions) would make use of a controlled flow-through system to investigate the direct effect of copper, *M. aeruginosa* and temperature on *D. magna*, as well as the indirect effects of copper and temperature on green algae and *M. aeruginosa* in a full factorial design. In order to increase ecological realism (i.e. mimicking natural scenarios where copper-based algaecides are used to combat cyanobacterial blooms) such an experiment should start with a daphnid-algae system to which first cyanobacteria are added and thereafter copper in a pulse exposure. In addition measurements of food intake by *D. magna*, toxin content (uptake and excretion) and nutritional quality of the cyanobacteria could help to identify the mechanistic basis of the G x G x E interactions.

<u>Research question 6</u>: Are 21-day Cu NOEC concentrations derived under optimal conditions protective under time-variable non-optimal conditions at the population level?

In chapter 5 a population experiment was carried out to compare the total population density dynamics of a *D. magna* population exposed to regulatory and environmentally relevant copper concentrations, equivalent to or lower than the 21-day copper reproduction NOEC concentration for the studied clone (Hochmuth et al., 2014, Hochmuth et al., 2016) under different realistic global change conditions (notably the addition of *M. aeruginosa* to the diet and a temperature increase of 4°C). Populations exposed to copper at the 21d NOEC level (44µg Cu/L) and *M. aeruginosa* under a 4°C temperature increase went extinct, despite the same diet and temperature having no significant effect on population density at control (2.4µg Cu/L) copper levels. This illustrates a case of 'ecological surprise', i.e. multiple stressors having combined effects that are not expected from the effects of the single stressors (Paine et al., 1998) and substantiates the claim that the current focus of ERA on single substances is not sufficiently conservative as the potentially interactive effects of chemical stressors with natural stressors cannot be

predicted form individual of the chemicals alone. The population level effects in chapter 5 are also in confirmation with the individual level effects in chapter 4, as a diet composed of only 10% *M. aeruginosa* (= 21d NOEC, see Appendix C Figure C.2) resulted in copper 21d EC_{50} values lower than the copper 21d NOEC under standard conditions (Figure 4.3A, Appendix C Table C.3) and therefore demonstrate that if ERA were to include the individual effects of natural stressors, interactive effects could actually be extrapolated from the individual to the population level (and as shown here: extinctions forecasted).

Important implications for ERA

The combined results of chapter 4 and 5 suggest that Cu NOECs derived from standard ecotoxicity tests may not be protective in systems that experience *M. aeruginosa* blooms and support the call to incorporate combined effects of stressors into ERA practice. The Water Framework Directive (WFD) aims to establish the basic principles of sustainable water policy in the European Union, with the aim to identify priority hazardous substances for the aquatic environment on the basis of scientific risk assessment (RA) and setting common environmental quality standards (EQS) and limit the emission values for chemicals (EC, 2011, WFD technical guidance). However, the WFD does not provide details on the assessment of chemical mixtures or mixture effects, despite the fact that the EQS guidance document recognizes that in some circumstances an EQS for mixtures may be preferable to deriving EQSs for the individual constituent substances (EC, 2011). In 2015, the Environmental Quality Standards Directive introduced the Development of the first Watch List of substances to support the identification of priority substances for regulation under the Water Framework Directive. However neither copper nor *M. aeruginosa* figure on this list.

8.3 Implications of the models for ERA and their potential shortcomings

"Essentially, all models are wrong, but some are useful" is a common aphorism in statistics. Below the strengths and weaknesses of the two main models used in this PhD dissertation and their implications for ecological risk assessment and ecology are discussed.

<u>Research question 1</u>: Can the combined effects of copper and cyanobacteria be predicted using CA/IA reference models, based on the known effects of the individual exposures?

The combined results from chapter 2 and 4 demonstrate that the CA reference model was consistently more accurate at predicting the combined effects of copper and *M. aeruginosa*, while the IA delivered more accurate predictions for the combined effects of copper and the other 4 cyanobacterial genera. Further, the CA model consistently overestimated the combined effects, while the IA model consistently underestimated the combined effects. This demonstrates that the CA reference model is always more conservative than the IA reference model, as it predicts higher toxicity of the combined effects compared to IA. As the IA reference model uses individual effects of the mixture components to calculate the expected mixture effect, it implies that agents present at doses below their individual effect thresholds (i.e. NOECs) will not contribute to the joint effect of the mixture. According to the CA reference model, on the other hand, all components of the mixture contribute to the mixture toxicity in direct proportion to their concentration in the mixture and their individual toxicity. Hence, whether the individual concentrations of the components in the mixture are above or below their individual effect thresholds (NOECs) does not matter. Consequently, the CA model can be used as a conservative approach in ecological risk assessment.

Limitations of the CA and IA references models

Currently the use of the CA and IA reference models can only infer interaction by comparing the model predictions to the empirical observations (Belden and Lydy, 2006). This is a major hurdle for ERA, as interactive effects can only be pragmatically resolved through experimental validation and quantitatively comparing the effects predicted by both models with observed effects. Under this framework ecological surprises, such as extinction events cannot be predicted *in silico*, i.e. prospectively.

Future research using mechanistic models

Predictive models that are based on mechanistic understanding, such as the DEB-IBM developed in chapter 6, are promising tools to move from a retrospective to a prospective risk assessment. Depending on the PMoA selected in DEB-IBM, the model was able to predict the extinction of the populations. In the case of copper and *M. aeruginosa* the correct PMoA would first have to be determined experimentally using mode of action driven analysis. Alternatively, or as a first step, the inclusion of more endpoints (e.g. growth, metabolic rates) could aid in the correct identification of the PMoA by principal of exclusion. For instance the results in chapter 3 (*M. aeruginosa* having an effect on length after 21 days) and chapter 4 (both copper and M. aeruginosa having an effect on length after 21 days), allowed the exclusion of reproduction as a PMoA (as both Cu and *M. aeruginosa* had no effect on length).

Research question 7: Can a mechanistic model (DEB-IBM) extrapolate the effects at the individual level to more ecologically relevant effects at the population level?

In chapter 6 a mechanistic model (DEB-IBM), was calibrated using standard ecotoxicity data from chapter 4 on individual-level reproduction of *D. magna* under control conditions. This model was used to extrapolate to population level effects of *D. magna* under combined exposure to copper and *M. aeruginosa* under changing environmental conditions. To validate the model an independent data-set on population density (presented in chapter 5) was used. The DEB-IBM presented in chapter 6 serves as a proof of principle that mechanistic models are an asset for ERA, as they can effectively translate standard ecotoxicity tests to more ecologically relevant scenarios. The results have demonstrated that process-based toxicity models (DEB-IBM) that are fitted on data from short-term life-table experiments can be used to make reliable predictions of population dynamics under ecologically realistic, time-variable, multiple stressor scenarios, including conditions of projected global change. The present study is novel for two reasons: Firstly, to our knowledge it is the first implementation of a time variable DEB-IBM model with fluctuating temperature. Secondly, this is the first attempt to incorporate the combined effects of stressors using the CA reference model into the DEB-IBM framework.

Differences in the experimental design on model predictions

The predictions of population density made by the DEB-IBM are not an absolute representation of the real observations, as not all underlining interactions are fully captured by the model. Although some aspects have an important impact on the further applicability of the DEB-IBM, their implications on the results presented in this dissertation are expected to be limited. For instance, the model currently doesn't account for effects of copper on algae and cyanobacteria. In the context of this research however, the consequences are expected to be minor as algae and a cyanobacteria are fed (i.e. replaced) daily. As the DEB-IBM model incorporates stress as effect and not as a response, it is based on the CA model and can't make predictions according to the IA model. The results from this research are not compromised, as the use of the CA concept is a logical extension of the previous chapters and the fact that the model predictions followed the observed trend generally well, confirms the use of CA addition as an appropriate reference model here.

Other assumptions may have had consequences on how well the model reflect the real data. Because *M. aeruginosa* is both a food source and a stressor to *D. magna*, additional assumptions had to be made. The harmful effect of *M. aeruginosa* was incorporate in the estimated EC_{50} for reproduction and accounted for all potential modes of action (toxins, feeding inhibition and low food quality). In line with the standard toxicity tests in previous chapters the total food content (in mg C/L) was assumed to remain constant throughout, and expressed as green algae cells. In other words, the model assumes *M. aeruginosa* to be equally nutritious as the algae. If we had used the same algae as in the development of the original model this last assumption would actually suggest that the effects of *M. aeruginosa* could be predominantly attributed to toxin production and not the lack of nutritional quality. The biggest difference between our experimental design and that for the model development is that we used a different *D. magna* clone and a different algal species. As was described in the method section in chapter 6, we re-calibrated the model by optimizing the species-specific feeding parameters, as the parameter values for our clone and algae could not be estimated.

Improvements to improve the ecological realism and increase the applicability of the DEB-IBM

If information were available on the effect of copper (i.e. EC_{50} or LC_{50}) for *P. subcapitata* and for *M. aeruginosa*, the DEB-IBM could relatively easily be extended to include the resulting indirect effects on *D. magna*. The model could then also be adapted to predict the outcome of a suggested future experiment (flow-through system with pulse exposure) proposed in the discussion of research question 5.

Current DEB-IBM model doesn't allow for effects of copper on algae and cyanobacteria

The DEB-IBM framework presented in this thesis focused solely on a single species, thereby neglecting possible interactions with other species. However, the absence of interactions between species is one of the main criticisms on current ERA methods (Rohr et al., 2006, Clements and Rohr, 2009). Future efforts should be directed towards incorporating interactions between species (e.g. competition, predation, food chain dynamics) to move even further from population to ecosystem level effects (Grimm et al., 2009) (De Laender et al., 2014). One recent modelling effort demonstrated that adding interspecific competition to individual-based models increased recovery times following chemical stress up to three times (Kattwinkel and Liess, 2014). DEB-IBM is well suited as such a model, as separate DEB-IBMs could serve as building block and hold the potential to be further enhanced based on specific needs.

The present DEB-IBM does not allow for microevolutionary effects

Future efforts should be made to include genetic variability in tolerance to copper and zinc in the DEB-IBM developed in chapter 6, to predict the population level response to metal selection from chapter 7, based on the dose response data collected for different clones within the population. Thus far there are two published studies where mechanistic models were successfully applied to quantify multigenerational effects to uranium, one in *Chironomus riparius* (Beaudouin et al., 2012) and one in *Caenorhabsitis elegans* (Goussen et al., 2015). DEB-IBM seems an appropriate framework for understanding and quantifying long term selection and tolerance mechanisms in a population under toxic stress, as natural

selection occurs at the level of the individual, which shapes the life-history traits of a species, and ultimately drives dynamics at higher levels of biological organisation.

Future applications for DEB-IBMs

Before mechanistic models such as DEB-IBM can become part of the accepted ERA tools, modelling needs to improve in certain key areas. Risk assessors need to be convinced that the model captures the actual patterns that are observed in real systems (Grimm and Martin, 2013). In order to improve their use and acceptance, models need to be validated by reliable scientific data sets or surveys (Grimm and Martin, 2013). The DEB-IBM developed in chapter 6 can be used to explore properties of both individual life history traits (survival, reproduction, growth) and population dynamics (population density, size structure daphnia-algae dynamics), which emerge from the set of DEB parameters of a species, and their interaction with environmental variables such as food density and chemical stressors. Potential users do not require extensive programming skills or an in depth technical understanding of DEB theory, as only the standard DEB parameters and environmental conditions need to be adjusted.

Because the DEB-IBM model is based on DEB, which is a generic theory, the model can be calibrated to any species, as long as the species-specific DEB parameters are available in the literature or experimentally determined. There are currently two thorough reviews and guides for parameterizing a DEB model for different species (van der Meer, 2006, Kooijman et al., 2008). Mechanistic models such as DEB-IBM offer a pragmatic advantage because shifting from higher to lower levels of biological organisation decreases the time and costs required to collect the data required. The individual level is of key interest as it is possible to work with mass and energy balances, and because individuals are the units of natural selection and the building blocks of populations and ecosystems. It is also much more practical to collect data on individuals than populations. Ideally, the large existing amount of historical ecotoxicology data could be used for modelling purposes and the resulting models can reduce the need for additional ecotoxicological testing and the amount of test animals needed.

8.4. Microevolutionary effects of chemicals in a global change context

It is the genetic variability in life-history traits under stress within a population that sets the scope for microevolutionary responses under exposure to that stress (Klerks et al. 2011 ; Messiaen et al. 2013). Microevolution can be defined as the change in allele frequencies that occurs over time within a population. The process of genetic adaptation to chemicals is therefore a double-edged sword. Current ecological risk assessment practices may be under-protective, as populations adapted to a given stressor may be more sensitive to additional stressors than non-adapted populations (cost-of-tolerance). Conversely, as ERA is based on populations with no or limited genetic variability, it may be overprotective, as natural selection favours those genotypes that are more tolerant to a stressor and allows them to replace less tolerant genotypes in a population. Below the microevolutionary findings are discussed in the context of ecological risk assessment

<u>Research question 8</u>: Can a genetically diverse populations adapt to lethal metal concentrations (equivalent to the 8-day LC50)?

In chapter 7, a 10 week microevolution experiment was conducted with a genetically diverse natural *D. magna* population that was exposed to Cu and Zn. Both Cu- and Zn-selected populations developed a significantly higher metal tolerance (i.e. genetic adaptation), indicated by higher reproduction probabilities of clonal lines in Cu and Zn exposures, than observed for the original and control populations. The results demonstrate that natural *D. magna* populations can adapt rapidly, after only 10 weeks (≈7 generations) to high concentrations (8 day LC50) of both copper and zinc, with initially strong effects on population density. Interestingly, the same *D. magna* population was observed to recover successfully in terms of total population density after selection under zinc stress but not after selection under copper stress, despite initial effects being stronger in the former than in the latter selection treatment. This observation not only warns against generalizations across stressors but invites for a deeper investigation on the genetic basis of copper and zinc adaptation in the studied population.

Limitations of the study design ecological relevance of the test concentrations

The concentrations of copper and zinc (8-day LC50) for which adaptation was observed have little relevance for ERA as they are higher than 21-day NOEC concentrations for reproduction in the same medium (70 vs 45µg cu/L, 428 vs. 630µg Zn/L).

Do microevolutionary effects already occur around more regulatory relevant concentrations?

Microevolutionary effects attract the attention of ecotoxicologists because they jeopardize the application of one of the foundation of ecotoxicology, the dose response curve. Attention from regulators to consider microevolutionary effects in ERA, or the derivation of EQS, on the other hand, has remained limited. In ERA, the hazardous concentration for 5% of the species (HC5), derived from species sensitivity distributions, is typically used to establish EQSs (Posthuma et al., 2002). Future research should therefore focus on determining if microevolutionary effects in copper and zinc already occur around regulatory relevant concentrations. A recent review assessed the regulatory relevance of microevolutionary effects of cadmium based on a comparison of concentrations at which microevolutionary effects have been reported in the literature and conventionally derived ecotoxicological threshold concentrations (De Coninck et al., 2014). The authors found reports of microevolutionary effects of cadmium at hardness-normalized concentrations that were at least 1.5 times higher than the HC5 of 0.34mg Cd/L. This suggests that there is no immediate need to consider microevolutionary effects of cadmium in ecological risk assessments of freshwater environments. A similar approach should be followed for copper and zinc to gain insight at which (bioavailable) metal concentrations natural selection (and adaptation) actually occurs.

<u>Research question 9</u>: Do populations display lower genetic diversity after adaptation than prior to selection?

Although natural populations can harbour evolutionary potential to adapt genetically to chemical stressors, it is often thought that natural selection leads to a general reduction of genetic diversity and involves costs. Microsatellite genotyping revealed a decrease in clonal diversity but no change in allelic richness (liked to excess heterozygosity) in the Cu- and Zn-adapted populations compared to the control and original populations. It is allelic diversity that primarily determines a population's ability to respond

to long-term selection over many generations (Allendorf, 1986), nevertheless, whether clonal diversity or allelic richness (gene diversity) is the biodiversity-based protection goal is a decision to be made by water quality managers. The heterozygote excess observed In the Cu- and Zn-selected populations may be a consequence of an increased fitness advantage of heterozygote genotypes under Cu or Zn stress. This type of selection prescribes that, while there is no single “best” allele, the heterozygotes in a population often have a fitness advantage over the homozygotes. There are numerous examples of so-called heterozygote-fitness correlations in field studies (HFCs, see (Chapman et al., 2009) for a review), yet there is lack of data on the effect of environmental stress on these HFCs. We are only aware of two experimental studies with *Daphnia*, which provides contrasting results. Hebert and colleagues found that outcrossed heterozygote clones of homozygote parents were more tolerant to temperature and salinity stress (Hebert et al., 1982). In another study clonal heterozygosity had a negative effect on survival under high and low conductivities as well as at low pH (Jose and Dufresne, 2010). This excess heterozygosity in metal-selected populations has important consequences for ERA, as it may act as a mechanism to maintain allelic richness under multi-generational chemical exposure.

Limitations of using microsatellite markers

Microsatellite markers are neutral markers that do not necessarily reflect the genetic variation at the genes under selection. Our study could be screenshot of an incomplete selective ‘sweep’ or evolution in action. The results from chapter 7 suggest that under experimental conditions natural selection favours heterozygote genotypes. Alternatively, the fact that heterozygous individuals possess a greater diversity of alleles, could make them better suited to cope with environmental stochasticity, the so-called ‘episodic heterozygote advantage (Samollow and Soule, 1983). While it appears likely, we cannot conclude that the heterozygote excess was a result of direct selection for increased heterozygosity (balancing selection), i.e. to maintain genetic variation.

Future research should consider other molecular techniques, such as selective sweep approaches or analysis of outlier loci in genome scans that identify genes or genomic regions linked with genes under selection, would be more appropriate (Coutellec and Barata, 2013). Future studies may want to consider more loci, as genomic heterozygosity was inferred by a few loci only. The finding of

heterozygote excess in metal adapted populations suggests that balancing selection may play role in metal adaptation and deserves further investigation.

Limitations of only considering asexual reproduction

It remains to be tested if this heterozygote excess is maintained after recombination events during subsequent sexual cycles. Although microevolutionary effects during clonal selection may be rapid (as evidenced in chapter 7), it is uncertain to what extent the 'selection of the fittest' necessarily contributes to the genetic composition of future generations. This is because daphnids alternate between clonal reproduction and sexual reproduction and the fact that ephippial recruitment events have the capacity to reset the evolutionary trajectory of active populations (Hembre and Megard, 2006). **Future research should determine if adaptation is preserved after genetic recombination during periods of sexual reproduction.** This could be achieved by hatching the sexually-produced ephippia collected during a selection experiment and comparing the tolerance of those hatchlings to adapted asexual population.

Research question 10: Is adaptation to chemical stressors associated with costs of adaptation?

In a follow-up study with the Zn-adapted populations, no effect of Zn selection on the tolerance to heat and *M. aeruginosa* was observed. Limited to no evidence of either a cost or a gain associated with zinc adaptation was found in a follow-up experiment where the Zn-adapted populations where exposed to novel stressors. The only evidence of cross-tolerance (i.e. a fitness gain) was a higher 21-day Cd EC_{20} of reproduction in the Zn-adapted populations. Contrary to the genetic erosion hypothesis, no clear costs of Zn adaptation upon exposure to the new stressors were observed. The only potential cost of adaptation was noted at cadmium concentrations higher than the EC_{50}.

Only the initial tolerance to additional stressors was tested not the adaptive potential

Adaptation to toxic cyanobacteria may play an important role too. It has already been demonstrated that *Daphnia* frequently exposed to harmful algal blooms also develop a higher tolerance to cyanobacteria (Gustafsson and Hansson, 2004). **Future experiments should also consider investigating the adaptive potential response to novel stressors rather than initial tolerance alone**. However it is not possible to study all possible combinations of stressors (which are only predicted to increase in the

context of global change). ERA therefore urgently needs robust tools that identify the imprint of microevolution on populations, as well as thorough mechanistic understanding of the measured endpoints to (i) interpret them in an ecological way and (ii) translate them to ESQs in ERA. As it will be impossible for ERA to tackle an almost infinite number of combination of stressors, the application of tools from the –omics field (e.g. biomarkers in transcriptomics, proteomics and metabolomics), as well as AOPs (mentioned above) could shed light on the mechanistic basis on the sub-organismal level.

Box 8.2. Overall contribution of this PhD dissertation to ecological risk assessment.

This PhD dissertation has identified 3 major outcomes relevant for ERA of chemicals as they hold the potential to improve ecotoxicology practices by increasing the ecological realism:

1. **Combined effects of chemicals and natural stressors**

PNECs or EQSs based on copper NOECs derived under standard conditions may not be protective enough in systems that experience *M. aeruginosa* blooms. Therefore it might become necessary to derive separate EQSs for mixtures of copper and *M. aeruginosa* for eutrophic systems, especially in the context of global change projections.

2. **DEB-IBM for extrapolations from individual to population level effects**

DEB-IBM can predict the combined effects of copper and *M. aeruginosa* at the population level (even extinction) based on the observed effects of the individual stressors at the individual level.

3. **Microevolutionary effects**

Clonal populations can adapt rapidly to chemical stress without loss of genetic diversity and without major costs of tolerance in subsequent exposure to additional stressors.

Summary

Summary

The current approach followed in ecological risk assessment (ERA) of chemicals suffers several limitations. For instance, environmental protection goals tend to target populations or higher levels of organization, whereas ERA relies on standardized laboratory tests in which effects are measured on individual. From these tests, the threshold concentration of a chemical, below which no population-level effects should occur, is derived. In addition, these laboratory tests are mostly conducted for individual substances with laboratory populations with limited genetic variability under optimal conditions, whereas natural populations are not only genetically diverse, but they also have to cope with multiple stressors and varying environmental conditions. There is however a pressing need to evaluate the combined effects of stressors, as research suggests that mixtures at No-Observed-Effect-Concentration (NOEC) levels of individual substances may cause adverse effects when they are combined. With global change projections, co-occurrences of natural and chemical stressors are only predicted to increase. The aim of this thesis was to investigate the combined effect of metals (copper and zinc) with natural stressors (harmful algal blooms and global warming) at different organization levels (individual vs. population), and time-scales (short term vs. long term) in the freshwater model organism *Daphnia magna*.

In chapter 2 the response of *D. magna* to the combined effects of copper and 5 cyanobacterial genera was predicted using 2 widely used reference models, i.e. the Concentration Addition (CA) reference model for similarly acting stressors and the Independent Action (IA) reference model for dissimilarly acting stressors.4 major findings were noted: (1) The interaction type differed between the *Microcystis aeruginosa* + copper mixture (non-interaction according to CA and synergism according to IA) and the 4 other cyanobacteria + copper mixtures (antagonism according to CA and non-interaction according to IA). (2), Interactive effects were predicted differently by both reference models. More specifically, mixtures of Cu and *Microcystis aeruginosa* were synergistic with IA, whereas non-interaction was observed with CA, while the remaining 4 cyanobacteria + copper combinations non-interaction was predicted according to IA and antagonism according to CA. (3) Both reference models provided reasonable predictions for all observed combined effects. Despite IA providing a more accurate fit to the data (with the exception of *M. aeruginosa*), CA consistently delivered more conservative predictions for the combined effects of copper and cyanobacteria mixtures. Thus, CA could serve as a conservative model to account for mixture toxicity of cyanobacteria and copper in water quality management, as it gives rise to conservative predictions of mixed stressor toxicity at sub-lethal effect levels in *Daphnia*

magna. (4) Finally, and in accordance with other studies of cyanobacteria + chemical mixtures, we did not detect any strong synergistic effects of copper and cyanobacteria mixtures on *D. magna.* Of the tested genera *Microcystis* was the most harmful in combination with copper, but the combined effects could be predicted with the references models based on the individual level effects.

In chapter 3 the effect of temperature on the harmfulness of 6 different cyanobacterial genera was assessed in a standard ecotoxicity test. More specifically two questions were answered, (i) whether *D. magna* becomes more or less sensitive to the harmful effects of cyanobacteria and (ii) whether the different cyanobacteria genera are more harmful to *D. magna* than starvation alone. The results suggest that higher temperatures, related to global warming, may increase the sensitivity of *D. magna* to the presence of some cyanobacteria (*Anabaena* and *Oscillatoria*) in their diet, while the harmful effects of others (*Microcystis, Nodularia and Aphanizomenon*) may diminish at higher temperatures. No effect of temperature on the sensitivity of *D. magna* to *Cylindrospermopsis* was observed. Further, the findings from chapter 2 suggest that *Microcystis* is the only strain for which the harmful effects can at least partly be attributed to toxin production, as it was the only strain to cause significantly greater mortality to *Daphnia* than starvation alone.

The combined results from chapters 2 and 3 suggested that *M. aeruginosa* was the most harmful cyanobacterial genus (i.e. the only cyanobacteria with a synergistic interaction with copper according to the IA model and the only cyanobacteria genus that was more harmful than starvation alone), and because the harmfulness of *M. aeruginosa* to *D. magna* decreased with temperature (chapter 3), chapter 4 investigated whether the combined effects of copper and *M. aeruginosa* would under less optimal environmental conditions (temperature and the total food concentration). A standard ecotoxicity test was carried out with mixtures of copper and *M. aeruginosa* at 3 different temperatures (15°C, 19°C, 23°C) and 2 different total food concentrations (0.8mg C/L and 2.5mg C/L). The interactive effects between copper and *M. aeruginosa*, i.e. synergism according to IA and non-interaction according to CA, was not affected by temperature and total food concentration. In line with chapter 2, CA gave rise to more accurate predictions of mixture toxicity than IA and we therefore confirm the former model's suitability as a suitable tool for evaluating mixture toxicity of copper and *M. aeruginosa* under the temperature and food concentrations tested. Further, the 21-day median effective concentration for copper based on

reproduction varied between 20 and 100 µg/L and the results indicate that the percentage of *M. aeruginosa* explained 76% of the variance in the Cu median effective concentration for reproduction (EC_{50}), suggesting that the effects of the temperature and the total food were much less important (together explaining 11% of the variance of the 21-day EC_{50}). Further, a diet composed of only 10% *M. aeruginosa* results in copper EC_{50} values close to the copper NOEC under standard conditions, which confirms that mixtures at NOEC levels of individual substances can result in adverse effects and indicates that ecological risk assessment of copper should consider specific situations where harmful *M. aeruginosa* blooms can co-occur with elevated copper exposure.

In chapter 5 a population experiment was carried out to compare the total density of a *D. magna* population exposed to regulatory and environmentally relevant copper concentrations under more realistic global change conditions. These conditions were equivalent to a seasonal increase of temperature and the proportion of the total diet consisting of the harmful cyanobacterium *M. aeruginosa* under current temperature conditions, as well as a 4°C temperature increase predicted under global change. Populations exposed to the copper and *M. aeruginosa* under a 4°C temperature increase went extinct, even though the effects of the individual stressors were limited. Two main conclusions were drawn: (1) The findings highlight the need to consider the combined effects of multiple stressors in ERA, as the population level extinction could not have predicted from the effect of copper alone. This is an example of an ecological surprise, i.e. multiple stressors having combined effects that are not expected from the effects of the single stressors. (2) The results presented in chapter 5 suggest that individual level effects measured in standard ecotoxicity tests (as in chapter 4) can be translated to more complex realistic effects at the population level (as in chapter 5).

In chemical risk assessment, the ecological effects of stressors are often inferred from observations made at the level of the individual. In chapter 6 the hypothesis was tested that a mechanistic model (DEB-IBM) could extrapolate the effects at the individual level (using reproduction as an endpoint) to the effects at more ecologically relevant endpoints at the population level (such as total abundance). The results from this chapter have shown that the combination of standard ecotoxicity endpoints and an existing DEB-IBM model is able to deliver reasonable predictions for the combined effects of copper and *M. aeruginosa* on population abundance under global change conditions. The modelling simulations

from chapter 6 also illustrate that the DEB-IBM can be applied for combined effects that follow the Concentration Addition reference model.

In chapter 7, the microevolutionary effects of copper and zinc were assessed on the basis of *3 hypotheses*: (i) that genetically diverse populations can adapt to lethal metal concentrations (equivalent to the 8-day LC50), (ii) that populations display lower genetic diversity after adaptation than prior to selection, and (iii) that adaptation to chemical stressors is associated with a cost of adaptation. A 10 week microevolution experiment was conducted with a genetically diverse and representative sample of a natural *Daphnia magna* population that was exposed to copper and zinc. Both Cu- and Zn-selected populations developed a significantly higher metal tolerance (i.e. genetic adaptation), indicated by higher reproduction probabilities of clonal lines in Cu and Zn exposures than observed for the original and control populations. We have further demonstrated that these populations can recover fully in the case of zinc but not for copper, suggesting that the natural *D. magna* population used in this study has a higher capacity to adapt to zinc than to copper. This warns against generalizations, as it suggests that observations of the evolutionary consequences of long-term exposures to lethal effect concentrations (8-day LC50s in our study) of a given pollutant cannot be extrapolated to other substances. The results suggests only limited costs of adaptation, as (1) the reproduction under control conditions didn't differ between the control and metal-adapted populations, and (2) as no effect of Zn-adaptation was observed on the tolerance to high temperature and cyanobacteria in a follow-up study with the Zn-adapted populations. However, higher tolerance to Cd was observed in the Zn-adapted than in the non-selected populations (but only if the 20% effective concentration of Cd was considered).

Finally, chapter 8 provides an overview of the main conclusions reached and the limitations of the results in light of the research questions put forward in the introduction, and provides suggestions for further research.

Samenvatting

Het huidige systeem voor de ecologische risicoschatting van chemicaliën is gelimiteerd door enkele duidelijke gebreken. Ecologische risicoschattingen hebben als doel effecten van chemicaliën op populaties en gemeenschappen te vermijden. Typisch wordt dit risico geschat op basis van gestandaardiseerde blootstellingexperimenten waarin enkel een beperkt aantal individuen worden getest. Bovendien worden deze blootstellingexperimenten uitgevoerd voor individuele stoffen en onder optimale omstandigheden op populaties met beperkte genetische variabiliteit. Natuurlijke populaties worden nochtans gekenmerkt door een hoge genetische diversiteit, blootstelling aan meerdere stressoren en door de tijd variërende, sub-optimale omstandigheden. Onderzoek heeft aangetoond dat de combinatie van verschillende stressoren (metaal)toxiciteit sterk kan beïnvloeden en verwacht wordt dat de combinatie van stressoren nog belangrijker zal worden in de toekomst door de klimaatsverandering. Het doel van dit proefschrift was daarom het onderzoeken van de gecombineerde effecten van metaaltoxiciteit (koper en zink) en andere stressoren (cyanobacteriën en hogere temperaturen) voor verschillende organisatieniveaus (individu en populatie) en verschillende termijnen (korte termijn vs. lange termijn) op de watervlo *Daphnia magna*.

In hoofdstuk 2 werd *D. magna* blootgesteld aan een combinatie van koper en één van vijf geselecteerde soorten cyanobactieriën. Verwacht werd dat de toxiciteit van deze mengsels afhankelijk was van de gebruikte cyanobacteriënsoort en dus van de mode of action van de toxines. De toxiciteit van deze mengsels werd geëvalueerd aan de hand van twee vaak-gebruikte referentiemodellen: concentratie-additie (CA) en independent action (IA). Vier resultaten vielen hierbij op: (1) De interactie tussen koper en *Microcystis aruginosa* was verschillend van de interactie tussen koper en de vier andere geteste cyanobacteriën. Mengsels van koper en *M. aruginosa* waren synergetisch volgens IA, terwijl geen interactie werd waargenomen werdt volgens CA. Voor de vier andere geteste cyanobacteriën waren de interacties met koper afwezig volgens IA en antagonistisch volgens CA. (2) Verschillende interacties voor hetzelfde mengsel werden dus voorspeld door beide referentiemodellen. (3) De effecten van elk mengsel werden door beide referentiemodellen behoorlijk voorspeld. Het IA model benaderde in het algemeen de observaties beter terwijl de voorspellingen door het CA model steeds conservatiever waren. Het CA model lijkt dus het meest geschikt voor gebruik in waterkwaliteitsbeheer. (4) Het ontbreken van synergistische interacties tussen koper en cyanobacteriën op *D. magna* is in overeenstemming met andere studies van mengsels met cyanobacteriën. Van alle geteste mengsels

had het mengsel van koper met *M. aruginosa* de grootste effecten op *D. magna*. De toxiciteit van dit mengsel kon echter accuraat voorspeld worden aan de hand van de beschikbare referentiemodellen.

In hoofdstuk 3 werd het effect van temperatuur op de schadelijkheid van zes verschillende cyanobactieriën in een standaard ecotoxiciteitstest onderzocht. Twee onderzoeksvragen stonden hierbij centraal: (1) het effect van temperatuur op de toxiciteit van cyanobacteriën voor *D. magna* en (2) voor welke cyanobacteriën is de toxiciteit niet enkel het gevolg van verhongering van *D. magna*. Een hogere temperatuur versterkte de negatieve effecten van twee cyanobacteriën (*Anabaena* and *Oscillatoria*) terwijl de toxiciteit afnam voor drie andere soorten (*Microcystis, Nodularia and Aphanizomenon*). Temperatuur had geen effect op de toxiciteit van *Cylindrospermopsis*. In lijn met de resultaten van hoofdstuk 2, werden enkel voor *Microcystis* negatieve effecten vastgesteld die niet aan verhongering alleen konden toegewezen worden. Dit suggereert dat, althans gedeeltelijk, de effecten van *Microcystis* op *D. magna* het gevolg zijn van de productie van toxines.

In hoofdstuk 4 werd de mengseltoxiciteit van *M. aeruginosa* met koper verder onderzocht. Volgend uit de resultaten van hoofdstuk 2 en 3, was de centrale hypothese in hoofdstuk 4 dat de mengseltoxiciteit van koper en *M. aeruginosa* op *D. magna* zou variëren met de temperatuur en de totale voedselconcentratie. Standaard ecotoxiciteitstesten werden uitgevoerd met mengsels van koper en *M. aeruginosa* bij drie verschillende temperaturen (15°C, 19°C en 23°C) en twee verschillende totale voedselconcentraties (0.8mg C/L and 2.5mg C/L). De gevonden interacties tussen koper en *M. aeruginosa* (synergisme volgens IE, geen interactie volgens CA) waren onafhankelijk van de temperatuur en de totale voedselconcentratie. Net zoals in hoofdstuk 2 was de accuraatheid van het CA model hoger dan van het IA model voor de mengseltoxiciteit van *M. aeruginosa* met koper. Het CA model werd daarom als een geschikte methode beschouwd om de mengseltoxiciteit van koper en *M. aeruginosa* bij de hier geteste temperaturen en voedselconcentratie te voorspellen. De 21-dagen mediane effectconcentratie van koper voor reproductie varieerde van 20 tot 100 µg/L. Het percentage *M. aeruginosa* in het voedsel verklaarde 76% van de variatie in deze mediane koper effectconcentratie voor reproductie (EC_{50}). Dit suggereert dat de invloed van temperatuur en totale voedselconcentratie (samen 11% van de EC_{50} variatie) veel minder belangrijk waren dan het voedselpercentage *M. aeruginosa*. Daarnaast werden EC_{50} waarden dicht bij de NOEC van koper gevonden wanneer het

voedsel slechts 10% *M. aeruginosa* bevatte. Dit resultaat bevestigt dat mengseleffecten kunnen optreden bij NOEC-concentraties (bepaald bij standaardcondities) van de individuele stressoren in het mengsel. Ecologische risicoschattingen van koper dienen dus in rekening te brengen wanneer *M. aeruginosa* bloeien samen voorkomen met kopervervuiling.

In hoofdstuk 5 werd een populatie-experiment uitgevoerd om de effecten van koper op *D. magna* populaties bij regulatorische en mileurelevante concentraties na te gaan bij realistische omgevingcondities. Deze omgevingscondities stemden overeen met een seizoenale toename in temperatuur en het aandeel *M. aeruginosa* in het voedsel, en dit bij zowel huidige temperatuurcondities als bij een gemiddelde toename van 4°C door klimaatsverandering. Ondanks beperkte effecten van individuele stressoren, werd totale extinctie van de *D. magna* populaties geobserveerd bij blootstelling aan het mengsel van koper en *M. aeruginosa* bij verhoogde temperaturen (klimaatsverandering). Om te testen of synergistische effecten op individu-niveau ook observeerbaar zijn op populatie-niveau, werden de resultaten van het populatie-experiment vergeleken met de resultaten van de standaard ecotoxiciteitstesten in hoofdstuk 4. Twee algemene conclusies werden hierbij vastgesteld: (1) Deze resultaten benadrukken het belang van mengseltoxiciteit voor ecologische risicoschattingen. De extinctie van *D. magna* in de populatie-experimenten was niet voorspelbaar op basis van ecotoxiciteitstesten met koper alleen. (2) De vergelijking tussen de experimenten op individu- en populatieniveau suggeren dat de effecten op individu-niveau inderdaad kunnen vertaald worden in meer complexe effecten op populatie-niveau.

Zoals reeds aangehaald worden de ecologische effecten van stressoren in ecologische risicoschattingen vaak bepaald aan de hand van observaties op individu-niveau. In hoofdstuk 6 werd nagegaan of een mechanistisch model (DEB-IBM) kan gebruikt worden om de effecten van individu-niveau (reproductievermindering) te extrapoleren naar meer ecologisch relevante eindpunten (zoals abundantie) op populatieniveau. De resultaten van dit hoofdstuk tonen aan dat met het DEB-IBM model relevante voorspellingen kunnen gemaakt worden voor de effecten van koper en M. aeruginosa op *D. magna* populaties bij veranderende klimaatcondities. Verder werd ook aangetoond dat het DEB-IBM model in staat is mengseltoxiciteit te voorspellen aan de hand van het concentratie-additie referentie model.

In hoofdstuk 7 werden de micro-evolutionaire effecten van koper en zink bestudeerd op basis van drie hypotheses: (1) genetisch diverse populaties kunnen zich aanpassen aan letale metaalconcentraties (equivalent aan de 8-dagen LC_{50}), (2) populaties zijn genetisch minder divers na adaptatie aan chemische stress en (3) adaptatie aan chemische stress gaat gepaard met kosten van adaptatie. Gedurende 10 weken werd een micro-evolutie experiment uitgevoerd met een genetisch divers en representatief staal van een natuurlijke *D. magna* populatie die werd blootgesteld aan koper en zink. Zowel populaties blootgesteld aan koper als aan zink werden toleranter voor blootstelling aan deze metalen, genetische adaptatie trad dus op. Dit kon duidelijk geobserveerd worden in de hogere reproductie bij metaalstress voor klonen uit deze blootgestelde populaties in vergelijking met controlepopulaties. Verder werd aangetoond dat deze populaties, in het geval van zink, zich volledig konden herstellen. Dit werd niet waargenomen voor koper, wat er op wijst dat de natuurlijke populatie gebruikt in deze experimenten een hogere capaciteit heeft om zich aan te passen aan zinkblootstelling dan aan koperblootstelling. Veralgemeningen over de adaptatie aan chemische stress op basis van de resultaten van één stressor zijn dus te vermijden. De kosten die gepaard gaan met adaptatie lijken beperkt te zijn: (1) de reproductie bij controlecondities was gelijkaardig voor controle populaties en populaties geadapteerd aan de metalen en (2) adaptie aan zink had geen effect op de tolerantie voor hoge temperaturen of cyanobacteriën, zoals aangetoond in een vervolgexperiment met de zink-geadapteerde populaties. In dit vervolgexperiment werd wel een hogere tolerantie voor cadmium vastgesteld in de zink-aangepaste populaties in vergelijking met de niet-geadapteerde populaties maar enkel bij de EC_{20} van cadmium.

Tot slot gaf hoofdstuk 8 een overzicht van de belangrijke conclusies in dit proefschrift en hoe zij hebben geantwoord op bepaalde problemen naar voren gebracht in de inleiding. Verder werd er ook aandacht besteed aan de uitdagingen die ecologische risicoschatting nog te wachten staan.

A

Supplementary material for Chapter 2

Appendix A

A.1. Green algae culture medium. All components are dissolved in carbon filtered city tap water (Bold, 1978)

Components	Concentration (g/L or mL/L)
I. Fe Stock	Stock Concentration (g/L)
$Fe(NH_4)_2(SO_4)2{\cdot}6H_2O$	0.702 g
Na_2EDTA	0.66 g
II. Metal Mix	Stock Concentration (g/L)
H_3BO_3	1.14 g
$FeCl_3{\cdot}6H_2O$	0.049 g
$MnSO4{\cdot}4H_2O$	0.164 g
$ZnSO_4{\cdot}7H_2O$	0.022 g
$CoSO_4{\cdot}7H_2O$	0.048 g
Na_2EDTA	1 g
III. Vitamin Stock	Stock Concentration (g/L)
Vit B12	0.01 g
Vit B1	0.5 g
Vit H	0.005 g
ES-Medium Provasoli	Stock Concentration (g/L or mL/L)
$NaNo_3$	3.5 g
Na2glcinophosphate	0.5 g
I. Fe Stock	250 mL
II. Metal Mix	250 mL
III. Vitamin Stock	2 mL
Walne-medium	Stock Concentration (g/L)
$FeSO_4{\cdot}7H_2O$	0.278 g
$NaH_2PO_4{\cdot}2H_2O$	3 g
$NaNO_3$	30 g
$MnCl_2{\cdot}4H_2O$	0.47 g
Per litre carbon filtered water add 10 mL ES-Provasoli medium and 5 mL Walne-medium	

Table A.2. Medium composition of $BG11_0$. All components are dissolved in deionized H_2O (Allen, 1968).

Components	Concentration (g/L)	Trace Components	Concentration (mg/L)
$NaNO_3$	1.5	H_3BO_3	2.86
$NaHCO_3$	0.42	$MnCl_2.4H_2O$	1.81
K_2HPO_4	0.04	$ZnSO_4.7H_2O$	0.222
$MgSO_4.7H_2O$	0.075	$Na_2MoO_4.2H_2O$	0.39
$CaCl_2.2H_2O$	0.036	$CuSO_4.5H_2O$	0.079
Citric acid ($C_6H_8O_7$)	0.006	$Co(NO_3)_2.6H_2O$	0.0494
Ferric ammonium citrate	0.006		
EDTA	0.001		
Na_2CO_3	0.04		

Table A.3. Medium composition of BG11. All components are dissolved in deionized H_2O (Allen 1968).

Components	Concentration (g/L)	Trace Components	Concentration (mg/L)
$NaNO_3$	1.5	H_3BO_3	2.86
K_2HPO_4	0.04	$MnCl_2.4H_2O$	1.81
$MgSO_4.7H_2O$	0.075	$ZnSO_4.7H_2O$	0.222
$CaCl_2.2H_2O$	0.036	$Na_2MoO_4.2H_2O$	0.39
Citric acid ($C_6H_8O_7$)	0.006	$CuSO_4.5H_2O$	0.079
Ferric ammonium citrate	0.006	$Co(NO_3)_2.6H_2O$	0.0494
EDTA	0.001		
Na_2CO_3	0.04		

Table A.4. Medium Composition of Z8. All components are dissolved in deionized H_2O unless stated otherwise (Kotai, 1972b)(Kotai 1972).

Components	Concentration (g/L)	Components	Concentration (mg/L)
$NaNO_3$ [a]	0.467	$(NH_4)_6.Mo_7O_{24}.4H_2O$ [d]	0.0088
$MgSO_4.7H_2O$ [a]	0.025	KBr [d]	0.012
$Ca(NO_3)_2.4H_2O$ [a]	0.059	KI [d]	0.04083
K_2HPO_4 [b]	0.031	$ZnSO_4$ [d]	0.0287
Na_2CO_3 [b]	0.021	$Co(NO_3)_2.6H_2O$ [d]	0.0146
$FeCl_3.6H_2O$ [c*]	0.0028[1]	$CuSO_4.5H_2O$ [d]	0.0125
EDTA-Na_2 [c*]	0.0037[2]	H_3BO_3 [d]	3.1

Components with the same letter in superscript can be combined in one stock solution.

[*] 2.80 g FeCl3•6H2O dissolved in 100 mL 0.1 N HCl to make an Fe-solution and 3.90 g EDTA-Na_2 dissolved in 100 mL 0.1 N NaOH to make an EDTA-solution. 10 mL of the Fe-solution are dissolved in circa 900 mL deionized H_2O to which 9.5 mL of the EDTA-solution is added, and filled up to one litre. Of this diluted combined Fe-solution and EDTA-solution 10mL is added per each L of Z8 medium.

Table A.5. Estimated model parameters used in the data analysis for the Xinb3 clone. *Ana=Anabaena, Aph=Aphanizomenon, Cyl=Cylindrospermopsis, Mc=Microcystis, Osc=Oscillatoria*, *s* is the slope of the concentration response curve, EC_{50} is the 50% effect concentration, IA is the Independent Action reference model (Equation 2.2), CA is the Concentration Addition reference model (Equation 2.3), IASA/CASA (reference models Equation 2.5 and Equation 2.6 including deviation parameter *a* to quantify synergism where *a*<0, or antagonism where *a*>0), SSE is sums of squared errors, AIC is Aikaike Information Criterion. **p* value <0.05 of the *F*-test to compare the nested models indicates a significant deviation from non-interaction. SSE is used to compare nested CA and CASA (or IA and IASA) models, while AIC is used to compare non nested CA and IA models.

	Ana	***Aph***	***Cyl***	***Mc***	***Osc***
s_{Cu}					
IA	7.332	11.405	11.380	9.242	12.845
IASA	8.584	9.440	11.785	11.502	12.698
CA	7.666	6.754	18.484	9.920	14.508
CASA	9.878	7.052	15.151	11.260	13.246
s_{Cyano}					
IA	2.008	3.558	2.729	5.613	1.401
IASA	1.890	3.295	2.417	7.016	2.199
CA	1.641	3.735	2.962	4.226	1.003
CASA	2.456	3.887	2.762	4.790	2.510
Cu EC_{50} (ug/L)					
IA	99.121	93.935	102.891	71.135	99.794
IASA	98.048	91.781	103.763	86.225	100.896
CA	109.532	110.126	116.432	87.961	105.455
CASA	99.713	91.595	113.196	90.328	108.621
Cyanobacteria EC_{50} (% of diet)					
IA	50.818	76.348	73.305	22.164	40.509
IASA	47.298	70.913	77.661	22.185	44.938
CA	76.648	80.785	115.218	24.588	66.954
CASA	51.108	91.337	86.507	23.826	49.590
***a* deviation parameter**					
IASA	-0.039	1.491	0.393	-7.369	0.861
CASA	1.266	2.013	1.587	0.068	1.047
***p* value (*F*-test)**					
IA/ IASA	0.777	0.357	0.520	3.13e-10*	0.505
CA/CASA	6.515e-06*	6.922e-07*	3.421e-04*	0.521	5.693e-05*
SSE					
IA	1807.537	3859.238	6166.729	6161.685	3053.506
IA/IASA	1800.123	3694.999	6037.257	816.152	2984.593
CA	5557.229	16426.940	12070.170	552.966	6488.326
CA/CASA	1964.284	5088.905	6260.156	541.419	2831.637
AIC					
IA	187.968	206.931	218.648	218.628	201.076
IA/IASA	189.865	207.843	220.118	170.090	202.506
CA	216.0464	243.142	235.4373	158.3574	219.919
CA/CASA	192.0471	215.8455	221.024	159.8299	201.1903

Table A.6. Estimated model parameters used in the data analysis for the Iinb1 clone. *Ana=Anabaena, Aph=Aphanizomenon, Cyl=Cylindrospermopsis, Mc=Microcystis, Osc=Oscillatoria*, *s* is the slope of the concentration response curve, EC_{50} is the 50% effect concentration, IA is the Independent Action reference model (Equation 2.2), CA is the Concentration Addition reference model (Equation 2.3), IASA/CASA (reference models Equation 2.5 and Equation 2.6 including deviation parameter *a* to quantify synergism where *a*<0, or antagonism where *a*>0), SSE is sums of squared errors, AIC is Aikaike Information Criterion. **p* value <0.05 of the *F*-test to compare the nested models indicates a significant deviation from non-interaction. SSE is used to compare nested CA and CASA (or IA and IASA) models, while AIC is used to compare non nested CA and IA models.

	Ana	***Aph***	***Cyl***	***Mc***	***Osc***
Cu slope					
IA	39.288	17.791	7.028	11.984	2.703
IASA	36.114	14.588	6.234	12.210	2.352
CA	9.152	6.452	8.960	6.460	2.440
CASA	9.636	8.911	6.816	6.199	4.482
Cyanobacteria slope					
IA	1.985	1.523	1.926	2.021	4.674
IASA	1.955	0.992	1.588	2.240	3.183
CA	2.393	0.732	1.869	3.715	2.086
CASA	5.077	1.611	2.711	2.816	1.693
Cu EC_{50} (ug/L)					
IA	78.759	81.837	81.370	56.359	73.390
IASA	76.435	77.487	77.711	50.966	68.070
CA	92.039	83.322	96.364	58.051	86.996
CASA	86.108	76.821	84.110	55.512	78.241
Cyanobacteria EC_{50} (% of diet)					
IA	52.665	35.451	51.911	15.706	42.204
IASA	45.553	25.193	51.754	27.446	40.591
CA	64.800	42.282	70.006	33.799	48.635
CASA	64.027	42.204	55.642	30.948	41.210
***a* deviation parameter**					
IASA	1.223	2.725	0.964	-4.570	0.915
CASA	1.042	1.604	1.225	0.078	1.918
***p* value (*F*-test)**					
IA/ IASA	0.140	0.137	0.411	2.292e-05*	0.678
CA/CASA	9.844e-03*	6.158e-05*	6.817e-03*	0.428	1.885e-02*
SSE					
IA	3798.862	2064.455	5315.825	3338.390	9343.703
IA/IASA	3398.267	1843.400	5134.702	1333.894	9261.569
CA	13129.020	7503.556	9206.041	2025.151	12789.100
CA/CASA	9332.444	3299.676	6327.305	1961.043	10015.180
AIC					
IA	206.537	191.291	214.936	203.306	229.037
IA/IASA	205.751	190.459	216.070	182.372	230.816
CA	237.540	223.553	228.665	190.810	236.884
CA/CASA	231.006	205.015	221.291	192.006	232.772

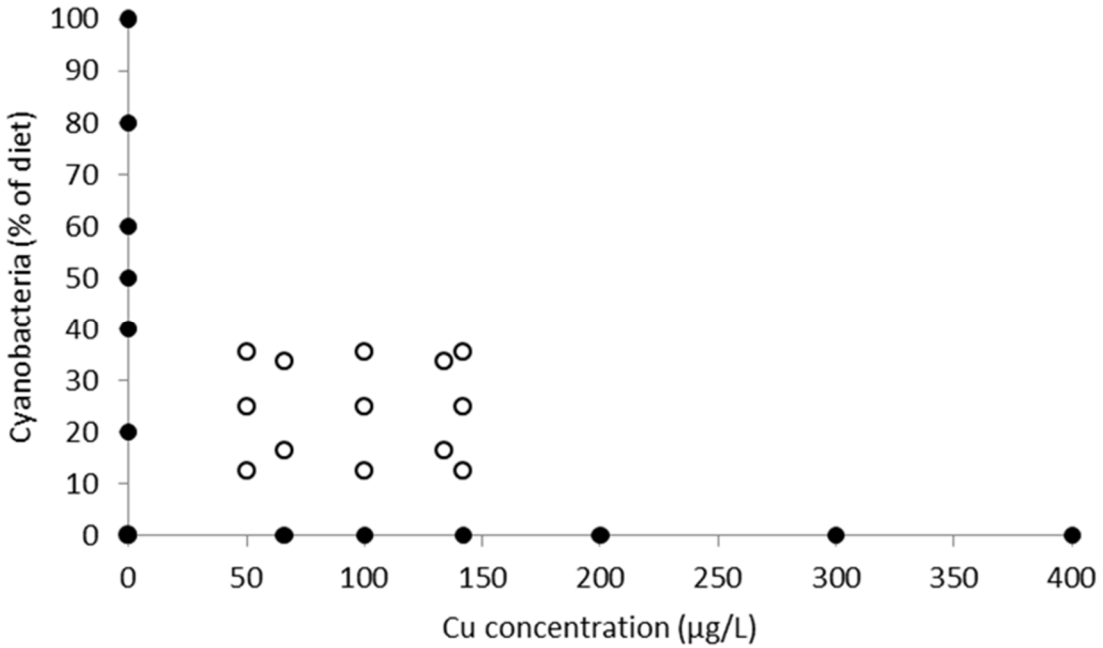


Figure A.1. Experimental design consisting of the single stressor treatments (black circles) and mixture combinations (white circles) based on the central composite design representing each binary combination of Cu and cyanobacteria in the experiments. Concentrations shown are nominal.

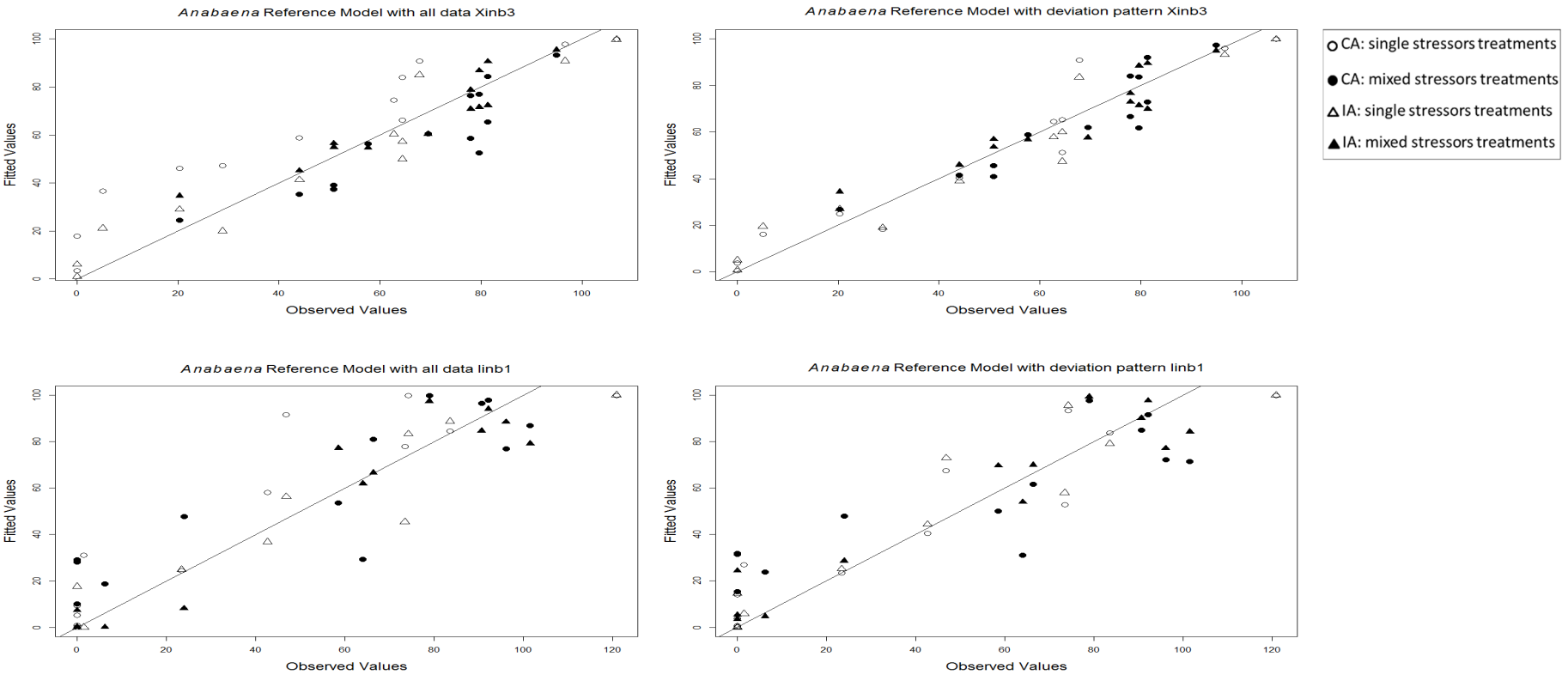


Figure A.2. Mean observed versus fitted values for total reproduction of the Xinb3 (upper figures) and Iinb1 (lower figures) clones after exposure to Cu and *Anabaena*. The fitted values are derived from the estimated model parameters from Tables A.5 (Xinb3) and A.6 (Iinb1). Both singles stressor data, as well as mixture stressor data were used for the model fitting. Points above the 1:1 line (higher predicted reproduction compared to observed reproduction) indicate synergism, while points below the regression line (lower predicted reproduction compared to observed reproduction) suggest antagonistic effects between copper and the cyanobacteria. A statistically significant improved fit of the reference model with the deviation pattern (Equation 2.5 and Equation. 2.6, right figures) compared to the reference model (Equation 2.2 and Equation. 2.3, left figures) is visually indicated by a an improved match of the fitted values with the observed values (points are closer to the 1:1 line in the right figures than in the left figures).

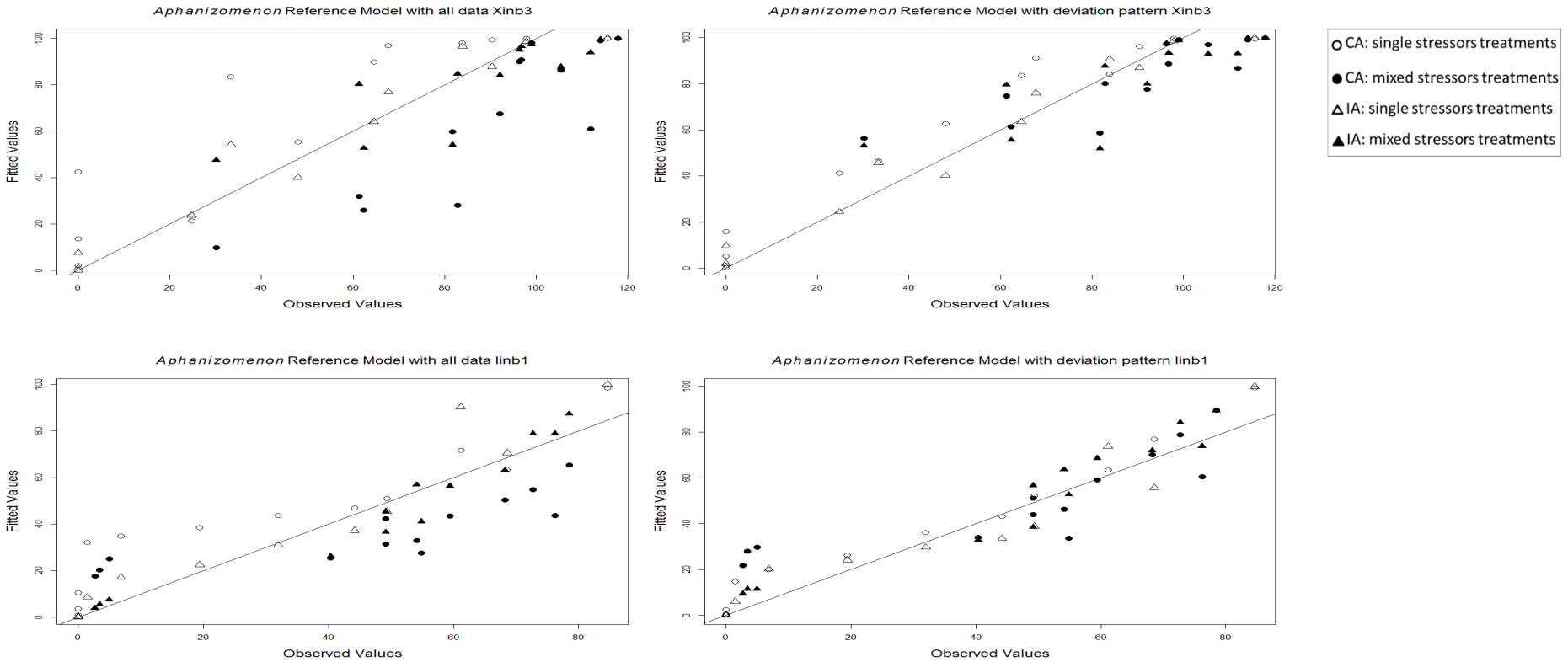


Figure A.3. Mean observed versus fitted values for total reproduction of the Xinb3 (upper figures) and Iinb1 (lower figures) clones after exposure to Cu and *Aphanizomenon*. The fitted values are derived from the estimated model parameters Tables A.5 (Xinb3) and A.6 (Iinb1). Both singles stressor data, as well as mixture stressor data were used for the model fitting. Points above the 1:1 line (higher predicted reproduction compared to observed reproduction) indicate synergism, while points below the regression line (lower predicted reproduction compared to observed reproduction) suggest antagonistic effects between copper and the cyanobacteria. A statistically significant improved fit of the reference model with the deviation pattern (Equation 2.5 and Equation. 2.6, right figures) compared to the reference model (Equation 2.2 and Equation. 2.3, left figures) is visually indicated by a an improved match of the fitted values with the observed values (points are closer to the 1:1 line in the right figures than in the left figures).

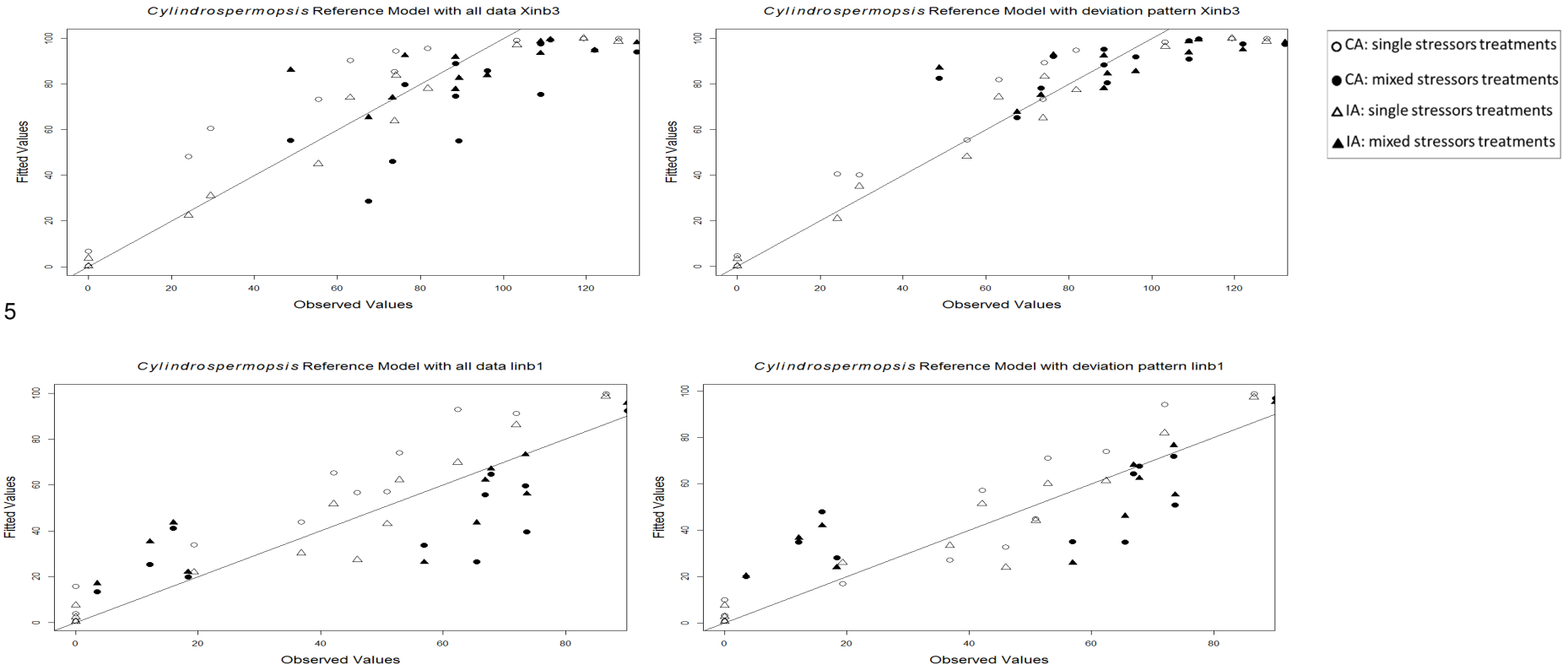


Figure A.4. Mean observed versus fitted values for total reproduction of the Xinb3 (upper figures) and Iinb1 (lower figures) clones after exposure to Cu and *Cylindrospermopsis*. The fitted values are derived from the estimated model parameters from Tables A.5 (Xinb3) and A.6 (Iinb1). Both singles stressor data, as well as mixture stressor data were used for the model fitting. Points above the 1:1 line (higher predicted reproduction compared to observed reproduction) indicate synergism, while points below the regression line (lower predicted reproduction compared to observed reproduction) suggest antagonistic effects between copper and the cyanobacteria. A statistically significant improved fit of the reference model with the deviation pattern (Equation 2.5 and Equation. 2.6, right figures) compared to the reference model (Equation 2.2 and Equation. 2.3, left figures) is visually indicated by a an improved match of the fitted values with the observed values (points are closer to the 1:1 line in the right figures than in the left figures).

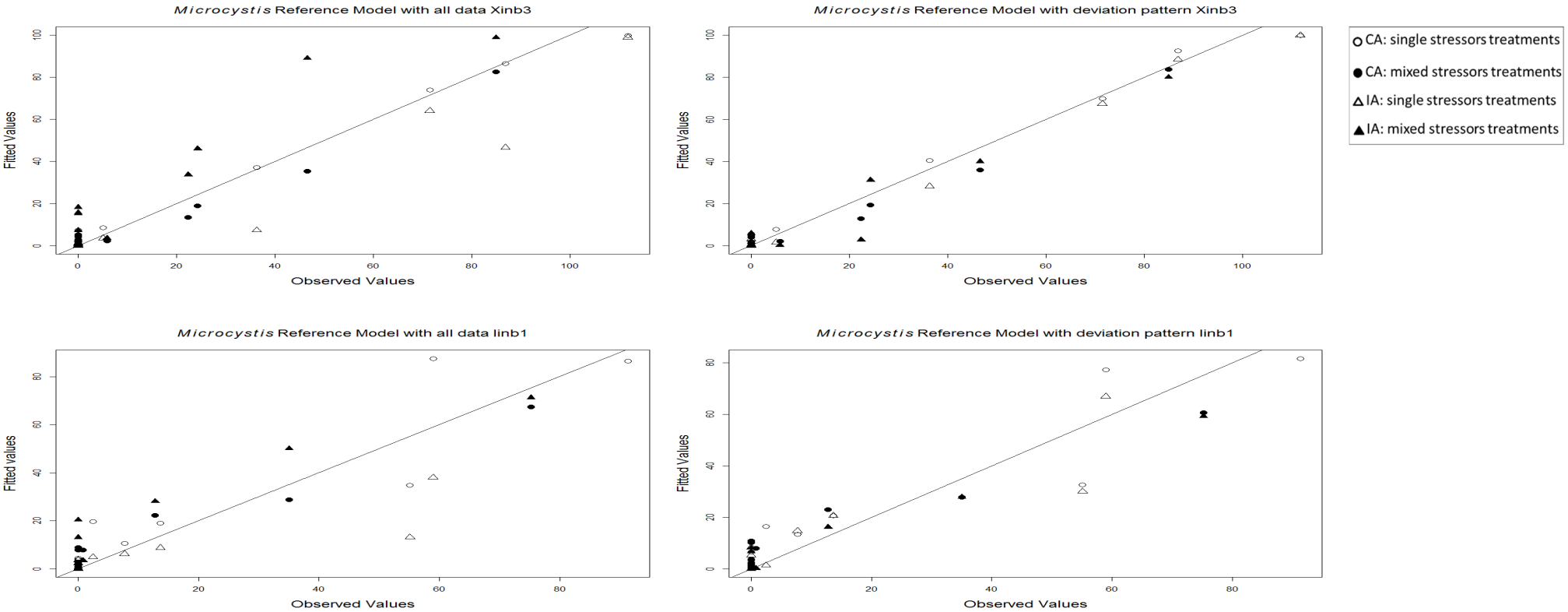


Figure A.5. Mean observed versus fitted values for total reproduction of the Xinb3 (upper figures) and linb1 (lower figures) clones after exposure to Cu and *Microcystis*. The fitted values are derived from the estimated model parameters from Tables A.5 (Xinb3) and A.6 (linb1). Both singles stressor data, as well as mixture stressor data were used for the model fitting. Points above the 1:1 line (higher predicted reproduction compared to observed reproduction) indicate synergism, while points below the regression line (lower predicted reproduction compared to observed reproduction) suggest antagonistic effects between copper and the cyanobacteria. A statistically significant improved fit of the reference model with the deviation pattern (Equation 2.5 and Equation. 2.6, right figures) compared to the reference model (Equation 2.2 and Equation. 2.3, left figures) is visually indicated by a an improved match of the fitted values with the observed values (points are closer to the 1:1 line in the right figures than in the left figures).

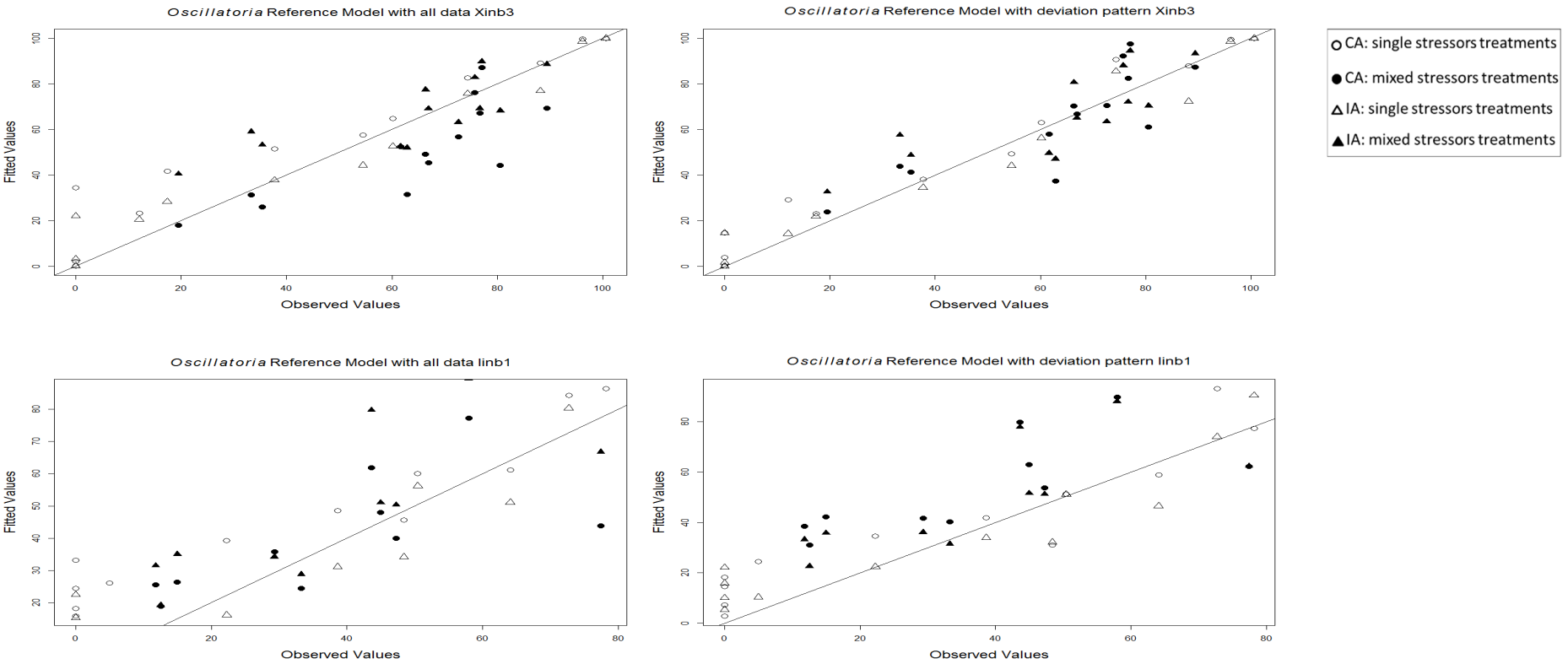


Figure A.6. Mean observed versus fitted values for total reproduction of the Xinb3 (upper figures) and Iinb1 (lower figures) clones after exposure to Cu and *Oscillatoria*. The fitted values are derived from the estimated model parameters from Tables A.5 (Xinb3) and A.6 (Iinb1). Both singles stressor data, as well as mixture stressor data were used for the model fitting. Points above the 1:1 line (higher predicted reproduction compared to observed reproduction) indicate synergism, while points below the regression line (lower predicted reproduction compared to observed reproduction) suggest antagonistic effects between copper and the cyanobacteria. A statistically significant improved fit of the reference model with the deviation pattern (Equation 2.5 and Equation. 2.6, right figures) compared to the reference model (Equation 2.2 and Equation. 2.3, left figures) is visually indicated by a an improved match of the fitted values with the observed values (points are closer to the 1:1 line in the right figures than in the left figures

B

Supplementary material for Chapter 3

Table B.1. Dose response parameters (-95%/+95% confidence interval) for the full dose response of total reproduction for daphnids exposed to each cyanobacteria species at each temperature.

	15℃	**19℃**	**23℃**
Anabaena			
EC_{10}	61.28 (44.34/84.68)	20.60 (16.99/24.98)	13.35 (10.51/16.94)
EC_{50}	74.09 (64.44/85.19)	42.26 (39.58/45.13)	31.02 (27.89/34.52)
Slope	11.57 (-2.841/25.99)	3.059 (2.406/3.162)	2.605 (2.047/3.162)
Aphanizomenon			
EC_{10}	5.77 (2.06/16.14)	15.59 (6.95/34.97)	32.45 (20.34/51.76)
EC_{50}	19.09 (12.14/30.01)	56.73 (44.87/71.74)	95.35 (74.97/121.28)
Slope	1.7 (0.626/2.775)	1.7 (0.693/2.707)	2.038 (0.911/3.166)
Cylindrospermopsis			
EC_{10}	52.11 (38.38/70.75)	31.1 (22.36/43.27)	36.96 (22.92/59.61)
EC_{50}	75.3 (66.21/85.63)	76.68 (67.47/87.14)	87.97 (70.63/109.58)
Slope	5.969 (1.485/10.45)	2.435 (1.477/3.392)	2.532 (0.909/4.154)
Microcystis			
EC_{10}	10.22 (7.36/14.18)	30.99 (24.42/39.32)	29.03 (22.67/37.17)
EC_{50}	20.27 (17.94/22.91)	39.06 (36.47/41.84)	42.27 (38.84/45.99)
Slope	3.207 (1.975/4.44)	9.484 (1.578/17.39)	5.849 (2.723/8.975)
Nodularia			
EC_{10}	15.15 (9.95/23.08)	31.42 (25.06/39.4)	32.74 (28.46/37.67)
EC_{50}	22.02 (18.67/25.97)	41.36 (38.11/44.89)	49.98 (47.45/52.64)
Slope	5.88 (-2.52/14.28)	7.997 (2.639/ 13.35)	5.195 (3.66/6.73)
Oscillatoria			
EC_{10}	25.57 (16.49/39.67)	18.08 (12.45/26.26)	6.58 (3.11/13.9)
EC_{50}	47.34 (40.87/54.85)	39.98 (34.97/45.71)	21.37 (16.06/28.44)
Slope	3.568 (1.393/5.743)	2.769 (1.733/3.806)	1.864 (1.025/2.702)

Table B.2. Summary of the p-values from the pairwise comparisons in the Wheeler ratio test for each cyanobacteria species at different temperatures.

	EC_{50}		Slope	
	Ana 15℃	Ana 19℃	Ana 15℃	Ana 19℃
Ana 19℃	<0.0001		0.002	
Ana 23℃	<0.0001	<0.0001	0.0005	NS
	Aph 15℃	Aph 19℃	Aph 15℃	Aph 19℃
Aph 19℃	<0.0001		NS	
Aph 23℃	<0.0001	0.003	NS	NS
	Cyl 15℃	Cyl 19℃	Cyl 15℃	Cyl 19℃
Cyl 19℃	NS		0.01	
Cyl 23℃	NS	NS	0.03	NS
	Mc 15℃	Mc 19℃	Mc 15℃	Mc 19℃
Mc 19℃	<0.0001		0.003	
Mc 23℃	<0.0001	NS	0.03	NS
	Nod 15℃	Nod 19℃	Nod 15℃	Nod 19℃
Nod 19℃	<0.0001		NS	
Nod 23℃	<0.0001	0.009	NS	NS
	Osc 15℃	Osc 19℃	Osc 15℃	Osc 19℃
Osc 19℃	NS		NS	
Osc 23℃	<0.0001	0.0002	0.05	NS

Table B.3. Summary of the p-values from the pairwise comparisons in the Wheeler ratio test for all cyanobacteria at each temperature.

	15℃					19℃					23℃				
EC_{10}	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***
Aph	<0.0001					NS					0.001				
Cyl	NS	<0.0001				0.04	NS				0.0002	NS			
Mc	<0.0001	NS	<0.0001			0.01	NS	NS			<0.0001	NS	NS		
Nod	<0.0001	NS	<0.0001	NS		0.007	NS	NS	NS		<0.0001	NS	NS	NS	
Osc	NS	0.01	0.01	0.002	NS	NS	NS	0.04	0.02	0.015	NS	0.0005	0.0002	0.0003	<0.0001
EC_{50}	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***
Aph	<0.0001					0.02					<0.0001				
Cyl	NS	<0.0001				<0.0001	0.03				<0.0001	NS			
Mc	<0.0001	NS	<0.0001			NS	0.004	<0.0001			<0.0001	<0.0001	<0.0001		
Nod	<0.0001	NS	<0.0001	NS		NS	0.02	<0.0001	NS		<0.0001	<0.0001	<0.0001	0.002	
Osc	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	NS	0.02	<0.0001	NS	NS	0.02	<0.0001	<0.0001	<0.0001	<0.0001
Slope	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***	***Ana***	***Aph***	***Cyl***	***Mc***	***Nod***
Aph	0.0001					0.03					NS				
Cyl	NS	0.001				NS	NS				NS	NS			
Mc	0.005	NS	NS			0.0006	<0.0001	0.0002			0.0009	0.0009	0.02		
Nod	NS	0.02	NS	NS		0.0007	<0.0001	0.0002	NS		<0.0001	0.0004	0.02	NS	
Osc	0.02	0.04	NS	NS	NS	NS	NS	NS	0.0005	0.0007	NS	NS	NS	<0.0001	<0.0001

Table B.4. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Anabaena* (+/-95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Anabaena* parameters**				**PwC 15℃-19℃**		**PwC 15℃-23℃**		**PwC 19℃-23℃**	
length	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	2967	3326	3389	-583.2/0.669	0.106	-773.9/-84.01	0.09	-446.7/128	0.507
Slope	-5.058	-5.813	-7.83	-4.313 ; 4.886	0.778	-1.991/9.222	0.621	0-1551/8.635	0.621
r_m	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.1325	0.3259	0.3600	-0.236 /-0.172	<0.0001*	-0.2799/-0.1924	0.0066*	-0.0706/0.0098	0.1636
Slope	-0.0003	-0.0018	-0.0024	0.001/0.0022	<0.0001*	0.0013/0.0034	0.0066*	-0.0002/0.0017	0.1836
Age 1st brood	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	17	10	9	6 /-8	<0.0001*	7.3 /9.5	<0.0001*	1/2	0.0033*
Slope	0.025	0	0	0.008/0.05	0.075	-0.013/0.04	0.548	-0.029/0	0.548
***1st* brood size**	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	7.2	14	12.5	-10/ -3	<0.0001*	-8.2/-2.3	0.01*	-2.1/4.5	0.4307
Slope	0.02	-0.125	-0.133	0.07/0.215	<0.0001*	0.09/0.23	0.0067*	-0.05/0.088	0.7596

Table B.5. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Aphanizomenon* (+/- 95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Aphanizomenon* parameters**				**PwC 15°C-19°C**		**PwC 15°C-23°C**		**PwC 19°C-23°C**	
length	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	3041	3364	3275	-585.2/ -75.53	0.03*	-529.8/-8.477	0.0868	-103.1/188.4	0.568
Slope	-9.861	-6.42	-4.612	-7.312 /0.5204	0.2136	-9.619 /-0.761	0.0801	-4.365/1.168	0.26
r_m	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.1987	0.3164	0.3567	-0.1865 /-0.0655	<0.0001*	-0.2345/-0.1069	<0.0001*	-0.0747/-0.0136	0.0067*
Slope	-0.0017	-0.0003	-0.0004	-0.0025/-0.0003	0.04*	-0.0024/-4.37e-05	0.0734	-0.0004/0.0008	0.7613
Age 1st brood	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	16.3	9.5	8	3.7 /8.7	<0.0001*	5.9/13.5	<0.0001*	1 /3	<0.0001*
Slope	0.0063	0	0	-0.2/0.08	1	-0.2/0.078	1	-0.017/0	1
***1st* brood size**	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	17	13.2	7.5	-3.7/6.25	0.3589	1.625/11.5	0.02336*	2.75 /8.042	<0.0001*
Slope	-0.2	-0.06	0	-0.21/-0.05	<0.0001*	-0.26/-0.1	<0.0001*	-0.092/0	0.0534

Table B.6. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Cylindrospermopsis* (+/-95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Cylindrospermopsis* parameters**				**PwC 15℃-19℃**		**PwC 15℃-23℃**		**PwC 19℃-23℃**	
length (µm)	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	3095	3457	3295	-572.1/-191.3	<0.0001*	-359.801/4.703	0.063*	47.568/339.831	0.0134*
Slope	-3.216	-4.789	-4.047	-1.357/5.15	0.284	-2.539/3.995	0.601	-3.336/0.915	0.3673
r_m	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.1925	0.3341	0.3268	-0.173/-0.1076	<0.0001*	-0.17/-0.089	<0.0001*	-0.033/4.6e-02	0.7813
Slope	-0.0003	-0.0009	-0.0004	8.79e-06/0.0012	0.1	-0.0007/0.0007	0.9215	-0.0012/-5.3e-05	0.09
Age 1st brood	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	15	10	8	3.41/6.38	<0.0001*	5.9/10.3	<0.0001*	0.75/2	0.0017*
Slope	0	0	0	-0.033/0.033	1	-0.017/0.078	1	0/0.025	1
***1st* brood size**	**15℃**	**19℃**	**23℃**	**CI (-95/+95)**	p-value	**CI (-95/+95)**	p-value	**CI (-95/+95)**	p-value
Intercept	16.5	13.8	6	-2.17/7.17	0.3456	6.17/13.5	<0.0001*	4/10.38	<0.0001*
Slope	-0.061	-0.075	0	-0.067/0.1	0.7329	-0.12/0.025	0.283	-0.12/-0.0125	0.0651

Table B.7. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Microcystis* (+/-95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Microcystis* parameters**				**PwC 15°C-19°C**		**PwC 15°C-23°C**		**PwC 19°C-23°C**	
length	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	2990	3536	3332	-819.594/-72.949	0.0201*	-627.7/100.1	0.2872	-91.92/472.6	0.2872
Slope	-20.37	-18.46	-13.65	-6.38/1.403	0.2571	-9.972/-3.734686	<0.0001	-8.05/-1.11	0.0334*
r_m	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.1908	0.3152	0.3507	-0.2219/-0.0672	<0.0001*	-0.2318/-0.1188	<0.0001*	-0.112/0.057	0.3472
Slope	-0.0026	-0.0034	-0.0035	-0.0006/0.0027	0.5944	-0.0002/0.002	0.2805	-0.0019/0.0017	0.8514
Age 1st brood	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	13.7	9	7.8	3.5/7.7	<0.0001*	5/9.2	<0.0001*	-0.333/3.4	0.102
Slope	0.09	0.05	0.075	-0.03/0.09	1	-0.05/0.075	1	-0.05/0.025	1
***1st* brood size**	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	10	11	7.5	-8/7.5	0.8731	-2.07/9	0.4007	-2.7/9	0.3255
Slope	-0.2	-0.2	-0.1	-0.2/0.17	0.9799	-0.23/0.05	0.3172	-0.2/0.07	0.3038

Table B.8. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Nodularia* (+/-95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Nodularia* parameters**				**PwC 15°C-19°C**		**PwC 15°C-23°C**		**PwC 19°C-23°C**	
length	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	2994	3158	3268	-683.2/5.398	0.1902	-559.161/32.747	0.1902	-228.3/378.1	0.768
Slope	-15.68	-13.55	-10.96	-8.486/2.323	0.4408	-8.68/-1.621	0.03*	-6.997/1.625	0.4408
r_m	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.2172	0.2881	0.325	-0.1433/-0.0209	0.0134*	-0.1752/-0.0638	<0.0001*	-0.075/0.0063	0.0935
Slope	-0.002	-0.0015	-0.001	-0.0029/0.0025	1	-0.0034/0.0016	1	-0.002/0.0006	0.187
Age 1st brood	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	13	8.5	8	1 ; 5.5	0.0234*	1.5/6	0.0051*	-0.25/2	0.222
Slope	0	0.05	0	-0.1//0.125	0.928	-0.075/0.175	0.989	0/0.075	0.2754
***1st* brood size**	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	7	7.5	6.5	-7.5/7.25	1	-5/8.5	1	-2/6	1
Slope	-0.05	-0.05	-0.05	-0.35/0.3	1	-0.35/0.3	1	-0.15/0.1	1

Table B.9. Results of the pairwise comparisons (PwC) of the slopes and intercepts of the non-parametric Theil-Sen regression models for *Oscillatoria* (+/-95% confidence interval around each PwC). * indicates significantly different slopes (i.e. interactive effects of temperature and cyanobacteria) at $p < 0.05$. The Bonferroni-Holm correction method was used to adjust the p-values for multiple comparisons. A summary of the *p*-values of the pairwise comparisons (PwC) of the slopes was already given in the manuscript (Table 3.2). For a summary of the Spearman's rho correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints we refer to Appendix B Table B.10.

***Oscillatoria* parameters**				**PwC 15°C-19°C**		**PwC 15°C-23°C**		**PwC 19°C-23°C**	
length	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	3130	3282	3277	-332.038/20.436	0.2403	-305.41/50.97	0.3004	-123.3/176.2	0.6578
Slope	-5.000	-5.802	-6.555	-1.811/4.648	1	-1.192/5.512	1	-2.222/3.193	1
r_m	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	0.2086	0.3174	0.3324	-0.1392/-0.0478	<0.0001*	-0.199/-0.045	0.0066*	-0.1028/0.0397	0.4307
Slope	-0.0009	-0.0021	-0.0035	0.0001 ; 0.0018	0.0301*	0.0013/0.0037	<0.0001*	0.0005/0.0026	0.0134*
Age 1st brood	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	15	8.7	5.3	3.4/7.8	<0.0001*	6 /11.2	<0.0001*	1 /5.2	0.0017*
Slope	0	0.033	0.067	-0.075/0.008	0.1336	-0.13/0.035	0.05*	-0.08/0	0.0934
***1st* brood size**	**15°C**	**19°C**	**23°C**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**	**CI (-95/+95)**	**p-value**
Intercept	16.8	13.0	4.6	-2.25/11	0.1936	6.5/17	0.01*	2/12.6	0.01*
Slope	-0.133	-0.15	-0.04	-0.133/0.1	0.7212	-0.188/0	0.1202	-02/0.04	0.2805

Table B.10. Summary of the Spearman's *Rho* correlation coefficient and associated p-value to test for an effect of cyanobacteria concentration on the endpoints.

Anabaena	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.659	0.0008*	-0.870	<0.0001*	-0.914	<0.0001*
r_m	-0.4324	0.0944	-0.963	<0.0001*	-0.897	<0.0001*
Age 1st brood	0.755	0.0021*	0.551	0.0022*	0.669	0.0046*
1st brood size	0.2239	0.42237	-0.767	<0.0001*	-0.859	<0.0001*
Aphanizomenon	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.770	<0.0001*	-0.865	<0.0001*	-0.961	<0.0001*
r_m	-0.764	0.0006*	-0.535	0.0011*	-0.529	0.001*
Age 1st brood	0.1005	1	-0.1011	1	0.371	0.057
1st brood size	-0.828	<0.0001*	-0.5532	0.0015*	-0.0842	0.636
Cylindrospermopsis	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.477	0.0088*	-0.782	<0.0001*	-0.689	<0.0001*
r_m	-0.294	0.1285	-0.871	<0.0001*	-0.486	0.004*
Age 1st brood	0.0335	0.866	0.721	<0.0001*	0.201	0.44
1st brood size	-0.348	0.1398	-0.597	0.0012*	-0.128	0.4763
Microcsytis	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.945	<0.0001*	-0.972	<0.0001*	-0.961	<0.0001*
r_m	-0.950	<0.0001*	-0.834	<0.0001*	-0.943	<0.0001*
Age 1st brood	0.767	0.0014*	0.783	<0.0001*	0.900	<0.0001*
1st brood size	-0.634	0.0149*	-0.619	0.0124*	-0.6287	0.0051*
Nodualaria	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.871	<0.0001*	-0.919	<0.0001*	-0.946	<0.0001*
r_m	-0.514	0.0877	-0.913	<0.0001*	-0.767	<0.0001*
Age 1st brood	0.138	0.667	0.887	<0.0001*	0.845	<0.0001*
1st brood size	-0.05	0.8708	-0.363	0.606	-0.305	0.606
Oscillatoria	**15℃**	**p-value**	**19℃**	**p-value**	**23℃**	**p-value**
length	-0.622	0.0026*	-0.797	<0.0001*	-0.895	<0.0001*
r_m	-0.767	<0.0001*	-0.908	<0.0001*	-0.792	<0.0001*
Age 1st brood	-0.159	0.4918	0.670	0.0004*	0.799	<0.0001*
1st brood size	-0.624	0.0057*	-0.438	0.05*	-0.338	0.085

* indicates a significantly correlation between cyanobacteria concentration and endpoint at $p < 0.05$.

The Bonferroni-Holm correction method was used

C

Supplementary material for Chapter 4

Table C.1. Summary table of the measured Cu concentrations. ± indicates the standard error (SE) around the mean values. All measurements are given as weighted averages as described in the OECD testing guideline and the standard error is calculated over all measurements in the clean medium prior to adding *Daphnia* and algae and in the old medium after renewal.

Exposure medium	total Cu µg/L	dissolved Cu µg/L
Control	3.54 ± 0.13	2.17 ± 0.09
Cu 1	45.09 ± 1.22	28.43 ± 1.41
Cu 2	59.41 ± 1.60	39.34 ± 1.94
Cu 3	83.85 ± 2.00	55.16 ± 2.35
Cu 4	115.77 ± 2.02	70.62 ± 3.35
Cu 5	161.00 ± 3.74	102.50 ± 4.14
Cu 6	208.14 ± 7.69	131.70 ± 8.28
Cu 7	289.61 ± 6.76	174.47 ± 4.55

Table C.2. Estimated model parameters. *s* is the slope of the concentration response curve, EC_{50} is the 50% effect concentration, *IA* is Independent Action reference model (Eq. 2.2), *CA* is Concentration Addition reference model (Eq. 2.3), IASA/CASA (reference models Eq. 2.5 and Eq. 2.6 including deviation parameter *a* to quantify synergism where a<0, or antagonism where a>0), *Mc* is *Microcystis aeruginosa*, *SSE* is the sums of squared errors, *AIC* is the Aikaike Information Criterion. *p value <0.05 of the F-test to compare the nested models indicates a significant deviation from non-interaction.

	0.8mg C/L			***2mg C/L***		
	15℃	***19℃***	***23℃***	***15℃***	***19℃***	***23℃***
s_{Cu}						
IA	8.75	5.84	7.37	4.33	2.03	3.17
IASA	7.06	7.05	5.02	6.76	4.57	3.41
CA	4.76	6.99	5.65	6.03	8.60	5.17
CASA	5.78	6.42	5.27	5.20	8.27	5.60
s_{Mc}						
IA	3.07	2.43	1.18	2.24	2.25	2.52
IASA	3.80	3.31	5.02	2.13	3.44	3.16
CA	4.51	3.96	3.24	2.77	3.20	3.96
CASA	4.12	4.61	3.81	3.26	2.83	3.84
Cu EC_{50} (ug/L)						
IA	62.00	57.93	52.66	64.20	99.25	83.06
IASA	61.02	67.77	53.86	68.12	98.13	83.56
CA	66.53	75.69	54.78	62.79	99.93	85.67
CASA	65.51	77.05	54.24	64.47	101.92	88.51
Mc EC_{50} (% of diet)						
IA	12.44	27.75	46.15	12.83	21.18	20.56
IASA	20.23	29.29	48.42	18.94	25.29	24.31
CA	19.81	35.16	49.07	19.40	21.52	49.11
CASA	17.85	38.33	52.60	22.46	22.88	30.47
***a* deviation parameter**						
IASA	-4.07	-3.353	-4.95	-4.472	-4.867	-3.2346
CASA	0.723	-0.223	-0.199	-0.420	-0.3814	-0.1072
***p* value (*F*-test)**						
IA/ IASA	2.58e-06*	9.41e-06*	1.01e-07*	2.27e-07*	7.42e-10*	0.0003*
CA/CASA	0.0370*	0.2564	0.8197	0.5393	0.0868	1
SSE						
IA	4974.6	13376.6	8393.3	4298.5	13910.7	8030.4
IASA	2162.5	6384.3	2884.9	1566.9	3338.7	4927.1
CA	2730.1	3953.0	3360.2	1206.8	2516.9	3869.1
CASA	2318.1	3765.5	3353.6	1189.8	2253.3	3868.9
AIC						
IA	262.3	294.0	279.0	257.6	295.2	290.4
IASA	237.6	272.3	246.9	227.3	251.5	264.0
CA	243.1	254.9	249.7	217.0	240.5	254.3
CASA	239.9	255.4	251.7	218.5	239.0	256.3

Table C.3. Summary table of the single stressor concentration response parameter values slope and EC50 (µg/L) of copper (± standard error). Single dose response curves can be accessed in Figure C.1.

Total food concentration	Temp	Cu EC_{50} 0% Mc	Cu Slope 0% Mc	Cu EC_{50} 10% Mc	Cu Slope 10% Mc	Cu EC_{50} 20% Mc	Cu Slope 20% Mc	Cu EC_{50} 40% Mc	Cu Slope 40% Mc
0.8mg C/L	15℃	65.75 ± 1.05	12.8 ± 6.43	40.75 ± 1.07	16.15 ± 28.51	28.56 ± 1.51	28.81 ± 2822	-	-
0.8mg C/L	19℃	75.74 ± 1.05	12.53 ± 6.11	50.51 ± 1.06	12.15 ± 5.86	35.64 ± 1.06	5.62 ± 1.21	29.72 ± 1.08	5.78 ± 1.41
0.8mg C/L	23℃	57.61 ± 1.04	5.68 ± 1.27	41.91 ± 1.05	16.22 ± 11.78	38.96 ± 1.06	5.84 ± 1.41	29.24 ± 2.03	20.29 ± 19.61
2mg C/L	15℃	64.09 ± 1.08	6.40 ± 3.02	43.70 ± 1.14	5.86 ± 3.62	19.37 ± 1.53	2.25 ± 1.42	-	-
2mg C/L	19℃	102.29± 1.04	8.68 ± 3.06	55.76 ± 1.29	32.16 ± 552	36.14 ± 1.11	3.84 ± 1.16	23.52 ± 1.27	3.67 ± 2.63
2mg C/L	23℃	87.10 ± 1.08	3.76 ± 0.93	60.15 ± 1.02	10.37 ± 1.85	46.98 ± 1.07	4.69 ± 1.08	35.53 ± 1.11	5.61 ± 2.53

Table C.4. Summary table of the single stressor concentration response parameter values slope and EC_{50} (% of total diet of *M. aeruginosa* (± standard error). Single dose response curves can be accessed in the Figure C.2.

Total food concentration	Temperature	EC_{50} (% of diet)	Slope
0.8mg C/L	15℃	17.60 ± 1.15	4.72 ± 3.08
0.8mg C/L	19℃	31.59 ± 1.15	2.61 ± 0.80
0.8mg C/L	23℃	46.40 ± 1.12	6.53 ± 4.34
2mg C/L	15℃	22.0 ± 1.08	7.00 ± 5.59
2mg C/L	19℃	27.93 ± 1.17	1.85 ± 0.53
2mg C/L	23℃	26.92 ± 1.05	3.74 ± 0.55

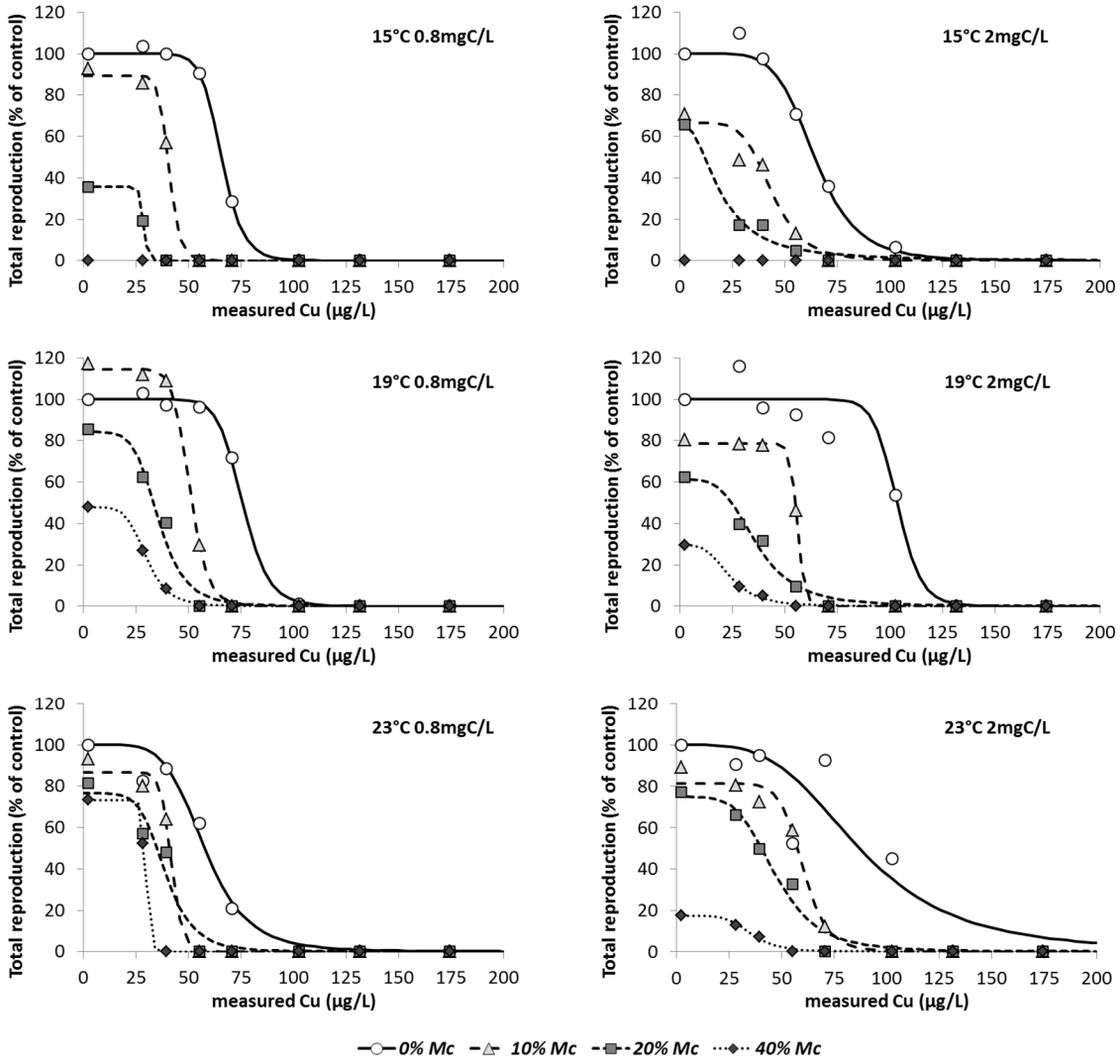


Figure C.1. Dose response curves of total reproduction after 21 days (control averaged) for Cu toxicity (dissolved Cu concentration) under 4 different concentrations of *Microcystis aeruginosa* (0, 10, 20 and 40% of the total diet), 3 different constant temperatures (15°C, 19°C, 23°C) and 2 total diet concentrations (0.8mgC/L and 2mgC/L). Please note that no reproduction occurred at 80% *Microcystis*, as well as at 15°C and 40% *Microcystis aeruginosa* at both 0.8 and 2mg C/L.

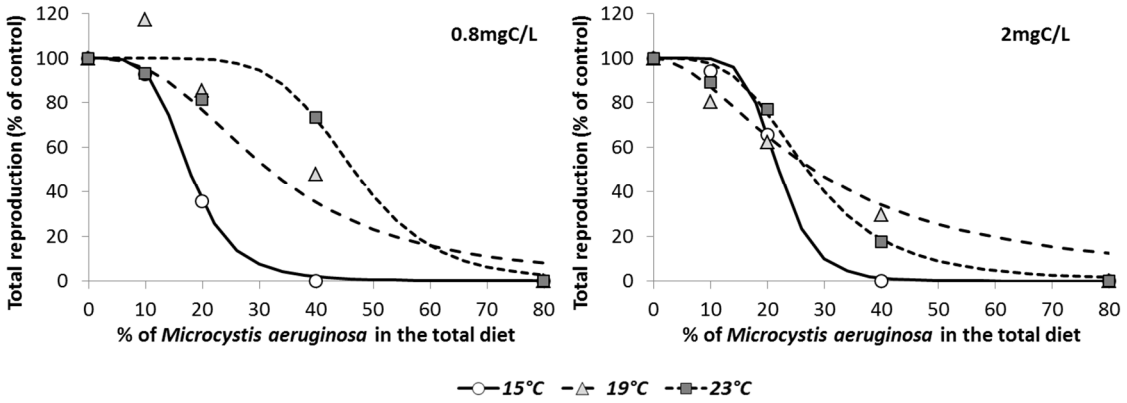


Figure C.2. Dose response curves of total reproduction after 21 days (control averaged) for cyanobacteria toxicity under 3 different constant temperatures (15℃, 19℃, 23℃) and 2 total diet concentrations (0.8mgC/L and 2mgC/L).

D

Supplementary material for Chapter 6

Table D.1. Parameters of the DEB model for *D. magna* along with the confidence intervals determined via profile likelihoods. The unit for time (t) is days, for structural length of animals (L) in mm, for the abundance of prey (cells/L), and for length of the environment (l) in cm. A dot over a symbol indicates a rate parameter (two dots represent a rate, t^{-2}). Curly brackets around a symbol represent the parameter is per unit surface area (see Kooijman 2010 for the full explanation). For *M* 0.9 was used as this was determined by Martin et al. 2013a. Assimilation was adjusted for all 8 primary DEB parameters, as well as for J_{XAm}. The half-saturation coefficient *K* was calibrated separately. The Arrhenius factor was applied to: U^B_H , U^P_H ,k_m, k_j, J_{XAm} and moltime.

DEB parameters				
symbol	**Description**	**dimension**	**Value**	**95% confidence interval**
κ	Fraction of mobilized energy to soma	-	0.678	.657-.700
κ_R	Fraction of reproduction energy fixed in eggs	-	0.95	Fixed value
$\dot{k}_m$	Somatic maintenance rate coefficient	t^{-1}	0.3314	0.327 - 0.336
$\dot{k}_j$	Maturity maintenance rate coefficient	t^{-1}	0.1921	0.150-0.236
U_H^b	Scaled maturity at birth	tL^2	0.1108	0.0989 - 0.123
U_H^p	Scaled maturity at puberty	tL^2	2.555	2.36 - 2.844
$\dot{v}$	Energy conductance	Lt^{-1}	18.1	17.89 - 18.3
g	Energy investment ratio	-	10	Fixed value
Ageing parameters				
$\ddot{h}_a$	Weibull ageing acceleration	t^{-2}	3.04E-6	1.70E-6 - 4.60E-6
s_G	Gompertz stress coefficient	-	.019	.00911-.0273
Prey dynamics parameters				
$\{\dot{J}_{XAm}\}$	Surface-area-specific max ingestion rate	$\#L^{-2}t^{-1}$	3.80E+05	3.7E+5 - 4.0E+5
K	Half-saturation coefficient	$\#l^{-3}$	1585	1571 - 1600
***Daphnia* specific parameter values**				
Molt-time	Time between reproductive events	t	2.8	-
V_{crit}	Proportion of structural mass below which *Daphnia* experience starvation mortality	-	0.4	-
M	Reserve dependent mortality coefficient	t^{-1}	Varied	-

Table D.2. Core DEB parameters of the DEB-IBM model for the DEB-IBM model for *Daphnia magna* and their link to various PMoAs through the stress level.

DEB parameters

Symbol	Description	Dimension	Value	PMoA
κ	Fraction of mobilized energy to soma	-	0.678	-
κ_R	Fraction of reproduction energy fixed in eggs	-	0.95	Reproduction costs and embryonic hazard
$\dot{k}_m$	Somatic maintenance rate coefficient	t^{-1}	0.3314	Maintenance costs, growth costs
$\dot{k}_j$	Maturity maintenance rate coefficient	t^{-1}	0.1921	Maintnenace costs
U_H^b	Scaled maturity at birth	tL^2	0.1108	-
U_H^p	Scaled maturity at puberty	tL^2	2.555	-
$\dot{v}$	Energy conductance	Lt^{-1}	18.1	-
g	Energy investment ratio	-	10	Growth costs
f	Scaled functional response	-	0-1	Feeding/assimilation

Table D.3. Sum of the toxic units of copper and *M. aeruginosa* under the different exposures from chapter 4.

Sum of TU			
Temperature			
Control	**2µg Cu/L +MC**	**25µg Cu/L +MC**	**44µg Cu/L +MC**
t0	0.345	0.647	0.897
t1	0.424	0.726	0.977
t2	0.504	0.805	1.056
t3	0.583	0.884	1.135
t4	0.662	0.963	1.214
+4°C	**2µg Cu/L +2xMC**	**25µg Cu/L +2xMC**	**44µg Cu/L +2xMC**
t0	0.662	0.963	1.214
t1	0.820	1.121	1.372
t2	0.978	1.280	1.531
t3	1.137	1.438	1.689
t4	1.295	1.596	1.847
Copper only	**2.171547**	**25**	**44**
t0-t4	0.029	0.330	0.581

Table D.4. Stress levels corresponding to the sum of the toxic units for the feeding PMoA.

Feeding	**A**	**B**	**SSE**
Temperature	0.5162	-0.1945	5968
Control	**2µg Cu/L +MC**	**25µg Cu/L +MC**	**44µg Cu/L +MC**
t0	-0.016	0.139	0.269
t1	0.025	0.180	0.310
t2	0.065	0.221	0.350
t3	0.106	0.262	0.391
t4	0.147	0.303	0.432
Change	**Cu2Mc**	**Cu25Mc**	**Cu44MC**
t0	0.147	0.303	0.432
t1	0.229	0.384	0.514
t2	0.311	0.466	0.596
t3	0.392	0.548	0.677
t4	0.474	0.630	0.759
Copper only	**2.171547**	**25**	**44**
t0-t4	-0.180	-0.024	0.105

Table D.5. Stress levels corresponding to the sum of the toxic units for the maintenance PMoA.

Maintenance PMoA		**A**	**B**	**SSE**
Temperature		4.633	-1.377	16951
Control	**2µg Cu/L +MC**		**25µg Cu/L +MC**	**44µg Cu/L +MC**
t0		0.222	1.619	2.781
t1		0.589	1.985	3.148
t2		0.956	2.352	3.514
t3		1.322	2.719	3.881
t4		1.689	3.085	4.248
Change	**Cu2Mc**		**Cu25Mc**	**Cu44MC**
t0		1.689	3.085	4.248
t1		2.422	3.819	4.981
t2		3.156	4.552	5.714
t3		3.889	5.285	6.448
t4		4.622	6.019	7.181
Copper only		**2.171547**	**25**	**44**
t0-t4		-1.244	0.152	1.314

Table D.6. Stress levels corresponding to the sum of the toxic units for the growth PMOA.

Growth		**A**	**B**	**SSE**
Temperature		3.639	-1.882	6644
Control	**2µg Cu/L +MC**		**25µg Cu/L +MC**	**44µg Cu/L +MC**
t0		-0.626	0.471	1.384
t1		-0.338	0.759	1.672
t2		-0.050	1.047	1.960
t3		0.238	1.335	2.248
t4		0.526	1.623	2.536
Change	**Cu2Mc**		**Cu25Mc**	**Cu44MC**
t0		0.526	1.623	2.536
t1		1.102	2.199	3.112
t2		1.678	2.775	3.688
t3		2.254	3.351	4.264
t4		2.830	3.927	4.840
Copper only		**2.171547**	**25**	**44**
t0-t4		-1.778	-0.681	0.232

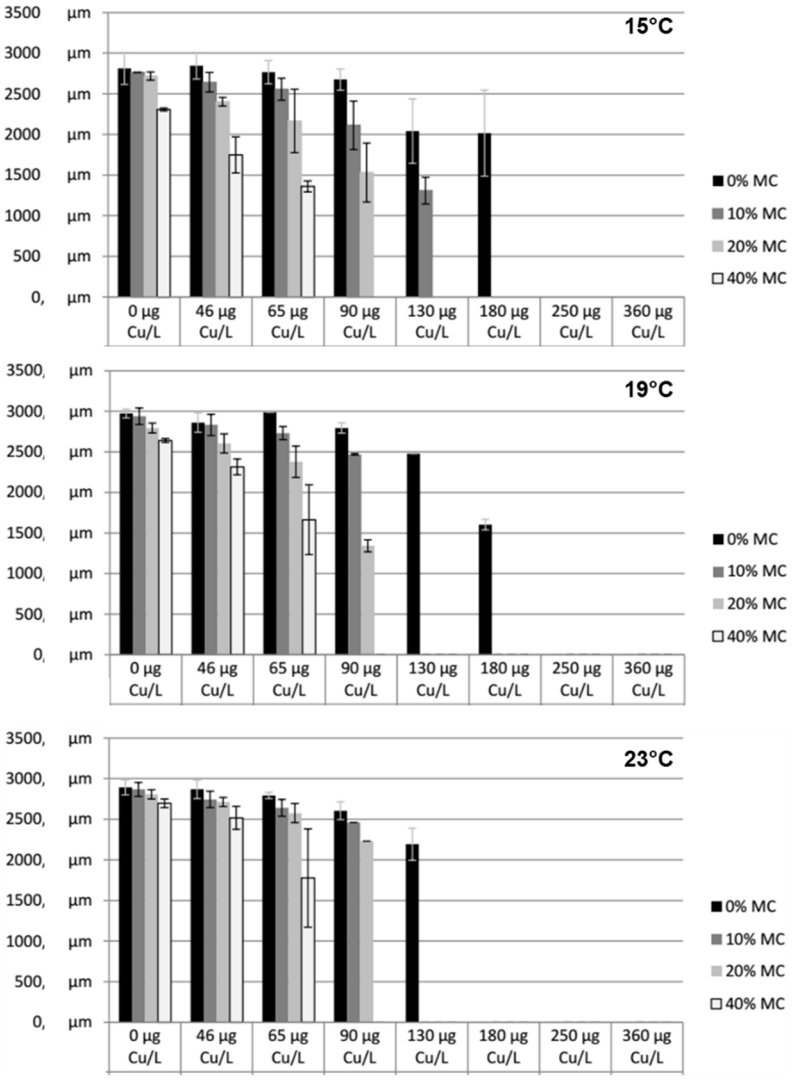


Figure D.1. Mean length (+/- SD) of *D. magna* after 21 days under copper and *M. aeruginosa* exposure at a total food concentration of 0.8 mgC/L.

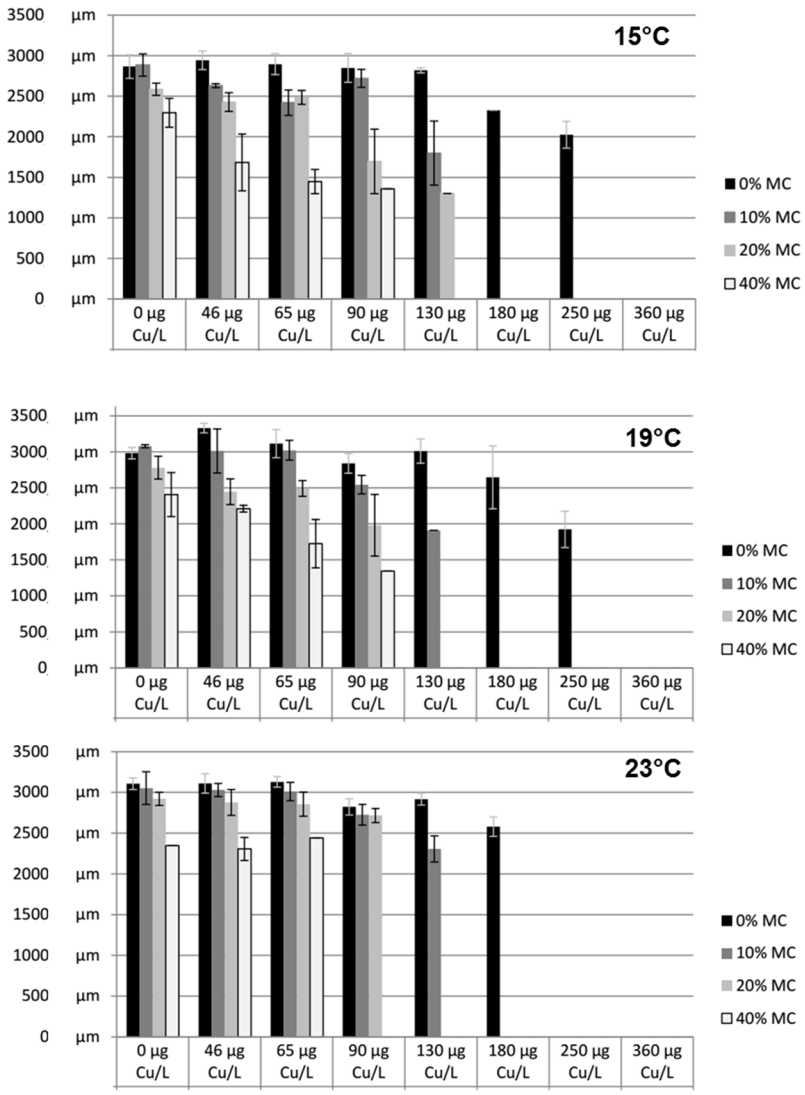


Figure D.2. Mean length (+/- SD) of *D. magna* after 21 days under copper and *M. aeruginosa* exposure at a total food concentration of 2 mgC/L.

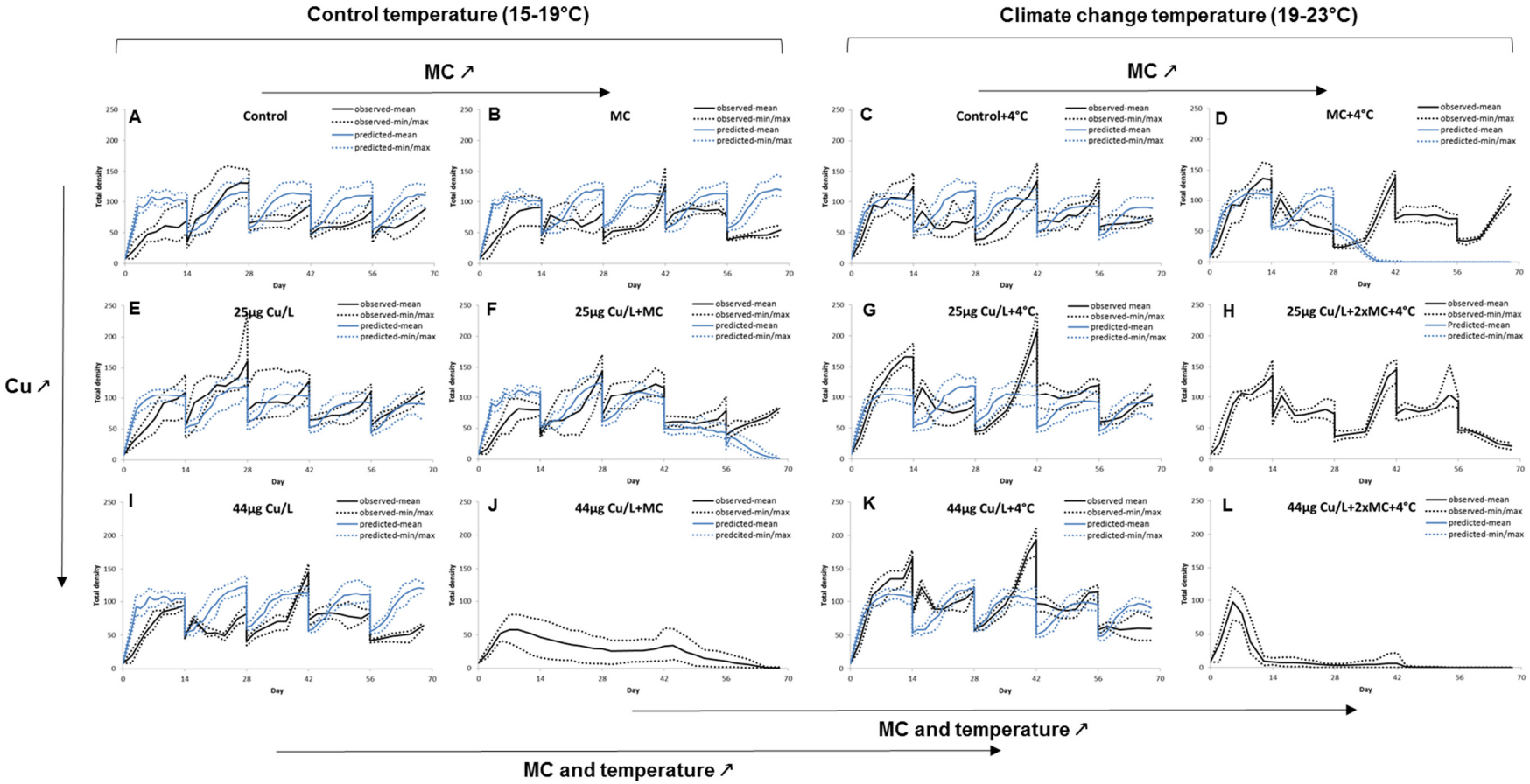


Figure D.3. Observed and predicted population dynamics for *D. magna* under copper (Cu) and *M. aeruginosa* (MC) stress using the growth PMoA. Full lines indicate mean total abundance (observations: = black, predictions = blue), while the dotted lines indicate the minimum and maximum observations (n = 4,) and predictions (n = 10). Arrows indicate increasing levels of stress. With the exception of 25µg Cu/L+MC, the DEB-IBM predicted that the populations would go extinct immediately in all mixture treatments.

E

Supplementary material for Chapter 7

Table E.1. Physico-chemical characteristics of the KNO17 pond at the time of ephippia collection in October 2011.

Latitude	51° 21' 01.97"N
Longitude	03° 19' 49.58"E
pH	7.72
Temperature (℃, daytime)	12.5
O_2 (mg/L)	12.32
Conductivity (µS/cm)	621
Surface area (m^2)	754
IC (mg/L)	dissolved: 47.95mg/L, total: 49.7mg/L
NPOC (mg/L)	dissolved: 30.3mg/L, total: 31.1mg/L
Daphnids present in the water column	no
Measured Cu (µg/L)	Dissolved: 0.3µg/L, total: 0.4µg/L , below quantification limit (<1µg/L)
Measured Zn (µg/L)	Below detection limit (<10µg/L) and quantification limit (<20µg/L)

Table E.2. Arrangement in multiplexes, annealing temperature in °C (Tm), labelling dye, PCR range size and bibliographic reference for the microsatellite loci used in this genotyping study. Taken from the supplementary material of Orsini et al. (2012).

Locus	Multiplex	Tm	Labeling	PCR range in bp	bibliographic reference
B008	M01	56	VIC	148-174	Jansen et al. 2010
B030	M01	56	PET	150-174	Jansen et al. 2010
B045	M01	56	NED	110-130	Jansen et al. 2010
B050	M01	56	6FAM	220-250	Jansen et al. 2010
B064	M01	56	6FAM	130-160	Jansen et al. 2010
B074	M01	56	NED	180-210	Jansen et al. 2010
B096	M01	56	VIC	225-245	Jansen et al. 2010
B107	M01	56	PET	242-290	Routtu et al. 2010
A001	M03	56	VIC	400-430	Routtu et al. 2010
B010	M03	56	NED	104-132	Jansen et al. 2010
B133	M03	56	PET	170-190	Jansen et al. 2010
B150	M03	56	VIC	157-187	Jansen et al. 2010
B164	M03	56	NED	189-219	Jansen et al. 2010

Table E.3. Summary table of the chemical analysis for the micro-evolution experiment. All measurements are given as averages in the clean medium prior to adding *Daphnia* and algae and in the old medium after renewal. ± indicates the standard error (SE) around the mean values. C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations.

Exposure medium	total Cu µg/L	dissolved Cu µg/L	total Zn µg/L	dissolved Zn µg/L	total organic carbon mg/L	dissolved organic carbon µg/L	pH
Selection experiment							
C	6.45 ± 0.25	4.23 ± 0.26	28.9 ± 0.2	26.3 ± 0.46	7.06 ± 0.48	6.31 ± 0.49	7.63 ± 0.01
Cu	140.1 ± 4.49	71.3 ± 3.20	28.9 ± 0.2	26.3 ± 0.46	9.01 ± 1.00	8.22 ± 0.98	7.58 ± 0.01
Zn	6.45 ± 0.25	4.23 ± 0.26	628.8 ± 10.5	496.4 ± 14.9	6.98 ± 0.66	6.61 ± 0.63	7.45± 0.01
Common garden experiment							
C	6.71 ± 0.53	4.45± 0.83	29.8 ± 0.75	21.3 ± 0.92	3.26 ± 0.18	2.58 ± 0.20	7.66 ± 0.02
Cu	146 ± 10.2	125 ± 38.4	29.8 ± 0.75	21.3 ± 0.92	3.71 ± 0.3	3.38 ± 0.20	7.75 ± 0.02
Zn	6.71 ± 0.53	4.45 ± 0.83	743 ± 8.08	609 ± 35.5	3.38 ± 0.2	2.85 ± 0.19	7.66 ± 0.02

Table E.4. Summary table of the chemical analysis of the cadmium tolerance experiment. All measurements are given as averages in the clean medium prior to adding *Daphnia* and algae/cyanobacteria and in the old medium after renewal. ± indicates the standard error (SE) around the mean values. < dl = below detection limit.

Exposure medium	**total Cd µg/L**	**dissolved Cd µg/L**	**total organic carbon mg/L**	**dissolved organic carbon mg/L**	**pH**
0µg Cd/L	< dl	< dl	5.15 ± 0.78	4.11 ± 0.44	7.64 ± 0.02
Cd 1	2.10 ± 0.16	1.23 ± 0.06	4.66 ± 0.72	4.60 ± 0.51	7.67 ± 0.02
Cd 2	5.02 ± 0.32	2.74 ± 0.13	4.48± 0.62	4.18 ± 0.49	7.67 ± 0. 09
Cd 3	11.07 ± 0.28	6.34 ± 0.31	4.53 ± 0.49	3.99 ± 0.36	7.70 ± 0.06
Cd 4	23.64 ± 0.64	11.83 ± 0.61	4.68 ± 0.58	4.42 ± 0.43	7.73 ± 0.05
Cd 5	54.36 ± 1.35	27.25 ± 1.61	4.94 ± 0.64	4.18 ± 0.42	7.71 ± 0.05

Table E.5. Pairwise comparisons of total population density among selection treatments over time. An asterisk (*) indicates a significant difference between selection treatments, based on the Wald Chisquare test wit Holm adjustment for multiple testing. We included different weights for the fixed effects to allow for unequal variances; we did not include a correlation structure correlating abundance at time point t+1 per aquaria with the abundance at time point t as it did not significantly improve the model fit (Likelihood ratio test). C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations.

Week	C vs. Cu	C vs. Zn	Cu vs. Zn
1	< 0.0001*	< 0.0001*	< 0.0001*
2	0.0016*	< 0.0001*	0.1675
3	< 0.0001*	< 0.0001*	0.1088
4	0.0312*	0.0043*	0.9737
6	< 0.0001*	0.2304	< 0.0001*
8	0.0425*	0.3552	0.1336

Table E.6. Summary table of the cadmium concentration response parameters for each selection treatment (-95% CI - +95% CI). O = original population, C = control populations, Zn = Zn-selected populations.

	EC_{10}	EC_{20}	EC_{50}	EC_{80}	EC90	Slope
reproduction						
O	0.96 (0.69-1.34)	1.45 (1.14-1.83)	2.90 (2.51-3.35)	5.80 (4.63-7.28)	8.71 (6.35-11.95)	2.00 (1.48-2.52)
C	1.26 (1.00-1.57)	1.64 (1.40-1.93)	2.60 (2.38-2.84)	4.12 (3.54-4.79)	5.39 (4.33-6.69)	3.02 (2.18-3.86)
Zn	1.64 (1.37-1.97)	2.04 (1.81-2.30)	2.96 (2.76-3.17)	4.29 (3.68-5.00)	5.34 (4.29-6.63)	3.72 (2.53-4.92)
survival						
O	1.25 (0.71-2.19)	2.10 (1.40-3.15)	5.12 (4.03-6.5)	12.48 (8.56-18.21)	21.03 (12.36-35.78)	1.55 (1.01-2.10)
C	1.83 (1.15-2.90)	2.49 (1.77-3.51)	4.23 (3.37-5.30)	7.17 (5.19-9.91)	9.77 (6.31-15.13)	2.62 (1.41-3.83)
Zn	1.60 (1.08-2.38)	2.23 (1.66-2.98)	3.91 (3.22-4.74)	7.57 (6.13-9.35)	11.01 (8.25-14.70)	2.46 (1.53-3.40)

Table E.7. Table of p-values of the pairwise Wheeler ratio comparisons among the different selection treatments for different dose response parameters (EC_{20}, EC_{50}, EC_{80} and slope) and endpoints (reproduction and survival) of the cadmium tolerance test. An asterisk (*) indicates significant differences ($p < 0.05$) between two selection treatments for a given endpoint and parameter combination. O = original population, C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations, ns = non-significant.

Reproduction			**Survival**		
EC_{20}					
	C	Zn		C	Zn
O	ns	0.04*	O	ns	ns
Zn	0.02*		Zn	ns	
EC_{50}					
	C	Zn		C	Zn
O	ns	ns	O	ns	ns
Zn	0.04*		Zn	ns	
EC_{80}					
	C	Zn		C	Zn
O	0.02*	0.04*	O	0.04*	0.03*
Zn	ns		Zn	ns	
Slope					
	C	Zn		C	Zn
O	0.02*	0.0009*	O	0.04*	0.05*
Zn	ns		Zn	ns	

Table E.8. Bootstrap test for differences in genotypic (i.e. clonal) diversity (Simpson index) between pairs of populations. O = original population, C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations, aquaria replicates 1-4. Significant differences ($p < 0.05$) are indicated in bold, below diagonal: p(A>=B), above diagonal: p(B<=A).

A \ B	O	C1	C2	C3	C4	Cu1	Cu2	Cu3	Cu4	Zn1	Zn2	Zn3	Zn4
O		0.7365	0.8861	0.5236	0.5383	0.0609	**0.0322**	**0.0431**	**0.0003**	**0.0186**	**0.0478**	**0.0027**	0.2216
C1	0.2636		0.2075	0.7159	0.6821	0.1017	**0.054**	0.0593	**0.0014**	**0.0192**	0.0588	**0.0056**	0.4213
C2	0.114	0.7926		0.8737	0.8822	0.1785	0.1087	0.0987	**0.0032**	**0.0362**	**0.1121**	**0.0118**	0.7394
C3	0.4765	0.2842	0.1284		0.4954	0.067	**0.0404**	**0.0447**	**0.0008**	**0.0157**	**0.0428**	**0.0033**	0.2406
C4	0.4618	0.3274	0.1191	0.5047		0.0692	**0.0348**	**0.0421**	**0.0008**	**0.0156**	**0.0472**	**0.003**	0.2284
Cu1	0.9392	0.8984	0.8216	0.9363	0.9309		0.4057	0.3346	**0.0149**	0.1235	0.3635	**0.0506**	0.8956
Cu2	0.9679	0.9461	0.8915	0.9603	0.9653	0.5944		0.3942	**0.0203**	0.1607	0.4781	0.0726	0.94
Cu3	0.957	0.9408	0.9015	0.9557	0.958	0.666	0.6081		**0.0356**	0.2195	0.5677	0.1095	0.9375
Cu4	0.9998	0.9987	0.9969	0.9993	0.9993	0.9852	0.9798	0.9646		0.8486	0.97	0.7332	0.9988
Zn1	0.9815	0.9809	0.9639	0.9845	0.9845	0.8803	0.8394	0.7809	0.1522		0.8191	0.3312	0.9805
Zn2	0.9523	0.9413	0.8924	0.9573	0.9533	0.6366	0.5225	0.4324	**0.0301**	0.181		0.0845	0.934
Zn3	0.9974	0.9945	0.9888	0.9968	0.9971	0.9495	0.9275	0.8911	0.2669	0.6689	0.9189		0.9923
Zn4	0.7785	0.5788	0.2706	0.7612	0.7717	0.1056	0.0601	0.0627	**0.0013**	**0.0196**	0.0697	**0.0085**	

Table E.9. Pairwise G'ST comparisons below the diagonal and 95% bootstrapped confidence interval above the diagonal. C = control populations, O = original population, Cu = Cu-selected populations, Zn = Zn-selected populations, aquaria replicates 1-4. Significant differences ($p < 0.05$) are indicated in bold.

	O	**C1**	**C2**	**C3**	**C4**	**Cu1**	**Cu2**	**Cu3**	**Cu4**	**Zn1**	**Zn2**	**Zn3**	**Zn4**
O		-0.02 to 0.07	0 to 0.11	-0.02 to 0.06	-0.03 to 0.04	0.04 to 0.18	0.01 to 0.13	0.06 to 0.20	0.12 to 0.24	0.02 to 0.14	0.12 to 0.24	0.12 to 0.27	-0.02 to 0.09
C1	0.01		-0.01 to 0.11	-0.01 to 0.13	-0.02 to 0.07	0.02 to 0.16	0.01 to 0.14	0.01 to 0.16	0.11 to 0.25	0 to 0.14	0.09 to 0.28	0.11 to 0.23	-0.03 to 0.08
C2	0.05	0.04		0.00 to 0.018	-0.02 to 0.09	0.05 to 0.20	0 to 0.1	0.04 to 0.18	0.04 to 0.14	0.03 to 0.14	0.15 to 0.33	0.13 to 0.33	0 to 0.1
C3	0.01	0.05	0.09		-0.01 to 0.12	0.02 to 0.16	0 to 0.15	0.07 to 0.24	0.11 to 0.28	0.03 to 0.23	0.14 to 0.26	0.13 to 0.29	-0.01 to 0.13
C4	0.00	0.02	0.03	0.05		0.07 to 0.22	0.01 to 0.13	0.07 to 0.23	0.11 to 0.26	0 to 0.13	0.14 to 0.32	0.13 to 0.32	-0.03 to 0.11
Cu1	**0.10**	**0.08**	**0.12**	**0.09**	**0.13**		0.04 to 0.18	0.05 to 0.21	0.11 to 0.25	0.06 to 0.20	0.16 to 0.32	0.21 to 0.40	0.04 to 0.21
Cu2	**0.06**	**0.06**	0.04	0.07	**0.08**	**0.11**		-0.02 to 0.12	-0.01 to 0.08	0.03 to 0.18	0.11 to 0.28	0.11 to 0.28	0.01 to 0.11
Cu3	**0.12**	**0.08**	**0.10**	**0.15**	**0.14**	**0.12**	0.03		0.04 to 0.17	0.02 to 0.15	0.13 to 0.28	0.18 to 0.28	0.05 to 0.17
Cu4	**0.18**	**0.17**	**0.08**	**0.19**	**0.19**	**0.17**	0.03	**0.10**		0.09 to 0.20	0.16 to 0.35	0.18 to 0.35	0.09 to 0.20
Zn1	**0.07**	0.06	**0.08**	**0.12**	0.06	**0.12**	**0.10**	**0.08**	**0.16**		0.12 to 0.25	0.19 to 0.32	0.01 to 0.17
Zn2	**0.18**	**0.18**	**0.20**	**0.20**	**0.23**	**0.24**	**0.19**	**0.20**	**0.26**	**0.18**		0.03 to 0.14	0.07 to 0.20
Zn3	**0.20**	**0.19**	**0.23**	**0.21**	**0.23**	**0.30**	**0.20**	**0.23**	**0.29**	**0.26**	**0.08**		0.01 to 0.18
Zn 4	0.02	0.02	0.03	0.05	0.03	**0.11**	**0.06**	**0.10**	**0.14**	**0.08**	**0.13**	**0.09**	

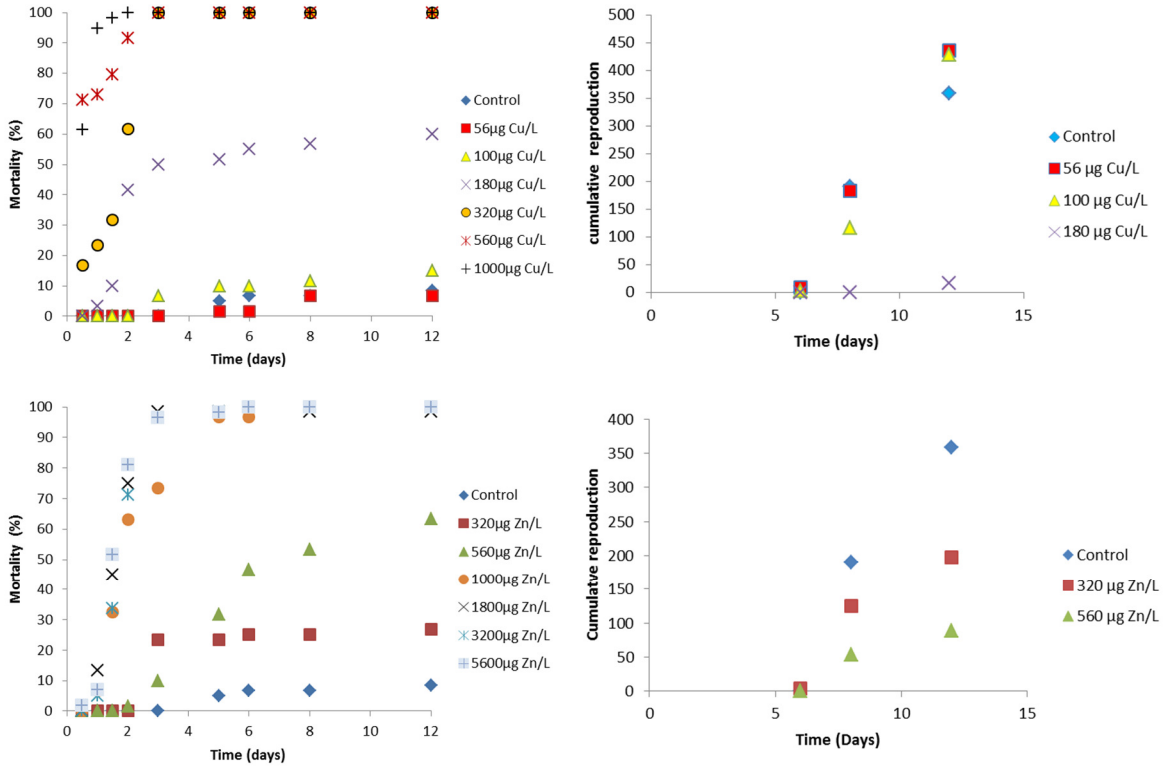


Figure E.1. Mortality and cummulative reproduction (no. of juveniles) of a subset of 20 clones of the KNO17 population exposed to a range of nominal Cu and Zn concentrations for 12 days. This is data from a pilot experiment using animals cultured under identical conditions as described for the main experiments of this study, which we used to determine the Cu and Zn concentrations for the selection experiment. We used the same initial population density (1 individual in 50 mL) and feeding regime (0.8mg C/L of *Pseudokirchneriella subcapitata*) as in the actual selection experiment. We postulated that micro-evolutionary effects should be most rapidly induced at lethal concentrations. We therefore based our choice for the metal concentrations at which to carry out our selection experiment on concentrations where 50% of clones died after 8 days as this time point typically marks the onset of reproduction. For copper we picked the nominal concentration of 180 µg Cu/L and for zinc 560 µg Zn/L. The data on cumulative reproduction after 12 days also indicate that little reproduction occurs at these concentrations, which lead us to expect strong selection to occur around these concentrations.

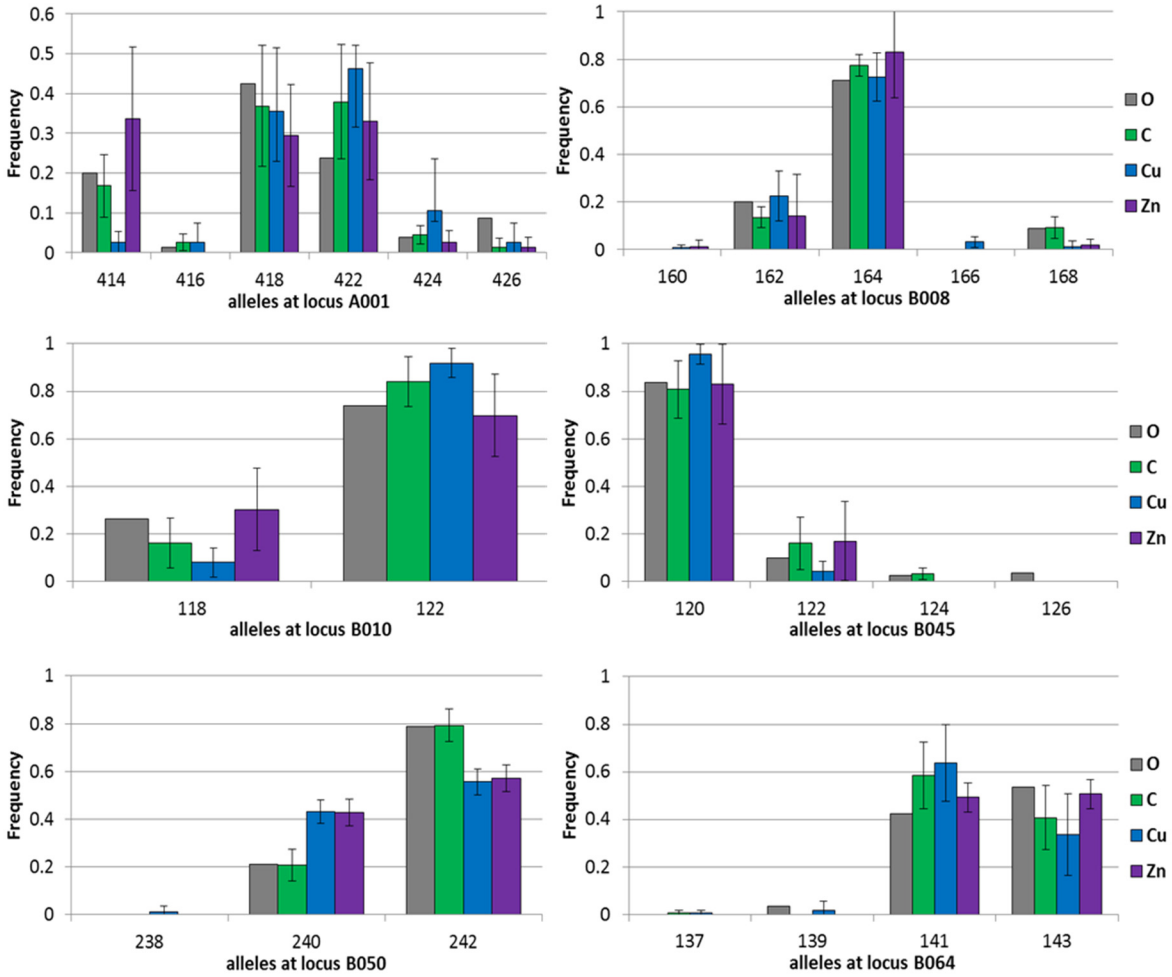


Figure E.2. Allele frequencies at 6 out of the 12 microsatellite markers (A001, B008, B010, B045, B050, B064) used for the data analysis under the different selection treatments. O = original population, C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations. Error bars indicate the 95% confidence interval around the mean allele frequency of the 4 aquaria replicates.

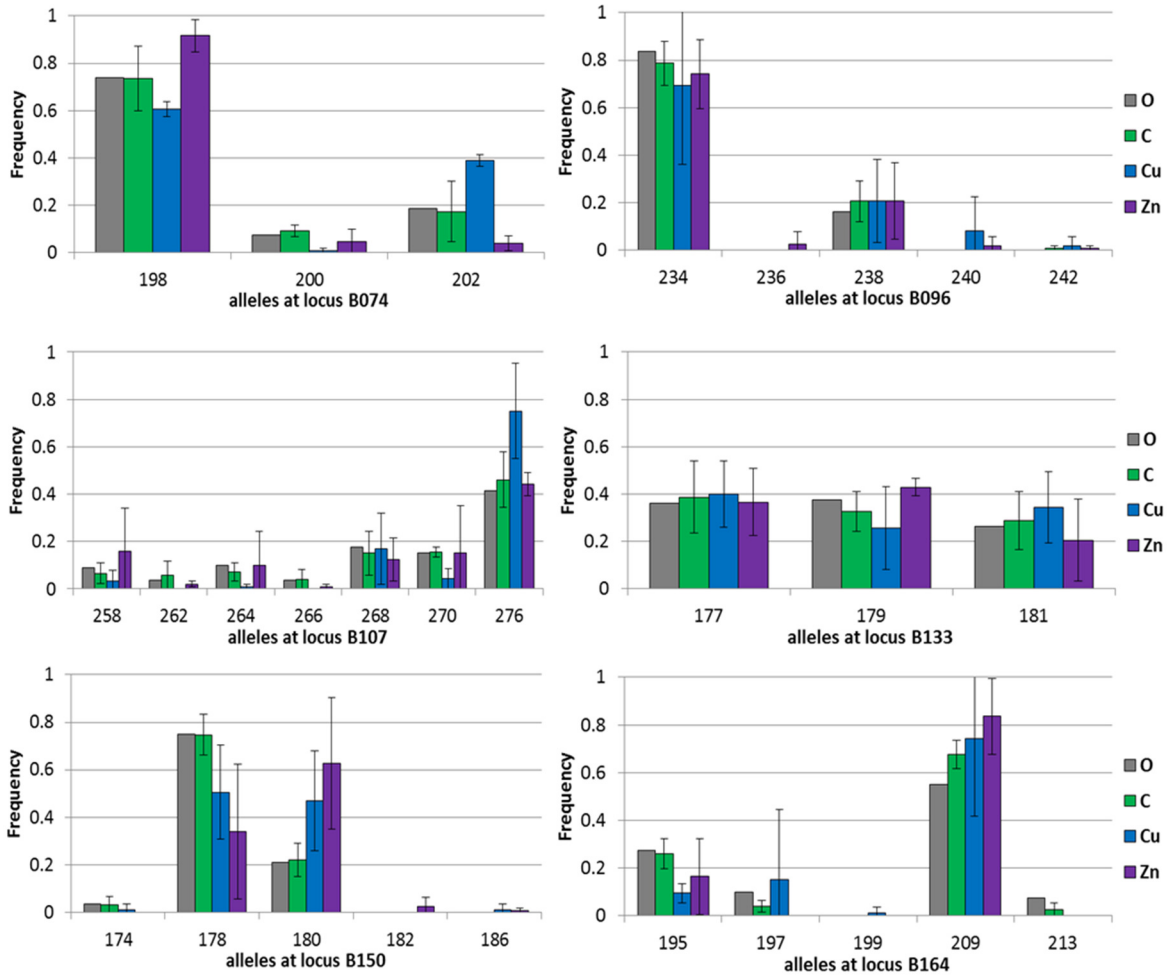


Figure E.3. Allele frequencies at 6 out of the 12 microsatellite markers (B074, B096, B107, B133, B150, B164) used for the data analysis under the different selection treatments. O = original population, C = control populations, Cu = Cu-selected populations, Zn = Zn-selected populations. Error bars indicate the 95% confidence interval around the mean allele frequency of the 4 aquaria replicates.

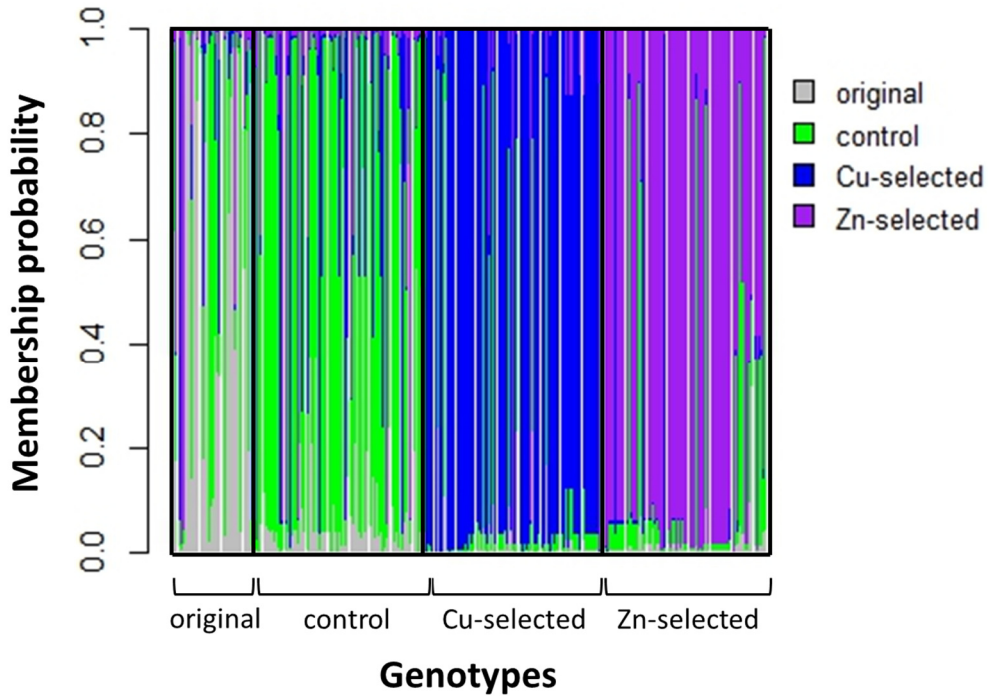


Figure E.4. Membership probability of the different multilocus genotypes to the different populations. Multilocus genotypes were grouped together according to their selection treatment along the x axis and the membership probability per multilocus genotype of belonging to each selection treatment is provided along the y-axis. This analysis was carried out in the R package *adegenet*.

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PROFESSIONAL EMPLOYMENT

June 2011- Present	PhD Student at the Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Belgium
March-December 2015	Internship and interim contracts at DG Mare, European Commission, Brussels

EDUCATION

April 2010 - May 2011	Postgraduate Certificate in Marine Sciences University of Tasmania, Hobart, Australia
September 2005 - July 2009	Bachelor (First Class Honours) in Marine Biology, University of Aberdeen, Aberdeen, United Kingdom Bachelor thesis: Do North Sea herring (*Clupea harengus*) down-regulate their fecundity through atresia in response to lowered fat content? Promoter: Dr. Tara Marshall

GRANTS AND AWARDS

- AFR-PhD grant from the Fonds National de la Recherche, Luxembourg (AFR-PhD Grant Agreement PhD 2011-1, Project Reference 1330121)
- Poster award from the European Science foundation

PUBLICATIONS AND INDUSTRY REPORTS

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. 2015. Temperature and food concentration have limited influence on the mixture toxicity of copper and *Microcystis aeruginosa* to *Daphnia magna*. 2016. Environmental Toxicology and Chemistry 35 (3): 742-749.

Hochmuth, J.D., De Meester, L., Pereira, C.M., Janssen, C.R., De Schamphelaere, K.A.C. 2015. Rapid adaptation of a *Daphnia magna* population to metal stress is associated with heterozygote excess. Environmental Science & Technology 49: 9298-9307.

Nys, C., Asselman, J., Hochmuth, J.D., Janssen, C.R., , Blust, R., Smolders, E., De Schamphelaere, K.A.C. 2015. Mixture toxicity of nickel and zinc to *Daphnia magna* is noninteractive at low effect sizes but becomes synergistic at high effect sizes.

Hochmuth, J.D., De Schamphelaere, K.A.C. 2014. The effect of temperature on the sensitivity of *Daphnia magna* to cyanobacteria is genus dependent. 2014. Environmental Toxicology and Chemistry 33(10): 2333-2343.

Asselman, J.*, Hochmuth, J.D.*, De Schamphelaere, K.A.C. 2014. A comparison of the sensitivities of *Daphnia magna* and *Daphnia pulex* to six different cyanobacteria species. (*shared first authorship). Harmful Algae 39: 1-7.

Hochmuth, J.D., Asselman, J., De Schamphelaere, K.A.C. 2014. Are interactive effects of harmful algal blooms and copper pollution a concern for water quality management? 2014. Water Research 60: 41-53.

Green B.S., Gardner C., Hochmuth J.D., Linnane A. 2014. Environmental effects on fished lobsters and crabs. Reviews in Fish Biology and Fisheries 24: 613-638.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C, June 2013. Final Report prepared for the International Zinc Association and the International Copper Association. Determining the

potential impact of genetic diversity on metal risk assessment. TIER 2 – Testing cases of significant evolutionary potential observed (in TIER 1) under more realistic conditions.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C, December 2012. Final Report prepared for the International Zinc Association and the International Copper Association. Determining the potential impact of genetic diversity on metal risk assessment. TIER 1 – Screening the evolutionary potential of natural Daphnia magna populations under Cu and Zn stress.

PLATFORM PRESENTATIONS

Hochmuth, J.D., De Meester, L., Pereiera C.M., Janssen, C.R., De Schamphelaere, K.A.C. 13th May 2014. Cu and Zn selection leads to adaptation and heterozygote excess in a natural *Daphnia magna* population without affecting tolerance to novel stressors. SETAC Europe 24th Annual meeting Basel, Switzerland.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. 13th May 2013. Micro-evolutionary response in a natural *Daphnia magna* population under Cu and Zn stress. SETAC Europe 23rd annual meeting, Glasgow, UK.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. 2012. Is there a potential for wild *Daphnia magna* populations to undergo selection at conventionally derived no observed effect concentrations of chemicals? Poster Spotlight Presentation. 6th SETAC World Congress SETAC Europe 22nd annual meeting, Berlin, Germany.

POSTER PRESENTATIONS

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. May 27 2013. Micro-evolutionary response in a natural *Daphnia magna* population under Cu and Zn stress. EuroEEFG Conference, Noordwijkerhout, Netherlands.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. 2013. February 8 Micro-evolutionary response in a natural *Daphnia magna* population under Cu and Zn stress. 18th National Symposium on Applied Biological Sciences, Gent Belgium.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. February 5 2013. Micro-evolutionary response in a natural *Daphnia magna* population under Cu and Zn stress. 7th Symposium on Eco-Evolutionary Dynamics, Leuven, Belgium.

Hochmuth, J.D., Janssen, C.R., De Schamphelaere, K.A.C. 2012. Is there a potential for wild *Daphnia magna* populations to undergo selection at conventionally derived no observed effect concentrations of chemicals? 6th SETAC World Congress SETAC Europe 22nd annual meeting, Berlin, Germany.

Hochmuth, J.D., Asselman, J., De Schamphelaere, K.A.C. 2012. Do mixture effects of metal stress (Cu) and natural stress (cyanobacterial toxins) add up in *Daphnia magna*? 6th SETAC World Congress SETAC Europe 22nd annual meeting, Berlin, Germany.

ATTENDED CONFERENCES AND WORKSHOPS

11-15 May 2014	SETAC Europe, 24th Annual Meeting, Basel, Switzerland.
26-30 May 2013	EuroEEFG Conference, Noordwijkerhout, Netherlands.
12-16 May 2013	SETAC Europe, 23th Annual Meeting, Glasgow, UK.
15-23 April 2013	Dynamic Energy Budget (DEB) workshop, Texel, Netherlands.
8 February 2013	National Symposium on Applied Biological Sciences, 18th Edition, Ghent, Belgium.
5-7 February 2013	7th Symposium on Eco-Evolutionary Dynamics, Leuven, Belgium.
20-24 May 2012	SETAC Europe, 6th World Congress, Berlin, Germany.

MEMBERSHIP OF PROFESSIONAL ORGANIZATIONS

Member of the Society of Environmental Toxicology and Chemistry (SETAC).