Necessity is the mother of invention

Plato

(Greek philosopher, 429/423 BC – 348/347 BC)

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TRANSCRIPTOMIC PROFILES AND FITNESS OF *DAPHNIA* EXPOSED TO CYANOBACTERIA AND INTERACTIONS WITH INSECTICIDES.

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch Title: Genexpressieprofielen en reproductiepotentieel van *Daphnia* blootgesteld aan cyanobacterien en interacties met insecticiden

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Acknowledgement

About 150 years ago Charles Dickens started his most famous work, "*A tale of two cities*", as follows: "*It was the best of times, it was the worst of times."* Although Charles Dickens may have written those words to characterize the relationship between France and England at the end of the French Revolution, it perfectly describes four years of doctoral study. To me, it reminds me of that feeling when your first paper is accepted or when you just received your very first scientific prize. But it also brings back memories of those long hours in the lab that still resulted in a failed experiment, those inexplicable errors in a programming code that you just cannot seem to resolve.

Four years of doctoral study are however not spent in isolation and Dickens' continuation, i.e. *"It was the age of wisdom, it was the age of foolishness."*, perfectly highlights that. It reminds me of the guidance that I have benefitted from with Karel as my promoter. He often mentioned that he was but a novice in omics research and bioinformatics. Yet, this often put me on the spot as he was extremely critical about any new methodology, experimental design or analysis. He has instilled in me a critical and analytical way of thinking that has been extremely invaluable in my scientific research. Although he has always given me enough freedom and opportunities to pursue my own scientific interests and research, he always steered me in the right direction. He stimulated me to not foolishly and unreservedly put my faith in commonly used techniques or practices but rather critically evaluate them and extract the good parts and make it better. This perfectly fits with Dickens next phrase, "*It was the epoch of belief, it was the epoch of incredulity."*

"It was the season of light, it was the season of darkness." A PhD is achieved with ups and downs, it feels like a free ride on an emotional rollercoaster. Luckily for me, that rollercoaster was always filled with caring and loving people. Friends and family made sure that even in the darkest times there was light brightly shining through. The friends of the GSR, the friends of the Rooiegemlaan, and An-Sofie in particular, were always there to lighten up the atmosphere. But, the brightest torch of all was always carried by my close family. I never knew who was more enthusiastic about an accepted talk at a conference, a new research opportunity abroad, me or my parents? It is unique to have parents who give their unwavering support to their children regardless of their choices, but I was lucky enough to have two. But it does not end there, indeed, I seem to have won the lottery with my husband Dieter.

Dieter, my greatest fan and my severest critic, has not only been a beacon of love and support but a fountain of wisdom that has been my scientific first aid kit for the last four years.

"It was the spring of hope, it was the winter of despair." Unlike in Charles Dickens pessimistic view, in my optimistic way of thinking winter comes before the spring, despair before hope. It is so characteristic for science, failure followed by solution, Eureka!. Or in other words, in a good environment, everything will fall into place eventually. Whether I was in Ghent, in the GhenToxLab, in Bloomington, in the Shaw Lab or in Notre Dame, in the Pfrender lab, I was always able to conduct my research in constructive and enthusiastic environment. There was always a helping hand in the lab or in the office when needed, always someone ready to help out when you were ill or could not make it in the weekend. I remember the nightly hours with Jackie in the Pfrender lab to finish all the microarrays or the whiteboard discussion in the Shaw lab with Chip to resolve the mystery of the MessageAmp kits or the determination of Nancy to detect those extremely low concentrations of pesticides with the GC in the GhenToxLab. All of the labs I have worked in where also extremely social environments. From birthday cakes and donuts to after work drinks and team buildings, work hard, play hard.

"*We had everything before us, we had nothing before us."* Four years later, now at the end of the PhD, I think the future lies brightly ahead. I have met so many amazing people at UGent, IU or Notre Dame, through the Daphnia Genomics Consortium and through SETAC who I am sure will cross my path again in the future. And to end with a final quote: "*I can`t tell where journey will end, but I know where to start."*

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List of abbreviations

AIC	Aikaike Information Criterion
ANA	*Anabaena*
ANOVA	Analysis of Variance
AOP	Adverse Outcome Pathway
APH	*Aphanizomenon*
ATP	Adenosine Triphosphate
BMAA	Beta-Methylamino-L-alanine
CA	Concentration Addition
cDNA	Complementary DeoxyriboNucleic Acid
CICCM	Cawthron Institute Culture Collection of Micro-algae
CYL	*Cylindrospermopsis*
DE	Differentially Expressed
DNA	DeoxyriboNucleic Acid
EC	Enzyme Classification
EC50	Effect Concentration causing 50% effect
ECx	Effect Concentration causing x% effect
EFSA	European Food Safety Authority
ELISA	Enzyme-linked Immunosorbent Assay
EU	European Union
FAME	Fatty Acid Methyl Esther
FDR	False Discovery Rate
GC	Gas Chromatography
GEO	Gene Expression Omnibus
GLM	General Linearized Model
GO	Gene Ontology
IA	Independent Action
IRAC	Insecticide Resistance Action Committee
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Kegg Orthology
KOG	Eukaryotic Orthology Groups
LIMMA	Linear Models for Microarray Data
LPS	Lipopolysaccharide
Mb	Megabase
MC	*Microcystis*

MTBE	Methyl-tert-butylether
NA	Not Available
NADH	Nicotinamide Adenine Dinucleotide
NCBI	National Centre for Biotechnology Information
NIH	National Institutes of Health
NOD	*Nodularia*
OECD	Organisation for Economic Cooperation and Development
OSL	*Oscillatoria*
PCB	Polychlorinated biphenyl
PCC	Pasteur Culture Collection
PCR	Polymerase Chain Reaction
PPP	Plant Protection Product
PUFA	Poly Unsaturated Fatty Acids
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SCCAP	Scandinavian Culture Collection for Algae and Protozoa
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
Sig	Significance
SPE	Solvent-phase Extraction
SSE	Sum of Squared Errors
US EPA	United States Environmental Protection Agency
USA	United States of America
UTEX	University of Texas Culture Collection
UV	Ultraviolet
WGCNA	Weighted Gene Co-Expression Network
WHO	World Health Organization

Chapter 1

General introduction and conceptual framework

Organisms inhabit complex environments leaving them exposed to various stressors of both anthropogenic and natural origin. This contradicts current standard ecotoxicity experiments where organisms are exposed to a single stressor under controlled conditions. The definition of stress in this dissertation follows Bradshaw and Hardwick (1989), Calow (1989), Heugens et al. (2001), Koehn and Bayne (1989), Sibly and Calow (1989) where stress is defined as *"an environmental change that affects the functioning of organisms (i.e., growth and reproduction), leading to reduced fitness"*. In environmental reality, the response of organisms is therefore often the result of complex interactions that cannot be easily disentangled into effects attributed to each individual stressor. Indeed, the effects of multiple stress might be larger (synergistic effect) or smaller (antagonistic effect) than the sum of the effects of each of the individual stressors (Van Gestel et al. 2010).

1.1 Interaction effects

In the last decade, an increasing number of studies have addressed this discrepancy between laboratory settings of "*single stress*" versus the environmental reality of "*multiple stress*". Holmstrup et al. (2010) reviewed 159 studies which focused on interactions between anthropogenic and natural stressors. In the majority of these studies synergistic effects were observed. For combinations of heat stress and chemical stress, synergistic effects, i.e. more negative effects than expected, were observed in almost 80% of the studies. For example, Heugens et al. (2003) observed increased mortality in acute toxicity experiments with *Daphnia* exposed to cadmium when the temperature was higher than the thermal tolerance of the *Daphnia.* In addition, uptake rates of cadmium were observed to be higher at 20°C than at 10°C suggesting increasing cadmium accumulation with increasing temperature. Heugens et al. (2003) conclude that the synergistic effect, i.e. increased mortality, depends upon the temperature which influences both the thermal tolerance as well as the uptake rate of cadmium. Antagonistic effects, i.e. more positive effects than expected, were observed in less than 10% of the studies. For example, Perschbacher (2005) observed decreasing copper toxicity with increasing temperature in the catfish *Ictalurus punctatus* which resulted in increased survival. They speculated a better functioning of resisting mechanism for copper toxicity with increasing temperatures, which resulted in an increased survival of the fish at 27°C compared to 23°C.

Heugens et al. (2001) studied interaction effects from a different perspective. They reanalyzed experimental data available in literature and used mathematical equations to model relationships

between natural and chemical stressors. Heugens et al. (2001) observed that in about 70% of the analyzed studies toxicity of the chemical increases with increasing temperature. In general, Heugens et al. (2001) observed that organisms living in conditions close to their thermal tolerance were more susceptible to the toxicity of a chemical than organisms living in optimal conditions. Similarly for nutritional state, they observed that increasing starvation, i.e. decreasing nutritional state, increased the toxicity of the chemical in 80% of the analyzed studies. Furthermore, transgenerational effects were also observed for *Daphnia* species. Heugens et al. (2003) concluded that daphnids from well-fed mothers were more susceptible to toxicants than daphnids from poor-fed mothers indicating that interactions or multiple stress can affect not only the current generation but also future generations.

Different results were obtained for interactions with salinity. Overall, increasing salinity resulted in a decrease of metal toxicity due to a decreased bio-availability of the metal. In contrast, increasing salinity resulted in an increased toxicity of organophosphate pesticides due to increased accumulation. No clear relationship was observed between salinity and the toxicity of other chemicals. Laskowski et al. (2010) found significant interactions between chemicals and environmental factors in over 60% of the studied cases without specifying the direction of the interaction effects.

The majority of reported studies discuss effects of combinations of two stressors. Yet, many more possible combinations exist. Laskowski et al. (2010) report two papers studying three-factor interactions. A detailed study by Coors and De Meester (2008) focused on the potential three way interactions between predation, parasitism and pesticides on *Daphnia magna*. They observed synergistic effects on survival, i.e. decreased survival, between pesticide exposure and parasite challenge. According to Coors and De Meester (2008), this suggests a potential immunomodulatory activity of the pesticide. In contrast, predation threat showed antagonistic effects when combined with either a parasite challenge or a pesticide exposure on the number of offspring in the first brood. Although all three stressors together did not result in synergistic or antagonistic effects on the age and size of maturity, the combined effects of these three stressors resulted in a considerable delay in the onset of reproduction compared to control conditions. These results clearly indicate that adding only a third factor already significantly complicates the potential effects and interactions on the organism.

Interaction effects under multiple stress conditions may thus significantly complicate risk assessment approaches of chemicals (Hooper et al., 2013; Moe et al., 2013), which are currently still primarily

focused on a chemical-by-chemical basis (Landis et al., 2013; Van Gestel et al, 2010). Consequently, there is a need for additional knowledge concerning these combined and interaction effects to improve current risks assessment practices. Indeed, without taking interaction effects into consideration, predicted effects based on single substance datasets may over- or underestimate the effects actually occurring in the environment (Moe et al., 2013; Van Gestel et al., 2010). Although adding additional safety factors may alleviate the potential negative impact on the aquatic ecosystem, unfounded safety factors may hamper economic activities. Therefore, there is a need for a science-based approach that will adequately assess the risks of complex environments.

At present, three main factors are still lacking to construct an adequate scientific framework for the effects of multiple stress. The first is the lack of a general statistical framework and terminology. The second is the lack of predictive or mechanistic models for interaction effects. The third factor is the lack of a comprehensive database that allows easy comparison across different multiple stress combinations.

1.1.1 A defined statistical framework for combined effects

Despite the growing body of literature on interaction effects under multiple stress conditions, a general statistical framework remains to be adopted. Studies described by Laskowski et al. (2010) used six different statistical methodologies: analysis of variance (ANOVA), general linear model (GLM), Kruskal-Wallis test, χ^2 test and two MixToxmodels as developed by Jonker et al. (2005). Holmstrup et al. (2010) also observed a wide variety of statistical methods without referring to the specific methods used in each study.

The majority of these statistical models are based upon two conceptual models which are Loewe additivity or concentration addition (CA) and Bliss independence or independent action (IA) also referred to as response addition (Jonker et al., 2005). The two models differ both mathematically and conceptually. Concentration addition (eq 1.1) assumes that the studied stressors have a similar mechanism of action (Boedeker et al., 1992). The null hypothesis states that the relative toxicity of the mixture equals the relative toxicity of the individual components (Jonker et al., 2005). In equation 1.1. n is the number of stressors in the mixture, c_i is the concentration of component i in the mixture and EC_{xi} is the concentration of component i that alone would cause the same effect x as the mixture. However, the interpretation of similar mechanism of action between stressors vastly differs (Faust et

al., 2003). A strict pharmacological viewpoint defines similar mechanism of action as interacting with the same molecular target site (Pöch, 1993), whereas from a broad phenomenological viewpoint a similar mechanism of action may also be causing a common toxicological response (Berenbaum, 1989). As a consequence, concentration addition has therefore been suggested to be applicable for all chemicals (Berenbaum, 1989).

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1 \quad \text{(eq 1.1.)}$$

Independent action (eq 1.2) assumes that stressors have dissimilar mechanisms of action and thus interact with different molecular targets (Pöch, 1993). The null-hypothesis of this model states that the probability of the response of one compound is independent of the probability of response of the other component (Jonker et al., 2005). The effect of the mixture $E(c_{mix})$ is calculated from the product of the effects of the individual components $E(c_i)$. Therefore, the effects of each of the stressors are independent of the other one from a probabilistic point of view (Faust et al., 2003).

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \quad \text{(eq 1.2)}$$

The data requirements for both models are quite different. Concentration addition requires a concentration response curve for each individual stressor to correctly estimate the EC_{xi} whereas independent action requires at least the response of the individual stressor alone at the same concentration as tested in the mixture. When testing few mixture combinations, independent action is therefore less time consuming and requires less data whereas concentration addition always requires a full dose response curve. However, concentration addition applied to dissimilar acting stressors often overestimates the effects (Backhaus et al., 2000; Faust et al., 2003). This overestimation is increasingly being accepted from a risk assessment point of view given the precautionary principle even though the independent action model may be more accurate in some cases (Backhaus et al., 2000; Cedergreen et al., 2008; Faust et al., 2003). Furthermore, both models are conceptually and mathematically very different and cannot be easily compared (Jonker et al., 2005). These models can also not be compared statistically and only qualitative comparisons such as the Aikaike Information Criterion (AIC) can be made (Jonker et al., 2005).

In addition, terminology differs between the two models but is often used interchangeably. For example, effects are often referred to as additive in terms of concentration addition even when analyzed with the independent action concept or with an ANOVA model (Cerbin et al., 2010; Coors and De Meester, 2008). To avoid confusion, it is therefore necessary to either place such statements in the context of the reference model or to refer to more general terminology such as no interaction or absence of interaction. Also, mixture toxicity and interaction effects are often used interchangeably although they are quite different. Mixture toxicity refers to the toxicity of the mixture which is most often more toxic than either of the compounds alone or in other words mixture toxicity refers to the combined effects of the stressors. In contrast, interaction effects refer to a statistical context in which interaction occurs because the predicted toxicity of the mixture tested differs from the observed toxicity of that same mixture. As a consequence, the mixture toxicity can be higher than the toxicity of the compounds alone without the presence of interaction effects or mixture toxicity can be lower than the toxicity of the compounds alone with the presence of interaction effects. To avoid confusion, this dissertation will use the terminology of combined and interaction effects rather than mixture toxicity.

1.1.2 Predictive and mechanistic models for interaction effects

Currently, neither the concentration addition model nor the independent action model allow predicting the occurrence of interaction (Belden and Lydy, 2006). Both models can only predict the toxicity of the combination of stressors under the hypothesis of non-interaction. Interactions are thus detected by comparing the observed response versus the predicted response which requires actual experiments. This has of course significant consequences for risk assessment as it means that interaction effects can only be incorporated by testing each potential combination of stressors, which is unfeasible. Nevertheless, current models do allow for incorporation of combined effects under the hypothesis of no interaction.

The lack of predictive models could potentially be attributed to the lack of mechanistic knowledge. Most studies regarding interactions focus on life history responses (Cedergreen et al., 2008; Faust et al., 2003; Jonker et al., 2005). Yet, these types of data may not contain sufficient information to enhance the current mechanistic understanding of interaction effects which is crucial in developing predictive models. New emerging technologies are currently being used to improve our understanding of interaction effects and will be discussed further on (Altenburger et al., 2012).

1.1.3 The need for comprehensive comparative datasets

Any general accepted statistical framework or mechanistic model needs to be validated across a comprehensive dataset. At present, few studies have generated such datasets. Faust et al. (2003) and Backhaus et al. (2000) investigated a rather large number of compounds but in a single multicomponent mixture at various concentrations of the different compounds. Cedergreen et al. (2008) were able to compare seven mixture combinations with the same statistical models, CA and IA. In reviews by Holmstrup et al. (2010), Heugens et al. (2001) and Laskowski et al. (2010), a large number of multiple stress studies were collected but given the differences in experimental design no statistical comparison was possible with a single model across all the collected studies as insufficient data was available about each of the studies. Clearly, there is a need to generate larger datasets standardized in experimental design to allow extensive comparisons of statistical and mechanistic models. Results of such comparisons can then be used to suggest and validate general frameworks that can be subsequently applied in risk assessment.

Based on recent reviews and literature, interaction effects under multiple stress conditions occur in at least half of the investigated studies. Yet, a generalized statistical framework as well as predictive models are lacking. These are however necessary to incorporate effects of multiple stress in risk assessment, but they first need to be scientifically validated across a comprehensive comparative dataset which is currently lacking.

1.2 Cyanobacteria

Cyanobacteria are unicellular organisms, also referred to as blue green algae. Unlike other prokaryotes, cyanobacteria have the ability to carry out oxygen-producing photosynthesis (Schopf, 2000). Therefore, some botanists consider them as a division of algae while their cellular and organismal morphology resembles that of bacteria (Stanier et al., 1971). At present, they are classified as bacteria with about 150 genera and more than 2000 species (van Apeldoorn, 2006; Fristachi and Sinclair, 2008). They have been dated back to more than 3 billion years ago based upon fossil evidence in Western Australia (Schopf, 2000). The conditions of the earth were then vastly different from the current environment. Anoxia, increased UV exposure and increased temperature compared to current conditions together with high levels of iron, methane and sulfur were environmental factors

shaping cyanobacteria evolution (Paul, 2008). This evolutionary history has been put forward as an explanation to why cyanobacteria currently thrive in extreme environments or under high environmental stress (Paul, 2008). Cyanobacteria have gained interest over the last century due to their toxicity which resulted in poisonings of fish, wild life and livestock in freshwater environments (Codd, 1995; Duy et al., 2000) as well as due to their presence in bloom formation (Peperzak, 2003).

1.2.1 Impact of cyanobacteria on the environment

Cyanobacteria and in particular cyanobacterial blooms can significantly impact freshwater environments due to a variety of factors. Cyanobacteria are known to produce a wide range of secondary metabolites of which some can be extremely toxic (Chorus et al., 2001; Gerwick et al., 2001). At present, toxin production by cyanobacteria has been estimated to include 40 genera, the main ones are *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya*, *Microcystis*, *Nostoc* and *Oscillatoria* (van Apeldoorn et al., 2007). A variety of different types of toxins has been identified and is summarized in Table 1.1. Specific guidelines with regards to drinking water quality have been derived for some toxins (Table 1.1) although an overall guideline is also available. The World Health Organization (WHO) defines risk levels based upon cyanobacteria cells/ml, where 20,000 cells/ml defines low risk, 100,000 cells/ml moderate risks and bloom formation is defined as high risk (WHO, 2005). In addition to toxin production, cyanobacterial blooms can impact aquatic ecosystems through a variety of factors. First, adverse tastes and odors hamper the use of water bodies with cyanobacterial blooms for both drinking water and recreational use (Paerl et al., 2001). Second, cyanobacterial blooms may “rob” the underlying water layers of oxygen, causing physicochemical changes in the water leading to hypoxia and anoxia which will kill most fauna (Jankowski et al., 2006; Paerl et al., 2001). Indeed, at high bloom densities, nutrients and resources are rapidly depleted which will ultimately lead to a sudden decline in biomass, which in turn leads to decaying scums that consume large amounts of oxygen (Paerl et al., 2001). Third, cyanobacterial blooms may deplete nutrient resources in the water body (O’Neil et al., 2012; Paerl et al., 2001). Fourth, cyanobacteria outcompete phytoplankton species generally abundant in lakes and ponds which directly affects zooplankton species for which the phytoplankton serves as a food source (Paerl et al., 2001). Therefore, cyanobacteria and cyanobacterial blooms have been identified as significant threats to aquatic ecosystems and even human health (Carmichael et al., 1985; Carmichael, 2001; Paerl et al., 2001).

Table 1.1 Overview of cyanobacterial toxins, their chemical structure, mechanism of action and the drinking water guideline and source organisms (van Apeldoorn et al., 2007). NA = not available

Name	Chemical structure	Mechanism of action	Drinking water guideline	Genera know to produce the toxins
Microcystin	Cyclic peptide	Eukaryotic protein serine/threonine phosphatases 1A and 2A inhibitor	1 µg/L[1]	*Microcystis, Anabaena, Oscillatoria, Nostoc*
Nodularin	Monocyclic pentapeptide	Eukaryotic protein serine/threonine phosphatases 1A and 2A inhibitor	10 µg/L[2]	*Nodularia*
Anatoxin-a	Alkaloid	Stereospecific nicotinic agonist	1 µg/L[3]	*Anabaena, Aphanizomenon, Cylindrospermopsis, Oscillatoria*
Anatoxin-a(s)	Guanidium methyl phosphate ester	Choline esterase inhibitor	1 µg/L[3]	*Anabeana, Aphanizomenon*
Cylindrospermopsin	Tricyclic alkaloid	Cytotoxin: irreversible protein synthesis inhibition	1 µg/L[3]	*Cylindrospermopsis, Aphanizomenon, Umezaka, Rhadiopsis*
Saxitoxin	Carbamate alkaloid	Voltage gated Sodium channel antagonist	3 µg/L[3]	*Anabaena, Aphanizomenon, Oscillatoria*
Aplysiatoxin	Phenolic bislacton	Protein kinase C activator – tumor promoter	NA	Marine species such as *Lyngbia*
Lyngbyatoxin	Isomer of teleocidin A	Not determined	NA	*Lyngbia*

1 WHO, 2005

2 Fitzgerald et al., 1999.

3 Burch, 2008

1.2.2 Cyanobacteria as an emerging threat

Over the last decade, cyanobacteria have again gained interest due to the increased incidence and frequency of cyanobacterial blooms in water bodies (Carmichael, 2008) (Fig. 1.1). This increase has been attributed to a variety of factors of which climate change and eutrophication are the most prominent (Schiedek et al., 2007; Paerl and Huisman, 2008; Paerl and Huisman, 2009) (Fig. 1.2.). Climate change conditions stimulate cyanobacterial growth primarily through increased temperature as cyanobacteria grow better at higher temperatures compared to other phytoplankton species (Paerl and Huisman, 2008). The best example is *Cylindrospermopsis raciborskii*, originally characterized as a (sub) tropical species, which now occurs as far north as Germany (Wiedner et al., 2007).

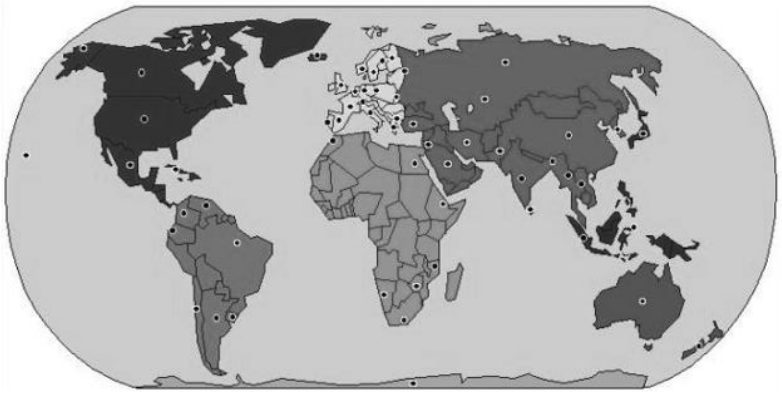


Figure 1.1 Countries reporting cyanobacterial blooms (From Fristachi and Sinclair, 2008)

Furthermore, increased temperature in surface waters reduces vertical mixing and thus increases stratification (Jöhnk et al., 2008). Given the buoyancy of cyanobacteria, they will float upward under stratifying conditions and accumulate in blooms (Jöhnk et al., 2008). In contrast, other phytoplankton species which are often non-buoyant will be suppressed by cyanobacteria through the competition for light (Jöhnk et al., 2008). Field and modelling studies by Jöhnk et al. (2008) indicated that artificial mixing of these water bodies was able to suppress cyanobacterial growth

In addition to changes in temperature, climate change conditions also consist of altered rainfall patterns, floods and storms (Paul, 2008). These changes may in turn influence nutrient entry and use in water bodies and increased nutrient loads will stimulate cyanobacterial bloom forming (Carpenter et al., 1992; Downing et al., 2001; Paerl and Huisman, 2009). Increased nutrient loading is not only mediated through climate change conditions but also through anthropogenic factors such as urbanization, industrialization and agriculture (Paerl and Huisman, 2009). Managing these nutrient loads may in turn help reducing cyanobacterial bloom formation (Heisler et al., 2008).

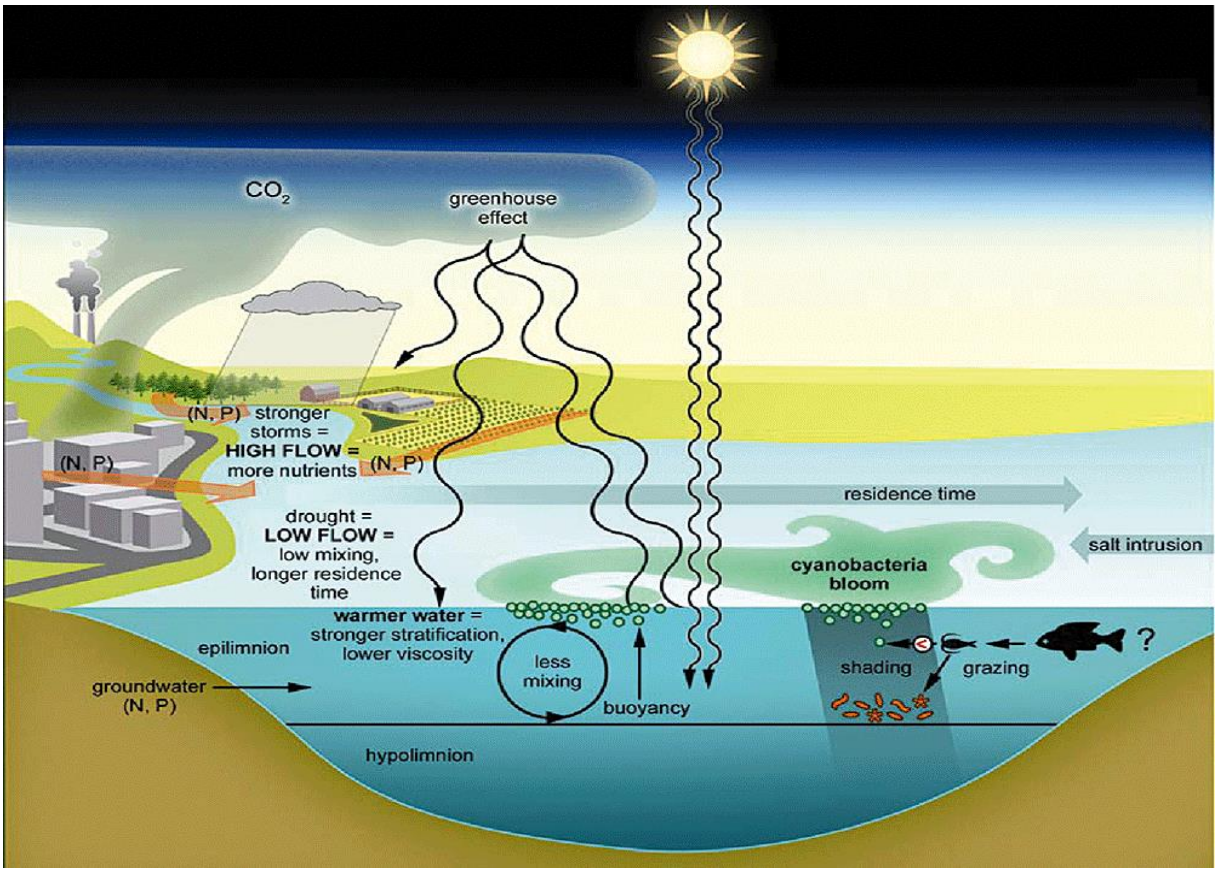


Figure 1.2 Visual summary of natural and anthropogenic factors stimulating cyanobacterial bloom formation (From Paerl and Huisman, 2009).

1.2.3 Effects of cyanobacteria on zooplankton species

Although cyanobacteria affect a great number of species, they are of particular concern to zooplankton as cyanobacteria outcompete other green algae normally serving as a food source for zooplankton (Dehn, 1930). Responses of zooplankton feeding on cyanobacteria have been widely reported in literature and consist of negative effects on survival, fecundity and body size (Kirk and Gilbert, 1992; Koski et al., 1999; Kozlowsky-Suzuki et al., 2003; Ojaveeret al., 2003) although some studies have reported tolerance to cyanobacteria for some *Daphnia* isolates (De Coninck et al., 2014; Gustafsson and Hansson, 2004). Effects of cyanobacteria on zooplankton have been primarily attributed to three main factors: lack of essential nutrients such as fatty acids or sterols, impairment or inhibition of feeding, i.e. mechanical interference, and toxin production (Demott and Müller-Navarra, 1997; Haney et al., 1994; Lürling, 2003). Current literature (Lürling, 2003; Rohrlack, 1999) remains undecided whether the effect of cyanobacteria on zooplankton can be contributed to only one of these factors or a combination of them. Two recent studies conducted by Wilson et al. (2006) and Tillmans et al.

(2008) further confirmed these findings. Both groups conducted a meta-analysis of published laboratory experiments with cyanobacteria and zooplankton. Wilson et al. (2006) observed that cyanobacteria were indeed poor food quality to zooplankton but found no differences between toxin and non-toxin producing cyanobacteria on population growth rates. They did observe differences between cyanobacteria with different morphologies on population growth rates. In contrast, survival rates of the grazers were more significantly impacted by toxin producing cyanobacteria than non-toxin producing cyanobacteria although this may be caused by a single *Microcystis* strain PCC7806. Overall, Wilson et al. (2006) could not make any conclusive statements given the significant overrepresentation in the data of studies with the cyanobacteria PCC7806. Tillmans et al. (2008) confirmed these findings in a subsequent meta-analysis. Furthermore, 21 of the 29 studied zooplankton species maintained positive growth rates when fed a diet containing cyanobacteria thus not supporting the hypothesis of potential mechanical interference. Tillmans et al. (2008) did observe a large species-specific variation between the different zooplankton species. Overall, it remains unclear which factors of cyanobacteria are primarily driving adverse effects on zooplankton.

1.2.4 Interactions between cyanobacteria and other stressors

The complexity of the aquatic environment where organisms face a variety of stressors has been discussed in section 1.1. Cyanobacteria may also be part of such multiple stress conditions and even interact with other stressors. Yet, despite the plethora of studies available on natural and chemical stressors as summarized by Holmstrup et al. (2010) and Laskowski et al. (2010), little to no biotic stressors were included. Potential interaction effects with cyanobacteria were not reported.

Literature search resulted in eleven studies concerning the potential interaction effects between cyanobacteria and other stressors published since 2010 (Table 1.2.). Nine of the eleven studies were conducted with *Daphnia* species (Table 1.2). Five studies focused on *Microcystis aeruginosa*, two on *Cylindrospermopsis raciborskii* and one on *Nodularia spumigena* (Table 1.2). Exposure to cyanobacterial toxins rather than cyanobacteria was conducted in three studies with microcystins (Table 1.2). Interactions with chemicals were studied in five cases of which four observed interaction effects (Table 1.2). However, in the study of De Coninck et al. (2013b), antagonistic interaction effects between cadmium and *Microcystis aeruginosa* on the total reproduction were only observed for one of the twenty studied *Daphnia magna* genotypes. For all other genotypes no significant interaction effects

were observed. Nevertheless, for all observed genotypes, reproduction of organisms exposed to both stressors was always lower than the reproduction of organisms exposed to each stressor alone. Bernatowicz and Pijanowska (2011) also observed both presence and absence of interaction effects on the number of eggs produced by organisms of genotypes of *Daphnia longispina* exposed to *Cylindrospermopsis raciborskii* and polychlorinated biphenyls (PCB). Turja et al. (2013) observed significant interaction effects on three of the four studied biomarkers, i.e. glutathione S-transferase, catalase and glutathione peroxidase, in *Gammarus oceanicus* when exposed to mixtures of *Nodularia spumigena* and benzo[a]pyrene whereas Cerbin et al. (2010) observed a synergistic effect only on the size at first reproduction.

Combinations with abiotic stressors resulted in interaction for four of the five studies (Table 1.2). Yang et al. (2011) observed interaction effects between microcystins and nitrite for two of the eight observed endpoints, time to first batch of eggs and first clutch. For all other endpoints no interactions were observed. A second study by Yang et al. (2012) found both synergistic and antagonistic effects on different endpoints of *Daphnia magna* when exposed to ammonia and microcystin. All endpoints were related to reproduction. In particular, synergistic effects were observed on the endpoint, time to first eggs whereas antagonistic effects were observed on the total offspring per female. Bednarska et al. (2011) observed interactions for some genotypes between temperature and *Cylindrospermopsis raciborskii* on *Daphnia magna*. In particular, they observed a large difference between the control and cyanobacterial treatment for the age at first reproduction at 20°C for some genotypes but this difference was significantly smaller at 24°C. This indicates that at higher temperatures the age at first reproduction is more comparable between green algae and cyanobacteria than at lower temperatures. Sun et al. (2012) observed interaction effects between ammonia and microcystin on antioxidant responses of *Hypophthalmythys nobilis* larvae.

Interaction with biotic stress was studied in one paper by Pires et al. (2011) who observed interaction effects between *Microcystis aeruginosa* and bacterial lipopolysaccharides (LPS) on life history responses of *Daphnia galeata* that depended on the type of *Microcystis* strain.

Interaction effects are clearly present between cyanobacteria and other stressors yet these effects vary significantly between species and even genotypes of both the exposed organism as well as the cyanobacterial strains. At present however the current literature contains too few studies to make

strong conclusions. Furthermore, the studies are biased towards *Microcystis*. In addition, given the small number of studies, only a very limited set of potential interacting stressors has been investigated.

Table 1.2 Overview of all published studies concerning combined effects of cyanobacteria and other stressors. Full reference to each study can be found in the reference list. PCB= polychlorinated biphenyls, LPS = lipopolysaccharides. GLM = generalized linear model. ANOVA = analysis of variance. ANOVA (=IA) means that ANOVA was conducted on log transformed data which is the same as applying the independent action model (IA).

Stressor 1	Stressor 2	Interaction	Organism	Statistical model	Reference
M. aeruginosa	Carbaryl	Yes	*D. pulicaria*	Factorial regression	Cerbin et al. (2013)
M. aeruginosa	Temperature	No	*D. galeata*	ANOVA	de Senerpont Domis et al. (2013)
M. aeruginosa	Cadmium	No	*D. pulex*	ANOVA (=IA)	De Coninck et al. (2014)
M. aeruginosa	Cadmium	Yes	*D. magna*	ANOVA (=IA)	De Coninck et al. (2013)
M. aeruginosa	LPS	Yes	*D. galeata*	GLM	Pires et al. (2011)
Microcystin	Nitrite	Yes	*D. obtusa*	ANOVA	Yang et al. (2011)
Microcystin	Amonia	Yes	*D. magna*	ANOVA	Yang et al. (2012)
Microcystin	Amonia	Yes	*H. nobilis*	ANOVA	Sun et al. (2012)
C. raciborskii	Temperature	Yes	*D. magna*	ANOVA (=IA)	Bednarska et al. (2011)
C. raciborskii	PCB	Yes	*D. longispina*	GLM	Bernatowicz et al. (2011)
N. spumigena	Benzo[a]pyrene	Yes	*G. oceanicus*	ANOVA (=IA)	Turja et al. (2013)

Cyanobacteria are an increasing threat to aquatic ecosystems due to the increase of cyanobacterial blooms under anthropogenic and climate change conditions. These harmful blooms can have significant effects on aquatic organisms. Yet, the primary driving factors of these adverse effects remain unclear. Furthermore, cyanobacteria have the potential to interact with other stressors in the environment leading to possible detrimental synergistic effects. At present, too few studies have investigated these interactions to make any strong conclusions.

1.3 Mechanistic research and emerging technologies

Highthrougput molecular technologies have fuelled a revolution in biological research. Since the publication of the *Haemophilus influenza* genome by Fleischmann et al. (1995), over 200 eukaryotic genomes and more than 2000 bacterial genomes have been fully sequenced and incorporated in the database of the National Center for Biotechnology Information (NCBI, 2014). With over 10,000

ongoing genome projects in the NCBI database and the recent announcement of Illumina to deliver the $1000 human genome (Illumina, 2014), the omics era has truly begun. Indeed, parallel to development of genomics, other omics technologies have emerged (Fig. 1.3). At present, omics technologies now cover the entire aspect from DNA to functional metabolite in the cell (Fig. 1.3). As a result, mechanistic research is thriving under the influence of these technologies. Omics technologies have revolutionized plant breeding (Langridge and Fleury, 2011) and medicine (Fin, 2007) and now tackle the field of ecology and ecotoxicology.

The development of microarray technology has been the main driver for this evolution in the field of environmental science (Poynton and Vulpe, 2009; Schirmer et al., 2010; Van Aggelen et al., 2010). It allowed researchers to investigate the effects of stressors on their organism of choice without the requirement of a fully sequenced genome, which was lacking for most model organisms in ecology and ecotoxicology. At present, microarray data for quite a number of ecotoxicological models is already available. Gene expression analysis in *Folsomia candida* revealed 142 genes involved in the response to heat stress (Nota et al., 2010). The effects of nanoparticles have been studied on a variety of organisms including *Hyalella azteca* (Poynton et al., 2013), *Daphnia magna* (Poynton et al., 2011) and *Danio rerio* (Griffitt et al., 2008). Nanoparticles primarily affected genes related to chitinases in *Hyalella azteca* and *Daphnia magna* whereas genes with functions in cell apoptosis were differentially regulated in *Danio rerio*. A query into the gene expression omnibus (GEO) database resulted in microarrays for other ecotoxicological models such as *Anguilla, Fundulus, Gasterosteus, Mytilus, Pimephales* and *Tigriopus* (GEO, 2014).

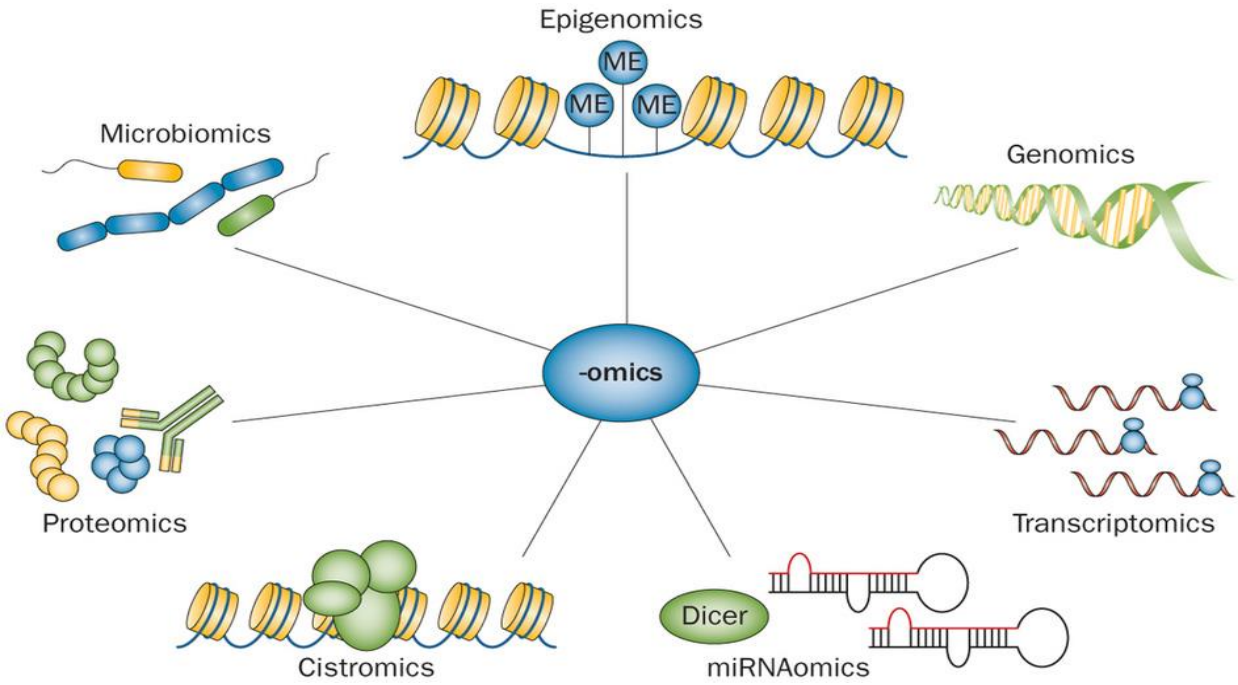


Figure 1.3 Overview of different types of omics. (From Weaver et al., 2014)

These recent advances in ecotoxicology often referred to as ecotoxicogenomics or environmental genomics require some nuance. Although the significant benefit of these studies in understanding the mechanisms of stress response of organisms remains unchallenged, several aspects still need to be addressed. First, despite the wealth of information in microarrays, there is often no straightforward link between gene expression and toxicological responses at the organismal level (Van Straalen and Feder, 2011). Second, genes constantly modify their expression even when comparing standard or "normal" conditions. It is therefore crucial to distinguish between these "neutral" genes and genes that respond to the environment under study (Van Straalen and Feder, 2011). Third, there exists a large variety in analysis and presentation of omics data between different studies (Ankley et al., 2006).

These challenges are currently being addressed by new frameworks such as the adverse outcome pathway (AOP) framework (Fig 1.4). Adverse outcome pathways consist of a molecular initiating event in which a certain chemical, or stressor in general, interacts macro-molecularly with a biological target. Then, a series of cellular and organ responses will ultimately lead to organismal responses, e.g. reduced reproduction or growth, and population responses. Adverse outcome pathways can thus be seen as a framework developed to provide clear links between molecular changes and organismal responses which tackles two of the three challenges raised in the paragraph above. Furthermore, the development of this framework within standard regulatory practices will naturally lead to a more

uniform analysis and presentation of results. Currently, a collaborative effort is ongoing through the AOP wiki (OECD, 2014b). In addition, OECD has developed a guideline on developing and assessing adverse outcome pathways (OECD, 2013). In this guideline, OECD clearly advocates that AOPs are a framework that incorporates information from various existing methods and links those to higher biological endpoints rather than a completely new methodology. Current AOPs under development span a variety of modes of actions of chemicals including acetylcholine esterase inhibition and mitochondrial toxicity (OECD, 2014b).

In addition to providing potential links between molecular events and apical effects, AOPs may alleviate the pressure on environmental regulation which has to asses an ever increasing number of chemicals, preferably with a greater speed and better accuracy as they are able to process information from emerging technologies such as omics within a defined regulatory framework (Ankley, 2010).

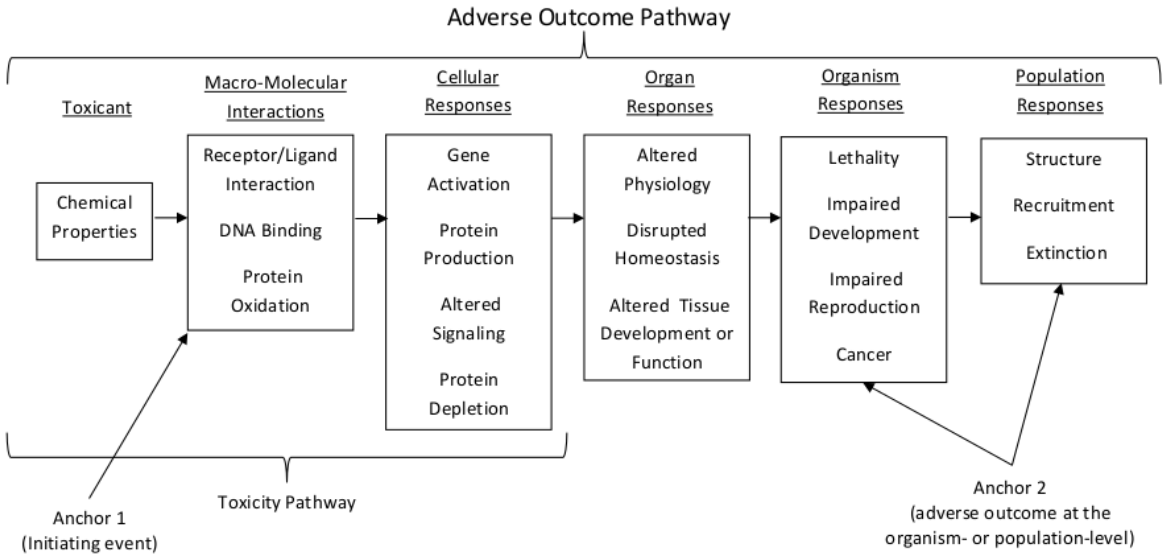


Figure 1.4 Conceptual diagram of adverse outcome pathways (AOP). The first three boxes are the parameters that define a toxicity pathway, as described by the National Research Council. (Adapted from Ankley et al. 2010)

Next to the challenges raised for the application of omics in ecotoxicology, opportunities were also identified. Antczak et al. (2013) used machine learning techniques to distinguish transcriptomic profiles originating from different classes of chemicals, suggesting that transcriptomic signatures indicative of toxicants could be archived and used as barcodes to identify chemicals in the environment. Several studies have focused on elucidating responses to multiple stress conditions by using microarrays, e.g. Hook et al. (2008), Garcia-Reyero et al. (2009), Vandenbrouck et al. (2009). Most of them have been

summarized by Altenburger et al. (2012). This review study structured current knowledge regarding mixture effects, which primarily originates from non-molecular studies, within an omics framework (Fig. 1.5).

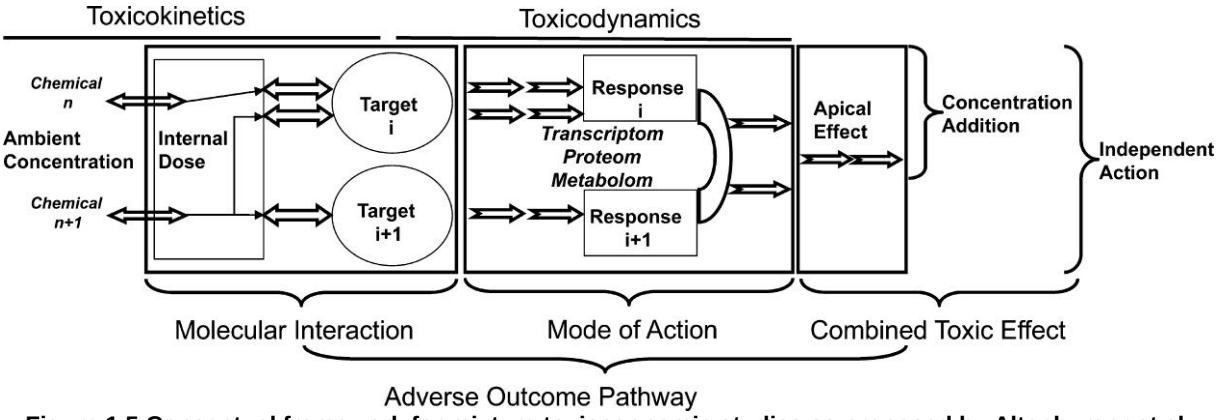


Figure 1.5 Conceptual framework for mixture toxicogenomic studies as proposed by Altenburger et al. (2012) (From Altenburger et al. (2012))

Altenburger et al. (2012) identified 41 papers using mainly molecular technologies such as microarrays and quantitative polymerase chain reaction (qPCR) to study mixture effects. All papers discussed mixtures of chemicals varying from binary mixtures to complex environmental samples consisting of multiple stressors. Six studies reported on chronic effects whereas the other studies conducted short term exposures. The majority of the studies focused on responses in fish organs although some studies investigating invertebrates such as *Daphnia*. Responses of mammalian cell lines were also reported. Although it seems quite a large number of studies investigated mixtures, Altenburger et al. (2012) remarked that none of them explicitly tested a mixture hypothesis. Often qualitative methods were used to compare treatments rather than specifically testing for interaction effects. Altenburger et al. (2012) observed that although authors referred to synergistic or antagonistic effects, this was often a comparison of observations versus the authors` expectation rather than through an explicit statistical hypothesis such as independent action or concentration addition. Hence, it was difficult to interpret these results. Altenburger et al. (2012) further pointed out that the use of terminology was not straightforward and different studies used similar terminology to describe different effects with different interpretations. The lack of consistent and straightforward terminology has also been observed in non omics mixture studies (section 1.1.1). Altenburger et al. (2012) suggested that research on interaction effects with omics technologies could be improved by defining explicit null hypotheses. Also, knowledge on interaction effects could be significantly improved by including concentration response

data according to Altenburger et al. (2012). They further recommend the use of predicted expression values of combined effects through the conceptual models of concentration addition and independent action. Finally, Altenburger et al. (2012) emphasized the need to validate exposure concentrations analytically in any type of study.

The rapid development of high throughput omics technologies is revolutionizing ecotoxicology. Applications vary from purely mechanistic research to transcriptomic profiling to identify chemicals and interaction effects. Yet, some challenges remain to be addressed. Linking molecular responses to biological outcomes remains difficult but adverse outcome pathways prove to be a promising framework. Potential interaction effects are already studied in a diversity of studies but lack explicit hypothesis testing and standard conceptual models such as concentration addition and independent action.

1.4 Model organism*: Daphnia pulex*

Daphnia pulex or the American water flea is one of the most common species of *Daphnia*. Daphnids are small crustaceans, present in a large variety of lakes and ponds across a wide geographical range (Lampert, 2006). It has been used as a model organism for ecological and ecotoxicological research for more than 100 years (Lampert, 2011; Weismann, 1880). Literature has increased constantly and a current literature search results in over 4000 papers in the last 50 years concerning *Daphnia*. *Daphnia* has also been a standard model organism in ecotoxicity testing guidelines issued by both the OECD (2008) and US EPA (1996). The reason for the success of the waterflea has been attributed to a variety of factors (Lampert, 2006). Some of the most important ones are its ecological position, life cycle, physical properties and species diversity. Daphnids are predators of phytoplankton while being at the same time a prey to fish, giving them a unique and central position in the foodweb. They are cyclic parthenogens, meaning that they reproduce both sexually as well as asexually (Fig. 1.6). In the case of *Daphnia*, the asexual cycle in which mothers produce only genetically identical daughters generally occurs. Only under stressful conditions will parthenogenetic females produce sons which can in turn mate with the females to produce a resting egg or ephiphium (Lampert, 2006). The resting egg is a dormant stage and will hatch when conditions become favorable again (Lampert, 2006). This unique life cycle off course has many advantages. First, genetic variability can be excluded or included in the experimental design. Second, parthenogenetic reproduction means only one daphnid is needed

to start-up a culture and allows for easy culture maintenance. Third, the consequences of both parthenogenetic and sexual reproduction can be studied. Fourth, the ephiphium can survive in sediment layers for over decades making it possible to resurrect *Daphnia* from the past and study past populations (Orsini et al., 2013). Daphnids are small, transparent organisms with a short generation time, i.e. 7 to 15 days, which makes them ideal organisms to culture in the lab. Finally, the genus of *Daphnia* contains over 100 different species that demonstrate a wide range of phenotypic and genotypic plasticity.

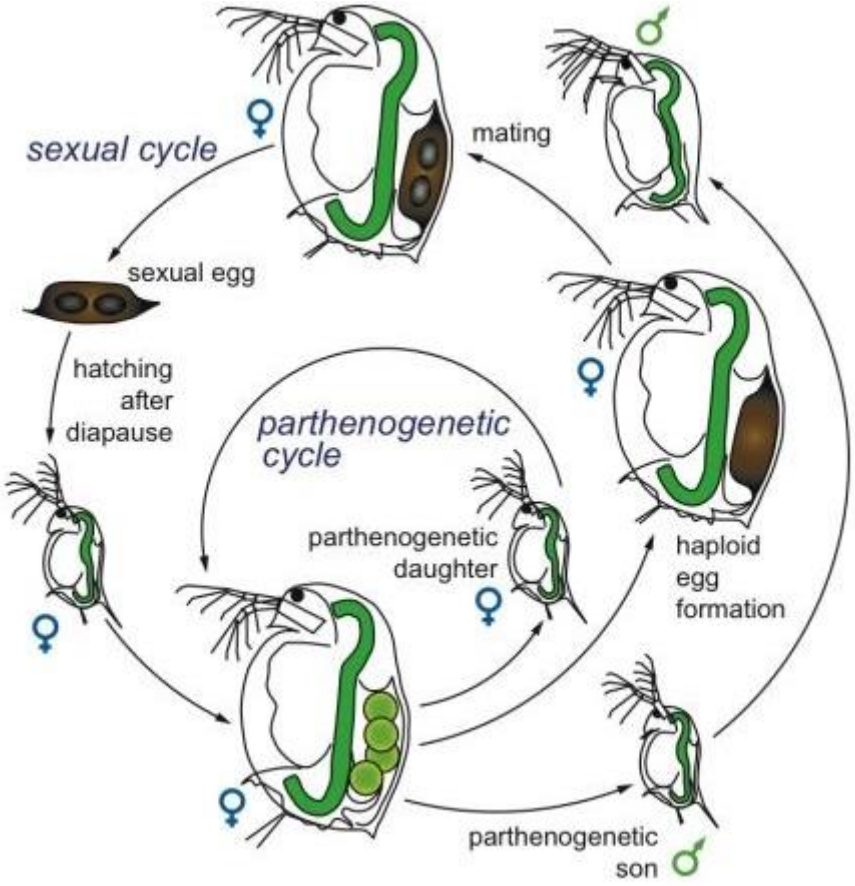


Figure 1.6 Schematic representation of the cyclic parthenogenetic life cycle of *Daphnia* (From Ebert, 2005)

The recent evolution in molecular technologies has propelled ecological and environmental research to tackle longstanding hypotheses with a new biological level of understanding. This evolution has greatly benefitted *Daphnia*, which is now emerging as a true model organism in ecological and environmental genomics (Ebert, 2011). The well-known ecology and use in ecotoxicology made *Daphnia* an ideal candidate for genome sequencing. The fully sequenced genome was published in 2011 (Colbourne et al.) and revealed a unique genomic structure unlike any other known genomic

model. *Daphnia* has an inflated number of duplicated genes due to a disproportionate expansion of gene families within the genome (Colbourne et al., 2011). Groups of these gene families cluster non randomly within specific metabolic pathways such as the sphingolipid biosynthesis (Colbourne et al., 2011). Expression patterns of these gene families within these specific pathways suggest non independent functional divergence. More than a third of the genes are specific to the *Daphnia* lineage and have no known detectable homology with any other gene in all available proteomes (Colbourne et al., 2011). Expression studies highlighted that these lineage-specific genes are more susceptible to ecological conditions and that gene duplicates demonstrate divergent expression patterns (Colbourne et al., 2011). Indeed, Colbourne et al. (2011) observed an evolutionary diversification of duplicated genes. They observed that divergence in expression pattern between gene duplicates corresponds with the age in many gene families. In other words, recent gene duplicates have very similar expression patterns whereas genes that duplicated a long time ago have very different expression patterns. However, they also observed that quite a number of recently arisen paralogs differ in their expression in at least one condition despite having nearly identical sequences. Colbourne et al. (2011) conclude that paralogous genes often diverge in gene expression pattern upon exposure to environmental conditions either at the time of duplication or soon after. This tight interaction between the genome and the environment has made *Daphnia* particularly suitable to study such interactions (Altshuler et al., 2011; Shaw et al., 2008). Despite the small genome size of only 200 megabases (Mb), it has over 30000 genes with a reduced intron size resulting in an average gene span of more than 1000 basepairs shorter than the average gene length in *Drosophila* (2300 basepairs versus 4000 basepairs) (Colbourne et al. 2011). However, the average protein length is similar in size between these two species. This is due to a reduced average intron size, i.e. 170 basepairs versus 660 basepairs in *Drosophila*. Furthermore, of all sequenced arthropods, *Daphnia* shares the highest number of genes with humans. Given all these features, *Daphnia pulex* is now recognized by the National Institutes of Health (NIH) as a model organism for biomedical research (NIH, 2014).

***Daphnia pulex* can be considered as a true environmental genomics model organism. The unique genome structure has highlighted its environmental relevance and is fully complemented with its well-known ecology and ecotoxicological responses.**

1.5 Model stressors

1.5.1 Cyanobacteria

Six species of cyanobacteria were selected representing the six main genera of toxic cyanobacteria (van Apeldoorn et al., 2007). These species are classified in different orders based upon their morphology (Table 1.3, Fig. 1.7). The following subsections will briefly situate each species geographically, its prevalence and importance in bloom formation as well as summarize the main effects on *Daphnia*.

Table 1.3 Overview of selected cyanobacteria classified within their order and the corresponding morphology.

Species	Order	Morphologic characteristic of the classification order
Anabaena lemmermannii	Nostocales	Multicellular, trichal, heterocysts present, filamentous
Aphanizomenon sp.	Nostocales	Multicellular, trichal, heterocysts present, filamentous
Cylindrospermopsis raciborskii	Nostocales	Multicellular, trichal, heterocysts present, filamentous
Microcystis aeruginosa	Chroococcales	Unicellular, isopolar, colony forming
Nodularia sp.	Nostocales	Multicellular, trichal, heterocysts present, filamentous
Oscillatoria sp.	Oscillatoriales	Multicellular, trichal, heterocysts not present, filamentous

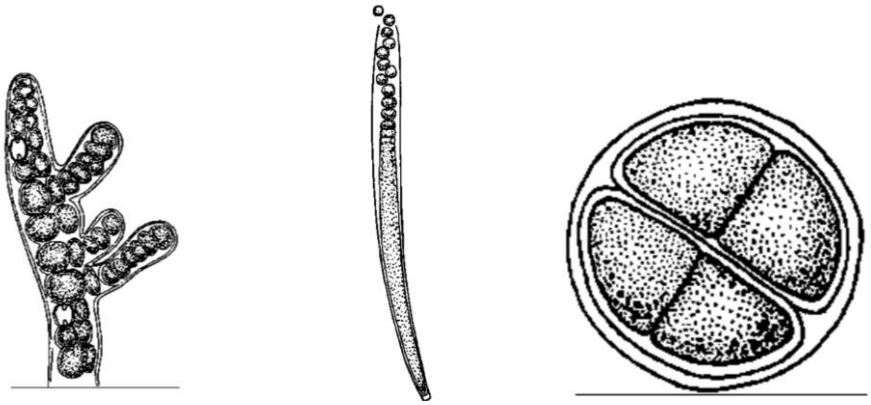


Figure 1.7 Morphology of cyanobacteria. From left to right: Nostocales – Oscillatoriales – Chroococcales (From Mur et al., 1999).

1.5.1.1 Anabaena lemmermanii

Strains of the *Anabaena* genus are filamentous nitrogen fixing cyanobacteria (Gugger et al., 2002). They can produce certain toxins such as neurotoxins, anatoxin-a and anatoxin-a(s), and saxitoxins (Table 1.1, Gugger et al., 2002). Some species of *Anabaena* have been known to produce a wide variety of hepatotoxins such as microcystins (Sivonen et al., 1992). *Anabaena* strains are in general closely related to *Aphanizomenon* strains. They have a very similar morphology, only the trichomes are genus-specific (Gugger et al., 2002).

Blooms of *A. lemmermannii* have been observed in the Baltic Sea although these blooms have in general been found to be non-toxic (Sivonen et al., 1989). However, toxin producing blooms of *A. lemmermannii* have been identified as the cause of bird kills in Danish lakes (Onodera et al., 1997). Onodera et al. (1997) identified the toxin as anatoxin-a(s). Although the majority of studies have reported *A. lemmermannii* blooms in the North of Europe (Cronberg, 1999; Ekman-Ekebom et al., 1992), they have a wide thermal distribution and have been detected in the Mediterranean area as well (Cook et al., 2004).

Some studies have investigated the effects of *Anabaena* species on *Daphnia*. Chow-Fraser and Sprules (1986) observed lower filtering rates of *Daphnia pulex* exposed to *Anabaena* sp. when compared to green algae. Gilbert and Durant (1990) observed reduced feeding of *D. pulex* and *D. galeata mendotae* on green algae when exposed to *Anabaena flos-aqua*. They identified two main causes, mechanical interference and increased food availability of *A. flos-aqua*. Von Elert et al. (2003) observed reduced growth of *D. galeata* when fed with *A. variabilis*. However when the diet was supplemented with poly unsaturated fatty acids (PUFAs) and sterols, an increase in growth was observed indicating that *A. variabilis* lacks sufficient PUFAs and sterols to sustain the growth of *D. galeata*. No studies with *A. lemmermannii* were found. Overall, *Anabaena* species seem to affect *Daphnia* species through mechanical interference in feeding and reduced food quality. No indications of potential toxins leading to these effects were found.

1.5.1.2 Aphanizomenon sp.

The genus *Aphanizomenon* is very similar to the *Anabaena* genus as discussed in section 1.5.1.1. The major differences are the trichome structure and toxin production. *Aphanizomenon* strains primarily produce saxitoxins and anatoxin-a (Table 1.1, Gugger et al., 2002). Some strains are also known to produce alkaloid cytotoxins such as cylindrospermopsins (Table 1.1, Gugger et al., 2002).

Aphanizomenon has been primarily associated with paralytic shellfish poisoning (Ballot et al., 2010; Mahmood and Carmichael, 1986; Pereira et al., 2000). Blooms of *Aphanizomenon* have a wide geographical distribution being reported in the Baltic Sea (Sivonen et al., 1989), in drinking reservoirs in Portugal (Pereira et al., 2000) and in tropical lakes in Australia (Shaw et al., 1999). Blooms in the Baltic Sea were not toxic whereas blooms in Portugal and Australia were reported to produce several toxins.

Effects of *Aphanizomenon* species on *Daphnia* were attributed to nutritional quality, toxins and combinations of both. Noguiera et al. (2004a) fed *Daphnia magna* with *A. issatschenkoi* and observed reduced growth and survival due to the presence of paralytic shellfish toxins. Another study by Noguiera et al. (2006) with a cylindrospermopsin producing *A. ovalisporum* observed effects on *Daphnia magna* that could be attributed to both low nutritional value and toxin production. These conclusions were made based on comparison with both a fed and unfed control treatment. Lampert (1981) observed no effects on *Daphnia pulicaria* when fed a diet consisting of green algae and *A. gracile*. However, a diet containing only *A. gracile* did have negative effects, indicating more an effect of low food quality rather than toxins.

1.5.1.3 Cylindrospermopsis raciborskii

Cylindrospermopsis raciborskii has by far been the most studied species within the genus of *Cylindrospermopsis*. This has two main reasons. First, *C. raciborskii* has been characterized as a cylindrospermopsin producer and this specific toxin (Table 1.1) was implicated in one the most severe human poisonings by cyanotoxins in Australia referred to as the Palm Island Mystery Disease (Blyth, 1980). Second, although it was characterized as only a tropical species, it has in less than ten years gained a wide global distribution and has been referred to as an invasive species (Neilan et al., 2003). Several authors have commented on this aspect and have pointed to the huge morphological and physiological plasticity of this adaptive species (Neilan et al., 2003; Padisák, 1997).

Studies with *Daphnia* again observed effects of both low quality food and toxins. Noguiera et al. (2004b) observed significant effects on growth and survival of juveniles of *D. magna* when exposed to toxin producing *C. raciborskii*. Effects were significantly less pronounced when exposed to non-toxin producing *C. raciborskii*. On the other hand, Soares et al. (2009) observed primarily feeding inhibition effects and no toxin effects of a toxin producing *C. raciborskii* strain on *D. magna*. Panosso and Lürling (2010) noted significant effects on feeding due to low food quality when *D. magna* was exposed to *C. raciborskii*.

1.5.1.4 Microcystis aeruginosa

Microcystis aeruginosa is by far the most common and best studied cyanobacterium (Fristachi and Sinclair, 2008). *Microcystis* primarily produces microcystins (a group of toxic cyclic heptapeptides, Table 1.1) and aeruginosins - a group of toxins with a peptide-like structure without any standard L-amino acids (Ersmark et al., 2008; Sandler et al., 1998; van Apeldoorn et al., 2007).

The global occurrence of *Microcystis* has resulted in a large body of scientific literature regarding *Microcystis* and microcystins. Toxicity of microcystins has been studied in a huge variety of organisms ranging from aquatic invertebrates to fish and mammals and even plants (van Apeldoorn et al., 2007). Microcystin is at present the only toxin for which the World Health Organization has established a drinking water limit (Table 1.1).

The effects of *Microcystis* on *Daphnia* have been well studied (Demott et al., 1991; Lürling, 2003; Nizan et al., 1986). Furthermore, some studies were even able to observe tolerance to *Microcystis* in some isolates of *Daphnia* (De Schamphelaere et al., 2011; Gustafsson and Hansson, 2004). Despite the large amount of research, no consensus is reached regarding the factors driving the negative effects on zooplankton (Lürling, 2003; Rohrlack et al., 1999).

1.5.1.5 Nodularia sp.

Strains of the *Nodularia* genus mainly occur in brackish and coastal environment, although some are found in freshwater environments (Beattie et al., 2000; Bolch et al., 1999). Blooms of this genus have been reported worldwide, Australia, Baltic Sea, North America, (Bolch et al., 1999). Not all species produce toxins. Some species, such as *N. harveyana* are known to never produce toxins or even associated with toxic blooms (Bolch et al., 1999). Others such as *N. spumigena* appear to be always

toxic (Bolch et al., 1999). Poisonings have been reported in North of Germany, west of South Africa and Sweden (Edler et al., 1985; Nehring, 1993; Van Halderen et al., 1995)

Studies with *Daphnia* are scarce. Literature search revealed a study by Demott et al. (1991) who exposed three species of *Daphnia* to toxins from *N. spumigena* and observed feeding inhibition for all three species. No studies were found in which *Daphnia* were exposed to actual cells of *Nodularia* sp.

1.5.1.6 Oscillatoria sp.

Oscillatoria strains have been reported in both freshwater and coastal waters (Sivonen et al., 1990). Although most blooms have been reported in Northern Europe (Scandinavia, northern region of the Netherlands, Scotland), some *Oscillatoria* blooms have occurred in warmer regions such as South Australia (Buijse et al., 1993; Hayes and Burch, 1989; Sano et al., 1998; Sivonen et al., 1990). Reports of specific poisonings as a result of an *Oscillatoria* bloom are limited. Literature search revealed a case of dog poisoning in Scotland (Edwards et al., 1992).

Effects on *Daphnia* were studied mainly by Repka. Repka published a series of studies in which the effects of *Oscillatoria* on several *Daphnia* species, including *D. galeata* and *D. cucullata*, were studied. For all daphnids, Repka observed lower reproduction on diets containing *Oscillatoria* (Repka, 1996; Repka, 1997). However, despite the lower reproduction daphnids were still able to maintain positive growth suggesting that nutrition quality might be more prominent than toxin production.

The six selected cyanobacterial strains cover a diverse range of morphologies, geographical distributions, habitats and toxin production. All have been reported in cyanobacterial blooms that have often been correlated with poisoning of humans and animals. The majority of the studies with *Daphnia* focus on the effect of *Microcystis*. Overall, no primary driving factor of the adverse effects on *Daphnia* could be identified as these factors differed between feeding inhibition, food quality and toxin production across the different studies

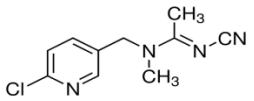
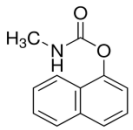
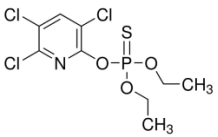
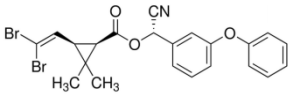
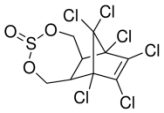
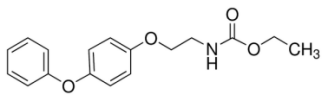
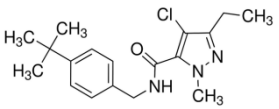
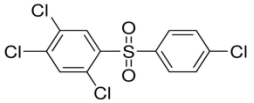
1.5.2 Pesticides

The increasing global population has required a substantial increase in agricultural activity which has in turn a significant impact on the environment. This has resulted in the nineties in an increased use of plant protection products (PPPs) such as endosulfan, carbaryl and diazinon with problematic residues in surface waters as a consequence due to discharge and spray drift (Eurostat, 2007). Since 2000 however, a more restrictive policy has been enforced at both national and international level with a focus on sustainability and rational use of PPPs and the prohibition of certain persistent PPPs. Nevertheless, even years after the prohibition of certain persistent PPPs, residues of these PPPs are still present in surface waters (MIRA, 2007).

Plant protection products may also interact with other stressors in the environment and significantly increase the potential impact on aquatic organisms. Interactions between PPPs and biotic stressors have been reported by several studies. Coors and De Meester (2008) observed significant interaction effects between carbaryl and the parasite *Pasteuria ramosa* on *Daphnia magna.* Hanazato and Dodson (1995) observed synergistic interactions between *Chaoborus* kairomones and carbaryl on *Daphnia pulex*. But amphibians are also susceptible to such interaction effects. *Hyla versicolor* is more susceptible to carbaryl under predator-induced stress (Relyea and Mills, 2001). Relyea (2004) also observed synergistic impacts of malathion and predatory stress on six species of *Rana sylvatica*.

Furthermore, agricultural areas are often confronted with eutrophication (Ulén et al., 2007) which may give rise to increased cyanobacterial blooms (section 1.2.2). The potential interaction between these natural and chemical stressors remains largely unknown.

Table 1.4 Insecticides with their chemical structure, classification, mode of action and the approval of use in European Union (EU) and the United States (USA).

Insecticide	Chemical structure[1]	Classification[2]	Mode of action[2]	Approval[3,4]	PEC[3] / MAC[5,6,7] (µg/L)
Acetamiprid		Neonicotinoid	Agonist of nicotinic acetylcholine receptor – Nerve action: hyperexcitation Acetamiprid will mimic the action of acetylcholine at the nicotinic acetylcholine receptor	EU: Yes USA: Yes	7 / NA
Carbaryl		Carbamate	Inhibitor of acetylcholine esterase – Nerve action: hyperexcitation Acetylcholine esterase normally terminates the action of acetylcholine at the nerve synapse.	EU: No USA: Yes	0.6-45 / 2.1
Chlorpyrifos		Organophosphate	Inhibitor of acetylcholine esterase – Nerve action: hyperexcitation Acetylcholine esterase normally terminates the action of acetylcholine at the nerve synapse.	EU: Yes USA: Yes	0.3 / 0.01
Deltamethrin		Pyrethroid	Modulator of sodium channels – Nerve action: hyperexcitation Deltamethrin will keep the sodium channels, which are involved in the propagation of action potentials, open.	EU: Yes USA: Yes	0.02-0.04 / 0.004
Endosulfan		Cyclodiene organochlorine	Antagonist of GABA-gated chloride channels – Nerve action: hyperexcitation GABA is the major inhibitory neurotransmitter in insects	EU: No USA: Yes	0.2-8 / 0.1
Fenoxycarb		Fenoxycarb	Juvenile hormone mimic – Growth regulation Fenoxycarb will disrupt and prevent metamorphosis	EU: Yes USA: Yes	22-87 / NA
Tebufenpyrad		METI insecticide	Inhibitor of mitochondrial complex I electron transport – Energy metabolism Tebufenpyrad will prevent the usage of energy by the cells	EU: Yes USA: Yes	0.3 -10/ NA
Tetradifon		Tetradifon	Inhibitor of mitochondrial ATP synthase – Energy metabolism	EU: No USA: No	NA

1 Sigma Aldrich, 2014 www.sigmaaldrich.com

2 IRAC, 2009.

3 EU pesticide database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

4 US EPA: http://ppis.ceris.purdue.edu/

5 Waterframework directive: http://ec.europa.eu/environment/water/water-framework/

6 US EPA: National Water Quality Criteria http://water.epa.gov/scitech/swguidance/standards/criteria/current/index.cfm#cmc

7 Canadian freshwater guidelines for the protection of aquatic life: http://ceqg-rcqe.ccme.ca/

Eight insecticides were selected spanning a variety of chemical structures, modes of action and classifications (Table 1.4). Given the impact of insecticides in the environment, a large body of literature is available describing the effects of all these insecticides. Here, a selection of studies in relation with *Daphnia* that sketch the current research field will be briefly discussed.

The effects of carbaryl on *Daphnia* are probably the best studied out of all insecticides. Jansen et al. (2013) reported on the gene expression changes in *Daphnia* upon exposure to carbaryl. They observed significant repression of cuticle genes and effects on gene transcription and translation. Other literature reports the effect of carbaryl on *Daphnia* in combination with a selection of natural stressors such as predators (Coors and De Meester, 2008), parasites (De Coninck et al., 2013b) and even *Microcystis* (Cerbin et al., 2010). Similar studies were found for chlorpyrifos, also an acetylcholine esterase inhibitor. They focused on interactions with both natural stress, toxic algal blooms (Daam et al., 2011), and other organophosphates (Li and Tan, 2011) and even other insecticides (Loureiro et al., 2010). The effects of juvenile hormone analogs such as fenoxycarb are also well studied as a model for effects of endocrine disrupting chemicals (Tatarazako and Oda, 2007). Juvenile hormone analogs are also of particular interest as they seem to induce male production (Lampert et al., 2012; Olmstead and LeBlanc, 2003). Pyrethroids and their effects on *Daphnia* have been studied since the 1980s (Day and Kaushik, 1987). Current research focusses on effects of deltamethrin in sex differentiation and embryonic development (Toumi et al., 2013) and potential interactions with metals (Barata et al., 2006). Effects of endosulfan in contrast do not impact sex differentiation in *Daphnia* (Zou and Fingerman, 1997). Like carbaryl, endosulfan has been studied together with predation stress. In the study by Barry (2000), endosulfan inhibited phenotypic plasticity of *Daphnia pulex* decreasing the probability of survival under predation stress.

Literature on the potential effects of the three other insecticides on *Daphnia* is much more limited. No studies were found describing effects of acetamiprid on *Daphnia*. But Qi et al. (2013) studied the

effects of guadipyr, another neonicotinoid, on *D. magna*. Effects were observed on growth and reproduction. No studies were found that solely focused on the effects of tebufenpyrad on *Daphnia*. One study (Beketov et al., 2011) reported on the potential effects of tebufenpyrad on *Daphnia* within a large set of pesticides. Beketov et al. (2011) observed antagonistic effects on *Daphnia* after exposure to tebufenpyrad and increased UV radiation. Studies on tetradifon were primarily reported by Villaroel et al. They (Villaroel et al., 1999) observed significant effects of tetradifon on the feeding behavior of *Daphnia magna*. Further study (Villaroel et al., 2008) revealed reduced reproduction in mature offspring, F1 generation, of parental exposed animals. Villaroel et al. (2009) also observed a good correlation between decreased energy budget and effects of tetradifon on life history parameters such as survival, growth and reproduction.

Insecticides can have significant effects on the life history of *Daphnia* varying from effects on reproduction and survival to effects on male production and sex development. Quite a number of studies are available for most insecticides with a well-known mode of action. Less is however known about newer insecticide classes such as neonicotinoids or newer insecticides such as tebufenpyrad and tetradifon. Interaction effects with other stressors have been studied for both chemical and natural stressors although few studies so far have focused on the combined effects with cyanobacteria.

1.6 Conceptual Framework

Aquatic ecosystems are complex environments where organisms interact with a heterogeneous group of stressors from anthropogenic and natural origin. Yet, current chemical risk assessment practices fail to include these combined effects of stressors and their potential interaction as they primarily use a chemical-by-chemical approach. The lack of sufficient comprehensive data in literature and the lack of predictive models further impede the incorporation of combined and interaction effects in environmental legislation. The occurrence and the frequency of combined and interaction effects in the environment are not unlikely to increase significantly in the future.

Anthropogenic factors and climate change conditions stimulate bloom formation of potential toxic cyanobacteria. These organisms are an emerging concern for both environmental and public health. Although effects on mammals are well documented and understood, the mechanisms driving effects

on zooplankton species remain unclear and research is largely biased towards effects of *Microcystis*. Therefore, **chapter 2** has focused on the effects of six cyanobacteria species, representing six main genera, on the life history of *Daphnia*. Effects of cyanobacteria were studied across the full concentration response curve which will allow to accurately compare the effects of diverse group of cyanobacteria. In addition, the comparison with a starvation response will allow to assess the impact of nutritional factors affecting cyanobacterial toxicity.

Cyanobacteria are likely to occur in environments together with other stressors leading to multiple stress conditions and altered responses of *Daphnia* exposed to cyanobacteria under such multiple stress conditions. Indeed, the eutrophication of water bodies, known to enhance bloom formation, often occurs in agricultural areas, which may give rise to unknown interaction effects with plant protection products. In particular, insecticides can severely affect aquatic invertebrates and two studies have shown interaction between insecticides and cyanobacteria on *Daphnia*. Given that each study focused on specific combinations of stressors, conclusions cannot be generalized and the potential interaction effects for insecticides and cyanobacteria in general remain largely unknown. In **chapter 3**, these potential interaction effects were studied by exposing *Daphnia* to binary combinations of a selection of cyanobacteria and insecticides. Combined and interaction effects were evaluated within defined statistical frameworks with the two conceptual models concentration addition and independent action under standard conditions of 21 day exposure. Evaluation within the two defined frameworks will allow testing different hypotheses about the interaction effects due to the different mathematical background of these models as well as allowing a thorough comparison of the models from a mechanistic and a risk assessment point of view.

Such experiments are however labor intensive and time consuming. **Chapter 4** therefore focused on studying the effects on life history of a comprehensive set of 48 binary combinations of insecticides and cyanobacteria under a shorter exposure time and with reduced experimental design. Statistical evaluation of the effects is therefore only possible within the independent action framework. This approach was however evaluated by comparing the results of **chapters 3 and 4** for those combinations that were repeated. Thus, it would be possible to evaluate the impact of shorted exposure time and reduced experimental design on interaction effects as well as estimate whether findings from chapter 3 can be extrapolated to other combinations of insecticides and cyanobacteria.

The evolution of high throughput molecular technologies has enhanced the mechanistic understanding of organismal responses under stress. Mechanistic understanding is not only crucial to identify the driving factors of stress response but may also aid in building predictive models. **Chapter 5** identified the potential of these technologies by studying the stress response of *Daphnia pulex* to *Microcystis aeruginosa* with high throughput microarrays. A comprehensive set of pathway tools was developed specifically taking into account the unique *Daphnia* genome within **chapter 5** to identify crucial pathways and gene networks involved in response to *Microcystis*.

Chapter 6 further applied microarray technology to study the response of *Daphnia* to the five other studied cyanobacteria in **chapter 2**. The pathway tools developed in **chapter 5** were used to compare similarities and differences across stress responses to each of these cyanobacteria at different levels of molecular organization and identify the main driving factors of adverse effects on *Daphnia*. In particular, chapter 6 aimed elucidate the differences and similarities between the effects of the different cyanobacteria at life history level by studying the effects at the molecular level.

In **chapter 7**, the molecular responses of daphnids to the 48 binary combinations, used in **chapter 4,** were studied. Combined and interaction effects are determined within standard statistical frameworks along with a priori defined hypotheses of non-interaction at the gene level. Genes, for whom interaction effects were observed, were then subjected to a functional analysis to identify potential mechanisms of interactions as well as crucial pathways involved in the interaction effects.

Ultimately, **Chapter 8** integrated all data from **chapters 4 and 7** by building comprehensive gene networks on the transcriptomic data generated in **chapter 7** and then integrating this data with life history responses and interaction effects defined in **chapter 4.** Integration of the data within an overall gene network will allow identifying factors driving interactions across the entire data set. These key modules of genes driving life history responses and interaction effects at life history level were functionally analyzed to help understand the mechanisms and pathways driving these effects.

Chapter 9 will give a final overview of the main conclusions reached throughout this dissertation and how they have answered the concerns and research gaps put forward in the introduction. It will also address the challenges that environmental research will still need to face in the future.

Chapter 2

A comparison of the sensitivity of *Daphnia pulex* to different cyanobacterial species

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Jana Asselman*, Jennifer Hochmuth*, Karel AC De Schamphelaere. 2014. A comparison of the sensitivities of *Daphnia magna* and *Daphnia pulex* to six different cyanobacteria species. Accepted in Harmful Algae. *These authors contributed equally.

2.1 Introduction

A large body of literature has discussed the significant environmental impact of cyanobacteria on aquatic organisms and freshwater invertebrates in particular (Codd, 2005; Falconer, 2001; Paerl et al., 2001; Sellner, 1997). As a consequence, the results of these studies are slowly being integrated in risk assessment literature and even further in environmental legislation. Current risk assessments focus on cyanobacterial toxins primarily and cyanobacteria secondarily in terms of toxicity to humans and other mammals (Codd et al., 2005). Such an approach can allow sufficient protection of human health and livestock but may not be recommended to ensure sufficient protection for aquatic invertebrates.

Indeed, studies of Wilson et al. (2006) and Tillmanns et al. (2008), both conducting a meta-analysis of laboratory experiments, could not support cyanobacterial toxins as a primary driver for the negative effects on zooplankton species. In particular, they did not observe significant differences between toxin producing and non-producing strains of cyanobacteria. Although a significant bias towards *Microcystis aeruginosa* was present in these datasets, they do indicate that concentrations of cyanobacterial toxins may not be suitable as the basis for water quality criteria to adequately protect zooplankton species. Potential other factors driving adverse effects on zooplankton according to Wilson et al. (2006) and Tillmanns et al. (2008) could be morphology of the cyanobacteria and nutritional deficiency of cyanobacteria compared to green algae. These hypotheses stem from, among others, the variability of effects of different cyanobacterial species. Nutritional deficiency of cyanobacteria has been reported by other authors and related to the lack of polyunsaturated fatty acids and sterols (Demott and Müller-Navarra, 1997; Von Elert et al., 2003). Yet, no conclusive evidence has been reported and the nutritional deficiency across different cyanobacterial species remains to be compared.

A confirmation of these hypotheses was not possible with a current meta-analysis due to the earlier mentioned bias towards *Microcystis aeruginosa*. Furthermore, few concentration response data are available (Hietala et al., 1997; Lürling, 2003; Wilson et al., 2006; Soares et al., 2009). Such data are crucial from a risk assessment point of view to allow translation into a protective regulation. In addition, field evidence highlights that both the total concentration of cyanobacteria and the percentage of cyanobacteria relative to the total phytoplankton bio-volume differ considerably with season and year (Wagner and Adrian 2009, Sondergaard et al. 2011). Thus, ecological reality requires experimental

concentration response data to correctly estimate potential risks of cyanobacterial blooms to aquatic invertebrates.

Therefore, the aim of this chapter was to investigate the effects on the reproduction of *Daphnia pulex* of six different cyanobacteria at various concentrations of these cyanobacteria in the diet. The six different selected cyanobacteria were: a *Microcystis* strain, *Cylindrospermopsis* strain, *Aphanizomenon* strain, *Anabaena* strain, *Nodularia* strain and *Oscillatoria* strain. The motivation of this choice is described in detail in Chapter 1 (section 1.5.1) and is attributed, among others, to their reported presence in harmful algal blooms. *D. pulex* was exposed to each of these six cyanobacteria species individually. A range of different concentrations in the diet was selected to be able to determine the full concentration response curve upon exposure to each cyanobacteria species. These concentration response curves can then be used to estimate effect concentrations (EC_x) for each specific cyanobacterium. With these concentration response data, two hypotheses can be tested. First, estimated parameters describing the observed concentration response curves can be used to test whether concentrations responses curves to different cyanobacteria are significantly different from one another. Second, the concentration response curves can also be compared to a starvation control to test whether the nutritional deficiency is significantly different from a starvation response. Furthermore, the concentration response curves form a first scientific basis to draft regulations that adequately protect aquatic invertebrates. In addition, the experimental design includes no bias towards any of the cyanobacteria and will therefore be able to shed more light on the cyanobacterial factors driving these negative effects on zooplankton.

2.2 Materials and methods

2.2.1 Experimental organisms

Daphnia pulex stock culture females were obtained from isoclonal laboratory cultures of an isolate from the Shaw laboratory (Indiana University, Bloomington, IN, USA; Shaw, 2007). This isolate belonged to the same isoclonal population as the isolate used in the genome sequencing (Colbourne et al., 2011). Animals were cultured in COMBO medium without nitrogen and phosphorous stocks (Shaw et al., 2007) under a photoperiod of 16:8h light:dark in a climate control chamber at 20±1°C. They were fed daily with 2 mg dry weight L^{-1} of an algal mixture consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 cell number ratio. These animals were

synchronized in terms of age and reproduction and used as brood mothers. Neonates (less than 24 hours old) originating from these brood mothers were randomly assigned to experimental treatments.

All cyanobacteria originated from certified culture collections (Table 2.1) and were cultured in modified referenced culture media to allow optimal growth (Allen, 1968; Kotai, 1972) as recommended by the respective culture collections. Cyanobacteria were cultured under standardized conditions in a sterile environment. Cultures were incubated in 6L volumetric flasks at 20±1°C under constant light conditions (14 µmol photon/m²/s) with gentle aeration and allowed to grow until mid-log phase, which had been assessed for each species during the optimization of the culture growth. Afterwards, cultures were concentrated by centrifugation and cleaned by resuspension and centrifugation using COMBO medium three times before use. Density of the cultures was determined with a counting chamber. In addition, the dry weight was determined on a given subsample of 2 mL of the concentrated culture. This subsample was dried in an oven for 24 hours at 40°C and then the weight of the completely dried out mass was determined and converted to a ratio of dry weight per mL. Based on this ratio, animals were fed with the concentrated culture in the experimental treatments. Additionally cyanobacterial cultures were set up under the same standard conditions to determine the fatty acid methyl ester (FAME) profile for each cyanobacterium. The green algae mixture of *P. subcapitata* and *C. reinhardtii* served as a reference sample. All cell cultures, cyanobacterial cultures and green algal cultures, were centrifuged until no more water could be poured off, resulting in a thick paste. Fatty acid composition of the cyanobacteria was determined as described in De Schamphelaere et al. (2007). Briefly, the samples were dried and subjected to direct acid catalyzed transesterification according to the procedure of Lepage and Roy (1984). FAME were extracted with hexane and were prepared for injection in the gas-chromatograph after evaporation of the solvent. Preparation for injection consisted of dissolution of the sample in iso-octane. Quantitative determination was obtained through a Chrompack CP9001 gas-chromatograph with a polar capillary column, BPX70 (SGE, Australia) and a flame ionization detection method.

Daphnia are non-selective filter feeders and will therefore ingest any particle ranging from 1 to 50 µm, occasionally even 70 µm (Ebert, 2005). Therefore, cyanobacterial cells were measured during microscopic observation to ensure their size did not exclude them from being a food source to *Daphnia*.

Table 2.1 List of cyanobacterial strains obtained from different culture collections (CC) with their respective identification number (ID), culture medium (CM), cell length or diameter and smallest trichome length measured if applicable. Full composition of culture media can be found in Appendix A Tables A.1, A.2 and A.3.

Species	CC	ID	CM[b]	Cell/Trichome length	Toxin production
Anabaena lemmermannii	SCCAP[a]	K-0599	Z8	3 µm / 35 µm	Anatoxin-a(s)[e]
Aphanizonmenon sp.	CICCM[a]	CAWBG01	$BG11_0$	12 µm / 24 µm	Saxitoxin[f]
Cylindrospermopsis raciborskii	UTEX[a]	LB 2897	Z8	8 µm / 38 µm	Cylindrospermopsin
Microcystis aeruginosa	PCC[a]	PCC7806	$BG11_0$	4 µm / NA[c]	Microcystin[g]
Nodularia sp.	PCC	PCC7804	$BG11_0$	4 µm / 31 µm	Nodularin[h]
Oscillatoria sp.	PCC	PCC6412	BG11	12 µm / 32 µm	Anatoxin-a[i]

[a] Scandinavion Culture Collection for Algae and Protozoa (SSCAP) Cawthorn Institute Culture Collection of Microalgae (CICCM), Pasteur Culture Collection (PCC), University of Texas (UTEX).

[b] Culture media were composed according to Allen (1968) and Kotai (1972)

[c] Not applicable as *M. aeruginosa* does not form trichomes

[e] Henriksen et al., 1997.

[f] Mahmood and Carmichael, 1986.

[g] Pearson et al., 2004.

[h] Beattie et al., 2000.

[i] Aráoz et al., 2005.

2.2.2 Experimental design

The experiment consisted of six treatments for each cyanobacterium. In these treatments, the diet of the animals consisted of 0% (control), 5%, 10%, 20%, 40% and 80% of cyanobacteria cell suspension on a dry weight basis. In all treatments, diets were supplemented with the same green algae mixture as used in the culturing conditions to obtain a final feeding concentration of 2 mg dry weight L^{-1} (i.e. 100% (control), 95%, 80%, 60% and 20% of green algae cell suspension). To investigate possible effects of starvation one additional treatment was added in which only green algae served as the food source and no cyanobacteria were included (i.e. 100%, 95%, 80%, 60% and 20% green algae). Possible starvation effects could be attributed to the morphology of the cyanobacteria and as a consequence potential inhibition of the feeding apparatus of the daphnids and the nutritional quality of the cyanobacteria. All treatments consisted of four individuals, placed in separate experimental vessels, hereafter referred to as four replicates. The entire experiment lasted for 21 days and was repeated twice, hereafter referred to as cyanobacteria/starvation 1 or 2. During the experiments, animals were monitored daily for reproduction and survival. Medium was renewed three times a week while pH was simultaneously monitored and varied at most 0.2 units from control treatments.

2.2.3 Data treatment and statistical analyses

All data analysis was conducted with the statistical software R (R Development Core Team, 2011, version 3.0.1). The response variable was the total reproduction after 21 days for each surviving organism, a standard parameter in toxicity testing in risk assessment (OECD, 2008) and identified as the most sensitive endpoint for exposure to *Microcystis* (Lürling and Beekman, 2006). Concentration response curves were fitted to each dataset (i.e. all response data for one of the cyanobacteria) with the *drc* package (Ritz and Streibig, 2005, version 2.3-7). For each cyanobacterium, two concentration response curves were modelled as the experiment was conducted twice. The log logistic function (Finney, 1971) was chosen as it provided the best fit overall. It was characterized by three parameters, i.e. the maximum response k, the median effect concentration EC_{50} and the slope s:

$$y = \frac{k}{(1+(\frac{x}{EC_{50}})^{s})} \qquad \text{(eq. 2.1)}$$

with:

y = response of measured endpoint (here: total reproduction)

k = response of measured endpoint at x = 0

s = slope parameter

x = concentration

EC_{50} = median effect concentration, resulting in a decline of 50% of the response variable relative to control treatment

Equation 2.1 is a simplification of the original log logistic function which was defined as follows:

$$y = \frac{k}{(1+e^{s*\log(x-EC_{50})})} \qquad \text{(eq 2.2)}$$

The parameters in eq. 2.1 are estimated using non-linear least squares which uses the function optim in R, a general purpose optimization based on gradient algorithms (Ritz and Streibig, 2005). Kruskal-Wallis Rank Sum tests were conducted to determine significant differences between concentration response curves (Hollander and Wolfe, 1973). Therefore, the three parameters of the concentration

response curves (eq. 2.1) were compared across the responses to different cyanobacteria. This non parametric alternative was chosen due to a violation of assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene test) (Fox, 2008; Royston, 1982), that are generally required for parametric statistics.

2.3 Results

Exposures to the different cyanobacteria resulted in a decline of the total reproduction with increasing amount of cyanobacteria in the diet (Appendix A Fig. A.1). Similarly, decreasing the amount of green algae or increased starvation of the *Daphnia* also resulted in an increased decline of total reproduction in the starvation treatment (Appendix A Fig. A.1). No significant differences were observed for any of the three parameters, modelling the response of total reproduction of all four replicates in all six treatments in the two repeated experiments, between the different exposures (Table 2.2, p_k=0.08, p_{EC50}=0.69, p_s=0.53). Fitted concentration response curves as well as the raw data are represented in Appendix A Fig A.1. The median effect concentrations varied between 30% and 50% of the total diet for cyanobacterial treatments as well as the starvation treatment, which means that at this range in the diet animals reproduced approximately half the amount of offspring compared to a diet containing 100% of green algae (Table 2.2). The lack of significance for the different values of the maximum response k is not surprising as it refers to the maximum reproduction under control conditions, i.e. 100% of green algae in the diet, which should be consisted across all experiments. The lack of significant differences for the values of the slope parameter s indicates that the decline in reproduction with decreasing concentrations of green algae in the diet is comparable across all experiments.

Table 2.2 Estimated value and standard error of each parameter in equation 2.1, i.e. maximum response *k*, median effect concentration (EC_{50}) and slope parameter *s*, for the concentration response data represent per repeated experiment labelled 1 or 2 for each cyanobacteria. Each repeated experiment consisted of six concentrations and each concentration consisted of four replicates.

	Maximum response k	EC50 (% of diet)	Slope parameter s
Anabaena 1	33.6 ± 2.98	52.5 ± 12.8	2.14 ± 1.29
Anabaena 2	25.3 ± 2.15	31.5 ± 9.86	0.89 ± 0.25
Aphanizomenon 1	32.1 ± 1.31	48.6 ± 5.70	1.42 ± 0.28
Aphanizomenon 2	36.2 ± 2.13	36.4 ± 6.38	1.18 ± 0.27
Cylindrospermopsis 1	37.7 ± 1.90	32.9 ± 4.20	1.78 ± 0.37
Cylindrospermopsis 2	33.2 ± 1.08	31.7 ± 3.31	1.10 ± 0.12

(Table 2.2 cont.)	Maximum response k	EC50 (% of diet)	Slope parameter s
Microcystis 1	29.4 ± 0.81	42.3 ± 2.73	3.09 ± 0.51
Microcystis 2	38.7 ± 1.37	43.3 ± 3.57	3.51 ± 1.11
Nodularia 1	31.7 ± 2.22	31.6 ± 5.41	2.12 ± 0.62
Nodularia 2	29.1 ± 1.29	42.7 ± 4.58	1.93 ± 0.44
Oscillatoria 1	33.4 ± 1.23	38.3 ± 3.16	2.52 ± 0.53
Oscillatoria 2	33.8 ± 1.49	31.9 ± 3.39	2.14 ± 0.40
Starvation 1	30.7 ± 1.12	46.4 ± 5.47	6.01 ± 4.59
Starvation 2	36.2 ± 1.38	49.2 ± 3.87	4.55 ± 1.49

No effects on survival time were observed as even in the treatments with the lowest percentage of green algae in the diet, animals survived (Fig. 2.1). This indicates no effect on survival of *Daphnia pulex* even when cyanobacteria make up the majority of the diet.

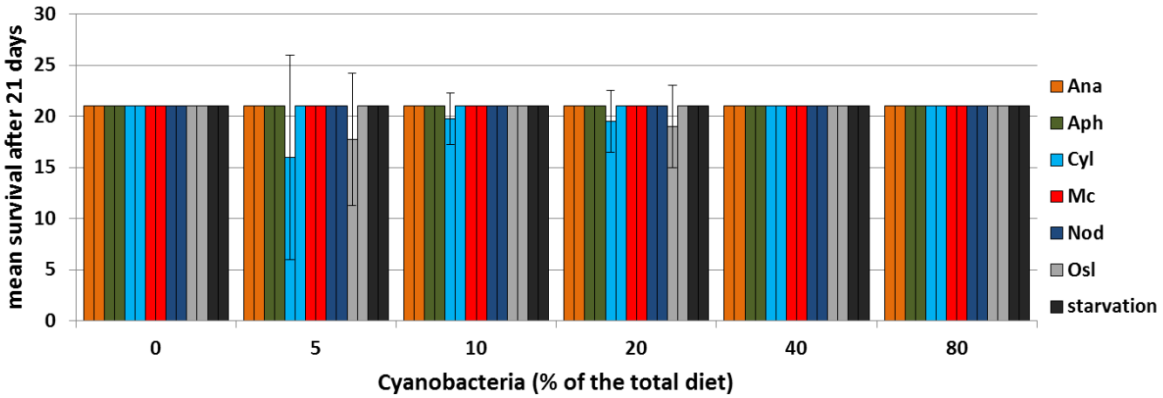

Figure 2.1 Mean survival time in the 21-day life history experiment. Two identically coloured bars represent the repeated experiments 1 and 2. Error bars indicate the standard deviation within each repeated experiment. When no error bars are plotted, all replicate animals in that treatment survived. Ana = *Anabaena*, Aph = *Aphanizomenon*, Cyl = *Cylindrospermopsis*, Mc = *Microcystis*, Nod = *Nodularia*, Osl = *Oscillatoria*.

The effects on reproduction could be attributed to a reduced number of clutches for organisms exposed to the lowest percentage of green algae as well as an increase in the time to the first clutch (Fig. 2.2). Both observations were only made for the lowest percentage of green algae as for high percentages of green algae, i.e. 5 to 20%, little difference could be observed between the treatment and the control. The size of the first clutch in treatments with lower percentages of green algae was also smaller (Fig 2.2).

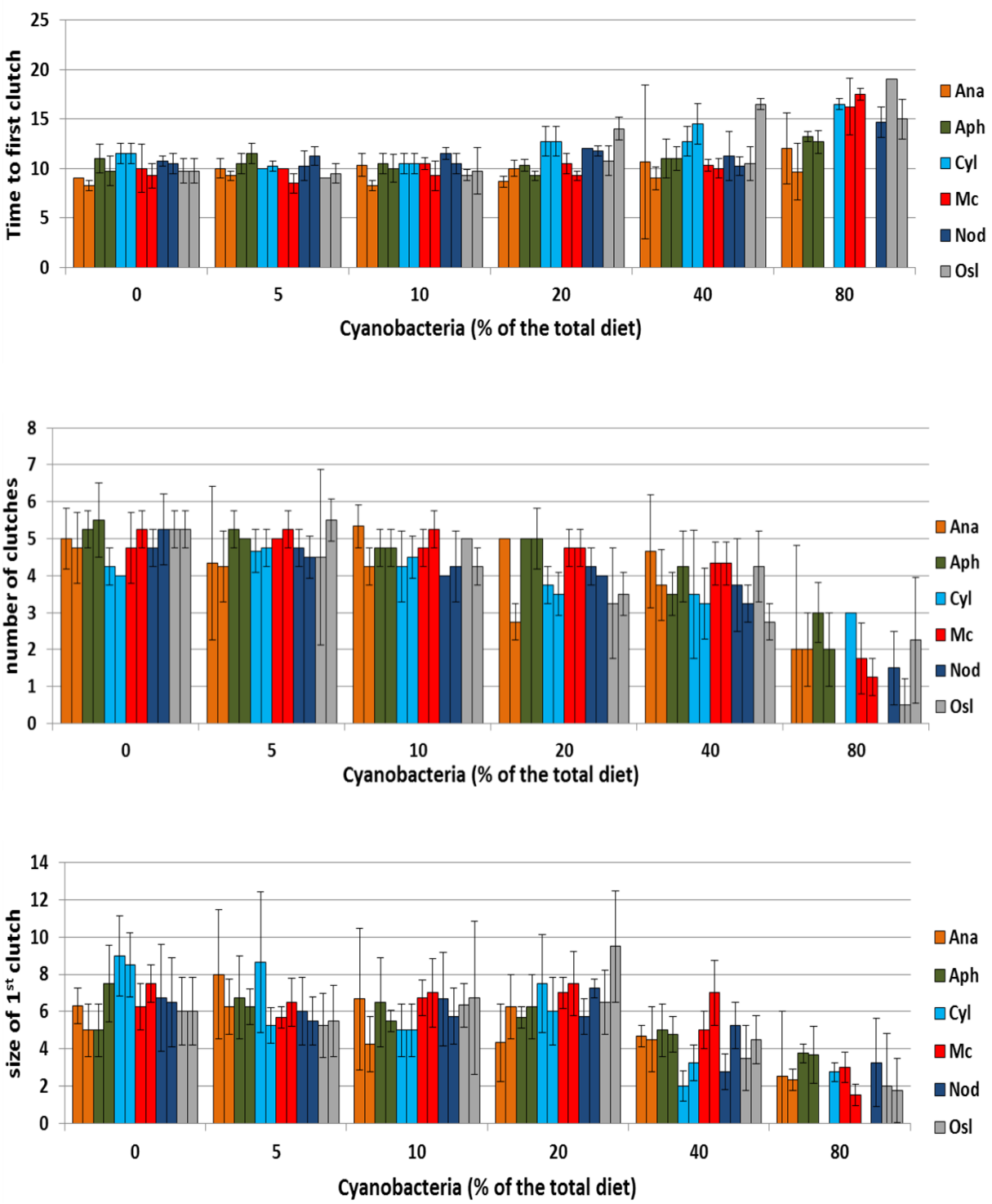

Figure 2.2 Time first clutch (top), number of clutches (middle) and size of 1st clutch (bottom) in the 21-day life history experiment. Two identically coloured bars represent the repeated experiments 1 and 2. Error bars indicate the standard deviation within each repeated experiment. When no error bars are plotted, the same value has been reported for all replicate animals in that treatment for that parameter. Ana = *Anabaena*, Aph = *Aphanizomenon*, Cyl = *Cylindrospermopsis*, Mc = *Microcystis*, Nod = *Nodularia*, Osl = *Oscillatoria*.

The fatty acid methyl ester profiles of all cell suspensions can be visually distinguished from one another (Appendix A Fig. A.3.). The total FAME content is in line with results from Piorreck et al. (1984), who also observed about three times more fatty acid methyl esters in green algae than in various cyanobacteria including *Microcystis aeruginosa* and *Oscillatoria.* Furthermore, the content of the specific fatty acids (Appendix A Fig. A.3) is line with values reported by Gugger et al. (2002) who studied cellular fatty acid in six genera of cyanobacteria, including *Anabaena, Aphanizomenon, Microcystis* and *Nostoc*. This could also be seen from the total omega-3 and omega-6 fatty acid content (Fig. 2.1). The total omega-3 content is five to ten times higher in green algae compared to cyanobacteria, excluding *Anabaena.* The omega-3 content in *Anabaena* is 1.5 to five times higher than that of other cyanobacteria, but still only half of the omega-3 content in green algae. No clear visual distinctions could be made between cyanobacterial FAME profiles on one hand and the green algae profile on the other hand. However, the total FAME content in green algae cultures was at least 1.5 times higher than the total FAME content of the cyanobacterial cultures.

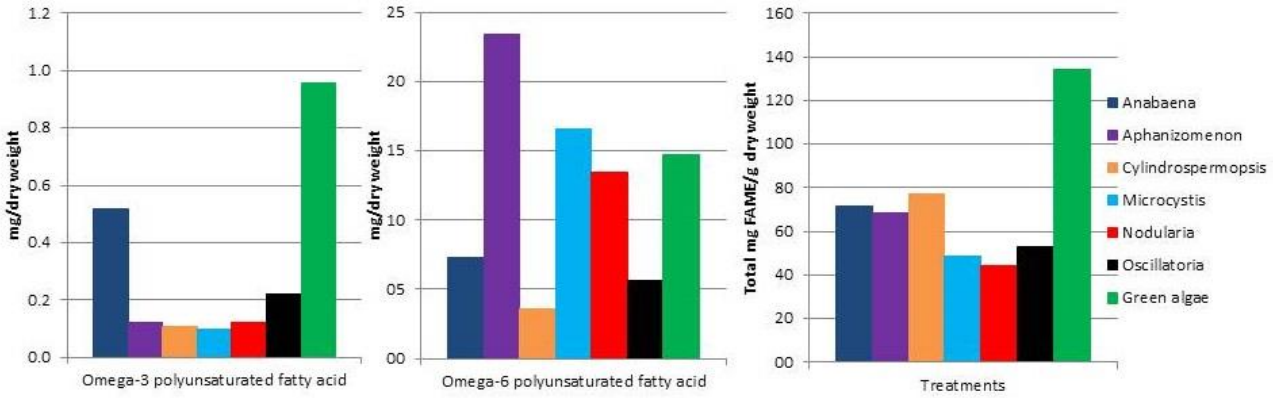

Figure 2.3 Bar chart of the total omega-3 and omega-6 poly unsatured fatty acid content for each of the food suspensions as well as the total fatty acid methyl ester (FAME) content.

2.4 Discussion

The results indicate overall negative effects of cyanobacteria on the reproduction of *Daphnia pulex*, even when cyanobacteria make up less than 50% of the diet, i.e. the median effect concentration varied between 30-50% of cyanobacteria in the diet. Furthermore, no clear difference between effects of different cyanobacteria on reproduction of *D. pulex* could be demonstrated. These findings are in line with conclusions made by Wilson et al. (2006) and Tillmanns et al. (2008) which found no evidence that toxin production would serve as a primary driver affecting zooplankton. More

importantly, in contrast to previous studies, current results did not demonstrate a significant bias towards *Microcystis aeruginosa* but covered a broader range of cyanobacterial species.

The lack of significant differences could be attributed to a lack of repeatability of the experiments given that the variation between the cyanobacteria is comparable to the variation within repeated experiments for some median effect concentrations and some slope parameters. However, this may also be a consequence of the selected diet ratios that were tested. Indeed, if the actual median effect concentration lies between two tested concentrations, the standard error on the median effect concentration increases with the size of the interval. Given that the median effect concentration is estimated between 30-50% and the tested concentrations were 20, 40 and 80%, the interval may not have been optimally chosen to estimate this parameter. To exclude the lack of repeatability, Wilcoxon Rank Sum tests were conducted to compare individual diet ratios across the different experiments, rather than estimating the dose response. No significant differences were observed between the effects at 20% of cyanobacteria in the diet ($p=0.13$) nor at 80% of cyanobacteria in the diet ($p=0.14$) in the different experiments. However, significant differences were observed at a concentration of 40% in the diet ($p=0.005$). This suggests that the lack of significant differences is not due to a lack of repeatability but rather that significant differences are only observed in a narrow range of the diet ratio around the 40% value.

The overall effects could be attributed to a general starvation effect given the lack of significant differences between the starvation concentration response data and any of the cyanobacteria concentration response data for the full concentration response curve as well as for the majority of the tested concentrations. These results suggest therefore common shared characteristics across the different cyanobacteria resulting in a similar concentration response curve related to nutritional quality. Furthermore, it underlines that, regardless of the potential of other mechanisms of toxicity of cyanobacteria, the lack of good quality food drives the effect on *D. pulex* at the organismal level for the majority of the diet ratios. Further mechanistic research is needed to assess the driving factors of significant differences around the 40% diet range. The analysis of the FAME profiles revealed a large difference in total FAME content between cyanobacteria cultures on one hand and green algae on the other hand which may have a significant impact on their nutritional quality for zooplankton. The key factors for good nutritional quality in cladocera such as *Daphnia* are among others the total content of omega-3 polyunsaturated fatty acids in the food (Von Elert, 2004). The omega-3 content was also

different in green algae compared to cyanobacteria although *Anabaena* seems to be in-between the green algae and the other cyanobacteria in terms of total omega-3 content. Furthermore, alpha-linoleic acid (18:3(n-3)) is often referred to as one of the crucial omega-3 amino acids to influence growth (Von Elert, 2004). Yet, *Anabaena* and *Cylindrospermopsis* contained at least 1.5 times as much alpha-linoleic acid than green algae (Appendix A Fig. A.3). Despite the differences in fatty acid content and omega-3 content, these results are thus not in line with the general knowledge concerning the specific fatty acids limiting *Daphnia* growth. Here, a molecular analysis of the mechanisms affected by cyanobacteria in *D. pulex* would be crucial to investigate how and whether nutritional quality is the only mechanism driving cyanobacterial toxicity.

From a risk assessment perspective, these results do not support the use of cyanobacterial toxins in regulations to protect zooplankton as they do not seem to drive detrimental effects on zooplankton species in contrast to effects on human health or livestock. The use of cyanobacterial concentrations rather than toxin concentrations may be a more suitable alternative or added value in particular given the lack of differences for the majority of the concentrations and the significant differences around the 40% diet ratio. In addition, the nutritional quality of cyanobacteria relatively to green algae could be used to indicate the lack of nutrition in terms of cyanobacteria concentrations.

These results also seem to suggest that a general risk assessment for all cyanobacteria could be drafted as the concentration response curves do not differ significantly between different cyanobacteria. However, *D. pulex* is but a single species and effects are known to vary considerably between different zooplankton species (DeMott et al., 1991). Tillmans et al. (2008) did observe significant differences between the effects of different cyanobacteria on *D. magna*. Pattinson et al. (2003) observed that *Daphnia lumholtzi* was less susceptible to cyanobacterial stress than *Daphnia parvula* and *Daphnia mendotae*. As a consequence, results should not be straightforwardly extrapolated from one species to another but rather be used in a multiple species approach to fully incorporate these variations.

2.5 Conclusion

The current chapter highlights that concentration response curves of *Daphnia pulex* exposed to different cyanobacteria remained conserved across the six studied cyanobacteria. This indicates that cyanobacterial toxins are unlikely to drive adverse effects on zooplankton. Given the overall lack of significant differences between cyanobacterial treatments and starvation treatments, the effects on the fitness of *Daphnia pulex* were most likely driven by common lack of nutritional quality across all cyanobacteria. Nevertheless significant differences were observed at a concentration of 40% cyanobacteria in the diet. Further mechanistic research within this concentration range is however needed to underpin the cause of these differences. Fatty acid methyl ester profiles revealed differences in total FAME content and omega-3 content but were in contrast with general expectations in literature.

Adverse effects on *Daphnia pulex* depended upon the concentration of cyanobacteria in the diet. This underlines the importance of including cyanobacteria concentrations in risk assessments to sufficiently protect zooplankton species. A proposed approach consists of using cyanobacterial concentration response curves across multiple zooplankton species to also incorporate potential interspecies variation.

Chapter 3

Combined and interaction effects of cyanobacteria and insecticides across full concentration responses

Redrafted after:

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Jana Asselman, Colin R Janssen, Guy Smagghe, Karel AC De Schamphelaere. 2014. Ecotoxicity of binary mixtures of *Microcystis aeruginosa* and insecticides to *Daphnia pulex*. Environmental Pollution 188, 56–63.

3.1 Introduction

Over the last decade, significant progress has been made in environmental risk assessment. The development of structural frameworks addressing combined effects has brought risk assessment closer to environmental reality (Altenburger et al., 2013). Yet, several obstacles still need to be addressed.

First, the consequences of interaction effects between biotic and chemical stressors remain largely unknown as regulatory research efforts stay focused on combinations of chemicals. Pioneer studies have been reported in section 1.1. However, as discussed in section 1.1, these studies address interaction effects between specifically selected stressors widely scattered across the research landscape and are mainly focused on abiotic stressors (Holmstrup et al. 2010).

Second, statistical methods to evaluate combined and interaction effects vary considerably between studies. Most commonly used models are the independent action model (Bliss independence) and the concentration addition model (Loewe additivity) (Bliss, 1939; Loewe, 1928). Some studies (Cerbin et al., 2010; De Coninck et al., 2013b) adhere to general ANOVA principles, which is mathematically equivalent to independent action after log transformation of the data (De Coninck et al., 2013b). At present, neither of the two reference models can be unequivocally selected as the best model (Backhaus et al., 2004; Cedergreen et al., 2008; Jonker et al., 2005).

Third, current statistical models can quite accurately predict combined effects when no interactions are present. However, as highlighted in chapter 1 (section 1.1.2) significant deviations such as synergisms and antagonisms cannot be predicted and can only be assessed by conducting full concentration response experiments of the mixtures. Given the scale and time of these types of experiments, it is impossible to conduct them on all possible stressor combinations. Hence, alternative approaches are needed.

Here, *Daphnia pulex* was exposed for 21 days to binary combinations of *Microcystis* and five insecticides (carbaryl, chlorpyrifos, fenoxycarb, tebufenpyrad and tetradifon) and binary combinations of carbaryl and four cyanobacteria (*Aphanizomenon*, *Cylindrospermopsis*, *Microcystis* and *Oscillatoria*). The aim of this chapter was therefore threefold. First, statistical models were applied on this selection of binary combinations to gain crucial knowledge concerning these specific groups of

toxicants. Here, insecticides and cyanobacteria were chosen due to their individual significant impact on aquatic ecosystems as well as their potential interaction effects as discussed in chapter 1 (section 1.2.4). Due to experimental constraints, one insecticide and one cyanobacterium were selected: carbaryl, an acetylcholine esterase inhibiting insecticide, and *Microcystis aeruginosa*, a cyanobacterium. Acetylcholine esterase inhibitors in general and carbaryl in particular are among the most extensively used pesticides and have been detected in freshwater environments (Hapeman et al., 2002; Murray et al., 2010). *Microcystis aeruginosa* and its potential effects on the aquatic system and *Daphnia* species in particular have been extensively studied (Lürling, 2003; Rohrlack et al., 1999). Second, experimental data was analyzed with both independent action (IA) and concentration addition (CA) to allow extensive and consistent comparison between the reference models. Third, carbaryl was combined with several cyanobacteria, including *Microcystis*, and *Microcystis* was combined with several pesticides, including carbaryl. Insecticides spanned a variety of modes of actions (section 1.5.2) and cyanobacteria differed in characteristics (section 1.5.1). Thus, two hypotheses can be tested: interaction effects of carbaryl and cyanobacteria are comparable between different cyanobacteria and interaction effects of *Microcystis* and pesticides are comparable between pesticides with similar modes of action and different between pesticides with dissimilar modes of action. Comparable interaction effects between different cyanobacteria combined with the same pesticide are expected given the similar concentration response curves reported in chapter 2. Overall, it was anticipated that this selection would allow an enhanced understanding of the mechanisms of interaction effects across a defined group of stressors.

3.2 Materials and methods

3.2.1 Experimental organisms and cyanobacteria

The experimental organisms originated from *D. pulex* cultures as described in section 2.2.1. Likewise, cyanobacteria culture conditions were also described in section 2.2.1.

3.2.2 Experimental design

Chronic toxicity experiments were conducted based on the central composite design (Fig. B.1), commonly used in evaluating and assessing mixture data and which allows optimal evaluation of both the independent action as well as the concentration addition model (Jonker et al., 2005; Lock and

Janssen, 2002), with the addition of five points for each of the two single stressors (Fig. 3.1, 3.2). This allowed simultaneous evaluation of the effects of the individual stressors and the mixtures as recommended by De Laender et al (2009). Each design point was replicated three times, i.e. three daphnids per treatment, each in an individual vessel. A solvent control (0.016% of pure ethanol) was added to exclude potential effects of the solvent in which insecticides were dissolved. Control and solvent control were replicated five times. Concentration ranges of the insecticide only treatments were determined based upon literature review and preliminary experiments with the single compounds. They were selected in such a manner to cover the range of different EC_{50}s (effect concentration causing a 50% decline in reproduction) reported in literature and observed in preliminary experiments. For each of the cyanobacteria, the concentrations were given as a percentage between 5% and 80% of the diet (on a dry weight per liter basis) supplemented with a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* (3:1 cell based ratio) to a final target concentration of 2 mg dry weight L^{-1}. This was based upon the results in chapter 2 (section 2.3).

Animals were exposed for a period of 21 days. The experiment was conducted in 25 mL glass vessels with one neonate per vessel. All animals were fed daily with a final feeding concentration of 2 mg dry weight L^{-1} of algae suspension. In each treatment, animals were monitored daily for survival and reproduction. If the adult reproduced, neonates were counted and removed from the vessel. The medium was renewed three times per week.

During all experiments, pH of old and new media was monitored at regular intervals and varied at most 0.2 units from control treatments (pH=7.00± 0.2). In addition, samples of the medium and stock solutions were taken for insecticide concentration analysis.

3.2.3 Chemical stock solutions and analysis

All insecticides were obtained from Sigma-Aldrich (Bornem, Belgium) in pure powder form, analytical grade (i.e. purity ≥99%). Due to low water solubility of the compounds, stock solutions were made by dissolving the insecticides in pure ethanol (analytical grade, Sigma-Aldrich, Bornem, Belgium).

All samples were stored in the dark at -20°C in glass tubes to prevent degradation until analysis with gas chromatography – mass spectrometry (GC-MS: Trace GC 2000 series, Thermoquest; Polaris, Finnigan/Thermoquest). For all insecticides, an apolar SLBTM-5ms column (Supelco, Sigma-Aldrich)

was used. Extraction and elution were performed by Solid Phase Extraction according to the manufacturer's notes (Waters and Phenomenex). Finally, the insecticide was eluted with tert-methyl-butyl-ether (MTBE) (Sigma Aldrich, purity ≥ 99.9%). During the procedure, recipients and glassware were rinsed with MTBE to avoid retention of insecticide residues on the glass or column wall. For each insecticide, a separate internal standard was used to control and correct for losses during the extraction and elution procedures (Appendix B.2). To control for the injection itself, a recovery standard was added after the solid phase extraction (Appendix B.2). For each solid phase extraction, a blank (no insecticide) and a spike (a given concentration of insecticide added to control medium from a certified solution) were added to the analysis. Quality criteria for blank and spike were no detection of the insecticide and more than 90% detection of the added amount of insecticide, respectively. Across all insecticides, recovery of the spike was always between 90 and 115%. Based on OECD guideline 211 (OECD, 2008), the time weighted means of measured insecticide concentrations were used for all further data analysis. All subsequent figures and tables therefore use time weighted means of measured insecticide concentrations and not nominal concentrations (Fig. 3.1-3.2).

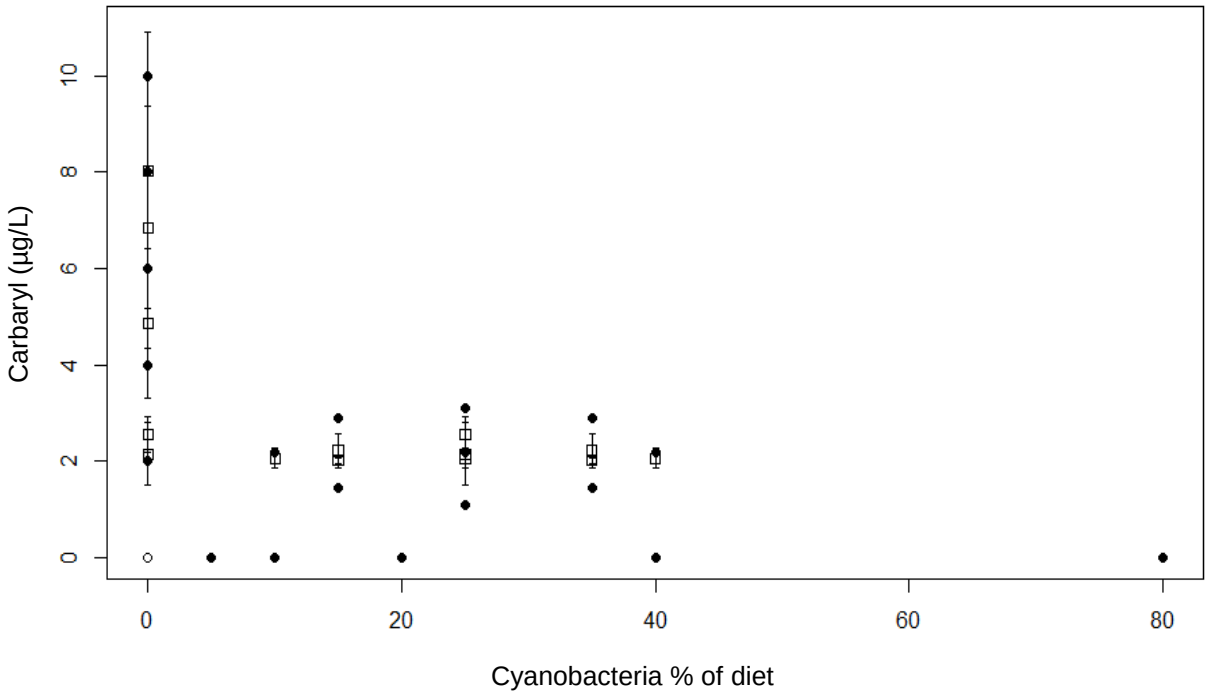


Figure 3.1 Experimental designs for the binary mixture combinations with carbaryl: Nominal concentrations are represented by filled circles, measured concentrations are represented by open squares with error bars representing standard deviation. Control treatment is represented by an open circle.

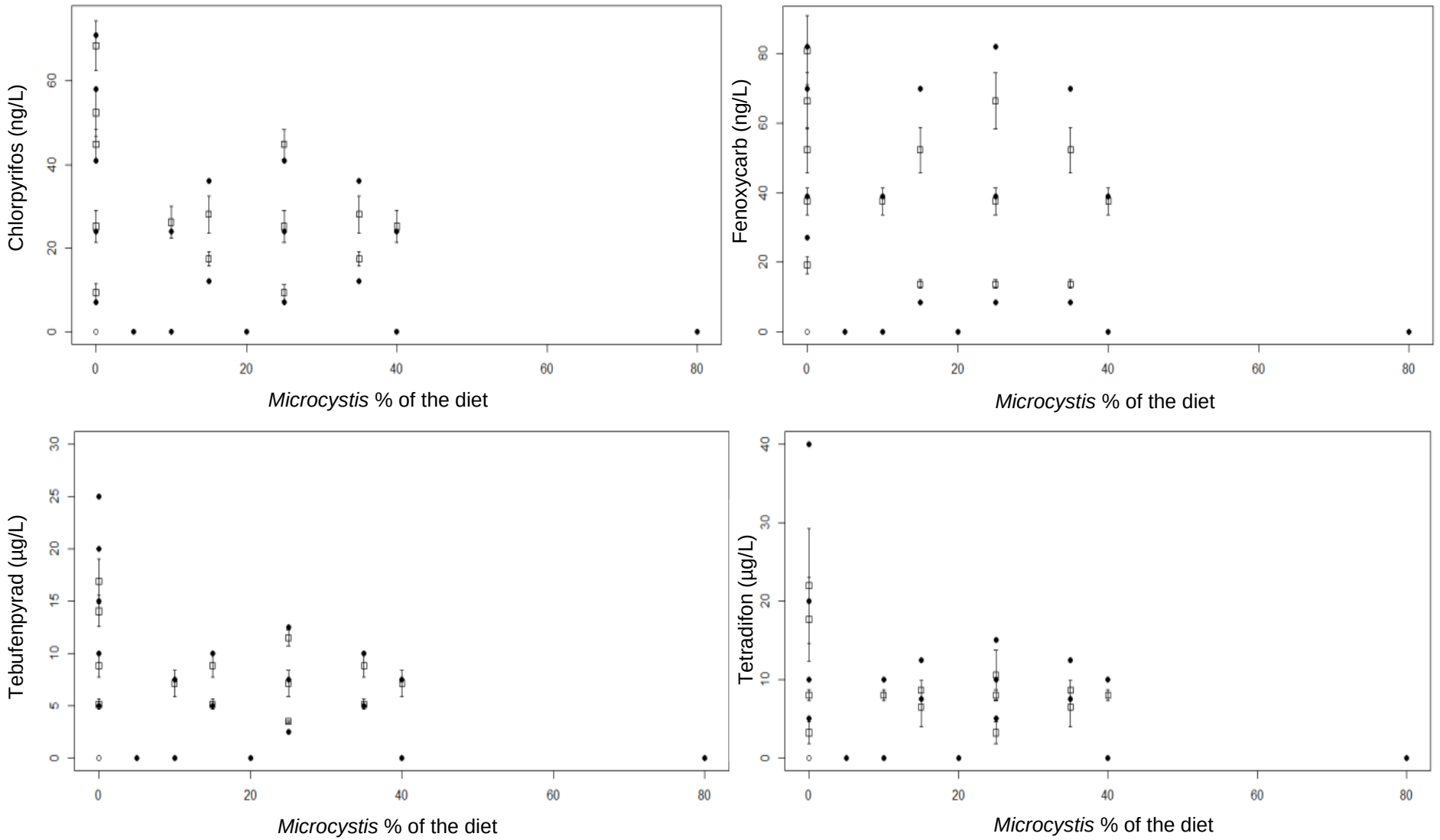


Figure 3.2 Experimental designs for the binary mixture combinations with *Microcystis*: Nominal concentrations are represented by filled circles, measured concentrations are represented by open squares with error bars representing standard deviation. Control treatment is represented by an open circle.

3.2.4 Statistical analysis

The chosen response variable to determine combined and interaction effects was the total number of offspring per surviving female at the end of the experiment. Females that did not survive the entire 21 day period were excluded from the analysis. In tebufenpyrad and tetradifon treatments, animals in the highest concentration, i.e. 25 µg/L and 40 µg/L, all died within the first week. Therefore, these concentrations were not taken into account in data analysis or in the concentration measurements. First, results from control treatments and solvent control treatments were analyzed and compared to exclude solvent effects. Comparison was done using a t-test after verifying assumptions of normality and homoscedasticity by the Shapiro-Wilk test (Royston, 1982) and Levene test (Fox, 2008), respectively.

The analysis of the binary mixture experiments was conducted with an in-house developed R-code (R Development Core Team, 2008, version 3.0.1) (Appendix B.3). This code evaluates mixture experiments with both the concentration addition and the independent action reference models based on Jonker et al. (2005). It uses the log logistic concentration response function (eq. 2.1) and subsequent derived equations for both concentration addition (eq. 3.1) and independent action (eq. 3.2) reference models as described by Jonker et al. (2005) and listed in the R-code (Appendix B.3)

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 and this is expressed as follows:

$$\frac{x_1}{{f_1}^{-1}(y)} + \frac{x_2}{{f_2}^{-1}(y)} = \exp(G) \quad \text{(eq. 3.1)}$$

Here, *x* is the concentration of the stressor and indexes 1 and 2 denote stressors 1 and 2 in the mixture. *Y* is the response variable and f^{1} denotes the inverse of the response function, i.e. the inverse of eq. 2.1. *G* refers to the deviation function (eq. 3.3, as defined in Jonker et al., 2005) and the value of *G* equals 0 under the hypothesis of no interaction.

Under the independent action reference model, the response to stressor 1 is assumed to act independently from stressor 2 and this is mathematically expressed as follows:

$$y = k\phi\left(\Phi^{-1}\left[k\left(\frac{1}{1+(\frac{x_1}{x_{50_1}})^{s_1}}\right)\left(\frac{1}{1+(\frac{x_2}{x_{50_2}})^{s_2}}\right)\right]+G\right) \text{ (eq. 3.2)}$$

In which indexes 1 and 2 again denote stressor 1 and 2 in the mixture and ϕ refers to the cumulative standard normal distribution function. The concentration of each stressor is represented by *x* and x_{50} denotes the median effect concentration, resulting in a decline of 50% of the response variable relative to control treatment. S represents the slope of the response function for either stressor 1 or stressor 2 while k is the response of the endpoint at x=0, i.e. the response of the control. All other parameters are identical to those in eq. 3.1.

The deviation function *G* is defined identically for both reference models and is as follows (Jonker et al., 2005):

$$G = a\left(\frac{\frac{x_1}{x_{50_1}}}{\frac{x_1}{x_{50_1}}+\frac{x_2}{x_{50_2}}}\right)\left(\frac{\frac{x_2}{x_{50_2}}}{\frac{x_1}{x_{50_1}}+\frac{x_2}{x_{50_2}}}\right) \text{ (eq. 3.3)}$$

In which *a* is the parameter that quantifies deviations from the reference model. All other parameters are identical to the ones in equations 3.1 and 3.2 (From Jonker et al., 2005). The more positive the deviation parameter, the more antagonistic the mixture deviates from the reference model. The more negative the deviation parameter, the more synergistic the mixture deviates from the reference model.

The data was analyzed in three steps. During all steps, nonlinear least-squares estimation was used through the nls function in R, using the default Gauss-Newton algorithm. Step one fits the reference model (IA or CA) to the data from individual stressor treatments only and not from the mixture treatments (i.e. eq. 3.1 or 3.2 in which *G*=0, because the parameter *a* is not included in this step and is therefore set to zero in eq.3.3, i.e. no interaction) and uses fitted models to make predictions for the combined treatments. Step two then fits the reference model (IA or CA) to all the data from all individual and all mixture stressors treatments (i.e. eq. 3.1 or 3.2 in which *G*=0 because the parameter *a* is not included in the model yet and is therefore set to zero in eq. 3.3, i.e. still no interaction). In step three the reference model (IA or CA) is extended with the deviation parameter *a* to quantify deviations from non-interaction (i.e. eq. 3.1 or 3.2 in which *G* is not zero anymore as the parameter *a* is added to the model based on eq. 4). The significance of the addition of the deviation parameter to the reference

model is statistically determined through an F-statistic (Jonker et al., 2005). Alternatively the Aikaike Information Criterion (AIC) can be used to give an indication of the best model fit although it is not a true statistical measure. To exclude the possibility that a single design point would affect conclusions, models of steps two and three were subsequently fitted and analyzed by each time leaving out one design point. No design point significantly influenced statistical conclusions as leaving one design point out did not alter the p-value (Appendix B Table B.1).

3.3 Results

Reproduction in control treatments was not significantly different from reproduction in solvent control treatments (p=0.57). Thus an effect of the solvent on the test organism can be excluded. Mortality in control treatments was less than 10% for all experiments. Measured concentrations of insecticides are reported in Fig. 3.1 and Fig. 3.2. The effect concentration for the total reproduction per surviving female resulting in a 50% decline of the total reproduction (EC50) was for all insecticides within the tested concentration range as can be seen from the single dose response data (Appendix B Fig. B.2-B.9) and the model estimates of step 1, using only single stressor data (Table 3.1). Fenoxycarb and chlorpyrifos were the most toxic, both having an EC50 around 70 ng/L whereas tetradifon and tebufenpyrad were significantly less toxic with EC50s around 10 µg/L (Table 3.1). The estimated EC50 for carbaryl (Table 3.2) was comparable between the different experiments, ranging between 1.93 and 6.16 µg/L.

The estimated EC50 for *Microcystis* ranged from 30% to 60% of the total diet between experiments (Table 3.1). In the experiments with fenoxycarb and tetradifon (Appendix B Fig. B.3 and B.5), animals exposed to 80% of *Microcystis* either died or survived without reproducing whereas in experiments with chlorpyrifos and tebufenpyrad (Appendix B Fig. B.2 and B.4), animals exposed to 80% of *Microcystis* were able to reproduce, albeit very little. In the mixture treatments, animals did not survive in the combination of chlorpyrifos and *Microcystis* containing the highest chlorpyrifos concentration (Appendix B Fig. B.2). In the three other combinations, at least one animal survived in each mixture combination (Appendix B Fig. B.3-B.5). However for combinations of carbaryl and *Microcystis* and carbaryl and *Oscillatoria*, animals did not reproduce in the mixture combination with the highest concentration of cyanobacteria (Appendix B Fig. B.8-B.9). Based on the estimated EC50 for each cyanobacterium (Table 3.2), *Microcystis* can be considered the most toxic followed by

Aphanizomenon, whereas *Cylindrospermopsis* and *Oscillatoria* have comparable toxicity and are the least toxic.

A first indication of combined effects of the binary mixtures is represented in Fig. 3.3 (left panel A-D, M-P). Here, predictions of the mixture data were made with the reference models based upon the single stressor data only (step 1). From these predictions, it indicates no clear interaction effects for binary combinations of *Microcystis* and chlorpyrifos, fenoxycarb or tetradifon and carbaryl and *Cylindrospermopsis*. Indeed, in Fig. 3.3 A, B, D and N, both single stressor data points (open circles and triangles) and mixture data points (filled circles and triangles) lie close to the 1:1 line which means that the fitted (single data points) and predicted (mixture points) values closely match the observed values. In contrast, for binary combinations of *Microcystis* and carbaryl or tebufenpyrad (Fig. 3.3 C and O), the mixture points all lie below the 1:1 line and there predicted values are quite different from the observed values. This indicates an antagonistic effect as observed reproduction is larger than reproduction predicted with the CA and IA reference models. The same observations can be made for binary combinations of carbaryl and *Aphanizomenon* or *Oscillatoria* (Fig. 3.3 M and P).

Fitting both the CA and IA reference models to the entire dataset (Fig. 3.3 middle panel E-H, Q-T, i.e. step2), results in similar conclusions as the ones obtained from step1 (i.e. model fit to only the single stressor data), i.e. no clear interactions as all observations lie close to the 1:1 line for binary combinations of *Microcystis* and chlorpyrifos, fenoxycarb or tetradifon and carbaryl and *Cylindrospermopsis* (Fig. 3.3 E, F, H and R). For binary combinations of *Microcystis* and carbaryl or tebufenpyrad and binary combinations of carbaryl and *Aphanizomenon* or *Oscillatoria* (Fig. 3.3 G, Q, S and T), the mixture points now fit better as they lie closer to the 1:1 line but the single points have moved further from the 1:1 line indicating this is not a good model fit. Extending the reference models with the deviation parameter *a* (eq. 3.3) does not improve the results clearly for *Microcystis* and chlorpyrifos or tetradifon and carbaryl and *Cylindrospermopsis* (Fig. 3.3 I, L and V). For the combination of *Microcystis* and fenoxycarb, i.e. Fig. 3.3 J, the fit becomes only slightly better as all points move closer to the 1:1 line. For binary combinations of *Microcystis* and carbaryl or tebufenpyrad and binary combinations of carbaryl and *Aphanizomenon* or *Oscillatoria*, (Fig. 3.3 K, U, W and X) both single and mixture points are now closer to the 1:1 line compared to the previous reference model not containing the deviation parameter (Fig. 3.3 G, Q, S and T).

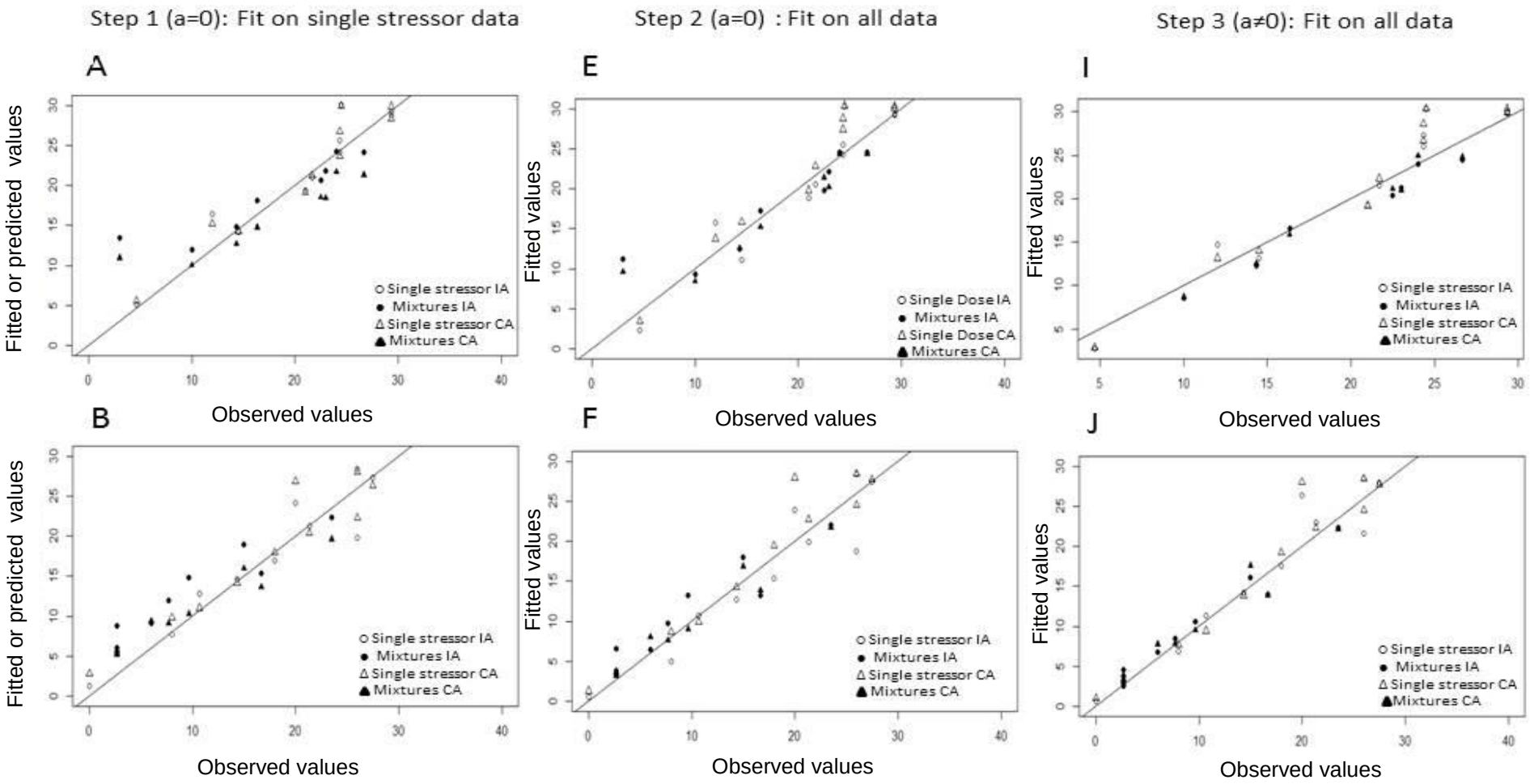


Figure 3.3 Part 1 Mean observed versus fitted values for models for total reproduction for each binary experiment: chlorpyrifos x *Microcystis* (first row: A,E,I), fenoxycarb x *Microcystis* (second row: B,F,J). Circles depict the independent action model fits (equation 3.2), triangles depict the concentration addition model fits (equation 3.1). Open symbols denote the single stressor treatments, full symbols denote the mixed stressor treatments. For all models, the 1:1 line is plotted.

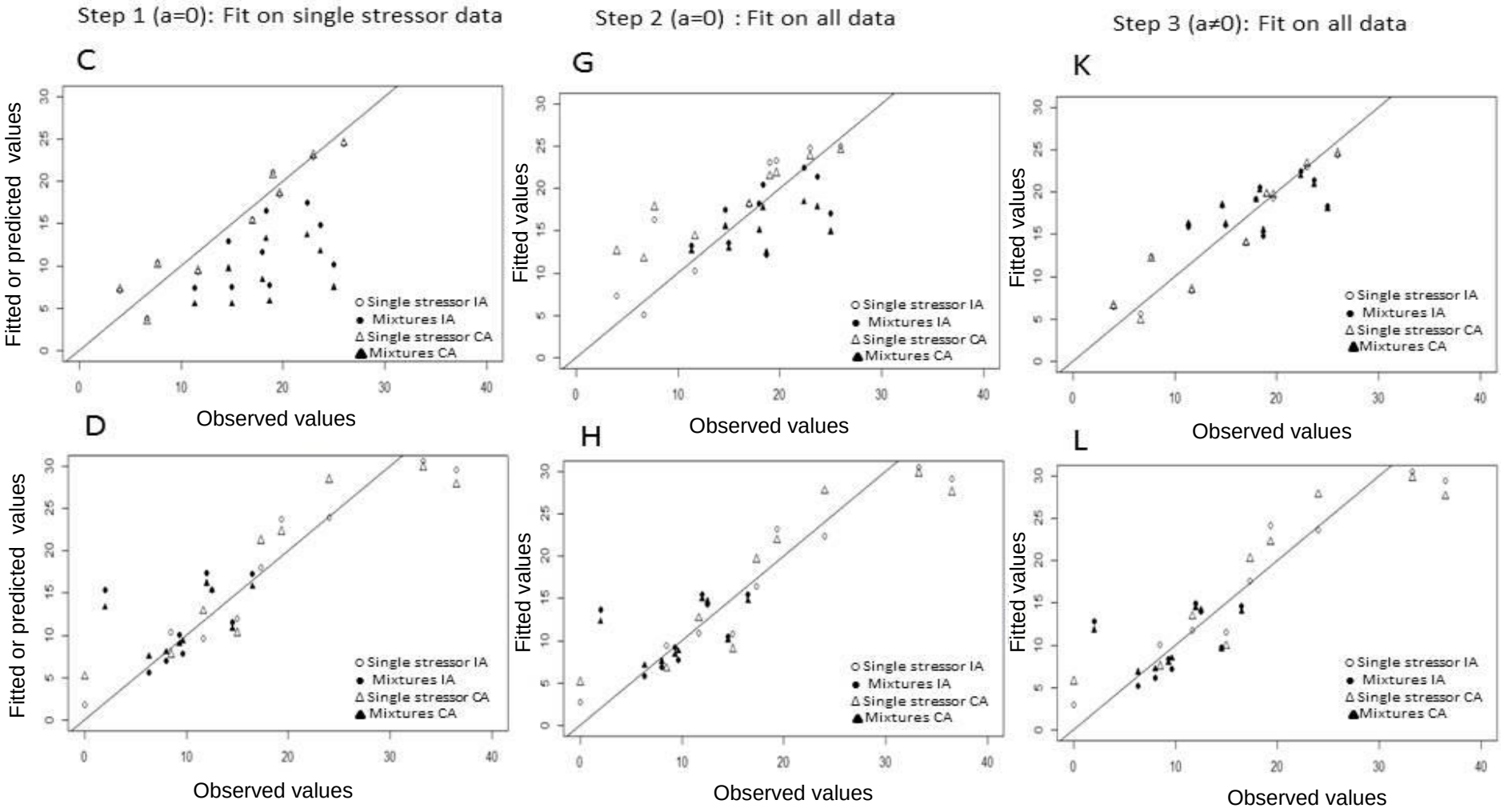


Figure 3.3 Part 2 Mean observed versus fitted values for models for total reproduction for each binary experiment: tebufenpyrad x *Microcystis* (first row: C,G,K), tetradifon x *Microcystis* (second row: D,H,L). Circles depict the independent action model fits (equation 3.2), triangles depict the concentration addition model fits (equation 3.1). Open symbols denote the single stressor treatments, full symbols denote the mixed stressor treatments. For all models, the 1:1 line is plotted.

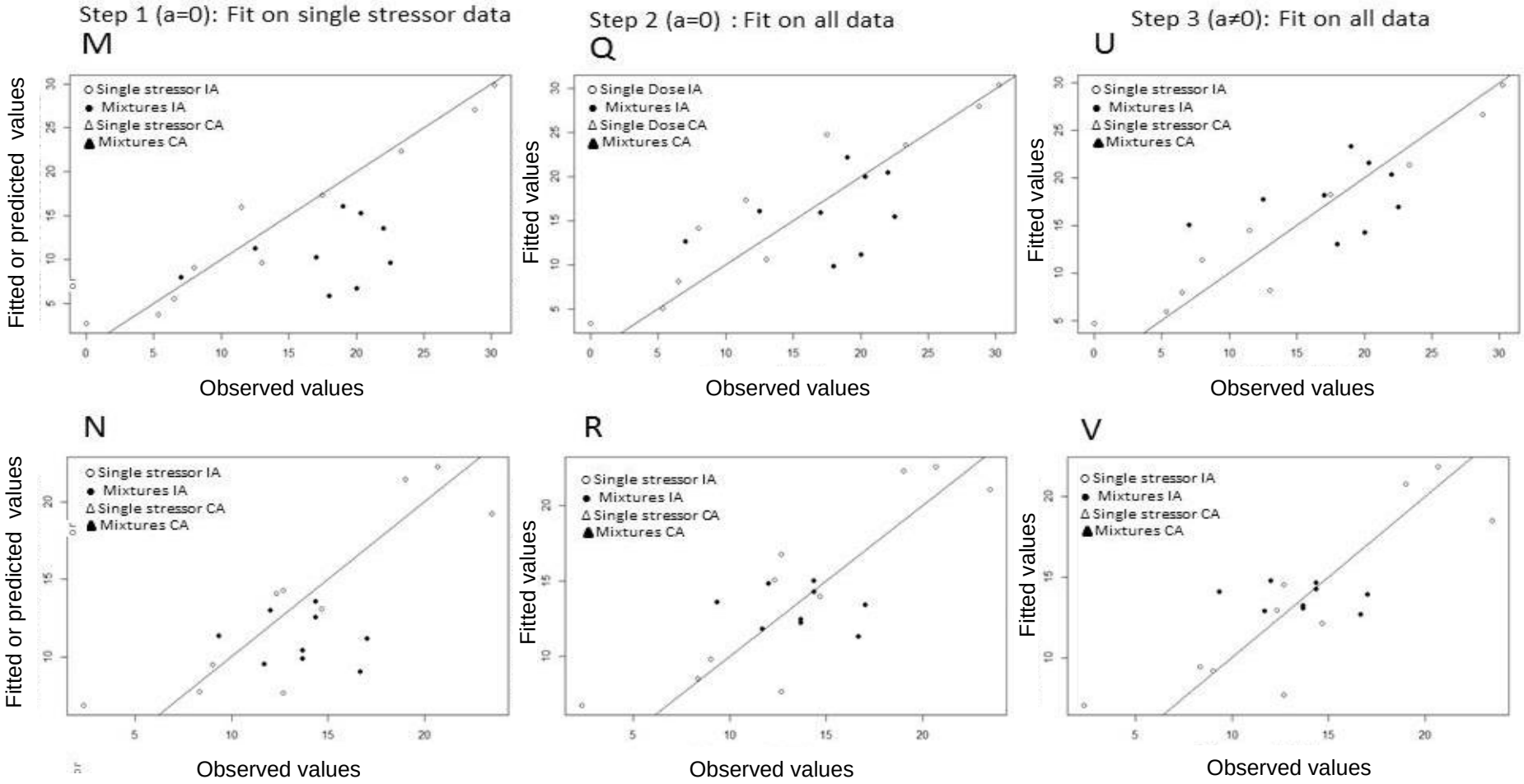


Figure 3.3 Part 3 Mean observed versus fitted values for models for total reproduction for each binary experiment: carbaryl x *Aphanizomenon* (first row: M,Q,U), carbaryl x *Cylindrospermopsis* (second row: N,R,V). Circles depict the independent action model fits (equation 3.2), triangles depict the concentration addition model fits (equation 3.1). Open symbols denote the single stressor treatments, full symbols denote the mixed stressor treatments. For all models, the 1:1 line is plotted.

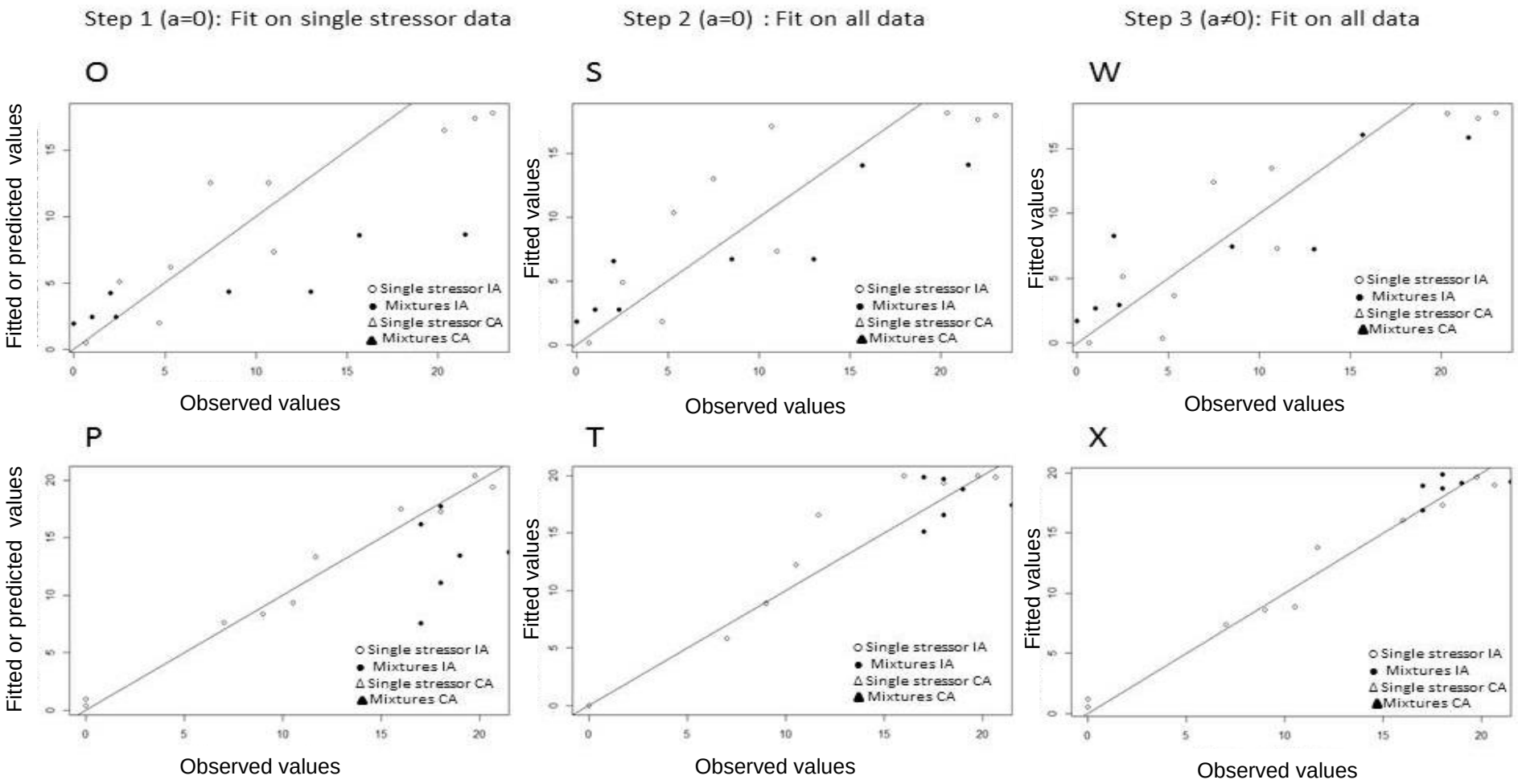


Figure 3.3 Part 4 Mean observed versus fitted values for models for total reproduction for each binary experiment: carbaryl x *Microcystis* (first row: O,S,W), carbaryl x *Oscillatoria* (second row: P,T,X). Circles depict the independent action model fits (equation 3.2), triangles depict the concentration addition model fits (equation 3.1). Open symbols denote the single stressor treatments, full symbols denote the mixed stressor treatments. For all models, the 1:1 line is plotted.

Based on statistical comparisons between the reference model and the reference model extended with the deviation parameter, three combinations (i.e. *Microcystis* combined with chlorpyrifos or tetradifon and carbaryl combined with *Cylindrospermopsis*) adhered to non-interaction based on either the concentration addition model or the independent action model (Table 3.1, Table 3.2, p-value>0.05). This means that for these three combinations, the deviation parameter *a* is not significantly different from zero, making the deviation function *G* (eq. 3.3) zero in both the independent action (eq. 3.2) as well as the concentration addition model (eq. 3.1). In contrast, four other binary combinations, i.e. *Microcystis* and carbaryl or tebufenpyrad and carbaryl and *Aphanizomenon* or *Oscillatoria,* resulted in antagonistic effects on the reproduction of *D. pulex* based on both reference models (Table 3.1, Table 3.2, p-value<0.05). For binary combinations of fenoxycarb and *Microcystis* a synergistic deviation was observed when analyzed with the independent action model (Table 3.1, Table 3.2, P-value<0.05). Analysis with the concentration addition model for this combination concluded non-interaction (Table 3.1, Table 3.2, p-value>0.05), meaning the deviation parameter *a* becomes zero, resulting in the sum of toxic units equaling 1 in eq. 3.1. The sum of squared errors (SSE) for the independent action reference model (618.4) was slightly higher than the SSE for the concentration addition model (536.9). However, addition of the deviation parameter to the independent action model reduced the SSE to 494.2, which is slightly lower than the SSE of the concentration addition reference model. The Aikaike Information Criteria (AIC) were comparable between the best model based on concentration addition (268.58) and the best model based on independent action (268.441).

Table 3.1 Estimated model parameters and their standard error: EC50 (50% effect concentration), s (slope parameter), and *a* (deviation parameter to quantify mixture interaction) for each of the different steps: IA (independent action, equation 3.2) or CA (concentration addition, equation 3.1)-model step 1 (reference model based on data from single stressors treatments only), IA or CA-model step 2 (reference model based on data from all treatments), IA or CA-model step 3 (reference model including the deviation parameter *a* to quantify mixture interaction, equation 3.3) per cyanobacteria. The reported p value is for the F-test that compared the nested models from step 2 and step 3. P-value <0.05 indicates a significant deviation from the reference model (i.e. an interaction effect). EC50 of the insecticide has SI units of µg L^{-1} for tebufenpyrad and tetradifon and ng L^{-1} for chlorpyrifos and fenoxycarb.

	Chlorpyrifos	Fenoxycarb	Tebufenpyrad	Tetradifon
Slope parameter (s) Insecticide:				
IA: step 1	1.52 ± 0.51	1.33 ± 0.44	2.60 ± 0.57	1.00 ± 0.25
IA: step 2	1.56 ± 0.47	1.51 ± 0.33	2.70 ± 0.52	0.93 ± 0.23
IA: step 3	2.24 ± 0.71	2.01 ± 0.43	2.25 ± 0.50	0.99 ± 0.24
CA: step 1	1.98 ± 0.30	2.26 ± 0.50	2.11 ± 0.37	1.85 ± 0.37
CA: step 2	3.06 ± 0.39	3.19 ± 0.34	2.86 ± 0.61	1.81 ± 0.27
CA: step 3	3.04 ± 0.39	3.27 ± 0.37	2.16 ± 0.38	1.75 ± 0.29
Slope parameter (s) *Microcystis*:				
IA: step 1	2.13 ± 0.37	2.98 ± 1.02	1.55 ± 0.42	2.89 ± 0.76
IA: step 2	2.76 ± 0.46	3.44 ± 0.63	2.99 ± 0.85	2.48 ± 0.57
IA: step 3	2.96 ± 0.51	3.71 ± 0.64	1.66 ± 0.50	2.55 ± 0.63
CA: step 1	1.98 ± 0.30	2.26 ± 0.50	2.11 ± 0.37	1.85 ± 0.37
CA: step 2	3.06 ± 0.39	3.19 ± 0.34	2.86 ± 0.61	1.81 ± 0.27
CA: step 3	3.04 ± 0.39	3.27 ± 0.37	2.16 ± 0.38	1.75 ± 0.29
EC50 (Insecticide):				
IA: step 1	75.53 ± 14.65	69.37 ± 10.16	10.98 ± 0.94	11.23 ± 2.00
IA: step 2	71.74 ± 13.82	57.80 ± 5.17	11.67 ± 0.81	9.18 ± 1.45
IA: step 3	66.36 ± 8.76	65.97 ± 5.03	10.35 ± 1.01	10.59 ± 1.94
CA: step 1	68.23 ± 6.69	66.09 ± 5.66	10.58 ± 1.05	12.19 ± 1.75
CA: step 2	64.23 ± 4.49	66.44 ± 3.15	13.83 ± 1.11	10.86 ± 1.28
CA: step 3	62.48 ± 4.56	65.41 ± 3.32	10.37 ± 1.03	11.55 ± 1.56
EC50 (*Microcystis*) (% of diet):				
IA: step 1	37.61 ± 3.56	28.74 ± 3.93	55.40 ± 9.51	30.48 ± 3.13
IA: step 2	32.75 ± 1.89	25.55 ± 1.35	64.11 ± 7.41	31.51 2.83
IA: step 3	36.42 ± 2.94	29.37 ± 1.90	58.30 ± 9.96	33.17 ± 3.50
CA: step 1	37.66 ± 3.67	30.04 ± 4.59	54.62 ± 7.65	33.61 ± 5.49
CA: step 2	41.06 ± 2.34	30.76 ± 1.78	88.07 ± 12.45	32.87 ± 3.92
CA: step 3	37.97 ± 3.17	29.58 ± 2.33	57.74 ± 8.30	34.54 ± 5.27
Deviation parameter *a*:				
IA: step 3	-1.51 ± 0.86	-2.10 ± 0.63	2.37 ± 0.88	-0.74 ± 0.67
CA: step 3	0.37 ± 0.33	0.23 ± 0.32	2.703 ± 0.64	-0.44 ± 0.64
Conclusion IA:	Non-interaction	Synergism	Antagonism	Non-interaction
P-value (IA: step 2 / IA: step 3)	0.1035	0.001584	0.03555	0.2813
Conclusion CA:	Non-interaction	Non-interaction	Antagonism	Non-interaction
P-value (CA: step 2 / CA: step 3)	0.2853	0.4851	<0.00001	0.4793

Table 3.2 Estimated model parameters and their standard error: EC50 (50% effect concentration), s (slope parameter), and *a* (deviation parameter to quantify mixture interaction) for each of the different steps: IA (independent action, equation 3.2) or CA (concentration addition, equation 3.1)-model step 1 (reference model based on data from single stressors treatments only), IA or CA-model step 2 (reference model based on data from all treatments), IA or CA-model step 3 (reference model including the deviation parameter *a* to quantify mixture interaction, equation 3.3) per cyanobacteria. The reported p value is for the F-test that compared the nested models from step 2 and step 3. P-value <0.05 indicates a significant deviation from the reference model (i.e. an interaction effect). Aph=*Aphanizomenon*, Cyl=*Cylindrospermopsis*, MC=*Microcystis*, Osl=*Oscillatoria*.

	Aph	Cyl	MC	Osl
Slope parameter (s) Carbaryl:				
IA: step 1	1.56 ± 0.63	1.01 ± 0.38	3.43 ± 1.81	2.88 ± 2.58
IA: step 2	2.07 ± 0.57	1.16 ± 0.35	3.79 ± 2.34	11.16 ± 7.69
IA: step 3	1.26 ± 0.48	0.82 ± 0.40	3.36 ± 1.98	2.58 ± 1.66
CA: step 1	1.28 ± 0.29	1.24 ± 0.29	2.77 ± 0.87	1.94 ± 0.84
CA: step 2	1.14 ± 0.26	1.05 ± 0.31	2.93 ± 0.91	2.29 ± 0.86
CA: step 3	1.32 ± 0.25	1.08 ± 0.29	3.43 ± 0.84	1.89 ± 0.67
Slope parameter (s) Cyanobacteria:				
IA: step 1	1.35 ± 0.33	1.72 ± 0.60	2.09 ± 0.75	1.40 ± 1.15
IA: step 2	1.23 ± 0.34	2.24 ± 0.85	3.54 ± 1.04	2.60 ± 1.09
IA: step 3	1.26 ± 0.31	1.33 ± 0.53	3.48 ± 1.01	1.55 ± 0.72
CA: step 1	1.28 ± 0.29	1.24 ± 0.29	2.77 ± 0.87	1.94 ± 0.84
CA: step 2	1.14 ± 0.26	1.05 ± 0.31	2.93 ± 0.91	2.29 ± 0.86
CA: step 3	1.32 ± 0.25	1.08 ± 0.29	3.43 ± 0.84	1.89 ± 0.67
EC50 (Carbaryl) (µg L^{-1}):				
IA: step 1	2.18 ± 0.64	3.52 ± 0.72	5.96 ± 0.69	2.31 ± 0.63
IA: step 2	3.52 ± 0.49	3.87 ± 0.64	6.16 ± 0.80	2.60 ± 0.13
IA: step 3	2.44 ± 0.70	3.05 ± 0.84	6.07 ± 0.80	2.29 ± 0.44
EC50 (Carbaryl) (µg L^{-1}):				
CA: step 1	1.93 ± 0.60	3.64 ± 0.60	6.11 ± 0.83	2.25 ± 0.65
CA: step 2	3.07 ± 0.70	4.27 ± 0.74	7.04 ± 1.16	3.45 ± 0.75
CA: step 3	2.48 ± 0.54	3.36 ± 0.68	6.08 ± 0.76	2.14 ± 0.52
EC50 (Cyanobacteria) (% of diet):				
IA: step 1	35.31 ± 6.23	54.87 ± 9.67	14.54 ± 2.77	59.22 ± 26.53
IA: step 2	44.51 ± 8.55	64.05 ± 10.70	21.59 ± 1.93	73.28 ± 13.29
IA: step 3	33.69 ± 6.14	62.18 ± 15.45	13.45 ± 2.24	66.69 ± 17.38
CA: step 1	38.16 ± 7.35	58.48 ± 13.35	13.70 ± 2.28	61.82 ± 15.43
CA: step 2	48.22 ± 9.94	82.70 ± 25.26	26.60 ± 3.76	97.02 ± 25.82

(Table 3.2 cont.)	Aph	Cyl	MC	Osl
CA: step 3	33. 41 ± 2.67	65.19 ± 18.80	13.25 ± 2.13	64.12 ± 13.53
Deviation parameter *a*:				
IA-model 3	2.50 ± 0.95	1.91 ± 0.99	7.77 ± 3.76	5.49 ± 2.62
CA-model 3	2.67 ± 0.89	1.86 ± 1.09	5.31 ± 1.66	6.07 ± 3.08
Conclusion IA:	Antagonism	Non-interaction	Antagonism	Antagonism
P-value (IA: step 2 / IA: step 3)	0.0071	0.079	0.0073	0.0183
Conclusion CA:	Antagonism	Non-interaction	Antagonism	Antagonism
P-value (CA: step 2 / CA: step 3)	0.0023	0.055	<0.0001	<0.0001

3.4 Discussion

Interaction effects between chemical and natural stressors have been demonstrated for a variety of combinations (Holmstrup et al., 2010). Here, research was focused on a specific group of stressors, i.e. cyanobacteria and insecticides. Four out of eight combinations of cyanobacteria and insecticides demonstrated a significant antagonistic interaction on the reproduction of *Daphnia pulex*, i.e. the reproduction upon exposure to these combinations was higher than expected based upon the reproduction upon exposure to the stressors alone. Three combinations demonstrated no interaction effects at all whereas a single combination demonstrated no interaction effects with the concentration addition model and a significant synergistic interaction with the independent action model.

Different interaction effects were observed for insecticides with different modes of action. Combinations of chlorpyrifos and *Microcystis* affected reproduction of *Daphnia* differently than combinations of carbaryl and *Microcystis*. This suggests that even for insecticides that target the same enzyme (i.e. acetylcholine esterase), the observation of interaction or non-interaction effects cannot be extrapolated from one insecticide to another. However, inhibition of acetylcholine esterase by organophosphates such as chlorpyrifos is less reversible and hence longer-lasting than inhibition of acetylcholine esterase by carbamates (Pope et al., 2005). This difference in recovery time may be a potential explanation for the shift in combined effects with cyanobacteria from antagonistic interaction with carbaryl (a carbamate) to non-interaction with chlorpyrifos (an organophosphate). Alternatively, choline esterase inhibitors have been shown to target other molecules than acetylcholine esterase (Pope et al., 2005). Differences in the ability to target other molecules could also be a potential

explanation for the differences in interaction effects observed for carbaryl and chlorpyrifos. Likewise, differences in metabolic activation and degradation between organophosphates and carbamates (Fukuto, 1990) may also be a potential cause for the difference in interaction pattern with *Microcystis*.

Furthermore, the antagonistic interaction between *Microcystis* and carbaryl contrasts with previous results of Cerbin et al. (2010), who observed a synergistic pattern. However, the study of Cerbin et al. (2010) and the present study differ in length of exposure period as well as endpoint. Cerbin et al. (2010) exposed animals only until the first clutch, whereas the present study used a continuous exposure of 21 days. In addition, the endpoints observed were different and different *Daphnia* clones were used in the two studies, which may have differed in sensitivities toward the stressors used. This has already been demonstrated for *Microcystis* stress (Hietala et al., 1995).

Likewise, tebufenpyrad and tetradifon, both are targeting the oxidative phosphorylation albeit through different molecular mechanisms, demonstrated different interaction effects with *Microcystis*. Combinations with tebufenpyrad were antagonistic whereas combinations with tetradifon were adhering to non-interaction. Tetradifon inhibits ATP-synthases while tebufenpyrad inhibits NADH:ubiquinone reductase activity in complex I of the mitochondrial respiration (IRAC, 2009; Sherer et al., 2006). Again, subtle differences in the molecular target between two insecticides lead to vastly different conclusions in terms of combined and interaction effects.

These observations, i.e. different interaction effects for insecticides with closely related molecular targets (e.g. carbaryl – chlorpyrifos, tetradifon – tebufenpyrad) when combined with the same stressor (here: *Microcystis*) in a binary mixture, suggest a potential mechanistic basis for interaction effects that may well be detectable at the molecular level. However, the differences in molecular mechanisms take place at the macro-molecular level and may not be distinguishable at the pathway level (e.g. tetradifon and tebufenpyrad both affect the oxidative phosphorylation).

Furthermore, two very different pesticide, carbaryl and tebufenpyrad (Table 1.4), demonstrated similar antagonistic interaction effects when combined with *Microcystis.* At present, it is still unclear to what extent the antagonistic effects caused by these two combinations are similar. Indeed, these insecticides have little in common in terms of molecular targets (IRAC, 2009) yet they do seem to affect biological processes that are also affected by *Microcystis*. A recent study by Jansen et al. (2013) indicated a significant effect of carbaryl on NADH:ubiquinone reductase after exposing *Daphnia*

magna for 96 hours to 5.6 µg/L of carbaryl. These findings do indicate that the occurrence of antagonistic interaction effects with *Microcystis* for both tebufenpyrad and carbaryl could be potentially caused by interactions with NADH:ubiquinone reductase.

Three out of four cyanobacteria acted antagonistically when combined with carbaryl. The fourth cyanobacteria, *Cylindrospermopsis*, did not interact with carbaryl although the p-value bordered on the 5% significance level and the *a*-value was positive. Overall, different cyanobacteria act quite similar to one another when combined with carbaryl. This is in line with the results obtained in chapter 2 (section 2.3) where no significant differences between the effects of different cyanobacteria on *Daphnia* were observed across the full dose response curve. Although carbaryl can cause oxidative stress and cell lysis in cyanobacteria, the potential effect of carbaryl on the cyanobacteria itself can be excluded. Indeed, the concentrations needed to elicit such a response are a factor 1000 higher than the concentrations used in the present study (Habib et al., 2011). The targeted mode of action of carbaryl is the inhibition of the enzyme acetylcholineesterase. Effects on NADH:ubiquinone reductase have also been reported in *Daphnia* (Jansen et al., 2013), yet no literature is available on the effects of cyanobacteria other than *Microcystis* on NADH:ubiquinone reductase. A study by Lethonen et al. (2003) demonstrated potential effects of nodularin, a cyanobacterial toxin, on the acetylcholineesterase enzyme activity in the clam *Macoma balthica*, which is also the main mechanism of toxicity of carbaryl. Potential antagonistic effects might also be the result of a similar biotransformation or detoxification process for both stressors. Indeed, cyanotoxins are primarily biotransformed through glutathione-S-transferase and cytochrome P450 (Wiegand and Pflugmacher, 2005). Furthermore, induction of glutathione-S-transferase activity as well as cytochrome 1A has been observed in *Oncorhynchus mykiss* exposed to carbaryl (Ferrari et al., 2007). Hence, molecular and biochemical research is needed to fully understand the mechanisms leading to these antagonistic interactions.

For the combination of *Microcystis* and fenoxycarb, different statistical conclusions were drawn with the two different reference models (Table 3.2). Such differences have been reported in literature and attributed to among others the different mathematical background of the reference models (Dresher and Boedeker, 1995; Jonker et al., 2005). Indeed, independent action hypothesizes that the probability of response to one stressor is independent from the probability of response to the other stressor (Jonker et al., 2009). In contrast, concentration addition hypothesizes that the relative toxicity of the

combined stressors is the same as the relative toxicity of the individual stressors (Jonker et al., 2009). Furthermore, this specific binary combination further confirms the general agreement in literature that the concentration addition reference model provides more conservative estimates of mixture toxicity for risk assessment than independent action reference model (Altenburger et al., 1996; Faust and Schlolze, 2004). However, like Cedergreen et al. (2008), these findings and recommendations are not based on a greater accuracy of concentration addition compared to independent action. Therefore, from a mechanistic point of view, the current data and subsequent analysis cannot fully exclude or confirm synergistic interactions between fenoxycarb, a juvenile hormone analog, and *Microcystis* as there is too little knowledge about how biological pathways are affected by these two stressors other than the primary molecular targets. At present, the two reference models, CA and IA, are sometimes compared by determining whether the data falls into the 95% confidence interval from one model rather than the other (Dias da Silva et al., 2013). However, when both model fits are similar (as is the case for this combination, Fig. 3.2 F and J) and their parameter values overlap (Table 3.1), again no conclusion can be made to select one model above the other. Requirements of accuracy are the primordial driver for pharmacokinetic studies or mechanistic studies, especially in human toxicology. Indeed, in those studies, the aim is to find the most accurate model for a given mixture. Based on the data in this study, both models are equally valid for these types of studies and one model cannot be selected above the other based on accuracy. The development of AOPs for different compounds may aid in model selection in the future as the similar or dissimilar mode of action at the molecular and even at the organismal level will become clearer and comparison will be more straightforward.

For risk assessment, however, the requirements of adequate protection of the aquatic ecosystems are more important than the requirement for an accurate mechanistic model. Overall, concentration addition always provided effect predictions that are conservative from a risk assessment point of view compared to the observed effects, i.e. the predicted effects are always as large as or larger than the observed effects. As a consequence, the protection of the ecosystem has a high probability of success with a conservative model such as the concentration addition model. Hence, risk assessment of combined and interaction effects for combinations of insecticides and cyanobacteria based on concentration addition model predictions will likely result in a sufficient protection of the aquatic ecosystem. Such an assessment will be necessary in the future when climate change conditions will

stimulate cyanobacterial blooms and as consequence combined exposure to cyanobacteria and insecticides (Moe et al., 2012; Paerl and Huisman, 2009).

Overall, these results clearly demonstrate the importance of combined and interaction effects in aquatic ecosystems. This has important implications for current regulatory risk assessment that mainly focuses on single substances. Based on the results of the present study, the concentration addition model can serve as a protective scenario in risk assessment of insecticides and cyanobacteria at sublethal effect levels for the observed endpoint reproduction.

3.5 Conclusion

Insecticides with different molecular targets showed different interaction patterns when combined with *Microcystis* on the reproduction of *Daphnia pulex*. In contrast, different cyanobacteria showed similar interaction patterns when combined with carbaryl on the reproduction of *Daphnia pulex*. Four out of eight combinations showed antagonistic deviation patterns, three showed no interaction patterns whereas one yielded different patterns depending on the reference models used.

These results demonstrated that interaction effects cannot be generalized for insecticides targeting the same pathway and even for insecticides targeting the same enzyme. In contrast, results may potentially be generalized across different cyanobacteria combined with the same insecticide. Yet, further mechanistic research is needed.

Overall, concentration addition provided more conservative predictions of effects than independent action. Furthermore, these effect predictions were always conservative compared to the observed effects which suggest using the concentration addition model to ensure an adequate protection of the aquatic ecosystem.

Chapter 4

Combined effects of cyanobacteria and insecticides across a large set of combinations

4.1 Introduction

The impact of interaction effects between natural and chemical stressors and cyanobacteria and insecticides in particular, has been amply discussed in chapter 1 (sections 1.1, 1.2.4 and 1.5.2). Chapter 3 further focused on this topic by addressing the combined effects of a selection of cyanobacteria combined with carbaryl and a selection of insecticides combined with *Microcystis*. Although new and important conclusions could be made, the question remains to what extent the results can be extrapolated across a wider set of insecticides and cyanobacteria.

Here, the dataset will be expanded to cover a wide variety of cyanobacteria and insecticides (section 1.5) in 48 binary combinations. By assessing combined effects across a large dataset, patterns of mixture toxicity will become clearer and it may be possible to infer hypotheses based upon *a priori* mechanistic knowledge regarding the mechanisms of toxicity of the different stressors. For example, based on the results of chapters 2 and 3, different cyanobacteria affect *Daphnia* in a similar manner and therefore interaction effects could be potentially extrapolated from one cyanobacterium to another. In contrast, insecticides often have different modes of action and the results from chapter 3 indicated that effects cannot be straightforwardly extrapolated from one insecticide to another. Furthermore, a large dataset will allow for more powerful conclusions with regards to a potential generalization or extrapolation of combined and interaction effects to other stressors which may form a scientific basis for risk assessment frameworks as well as enhance our understanding of how organisms responds to combinations of stressors.

4.2 Materials and methods

4.2.1 Experimental organisms and cyanobacteria

The experimental organisms originated from *D. pulex* cultures as described in section 2.2.1. Likewise, cyanobacteria culture conditions were also described in section 2.2.1.

4.2.2 Experimental design

All exposures were conducted inside a climate controlled room at a constant temperature (20 ± 1°C) and photoperiod (16:8h light-dark). The experimental design is depicted in Fig. 4.1. Insecticide and mixture treatments consisted of five replicate beakers. For control and cyanobacterial treatments, this

number was doubled to ten replicate beakers to ensure sufficient RNA for subsequent binary mixture comparisons with microarrays (chapter 7) as pesticides were simultaneously tested in groups of four sharing a control and cyanobacterial treatment to conserve time and resources. Thirty neonates (less than 24 hours old) were placed in each of the borosilicate beakers containing 1.5 L no N, no P COMBO medium (Shaw et al., 2007). On the fourth day, these animals were randomly assigned to a control or a cyanobacteria treatment and exposed for ten days. Given the results of chapter 2, the same concentration of cyanobacteria in the diet was used in all treatments. For the insecticide treatments, the same effect concentration was chosen for all treatments, i.e. half of the EC50. This concentration was selected as it was deemed high enough to elicit a toxic response but low enough to allow quantification of potential synergisms in the mixture treatment. (i.e. if the concentration in the mixture treatment is too high, the effect approaches 100% which makes it impossible to quantify potential synergisms as they would be larger than 100%).

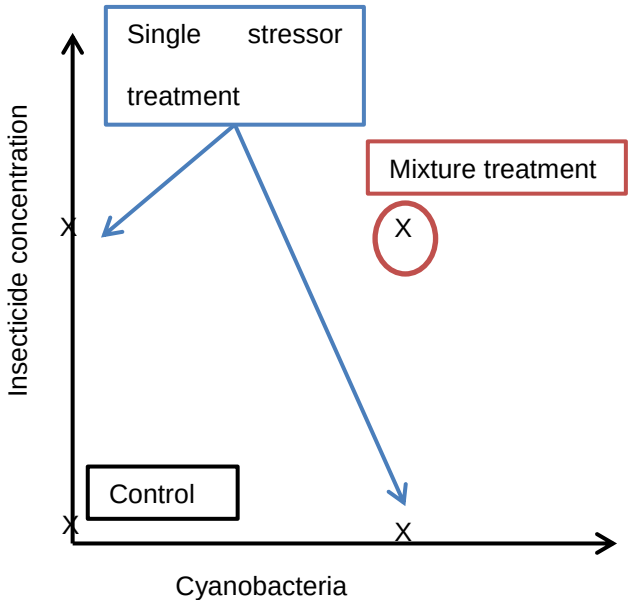


Figure 4.1 Experimental design for each binary mixture combination.

Animals were fed daily with a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio at a rate of 2 mg dry weight L^{-1} in control conditions. In cyanobacteria treatments, this diet was contaminated with 50% of a respective cyanobacterium based upon the results of chapter 2 (section 2.3). Insecticide treatments contained a given amount of one of the eight insecticides (section 1.5.2), based upon preliminary life history experiments (Table 4.1). Mixture treatments consisted of COMBO medium with a specific insecticide concentration and were given a

diet contaminated with 50% of a respective cyanobacterium. For all treatments, medium was renewed every two days. At the same time, reproduction and survival were monitored. If the animals reproduced, neonates were counted and removed from the beaker. At the end of the experiment, RNA was extracted from adult exposed animals for gene expression analysis at the end of the experiment. Results of gene expression analysis will be discussed in chapter 7.

Table 4.1 The observed effect concentration for total reproduction (EC50) from preliminary life history experiments per insecticide and the final concentration of insecticide used in both insecticide and mixture treatments.

Insecticide	Observed EC50	Final Concentration	Measured Concentration
Acetamiprid	30.34 µg/L	15 µg/L	17.26 ± µg/L
Carbaryl	0.50 µg/L	0.25 µg/L	0.16 ± 0.03 µg/L
Chlorpyrifos	31.33 ng/L	16 ng/L	21.12 ± ng/L
Deltamethrin	0.45 ng/L	0.23 ng/L	NA
Endosulfan	1.00 µg/L	0.5 µg/L	2.06 ± µg/L
Fenoxycarb	2.00 ng/L	1 ng/L	NA
Tebufenpyrad	6.06 µg/L	3 µg/L	3.12 ± 0.91 µg/L
Tetradifon	8.09 µg/L	4 µg/L	3.91 ± 0.81 µg/L

Samples for concentration analysis of insecticides were taken with every medium renewal of both old and new media. At the same time, pH was measured for all treatments to ensure that pH never differed more than 0.2 units from control treatments (pH=7.00± 0.2).

4.2.3 Statistical analysis

Total reproduction per replicate beaker was analyzed for all replicates. Significant differences between treatments were analyzed by comparing the reproduction relative to control reproduction across treatments. As data was not normally distributed, Kruskal Wallis Rank sum test (Hollander and Wolf, 1973) was used to compare reproduction across all treatments. Subsequent pairwise comparisons were executed with a Mann-Whitney U-test (Bauer, 1972). Analysis of variance with two factors was performed to determine interaction effects for each binary combination of cyanobacteria and insecticides on the log transformed total reproduction. Log transformation of the data is essential to test the hypothesis of independent action through an analysis of variance as described in De Coninck et al. (2013a). Assumptions of normality and homoscedasticity were verified on the log transformed

data with the Shapiro-Wilk test (Royston, 1982) and the Levene test (Fox, 2008). All p-values were corrected for multiple testing with the Benjamini-Hochberg false discovery rate (FDR) procedure at the 5% significance level (Benjamini and Hochberg, 1992). Deviation from non-interaction was quantified according to De Coninck et al. (2013a):

$$Deviation\ a = \log\left(\frac{\text{observed reproduction}}{\text{predicted reproduction}}\right) \text{ (eq 4.1)}$$

Here, predicted reproduction is determined based upon the independent action model as originally formulated by Bliss, eq. 1.2, and thus estimated from the reproduction observed in the single treatments. In equation 4.1, the deviation parameter a will be positive when observed reproduction is larger than predicted reproduction, which is an antagonistic deviation. The deviation parameter will be negative when observed reproduction is smaller than the predicted reproduction, which is a synergistic deviation.

4.2.4 Chemical analyses

Samples for insecticide concentrations were analyzed as described in chapter 3 (section 3.2.3). Solvent phase extraction procedures are detailed per insecticide in Appendix B.2.

4.3 Results

Effects on reproduction were expected to be comparable between the cyanobacterial treatments based upon the results from chapter 2. However, significant differences were observed (Fig. 4.2, Table 4.2). *Anabaena* and *Cylindrospermopsis* were the least toxic whereas *Microcystis* was the most toxic for the reproductive capacity of *Daphnia pulex* (Fig. 4.2). Indeed, exposure to *Anabaena* or *Cylindrospermopsis* resulted in a decline of reproduction with 20% compared to unexposed animals whereas exposure to *Microcystis* resulted in a decline of reproduction with about 75% compared to unexposed animals. Across the different cyanobacterial treatments, effects differed at most threefold (Fig. 4.2). In insecticide treatments, animals were exposed to half of the EC50, expecting in general an effect between 20-25%, i.e. a decline in reproduction with about 20-25% compared to unexposed animals. For most insecticides, effects were within this range (Fig. 4.3). Effects between different insecticide treatments differed at most by a factor of 1.5 (Fig. 4.3). Effects of endosulfan and tetradifon were more toxic as reproduction in treatments was about 60% of control reproduction (Fig. 4.3).

Endosulfan was significantly more toxic than all other insecticides excluding tetradifon whereas tetradifon was significantly different from 5 insecticides, i.e. acetamiprid, chlorpyrifos, deltamethrin and fenoxycarb (Table 4.2).

Interaction effects were observed in eighteen of the forty-eight binary mixture combinations (Table 4.4, Appendix C Table C.1). All insecticides interacted significantly with *Aphanizomenon* on the total reproduction of *Daphnia*. In contrast, no interaction effects were observed for combinations of *Anabaena* and insecticides. For the binary combinations with other cyanobacteria, a complex interaction pattern emerged. In all interactions with insecticides and *Aphanizomenon*, the observed reproductive response was significantly larger than the predicted reproductive response (Fig. 4.4 B). This demonstrates an antagonistic effect on the reproduction of *Daphnia*. Combinations of insecticides with other cyanobacteria also resulted in significant antagonistic effects (Fig. 4.5-4.6, Table 4.4, Appendix C Table C.1). Five combinations had a negative deviation parameter indicating a synergistic trend, only the combination of tebufenpyrad and *Cylindrospermopsis* (Fig. 4.5 A) demonstrated a significant synergistic effect as observed reproduction was significantly smaller than predicted reproduction.

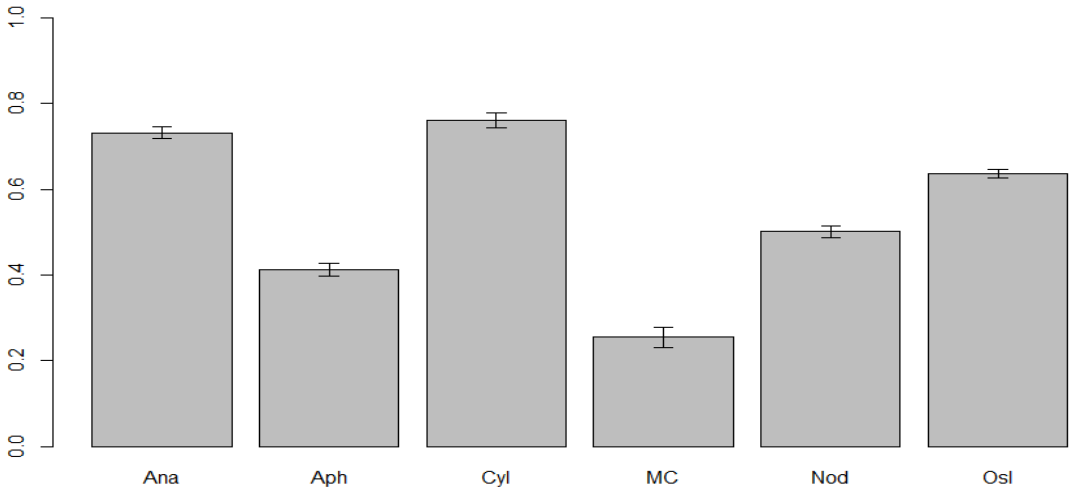


Figure 4.2 Effect of cyanobacterial treatments (all 50% of the total diet) on reproduction of Daphnia pulex relative to control (i.e. control response=1). (Ana=*Anabaena*, Aph=*Aphanizomenon*, Cyl=*Cylindrospermopsis*, MC=*Microcystis*, Nod=*Nodularia*, Osl=*Oscillatoria*). Error bars represent standard error.

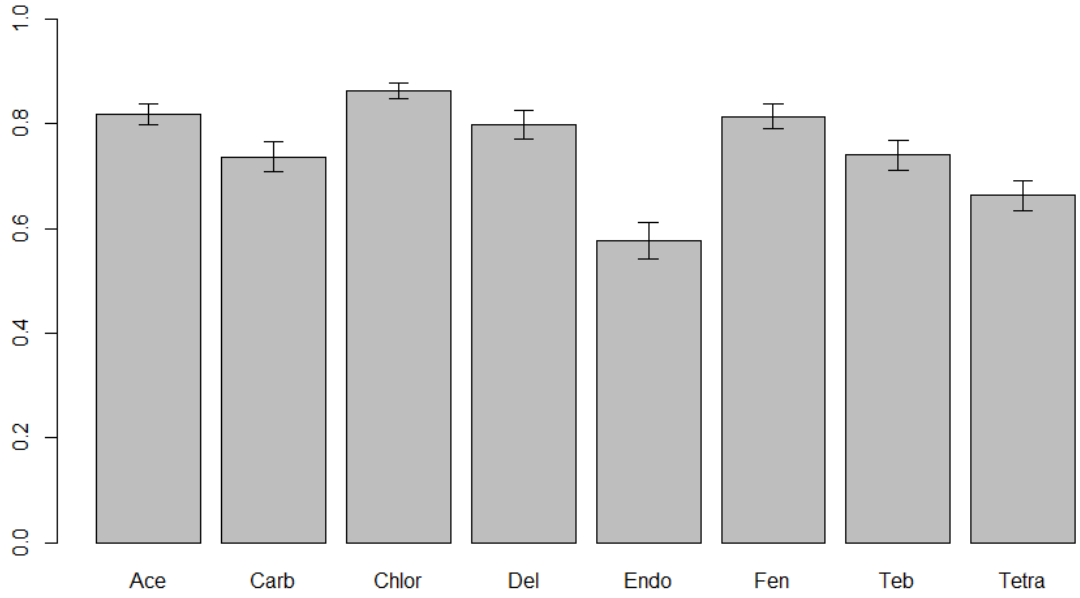


Figure 4.3 Effect of insecticide treatments on reproduction of Daphnia pulex relative to control. (i.e. control responses = 1). (Ace=Acetamiprid, Carb=Carbaryl, Chlor=Chlorpyrifos, Del=Deltamethrin, Endo=Endosulfan, Fen=Fenoxycarb, Teb=Tebufenpyrad, Tetra=Tetradifon). Error bars represent standard error.

Table 4.2 Benjamini-Hochberg corrected p-values for pairwise comparisons between all cyanobacterial treatments. P-values smaller than 0.05 are represented in bold italic.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Anabaena		***<2 e-16***	***6.34 e-3***	***<2 e-16***	***<2 e-16***	***<2 e-16***
Aphanizomenon	***<2 e-16***		***<2 e-16***	***1.25 e-12***	***1.86 e-11***	***<2 e-16***
Cylindrospermopsis	***6.34 e-3***	***<2 e-16***		***<2 e-16***	***<2 e-16***	***<2 e-16***
Microcystis	***<2 e-16***	***1.25 e-12***	***<2 e-16***		***<2 e-16***	***<2 e-16***
Nodularia	***<2 e-16***	***1.86 e-11***	***<2 e-16***	***<2 e-16***		***<2 e-16***
Oscillatoria	***<2 e-16***	***<2 e-16***	***<2 e-16***	***<2 e-16***	***<2 e-16***	

Table 4.3 Benjamini-Hochberg corrected p-values for pairwise comparisons between all insecticide treatments. P-values smaller than 0.05 are represented in bold italic.

	Acetamiprid	Carbaryl	Chlorpyrifos	Deltamethrin	Endosulfan	Fenoxycarb	Tebufenpyrad	Tetradifon
Acetamiprid		7.72 e-02	9.02 e-02	9.82 e-01	***1.00 e-06***	7.51 e-01	***4.87 e-02***	***2.04 e-04***
Carbaryl	7.72 e-02		***9.30 e-04***	15.8 e-01	***1.81 e-03***	7.08 e-02	9.82 e-01	7.85 e-02
Chlorpyrifos	9.02 e-02	***9.30 e-04***		8.04 e-02	***<2 e-16***	1.54 e-01	***2.19 e-04***	***<2 e-16***
Deltamethrin	9.82 e-01	15.8 e-01	8.04 e-02		***7.00 e-06***	6.60 e-01	4.47 e-02	***9.33 e-04***
Endosulfan	***1.00 e-06***	***1.81 e-03***	***<2 e-16***	***7.00 e-06***		***<2 e-16***	***4.17 e-04***	1.12 e-01
Fenoxycarb	7.51 e-01	7.08 e-02	1.54 e-01	6.60 e-01	***<2 e-16***		3.37 e-02	**2.19 e-04**
Tebufenpyrad	***4.87 e-02***	9.82 e-01	***2.19 e-04***	***4.47 e-02***	***4.17 e-04***	***3.37 e-02***		6.74 e-02
Tetradifon	***2.04 e-04***	7.85 e-02	***<2 e-16***	***9.33 e-04***	1.12 e-01	***2.19 e-04***	6.74 e-02	

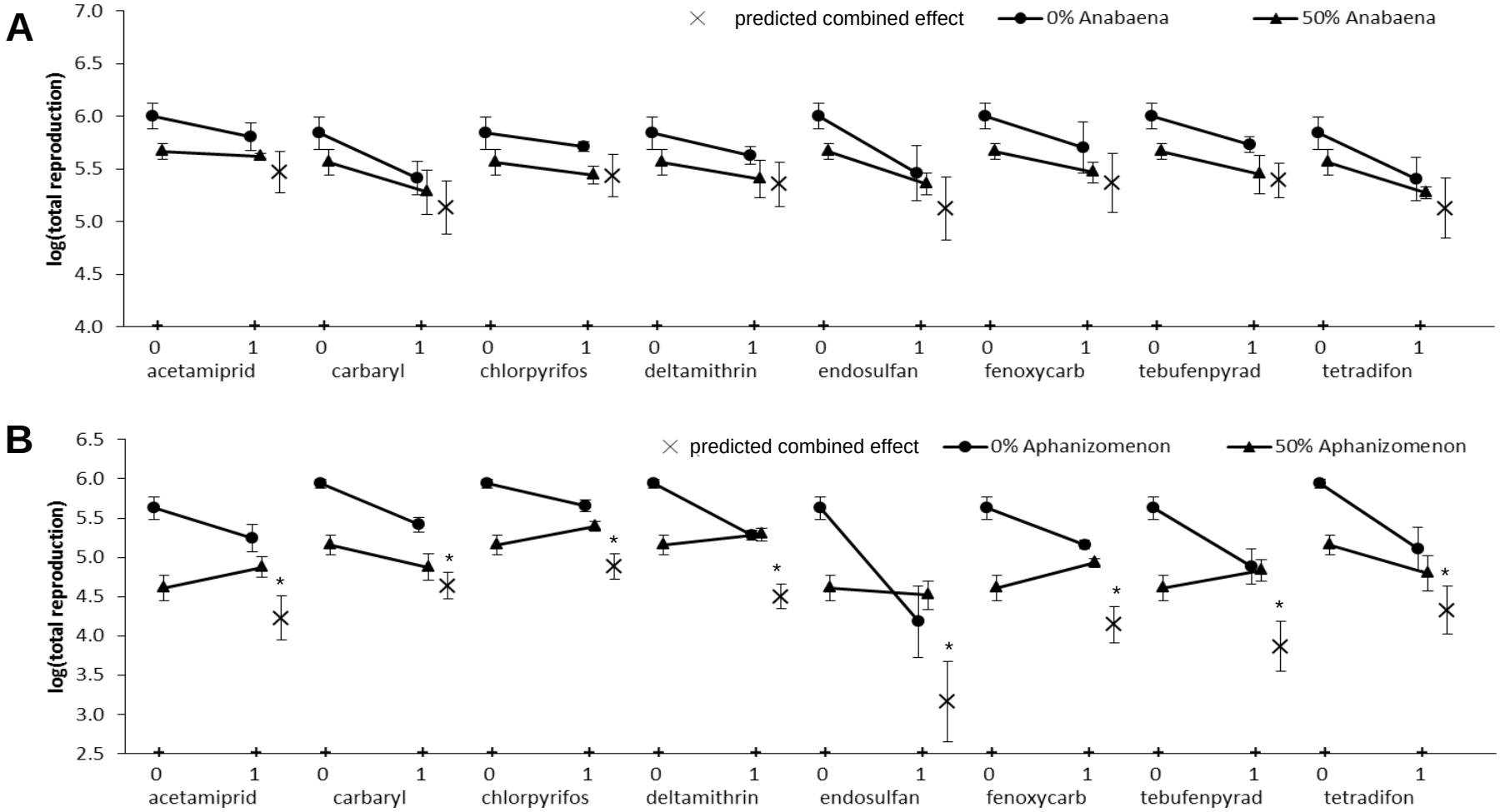


Figure 4.4 Log transformed reproduction for each treatment grouped per cyanobacteria, i.e. *Anabaena* (A) and *Aphanizomenon* (B). Absence (0) or presence (1) of insecticide is denoted on the y-axis. Absence or presence of the cyanobacteria (*Anabaena* or *Aphanizomenon*) are denoted by circles or triangles, respectively. The observed combined effect is thus represented by a triangle at the 1 postion. Predicted combined effects are represented with an x, significant difference between predicted and observed combined effect is highlighted by an asterix. Error bars represent standard error.

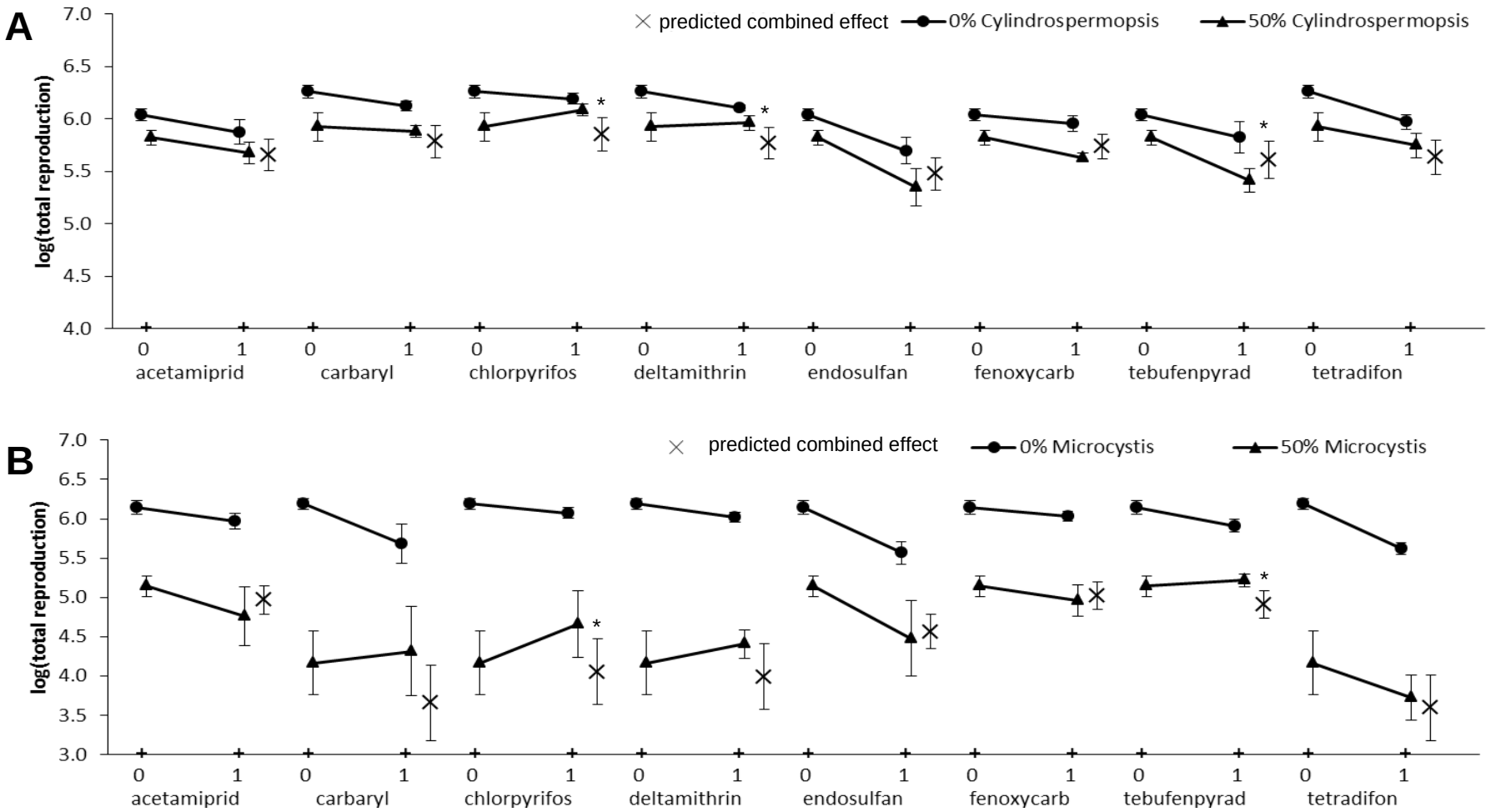


Figure 4.5 Log transformed reproduction for each treatment grouped per cyanobacteria, i.e. *Cylindrospermopsis* (A) and *Microcystis* (B). Absence (0) or (1) presence of insecticide is denoted on the y-axis. Absence or presence of the cyanobacteria (*Cylindrospermopsis* or *Microcystis*) are denoted by circles or triangles, respectively. The observed combined effect is thus represented by a triangle at the 1 postion. Predicted combined effects are represented with an x, significant difference between predicted and observed combined effect is highlighted by an asterix. Error bars represent standard error.

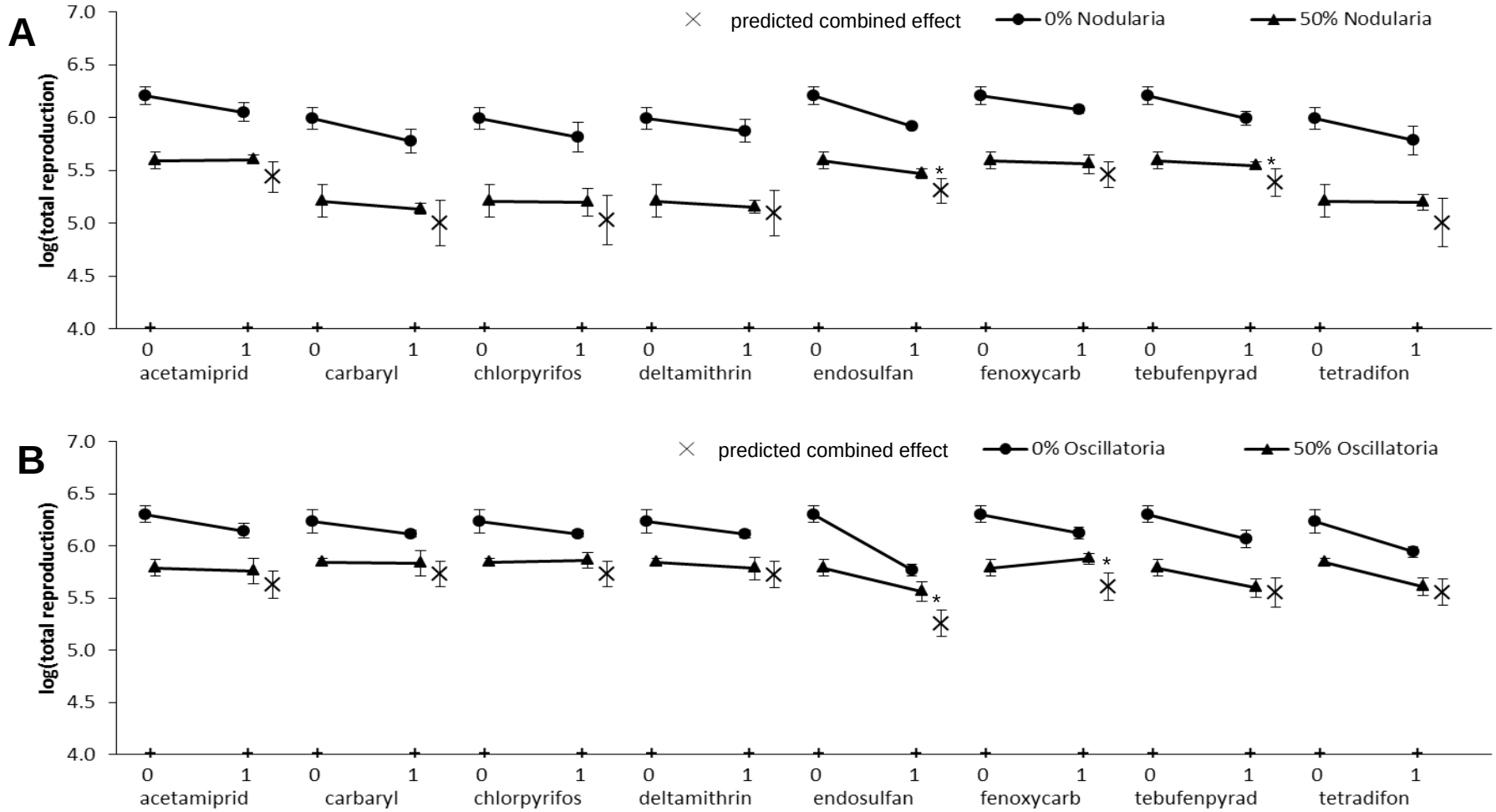


Figure 4.6 Log transformed reproduction for each treatment grouped per cyanobacteria, i.e. *Nodularia* (A) and *Oscillatoria* (B). Absence (0) or presence (1) of insecticide is denoted on the y-axis. Absence or presence of the cyanobacteria (*Nodularia* or *Oscillatoria*) are denoted by circles or triangles, respectively. The observed combined effect is thus represented by a triangle at the 1 postion. Predicted combined effects are represented with an x, significant difference between predicted and observed combined effect is highlighted by an asterix. Error bars represent standard error.

Table 4.4 The deviation parameter *a* of log transformed total reproduction of observed combined effects versus predicted combined effects for each combination. Deviation parameters with p-values smaller than 0.05, after Benjamini-Hochberg correction, are represented in bold italic. The color code gives a visual indication of the interaction effect: the darker the green or red, the more antagonistic (green) or synergistic (red) the combination, the lighter the closer to non-interaction.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	0.15	***0.65***	0.02	-0.21	0.16	0.13
Carbaryl	0.14	***0.24***	0.09	0.66	0.14	0.10
Chlorpyrifos	0.01	***0.51***	***0.23***	***0.60***	0.17	0.13
Deltamethrin	0.05	***0.79***	***0.19***	0.41	0.06	0.06
Endosulfan	0.23	***1.35***	-0.13	-0.09	***0.16***	***0.31***
Fenoxycarb	0.10	***0.79***	-0.11	-0.06	0.10	***0.27***
Tebufenpyrad	0.06	***0.97***	***-0.19***	***0.31***	***0.16***	0.05
Tetradifon	0.15	***0.47***	0.11	0.13	0.20	0.05

4.4 Discussion

In contrast to overall results of chapter 2 (section 2.3), individual cyanobacteria treatments differed significantly from one another. In chapter 2, results were also analyzed by comparing each diet ratio separately and significant differences were observed at 40% cyanobacteria in the diet. No significant differences were observed at 20% and 80% of cyanobacteria in the diet in chapter 2. Here, animals were exposed to 50% of cyanobacteria in the diet. These results confirm that significant differences are indeed observed within a narrow range of proportions of cyanobacteria in the diet. Furthermore, it highlights that for assessing and comparing risks of single stressors, full concentration response curves are crucial as observing effects at only one concentration may lead to different interpretations. The potential cause for these significant differences may be attributed to several factors. First, these six cyanobacteria are known to produce different toxins (Table 2.1) which may differ in toxicity and the concentration of toxin produced may differ between the different species. Differences in toxicity of the different toxins have not been reported so far for *Daphnia* but LD50 values for mouse are available for all six produced toxins (Van Apeldoorn et al., 2007). Microcystins, nodularins, anatoxin-a(s) and saxitoxins are the most toxic with LD50s varying from 10-60 µg/kg body weight whereas cylindrospermopsins and anatoxin-a are significantly less toxic with LD50s varying from 300-400 µk/kg bodyweight. These values do not agree with the overall trend observed in Fig. 4.2 for which Anabaena, an anatoxin-a(s) producer, and *Cylindrospermopsis*, a cylindrospermopsin producer, were

the least toxic to the *Daphnia*. Although sensitivity can be rarely extrapolated in a straightforward manner from mouse to *Daphnia,* it does suggest that toxins do not primarily drive these differences, which is in agreement with literature (Wilson et al., 2006). Second, these cyanobacteria differ in their morphology (Table 1.3. Fig. 1.7) which may lead to different biological effects on the exposed *Daphnia*. Again, the different morphologous classes from Table 1.3 do not overlap with the differences observed in Fig. 4.2. Third, their PUFA content also differs (Fig. 2.3). Again, similar conclusions can be drawn as for the other two factors, no clear overlap between the PUFA content and the effect on reproduction can be observed.

Differences in effect between the different insecticide treatments were also observed but could be attributed to the nonlinear slope of the concentration response curve resulting in potential different effects at half of the EC50 concentration. In addition, slight differences in nominal and measured concentration may also account for the differences (Table 4.1). Furthermore, this has no influence on the interaction effects given that interaction effects are estimated by comparing the response to the insecticide alone with the response of the mixture alone without relation to the original EC50/2 estimate or any other insecticide.

Conclusions related to combined and interaction effects were similar for four of the eight combinations previously tested in life history experiments (section 3.3). Indeed, mixtures of *Aphanizomenon* and carbaryl and mixtures of *Microcystis* and tebufenpyrad were antagonistic in both experiments. For combinations of *Microcystis* and tetradifon and *Cylindrospermopsis* and carbaryl, no interaction effects could be detected in the two experiments.

Combinations of *Microcystis* and carbaryl were significantly antagonistic in previous life history experiments. Here, this antagonistic deviation could not be statistically confirmed although the p-value was close to the significance level (i.e. 0.05). This discrepancy may be in part attributed to a strong multiple testing correction as the p-value was significant prior to this correction.

For the three other combinations effects differed between the life history experiments and these exposures. First, combinations of chlorpyrifos and *Microcystis* showed an antagonistic deviation not detectable during the life history experiments where no interaction effects were observed. Second, combinations of fenoxycarb and *Microcystis* were synergistic in the 21 day life-history experiments, but this synergism was not observed in these exposures. Although for this combination, synergism was

only observed with the independent action model and not with the concentration addition model (section 3.3). Last, combinations of *Oscillatoria* and carbaryl showed an antagonistic deviation in the 21 day life-history experiments which was not observed in this chapter.

These differences between the previous chapter and the current chapter could in part be attributed to experimental design and analysis: exposure duration, exposure concentration and statistical analysis. Differences in response at different exposure times and endpoints have been already demonstrated by Alda et al. (2006) for a fungicide and a chlorinated aromatic hydrocarbon and by Jager et al. (2006) for cadmium. Differences in mixture effects due to exposure time have already been discussed by Baas et al. (2007) and Van Gestel and Hensbergen (1997). However, the data from chapter 3 were re-analyzed in the exact same manner but only including reproduction up and until day 14. This analysis did not result in different conclusions compared to the analysis after 21 days (Appendix C Tables C.2-C.3, Tables 3.1-3.2). Again, these results suggest other factors than exposure time influencing these differences. Regardless of the cause, it indicates that the toxicity of a mixture is a complex process dependent upon a variety of factors but seems to remain consistent over the duration of the exposure. As a consequence, generalization across concentrations of combined and interaction effects at the life history level seems difficult.

In chapter 3, similar interaction effects were observed for different cyanobacteria combined with the same insecticide and different interaction effects for different insecticides combined with the same cyanobacteria. Here, similar interaction effects were observed for all insecticides combined with *Aphanizomenon* and no interaction effects were observed for any combination of an insecticide with *Anabaena.* Also, antagonistic trends were observed for insecticides combined with *Nodularia* and *Oscillatoria* despite the lack of significance in the majority of these combinations. The results suggest that extrapolation of interaction effects from one insecticide to the other depends upon the cyanobacteria used or insecticide used. Indeed, for *Aphanizomenon* and *Anabaena* effects are consistent whereas for *Microcystis* and *Cylindrospermopsis*, different interactions are observed for combinations with different insecticides. Combinations of *Nodularia* and *Oscillatoria* have a general similar antagonistic trend across all insecticides yet only a few can be confirmed statistically. Furthermore, correlation analysis of deviation parameters for each insecticide combined with the six cyanobacteria indicated no significant correlations except for endosulfan and fenoxycarb (Appendix C Table C.3). Thus, interaction effects of endosulfan and fenoxycarb when combined with the same

cyanobacteria were similar for these two insecticides. This correlation cannot be explained from a mode of action point of view as the modes of action are very different (section 1.5.2). However, insecticides can affect other processes than their known molecular target (Pope, 1999) which may explain the correlation although mechanistic confirmation is needed. Also, different interaction effects of different cyanobacteria combined with the same insecticide were observed. Thus, when looking at a larger group of stressors, interaction effects of cyanobacteria combined with insecticides cannot always be extrapolated to other cyanobacteria. Correlation analysis confirmed no significant correlations between the different cyanobacteria (Appendix C Table C.4). Cedergreen et al. (2009) studied the reproducibility of binary mixture studies by replicating binary mixture experiments. They concluded increased variability when the complexity of the test organism, e.g. unicellular organisms such as bacteria or algae are less complex than multicellular organisms such as *Daphnia*, increases leading to less reproducible conclusions. Furthermore, Cedergreen and Streibig (2005) also found differences in mixture effects on different endpoints.

Overall, these results in combination with the previous results from chapters 2 and 3 indicate that life history data is insufficient to understand mechanisms of combined and interaction effects. At the life history level, interaction seems a complex trait dependent upon exposure concentration and experimental design. Confounding factors such as variability between experiments and differences in statistics may further complicate the matter. Alternative approaches have been suggested by Borgert et al. (2004) and Jager et al. (2010). Borgert et al. (2004) suggest a thorough characterization of toxicodynamics and kinetics in combinations with the general mode of action of the chemical to help elucidate interaction effects. In contrast, Jager et al. (2010) suggest biology based models that use a dynamic energy budget concept. Yet, both concepts require *a priori* available estimates of parameters and large data sets which is neither always feasible nor available for every toxicant. In addition, both concepts do not focus on a clear molecular understanding of mechanisms which may be crucial given the subtle differences in the data presented here (e.g. different effects for closely related insecticides). In conclusion, an integrative approach using both life history data and molecular data may be a way forward.

4.5 Conclusion

Life history observations across a large set of combinations of cyanobacteria and insecticides indicated interaction to be a complex trait dependent upon several factors. Both exposure concentration and experimental design significantly altered conclusions drawn in relation to combined and interaction effects together with other confounding factors such as biological variability, experimental design and statistics. As a consequence, studying combined and interaction effects at the life history level are insufficient to attain a clear insight in the dynamics and processes leading to interactions. A consolidated approach combining both life history and molecular studies is the next logical step.

Chapter 5

Identification of pathways, gene networks and paralogous gene families in *D. pulex* responding to exposure to the toxic cyanobacterium *Microcystis aeruginosa*.

Redrafted after:

Asselman J, De Coninck DIM, Glaholt S, Colbourne JK, Janssen CR, Shaw JR, De Schamphelaere KAC. 2012. Identification of pathways, gene networks and paralogous gene families in Daphnia pulex responding to exposure to the toxic cyanobacterium Microcystis aeruginosa. Environ Sci Technol 46, 8448-8457.

5.1 Introduction

The most common and best studied cyanobacterium is *Microcystis aeruginosa* (Fristachi and Sinclair, 2008). The effects of *Microcystis* stress on *Daphnia* at physiological and life-history level have been studied since the 1980s (Demott et al., 1991; Gustafsson and Hansson., 2004; Nizan et al., 1986) and have primarily been related to three different factors: lack of essential nutrients such as essential fatty acids or lipids (Haney et al., 1995; Lürling, 2003; Nizan et al., 1986), deterring feeding (Demott et al.,1991, Lürling, 2003), or toxin production (Demott et al., 1991; Lürling, 2003; Rohrlack et al., 1999). Current literature (Lürling, 2003; Rohrlack et al., 1999) remains undecided whether the effect of *Microcystis* on *Daphnia* can be contributed to only one of these factors or a combination of them.

The goal of this chapter was therefore to investigate the effects of cyanobacterial stress, i.e. *M. aeruginosa*, on the transcriptome of *Daphnia pulex*. The transcriptional stress response of *D. pulex* feeding on *M. aeruginosa* will be described by using a comprehensive transcriptome microarray. Such an array will allow identifying pathways or gene networks that characterize the response of *Microcystis* stress. Using microarrays to characterize stress response in *Daphnia* has been done before (e.g.: Poynton et al. (2007, 2008 and 2011), Heckmann et al. (2008) and Soetaert et al. (2007)). All these studies use acute or short-term exposure which contrasts ecological reality where exposure is of a more chronic nature.

In this chapter, microarrays will be implemented to assess chronic toxicity of *M. aeruginosa*. As a consequence, the results of this chapter will not only serve to elucidate the mechanisms of *Microcystis* toxicity but also as a proof-of-principle concept to assess molecular mechanisms of chronic toxicity response in *Daphnia*.

An additional aim of this chapter was to develop a tailor-made bioinformatics pathway pipeline for *Daphnia pulex* microarrays. Such a framework is necessary given the specific characteristics of the *Daphnia* genome (Colbourne et al., 2011): a very high number of lineage specific genes (i.e. they have no detectable sequence homology to genes in any of the current genome databases) and a very high number of duplicated genes.

5.2 Materials and methods

5.2.1 Experimental organisms

Daphnia pulex were obtained from isoclonal laboratory cultures of an isolate, originating from the Basshaunt Lake, Dorset region, Ontario, Canada. Culture conditions were already described in section 2.2.1. Cultures were fed daily with *Ankistrodesmus falcatus* at a rate of 1.5 mg dry weight L^{-1}. For experiments, neonates (< 24 h old) were isolated from unexposed maintenance cultures.

The cyanobacterial strain used was a microcystin producing *Microcystis aeruginosa* strain (UTEX LB2385). Culture conditions were described in section 2.2.1.

5.2.2 Experimental design

Animals were exposed in 1 L polyethylene beakers (18 neonates per beaker) for a period of sixteen days under a constant photoperiod (16:8h light dark) and constant temperature of 20 ± 1°C. Both control and exposed treatments consisted of four biological replicates, i.e. four beakers. All animals were fed with a diet in which the final feeding concentration was 1.5 mg dry weight L^{-1}. The diet of the exposed animals contained 50% of *Microcystis aeruginosa* and 50% of *Ankistrodesmus falcatus*, control diet consisted of 100% *A. falcatus*. This ratio was based on De Schamphelaere et al. (2011), where it resulted in a decline of 50% in reproduction in exposed animals. During the experiment, pH of the media was monitored on regular intervals. At the end of the experiment, animals were isolated for gene expression analysis.

5.2.3 mRNA extraction, labelling and hybridization.

RNA was extracted with the RNeasy kit and Qiashredder (Qiagen, Venlo, Netherlands) following manufacturer's protocol. All animals (18 in total) from one beaker were pooled into one sample and will further be referred to as one biological replicate. DNA contamination was removed by a DNAse treatment (Qiagen, Venlo, Netherlands). RNA quantity and quality were determined with the spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, DE, USA) and with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) respectively. Samples were stored at -80°C until RNA amplification.

The microarray protocol follows detailed instructions by Lopez and Colbourne, 2011. Samples were amplified using a T7-based RNA amplification technique. One microgram of total RNA was amplified with the MessageAmp II aRNA Amplification kit (Ambion, Applied Biosystems, Carlsbad, CA, USA) following manufacturer's protocol. Quantity and quality of the amplified RNA were determined with the spectrophotometer and Bioanalyzer 2100.

Double stranded cDNA was synthetized with SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) following clean up (alkaline hydrolysis and Qiaquick columns, Qiagen). Concentration and integrity of the cDNA were determined with Nanodrop spectrophotometer and Bioanalyzer 2100.

Samples were labeled with Dual-Color DNA Labeling Kit (Roche NimbleGen, Madison, WI, USA) following manufacturers protocol. Quantity and quality of the samples were again determined with the spectrophotometer and Bioanalyzer 2100.

The microarray design (Appendix D Table D.1) consisted of four arrays, each containing two samples, i.e. a control and a *Microcystis* exposed sample. Different biological replicates were used for each array and dye swaps were conducted. All eight labeled samples were pooled according to the design (i.e. one control biological replicate was pooled with one *Microcystis* exposed replicate), resulting in four pools to be hybridized to four arrays. Each pool was dried and resuspended in hybridization buffer according to Roche NimbleGen's User Guide for Expression Analysis for Cy-labeled cDNA derived from Eukaryote systems. Subsequent hybridization of each of these pools on the respective arrays followed the same protocol (Lopez and Colbourne, 2011) and was executed with the NimbleGen Hybridization Kit (Roche NimbleGen, Madison, WI, USA). After hybridization the slides were washed with NimbleGen Hybridization Wash Buffers (Roche NimbleGen, Madison, WI, USA). The microarray itself is a transcriptome array developed by the Centre for Genomics and Bioinformatics (Indiana University, Bloomington, IN, USA) and is in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus under the accession number (GEO: GPL11278). Finally, arrays were scanned with the NimbleGen MS 200 Microarray Scanner to measure fluorescence and images were processed with NimbleScan 2.6 Software and deposited in (GEO: GSE36635).

5.2.4 Image analysis and data processing

Microarray images were analyzed with the statistical software package R (R Development Core Team, 2011, version 3.0.1) and Bioconductor (Gentleman et al., 2004). The LIMMA (Smyth, 2004, version 3.16.7) package was used with additions and modifications according to Colbourne et al. (2011). All signal distributions were quantile normalized across arrays, samples and replicates. Differential expression of a gene was determined based on the mean M-value of probes that represent the gene in question. The M-value for a gene was defined as the log2 ratio of the expression in the exposed animals and the expression of the animals in the control treatment. Linear models were constructed with lmFit function, which fits multiple linear models using least-squares and empirical Bayes Statistics were implemented with eBayes function, which computes moderated t-statistics after empirical Bayes moderation of the standard errors towards a common value (Smyth, 2004). Benjamin-Hochberg method (Benjamini and Hochberg, 1995) was implemented to adjust p-values for multiple testing at a 95% significance level.

5.2.5 Analysis of gene-lists

The analysis of the gene lists was combined with annotation information on each gene available through wFleabase.org (Colbourne et al., 2005), KEGG database (Kanehisa et al., 2010) and KOG (clusters of eukaryotic orthologous groups) database (Tatusov et al., 2003) in R. Annotation information from wFleabase.org including KOG annotation, and enzyme classification (EC) numbers, was downloaded in batch and combined with gene expression lists in R. Annotation information from the KEGG database was obtained with KAAS (Moriya et al., 2007), for which all protein sequences of the draft genome sequence were uploaded to the KAAS server. All results were stored in a txt file for further use in R. Hence, the gene lists were analyzed in three different steps: KOG grouping analysis, pathway analysis and analysis of paralogous gene families. To assess the impact of duplicated genes, both KOG and KEGG analysis were executed once with and once without duplicated genes (i.e. only single copy genes were considered in the latter analysis). Duplicated genes were excluded based on their grouping into a paralog family as defined on wFleabase.org, which has used OrthoMcl (http://wfleabase.org/release1/current_release/gene-predictions/dpulex1_gnomon_ paralog_mcl2ids.tab). KOG analysis was executed based on KOG classification as defined by the Joint Genome Institute (http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Dappu1) where p-value was

calculated with a Fisher`s exact Test (Fisher, 1922) and corrected for multiple testing with the Benjamin-Hochberg method (Benjamini and Hochberg, 1995). Pathway analysis with KEGG reference pathway maps revealed differential expression of pathways, where p-values were calculated with a Fisher`s exact Test and corrected with Benjamin-Hochberg method for multiple testing. KEGGSOAP package (Kanehisa et al., 2010) was used in R to query KEGG databases for full pathway annotation. Pathway analysis was executed with both annotated enzyme classification number and KEGG Orthology (KO) classification as input identifiers. A global metabolic pathway map was created within KEGG through KEGGSOAP from R. In addition, gene lists were analyzed with Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). The input identifier for Ingenuity Pathway Analysis was the UniProt accession number for each gene. Genes from the dataset that had an absolute M-value larger than 1 and a q-value <0.05, were associated with biological functions in the Ingenuity Knowledge Base and were included in the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) a ratio of the number of molecules from the data set that mapped to the pathway divided by the total number of molecules that mapped to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. Significantly expressed genes were screened for the overrepresentation of gene families. All genes were grouped according to annotation information available through wfleabase.org, excluding lineage specific genes as no annotation information was available. The representation of these groups in the genome was compared with their representation within the significantly expressed genes through a Fisher`s exact test. Finally, overrepresentation of paralog families in the differentially expressed (DE) gene set was studied in a similar manner. Again Fisher`s exact test and Benjamin-Hochberg method were used to determine p-values at a 95% significance level.

5.2.6 Validation of the microarray results through quantitative real-time PCR

Microarray results were validated with real-time qPCR. We selected six significantly regulated genes and one reference gene from different pathways/ gene families: trypsin (Dappudraft_224995), ATP-synthase (Dappudraft_230756), apoptosis inducing factor (Dappudraft_327425), neurexin IV (Dappudraft_227614), presenilin 2 (PSEN2) (Dappudraft_306694), serine/threonine kinase (Dappudraft_259493), reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(Dappudraft_302823) based upon Asselman et al. (2012). For each of these genes, three biological replicates, i.e. three independently collected RNA samples, were run in duplicate, i.e. technical replication, on a single qPCR plate. Samples included replicate RNA from the microarray as well as independent biological replicates. RNA was extracted using the same protocols as described above for the microarray samples. Primers were designed with PrimerQuest (IDT technologies, Coralville, IA, USA) and are listed in Appendix D Table D.2. Reverse transcription was conducted with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's protocol. A total of one µg RNA was reverse transcribed using random hexamer primers. Quality of the RNA and cDNA were determined using the same methods as for the microarray samples. Real-time qPCR was conducted with the Roche Lightcycler 480 DNA SYBR Green I Master kit according to manufacturer's protocol on the Roche LightCycler II 480. A total of five µL of each cDNA sample was added to 35 µL of mastermix. Plate design included negative (both no template and no primer controls) and positive controls as well as standard curves with 2-fold dilution series of a single cDNA sample. The amplification steps consisted of 45 cycles (10s at 95°C, 20s at 59°C, 30s at 72°C) preceded by one cycle at 95°C for five minutes and followed by a melt curve analysis. Samples were analyzed with the Roche Lightcycler corresponding software release 1.5.0. Analysis consisted of quality analysis of the melt curves and Ct values for each sample were normalized with the reference gene according to Pfaffl (2001). qPCR results were compared with microarray results with Pearson's Coefficient of Correlation. Assumptions (e.g. normality) were verified prior to using the correlation statistic (Sigmaplot 12, Systat Software).

5.3 Results

At a false discovery rate of 5%, the microarray experiment (GSE36635) revealed 2247 differentially expressed (DE) genes (7.6% of the array) in response to *Microcystis*, of which 17% are lineage specific and 49% are gene duplicates (paralogs) (Fig. 5.1). qPCR confirmed the expression obtained with the microarray, both in magnitude and direction for six DE genes (Fig. 5.2) with a Pearson correlation coefficient of 0.982 ($p<0.01$).

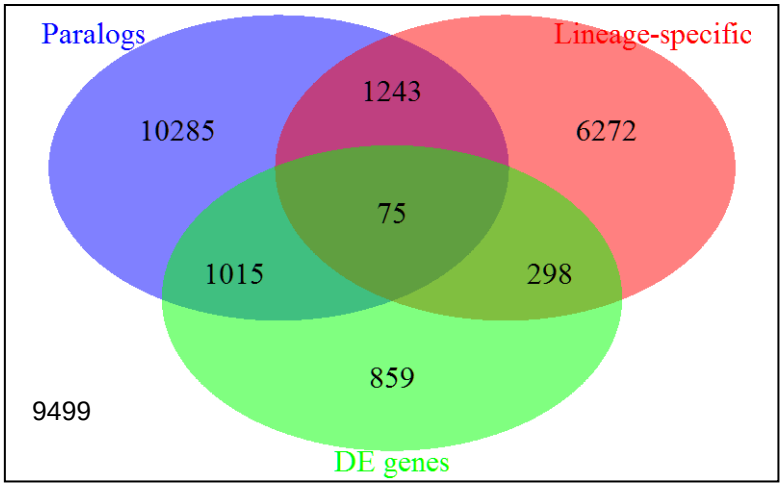


Figure 5.1 Venn diagram of the microarray analysis of all genes (29546) for which probes are printed on the microarray. Lineage-specific genes are genes having no sequence homology to genes in the currently available genome databases. Paralogues are genes assigned to a gene family as defined on wFleabase.org. Differently expressed (DE) genes are defined at a false discovery rate (FDR) of 5%.

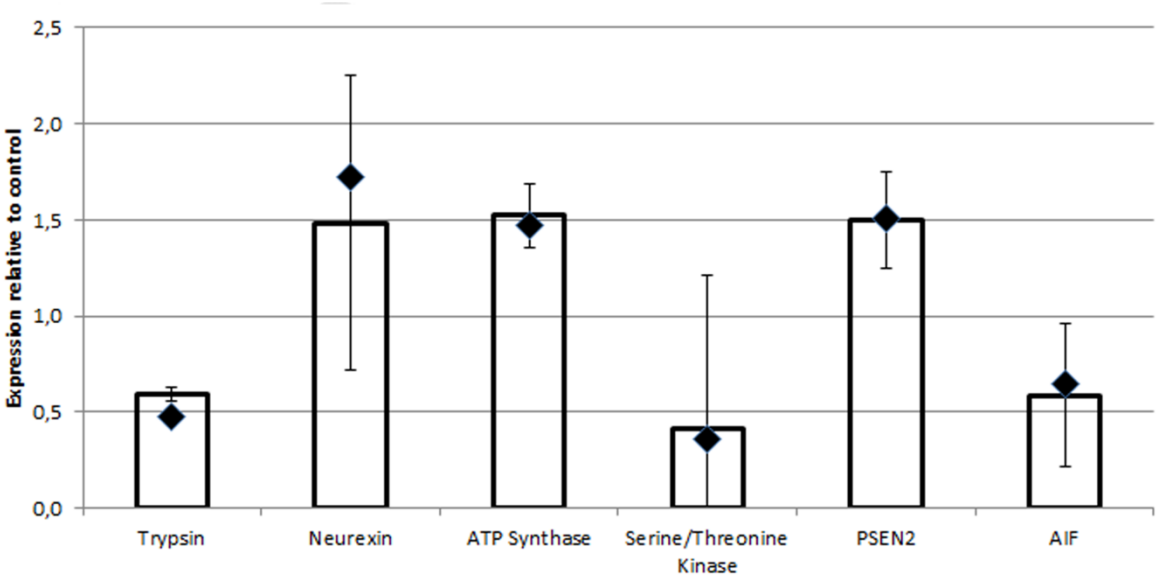


Figure 5.2 Confirmation of the array results with qPCR. Array results are represented with a diamond. Bars represent mean expression determined with qPCR and the corresponding standard deviation for each gene, expression in control samples was set to one.

First, the functional eukaryotic orthology groups (KOG) analysis of these results indicated a complex pattern of over and underrepresentation in the different KOG groups and subgroups (Table 5.1, Appendix D Table D.3). This pattern differed between groups and subgroups. For seven of these KOG subgroups (Table 5.1), results depended on whether or not duplicated genes where included in the analysis. These belonged to three major groups: cellular processes and signaling, information storage

and processing, and metabolism. Six of these were significantly overrepresented by DE genes when accounting for duplicated genes. Five of these KOGs were involved in metabolic functions whereas one was involved in information storage and processing. One other, containing genes involved in cellular processes and signaling, was significantly overrepresented by DE genes when duplicated genes were excluded from the analysis (Table 5.1). This contrasts with the five KOG subgroups where results were significant independent of the method used (Table 5.1). Two of these groups had no known functional annotation, either poorly characterized or lineage specific genes. These differences clearly indicate the importance of accounting for duplicated genes.

Table 5.1. Gene counts, number of differentially expressed (DE at 5% FDR) genes and DE single copy genes in the different KOG groups and significant KOG functions provided by the Joint Genome Institute (http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Dappu1) for all the genes on the array. In each column header, the total number of genes in that gene set is listed. Genes with no KOG are indicated in the final row. KOG functions with proportions differing significantly (p<0.05, based on Fisher`s exact test with multiple testing correction) from the total gene set are indicated with *, p-value is given between parentheses. In addition to the counts, O and U indicate respectively over- and underrepresentation of that group or function in the DE set. Full list of KOG functions is presented in Appendix D Table D.3.

KOG Classification (Function ID)	N° genes (29546)	N° significant genes (2247)		N° significant single copy genes (1157)	
Cellular processes & signaling	5561	518* (p<0.01)	O	245* (p<0.01)	O
1.3 Posttranslational modification, protein turnover, chaperones (O)	1256	114		56* (p=0.02)	O
1.4 Signal transduction mechanisms (T)	2188	219* (p<0.01)	O	91* (p<0.01)	O
Information storage & processing	3261	253		170* (p<0.01)	O
2.3 Translation, ribosomal structure and biogenesis (J)	509	91* (p<0.01)	O	67* (p<0.01)	O
2.5 Replication, recombination and repair (L)	409	16* (p<0.01)	U	8	
Metabolism	3197	389* (p<0.01)	O	111* (p<0.01)	O
3.1 Energy production and conversion (C)	305	45* (p<0.01)	O	26* (p<0.01)	O
3.3 Amino acid transport and metabolism (E)	536	56 * (p=0.03)	O	9	
3.5 Carbohydrate transport and metabolism (G)	636	92* (p<0.01)	O	23	
3.7 Lipid transport and metabolism (I)	489	64* (p<0.01)	O	15	
3.8 Inorganic ion transport and metabolism (P)	309	36* (p=0.03)	O	9	
3.9 Secondary metabolites biosynthesis, transport and catabolism (Q)	167	23* (p=0.01)	O	3	
Poorly Characterized	3527	288		154	
4.2 Function Unknown (S)	1061	113* (p<0.01)	O	73* (p<0.01)	O
No KOG id available	14018	799* (p<0.01)	U	477 * (p<0.01)	U
5.1 Lineage specific genes	7888	373* (p<0.01)	U	298* (p<0.01)	U

Second, we identified four pathways or gene networks as defined by KEGG (Kanehisa et al., 2010) significantly regulated by *Microcystis* exposure, i.e. ribosome, oxidative phosphorylation, mitochondrial dysfunction and protein export (Table 5.2). All significantly affected pathways are enriched by up-regulated genes, although all but the mitochondrial dysfunction did contain at least one gene that was significantly down-regulated (Table 5.2). Furthermore, the number of repressed genes decreased to zero in the ribosome and the oxidative phosphorylation when analyzing the data without the duplicated genes (Table 5.2). Ingenuity Pathway Analysis software (Ingenuity Systems, California, USA), revealed similar p-values (Table 5.2). For all pathways except the protein export, analysis with or without duplicated genes resulted in the same outcome. In contrast, the protein export pathway was only significant when analyzed without accounting for duplicated genes (Table 5.2).

Table 5.2 The significantly regulated pathways (as defined by KEGG (2011) and Ingenuity©): the number of genes annotated to pathways, number of significantly upregulated and downregulated genes after *Microcystis* exposure and the p-value of enrichment tests for the pathway. P-values in parentheses are determined using Ingenuity©. Analysis was executed with and without duplicates (denoted as with/without). Maximum, minimum and median values are given for the number of genes in the pathway.

Pathway	N° of genes in pathway	N° of genes (q<0.05 & M>0)	N° of genes (q<0.05 & M<0)	P-value	Maximum M-value	Minimum M-value	Median of M-value
Ribosome	351/169	50/41	8/0	<0.01/<0.01 (<0.01/0.01)	1.67/1.37	-1.32/-0.97	0.80/0.33
Oxidative phosphorylation	148/96	30/26	1/0	<0.01/<0.01 (<0.01/<0.01)	1.30/1.30	-1.11/-0.46	0.81/0.38
Mitochondrial Dysfunction	107/66	24/22	0/0	<0.01/<0.01 (<0.01/<0.01)	1.16/1.16	-0.44/-0.23	0.38/0.55
Protein Export	61/31	8/8	3/1	0.12/<0.01 (0.15/<0.01)	1.37/1.37	-0.97/-0.88	0.01/0.23

Third, analysis of all 2356 paralog clusters, including lineage specific genes, resulted in six clusters that were significantly overrepresented in the DE gene set (Table 5.3). In contrast, the singly copy genes were underrepresented in the DE gene set (Table 5.3). Also, we observed almost no up-regulated genes in these six clusters, whereas the majority of the singly copy DE genes were up-regulated (Table 5.3).

Table 5.3 Representation of paralogous gene families for which a significant number of genes were differentially regulated (Fisher`s exact test, p<0.05): listing the total numbers of genes in the families in the genome, numbers of significantly up-regulated genes (M>0) and down-regulated (M<0) genes. For each gene family maximum, minimum and median M-value are represented. Lineage specific genes are represented between parentheses. Grouping of gene families was definied by wfleabase, using OrthoMcl (http://wfleabase.org/release1/current_release/genepredictions/dpulex1_gnomon_paralog_mcl2ids.tab).

Paralog Cluster ID	N° of genes in the genome	N° of genes (q<0.05 & M>0)	N° of genes (q<0.05 & M<0)	P-value	Maximum M-value	Minimum M-value	Median of M-value
Omcl0	169(11)	6	27(1)	<0.01	1.83	-2.06	-0.28
Omcl242	11(1)	0	6	0.02	0.03	-2.14	-1.15
Omcl485	6(0)	0	5	<0.01	0.40	-1.08	-0.76
Omcl6	76(7)	1	23(3)	<0.01	0.52	-1.30	-0.44
Omcl61	28(0)	0	12	<0.01	0.00	-1.29	-0.76
Omcl8	82(13)	1	28(3)	<0.01	0.70	-0.94	-0.38
Single Copy genes	16928(6570)	718 (169)	439(129)	<0.01	3.29	-2.77	0.02

Last, out of a total of 4354 annotated paralogous gene families, we observed eight paralogous gene families where a significant majority of the genes were differentially regulated by *Microcystis* exposure (Table 5.4, Appendix D Tables D.4-D.11). We observed gene families related to protein metabolism, energy metabolism, signal transduction, programmed cell death and the digestive system (Table 5.4).

Table 5.4. Representation of paralogous annotated gene families for which a significant number of genes were both in magnitude and direction differentially regulated (Fisher`s exact test, p<0.05): listing the total numbers of the genes within families in the genome, numbers of significantly up-regulated genes (M>0) and down-regulated (M<0) genes. For each gene family maximum, minimum and median M-value are represented.

Gene Function	N° of genes in the genome	N° of genes (q<0.05 & M>0)	N° of genes (q<0.05 & M<0)	P-value	Maximum M-value	Minimum M-value	Median of M-value
Serine/threonine protein kinase	66	1	13	<0.01	1.15	-1.17	-0.40
40S Ribosomal protein	34	16	0	<0.01	1.32	0.64	0.87
60S Ribosomal protein	48	16	0	<0.01	1.21	0.62	0.81
Mitochondrial ribosomal protein	53	31	0	<0.01	1.19	0.62	0.8
NADH:ubiquinone oxidoreductase	26	11	0	<0.01	1.04	0.67	0.82
Neurexin IV	50	2	13	<0.01	1.41	-2.06	-1.01
Apoptosis Inducing Factor	25	0	11	<0.01	-0.63	-0.87	-0.74
Trypsin	255	12	20	0.02	1.86	-2.33	-0.1

5.4 Discussion

In this chapter, microarray technology was implemented to study the response of the recently sequenced micro-crustacean *D. pulex* exposed to the environmental stressor *Microcystis aeruginosa*.

Four pathways/gene networks (Table 5.2) and eight paralogous gene families (Table 5.4) were affected by *Microcystis* and correspond with the significant over or underrepresentation of KOG groups in the differentially expressed gene set (Table 5.1). These KOG groups indicate a broad range of functional networks that are potentially affected by *Microcystis*. Yet, the identified pathways/gene networks are essential to understand interactions and relations among genes through their responses in these pathways and networks and to identify primary mechanisms in the stress response. The expression pattern of representative genes in these identified pathways and families were validated by qPCR (Fig. 5.2), which confirms the validity of the conclusions from the microarray analysis and emphasizes the potential of this technology in environmental genomics. In addition, the fact that different results (Table 5.1-5.2) were obtained using different analysis methods, tailored to account for

the unique structure of the *D. pulex* genome (i.e. duplicated genes), emphasizes the necessity for these methods. Indeed, the elevated number of paralogous gene families in the *D. pulex* genome had a clear impact on the analysis of the list of the DE genes and should be taken into account in further gene transcription studies with this species. Collectively, these results demonstrate that duplicated genes can either be those that are primarily responsive to a stressor (Table 5.1, KOG groups 2.5, 3.3, 3.5, 3.7, 3.8, 3.9) or they can mask potential effects on singly copy genes if they are less responsive (Table 5.1, KOG group 1.3). Indeed, in small pathways with few enzymes, duplicated genes, all encoding the same enzyme, can mask potential effect of the single copy genes which are smaller in number.

Four pathways/networks were identified that are significantly overrepresented after chronic *Microcystis* exposure. First, differential regulation of the ribosome (Table 5.1, KOG group 2.4; Table 5.2), including three DE paralogous gene families in this network (40S, 60S and mitochondrial ribosomal proteins; Table 5.4, Appendix D Tables D.5-D.7), suggests an impact of Microcystis on protein synthesis of *D. pulex*. The differential regulation of ribosomes in *D. pulex* has already been observed after exposure to stressful conditions such as metal stress, oxidative stress, and carbamates (Pereira et al., 2010; Vandegehuchte et al., 2010). Interestingly, the direction of the expression in these studies is at odds with the results here, in which the majority of the genes of network and all paralogous gene families were upregulated, whereas the other studies observed downregulation. Nevertheless, the significant overrepresentation observed here does indicate a clear impact on the protein synthesis. Furthermore, Pereira et al. (2010) observed a downregulation of the ribosomes upon exposure to the insecticide methomyl, which belongs to the same family as carbaryl, i.e. the carbamates. Indeed, these findings could be a potential explanation for the observed antagonistic interaction between *Microcystis* and carbaryl in chapter 3 as a carbamate and *Microcystis* both affect the ribosome but in the opposite direction. This off course depends upon whether the effects from methomyl can be extrapolated to carbaryl.

The second identified pathway/network is the oxidative phosphorylation, involved in energy production and conversion (Table 5.1 KOG group 3.1, Table 5.2) and including all genes of the paralogous gene family encoding NADH:ubiquinone oxidoreductases (Table 5.4, Appendix D Table D.8). A plausible explanation for the overrepresentation of genes in the oxidative phosphorylation is the additional requirement for energy of the organism, because of a general stress response. A general stress

response has often been observed upon exposing *Daphnia* to a variety of oxidative stressors, e.g. paraquat, cadmium, UV-radiation (Shaw et al., 2007; Barata et al., 2005; Poynton et al., 2007; Vega and Pizarro, 2000) and is also a major response mechanism following microcystin exposure (Campos and Vasconcelos, 2010; Amado and Monserrat, 2010). This leads to the hypothesis that *D. pulex* requires additional energy to cope with the *Microcystis* stress, for instance to support increased protein synthesis (cfr up-regulated ribosomes, up-regulated protein export, Table 5.2), or to cope with misfolded proteins (Table 5.1 KOG group 1.3, Table 3 serine/protein kinases). In addition, readers are referred to the review by Amado and Monserrat (2010), who provide an overview of oxidative stress related to microcystin exposure in several aquatic species. Based on a compilation of evidence, these authors postulated an interacting mechanism between oxidative stress and glutathione-S-transferase levels in the cell that consequently affects the mitochondria. Here, one out of the twelve glutathione-S-transferases was up-regulated, yet it was the only one belonging to KOG cluster KOG0868 (Appendix D Table D.12). This points to a differential response of genes with the same protein annotation, yet belonging to a different KOG cluster, to an environmental stressor and suggests different roles for these glutathione-S-transferases in stress response. These results show how future studies investigating gene responses under a variety of environmental stress conditions can help to ecologically annotate genes in expanded gene families, of which glutathione-S-transferases are just one example.

The third significantly overrepresented pathway was the mitochondrial dysfunction pathway. This pathway in combination with the effects on the oxidative phosphorylation suggests another possible reason for the effects on the latter. *Microcystis*, and more specifically microcystins, are known to affect mitochondria and the oxidative phosphorylation. Several studies have investigated these effects in a wide range of species (e.g. rats, rabbits, bighead carp, goldfish) (Zhao et al., 2008; Qui et al., 2009; La-Salete et al., 2008; Ding et al., 1998; Zhang et al., 2007; Li et al., 2005). Campos and Vasconcelos (2010) summarized current literature and postulated a general mechanism of microcystin toxicity to mitochondria, yet the exact target and interacting proteins leading to these effects remain unknown. La-Salete et al. (2008) observed a decrease and inhibition of the mitochondrial membrane potential as the result of an interaction of the oxidative phosphorylation with microcystins when rat kidneys were exposed to microcystins. A study with *Daphnia magna* (Chen et al., 2005) exposed to pure microcystin-LR demonstrated broken and blurry mitochondria. These observations correspond well

with the results in this chapter, i.e. effects on the oxidative phosphorylation pathway and on the mitochondrial dysfunction pathway (Table 5.2). The dysfunctioning of mitochondria - and more specifically complex I to which the NADH:ubiquinone oxidoreductase paralogous gene family belongs (Table 5.4) - is often associated with generation of reactive oxygen species as well as the activation of the mitochondrial apoptosis-inducing pathway (Chomova and Racay, 2010). Although induced apoptosis in mitochondria is often associated with microcystin exposure in a variety of species (Campos and Vasconcelos, 2010; Vega and Pizarro, 2000), here eleven apoptosis inducing factors were significantly down-regulated (Table 5.4, Appendix D Table D.10). These apoptosis inducing factors have a wide range of functions, including scavenging free radicals and inducing apoptosis, depending on the environmental conditions. The mechanisms behind these functions are tightly regulated by pro- and anti-apoptotic proteins that control the release of apoptosis inducing factors and thus the subsequent induction of apoptosis pathways (Saelens et al., 2004). For a thorough explanation we refer to the available literature (Vega and Pizarro, 2000; Saelens et al., 2004). Here, pro- and anti-apoptotic proteins, i.e. Bcl2, Bax and apoptosis inhibitors (IAP), were not significantly regulated (Appendix D Table D.13). Differential regulation of these proteins would induce programmed cell death pathways, resulting in the release of, among others, apoptosis inducing factors (Vega and Pizarro, 2010). The lack of DE of these proteins suggests that the DE of the apoptosis inducing factors is not correlated with the function in programmed cell death. A potential hypothesis can therefore be that their differential expression is more related to their function in oxidative stress, i.e. scavenging free radicals than in apoptosis. In addition, both explanations put forward above for the DE of the oxidative phosphorylation may be complementary and the overall impact may well be an interaction between the two.

In addition to the gene network analysis, the representation of annotated paralogous gene families in the DE gene list was analyzed (Table 5.4). Paralogous gene families are of particular interest as it has been suggested that the maintenance of these duplicated genes over the course of evolution is non-random in *Daphnia* (Colbourne et al., 2011). Moreover, in some cases, it has been shown that members of the same gene family can respond differently to environmental stress (Table 5.4, Colbourne et al., 2011). Thus, studying such paralogous gene families under a broad range of environmental conditions will provide essential information of the functional consequences of gene duplication. This analysis returned eight paralogous gene families that are overrepresented in the DE

gene list. Five of these could be associated with the pathways and networks in Table 5.2 and have already been discussed above. Three others are serine/threonine protein kinases, neurexin IV and trypsins. First, based on their KOG ID, serine/threonine protein kinases (KOG1027, Appendix D Table D.4) are sensors of the unfolded protein response pathway. They play a primordial role in the homeostatic regulation of protein folding as well as the stress response to cope with an increased number of unfolded proteins (Walter and Ron, 2011). The differential expression of these genes suggests an impact of *Microcystis* on protein folding and potential accumulation of misfolded proteins. This is supported by the observed significant overrepresentation of single copy genes involved in posttranslational modification, protein turnover and chaperones (Table 5.1). Second, neurexin IV proteins (Table 5.4, Appendix D Table D.9, KOG3516) are involved in signal transduction mechanisms (Table 5.1 KOG group 1.4). Studies on Neurexin IV, in *Drosophila melanogaster*, have detailed the importance of this protein in the nervous system, more specifically in adhesive cell-cell contact (Baumgartner et al., 1996; Stork et al., 2009). The DE of a part of this gene family is a potential indication of divergent roles for the members of this gene family. Finally, trypsins (Table 5.4, Appendix D Table D.11, KOG3627) are involved in the amino acid transport and metabolism (Table 5.1: KOG group 3.3). Aeruginosins, toxins produced by *M. aeruginosa* (Cadel-Six et al., 2008; Ishida et al., 1999), are known to inhibit the serine proteases such as trypsins at the protein level through direct interaction with the protein (Ishida et al., 1999). Inhibition of trypsins is supported by a study of Czarnecki et al. (2006), who also reported inhibited trypsin activity by *Microcystis* in *Daphnia*. Trypsins represent one of the most important components in the digestive system in *Daphnia* (Schwarzenberger et al., 2010). In addition, it has been suggested (Czarnecki et al., 2006) that *Microcystis* strains could strongly inhibit digestive activity in *Daphnia* through the inhibition of trypsins. As a result, this could lead to reduced food assimilation, as demonstrated by Rohrlack et al. (2004). Here, DE genes encoding trypsins were regulated in both directions, i.e. up-regulation and down-regulation. Although, DE at the transcriptional level cannot be straightforwardly related to effects at the protein level, Agrawal et al. (2005) and Schwarzenberger et al. (2010) did show differential sensitivity of *Daphnia* trypsins to *Microcystis* strains at both RNA and protein level. More precisely, they showed inhibition of certain trypsins, but also an increased activity of non-inhibited trypsins. Additional research is needed to test if effects of *M. aeruginosa* at the protein level correlate with effects on the transcription of these trypsins.

Finally, thirty-five percent of the *D. pulex* genome contains lineage specific genes with no detectable homolog to any gene in the current databases (Colbourne et al., 2011). Here, a total of 273 DE lineage-specific genes (Fig. 5.1), of which 75 were member of a paralogous gene family, were observed. To gain information about these lineage specific genes, the overrepresentation of all paralogous gene families in the DE gene list was studied. These results (Table 5.3) indicated six overrepresented clusters, of which four contained lineage specific genes. The majority of the genes in these clusters were repressed after *M. aeruginosa* exposure, including the lineage specific genes. Although the precise functions of these DE lineage-specific genes in these clusters still remain unknown, it can at least be concluded that their response to *M. aeruginosa* is similar to the response of the majority of the genes in that cluster. Using this type of information, from exposures to a broad range of environmental conditions, could aid the future annotation of these lineage specific genes.

5.5 Conclusion

In conclusion, the implementation of whole transcriptome microarray technology to study the response to the natural stressor *Microcystis aeruginosa* in *Daphnia pulex* resulted in identifying a characteristic stress response pattern. This pattern consisted of four major pathways/ gene networks as well as eight paralogous gene families that were significantly affected. Some of these could explain why fitness is reduced (e.g. oxidative phosphorylation, trypsins) based on energy budget considerations. For others, a link with fitness remains to be established.

Moreover, this chapter underlines the need to take into account the specific and unique *D. pulex* genome structure in expression studies, because it contains an elevated number of duplicated genes as well as lineage specific genes which may influence conclusions drawn. Finally, this chapter suggested a first approach to start functionally annotating these genes in environmentally relevant conditions. Further advancement of molecular tools in *D. pulex* research would support such an effort.

Chapter 6

General and specific molecular mechanisms of cyanobacterial toxicity in *Daphnia pulex*

6.1 Introduction

The evolution in molecular technologies has propelled ecological and environmental research to tackle longstanding hypotheses with a new biological level of understanding. This evolution has greatly benefitted *Daphnia*, now emerging as a true model organism in ecological and environmental genomics (Ebert, 2011). The well-known ecology and unique genomic structure, described in detail in section 1.4, make *Daphnia* the perfect model of study. Gene expression analysis conducted in chapter 5 highlighted that *Daphnia`s* lineage-specific genes are susceptible to ecological conditions and that gene duplicates demonstrate divergent expression patterns. This tight interaction between the genome and the environment has made *Daphnia* particularly suitable to study such interactions.

Genome-environment interactions become increasingly complex with regards to biotic stressors. These stressors, in particular cyanobacteria, can often not be straightforwardly linked to a single molecular target or mode of action. Cyanobacteria are a complex form of stress as they can produce toxins, which can be compared to chemical toxicants, but they also can inhibit feeding responses and serve as a food source for zooplankton species. As discussed in previous chapters (chapters 1-3), interactions between cyanobacteria and *Daphnia* have been studied extensively. Yet, despite the extensive research, no conclusive mechanism of cyanobacterial stress has been put forward (Rohrlack et al., 1999; Schwarzenberger et al., 2010; Von Elert et al., 2003). Results from chapters 2-4 suggest a novel approach is necessary.

Systems biology approaches have been successfully applied in the past with *Daphnia* (chapter 5, Heckmann et al., 2008; Latta et al., 2012) to link molecular responses to higher organismal responses. The previous chapter identified main mechanisms of stress in *Daphnia pulex* exposed to *M. aeruginosa*. Here, gene expression analysis will be applied in a wider context by focusing on five different cyanobacterial species and their potential effects on *Daphnia pulex*. The aim of the chapter is to answer two crucial questions by formulating the following research hypotheses. First, transcriptomic profiles of *D. pulex* exposed to different cyanobacteria can identify mechanisms of cyanobacterial toxicity. Transcriptomic profiles are being increasingly used to identify the stress/chemical an organism was exposed to. Indeed, Antczak et al. (2013) have implemented machine learning methods to distinguish between different classes of chemical. These machine learning methods may help environmental risk assessment by identifying the chemicals or stressors that are causing adverse

effects. Second, transcriptomic profiles can distinguish between species-specific and general cyanobacterial stress responses. Current identification and classification focusses on identifying the general stress response for certain groups of chemicals, i.e. anti-inflammatory drugs (Heckmann et al., 2008) or narcotics (Dom et al., 2012). Less attention is given to the potential differences between general responses indicative of a group of stressors and specific responses unique to each stressor within that same group. From a systems biology perspective, these questions will be answered at the gene level and at the higher functional level of gene annotations and pathways. The integration of these two levels is crucial to fully link molecular responses to higher level effects.

6.2 Material and methods

6.2.1 Experimental organisms

The experimental organisms originated from *D. pulex* cultures as described in section 2.2.1. Likewise, cyanobacteria culture conditions (i.e. for *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia* and *Oscillatoria*) were also described in section 2.2.1.

6.2.2 Experimental design

All exposures were conducted as described in section 4.2.2 to ensure enough RNA material for all hybridizations, a control treatment was set up for each cyanobacteria treatment. Each treatment consisted of four biological replicates. For each cyanobacterium, exposures were conducted twice, i.e. the entire experimental set-up was repeated independently, which resulted in a total of eight biological samples or replicates per treatment.

6.2.3 Gene expression analysis

Gene expression patterns were assessed following detailed procedures described in section 5.2.3 and section 5.2.4. Briefly, RNA was extracted, amplified and reverse transcribed to cDNA. Samples were hybridized to whole transcriptome Nimblegen arrays. For each cyanobacterium, treatment samples were hybridized together with control samples to allow for direct comparisons. Per exposure, eight samples, four controls and four treatments, were hybridized which results in sixteen samples or eight comparisons per cyanobacteria, including dye swaps, as experiments were duplicated. Data was analyzed in LIMMA (version 3.16.7) which constructed linear models with least-squares and calculated

moderated t-statistics after empirical Bayes moderation of the standard errors. Analysis was conducted on all data simultaneously and a gene expression list was generated for each cyanobacterium, containing relative expression values (M-values), i.e. $\log_2$ expression in the treatment versus the $\log_2$ expression in the control, and q-values, Benjamini-Hochberg corrected p-values.

6.2.4 Analysis of gene expression lists

The analysis of the five gene expression lists or profiles, one for each cyanobacterium, continued in R (version 3.0.1), where they were combined with annotation information about each gene as available through wfleabase.org (Colbourne et al., 2005). A schematic diagram of the analysis can be found in Fig. 6.1. For each expression profile, significant genes, defined as having a q-value, i.e. Benjamini-Hochberg corrected p-value, smaller than 0.05, with their full annotation information were extracted from the expression profiles for further analysis. Venn diagrams were then constructed with the R package Venn diagram (version 1.6.5) to determine the number of shared genes, shared functional annotations and shared gene ontology (GO) terms within the lists of significant genes. GO terms and functional annotations were defined as shared when there was at least one gene present with that functional annotation or GO term in each of the five lists. Concordant and discordant expression of the functional annotations was determined by bootstrapping all significant gene lists and randomly assigning genes to each functional annotation. Concordant expression means that the variation in expression of the genes in the functional annotation under study is smaller than expected. Disconcordant expression means that the variation in expression of the genes in the functional annotation under study is larger than expected. Therefore, the means of relative gene expression (M-values) were first calculated for each functional annotation for each gene list, resulting in five means for each functional annotation. Second, an overall standard deviation was calculated from the five means for each functional annotation. This standard deviation was compared with the standard deviation generated by bootstrapping. If the actual standard deviation of the functional annotation fell outside the 95% confidence interval of the bootstrap data, expression was determined concordant or discordant. Concordant expression was defined as having a standard deviation lower than the 2.5 percentile, whereas disconcordant expression was defined as having a standard deviation higher than the 97.5 percentile. The bootstrap procedure was conducted only on significant genes. For each functional annotation within the significant genes, the number of genes with that functional annotation was determined. The same number of genes was then selected at random from the significant genes.

This procedure was done for all the functional annotations within the significant gene list. The mean and the standard deviations were then determined in the exact same manner as for the actual data. The entire bootstrap procedure was repeated 1000 times which resulted in 1000 standard deviations for each functional annotation.

In addition to the analysis represented in Fig. 6.1, gene lists were also analyzed for enrichment of pathways as described in section 5.2.5.

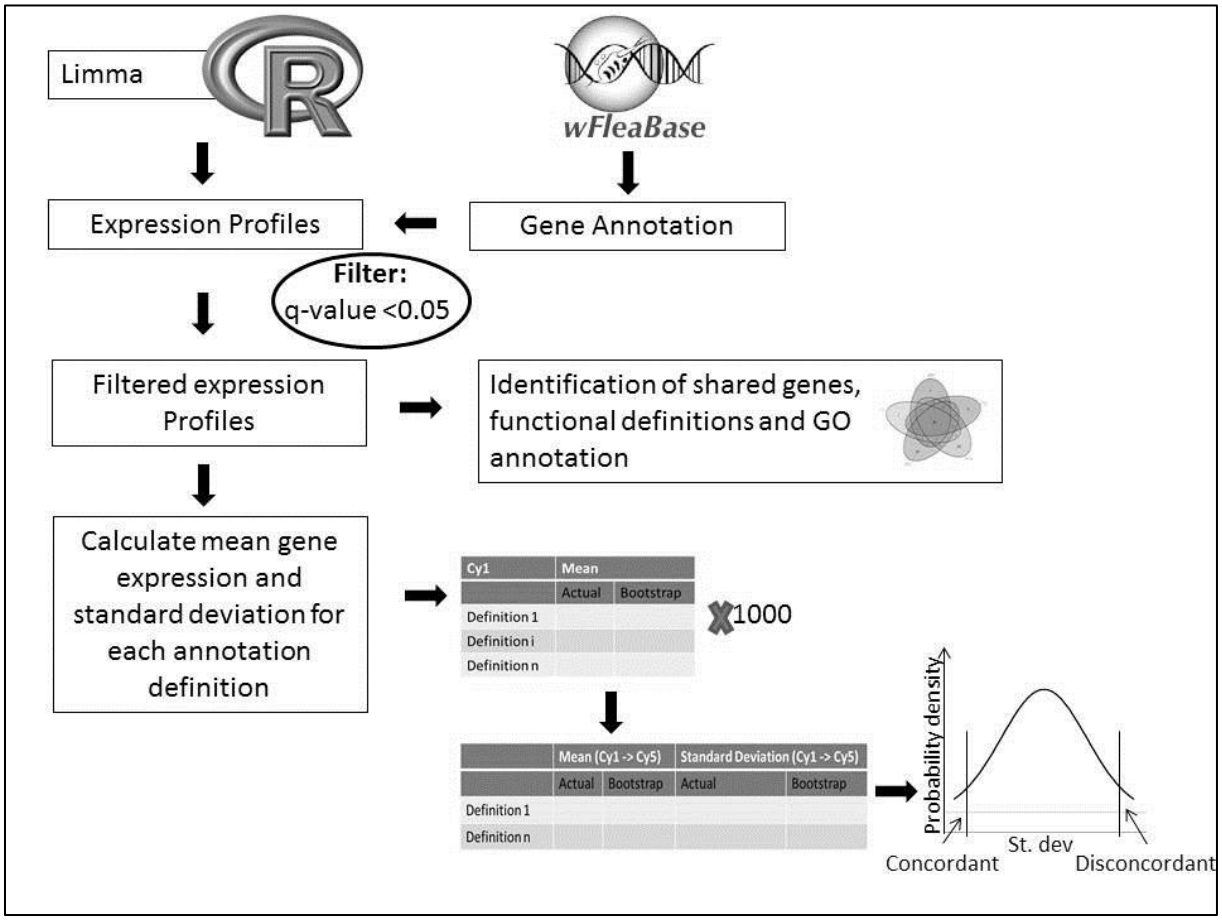


Figure 6.1 Schematic representation of the analysis of expression profiles of Daphnia following exposure to cyanobacteria (Cy). GO= gene ontology, St. dev. = standard deviation.

6.3 Results and discussion

Transcriptomic profiles of *Daphnia* exposed to the five cyanobacteria identified distinct responses to each cyanobacterium (Fig. 6.2). Few genes, i.e. 22, were significantly regulated in all cyanobacterial exposures (Fig. 6.3, Appendix E Table E.1). The functional annotations of shared significantly regulated genes were diverse ranging from cytochrome P450 to trypsins and neurexins (Appendix E Table E.1). They also corresponded well with the functions of significantly regulated genes in response to *Microcystis aeruginosa* (section 5.3). Out of the 22 shared genes, 12 were shared with the transcriptomic profile of *Daphnia pulex* exposed to *Microcystis aeruginosa* described in section 5.3 (Appendix E Table E.1). All 22 shared genes had positive M-values (Fig. 6.3) indicating induction of gene expression upon exposure to cyanobacteria, i.e. expression in the exposure was higher than in the control. The majority of the genes had an M-value between 1 and 1.5. Five of the 22 shared genes had an M-value larger than 1.5 whereas five others had an M-value smaller than 1.

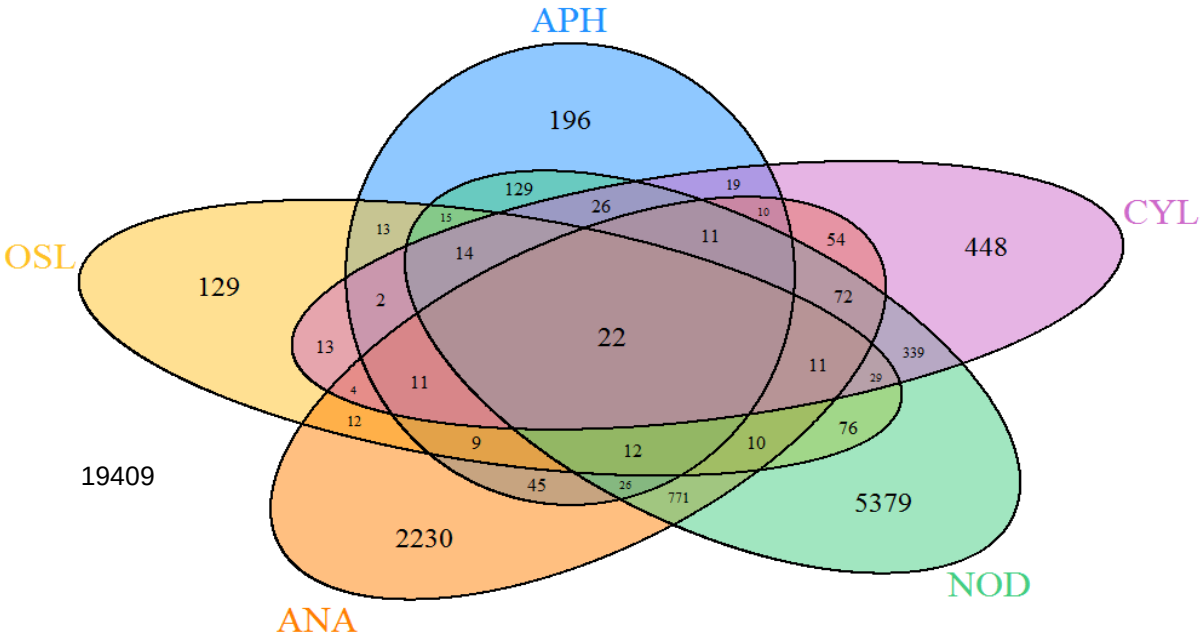


Figure 6.2 Venn diagram of genes significantly regulated (q-value<0.05) by each of the five cyanobacteria compared to control conditions. The sum of the numbers of genes in the subsets of each oval is the total number of genes significantly regulated by the cyanobacterium corresponding to that oval. 19409 genes were not significantly regulated by any of the cyanobacteria. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

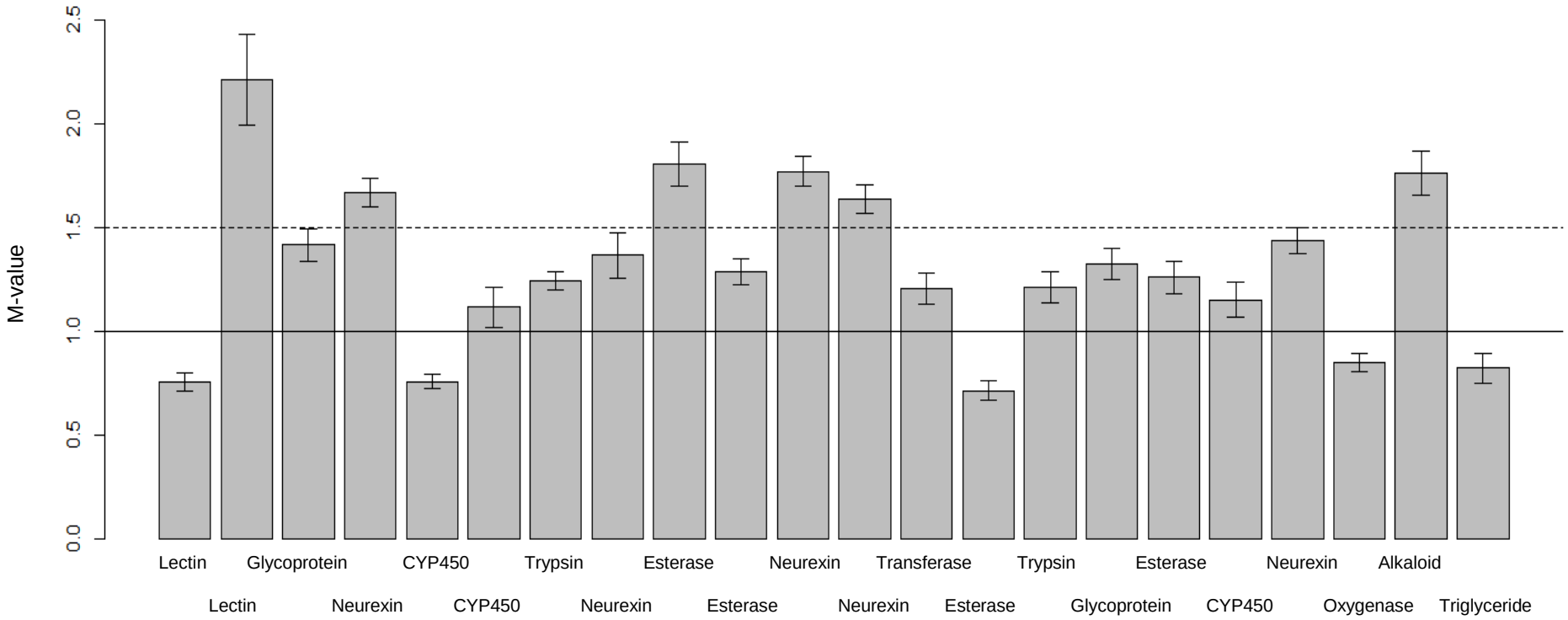


Figure 6.3 Significant genes (q-value<0.05) shared among all transcriptomic profiles. The mean M-value (log_2 (Cyano)/(Control)) across all five transcriptomic profiles is plotted, error bars represent standard deviation per gene across the five profiles. Gene IDs can be found in Appendix E Table E.1.

Although few genes were shared by the different cyanobacterial treatments, a high number of functional annotations were shared by the cyanobacterial treatments. Analysis of the number of functional annotations and gene ontology (GO) terms in the significant gene lists, revealed 56 functional annotations and 80 GO terms within the significant gene lists that were shared among all five cyanobacterial treatments (Fig. 6.4, Fig. 6.5). The shared functional annotations comprised a variety of functions such as chitinase, collagen, cytochrome P450 and glutathione-S-transferase (Appendix E Table E.2). These annotations corresponded well with the shared GO terms (Appendix E Table E.3). Further analysis revealed that functional annotations and GO terms shared by all cyanobacterial treatments covered on average more significant genes per annotation or GO term than unique annotations or GO terms, which suggests an overrepresentation of duplicated genes (Table 6.1).

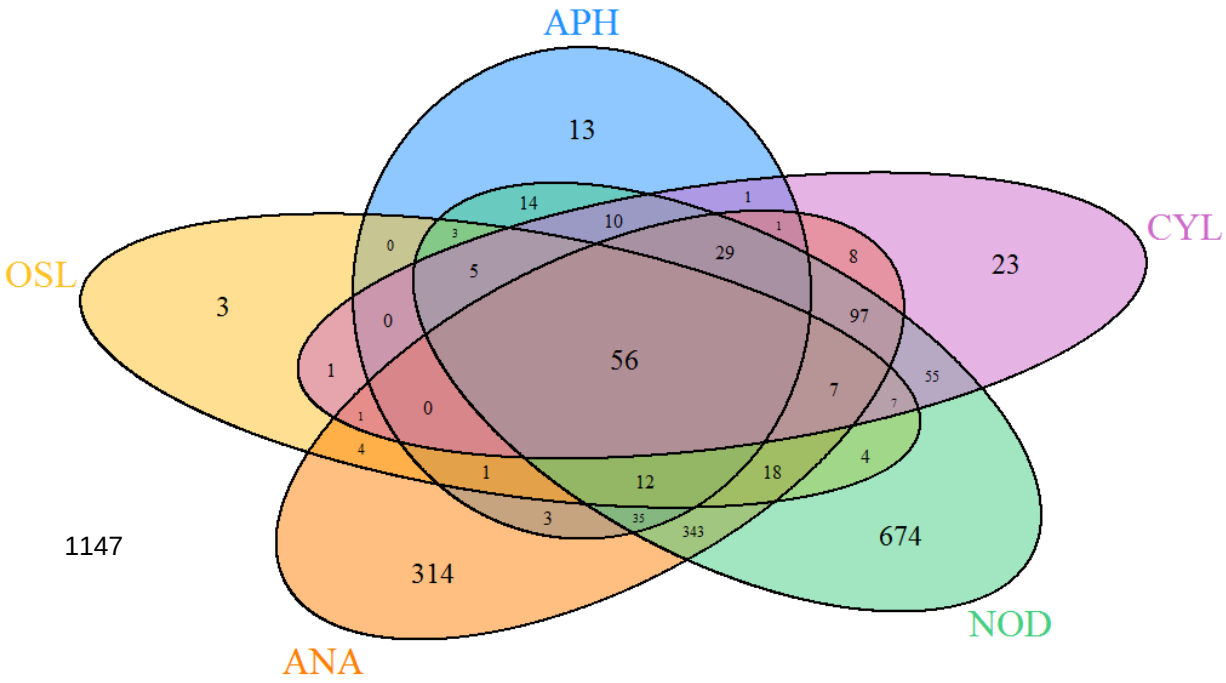


Figure 6.4 Venn diagram of functional annotations shared by all five cyanobacteria treatments derived from the significant genes in each of the five treatments (q-value<0.05). The sum of the numbers of functional annotations in the subsets of each oval is the total number of functional annotations that could be matched to the significantly regulated genes upon exposure to the cyanobacteria corresponding to that oval. 1147 annotation definitions were not matched to any of the significant genes in any cyanobacterial treatment. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

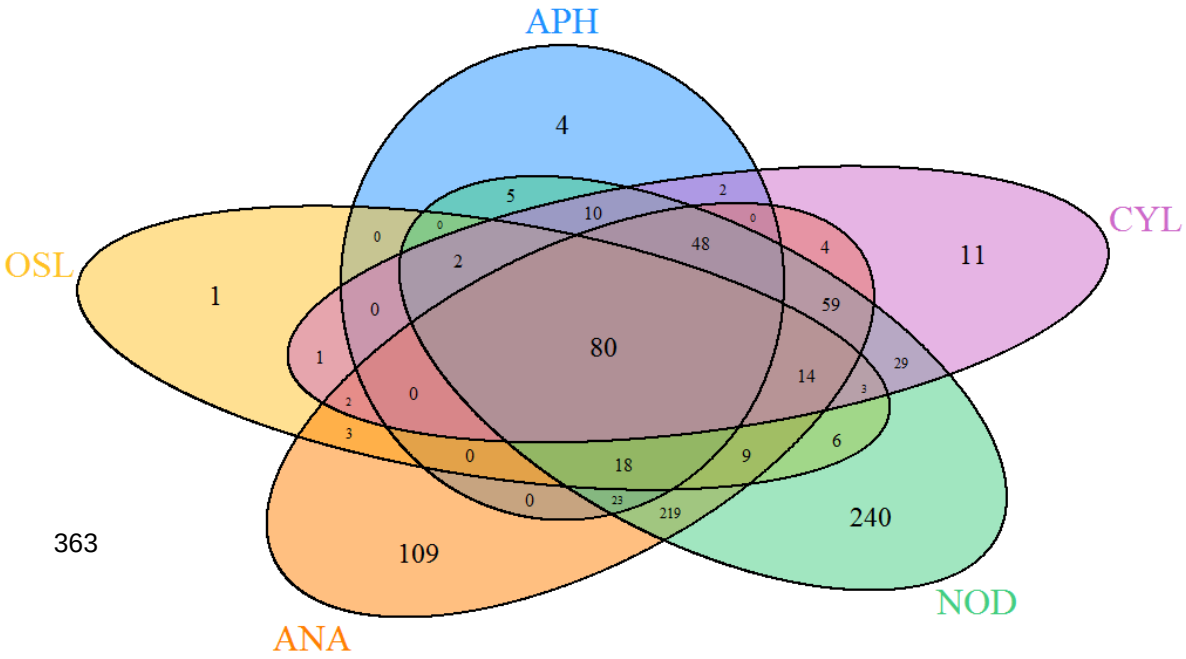


Figure 6.5 Venn diagram of shared gene ontology (GO) terms by all five cyanobacteria treatments derived from the significant genes in each of the five treatments (q-value<0.05). The sum of the numbers of GO terms in the subsets of each oval is the total number of GO terms of the genes significantly regulated by each of the five cyanobacteria. 363 GO terms were not matched to any of the significant genes in any cyanobacterial treatment. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

Table 6.1 Number of significant (sig) genes (q-value<0.05) with functional annotations or Gene Ontology (GO) terms that are shared by all cyanobacterial treatments and with functional annotations or GO terms unique for each cyanobacterial treatment. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

	APH	OSL	ANA	NOD	CYL
N° of sig genes in 56 functional annotations shared with all treatments	207	171	472	908	270
Average number of genes per shared functional annotation	4	3	8	16	5
N° of sig genes within unique functional annotations for each treatment	18	3	372	1013	25
Average number of genes per unique functional annotation	1	1	1	2	1
N° of sig genes with annotation definition	377	251	2180	4201	644
N° of sig genes in 80 GO term shared with all treatments	200	184	1013	2134	344
Average number of genes per GO term	2	2	10	22	3
N° of sig genes within GO term unique for each treatment	2	1	95	234	8
Average number of genes per GO term	0.5	1	0.8	0.9	0.7
N° of sig genes with a GO term	258	197	1236	2532	410

The discrepancy between the number of shared genes, only 22, on one hand and the number of shared functional annotations (56) and GO terms (80) on the other hand, could in part be attributed to the high number of duplicated genes within the *Daphnia* genome (Colbourne et al., 2011). In particular, the expression of paralogous genes may differ between the different treatments but due to their close sequence similarity, they are still assigned to the same functional annotation and GO term. Colbourne et al. (2011) noted a condition-specific diversification of expression patterns. Here, a similar conclusion could be made based on the high percentage of duplicated genes within functional annotations shared by all cyanobacterial treatments (Table 6.2).

Table 6.2 Proportions of duplicated genes within different types of significant genes (q-value <0.05) having a functional annotation, calculated for each cyanobacterial treatment separately. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

Groups:	APH	OSL	ANA	NOD	CYL
% of duplicated genes within all functional annotations	65.9%	67.7%	72.1%	81.0%	70.2%
% of duplicated genes within functional annotations shared by all profiles	88.4%	83.6%	97.7%	99.6%	93.7%
% of duplicated genes within unique functional annotations	22.2%	0%	26.1%	47.9%	16.0%

Further analysis of the data on duplicated and non-duplicated genes separately supported the hypothesis that the discrepancy between the number of shared genes and the number of shared functional annotations can be attributed to paralogous genes. Indeed, paralogous genes were more likely to be shared between the cyanobacterial treatments than non-duplicated genes (Table 6.3, Appendix E Table E.4). In contrast, non-duplicated genes had a higher chance of being unique to only one of the cyanobacteria treatments than duplicated genes (Table 6.3, Appendix E Table E.4). Only the gene expression upon exposure to *Oscillatoria* did not have significant differences between proportions of duplicated and non-duplicated genes in shared and unique functional annotations and GO terms (Table 6.3). No difference was observed for either functional level (functional annotation and GO term) between duplicates and tandem-duplicates for *Aphanizomenon* and *Oscillatoria* (Table 6.4, Appendix E Table E.5). For *Anabaena* and *Nodularia*, significant differences were only observed at the level of Gene Ontology terms whereas for *Cylindrospermopsis*, significant differences were only observed at the gene level (Table 6.4, Appendix E Table E.5). Significant duplicated genes had a higher probability of being unique to *Cylindrospermopsis* than tandem-duplicates (Table 6.4, Appendix

E Table E.5). *Anabaena* and *Nodularia* stress resulted in more GO terms of significant duplicated genes being shared than GO terms of tandem duplicated genes.

Table 6.3 P-values, corrected for multiple testing, of Fisher`s exact tests comparing the proportion of shared and unique significantly regulated (q-value<0.05) genes, annotation definitions and gene ontology (GO) terms between duplicated and non-duplicated genes, based upon data from Fig. 6.2. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

	APH	OSL	ANA	NOD	CYL
Proportion of significantly regulated genes shared by all treatments versus unique for each treatments	<0.01	0.06	<0.001	<0.001	<0.001
Proportion of annotation definitions within significant gene lists shared by all treatments versus unique for each treatment	<0.001	0.09	<0.0001	<0.0001	<0.01
Proportion of GO terms within significant gene list shared by all treatments versus unique for each treatment	<0.01	1.00	<0.0001	<0.0001	0.03

Table 6.4 P-values, corrected for multiple testing, of Fisher`s exact tests comparing the proportion of shared and unique significantly regulated (q-value<0.05) genes, annotation definitions and gene ontology (GO) terms between duplicated and tandem-duplicated genes, based upon data from Appendix E Table E.2. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL)

	APH	OSL	ANA	NOD	CYL
Proportion of significantly regulated genes shared by all treatments versus unique for each treatments	0.95	0.86	0.53	0.08	<0.001
Proportion of annotation definitions within significant gene lists shared by all treatments versus unique for each treatments	0.75	1.00	0.75	1.00	0.75
Proportion of GO terms within significant gene list shared by all treatments versus unique for each treatments	0.16	0.08	<0.01	<0.01	0.04

All but one of the 56 annotation definitions shared by all cyanobacterial treatments were also identified in the transcriptomic profile of *Microcystis aeruginosa* stress (section 5.3). Like exposure to *Microcystis*, exposure to all other cyanobacteria resulted in significant effects on genes involved in detoxification, i.e. cytochrome P450 gene families, glutathione-S-transferases, which is represented by both the shared annotation definitions as well as the GO terms. Given that only three cytochrome P450 genes were shared across all five cyanobacteria, it indicates that these cyanobacteria produce comparable toxic metabolites that are similar enough to induce the same type of cytochrome P450s but different enough to induce different paralogous genes upon exposure to each cyanobacteria. This is also supported by a review by Wiegand and Pflugmacher (2005) who discussed the different

biotransformation and degradation of cyanobacterial toxins through cytochrome P450 and glutathione-S-transferase. The same conclusion can also be drawn for serine/threonine kinases which have also been discussed in section 5.4 in relation to *Microcystis* exposure. As discussed in section 5.4, these proteins are involved in protein folding and may help to cope with misfolded proteins due to increased cyanobacterial stress.

In contrast, only a limited number of these annotation definitions were identified in transcriptomic profiles under cadmium (De Coninck et al., 2014), i.e. 9 annotation definitions, and salinity (Latta et al., 2013) stress, i.e. 14 annotation definitions (Appendix E Table E.6). Only five annotation definitions were shared between the transcriptomic profiles in response to five cyanobacteria, salinity and cadmium, seven conditions in total (Appendix E Table E.6). These shared annotation definitions also only cover a limited part of the shared GO terms (Appendix E Table E.3). This observation suggests that the majority of the 56 annotation definitions and GO terms common to all cyanobacterial treatments were specific to cyanobacterial exposure, regardless of the cyanobacteria species, rather than a general stress response. The annotation definitions and corresponding GO terms shared with salinity and cadmium profiles primarily pertain to functions identified in literature with general stress response: chitinases, trypsins, von Willebrand factor (Heckman et al., 2008; Poynton et al., 2007). Surprisingly, while trypsins have been primarily reported in relation with cyanobacterial stress and nutritional quality (section 5.4), they are also differentially regulated upon cadmium and salinity stress. No clear explanation can be given at this point, in particular as trypsins are differentially regulated and not solely up or downregulated. Furthermore, given that few trypsin genes are shared, it indicates again a functional diversification of these paralogous genes in which their different roles depend upon the environment as suggested by Colbourne et al (2011). Some annotation definitions not related to general stress response but more specific were also identified as shared between the different stressors. Both salinity and cyanobacterial stress affect kainite type ion channel receptors. Such a response correlates with the known function of certain cyanobacterial toxins such as beta-Methylamino-L-alanine (BMAA), known to stimulate kainite receptors (Rao et al., 2006). In addition, neurexin IV was also shared by both salinity and cyanobacterial stress and is involved in signal transduction (Baumgarter et al., 1996; Stork et al., 2009) yet its function under these two stress conditions remains to be elucidated.

At the pathway level, only the starch and sucrose metabolism was shared by all cyanobacterial treatments. The steroid hormone biosynthesis, detoxification through cytochrome P450 and the arachidonic acid metabolism were shared by all cyanobacterial treatments excluding *Anabaena* (Table 6.6). The lack of significance of the detoxification pathway through CYP450 upon exposure to *Anabaena* is surprising given the presence of high number of significantly regulated genes belonging to the CYP450 family compared to the other cyanobacteria (Appendix E Table E.2).

The shared pathways correlate well with known mechanisms of cyanobacterial stress: lack of sterols (Von Elert et al., 2003) and fatty acids (Brett et al., 2006) and production of toxins (Codd et al., 2005). Most likely neither of these mechanisms is a unique driver of cyanobacterial stress, but a cyanobacterial species-specific combination of these mechanisms underlies their stress. Again, a discrepancy between the gene level and a higher functional level was clear. Only 22 genes were shared between all cyanobacterial treatments with a diverse set of functions (Fig. 6.2.) yet quite a number of pathways were shared between all cyanobacterial treatments (Fig 6.6). This again indicates that cyanobacterial treatments regulated different genes with a similar function, i.e. belonging to the same pathway.

Table 6.6 Statistically enriched pathways (p-value<0.05) with significantly regulated genes (q-value<0.05) for each cyanobacterial treatment. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL). Blank or white cells denote a p-value larger than 0.05, i.e. no significant enrichment in that pathway for that cyanobacterial treatment, and filled or gray cells denote a p-value smaller than 0.05, i.e. significant enrichment in that pathway for that cyanobacterial treatment.

	APH	OSL	ANA	NOD	CYL
Arachidonic acid metabolism	■	■		■	■
Ascorbate and aldarate metabolism		■			■
Adipocytokine signaling pathway				■	
Detoxification through cytochrome P450	■	■		■	■
Glutathione metabolism	■				
Histidine metabolism	■			■	
Pentose and glucuronate metabolism		■			■
Ribosomes				■	
Starch and sucrose metabolism	■	■	■	■	■
Steroid biosynthesis	■	■		■	■
Tryptophan metabolism					■

Effects on genes can also be integrated with higher functional levels through their expression patterns. Here, we observed high similarities at the functional levels (Fig. 6.4-6.5: GO terms and annotation definitions) yet few shared genes (Fig. 6.3). Results have already attributed this to disproportionate high number of duplicated genes in shared GO terms and functional annotations. Furthermore, the results suggest a potential functional diversification of these duplicated genes with some individual genes being primarily responsive to one cyanobacterium while others seem to be primarily affected by another cyanobacterium.

Expression patterns may shed further light on these findings in terms of the regulation of these duplicated genes. Here, we observed significant concordant expression, i.e. smaller standard deviation in gene expression than expected between all cyanobacterial treatments for eight annotation definitions (Table 6.7). For two functional annotations, acyl-CoA synthetase and type I phosphodiesterase-nucleotide pyrophosphatase, gene expression was discordant, i.e. larger deviation in gene expression than expected between all cyanobacterial treatments. The concordant expression of these eight functional annotations indicated a strong and tight regulation of these functions among the different cyanobacterial treatments even though the specific significantly regulated genes with these functions were different in different cyanobacterial treatments. This tentatively suggests that the function remains conserved, given the concordant regulation, but is mediated through different genes dependent upon the environmental stressor.

In contrast, the discordant expression of acyl-CoA synthetase and pyrophosphatases indicated that although all cyanobacteria affected these functions, the effects were widely different among the different cyanobacterial treatments. Expression of acyl-coA synthetases, the rate-limiting enzyme in the fatty acid synthesis (Zinke et al., 2002), most likely differed widely in expression due to the different fatty acid composition of the cyanobacteria (section 2.3). In addition, the discordant expression may also imply that the functions may have diversified to such an extent that they do not fully correspond with the given annotation anymore. The majority of shared functional annotations lacked clear concordant or discordant expression patterns. This means that although the genes within these functional annotations all share a similar conserved function, the regulation of this gene function differs upon exposure to different cyanobacteria. This suggests a complex pattern of conserved mechanistic functions under cyanobacterial stress response and cyanobacteria specific effects on these functions.

Table 6.7 Annotation definitions with concordant expression across all transcriptomic profiles.

Annotation definition
Alpha-amylase
Lipid exporter ABCA1 and related proteins ABC superfamily
Neurexin IV
Peroxidase-oxygenase
Predicted transporter
Triglyceride lipase-cholesterol esterase
von Willebrand factor and related coagulation proteins
Zinc carboxypeptidase

6.4 Conclusion

Complex response patterns of *Daphnia* to cyanobacterial stress were identified that differ across levels of molecular organization. These patterns suggest potential mechanisms of cyanobacterial stress. Some were conserved across the responses to the different cyanobacterial stressors whereas others were species specific. The observed discrepancy between the different levels of molecular biological organization underlines the necessity of an integrative approach. Furthermore, the incorporation of the unique features of the genome under study in the analysis benefitted the integration of these different levels of organization in a true systems biology approach. Overall, cyanobacterial stress targeted mainly similar mechanisms and pathways regardless of the cyanobacterial species. Yet, the effects on these mechanisms and pathways were species-specific and were mediated at the gene level through different genes.

Chapter 7

Combined effects of cyanobacteria and insecticides at the transcriptomic level

7.1 Introduction

Combined and interaction effects complicate environmental risk assessment. Yet, they are an undeniable part of ecological reality. Research efforts focused on organismal or life history effects remain insufficient to elucidate the complexity of these effects and to unravel mechanisms that potentially could explain the occurrence of interaction effects.

In chapters 3 and 4, combined and interaction effects of insecticides and cyanobacteria were extensively studied at the organismal level. Results could be summarized as complex patterns of interaction effects across stressors that were insufficient to fully explain the occurrence of these effects.

Here, a transcriptomics approach will be used to study combined and interaction effects at the transcriptomic level. The usefulness of such an approach has been discussed in section 1.3 and has been demonstrated for the effects of cyanobacteria on *Daphnia* in chapters 5 and 6. As mentioned in section 1.3, few studies have investigated combined effects at the transcriptomic level after chronic exposure of *Daphnia* (De Coninck et al., 2014; Stanley et al., 2013).

This chapter will build further on the transcriptomic approaches described in chapter 5 and 6. Whole transcriptome arrays were used to query effects on gene expression of both single and combined treatments of cyanobacteria and insecticides. As a consequence, mechanisms of interaction can be studied at the gene expression level, which will allow characterization of combined and interaction effects at this level. As mentioned in section 1.3, studies discussing combined and interaction effects at the transcriptomic level do not generally estimate these effects through standard mixture toxicity models such as multiplicative or additive models (section 1.1.1, section 1.3). Without the use of a standard mixture toxicity model or framework, it is impossible to compare and potentially generalize effects across studies.

Therefore, this chapter will focus on studying combined and interaction effects at the transcriptomic level within a defined statistical framework using a standard mixture toxicity model based upon the framework developed by De Coninck et al. (2014). The selection of 48 combinations will not only aid in understanding the complex mechanisms driving interaction effects but also requires new

methodologies to compare interaction effects between these combinations at the whole transcriptomic level rather than estimating interaction effects gene-by-gene for each combination.

7.2 Material and Methods

7.2.1 Experimental organisms

The experimental organisms originated from *D. pulex* cultures as described in section 2.2.1. Likewise, cyanobacteria culture conditions were also described in section 2.2.1.

7.2.2 Experimental design

Biological tissue originated from exposures described in chapter 4 (section 4.2.2). Exposures followed a two factorial design depicted in Fig. 4.1. Briefly, *Daphnia* were exposed to control treatment, an insecticide treatment with a concentration of half the EC50, a cyanobacteria treatment in which the diet was contaminated with 50% of cyanobacteria, and a mixture treatment containing 50% of cyanobacteria in the diet and having a concentration of half the EC50 for each insecticide. In total, 48 combinations were tested.

7.2.3 mRNA extraction, labelling and hybridization.

RNA extractions were conducted as detailed in section 5.2.3. Labelling and hybridization procedures followed similar protocols as described in section 5.2.3 although the labelling design differed. The labelling design followed a standard loop design for each binary mixture (Fig. 7.1) to allow optimal comparison of single and combined effects within each mixture. Different biological replicates were used on each array. No technical replicates were used as sufficient biological replicates were available (i.e. four per treatment) for replication and dye swaps.

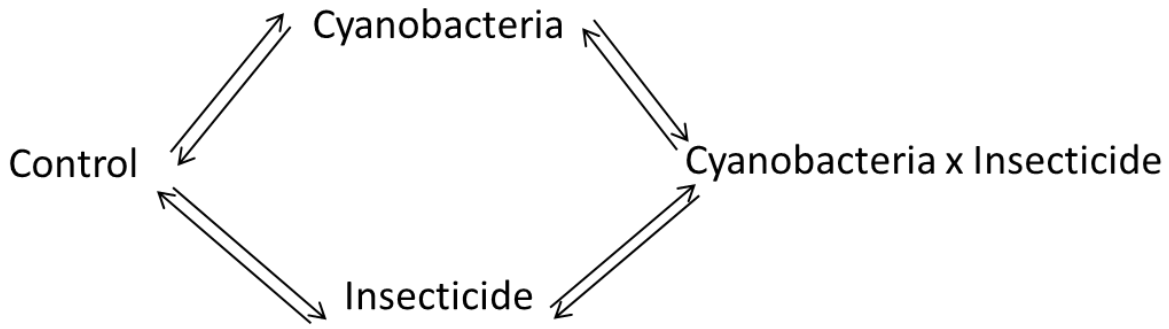


Figure 7.1 Labelling design for each binary combination. Arrows represent microarrays and point to red labelled (Cy5) sample. The sample at the base of the arrow is then labelled green (Cy3).

7.2.4 Image analysis and data processing

Image and data processing followed general concepts described in section 5.2.4. However, given the different experimental setup, a different approach to present the data and to construct the linear model was used. Specifically, a separate channel analysis, i.e. each channel or each colour was first analysed separately, was conducted rather than a log ratio analysis, i.e. both channels or colours were analysed together as a log-ratio of the Cy5 labelled sample versus the Cy3 labelled sample. As described by Smyth and Altman (2013), a separate channel analysis will improve detection power as it will include more information than a standard log ratio analysis. In simple paired designs with only two treatments, such as described in chapters 5 and 6, no information can be gained from a separate channel analysis and both models will yield the same test statistic (Smyth and Altman, 2013). However, in more complex designs such as here, where for example cyanobacteria treatments or insecticide treatments serve as a common reference to control and mixture treatments, a significant improvement can be made through separate channel analysis. In particular, separate channel analysis includes the information captured by the A-value, i.e. average log intensity or half of the sum of the $\log_2$ expressions of Cy3 and Cy5 that is ignored in a log ratio analysis. It consists of processing the data as separate single channels and using standard techniques for normalization and quality assessment as described in section 5.2.4. Subsequently, the intra-spot correlation, the correlation between the Cy5 and Cy3 labelled sample for that spot on the array, is determined which together with a standard design matrix and the microarray data is the input for the subsequent linear model analysis for separate channels. In R (version 3.0.1), this analysis can be done using the function lmscFit.

Subsequent analysis consisted of Bayes statistics to calculate the moderated t-statistic and Benjamini-Hochberg correction as described in section 5.2.4.

No independent qPCR validation was conducted. This was less important as data will not be analysed on a gene-by-gene basis per dataset but will rather be focussed on identifying genes across datasets. As a consequence, each dataset serves as type of validation for the other dataset.

7.2.5 Interaction effects at the transcriptomic level: the independent action model

The independent action model described in section 3.2.4 can also be used to predict effects at the transcriptomic level under the assumption of non-interaction. Based on De Coninck et al. (2013a), the independent action model under the assumption of non-interaction and as originally defined by Bliss (1939), can also be written as follows (Appendix F.3 gives the full deduction) :

$$\frac{Y_{mix}}{Y_{Ctr}} = \frac{Y_{pesticide}}{Y_{Ctr}} * \frac{Y_{cyano}}{Y_{Ctr}} \text{ (eq. 7.1)}$$

Where Y_{Ctr},Y_{Cyano},Y_{mix} and $Y_{Pesticide}$ is the response of the control, cyanobacteria, mixture and pesticide treatment respectively. After log-transformation, this multiplicative model becomes additive:

$$log\left(\frac{Y_{mix}}{Y_{Ctr}}\right) = log\left(\frac{Y_{pesticide}}{Y_{Ctr}}\right) + log(\frac{Y_{cyano}}{Y_{Ctr}}) \text{ (eq. 7.2)}$$

In microarray studies, the logarithm of the response of a treatment versus a control treatment can be defined as an M-value, i.e. logratio, and its corresponding standard error (SE). Therefore eq. 7.2 can also be written as follows for microarray studies:

$$M_{mix} \pm \text{SE} = M_{pesticide} \pm \text{SE} + M_{cyano} \pm \text{SE} \text{ (eq. 7.3)}$$

Eq. 7.3 can be straightforwardly analysed with LIMMA (version 3.16.7) as described in De Coninck et al. (2014). The mixture effect is then defined as the observed effect of the mixture treatment versus the control treatment. The interaction effect (M_{int}) was defined as the effect of the mixture treatment (M_{mix}) minus the effects of the cyanobacteria (M_{cyano}) and insecticide treatment ($M_{insecticide}$), both normalized versus control treatment. Under the hypothesis of no interaction, equation 7.3 should equal zero (or M_{int} = 0) when all terms in the equation are transferred to one side:

$$M_{mix} \pm \mathrm{SE} - M_{pesticide} \pm \mathrm{SE} - M_{cyano} \pm \mathrm{SE} = M_{int} \pm \mathrm{SE} \text{ (eq. 7.4)}$$

Mathematically, the genes not adhering to eq. 7.3 are defined as genes with a significant interaction effect and the deviation from zero can be seen as the deviation from non-interaction (M_{int}). The LIMMA analysis results in a deviation value (M_{int}) and a p-value, based on moderated t-statistics after empirical Bayes moderation of standard errors, indicating the significance of the deviation from non-interaction for each gene. Genes demonstrating an interaction effect can be labelled as synergistic or antagonistic based upon their M-value. If the observed M-value (M_{mix}) is smaller than the predicted M-value (M_{cyano} + $M_{insecticide}$), the effect on the gene is smaller than predicted or antagonistic. If the observed M-value (M_{mix}) is larger than the predicted M-value (M_{cyano} + $M_{insecticide}$), the effect on the gene is larger than predicted or synergistic.

These results are then subjected to empirical Bayes statistics and Benjamini-Hochberg correction as described in section 5.2.4. Interaction effects are thus quantified in terms of statistically significantly deviation from non-interaction defined by M_{int}. Alternatively to Benjamini-Hochberg correction, confidence intervals can be used to additionally filter the data after empirical Bayes moderation of standard errors. Indeed, the requirement for a gene to demonstrate a significant interaction effect would then be no overlap of the confidence intervals of the predicted M-value (M_{cyano} + $M_{insecticide}$) and observed M-value (M_{mix}) rather than a cut-off q-value. Here, the confidence intervals of 95% and 90% were selected. M-values are also often used as alternative to multiple testing (Hampton and Stanton, 2010). Here, several cut-off values were used, i.e. absolute M_{int}-value larger than 1, 2 and 3. Biologically this implies that the predicted effect of the cyanobacteria and the pesticide differs by a factor of 2, 4 or 8 from the observed effect of the cyanobacteria and the pesticide, which indicates a large interaction effect.

The benefits and disadvantages of each of the three methods, i.e. Benjamini-Hochberg correction, confidence interval filtering and M-value cut-off, will be discussed in the results section. The analysis was conducted on all 48 combinations separately and in the end gives an M-value, deviation from non-interaction, for each gene with a corresponding p-value for each combination. The significance of the deviation from non-interaction is determined by the p-value and the subsequent correction, filter or cut-off value. As a result, the gene list from the Limma ANOVA analysis will be filtered in six different ways resulting in six different filtered gene lists as illustrated in Fig. 7.2 for each combination.

Lastly, the pattern of the genes with an interaction effect based upon the gene list selected as the best gene list out of the six gene lists, were visualized through interaction plots as described by De Coninck et al. (2014).

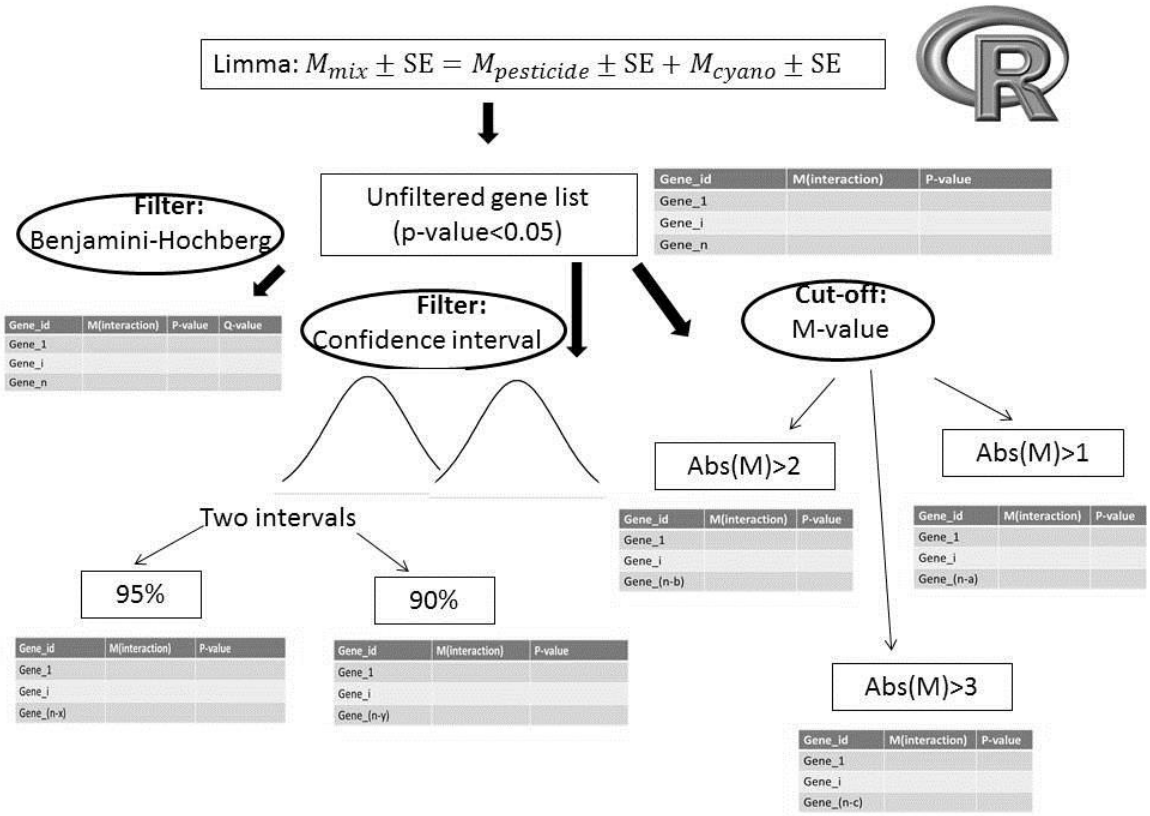


Figure 7.2 Schematic overview of the analysis and the different methods to process the gene list afterwards. *n* refers to the total number of genes with a significant p-value. *A*, *b*, *c*, *x* and *y* refer to the numbers falling below the cut-off M-value or within overlapping confidence intervals.

For each combination, the number of genes with no interaction, a synergistic or antagonistic deviation was calculated. However, to compare effects between combinations an overall measure of interaction is recommended. No relevant literature was found, therefore two potential measures will be suggested here. First, a measure based upon the numbers of synergistic and antagonistic genes. These numbers were then used to determine the overall synergistic or antagonistic deviation from non-interaction for the specific combination as followed:

$$Deviation = log_2\left(\frac{\#\ synergistic\ genes}{\#\ antagonistic\ genes}\right) \quad \text{(eq. 7.5)}$$

The metric in eq. 7.5 allows quantifying deviations similarly as the deviation parameter defined by Jonker et al. (2005) for life history data. The metric becomes more positive as the number of synergistic genes increases and becomes more negative when the number of antagonistic genes increases. If any of the two numbers is zero, it will be set to one to allow calculating the log ratio. As a consequence, if both are zero, the ratio will be one and the deviation parameter will be zero indicating non-interaction, similarly to the deviation parameter of Jonker et al. (2005).

Second, a measure based upon the M-value of each gene defined as synergistic or antagonistic. The M-value is a deviation from non-interaction for that gene and may be a suitable measure to quantify overall interaction as followed:

$$Deviation = \sum_1^n M_i \quad \text{(eq. 7.6)}$$

In which *n* is the number of genes with a significant deviation from non-interaction, M_i is defined as M_{int} in eq. 7.4 of the i^{th} gene. As the M-value itself is a $\log_2$ value, the deviation parameter will also be. Similar to eq. 7.5, the more negative the deviation parameter, the more antagonistic the deviation. In contrast, the more positive the parameter, the more synergistic the deviation will be. Indeed, the deviation M-value, M_{int}, originates from the contrast from eq. 7.4. In this equation, if $M_{mix} - M_{cyano} - M_{pesticide}$ is positive, it means that M_{mix} is larger than $M_{cyano} + M_{pesticide}$, which indicates synergism. However, if $M_{mix} - M_{cyano} - M_{pesticide}$ is negative, it means that M_{mix} is smaller than $M_{cyano} + M_{pesticide}$, which indicates antagonism. Both methods will be compared and discussed in the following sections.

7.2.6 Analysis of gene lists

Analysis of the gene lists generated under 7.2.4 and 7.2.5 was previously described in section 5.2.5. Similarly, analysis was conducted with all genes, without duplicated genes and with only duplicated genes to assess the impact of gene duplication on the biological interpretation.

7.3 Results and discussion

The results and discussion section consists of three parts. In the first part, the different approaches from Fig. 7.2 will be discussed, i.e. Benjamini-Hochberg (BH) correction, filtering based upon confidence intervals and the cut-off M_{int}-value. All these methods resulted in gene lists in which each gene was determined as significant from non-interaction or adhering to non-interaction. The second

part consists of comparing the two deviation measures to quantify interaction, i.e. eq. 7.5 and eq. 7.6. The third part consists of a functional analysis of genes significantly different from non-interaction.

7.3.1 Interaction effects at the gene level

The two-way ANOVA identified combinations with genes demonstrating an interaction effect for all six filtering approaches (Table 7.1, Appendix F Tables F.1-F.6). BH correction and an absolute M_{int}-value cut-off of 3 identified the most combinations without any genes deviating significantly from non-interaction, thus being the most stringent. In contrast, an absolute M_{int}-value cut-off of 1 was the least stringent as it detected deviations from non-interaction for genes in all combinations (Table 7.1). The number of genes with a significant interaction effect varied enormously across combinations when subjected to BH correction, with a single significant gene identified for the combination tebufenpyrad and *Aphanizomenon* and 6786 genes identified for the combination carbaryl and *Anabaena* (Appendix F Table F.1). The cut-off absolute M_{int}-value of 1 also showed large variation with 5253 genes with a significant interaction effect for the combination of carbaryl and *Anabaena* and only 100 genes with a significant interaction effect for the combination of fenoxycarb and *Aphanizomenon*. Confidence interval based approaches and a cut-off absolute M_{int}-value of 2 showed less variation as genes with an interaction effect varied between one and 150-300. The cut-off absolute M_{int}-value of 3 showed very little variation with at most twenty genes with a significant interaction effect.

Table 7.1 Overview of the number of combinations out of a total of 48 with no significant genes (i.e. M_{int} not significant) for all six approaches (Benjamini-Hochberg (BH) correction, 95% and 90% confidence intervals (CI), absolute (abs) M_{int}-value cut-off of 1, 2 and 3). The minimum number, the maximum number, mean and median of significant genes (i.e. M_{int} significant) across all combination for combinations with at least one significant gene for all six approaches are shown.

	BH-correction	95% CI	90% CI	Abs(M)≥1	Abs(M)≥2	Abs(M)≥3
Combinations with no sig genes	13	6	1	0	1	13
Minimum number of sig genes per combination	1	1	3	100	1	1
Mean number of sig genes per combination	874	19	53	729	40	4
Median number of sig genes per combination	107	13	35	460	24	3
Maximum number of sig genes per combination	6786	157	327	5253	329	20

Comparing the number of genes deviating significantly from non-interaction across combinations with the Benjamini-Hochberg correction may be biased. Indeed, the experimental design will influence the power to detect significant genes and this may differ between studies. Although differences in experimental design were not a concern for these experiments as they were all conducted with a similar experimental design, other concerns do play a role. First, a gene may be differentially corrected for multiple testing in two datasets depending upon the rank of that gene in the overall gene list and regardless of the value for M_{int} (Pawiton et al., 2005). Second, statistical significance does not always correspond with biological significance, a gene may be statistical significant with a fold change of 0.5 whereas biological significance often requires a fold change of at least 1 or 2 (Hampton and Stanton, 2010). Third, very small p-values are rare in microarray studies using biological replicates and as a consequence multiple testing procedures may limit biological findings. These aspects are discussed by Hampton and Stanton (2010) who re-analysed and compared microarray studies investigating genes underlying cystic fibrosis. They concluded that integrating ANOVA analysis with less restrictive procedures such as cut-off M_{int}-values that underline biological rather than statistical findings improves the data analysis. Based upon these arguments, the Benjamini-Hochberg correction was discarded.

Distributions of the p-value of all genes for all combinations again confirmed the cut-off absolute M_{int} -value of 1 as the least stringent method (Fig. 7.3). The other four methods were much more conservative which can be seen from the small difference between the first and third quartile, resulting in a very “flat” box (Fig. 7.3). The length and density of the whiskers do indicate some differences between the methods that were less pronounced (Fig. 7.3). Evaluation of the boxplots of the Benjamini-Hochberg corrected p-values confirmed that this correction was too conservative and would exclude important biological findings, i.e. quite a number of genes with no 95% overlapping confidence intervals obtained a BH-corrected p-value larger than 0.05. Density plots of the M_{int}-value of all genes for all combinations supported these conclusions although they did highlight the very restrictive approach of an absolute M_{int}-value cut-off of 3 resulting in a skewed density plot (Fig. 7.4). Taking into account the observations made concerning Table 7.1, the absolute M_{int}-value cut-offs of 1 and 3 were discarded as less suitable methods: the first for being not restrictive enough, the second for being too restrictive.

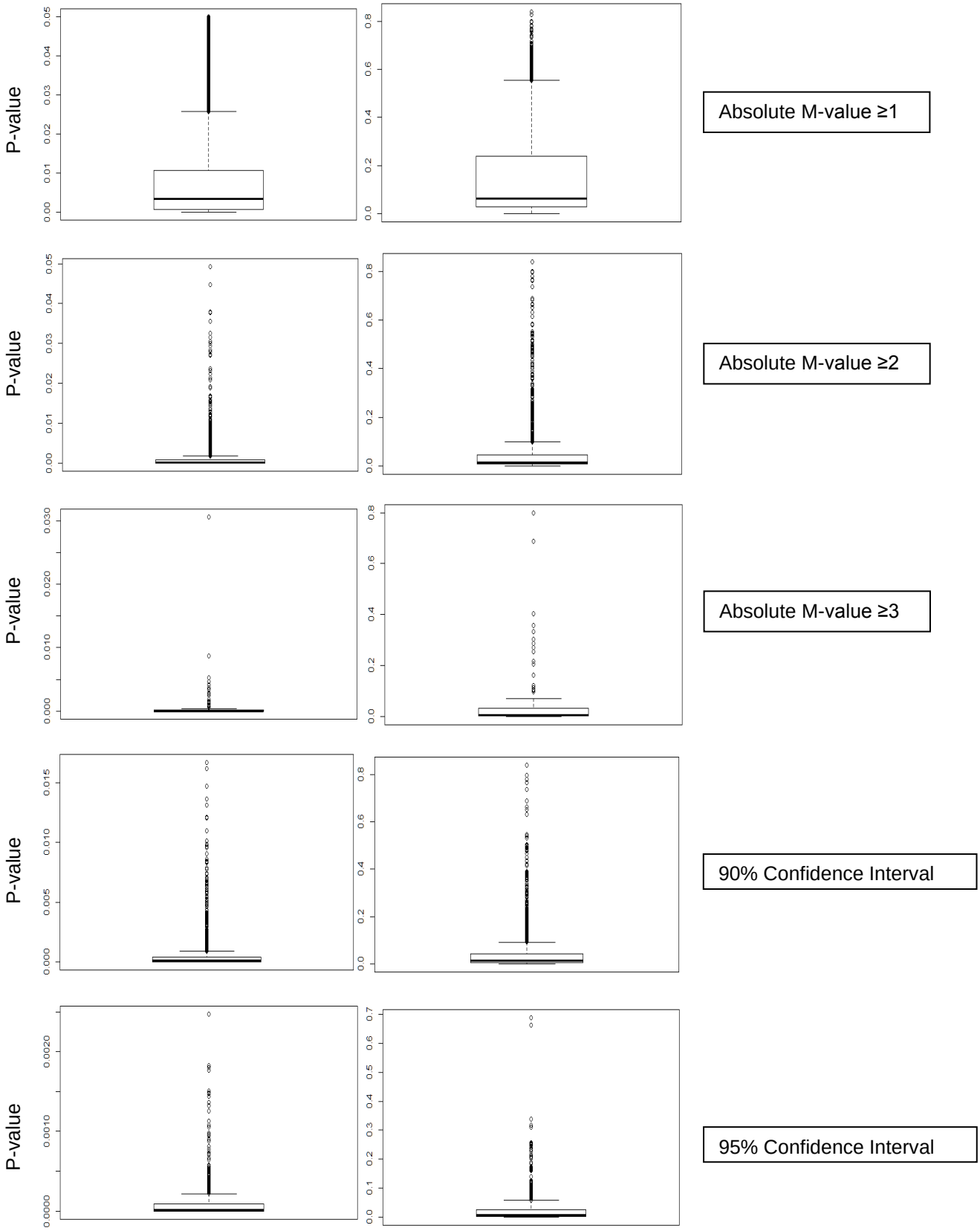


Figure 7.3 Boxplots of the p-values (left) and the Benjamini-Hochberg corrected-values (right) of all genes for all combinations for each of the five filtering methods.

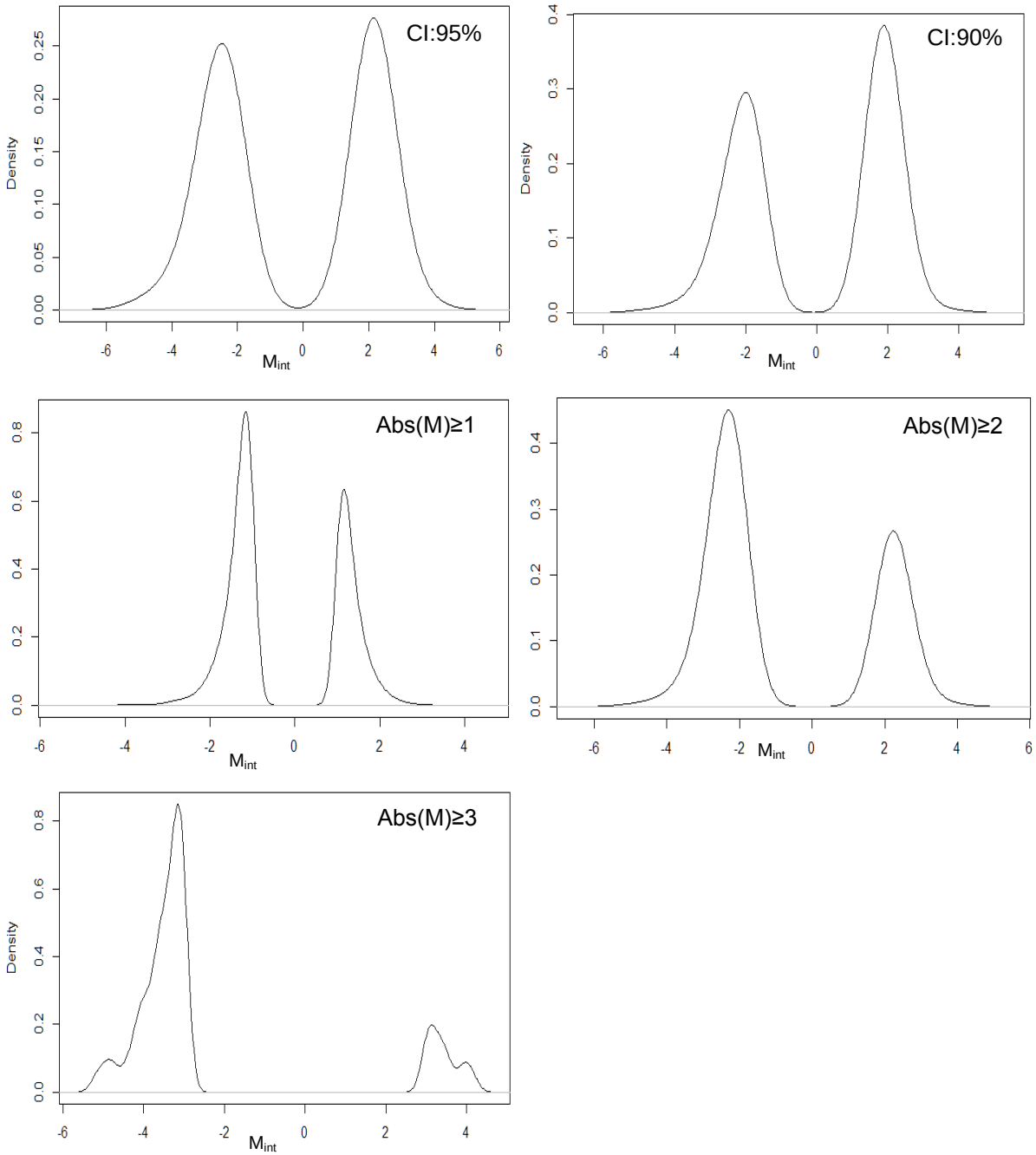


Figure 7.4 Density plots of all the M_{int}-values for all combinations for each of the five remaining filtering methods. (Abs=Absolute, CI=Confidence Interval).

Based upon the results in Table 7.3, Fig. 7.3 and 7.4, three of the six filtering methods were excluded from further research. Of the three remaining methods, i.e. absolute M_{int}-value cut-off of 2, confidence intervals of 90 and 95%, not one could be selected above the other two or discarded in favour of the other two based upon the results presented here.

7.3.2 Interaction effects at the transcriptome level

Interactions at the transcriptomic level have not been discussed before in literature within a defined statistical framework. Here, two newly proposed measures of quantifying deviation were evaluated. The first measure, eq. 7.5, quantified deviation as the $\log_2$ of the number of significantly antagonistic significantly genes divided by the number of synergistic genes. The second measure, eq. 7.6, quantified deviation as the sum of the M_{int}-values of all genes significantly deviating from non-interaction.

Both deviation measures had a very different distribution (Table 7.2), which was to be expected given the different mathematical properties of these measures. The deviation measure based upon the number of synergistic and antagonistic genes had off course a much narrower distribution as it is the result of a division whereas the deviation measure based upon the summation of M_{int}-values had a much wider distribution. For all filtering approaches, means and medians of deviation parameters based upon the log ratio of synergistic versus antagonistic genes, were positive indicating synergism (Table 7.2, Appendix F Tables F.7-F.12). In contrast, the means and medians of deviation parameters based upon the sum of the value varied in sign for the confidence interval approaches but indicated both antagonistic for the filtering based on the cut-off M_{int} -value of 2.

The number of combinations identified as synergistic or antagonistic was more consistent with the deviation measure based upon the summation of the M_{int}-value than the deviation measure based upon the $\log_2$ ratio of the number of antagonistic and synergistic genes across the different filtering approaches (Table 7.3). Filtering the data with no overlapping 95% confidence intervals was the most consistent filter approach of three, given similar results with the two deviation measures (Table 7.3). In contrast, filtering the data based upon an absolute M_{int}-value cut-off of 2 was the least consistent approach. Both deviation measures drew different conclusions based upon the same dataset. The approach using no overlapping 90% confidence intervals also drew different conclusions with the two deviation measures. The difference in conclusions with the two deviation measures can be clarified mathematically. Indeed, the summation of M_{int} -values indicated an overall antagonistic trend in the data for the majority of the combinations whereas the $\log_2$ ratio of the numbers of synergistic and antagonistic genes indicated a synergistic trend for the majority of the combinations. Taking into account the different mathematical properties of each of these deviation measures, the 90%

confidence interval filtering and the absolute M_{int} -value cut-off of 2 seemed to select more genes with a positive M_{int}-value than a negative M_{int}-value. However, the majority of these genes with a positive sign seemed to have a rather small M-value whereas the genes with a negative sign seemed to have larger M-values.

Table 7.2 Overview of the minimum, mean, median and maximum deviation across all 48 combinations for the two deviation measures per filtering approach, based upon the results in Appendix F Tables F.7-F.12.

Deviation measure:	$\log(\frac{\#\ synergistic\ genes}{\#\ antagonistic\ genes})$				$\sum_{1}^{n} M_i$			
Filtering approach:	Min	Mean	Median	Max	Min	Mean	Median	Max
95% Confidence Interval	-5.26	0.68	0.34	5.67	-115	-3.93	3.87	302
90% Confidence Interval	-4.52	0.76	0.10	6.77	-207	0.85	-8.09	483
Absolute M_{int} -value equal or larger than 2	-5.78	1.3	0.85	5.78	-578	-30.7	-12.1	283

Table 7.3 Overview of the number of antagonistic and synergistic combinations for each deviation measure per filtering approach, based upon the results in Appendix F Tables F.7-F.12.

Deviation measure:	$\log(\frac{\#\ synergistic\ genes}{\#\ antagonistic\ genes})$		$\sum_{1}^{n} M_i$	
Filtering approach:	Antagonistic combinations	Synergistic combinations	Antagonistic combinations	Synergistic combinations
95% Confidence Interval	34	5	30	12
90% Confidence Interval	18	28	30	17
Absolute M_{int} -value equal or larger than 2	10	34	36	11

Overall, the approach of no overlapping 95% confidence intervals and the deviation parameter based upon the sum of the M_{int}-values seemed to be the most consistent approaches and these were used together for further analysis. The synergistic combinations, i.e. 12 combinations (25%) were scattered across the matrix with the most severe ones being *Anabaena* and carbaryl and *Oscillatoria* and fenoxycarb (Table 7.4). Four out of the twelve synergistic combinations were combinations with *Oscillatoria*. The most severe antagonistic combinations were *Oscillatoria* and tetradifon and *Cylindrospermopsis* and endosulfan. The distribution of the deviation parameter (Table 7.4), defined in eq. 7.6, was not significantly different between combinations with different cyanobacteria (p=0.85) or between combinations with different insecticides (p=0.78). This indicates that differences between cyanobacteria alone or differences between insecticides alone did not explain the presence or absence of genes with an interaction effect.

Table 7.6 Deviation from non-interaction as defined by equation 7.6, i.e. sum of the M-values of the significant genes deviating from non-interaction. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05, and the absolute M_{int}-value, i.e. larger than 2. The darker the red, the more synergistic the deviation is. The darker the green, the more antagonistic, the observed deviation is.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	-10.6	0	-28.2	-10.6	-7.17	9.21
Carbaryl	99.4	-1.23	-16.8	0.07	-5.45	-57.4
Chlorpyrifos	-52.7	8.57	-1.10	0.71	0	-35.0
Deltamethrin	0	-16.9	1.84	-3.29	0	17.5
Endosulfan	0	-9.61	-91.2	-0.68	-32.4	21.6
Fenoxycarb	-8.12	3.83	-4.45	-19.1	-14.5	302
Tebufenpyrad	14.8	-1.90	-45.9	0	-32.9	-7.53
Tetradifon	-0.05	-26.7	-28.2	23.9	-7.59	-115

The behavior of the genes with a significant interaction effect was represented in interaction plots (Fig. 7.5, based on De Coninck et al., 2014) for the most abundant patterns covering around 70% of the genes with an interaction effects. All other patterns can be found in Appendix F Fig. F.1-F.2. Figure 7.5 clearly indicates that these genes can have very distinct patterns. Furthermore, the current interpretation generally used in life history experiments of synergism and antagonism seemed insufficient to fully explain the diversity of patterns here. Indeed, the antagonistic pattern (Fig. 7.5 D) and the third synergistic pattern (Fig. 7.5 G) genes were each other's mirror image, yet they were defined differently. An alternative would be to compare absolute observed and predicted M_{int}-values instead. Although this would classify the current patterns with the same terminology, it may still be insufficient to really address the complexity of the effects. For example, in the antagonistic interaction plot in Fig. 7.5 C, the predicted effect was positive but smaller in absolute value than the observed effect which was negative. Using the actual M_{int}-values will classify this as antagonistic, whereas using absolute values will result in a synergistic effect. Yet, both terminologies seem insufficient as the pattern is clearly different from the other patterns which indicated more 'obvious' synergisms or antagonisms such the antagonistic interaction plot (Fig. 7.5 B) or the synergistic plot (Fig. 7.5 F). Defining synergisms and antagonisms at the gene level is therefore not recommended with the current terminology which was inadequate to describe all patterns properly.

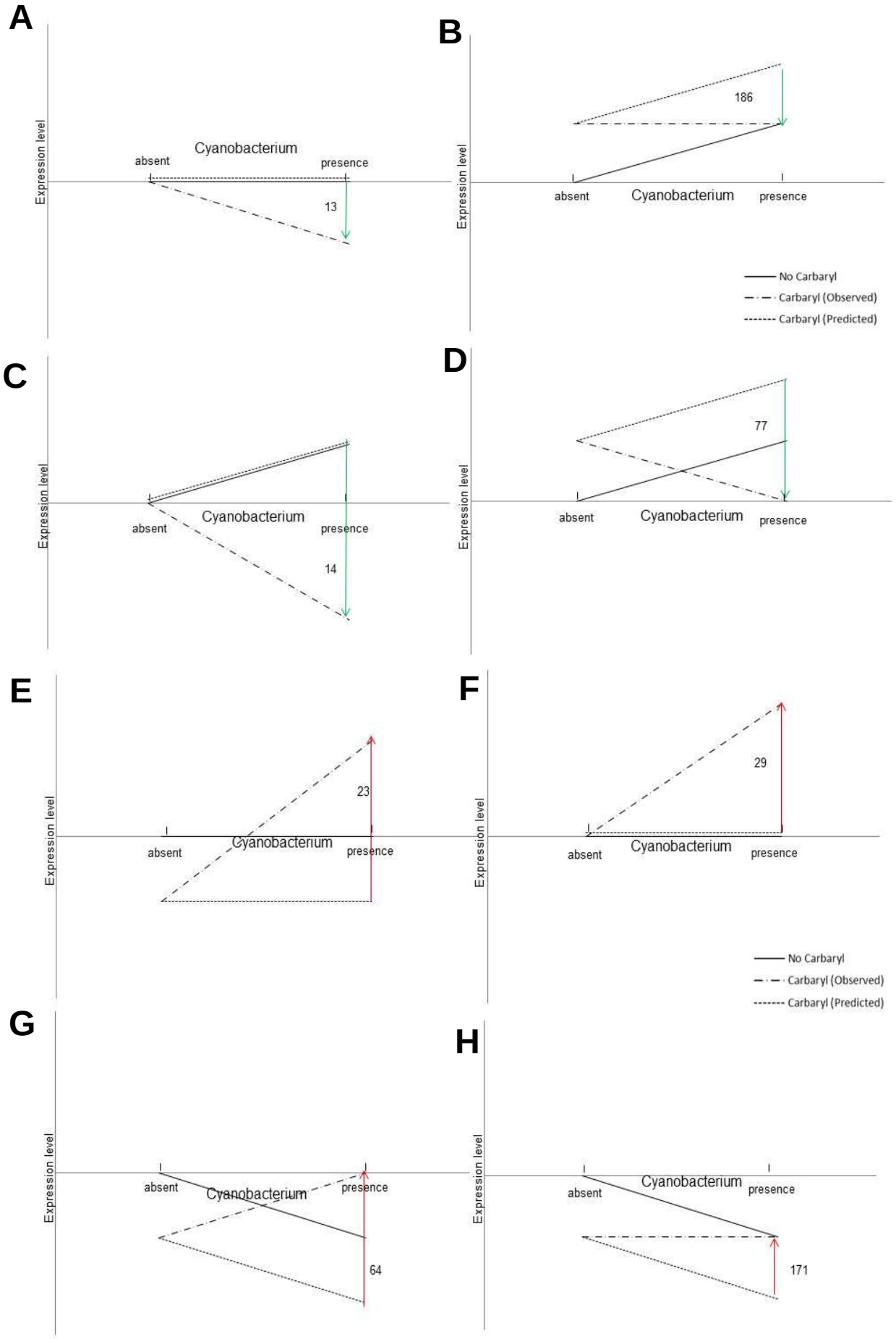

A
Expression level
Cyanobacterium
absent
presence
13
B
186
Expression level
absent
Cyanobacterium
presence
No Carbaryl
Carbaryl (Observed)
Carbaryl (Predicted)
C
Expression level
absent
Cyanobacterium
presence
14
D
77
Expression level
absent
Cyanobacterium
presence
E
23
Expression level
absent
Cyanobacterium
presence
F
29
Expression level
absent
Cyanobacterium
presence
No Carbaryl
Carbaryl (Observed)
Carbaryl (Predicted)
G
Expression level
absent
Cyanobacterium
presence
64
H
Expression level
absent
Cyanobacterium
presence
171

Figure 7.5 Interaction plots for genes with an interaction effect upon cyanobacterium exposure (presence vs. absence) under no insecticide (solid line), addition of the insecticide (dash-dot line) and predicted addition of the insecticide (dashed line). Arrows indicate the difference in expression level between observed and predicted expression of the significant genes. Green arrows indicate antagonism, red arrows indicate synergism. Horizontal axis intersects vertical axis at no differential expression. For instance, in the top left panel, genes are not significantly regulated upon exposure to a cyanobacterium and also upon exposure to an insecticide alone. The predicted expression level under exposure of insecticide and a cyanobacterium is no regulation whereas in reality the genes are being downregulated upon exposure to both. Observed expression is more negative than the predicted expression and antagonism can be concluded. Numbers indicate the number of genes showing the particular expression pattern.

7.3.3 Functional analysis and annotation of genes with an interaction effect

Transcriptomic analysis of section 7.3.2 revealed 812 genes demonstrating an interaction effect of which 558 were unique to a single combination (Fig. 7.6) with 95% confidence interval filtering approach. Thirteen genes were present in at least five combinations of which two genes were present in 10 and 13 combinations respectively (Appendix F Table F.13). Thirty-five out of 141 genes were classified in some combinations as synergistic but showed clear antagonistic effects in other combinations (Appendix F Table F.14). Functional analysis of the 812 genes with an interaction effect revealed a diverse set of metabolic functions (Fig. 7.7), including oxidative phosphorylation, fatty acid elongation and biosynthesis, energy metabolism.

The 95% confidence interval filtering approach was the most consistent across all combinations but does not fully guarantee the lack of false positives. This is especially a concern when looking at individual genes. Therefore, the BH-corrected p-value of these 812 genes demonstrating an interaction effect was evaluated. 115 genes had a BH-corrected p-value larger than 0.05 of which 64 had a BH-corrected p-value of 0.1. Of the 115 genes with a BH-corrected p-value larger than 0.05, 80 genes occur multiple times in the total set of 812 genes and have a BH-corrected p-value smaller than 0.05 in the majority of the combinations. Only 23 genes occur only once and thus have always a BH-corrected p-value larger than 0.05. Only 6 genes occur twice with a BH-corrected p-value larger than 0.05. This is means that only 29 unique genes can be a false positive whereas all other 529 unique genes are truly interacting for at least one combination at the 5% significance level.

Significant overrepresentation of genes with an interaction effect was observed in pathways related to nutritional quality and detoxification (Table 7.5). The same results were obtained when analysis was performed with or without the duplicated genes. Enrichment analysis of functional annotations identified genes with functions such as trypsins, cytochrome P450, transcription factors and ribosomal proteins (Table 7.6, Fig. 7.7). The functional annotation definition “NA”, indicating lineage-specific genes, was the only definition significantly underrepresented than expected by genes with an interaction effect. Analysis without the 115 genes with a BH-corrected p-value resulted in the same conclusions for all pathways in Table 7.5 and the majority of the annotation definitions in Table 7.6. Three definitions became not significant when leaving out the 115 genes with a BH-corrected p-value larger than 0.05. Xaa-Pro aminopeptidase and Glycosylphosphatidylinositol anchor synthesis protein both only had a single gene demonstrating an interaction effect when excluding the 115 genes which resulted in a p-value of 0.07 and 0.10 respectively. Also, excluding the 115 genes resulted in only eight trypsins with an interaction effect which lead to p-value 0.15.

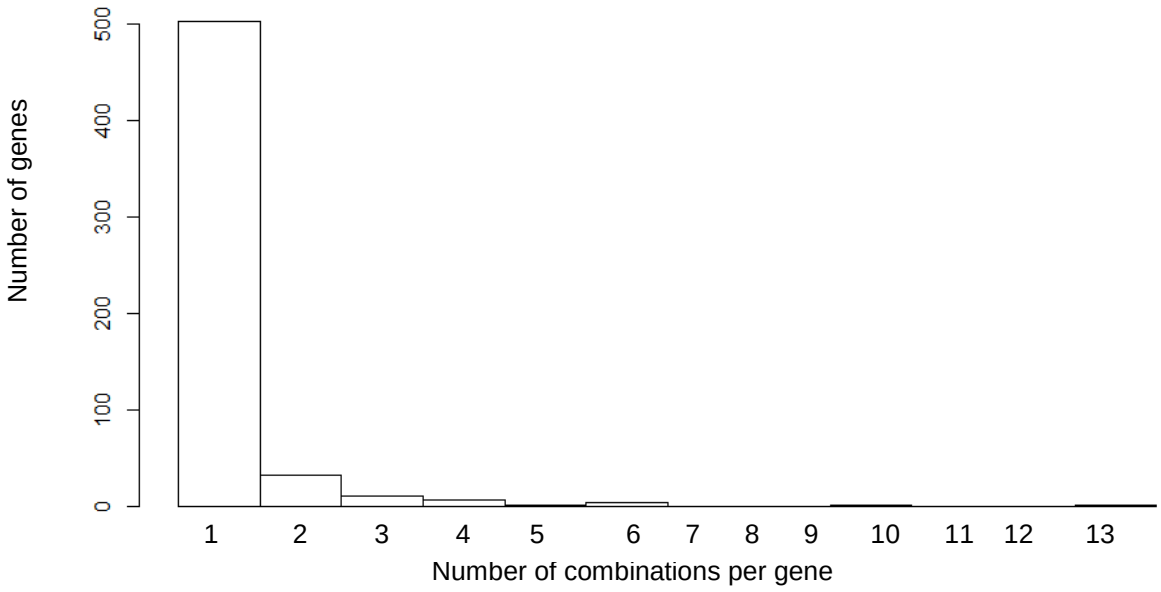


Figure 7.6 Histogram of the number of combinations for which a gene was determined as having a significant interaction effect.

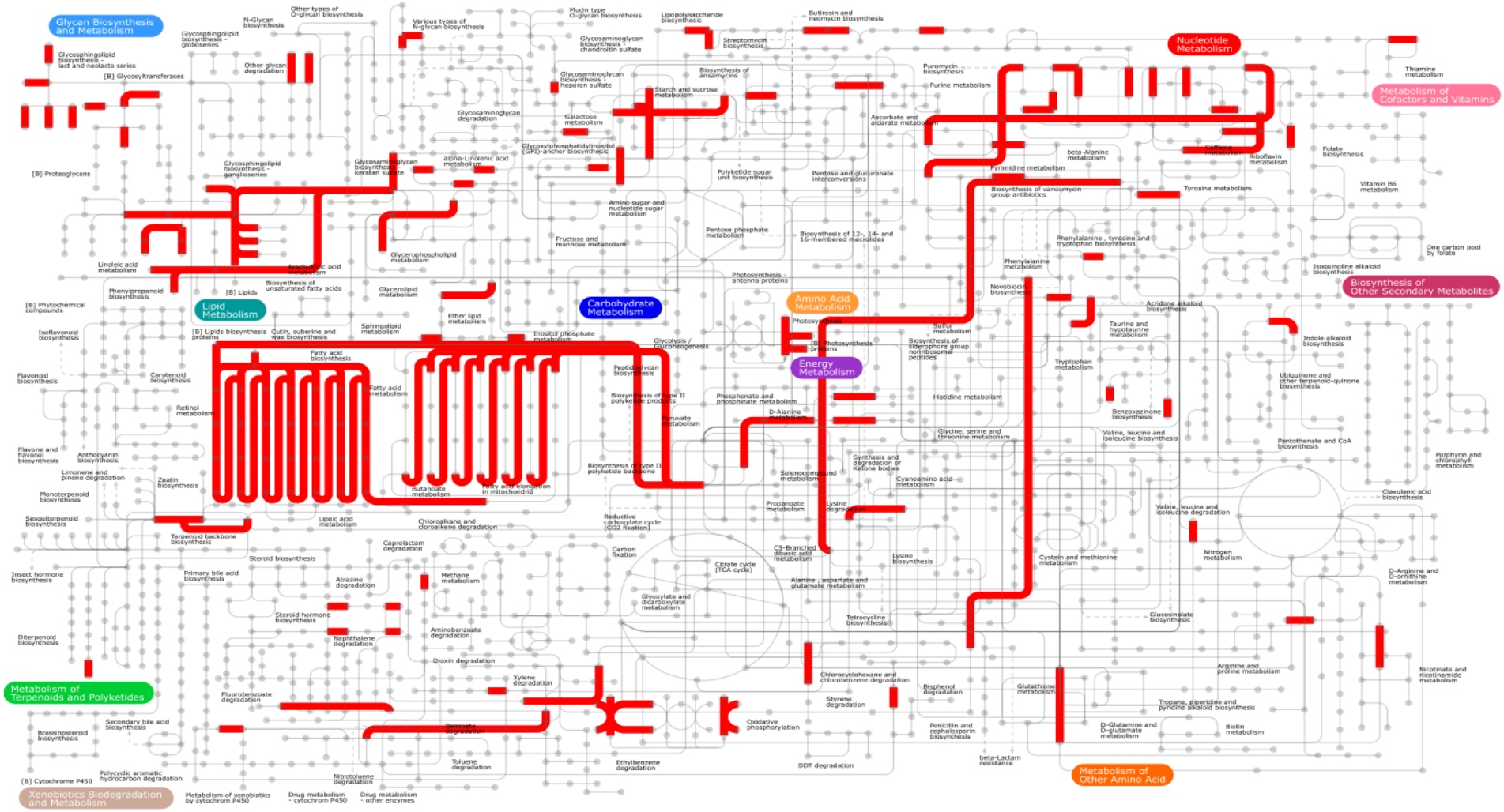


Figure 7.7 Metabolic pathway map from KEGG database (http://www.genome.jp/kegg-bin/show_pathway?map01100) modified with all 812 genes with an interaction effect, denoted in red. This figure gives a general overview of the entire metabolic pathway map and the red lines denote how many pathways contain genes with an interaction effect without specifically highlighting any particular pathway.

Table 7.5 List of significantly overrepresented pathways with their KEGG ID, name, Benjamini-Hochberg corrected p-value obtained after Fisher`s exact test and the number of genes with an interaction effect within the pathway.

Pathway ID	Name	P-value	N° of genes with interaction	N° of genes in pathway
Map00071	Fatty acid degradation	0.021	5	29
Map00590	Arachidonic acid metabolism	0.012	5	29
Map00591	Linoleic acid metabolism	0.029	4	11
Map00983	Detoxification	0.012	6	22

Table 7.6 List of annotation definitions with significantly different proportions, the Benjamini-Hochberg corrected p-value obtained after Fisher`s exact test, the number of genes with an interaction effect within the annotation and the total number of genes within the annotation. For all annotation definitions in normal font a significant overrepresentation was observed. Annotation definitions in bold font had significantly smaller proportion of genes with an interaction effect than expected by chance. NA=Not available, i.e. no known homology or in other words lineage specific genes.

Name	P-value	N° of genes with interaction	N° of genes in annotation
Xaa-Pro aminopeptidase	0.002	2	4
Predicted metallothionein	0.003	2	5
Glycosylphosphatidylinositol anchor synthesis protein	0.005	2	6
Predicted transmembrane-protein (immunoglobulin family)	0.005	3	19
Glucose dehydrogenase family	0.006	3	20
Transcription factor LIMP-1-PRDI-BF1	0.009	2	8
Trypsin	0.010	11	255
Uncharacterized conserved protein	0.010	21	613
Serine-threonine protein kinase	0.011	5	71
Collagens type IV and type XIII related proteins	0.011	9	192
60S ribosomal protein	0.016	4	51
Cytochrome P450 CYP3-CYP5-CYP6-CYP9 subfamilies	0.024	2	14
FOG RRM domain	0.033	5	94
NA	0.036	244	14235

Some of these functions are in line with observations by Vandenbrouck et al. (2009). In their study with cadmium-nickel and lead-nickel mixtures, interaction effects were observed for genes coding for structural components such as collagens. Also in the study of Vandenbrouck et al (2010) with mixtures of pyrene, fluoranthene, gene ontology analysis reported a variety of metabolic functions including

protein localization and chitin metabolic processes. The same conclusions could be drawn from the study by Garcia-Reyero et al. (2012) who also observed a diverse set of metabolic functions present only in exposures to mixture of munitions constituents and not their single treatments. De Coninck et al. (2014) observed primarily interaction effects on the ubiquinone biosynthesis, serine-threonine protein kinases and collagens type IV and type XIII when *Daphnia* were exposed to both *Microcystis* and cadmium. No comparisons could be made with the study by David et al. (2011) focusing on mixtures of sodium dichromate and benzo-a-pyrene as they only investigated responses to the mixtures without reference to effects of single stressors.

The responses found here together with those in literature indicate that genes with an interaction effect cover a wide array of metabolic functions and are involved in a broad stress response involving a variety of affected pathways and mechanisms. These observations were in agreement with observations in human toxicology (Sen et al., 2007). Interaction effects therefore do not seem to occur from specific interaction at target sites or molecular events but rather from the complex interplay of the entire stress response in the organism. It seems therefore necessary to incorporate interactions between genes and pathways in analysis of mixture toxicity data to really underpin the complexity of these effects and potentially identify those that drive phenotypic effects.

7.4 Conclusion

Transcriptomic profiling of 48 mixture combinations were evaluated on a gene-by-gene basis and on a whole transcriptome level with three different filtering approaches and two different measures of deviation. Overall, filtering the data based upon no overlapping 95% confidence intervals between observations and predictions and subsequently calculating the sum of M_{int}-values of all genes with significant deviations from non-interaction was the most consistent approach allowing identification of statistical and biological significant genes. Genes with a significant interaction effect across all combinations showed a diverse pattern of expression in both synergistic and antagonistic directions. Current terminology of synergistic and antagonistic effects seems insufficient to fully explain the changes in expression of genes demonstrating an interaction effect. Functional analysis of these genes revealed a diverse set of metabolic functions and pathways indicating that interaction effects trigger a complex general stress response in the organism.

Chapter 8

Integration of transcriptomic and organismal data to study combined effects of cyanobacteria and insecticides

8.1 Introduction

Within this dissertation, chapters 2 to 4 have discussed the effects at the life history level while chapters 5 to 7 have focused on the effects at the molecular level. Results from all these previous chapters indicated that both life history and molecular responses cannot fully explain the complexity of interaction effects. Therefore, this final chapter will focus on integrating all data to identify those genes and mechanisms driving effects at life history level which in turn will aid to elucidate combined and interaction effects.

Few studies have addressed interaction effects with an integrative approach and of those, most are discussed in section 7.4. Yet, even less studies have fully integrated approaches at both life history and molecular level (Altenburger et al., 2012). Often, integration of molecular and life history data is comparative and descriptive. Indeed, most studies describe gene expression changes in pathways and networks as potential causes of observed fitness effects yet fail to directly correlate these two levels quantitatively. Few studies use the full potential of these two levels of biological organization to acquire new insights. Vandenbrouck et al. (2009) integrated suborganismal responses such as energy reserves with gene expression to understand responses to metal mixtures. Ghazalpour et al. (2006) correlated gene expression responses with the body weight of mice using weighed gene network co-expression analysis.

The potential benefits of integrating multiple levels of biological organization have stimulated environmental research towards a new vision (Villeneuve and Garcia-Reyero, 2011). Current advances in environmental science have put forward adverse outcome pathways as a molecular framework with high potential for risk assessment. Adverse outcome pathways are frameworks starting from molecular initiation events to a final adverse outcome at organismal level or sometimes even at population level as described previously (section 1.3) and can be summarized as a full integration of all levels of biological organization. Although adverse outcome pathways are becoming increasingly popular, only few are currently being constructed (OECD, 2014). The integrative approach in this chapter therefore hopes to identify key genes and gene networks that are driving effects at higher levels of functional organization. Such genes may form a crucial part of any adverse outcome pathway and help assist in further developing these frameworks and incorporating them in risk assessment. Furthermore, by identifying mechanisms involved in interaction effects, this approach

could pave the way for integrating interaction effects in risk assessment through new frameworks such as adverse outcome pathways. In addition, it will also address the current limitations that need to be improved prior to a general integration in regulatory frameworks.

8.2 Material and methods

8.2.1 Experimental design and data origin

The data used in this chapter originate from experiments discussed in chapters 4 and 7. Briefly, life history data was obtained from the experiment and analysis described in section 4.2. Molecular data was obtained from the experiment and analysis described in section 7.2. Explorative analysis of the two datasets, i.e. molecular and phenotypic data, consisted of standard Pearson`s correlation tests conducted in R (version 3.0.1) using the deviation parameter at the life history level (eq. 4.1) and the deviation parameter at the molecular level, summation of M-values of all genes with an interaction effect (eq. 7.5).

8.2.2 Weighted gene co-expression analysis (WGCNA)

Here all data, both molecular and life history responses, was integrated in a weighted gene co-expression analysis (Langfelder and Horvath, 2008). The WGCNA (version 1.27-1) has been successfully applied in vertebrate studies where it was able to correlate transcriptomic profiles with disease signatures (Korade and Mirnics, 2011; Wang et al., 2009). The analysis was conducted according to the general framework from Langfelder and Horvath (2008), represented in Fig. 8.1. Two types of networks were constructed. First, all expression data of all 48 combinations for single and combined exposures, i.e. 144(=48*3) expression profiles, were selected for the analysis. Expression profiles consisted of the M-values as generated through Limma analysis (chapter 7) for each gene on the array across all combinations. As recommended by Langfelder and Horvath (2008), only a subset of the genes was selected for the construction of gene networks to avoid noise and reduce computational requirements. For each gene, the variance across all 144 expression profiles was calculated and the 8000 genes with the largest variance were selected for further analysis upon recommendation by Langfelder and Horvath (2008) which in terms of data storage also corresponds to the maximum available memory in most standard desktops. As depicted in Fig. 8.1, gene networks were then constructed based upon the expression information in all profiles.

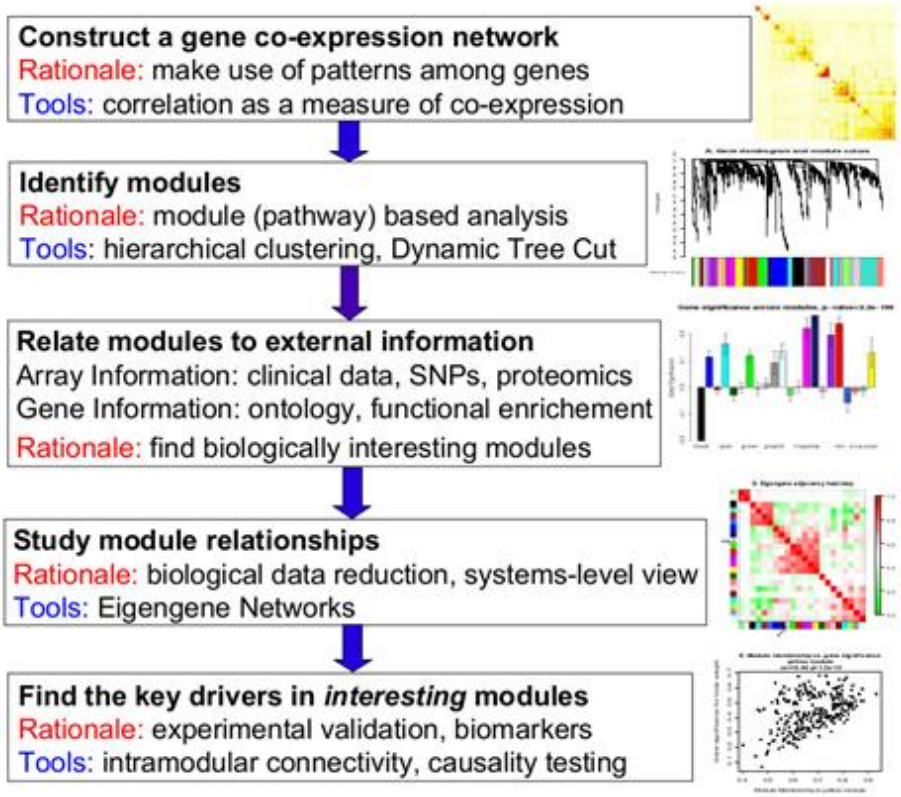


Figure 8.1 Analysis pipeline for weighted gene co-expression networks (From Langfelder and Horvath *BMC Bioinformatics* 2008, 9:559).

The WGCNA searches for and identifies clusters of highly correlated genes after network construction. In particular, gene networks can be represented as follows according to Langfelder and Horvath (2008):

$$X = [x_{ik}] = \begin{pmatrix} x_{1,} \cdot \\ x_i, \cdot \\ x_n, \cdot \end{pmatrix} \text{ (eq. 8.1)}$$

In eq. 8.1 *X* represents an *n* x *m* matrix representing *n* nodes (i=1,…, n) or genes and *m* samples or treatments (k=1,.., m). Co-expression is then defined by the absolute correlation coefficient between the expression profiles (all $M_{treatment/control}$ values of node *i* across all *m* treatments) of node *i* and node *i+1*, here gene *i* and gene *i+1(=j)* (Langfelder and Horvath, 2008):

$$s_{ij} = |cor(x_{i,\cdot} x_{j,\cdot})| \text{ (eq. 8.2)}$$

Genes are then clustered into modules based upon the adjacency *a* between genes *i* and gene *j* and is defined as follows (Langfelder and Horvath, 2008):

$$a_{ij} = s_{ij}^{\beta} \text{ (eq.8.3)}$$

In eq. 8.3 β is the soft thresholding power selected based upon the scale free topology criterion, generally used to describe biological networks (Zhang and Horvath, 2005). β is defined as a soft threshold as it is a continuous parameter rather than a binary parameter which is defined as a hard threshold. Indeed, a hard threshold would require the correlation coefficient between two genes to cross a certain specified threshold after which the two genes would be correlated. However, gene expression and correlation is not a black and white call but rather a continuous parameter which expresses the degree of correlation between two genes which in turn is represented by the soft thresholding power. The selection of the soft thresholding power based upon the scale free topology criterion is illustrated in Figure 8.2. Indeed, the lowest soft thresholding power was selected for which the curve in Figure 8.2 flattens after reaching a high value as the higher the value the lower the mean connectivity of the network and the higher the computational requirements. In Figure 8.2, a soft thresholding power of 16 and 24 would have been the most appropriate for the left and right plot respectively.

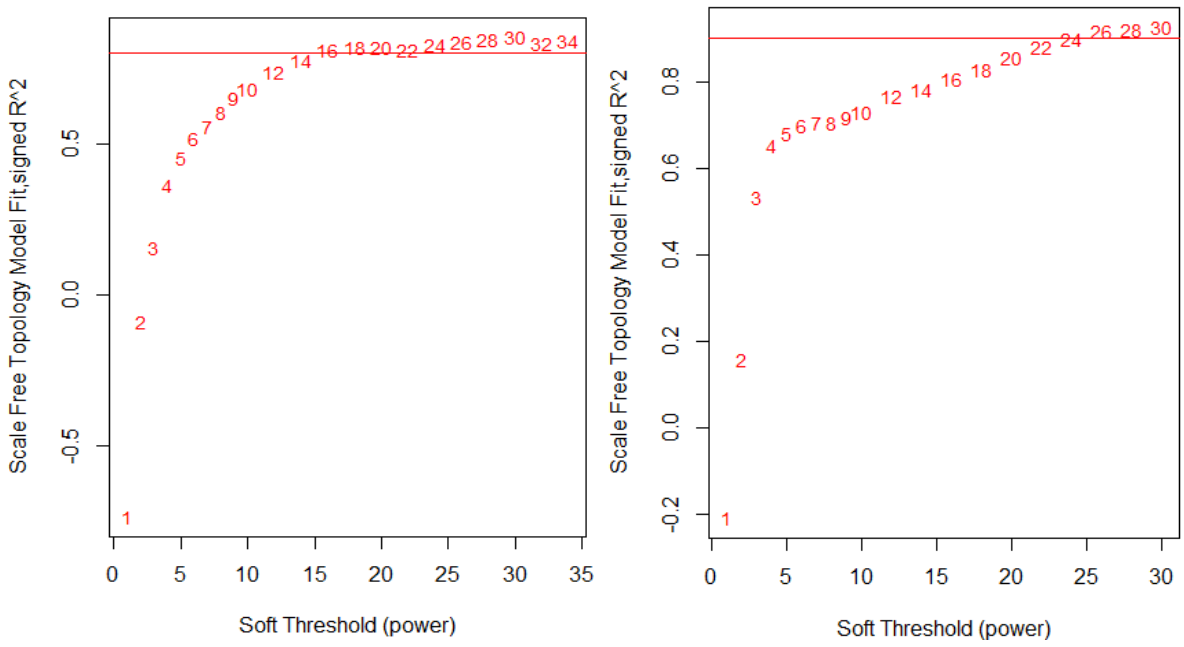


Figure 8.2 The scale free topology fit for various soft thresholding powers. The line intersects at a scale free topology fit of R^2=0.8 in the left figure and at a scalfe free topology fit of R^2=0.9 in the right figure.

After construction of the adjacency matrix for all genes, clustering is used to cluster genes into modules. Several clustering methods are available within the WGCNA package. Here, the default method was used, which is average linkage hierarchical clustering using the standard R function hclust. Thus, branches of the dendogram generated with hierarchical clustering correspond to the modules. Within the hierarchical clustering, the function dynamic tree cut is used which implements a variable cut height value and subsequently re-examines the clusters and potentially re-assigns clusters. Afterwards, modules whose eigengenes are highly correlated are merged. The expression profile of each module can then be defined by the eigengene of the module which is defined within the WGCNA package as the first principal component of the expression matrix of the module. Here, a soft thresholding power was used, rather than a hard thresholding power, so the module membership of a gene was fuzzy measure rather than a binary measure:

$$K_{cor,i}^{q} := cor(x_i, E^q) \quad \text{(eq. 8.4)}$$

Where x_i is the expression profile of gene *i* (columvector with *m* columns) and *E* the eigengene of module *q* (column vector with *m* columns) and the module membership *K* lies thus within [-1, 1] and specifies how close gene *i* is to module *q*.

Afterwards, the generated gene network consists of modules of highly connected genes that can be correlated with external information. Here, external information consisted of the reproductive output relative to a control per treatment, averaged across biological replicates, and the deviation parameter as defined in eq. 4.1 and represented in Table 4.4 for deviations at life history level, i.e. mixture interaction effect. Correlation with external traits is defined as the gene significance *GS*:

$$GS_i = |cor(x_i, T)| \quad \text{(eq. 8.5)}$$

In which *GS* is the gene significance of gene *i,* x_i the expression profile of gene *i* and *T* the external information, e.g. reproduction data. The statistical significance of the *GS* measure for gene *i* is defined by the p-value of the Pearson correlation test. Eq. 8.5 can also be modified to determine the module significance, i.e. correlation between a module and the trait of interest in which x_i then represents the expression profile of the eigengene.

Second, all expression profiles for the mixture data, i.e. 48 profiles, were used for network construction. Again, a subset of genes was selected. Due to a much smaller dataset than above, no selection criterion was necessary as the use of all genes was not limited by computational requirements. Again, based on the gene list, here containing all genes, gene networks were constructed and were correlated with external information after construction. The same external information was related to the gene networks, i.e. reproductive output and deviation at life history level.

In both networks, the identified key modules, i.e. modules that correlated significantly with the reproductive output or with the deviation at life history level, were analyzed for overrepresentation of pathways, paralog clusters and functional annotations as described in section 5.2.5. Additionally, for each significant module, gene significance and module membership were correlated for all genes in the module. A significant positive correlation indicates that genes with high absolute gene significance also have a high absolute module membership. In other words, genes that are highly correlated with the trait of interest are also the most important genes in the network, i.e. hub genes. Langfelder and Horvath (2008) define these genes as highly interconnected genes and such genes are often representatives of the modules they were assigned to, described as key drivers, based upon eq. 8.4. Here, for each module, hub genes were selected based upon Langfelder and Horvath (2008) as the 10 genes with the highest module membership defined through eq. 8.4. Modules were visualized with Cytoscape by using their hub genes (Cline et al., 2007).

8.3 Results and discussion

Simpler models are often preferred to complex networks in any analysis due to ease of interpretation and data requirements. Yet, no significant correlation was observed between the suggested deviation measure which sums the M_{int}-values of all genes with a significant interaction effect, (eq. 7.6, Table 7.4) at the transcriptomic level, and the deviation from non-interaction at the life history level (eq. 4.1, Table 4.4), defined as the difference between the log transformed predicted and observed effects on reproduction (Fig. 8.3). Clearly, deviations at the transcriptional level as defined in section 7.2.5 did not sufficiently explain deviations at the life history level and more appropriate parameters need to be identified. The lack of correlation could have two different causes. First, the developed measure at the transcriptomic level might not have been appropriate to quantify deviations at transcriptomic level that are indicative of deviations from non-interaction at life history level. Second, transcriptomic information

or data may not be sufficient to explain deviations from non-interaction at life history level. In the first case, a more suitable measure needs to be defined. In the second case, additional data generation at other functional levels is needed, e.g. proteomic or metabolomic data.

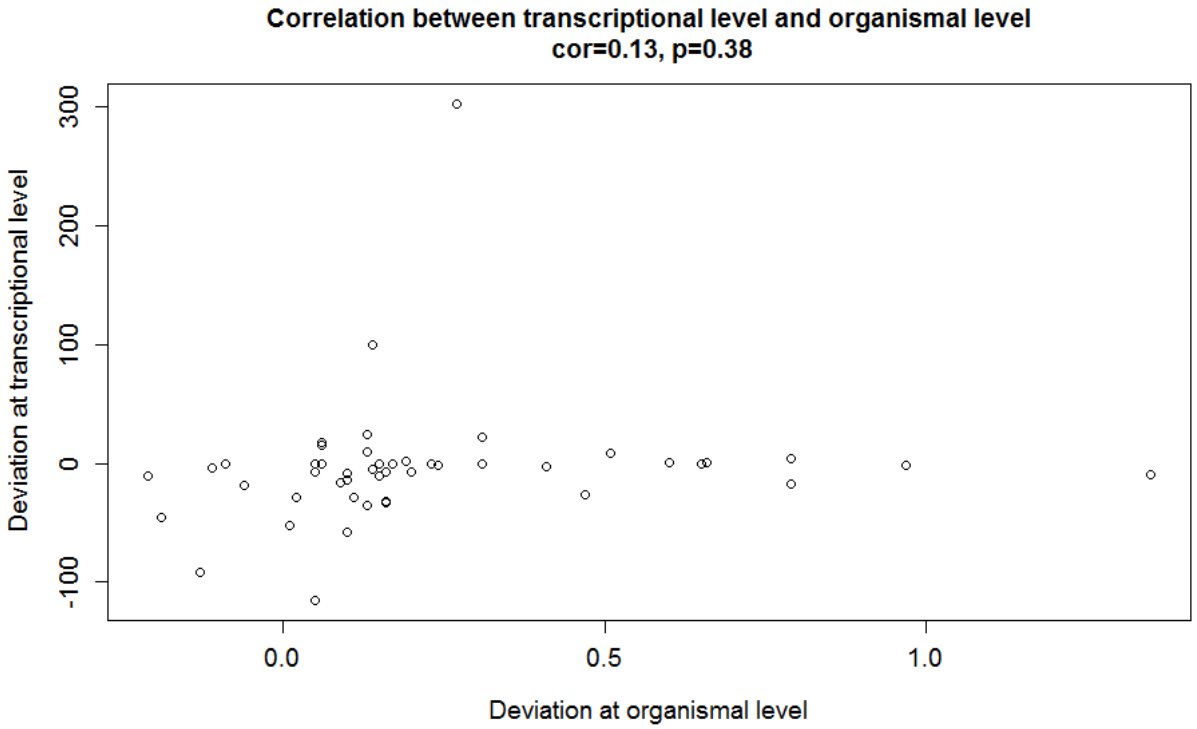


Figure 8.3 Correlation between the deviation observed at the organismal level and at the transcriptional level.

Therefore, we will focus on identifying potential patterns in transcriptomic data that can be correlated with or be representative of deviation from non-interaction at life history level. Identification of these patterns will confirm that transcriptomic data does contain suitable information to quantify deviations at life history level and that subsequently, more appropriate measures than the one suggested in chapter 7 can be developed. If no such patterns are identified, it will confirm that additional information at other functional levels is essential to quantify deviations at life history level.

Pattern identification was conducted through the construction of gene networks based upon transcriptomic profiles (section 8.2). Subsequent analysis through gene networks did reveal significant correlations between the eigengene of the network modules and life history traits (Fig. 8.4). Both networks, i.e. the network constructed with all 144 profiles and the network constructed with 48 mixture profiles, resulted in a comparable number of modules (15 versus 16). Both networks contained

modules that significantly correlated with the life history traits, i.e. reproduction and deviation from non-interaction (Fig. 8.4). More and stronger correlations were observed for the network developed based on 48 mixture profiles and the reproductive output than for the network developed based on all 144 profiles and the reproductive output. Only one module correlated significantly with reproductive output in the network developed based on 144 profiles (Fig. 8.4). In contrast, no significant correlations with the deviation from non-interaction at life history level were observed for the network with 48 mixture profiles whereas several modules of the network with all 144 profiles did correlate significantly with this life history parameter (Fig. 8.4).

Overall, this pattern demonstrated that expression of genes in profiles of exposure to single stressors is necessary in addition to expression of genes in the mixture profiles to identify factors driving interaction. Indeed, only the network profile containing both single and mixture profiles contained modules significantly correlated with deviation from non-interaction at life history. In contrast, no module of the network without these single stress profiles and only the mixture profiles correlated significantly with interaction at life history. Further study should focus on determining whether these driving factors can also be identified based upon expression profiles of single stressors alone. If this would be the case, expression profiles may serve as a basis for predictive models regarding mixture toxicity. The present study did not allow building such gene networks only with single stressor profiles given that there were only fourteen single stressors available which is a too small number to build reliable gene networks. Indeed, due to the size of the data, a limited number of profiles often require a high soft thresholding power (Langfelder and Horvath, 2006). However, the higher the soft thresholding power, the higher computational requirement needed to construct weighted gene networks. At present, the 48 profiles required a soft thresholding power of 24 which is close to the upper limit of current computational requirements and algorithms programmed within the WGCNA package.

Gene network analysis was able to identify significant correlations between network modules and life history traits, i.e. deviation from non-interaction and the reproductive output relative to control. Information on the expression profile of the individual stressor in addition to the mixture expression profile seems crucial in identifying gene modules that correlate with deviations from non-interaction.

Network based on all profiles		
Module	Reproduction	Interaction
MEBrown	**0.17 (0.04)**	**-0.25 (0.033)**
MEyellow	0.16 (0.05)	-0.1 (0.2)
MEcyan	-0.01 (0.9)	-0.07 (0.3)
MEred	-0.11 (0.2)	0.03 (0.7)
MEpink	-0.15 (0.07)	-0.07 (0.4)
MEblack	-0.02 (0.8)	**-0.3 (2e-04)**
MEblue	2e-04 (1)	-0.16 (0.05)
MEgreenyellow	-0.01 (0.9)	0.15 (0.07)
MEsalmon	0.11 (0.2)	**0.26 (0.002)**
MEmagenta	-0.1 (0.2)	0.09 (0.3)
MEgreen	-0.16 (0.06)	**0.26 (0.002)**
MEpurple	-0.15 (0.08)	**0.18 (0.03)**
MEtan	0.09 (0.3)	-0.17 (0.06)
MEturquoise	0.04 (0.6)	0.08 (0.3)
MEgrey	-0.09 (0.3)	**0.22 (0.009)**

Network based on mixture profiles		
Module	Reproduction	Interaction
MEBrown	**0.37 (0.01)**	-0.24 (0.1)
MEturqouise	0.083 (0.6)	0.09 (0.6)
MEyellow	**0.31 (0.03)**	-0.1 (0.5)
MEmagenta	0.22 (0.1)	-0.09 (0.5)
MEtan	**0.3 (0.04)**	-0.17 (0.3)
MEpink	-0.28 (0.05)	0.16 (0.3)
MEsalmon	**-0.59 (1e-05)**	0.28 (0.06)
MEcyan	-0.04 (0.8)	0.17 (0.2)
MEgreenyellow	0.05 (0.8)	0.23 (0.1)
MEgreen	-0.14 (0.4)	0.22 (0.1)
MEpurple	-0.1 (0.5)	0.07 (0.68)
MEblack	-0.26 (0.08)	0.04 (0.8)
MEred	-0.27 (0.06)	-0.09 (0.6)
MEblue	-0.11 (0.4)	-0.14 (0.4)
MEmidnightblue	**-0.36 (0.01)**	-0.12 (0.4)
MEgrey	0.05 (0.7)	0.11 (0.5)

Figure 8.4 Heatmap of the modules (ME) within the gene network developed based on all expression data (left) and the network developed based on only the mixture data (right) and their correlation with external traits, reproduction at life history level (labelled Reproduction) and deviation from non-interaction (labelled Interaction). For each module-trait combination in each network, the correlation value is represented with the corresponding p-value between brackets. Significant correlations are printed in bold. The more negative the correlation coeficient, the darker the green, the more positive the correlation coefficient the darker the red.

For each network five to six modules were significantly correlated with reproduction or deviation from non-interaction at life history level and these modules were then selected for functional analysis. Modules size differed largely between the different modules and different networks (Table 8.1). Modules with colors green, midnightblue, purple, salmon and tan were too small for functional analysis and were therefore not included in the functional analysis.

Table 8.1 List of modules significantly correlated with external traits and the number of genes in each of these modules as well as the network they belong to.

Module Color	N° of genes	Network	Module Color	N° of genes	Network
Brown	1020	All profiles	Brown	1625	Mixture profiles
Black	123	All profiles	Midnightblue	31	Mixture profiles
Salmon	48	All profiles	Salmon	57	Mixture profiles
Green	479	All profiles	Yellow	1338	Mixture profiles
Grey	1541	All profiles	Tan	57	Mixture profiles
Purple	64	All profiles			

In the network with all expression profiles, modules brown, green and grey were significantly correlated with deviations from interaction on life history. In all modules, genes related with the amino acid metabolism were overrepresented as well as lineage specific genes (Table 8.2, Table 8.3). The brown and grey modules also contained genes in relation with signal transduction, i.e. G protein coupled receptors, and histones, structural components of chromatin, MAPK signaling. In contrast, the green module contained genes related to hormone biosynthesis, transcription and proteolysis. The grey module contained genes related to digestion such as trypsins and genes with a still unknown function in *Daphnia* such as Neurexin IV and speckle-type POZ (pox virus and zinc finger) proteins (Bardwell and Treisman, 1994).

Table 8.2 List of significantly overrepresented pathways with their KEGG ID in modules of the gene network based on all 148 expression profiles. The Benjamaini-Hochberg corrected p-value obtained after Fisher`s exact test, the number of module genes belonging to each pathway and the color of the module are shown.

Pathway ID	Name	P-value	N° of module genes	Module Color
Map00620	Pyruvate metabolism	0.021	6	Brown
Map00350	Tyrosine metabolism	0.024	11	Brown
Map00340	Histidine metabolism	0.014	6	Green
Map03022	Basal transcription factors	0.0010	4	Green
Map00260	Glycine, serine and threonine metabolism	0.017	5	Green
Map00520	Amino sugar and nucleotide sugar metabolism	0.038	6	Green
Map00981	Insect hormone biosynthesis	0.046	5	Green
Map00340	Histidine metabolism	0.017	14	Grey
Map04010	MAPK Signalling	0.003	7	Grey
Map04916	Melanogenesis	0.004	7	Grey

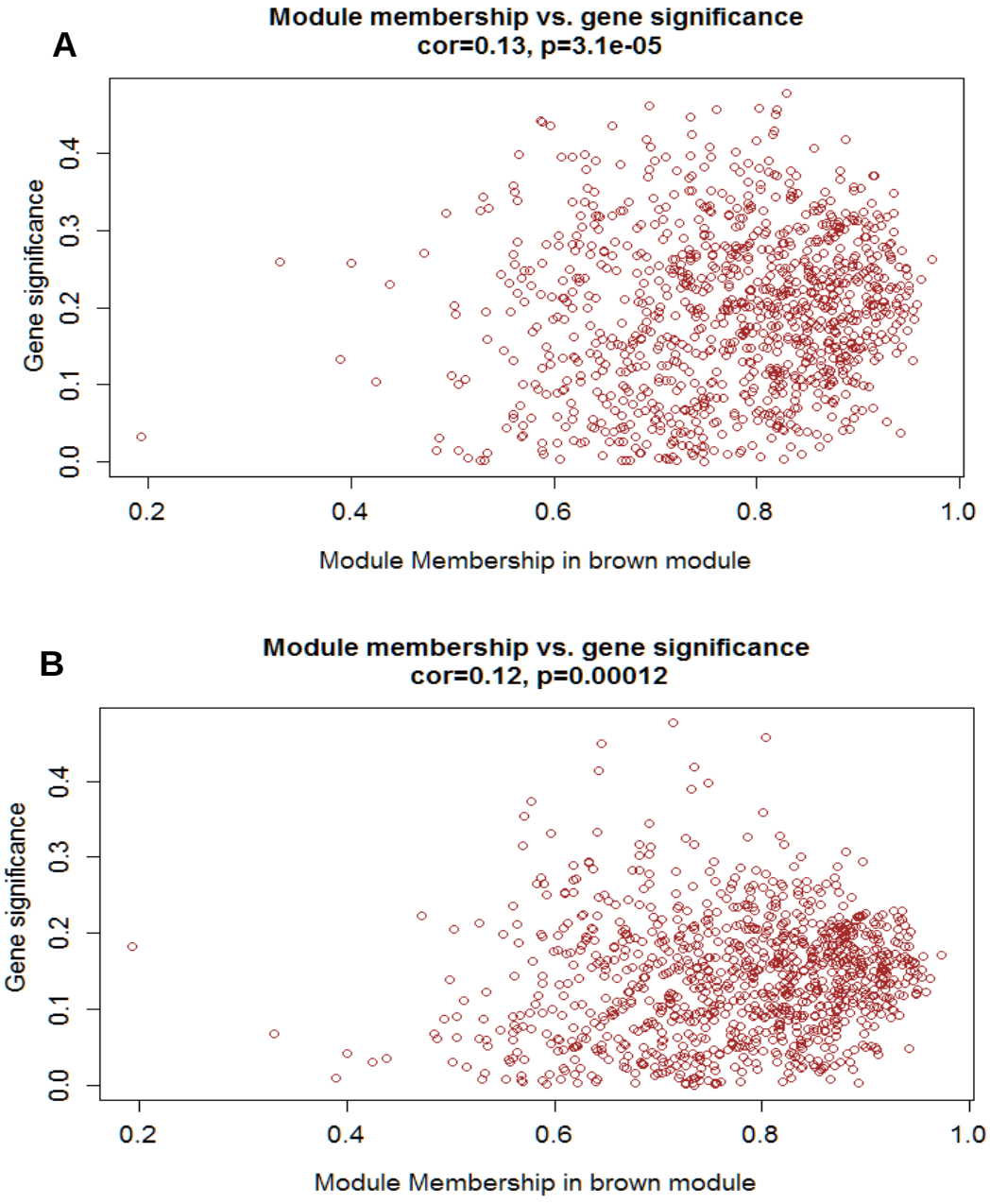
Table 8.3 Number of module genes in functional annotations significantly overrepresented with module genes of the gene network based all 144 expression profiles together with the Benjamini-Hochberg corrected p-value from Fisher`s exact test and the color of the module.

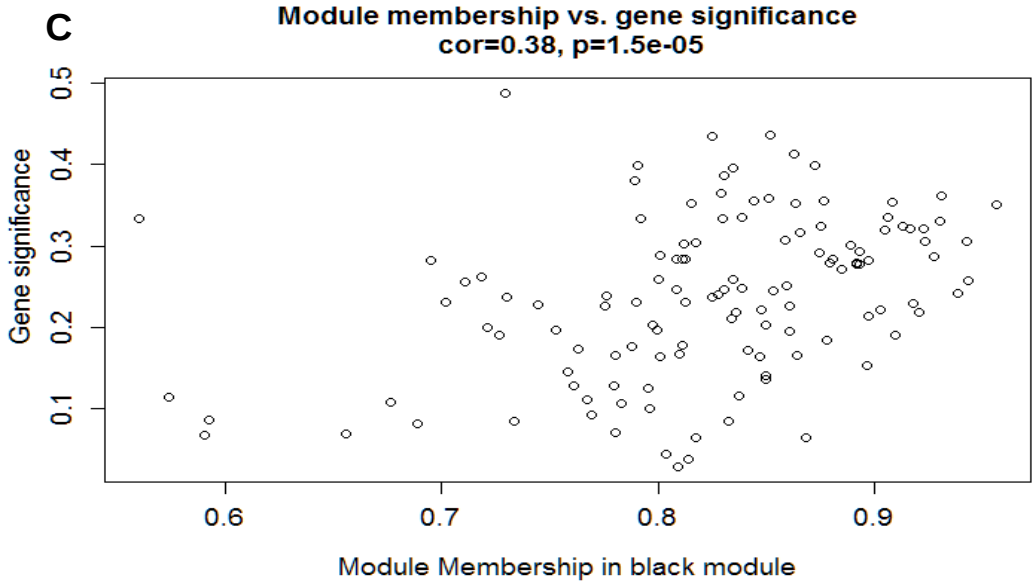
Annotation defintion	P-value	N° of module genes	Module color
NA (no functional annotation available)	8.77 e-09	401	Brown
Uncharacterized conserved protein	6.95 e-05	41	Brown
Histone H4	0.0029	4	Brown
G-protein coupled receptors	0.013	5	Brown
NA (no functional annotation available)	0.0049	200	Green
E3 ubiquitin-ligase	0.016	3	Green
NA (no functional annotation available)	3.70 e-08	637	Grey
Speckle type POZ proteins	0.002	17	Grey
Trypsin	0.010	23	Grey
Neurexin IV	0.014	7	Grey
FOG-7 Transmembrane receptor	0.015	15	Grey
Chitinase	0.023	14	Grey

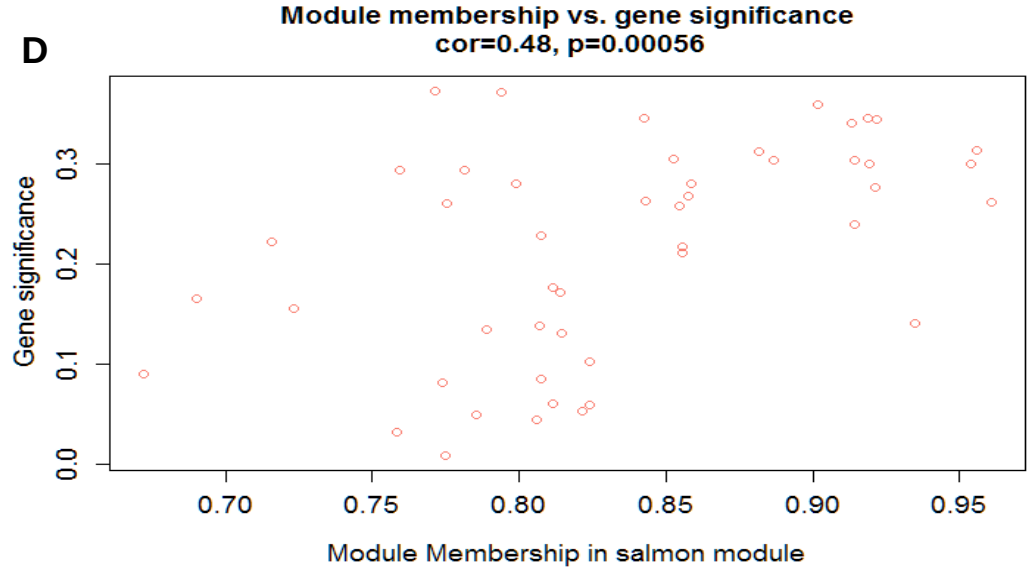
The brown, the green and the grey module correlated all significantly with the deviation from non-interaction at life history level but in the opposite direction. In other words, the green and the grey module correlated positively, meaning that changes in gene expression correlate positively with changes in deviation from non-interaction. The brown module, in contrast, correlated negatively, meaning that changes in gene expression correlated negatively with changes in deviations from non-interaction. In terms of functional annotation, these results indicated that expression changes of genes involved in hormone biosynthesis, transcription, digestion as well as proteolysis correlated positively with changes in the deviation from non-interaction at life history level. However, expression changes in genes involved in the energy metabolism, tyrosine metabolism as well as chromatin structure correlated negatively with changes in deviation of interaction but correlated positively with changes in the reproduction of the organism, given the significant correlation of the brown module with reproduction.

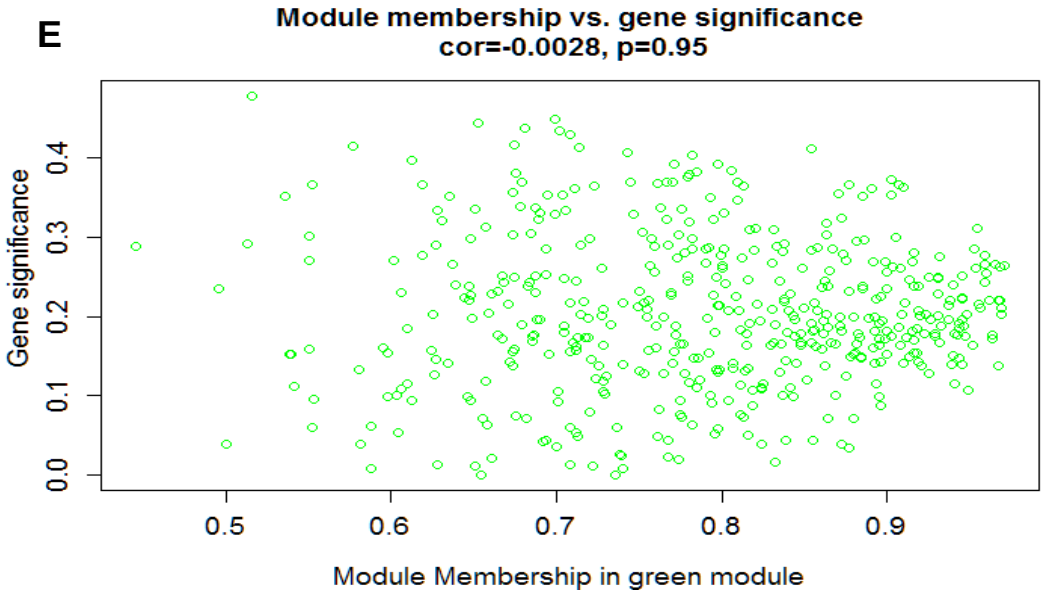
In addition to the functional analysis, genes within all significantly correlated modules were analyzed for potential correlations between gene significance and module membership. This was particularly interesting for modules which are too small in size for a functional analysis. Indeed, positive significant correlation indicates that genes have both high gene significance as well as a high module

membership, indicating that the genes with the highest correlation with the trait of interest are also the most important genes in the modules, i.e. hub genes. The green, grey and purple modules did not have significant correlations between gene significance and module membership (Fig. 8.5 E, F, G) whereas a high significant correlation was observed in the black and salmon modules (Fig. 8.5. C, D). In the brown module, gene significance correlated significantly although the correlation value was not very high with module membership for both traits, i.e. reproduction and deviation from non-interaction (Fig. 8.5 A, B).




C
Module membership vs. gene significance
cor=0.38, p=1.5e-05
Gene significance
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Module Membership in black module


D
Module membership vs. gene significance
cor=0.48, p=0.00056
Gene significance
0.0
0.1
0.2
0.3
0.70
0.75
0.80
0.85
0.90
0.95
Module Membership in salmon module


E
Module membership vs. gene significance
cor=-0.0028, p=0.95
Gene significance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Module Membership in green module

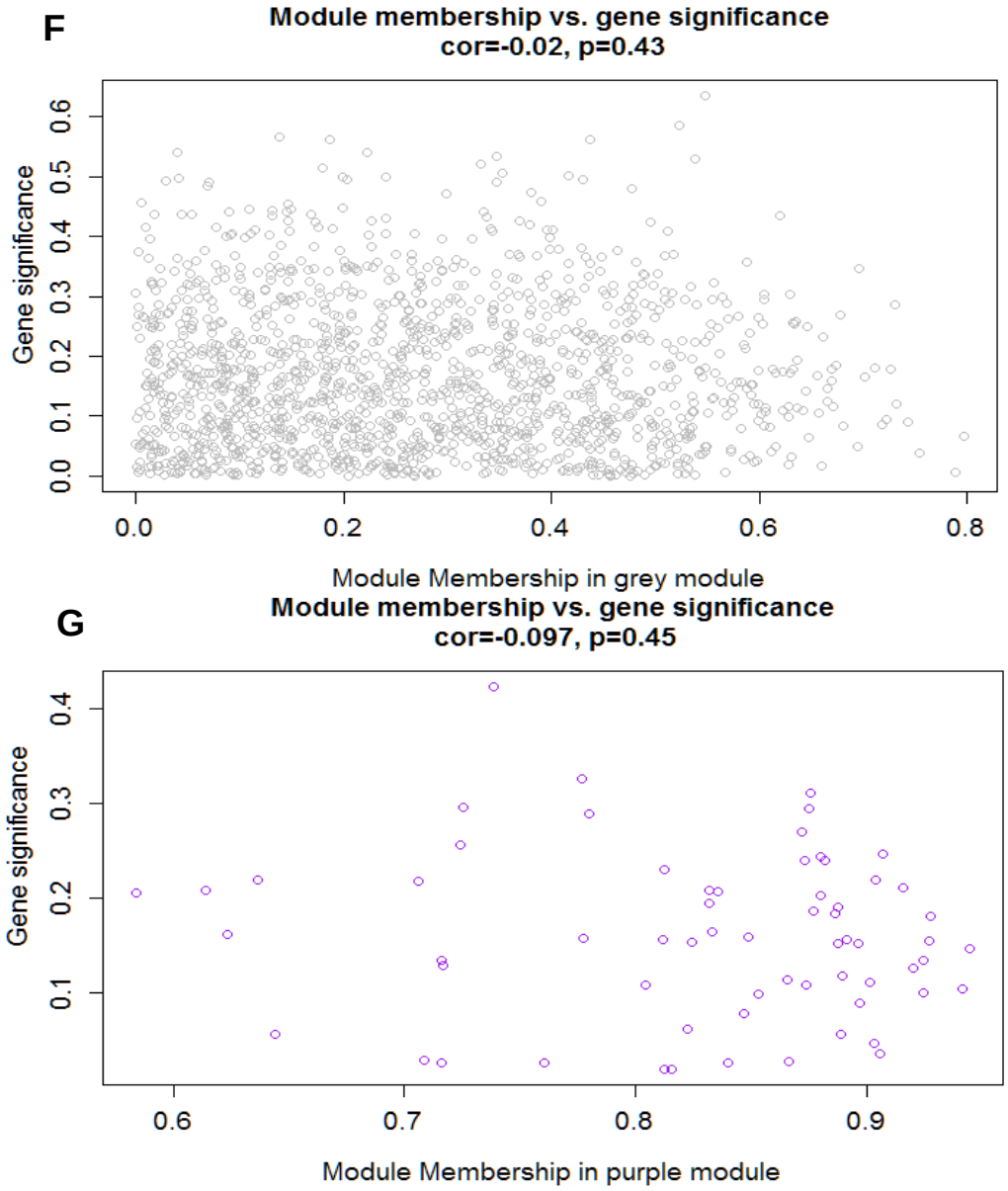


Figure 8.5 Correlation between gene significance and module membership for modules within the network based on all 148 expression profiles. For all modules correlations were made between the gene significance for the deviation from interaction. For the brown module, correlations were also made between the gene significance for reproduction (A).

Thus, for the black and salmon modules, the hub genes were also the genes with the highest correlation with the trait of interest. The function of these genes (Table 8.4) was quite diverse for the black module whereas the salmon module contained only one annotated hub gene, i.e. RNA polymerase II.

Table 8.4 Functional annotation of the ten most connected genes or hub genes in the black and salmon modules of the network based on all 148 expression profiles. NA= no functional annotation available, i.e. lineage specific gene.

Module	Gene_id	Functional Annotation
Black	JGI_V11_118253	Nucleolar GTPase-ATPase p130
Black	JGI_V11_114513	NA
Black	JGI_V11_249001	RNA polymerase II
Black	JGI_V11_109902	RNA polymerase II
Black	JGI_V11_186885	NA
Black	JGI_V11_299612	Golgi integral membrane protein
Black	JGI_V11_209116	Protein-tyrosine sulfotransferase TPST1-TPST2
Black	JGI_V11_122224	Transcription factor containing C2HC-type Zn finger
Black	JGI_V11_196799	Predicted NAD synthase contains CN-hydrolase domain
Black	JGI_V11_232534	NA
Salmon	JGI_V11_242810	NA
Salmon	JGI_V11_234827	RNA polymerase II
Salmon	JGI_V11_306978	NA
Salmon	JGI_V11_266550	NA
Salmon	JGI_V11_308817	NA
Salmon	JGI_V11_329725	NA
Salmon	JGI_V11_261759	NA
Salmon	JGI_V11_331152	NA
Salmon	JGI_V11_310096	NA
Salmon	JGI_V11_263306	NA

A visual representation of the significant gene modules by using the hub genes of each of these modules revealed some interesting features (Fig. 8.6). First, hub genes of the purple and the green gene network were interconnected with the genes in each of these respective modules but also with each other. The hub genes in the salmon, black and brown modules were only connected with the hub genes within their own module. In contrast, the grey module had few connections between its hub genes and some hub genes were not even connected with each other. Also six of the ten hub genes overlapped in their network topology two by two which was why only seven hub genes were represented. This suggests that the hub genes of the grey module were each extremely interconnected with other groups of genes within the grey module and not with other hub genes.

Gene modules significantly correlating with deviation from non-interaction at life history consisted of a variety of pathways and gene functions. Modules correlating positively with the interaction parameter primarily consisted of genes involved in hormone biosynthesis, transcription, and digestion. The module correlating negatively with deviation from non-interaction at life history consisted of genes involved in energy metabolism, tyrosine metabolism as well as chromatin structure. Functional analysis revealed both larger metabolic pathways as specific gene functions such as neurexin IV and trypsins. Two of the five modules could be represented by the hub genes as a significant correlation was observed between the gene significance and the module membership. The intramodular connectivity of each of the modules differed significantly as some, i.e. green and purple module, were extremely connected whereas the grey module showed very few connections.

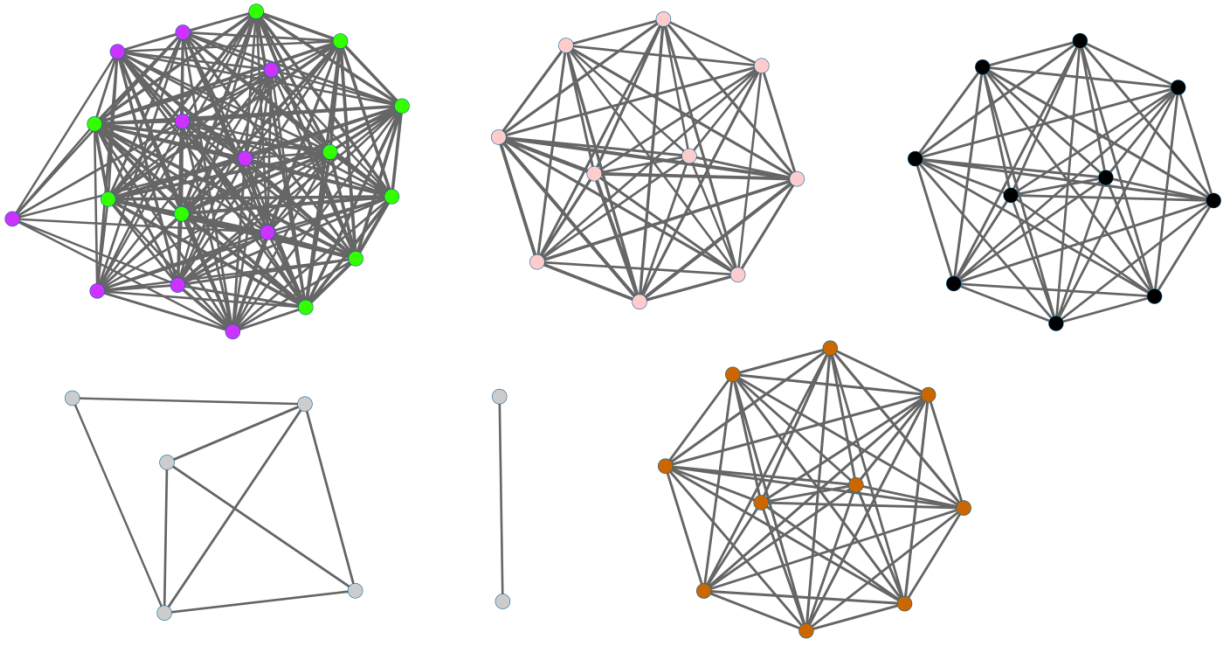


Figure 8.6 Visual representation of the hub genes for each of the significantly correlated modules within the network with all expression profiles. Colors correspond with module colors. The thickness of the edge, i.e. the line which connect the nodes, represents the adjancency between the genes (equation 8.3).

The network based on only the 48 mixture profiles contained only modules correlating significantly with reproduction. The two modules that could be functionally analyzed were significantly overrepresented with genes involved in the amino acid metabolism and the hormone biosynthesis (Table 8.6). The brown module also contained genes involved in the fatty acid metabolism whereas the yellow module

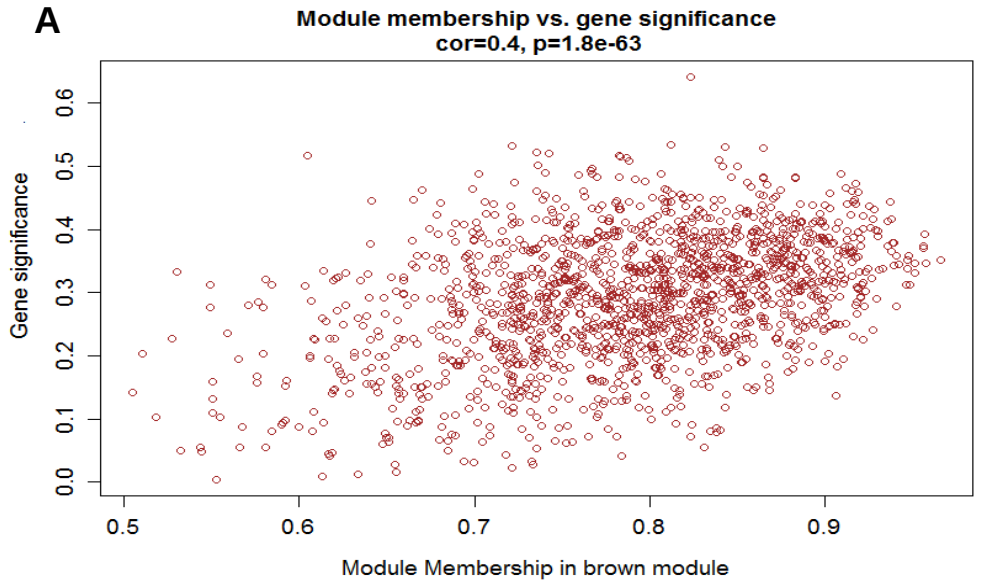
contained genes related to steroid synthesis (Table 8.5, Table 8.6). Analysis of overrepresentation of functional annotations also revealed a high proportion of lineage specific genes (Table 8.6).

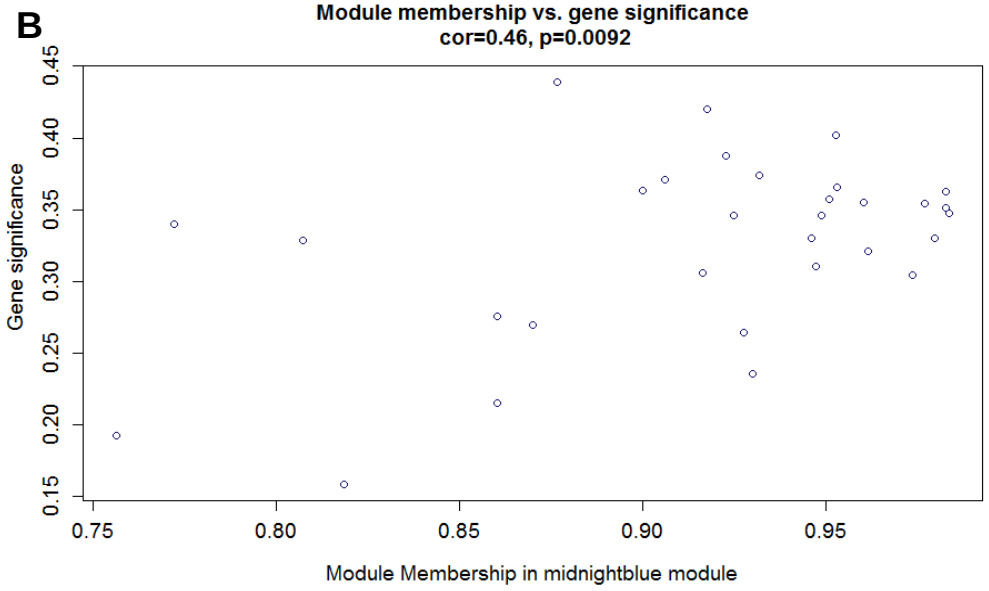
Table 8.5 List of pathways with their KEGG ID significantly overrepresented with genes of the modules of the gene network based on only the 48 mixture profiles. The Benjamini-Hochberg corrected p-value obtained after Fisher`s exact test, the number module genes belonging to each pathway and the module color are shown.

Pathway ID	Name	P-value	N° of module genes	Module Color
Map00260	Glycine, serine and threonine metabolism	0.0004	14	Brown
Map00981	Insect hormone biosynthesis	0.004	15	Brown
Map01040	Biosynthesis of unsaturated fatty acids	0.006	12	Brown
Map00520	Amino sugar and nucleotide sugar metabolism	0.006	17	Brown
Map000620	Pyruvate metabolism	0.022	8	Brown
Map00340	Histidine metabolism	0.024	13	Brown
Map00350	Tyrosine metabolism	0.033	15	Brown
Map00981	Insect hormone biosynthesis	0.003	13	Yellow
Map00240	Pyrimidine metabolism	0.004	16	Yellow
Map00100	Steroid biosynthesis	0.012	6	Yellow
Map00340	Histidine metabolism	0.024	11	Yellow
Map00480	Glutathione metabolism	0.039	14	Yellow

Table 8.6 Number of module genes in functional annotations significantly overrepresented for genes of modules of the gene network based on only the 48 mixture profiles together with the Benjamini-Hochberg corrected p-value from Fisher`s exact test and the color of the module.

Annotation defintion	P-value	N° of module genes	Module color
NA (no functional annotation available)	8.35 e-08	637	Brown
Fatty acyl-coenzyme A elongase	0.009	5	Brown
Uncharacterized conserved protein	0.004	50	Brown
Mitochondrial ribosomal proteins	0.006	6	Brown
Phosphatidylinositol transfer protein	0.038	6	Brown
NA (no functional annotation available)	4.8 e-25	461	Yellow
Predicted membrane proteine	0.0002	10	Yellow
Uncharacterized conserved protein	0.0008	46	Yellow
Proteins with POZ domains involved in signal transduction	0.018	6	Yellow
FOG leuchine rich repeat	0.018	7	Yellow


A
Module membership vs. gene significance
cor=0.4, p=1.8e-63
Gene significance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.5
0.6
0.7
0.8
0.9
Module Membership in brown module


B
Module membership vs. gene significance
cor=0.46, p=0.0092
Gene significance
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.75
0.80
0.85
0.90
0.95
Module Membership in midnightblue module


C
Module membership vs. gene significance
cor=0.47, p=0.00023
Gene significance
0.1
0.2
0.3
0.4
0.80
0.85
0.90
0.95
Module Membership in tan module

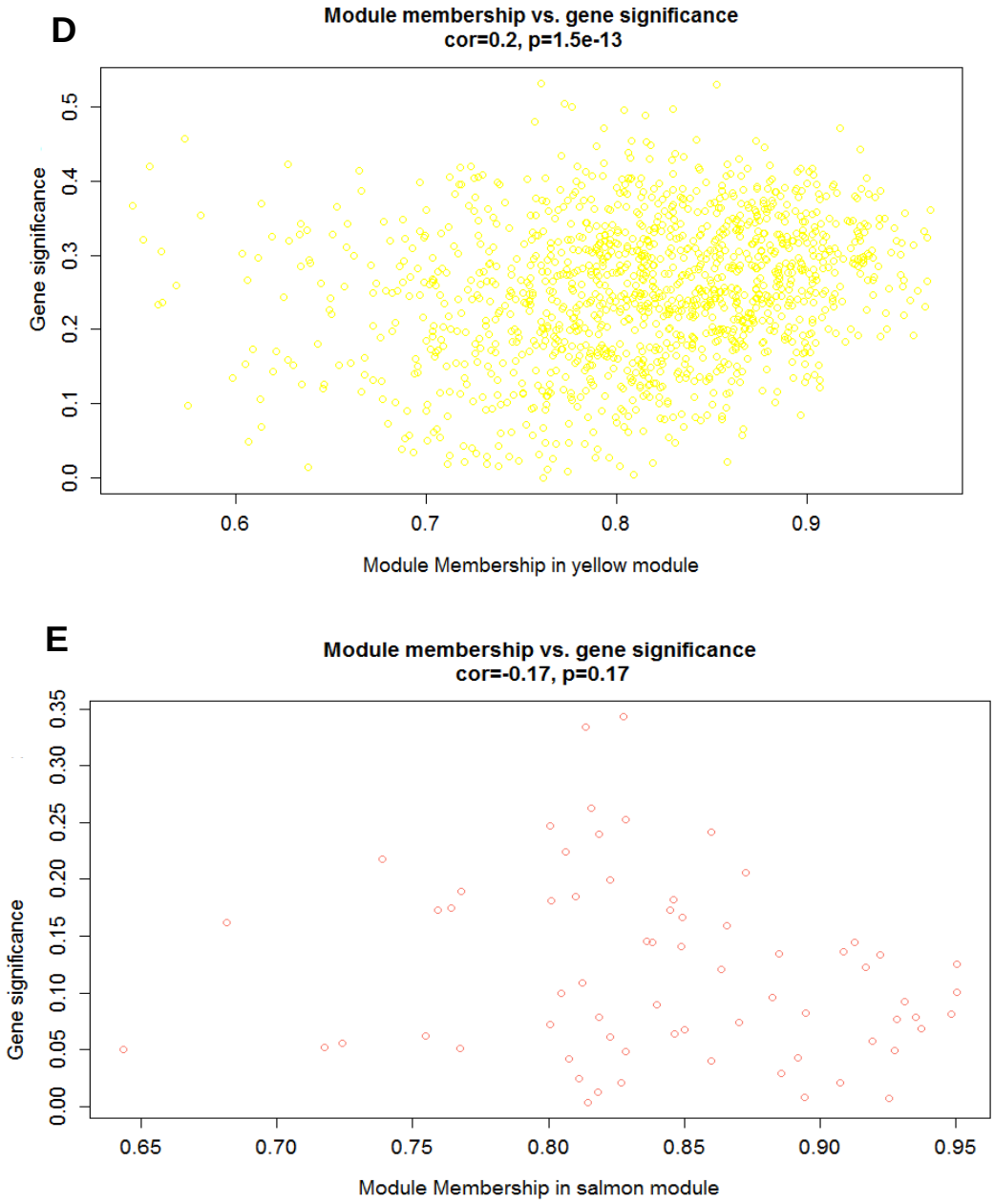


Figure 8.7 Correlation between gene significance and module membership for modules within the network based on only the 48 mixture expression profiles. For all modules correlations were made between the gene significance for reproduction.

Correlations of gene significance and module membership were significant for four of the five modules (Fig. 8.7 A, B, C, D). Only in the salmon module (Fig. 8.7 E), no significant correlation was observed. Functions of the hub genes in these four modules consisted primarily of lineage-specific genes and uncharacterized proteins (Table 8.7). Additional functions were among others RNA polymerases, FOG domain proteins, collagens and ABC superfamily related proteins.

Table 8.7 Functional annotation of the ten most connected genes or hub genes in the black, midnightblue, tan and purple modules of the network based on only the 48 mixture expression profiles.

Module	Gene_id	Functional Annotation
Brown	JGI_V11_187192	3-Methylcrotonyl-CoA carboxylase
Brown	JGI_V11_205678	Uncharacterized conserved protein
Brown	JGI_V11_228142	NA
Brown	JGI_V11_299660	NA
Brown	JGI_V11_299669	Cell growth regulatory protein
Brown	JGI_V11_304743	NA
Brown	JGI_V11_306103	NA
Brown	JGI_V11_307765	Uncharacterized conserved protein
Brown	JGI_V11_337141	NA
Brown	JGI_V11_93995	Membrane coat complex Retromer subunit
Midnightblue	JGI_V11_119702	Collagens -type IV and type XIII-related proteins
Midnightblue	JGI_V11_120485	NA
Midnightblue	JGI_V11_122685	NA
Midnightblue	JGI_V11_123715	Alternative splicing factors
Midnightblue	JGI_V11_124343	NA
Midnightblue	JGI_V11_305186	Lipid exporter ABCA1 and related proteins ABC superfamily
Midnightblue	JGI_V11_66764	Multidrug-pheromone exporter ABC superfamily
Midnightblue	JGI_V11_67653	Meprin A metalloprotease
Midnightblue	JGI_V11_7071	NA
Midnightblue	JGI_V11_7140	RNA polymerase II transcription termination factor superfamily
Tan	JGI_V11_101682	NA
Tan	JGI_V11_104443	Predicted transmembrane protein (immunoglobulin family)
Tan	JGI_V11_110605	FOG Immunoglobulin C-2 Type-fibronectin type III domains
Tan	JGI_V11_241465	NA
Tan	JGI_V11_243781	NA
Tan	JGI_V11_304016	NA
Tan	JGI_V11_304032	NA
Tan	JGI_V11_309130	NA
Tan	JGI_V11_46113	FOG Zn-finger
Tan	JGI_V11_92217	Uncharacterized conserved protein
Yellow	JGI_V11_203235	NA
Yellow	JGI_V11_208467	Cell-cycle nuclear protein contains WD-40 repeats
Yellow	JGI_V11_307124	NA
Yellow	JGI_V11_313246	NA

Module *(Table 8.7 cont.)*	Gene_id	Functional Annotation
Yellow	JGI_V11_317426	NA
Yellow	JGI_V11_323650	NA
Yellow	JGI_V11_325783	RNA polymerase II
Yellow	JGI_V11_45609	Protein kinase containing WD40 repeats
Yellow	JGI_V11_52570	DNA helicase
Yellow	JGI_V11_95969	NA

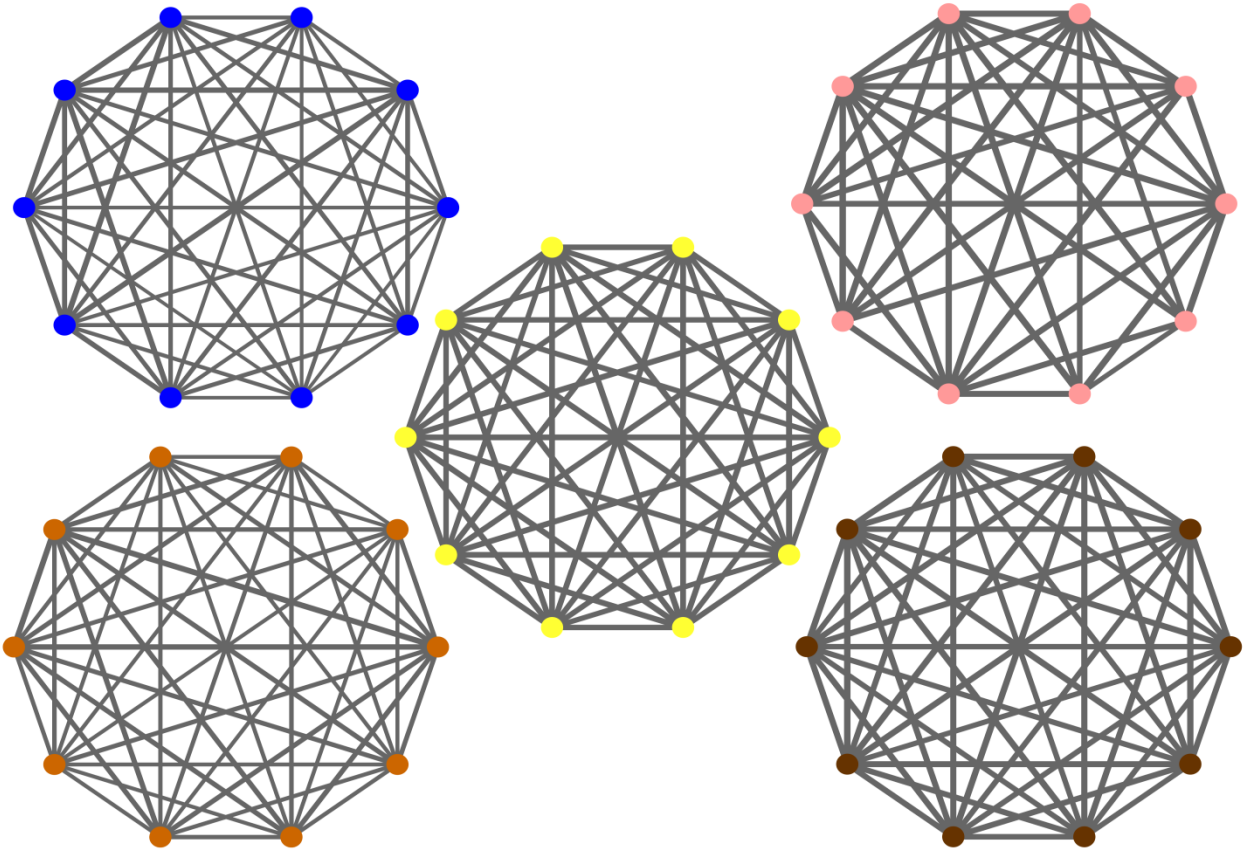


Figure 8.8 Visual representation of the hub genes for each of the significantly correlated modules within the network with only mixture expression profiles. Colors correspond with module colors. The thickness of the edge, i.e. the line which connects the nodes, represents the adjancency between the genes (equation 8.3).

Visual representation of the significant modules based on their hub genes indicated five tightly connected modules with no overlap between the modules.

Five gene modules within the network based on only the 48 mixture profiles correlated significantly with the reproductive output. The majority of these modules could be represented by their hub genes due to a significant correlation between gene significance and module membership. Functional analysis indicated overrepresentation of genes involved in the amino acid metabolism and the hormone biosynthesis. Specific gene functions in the modules were among others speckle POZ proteins and phosphatidyl inositol transfer proteins.

Functional analysis of the modules within the two constructed gene networks that could be significantly correlated with traits identified key pathways potentially driving these traits. Genes involved in hormone biosynthesis were overrepresented in modules that correlated significantly with reproduction or with deviations from non-interaction at life history level. Hormones in *Daphnia* play a crucial role in the molt cycle and thus reproduction (Chang et al., 1993). Furthermore, deviation from non-interaction at life history level was measured as how much observed reproduction deviates from predicted reproduction. Deviations or changes in reproduction occur with variations in molt cycle and thus variations in biosynthesis of these hormones, e.g. variation in ecdysteroids such as 20-hydroxoyecydsone and the molt-inhibiting hormone (Leblanc, 2006). However, it was unclear at present whether genes involved in hormone biosynthesis were a direct or indirect measure of deviations from non-interaction. Indeed, the effect on these genes may have been indirectly mediated through other pathways or pesticides and cyanobacteria may both have acted directly on these pathways. Time series analysis of gene expression data should be able to distinguish in the future between direct and indirect consequences of interactions. Nevertheless, these results indicated that gene expression measurements may be suitable predictors of occurring higher level effects given the clear correlation between known pathways affecting reproduction and the changes in reproductive output.

The overrepresentation of genes involved in the amino acid metabolism in modules significantly correlating with both reproductive output and deviation from non-interaction was less straightforward. It correlated well off course with the RNA polymerases which had been identified as hub genes in several modules (Tables 8.4, 8.7) as well as the presence of other genes involved in transcription (Tables 8.3-8.4). Overall, this indicates that deviation from non-interaction was characterized by changes in gene transcription and protein metabolism, i.e. requirement of changes in protein

production to cope with the deviations from non-interaction or changes in reproduction. Again, the same question arises whether these changes are the direct or indirect result of an interaction.

In addition to changes in large metabolic pathways, more specific gene functions were also overrepresented in the modules correlating significantly with life history traits. Tables 8.3 and 8.6 list a diverse set of functions that could not be straightforwardly linked to larger groups of pathways or metabolism. Trypsins and neurexin IV genes could be indicative of the specific interactions with cyanobacteria as these functional annotations were also overrepresented upon exposure to cyanobacteria alone (chapters 5 and 6). Given their specificity and the prior knowledge concerning their importance in exposure to cyanobacteria, these genes are a likely candidate for a quantitative predictor at the transcriptional level that corresponds to interactions at life history level. Other functional annotations included speckle type POZ proteins, lineage specific and uncharacterized proteins which only offer limited additional understanding due to their lack of sufficient annotation at the invertebrate level.

At present, it was not possible to identify a single measure at the transcriptional level that correlates with interactions at the life history level. This could be attributed to the difficulty of selecting the appropriate parameters or quantifiers for such a relationship rather than the actual data information. Indeed, network construction of expression profiles and unsupervised clustering methods have identified groups of genes that correlated significantly with interactions at the life history level. These group of genes or modules could only be identified when using both single and mixture expression profiles. The absence of single expression profiles resulted in no significant correlations with interaction at the life history level, which confirms the importance of including effects of single stressors. Whether these significant correlations between gene modules and interactions at life history level could also be identified using single stressor profiles alone, remains to be investigated as the current study did not have enough single stressor profiles to do so.

Interactions at life history level seemed to be driven by changes in larger metabolic pathways such as hormone biosynthesis and amino acid metabolism as well as specific gene functions such as trypsins and neurexin IVs. This confirmed the validity of transcriptional profiles to identify pathways and mechanisms involved in interaction effects despite the large amount of lineage specific genes present in significant modules.

In addition to interactions at life history level, expression profiles were also correlated with reproductive output. Significant correlations with gene modules were primarily observed when using only mixture expression profiles. This may be attributed to the repetition of single treatments within the network using all expression profiles. Indeed, all 144 profiles consisted of 48 unique mixture profiles, i.e. 48 unique treatments, and 96 single stressor profiles of which eight profiles were present for each of the six cyanobacteria and six profiles were present for each of the eight insecticides due to the different binary combinations. Effects on reproductive output could be described by major metabolic pathways, i.e. hormone biosynthesis, amino acid and fatty acid metabolism. Specific functional annotations were less pronounced and primarily consisted of lineage specific genes. In contrast to modules significantly correlating with interactions, genes in the majority of the modules significantly correlating with reproduction had both high gene significance and a high module membership. As a consequence, modules significantly correlating with reproductive output could be represented by their hub genes in addition to their eigengene. This was also confirmed in the visualization of the gene modules, where modules correlating with reproduction were quite similar to one another and all well connected (Fig. 8.8). In contrast, modules significantly correlating with deviations from non-interaction were very diverse as some were extremely connected and others had few connections (Fig. 8.7). These observations underline the complexity of interaction effects which could not be uniformly grouped at any level, life history level or transcription level.

The identified modules and the corresponding genes within these modules can already be used within adverse outcome pathway frameworks as these genes are correlated with apical effects. This may well be a first step towards developing an adverse outcome pathway framework for the risk assessment of mixtures. Indeed, the current gene set can be used to query available microarray data bases for additional expression data in other exposure studies to further validate these findings. Further study is needed to identify whether the apical effects are directly mediated through these genes or whether other genes are involved as discussed above as this will influence how this data is incorporated within the adverse outcome pathway framework.

At present, it was not possible to identify the sequence of transcriptional changes or chain of response to the stressors. Two potential approaches could in the future help to identify these changes. First, time series analysis of gene expression data is the most straightforward way to identify which pathway is perturbed or affected first and in turn affects another pathway initiating a series of transcriptional

changes that will ultimately result in effects at life history level. Second, the current results may be integrated into one large pathway in which distance measures between genes are used as an estimate of steps needed to go from gene *x* to gene *y*. This would require a full integration of all current pathway and biological knowledge which may in the case of *Daphnia* be insufficient to build a complete transcriptomic pathway or map and would lead to the introduction of uncertain paths or even gaps requiring advanced computational methods.

Lastly, this chapter has illustrated the potential of weighted gene network co-expression analysis to correlate gene expression changes with apical effects. While this dissertation focused on multiple stressors and cyanoabacteria and insecticides in particular, these analyses may also be applied for identifying genes mediating effects of endocrine disruption or nonpolar narcosis which are adverse outcome pathways under development (OECD, 2014a).

8.4 Conclusion

Transcription profiles were incorporated into gene networks of which gene modules correlated significantly with external traits. Significant correlations between modules and deviation from non-interaction could only be identified when using both single and mixture expression profiles. The absence of single expression profiles resulted in no significant correlations with deviation from non-interaction at the life history level, which confirmed the importance of including effects of single stressors. Functional analysis of gene modules identified pathways such as hormone biosynthesis and amino acid metabolism as well as significant proportion of lineage specific genes. Interaction effects were also characterized by specific gene functions present in the significantly correlated modules such as trypsins and neurexins IV. In addition, the identified modules can form a first foundation to incorporate effects of multiple stress in adverse outcome pathways. Overall, modules describing interaction effects were more diverse in terms of module structure and connectivity than modules describing reproduction effects which underlined the complexity of interaction effects.

Chapter 9

General Conclusion and further research perspectives

9.1 General conclusion

Effects of cyanobacteria on zooplankton species remain, despite the large body of literature, poorly understood. Meta-analyses reveal no clear impact of cyanobacterial toxins but rather point to nutritional quality and cyanobacterial morphology. Given the significant bias towards *Microcystis* species however, no definitive conclusions can be drawn. **Chapter 2** has addressed these caveats by studying the concentration response curves of *Daphnia pulex* exposed to six different cyanobacteria. These curves were conserved across the six studied cyanobacteria and were not significantly different from starvation treatments while for a limited range of diet ratios, i.e. at 40% of cyanobacteria in the diet, effects differed significantly. This indicated a potential common lack of nutritional quality across all cyanobacteria similarly affecting the fitness of *D. pulex* rather than effects of different cyanobacterial toxins for the majority of the diet ratios tested. Fatty acid methyl ester profiles further revealed differences in total FAME content and omega-3 content but were in contrast with general expectations based on literature and did not explain the observed overall similarities nor the specific differences observed at 40% of cyanobacteria in the diet.

Adverse effects occurred at concentrations of cyanobacteria in the diet that can be lower than concentrations in cyanobacterial blooms. This underlined the importance of including cyanobacteria concentrations in risk assessments to sufficiently protect zooplankton species. A proposed approach consisted of using cyanobacterial concentration response curves across multiple zooplankton species to fully incorporate potential interspecies variation. Indeed, the use of concentration response curve will allow generalizing risk assessments across a potentially large group of cyanobacteria for the majority of the tested concentrations yet also allowing for a specific or more stringent policy for those concentration ranges where some cyanobacteria are more toxic than the other.

Mechanistic research is currently making significant progress through the development of high throughput technologies such as microarrays and may help elucidate the driving factors of cyanobacterial effects on *Daphnia*. **Chapter 5** therefore implemented whole transcriptome microarray technology to study the response of *D. pulex* to the natural stressor *Microcystis aeruginosa*. The stress response pattern consisted of four major pathways or gene networks as well as eight paralogous gene families. Differential regulation of the ribosome, including three paralogous gene families encoding 40S, 60S, and mitochondrial ribosomal proteins, suggested an impact of *Microcystis* on protein

synthesis of *D. pulex*. In addition, differential regulation of the oxidative phosphorylation pathway (including the NADH:ubiquinone oxidoreductase gene family) and the trypsin paralogous gene family (a major component of the digestive system in *D. pulex*) could explain why fitness is reduced based on energy budget considerations. Moreover, **chapter 5** underlined the need to take into account the specific and unique *D. pulex* genome structure in expression studies, which may influence conclusions drawn. This genomic structure is characterized by, among others, a high number of lineage specific or unknown genes (Colbourne et al., 2011). **Chapter 5** further highlighted how these genes can be functionally annotated in environmentally relevant conditions.

Chapter 6 used high throughput microarray technology to identify and compare complex response patterns of *Daphnia* to six different cyanobacterial species. Both mechanisms that were specific to a single cyanobacterial stressor as well as mechanisms that were conserved across all cyanobacterial stressors were identified. The similarities and differences between these patterns across cyanobacterial stressors depended upon the level of functional biological organization, i.e. genes, functional annotations and pathways. The observed discrepancy between these levels underlined the necessity of such a research approach. Indeed, a total of 56 gene functions or functional annotations was shared by all cyanobacterial stress exposures whereas only 22 genes were shared across all conditions. Functional annotations comprised functions such as cytochrome P450, chitinases, collagens and neurexin IV. At the pathway level, only the starch and sucrose metabolism was shared by all cyanobacterial treatments. The steroid hormone biosynthesis, detoxification through cytochrome P450 and the arachidonic acid metabolism were shared by all cyanobacterial treatments excluding *Anabaena*. Furthermore, the incorporation of the unique features of the genome under study, i.e. the high gene duplication, in the analysis benefitted the integration of these different levels of organization in a true systems biology approach. Indeed, paralogous genes were more likely to be shared between the cyanobacterial treatments than non-duplicated genes. In contrast, non-duplicated genes had a higher chance of being unique to only one of the cyanobacteria treatments than duplicated genes. Overall, cyanobacterial stress targeted mainly similar mechanisms regardless of the cyanobacterial species. Yet, the regulation of these mechanisms was species-specific as these mechanisms were affected through the regulation of different genes albeit all having the same functional annotation. In addition, this chapter has also highlighted the functional diversification of duplicated genes under similar but still distinguishable forms of stress, i.e. cyanobacteria.

Environmental reality contrasts laboratory experiments as *Daphnia* are rarely exposed to cyanobacteria alone. Interaction effects between cyanobacteria and insecticides have been reported in literature for a limited set of combinations. **Chapter 3** studied the effects of different cyanobacteria when combined with different insecticides on the reproduction of *Daphnia pulex*. Different interaction patterns were observed for insecticides with different molecular targets when combined with *Microcystis*. In contrast, different cyanobacteria showed similar interaction patterns when combined with carbaryl. Four out of eight combinations showed antagonistic deviation patterns, three showed no interaction patterns whereas one yielded different patterns dependent on the reference model. The independent action model concluded synergism whereas the concentration addition model concluded no significant deviations from non-interaction. Overall, concentration addition provided more conservative predictions of effects than independent action. Therefore, the concentration addition model is preferred over the independent action model from a risk assessment point of view as it provided more conservative predictions. This is in agreement with observation from literature. Nevertheless, from a mechanistic point of view, neither model can be selected above the other without detailed knowledge concerning the modes of action of stressors under study. **Chapter 3** clearly highlighted that interaction effects cannot be generalized across modes of actions of insecticides whereas results may potentially be generalized across different cyanobacteria combined with the same insecticide.

Life history observations across a large set of combinations of cyanobacteria and insecticides, studied in **chapter 4**. First, **Chapter 4** confirmed the results of **chapter** 2. Indeed, for a narrow range of concentrations of cyanobacteria in the diet, effects do differ significantly between the different cyanobacteria. The results of the studied combinations indicated the occurrence of interaction effects to be a complex trait dependent upon several factors. Indeed, conclusions regarding interaction effects depended on exposure concentration and exposure design. Results from **chapter 3** could be extrapolated in a straightforward manner to the different experimental design in **chapter** 4. Further analysis of the data indicated that exposure conditions and exposure concentration rather than exposure time affected the conclusions concerning interaction. Indeed, conclusions about interaction effects observed in **chapter 3** remained conserved after 14 and 21 days suggesting potential extrapolation of interaction effects over different time points. In conclusion, studies on life history level

without further mechanistic research seem to be insufficient to attain a clear insight in the dynamics and processes leading to interactions.

Therefore, **chapter 7** focused on the transcriptomic profiling of 48 binary combinations of cyanobacteria and insecticides. Transcriptomics have been used to study interaction effects but often lack statistical frameworks and *a priori* defined hypotheses. **Chapter 7** put forward and applied three different approaches and two different measures of deviation. Overall, filtering the data based upon no overlapping 95% confidence intervals between observations and predictions and then calculating the sum of M_{int}-values of all genes with significant deviations from non-interaction was the most consistent approach allowing identification of statistical and biological significant genes. Genes with a significant interaction effect across all combinations showed a diverse pattern of expression in both synergistic and antagonistic directions. This approach selected overall genes which remained significant after benjamini-hochberg correction which reduced the chances of false positives within the selected gene set. Nevertheless, the generally accepted terminology of synergism and antagonism at the life history level was insufficient to explain all possible combinations and patterns of genes demonstrating an interaction effect in terms of clear synergism or antagonisms which suggests that alternative terminology is needed. Functional analysis of all genes with a significant interaction effect revealed a diverse set of metabolic functions and pathways which indicated that interaction effects trigger a complex general stress response in the organism, involving among others trypsins, collagens and cytochrome P450 genes rather than specific pathways or genes particular to the molecular modes of action insecticides or cyanobacteria.

Molecular high throughput technologies are increasingly being used in research. Yet, clear and direct links between molecular and life-history effects remain difficult to establish. Therefore, **chapter 8** incorporated the transcription profiles of **chapter 7** into gene networks. The modules of these networks were then in turn correlated with life history parameters and interaction parameters at both the life history level, defined in **chapter 4**, and the molecular level, defined in **chapter 7**. Significant correlations between modules and deviation from non-interaction could only be identified when using both single and mixture expression profiles. The gene networks without single expression profiles did not contain any modules significantly correlating with interaction at the life history level, which confirms the importance of including effects of single stressors. Functional analysis of gene modules identified that pathways such as hormone biosynthesis and amino acid metabolism as well as a significant

proportion of lineage specific genes were correlated with reproductive toxicity. Interaction effects were also characterized by specific gene functions present in the significantly correlated modules such as trypsins and neurexins IV. Overall, modules describing interaction effects were more diverse in terms of module structure and connectivity than models describing reproduction effects which underlined the complexity of interaction effects.

9.2 Applicability of the results and future research perspectives

While scientists have advocated the need for knowledge concerning interaction effects, legislation has remained focussed on the effects of single chemicals without regards to potential interaction effects. The increase in scientific publications regarding interaction effects has pushed regulatory bodies to take a stand. The European Commission has declared its intent to focus on priority mixtures, assessing the impact of these mixtures on the environment, filling knowledge and data gaps in mixture toxicity (European Commission, 2012). Results of **chapters 3 and 4** have started to fill these knowledge and data gaps for combinations of cyanobacteria and pesticides. In addition, the data itself can be used to test and validate predictive models that can be implemented in risk assessment. In the opinion of three scientific European committees on "*the toxicity and assessment of chemical mixtures*", several criteria are proposed to help tackle the risk assessment of an almost infinite number of possible mixture combinations in the environment (SCHER, SCCS, SCENIHR, 2012). The results of **chapters 7 and 8** can be applied within two of these criteria. The first criterion is known information of potential interactions. While **chapters 3 and 4** have provided information about the interaction of 48 binary combinations at life history level, results of **chapters 7 and 8** have provided additional mechanistic information about these combinations that may be applied to new similar combinations with other cyanobacteria or other insecticides. The second criterion is predictive information that chemicals act similarly. Mechanistic information gained from new technologies such as microarrays can add substantial mechanistic information that may help generating predictive information when combined with toxicodynamics and toxicokinetics (Villeneuve and Garcia-Reyero, 2011). Therefore, the microarray data generated in **chapters 7 and 8** can be subsequently queried and used to extract the necessary information. In this dissertation, a primary focus was the explorative analysis within defined statistical frameworks and linking the results of this analysis with life history traits. Future opportunities exist to query the data with more advanced techniques such as kernel methods or other

machine learning techniques (Brown et al., 2000). In addition, more information can be integrated to actually tackle gene interactions (Husmeier, 2003). Possibilities can be to combine pathway information and distance measures to link all pathways and genes with each other (Pavlopoulos et al., 2011). For example, the distance measure can be used to quantify the number of enzymes or products between genes. Interactions between genes can then be studied at a new level of organisation (Croes et al., 2006).

In addition to the need for adequate testing of chemicals and combined and interaction effects, environmental regulation also requires a minimal of animal testing to achieve these goals. Alternatives to animal testing are not only necessary from an ethical point of view but are also required to achieve adequate screening of chemicals within a given timeframe (Collins et al., 2008). Indeed, animal models, mammal models in particular, will only allow between 100-10000 tests a year whereas *in vitro* screening and alternative methods, e.g. predictive models or molecular screening libraries, could reach these numbers in a day (Collins et al., 2008). *In vitro* alternatives are only feasible if sufficient mechanistic knowledge is available to allow prediction and extrapolation. Ankley et al. (2010) proposed adverse outcome pathways (AOP) as a framework summarizing mechanistic knowledge from the molecular initiating event to the eventual adverse outcome at life history level. The molecular results obtained in **chapters 5 to 8** are a first step to building such AOPs not only for chemicals but also for natural stressors.

Indeed, emerging natural stressors such as cyanobacteria have significant impacts on aquatic ecosystems and human health. The 2006 bathing water directive of the European Commission (European Commission, 2006) stresses the potential effects of cyanobacteria in general but without clear guidelines. Results from **chapter 2** can be integrated in these directives and others relating to environmental health in general to help set guidelines and criteria for cyanobacteria. Indeed, **chapter 2** provided full concentration response curves of six common cyanobacterial species and demonstrated that these curves remain conserved across the different species for the majority of the concentrations. These observations can help to draft general guidelines for cyanobacteria that ensure minimal effects on zooplankton species. The mechanistic results of **chapters 5 and 6** can also be integrated to better understand cyanobacterial toxicity on zooplankton species within adverse outcome pathways.

Despite the efforts in environmental regulation, several challenges remain to be addressed. First, the exposure time remains a factor under continuous debate in science and in risk assessment. Often, acute exposures are conducted because they require less time and resources. The question however remains whether acute exposure conditions are relevant to determine chronic effects in the environment. The OECD has developed standard test guidelines for *Daphnia* testing in both acute and chronic settings (OECD, 1984; OECD, 2008). They have defined 48 hours as acute exposure to test immobilisation and 21 days as a chronic exposure to test reproduction. However, results from **chapters 2, 3 and 4** show that similar conclusions were made with regards to research questions after 14 and 21 days of exposure. If these results can be extrapolated, it would mean a significant increase in available resources and time as it reduces the chronic exposure assay with seven days. At present however, the current results represent a too small dataset to make such extrapolations and further research is necessary. For molecular assays, the same concerns arise. **Chapters 5 to 8** have focussed on chronic exposure to assess gene expression changes whereas previous studies have primarily focussed on acute exposures. Comparisons between the current results and literature to determine the most appropriate exposure time are not feasible. Indeed, the chronic exposures presented here refer to exposures where daphnids were exposed for ten days from juvenile till adult life stage. In contrast, acute exposures typically expose adults for a period ranging from 24 to 96 hours. As a consequence, it is impossible to define the most appropriate exposure time. There is therefore a need to generate time-course data that study the changes in expression across different time periods (Van Straalen and Feder, 2011). Asselman et al. (2013) observed time-dependent expression of metallothioneins upon exposure to copper and cadmium. Furthermore, the most appropriate time point to measure gene expression may differ from gene to gene as they have different metabolic functions and gene expression of one gene may often be a consequence of gene expression of another gene.

Second, the concentration of exposure significantly affects conclusions made about interaction effects as demonstrated in **chapter 4**. Full concentration response curves overcome these issues but are resource-demanding. Furthermore, the question remains to determine how many concentrations need to be tested to draw correct conclusions. These concerns are extremely valid in molecular studies as little concentration response data is available due to the high amount of resources needed. Yet, if we want to fully understand the concentration response relationship, such data is needed as advocated

already by Altenburger et al. (2012). When effects differ at different concentrations, in particular for interaction effects and molecular effects, it is unclear to what extent, if at all, these results can be extrapolated to environmentally relevant concentrations. This confirms the need that without clear understanding of concentration response data in complicated settings such as mixtures, research should focus on testing environmentally relevant concentrations. Here, concentrations for the studied pesticides were within or below the ranges of predicted environmental concentrations (PEC) or the maximum allowed concentrations (MAC) except for acetamiprid (Table 1.4) which indicates that the effects observed here can also occur in the actual environment with significant effects on the reproduction of *Daphnia*. The higher concentration of acetamiprid was selected due to the low sensitivity of *Daphnia* (Beketov and Liess, 2008) but nevertheless acetamiprid may serve as a mechanistic model stressor for other neonicotinoids.

Third, environmental risk assessment often focusses on populations with little to no genotypic diversity, without taking into account the potential genetic variation within the studied species or genus. This variation can significantly change conclusions on interaction effects. De Coninck et al. (2013b) observed a variation of interaction effects for different genotypes of *Daphnia* exposed to *Microcystis* and cadmium. The same observation was made when exposing two genotypes to carbaryl and *Pasteuria ramosa* (De Coninck et al., 2013a). More research is needed to determine how genetic diversity can be integrated in environmental risk assessment. Kramer et al. (2011) proposed an extension of adverse outcome pathways from organismal level to population level but it remains a challenge even in such frameworks to implement genetic diversity and its potential consequences on stress response.

Nevertheless, results from **chapter 3 and 4** can also have direct implications on risk management. Indeed, guidelines could be drafted to prefer insecticides known to have antagonistic interactions with cyanobacteria in those periods where water conditions are favourable for cyanobacterial bloom formation. Combined effects would therefore be minimized if a bloom actually occurs. If possible, depending on the agricultural needs and the potential persistence of the insecticides, a general guideline could be drafted to prefer use of insecticides known to have antagonistic interactions with cyanobacteria on *Daphnia*. In addition, new pesticides could be screened and selected for antagonistic interactions with cyanobacteria during the development phase to reduce environmental impact of these pesticides.

Finally, the molecular techniques used in **chapters 5 to 8** have highlighted the unique features of the *Daphnia* genome, i.e. high gene duplication and lineage specific genes. Although the results presented here have focussed on responses of known genes and pathways, the data contains a wealth of potential environmental annotation information of gene duplicates and lineage specific genes. The current dataset, combined with other molecular data, can aid in elucidating the gene functions of these gene duplicates and lineage specific genes under a variety of environmental stress responses. Results of **chapter 5 and 6** in particular have demonstrated methods to start annotating these genes environmentally while taking into account the specific structure of the *Daphnia* genome. *Daphnia* was the first fully sequenced crustacean genome. Currently, other sequencing projects are ongoing, e.g. *Artemia* genome (*Artemia* Genome Workshop, 2013), and these can only benefit from the availability of the *Daphnia* genome and the knowledge and bioinformatic pathway tools generated in this dissertation. Likewise, the availability of other crustacean genomes will allow for a better definition of the currently defined lineage specific genes and will give a broader insight into the function of gene duplication and evolutionary diversification of these duplicated genes.

References

Agrawal MK, Zitt A, Bagchi D, Weckesser J, Bagchi SN, von Elert E. 2005. Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. Environ Toxicol 20, 314-322.

Alda Alvarez O, Jager T, Nunez Coloa B, Kammenga JE. 2006. Temporal dynamics of effect concentrations. Environ Sci Technol 40, 2478-2484.

Allen MM. 1968. Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4, 1-4.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M. 2013. Simplifying complexity: Mixture toxicity assessment in the last 20 years. Environ Toxicol Chem 32, 1685-1687.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chenicals. Environ Toxicol Chem 19, 2341-2347.

Altenburger R, Boedeker W, Faust M, Grimme LH. 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. Food Chem Toxicol 34, 1155-1157.

Altenburger R, Scholz S, Schmitt-Jansen M, Busch W, Escher BI. 2012. Mixture toxicity revisited from a toxicogenomic perspective. Environ Sci Technol 46, 2508-2522.

Altshuler I, Demiri B, Xu S, Constantin A, Yan ND, Cristescu ME. 2011. An integrated multi-disciplinary approach for studying multiple stressors in freshwater ecosystems: *Daphnia* as a model organism. Integr Comp Biol 51, 623-633.

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW et al. 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 29, 730-741.

Ankley GT, Daston GP, Degitz SJ, Denslow ND, Hoke RA, et al. 2006. Toxicogenomics in regulatory ecotoxicology. Environ Sci Technol 40, 4055-4065.

Antczak P, Jo HJ, Woo Seonock, Scanlan L, Poynton H, Loguinov A, Chan S, Falciani F, Vulpe C. 2013. Molecular toxicity identification evaluation (mTIE) approach predicts chemical exposure in *Daphnia magna*. Environ Sci Technol 47, 11747-11756.

Aráoz R, Nghiêm H., Rippka R, Palibroda N, Tandeau de Marsac N, Herdman M. 2005. Neurotoxins in axenic oscillatorian cyanobacteria: coexistence of anatoxin-a and homoanatoxin-a determined by ligand-binding assay and GC/MS. Microbiol 151, 1263-1273.

Asselman J, Glaholt SP, Smith Z, Smagghe G, Janssen CR, Colbourne JK, Shaw JR, De Schamphelaere KAC. 2012. Functional characterization of four metallothionein genes in *Daphnia pulex* exposed to environmental stressors. Aquat Toxicol 110-111, 54-65.

Asselman J, Shaw, JR, Glaholt SP, Colbourne JK, De Schamphelaere KAC. 2013. Transcription patterns of genes encoding four metallothionein homologs in Daphnia pulex exposed to copper and cadmium are time- and homolog-dependent. Aquat Toxicol 142-143, 442-430.

Baas J, Van Houte BPP, Van Gestel CAM, Kooijman SALM. 2007. Modelling the effects of binary mixtures on survival in time. Environ Toxicol Chem 26, 1320-1327.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. Environ Toxicol Chem 19, 2348 -2356.

Backhaus T, Faust M, Scholze M, Gramatica P, Vighi M, Horst Grimme L. 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. Environ Toxicol Chem 23, 258-264.

Ballot A, Fastner J, Wiedner C. 2010. Paralytic shellfish poisoning toxin-producing cyanobacterium *Aphanizomenon gracile* n Northeast Germany. Appl Environ Microbiol 76, 1173-1180.

Barata C, Baird DJ, Nogueira AJA, Soares AMVM, Riva MC. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquat Toxicol 78, 1-14.

Barata C, Varo I, Navarro JC, Arun S, Porte C. 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comp. Biochem. Physiol. 140 C, 175-186.

Bardwell VJ,Treisman R. 1994. The POZ domain: a conserved protein-protein interaction motif. Genes Dev. 8, 1664-1677.

Barry MJ. 2000. Effects of endosulfan on *Chaoborus*-induced life-history shifts and morphological defenses in *Daphnia pulex*. J Plankton Res 22, 1705-1718.

Baumgartner S, Littleton JT, Broadie K, Bhat MA, Harbecke R, Lengyel JA, Chiquetehrismann R, Prokop A, Bellen HJ. 1996. A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. Cell 87,1059-1068.

Bauer DF. 1972. Constructing confidence sets using rank statistics. Journal of the American Statistical Association 67, 687-690.

Beattie KA, Kaya K, Codd GA. 2000. The cyanobacterium *Nodularia* PCC7804, of freshwater origin, produces [L-Har^2]nodularin. Phytochem 54, 57-61.

Bednarska A, Łoś J, Dawidowicz P. 2011. Temperature-dependent effect of filamentous cyanobacteria on *Daphnia magna* life history traits. J Limnol 70, 353-358.

Beketov MA, Liess M. 2008. Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. Environ Toxicol Chem 27, 461-470.

Beketov MA, Speranza A, Liess M. 2011. Ultraviolet radiation increases sensitivity to pesticides: synergistic effects on population growth rate of *Daphnia magna* at low concentrations. Bull Environ Contam Toxicol 87, 231-237.

Belden JB, Lydy MJ. 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ Toxicol Chem 25, 623-629.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57, 289-300.

Berenbaum MC. 1989. What is synergy. Pharmacol. Rev 41, 93-141.

Bernatowicz P, Pijanowska J. 2011. *Daphnia* response to biotic stress is modified by PCBs. Ecotoxicol Environ Safe 74, 711-718.

Bliss CI. 1939. The toxicity of poisons applied jointly. Ann Appl Biol 26, 585-615.

Blyth S. 1980. Palm Island mystery disease. Med J Aust 2, 40-42.

Boedeker W, Altenburger R, Faust M, Grimme LH. 1992. Synopsis of concepts and models for the quantitative analysis of combination effects: from biometrics to ecotoxicology. Arch Complex Environ Studies 4, 45-53.

Bolch CJS, Orr PT, Jones GJ, Blackburn SI. 1999. Genetic, morphological and toxicological variation among globally distributed strains of *Nodularia* (cyanobacteria). J Phycol 35, 339-355.

Borgert CJ, Quill TF, McCarty LS, Mason AM. 2004. Can mode of action predict mixture toxicity for risk assessment? Toxicol Appl Pharmacol 201, 85-96.

Bradshaw AD, Hardwick K. 1989. Evolution and stress - genotypic and phenotypic components. Biol J Linnean Soc 37, 137-155.

Brett MT, Müller-Navarra DC, Ballantyne AP, Ravet JL, Goldnan CR. 2006. *Daphnia* fatty acid composition reflects that of their diet. Limnol Oceanogrp 51, 2428-2437.

Brown MPS, Grundy WN, Lin D, Cristianini N, Walsh C, Furey TS, Ares MJ, Haussler D. 2000. Knowledge-based analysis of microarray gene expression data by using support vector machines. Proc Natl Acad Sci USA 97, 262-267.

Buijse AD, Van Eerden MR, Dekker W, Van Densen WLT. 1993. Elements of a trophic model for IJsselmeer (The Netherlands), a shallow euthropic lake. In: Trophic models for Aquatic ecosystems. Christensen V and Pauly D, Eds. International Center for Living Aquatic Resource Management. 390p.

Burch, M.D. 2008. Chapter 36: Effective doses, guidelines and regulations. In Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. H.K. Hudnell (ed.). 950p. http://www.epa.gov/cyano_habs_symposium/monograph/Ch36.pdf

Cadel-Six S, Dauga C, Castets AM, Ripka R, Bouchier C, Tandeau de Marsac N, Welker M. 2008. Halogenase genes in nonribosomal peptide synthetase gene clusters of *Microcystis* (Cyanobacteria): Sporadic distribution and evolution. Mol Biol Evol 25, 2031-2041.

Calow P. 1989. Proximate and ultimate responses to stress in biological systems. boil J Linnean Soc 37, 173-181.

Campos A, Vasconcelos V. 2010. Molecular mechanisms of microcystin toxicity in animal cells. Int J Mol Sci 11, 268-287.

Carmichael WW. 2001. Health effects of toxin-producing cyanobacteria, "The CyanoHabs". Hum Ecol Risk Assessement 7, 1393-1407.

Carmichael WW. 2008. A world overview-one-hundred-twenty-seven years of research on toxic cyanobacteria--Where do we go from here? In Cyanobacterial Harmful Algal Blooms: State of the Science Research Needs; Advances in Experimental Medicine and Biology, Vol. 619; Hudnell, K. H., Ed.; Springer: New York, p 43-103.

Carmichael WW, Jones CLA, Mahmood NA, Theiss WC. 1985. Algal toxins and water-based diseases. CRC Crit Rev Envir Control 15, 275-313.

Carpenter SR, Fisher SG, Grimm NB, Kitchell JF. 1992. Global change and freshwater ecosystems. Annu Rev Ecol Syst 23, 119-139.

Cedergreen N, Christensen AM, Kamper A, Kudsk P, Mathiassen SK, Streibig JC, Sorensen H. 2008. A review of indepdent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environ Toxicol Chem 27, 1621-1632.

Cedergreen N, Kudsk P, Mathiassen SK, Sorensen H, Streibig JC. 2009. Reproducibility of binary-mixture toxicity studies. Environ Toxicol Chem 26, 149-156.

Cedrgreen N, Streibig JC. 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environ Toxicol Chem 24, 1676-1683.

Cerbin S, Kraak MHS, de Voogt P, Visser PM, Van Donk E. 2010. Combined and single effects of pesticide carbaryl and toxic *Microcystis aeruginosa* on the life history of *Daphnia pulicaria*. Hydrobiologia 643, 129-138.

Chang ES, Bruce MJ, Tamone SL. 1993. Regulation of crustacean molting: A multi-hormone system. Am Zool 33, 324-329.

Chen W, Song L, Ou D, Gan G. 2005. Chronic toxicity and responses of several important enzymes in Daphnia magna on exposure to sublethal microcystin-LR. Environ Toxicol 20, 323-330.

Chorus I (ed). 2001. Cyanotoxins: occurrence, causes, consequences. Springer-Verlag, Berlin, 357 p.

Chomova M, Racay P. 2010. Mitochondrial complex I in the network of known and unknown facts. Gen Physopm Biophys 29, 3-11.

Chow-Fraser P, Sprules GW. 1986. Inhibitory effect of *Anabaena* sp. on in situ filtering rate of *Daphnia*. Can J Zool 64, 1831-1834.

Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, et al. 2007. Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc. 2, 2366-2382.

Codd GA. 1995. Cyanobacterial toxins: Occurrence, properties and biological significance. Water Sci Technol 32, 149-156.

Codd, G.A., Morrison, L.F., Metcalf, J.S. 2005. Cyanobacterial toxins: risk management for health protection. Toxicol. Appl. Pharmacol. 203, 264-272.

Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A et al. 2011. The ecoresponsive genome of *Daphnia pulex*. Science 331, 555-561.

Colbourne JK, Singan VR, Gilbert DG. 2005. wFleaBase: the Daphnia genome database. BMC Bioinformatics, 6:45.

Collins FS, Gray GM, Bucher JR. 2008. Transforming environmental health protection. Science 319, 906-907.

Cook CM, Vardaka E, Lanaras T. 2004. Toxic cyanobacteria in Greek freshwaters, 1987-2000: Occurence, toxicity and impacts in the Mediterranean region. Acta Hydrochim Hydrobiol 32, 107-124.

Coors A, De Meester L. 2008. Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. J Appl Ecol 45, 1820-1828.

Croes D, Couche F, Wodak SJ, van Helden J. 2006. Inferring meaningful pathways in weighted metabolic networks. J Mol Biol 356, 222-236.

Cronberg G. 1999. Qualitative and quantitative investigations of phytoplankton in Lake Ringsjön, Senia, Sweden. Hydrobiologia 404, 27-40.

Czarnecki O, Henning M, Lippert I, Welker M. 2006. Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). Environ Micorbiol 8, 77-87.

Daam MA, Cerejeira MJ, Van den Brink PJ, Brock TCM. 2011. Is it possible to extrapolate results of aquatic microcosm and mesocosm experiments with pesticides between climate zones in Europe? Environ Sci Pollut Res 18, 123-126.

David RM, Dakic V, Williams TD, Winter MJ, Chipman KJ. 2011. Transcriptional responses in neonate and adult *Daphnia magna* in relation to relative susceptibility to genetoxicants. Aquat Toxicol 104, 192-204.

Day K, Kaushik NK. 1987. An assessment of the chronic toxicity of the synthetic pyrethroid, fenvalerate, to *Daphnia galeata mendotae*, using life tables. Environ Pollut 44, 13-26.

De Coninck DIM, Asselman J, Glaholt S, Janssen CR, Colbourne JK, Shaw JR, De Schamphelaere KAC. 2014. Genome-wide transcription profiles provide insights in genotype-dependent mechanisms of tolerance to two interacting stressors in Daphnia. Accepeted in Environmental Science and Technology.

De Coninck DIM, De Schamphelaere KAC, Jansen M, De Meester L, Janssen CR. 2013a. Interactive effects of a bacterial parasite and the insecticide carbaryl to life-history and physiology of two *Daphnia magna* clones differing in carbaryl sensitivity. Aquat Tox 130-131, 149-159

De Coninck DIM, Janssen CR, De Schamphelaere KAC. 2013b. An investigation of the inter-clonal variation of the interactive effects of cadmium nd *Microcystis aeruginosa* on the reproductive performance of *Daphnia magna*. Aquat Toxicol 140-141, 425-431.

De Laender F, Janssen CR, De Schamphelaere KAC. 2009. Non-simultaneous ecotoxicity testing of single chemicals and their mixture results in erroneous conclusions about the joint action of the mixture. Chemosphere 76, 428-432.

Dehn M.1930 Untersuchungen über die Verdauung bei Daphnien. Zeitschrift für vergleichende Physiologie 13, 334-358.

DeMott WR, Müller-Navarra D. 1997. The importance of highly unsaturated fatty acids in zooplankton nutrition: evidence from experiments with *Daphnia*, a cyanobacterium and lipid emulsions. Freshwater Biol 38, 649-664.

DeMott WR, Zhang QX, Carmichael WW. 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnol Oceanogr 36, 1346-1357.

Deneer JW. 2000. Toxicity of mixtures of pesticides in aquatic systems. Pest Manage Sci 56:516-520.

De Schamphelaere KAC, Forrez I, Dierckens K, Sorgeloos P, Janssen CR. 2007. Chronic toxicity to dietary copper to *Daphnia magna*. Aquat Toxicol 81, 409-418.

De Schamphelaere KAC, Glaholt S, Asselman J, Messiaen M, De Coninck D, Janssen CR, Colbourne JK, Shaw JR. 2011. Will genetic adaptation of natural populations to chemical pollution result in lower or higher tolerance to future climate change? Integr Environ Assess Manag 7, 141-143.

de Senerpont Domis LN, Bartosiewicz M, Davis C, Cerbin S. 2013. The effect of small doses of toxic cyanobacterial food on the temperature response of *Daphnia galeata*: is bigger better? Fresh Biol 58, 560-572.

Dias da Silva D, Silva E, Carvalho F, Carmo H. 2013. Mixtures of 3,4-methylene-dioxymethamphetamine (ectasy) and its major human metabolites act additively to induce significant toxicity to liver cells when combined at low, non-cytotoxic concentrations. J Appl Toxicol, DOI: 10.1002/jat.2885

Ding WX, Shen HM, Zhu HG, Ong CN. 1998. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ Res 78, 12-18.

Dom N, Vergauwen L, Vandenbrouck T, Jansen M, Blust R, Knapen D. 2012. Physiological and molecular effect assessment versus physico-chemistry based mode of action schemes: *Daphnia magna* exposed to narcotics and polar narcotics. Environ Sci Technol 46, 10-18.

Downing JA, Watson JB, McCauley E. 2001. Predicting cyanobacterial dominance in lakes. Canadian Journal of Fisheries and Aquatic Sciences 58, 1905-1908.

Dresher K, Boedeker W, 1995. Assessment of the combined effects of substances: the relationship between concentration addition and independent action. Biometrics 51, 716-730.

Duy TN, Lam PKS, Shaw G, Connell DW. 2000. Toxicology and risk assessment of freshwater cyanobacterial (bluegreen algal) toxins in water. Rev Environ Contam Toxicol 163, 113-186.

Ebert D. 2005. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. Bethesda (MD): National Center for Biotechnology Information (US); Available from: http://www.ncbi.nlm.nih.gov/books/NBK2036/

Ebert D. 2011. A genome for the environment. Science 331, 539-540.

Edler L, Fernö S, Lind MG, Lundberg R, Nilsson PO. 1985. Mortality of dogs associated with a bloom of the cyanobacterium *Nodularia spumigena* in the Baltic Sea. Ophelia 24, 103-109.

Edwards C, Beatti KA, Scrimgeour CM, Codd GA. 1992. Identification of anatoxin-A in benthic cyanobacteria (blue-green algae) and in associated dog poisonings at Loch Insch, Scotland. Toxicon 30, 1165-1175.

Ekman-Ekebom M, Kauppi M, Sivonen K, Niemi M, Lepistö L. 1992. Toxic cyanobacteria in some finnish lakes. Env Toxic Water 7, 201-213.

Emler S, Scholze M, Kortenkamp A. 2011. The suitability of concentration addition for predicting the effects of multi-component mixtures of up to 17 anti-androgens zith varied structural features in an *in vitro* AR antagonist assay. Toxicol Appl Pharmacol 257, 189-197.

Ersmark K, Del Valle JR, Hanessian S. 2008. Chemistry and biology of the aeruginosin family of serine protease inhibitors. Angew Chem Int Ed Engl 47, 1202-1223.

EPA US: Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th addition. Washington, DC: U.S. EPA, Office of Water; 2002:275.

European Comission. 2006. Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC.

European Comission. 2012. Environment: A new approach to assessing toxic chemical mixtures. European Comission IP/12/541. http://europa.eu/rapid/press-release_IP-12-541_en.htm

Eurostat. 2007. The use of plant protection products in the European Union, Data 1992-2003. Eurostat, Statistical Books, 215p.

Falconer IR. 2001. Toxic cyanobacterial bloom problems in Australian waters: risks and impacts on human health. Phycologia 40, 228-233.

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighli M, Grimme LH. 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat toxicol 63, 43-63.

Faust, M., Scholze, M. 2004 Competing concepts for the prediction of mixture toxicity: Do the differences matter for regulatory purposes? EVKI-CT-1999-00012, European Union project BEAM, final. European Commission, Brussels, Belgium.

Ferrari A, Venturino A, Pechén de D'Angelo AM. 2007. Effects of carbaryl and zinphos methyl on juvenile rainbow trout (Oncorhynchus mykiss) detoxifying enzymes. Pestic Biochem Physiol 88, 134-142.

Fisher RA. 1922. On the interpretation of χ^2 from contingency tables, and the calculation of P. J R Stat Soc 85, 87-94.

Fin WG. 2007. Diagnostic pathology and laboratory medicine in the age of “omics”: a paper from the 2006 William Beaumont Hospital Symposium on Molecular Pathology. J Mol Diagn 9, 431-436.

Finney DJ. 1971. Probit analysis. Cambridge, Cambridge University Press. 272p.

Fitzgerald DJ, Cunliffe DA, Burch MD. 1999. Development of health alerts for cyanobacteria and related toxins in drinking water in South Australia. Environ Tox 14, 203-209.

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM and et al. 1995. Whole-genome random sequencing and assessmbly of *Haemophilus influenzae* Rd. Science 269, 496-512.

Fox J. 2008. Applied Regression Analysis and Generalized Linear Models, Second Edition. Sage. 688p.

Fristachi A, Sinclair JL. 2008. Occurrence of cyanobacterial harmful algal blooms. In Cyanobacterial Harmful Algal Blooms: State of the Science Research Needs Series: Advances in Experimental Medicine and Biology, Vol 619, Hudnell KH, Ed., Springer: New York 2008; 43-105.

Fukuto TR, 1990. Mechanism of action of organophosphorus and carbamates insecticides. Environ Health Perspect 87, 245-254.

Garcia-Reyero N, Escalon BL, Loh P., Laird JG, Kennedy AJ, Berger B, Perkins EJ. 2012. Assessment of chemical mixtures and groundwater effects on *Daphnia magna* transcriptomics. Environ Sci Technol 46, 42-50.

Garcia-Reyero N, Kroll KJ, Liu L, Orlando EF, Watanabe KH, Sepulveda MS, Villeneuve DL, Perkins EJ, Ankley GT, Denslow ND. 2009. Gene expression responses in male fathead minnows exposed to binary mixtures of an estrogen and antieestrogen. BMC Genomics 10, 308-325.

Gene Expression Omnibus. 2014. http://www.ncbi.nlm.nih.gov/geo/

Gentleman RC, Carey VJ, Bates DM, Bolstad B et al. 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biology 5: R80.

Gerwick WH, Tan LT, Sitachitta N. 2001. Nitrogen-containing metabolites from marine cyanobacteria. In:Cordell GA (ed) lkaloids: Chemistry and Biology,Vol. 57 Academic Press, NY, 75-184.

Gilbert JJ, Durand MW. 1990. Effect of *Anabaena flos-aqua* on the abilities of *Daphnia* and *Keratella* to feed and reproduce on unicellular algae. Freshwater Biol 24, 577-596.

Ghazalpour A, Doss S, Zhang B, Wang S, Plaisier C, Castellanos R, Brozell A, Schadt EE, Drake TA, Lusis AJ, Horvath S. 2006. Integrating genetic and network analysis to characterize genes related to mouse weight. Plos Genet 2:e130.

Griffitt RJ, Hyndman K, Denslow ND, Barber DS. 2009. Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanopartciles. Toxicol Sci 107, 404-415.

Gugger M, Lyra C, Henriksen P, Couté A, Humber J, Sivonen K. 2002. Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. IJSEM 52, 1867-1880.

Gugger M, Lyra C, Suominen I, Tsitko I, Humbert J, Salkinoja-Salonen MS, Sivonen K. 2002. Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena, Aphanizomenon, Microcystis, Nostoc* and *Planktothrix* (cyanobacteria). Int J Syst Evol Microbiol 52, 1007-1015.

Gustafsson S, Hansson L-A. 2004. Development of tolerance against toxic cyanobacteria in *Daphnia*. Aquatic Ecology 38, 37-44.

Habib K, Kumar S, Manikar N, Zutshi S, Fatma T. 2011. Biochemical effect of carbaryl on oxidative stress, antioxidant enzymes and osmolytes of cyanobacterium Calothirx brevissima. Bull Environ Contam Toxicol 87, 615-620.

Hampton TH, Stanton BA. 2010. A novel approach to analyze gene expression data demonstrates that the ΔF508 mutation in CFTR downregulates the antigen presentation pathway. Am J Physiol-Lung C 298, 473-482.

Hanazato T, Dodson SI. 1995. Synergistic effects of low-oxygen concentration,predator kairomone, and a pesticide on the cladoceran *Daphnia pulex*. Limnol Oceanogr 40, 700-709.

Haney JF, Forsyth DJ, James MR. 1994. Inhibition of zooplankton filtering rates by dissolved inhibitors produced by naturally occurring cyanobacteria. Arch Hydrobiol 132, 1-13.

Haney JF, Sasner JJ, Ikawa M. 1995. Effects of products released by *Aphanizomenon flos-aquae* and purified saxitoxin on the movements of *Daphnia carinata* feeding appendages. Limnol Oceanogr 40, 263-272.

Hapeman CJ, Dionigi CP, Zimba PV, McConnell LL. 2002. Agrochemical and nutrient impacts on estuaries and other aquatic ecosystems. J Agric Food Chem 50, 4382-4384.

Hayes KP, Burch MD 1989. Odorous compounds associated with algal blooms in south australian waters. Water Res 23, 115-121.

Heckmann L-H, Sibly RM, Connon R, Hooper HL, Hutchinson TH, Maund SJ, Hill CJ, Bouetard A, Callaghan A. 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biol 9:R40.

Heisler J, Glibert PM, Burkholder JM, Anderson DM, Cochlan W, Dennison WC, Dortch Q, Gobler CJ, Heil CA, Humphries E, Lewitus A, Magnien R, Marshall HG, Sellner K, Stockwell DA, Stoecker DK, Suddleson M. 2008. Eutrophication and harmful algal blooms: A scientific consensus, Harmful Algae 8, 3-13.

Henriksen P, Carmichael WW, Am J, Moestrup ø. 1997. Detection of an anatoxin-a(s)-like anticholinesterase in natural blooms and cultures of cyanobacteria/blue-green algae from danish lakes and in the stomach contents of poisoned birds. Toxicon 35, 901-913.

Heugens EH, Hendriks AJ, Dekker T, Van Straalen NM, Admiraal W. 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit Rev Toxicol 31, 247-284.

Heugens EH, Jager T, Creyghton R, Kraak MH, Hendriks AJ, van Straalen NM, Admiraal W. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. Environ Sci Technol 37, 2145-2151.

Hietala J, Lauron-Maatta C, Walls M. 1997. Life history responses of *Daphnia* clones to toxic *Microcystis* at different food levels. J Plankt Res 19, 917-926.

Hietala J, Reinikainen M, Walls M. 1995. Variation in life history responses of *Daphnia* to toxic *Microcystis aeruginosa*. J Plankton Res 17, 2307-2318.

Holmstrup M, Bindesbøl A-M, Oostingh GJ, Duschl A, Scheil V, Köhler H-R, Loureiro S, Soares AMVM, Ferreira ALG, Kienle C, Gerhardt A, Laskowski R, Kramarz PE, Bayley M, Svendsen C, Spurgeon DJ. 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci Total Environ 408, 3746-3762.

Hook SE, Skillman AD, Gopalan B, Small JA, Schultz IR. 2008. Gene expression profiles in rainbow trout, *Onchorynchus mykiss*, exposed to a simple chemical mixture. Toxicol Sci 102, 42-60.

Hooper MJ, Ankley GT, Cristol DA, Maryoung LA, Noyes PD, Pinkerton KE. 2013. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environ Toxicol Chem 32, 32-48.

Husmeier D. 2003. Sensitivity and specificity of inferring genetic regulatory interactions from microarray experiments with dynamic Bayesian networks. Bioinformatics 19, 2271-2282.

Illumina. 2014. http://www.illumina.com/systems/hiseq-x-sequencing-system.ilmn

Insecticide Resistance Action Committee (IRAC), 2009. Mode of Action Classification. Available online:

http://www.irac-online.org/wpcontent/uploads/2009/09/pp_moa_structure_poster_v2.7_16oct09.pdf

Ishida K, Okita Y, Matsuda H, Okino T, Murakami M. 1999. Aeruginosins, protease inhibitors from the cyanobacterium *Microcystis aeruginosa*. Tetrahedron 55, 10971-10988.

Jager T, Heugens EHW, Kooijman SALM. 2006. Making sense of ecotoxicological test results: towards application of precess-based models. Ecotoxicology 15, 305-314.

Jager T, Vandenbrouck T, Baas J, De Coen WM, Kooijman ALM. 2010. A biology-based approach for mixture toxicity of multiple endpoints over the life cycle. Ecotoxicology 19, 351-361.

Jankowski T, Livingstone DM, Buhrer H, Forster R, Niederhauser P. 2006. Consequences of the 2003 European heat wave for lake temperature profiles, thermal stability, and hypolimnetic oxygen depletion: Implications for a warmer world. Limnol Oceanogr 51, 815-819.

Jansen M, Vergauwen L, Vandenbrouck T, Knapen D, Dom N, Spanier Ki, Cielen A, De Meester L. 2013. Gene expression profiling of three different stressors in the water flea *Daphnia magna*. Ecotoxicology 22, 900-914.

Jöhnk KD, Huisman J, Sharples J, Sommeijer B, Visser PM, Stroom JM. 2008. Summer heatwaves promote blooms of harmful cyanobacteria. Global Change Biol 14, 495-512.

Jonker MJ, Svendsen C, Bedeaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ Toxicol Chem 24, 2701-2713.

Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M. 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res. 38, 355-360.

Kirk KL, Gilbert JJ. 1992. Variation in herbivore response to chemical defenses: zooplankton foraging on toxic cyanobacteria. Ecology 73, 2208-2217.

Koehn RK, Bayne BL. 1989. Towards a physiological and genetical understanding of the energetics of the stress response. Biol J Linnean Soc 37, 151-171.

Korade Z, Mirnics K. 2011. Wnt signaling as a potential therapeutic target for frontotemporal dementia. Neuron 71, 955-957.

Koski M, Engström J, Viitasalo M. 1999. Reproduction and survival of the calanoid copepod *Eurytemora affinis* fed with toxic and non-toxic cyanobacteria. Mar Ecol Prog Ser 186, 187-197.

Kotai J. 1972. Instructions for preparation of modified nutrient solution Z8 for algae. Norwegian Institute for Water Research, Oslo.

Kozlowsky-Suzuki B, Karjalainen M, Lehtiniemi M, Engström-Öst J, Koski M, Carlsson P. 2003. Feeding, reproduction and toxin accumulation by the copepods *Acartia bifilosa* and *Eurytemora affinis* in the presence of the toxic cyanobacterium *Nodularia spumigena*. Mar ecol Prog Ser 249, 237-249.

Kramer VJ, Etterson MA, Hecker M, Murphy CA, Roesijadi G, Spade DJ, Spromberg JA, Wang M, Ankley GT. 2011. Adverse outcome pathways and ecological risk assessment: Bridging to population-level effects. Environ Toxicol Chem 30, 64-76.

Lampert W. 1981. Inhibitory and toxic effects of blue-green algae on *Daphnia*. Internationale Revue der gesamten Hydrobiologie 66, 285-298.

Lampert W. 1987. Laboratory studies on zooplankton-cyanobacteria interactions. New Zealand Journal of Marine and Freshwater Research 21, 483-490.

Lampert W. 2006. *Daphnia:* Model herbivore, predator and prey. Pol J Ecol 54, 607-620.

Lampert W. 2011. *Daphnia*: Development of a model organism in ecology and evolution. In: Kinne O. (Ed.), Excellence in ecology, Book 21. International Ecology Institute, Oldendorf/Luhe.

Lampert W, Lampert KP, Larsson P. 2012. Induction of male production in clones of *Daphnia pulex* by the juvenoid hormone methyl farnesoate under short photoperiod. Comp Biochem Physiol, Part C: Toxicol Pharmacol 156, 130-133.

Landis WG, Durda JL, Brooks ML, Chapman PM, Menzie CA, Stahl RG, Stauber JL. 2013. Ecological risk assessment in the context of global climate change. Environ Toxicol Chem 32, 79-92.

Langfelder P, Horvath S. 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9:559.

Langridge P, Fleury D. 2011. Making the most of 'omics' for crop breeding. Trends Biotechnol 29, 33-40.

La-Salete R, Oliveira MM, Palmeira CA, Almeida J, Peixoto FP. 2008. Mitochondria a key role in microcystin-LR kidney intoxication. J Appl Toxicol 28, 55-62.

Laskowski R, Bednarska AJ, Kramarz PE, Loureiro S, Scheil V, Kudłek J, Holmstrup M. 2010. Interactions between toxic chemicals and natural environmental factors: A meta-analysis and case studies. Sci Total Environ 408, 3763-3774.

Latta LC, Weider LJ, Colbourne JK, Pfrender ME. 2012. The evolution of salinity tolerance in *Daphnia*: a functional genomics approach. Ecol Lett 15, 794-802.

Leblanc GA. 2006. Crustacean endocrine toxicology: a review. Ecotoxicology, 16, 61-81.

Lethonen KK, Kankaanpää H, Leiniö S, Sipiä VO, Pflugmacher S, Sandberg-Kilpi E. 2003. Accumulation of nodularin-like compounds from the cyanobacterium Nodularia spumigena and changes in acetylcholinesterase activity in the clam Macoma balthica during short-term laboratory exposure. Aquat Toxicol 64, 461-476.

Li L, Xie P, Chen J. 2005. In vivo studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the phytoplanktivorous bighead carp i.p.-injected with extracted microcystins. Toxicon 46, 533-545.

Li S, Tan Y. 2011. Hormetic response of cholinesterase from *Daphnia magna* in chronic exposure to triaszophos and chlorpyrifos. J Environ Sci 23, 852-859.

Lock K, Janssen CR. 2002. Mixture Toxicity of Zinc, Cadmium, Copper, and Lead to the Potworm *Enchytraeus albidus*. Ecotox Environ Saf 52,1-7.

Loewe S. 1928. Die quantitativen probleme der pharmakologie. Ergebn Physiol 27, 47-187.

Lopez JA, Colbourne JK Technical Report; The Center for Genomics and Bioinformatics, Indiana University, Bloomington, Indiana, 2011. DOI http://dx.doi.org/10.2506/cgbtr-201102

Loureiro S, Svendsen C, Ferreira ALG, Pinheiro C, Ribeiro F, Soares AMVM. 2010. Toxicity of three binary mixtures to *Daphnia magna*: comparing chemical modes of action and deviations from conceptual models. Environ Toxicol Chem 29, 1716-1726.

Lürling M. 2003. Effects of microcystin-free and microcystin-containing strains of the cyanobacterium *Microcystis aeruginosa* on growth of the grazer *Daphnia magna*. Environ Toxicol 18, 202-210.

Lürling M, Beekman W. 2006. Growth of *Daphnia magna* males and females fed with the cyanobacterium *Microcystis aeruginosa* and the green alga *Scenedesmus obliquus* in different proportions. Acta Hydrochim. Hydrobiol 34, 375-382.

Mahmood NA, Carmichael WW. 1986. Paralytic shellfish poisons produced by the freshwater cyanobacterium *Aphanizomenon flos-aqua* NH-5. Toxicon 24, 175-186.

Miller RG. 1981 Simultaneous Statistical Inference. Springer. 272p.

MIRA Milieu- en natuurrapport Vlaanderen. Achtergronddocument 2007. Verspreiding van bestrijdingsmiddelen. Claeys S, Steurbaut W, Theuns I, De Cooman W, De Wulf E, Eppinger R, D'hont D, Dierckxens C, Goemans G, Belpaire C, Wustenberghs H, den Hond E, Peeters B, Overloop S. Vlaamse Milieumaatschappij, www.milieurapport.be

Moe SJ, De Schamphelaere KAC, Clements WH, Sorensen MT, Van den Brink PJ, Liess M. 2013. Combined andinteracting effects of global climate change and toxicants on populations and communities. Environ Tox Chem 32, 49-61.

Moriya Y, Itoh M, Okuda S, Yoshizawa A, Kanehisa M. 2007. KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res. 35, 182-185.

Mur L, Skulberg OM, Utkilen H. Cyanobacteria in the environment, in: Chorus I and Bartram J (Eds), Toxic Cyanobacteria in Water. A Guide to Their Public Health Consequences, Monitoring and Management, E & FN Spon on behalf of WHO, London 1999, 15-40.

Murray KE, Thomas SM, Bodourt AA. 2010. Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ Pollut 158, 3462-3471.

Hollander M, Wolfe DA. 1973. Nonparametric Statistical Methods. New York: John Wiley & Sons. Pages 115-120.

National Center for Biotechnology Information. 2014. http://www.ncbi.nlm.nih.gov/genome/browse/

National Institutes for Health. 2014. http://www.nih.gov/science/models/

Neilan BA, Saker ML, Fastner J, Törökné A, Burns BP. 2003. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. Mol Ecol 12, 133-140.

Nehring S. 1993. Mortality of dogs associated with a mass development of *Nodularia spumigena* (Cyanophyceae) in a brackish lake at the German North Sea coast. J Plankton Res 15, 867-872.

Nizan S, Dimentman C, Shilo M, 1986. Acute toxic effects of the cyanobacterium *Micocystis aeruginosa* on *Daphnia magna*. Limnol Oceanogr 31, 497-502.

Nogueira ICG, Lobo-da-Cunha A, Vasconcelos VM. 2006. Effects of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* (cyanobacteria) ingestion on *Daphnia magna* midgut and associated diverticula epithelium. Aquat Toxicol 80, 194-203.

Nogueira ICG, Pereira P, Dias E, Pflugmacher S, Wiegand C, Franca S, Vasconcelos VM. 2004a. Accumulation of paralytic shellfish toxins (PST) from cyanobacterium *Aphanizomenon issatschenkoi* by the cladoceran *Daphnia magna*. Toxicon 44, 773-780.

Noguiera ICG, Saker ML, Pflugmacher S, Wiegand C, Vasconcelos VM. 2004. Toxicity of the cyanobacterium *Cylindrospermopsis raciborskii* to *Daphnia magna*. Environ Toxicol 19, 453-159.

Nota B, Van Straalen NM, Ylstra B, Roelofs D. 2010. Gene expression microarray analysis of heat stress in the soil invertebrate *Folsomia candida*. Insect Mol Biol 19, 315-322.

OECD. 2014a. Lists of projects on the AOP development programme workplan. http://www.oecd.org/env/ehs/testing/listsofprojectsontheaopdevelopmentprogrammeworkplan.htm

OECD. 2014b. http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm#AOP_wiki

OECD. 2013.Guidance document on developping and assessing adverse outcome pathways.

OECD. 2008. Guidelines for testing of chemicals, *Daphnia magna* reproduction test.

OECD. 1984. Guidelines for testing of chemicals, *Daphnia* sp, acute immobolisation and reproduction test.

Ojaveer E, Simm M, Balode M, Purina I, Suursaar U. 2003. Effect of *Microcystis aeruginosa* and *Nodularia spumigena* on survival of *Eurytemora affinis* and the embryonic and larval development of the Baltic herring *Clupea harengus* membras. Environ Toxicol 18, 236-242.

Olmstead AW, LeBlanc GA. 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ Health Perspect 111, 919-924.

O'Neil JM, Davis TW, Burford MA, Gobler CJ. 2012. The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. Harmful Algae 14, 313-334.

Onodera H, Ohsima Y, Henriksen P, Yasumoto T. 1997. Confirmation of anatoxin-a(s), in the cyanobacterium *Anabaena lemmermannii* as the cause of bird kills in Danish lakes. Toxicon 35, 1645-1648.

Orsini L, Schwenk K, De Meester L, Colbourne JK, Pfrender ME, Weider LJ. 2013. The evolutionary time machine: using dormant propagules to forecast how populations can adapt to changing environments. Trends Ecol Evol 28, 274-282.

Padisák J. 1997. *Cylindrospermopsis raciborskii* (Woloszyńska) Seenaya et Subba Ruja, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology Archiv Hydrobiologie 107, 563-593.

Paerl, H.W., Fulton III, R.S., Moisander, P.H., Dyble, J. 2001. Harmful Freshwater Algal Blooms, With an Emphasis on Cyanobacteria," The Scientific World Journal, 1, 76-113.

Paerl HW, Huisman J. 2008. Blooms like it hot. Science 320, 57-58.

Paerl HW, Huisman J. 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microbiol Rep 1, 27-37.

Panosso R, Lürling M. 2010. *Daphnia magna* feeding on *Cylindrospermopsis raciborskii*: the role of food composition, filament length and body size. J Plankton Res 32, 1393-1404.

Pavlopoulos GA, Secrier M, Moschopoulos C, Soldatos TG, Kossida S, Aerts J, Schneider R, Bagos PG. 2011. Using graph theory to analyze biological networks. BioData Mining 4:10.

Pawiton Y, Michiels S, Koscielny S, Gusnanto A, Ploner A. 2005. False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21, 3017-3024.

Pattinson KR, Havel JE, Rhodes RG. 2003. Invasibility of a reservoir to exotic *Daphnia lumholtzi*: experimental assessment of diet selection and life history responses to cyanobacteria. Freshwater Biol 48, 223-246.

Paul VJ. 2008. Global warming and cyanobacterial harmful algal blooms. In Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs; Advances in Experimental Medicine and Biology, Vol. 619; Hudnell, H. K., Ed.; Springer: New York; 239-257.

Pearson LA, Hisbergues M, Börner T, Dittmann E, Neilan BA. 2004. Inactivation of an ABC Transporter Gene, *mcyH*, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC7806. Appl Environ Microbiol 70, 6370-6378.

Peperzak L. 2003. Climate change and harmful algal blooms in the North Sea. Acta Oecol 24, 139-144.

Pereira JL, Hill CJ, Sibly RM, Bolshakov VN, Goncalves F, Heckmann LH, Callaghan A. 2010. Gene transcription in *Daphnia magna*: Effects of acute exposure to a carbamate insecticide and an acetanilide herbicide. Aquat Toxicol 97, 268-276.

Pereira P, Onodera H, Andrinolo D, Franca S, Araújo F, Lagos N, Oshima Y. 2000. Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aqua*, isolated from Montargil reservoir Portugal. Toxicon 38, 1689-1702.

Perschbacher PW. 2005. Temperature effects on acute copper toxicity to juvenile channel catfish *Ictalurus punctatus*. Aquaculture 243, 225-228.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45, DOI 10.1093/nar/29.9.e45.

Pires LMD, Sarpe D, Brehm M, Ibelings BW. 2011. Potential synergistic effects of microcystins on bacterial lipopolysaccharides on life history traits of *Daphnia galeata* raised on low and high food levels. Aquat Toxicol 104, 230-242.

Piorreck M, Baasch KH, Pohl P. 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry 23, 207-216.

Pöch G. 1993. Combined effects of drugs and toxic agents. In: Modern Evaluation in Theory and Practice. Springer, Wien.

Pope CN. 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity. J Toxicol Environ Health Part B: Crit Rev 2, 161-181.

Pope, C., Karanth, S., Liu, J. 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ Toxicol Pharmacol 19, 433-446.

Poynton HC, Lazorchak J, Impellitteri C, Blalock B, Smith ME, Struewing K, Unrine J, Roose D. 2013. Toxicity and transcriptomic analysis in *Hyalella Azteca* suggest increased exposure and susceptibility of epibenthic organisms to zinc oxide nanoparticles. Environ Sci Technol 47, 9453-9460.

Poynton HC, Lazorchak J, Impellitteri C, Smith ME, Rogers K, Patra M, Hammer K, Allen J, Vulpe C. 2011. Differential gene expression in *Daphnia magna* suggests distinct modes of action and bioavailability for ZnO nanoparticles and Zn ions. Environ Sci Technol 45, 762-768.

Poynton HC, Loguinov AV, Varshavsky JR, Chan S, Perkins EJ, Vulpe CD. 2008. Gene expression profiling in *Daphnia magna* Part I: Concentration-dependent profiles provide support for the No Observed Transcriptional Effect Level. Environ Sci Technol 42, 6250-6256.

Poynton HC, Varshavsky JR, Chang B, Cavigiolio G, Chan S, Holman PS, Loguinov AV, Bauer DJ, Komachi K, Theil EC, Perkins EJ, Hughes O, Vulpe CD. 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ Sci Technol 41, 1044-1050.

Poynton HC, Vulpe CD. 2009. Ecotoxicogenomics: Emerging technologies for emerging contaminants. J American Water Resources Association 45, 83-96.

Qi S, Wang C, Chen X, Qin Z, Li X, Wang C. 2013. Toxicity assessments with *Daphnia magna* of Guadipyr, a new neonicotinoid insecticide and studies of its effect on acetylcholinesterase (AChE), glutathione S-transferase (GST), catalase (CAT) and chitobiase activities. Ecotox Environ Saf 98, 339-344.

Qui T, Xie P, Liu Y, Li G, Xiong Q, Hao L, Li H. 2009. The profound effects of microcystin on cardiac antioxidant enzymes, mitochondrial function and cardiac toxicity in rat. Toxicol. 257, 86-94.

Rao SD, Banack SA, Cox PA, Weiss JH. 2001. BMAA selectively injures motor neurons via AMPA/kainite receptor activation. Experimental Neurology 20, 224-252.

R Developmental Core Team. 2005. R: A language and environment for statistical computing, Reference index Version 2.12.2. R Foundation for Statistical Computing, Vienna. http://www.R-project.orgRelyea R, Hoverman J. 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecol Lett 9, 1157-1171.

Relyea RA, Mills N. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proc Natl Acad Sci 98, 2194-2196.

Relyea RA. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environ Toxicol Chem 23, 1080-1084.

Repka S. 1996. Inter- and intraspecific differences in *Daphnia* life histories in response to two food sources: the green alga *Scenedesmus* and the filamentous cyanobacterium *Oscillatoria*. J Plankton Res 18, 1213-1223.

Repka S. 1997. Effects of food type on the life history of *Daphnia* clones from lakes differing in trophic state. II. *Daphnia cucullata* feeding on mixed diets. Freshwater Biol 38, 685-692.

Ritz, C., Streibig, J. C. 2005. Bioassay analysis using R. J Stat Softw 12, 1-22.

Rohrlack T, Dittmann E, Henning M, Börner T, Kohl J. 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Appl Environ Microbiol 65, 737-739.

Rohrlack T, Christoffersen K, Kaebernick M, Neilan BA. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in Daphnia pulicaria. Appl Environ Microbiol 70, 5047-5050.

Royston P. 1982. An extension of Shapiro and Wilk's W test for normality to large samples. Appl Stat, 31, 115-124.

Saelens X, Festjens N, Vande Walle L, van Gurp L, van Loo G, Vandenabeele P. 2004. Toxic proteins released from mitochondria in cell death. Oncogene 23, 2861-2874.

Sandler B, Murakami M, Clardy J. 1998. Atomic structure of the trypsin -aeruginosin 98-B complex J. Am. Chem. Soc. 120, 595-596.

Sano T, Beattie KA, Codd GA, Kaya K. 1998. Two-(Z)-dehydrobutyrine-containing microcystins from a hepatotoxic bloom of *Oscillatoria agardhii* from Soulseat Loch, Scotland. J Nat Prod 61, 851-853.

SCHER, SCCS, SCENIHR. Opinion on the toxicity and assessment of chemical mixtures, 2012.

Schiedek D, Sundelin B, Readman JW, Macdonald RW. 2007. Interactions between climate change and contaminants. Mar Pollut Bull 54, 1845-1856.

Schirmer K, Fischer BB, Madureira DJ, Pillai S. 2010. Transcriptomics in ecotoxicology. Anal Bioanal Chem 397, 917-923.

Schopf JW. 2000. The fossil record: tracing the roots of the cyanobacterial lineage. In: Whitton BA, Potts M (eds) The ecology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, 13-35.

Schwarzenberger A, Zitt A, Kroth P, Mueller S, Von Elert E. 2010. Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. BMC Physiol 10:6.

Sellner, K.G. 1997. Physiology, ecology, and toxic properties of marine cyanobacteria blooms. Limnol. Oceanogr. 42, 1089-1104.

Sen B, Mahadevan B, DeMarini DM. 2007. Transcriptional responses to complex mixtures - a review. Mut Res/Rev in Mut Res 636, 144-177.

Shaw GR, Sukenik A, Livne A, Chiswell RK, Smith MJ, Seawright AA, Norris RL, Eaglesham GK, Moore MR. 1999. Blooms of the cylindrospermopsin containg cyanobacterium, *Aphanizomenon ovalisporum* (Forti), in newly constructed lakes, Queensland, Australia. Environ Toxicol 14, 167-177.

Shaw JR, Colbourne JK, Davey JC, Glaholt SP, Hampton TH, Chen CY, Folt CL, Hamilton JW. 2007. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. BMC Genomics 8:477.

Shaw JR, Pfrender ME, Eads BD, Klaper R, Callaghan A, Sibly RM, Colson I, Jansen B, Gilbert D, Colbourne JK. 2008. *Daphnia* as an emerging model for toxicological genomics. Adv Experiment Biol 2, 165-219.

Sherer, T.B., Richardson, J.R., Testa, C.M., Seo, B.B., Panov, A.V., Yagi, T., Matsuno-Yagi, A., Miller, G.W., Greenamyre, J.T., 2006. Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinsons's disease. J Neurochem 100, 1469-1479.

Sibly RM, Calow P. 1989. A life-cycle theory of responses to stress. Biol J Linnean Soc 37, 101-116.

Sigma Aldrich. 2014. http://www.sigmaaldrich.com

Sivonen K, Kononen K, Carmichael WW, Dahlem AM, Rinehart KL, Kiviranta J, Niemelä SI. 1989. Occurrence of the hepatotoxic cyanobacterium Nodularia spumigena in the Baltic Sea and structure of the toxin. Appl Environ Microbiol 55, 1990-1995.

Sivonen K, Namikoshi M, Evans WR, Carmichael WW, Sun F, Rouhiainen L, Luukkainen R, Rinehart KL. 1992. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. Appl Environ Microbiol 58, 2495-2500.

Sivonen K, Niemelä SI, Niemi RM, Lepistö L, Luoma TH, Räsänen LA. 1990. Toxic cyanobacteria (blue-green algae) in Finnish fresh and coastal waters. Hydrobiologia 190, 267-275.

Smyth GK. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, 1; DOI 10.2202/1544-6115.1027.

Smyth GK, Altman NS. 2013. Separate-channel analysis of two-channel microarrays: recovering inter-spot information. BMC Informatics 14:165.

Soares MCS, Lürling M, Panosso R, Huszar V. 2009. Effects of the cyanobacterium *Cylindrospermopsis raciborskii* on feeding and life-history characteristics of the grazer *Daphnia magna*. Ecotoxicol Environ Safe 72, 1183-1189.

Soetaert A, Vandenbrouck T, van der Ven K, Maras M, van Remortel P, Blust R, De Coen WM. 2007. Molecular responses during cadmium-induced stress in *Daphnia magna*: Integration of differential gene expression with higher-level effects. Aquatic Toxicol 83, 212-222.

Sondergaard, M., S. E. Larsen, T. B. Jorgensen, and E. Jeppesen. 2011. Using chlorophyll a and cyanobacteria in the ecological classification of lakes. Ecol. Indic. 11:1403-1412.

Stanier RY, Kunisawa R, Nadel M, Cohen-Bizarre G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol Rev 35, 171-205.

Stanley JK, Perkins EJ, Habib T, Sims JG, Chappel P, Escalon L, Wilbanks M, Garcia-Reyero N. 2013. The good, the bad, and the toxic: approaching hormesis in *Daphnia magna* exposed to an energetic compound. Environ Sci Technol 47, 9424-9433.

Stork T, Thomas S, Rodrigues F, Silies M, Naffin E, Wenderdel S, Klambt C. 2009. *Drosophila* neurexin IV stabilizes neuron-glia interactions at the CNS midline by binding to Wrapper. Development 136, 1251-1261.

Sun H, Lü K, Minter EJA, Chen Y, Yang Z, Montagnes DJS. 2012. Combined effects of ammonia and microcystin on survival, growth, antioxidant responses, and lipid peroxidiation of bighead carp *Hypophthalymythys nobilis* larvae. J Harzar Mater 221-222, 213-219.

Tatarazako N, Oda S. 2007. The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. Ecotoxicology 16, 197-203.

Tatusov R, Fedorova N, Jackson J, Jacobs A, Kiryutin B et al. 2003. The COG database: an updated version includes eukaryotes. BMC Bioinf. 4: 41.

Tillmanns, A. R., A. E. Wilson, F. R. Pick, Sarnelle, O. 2008. Meta-analysis of cyanobacterial effects on zooplankton population growth rate: species-specific responses. Fund. Appl. Limnol. 171, 285-295.

Toumi H, Boumaiza M, Millet M, Radetski CM, Felten V, Fouque C, Férard JF. 2013. Effects of deltamethrin (pyrethroid insecticide) on growth, reproduction, embryonic development and sex differentiation in two strains of *Daphnia magna* (Crustacea, Cladocera). Sci Total Environ 458-460, 47-53.

Turja R, Guimarães L, Nevala A, Kankaanpää H, Korpinen S, Lehtonen KK. 2013. Cumulative effects of exposure to cyanobacteria bloom extracts and benzo[a]pyrene on antioxidant defence biomarkers in *Gammarus oceanicus* (Crustacea: Amphipoda). Toxicon 78, 68-77.

Ulén B, Bechmann M, Fölster J, Jarvie HP, Tunney H. 2007. Agriculture as a phosphorus source for eutrophication in the north-west European countries, Norway, Sweden, United Kingdom and Ireland: a review. Soil Use Manage 23, 5-15.

United States Environmental Protection Agency. 1996. Ecological Effects Test Guidelines OPPTS 850.1300 Daphnid Chronic Toxicity Test. US EPA, 12p.

Van Aggelen, G, Ankley T, Baldwin WS, Baerden DW et al. 2010. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: hurdles, achievements and future outlook. Environ Health Perspect 118, 1-5.

van Apeldoorn ME, van Egmond HP, Speijers GJA, Bakker HJI. 2007. Toxins of cyanobacteria. Mol Nutr Food Res 51, 7-60.

Vandegehuchte MB, Vandenbrouck T, De Coninck D, De Coen WM, Janssens CR. 2010. Gene transcription and higher-level effects of multigenerational Zn exposure in Daphnia magna. Chemosphere 80, 1014-1020.

Vandenbrouck T, Jones OAH, Dom N, Griffin JL, De Coen W. 2010. Mixtures of similarly acting compounds in *Daphnia magna*: From gene to metanolite and beyond. Environ Int 36, 254-268.

Vandenbrouck T, Soetaert A, van der Ven K, Blust R, De Coen W. 2009. Nickel and binary metal mixture responses in *Daphnia magna*: molecular fingerprints and suborganismal effects. Aquat Toxicol 92, 18-29.

Van Gestel CAM, Hensbergen PJ. 2009. Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidaa) in relation to bioavailability in soil. Environ Toxicol Chem 16, 1177-1186.

Van Gestel CAM, Jonker M, Kammenga JE, Laskowski R, Svendsen C. 2010. Mixture toxicity: Linking approaches from ecological and human toxicology. First ed., CRC Press, Florida.

Van Halderen A, Harding WR, Wessels JC, Schneider DJ, Heine EW, Van der Merwe J, Fourie JM. 1995. Cyanobacterial (blue-green algae) poisoning of livestock in the western Cape Province of South Africa. J S Afr Vet Assoc 66, 260-264.

Van Straalen NM, Feder ME. 2012. Ecological and Evolutionary Functional Genomics - how can it contribute to the risk assessment of chemicals? Environ Sci Technol 46, 3-9.

Vega MP, Pizarro RA. 2000. Oxidative stress and defence mechanisms of the freshwater cladoceran Daphnia longispina exposed to UV radiation. J. Photochem. Photobiol. B 54, 121-125.

Villaroel MJ, Ferrando MD, Sancho E, Andreu E. 1999. *Daphnia magna* feeding behavior after exposure to tetradifon and recovery from intoxication. Ecotox Environ Saf 44, 40-46.

Villaroel MJ, Ferrando MD, Sancho E, Andreu E. 2008. Population dynamics in *Daphnia Magna* as modified by chronic tetradifon stress. J Environ Sci Health, Part B 35, 221-227.

Villaroel MJ, Sancho E, Andreu-Moliner E, Ferrando MD. 2009. Biochemical stress response in tetradifon exposed *Daphnia magna* and its relationship to individual growth and reproduction. Sci Total Environ 407, 5537-5542.

Villeneuve DL, Garcia-Reyero N. 2011. A vision and strategy for predictive ecotoxicology testing in the 21st century. Environ Toxicol Chem 30, 1-8.

von Elert, E. 2004. Food quality constraints in *Daphnia*: interspecific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. Hydrobiologia 526, 187-196.

Von Elert E, Martin-Creuzburg D, Le Coz JR. 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (Daphnia galeata). Proc R Soc Lond B 270, 1209-1214.

Wagner, C., Adrian, R. 2011. Consequences of changes in thermal regime for plankton diversity and trait composition in a polymictic lake: a matter of temporal scale. Freshwater Biol. 56, 1949-1961.

Walter P, Ron D. 2011. The unfolded protein response: From stress pathway to homeostatic regulation. Science 334, 1081-1086.

Wang L, Tang H, Thayanithy V, Subramanian S, Oberg LA, Cunnignham LM, Cerhan JR, Steer CJ, Thibodeau SN. 2009. Gene networks and microRNAs implicated in aggressive prostate cancer. Cancer Res 69:9490.

Warne MSTJ, Hawker DW. 1995. The number of componets in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: the funnel hypothesis. Ecotox Environ Saf 31, 23-28.

Weaver JMJ, Ross-Innes C.S. Fitzgerald R.C. 2014. The –omics revolution and oesophageal adenocarcinoma. Nat Rev Gastroenterol Hepatol 11, 19-27.

Weismann A. 1880. Beiträge zur Naturgeschichte der Daphnoiden. Zeitschrift für wissenschaftliche Zoologie 33, 55-70.

Wiegand C, Pflugmacher S. 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol Appl Pharmacol 203, 201-218.

Wiedner C, Rücker J, Brüggeman R, Nixdorf B. 2007. Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia 152, 473-484.

Wilson AE, Sarnelle O, Tillmanns AR. 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analyses of laboratory experiments. Limnol Oceanogr 51, 1915-1924.

World Health Organization. 2005. Guidelines for safe recreational water environments Volume 1: coastal and fresh waters. 253p.

Yang Z, Xiang F, Minter EJA, Lü K, Chen Y, Montagnes DJS. 2011. The interactive effects of microcystin and nitrite on life-history parameters of the cladoceran *Daphnia obtuse*. J Hazar Mater 190, 113-118.

Yang Z, Lü K, Chen Y, DJS Montagnes. 2012. The interactive effects of ammonia and microcystins on life-history traits of the cladoceran *Daphnia magna*: Synergistic or antagonistic? PLOS One 7, e32285.

Zhang H, Zhang J, Chen Y, Zhu Y. 2007. Influence of intracellular Ca2+, mitochondria membrane potential, reactive oxygen species, and intracellular ATP on the mechanism of microcystin-LR induced apoptosis in Carassius auratus lymphocytes in vitro. Environ Toxicol 22, 559-564.

Zhang B, Horvath S: A General Framework for Weighted Gene Co-expression Network Analysis. Stat Appl Genet Mol Biol 2005, 4: Article 17.

Zhao Y, Xie P, Tanga R, Zhang X, Li L, Li D. 2008. In vivo studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comp Biochem Physiol C 148, 204-210.

Zinke I, Schütz CS, Katzenberger JD, Bauer M, Pankratz MJ. 2002 Nutrient control of gene expression in Drosophila microarray analyis of starvation and suger-dependent response. The EMBO Journal 21, 6162-6173.

Zou E, Fingerman M. 1997. Synthetic estrogenic agents do not interfere with sex differentiation but do inhibit molting of the cladoceran *Daphnia magna*. Bull Environ Contam Toxicol 58, 596-602.

Summary

Aquatic ecosystems are complex environments where organisms interact with a heterogeneous group of stressors from anthropogenic and natural origin. Yet, current risk assessment practices fail to include these combined effects of stressors and their potential interaction as they primarily use a chemical-by-chemical approach. The lack of sufficient comprehensive data in literature and the lack of predictive models further impede the incorporation of combined and interaction effect in environmental regulation.

Combined and interaction effects are likely to increase significantly in the future. Anthropogenic factors and climate change conditions stimulate bloom formation of potential toxic cyanobacteria. These organisms are an emerging concern for both environmental and public health. Although effects on mammals are well documented and understood, the mechanisms driving adverse effects on zooplankton species remain unclear and research is largely biased towards effects of *Microcystis*. Therefore, **chapter two** has focused on the effect of six cyanobacteria species, representing six main genera, on the life history of the *Daphnia*. Effects of cyanobacteria were studied across the full concentration response curve and compared with each other and with a starvation response. *Daphnia* were exposed to cells of cyanobacteria rather than cyanobacterial toxins to more closely adhere to environmental reality. The concentration response curves remained conserved across the six studied cyanobacteria and were not significantly different from starvation treatments. This indicated a potential common lack of nutritional quality across all cyanobacteria similarly affecting the fitness of *D. pulex* rather than effects of different cyanobacterial toxins.

Cyanobacteria are likely to occur in environments with other stressors leading to multiple stress conditions and altered responses of *Daphnia* exposed to cyanobacteria under such multiple stress conditions. Indeed, the eutrophication of water bodies, known to enhance bloom formation, often occurs in agricultural areas, which may give rise to unknown interaction effects with plant protection products. In particular, insecticides can severely affect aquatic invertebrates and two studies have shown interaction between insecticides and cyanobacteria on *Daphnia*. Given that each study focused on specific combinations of stressors, conclusions cannot be generalized across different cyanobacteria or insecticides and the potential interaction effects for insecticides and cyanobacteria in general remain largely unknown. In **chapter 3**, these potential interaction effects were studied by exposing *Daphnia* to binary combinations of a selection of cyanobacteria and insecticides. Combined and interaction effects were evaluated within defined statistical frameworks with the two conceptual

models, i.e. concentration addition and independent action, under standard conditions of 21 day exposure. Different interaction patterns were observed for insecticides with different molecular targets when combined with *Microcystis*. In contrast, different cyanobacteria showed similar interaction patterns when combined with carbaryl. Four out of eight combinations showed antagonistic deviations patterns, three showed no interaction patterns whereas one yielded different patterns dependent on the conceptual model. Independent action concluded synergistic deviations from non-interaction whereas concentration addition concluded no significant deviations from non-interaction. **Chapter 3** clearly highlighted that interaction effects cannot be generalized across modes of actions of insecticides whereas results may potentially be generalized across different cyanobacteria combined with the same insecticide. In agreement with general literature, the concentration addition conceptual model provided more conservative predictions of effects than independent action from a risk assessment point view.

Twenty-one day exposure experiments are however labor intensive and time consuming. **Chapter 4** therefore focused on studying the effects on life history of a comprehensive set of 48 binary combinations of insecticides and cyanobacteria under a shorter exposure time and with reduced experimental design. Statistical evaluation of the effects is therefore only possible within the independent action framework. This approach was however evaluated by comparing the results of **chapters 3 and 4** for those combinations that were repeated. The results in **chapter 4** indicated the occurrence of interaction effects to be a complex mechanism dependent upon several factors. Conclusions regarding interaction effects were significantly altered by exposure concentration and exposure design. As a consequence, studies on life history level without further mechanistic research seem to be insufficient to attain a clear insight in the dynamics and processes leading to interactions.

The evolution of high throughput molecular technologies has enhanced the mechanistic understanding of organismal responses under stress. Mechanistic understanding is not only crucial to identify the driving factors of stress response but may also aid in building predictive models. **Chapter 5** identified the potential of these technologies by studying the stress response of *Daphnia pulex* to *Microcystis aeruginosa* with high throughput microarrays. A comprehensive set of bioinformatics tools was developed specifically taken into account the unique *Daphnia* genome within **chapter 5** to identify crucial pathways and gene networks involved in response to *Microcystis*. The stress response pattern upon exposure to *Microcystis* consisted of four major pathways or gene networks as well as eight

paralogous gene families. Moreover, **chapter 5** underlined the need to take into account the specific and unique *D. pulex* genome structure in expression studies, which may influence conclusions drawn. This genomic structure is characterized by among others, a high number of lineage specific or unknown genes. **Chapter 5** also highlighted how these genes can be functionally annotated to environmentally relevant conditions.

Chapter 6 further applied microarray technology to study the response of *Daphnia* to the five other studied cyanobacteria in **chapter 2**. The bioinformatics tools developed in **chapter 5** were used to compare similarities and differences across stress responses to each of these cyanobacteria at different levels of molecular organization. Both mechanisms that were specific to a cyanobacterial stressor as well as mechanisms that were conserved across the cyanobacterial stressors were identified. The similarities and differences between these patterns depended upon the level of biological organization. The observed discrepancy between these levels underlined the necessity of such a research approach. Furthermore, the incorporation of the unique features of the genome under study in the analysis benefitted the integration of these different levels of organization in a true systems biology approach. Overall, cyanobacterial stress targeted mainly similar mechanisms regardless of the cyanobacterial species yet the effects on these mechanisms are species-specific at both the gene and organismal level.

In **chapter 7**, the molecular responses of animals to the 48 binary combinations, used in **chapter 4,** were studied. Transcriptomics have been used to study interaction effects but often lack statistical frameworks and a priori defined hypotheses. **Chapter 7** put forward and applied three different approaches and two different measures of deviation. Overall, filtering the data based upon no overlapping 95% confidence intervals between observations and predictions and then calculating the sum of M-values of all genes with significant deviations from non-interaction was the most consistent approach allowing identification of statistical and biological significant genes. Genes with a significant interaction effect across all combinations showed a diverse pattern of expression in both synergistic and antagonistic directions. However, the general accepted terminology of synergism and antagonism at the life history level was insufficient to explain the possible combinations and patterns of genes demonstrating an interaction effect in terms of clear synergism or antagonisms. Functional analysis of these genes revealed a diverse set of metabolic functions and pathways which indicated that interaction effects trigger a complex general stress response in the organism.

Molecular high throughput technologies are increasingly used in research yet clear and direct links between molecular and biological effects remain difficult to establish. Therefore, **Chapter 8** integrated all data from **chapters 4 and 7** by building comprehensive gene networks on different parts of the transcriptomic data generated in **chapter 7.** The modules of these gene networks were then in turn correlated with life history parameters and interaction parameters at both the life history level, defined in **chapter 4**, and the molecular level, defined in **chapter 7**. Significant correlations between modules and deviation from non-interaction could only be identified when using both single and mixture expression profiles. The absence of single expression profiles resulted in no significant correlations with interaction at the life history level, which confirms the importance of including effects of single stressors. Functional analysis of gene modules identified pathways such as hormone biosynthesis and amino acid metabolism as well as significant proportion of lineage specific genes. Interaction effects were also characterized by specific gene functions present in the significantly correlated modules such as trypsins and neurexins IV. Overall, modules describing interaction effects were more diverse in terms of module structure and connectivity than models describing reproduction effects which underlined the complexity of interaction effects.

Finally, **Chapter 9** gave an overview of the main conclusions reached throughout this dissertation and how they have answered the concerns and research gaps put forward in the introduction. It has also addressed the challenges that environmental research will still need to face in the future.

Samenvatting

Aquatische ecosystemen zijn complexe omgevingen waar organismen interageren met een heterogene groep stressoren van zowel antropogene als natuurlijke oorsprong. Echter, de huidige richtlijnen voor risicoschattingen slagen er niet in deze gecombineerde effecten van stressoren en de mogelijke interacties op te nemen en maken voornamelijk gebruik van een enkelvoudige benadering waarin iedere chemische stof apart wordt beoordeeld. Het ontbreken van voldoende uitvoerige gegevens in de literatuur en het gebrek aan voorspellende modellen belemmeren verder de incorporatie van zowel gecombineerde effecten als interactie effecten in de milieuwetgeving.

Gecombineerde effecten en interactie effecten zullen waarschijnlijk in de toekomst sterk toenemen. Antropogene factoren en toekomstige klimaatsveranderingen stimuleren de bloei vorming van potentieel toxische cyanobacteriën. Deze organismen zijn een opkomende bedreiging voor zowel het milieu als de volksgezondheid. Hoewel de effecten van cyanobacteriën op zoogdieren goed begrepen en gedocumenteerd zijn, blijven de mechanismen achter de effecten op zoöplankton soorten onduidelijk. Bovendien is onderzoek gebaseerd op de effecten van *Microcystis*. Daarom heeft hoofdstuk 2 zich gericht op het effect van zes soorten cyanobacteriën, representatief voor de zes belangrijkste genera, op de reproductie van *Daphnia*. Effecten van cyanobacteriën werden bestudeerd op basis van een volledige concentratie responscurve en vergeleken met elkaar en met de respons op uithongering. *Daphnia* werden blootgesteld aan cellen van cyanobacteriën in plaats van cyanobacteriële toxinen om nauwer aan te sluiten bij de ecologische realiteit. De concentratie respons curves waren gelijk tussen de zes onderzochte cyanobacteriën en waren dus niet significant verschillend van de uithongerings-respons. Dit duidt op een mogelijk gemeenschappelijke gebrek aan voedingswaarde in alle cyanobacteriën dat dan zo ook de reproductie van *Daphnia pulex* beïnvloedde eerder dan effecten van verschillende cyanobacteriële toxines.

Cyanobacteriën komen ook voor in omgevingen met andere stressoren wat kan leiden tot meervoudige stress en dus ook andere reacties van *Daphnia* die worden blootgesteld aan cyanobacteriën in combinatie met mogelijke andere stressoren. Inderdaad, de eutrofiëring van vijvers en meren, een factor die bloei vorming stimuleert, komt vaak voor in agrarische gebieden, die aanleiding kunnen geven tot onbekende interactie effecten met gewasbeschermingsmiddelen. In het bijzonder kunnen insecticiden ernstige gevolgen hebben op aquatische invertrebraten. Twee studies hebben al interacties tussen insecticiden en cyanobacteriën op *Daphnia* aangetoond. Aangezien elk van deze onderzoeken gericht is op specifieke combinaties van stressoren, kunnen conclusies niet

veralgemeend worden. Dus blijven ook de mogelijke interactie effecten van combinaties van insecticiden en cyanobacteriën in het algemeen nog grotendeels onbekend. In hoofdstuk 3, werden deze mogelijke interactie effecten bestudeerd door het blootstellen van *Daphnia* aan binaire combinaties van een selectie van cyanobacteriën en insecticiden. Gecombineerde effecten en interactie effecten werden geëvalueerd binnen gedefinieerde statistische modellen, op basis van twee conceptuele beschrijvingen: concentratie additie en onafhankelijke actie, onder standaard condities van 21 dagen blootstelling. Verschillende interactiepatronen werden waargenomen voor insecticiden met verschillende moleculaire targets in combinatie met *Microcystis*. Echter verschillende cyanobacteriën vertoonden vergelijkbare interactie patronen in combinatie met carbaryl. Vier van de acht combinaties vertoonden antagonistische afwijkingen, drie vertoonden geen interactie patronen terwijl voor één het interactie patroon afhankelijk was van het conceptuele model. Het onafhankelijk actie model besloot synergisme terwijl het concentratie additie model geen afwijkingen concludeerde. Hoofdstuk 3 gaf dus duidelijk aan dat de interactie effecten niet veralgemeend kunnen worden over werkingsmechanismen van insecticiden heen, terwijl de resultaten potentieel kunnen worden veralgemeend over verschillende cyanobacteriën heen gecombineerd met hetzelfde insecticide. In overeenstemming met de algemene literatuur, leverde het concentratie additie conceptueel model meer conservatieve voorspellingen van effecten op dan het onafhankelijke actie conceptueel model vanuit een risicoschattings-perspectief.

Eenentwintig dagen blootstellingexperimenten zijn echter arbeidsintensief en tijdrovend. Hoofdstuk 4 richtte zich daarom op het bestuderen van de effecten op de reproductie van *Daphnia* van een uitgebreide set van 48 binaire combinaties van insecticiden en cyanobacteriën onder een kortere blootstellingstijd en met beperkte experimentele opzet. Statistische evaluatie van de effecten was dus alleen mogelijk met het onafhankelijke actie model. Deze aanpak werd echter geëvalueerd door de resultaten van hoofdstuk 3 en 4 voor de combinaties die werden herhaald te vergelijken. De resultaten in hoofdstuk 4, toonden aan dat het optreden van interactie effecten een complex mechanisme is afhankelijk van verschillende factoren. Conclusies ten aanzien van interactie effecten waren significant gewijzigd door concentratie en de blootstellingstijd. Bijgevolg lijken studies op organismaal niveau zonder verder mechanistische onderzoek onvoldoende om een inzicht te krijgen in de dynamiek en processen die leiden tot interacties.

De evolutie van high throughput moleculaire technologieën hebben de mechanistische kennis van organismale responsen onder stress versterkt. Mechanistische kennis is niet alleen cruciaal voor de drijvende factoren van stress respons te identificeren, maar kan ook helpen bij het bouwen van voorspellende modellen. In hoofdstuk 5 werden de mogelijkheden van deze technologieën onderzocht door het bestuderen van de stress respons van *Daphnia pulex* aan *Microcystis aeruginosa* met high throughput microarrays. Een uitgebreide set van bioinformatica tools werd ontwikkeld in hoofdstuk 5 op maat van het unieke *Daphnia* genoom met als doel cruciale pathways en netwerken van genen betrokken bij de reactie op *Microcystis* te identificeren. De reactie op stress bij blootstelling aan *Microcystis* bestaat uit vier belangrijke pathways of gen- netwerken, alsook acht paraloge gen families. Bovendien, onderstreept hoofdstuk 5 de noodzaak om rekening te houden met de specifieke en unieke D. pulex genoomstructuur in expressie studies, gezien dit de gemaakte conclusies kan beïnvloeden. De genomische structuur wordt gekenmerkt door onder andere een groot aantal *daphnia*-specifieke of onbekende genen. Hoofdstuk 5 ging dieper in op hoe deze genen functioneel kunnen geannoteerd worden binnen een milieurelevante context.

Hoofdstuk 6 gebruikte microarray technologie om de respons van *Daphnia* aan de vijf andere onderzochte cyanobacteriën in hoofdstuk 2 te bestuderen. De ontwikkelde bioinformatica tools in hoofdstuk 5 werden gebruikt om gelijkenissen en verschillen tussen stress responsen te vergelijken met elk van deze cyanobacteriën op verschillende niveaus van de moleculaire organisatie. Zowel mechanismen die specifiek zijn voor een cyanobacteriële stressor als mechanismen die gelijk zijn voor de cyanobacteriële stressoren werden geïdentificeerd. De overeenkomsten en verschillen tussen deze patronen hing af van de niveaus van biologische organisatie. De waargenomen verschillen tussen deze niveaus benadrukte de noodzaak van een dergelijke onderzoeksaanpak. Bovendien werd de analyse positief beïnvloed door de integratie van de unieke kenmerken van het genoom binnen deze verschillende niveaus van organisatie in een echte sytems biology benadering. Cyanobacteriële stress was dus overwegend gericht op soortgelijke mechanismen onafhankelijk van de cyanobacteriën soorten echter de effecten op deze mechanismen zijn soort specifiek gezien deze mechanismen gereguleerd worden door verschillende genen bij blootstelling aan verschillende cyanobacteriën.

In hoofdstuk 7 werden de moleculaire profielen van de daphnias bij blootstelling aan 48 binaire combinaties van insecticiden en cyanobacteriën, die in hoofdstuk 4 getest werden, bestudeerd. Transcriptomics worden gebruikt om interactie-effecten te bestuderen maar missen vaak statistische

modellen en a priori gedefinieerde hypotheses. Hoofdstuk 7 stelde voor en implementeerde drie verschillende benaderingen om data te filteren en twee mogelijke predictors van deviatie van non-interactie. Het filteren van de data op basis van geen overlappende 95% betrouwbaarheidsintervallen tussen observaties en predicties was over de hele lijn de meest consistente aanpak. De som van alle M-waarden van alle genen met een significante deviatie van non-interactie was de meest consistente aanpak die toeliet om statistisch en biologisch significante genen te identificeren. Genen met significante interactie effecten over alle combinaties heen vertoonden een zeer diverse expressie patroon met zowel synergistische als antagonistische effecten. De huidige terminologie om synergismen en antagonismen te beschrijven was echter ontoereikend om de verschillende patronen van genen met een interactie effect te beschrijven. Functionele analyse van deze genen wees op een divers set van metabolische functies en pathways wat suggereerde dat interactie effecten kunnen leiden tot een complexe algemene reactie op stress in het organisme.

Moleculaire highthroughput technologieën worden steeds vaker gebruikt in het onderzoek maar het blijk echter nog steeds moeilijk om duidelijk en directe verbindingen tussen de moleculaire en biologische effecten te maken. Daarom integreerde hoofdstuk 8 alle gegevens van de hoofdstukken 4 en 7 door de constructie van uitgebreide netwerken van genen op verschillende delen van de transcriptomics gegevens die in hoofdstuk 7 gegenereerd werden. De modules van deze gen-netwerken werden dan op hun beurt gecorreleerd met organismale parameters en interactie parameters op zowel de organismaal niveau, parameters uit hoofdstuk 4, als het moleculaire niveau, beschreven in hoofdstuk 7. Significante correlaties tussen modules en deviatie van non-interactie konden enkel geïdentificeerd worden wanneer zowel expressieprofielen na blootstelling aan enkelvoudige stress als expressieprofielen na blootstelling aan mengsels beschouwd werden. Het weglaten van deze expressieprofielen na blootstelling aan enkelvoudige stress leidde tot geen significante correlaties met interactie op het organismale niveau. Dit bevestigde het belang om effecten van enkelvoudige stress in rekening te brengen. Functionele analyse van de modules identificeerde verscheidene pathways waaronder de hormoon biosynthese en het aminozuur metabolisme alsook een significant aandeel aan daphnia specifieke genen. Interactie effecten werden ook gekarakteriseerd door specifieke gen functies zoals trypsines en neurexines. Modules die correleren met interactie effecten waren in het algemeen meer diverse in zowel de structuur van de

module als de connectiviteit binnen de module in vergelijking met modules die significant correleren met effecten op reproductie. Dit benadrukt de complexiteit van interactie effecten.

Tot slot gaf hoofdstuk 9 een overzicht van de belangrijkste conclusies in dit proefschrift en hoe zij hebben geantwoord op bepaalde problemen en onderzoek hiaten naar voren gebracht in de inleiding. Verder werd er ook aandacht besteed aan de uitdagingen die milieuonderzoek nog te wachten staan.

Curriculum Vitae

Personalia

Name: Jana Asselman

Date of birth: October 8th, 1987

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Education

2010 - present PhD student in Bio-science Engineering

Ghent University, Belgium

PhD-dissertation: *"Transcriptomic profiles and fitness of Daphnia exposed to cyanobacteria and interactions with insecticides." (Promotor: Prof. Dr. ir. Karel De Schamphelaere)*

2008 - 2010 Master of Science in Bioscience Engineering, Cell- and genebiotechnology

Ghent University, Belgium

Master-thesis: *"Ecotoxicological and molecular characterization of metallothionein genes in Daphnia pulex" (Promotors: Prof. Dr. ir. Karel De Schamphelaere and Prof. Dr. Colin Janssen*)

2005 - 2008 Bachelor in Bioscience Engineering, Cell- and genebiotchenology

Ghent University, Belgium

1999 - 2005 Secondary school degree in Mathematics & Sciences

Sint-Vincentius à Paulo Instituut, Gijzegem, Belgium

Professional employment

2010 - present Scientific researcher at the Laboratory of Environmental Toxicology

Faculty of Bioscience Engineering, Ghent University, Belgium

Publications

Peer-reviewed papers (A1)

[1] De Coninck DIM*, **Asselman J***, Glaholt S, Janssen CR, Colbourne JK, Shaw JR, De Schamphelaere KAC. 2014. Genome-wide transcription profiles provide insights in genotype-dependent mechanisms of tolerance to two interacting stressors in *Daphnia*. Accepted in Environ Sci Technol. *These authors contributed equally

[2] **Asselman J***, Hochmuth JD*, De Schamphelaere KAC. 2014. A comparison of the sensitivities of Daphnia magna and Daphnia pulex to six different cyanobacteria species. Accepted in Harmful Algae. *These authors contributed equally.

[3] Hochmuth JD, **Asselman** J, De Schamphelaere KAC. 2014. Are interactive effects of harmful algal blooms and copper pollution a concern for water quality management? Water Research: in Press.

[4] **Asselman J**, Janssen CR, Smagghe G, De Schamphelaere, KAC. 2014. Ecotoxicity of binary mixtures of Microcystis aeruginosa and insecticides to Daphnia pulex. Environ Pollut 188, 56-63.

[5] **Asselman J**, Shaw JR, Glaholt SP, Colbourne JK, De Schamphelaere KAC. 2013. Transcription patterns of four metallothionein homologis in *Daphnia pulex* exposed to copper and cadmium are time- and homolog-dependent. Aquat Toxicol 142-143, 422-430

[6] **Asselman J,** Meys J, Waegeman W, De Baets B, De Schamphelaere KAC. 2013. Combined exposure to cyanobacteria and carbaryl results in antagonistic effects on the reproduction of *Daphnia pulex*. Environmental Toxicology and Chemistry 32, 2153-2158.

[7] **Asselman J**, De Coninck DIM, Glaholt S, Colbourne JK, Janssen CR, Shaw JR, De Schamphelare KAC. 2012. Identification of pathways, gene networks, and paralogous gene

families in *Daphnia pulex* responding to exposure to the toxic cyanobacterium *Microcystis aeruginosa*. Environmental Science & Technology 46:8448-8457.

[8] **Asselman J**, Glaholt SP, Smith Z, Smagghe G, Janssen CR, Colbourne JK, Shaw JR, De Schamphelaere KAC. 2012. Functional characterization of four metallothionein genes in *Daphnia pulex* exposed to environmental stressors. Aquatic Toxicology 110-111, 54-65.

Non-peer-reviewed papers (A4)

[9] De Schamphelaere KA, Glaholt S, **Asselman J**, Messiaen M, De Coninck D, Janssen CR, Colbourne JK, Shaw JR. 2011. Will genetic adaptation of natural populations to chemical pollution result in lower or higher tolerance to future climate change? Integrated Environmental Assessment and Management 7:141-143.

Platform presentations (presenting author)

[1] **Asselman J**, Pfernder M, Lopez J, Shaw J, Stock M, De Baets B, De Schamphelaere K. TRanscriptomics and multiple stress: can gene expression elucidateinteracting effects in response to multiple stressors? EMBO Conference on The mighty daphnia past present and future, 19-22 January 2014. Birmingham, UK.

[2] **Asselman J**, Pfrender M, Shaw J, Lopez J, Meys J, Waegeman W, De Baets B, De Schamphelaere K. Studying combined exposure of cyanobacteria and carbaryl on Daphnia pulex at both the life-history level and the molecular level. SETAC North America 34th Annual Meeting, 17-21 November 2013, Nashville, USA.

[3] **Asselman J**, De Coninck D, Pfrender M, Shaw J, Colbourne J, Lopez J, Glaholt S, Janssen C, De Schamphelaere K. Integrating Genomics into ecotoxicology. Artemia Genome Day, September 1st 2013, Ghent, Belgium.

[4] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere K. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacterial stressors. 9th International Symposium on Toxic Cyanobacteria, 11-16th August 2013, Pilaneseberg, South Africa.

[5] Vandegehuchte M, De Coninck D, **Asselman J**, Jansen M, Vandenbussche J, Vanhaecke L, Decaestecker E, De Schamphelaere K, Janssen C. Global DNA methylation in *Daphnia magna* is influenced by genotype and a wide variety of environmental stressors. 17th Pollutant Responses in Marine Organisms (PRIMO) conference, 5-8 May 2013, Faro, Portugal.

[6] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere K. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacterial stressors. SETAC Europe 23rd Annual Meeting, 16-20 May 2013, Glasgow, UK.

[7] De Coninck D, **Asselman J**, Glaholt S, Colbourne J, Janssen C, Shaw J, De Schamphelaere K. What transcriptomic mechanisms cause a cadmium-adapted *Daphnia pulex* genotype to be more tolerant to cyanobacterial stress than a non-adapted genotype? SETAC Europe 23rd Annual Meeting, 16-20 May 2013, Glasgow, UK.

[8] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere K. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacterial stressors. 18th National Symposium on Applied Biological Sciences, February 8 2013, Ghent, Belgium.

[9] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere K. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacterial stressors. 18th National Symposium on Applied Biological Sciences, February 8 2013, Ghent, Belgium.

[10] De Coninck D, **Asselman J**, Glaholt S, Colbourne J, Janssen C, Shaw J, De Schamphelaere K. Genomic mechanisms of co-tolerance of cadmium-adapted *Daphnia pulex* populations to cyanobacterial stress. SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[11] Vandegehuchte M, De Coninck D, **Asselman J**, Jansen M, Trenti I, Vandenbussche J, Vanhaecke L, Decaestecker E, De Schamphelaere K, Janssen C. Global DNA methylation in *Daphnia magna* is influenced by genotype and a wide variety of environmental stressors. SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[12] **Asselman J**,Smagghe G, Janssen CR, De Schamphelaere, KAC.interacting mixture toxicity effects of a cyanobacterial stressor and insecticides may be partly grouped according to insect mode of action. SETAC Europe 22nd Annual Meeting, 20-24 May 2012. Berlin, Germany.

[13] **Asselman J**, De Coninck D, Glaholt S, Colbourne J, Janssen C, Shaw J, De Schamphelaere K. Using the model organism *Daphnia pulex* to delineate molecular pathways responding to environmental stress. SETAC North America 32nd Annual Meeting, 13-17 November 2011, Boston, USA.

[14] De Schamphelaere K, **Asselman J**, Glaholt S, Colbourne J, Janssen C, Shaw J.Unravelling the genomic basis of the cross-tolerance fo Cd-adapted *Daphnia pulex* to cyanobacterial stress. SETAC North America 31st Annual Meeting, 7-11 November 2010, Portland, USA.

[15] De Schamphelaere K, **Asselman J**, Glaholt S, Colbourne J, Janssen C, Shaw J. Will genetic adaptation of *Daphnia pulex* to chemical stress result in lower or higher tolerance to climate change. SETAC Europe 20th Annual Meeting, 23-27 May 2010, Sevilla, Spain.

[16] De Schamphelaere K**, Asselman J**, Glaholt S, Choi J, Colbourne J, Janssen C, Shaw J. *Daphnia* that have genetically adapted to Cd stress are also tolerant to toxic cyanobacteria. SETAC North America 30th Annual Meeting, 19-23 November 2009, New Orleans, USA.

Poster presentations (presenting author)

[1] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere KAC. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacteria. 3rd Nucleotides2Networks Symposium, 24th September 2013, Ghent, Belgium.

[2] **Asselman J**, Pfrender M, Lopez J, Shaw J, De Schamphelaere KAC. Unraveling mode of actions and toxin profiles with high throughput microarrays: a case study in Daphnia exposed to different cyanobacteria. EUROCORES Program Ecological and Evolutionary Functional Genomics Conference, 26-30 May 2013, Noorwijkerhout, the Netherlands.

[3] De Coninck D, **Asselman J**, Glaholt S, Janssen CR, Shaw JR, De Schamphelaere KAC. Are metal adapted *Daphnia pulex* populations better armed against future climate change stressors

than non-adapted populations? SETAC Europe 23rd Annual Meeting, 16-20 May 2013, Glasgow, UK.

[4] De Coninck D, **Asselman J**, Glaholt S, Colbourne J, Janssen CR, Shaw JR, De Schamphelaere KAC. Genomic mechanisms of cross-tolerance between cadmium and cyanobacterial stress in the waterflea. 18th National Symposium on Applied Biological Sciences, 8 Feb 2013, Gent, Belgium.

[5] **Asselman J**, De Laender F, Janssen CR, De Schamphelaere K. Increasing the number of design points does not necesserarily reduce the chance of falsely classifying additive mixtures as synergisms or antagonisms in mixture toxicity experiments. SETAC Europe 22nd Annual Meeting, 20-24 May, 2012, Berlin, Germany.

[6] **Asselman J**, Janssen CR, De Schamphelaere K.interacting effects of combined chemical and natural stressors on Daphnia pulex. SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[7] De Coninck D, Fisher M, Glaholt S, **Asselman J**, Janssen CR, Shaw J, De Schamphelaere KAC. Conseauences of metal adaptation on sensitivity to future climate change stressors in *Daphnia pulex* populations. SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[8] Hochmuth J, **Asselman J**, De Schamphelaere KAC. Do mixture effects of metal stress (Cu) and natural stress (cyanobacteria) add up in *Daphnia magna*. SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[9] Trenti IT, Vandegehuchte MV, **Asselman J**, Janssen C, De Meyer T, De Schanphelaere KAC. Is there a functional role of DNA methylation in the stress response? SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany.

[10] De Coninck D, **Asselman J**, Glaholt S, Colbourne J, Janssen CR, Shaw JR, De Schamphelaere KAC. Challenges in genomic analyses with *Daphnia pulex*: A case study on cyanobacterial stress. 2nd Nucleotide2Networks Symposium, 5 Sept 2012, Gent, Belgium.

[11] **Asselman J**, Smagghe G, Janssen CR, De Schamphelaere, KAC. Effects of climate change on risk assessments: toxicity of binary mixtures of harmful cyanobacteria and insecticides on Daphnia pulex. Poster presentation at the 63rd International Symposium on Crop Protection, May 24, 2011, Ghent, Belgium

[12] **Asselman J**, De Coninck D, Glaholt S, Colbourne JK, Janssen CR, Shaw JR, De Schamphelaere K. Comparison of the transcriptomic response of a Cd-sensitive and a Cd-tolerant *Daphnia pulex* isolate to cyanobacterial stress. SETAC Europe 21st Annual Meeting, 15-19 May 2011, Milan, Italy.

[13] **Asselman J**, De Baets B, De Schamphelaere KAC. microarrays in support vector regression to predict the toxicity of mixtures in the aquatic environment. Kick-Off meeting Nucleotide2Networks Symposium, 5 May 2011, Gent, Belgium

[14] **Asselman J**, Glaholt S, Smith Z, De Geyter E, Smagghe G, Janssen CR, Colbourne JK, Shaw JR, De Schamphelaere KAC. Functional characterization of four metallothionein genes in Daphnia pulex exposed to environmental stressors. Poster Presentation at SETAC North America 31th Annual Meeting, 7-11 November, 2010, Portland, USA.

[15] **Asselman J**, Glaholt S, Colbourne JK, Janssen CJ, Shaw JR, De Schamphelaere KAC. Metallothionein expression in Daphnia pulex under stress: functional characterization of a gene with high ecotoxicological relevance. Poster Presentation at the Daphnia Genomics Consortium Meeting, 26-30 March, 2010, Leuven, Belgium.

Foreign study visits

10 August 2012 - 15 December 2012: Research visit at the Shaw Lab and the Center for Genomics and Bioinformatics, Indiana University, Bloomington, Indiana, USA. Travel grant awarded by the Research Fund Flanders (FWO).

Attended conferences and workshops

[1] EMBO Conference on "The mighty daphnia: past present and future", 19-22 January 2014, Birmingham, UK.

[2] MDIBL-NERC Environmental Genomics Course, 17-18 January 2014, Birmingham, UK.

[3] Society of Environmental Toxicology and Chemistry - North America (SETAC-North America), 34th annual meeting, 17-21 November 2013, Nashville, TN, USA

[4] 3rd Nucleotide2Networks Symposium, 24th September 2013, Ghent, Belgium

[5] Artemia Genome Workshop, 2 September, Ghent, Belgium.

[6] International Conference on Toxic Cyanobacteria, 11-16 August, 2013, Pilaneseberg, South Africa

[7] EUROCORES Program Ecological and Evolutionary Functional Genomics Conference, 26-30 May 2013, Noorwijkerhout, the Netherlands.

[8] Society of Environmental Toxicology and Chemistry - Europe (SETAC-Europe), 23rd annual meeting, 16-20 May 2013, Glasgow, UK.

[9] 18th National Symposium on Applied Biological Sciences, 8 Feb 2013, Gent, Belgium.

[10] Society of Environmental Toxicology and Chemistry - Europe (SETAC-Europe), 22nd annual meeting, 20-24 May 2012, Berlin, Germany.

[11] Society of Environmental Toxicology and Chemistry - Europe (SETAC-Europe), 22nd annual meeting, 20-24 May 2012, Berlin Germany: Short course in Statistical methods in ecotoxicology using R.

[12] Pharmaceutical Bioinformatics course, Uppsala University, August-October 2011.

[13] Environmental Genomics Short Course, 20-27 August 2011, Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, USA. (Assiting staff)

[14] International Symposium on Crop Protection, 24 May, 2011, Ghent, Belgium

[15] Society of Environmental Toxicology and Chemistry - Europe (SETAC-Europe), 21th annual meeting, 15-19 May 2011, Milan, Italy.

[16] Kick-Off meeting Nucleotide2Networks Symposium, 5 May 2011, Gent, Belgium.

[17] Environmental Genomics Short Course, 21-29 August 2010, Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, USA.

[18] *Daphnia* Genomics Consortium Meeting, 26-30 March 2010, Leuven, Belgium.

Membership of professional organizations

2010 - present Member of the Society of Environmental Toxicology and Chemistry (SETAC)

2010 - present Member of the *Daphnia* Genomics Consortium

Awards and Prizes

Best Student Presentation, International Conference on Toxic Cyanobacteria, Pilanseberg, South Africa, 11-15 August 2013.

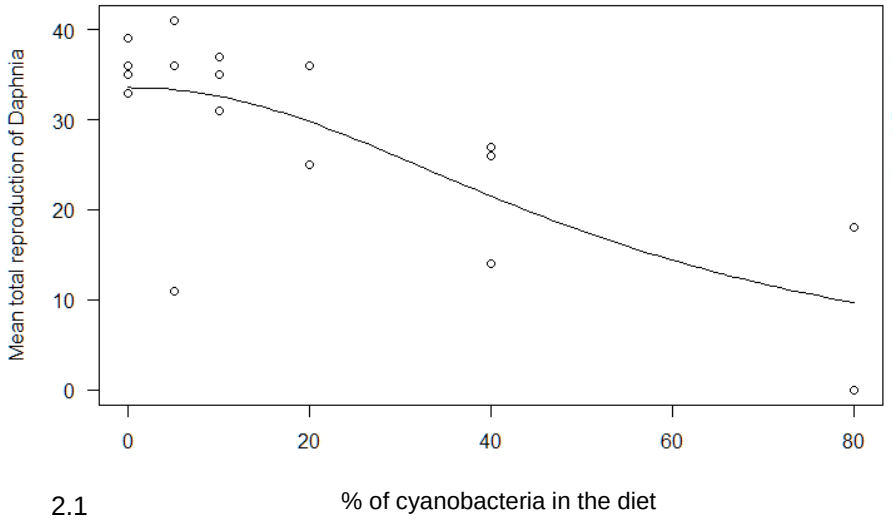
Best Poster Award, EUROCORES Program Ecological and Evolutionary Functional Genomics Conference 26-30 May 2013, Noorwijkerhout, the Netherlands.

Appendix A

Supplementary material for Chapter 2

A.1 Figures

1.1



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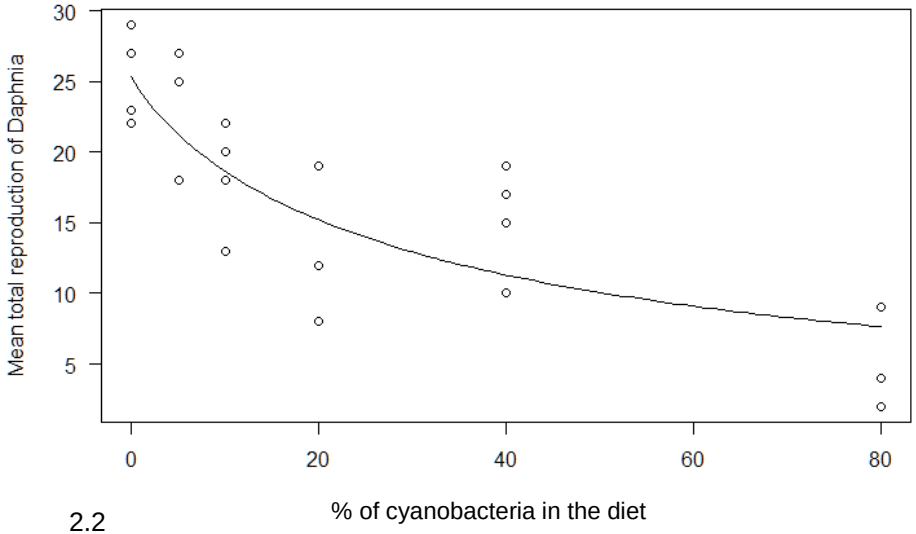

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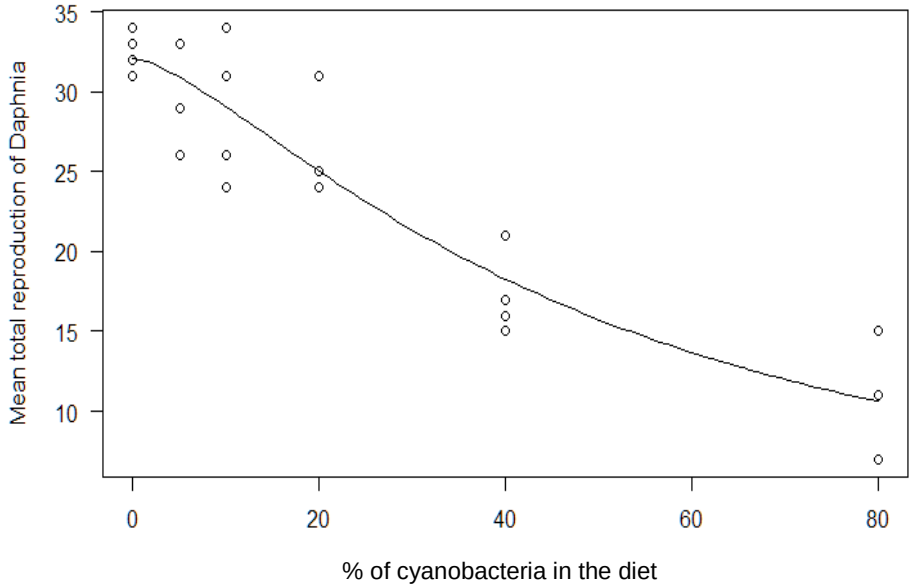

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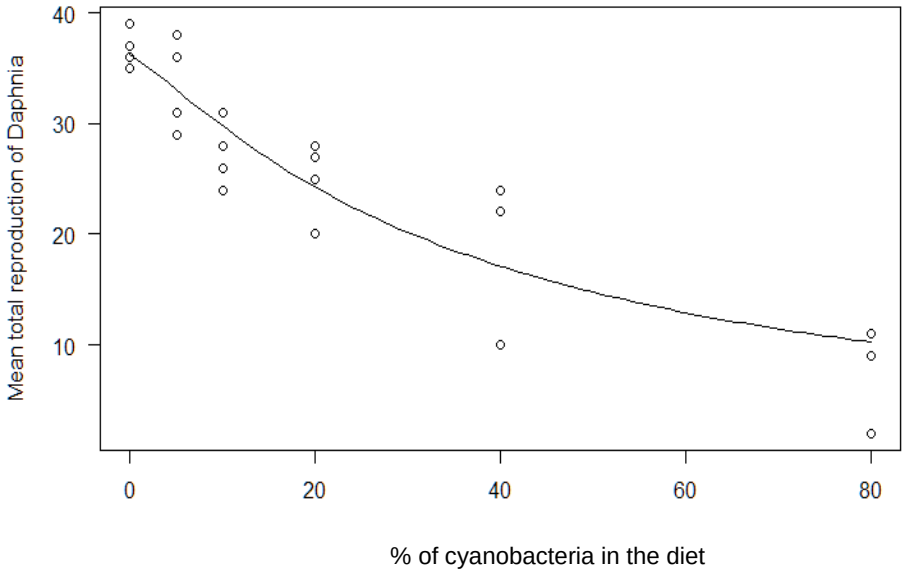

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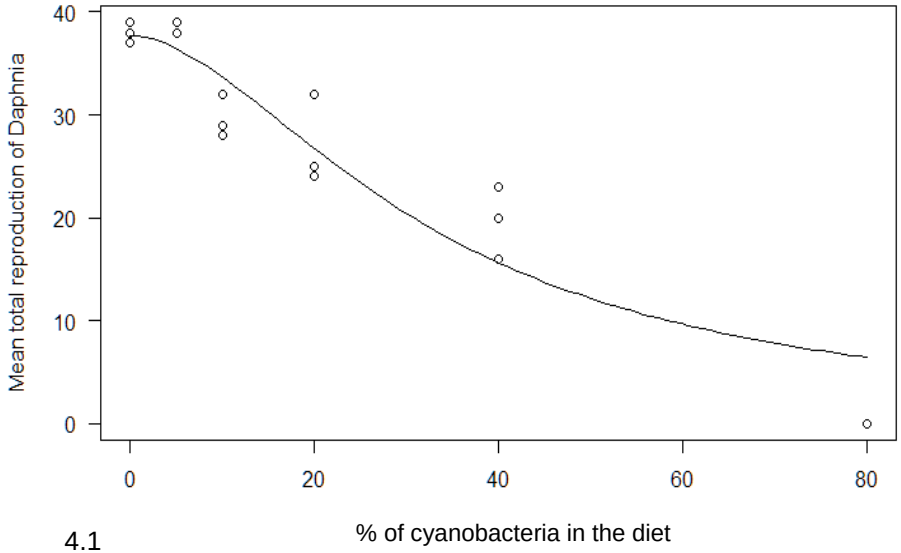
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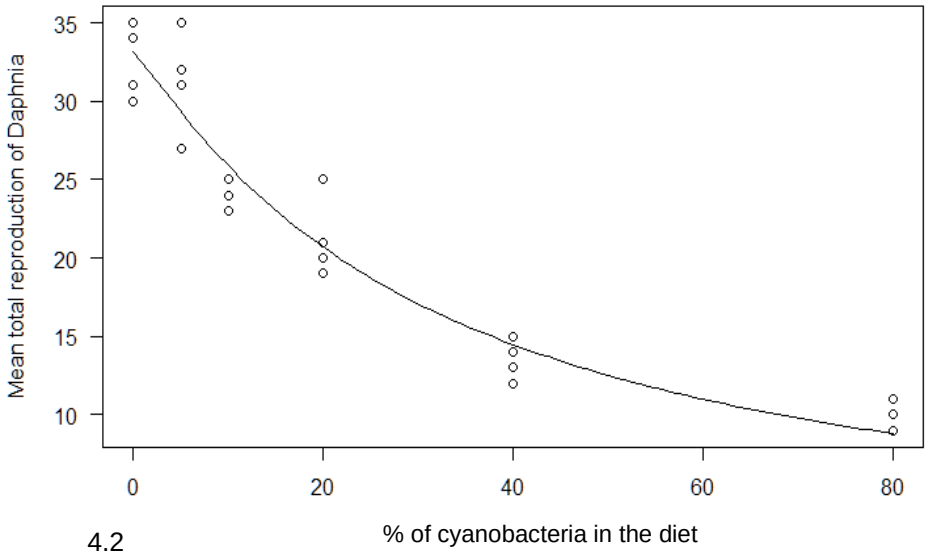
% of cyanobacteria in the diet

3.2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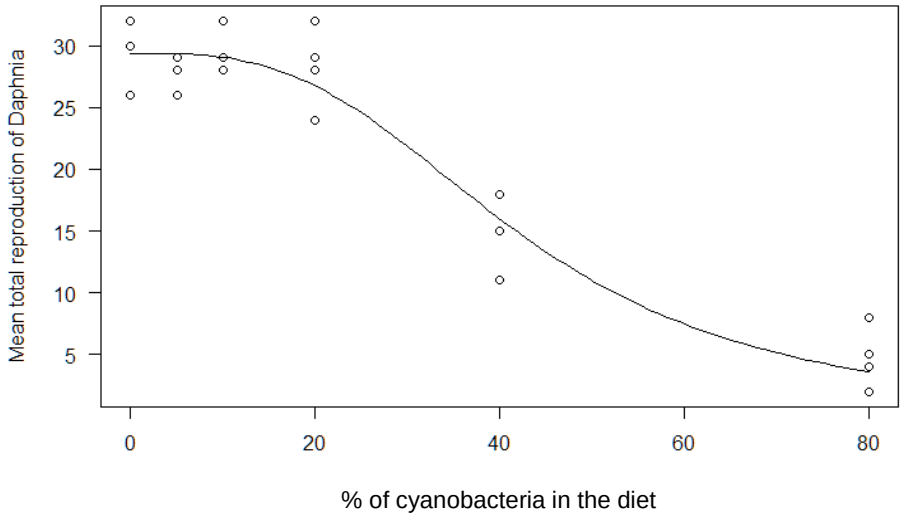
4.1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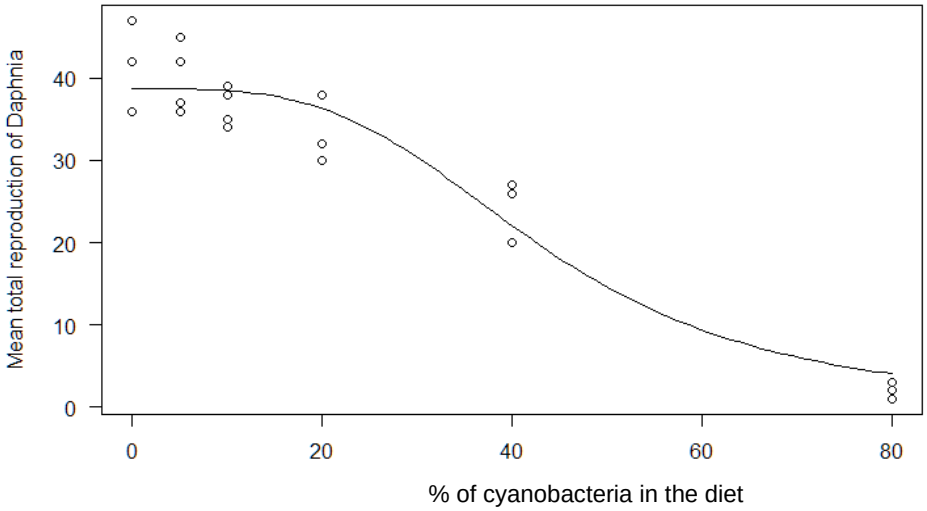
% of cyanobacteria in the diet

4.2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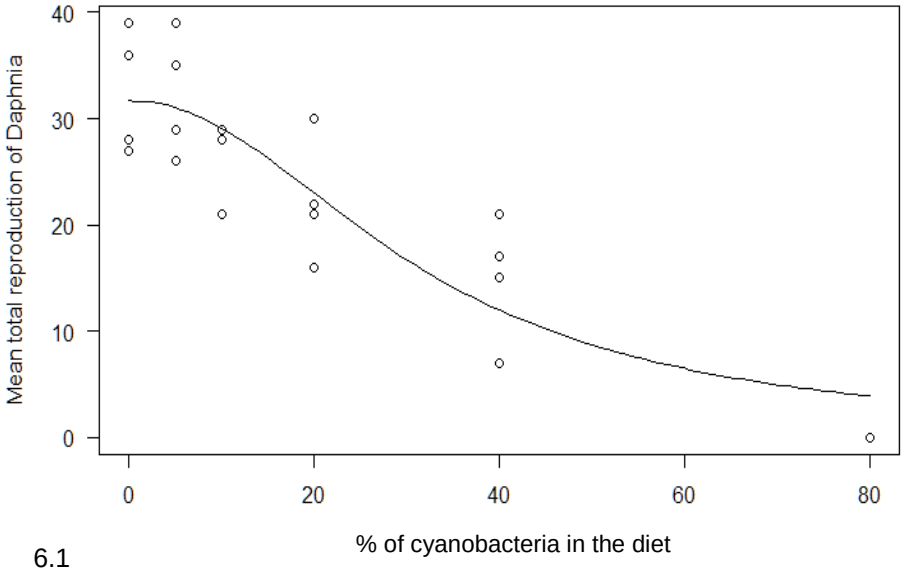
5.1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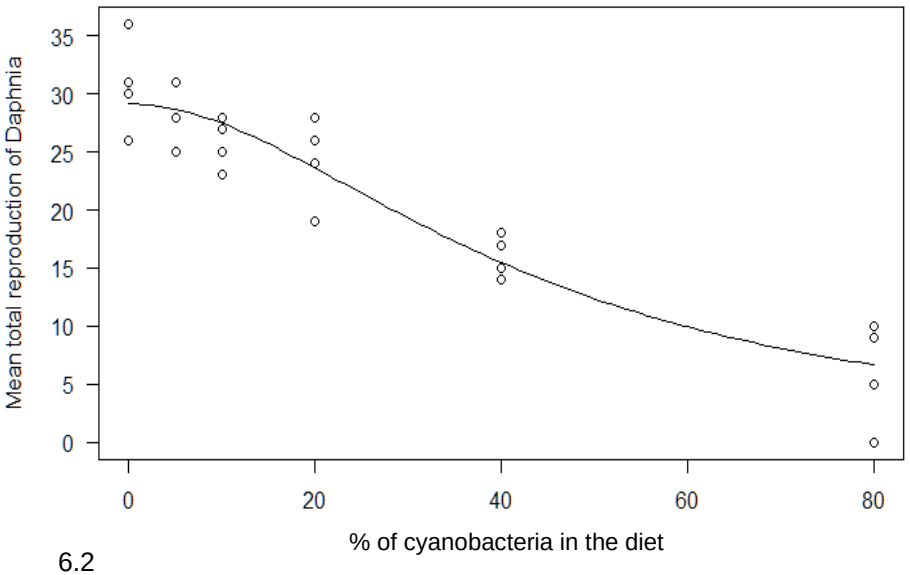
5.2


Mean total reproduction of Daphnia
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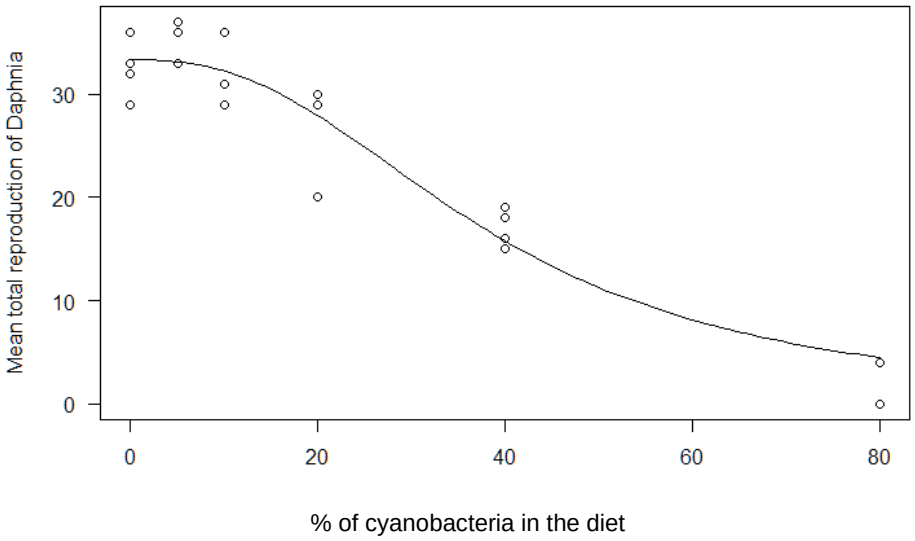
% of cyanobacteria in the diet

6.1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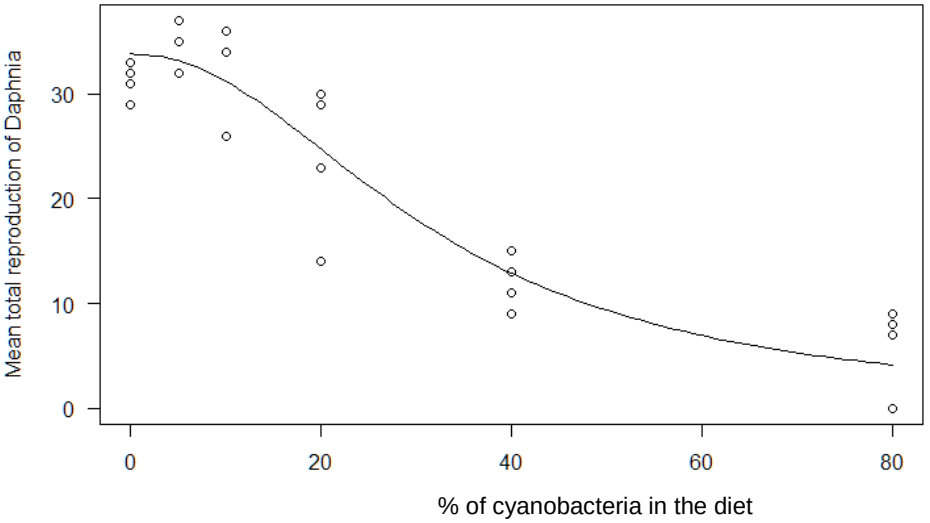
% of cyanobacteria in the diet

6.2


Mean total reproduction of Daphnia
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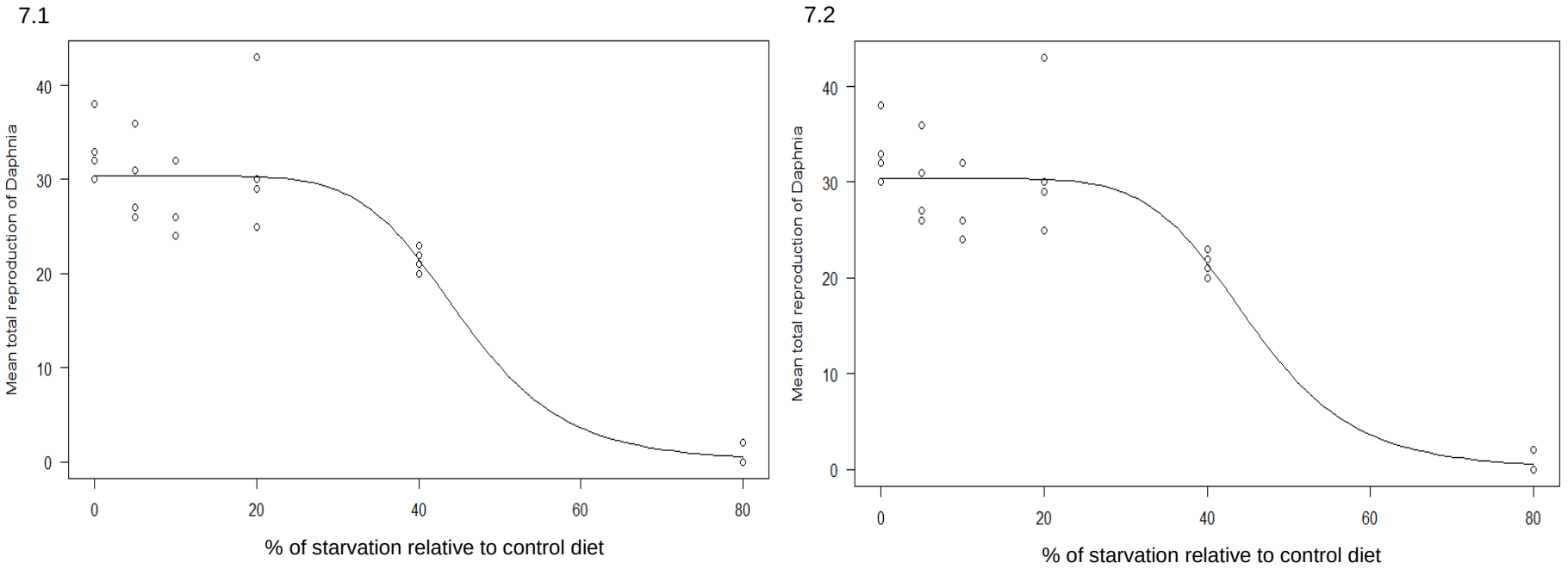


Figure A.1 Concentration reponse data (circles) and fitted concentration response curves for all cyanobacteria: *Anabaena* (1), *Aphanizonmenon* (2), *Cylindroserpmopsis* (3), *Microcystis* (4), *Nodularia* (5), *Oscillatoria* (6) and the starvation treatment (7). Numbers .1 and .2 denote repeated experiments. Circles represent observed total reproduction per organism for each treatment.

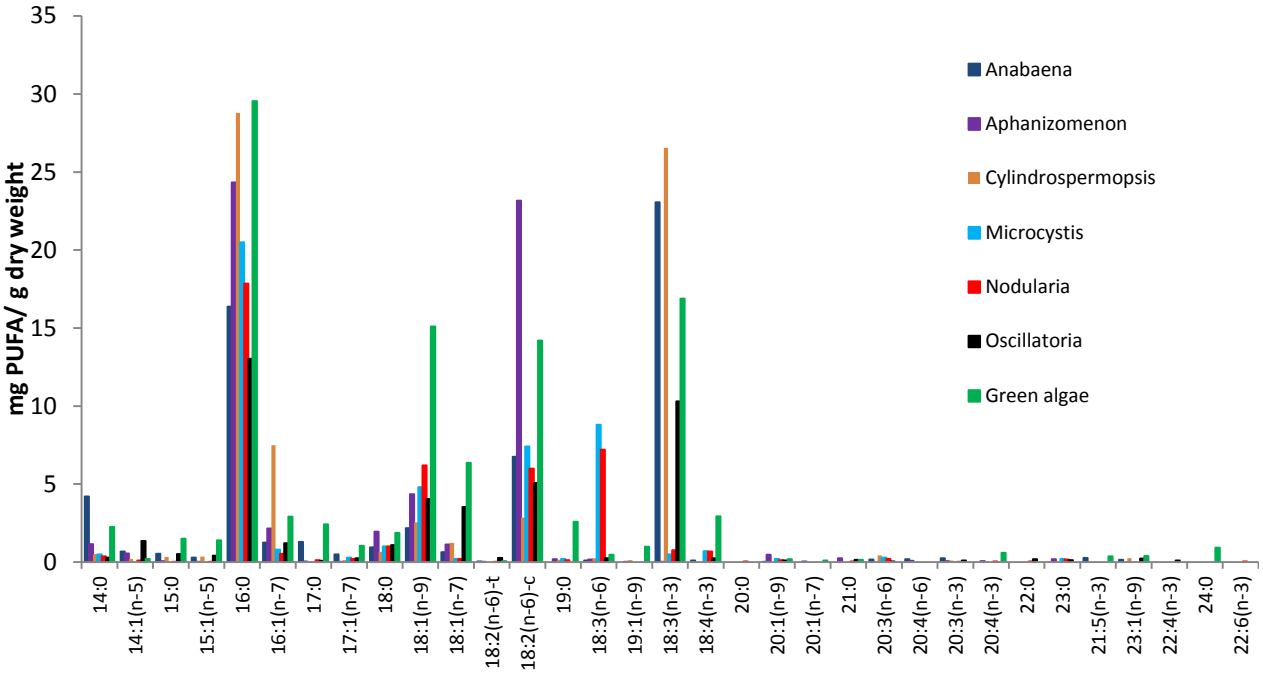


Figure A.3 Polyunsaturated fatty acid profile for all cell suspensions in milligram (mg) poly unsaturated fatty acid (PUFA) per gram (g) dry weight of the cell suspension.

A.2 Tables

Table A.1 Medium composition of $BG11_0$. All components are dissolved in 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.2 Medium composition of BG11. All components are dissolved in 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.3 Medium Composition of Z8. All components are dissolved in H_2O unless stated otherwise (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 fill up to one litre. Of this diluted combined Fe-solution and EDTA-solution 10mL is added per each L of Z8 medium.

Appendix B

Supplementary material for Chapter 3

B.1 Figures

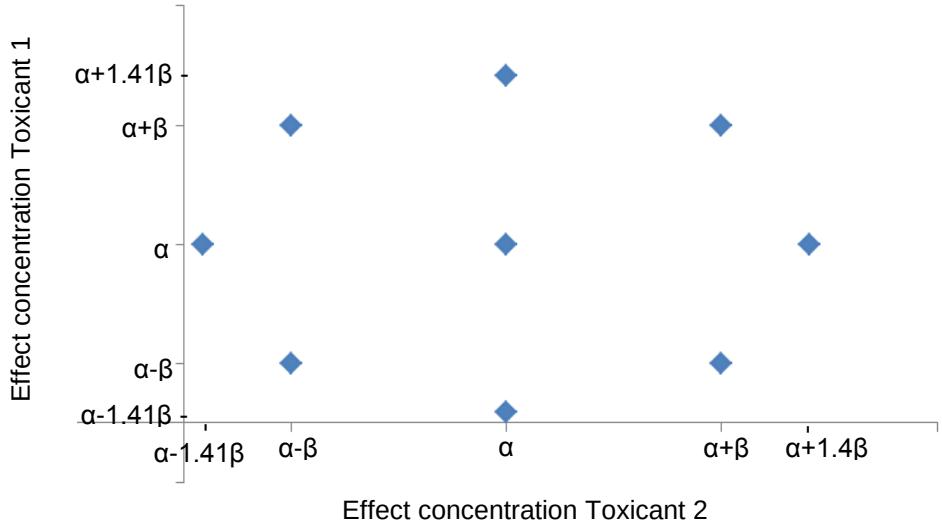


Figure B.1 Central composite design adapted from Lock and Janssen (2002). α = EC50/2 whereas β= EC50/2-EC10/2. EC50 is the effect concentration causing 50% decline in the monitored endpoint compared to control treatments. EC10 is the effect concentration causing 10% decline in the monitored endpoint compared to control treatments.

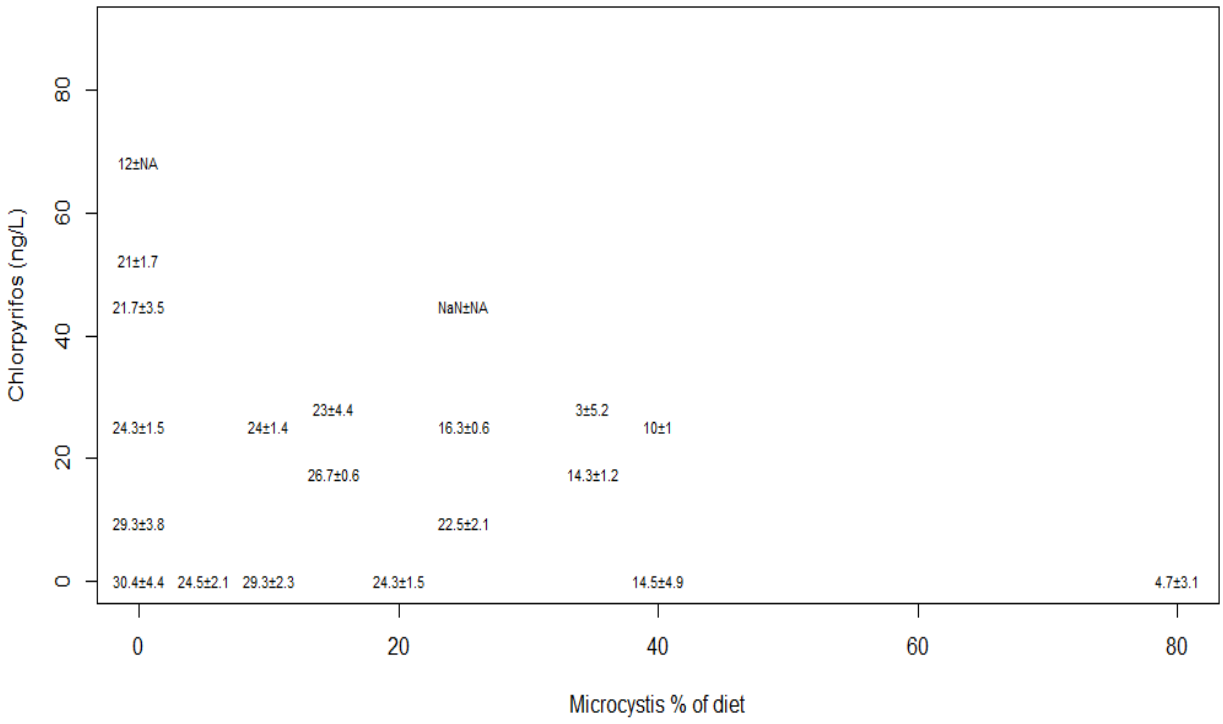


Figure B.2 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NaN means no animals survived the treatment. NA means no standard deviation could be computed due to less than two surviving replicates for that treatment.

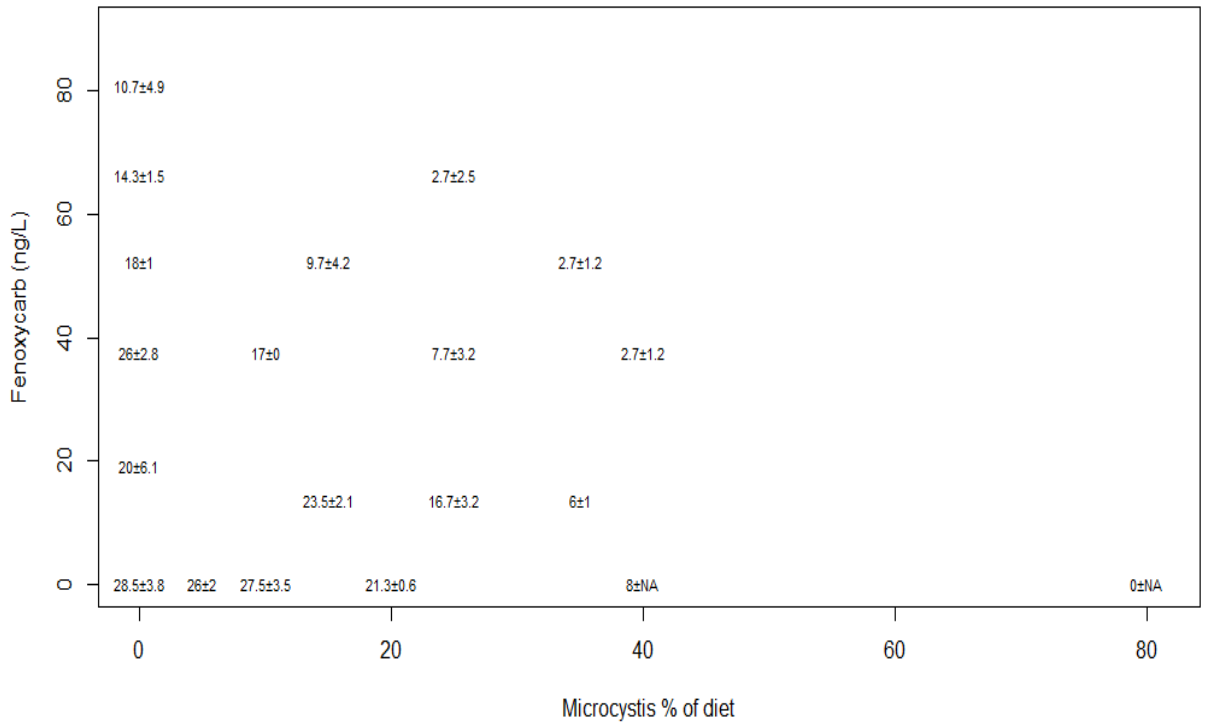


Figure B.3 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due to less than two surviving replicate for that treatment.

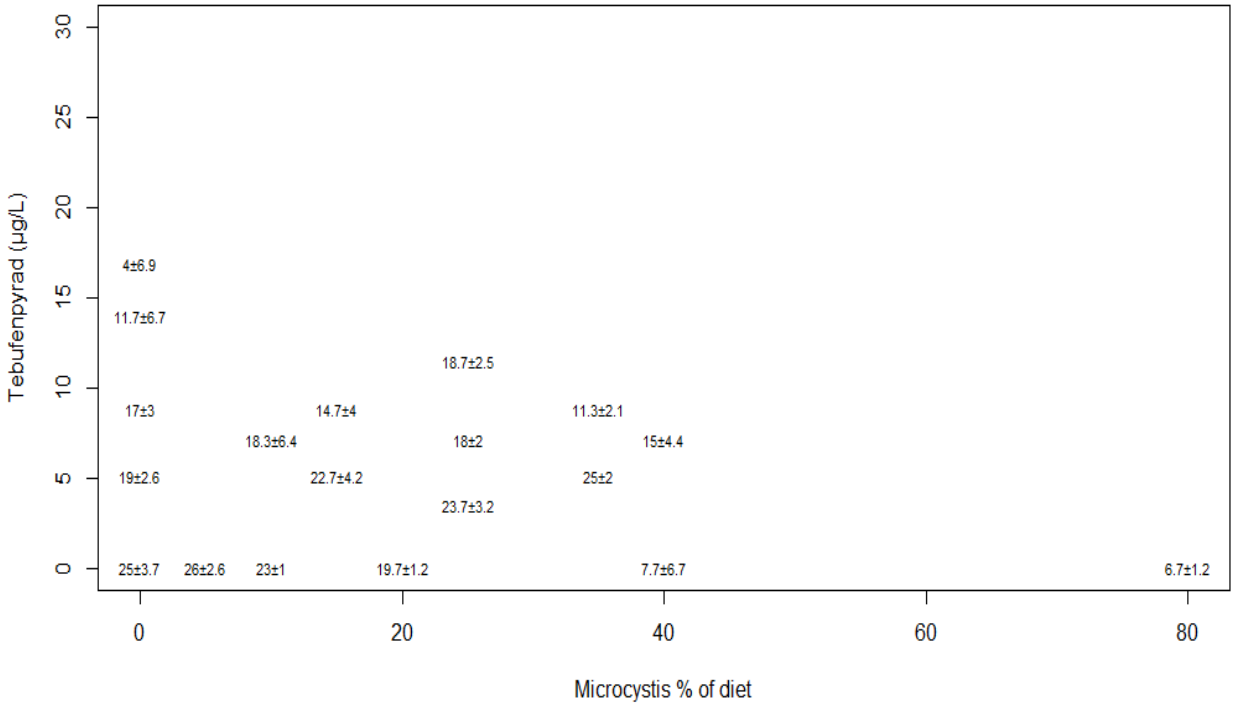


Figure B.4 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment.

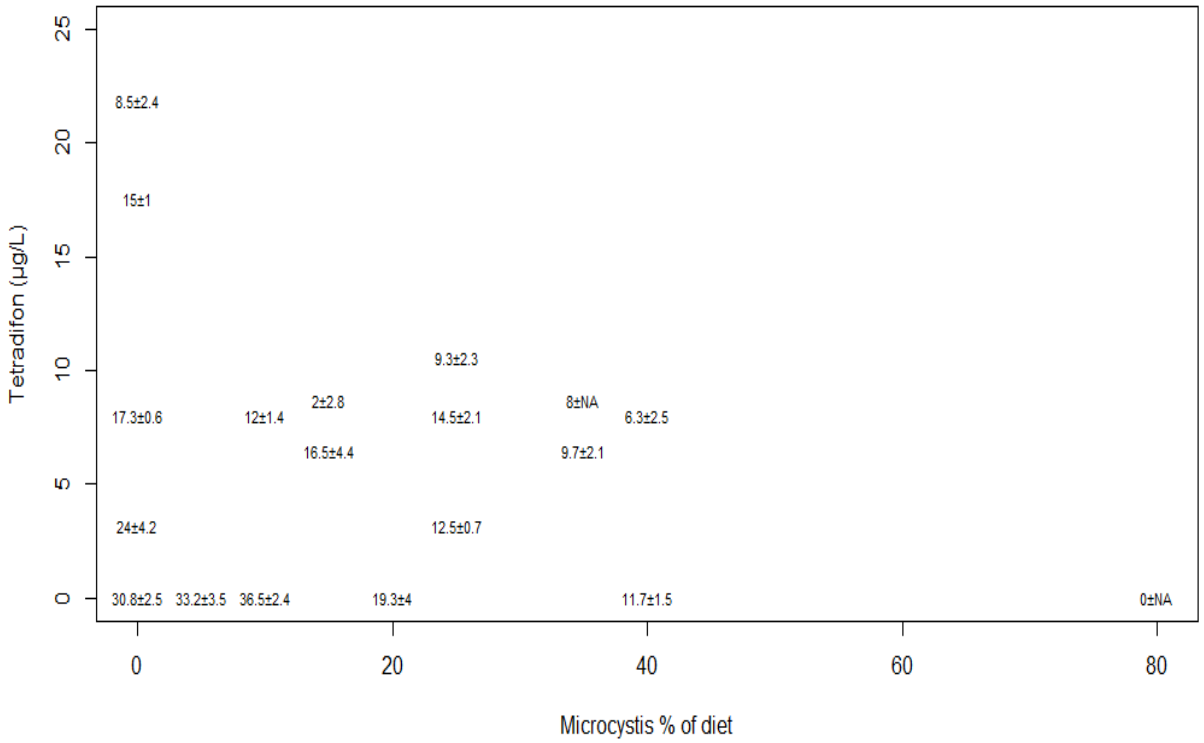


Figure B.5 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due less than two surviving replicates for that treatment.

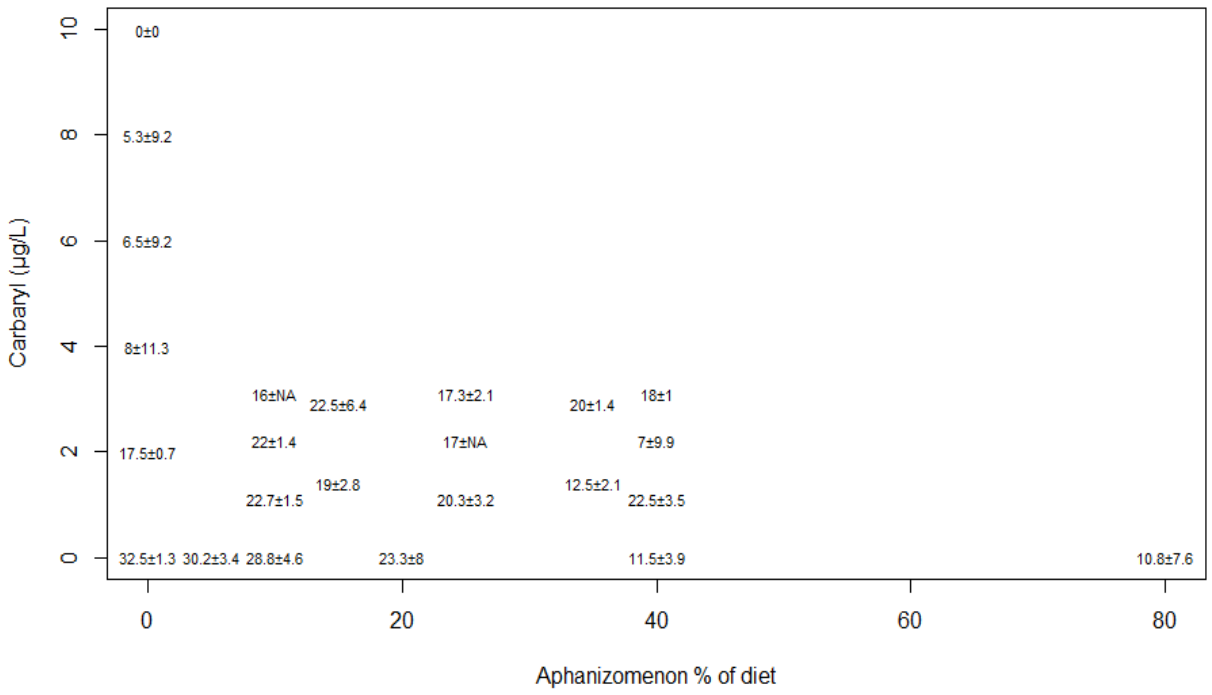


Figure B.6 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due to less than two surviving replicates for that treatment.

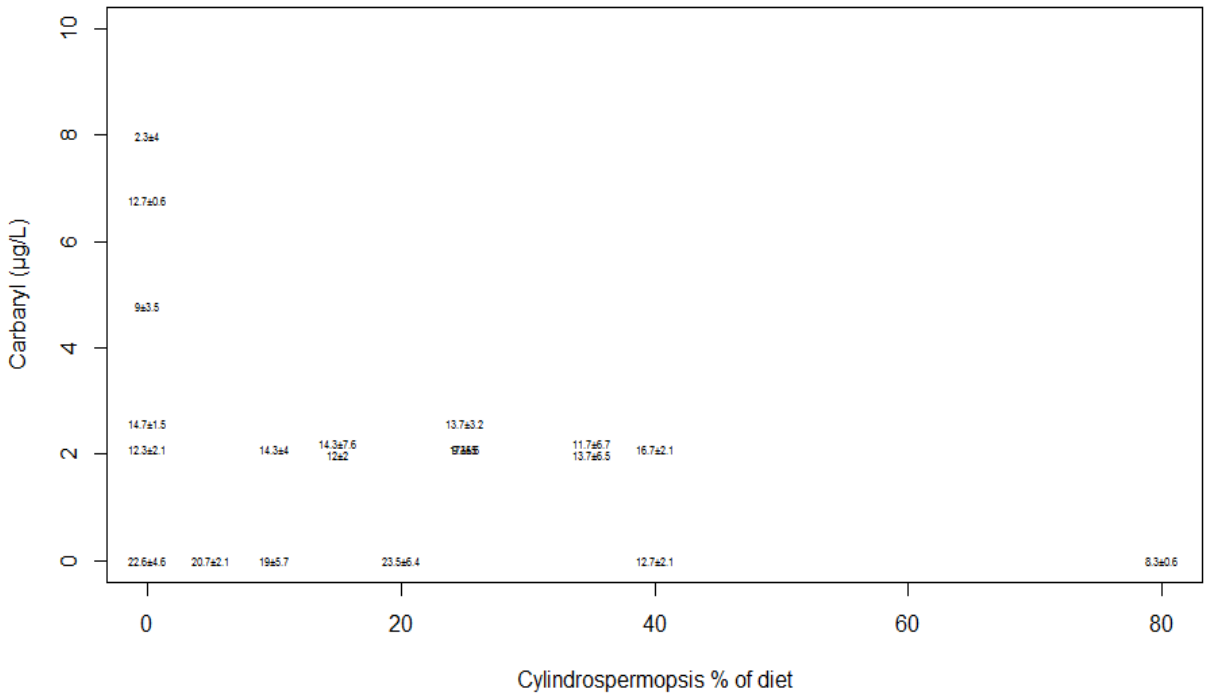


Figure B.7 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due to less than two surviving replicates for that treatment.

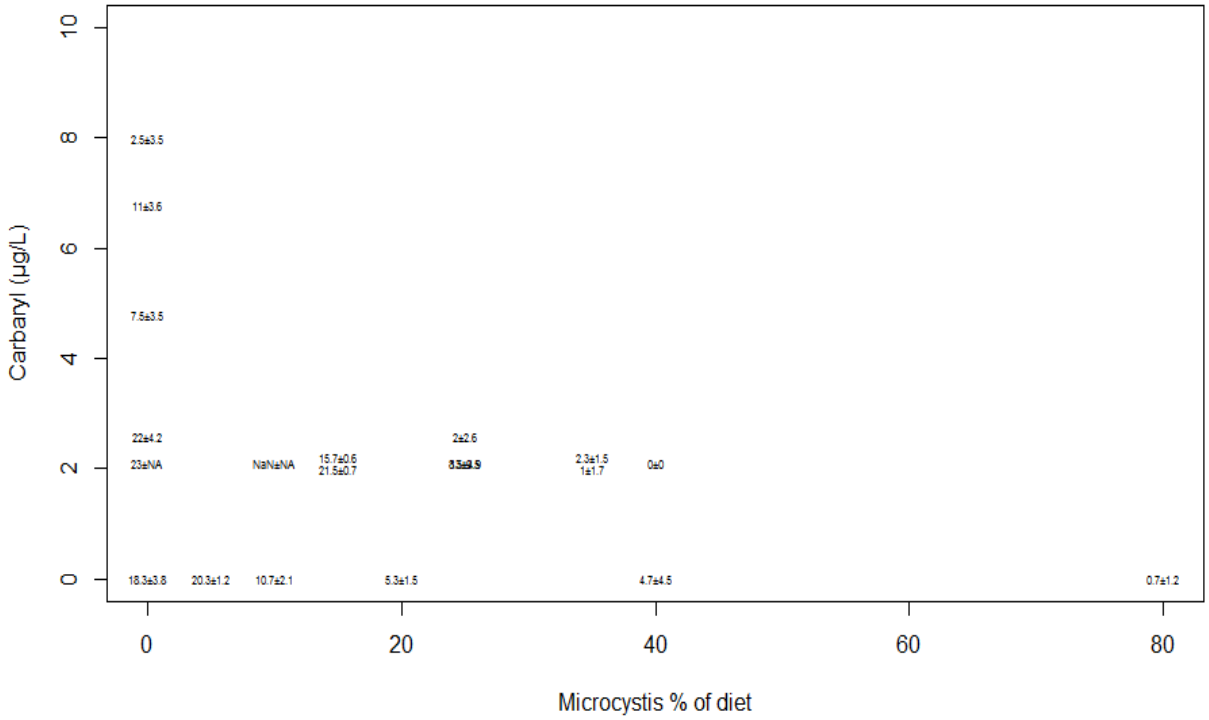


Figure B.8 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due to less than two surviving replicates for that treatment.

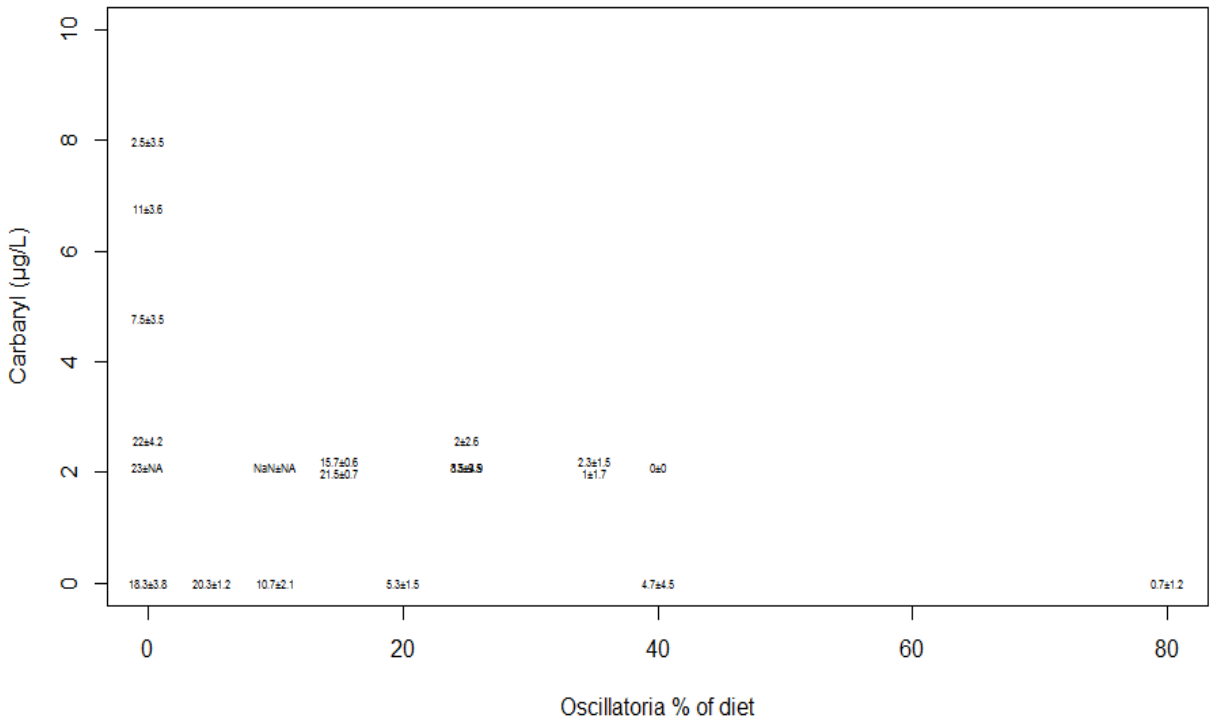


Figure B9 Mean total reproduction per surviving animal and the corresponding standard deviation per treatment. NA means no standard deviation could be computed due to less than two surviving replicates for that treatment.

B.2. Solid Phase Extractions (SPE) and Gas Chromatography (GC) procedures for insecticide concentration analysis:

B.2.1. General quality procedure

Blank	
Preparation:	Add 10 mL of culture medium (COMBO) to sample tube
SPE and GC:	Follow procedures as if it was a sample
Spike:	
Preparation:	Add 10 mL of culture medium (COMBO) to sample tube
	Add 15 µL of a 10 mg/L solution of the insecticide to be tested
SPE and GC	Follow procedures as if it was a sample

B.2.2: Acetamiprid

Type SPE Column:	100mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (Propoxur): 10 µL of 10 mg/L solution
SPE:	Condition by adding 3 ml MTBE on column
	Rinse by adding 3 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 3 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 3 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.3: Carbaryl

Type SPE Column:	30mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (Propoxur): 8 µL of 15 mg/L solution
SPE:	Condition by adding 1 ml MTBE on column
	Rinse by adding 1 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 1 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 1 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.4: Chlorpyrifos

Type SPE Column:	100mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (fenthion): 10 µL of 0.5 mg/L solution
SPE:	Condition by adding 3 ml MTBE on column
	Rinse by adding 3 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 3 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 5 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.5: Deltamethrin

Type SPE Column:	100mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (mirex): 10 µL of 0.5 mg/L solution
SPE:	Condition by adding 3 ml MTBE on column
	Rinse by adding 3 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 3 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 5 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.6: Endosulfan

Type SPE Column:	30mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (mirex): 10 µL of 15 mg/L solution
SPE:	Condition by adding 1 ml MTBE on column
	Rinse by adding 1 ml MeOH on column
	Equilibrate by filling column completely with H2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 2 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 1 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.7: Fenoxycarb

Type SPE Column:	100mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (propoxur): 10 µL of 2 mg/L solution
SPE:	Condition by adding 3 ml MTBE on column
	Rinse by adding 3 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 3 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 6 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.8: Tebufenpyrad

Type SPE Column:	30mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (propoxur): 10 µL of 10 mg/L solution
SPE:	Condition by adding 1 ml MTBE on column
	Rinse by adding 1 ml MeOH on column
	Equilibrate by filling column completely with H2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 2 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 1 ml MTBE
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.2.9: Tetradifon

Type SPE Column:	30mg/3mL
Preparation:	Weigh volume of sample
	Add internal standard (fention): 10 µL of 10 mg/L solution
SPE:	Condition by adding 3 ml MTBE on column
	Rinse by adding 3 ml MeOH on column
	Equilibrate by filling column completely with H_2O
	Add sample on column
	Rinse sample bottles with MeOH/H_2O 5/95
	Rinse column with 3 mL MeOH/H_2O 5/95 and dry 5 minutes under vacuum
	Place elution tubes under columns and elute with 3 ml MeOH/MTBE 10/90
GC Preparation:	Add MTBE to elution tubes to a final volume of 1 mL
	Remove H_2O by adding Na_2SO_4
	Add 750 µL from elution tube to a GC tube
	Add 7.5 µL of 10 mg/L reference standard
	Weigh empty sample bottle
	Store GC tubes in the dark at 4°C until GC analysis

B.3 R-code:

```
# Load in necessary libraries

library(nortest)

library(drc)

library(lattice)

### Data input ### File Input example below

Data<-read.table("Datafile.txt", header=TRUE, na.strings=NA,sep="\t",dec=".")

Datamean<-read.table("Datafile.txt",header=FALSE,na.strings=NA,sep="\t",dec=".")

### INDEPENDENT ACTION MODEL = IA ####

### CONCENTRATION ADDITION MODEL = CA ###

# Control observation are excluded as they will result in NAs

# E.g. dividing by 0 as for ((Insecticide/EI)+(Cyano/EC))^(-2)

# both Insecticide and Cyano are 0 in control terms, resulting in a final zero in denominator

# Although this is only for the deviation models, we still do is for standard model as well

# Otherwise the deviation and standard model will be fitted to other datasets

# Then statistical comparison is not so straightforward.

# Fit model only to single stressor data, no mixture data included

ModelIAsingle<-nls(Daphnia ~ mean(Data[1:19,3])*1/((1+(Insecticide/EI)^BI)*(1+(Cyano/EC)^BC)),

    data = Data[20:49,], start= list(BI= 1, BC=1,EI= 4, EC = 50),

    trace=TRUE, na.action=na.omit)

ModelCAsingle<-nls(Daphnia~ (mean(Data[1:19,3]))/(((Insecticide*EC+Cyano*EI)/(EI*EC))^B+1),

     data = Data[20:49,], start= list(B= 1, EI= 4, EC = 50),

     trace=TRUE, na.action=na.omit)

# Fit standard model to all data including mixtures

ModelIA<-nls(Daphnia~ mean(Data[1:19,3])*1/((1+(Insecticide/EI)^BI)*(1+(Cyano/EC)^BC)),

    data = Data[20:76,], start= list(BI= 1, BC=1,EI= 4, EC = 50),trace=TRUE, na.action=na.omit)

ModelCA<-nls(Daphnia~ (mean(Data[1:19,3]))/(((Insecticide*EC+Cyano*EI)/(EI*EC))^B+1),

          data = Data[20:76,], start= list(B= 1, EI= 4, EC = 50), trace=TRUE, na.action=na.omit)

# Synergism-Antagonism Model

ModelIAS<-   nls(Daphnia~mean(Data[1:19,3])*pnorm(qnorm(1/((1+(Insecticide/EI)^BI)*(1+(Cyano/EC)^BC)))+
              (a*(Insecticide/EI)*( Cyano/EC)*((Insecticide/EI)+(Cyano/EC))^(-2))), data = Data[20:76,], start=
              list(BI= 0.8, BC=2,EI= 4.15, EC = 65.90, a=0), trace=TRUE, na.action=na.omit)

ModelCAS<-nls(Daphnia~   (mean(Data[1:19,3]))/((((Insecticide*EC+Cyano*EI)/(EI*EC))/exp(a*(Insecticide/EI)*
            (Cyano/EC)*((Insecticide/EI)+(Cyano/EC))^(-2)))^B+1), data = Data[20:76,], start= list(B= 1, EI= 4,
            EC = 50, a=0), trace=TRUE, na.action=na.omit)
```

```
#### Comparing Standard model with Deviation model based on F-statistic
anova(ModelIA, ModelIAS)
anova(ModelCA, ModelCAS)
## Verifying assumptions for F-statistic
# Assumption of Normality of Residuals
shapiro.test(residuals(ModelIA))
shapiro.test(residuals(ModelIAS))
shapiro.test(residuals(ModelCA))
shapiro.test(residuals(ModelCAS))
# Assumption of Homoscedasticity of Residuals
concrange<-na.omit(Data[20:76,])
concrange<-concrange[,1]+concrange[,2]
leveneTest(residuals(ModelIactor(concrange) )
leveneTest(residuals(ModelIAS),as.factor(concrange) )
concrange<-na.omit(Data[20:76,])
concrange<-concrange[,1]+concrange[,2]
# Make model predictions for mixture concentrations based on model developped on single stressor data
PredictIAsingle<-predict(ModelIAsingle, Data[50:76,1:2])
# Plot mean model predictions versus observed mean mixture data
plot(Datamean[1:10,],unique(fitted(ModelIAsingle)),
  xlab='data', ylab='fitted values')
lines(Datamean[11:19,],unique(PredictIAsingle), type="p", pch=19)
abline(0,1)
# Plot all model predictions versus all observed mixture data
plot(na.omit(Data[20:49,3]),fitted(ModelIAsingle),
```

Example of data input:

Cyano(% of cyanobacteria in the diet)	Insecticide (Insecticide concentration	Daphnia (Total reproduction)
0	1	30
20	2	25

B.4: Tables

Table B.1 P-values for concentration addition (CA)/ independent action (IA) when leaving one design point out. Insecticide concentrations are represented as effect concentrations (EC) based upon the general central composite design in Fig. S1. Cyanobacteria concentrations are given in % of the diet. α = EC50/2 whereas β= EC50/2-EC10/2. EC50 is the effect concentration causing 50% decline in the monitored endpoint compared to control treatments. EC10 is the effect concentration causing 10% decline in the monitored endpoint compared to control treatments.

Design points		P-values							
Insecticide EC (Fig. S1)	Cyano (% of diet) (Fig.1-4)	Chlorpyrifos x *Microcystis*	Fenoxycarb x *Microcystis*	Tebufenpyrad x *Microcystis*	Tetradifon x *Microcystis*	Carbaryl x *Microcystis*	Carbaryl x Aphanizomenon	Carbaryl x Cylindroserpmopsis	Carbaryl x Oscillatoria
α - 1.41β	25	0.136/0.266	0.488/0.003	<0.001/<0.001	0.312/0.560	0.014/<0.001	0.013/0.006	0.126/0.136	<0.001/<0.001
α - β	15	0.104/0.430	0.638/0.002	<0.001/<0.001	0.314/0.461	0.045/<0.001	0.009/0.003	0.102/0.050	<0.001/<0.001
α - β	35	0.099/0.298	0.663/0.002	0.001/<0.001	0.211/0.449	0.015/<0.001	0.007/0.002	0.111/0.081	<0.001/<0.001
α	10	0.115/0.271	0.435/0.003	<0.001/<0.001	0.378/0.590	0.012/<0.001	0.013/0.007	0.105/.069	<0.001/<0.001
α	25	0.131/0.363	0.487/0.003	<0.001/<0.001	0.158/0.318	0.007/<0.001	0.012/0.004	0.057/0.214	<0.001/<0.001
α	40	0.087/0.342	0.503/0.002	<0.001/<0.001	0.263/0.512	0.015/<0.001	0.003/0.001	0.279/0.181	<0.001/<0.001
α + β	15	0.064/0.564	0.542/0.009	<0.001/<0.001	0.788/0.963	0.019/<0.001	0.017/0.008	0.088/0.063	<0.001/<0.001
α + β	35	0.603/0.135	0.491/0.003	<0.001/<0.001	0.281/0.479	0.015/<0.001	0.036/0.011	0.078/0.051	<0.001/<0.001
α + 1.41β	25	0.103/0.285	0.498/0.003	<0.001/<0.001	0.267/0.439	0.004/<0.001	0.037/0.010	0.099/0.070	0.004/<0.001

Appendix C

Supplementary material for Chapter 4

C.1 Tables

Table C.1 Results of the two-way analysis on log transformed total reproduction. For each effect, the Benjamini-Hochberg corrected p-value is represented. Interaction effects with a p-value smaller than 0.05 are represented in bold italic. The color code gives a visual indication of the interaction effect: the darker the green or red, the more antagonistic or synergistic the combination, the lighter the closer to non-interaction.

Insecticide	Cyanobacteria	Insecticide Effect	Cyanobacteria Effect	Interaction Effect	Log deviation
Acetamiprid	*Anabaena*	8.93 e-03	2.84 e-08	1.11 e-01	0.15
Carbaryl	*Anabaena*	1.06 e-05	3.96 e-04	3.20 e-01	0.14
Chlorpyrifos	*Anabaena*	1.92 e-02	1.34 e-06	9.38 e-01	0.01
Deltamethrin	*Anabaena*	4.20 e-03	2.27 e-05	6.58 e-01	0.05
Endosulfan	*Anabaena*	1.60 e-07	2.58 e-05	8.90 e-02	0.23
Fenoxycarb	*Anabaena*	1.19 e-04	1.31 e-06	4.24 e-01	0.10
Tebufenpyrad	*Anabaena*	2.66 e-05	6.42 e-08	5.92 e-01	0.06
Tetradifon	*Anabaena*	2.26 e-06	1.62 e-04	2.72 e-01	0.15
Acetamiprid	*Aphanizomenon*	3.61 e-01	1.76 e-13	***9.44 e-05***	0.65
Carbaryl	*Aphanizomenon*	1.48 e-08	6.12 e-15	***3.36 e-02***	0.24
Chlorpyrifos	*Aphanizomenon*	5.78 e-01	1.71 e-15	***1.14 e-06***	0.51
Deltamethrin	*Aphanizomenon*	4.04 e-07	4.12 e-14	***1.22 e-09***	0.79
Endosulfan	*Aphanizomenon*	3.43 e-08	4.83 e-07	***6.56 e-07***	1.35
Fenoxycarb	*Aphanizomenon*	1.89 e-01	2.55 e-14	***6.56 e-07***	0.79
Tebufenpyrad	*Aphanizomenon*	9.52 e-04	1.79 e-11	***6.56 e-07***	0.97
Tetradifon	*Aphanizomenon*	9.62 e-09	1.03 e-10	***4.38 e-03***	0.47
Acetamiprid	*Cylindrospermopsis*	8.96 e-05	1.80 e-07	7.62 e-01	0.02
Carbaryl	*Cylindrospermopsis*	2.25 e-02	1.92 e-09	2.84 e-01	0.09
Chlorpyrifos	*Cylindrospermopsis*	2.08 e-01	4.41e-08	1.48 e-02	0.23
Deltamethrin	*Cylindrospermopsis*	1.69 e-02	1.80 e-08	***3.36 e-02***	0.19
Endosulfan	*Cylindrospermopsis*	2.04 e-09	2.00 e-07	1.89 e-01	-0.13
Fenoxycarb	*Cylindrospermopsis*	1.40 e-05	3.42 e-11	8.43 e-02	-0.11
Tebufenpyrad	*Cylindrospermopsis*	2.34 e-08	7.85 e-09	***3.36 e-02***	-0.19
Tetradifon	*Cylindrospermopsis*	1.14 e-05	2.09 e-08	2.47 e-01	0.11
Acetamiprid	*Microcystis*	8.40 e-04	7.49 e-15	2.19 e-01	-0.21
Carbaryl	*Microcystis*	2.03 e-01	1.37 e-13	5.08 e-02	0.66
Chlorpyrifos	*Microcystis*	1.34 e-01	6.12 e-15	***3.76 e-02***	0.60
Deltamethrin	*Microcystis*	7.49 e-01	1.20 e-15	9.58 e-02	0.41
Endosulfan	*Microcystis*	4.04 e-07	1.46 e-12	6.51 e-01	-0.09
Fenoxycarb	*Microcystis*	8.94 e-03	1.20 e-15	5.92 e-01	-0.06

Insecticide *(Table C.1 cont.)*	Cyanobacteria	Insecticide Effect	Cyanobacteria Effect	Interaction Effect	Log deviation
Tebufenpyrad	*Microcystis*	7.33 e-02	1.20 e-15	***3.68 e-02***	0.31
Tetradifon	*Microcystis*	1.19 e-04	1.20 e-15	5.92 e-01	0.13
Acetamiprid	*Nodularia*	3.19 e-02	1.20 e-15	***3.36 e-02***	0.16
Carbaryl	*Nodularia*	9.32 e-03	6.17 e-15	2.27 e-01	0.14
Chlorpyrifos	*Nodularia*	9.99 e-02	6.51 e-14	1.89 e-01	0.17
Deltamethrin	*Nodularia*	9.49 e-02	4.02 e-15	5.89 e-01	0.06
Endosulfan	*Nodularia*	2.79 e-07	1.20 e-15	***2.54 e-02***	0.16
Fenoxycarb	*Nodularia*	1.40 e-02	1.20 e-15	1.89 e-01	0.10
Tebufenpyrad	*Nodularia*	2.38 e-04	1.20 e-15	***3.36 e-02***	0.16
Tetradifon	*Nodularia*	5.09 e-02	2.75 e-14	1.05 e-01	0.20
Acetamiprid	*Oscillatoria*	1.66 e-02	6.48 e-14	1.16 e-01	0.13
Carbaryl	*Oscillatoria*	6.93 e-02	2.22 e-11	2.06 e-01	0.10
Chlorpyrifos	*Oscillatoria*	1.09 e-01	3.18 e-12	8.43 e-02	0.13
Deltamethrin	*Oscillatoria*	1.37 e-02	4.70 e-12	4.60 e-01	0.06
Endosulfan	*Oscillatoria*	9.55 e-11	1.49 e-13	***1.94 e-04***	0.31
Fenoxycarb	*Oscillatoria*	1.45 e-01	1.59 e-14	***3.58 e-04***	0.27
Tebufenpyrad	*Oscillatoria*	1.43 e-06	6.17 e-15	5.57 e-01	0.05
Tetradifon	*Oscillatoria*	3.43 e-08	2.07 e-12	5.05 e-01	0.05

Table C.2 Estimated value and standard error for each parameter of equation 2.1, i.e. maximum response k, median effect concentration (EC50) and slope parameter s, for the concentration response data after fourteen days of exposure represented per cyanobacteria. Numbers denote repeated experiments.

	Maximum response k	EC50	Slope parameter s
Anabaena 1	19.89 ± 3.56	27.72 ± 17.38	1.00 ± 0.68
Anabaena 2	14.61 ± 1.15	63.89 ± 12.94	5.51 ± 4.51
Aphanizomenon 1	10.82 ± 1.10	94.34 ± 48.91	1.16 ± 0.64
Aphanizomenon 2	20.69 ± 3.39	42.66 ± 28.944	0.65 ± 0.46
Cylindrospermopsis 1	9.44 ± 1.22	19.15 ± 5.65	2.54 ± 1.08
Cylindrospermopsis 2	15.89 ± 1.76	49.20 ± 11.10	2.42 ± 2.17
Microcystis 1	13.42 ± 0.76	51.96 ± 7.45	6.28 ± 3.21
Microcystis 2	17.81 ± 1.18	50.89 ± 40.98	12.57 ± 4.35
Nodularia 1	9.98 ± 1.41	27.43 ± 10.34	1.78 ± 0.79
Nodularia 2	11.56 ± 1.14	43.44 ± 41.46	15.23 ± 7.43
Oscillatoria 1	18.39 ± 1.62	33.09 ± 6.75	2.84 ± 1.20
Oscillatoria 2	11.17 ± 1.15	21.21 ± 17.63	15.75 ± 12.39
Starvation 1	10.43 ± 0.89	46.31 ± 37.49	10.08 ± 5.87
Starvation 2	20.63 ± 1.52	51.06 ± 8.71	6.04 ± 4.32

Table C.3 Benjamini-Hochberg corrected p-values for pairwise correlation between deviations parameters for all insecticides. P-values smaller than 0.05 are represented in bold italic. Chlor=chlorpyrifos, Del=deltamethrin, Teb=tebufenpyrad, Tetra=tetradifon.

	Acetamiprid	Carbaryl	Chlor	Del	Endosulfan	Fenoxycarb	Teb	Tetra
Acetamiprid		0.54	0.96	0.45	0.06	0.08	0.23	0.52
Carbaryl	0.54		0.13	0.52	0.86	0.86	0.66	0.95
Chlor	0.96	0.13		0.12	0.73	0.73	0.29	0.53
Del	0.45	0.52	0.12		0.23	0.27	0.09	0.11
Endosulfan	0.06	0.86	0.73	0.23		***0.004***	0.09	0.09
Fenoxycarb	0.08	0.86	0.73	0.27	***0.004***		0.11	0.12
Teb	0.23	0.66	0.29	0.09	0.09	0.11		0.09
Tetra	0.52	0.95	0.53	0.11	0.09	0.12	0.09	

Table C.4 Benjamini-Hochberg corrected p-values for pairwise correlation between deviations parameters for all insecticides. P-values smaller than 0.05 are represented in bold italic.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Anabaena		0.46	0.31	0.12	0.45	0.22
Aphanizomenon	0.46		0.07	0.17	0.76	0.15
Cylindrospermopsis	0.31	0.07		0.15	0.84	0.25
Microcystis	0.12	0.17	0.15		0.80	0.17
Nodularia	0.45	0.76	0.84	0.80		0.88
Oscillatoria	0.22	0.15	0.25	0.17	0.88	

Table C.5 Estimated model parameters and their standard error after 14 days of exposure: EC50 (50% effect concentration), s (slope parameter), and a (deviation parameter to quantify mixture interaction) for each of the different steps: IA (independent action, Eq. 3.2) or CA (concentration addition, Eq. 3.1)-model step 1 (reference model based on data from single stressors treatments only), IA or CA-model step 2 (reference model based on data from all treatments), IA or CA-model step 3 (reference model including the deviation parameter a to quantify mixture interaction, Eq.3.3) per cyanobacteria. The reported p value is for the F-test that compared the nested models from step 2 and step 3. P <0.05 indicates a significant deviation from the reference model (i.e. aninteracting effect). EC50 of the insecticide has SI units of µg L^{-1} for tebufenpyrad and tetradifon and ng L^{-1} for chlorpyrifos and fenoxycarb.

	Chlorpyrifos	Fenoxycarb	Tebufenpyrad	Tetradifon
Slope parameter (s) Insecticide:				
IA: step 1	1.52 ± 0.51	1.26 ± 0.10	2.60 ± 0.60	1.00 ± 0.26
IA: step 2	1.42 ± 0.36	1.43 ± 0.32	2.87 ± 0.61	0.93 ± 0.23
IA: step 3	1.65 ± 0.48	1.85 ± 0.40	2.23 ± 0.52	0.99 ± 0.24
CA: step 1	1.98 ± 0.30	2.28 ± 0.47	2.16 ± 0.37	1.85 ± 0.37
CA: step 2	2.38 ± 0.29	3.09 ± 0.34	2.58 ± 0.60	1.81 ± 0.27
CA: step 3	2.34 ± 0.28	3.11 ± 0.36	2.11 ± 0.37	1.75 ± 0.29

Table C.5 cont.	Chlorpyrifos	Fenoxycarb	Tebufenpyrad	Tetradifon
Slope parameter (s) *Microcystis*:				
IA: step 1	2.13 ± 0.37	3.01 ± 1.03	1.78 ± 0.45	2.89 ± 0.76
IA: step 2	2.41 ± 0.34	3.38 ± 0.64	3.02 ± 0.85	2.48 ± 0.57
IA: step 3	2.57 ± 0.39	3.79 ± 0.68	1.74 ± 0.48	2.55 ± 0.63
CA: step 1	1.98 ± 0.30	2.28 ± 0.49	2.16 ± 0.37	1.85 ± 0.37
CA: step 2	2.38 ± 0.29	3.09 ± 0.34	2.58 ± 0.60	1.81 ± 0.27
CA: step 3	2.34 ± 0.28	3.11 ± 0.36	2.11 ± 0.37	1.75 ± 0.29
EC50 (Insecticide):				
IA: step 1	75.52 ± 14.65	69.84 ± 10.66	10.98 ± 0.99	11.23 ± 2.00
IA: step 2	74.86 ± 13.52	56.44 ± 5.36	11.93 ± 0.89	9.18 ± 1.45
IA: step 3	72.37 ± 11.71	66.28 ± 5.50	10.03 ± 1.04	10.59 ± 1.94
CA: step 1	68.23 ± 6.69	66.13 ± 5.47	10.63 ± 1.07	12.19 ± 1.75
CA: step 2	68.14 ± 5.46	65.49 ± 3.21	14.22 ± 1.39	10.86 ± 1.28
CA: step 3	64.87 ± 5.18	65.24 ± 3.51	9.99 ± 1.05	11.55 ± 1.56
EC50 (*Microcystis*) (% of diet):				
IA: step 1	37.61 ± 3.56	27.91 ± 3.27	39.46 ± 6.16	30.44 ± 3.14
IA: step 2	36.25 ± 2.09	25.73 ± 1.43	54.76 ± 6.40	31.51 ± 2.83
IA: step 3	37.95 ± 2.67	29.94 ± 1.95	44.84 ± 7.07	33.17 ± 3.49
CA: step 1	37.66 ± 3.66	29.78 ± 3.95	38.52 ± 5.46	33.62 ± 5.49
CA: step 2	42.42 ± 2.53	30.43 ± 1.82	79.03 ± 12.29	32.87 ± 3.92
CA: step 3	38.23 ± 3.10	30.15 ± 2.55	43.96 ± 6.34	34.54 ± 5.27
Deviation parameter *a*:				
IA: step 3	-0.71 ± 0.69	-2.27 ± 0.62	2.97 ± 0.85	-0.74 ± 0.67
CA: step 3	0.60 ± 0.35	0.07 ± 0.35	3.25 ± 0.65	-0.44 ± 0.64
Conclusion IA:	Non-interaction	Synergism	Antagonism	Non-interaction
P-value (IA: step 2 / IA: step 3)	0.30	6.05 e-04	0.003	0.28
Conclusion CA:	Non-interaction	Non-interaction	Antagonism	Non-interaction
P-value (CA: step 2 / CA: step 3)	0.09	0.87	1.35 e-07	0.48

Table C.6 Estimated model parameters and their standard error after fourteen days of exposure: EC50 (50% effect concentration), s (slope parameter), and a (deviation parameter to quantify mixture interaction) for each of the different steps: IA (independent action, Eq. 3.2) or CA (concentration addition, Eq. 3.1)-model step 1 (reference model based on data from single stressors treatments only), IA or CA-model step 2 (reference model based on data from all treatments), IA or CA-model step 3 (reference model including the deviation parameter a to quantify mixture interaction, Eq. 3.3) per cyanobacteria. The reported p value is for the F-test that compared the nested models from step 2 and step 3. P <0.05 indicates a significant deviation from the reference model (i.e. aninteracting effect). Aph=*Aphanizomenon*, Cyl=*Cylindrospermopsis*, MC=*Microcystis*, Osl=*Oscillatoria*.

	Aph	Cyl	MC	Osl
Slope parameter (s) Carbaryl:				
IA: step 1	0.26 ± 0.48	0.51 ± 0.48	3.12 ± 2.41	1.86 ± 1.03
IA: step 2	0.91 ± 0.37	0.71 ± 0.40	3.54 ± 3.13	3.91 ± 1.63
IA: step 3	0.15 ± 0.27	0.45 ± 0.50	3.08 ± 2.59	0.73 ± 0.26
CA: step 1	1.01 ± 0.22	0.97 ± 0.34	2.49 ± 1.01	0.65 ± 0.20
CA: step 2	0.80 ± 0.15	0.75 ± 0.32	2.28 ± 0.99	0.54 ± 0.17
CA: step 3	0.90 ± 0.15	0.81 ± 0.31	3.16 ± 1.05	0.59 ± 0.13
Slope parameter (s) Cyanobacteria:				
IA: step 1	1.17 ± 0.26	2.09 ± 0.85	1.92 ± 0.88	0.55 ± 0.19
IA: step 2	1.06 ± 0.24	2.74 ± 1.11	2.86 ± 1.14	0.34 ± 0.16
IA: step 3	1.14 ± 0.21	1.91 ± 0.90	3.16 ± 1.21	0.51 ± 0.16
CA: step 1	1.01 ± 0.22	0.97 ± 0.34	2.49 ± 1.01	0.65 ± 0.20
CA: step 2	0.80 ± 0.15	0.75 ± 0.32	2.28 ± 0.99	0.54 ± 0.17
CA: step 3	0.90 ± 0.15	0.81 ± 0.31	3.16 ± 1.05	0.59 ± 0.13
EC50 (Carbaryl) (µg L^{-1}):				
IA: step 1	3.34 ± 3.61	6.09 ± 3.76	6.87 ± 1.13	1.42 ± 0.47
IA: step 2	7.35 ± 2.95	4.82 ± 2.68	6.87 ± 1.16	2.20 ± 0.19
IA: step 3	2.43 ± 1.08	6.16 ± 2.93	6.89 ± 1.24	0.50 ± 0.22
CA: step 1	3.62 ± 1.09	5.05 ± 1.37	7.13 ± 1.38	0.41 ± 0.35
CA: step 2	5.92 ± 1.44	6.35 ± 2.09	8.79 ± 2.64	0.98 ± 0.54
CA: step 3	2.06 ± 0.86	4.90 ± 1.60	6.86 ± 1.15	0.33 ± 0.13
EC50 (Cyanobacteria) (% of diet):				
IA: step 1	39.17 ± 6.68	60.48 ± 11.07	16.58 ± 4.02	16.42 ± 5.52
IA: step 2	55.53 ± 10.39	67.33 ± 10.86	24.15 ± 2.93	35.48 ± 17.21
IA: step 3	40.51 ± 6.05	67.49 ± 14.51	14.66 ± 2.94	18.02 ± 5.42
CA: step 1	41.48 ± 8.39	78.63 ± 31.34	15.28 ± 3.13	16.78 ± 5.05
CA: step 2	57.18 ± 11.21	121.22 ± 70.51	29.76 ± 5.50	29.74 ± 10.44
CA: step 3	43.72 ± 7.66	95.03 ± 48.06	14.39 ± 2.79	17.03 ± 4.56
Deviation parameter *a*:				
IA-model 3	2.99 ± 0.52	1.49 ±1.12	8.08 ± 4.66	4.86 ± 0.86
CA-model 3	2.06 ± 0.86	1.70 ± 1.60	5.75 ± 2.27	9.11 ± 2.63
Conclusion IA:	Antagonism	Non interaction	Antagonism	Antagonism
P-value (IA: step 2 / IA: step 3)	8.56 e-05	0.28	0.024	3.22 e-04
Conclusion CA:	Antagonism	Non interaction	Antagonism	Antagonism
P-value (CA: step 2 / CA: step 3)	0.03	0.27	0.001	5.38 e-06

Appendix D

Supplementary material for Chapter 5

D.1 Tables

Table D.1 Labeling design of the microarray. For each array it is denoted which sample was labeled with Cy3 and which labeled with Cy5. Ctr = control sample, MC= *Microcystis* exposed sample.

Array	Cy3	Cy5
1	Ctr1	MC1
2	Ctr2	MC2
3	MC3	Ctr3
4	MC4	Ctr1

Table D.2 Primer sequences for qPCR validation

Apoptosis Inducing Factor	Sequence
Forward Primer	TGGCTGGAGAGAAGAATACTGGCA
Reverse Primer	TACACCAGTGATCGACCCAACCTT
ATP-synthase	Sequence
Forward Primer	ACAGCCTTGTTAAGTCTGCCAGGA
Reverse Primer	CCACAATGGTTCCTTTGCCAATGC
Glyceraldehyde-3--phosphatedehydrogenase	Sequence
Forward Primer	TGGGATGAGTCACTGGCATAC
Reverse Primer	GAAAGGACGACCAACAACAAAC
Neurexin IV	Sequence
Forward Primer	TGAACGGTGAGCAAACTGGGATTG
Reverse Primer	TGTCACCATGCAAATACGCTCCTG
Presinilin Enhancer 2	Sequence
Forward Primer	TGCCGAAAGTATTATTATGGAGGATTTGCT
Reverse Primer	AGCACCAATTCCTGAGCGAATGAC
Serine/Threonine Kinase	Sequence
Forward Primer	CAACCGGTCTTGCATGTCCAATCA
Reverse Primer	TCATCACTTGGGCTGGCTGATGTA
Trypsin	Sequence
Forward Primer	AAACAGCTGGAGACCCAACTCGAA
Reverse Primer	ACATGTCTTCGGGATTCCGCTCTT

Table D.3. Gene counts, number of differentially expressed (DE at 5% FDR) genes and DE single copy genes in the different KOG groups and functions provided by the Joint Genome Institute (http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Dappu1) for all the genes on the array. In each column

header, the total number of genes in that gene set is listed between parentheses. Genes with no KOG are indicated in the final row. KOG functions with proportions differing significantly from the total gene set are indicated with *, p-value is given between parentheses (significance level defined at p<0.05, based on Fisher`s exact test with multiple testing correction). In addition to the raw counts, O and U indicate respectively over- and underrepresentation of that group or function in the DE set.

KOG Classification (Function ID)	N° genes (29546)	N° significant genes (2247)		N° significant single copy genes (1157)	
Cellular processes & signaling	5561	518* (p<0.01)	O	245* (p<0.01)	O
Cell wall/membrane/envelope biogenesis (M)	198	16		6	
Cell Motility (N)	18	3		1	
Posttranslational modification, protein turnover, chaperones (O)	1256	114		56* (p=0.02)	O
Signal transduction mechanisms (T)	2188	219* (p<0.01)	O	91* (p<0.01)	O
Intracellular trafficking, secretion, and vesicular transport (U)	501	45		27	
Defense mechanisms (V)	387	50		31	
Extracellular structures (W)	264	17		11	
Nuclear structure (Y)	210	11		4	
Cytoskeleton (Z)	539	43		18	
Information storage & processing	3261	253		170* (p<0.01)	O
RNA processing and modification (A)	712	45		31	
Chromatin structure and dynamics (B)	375	22		10	
Translation, ribosomal structure and biogenesis (J)	509	91* (p<0.01)	O	67* (p<0.01)	O
Transcription (K)	1256	79		54	
Replication, recombination and repair (L)	409	16* (p<0.01)	U	8	
Metabolism	3197	389* (p<0.01)	O	111* (p<0.01)	O
Energy production and conversion (C)	305	45* (p<0.01)	O	26* (p<0.01)	O
Cell cycle control, cell division, chromosome partitioning (D)	514	50		19	
Amino acid transport and metabolism (E)	536	56 * (p=0.03)	O	9	
Nucleotide transport and metabolism (F)	126	11		3	
Carbohydrate transport and metabolism (G)	636	92* (p<0.01)	O	23	
Coenzyme transport and metabolism (H)	97	12		4	
Lipid transport and metabolism (I)	489	64* (p<0.01)	O	15	
Inorganic ion transport and metabolism (P)	309	36* (p=0.03)	O	9	
Secondary metabolites biosynthesis, transport and catabolism (Q)	167	23* (p=0.01)	O	3	
Poorly Characterized	3527	288		154	
General function prediction only (R)	2466	175		78	
Function Unknown (S)	1061	113* (p<0.01)	O	73* (p<0.01)	O
No KOG id available	14018	799* (p<0.01)	U	477 * (p<0.01)	U
Lineage specific genes	7888	373* (p<0.01)	U	298* (p<0.01)	U

Table D.4 Representation of all significant genes encoding serine/threonine protein kinases with their gene id, M-value and q-value.

Gene_id	M	Q
Dappudraft_260025	-1.167838252	0.004636829
Dappudraft_333324	-1.084448257	0.00546086
Dappudraft_222058	1.146186673	0.012547633
Dappudraft_259603	-0.861809389	0.013005852
Dappudraft_300401	-0.879055652	0.013043623
Dappudraft_327929	-0.867523257	0.014294608
Dappudraft_117153	-0.849548753	0.014982355
Dappudraft_102851	-0.847891239	0.015544494
Dappudraft_331153	-0.941185423	0.022081304
Dappudraft_103157	-0.805855252	0.024829462
Dappudraft_330244	-0.678801296	0.03467279
Dappudraft_35627	-0.924324297	0.01702005
Dappudraft_35689	-0.775677997	0.022135239

Table D.5 Representation of all significant genes encoding 40S ribosomal proteins with their gene id, M-value and q-value.

Gene_id	M	Q
Dappudraft_230714	-0.860191318	0.03358461
Dappudraft_308825	-0.660300638	0.037136694
Dappudraft_230521	-0.654487776	0.030334904
Dappudraft_129273	-0.91000268	0.01385579
Dappudraft_301703	-0.756584257	0.025632432
Dappudraft_306294	-0.645223001	0.03392859
Dappudraft_300540	-0.874099939	0.019719688
Dappudraft_309158	-1.228715997	0.019382698
Dappudraft_230600	-1.115467193	0.007737106
Dappudraft_230652	-0.836630087	0.013574141
Dappudraft_310174	-0.772921638	0.0291236
Dappudraft_92111	-0.96033012	0.008937795
Dappudraft_308217	-0.878130721	0.021520822
Dappudraft_128589	-1.035264907	0.022921808
Dappudraft_231413	-1.324992298	0.021561447
Dappudraft_230667	-0.641106828	0.039525013

Table D.6 Representation of all significant genes encoding 60S ribosomal proteins with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_302274	-0.797684846	0.022783629
Dappudraft_112232	-0.621636854	0.043380528
Dappudraft_300630	-0.855899385	0.028195789
Dappudraft_303528	-0.638250089	0.04370816
Dappudraft_301730	-0.637080074	0.043008032
Dappudraft_231518	-0.711774055	0.046067175
Dappudraft_230702	-0.785667304	0.0286711
Dappudraft_230579	-1.067247026	0.018101513
Dappudraft_304893	-1.106150764	0.01298175
Dappudraft_227532	-0.819838425	0.012873306
Dappudraft_230219	-0.803331319	0.015606794
Dappudraft_318183	-0.957472077	0.015118152
Dappudraft_230277	-0.801893212	0.022759173
Dappudraft_309347	-1.216220458	0.009151168
Dappudraft_303155	-0.927698424	0.048472193
Dappudraft_306617	-1.042297922	0.010971481

Table D.7 Representation of all significant genes encoding mitochondrial ribosomal proteins with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_301701	-0.681231051	0.043870753
Dappudraft_92746	-1.074203758	0.01046822
Dappudraft_127151	-0.745357675	0.024182779
Dappudraft_203941	-0.927077479	0.010387536
Dappudraft_230879	-0.779429704	0.021369521
Dappudraft_299906	-1.198314017	0.013330911
Dappudraft_231414	-0.775949638	0.029013526
Dappudraft_230080	-0.851131717	0.010138063
Dappudraft_301800	-0.760278683	0.024198046
Dappudraft_302746	-0.62378064	0.041112743
Dappudraft_299797	-0.777153422	0.03666647
Dappudraft_230221	-0.668864972	0.029130251
Dappudraft_303638	-0.860269098	0.015306692
Dappudraft_202590	-1.031490978	0.008478767
Dappudraft_235934	-0.735857841	0.022005555

Gene_id *(Table D.7 cont.)*	M	q
Dappudraft_308640	-0.857987823	0.016257231
Dappudraft_306295	-0.697098988	0.044635538
Dappudraft_304054	-0.796991413	0.020647025
Dappudraft_45963	-0.696473533	0.044843598
Dappudraft_92874	-0.704764805	0.041172091
Dappudraft_212601	-0.95004552	0.011364121
Dappudraft_301505	-1.006761256	0.009119092
Dappudraft_52198	-0.627335103	0.046665881
Dappudraft_54066	-0.924111396	0.022350937
Dappudraft_49671	-0.934347924	0.025547143
Dappudraft_47251	-1.061919222	0.011809093
Dappudraft_230842	-0.713552469	0.039346196
Dappudraft_308808	-0.874164157	0.013278267
Dappudraft_304832	-0.746501906	0.017715883
Dappudraft_201511	-0.958025435	0.019341063
Dappudraft_300200	-0.823671114	0.012529798

Table D.8 Representation of all significant genes encoding NADH:ubiquinone oxidoreductases with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_304291	-0.858995744	0.025536468
Dappudraft_299813	-0.94309662	0.018969062
Dappudraft_230517	-0.769376597	0.047371208
Dappudraft_231213	-0.819761192	0.026740597
Dappudraft_300905	-0.862170724	0.030811829
Dappudraft_57515	-0.665131598	0.04114799
Dappudraft_230160	-0.705074892	0.035607725
Dappudraft_301888	-0.765291003	0.016817111
Dappudraft_329128	-1.038609843	0.014654532
Dappudraft_319968	-0.956596337	0.010058282
Dappudraft_230203	-0.747899746	0.018330543

Table D.9 Representation of all significant genes encoding Neurexin IV proteins with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_112805	2.057113146	0.001458643
Dappudraft_15938	1.442465531	0.024437146
Dappudraft_18957	1.226031077	0.023267106
Dappudraft_19082	0.736019375	0.025239532
Dappudraft_224995	1.862206383	0.001978969
Dappudraft_227614	-1.276953909	0.016298837
Dappudraft_241573	0.849641647	0.028920004
Dappudraft_254743	-1.405455401	0.003987622
Dappudraft_25868	1.181945327	0.037356416
Dappudraft_27377	1.246886822	0.019353378
Dappudraft_28846	1.625758842	0.021475498
Dappudraft_307670	0.742128388	0.048094669
Dappudraft_316232	0.968238448	0.031815929
Dappudraft_316370	0.920783984	0.034623099
Dappudraft_316536	1.01333875	0.027158434

Table D.10 Representation of all significant genes encoding apoptosis inducing factors with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_101684	0.761409017	0.021088559
Dappudraft_110393	0.785333634	0.022529325
Dappudraft_114576	0.784084611	0.027761704
Dappudraft_241495	0.737738857	0.03511398
Dappudraft_241532	0.839590203	0.029226648
Dappudraft_244863	0.871002662	0.017093582
Dappudraft_254132	0.7096745	0.032287331
Dappudraft_273989	0.683210292	0.046739127
Dappudraft_319562	0.630629594	0.040974695
Dappudraft_43230	0.665554843	0.035756202
Dappudraft_67988	0.635347866	0.036722419

Table D.11 Representation of all significant genes encoding trypsins with their gene id, M-value and q-value.

Gene_id	M	q
Dappudraft_324510	-1.783511109	0.002361563
Dappudraft_307264	1.855196024	0.00405258
Dappudraft_318727	-1.427669124	0.006037718
Dappudraft_302655	-1.079625661	0.008579145
Dappudraft_26734	-0.895365775	0.010139564
Dappudraft_230054	1.054763006	0.010646843
Dappudraft_302564	-1.166617979	0.011170264
Dappudraft_49162	-1.218815268	0.01122611
Dappudraft_225444	-1.66922385	0.01135651
Dappudraft_215674	-1.802824347	0.011688464
Dappudraft_305245	-2.330179795	0.012911294
Dappudraft_316923	-0.802022448	0.014392303
Dappudraft_323225	0.896210955	0.014975322
Dappudraft_305317	1.117568506	0.015566071
Dappudraft_331736	-1.401798012	0.015952594
Dappudraft_319989	-1.661771994	0.017259358
Dappudraft_225511	-1.289506079	0.017323422
Dappudraft_305246	0.967602383	0.018623717
Dappudraft_306771	-1.014310732	0.020520895
Dappudraft_324053	0.853930356	0.02150227
Dappudraft_308787	1.37678192	0.023694267
Dappudraft_63727	0.715592805	0.026260312
Dappudraft_224273	-1.116861259	0.026818545
Dappudraft_323226	0.916442804	0.027143033
Dappudraft_104230	-1.799256125	0.027669029

Table D.12 Representation of all significant genes encoding glutathione-S-transferases with their gene id, M-value, q-value and KOG ID.

Gene ID	M-value	q-value	KOG ID
Dappudraft_15523	0,761329759	0,03099197	KOG0868
Dappudraft_205726	0,597296387	0,090511183	KOG1695
Dappudraft_196080	0,446879652	0,174472064	KOG0867
Dappudraft_95675	-0,341370785	0,219083444	KOG0867
Dappudraft_318232	-0,31463263	0,376413789	KOG0867
Dappudraft_200523	0,270482358	0,390270386	KOG0867
Dappudraft_219884	-0,2290158	0,432623386	KOG1695
Dappudraft_230826	-0,167060446	0,446883927	KOG0867
Dappudraft_230303	0,199841112	0,50924953	KOG1695
Dappudraft_255502	-0,399724484	0,641746293	KOG1695
Dappudraft_230650	-0,017844763	0,820711729	KOG1695
Dappudraft_230761	0,027026499	0,853910691	KOG1695

Table D.13 Representation of all significant genes encoding Bcl2, Bax and apoptosis inhibitors (IAP) with their gene id, M-value and q-value.

Gene ID	M-value	q-value
Dappudraft_319285	0,114322393	0,77278252
Dappudraft_329424	0,409667089	0,268325843
Dappudraft_306240	-0,952840551	0,014317754
Dappudraft _58422	-0,601298823	0,044295316
Dappudraft_307098	0,435279121	0,277481019
Dappudraft_204003	0,286216464	0,451265164
Dappudraft_256692	-0,238987239	0,58031401
Dappudraft _95322	-0,185334088	0,696223287

Appendix E

Supplementary material for Chapter 6

E.1 Figures

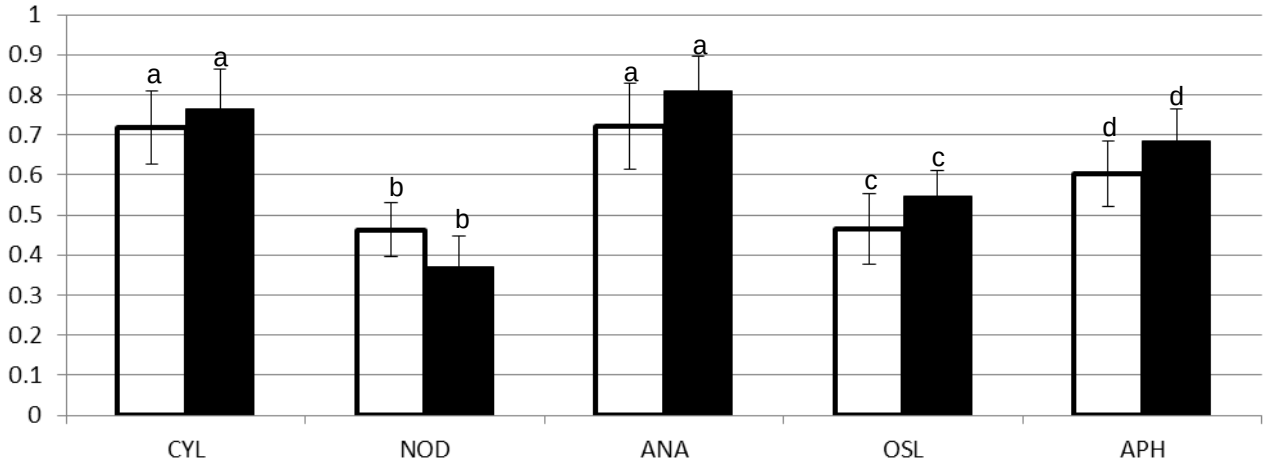
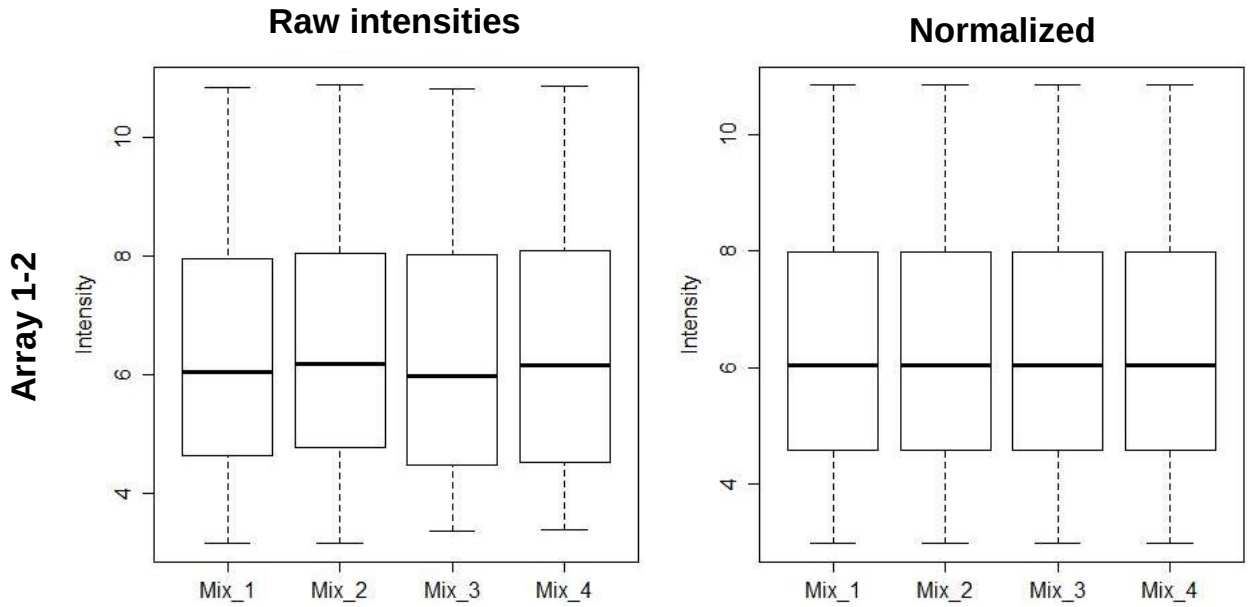
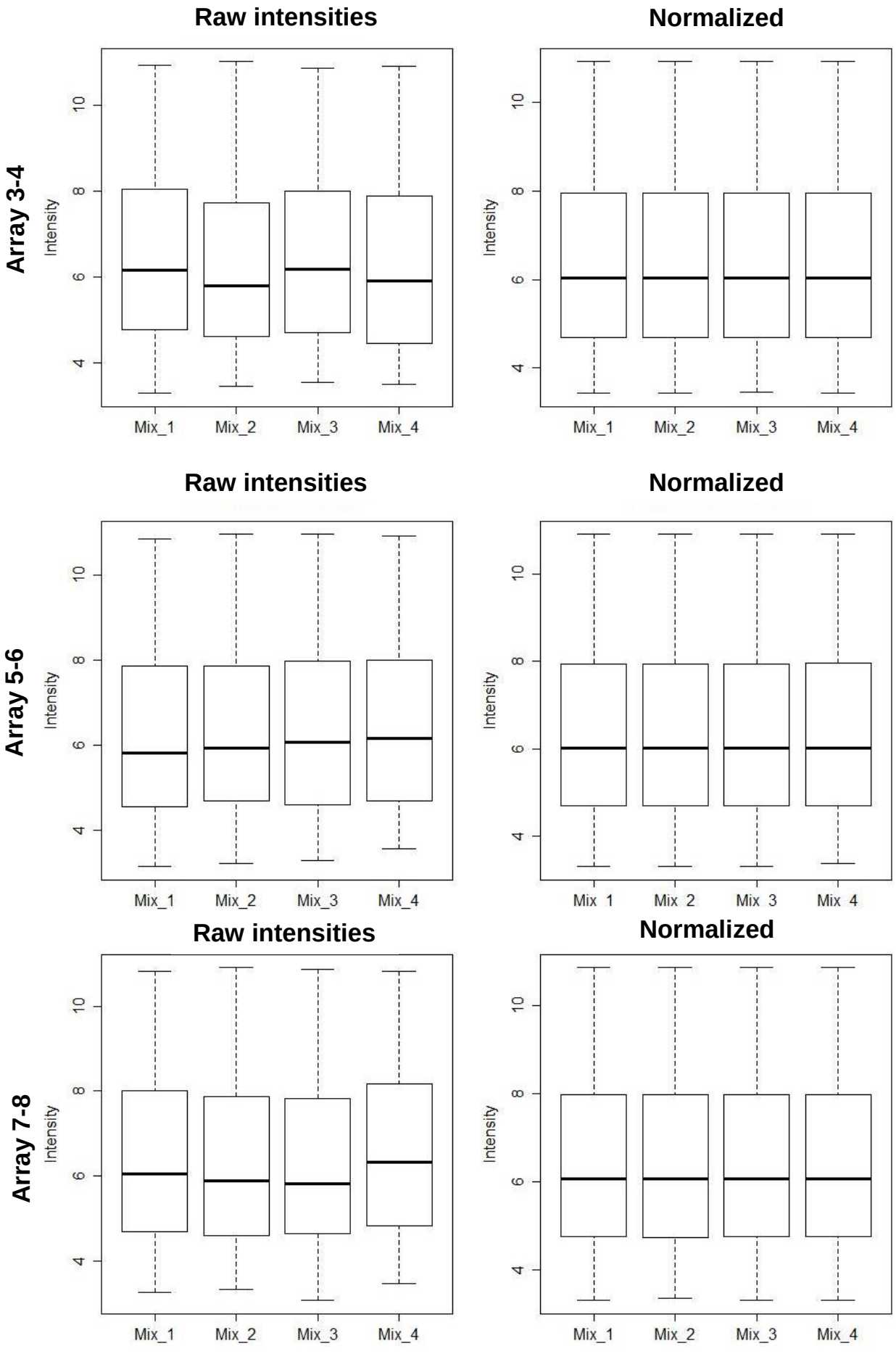


Figure E.1 Reproduction in exposed treatments (*Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Anabaena*: ANA, *Oscillatoria*: OSL, *Aphanizomenon*: APH) relative to respective control treatments. Error bars represent standard deviation per repeated experiment. Open and filled bars denote response of repeated experiments. Significant differences (p-value<0.05) are denoted with letters.

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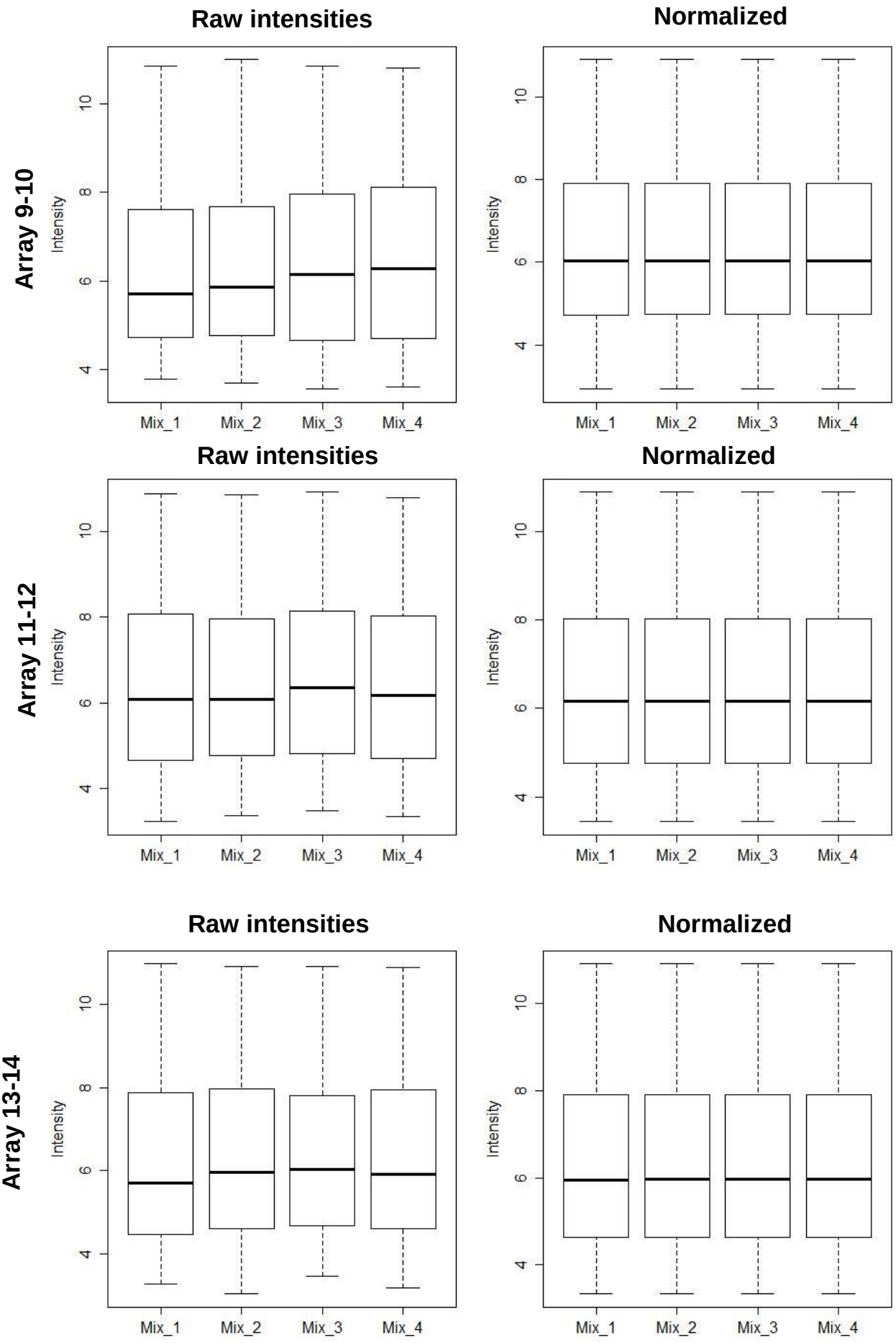
Mix 4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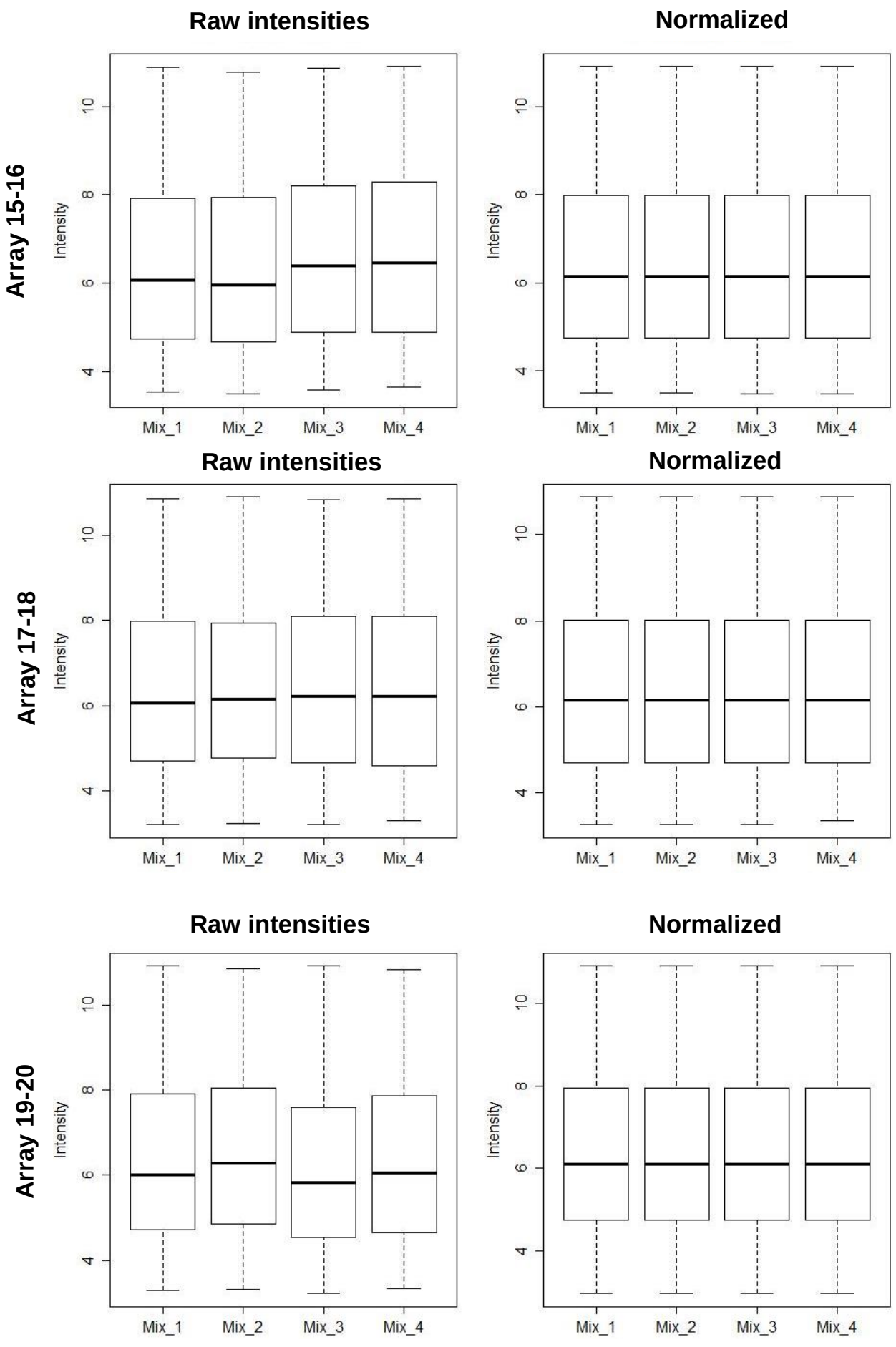
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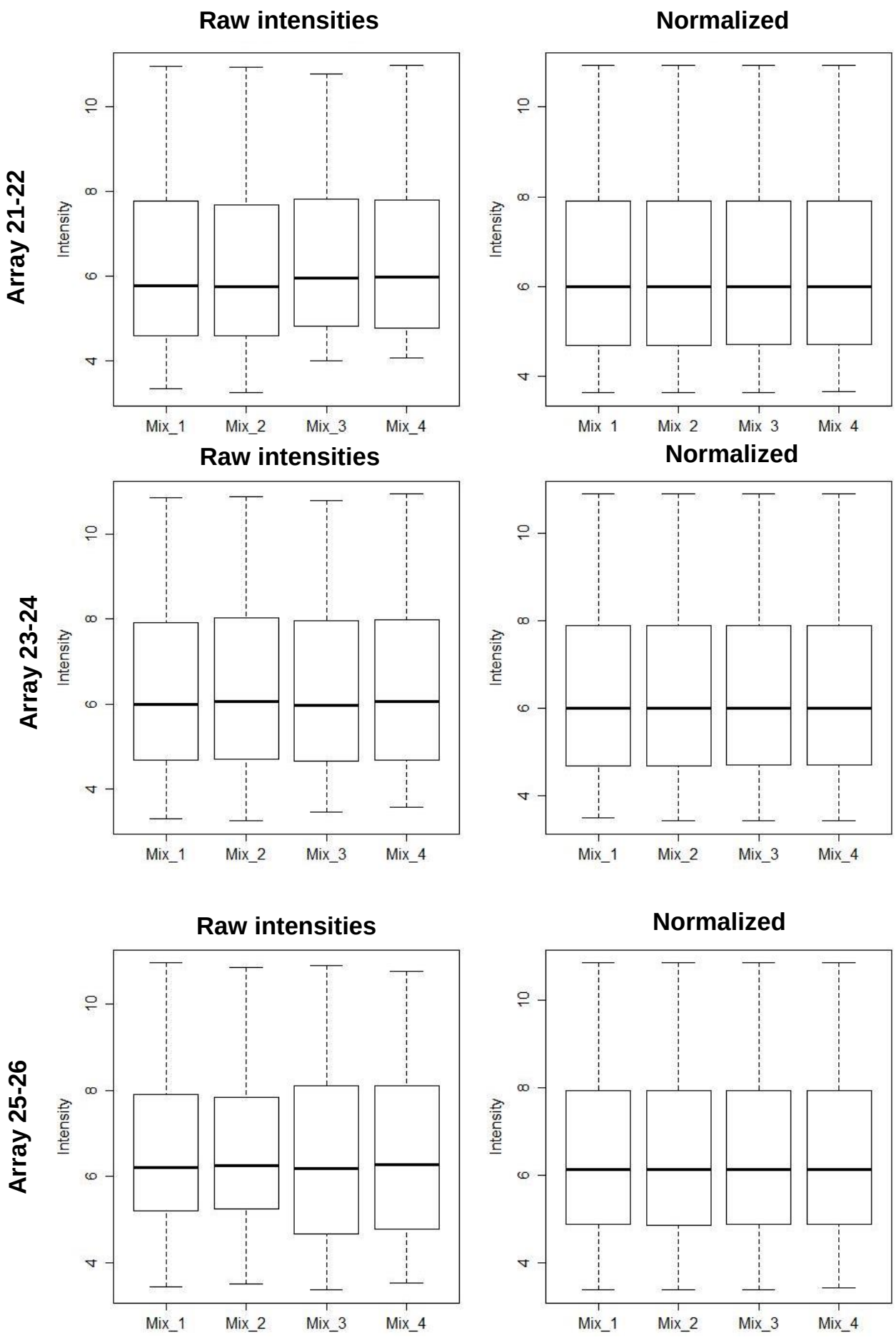
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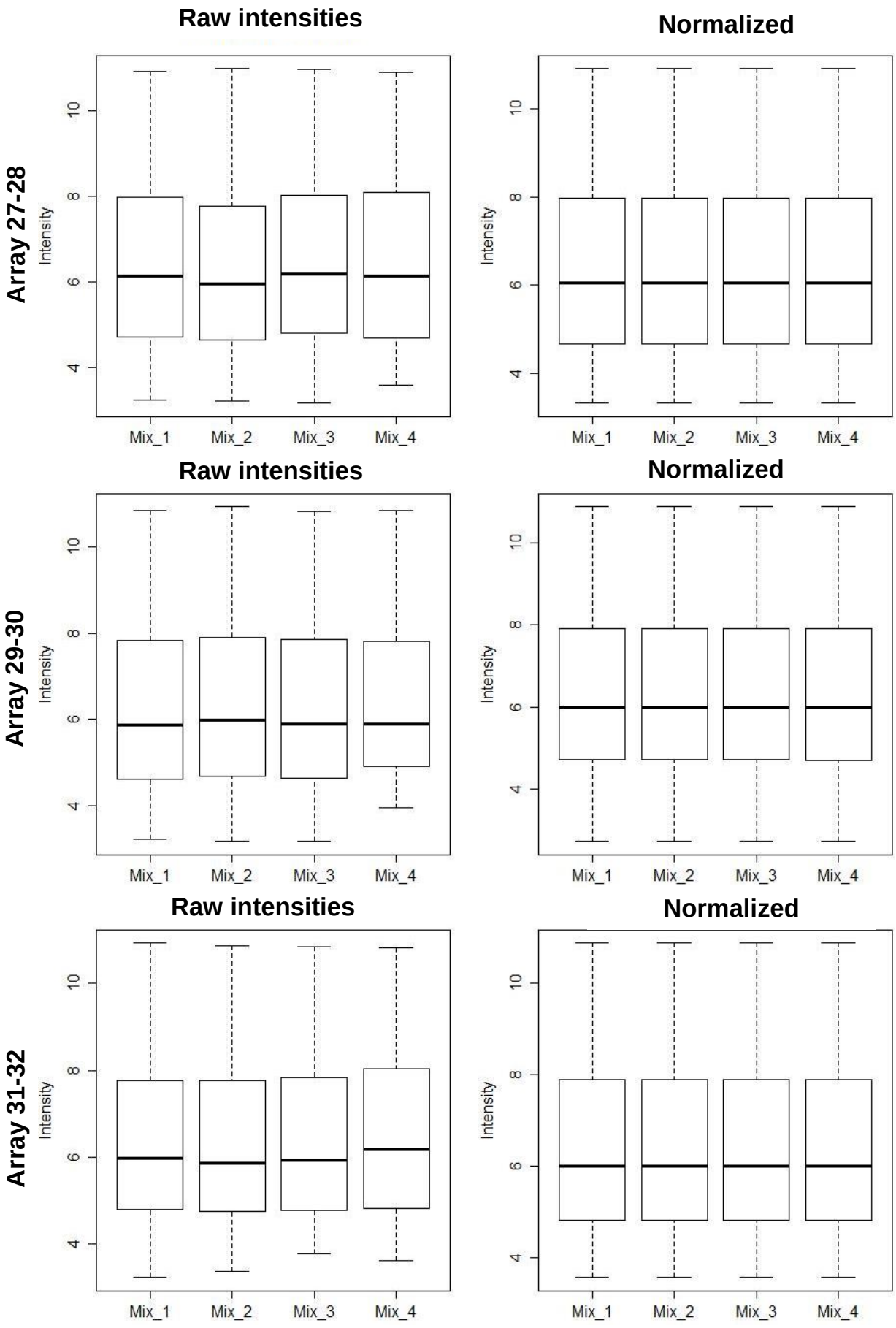
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Normalized
Intensity
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10
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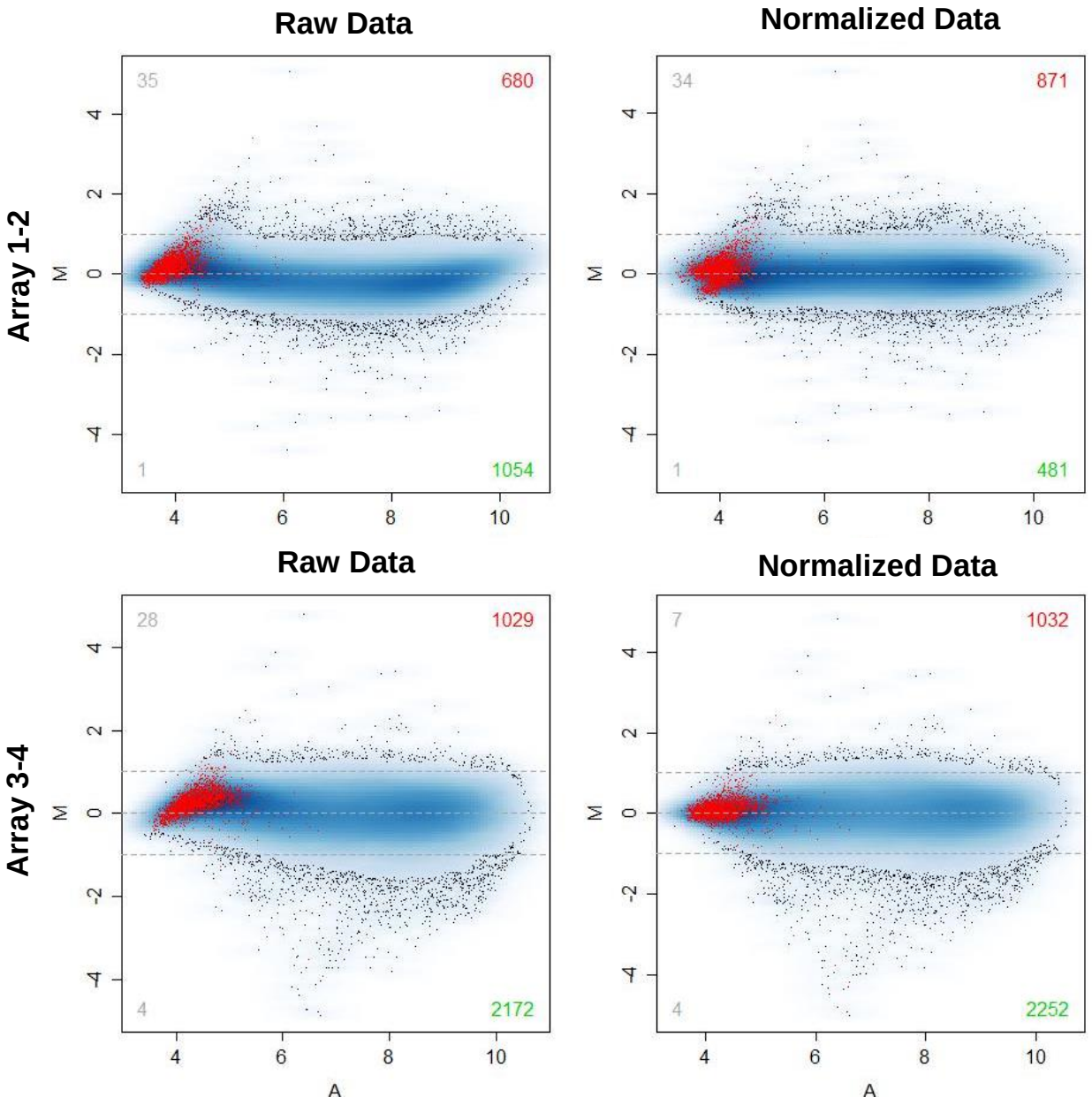
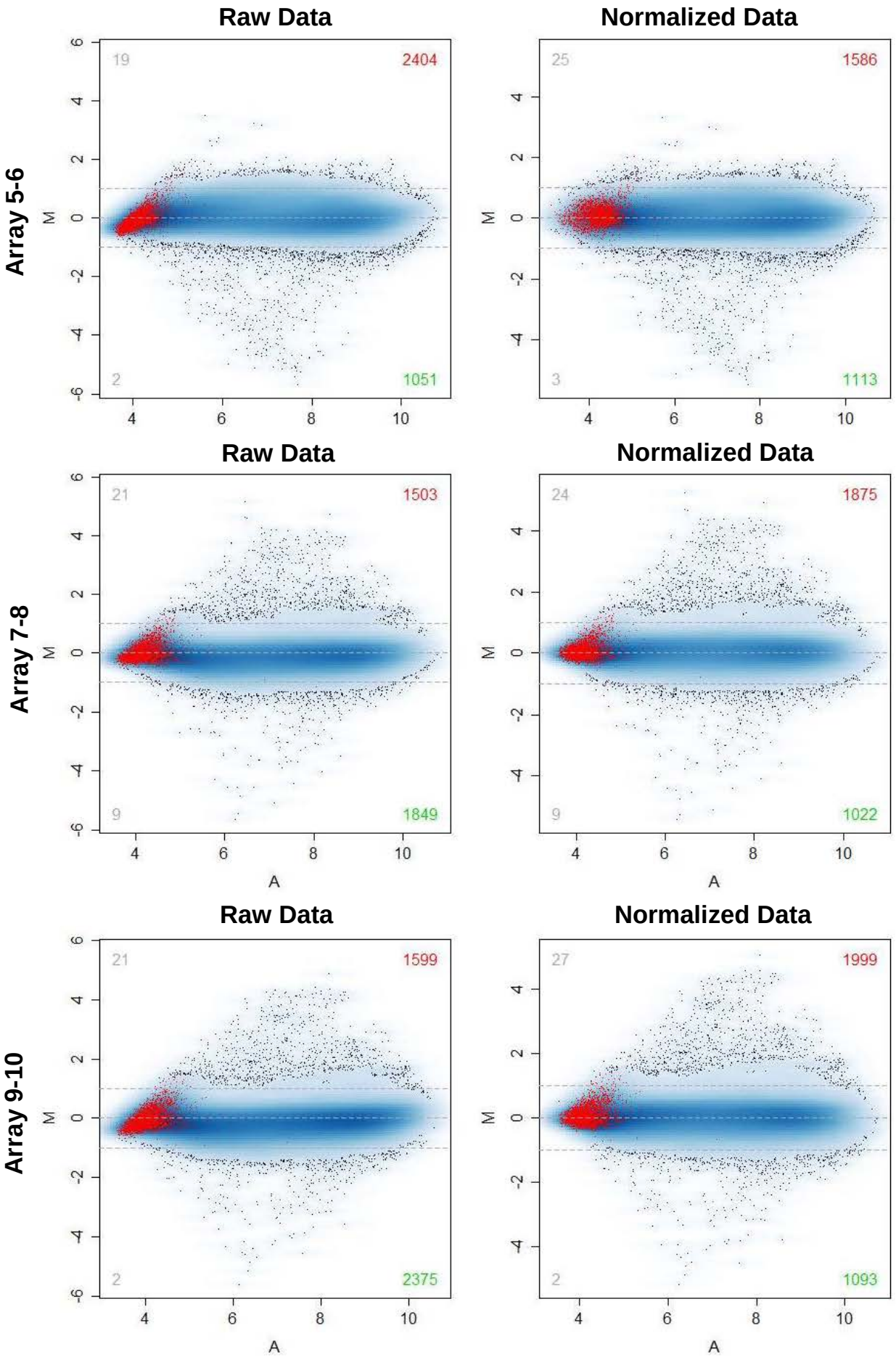
Mix_2
Mix_3
Mix_4

Figure E.2 Boxplots of the raw and normalized intensities. Arrays are represented in pairs as one array was always the identitical dye swap of the other. Per pair, four biological replicates are plotted labelled Mix 1 to 4. These four biological replicates represent a single mixture treatment out of the 48 combinations per array pair.

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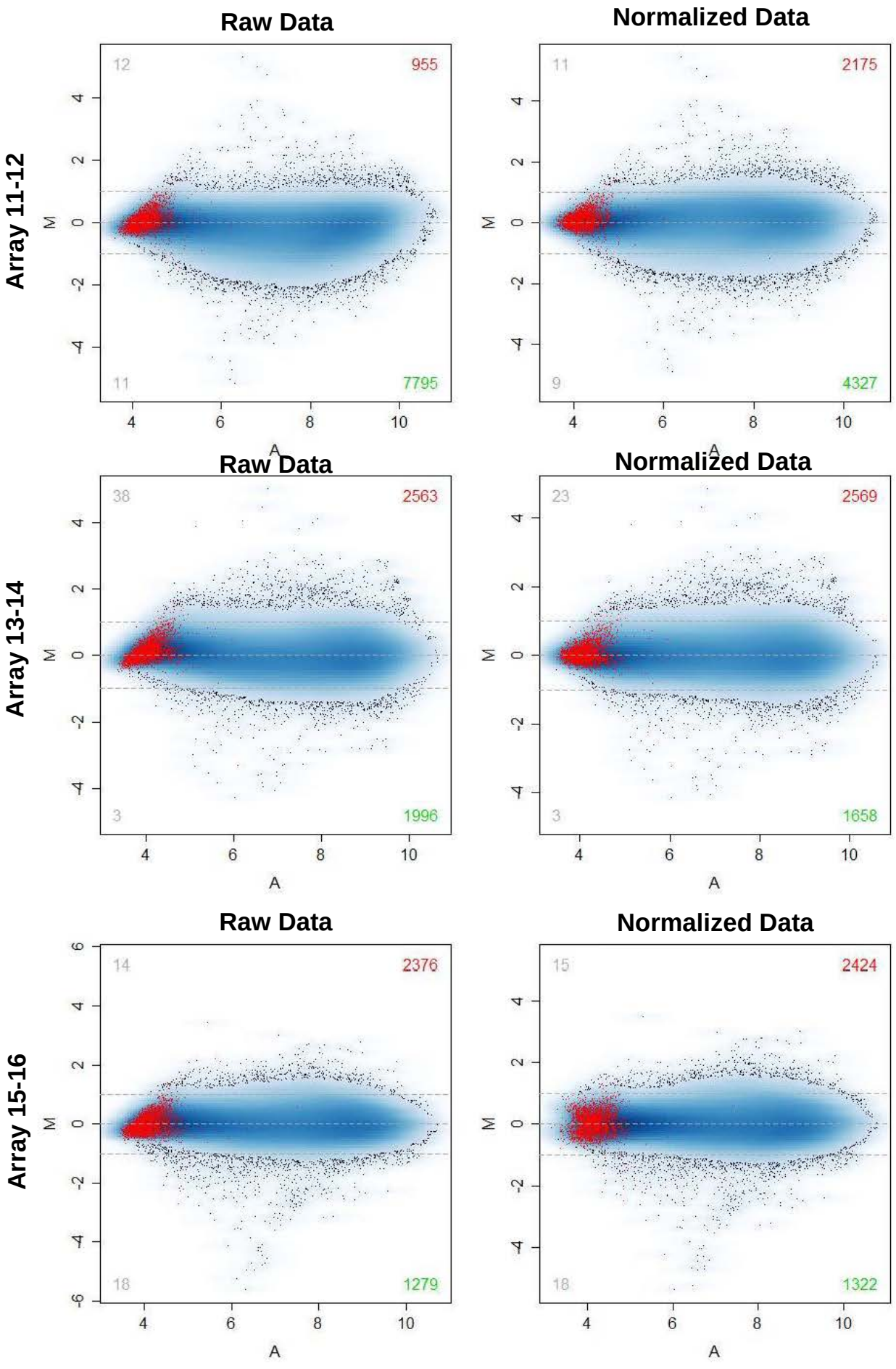
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1996
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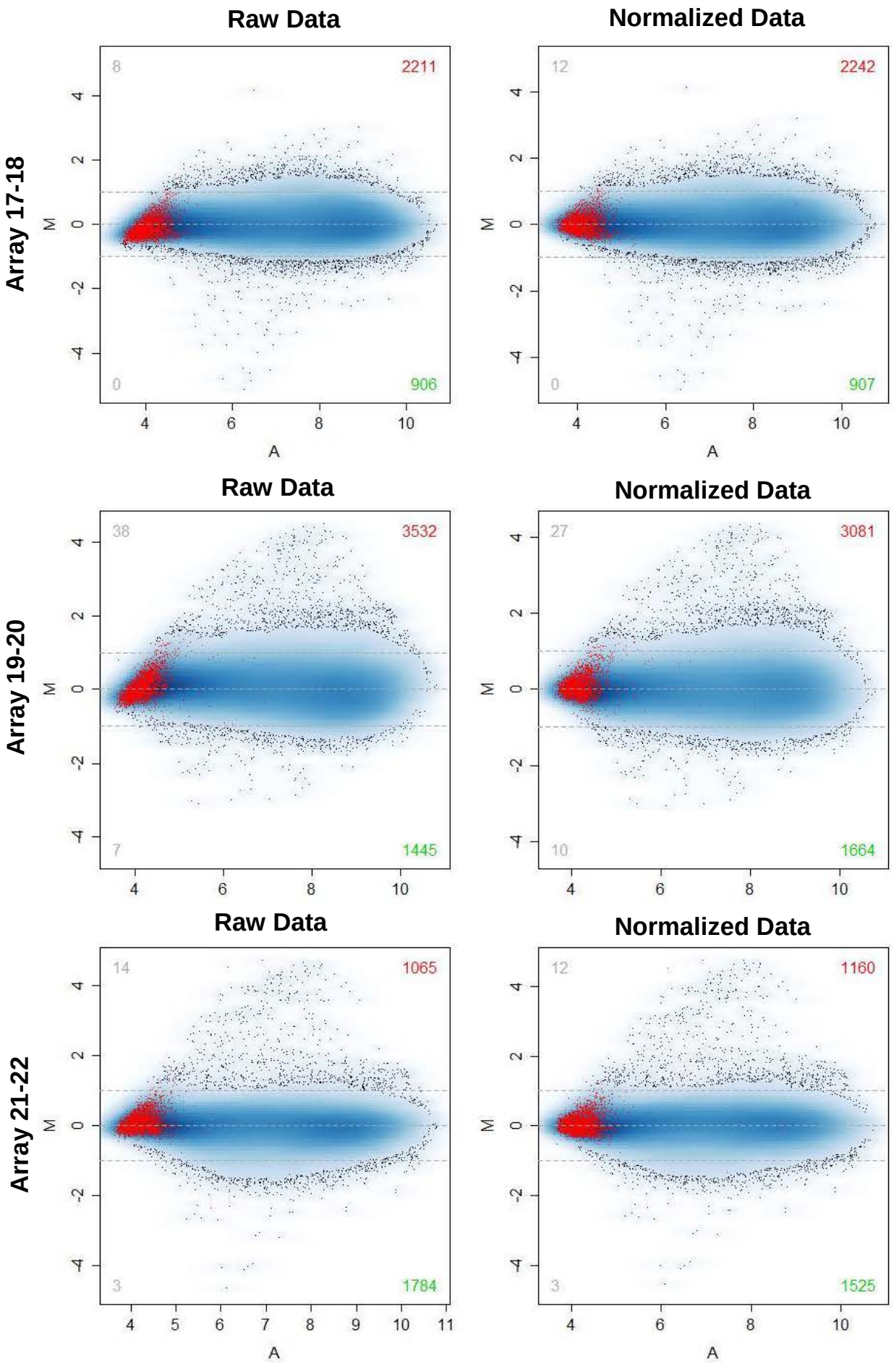
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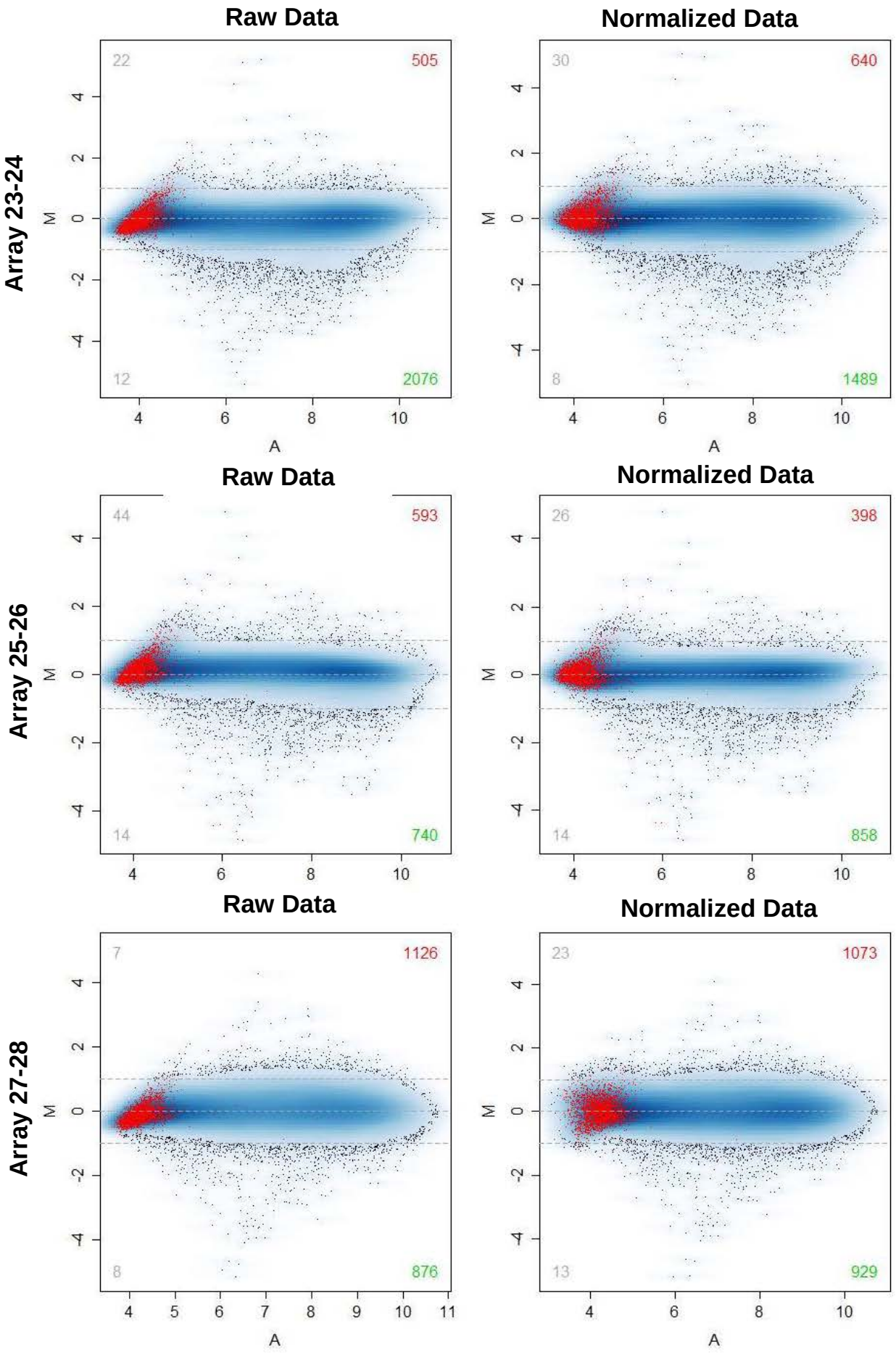
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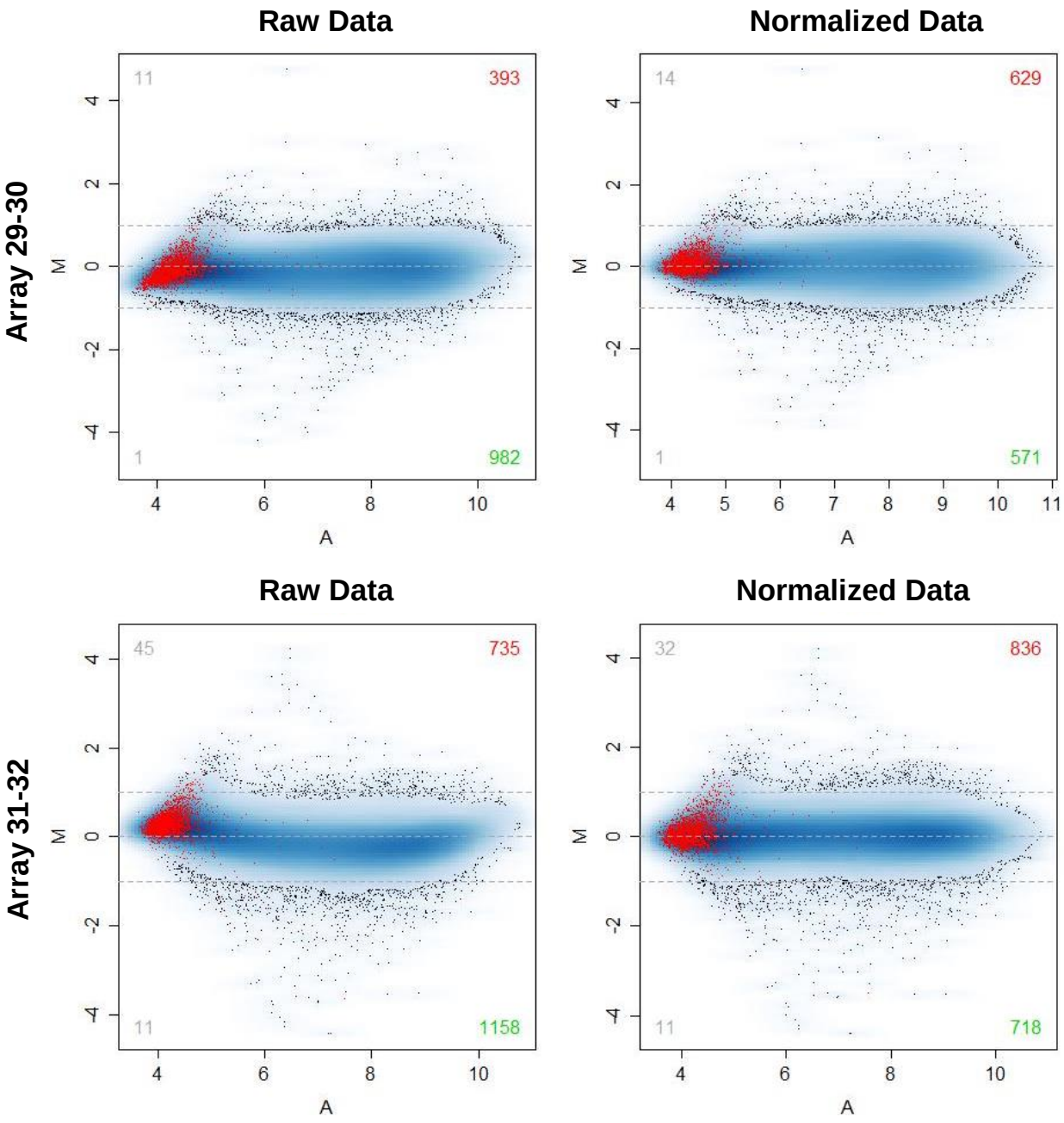


Figure E.3 MA plots of the raw and normalized M and A-values. Arrays are represented in pairs as one array was always the identitical dye swap of the other. Each MAplot is subarray of the complete array, one Cy3 labelled sample versus its corresponding Cy5 labelled sample . Each MAplot represent a single comparison out of 192 comparisons excluding dye swap based upon the labelling design in Fig. 7.1. For each MA plot the number of probes with an M-value larger than 1 is printed in red, the number of probes with an M-value smaller than -1 is printed in green. All probes representing genes are printed in blue dots, all random probes are printed in red. The number of random probes with an M-value larger than 1 is printed in grey in the top left corner while the number of random probes with an M-value smaller than -1 is printed in grey in the bottom left corner.

E.2 Tables

Table E.1 Genes with their JGI id and annotation definition that are shared among all transcriptomic profiles. X denotes genes that are also shared with the transcriptomic profile in response to *Microcystis aeruginosa* stress (section 5.3).

JGI ID	Annotation Definitions	Shared with *Microcystis*
JGI_V11_97232	Beta-beta-carotene 1515-dioxygenase	
JGI_V11_240263	Carboxylesterase and related proteins	x
JGI_V11_240264	Carboxylesterase and related proteins	x
JGI_V11_304160	Carboxylesterase and related proteins	
JGI_V11_100284	C-type Lectin	
JGI_V11_104167	C-type Lectin	
JGI_V11_194538	Cytochrome P450 CYP2 subfamily	
JGI_V11_309471	Cytochrome P450 CYP2 subfamily	
JGI_V11_192258	Cytochrome P450 CYP4-CYP19-CYP26 subfamily	
JGI_V11_15938	Neurexin IV	x
JGI_V11_224885	Neurexin IV	x
JGI_V11_27377	Neurexin IV	x
JGI_V11_28846	Neurexin IV	x
JGI_V11_50444	Neurexin IV	x
JGI_V11_122791	Plasma membrane Glycoprotein CD36	x
JGI_V11_307582	Plasma membrane Glycoprotein CD36	x
JGI_V11_97775	Predicted Alkaloid synthase	
JGI_V11_300798	Putative SAM-dependent rRNA methyltransferase	x
JGI_V11_307732	Triglyceride lipase-cholesterol esterase	x
JGI_V11_224273	Trypsin	x
JGI_V11_304515	Trypsin	
JGI_V11_99426	UDP-glucuronosyl and UDP-glucosyl transferase	x

Table E.2 Number of significant genes for each transcriptomic profile for each functional annotation. APH= *Aphanizomenon*, ANA=*Anabaena*¸CYL=*Cylindrospermopsis*, NOD=*Nodularia*, OSL=*Oscillatoria*.

Annotation Definitions	APH	ANA	CYL	NOD	OSL
Actin and associated proteins	1	9	1	15	1
Acyl-CoA synthetase	1	9	7	17	7
Alkaline phosphatase	1	3	3	4	2
Alpha-amylase	2	5	4	7	4
Anaphase promoting complex	1	3	1	5	1
Ankyrin	1	10	4	11	1
Aromatic-L-amino-acid-L-histidine decarboxylase	1	2	2	2	2
Beta-glucosidase lactase phlorizinhydrolase and related proteins	1	1	1	2	1
Beta beta-carotene 1515-dioxygenase and related enzymes	1	1	1	2	1
C-type lectin	12	11	15	49	7
Carbonic anhydrase	1	3	2	7	2
Carboxylesterase and related proteins	7	16	7	17	8
Chitinase	7	18	9	47	6
Collagens -type IV and type XIII- and related proteins	29	26	11	51	3
Conserved Zn-finger protein	3	3	1	14	1
Cytochrome P450 CYP11-CYP12-CYP24-CYP27 subfamilies	1	1	1	1	1
Cytochrome P450 CYP2 subfamily	4	8	6	8	5
Cytochrome P450 CYP3-CYP5-CYP6-CYP9 subfamilies	1	1	2	5	3
Cytochrome P450 CYP4-CYP19-CYP26 subfamilies	4	3	6	4	5
Fasciclin and related adhesion glycoproteins	1	4	1	10	5
FKBP-type peptidyl-prolyl cis-trans isomerase	1	3	1	3	1
FOG 7 transmembrane receptor	2	41	4	24	1
FOG Immunoglobulin C-2 Type-fibronectin type III domains	4	14	3	10	1
FOG Leucine rich repeat	2	13	6	16	3
FOG Zn-finger	4	27	5	45	1
Fucosyltransferase	3	6	4	30	1
Galactosyltransferases	2	3	2	14	2
Glutamate-gated kainate-type ion channel receptor subunit GluR5 and related subunits	1	5	1	28	1
Glutathione S-transferase	2	5	2	14	4
Heterogeneous nuclear ribonucleoprotein R -RRM superfamily-	1	1	1	1	1
Lipid exporter ABCA1 and related proteins ABC superfamily	3	4	1	3	1
Meprin A metalloprotease	1	1	6	13	4
Monocarboxylate transporter	1	3	1	6	1
Neurexin IV	15	24	23	23	21

Annotation Definitions *(Table E.2 cont.)*	APH	ANA	CYL	NOD	OSL
Permease of the major facilitator superfamily	3	1	2	9	1
Peroxidase-oxygenase	1	12	4	15	4
Phosphatidylinositol transfer protein SEC14 and related proteins	5	6	15	17	3
Plasma membrane glycoprotein CD36 and related membrane receptors	5	3	4	3	2
Predicted alkaloid synthase-Surface mucin Hemomucin	3	1	2	7	2
Predicted E3 ubiquitin ligase	3	12	5	38	1
Predicted lipoprotein	1	2	4	4	1
Predicted membrane protein contains DoH and Cytochrome b-561-ferric reductase transmembrane domains	2	5	8	14	1
Predicted transporter	1	2	1	5	1
Putative SAM-dependent rRNA methyltransferase SPB1	1	1	1	1	1
Renal dipeptidase	3	3	2	3	3
RNA polymerase II	10	20	2	31	1
Serine-threonine protein kinase	2	13	1	21	1
Serine-threonine protein kinase and endoribonuclease ERN1-IRE1 sensor of the unfolded protein response pathway	3	12	2	22	3
Serine proteinase inhibitor -KU family-	2	3	2	8	1
Transcription initiation factor TFIID	1	5	1	12	1
Triglyceride lipase-cholesterol esterase	1	1	2	5	1
Trypsin	24	32	40	108	18
Type I phosphodiesterase-nucleotide pyrophosphatase	1	1	1	1	1
UDP-glucuronosyl and UDP-glucosyl transferase	3	9	12	10	6
von Willebrand factor and related coagulation proteins	4	29	8	48	2
Zinc carboxypeptidase	6	12	6	18	7

Table E.3 List of Gene Ontology terms shared by all transcriptomic profiles

Gene Ontology (GO) Term
GO:0000151/C:ubiquitin ligase complex
GO:0000166/F:nucleotide binding
GO:0001584/F:rhodopsin-like receptor activity
GO:0003676/F:nucleic acid binding
GO:0003677/F:DNA binding
GO:0003700/F:transcription factor activity
GO:0003723/F:RNA binding
GO:0003824/F:catalytic activity
GO:0004089/F:carbonate dehydratase activity
GO:0004180/F:carboxypeptidase activity

Gene Ontology (GO) Term *(Table E.3 cont.)*
GO:0004182/F:carboxypeptidase A activity
GO:0004237/F:membrane dipeptidase activity
GO:0004263/F:chymotrypsin activity
GO:0004289/F:subtilase activity
GO:0004295/F:trypsin activity
GO:0004364/F:glutathione transferase activity
GO:0004386/F:helicase activity
GO:0004497/F:monooxygenase activity
GO:0004553/F:hydrolase activity. hydrolyzing O-glycosyl compounds
GO:0004556/F:alpha-amylase activity
GO:0004601/F:peroxidase activity
GO:0004672/F:protein kinase activity
GO:0004674/F:protein serine/threonine kinase activity
GO:0004713/F:protein-tyrosine kinase activity
GO:0004714/F:transmembrane receptor protein tyrosine kinase activity
GO:0004842/F:ubiquitin-protein ligase activity
GO:0005201/F:extracellular matrix structural constituent
GO:0005215/F:transporter activity
GO:0005319/F:lipid transporter activity
GO:0005506/F:iron ion binding
GO:0005509/F:calcium ion binding
GO:0005515/F:protein binding
GO:0005524/F:ATP binding
GO:0005529/F:sugar binding
GO:0005576/C:extracellular region
GO:0005581/C:collagen
GO:0005622/C:intracellular
GO:0005634/C:nucleus
GO:0005975/P:carbohydrate metabolism
GO:0006030/P:chitin metabolism
GO:0006118/P:electron transport
GO:0006355/P:regulation of transcription. DNA-dependent
GO:0006396/P:RNA processing
GO:0006457/P:protein folding
GO:0006468/P:protein amino acid phosphorylation
GO:0006486/P:protein amino acid glycosylation

Gene Ontology (GO) Term *(Table E.3 cont.)*
GO:0006508/P:proteolysis and peptidolysis
GO:0006519/P:amino acid and derivative metabolism
GO:0006520/P:amino acid metabolism
GO:0006629/P:lipid metabolism
GO:0006730/P:one-carbon compound metabolism
GO:0006810/P:transport
GO:0006869/P:lipid transport
GO:0006979/P:response to oxidative stress
GO:0007155/P:cell adhesion
GO:0007165/P:signal transduction
GO:0007169/P:transmembrane receptor protein tyrosine kinase signaling pathway
GO:0007186/P:G-protein coupled receptor protein signaling pathway
GO:0007242/P:intracellular signaling cascade
GO:0008026/F:ATP-dependent helicase activity
GO:0008061/F:chitin binding
GO:0008152/P:metabolism
GO:0008237/F:metallopeptidase activity
GO:0008239/F:dipeptidyl-peptidase activity
GO:0008270/F:zinc ion binding
GO:0008378/F:galactosyltransferase activity
GO:0008417/F:fucosyltransferase activity
GO:0008533/F:astacin activity
GO:0016020/C:membrane
GO:0016021/C:integral to membrane
GO:0016491/F:oxidoreductase activity
GO:0016567/P:protein ubiquitination
GO:0016758/F:transferase activity. transferring hexosyl groups
GO:0016787/F:hydrolase activity
GO:0016789/F:carboxylic ester hydrolase activity
GO:0016831/F:carboxy-lyase activity
GO:0016887/F:ATPase activity
GO:0017111/F:nucleoside-triphosphatase activity
GO:0031177/F:phosphopantetheine binding
GO:0042626/F:ATPase activity. coupled to transmembrane movement of substances

Table E.4 Venn diagrams for significantly regulated(q-value) duplicated genes and non-duplicated genes, the number of annotation definitions within the significantly regulated duplicated and non-duplicated genes and the number of gene ontology (GO) terms within the significantly regulated duplicated and non-duplicated genes for all transcriptomic profiles. (Aphanizomenon: APH, Anabaena: ANA, Cylindrospermopsis: CYL, Nodularia: NOD, Oscillatoria: OSL).

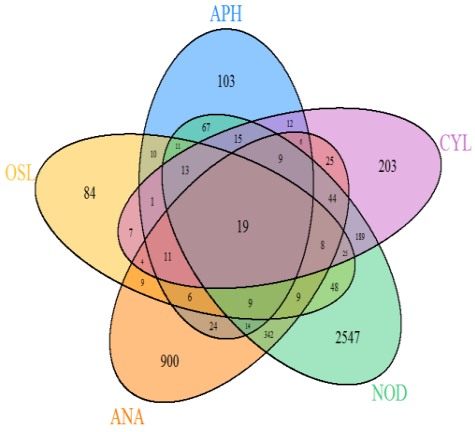
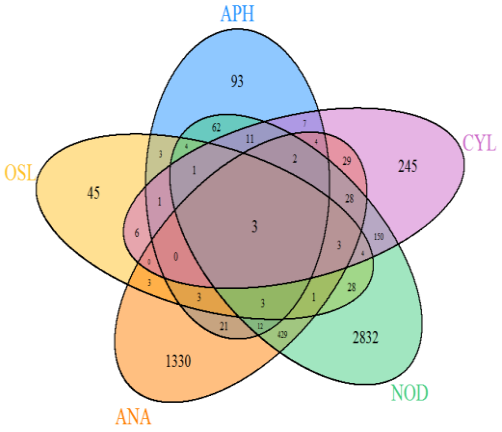
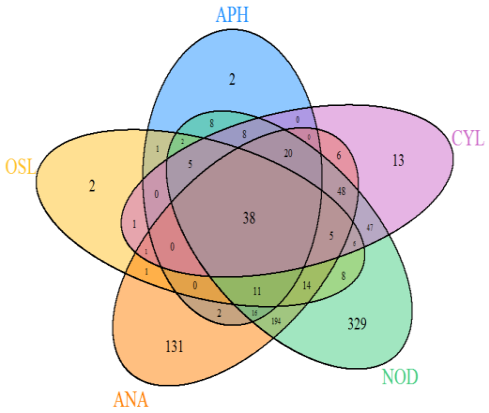
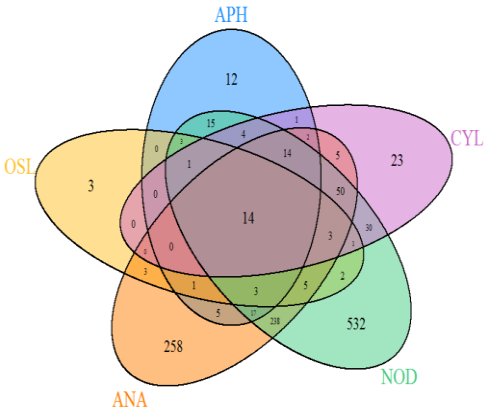
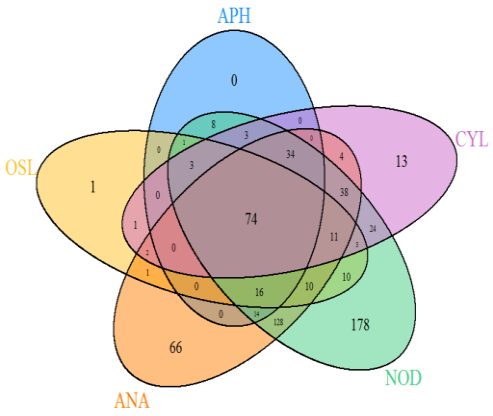
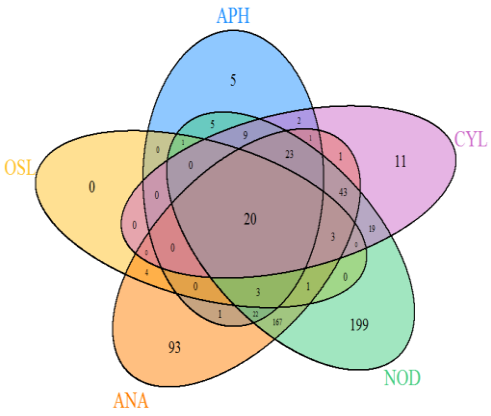
	Significant duplicated genes	Significant non-duplicated genes
Number of significant genes		
Number of significant annotation definitions		
Number of significant gene ontology (GO) terms		

Table E.5 Venn diagrams for significantly regulated(q-value) duplicated genes and tandem-duplicated genes, the number of annotation definitions within the significantly regulated duplicated and tandem-duplicated genes and the number of gene ontology (GO) terms within the significantly regulated duplicated and tandem-duplicated genes for all transcriptomic profiles. (Aphanizomenon: APH, Anabaena: ANA, Cylindrospermopsis: CYL, Nodularia: NOD, Oscillatoria: OSL).

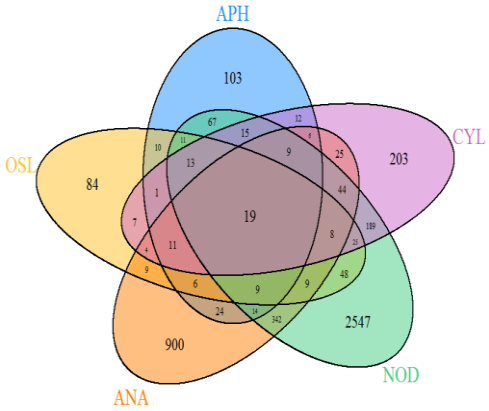
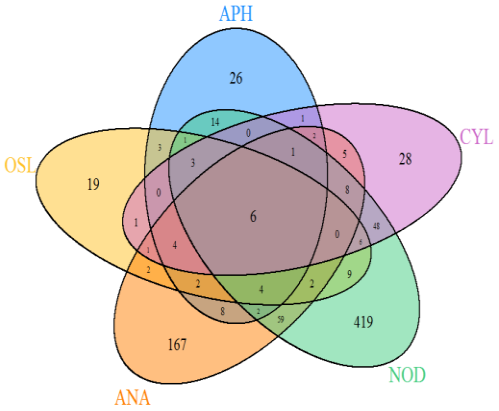
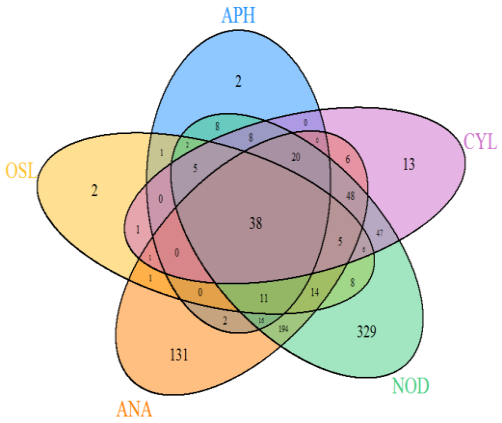
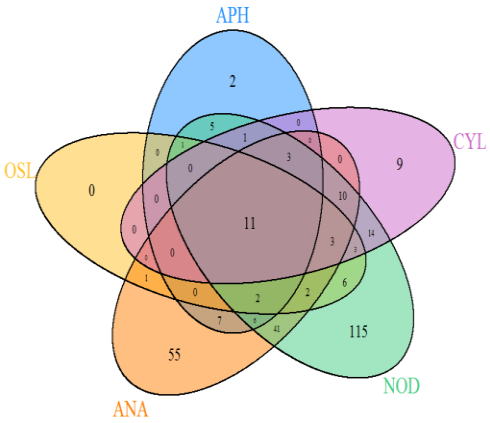
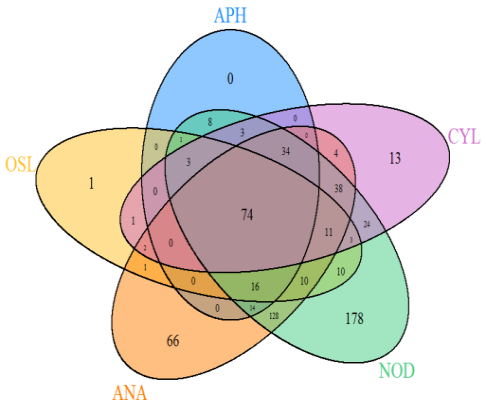
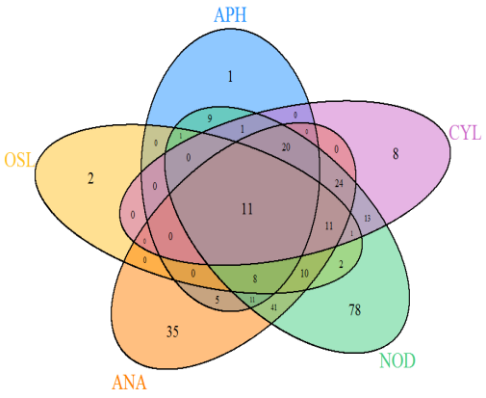
	Significant duplicated genes	Significant tandem-duplicated genes
Number of significant genes		
Number of significant annotation definitions		
Number of significant gene ontology (GO) terms		

Table E.6 Shared annotation definitions across all transcriptomic profiles and their presence (gray filled cells) or absence (blank cells) in transcriptomic profiles exposed to *Microcystis aeruginosa*, salinity or cadmium.

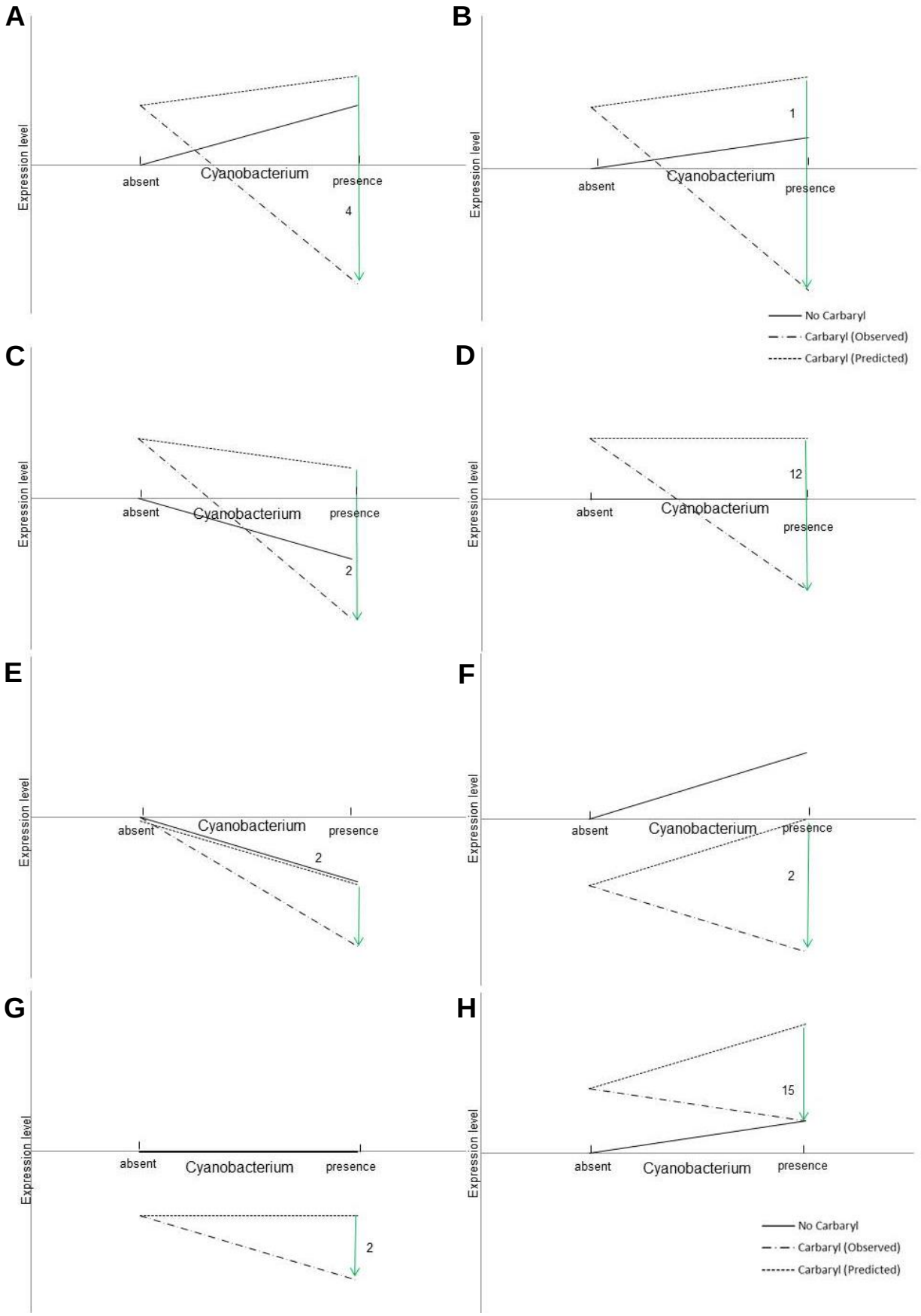
Annotation Definitions	MC	NaCl	Cd
Actin and associated proteins	■		
Acyl-CoA synthetase	■		
Alkaline phosphatase	■		
Alpha-amylase	■	■	
Anaphase promoting complex	■		
Ankyrin	■		■
Aromatic-L-amino-acid-L-histidine decarboxylase	■		
Beta-glucosidase lactase phlorizinhydrolase and related proteins	■		
Beta beta-carotene 1515-dioxygenase and related enzymes			
C-type lectin	■	■	
Carbonic anhydrase	■		
Carboxylesterase and related proteins	■	■	
Chitinase	■	■	■
Collagens -type IV and type XIII- and related proteins	■		■
Conserved Zn-finger protein	■		
Cytochrome P450 CYP11-CYP12-CYP24-CYP27 subfamilies	■		
Cytochrome P450 CYP2 subfamily	■	■	
Cytochrome P450 CYP3-CYP5-CYP6-CYP9 subfamilies	■		
Cytochrome P450 CYP4-CYP19-CYP26 subfamilies	■		
Fasciclin and related adhesion glycoproteins	■		
FKBP-type peptidyl-prolyl cis-trans isomerase	■		
FOG 7 transmembrane receptor	■		
FOG Immunoglobulin C-2 Type-fibronectin type III domains	■		
FOG Leucine rich repeat	■		
FOG Zn-finger	■		
Fucosyltransferase	■		
Galactosyltransferases	■		
Glutamate-gated kainate-type ion channel receptor subunit GluR5 and related subunits	■	■	
Glutathione S-transferase	■		
Heterogeneous nuclear ribonucleoprotein R -RRM superfamily-	■		
Lipid exporter ABCA1 and related proteins ABC superfamily	■		
Meprin A metalloprotease	■	■	■
Monocarboxylate transporter	■		
Neurexin IV	■	■	

Annotation Definitions *(Table E.6 cont.)*	MC	NaCl	Cd
Permease of the major facilitator superfamily			
Peroxidase-oxygenase			
Phosphatidylinositol transfer protein SEC14 and related proteins			
Plasma membrane glycoprotein CD36 and related membrane receptors			
Predicted alkaloid synthase-Surface mucin Hemomucin			
Predicted E3 ubiquitin ligase			
Predicted lipoprotein			
Predicted membrane protein contains DoH and Cytochrome b-561-ferric reductase transmembrane domains			
Predicted transporter			
Putative SAM-dependent rRNA methyltransferase SPB1			
Renal dipeptidase			
RNA polymerase II			
Serine-threonine protein kinase			
Serine-threonine protein kinase and endoribonuclease ERN1-IRE1 sensor of the unfolded protein response pathway			
Serine proteinase inhibitor -KU family-			
Transcription initiation factor TFIID			
Triglyceride lipase-cholesterol esterase			
Trypsin			
Type I phosphodiesterase-nucleotide pyrophosphatase			
UDP-glucuronosyl and UDP-glucosyl transferase			
von Willebrand factor and related coagulation proteins			
Zinc carboxypeptidase			

Appendix F

Supplementary material for Chapter 7

F.1 Figures


A
B
C
D
E
F
G
H
Expression level
absent
Cyanobacterium
presence
4
1
2
12
2
2
15
No Carbaryl
Carbaryl (Observed)
Carbaryl (Predicted)

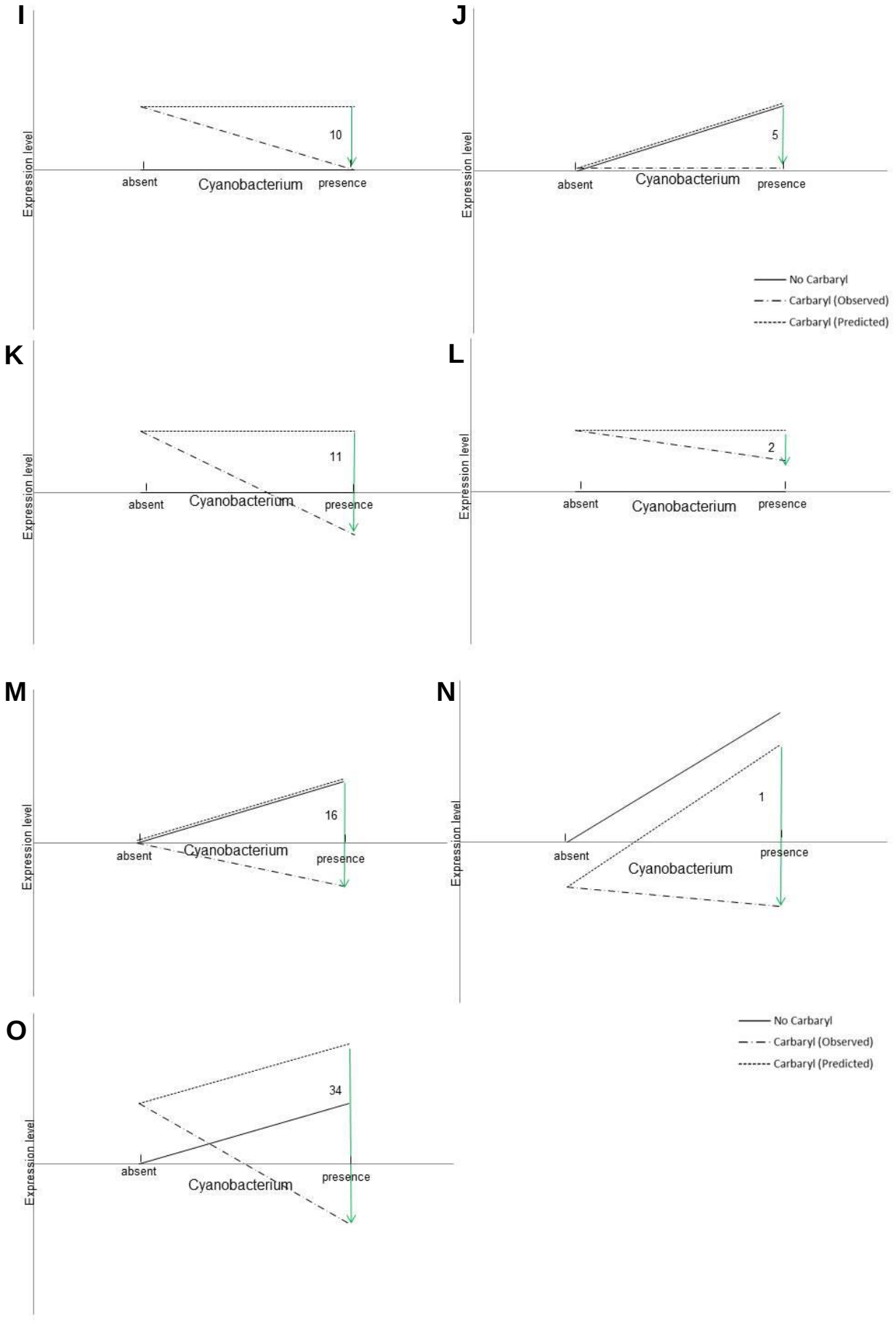
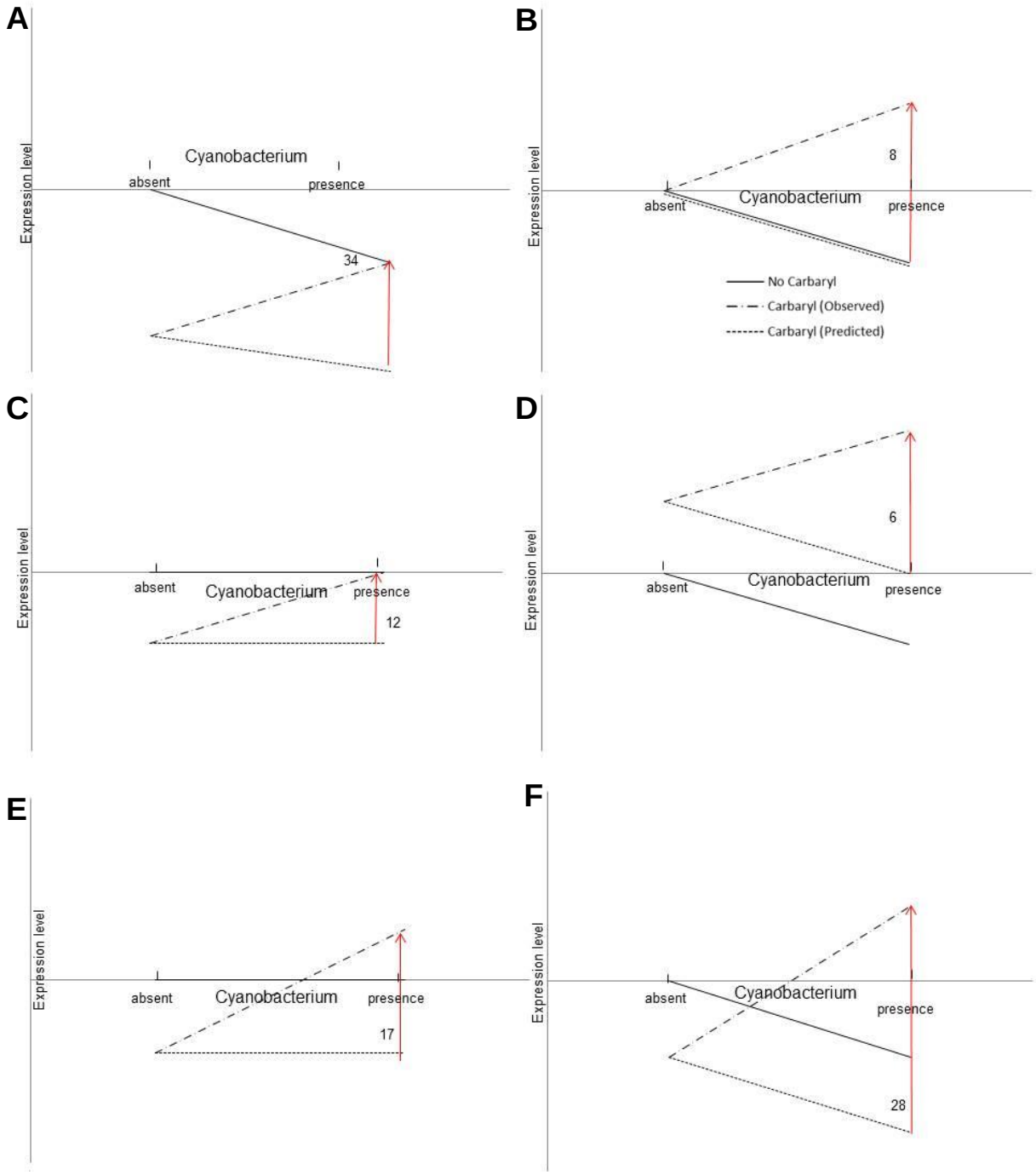

I
J
K
L
M
N
O
Expression level
absent
Cyanobacterium
presence
10
5
11
2
16
1
34
No Carbaryl
Carbaryl (Observed)
Carbaryl (Predicted)

Figure F.1 Reaction norms for antagonistic interacting genes upon cyanobacterium exposure (presence vs. absence) under no carbaryl (solid line), addition of carbaryl (dash-dot line) and predicted addition of carbaryl (dashed line). Green arrows indicate the difference in expression level between observed and predicted expression of the interacting genes. Horizontal axis intersects vertical axis at no differential expression. Numbers indicate the number of genes showing the particular expression pattern.



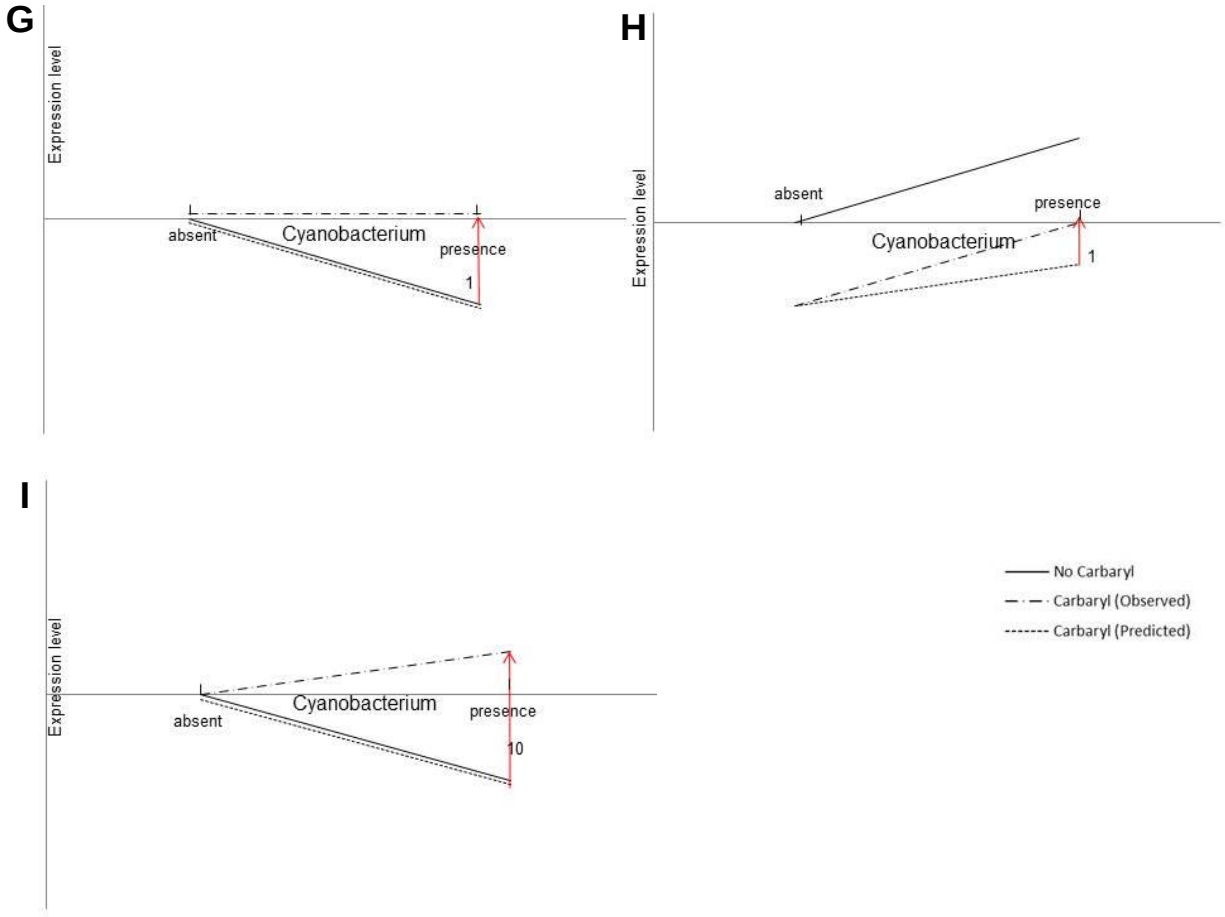


Figure F.2 Reaction norms for synergistic interacting genes upon cyanobacterium exposure (presence vs. absence) under no carbaryl (solid line), addition of carbaryl (dash-dot line) and predicted addition of carbaryl (dashed line). Red arrows indicate the difference in expression level between observed and predicted expression of the interacting genes. Horizontal axis intersects vertical axis at no differential expression. Numbers indicate the number of genes showing the particular expression pattern.

F.2 Tables

Table F.1 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with a Benjamini-Hochberg corrected p-value smaller than 0.05 for the interaction effect are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	16	0	558	676	5	1825
Carbaryl	6786	188	978	12	0	15
Chlorpyrifos	3786	24	5	0	0	0
Deltamethrin	0	647	115	0	0	0
Endosulfan	0	14	261	0	4789	53
Fenoxycarb	4	0	59	4	2369	1067
Tebufenpyrad	107	1	55	0	2971	70
Tetradifon	5	17	153	2417	9	514

Table F.2 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with p-value smaller than 0.05 for the interaction effect and with no overlap between the 95% confidence intervals of observed M-value and of the predicted M-value are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	7	0	26	13	3	52
Carbaryl	48	19	35	2	2	24
Chlorpyrifos	22	6	6	2	0	14
Deltamethrin	0	13	11	1	0	7
Endosulfan	0	9	31	2	35	18
Fenoxycarb	3	2	12	9	14	157
Tebufenpyrad	14	7	18	0	12	17
Tetradifon	4	17	14	35	8	52

Table F.3 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with p-value smaller than 0.05 for the interaction effect and with no overlap between the 90% confidence intervals of observed M-value and of the predicted M-value are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	16	6	74	59	13	152
Carbaryl	286	43	130	6	10	56
Chlorpyrifos	77	16	24	8	0	38
Deltamethrin	3	67	32	7	6	31
Endosulfan	3	17	59	11	111	36
Fenoxycarb	6	3	31	38	67	327
Tebufenpyrad	43	16	42	5	39	90
Tetradifon	9	43	35	192	19	112

Table F.4 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with p-value smaller than 0.05 for the interaction effect and with an absolute M-value larger than 1 are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	291	119	731	830	506	1110
Carbaryl	5253	305	804	348	563	229
Chlorpyrifos	2022	232	445	158	486	250
Deltamethrin	890	524	756	341	152	211
Endosulfan	287	171	510	405	1987	190
Fenoxycarb	274	100	462	772	1732	861
Tebufenpyrad	438	114	421	160	2560	550
Tetradifon	552	737	536	2765	458	392

Table F.5 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with p-value smaller than 0.05 for the interaction effect and with an absolute M-value larger than 2 are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	19	7	33	21	26	45
Carbaryl	329	18	72	6	12	27
Chlorpyrifos	55	11	7	7	12	18
Deltamethrin	13	16	38	8	3	7
Endosulfan	10	24	55	4	145	25
Fenoxycarb	9	0	37	25	82	112
Tebufenpyrad	29	11	36	1	248	20
Tetradifon	30	24	37	70	18	36

Table F.6 Results of the two-way analysis on log transformed total reproduction. For each effect, the number of genes with p-value smaller than 0.05 for the interaction effect and with an absolute M-value larger than 3 are represented.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	2	0	4	2	0	6
Carbaryl	3	3	4	0	3	1
Chlorpyrifos	5	3	0	0	0	1
Deltamethrin	0	0	2	1	0	2
Endosulfan	3	1	9	0	6	5
Fenoxycarb	1	0	5	3	4	10
Tebufenpyrad	2	0	7	0	20	4
Tetradifon	1	3	3	1	1	4

Table F.7 Deviation from non-interaction as defined by equation 7.3, i.e. log of the number of antagonistic genes divided by the number of synergistic genes. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and no overlap between the 95% confidence intervals of observed and predicted M-values.

	Anabaena	***Aphanizomenon***	***Cylindrospermopsis***	***Microcystis***	***Nodularia***	***Oscillatoria***
Acetamiprid	1.32	0	0.68	0.68	1.58	-0.33
Carbaryl	-3.91	-0.15	0.25	0	1	4.58
Chlorpyrifos	3.32	-2.32	1.58	0	0	3.81
Deltamethrin	0	1.74	-0.26	0	0	-2.81
Endosulfan	0	1	4.95	0	0.76	-1.81
Fenoxycarb	1.58	0	0.26	3	0.85	-5.26
Tebufenpyrad	-1.87	0.42	4.09	0	3.46	0.54
Tetradifon	0	1.7	2.58	-0.94	0.74	5.67

Table F.8 Deviation from non-interaction as defined by equation 7.4, i.e. sum of the M-values of the significant genes deviaiting from non-interaction. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and no overlap between the 95% confidence intervals of observed and predicted M-values.

	Anabaena	***Aphanizomenon***	***Cylindrospermopsis***	***Microcystis***	***Nodularia***	***Oscillatoria***
Acetamiprid	-10.6	0	-28.2	-10.6	-7.17	9.21
Carbaryl	99.4	-1.23	-16.8	0.07	-5.45	-57.4
Chlorpyrifos	-52.7	8.57	-1.10	0.71	0	-35.0
Deltamethrin	0	-16.9	1.84	-3.29	0	17.5
Endosulfan	0	-9.61	-91.2	-0.68	-32.4	21.6
Fenoxycarb	-8.12	3.83	-4.45	-19.1	-14.5	302
Tebufenpyrad	14.8	-1.90	-45.9	0	-32.9	-7.53
Tetradifon	-0.05	-26.7	-28.2	23.9	-7.59	-115

Table F.9 Deviation from non-interaction as defined by equation 7.3, i.e. log of the number of antagonistic genes divided by the number of synergistic genes. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and no overlap between the 90% confidence intervals of observed and predicted M-values.

	Anabaena	***Aphanizomenon***	***Cylindrospermopsis***	***Microcystis***	***Nodularia***	***Oscillatoria***
Acetamiprid	0	0	0.08	1.07	3.7	0.19
Carbaryl	-3.2	0.07	1.28	2.32	3	5.67
Chlorpyrifos	0.11	-2.81	3.32	-0.74	0	4.39
Deltamethrin	0	1.56	-0.55	2.32	0	-4.52
Endosulfan	0	0.17	5.88	-0.26	0.34	-1
Fenoxycarb	2	0	0.66	1.69	-0.13	-3.11
Tebufenpyrad	-2.62	0.74	5.25	0	1.54	-0.32
Tetradifon	1.81	-1.05	2.58	-1.03	-0.46	6.77

Table F.10 Deviation from non-interaction as defined by equation 7.4, i.e. sum of the M-values of the significant genes deviaiting from non-interaction. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and no overlap between the 90% confidence intervals of observed and predicted M-values.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	-4.46	-12.9	-30.6	-45.5	-28.5	-26.1
Carbaryl	483	-6.63	-125	-8.30	-13.9	-95.8
Chlorpyrifos	-26.5	22.2	1.99	5.49	0	-17.0
Deltamethrin	-2.58	-57.5	10.0	-8.27	12.3	30.1
Endosulfan	-3.90	-7.91	-150	0.19	-58.8	20.2
Fenoxycarb	-5.53	1.83	-17.3	-46.9	-11.4	478
Tebufenpyrad	59.9	-8.73	-87.3	4.79	-66.0	15.8
Tetradifon	-12.1	18.4	-60.4	126	3.28	-207

Table F.11 Deviation from non-interaction as defined by equation 7.3, i.e. log of the number of antagonistic genes divided by the number of synergistic genes. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and the absolute M-value, i.e larger than 2.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	0.15	2.81	2.49	1.68	4.7	0.32
Carbaryl	-1.22	0.32	2.81	2.32	3.58	4.7
Chlorpyrifos	1.04	-0.26	2.81	-1.32	3.46	2.32
Deltamethrin	0.22	0.36	1.12	0.74	-1.58	-2.58
Endosulfan	0	1	5.78	0	2.91	0.12
Fenoxycarb	-1	0	0.88	2.87	3.42	-5.78
Tebufenpyrad	-2.64	0.81	5.17	0	5.6	-0.58
Tetradifon	3.81	0.24	2.37	-1.22	0.32	5.17

Table F.12 Deviation from non-interaction as defined by equation 7.4, i.e. sum of the M-values of the significant genes deviaiting from non-interaction. Significance at the gene level was determined by the p-value, i.e. smaller than 0.05 for the interaction effect, and the absolute M-value, i.e larger than 2.

	Anabaena	*Aphanizomenon*	*Cylindrospermopsis*	*Microcystis*	*Nodularia*	*Oscillatoria*
Acetamiprid	-3.74	-16.9	-63.2	-28.5	-59.9	-13.4
Carbaryl	283	-8.79	-129	-9.11	-29.6	-60.8
Chlorpyrifos	-56.9	1.58	-15.6	6.97	-22.7	-29.8
Deltamethrin	-3.18	-4.59	-32.2	-6.11	6.89	13.9
Endosulfan	-2.19	-22.0	-144	-0.64	-269	-4.70
Fenoxycarb	6.71	0	-25.8	-44.0	-162	265
Tebufenpyrad	45.7	-7.13	-92.9	2.03	-578	11.3
Tetradifon	-58.8	-10.8	-61.8	59.8	-7.67	-90.9

Table F13 Genes represented by their JGI id and the number of combinations in which each gene was determinedinteracting, for genes with a frequence higher than 4.

Gene ID	Frequence
JGI_V11_104169	10
JGI_V11_108530	7
JGI_V11_16207	7
JGI_V11_236269	5
JGI_V11_263306	5
JGI_V11_274020	7
JGI_V11_308504	13
JGI_V11_313056	5
JGI_V11_314387	5
JGI_V11_320123	7
JGI_V11_328955	5
JGI_V11_43964	5
JGI_V11_97780	6
JGI_V11_104169	10
JGI_V11_108530	7
JGI_V11_16207	7
JGI_V11_236269	5
JGI_V11_263306	5
JGI_V11_274020	7
JGI_V11_308504	13
JGI_V11_313056	5

Table F14 Gene IDs of the genes that demonstrated both antagonistic and synergistic interaction in different combinations as well as the number of times a gene was defined as antagonistic or synergistic.

Gene ID	Antagonistic	Synergistic
JGI_V11_103668	1	1
JGI_V11_106059	1	3
JGI_V11_114507	1	1
JGI_V11_17148	1	1
JGI_V11_204027	1	2
JGI_V11_231626	1	1
JGI_V11_236269	1	4
JGI_V11_241311	1	1
JGI_V11_242681	1	1
JGI_V11_251597	1	1
JGI_V11_263306	3	2
JGI_V11_300401	2	1
JGI_V11_301410	1	1
JGI_V11_301602	3	2
JGI_V11_30204	1	1
JGI_V11_303449	1	1
JGI_V11_304104	3	1
JGI_V11_304311	1	1
JGI_V11_306630	1	1
JGI_V11_306763	2	1
JGI_V11_308504	12	1
JGI_V11_308817	1	1
JGI_V11_310452	1	1
JGI_V11_313112	2	1
JGI_V11_315160	1	1
JGI_V11_315713	1	2
JGI_V11_317504	3	1
JGI_V11_318090	1	1
JGI_V11_325284	1	1
JGI_V11_326128	2	1
JGI_V11_327371	1	1
JGI_V11_328069	1	1
JGI_V11_328955	1	4
JGI_V11_336639	1	2
JGI_V11_43964	1	4

F.3 Derivation of Independent Action Model:

Jonker et al. (2005) defined the independent action model within their conceptual framework to test deviations from non-interaction as follows:

$$y = k\Phi\left(\Phi^{-1}\left[k\left(\frac{1}{1+(\frac{x_1}{x_{50_1}})^{s_1}}\right)\left(\frac{1}{1+(\frac{x_2}{x_{50_2}})^{s_2}}\right)\right]+G\right) \text{ (eq.3.2)}$$

Parameters for this function were described in section 3.2.4. However, Jonker et al. (2005) derived this equation from the original Bliss independence model which is:

$$y = \mathrm{k}\prod_{i=1}^{n} q_i(c_i) \text{ (eq. F.1)}$$

In which y and k are the same parameters as in eq. 3.2, qi(ci) is the probability of non-response for stressor i based on the single response function, i.e. eq. 2.1) To calculate the response of the combination at a single given concentration under the hypothesis of no interaction (i.e. eq. F.1), Jonker et al. (2005) defined eq. F.1 as follows:

$$y = \mathrm{k} * y_1 * y_2 \text{ (eq. F.2)}$$

In which y and k are the same parameters as in eq. 3.2, y_1 and y_2 are the responses of the animal upon exposure to stressor 1 and stressor 2 alone at the same given concentration as in the mixture. These responses are determined by eq. 2.1. Jonker et al. (2005) also expresses the responses relative to control so when using the actual raw response data, eq. F.2 can be written as follows with K defined as the control response relative to the control:

$$\frac{Y}{Y_{Ctr}} = \frac{Y_{Ctr}}{Y_{Ctr}} * \frac{Y_1}{Y_{Ctr}} * \frac{Y_2}{Y_{Ctr}} \text{ (eq. F.3)}$$

As a consequence, when using raw data, k becomes 1. Finally, modifying the equation for combinations of pesticide and cyanobacteria results in the following equation wich equals eq. 7.1:

$$\frac{Y_{mix}}{Y_{Ctr}} = \frac{Y_{pesticide}}{Y_{Ctr}} * \frac{Y_{cyano}}{Y_{Ctr}} \text{ (eq. F.4)}$$