Interaction of *Daphnia magna* with cyanobacteria: New insights from using high-resolution LC-MS

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General introduction

Cyanobacteria are photoautotrophic prokaryotes which inhabit our planet for at least 3.5 billion years (Schopf and Packer 1987). They display numerous adaptive features which explain their immense evolutionary success: Cyanobacteria are able to harvest a broad wavelength range of sunlight due to the light harvesting phycobiliproteins phycocyanin, allophycocyanin and phycoerythrin (Glazer 1989). The blue-colored phycocyanins are the reason for the misleading but commonly used term blue-green algae. In addition, cyanobacteria acquire nutrients such as phosphorous and nitrogen, but also trace metals with a high efficiency and some species are even capable of fixating dinitrogen (N_2) from the atmosphere (Gallon 1992). This enables them to access a nitrogen source which is unavailable for most other organisms (Gehringer and Wannicke 2014). Furthermore, many cyanobacterial species contain gas vesicles which provide them with the ability to regulate their buoyancy in order to position themselves at depth with optimal light conditions (Walsby 1994). All of these attributes contribute to one of the most striking features of cyanobacteria, which is the ability to form blooms (El-Shehawy et al. 2012; Paerl and Paul 2012). In freshwaters, these blooms are usually dominated by cyanobacteria of the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix* and *Limnothrix* (Dokulil and Teubner 2000). Such cyanobacterial blooms are not only a nuisance with respect to the domestic, industrial and recreational usage of water bodies (Paerl and Huisman 2009; Stewart et al. 2006), but they can also be a serious hazard with regard to public health (Jochimsen et al. 1998). Cyanobacteria frequently produce large amounts and also a high variability of often toxic secondary metabolites, whose effects range from liver, digestive and skin diseases to neurological impairment and death (Carmichael et al. 2001; Cox et al. 2003; Paerl and Huisman 2009). Numerous other metabolites do not specifically display toxic properties with regard to vertebrates, but are important in the ecological context (Gademann and Portmann 2008). An important example are cyanobacterial depsipeptides which act as potent inhibitors of grazer gut proteases (Martin et al. 1993). The extent and frequency of cyanobacterial blooms has increased in recent years, due to worldwide increased anthropogenic impacts on freshwater ecosystems (Paerl and Huisman 2008; Woodward et al. 2010). While in general the consequences of human impact on ecosystems are diverse and difficult to predict, there is a general consensus that cyanobacteria are generally favored by anthropogenic impacts (O'Neil et al. 2012; Paerl and Huisman 2008; Paerl and Huisman 2009). The influences that are administrated by humans

include the acceleration of eutrophication processes through nutrient input and generally increased temperatures due to global warming which both have been repeatedly demonstrated to contribute to cyanobacterial bloom formation (O'Neil et al. 2012; Paerl and Huisman 2008; Paerl and Huisman 2009).

Besides being a nuisance and serious hazard to public health, harmful cyanobacterial blooms strongly impact freshwater ecosystems, especially with respect to herbivorous zooplankton (Ghadouani et al. 2003; Paerl and Huisman 2009; Wilson et al. 2006). In particular unselectively filter-feeding cladocerans of the genus *Daphnia* are affected due to their inability to discriminate between good and bad food items (Demott 1982). The diet of daphnids is mostly constrained by the food size. The edible size range usually lies within 0.1 to 55 µm due to the mesh size of the filter combs and the size of the carapace gape (Geller and Müller 1981) and single-celled cyanobacteria lie well within this size range.

There are about 150 *Daphnia* species described so far ranging in size from 0.2 to 5 mm. They occur ubiquitously in standing freshwaters worldwide, ranging from deep permanent lakes to small and shallow temporary ponds. *Daphnia* species constitute the major herbivores for algae and cyanobacteria in most freshwater ecosystems. Simultaneously, they are a major prey organism for invertebrate predators and planktivorous fish. Accordingly, daphnids play a key role in aquatic ecosystems with regard to the transfer of energy and carbon from primary producers to higher trophic levels (Persson et al. 2007; Von Elert et al. 2003). However, cyanobacteria have a low dietary quality for daphnids due to several reasons: Some cyanobacterial strains form filaments or colonies, which exceed the edible size of *Daphnia* and subsequently clog their filter apparatus due to mechanical interference (Porter and Mcdonough 1984). Moreover, cyanobacteria lack several nutritional components such as sterols and polyunsaturated fatty acids (PUFAs), which are essential for daphnids (Martin-Creuzburg et al. 2005; Martin-Creuzburg et al. 2008; Von Elert 2002; Von Elert et al. 2003). Finally, toxic cyanobacterial metabolites negatively affect *Daphnia* fitness parameters (Lürling and van der Grinten 2003; Rohrlack et al. 2001; Von Elert et al. 2012).

Due to these factors, the transfer of carbon and energy, particularly in eutrophic lakes is often not constrained by food availability, but by food quality.

Water resource managers are often concerned with the control of harmful cyanobacterial blooms. Strategies for bloom prevention include biomanipulative approaches (Hansson et al. 1998; Shapiro et al. 1975), which aim at food chain manipulations. There, higher trophic

levels (such as fish community structure and abundance) are altered in order to increase the size and abundance of herbivorous zooplankton (Shapiro et al. 1975; Shapiro and Wright 1984). Accordingly, the presence and abundance of *Daphnia* is of major importance for the success of food chain manipulation efforts (Leibold 1989). Therefore, a better understanding of the *Daphnia* - cyanobacteria interaction is not just of interest from the ecological perspective, but might as well provide practical benefits with respect to water quality management.

Experimental studies that addressed the interaction of daphnids with cyanobacteria reveal rather contradictory results. Several studies demonstrated the negative effects of cyanobacteria on zooplankton populations (Ghadouani et al. 2003; Tillmanns et al. 2008; Wilson et al. 2006). On the other hand, a suppression of cyanobacterial bloom formation due to the presence of *Daphnia* was observed in other studies (Chislock et al. 2013a; Sarnelle 2007). In agreement with this, several authors reported persistent coexistence of cyanobacteria and *Daphnia* in nature (Ka et al. 2012; Sarnelle 2007).

Daphnia species are able to adapt to cyanobacterial diets. The nutrient content in lakes is usually well correlated with the abundance of cyanobacteria. Hence, the investigation of *Daphnia* clones from distinct lakes that differ in their nutrient content revealed patterns of local adaptation of these daphnids with regard to cyanobacterial diets (Sarnelle and Wilson 2005). In addition, hatched individuals from ancient resting eggs derived from core samples of a lake that experienced a period of eutrophication revealed the rapid evolution of resistance against cyanobacteria (Hairston et al. 1999). Daphnids are also able to develop higher resistance against cyanobacterial diets on a short timescale and are capable of transferring this acquired resistance to the next generation via maternal effects (Gustafsson and Hansson 2004; Gustafsson et al. 2005; Schwarzenberger and Von Elert 2013). As a result of these adaptive processes, the sensitivity towards cyanobacterial diets differs strongly between *Daphnia* species (Tillmanns et al. 2008) and between genotypes of the same species (Chislock et al. 2013b; Jiang et al. 2013; Sarnelle and Wilson 2005). Such different degrees of adaptation are probably a main reason for the contradictory findings with regard to *Daphnia* - cyanobacteria interactions.

In conclusion, a deeper understanding of the physiology behind this interaction can be expected to be highly beneficial for practical purposes in water resource management and in addition deliver new insights from an ecological as well as from an evolutionary perspective.

A major goal of this dissertation was the investigation of these physiological mechanisms and their ecological implications.

In **chapter 1** I focused on the most frequent and best studied group of cyanotoxins, the microcystins (MCs) (Dittmann and Wiegand 2006). MCs are heptapeptides consisting of seven amino acids two of which are variable (referred to in the MC nomenclature by the last two letters according to the amino acid one letter abbreviation code) and contribute mostly to the variability of this group of toxins. Among about 80 described MCs, microcystin-LR (MCLR) (Fig.1) is the most frequent and best studied one (van Apeldoorn et al. 2007).

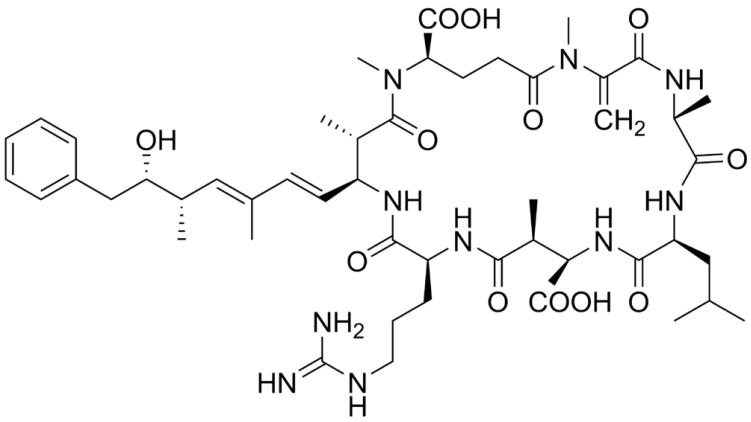

Fig.1: Chemical structure of microcystin-LR.

MCs are very potent toxins which inhibit the activity of eukaryotic protein phosphatases PP1 and PP2A (Honkanen et al. 1990; Runnegar et al. 1995). As a consequence, cells suffer from hyperphosphorylation of cell compounds leading to cell damage (Eriksson et al. 1990; Ohta et al. 1992). However, the toxicological effects of MCs are very diverse and only known to a limited extent (Dittmann and Wiegand 2006). A lot of studies showed pronounced negative effects of dissolved MCs as well as MC containing diets on *Daphnia* fitness parameters (Lürling 2003a; Rohrlack et al. 2001). Hence, differences in *Daphnia*'s ability to detoxify cyanobacterial secondary metabolites, such as MCLR might constitute an explanation for the huge variety within *Daphnia*'s sensitivity towards cyanobacteria in general and cyanobacterial toxins in particular.

With regard to MCLR detoxification in *Daphnia*, glutathione-S-transferase (GST) was assumed to be the key enzyme. GSTs catalyze the conjugation of cellular compounds with the tripeptide glutathione (GSH) (Hayes et al. 2005). As a consequence, these compounds

increase in hydrophilicity and are therefore easier to excrete. MCs constitute suitable substrates for GSTs (Kondo et al. 1992) and have been demonstrated to be of major importance for several aquatic organisms with regard to MC detoxification (Campos and Vasconcelos 2010; He et al. 2012; Li et al. 2014; Zhang et al. 2009). However, the knowledge about MCLR detoxification via GST in *Daphnia* is confined to one single *in vitro* study. In this study, the formation of an enzymatically formed MCLR-GSH conjugate could be shown after adding MCLR and GSH to a *Daphnia* crude extract (Pflugmacher et al. 1998). The question if this process is of relevance in the living organism remained unanswered to date. Thus, there was no resilient knowledge about MC detoxification in daphnids *in vivo* at the beginning of this study.

The purpose of the first study (**chapter 1**) was to investigate whether MCLR detoxification via GST actually occurs in living *Daphnia*. To test this, I conducted exposure experiments with *Daphnia magna* and the MCLR containing cyanobacterial strain *Microcystis aeruginosa* PCC7806. The novel approach here was not only to detect and quantify conjugation products of MCLR with GSH, but also to quantify unconjugated MCLR. The measurement of these compounds was carried out in all parts of an enclosed experimental setup (incubation media, cyanobacterial cells and *Daphnia* tissue). This approach allowed me to sum up the MCLR amounts of these different parts in order to obtain the overall MCLR amount within the experiment. I hypothesized that this overall MCLR amount should decrease in the presence of daphnids in case of significant biotransformation of MCLR in comparison to controls in the absence of daphnids. This accounts not only for conjugation with GSH catalyzed by GST, but for all other possible biotransformation or biodegradation processes as well. The detection and quantification of MCLR and MCLR conjugation products was carried out making use of liquid chromatography (LC) coupled with high resolution mass spectrometry (MS). Through this technique, I was able to detect very low amounts of target compounds with a very high mass accuracy of less than 3 parts per million (ppm) mass deviation. As a result, I was able to demonstrate that biotransformation processes are of minor importance in daphnids with regard to dietary MCLR. Instead, my findings suggest the presence of transport mechanisms capable of exporting unconjugated MCLR from the body of the daphnids.

The purpose of two follow up studies (**chapters 2** and **3**) was to further investigate these so far unknown transport mechanisms in *Daphnia*: Several studies demonstrated that cyanobacterial strains that do not produce MCs also have pronounced negative effects on *Daphnia* fitness parameters (Lürling 2003b; Schwarzenberger et al. 2009; Tillmanns et al.

2008). Therefore, it is important to take cyanobacterial compounds other than microcystins into account, in order to reveal the physiological background that might explain the differing sensitivities of daphnids against dietary cyanobacteria.

In **chapter 2** I used the mass spectral dataset that had already been obtained in the first study (**chapter 1**) in order to find out if such cyanobacterial compounds might be subjected to biotransformation or whether they would be exported out of *Daphnia* tissue similar to MCLR. For this purpose, I investigated the fate of three cyanopeptolines (CP-A, B and C) and four cyclamides (microcyclamide 7806A and aerucyclamides B, C and D) which can be found in the cyanobacterial strain *M. aeruginosa* PCC7806 as well. CPs are depsipeptides which act as potent inhibitors of the serine proteases trypsin and chymotrypsin (Gademann and Portmann 2008; Martin et al. 1993). These two groups of proteases account for more than 80 % of the proteolytic activity in the gut of *Daphnia magna* (Von Elert et al. 2004). Cyclamides represent another widely distributed class of cyanobacterial peptides, which had previously been reported to be detrimental for crustaceans (Ishida et al. 2000).

Two types of trans membrane transporters were identified that constitute promising candidates for the export of dietary MCLR. The human multi drug resistance (MDR) mechanism, also referred to as P-glycoprotein (P-gp), is associated with the export of detrimental substances out of mammalian cells (Kim 2002). A similar export system can be found in aquatic animals as well, where it is referred to as multi xenobiotic resistance (MXR) (Kurelec 1992). This MXR mechanism is of major importance for aquatic animals and has been hypothesized to be the main reason for the insensitivity of bivalves against MCs (Contardo-Jara et al. 2008). Moreover, the up-regulation of a P-gp transporter in the fish *Jenynsia multidentata* could be demonstrated in relation to an exposure to MCLR (Ame et al. 2009). Another putative transporter type might be represented by organic anion transporting polypeptides (Oatps). This transporter type is widely distributed among the animal kingdom and has been repeatedly shown to be able to transport MCs (Fischer et al. 2005; Hagenbuch and Meier 2003; Hagenbuch and Meier 2004). However, studies regarding the MC transport via Oatps are mostly confined to humans or other mammals (Feurstein et al. 2009; Fischer et al. 2005) and nothing is known about the function of this transporter type in *Daphnia*.

Prior studies confirmed the presence of P-gp related sequences in daphnids (Sturm et al. 2009). Here I was able to present first evidence for the presence of Oatps in *Daphnia* (**chapter 3**). In this study experiments were performed in order to find out if one of these or

both transporter types are actually involved in the export of dietary MCLR. For this purpose, two different experimental approaches were applied: In the first approach *D. magna* individuals were fed with a 100 % diet of *M. aeruginosa* PCC7806 in the presence or absence of specific inhibitors of P-gp or Oatp transporters dissolved in the surrounding medium. Thereafter, I measured the MCLR amount within the *Daphnia* tissue to check for differences due to inhibitor interference with respective transporters. I hypothesize that inhibition of these transporters should result in increased amounts of MCLR within the *Daphnia* tissue. In a second approach I used quantitative polymerase chain reaction (qPCR) in order to find out if daphnids react to MC containing diets with increased expression levels of Oatp or P-gp genes. A reaction on the gene expression level would constitute a fast inducible defense mechanism in the daphnids.

The necessity of fast inducible defense mechanisms in *Daphnia* is underlined by findings presented in **chapter 4**. There, I conducted an experiment with a time series of phytoplankton samples obtained from a small urban pond (Aachener Weiher, AaW) throughout a summer season. Cyanobacterial biomass in freshwater lakes or ponds often peaks in late summer or early fall (Sommer et al. 1986). Due to the fact that many cyanobacteria contain protease inhibitors (Gademann and Portmann 2008), Kuster et al (2013) could demonstrate a similar seasonal variation of the protease inhibition potential of natural phytoplankton samples from the AaW. These phytoplankton samples were used in order to find potential new protease inhibitors and unravel seasonal succession patterns within the cyanobacterial community. I performed mass spectral analyses to obtain a metabolomic profile for each phytoplankton sample. These data were then combined with the analyses of their inhibitory potential using multivariate statistics. This entirely new approach turned out to be very successful not only with respect to the detection of new putative protease inhibitors, but also for the unraveling of seasonal succession patterns of cyanobacteria.

As a whole, the studies within this dissertation are designed to shed light on the physiological basis of the interaction between *Daphnia* and cyanobacteria. The use of modern high resolution LC-MS technology allowed for deeper insights into detoxification and transportation processes in *Daphnia*. The widespread assumption of MCLR detoxification via GST is being challenged, while an alternative hypothesis is being investigated. This alternative hypothesis for the first time considers transport processes with respect to dietary cyanobacterial compounds. The usage of high resolution LC-MS technology also facilitated

the investigation of seasonal succession patterns in natural lake phytoplankton samples for which a new methodology has been developed.

Chapter 1

Dietary exposure of *Daphnia* to microcystins: No *in vivo* relevance of biotransformation

Abstract

Anthropogenic nutrient input into lakes has contributed to the increased frequency of toxic cyanobacterial blooms. *Daphnia* populations have been shown to be locally adapted to toxic cyanobacteria and are able to suppress bloom formation; little is known about the physiology behind this phenomenon. Microcystin-LR (MCLR) is the most widespread cyanobacterial toxin, and, based on *in vitro* experiments, it is assumed that the enzyme glutathione-S-transferase (GST) might act as the first step of detoxification in *Daphnia* by conjugating MCLR with glutathione.

In the present study *Daphnia magna* was fed a diet of 100 % *Microcystis aeruginosa* PCC7806, a cyanobacterial strain that contains MCLR in high amounts (4.8-5.6 fg cell^{-1}), in order to test for a possible conjugation of MCLR with GST in *Daphnia in vivo*. We used high-resolution LC-MS to analyze incubation water, cyanobacterial cells and *Daphnia* tissue for the presence of MCLR conjugation products as well as unconjugated MCLR.

Newly formed conjugation products were detected neither in *Daphnia* tissue nor in the incubation water. Moreover, the presence of *Daphnia* led to a decrease in unconjugated MCLR in the cyanobacterial cell fraction due to grazing, in comparison to a control without daphnids, which was well reflected by a similar increase of MCLR in the respective incubation water. As a consequence, the MCLR content did not change due to *Daphnia* presence within the entire experimental setup. In summary, MCLR ingestion by *Daphnia* led neither to the formation of conjugation products, nor to a decrease of unconjugated MCLR.

GST-mediated conjugation thus seems to be of minor relevance for microcystin (MC) tolerance in *Daphnia in vivo*. This finding is supported by the fact that GST activity in *Daphnia* feeding on the MC-containing wildtype or a MC-free mutant of *M. aeruginosa* PCC7806 revealed an identical increase of specific activity in comparison to a cyanobacteria-free diet. Therefore, the frequently observed induction of GST activity upon exposure to toxic cyanobacteria is not a specific MC effect but a general cyanobacterial effect. This suggests that GST in *Daphnia* is involved in an oxidative stress response rather than in the specific detoxification of MCs. Furthermore, our results indicate the presence of an efficient transport mechanism which efficiently removes unconjugated MCLR from the *Daphnia* tissue. Further studies are needed to elucidate the nature of this transport mechanism.

Introduction

Cyanobacteria can be regarded as a key taxon in many aquatic ecosystems. Particularly the increased input of nutrients into lakes has led to more frequently occurring cyanobacterial blooms (Dokulil and Teubner 2000; O'Neil et al. 2012) and high cyanobacterial abundances negatively affect herbivorous zooplankton, e.g. the unselective filter-feeder *Daphnia* (Ghadouani et al. 2003; Wilson et al. 2006). The strong impact of cyanobacteria on freshwater ecosystem services is in part due to the very low food quality of cyanobacteria for herbivorous zooplankton (Porter and Mcdonough 1984; Von Elert et al. 2003), but at least partly it can also be assigned to cyanobacterial secondary metabolites, a lot of which are known to have detrimental effects on zooplankton (Hansson et al. 2007; Rohrlack et al. 1999).

Among the various cyanotoxins known, microcystins (MCs) are the most frequent and best-studied ones. MCs are heptapeptides which mainly differ in amino acid moieties at two positions. MCs are known to be potent inhibitors of the protein phosphatases PP1 and PP2A (Honkanen et al. 1990; Runnegar et al. 1995), both of which play an important role in the dephosphorylation of cellular compounds. Accordingly, inhibition of PPs by MCs leads to hyperphosphorylation, causing severe cell damage (Eriksson et al. 1990; Ohta et al. 1992). However, the toxicological effects of microcystins are diverse and only understood to a limited extent (Dittmann and Wiegand 2006).

MCs as well as MC-containing cyanobacteria have pronounced negative effects on herbivorous zooplankton (Rohrlack et al. 2001). In particular daphnids are negatively affected by cyanobacteria producing MCs. As unselective filter feeders daphnids are not capable of discriminating between good and bad food items. However, cyanobacterial strains lacking microcystins also lead to reduced fitness of *Daphnia* (Lürling and van der Grinten 2003; Schwarzenberger et al. 2009; Tillmanns et al. 2008). The sensitivity to toxic cyanobacteria thereby differs among *Daphnia* species (Tillmanns et al. 2008) and also among clones of the same species (Jiang et al. 2013; Sarnelle and Wilson 2005).

We argue that the elucidation of the physiological background accounting for these differences in sensitivity to MCs is crucial for several reasons.

There are multiple strategies in water resource management aiming at the control of harmful cyanobacterial blooms, e.g. efforts to reduce nutrient intake or application of herbicides; the manipulation of food chains in freshwater ecosystems has also been proposed (Hansson et al. 1998; Shapiro et al. 1975). The basic concept usually involves the manipulation of higher

trophic levels, for example by adding piscivorous fish or by removing planktivorous fish, in order to increase the size and abundance of herbivorous zooplankton (Shapiro et al. 1975; Shapiro and Wright 1984). Hence, the biomass of *Daphnia* is of major importance for the success of food chain manipulation efforts (Leibold 1989) and has been shown to effectively suppress cyanobacterial biomass in eutrophic lakes (Chislock et al. 2013; Shapiro and Wright 1984). On the other hand, cyanobacteria can be regarded as a major factor explaining declines in *Daphnia* abundance due to increased eutrophication (Ghadouani et al. 2003). We argue that the differing sensitivity of *Daphnia* species or clones facing cyanobacterial toxins might be an important factor affecting the outcome and success of food chain manipulation efforts. Therefore, a deeper understanding of the way in which daphnids cope with cyanobacterial toxins on the physiological level might facilitate the improved application of such biomanipulative approaches.

In addition to that, it has been shown that *Daphnia* populations adapt to the presence of toxic cyanobacteria locally (Sarnelle and Wilson 2005) as well as temporally (Hairston et al. 1999). In light of this, elucidation of the physiological processes leading to reduced cyanotoxin sensitivity might allow us to better understand the local adaptation patterns and subsequently allow a mechanistic understanding of the microevolutionary processes in *Daphnia* populations.

Only recently Kozlowsky-Suzuki et al. (2012) showed a tendency of biodilution of MCs on the primary consumer level, when investigating MCs distribution in freshwater foodwebs in a meta analysis. This is in line with earlier findings by Ibelings et al. (2005), who revealed the absence of biomagnification of MCs through an aquatic food web. Together with MC's low hydrophobicity (Ward and Codd 1999) an effective MC detoxification or depuration mechanism on the primary consumer level could explain this pattern of MC distribution in lake food webs.

Pflugmacher et al. (1998) proposed a MC detoxification in daphnids by conjugation of MC with glutathione (GSH), which includes the involvement of glutathione-S-transferase (GST) as the first step of detoxification. This detoxification mechanism is assumed to be widespread in aquatic animals (Campos and Vasconcelos 2010) and could, if differently well developed, explain differences in sensitivity of *Daphnia* genotypes to MCs.

GSTs occur in a number of isoforms and have numerous functions, among which are the detoxification of xenobiotics and the conjugation of cell compounds that are generated due to

oxidative stress, e.g. lipid peroxides, with glutathione (GSH) (Hayes et al. 2005; Hellou et al. 2012; Ketterer and Meyer 1989).

An effect of MC (cell-bound or free) exposure of daphnids on their GST activity has been demonstrated several times (Chen et al. 2005; Ortiz-Rodriguez and Wiegand 2010). Moreover, an increase of GST activity due to MC exposure has been shown for a variety of other organisms, e.g. mammals (Buratti et al. 2013; Gehringer et al. 2004), fish (He et al. 2013; Sotton et al. 2012), crustaceans (Pflugmacher et al. 2005) and plants (Pflugmacher et al. 2001).

However, experimental evidence that MC conjugates were formed enzymatically by daphnids is unfortunately confined to one *in vitro* study making use of crude extracts of *Daphnia* tissue with the addition of external substrates (MCs and GSH) (Pflugmacher et al. 1998). In contrast, the formation of MC conjugation products *in vivo* due to MC exposure (dietary or directly injected into the organism) could be shown for a variety of aquatic organisms (He et al. 2012; Li et al. 2014; Zhang et al. 2009; Zhang et al. 2012), but not for daphnids. Hence, the question as to whether GST biotransformation of MCs in daphnids occurs *in vivo* remains unanswered.

It is noteworthy that MCs are known to cause oxidative stress in daphnids (Ortiz-Rodriguez and Wiegand 2010) as well as in other aquatic animals (Amado and Monserrat 2010; Campos and Vasconcelos 2010). Here we hypothesize that the frequently reported increase in GST activity in *Daphnia* due to MC exposure might be a consequence of oxidative stress and does not necessarily indicate MC-detoxification via biotransformation. In this respect, the demonstrated increase of MC conjugation products in crude animal tissue extracts provides insufficient evidence for the relevance of the GST detoxification of MCs in the living animal.

In the present study we investigate whether a cyanobacterial diet of the MCLR-containing strain *Microcystis aeruginosa* PCC7806 leads to the formation of MCLR conjugation products in daphnids *in vivo*. In a short term exposure experiment (24h) *Daphnia magna* was fed with 100 % MC-containing cyanobacterium *M. aeruginosa* PCC7806. Afterwards, the total amounts of MCLR-GSH and unconjugated MCLR in the incubation medium, the cyanobacterial cells and the *Daphnia* tissues were determined using high performance liquid chromatography (HPLC) coupled with high resolution electrospray ionization (ESI) mass spectrometry (MS). In addition, we screened for other putative metabolites of the GST detoxification pathway, namely MCLR-Cysteine (Cys), MCLR conjugates with the dipeptides Cys-Glu (cysteine and glutamic acid) or Cys-Gly (cysteine and glycine) and with mercapturic

acid of MCLR. We argue that all putative biotransformation processes must result in an overall decrease of the total amount of free MCLR in the experimental setup.

During a subsequent chase experiment, we kept the daphnids (from the short term exposure experiment) for another 54h in the absence of cyanobacteria in order to account for the release of free MCLR or biotransformation of MC that occurs with a time delay after ingestion of MCLR.

In a separate experiment we measured GST activities in daphnids fed with MC containing or MC free cyanobacteria.

Materials and Methods

Culture conditions

Daphnia magna clone P (P132.85; derived from a pond (Driehoek) in Heusden (Netherlands), N51°44'01", E5°08'17") (De Meester 1994) was cultured in 10 L of membrane-filtered (0.2 µm), aged tap water at 20 °C. This water, in the following referred to as medium, was used for all experiments, if not stated otherwise. *D. magna* clone P was chosen because it exhibits a very high tolerance to cyanobacterial diets in general (Schwarzenberger 2010). The animals were fed every second day with the green algae *Chlamydomonas klinobasis* (Chlorophyceae, strain 56, culture collection of the Limnological Institute at the University of Konstanz, Germany) (approximately 2 mg particulate organic carbon (POC) L^{-1}). The medium was replaced on a weekly basis. Animals of different sizes were chosen randomly from the culture for the experiments. Due to the high density of individuals in all experiments, this resulted in a more-or-less equal *Daphnia* fresh weight in the different treatments (data not shown). The green algae *C. klinobasis* and *Cryptomonas* sp. (Cryptophyceae, SAG 26.80; culture collection of algae at the University of Göttingen, Göttingen, Germany) were cultivated semi-continuously in cyanobacteria medium (Von Elert and Jüttner 1997) (constant light: 90 - 100 µE m^{-2} s^{-1}; at 20 °C) with a 20 % medium exchange every two days. *Microcystis aeruginosa* PCC7806 and a genetically engineered mutant form (mcy-) of the strain, which lacks the ability to produce MCs (Dittmann et al. 1997), were grown as chemostat cultures in cyanobacteria medium (constant light: 50 µE m^{-2} s^{-1}; at 20 °C; dilution rate 0.23 per day). The wildtype (WT) of strain PCC7806 contains the microcystins Microcystin-LR (MCLR, L = leucine, R = arginine) and D-Asp3-Microcystin-LR (D - aspartic acid on position 3) in high amounts, experimentally confirmed via LC-MS measurements (data not shown). Food

concentrations in terms of particulate organic carbon (POC) were estimated from photometric light extinction (470 nm) and from previously determined carbon extinction equations.

Enzyme activity measurements

Pretreatment and preparation of Daphnia *homogenates*

Three replicates, each with 50 adult individuals of *D. magna* of various sizes, were kept in 125 mL medium for 24 h. The animals were fed with *Microcystis aeruginosa* PCC7806 and its MC-deficient mutant form (mcy-) (each treatment with 5 mg POC L^{-1}). A food control received *Chlamydomonas klinobasis* (5 mg POC L^{-1}); a starvation control without any food was also performed. After a 24 h exposure the daphnids were transferred into 1.5 mL test tubes by filtering them through a 200 µm gauze, followed by three washing steps with 20 mL of ultrapure water. After removal of the ultrapure water, we added 250 µL 0.1 M potassium phosphate buffer containing 0.1 mM ethylene diamine tetra acetate (EDTA) at a pH of 6.5 to each *Daphnia* tissue sample. Samples were then immediately frozen at -80 °C. After thawing, the samples were homogenized using an electric homogenizer and then centrifuged (2 min, 11.000 g); the supernatant was used as enzyme extract in the enzyme activity measurements. To normalize enzyme activity, we determined the general protein concentration (mg protein mL^{-1}) of the samples photometrically using a Nanodrop (Thermo Scientific) at an absorption of 280 nm.

Glutathione-S-transferase activity measurements

Measurements of glutathione-S-transferase (GST) activity were carried out using the method of Habig et al. (1974) and Pflugmacher et al. (2005) with 2,4-Dinitrochlorobenzene (CDNB) as a substrate. Due to its small size and the absence of steric hindrance, CDNB acts as a substrate for almost all families of GSTs except for GSTs from the theta class (Meyer et al. 1991). We mixed 20 µL of *Daphnia* homogenate with 66 µL of 75 mM glutathione (GSH) in ultrapure water, 66 µL 30 mM CDNB in 100 % ethanol and 858 µL of 0.1 M potassium phosphate buffer at pH 6.5 containing 1 mM EDTA. Blanks were carried out with the same mixture using additional 20 µL of potassium phosphate buffer instead of *Daphnia* homogenate.

The change of absorption due to formation of conjugates with reduced GSH and CDNB was monitored every 30 seconds for seven minutes at 340 nm and 25 °C with a CaryWin UV 50 photometer; the increase of absorption was checked for linearity. Using Lambert-Beers law

with a given molar absorption coefficient for CDNB (ΔE_{CDNB}) of 9.6 L mmol^{-1} cm^{-1} (Habig et al. 1974), we calculated the decrease in CDNB concentration which reflects the amount of CDNB-GSH conjugate that has been produced during the seven-minute measurement. Subsequently we calculated the enzymatic activity given in nkat (nmol s^{-1}) per mg protein.

Exposure experiments

Short term exposure experiment (24h)

200 adult *D. magna* of various sizes were transferred into beakers with 500 mL medium containing *M. aeruginosa* PCC7806 (5 mg POC L^{-1}) and kept there for 24 hours. Controls were carried out in the absence of daphnids. At the beginning of the experiment and after 24 h, the *Microcystis* cell concentrations were determined using a Neubauer improved counting chamber. Treatments and controls were replicated threefold. After 24 hours the daphnids were removed from the medium using a 200 µm gauze, and the resulting cyanobacterial cell suspension was collected and processed immediately.

Chase experiment

The *D. magna* from the short term experiment were immediately transferred into the subsequent chase experiment, which lasted another 54 h. The transfer of animals in general was carried out by filtering all daphnids with a gauze (200 µm), rinsing them with approximately 10 mL of tap water to avoid experimental carryover of dissolved metabolites and adhering cells and subsequently transferring them to the next part of the experiment. The chase experiment was carried out in 200 mL medium for each experimental stage.

In the first 6 h, the animals were fed with a 100 % *Cryptomonas* sp. (5 mg POC L^{-1}) diet. This nutritious alga was chosen because of its brownish color. Concomitant microscopic investigations of randomly picked individuals therefore revealed a brownish gut content, indicating that the *Microcystis* cells in the gut had been replaced by *Cryptomonas* sp. within 6 h after termination of the short term exposure experiment.

Thereafter daphnids were transferred into fresh medium without food for another 48 h, with a first change of the medium after 24 h. Controls were carried out similarly, but in the absence of daphnids. To simulate experimental carryover that might happen due to transfer of daphnids, we transferred 1 mL of each control sample into the respective following one. Thereby, one ml represents a very conservative estimate, since the amount of medium

adhering to the daphnids is considerably less than a milliliter, and in addition the cyanobacterial cell density in this adhering volume of water is strongly diluted by the tap water washing step.

Sample preparation

Daphnia tissue, *Microcystis* and *Cryptomonas* cells and the different culture media from the short term exposure experiment and the chase experiment were subsequently used for the quantification of the content of MCLR-GSH conjugate, MCLR and for the detection of other putative conjugates of MC, namely MCLR-Cys, MCLR conjugates with the dipeptides Cys-Glu or Cys-Gly and with mercapturic acid of MCLR. We therefore obtained samples of incubation water, algal or cyanobacterial cells and *Daphnia* tissue, each of which required different extraction techniques. *Microcystis* and *Cryptomonas* cells were separated from the medium by repeated centrifugation (2 min, 11.500 g), thereby pooling the supernatants (water sample) and obtaining one pellet per sample (cell sample). In general we did not use subsamples, but always the entire samples for the quantification of MCLR or MCLR-GSH. Hence we did not differentiate between the medium, algal or cyanobacterial cells or daphnids and we determined total amounts, rather than concentrations. The amounts of MCLR-GSH and MCLR of all experimental fractions from the short term exposure experiment (*Microcystis* cells and medium) and the chase experiment (*Cryptomonas* cells, *Daphnia* tissue and incubation medium) were summed up to obtain the total amounts of these two metabolites within the entire experimental setup.

Preparation of cells and tissue – methanolic extraction

The cell pellets were resuspended in 10 mL 80 % MeOH, and 20 µL MC-HilR (Hil = homoisoleucine, R = arginine) (5 µg mL^{-1}) was added as an internal standard. The cells were then sonicated (10 min) and centrifuged again (2 min, 11.500 g). The supernatant was transferred into new test tubes and evaporated to dryness in a vacuum centrifuge and finally dissolved in 100 µL 100 % MeOH. After another centrifugation (2 min, 11.500 g), the supernatant was transferred into glass vials.

Daphnids were separated from the medium using a 200 µm gauze. After rinsing the animals three times with ultrapure water they were transferred into 1.5 mL test tubes, their fresh weight was measured, and the internal standard was added (20 µL, MC-HilR, 5 µg mL^{-1}). The daphnids were stored at -20 °C until further preparation. 100 µL 80 % MeOH were added

after thawing, and the tissue was homogenized using an electric homogenizer. After adding another 400 µL of 80 % MeOH, the samples were sonicated (5 min) and centrifuged (3 min, 11.500 g); the supernatant was then transferred into 1.5 mL test tubes. After evaporating to dryness in a vacuum, the centrifuge samples were redissolved in 100 µL 100 % MeOH and transferred into glass vials.

Extraction of water samples – solid phase extraction (SPE)

After separating the cells, the internal standard (20 µL MC-HilR, 5 µg mL^{-1}) and additionally 5 mL 100 % MeOH were added to the medium to obtain a 1 % methanolic solution. After that the water samples were subjected to solid-phase extraction (SPE) with a C_{18} cartridge (50 mL, BRAND) as according to the following protocol: 1. cleaning the cartridge (50 mL 100 % MeOH), 2. equilibration (50 mL 1 % MeOH), 3. adding of the complete sample (~500 mL with 1 % MeOH), 4. purification (50 mL 20 % MeOH), 5. elution (50 mL 80 % MeOH). The elute was evaporated to dryness using a rotary evaporator (40 °C and 30 mbar). The dried sample was redissolved in three times one milliliter of 100 % MeOH and subsequently evaporated to dryness in a vacuum centrifuge. Finally, the sample was redissolved in 100 µL MeOH and transferred into a glass vial.

Note that in all cases the entire sample was processed with a final nominal concentration of internal standard of 1 µg mL^{-1}. If not stated otherwise, MCLR and MCLR-GSH content is given in absolute numbers which represent the total content of the entire sample.

Liquid chromatography – mass spectrometry

All measurements were carried out using an Accela Ultra high pressure liquid chromatography (UHPLC) system (Thermo Fisher) consisting of a 1250 psi pump, an autosampler (AS) and a photo diode array (PDA) detector coupled with an Exactive Orbitrap mass spectrometer (MS) (Thermo Fisher) with electrospray ionization (ESI) in positive ionization mode. In all experiments we used a C_{18} column (Nucleosil, 125/2, 100-3) as the stationary phase and a gradient of acetonitrile (ACN) and ultrapure water, each containing 0.05 % trifluoracetic acid (TFA) at a temperature of 30 °C and a flow rate of 300 µL per minute as mobile phase: 0 min: 38 % ACN; 2 min: 40 % ACN; 12 min: 50 % ACN; 12.5 min: 100 % ACN; 15 min: 100 % ACN; 15.5 min: 38 % ACN 17 min: 38 % ACN. The injection volume of the sample was always 10 µL. Mass spectrometry measurements were carried out at a rate of one scan per second and a mass range of between 75 and 1500 Da (spray voltage

4.5 kV, capillary temperature 325 °C, sheath and aux gas nitrogen set to 45 and 15, respectively). For the identification of compounds we used extracted ion chromatograms for the respective specific masses of the different compounds (Tab.1) granting a mass deviation of 3 parts per million (ppm). Electrospray ionization resulted in adduct ions with one positive charge for MCLR (z = 1, $M + H^+$), whereas the ionization of MCLR-GSH and MCLR-Cys conjugates resulted in adduct ions with a double charge (z = 2, $M + 2H^+$), most likely due to the introduction of a second amino group via GSH or cysteine. MCLR and MCLR-GSH were additionally confirmed by retention times, since synthetic standards were available. The combination of a very narrow mass range, together with the known retention time allowed for a very high certainty with respect to proper detection. In case of metabolites that we could not detect in our samples (MCLR conjugates with the dipeptides Cys-Glu, Cys-Gly or with mercapturic acid) we screened for m/z values with the charges z = 1 and z = 2 in order to account for different degrees of ionization. The mass range was set to a granted deviation of 10 ppm in order to avoid false negatives. We used the Xcalibur software package (Thermo Fisher) for qualitative and quantitative analysis.

Tab.1 Monoisotopic masses, m/z values and retention times of microcystin-LR, microcystin-LR conjugates and the internal standard microcystin-HilR (GSH = glutathione, L = leucine, R = arginine, Hil = homoisoleucine).

Metabolite	**Monoisotopic mass [Da]**	**m/z with z = 1 [Da]**	**m/z with z = 2 [Da]**	**Retention time [min]**
Microcystin-LR	994.54877	995.55604	498.28166	4.19
Microcystin-LR-GSH	1301.63257	1302.63985	651.82356	2.21
Microcystin-LR-cysteine	1115.56852	1116.57579	558.79153	2.45
Microcystin-LR-glutamylcysteine	1244.61111	1245.61839	623.31283	not detected
Microcystin-LR-glycinylcysteine	1186.59305	1187.60033	594.3038	not detected
Mercapturic acid of MCLR	1157.57908	1158.58636	579.79682	not detected
Microcystin-HilR (internal standard)	1008.56442	1009.57169	505.28949	5.02

Quantification

Microcystin-LR

For the quantification of MCLR we conducted three different calibration curves (5 - 50 µg mL^{-1}, 0.25 - 2 µg mL^{-1} and 0 - 0.25 µg mL^{-1}). This was necessary due to differing MCLR amounts in the different sample types. As samples were always redissolved in a final

volume of 100 µL MeOH, the total MCLR content of the respective original sample could be obtained by dividing the measured concentration value by ten. As an internal standard we used a slightly modified MCLR variant which is elongated by one CH_2 unit at the leucine (L) amino acid moiety: MC-HilR (Hil = homoisoleucine, Enzo Lifesciences, Lörrach, Germany). MC-HilR is easy to differentiate in the mass spectrum while having very similar chemical characteristics as MCLR. As an external standard we used different concentrations of MCLR (Enzo Lifesciences, Lörrach, Germany). For the calculation of the ratios of internal and external peak areas, peaks obtained from the extracted ion chromatograms (MS) for the specific masses (Tab.1) of both substances were used (granted mass deviation < 3 ppm). The recovery rates for the internal standard differed strongly between the different matrices (medium = 43 %, cells = 89 % and *Daphnia* tissue = 63 %) and were obtained by calculating the average of internal standard peak areas of five randomly chosen samples of the respective matrix and comparing this value to the average peak area of the internal standard obtained from the calibration standards.

Microcystin-LR-GSH

To obtain a conjugate of MCLR with GSH as a reference compound we carried out a Michael reaction by mixing a known concentration of MCLR (100 µg mL^{-1}) with saturating concentrations of GSH (30 mg mL^{-1}) under alkaline conditions (pH 11.05) at 30 °C under shaking conditions for 3 h (Kondo et al. 1992). The same incubation was performed without addition of GSH. The samples were subsequently neutralized with 0.1 M HCl (pH 7.5) and internal standard (20 µL MC-HilR, 50 µg mL^{-1}) was added. We then processed the samples by solid-phase extraction (SPE) and measured them by LC-MS, thereby quantifying the amounts of MCLR in both samples. The addition of GSH led to a peak of MCLR-GSH and a strong decrease of MCLR. We quantified the decrease in MCLR and calculated the amount of MCLR-GSH based on the assumption that the magnitude of the decline of MCLR reflected an identical magnitude of increase of MCLR-GSH. The resultant MCLR-GSH concentration was used to establish a calibration curve for MCLR-GSH (0.0 - 2.53 µg mL^{-1}).

Microcystin-LR-GSH stability

To check for putative chemical or microbial degradation of the MCLR-GSH conjugate and subsequent putative release of MCLR, we dissolved the same amount of MCLR-GSH in 10 mL of medium, which we extracted immediately, whereas the other sample was kept for

24 h at room temperature prior to the extraction. Both treatments were carried out in triplicates. Extraction was carried out similar to the procedure described for the water samples. Afterwards we compared the MCLR-GSH peak areas between the two treatments.

Results

Ingestion of *Microcystis* cells by *D. magna*

We determined the number of cells ingested by *D. magna* by counting *M. aeruginosa* cell abundance at the beginning (0 h) and the end (24 h) of the short term exposure experiment. The presence of 200 *D. magna* individuals led to a significant reduction of *M. aeruginosa* cell abundance: 35 % from $11.41*10^5$ to $7.39*10^5$ cells mL^{-1} (t-test, $P = 0.009$), which corresponded to a food concentration of 3.2 mg POC L^{-1} after 24 h. As this final food concentration was far above the incipient limiting level (Lampert 1978), ingestion rates were assumed to have been constant during the experiment and were calculated to be 8.8 µg POC per individual in 24 h. The cell abundance of *M. aeruginosa* did not change in the control treatment without daphnids (t-test, $P = 0.349$) (Fig.1).

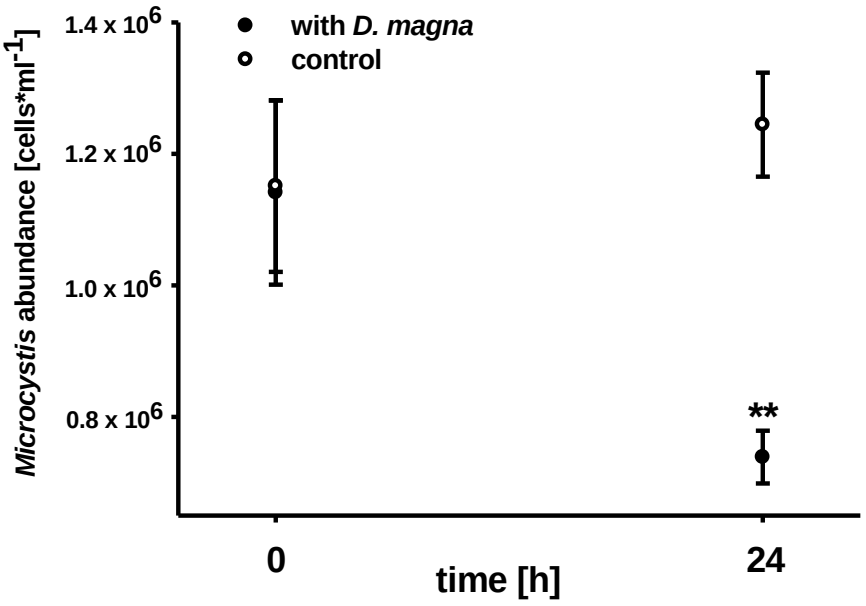

Fig.1 *Microcystis* cell abundances at the beginning (0 h) and the end (24 h) of the *D. magna* short term exposure experiment. Control without daphnids. Means +/- SD, n = 3, t-test, ** $P < 0.01$.

Glutathione-S-transferase activity

In order to test for changes in soluble GST (sGST) activity in *D. magna* in response to the presence of microcystin (MC) containing cyanobacteria, we measured sGST activities in *D. magna* tissues after exposure to a diet of *M. aeruginosa* PCC7806 for 24 hours. As controls we used daphnids exposed to the MC-deficient mutant form of *M. aeruginosa* PCC7806 (mcy-), daphnids fed with the green algae *Chlamydomonas klinobasis* and a no-food treatment. The GST activity measurements revealed significant differences between the treatments (one way ANOVA, F = 13.039, $P = 0.002$).

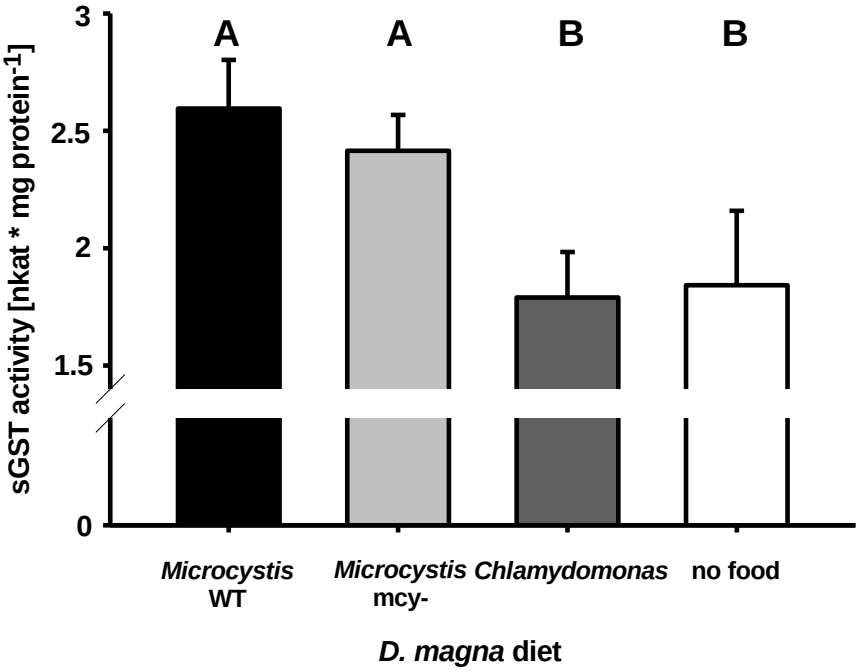

Fig.2 Soluble glutathione-S-transferase (sGST) activity of *D. magna* fed with a 100 % *M. aeruginosa* diet of the wildtype (WT) and the mutant form (mcy-). Controls were fed with 100 % of the green algae *Chlamydomonas klinobasis* or kept without food. Mean + SD, n = 3. Different letters indicate significantly different treatments. Tukey-test, $P < 0.05$, after one-way ANOVA.

The presence of food in general (*Chlamydomonas* diet versus no food) did not affect sGST-activity ($P = 0.998$, Tukey-test after one way ANOVA) (Fig.2). In comparison to the *Chlamydomonas* treatment, both cyanobacteria caused a significant ($P = 0.005$ for *M. aeruginosa* WT vs. *Chlamydomonas* and $P = 0.02$ for *M. aeruginosa* (mcy-) vs. *Chlamydomonas*, Tukey-test after one way ANOVA) increase of an average of 39 % sGST activity, from 1.8 nkat mg protein^{-1} to 2.5 nkat mg protein^{-1} (Fig.2). However, these increased

levels of sGST did not differ between the wildtype and the mutant strain of *M. aeruginosa* ($P = 0.690$, Tukey-test after one way ANOVA) (Fig.2).

Short term exposure experiment

In the short -term exposure experiment (0 - 24 h), we determined the total amounts of MCLR conjugates with GSH as well as unconjugated MCLR in the cell-free supernatant (incubation water) and in the fraction of the cyanobacterial cells. The results revealed significant differences in the different samples (*Microcystis* cells and medium) between the control (without daphnids) and the *Daphnia* treatment for MCLR-GSH (one way ANOVA, $F = 5.168$, $P = 0.028$) as well as unconjugated MCLR (one way ANOVA, $F = 25.105$, $P < 0.001$).

For each treatment the total amounts of MCLR-GSH and of unconjugated MCLR in the entire experimental setup were calculated from the respective total amounts obtained from the *Microcystis* cells and the medium, plus the amounts from the different fractions from the subsequent chase experiment, i.e. incubation medium, *Cryptomonas* cells and *Daphnia* tissue.

However, the different components of the chase experiment did not contain any MCLR-GSH conjugates.

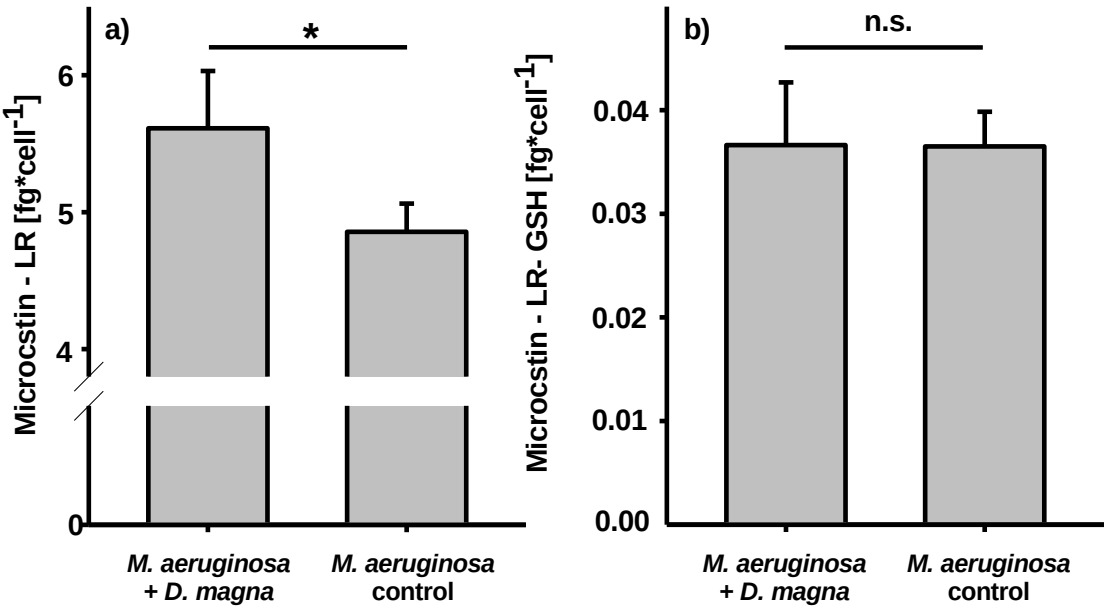

Fig.3 a) Microcystin-LR and b) Microcystin-LR-GSH amount per *Microcystis* cell from the *D. magna* short term exposure experiment (after 24 h) in the presence or absence of 200 *D. magna*. Means + SD, n = 3, t-test, * $P < 0.05$.

In addition to that, we determined the amount of MCLR-GSH and MCLR per *Microcystis* cell in both treatments. The presence of 200 *D. magna* individuals resulted in a significant increase of the MCLR content per cyanobacterial cell from 4.9 fg cell^{-1} to 5.6 fg cell^{-1} (t-test, $P = 0.049$) (Fig.3a). Surprisingly, we also found low amounts of MCLR-GSH conjugate (0.037 fg cell^{-1} for both treatments) within the *Microcystis* cells. However, the MCLR-GSH content did not change due to the presence of daphnids (t-test, $P = 0.958$) (Fig.3b).

The whole fraction of *Microcystis* cells from the *Daphnia* treatment had a lower total content of MCLR-GSH (13.5 ng) than the cells from the control (22.5 ng) ($P = 0.039$, Tukey-test after one way ANOVA), which is probably attributable to the ingestion of 35 % of the cyanobacteria. A similarly low amount of MCLR-GSH was found in the medium after 24 h of incubation, and this amount was not affected by the absence or presence of daphnids ($P = 0.679$, Tukey-test after one way ANOVA) (Fig.4). The total amount of MCLR-GSH decreased in the presence of *D. magna* (t-test, $P = 0.001$) (Fig.4).

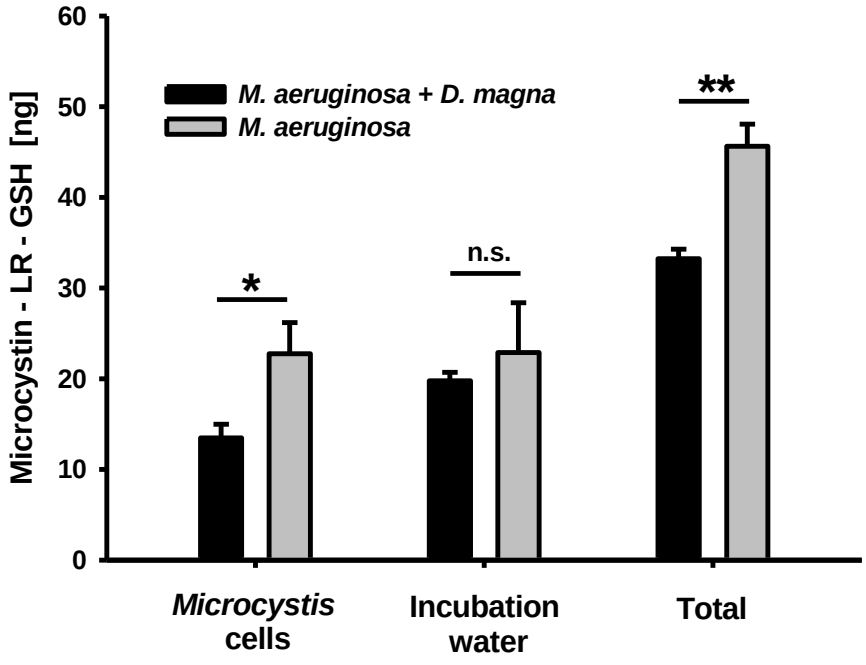

Fig.4 Total microcystin-LR-GSH amounts of the cyanobacterial cells, the incubation water (first incubation period; 0 – 24 h) and the summed total amounts from the *D. magna* short term exposure experiment in the presence (black bars) or absence (gray bars) of daphnids. Means + SD, n = 3. For *Microcystis* cells and incubation water: Tukey-test, * $P < 0.05$, after one-way ANOVA. For the summed total amounts: t-test, ** $P < 0.01$.

A 24 h incubation of synthetic MCLR-GSH in the medium led neither to a significant decline of the amount of the conjugate compared to a control that was extracted without such prior incubation (t-test, $P = 0.406$) nor to the presence of MCLR (not detected), which suggests that biologically formed MCLR-GSH conjugates are stable under the experimental conditions.

The presence of *D. magna* led to an increase in the total amount of unconjugated MCLR in the incubation water (3.0 µg) compared to the control water without daphnids (1.9 µg) ($P = 0.001$, Tukey-test after one way ANOVA) (Fig.5). However, the total amount of MCLR in the fraction of the cyanobacterial cells was significantly reduced from 3.1 µg in the control to 2.1 µg in the presence of *D. magna* ($P = 0.002$, Tukey-test after one way ANOVA). This could be partly attributed to a significant reduction of the cyanobacterial biomass in the *Daphnia* treatment (Fig.5).

A putative biotransformation of MCLR by *D. magna* should result in a lower total amount of free MCLR within the entire experimental setup; however, the total amount of free MCLR in the treatments was not affected by the presence of daphnids (t-test, $P = 0.382$).

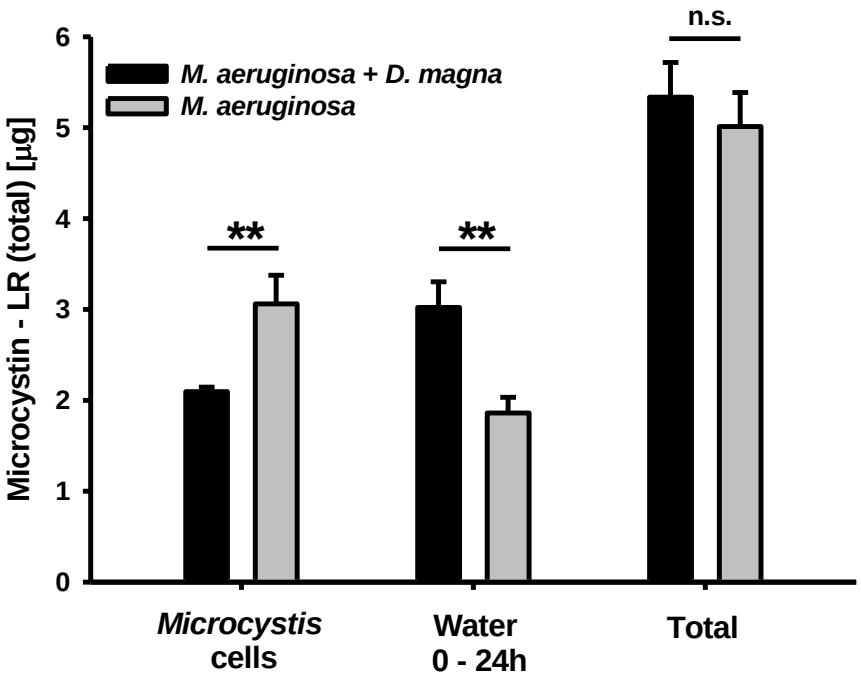

Fig.5 Total microcystin-LR amounts of the cyanobacterial cells, the incubation water (first incubation period: 0 - 24 h) and the summed total amounts from the *D. magna* feeding and chase experiment in the presence (black bars) or absence (gray bars) of daphnids. Means + SD, n = 3. For *Microcystis* cells and incubation water: Tukey-test, ** $P < 0.01$, after one-way ANOVA. For the summed total amounts: t-test, $P = 0.382$.

With regard to other putative conjugates of MCLR (MCLR-Cys, MCLR conjugates with the dipeptides Cys-Glu or with Cys-Gly or mercapturic acid) we only detected MCLR-Cys conjugates. However, MCLR-Cys was detectable only within cyanobacterial cells in amounts too low for quantification.

Chase experiment

To check whether MCLR had actually been assimilated by *D. magna* in the short term exposure experiment we additionally conducted a chase experiment in which those daphnids used in the 24 h *Microcystis* short term exposure experiment were kept for another 6 h in a medium containing *Cryptomonas* sp. which does not contain microcystins. Afterwards the animals were transferred into fresh medium without any food for 24 h twice. Total amounts of MCLR and MCLR-GSH conjugates were measured in all incubation media, as well as in *Daphnia* tissue and *Cryptomonas* cells.

The MCLR content determined in the incubation water, in the fraction of *Cryptomonas* cells and in the daphnids in the chase experiment was generally much lower than in the MC short term exposure experiment. Incubation water from the 24 - 30 h incubation period contained 134 ng MCLR, whereas we found 5 ng MCLR associated with the *Cryptomonas* cells, probably due to residual medium adhering on the cell surfaces. Incubation water from the from the 30 - 54 h period contained 57 ng MCLR, and the last water sample (54 - 78 h) still had a MCLR content of 14 ng (Fig.6).

It is noteworthy that this incubation water had never been in contact with cyanobacteria. As the MCLR amounts in the control samples (in the absence of daphnids) of the chase experiment were below the limit of quantification (0.25 ng), an accidental carryover of MCLR or MCLR–GSH in the *Daphnia* treatments can be ruled out. Therefore the results point at an at least partial assimilation of cyanobacterial MCLR by the daphnids with a subsequent, significantly delayed release. The daphnids had a total MCLR content of 8 ng (40 pg per individual) at the end of the experiment (Fig.6), and neither of the fractions contained detectable MCLR-GSH amounts.

Neither of the other putative conjugates of MCLR (MCLR-Cys, MCLR conjugates with the dipeptides Cys-Glu or with Cys-Gly or mercapturic acid) could be detected in samples of the chase experiment.

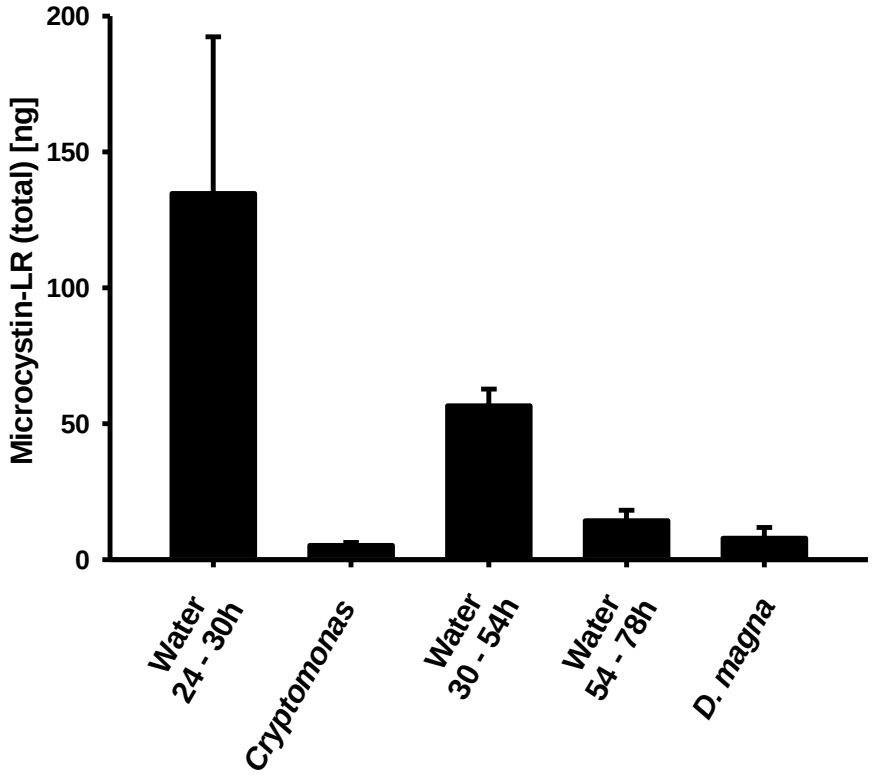

Fig.6 Total microcystin-LR (MCLR) amounts of the different incubation media, the *Cryptomonas* cells and the *Daphnia* tissue from the chase experiment carried out with the 200 *D. magna* from the short term exposure experiment (fed for 24 h with a 100 % *M. aeruginosa* diet, see Figs.4 and 5). The daphnids were incubated for another 6 h with *Cryptomonas* sp. and subsequently two times for 24 h without food. The controls without daphnids did not contain quantifiable MCLR amounts. Means + SD, n = 3.

Discussion

When comparing the GST activities of daphnids exposed to different food regimes, we found a general increase caused by a cyanobacterial diet compared to the controls (*Chlamydomonas* and no food), whereas the comparison between the MC-containing strain PCC7806 with its MC-deficient mutant form (mcy-) did not reveal any differences (Fig.2). Therefore, no direct MC effect on GST activity was observable. An earlier report of a positive effect of a MC-containing cyanobacterial diet on D*aphnia* GST activity (Guo and Xie 2011) did not include a MC-free cyanobacterial diet as a control. Therefore this effect cannot be attributed to MC and might instead be a general effect of cyanobacterial food. However, a direct MCLR exposure, with MCLR dissolved in the incubation medium, can lead to increased GST activity in juvenile daphnids (Ortiz-Rodriguez and Wiegand 2010).

In theory, MCs constitute fairly suitable substrates for the conjugation with GSH. The methyldehydroalanine (Mdha) moiety of MCs acts as a potent electrophile which facilitates the conjugation to GSH. For the same reason Mdha is also responsible for the inhibition of protein phosphatases (PPs) by MCs, since a covalent bond is established between the α,β-unsaturated carbonyl of Mdha and a Cys moiety of the PPs (Honkanen et al. 1990; Runnegar et al. 1995). In a similar way, MCs react with GSH or unbound Cys without enzymatic catalysis in a Michael-addition (Kondo et al. 1992). However, this non-enzymatic reaction requires pH values of above 10 and is therefore of minor importance in natural cellular processes.

Pflugmacher et al. (1998) was the first to point out the possibility of GST catalyzing the conjugation of MCs with GSH as the first step of detoxification in *Daphnia*. However, the fact that the MC-deficient mutant form (mcy-) of *M. aeruginosa* led to the same increase in *Daphnia* GST activity as the PCC7806 wildtype (WT) strain challenges the assumption that such increases of GST activity are mainly due to the role of GST in the detoxification of MCs. In this case one would expect higher GST activity in *Daphnia* when they feed on the PCC7806 wildtype than on the mutant. Since this was not the case, we argue that our data indicate an involvement of GST in a general oxidative stress response rather than a direct detoxification of MCs. In line with this, although the generation of oxidative stress is a general feature of MCs (Amado and Monserrat 2010), Dao et al. (2013) found an increase of sGST activity and other oxidative stress-response enzymes due to a cyanobacterial diet free of microcystins. Nonetheless, we cannot rule out a role of GSTs in MC detoxification, since GST's role as general oxidative stress response biomarker might eventually obscure the MC detoxification effect.

To directly address the issue of biotransformation of MCs, we conducted exposure experiments with daphnids and cyanobacteria in which we quantified the total MCLR-GSH and MCLR amounts in the entire setup, including the incubation water, the *Microcystis* cells and the *Daphnia* tissue. We argue that an involvement of GST in conjugation of MCLR would necessarily lead to an overall reduction of free MCLR in the experiment due to the presence of *Daphnia*. This, however, was not the case.

After the first 24 h of feeding on *M. aeruginosa* PCC7806, the presence of *D. magna* clearly led to a reduction of unconjugated MCLR in *Microcystis* cells by roughly 1/3 (Fig.5), which can be well explained by a comparable reduction of the cyanobacterial cell abundance due to *Daphnia* feeding activity (Fig.1). This decrease in cyanobacterial cell abundances is reflected

in a corresponding increase of free MCLR in the medium. The fact that the control medium also contained MCLR might be partly explained by cyanobacterial cell lysis and a release of MCLR in the medium during the centrifugation process in order to divide the cells from the medium. The overall MCLR amount in the two treatments did not differ; hence the presence of 200 *D. magna* individuals did not lead to a reduction of total free MCLR, which demonstrates that conjugation of MCLR to glutathione or any other biotransformation of the molecule is of minor relevance for the interaction of *Daphnia* with dietary MCLR (Fig.5).

This clearly forces us to reject the idea of an involvement of GST in the conjugation of MCLR with GSH in *Daphnia in vivo*. Instead, these findings suggest that the uptake is followed by a release of unconjugated MCLR. Interestingly, the presence of *D. magna* led to an increase of MCLR per *Microcystis* cell (Fig.3a). This may indicate an inducible defense mechanism of *M. aeruginosa* and is in line with earlier findings by Jang et al. (2003; 2007; 2008). However, it cannot be ruled out that this increase in MCLR within the *Microcystis* cells is not a direct effect of the presence of *Daphnia*. The daphnids used in the short term exposure experiment were fed with a *Chlamydomonas* diet prior to the experiment. Therefore, experimental carryover of components derived from *Chlamydomonas* in the guts of the daphnids cannot be excluded as the cause for the increase of MCLR content in the *Microcystis* cells.

We cannot distinguish whether the increase of free MCLR in the incubation water during the *Microcystis* short term exposure experiment was due to assimilation and subsequent release of MCLR by the daphnids or due to sloppy feeding (He and Wang 2006). Nonetheless, the chase experiment revealed significant MCLR amounts in the incubation water samples (24 h - 30 h, 30 h - 54 h and 54 h - 78 h) and in the *D. magna* tissue, although dietary MCLR had not been available for 54 h (Fig.6). Since the chase experiment started with a 6 h feeding period on *Cryptomonas* sp., it appears rather unlikely that these amounts of MCLR could result from *Microcystis* cells remaining in the *Daphnia* gut. However, we cannot entirely rule out this possibility. Nevertheless, our data suggest the possibility that daphnids assimilated MCLR from the *Microcystis* cells and released unconjugated MCLR with a significant time delay. The control incubation media in absence of daphnids did not contain quantifiable MCLR amounts, ruling out an effect of accidental carryover (data not shown). Rohrlack et al. (1999) showed that the presence of *Microcystis* in the medium might lead to ingestion inhibition in daphnids, which would subsequently result in reduced MCLR uptake by the animals.

However, in our experiments, daphnids ingested roughly 1/3 of all *Microcystis* cells (Fig.1) without reducing the amount of total MCLR (Fig.5).

In addition to the absence of a reduction of the total MCLR content, we also did not detect any other metabolite that might be produced along the GST detoxification pathway (MCLR-Cys, MCLR conjugates with the dipeptides Cys-Glu and Cys-Gly or mercapturic acid of MCLR) with the exception of MCLR-GSH, which we found in quantifiable amounts and MCLR-Cys which was only found in the *Microcystis* cells and only in very low non-quantifiable amounts.

Despite small amounts of MCLR-GSH could be found in the control *Microcystis* cells, there was no evidence in literature pointing at the presence of MCLR-GSH conjugates in MC-containing cyanobacteria. GSTs are present in cyanobacterial cells as well (Wiktelius and Stenberg 2007) and might be responsible for the presence of MCLR-GSH in cyanobacteria observed here. According to our measurements, this MCLR-GSH accounts for only about 7 ‰ of the MCLR amount and is therefore probably of minor importance. The presence of daphnids, however, does not lead to an increase in the overall amount of MCLR-GSH conjugates in the experiment. Again we found a reduction of MCLR-GSH within the *Microcystis* cell fraction in the presence of daphnids, whereas the MCLR-GSH content per cell was not changed. In contrast to MCLR, this decrease is not reflected in an increase of MCLR-GSH in the medium. Subsequently the overall amount of MCLR-GSH conjugate is not higher but lower in the presence of daphnids (Fig.4).

A possible explanation for this could be assimilation and subsequent degradation of MCLR-GSH conjugates by *Daphnia*, while a reduced production of MCLR-GSH of *Microcystis* due to grazing pressure cannot be ruled out either. In fact we do not know, if MCLR-GSH conjugates were assimilated by the daphnids or not, since no conjugate could be found in the *Daphnia* tissue. However, the amount of MCLR-GSH conjugate did not increase in the presence of daphnids, which, in line with the absence of a reduction of MCLR amounts, further supports the notion that conjugation of MCLR with GSH seems to be of minor importance in *Daphnia in vivo*.

We cannot rule out that MCs were bound to protein phosphatases, as MCs are known to inhibit protein phosphatases by covalently binding to Cys-moieties (Honkanen et al. 1990; Runnegar et al. 1995). MCs covalently bound to proteins were beyond the scope of this experiment; nevertheless, a significant binding of MCs to proteins should result in an overall decrease of unconjugated MCs, which was not observed.

In summary, ingestion of MCLR-containing cyanobacteria by daphnids a) did not lead to an increase in the daphnids GST activity in comparison to daphnids feeding on the MC-deficient mutant form of the same strain, b) did not decrease the overall MCLR amount in a 24 h exposure experiment compared to a control without daphnids, and c) neither increased the overall amount of MCLR-GSH conjugates nor led to the formation of other conjugates (or metabolites) of MC that might be associated with the GST pathway. These findings strongly indicate that GSTs are not directly involved in the MC detoxification process in *Daphnia*, nor is any other kind of biotransformation detectable, as any biotransformation would have resulted in a decrease of total free MCLR.

Nonetheless, it should be noted that these experiments were carried out with a laboratory clone that had not been in contact with cyanobacteria prior to the experiment at all. It is possible that a physiological response of daphnids to MC containing cyanobacteria, as it would be the conjugation to GSH, requires a certain amount of time exceeding the 24 h exposure used in our experiment. Further studies are needed addressing this issue by comparing clones with and without a history of prior exposure to MC containing cyanobacteria.

The finding that the decrease of cyanobacterial MCLR due to the presence of *Daphnia* is very well reflected in a similar increase of the MCLR content in the respective incubation medium (Fig.5) suggests an involvement of export mechanisms that are capable of transporting MCs without prior biotransformation. This indicates that, despite an unknown cyanobacterial history of the *D. magna* clone used here, an efficient export mechanism rather than a conjugation of MCLR contributes to the tolerance of MCLR.

A possible transport might take place via multi xenobiotic resistance proteins (P-glycoprotein, P-gp). An increased expression of P-gp was demonstrated in the freshwater mussel *Dreissena polymorpha* (Contardo-Jara et al. 2008) and also in the fish *Jenynsia multidentata* (Ame et al. 2009) as a result of direct MCLR exposure. However, so far no data are available for the function of this transporter class in *Daphnia*, although their existence has been confirmed (Sturm et al. 2009). Such transport mechanisms on the primary consumer level could deliver an explanation for the observed tendency of MCs to biodilute along foodwebs (Ferrao and Kozlowsky-Suzuki 2011; Kozlowsky-Suzuki et al. 2012), but these putative MC transport mechanisms, as well as other functional mechanisms helping *Daphnia* to cope with cyanotoxins in general and MCs in particular are still poorly understood. A better understanding of these physiological mechanisms might reveal new information which could

turn out to be very valuable for the successful application of biomanipulative approaches in lake water management. Especially studies focusing on the role of MC transport mechanisms in *Daphni*a are needed in order to shed light on their role in suppressing cyanobacterial blooms.

Conclusions

The data presented here clearly reject the currently assumed role of *Daphnia* GSTs in MCLR detoxification. The presence of 200 *D. magna* individuals that had fed on a 100 % *M. aeruginosa* PCC7806 diet (a strain containing MCLR) did not affect the overall amount of free MCLR. Furthermore, no increase of the MCLR-GSH conjugate could be detected. Although our data show that GST activity is increased after *M. aeruginosa* PCC7806 exposure, we could also show that there is no MC effect on GST activity, since the same increase was observed with the MC-deficient mutant form of this cyanobacterium. We therefore argue that GSTs are involved in a general oxidative stress response caused by a cyanobacterial diet rather than in the biotransformation of MCs. Since unconjugated MCs are released by the daphnids, a MC-transporter system which might be the crucial mechanism explaining *Daphnia*'s well-developed resistance to MCs seems to be present.

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Chapter 2

Physiological interaction of *Daphnia* and *Microcystis* with regard to cyanobacterial secondary metabolites

Abstract

Cyanobacterial blooms in freshwater ecosystems are a matter of high concern with respect to human health and ecosystem services. Investigations on the role of cyanobacterial secondary metabolites have largely been confined to microcystins, although cyanobacteria produce a huge variety of toxic or inhibitory secondary metabolites. Mass occurrences of toxic cyanobacteria strongly impact freshwater zooplankton communities; especially the unselective filter feeder *Daphnia*. Daphnids have been shown to successfully suppress bloom formation. However, the opposite situation, i.e. the suppression of *Daphnia* populations by cyanobacteria can be observed as well. To understand these contradictory findings the elucidation of the underlying physiological mechanisms that help daphnids to cope with cyanotoxins is crucial.

We fed *Daphnia magna* with the cyanobacterium *Microcystis aeruginosa* PCC7806 for 24 h and used high resolution LC-MS analytics to analyze the *Microcystis* cells, the *Daphnia* tissue and the surrounding medium in order to investigate the fate of seven investigated cyanobacterial compounds (cyanopeptolins A - C, microcyclamide 7806A and aerucyclamides - D). For none of these bioactive compounds evidence for biotransformation or biodegradation by *Daphnia* were found. Instead feeding and subsequent release experiments point at the importance of transport mechanisms in *Daphnia* with regard to the cyanopeptolins A and C and microcyclamide 7806A.

In addition we found hints for new inducible defense mechanism in *Microcystis* against predation by *Daphnia*. These putative defense mechanisms include the elevated production of toxic compounds other than microcystins, as could be demonstrated here for aerucyclamide B and D, cyanopeptolin B and microcyclamide 7806A. Moreover, our data demonstrate the elevated active export of at least one cyanobacterial compound (microcyclamide 7806A) into the surrounding medium as a response to grazer presence, which might constitute an entirely new not yet described cyanobacterial defense mechanism.

Introduction

Anthropogenic influence on freshwater ecosystems, especially increased nutrient loading and global warming, has led to more frequently occurring cyanobacterial blooms (Dokulil and Teubner 2000; O'Neil et al. 2012; Paerl and Huisman 2008). Cyanobacteria in high abundances are known to have a strong impact on freshwater ecosystems; for instance by negatively affecting herbivorous zooplankton (Ghadouani et al. 2003; Wilson et al. 2006). Accordingly, the cyanobacteria-zooplankton interaction has become a matter of increasing importance (Ger et al. 2014). Two factors are regarded as the main cause for these detrimental effects. First, cyanobacteria are of very low food quality for grazers, for example due to mechanical interference (Porter and Mcdonough 1984) or to low nutritional food quality (Von Elert et al. 2003; Von Elert 2004). Secondly, cyanobacteria are typically rich in secondary metabolites. These compounds are very diverse, and a lot of them are known to have toxic effects on herbivorous zooplankton (Hansson et al. 2007; Paerl et al. 2001).

However, not only do cyanobacteria negatively influence the zooplankton community; they also provide a serious hazard for the usage of surface waters as drinking water or for recreational purposes (Stewart et al. 2006). Therefore, a lot of effort has been put into the control of cyanobacterial blooms. Among different strategies in water resource management aiming at the control of harmful cyanobacterial blooms are food chain manipulations in order to increase size and abundance of herbivorous zooplankton (Hansson et al. 1998; Shapiro et al. 1975). The success of such manipulative approaches is thereby strongly influenced by presence and abundance of the unselective filter feeder *Daphnia* (Leibold 1989). However, while Chislock et al. (2013) could show that some daphnids are capable of efficiently reducing cyanobacterial biomass, the opposite situation, i.e. the decline of *Daphnia* abundance due to cyanobacteria, was also observed (Ghadouani et al. 2003). According to this, we argue that actually not only presence and abundance of *Daphnia* is of high importance, but also the question, which species or genotypes are present. Several studies revealed that the sensitivity of *Daphnia* towards cyanobacterial diets differs strongly between species (Kuster and Von Elert 2013; Tillmanns et al. 2008) and between clones within a species (Jiang et al. 2013; Sarnelle and Wilson 2005; Schwarzenberger et al. 2012). For that reason, the elucidation of the physiological background explaining differences in the sensitivity of daphnids to cyanobacterial diets is crucial.

In an earlier study we conducted an experiment focusing on the most prominent and best studied cyanobacterial toxin, i.e. microcystin-LR (MCLR) (Sadler and von Elert 2014). In that study we could reject the hitherto assumed detoxification mechanism for MCLR via glutathione-S-transferase (GST) in *Daphnia* (Pflugmacher et al. 1998), and we proposed the possibility that transport mechanisms might be the key factor explaining differing sensitivities in *Daphnia* towards cyanobacteria.

Several studies showed pronounced negative effects of MCLR on *Daphnia* fitness parameters (Lürling 2003; Rohrlack et al. 1999). On the other hand, Wilson et al. (2006) did not find a significant effect of MC on zooplankton growth rates, when correlating cyanobacterial toxicity and morphology with population growth rates of several zooplankton genera. With respect to *Daphnia magna*, another meta study could not detect significant differences between cyanobacterial diets with or without MCs (Tillmanns et al. 2008). In line with that, cyanobacterial strains that do not produce MCs also have pronounced detrimental effects on *Daphnia* fitness parameters (Lürling and van der Grinten 2003; Schwarzenberger et al. 2009; Tillmanns et al. 2008). However, even in cases where a correlation between MCs and zooplankton fitness could be demonstrated, the question whether these correlations reflect causality remains unanswered.

In conclusion, it appears reasonable not only to focus on MCs in order to investigate the physiological background of *Daphnia´*s differing sensitivity towards cyanobacterial diets, but to take into account further secondary metabolites, which also have putatively negative effects on *Daphnia* fitness.

We therefore re-examined an already existing dataset of mass spectrometry, derived from a 24 h feeding experiment performed with the unselective filter feeder *Daphnia magna* and the MC containing cyanobacterium *Microcystis aeruginosa* PCC7806.

In that experiment we fed *D. magna* with saturating concentrations of the MC containing cyanobacterium for 24 h. In a subsequent chase experiment the daphnids were kept for another 54 h in the absence of cyanobacteria to account for released MCLR or MCLR conjugation products with a significant time delay. All experimental units (namely all incubation media, the cyanobacterial cells and the *Daphnia* tissue) from the 24 h feeding and the subsequent 54 h chase experiment were subjected to LC-MS measurements in order to detect and quantify both MCLR and MCLR conjugation products. Based on these data, Sadler and von Elert (2014) rejected the idea of significant biotransformation of MCLR by *Daphnia*.

In the present study we investigate the fate of further cyanobacterial secondary metabolites other than microcystins, after ingestion by *Daphnia magna*. In particular, we try to answer the question, if any of these compounds are subjected to biotransformation or if *Daphnia* exports these compounds without prior biotransformation into the surrounding water.

For this purpose, we re-examined the already existing mass spectral dataset in order to detect and quantify relative amounts of seven different peptides (cyanopeptolins A - C (CP A, CP B, CP C; aerucyclamides B - D (AC B, AC C, AC D) and microcyclamide 7806A (MC 7806A)). We also calculated the relative amounts within all different experimental stages and parts and summed these up to obtain the overall relative amount.

We focused on two distinct classes of cyanobacterial peptides, namely the cyanopeptolins (CPs) and the cyclamides. The widely distributed cyanopeptolins act as protease inhibitors (Gademann and Portmann 2008; Martin et al. 1993) and are produced in a non-ribosomal pathway similar to MCs (Welker and von Dohren 2006). Their detrimental effects on zooplankton have been demonstrated repeatedly (Agrawal et al. 2005; Schwarzenberger et al. 2010; Von Elert et al. 2012). Cyclamides also constitute a widely distributed cyanobacterial peptide class, which is produced by a ribosomal pathway (Portmann et al. 2008a; Portmann et al. 2008b; Ziemert et al. 2008). Only little is known about the impact of cyanobacterial cyclamides on zooplankton, but cyclamides have been shown to have cytotoxic properties with regard to the crustacean *Thamnocephalus platyurus* (Ishida et al. 2000; Portmann et al. 2008a; Portmann et al. 2008b).

Besides the quantification of these compounds we also screened for putative conjugation products of the respective compounds with cysteine or glutathione. Finally, we determined the relative amounts of these compounds within the cyanobacterial cells in order to find out if the presence of *Daphnia* leads to changes of the compound composition within the cyanobacterium. Our findings revealed new insights into the question how daphnids deal with cyanobacterial compounds after ingestion and clearly highlight the possibility of efficient transport mechanisms not only for microcystins but also for other cyanobacterial compounds. Moreover, the presented data suggest the existence of further yet unknown inducible active anti grazer defense mechanisms in *Microcystis* against *Daphnia*.

Material and Methods

In the present study an already published mass spectrometry dataset has been re-examined. The experimental details are therefore already described in Sadler and von Elert (2014) in detail. Here, we only give a brief overview of the experimental setup.

Culture conditions

We cultured *Daphnia magna* clone P (P132.85; derived from a pond (Driehoek) in Heusden (Netherlands), N51°44'01", E5°08'17") (De Meester 1994) in membrane-filtered (0.2 µm), aged tap water at 20 °C. This water, in the following referred to as medium, was used for all experiments. *Microcystis aeruginosa* PCC7806 was grown as chemostat culture in cyanobacteria medium (Von Elert and Jüttner 1997) (constant light: 50 µE m^{-2} s^{-1}; at 20 °C; dilution rate 0.23 per day). *Cryptomonas* sp. (Cryptophyceae, SAG 26.80; culture collection of algae at the University of Göttingen, Göttingen, Germany) was cultivated semi-continuously in cyanobacteria medium (constant light: 90 - 100 µE m^{-2} s^{-1} at 20 °C) with a 20 % medium exchange every two days. Food concentrations in the experiment were set to 5 mg particulate organic carbon (POC) per liter and were estimated from photometric light extinction at 470 nm with previously determined carbon extinction equations.

Exposure experiments

Short term exposure experiment (24 h)

200 adult *D. magna* individuals of various sizes in 500 mL of medium were fed with a 100 % diet of *M. aeruginosa* PCC7806 for 24 h. Cyanobacterial cell density was determined with a Neubauer improved counting chamber at the beginning and the end of the experiment. Experiments were replicated threefold, and controls were carried out in absence of daphnids.

Chase experiment

The *D. magna* from the short term exposure experiment were immediately transferred into the subsequent chase experiment. In the first 6 h, the animals were fed with a 100 % *Cryptomonas* sp. diet. Thereafter daphnids were transferred into fresh medium without food for another 48 h, with a first change of the medium after 24 h. Controls were carried out similarly, but in the absence of daphnids. To simulate experimental carryover that might

happen due to transfer of daphnids, we transferred 1 mL of each control sample into the respective following one.

Sample preparation

Daphnia tissue, *Microcystis* and *Cryptomonas* cells and the different culture media from the short term exposure experiment and the chase experiment were subsequently used for the quantification of seven cyanobacterial compounds, namely cyanopeptolins A, B and C and the cyclamides microcyclamide 7806A and aerucyclamide B, C and D. Microcystin-HilR (MC-HilR; homoisoleucine, arginine) was used as internal standard. Algal and cyanobacterial cells were separated from the medium by repeated centrifugation (2 min, 11.500 g), thereby pooling the supernatants (water sample) and obtaining one pellet per sample (cell sample). In general not subsamples, but always the entire samples were used for the quantification of the cyanobacterial compounds. Hence we obtained total amounts instead of concentrations. The amounts of all compounds of all experimental fractions from the short term exposure experiment (*Microcystis* cells and medium) and the chase experiment (*Cryptomonas* cells, *Daphnia* tissue and incubation medium) were summed up to obtain the overall amounts of the compounds within the entire experimental setup.

The relative metabolite amount per cyanobacterial cell was obtained by dividing the relative metabolite amount of the entire cyanobacterial cell fraction by the total number of cyanobacterial cells counted within that sample.

Preparation of cells and tissue – methanolic extraction

The cell pellets were resuspended in 10 mL 80 % methanol (MeOH), and 20 µL MC-HilR (5 µg mL^{-1}) was added as an internal standard (see 2.3). The cells were then sonicated (10 min) and centrifuged again (2 min, 11.500 g). The supernatant was transferred into new test tubes and evaporated to dryness in a vacuum centrifuge and finally dissolved in 100 µL 100 % MeOH. After another centrifugation (2 min, 11.500 g), the supernatant was transferred into glass vials.

Daphnids were separated from the medium using a 200 µm gauze. After rinsing the animals three times with ultrapure water, they were transferred into 1.5 mL test tubes, their fresh weight was measured, and the internal standard was added (20 µL, MC-HilR, 5 µg mL^{-1}). After that, 100 µL 80 % MeOH were added, and the tissue was homogenized using an electric

homogenizer. After adding another 400 µL of 80 % MeOH, the samples were sonicated (5 min) and centrifuged (3 min, 11.500 g). Finally the supernatant was transferred into 1.5 mL test tubes, evaporated to dryness in a vacuum centrifuge and then redissolved in 100 µL 100 % MeOH.

Extraction of water samples – solid phase extraction (SPE)

After separating the cells, the internal standard (20 µL MC-HilR, 5 µg mL^{-1}) and additionally 5 mL 100 % MeOH were added to the medium to obtain a 1 % methanolic solution. Water samples were then subjected to solid-phase extraction (SPE) with a C_{18} cartridge (50 mL Bond Elute C_{18}, BRAND) as according to the following protocol: 1. cleaning the cartridge (50 mL 100 % MeOH), 2. equilibration (50 mL 1 % MeOH), 3. adding of the complete sample (~500 mL with 1 % MeOH), 4. purification (50 mL 20 % MeOH), 5. elution (50 mL 80 % MeOH). The eluate was evaporated to dryness using a rotary evaporator (40 °C and 30 mbar). The dried sample was redissolved in 100 % MeOH and subsequently evaporated to dryness in a vacuum centrifuge. Finally, the sample was redissolved in 100 µL MeOH and transferred into a glass vial.

Note that in all cases the entire sample was processed with a final nominal concentration of internal standard of 1 µg mL^{-1}.

Liquid chromatography – mass spectrometry

For the measurements we used an Accela Ultra high pressure liquid chromatography (U-HPLC) system (Thermo Fisher) coupled with an Exactive orbitrap mass spectrometer (MS) (Thermo Fisher) with electrospray ionization (ESI) in positive ionization mode. A C_{18} column (Nucleosil, 125/2, 100-3) was used as stationary phase, and a gradient of acetonitrile (ACN) and ultrapure water, each containing 0.05 % trifluoroacetic acid (TFA) at a temperature of 30 °C and a flow rate of 300 µL per minute as mobile phase: 0 min: 38 % ACN; 2 min: 40 % ACN; 12 min: 50 % ACN; 12.5 min: 100 % ACN; 15 min: 100 % ACN; 15.5 min: 38 % ACN 17 min: 38 % ACN. The injection volume of the sample was 10 µL. Mass spectrometry measurements were carried out at a rate of one scan per second and a mass range of between 75 and 1500 Da (spray voltage 4.5 kV, capillary temperature 325 °C, sheath and aux gas nitrogen set to 45 and 15, respectively).

For the identification of compounds we used extracted ion chromatograms for the respective specific masses of the different compounds granting a mass deviation of 3 parts per million (ppm). Electrospray ionization resulted in adduct ions with one positive charge for all seven compounds. We used the Xcalibur software package (Thermo Fisher) for qualitative and quantitative analysis.

Compound selection and quantification

The *M. aeruginosa* strain PCC7806 contains a whole set of already described secondary metabolites. Among those are the cyanopeptolins A – D (Martin et al. 1993), cyanopeptolin 963A (Bister et al. 2004), aerucyclamides A – D and microcyclamide 7806A (Portmann et al. 2008a; Portmann et al. 2008b). We screened for all of these compounds within our dataset and picked those for further analysis, that could be found in both the incubation water and the cyanobacterial cell fraction from the 24 h short term exposure experiment and displayed clear and distinct peaks within their respective extracted ion chromatograms. Those criteria were met by the cyanopeptolins A, B and C and the cyclamides aerucyclamide B, C, D and microcyclamide 7806A (Tab.1). We only quantified these compounds relatively; therefore we cannot draw conclusions about the absolute contents of respective compounds in any of the experimental parts. For the relative quantification we did not use the peak areas, but always the peak area ratios of the respective compound with the internal standard MC-HilR, thereby ruling out effects, due to varying extraction efficiencies. For the calculation of the ratios of internal and external peak areas, peaks obtained from the extracted ion chromatograms (MS) for the specific masses of both substances were used (granted mass deviation < 3 ppm). Peak area ratios of less than 0.02 or in other words peak areas of compounds which displayed values less than 2 % of the internal standard peak area could not be determined properly and were therefore regarded as the limit of quantification.

Tab.1 Monoisotopic masses, m/z values, sum formulas and retention times on a C-18 HPLC column for the seven investigated cyanobacterial secondary metabolites.

compound	monoisotopic mass [Da]	m/z with z = 1	sum formula	retention time [min]
Cyanopeptolin A	956.53312	957.54095	C_{46} H_{72} N_{10} O_{12}	5.45
Cyanopeptolin B	928.52642	929.53425	C_{46} H_{72} N_{8} O_{12}	4.91
Cyanopeptolin C	942.54207	943.54990	C_{47} H_{74} N_{8} O_{12}	5.30
Microcyclamide 7806A	534.22550	535.23333	C_{24} H_{34} N_{6} O_{6} S	3.23
Aerucyclamide B	532.19210	533.19993	C_{24} H_{32} N_{6} O_{4} S_{2}	13.79
Aerucyclamide C	516.21494	517.22277	C_{24} H_{32} N_{6} O_{5} S	12.07
Aerucyclamide D	586.14907	587.15690	C_{26} H_{30} N_{6} O_{4} S_{3}	7.55

Data analysis

To compare different compounds with regard to their distribution throughout the experiment, we transformed the data by setting the highest measured value for a respective compound within the 24 h exposure experiment to one. The data concerning the chase experiment and the compound content per cell were similarly transformed by setting the highest single value of each compound within the chase experiment or each compound per *Microcystis* cell to one. For the comparison of relative compound amounts in the *Microcystis* cell fraction and the incubation medium in the 24 h exposure experiment, we performed analysis of variance (one way ANOVAs). One way ANOVAs were also used for the comparison of different relative compound amounts within the different experimental parts in the chase experiment. Prior to the ANOVAs, Shapiro-Wilk-Normality tests and equal variance tests were performed and expected to give *P* values above 0.05. In cases where these criteria were not met, we performed a Kruskal-Wallis ANOVA for AC-D in the 24 h incubation experiment and single t-tests for the comparison of the different cyanopeptolins and MCLR in the chase period (54 h-78 h). For the comparison of the respective total amounts of each compound we carried out t-tests, which were also used for the comparisons of relative compound content per *Microcystis* cell.

Results

Relative compound amounts in the 24 h exposure experiment

In a 24 h exposure experiment we determined the relative amounts of seven secondary metabolites (cyanopeptolins A, B and C; aerucyclamides B, C and D and microcyclamide 7806A, Tab.1) within the fraction of the cyanobacterial cells, the incubation medium (0 h – 24 h), and we quantified the overall amounts for each of these compounds (referred to as total amount), consisting of the sum of these two fractions plus the amounts that were found in the subsequent chase experiment (Fig.1).

The CPs A, B and C, MC 7806A and AC D revealed significant differences between the different treatments (one way ANOVA CP A: $F = 18.001$, $P < 0.001$; CP B: $F = 12.73$, $P = 0.002$; CP C: $F = 19.877$, $P < 0.001$; MC 7806A: $F = 77.599$, $P < 0.001$; Kruskal-Wallis one way ANOVA on Ranks AC D $H = 8.641$, $P = 0.034$) (Fig.1). Aerucyclamides B and C did neither occur in differing amounts in the cyanobacterial cell fraction compared to the incubation medium nor reveal any differences due to the presence of *Daphnia* (one way ANOVA AC B: $F = 0.826$, $P = 0.516$; AC C: $F = 2.314$, $P = 0.153$) (Fig.1).

With regard to the total compound amounts, five compounds revealed no significant differences (t-test CP A: $P = 0.131$; CP B: $P = 0.358$; AC B: $P = 0.376$; AC C: $P = 0.984$; AC D: $P = 0.444$), whereas two compounds (CP C and MC 7806A) increased due to the presence of *Daphnia* (t-test MC 7806A, $P = 0.003$; CP C, $P = 0.022$) (Fig.1).

Cyanopeptolins

The presence of 200 individuals of *D. magna* led to a reduction of the relative CP A content in the cyanobacterial cells by 34 % (Tukey test after one way ANOVA, $P = 0.011$). This decrease is reflected by a corresponding increase of the relative CP A amount in the incubation media (Tukey test after one way ANOVA, $P = 0.002$). Accordingly, the total amount did not change due to *Daphnia* presence (t-test, $P = 0.131$) (Fig.1).

CP C exhibited a very similar pattern with a decrease of the relative CP C content in the cell fraction by 32 % (Tukey test after one way ANOVA, $P = 0.01$), which again was well reflected in a similar increase of the relative CP C content in the incubation medium due to the presence of *Daphnia* (Tukey test after one way ANOVA, $P = 0.002$) (Fig.1). Notwithstanding the CP A findings, this did not result in an unaltered total CP C amount, but

in a slightly increased total amount of CP C in the presence of daphnids (t-test, $P = 0.022$) (Fig.1).

The distribution of CP B differed from that of the other two cyanopeptolins insofar, as the presence of daphnids did not affect the relative CP B content (Fig.1). This accounts for the cyanobacterial cell fraction (Tukey test after one way ANOVA, $P = 0.287$) as well as for the incubation medium (Tukey test after one way ANOVA, $P = 0.910$). Detected differences therefore were only due to the differing compound amounts in the medium and in the cell fraction but never due to *Daphnia* presence. The total amount of CP B was also not affected by the presence of *Daphnia* (t-test, $P = 0.358$) (Fig.1).

Microcyclamide 7806A

In the case of MC 7806A content, the presence of daphnids did not lead to a significant change within the cyanobacterial cell fraction (Tukey test after one way ANOVA, $P = 0.559$) (Fig.1). Nevertheless, the presence of *Daphnia* caused an increase of the relative amount of MC 7806A in the incubation medium (Tukey test after one way ANOVA $P < 0.001$). The overall MC 7806A amount increased strongly (82.5 %) in the presence of daphnids (t-test $P = 0.003$) (Fig.1).

Aerucyclamides B, C and D

The amounts of all three investigated aerucyclamides (ACs B, C and D) were not affected by the presence of *Daphnia*, neither in the incubation medium nor in the cyanobacterial cell fraction (Fig.1). Accordingly, the overall amount was also not affected by the presence of *Daphnia* (Fig.1).

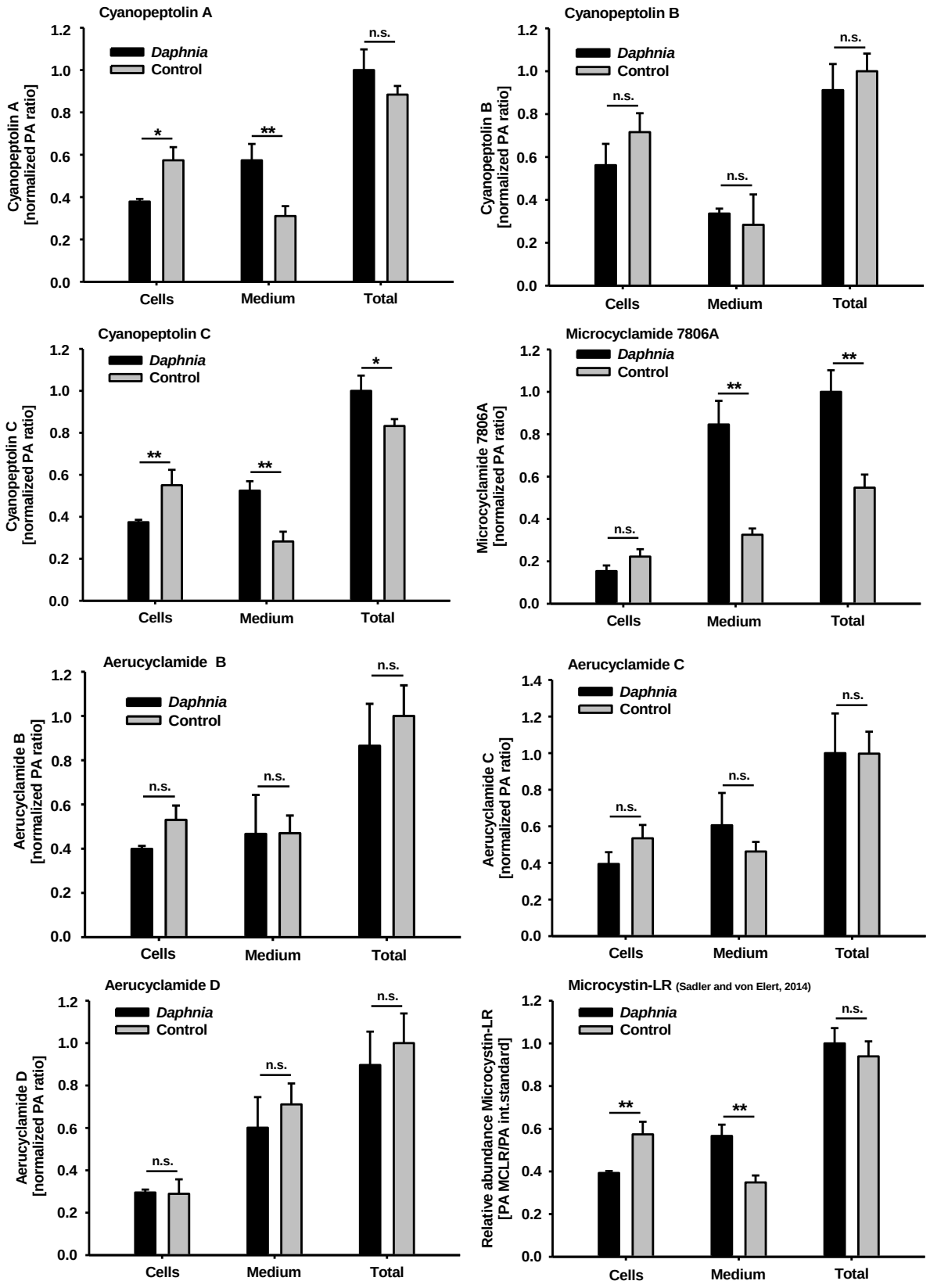

Fig.1 Relative amounts of seven cyanobacterial compounds (cyanopeptolin A, B, C, microcyclamide 7806A, aerucyclamide B, C, D) within the cyanobacterial cells, the incubation water (first incubation period: 0 – 24 h) and the total sum of the relative amounts from the *D. magna* 24 h exposure experiment and the subsequent chase experiment in the presence (black bars) or absence (gray bars) of daphnids. Relative amounts are given in peak

area ratios of the respective compound relative to the internal standard MC HilR. Values are additionally normalized by setting the highest value to one. Additionally depicted are earlier published findings for microcystin-LR (Sadler and von Elert 2014) also given as normalized peak area ratios. Means + SD, n = 3. For *Microcystis* cells and incubation water: Tukey-test, ** $P < 0.01$, * $P < 0.05$, after one-way ANOVA. For the summed total amounts: t-test, ** $P < 0.01$, * $P < 0.05$.

Relative compound amounts in the chase experiment

In the *Daphnia* treatment we detected significant amounts of the CPs A, B and C in all experimental parts (Tab.2, Fig.2). However, none of the ACs (B, C or D) or MC 7806A could be detected in any experimental part of the chase experiment (Tab.2, Fig.2). The CPs within the chase experiment were in general less abundant than in the prior 24 h exposure experiment (Tab.2). In the medium sample from the first chase period (24 h – 30 h) the relative CP amounts did not differ among each other and also not with regard to MCLR (Sadler and von Elert 2014) (one way ANOVA, $F = 0.445$, $P = 0.727$) (Fig.2). The medium sample from the second chase period (30 h – 54 h) revealed no significant differences between the relative CP amounts. However, the sample contained more CPs in comparison to MCLR, which was significant for the comparison of CP A with MCLR (Tukey test after one way ANOVA, $P = 0.027$) (Fig.2). The medium from the third incubation period (54 h – 78 h) contained in general less relative amounts of CPs and MCLR than the two prior incubation media. We again found higher relative amounts of CPs in comparison to MCLR, which was significant for the comparison of the CPs A and C with MCLR (t-test, $P = 0.015$ for CP A and $P = 0.024$ for CP C) (Fig.2). With regard to the compound content of the *Daphnia* tissue, we could not detect any differences among the CPs, but again relative CP amounts were higher compared to MCLR, which was significant for the comparison of CP B with MCLR (Tukey test after one way ANOVA, $P = 0.013$) and CP C with MCLR (Tukey test after one way ANOVA, $P = 0.024$) (Fig.2). The *Cryptomonas* cells contained very little relative amounts of CP A, CP C and MCLR and no CP B (Tab.2, Fig.2). The amounts of CP A, CP C and MCLR were probably caused by residual medium adhering to the *Cryptomonas* cell surfaces. In general, the distribution patterns of all three CPs looked very similar and did not differ from each other significantly. CPs were equally abundant in comparison to MCLR in the first incubation medium, but occurred in higher amounts in the latter chase phases compared with MCLR (Fig.2).

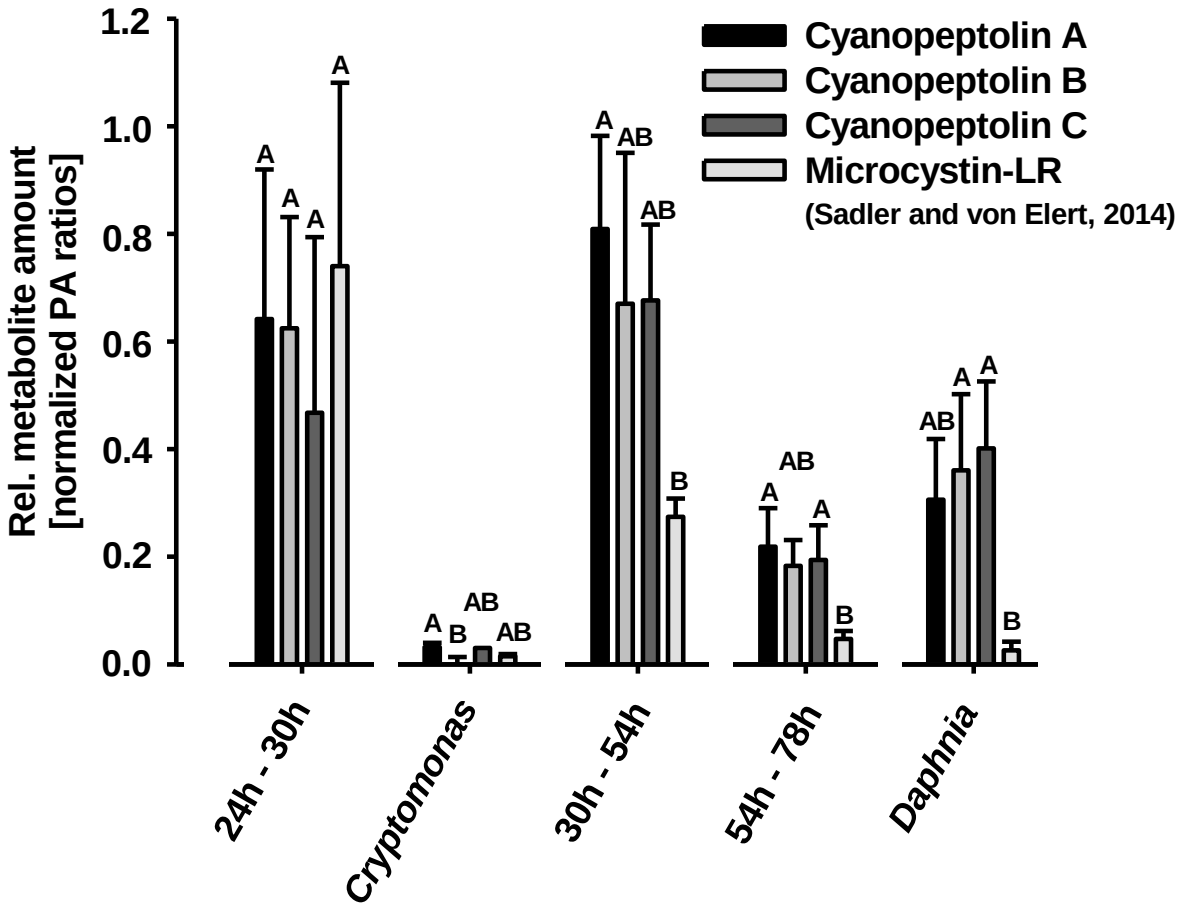

Fig.2 Relative amounts of cyanopeptolins A, B and C in the chase experiment. Depicted are the amounts in the different incubation media, the *Cryptomonas* cells and the *Daphnia* tissue carried out with the 200 *D. magna* from the 24 h exposure experiment (fed for 24 h with a 100 % *M. aeruginosa* diet, see Fig.1). The daphnids were incubated for another 6 h with *Cryptomonas* sp. and subsequently two consecutive times for 24 h without food. In the controls without daphnids the amounts of cyanopeptolins were below the limit of quantification. Relative amounts are given in peak area ratios of the respective compound relative to the internal standard MC-HilR. Values were additionally normalized by setting the highest value to one. Additionally depicted are earlier published findings for microcystin-LR (Sadler and von Elert 2014) also given as normalized peak area ratios. Means + SD, n = 3. Means + SD, n = 3. Different letters indicate significant differences within each experimental part. Tukey test, $P < 0.05$, after one way ANOVA.

Relative compound amounts within the cyanobacterial cells

When comparing the relative cellular concentrations of all seven compounds between the *Daphnia* treatment and the control, we found the general tendency that all compounds were elevated in the *Daphnia* treatment (percentaged increases: CP A 11.6 %, CP B 33.6 %, CP C 15.2 %, AC B 27.3 %, AC C 25.5 %, AC D 73.5 % and MC 7806A 18.1 %) (Fig.3).

However, only in case of ACs B and D this difference was significant (t-tests: CP A P = 0.102, CP B P = 0.14, CP C P = 0.097, AC B P = 0.017, AC C P = 0.198, AC D P = 0.008, MC 7806A P = 0.337) (Fig.3).

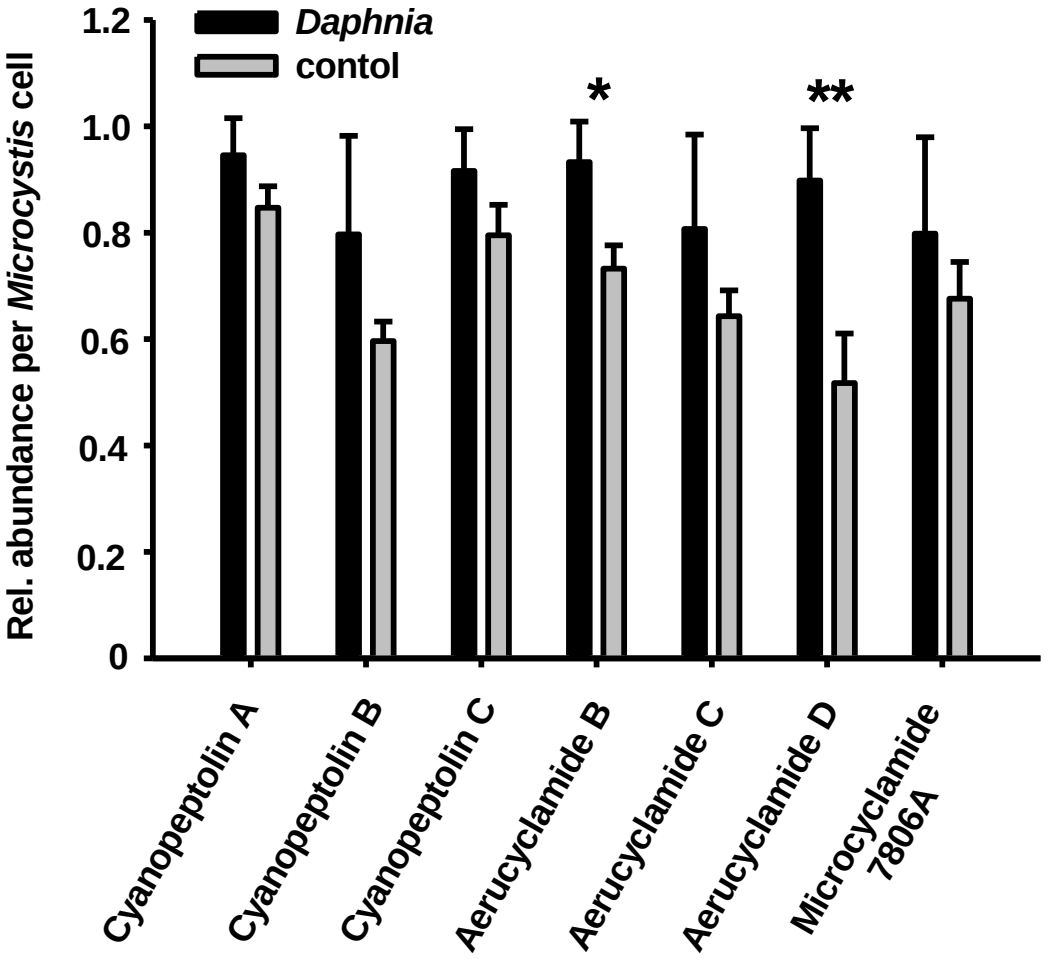

Fig.3 Relative amounts of seven cyanobacterial compounds (cyanopeptolin A, B, C, microcyclamide 7806A, aerucyclamide B, C, D) per cyanobacterial cell. Depicted are the relative amounts (peak area ratios of the respective compound relative to the internal standard) from cells kept in the presence (black bars) or absence (gray bars) of daphnids. Values were additionally normalized by setting the highest value to one. Means + SD, n = 3. t-test: * $P < 0.05$, ** $P < 0.01$.

Conjugation products

In addition to the quantification of the seven compounds we also searched for the exact masses of putative *Daphnia* conjugation products of the respective compounds with glutathione or cysteine. However, we could not detect any conjugation products with cysteine or glutathione for any of these seven compounds.

Tab.2 Relative compound amounts of the cyanopeptolins A, B and C and the cyclamides microcyclamide 7806A and aerucyclamide B, C and D given as peak area ratios of the respective compound with the internal standard MC-HilR within the different experimental parts and the overall sum of the relative amounts. Means ± SD, n = 3.

	Experimental part	Cyanopeptolin A	Cyanopeptolin B	Cyanopeptolin C	Microcyclamide 7806A	Aerucyclamide B	Aerucyclamide C	Aerucyclamide D
***Daphnia* 24 h - exposure experiment**	Incubation water 0h - 24 h	80.13 ± 10.8	15.63 ± 1.06	12.32 ± 1.05	0.83 ± 0.11	2.94 ± 1.11	28.64 ± 8.34	19.11 ± 4.58
	Microcystis cells	52.88 ± 1.80	26.12 ± 4.61	8.79 ± 0.27	0.15 ± 0.03	2.51 ± 0.09	18.63 ± 3.06	9.39 ± 0.43
***Daphnia* chase experiment**	Incubation water 24 h - 30 h	2.13 ± 0.92	0.22 ± 0.11	0.63 ± 0.28	not detected	not detected	not detected	not detected
	Cryptomonas cells	0.11 ± 0.03	not detected	0.04 ± 0.02	not detected	not detected	not detected	not detected
	Incubation water 30 h - 54 h	2.69 ± 0.58	0.24 ± 0.05	0.91 ± 0.38	not detected	not detected	not detected	not detected
	Incubation water 54 h - 78 h	0.73 ± 0.24	0.06 ± 0.02	0.26 ± 0.06	not detected	not detected	not detected	not detected
	Daphnia tissue	1.02 ± 0.37	0.13 ± 0.04	0.54 ± 0.19	not detected	not detected	not detected	not detected
***Daphnia* total**	Total amount	139.68 ± 13.62	42.39 ± 5.65	23.49 ± 1.70	0.99 ± 0.10	5.45 ± 1.20	47.27 ± 10.24	28.49 ± 5.00
control 24 h – exposure experiment	Incubation water 0h - 24 h	43.38 ± 6.48	13.20 ± 6.58	6.63 ± 1.09	0.32 ± 0.03	2.96 ± 0.50	21.84 ± 2.48	22.59 ± 3.14
	Microcystis cells	80.12 ± 8.70	33.29 ± 4.10	12.93 ± 1.73	0.22 ± 0.03	3.34 ± 0.41	25.28 ± 3.44	9.18 ± 2.17
control chase experiment	Incubation water 24 h - 30 h	not detected	not detected	not detected	not detected	not detected	not detected	not detected
	Cryptomonas cells	not detected	not detected	not detected	not detected	not detected	not detected	not detected
	Incubation water 30 h - 54 h	not detected	not detected	not detected	not detected	not detected	not detected	not detected
	Incubation water 54 h - 78 h	not detected	not detected	not detected	not detected	not detected	not detected	not detected
	Daphnia tissue	not detected	not detected	not detected	not detected	not detected	not detected	not detected
control total	Total amount	123.50 ± 5.68	46.48 ± 3.82	19.56 ± 0.76	0.54 ± 0.06	6.30 ± 0.87	47.12 ± 5.69	31.77 ± 4.45

Discussion

After ingestion by *Daphnia magna*, none of the seven investigated cyanobacterial secondary metabolites has been subjected to any kind of biotransformation or biodegradation within the animals. Instead, as already suggested for microcystins (Sadler and von Elert 2014), transporters seem to be of high importance for daphnids, when dealing with putatively detrimental dietary metabolites.

We re-examined an existing mass spectrometry dataset to investigate the fate of various secondary metabolites derived from the cyanobacterium *M. aeruginosa* PCC7806. The quantification of these metabolites represents a very powerful tool to test for putative biotransformation processes, since any such process would necessarily lead to an overall decrease of the respective metabolite. This is independent of the question whether the conjugation product itself is detectable or not.

Within the whole experimental setup, however, overall amounts of none of the investigated metabolites decreased due to the presence of daphnids (Fig.1). This clearly rejects the idea of significant processes of biotransformation or biodegradation by *Daphnia*.

Cyanopeptolins

24 h exposure experiment

The presence of *D. magna* led to a significant reduction of cyanobacterial cells by 35 % due to ingestion (Sadler and von Elert 2014). The amounts of the CPs A and C within the cyanobacterial cell fraction are similarly decreased by 34 % and 32 %, respectively (Fig.1), which therefore can be attributed to this decrease in cell abundance. The intracellular CP A and CP C concentrations were not influenced significantly by the presence of daphnids (Fig.3). The same accounts for the overall amounts of these metabolites, which either revealed no differences with regard to CP A or even a slight increase in the case of CP C (Fig.1). CPs A and C therefore very well reflect already published findings within this experimental setup for MCLR, where we found a decrease of MCLR within the cyanobacterial cell fraction, well reflected by a similar increase in the medium resulting in an unaltered overall MCLR amount (Fig.1). This was interpreted to point at the likely presence of a transport mechanism in *Daphnia*, capable of transporting unconjugated microcystins (Sadler and von Elert 2014). The same seems to be true for the CPs A and C. Similar to the case of MCLR, we can rule out that

the increase of CPs in the medium is caused by sloppy feeding only and would therefore not require any kind of transport. The chase experiment clearly showed (Fig.2) that CPs can be found in the latter incubation media and in the *Daphnia* tissue, which proves that CPs were taken up in the *Daphnia* tissue at least to a certain extent.

CPs are protease inhibitors and act against trypsins and chymotrypsins (Martin et al. 1993), and their function as grazer defense has been shown convincingly (Agrawal et al. 2005; Schwarzenberger et al. 2010; Von Elert et al. 2012). In the case of the CPs A and C, it seems that daphnids are capable of exporting these metabolites, thus explaining the increase of metabolite content in the medium (Fig.1). The observation that total amounts of these metabolites did not decrease (Fig.1), suggests, that *Daphnia* have no mechanism to biotransform or metabolize these substances, which would have resulted in overall lower amounts.

The distribution of CP B strongly differs from that of the CPs A and C, since we could not detect any differences between the *Daphnia* treatment and the control (Fig.1). In particular the amount of CP B in the fraction of cyanobacterial cells remained unchanged despite the ingestion of 35 % of the cells by *Daphnia*. This can be well explained by an increase of the mean cellular content of CP B in the cyanobacterial cells by approx. 33.6 % (Fig.3), which was almost significant. This effect strongly suggests that unknown chemical cues from actively grazing *Daphnia* induce an elevated content of CP B. Tonk et al. (2009) observed changes in the production of cyanopeptolins with nutrient limitation. Specifically they found decreased concentrations of the CPs A and C under phosphorous limited conditions, which appeared to be compensated by increased production of CP 970. In our case the almost significant increase in the cellular content of CP B was not related to a simultaneous decrease in any other of the investigated CPs, so that no evidence for a compensatory mechanism as proposed by Tonk et al. (2009) was found.

All investigated cyanopeptolins were additionally found in different parts of the subsequent chase experiment, namely in the different incubation media, the *Cryptomonas* cells and the *Daphnia* tissue (Fig.2).

Chase experiment

The results from the chase experiment provide strong evidence that CPs were taken up in the *Daphnia* tissue and subsequently released with a significant time delay. This implies the

existence of export mechanisms capable of transporting non-metabolized CPs. This export of CPs out of *Daphnia* tissue apparently happens with a lower efficiency than the export of MCLR.

In the incubation media from the chase experiment, relative CP amounts were always higher in comparison to MCLR, with exception of the first incubation period (24h - 30 h) (Fig.2). Since no cyanobacteria were present at any time during the chase experiment, the daphnids constituted the only source for the cyanobacterial compounds in these media. Although the first incubation period (24 h - 30 h) within the chase experiment was, with a duration of six hours, shorter than the latter two incubation periods, which lasted for 24 hours each (30 h - 54 h and 54 h - 78 h), the MCLR content in the early incubation medium was considerably higher than those in the two later incubation periods. This finding points at a high MCLR export efficiency. The cyanopeptolins were about equally abundant in the first (24 h - 30 h) and the second (30 h - 54 h) incubation medium despite the different duration times. However, the comparison between the relative amounts of the CPs with MCLR clearly shows, that CPs disappear from the *Daphnia* tissue with a lower efficiency; this is not only supported by the higher relative amounts of CPs in the later incubation media, but also by the higher relative amounts of CPs in the *Daphnia* tissue after termination of the experiment (Fig.2).

We cannot draw any definite conclusions what causes these differing export efficiencies among CPs and MCLR. It might be that MCs and CPs are both substrates for the same trans-membrane transporters with differing substrate binding properties. On the other hand CPs and MCs might be exported by entirely different transporters. Unfortunately, there is nothing known about transport processes in aquatic organisms with regard to cyanopeptolins. However, with respect to MC, it has been suggested for the mussel *Dreissena polymorpha* (Contardo-Jara et al. 2008) and for the fish *Jenynsia multidentata* (Ame et al. 2009) that multi xenobiotic resistance proteins (P-glycoproteins) are involved in the transport of MC.

Cyclamides

Microcyclamide 7806A

The distribution pattern of MC 7806A might hint at an active anti grazer defense in *Microcystis aeruginosa*. The data presented here suggest, that *M. aeruginosa* increases the production of MC 7806A and subsequently exports the compounds out of the cell as a response to the presence of *Daphnia*.

Despite a reduction of cyanobacterial biomass of 35 % (Sadler and von Elert 2014), the overall amount of MC 76806A increased by 82.5 % due to the presence of *Daphnia* (Fig.1). This overall increase must be due to synthesis of new MC 7806A during the 24 h incubation in the presence of *Daphnia*. However, since the relative concentration of MC 7806A per *Microcystis* cell did not increase (Fig.3), we argue that the cyanobacteria not only increased MC 7806A production but also exported the compound out of the cell. In line with that, the 82.5 % overall increase of MC 7806A is due to an increase of this compound within the medium and not caused by an increased amount within the cyanobacterial cell fraction (Fig.1). Although nothing specific is known about the toxicity of MC 7806A, cyclamides in general have cytotoxic properties (Ishida et al. 2000) and have been shown to be detrimental for crustaceans (Portmann et al. 2008a; Portmann et al. 2008b). Nothing however is known about the effects of cyclamides on daphnids.

Nevertheless, these findings constitute a first hint that the presence of actively grazing *Daphnia* induces an increased export of a cyanobacterial metabolite. As cyclamides are putatively detrimental to *Daphnia*, this induced synthesis and release might serve as an active anti-grazer defense. Although increased metabolite production due to grazer presence in cyanobacteria could be shown several times for microcystins (Jang et al. 2003; Jang et al. 2004; Jang et al. 2007; Jang et al. 2008), there are hitherto no reports about a grazer-induced export of compounds into the surrounding medium.

Since no MC 7806A could be found within any part of the subsequent chase experiment including the *Daphnia* tissue, we argue that daphnids are capable of exporting MC 7806A efficiently. The combined effects of metabolite export by daphnids and cyanobacteria provide a good explanation for the strong increase of the relative MC 7806A content in the medium.

Aerucyclamides

The ACs B, C and D did not show significant differences in any experimental part due to the presence of *Daphnia*. This finding was puzzling with regard to the significant reduction of cyanobacterial cells (35 %) by the daphnids (Sadler and von Elert 2014). However, as already argued above, the absence of a decrease of the respective compounds within the cyanobacterial cell fraction can be attributed to a simultaneously increased content in the remaining cells, as was observed for of ACs B and D. With regard to MCLR, earlier studies could show that cyanobacteria respond to the presence of grazers with increased compound production (Jang et al. 2003; Jang et al. 2004; Jang et al. 2007; Jang et al. 2008). It remains

unclear, why such an increase did not lead to an overall increase of the respective metabolite. Though it is tempting to speculate that this was caused by biotransformation of ACs by *Daphnia*, no evidences for a conjugation of ACs with cysteine or glutathione could be detected.

Cyanopeptolins and cyclamides

The distribution patterns of the different cyanobacterial secondary metabolites allow first insights into the nature of putative xenobiotic transport mechanisms in *Daphnia*.

The basic structure of CPs consists of a β-lactone ring of six amino acids, whereby the cyclisation is established by an ester bond between the β-hydroxy group of a threonin (prolin in some cases) with the carboxyl group of an amino acid at position six. The presence of the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) is characteristic as well (Martin et al. 1993). However, cyclamides (such as aerucyclamides or microcyclamides) have very different structural properties. Usually, they contain thiazol and oxazol units, which are typically arranged alternating with unmodified lipophilic amino acids, thereby forming a cyclic hexapeptide.

In general, the high variability of cyanobacterial secondary metabolites is attributable to their mode of synthesis. In that context, it is noteworthy that cyanopeptolins and cyclamides not only differ due to their structural properties but also due to their biochemical pathways of synthesis. Most cyanobacterial oligopeptides, such as microcystins and cyanopeptolins, are produced by large multifunctional enzyme complexes, including the non-ribosomal peptide synthetases (NRPSs) and polyketide sythases (PKS) (Welker and von Döhren 2006). In contrast, the biosynthesis of cyclamides takes place via a ribosomal pathway involving a precursor protein (Ziemert et al. 2008), similar to the patellamide pathway discovered in *Prochloron didemni*, a symbiotic cyanobacterium of the ascidian *Lissoclinum patella* (Schmidt et al. 2005).

While here the CPs by trend appear to be distributed similar to MCLR, the distribution of aerucyclamides shows a different pattern. These differences might be related to the different pathways of biosynthesis and to the different chemical structures. According to retention times on a reversed phase C_{18} column, the aerucyclamides investigated here are more lipophilic compounds than the cyanopeptolins (Tab.1). MC 7806A displays by far the lowest lipophilicity, which can be explained by the absence of an oxazoline ring, notwithstanding the

other investigated cyclamides. Since MC 7806A can be regarded as a hydrolysis product of AC C, it has been suggested that MC 7806A is generated during the employed extraction procedure and does not occur naturally in *M. aeruginosa* PCC7806 cells (Portmann et al. 2008b). Our results rather point at the actual existence of this compound in the cyanobacterium, since we found differing distributions of AC C and MC 7806A throughout the experiment. Our results show, that the less lipophilic compounds like cyanopeptolins, MCLR and MC 7806A are probably exported out of *Daphnia* tissue, whereas we could not detect such an effect with regard to the very lipophilic aerucyclamides. Therefore, one might speculate that more lipohilic compounds provide less suitable substrates for *Daphnia* trans membrane transporters.

However, while the data presented here provide strong evidence for the existence of transport mechanisms in *Daphnia* not only for microcystins, but also for cyanopeptolins and MC 7806A, they only deliver preliminary clues regarding the nature and properties of such trans membrane transporters. Only little is known about transport mechanisms in *Daphnia*. As already stated above the involvement of a multi xenobiotic resistance protein (P-glycoprotein) was suggested for bivalves (Contardo-Jara et al. 2008) and fish (Ame et al. 2009). However, in mammals organic anion transporter polypeptides (Oatps) seem to be of major importance with regard to MCLR transport into the liver and across the blood brain barrier (Feurstein et al. 2009; Fischer et al. 2005). Sturm et al. (2009) confirmed the existence of multi xenobiotic resistance proteins in the *Daphnia pulex* genome, but sequences similarities with known Oatp sequences can be found as well in the *D. pulex* genome (unpublished data).

Additional studies are necessary to further explore the idea of transport of cyanobacterial secondary metabolites in *Daphnia,* since transportation might be a key factor to understand *Daphnia´*s varying sensitivity towards cyanobacteria.

Conclusions

The data presented here provide clear evidence underlining the importance of transport processes for *Daphnia*, when confronted with putatively toxic dietary cyanobacterial compounds. At the same time we were able to reject the idea of significant processes of biotransformation or biodegradation for cyanobacterial compounds in daphnids due to the fact that within a closed experimental setup none of seven investigated metabolite abundances was

reduced by the presence of *Daphnia*. These results are in line with earlier findings with a similar outcome for the most prominent cyanotoxin MCLR.

Additionally, we report an increase in intracellular concentration of cyanobacterial compounds other than microcystins in the cyanobacterial cells caused by the presence of actively grazing *Daphnia*. This suggests, for the first time, a role of these compounds as anti-herbivore defense in cyanobacteria. In addition we report for the first time that the presence of *Daphnia* furthermore induces the elevated export of MC 7806A from the cyanobacterial cells into the surrounding medium, which suggests that this extracellular compound might constitute another not yet described line of anti-grazer defense in *Microcystis*.

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Chapter 3

Dietary microcystins in *Daphnia magna*

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The role of multi xenobiotic resistance (MXR) proteins and organic anion transporting polypeptides (Oatps)

Abstract

Cyanobacterial blooms in inland waters are an issue of increasing importance, due to anthropogenic nutrient input into water bodies and global warming. Especially the ability of cyanobacteria to produce huge amounts and a high variety of often toxic secondary metabolites emphasizes their crucial role in freshwater ecosystems. Therefore, understanding the interaction of cyanobacteria with its natural consumer, i.e. the unselective filter-feeder *Daphnia,* which may play a key role in the suppression of cyanobacterial blooms, is of pivotal interest. The most widespread and most investigated cyanobacterial toxins are the microcystins (MCs), which are detrimental for humans, but also for herbivorous zooplankton. A widely accepted assumption is that within aquatic consumers, such as *Daphnia* the enzyme glutathione-S-transferase (GST) is involved in the detoxification of MCs by conjugation of MCs to glutathione. However, recent findings rejected the importance of *Daphnia* GST for MC detoxification *in vivo* and pointed at the importance of trans-membrane transporters capable of exporting unconjugated MCs out of *Daphnia* tissue. In the present study we investigate the role and function of two transporter types, namely the multi xenobiotic resistance proteins (MXR or P-glycoprotein, P-gp) and the organic anion transporting polypeptides (Oatps). For this purpose we used high resolution LC-MS analytics to determine the amount of microcystin-LR (MCLR) within the tissue of *Daphnia magna* that fed for 24 h on a MC containing diet of the cyanobacterium *Microcystis aeruginosa*. This experiment was carried out with different putative inhibitors for P-gps and Oatps dissolved in the surrounding water. For each transporter type we identified an inhibitor leading to strongly increased MCLR amounts within the *Daphnia* tissue. The Oatp inhibitor taurocholate caused an increase of MCLR in the *Daphnia* tissue by 75 %, and the putative P-gp inhibitor cyclosporine A caused an even stronger increase of tissue MCLR by 106 %. These results provide clear and concise evidence for an involvement of both transporter types in the export of unconjugated MCLR out of *Daphnia* tissue. A quantitative PCR revealed no changes in expression of two Oatp and two P-gp related gene sequences upon 24 h exposure of *D. magna* to dietary MCs.

Nevertheless, our data strongly point at a crucial role of Oatp and P-gp transporters with regard to the export of MCLR in *Daphnia*.

Introduction

In recent years the extent and frequency of cyanobacterial blooms in standing freshwater bodies has increased, due to increasing anthropogenic influence, such as nutrient loading and global warming (Dokulil and Teubner 2000; O'Neil et al. 2012; Paerl and Huisman 2008). This development constitutes a serious hazard with regard to the usage of surface waters as source for drinking water, for recreational purposes and for various other ecosystem services (Stewart et al. 2006). Moreover, mass occurrences of cyanobacteria strongly affect the zooplankton community (Ghadouani et al. 2003; Wilson et al. 2006), especially the filter feeder *Daphnia*, due to its inability to discriminate between good and bad food items.

Accordingly, the interaction of cyanobacteria with *Daphnia* species has gained more and more attention (Ger et al. 2014; Tillmanns et al. 2008; Wilson et al. 2006) with rather ambiguous results. While some studies show that cyanobacteria have strong negative effects on *Daphnia* populations (Ghadouani et al. 2003), others find the opposite situation, such that *Daphnia* populations are capable of suppressing cyanobacterial bloom formation (Chislock et al. 2013). *Daphnia* populations can adapt to detrimental cyanobacteria spatially (Sarnelle and Wilson 2005) as well as temporally (Hairston et al. 1999). Accordingly, the sensitivity of daphnids towards cyanobacterial diets differs enormously between species (Tillmanns et al. 2008) and between different genotypes of the same species (Jiang et al. 2013b; Sarnelle and Wilson 2005; Schwarzenberger et al. 2012). In addition to that it could be shown that daphnids pre-exposed to toxic cyanobacteria develop an increased resistance against these cyanobacteria in a short time span, which can even be handed over to the next generation via maternal effects (Gustafsson and Hansson 2004; Gustafsson et al. 2005; Schwarzenberger and Von Elert 2013). These ambiguous outcomes in different studies investigating the *Daphnia* – cyanobacteria interaction could therefore be explained with the differing degree of tolerance of the respective *Daphnia* species or genotypes.

Obviously, a deep understanding of the underlying physiological mechanisms behind these phenomena is necessary in order to shed further light on the nature of the *Daphnia* – cyanobacteria interaction.

Another reason highlighting the importance of this interaction can be found in the field of applied sciences. Some strategies in water resource management include biomanipulative approaches aiming at the control of harmful cyanobacterial blooms (Shapiro et al. 1975; Hansson et al. 1998). These approaches usually include the manipulation of higher trophic

levels in order to increase size and abundance of herbivorous zooplankton, for instance by adding piscivorous fish or removing planktivorous ones (Shapiro et al. 1975; Shapiro and Wright 1984). It has been proposed that the presence or absence of *Daphnia* is of major importance for such biomanipulative approaches (Leibold 1989). Doubtlessly, an increased understanding of the physiological basis of the *Daphnia* - cyanobacteria interaction could positively influence the success of such biomanipulations.

In general, cyanobacteria are of minor food quality for daphnids due to several reasons. Among those are the mechanical interferences with *Daphnia*s' filter apparatus due to cyanobacterial colony formation (Porter and Mcdonough 1984), the lack of important nutritional compounds like sterols and poly unsaturated fatty acids (PUFAs) (Von Elert et al. 2003; Von Elert 2004) and the presence of cyanobacterial secondary metabolites with detrimental effects on *Daphnia* fitness parameters (Agrawal et al. 2005; Hansson et al. 2007; Von Elert et al. 2005).

Among the high number and variety of cyanobacterial secondary metabolites the microcystins (MCs) are the most frequent and best studied ones. MCs are heptapeptides and act as potent inhibitors of the protein phosphatases PP1 and PP2A (Runnegar et al. 1995; Honkanen et al. 1990). Inhibition of these enzymes subsequently leads to cellular hyperphosphorylation resulting in severe cell damage (Eriksson et al. 1990; Ohta et al. 1992). However, the toxicological effects of MCs are diverse and only known to a limited extent, especially with regard to aquatic organisms (Dittmann and Wiegand 2006). Nevertheless, MC containing diets have pronounced negative effects on fitness parameters of *Daphnia* (Rohrlack et al. 1999; Rohrlack et al. 2001). The question how daphnids deal with MCs on a physiological level might therefore deliver a good explanation for differing sensitivities towards cyanobacteria in general.

It is widely assumed that aquatic invertebrates detoxify MCs by conjugation of MCs with the tripeptide glutathione (GSH) via the enzyme glutathione-S-transferase (GST). This process was demonstrated to be of major importance for MC detoxification in several other aquatic organisms (Campos and Vasconcelos 2010; He et al. 2013; Li et al. 2014; Zhang et al. 2009). Moreover, Pflugmacher et al. (1998) showed *in vitro* the enzymatic formation of MC-GSH conjugates after adding GSH and MCs to crude *Daphnia* extracts.

However, in a recent study Sadler and von Elert (2014) rejected the relevance of GST conjugation in the living *Daphnia* and suggested the involvement of transport mechanisms for

the export of unconjugated MCs. Such transport mechanisms might constitute the key factor explaining *Daphnia'*s varying ability to cope with cyanobacterial toxins, such as MCs.

We identified two different transporter types which constitute promising candidates for the transport of cyanobacterial toxins in general and MCs in particular in *Daphnia*. The multi xenobiotic resistance mechanism (P-glycoprotein) (MXR, P-gp) has been shown to be of major importance for aquatic organisms (Kurelec 1992) and was proposed to be the reason for the insensitivity of bivalves towards cyanobacterial toxins (Contardo-Jara et al. 2008). P-gp related sequences can also be found in the genomes of *D. pulex* and *D. magna* (Campos et al. 2014; Sturm et al. 2009). The second transporter type that might be involved in the export of MCs could be the organic anion transporting polypeptides (Oatps). The superfamily of Oatp transporters consists of at least ten protein families distributed among very different zootaxa (Hagenbuch and Meier 2004). Although they are less well studied with regard to their function in organisms other than mammals, their capability to transport MCs across membranes has been demonstrated convincingly, especially within liver tissue (Fischer et al. 2005; Fischer et al. 2010) and across the blood-brain barrier (Feurstein et al. 2009; Fischer et al. 2005). Since we were able to detect Oatp related sequences within the *D. pulex* and *D. magna* genomes, we hypothesize that Oatps might play an important role in the MC detoxification process as well.

In the presented study we have used two different approaches to assess the relevance of both transport types in *Daphnia* with regard to MC detoxification. The first approach included the application of putative inhibitors of trans membrane transporters in order to find effects on *Daphnia'*s capability of cyanotoxin uptake or release. For this purpose daphnids were fed for 24 h with a 100 % diet of the MC containing cyanobacterium *Microcystis aeruginosa* PCC7806 in the presence or absence (control) of trans membrane transporter inhibitors dissolved in the water. Afterwards the daphnids were fed for another 6 h (chase period) with the green alga *Chlamydomonas klinobasis* in order to get rid of cyanobacteria in the gut. During this 6 h chase period the putative trans-membrane transporter inhibitor was present as well. We then used high resolution LC-MS analytics to detect and quantify microcystin-LR (MCLR) within the *Daphnia* tissue. Three different putative Oatp inhibitors (naringin, taurocholate and dipyridamole) and four different putative P-gp inhibitors (erythromycin, lidocaine, cyclosporin A (Cs A) and hydrocortisol) were applied in order to check whether interference with the respective transporters took place or not, which would be reflected in differing MCLR amounts within the *Daphnia* tissue.

In a second experiment we tried to figure out, if daphnids respond to MC containing diets with changes in the expression of the genes of these two transporter types. In order to do so we performed quantitative PCR (qPCR) with specific primers for two P-gp related and two Oatp related *D. magna* sequences. The qPCR runs were carried out with cDNA obtained from RNA that we extracted from *D. magna* individuals feeding for 24 h on *M. aeruginosa* PCC7806 wildtype (WT), or the mutant form of that strain that does not produce any MCs (mcy-), or the green alga *C. klinobasis* or from daphnids that were kept in the absence of food (starvation control). Accordingly, our experiment aimed at elevated or reduced transporter gene expression as additional evidence for the involvement of these transporters in a potential short term inducible defense mechanism in *Daphnia*.

Material and Methods

Culture conditions

Daphnia magna clone P (P132.85; derived from a pond (Driehoek) in Heusden (Netherlands), N51°44'01", E5°08'17") (De Meester 1994) was chosen, because it exhibits a very high tolerance to cyanobacterial diets in general (Schwarzenberger 2010). All experiments were carried out at 20 °C in membrane-filtered (0.2 µm), aged tap water, which we will refer to as medium in the following. The animals were fed every second day with the green algae *Chlamydomonas klinobasis* (Chlorophyceae, strain 56, culture collection of the Limnological Institute at the University of Konstanz, Germany). In order to avoid size- or stage specific effects, we randomly chose animals of different sizes from the culture for all the transporter inhibition experiments, which resulted in more or less equal *Daphnia* fresh weights in the different treatments. The green algae *C. klinobasis* was cultivated semi-continuously in cyanobacteria medium (Von Elert and Jüttner 1997) (constant light: 90 - 100 µE m^{-2} s^{-1}; at 20 °C) with a 20 % medium exchange every two days. The microcystin (MC) containing cyanobacterium *Microcystis aeruginosa* strain PCC7806 and its genetically engineered mutant form (mcy-), which does not produce any MCs (Dittmann et al. 1997), were grown as chemostat cultures in cyanobacteria medium (constant light: 50 µE m^{-2} s^{-1}; at 20 °C; dilution rate 0.23 per day). The wildtype (WT) of strain PCC7806 contains the microcystins Microcystin-LR (MCLR, L = leucine, R = arginine) and D-Asp3-Microcystin-LR (D - aspartic acid on position 3) in high amounts, experimentally confirmed via LC-MS measurements (data not shown). Food concentrations in terms of POC were estimated from

photometric light extinction (470 nm) and from previously determined carbon extinction equations.

Transporter inhibition experiments

Oatp inhibition

24 h feeding on Microcystis

For each of the four treatment we picked 50 *D. magna* individuals of different sizes and transferred them into 200 mL beakers filled with 100 mL medium containing *M. aeruginosa* PCC7806 (5 mg POC L^{-1}). Three of the four treatments contained a putative Oatp inhibitor (naringin, taurocholate and dipyridamole, Sigma-Aldrich) (Tab.1) at final concentrations of 10 µg mL^{-1} (naringin and dipyridamole) or 50 µg mL^{-1} (taurocholate). Preliminary experiments had shown that these inhibitor concentrations had no observable toxic effect on *D. magna*. The inhibitors were supplied by addition of 100 µL of a stock solution in ethanol (EtOH) (10 mg mL^{-1}: naringin and dipyridamole) or in ultrapure water (50 mg mL^{-1}: taurocholate) to the medium. Additionally, 100 µL 100 % EtOH were added to the control treatment resulting in a final concentration of 1 µl mL^{-1} in order to rule out effects due to EtOH addition. In order to determine the ingestion of *Microcystis* cells by *Daphnia* we determined the number of *Microcystis* cells in the samples at the beginning and at the end of the 24 h feeding experiment using a Neubauer improved counting chamber. After 24 h the daphnids were removed from the medium using a 200 µm gauze and processed immediately. All treatments were replicated threefold.

24 h feeding on Microcystis + 6 h chase on Chlamydomonas

The second experiment with regard to Oatp inhibition was carried out similarly with an additional 6 h feeding period (chase) on the nutritious green algae *C. klinobasis*. For this purpose the daphnids were removed from the prior medium using a 200 µm gauze, rinsed 3 times thoroughly with approximately 10 mL tap water and transferred into new 200 mL beakers now containing 100 mL medium with *C. klinobasis* (5 mg POC L^{-1}) and the respective Oatp inhibitor at the same concentration as during the first 24 h feeding period. After the 6 h chase time the daphnids were removed from the medium using a 200 µm gauze and processed immediately.

P-gp inhibition

The P-gp inhibition experiment was carried out similar to the Oatp inhibition experiment including the 6 h *Chlamydomonas* feeding period (chase time). Here we used the P-gp inhibitors erythromycin, lidocaine, cyclosporin A (Cs A) and hydrocortisol (Sigma-Aldrich) (Tab.1) at final exposure concentrations of 1 µg mL^{-1} (erythromycin and lidocaine), 10 µg mL^{-1} (cyclosporin) or 15 µg mL^{-1} (hydrocortisol). Noteworthy, all these substances, although depicted as P-gp inhibitors might also have an inhibitory effect on Oatps as has been demonstrated for human OATPs (Cvetkovic et al. 1999; Giacomini et al. 2010). Experiments were again carried out in triplicates, with the exception of the Cs A treatment in which one replicate had to be discarded due to an unexplained total lack of feeding activity resulting in unnaturally high MTL values (for MTL value see p.83). The substances were added by pipetting 100 µL of stock solutions in EtOH (1 mg mL^{-1}: erythromycin and lidocaine; 10 mg mL^{-1}: Cs A; 15 mg mL^{-1}: hydrocortisol) to the medium. The 24 h feeding period on *Microcystis* and the chase period were carried out in the presence of the respective putative P-gp inhibitor. A control treatment received 100 µL of 100 % EtOH. Again *Microcystis* cell ingestion by *Daphnia* was determined by using a Neubauer improved counting chamber comparing the cell number in the different treatments after the first 24 h with the cell count in a control treatment after 24 h in the absence of daphnids. After the 6 h chase period the daphnids were removed from the medium and immediately processed.

Sample preparation – methanolic extraction

After removing the daphnids from the medium, the animals were rinsed extensively with three times about 5 mL of ultrapure water. Afterwards all individuals of one replicate of a treatment were transferred into 1.5 mL test tubes, their fresh weight was determined and 10 µL of an internal standard (microcystin-Hil (homoisoleucine) R (arginine), 5 µg mL^{-1}) (Enzo Life Sciences) were added. Subsequently 100 µL of 80 % methanol (MeOH) were added, and the tissues were homogenized using an electric homogenizer. Then another 400 µL of 80 % MeOH were added, the samples were sonicated (5 min) and centrifuged (3 min, 11.500 g). Subsequently the supernatant was transferred into a new 1.5 mL test tube and evaporated to dryness in a vacuum centrifuge. Finally, the samples were redissolved in 100 µL 100 % MeOH and transferred into glass vials, with a final internal standard concentration of 0.5 µg mL^{-1}.

Tab.1 List of putative Oatp and P-glycoprotein (P-gp) inhibitors used within the experiments

Oatp inhibitors	**sum formula**	**M_W [Da]**	**reference**
naringin	$C_{27}H_{32}O_{14}$	580.54	Bailey et al. (2007)
taurocholate	$C_{26}H_{45}NO_7S$	515.7	Karlgren et al. (2012)
dipyridamole	$C_{24}H_{40}N_8O_4$	504.63	Karlgren et al. (2012)
P-gp inhibitors			
erythromycin	$C_{37}H_{67}NO_{13}$	733.93	Liu et al. (2003)
lidocaine	$C_{14}H_{22}N_2O$	234.34	Hunter and Hirst (1997), Varma et al. (2003)
cyclosporin A	$C_{62}H_{111}N_{11}O_{12}$	1202.61	Hunter and Hirst (1997), Liu et al. (2003), Varma et al. (2003)
hydrocortisol	$C_{21}H_{30}O_5$	362.47	Vankalken et al. (1993)

Liquid chromatography - mass spectrometry

Detection and quantification of microcystin-LR (MCLR) was performed on an Accela ultra high pressure liquid chromatography system (UPLC) (Thermo Fisher), which consisted of a 1250 psi pump, an autosampler (AS) and a photo diode array (PDA). The UPLC was equipped with a C_{18} column (Nucleosil, 125/2, 100-3, Macherey and Nagel) as stationary phase and operated with a gradient of acetonitrile (ACN, HPLC grade) and ultrapure water, each containing 0.05 % trifluoracetic acid (TFA) as mobile phase: 0 min: 38 % ACN; 2 min: 40 % ACN; 12 min: 50 % ACN; 12.5 min: 100 % ACN; 15 min: 100 % ACN; 15.5 min: 38 % ACN 17 min: 38 % ACN. The runs were carried out at a column temperature of 30° C, a flow rate of 300 µL min^{-1} and an injection volume of 10 µL. The UPLC system was coupled with an Exactive orbitrap mass spectrometer (MS) (Thermo Fisher) with electrospray ionization (ESI), which was operated in positive ionization mode. Mass spectrometry measurements were carried out at a scan rate of one per second and a mass range between 75 and 1500 Da (spray voltage 4.5 kV, capillary temperature 325 °C, sheath and aux gas nitrogen set to 45 and 15, respectively). For the identification of MCLR we used extracted ion chromatograms for the respective specific mass (m/z = 995.55604) granting a mass deviation of 3 parts per million (ppm). Electrospray ionization resulted in adduct ions with one positive charge for MCLR (z =1, $M+H^+$).

Quantification of microcystin-LR and data transformation

For the quantification of MCLR we conducted two calibration curves (0.25-2 µg mL^{-1} and 0.-0.25 µg mL^{-1}) in order to account for differing MCLR amounts in the different samples. As already mentioned above we used microcystin-HilR (MC-HilR) as an internal standard. MC-HilR is a slightly modified variant of MCLR, which is elongated by one CH_2 unit at the leucine (L) amino acid moiety: Therefore it is easy to differentiate from actual MCLR in the mass spectrum while having very similar chemical characteristics. As an external standard we used different concentrations of MCLR (Enzo Life Sciences). For the calculation of the ratios of internal and external peak areas, peaks obtained from the extracted ion chromatograms (MS) for the specific masses of both substances were used (granted mass deviation < 3 ppm). Samples were always redissolved in a final volume of 100 µL MeOH. Therefore, the total MCLR content of the entire *Daphnia* tissue sample could be obtained by dividing the measured concentration value by ten. We calculated a microcystin tissue level (MTL) for each *Daphnia* sample according to formula 1.

(1) $$MTL_{sample} = \frac{MCLR_{total}\,[ng] \times Daphnia\ FW_{total}^{-1}\,[mg]}{No.\ of\ ing.\ cells_{sample} \times No.\ of\ ing.\ cells_{max}^{-1}}$$

MTL: microcystin tissue level

$MCLR_{total}$: total MCLR amount within an entire *Daphnia* tissue sample

Daphnia FW_{total}: fresh weight of an entire *Daphnia* tissue sample

No. of ing. $cells_{sample}$: total number of ingested cells by 50 *Daphnia* individuals over 24 h for one sample

No. of ing. $cells_{max}$: number of ingested cells by 50 *Daphnia* individuals over 24 h for the sample with the highest ingestion

We normalized the total MCLR tissue content to the fresh weight of the respective sample to obtain the MCLR amount per mg *Daphnia* fresh weight (FW). In addition to that we

determined the total number of ingested cells in each single sample and calculated an ingestion normalization factor by dividing this value by the number of ingested cells from the sample with the highest ingestion within the entire experimental setup. MCLR per mg *Daphnia* fresh weight was then additionally normalized by dividing the value by the ingestion normalization factor (formula 1) which resulted in the MTL value. Differences in the MTL value are therefore not attributable to different *Daphnia* fresh weights or differing degrees of *Microcystis* cell ingestion. Accordingly MTL values should be interpreted as either differing MCLR uptake efficiencies or MCLR export efficiencies.

Quantitative PCR

Experimental setup

In order to test the effect of the exposure to dietary MCLR on *D. magna* we performed feeding experiments with *D. magna* and *M. aeruginosa* PCC7806 WT and its MCLR deficient mutant form (mcy-). Six five day old individuals of *D. magna* were transferred into 100 mL medium containing either *M. aeruginosa* PCC7806 WT or the mutant form of that strain (mcy-) (5 mg POC L^{-1}). As controls we used six 5 day old daphnids feeding either on *C. klinobasis* (5 mg POC L^{-1}) or kept in the absence of food (no food control). After 24 h the daphnids were removed from the medium using a 200 µm gauze and immediately stored at -80° C. All treatments were replicated threefold.

Sequence selection and primer design

In a first step we screened for Oatp and P-glycoprotein related sequences within the fully available *Daphnia pulex* genome (wFlea base) by keyword search. The obtained sequences (P-gp related: Dappudraft_127589; Oatp related: Dappudraft_303505 and Dappudraft_545555) were blasted against the *D. magna* EST database to obtain specific *D. magna* sequences (P-gp: isotig_21462 and isotig_20574; Oatp: isotig_20705 and isotig_16106). Those sequences were then used to design *D. magna* specific primers (Tab.2). The primers were designed using Primer 3 (v.2.3.5), and their quality was checked with Netprimer (Premier Biosoft). Additionally, the primers were checked with a standard PCR reaction after which all four primer pairs produced clear single products checked on an 1.2 % agarose gel.

Tab.2 Oatp and P-glycoprotein (P-gp) related *Daphnia* sequence IDs and their primer pairs as used in the qPCR reaction

Name	gene ID		forward primer (5´-3´)	reverse primer (5´-3´)
	D. pulex genome	*D. magna* EST		
Oatp 1	Dappu303505	isotig 20705	CTCGTCCTCGGGAAAAGTTA	AGGGGCATGTTGGCTTTAC
Oatp 2	Dappu 54555	isotig 16106	ATTGGACCGATTTGCGTAGT	TCGCCCCTCACTTCATTTAC
P-gp 1	Dappu127589	isotig21462	CACTTTGGCGGATTTTTGTA	CCTCGGTTATTCTGGCTCTC
P-gp 2	Dappu127589	isotig20574	GCTTTTCTTGCGGCTTCTAT	TCACAAATGGGCATCGTATC

quantitative PCR

RNA was extracted and purified making use of the Nucleospin RNA II kit (Macherey and Nagel). The resulting RNA concentrations were measured with a Nanodrop (ND-100 v.3.7, Thermo scientific), subsequently reverse-transcribed (high capacity cDNA reverse transcription kit, Applied Biosystems) and stored at -20°C. Data acquisition for relative gene expression was carried out using a 7300 qPCR system (Applied Biosystems). Each qPCR reaction contained 5 ng of the respective cDNA template, 10 µL SYBR green PCR master mix and 2.5 µM of each primer in a final volume of 20 µL. The qPCR method started with a 10 minute period of 95° C followed by 40 cycles with 95° C for 15 s, 55° C for 30 s and 68° C for 30 s. These were followed by a final dissociation step of 95° C for 15 s, 55° C for 30 s, 68° C for 30 s and 95° C for 15 s. As endogenous controls we used the genes 28S ribosomal RNA (28S), TATA box binding protein (TBP) and succinate dehydrogenase (SucDH), which are known to be constitutively expressed in *Daphnia magna* (Heckmann et al. 2006; Schwarzenberger et al. 2009). The baseline and threshold for the threshold (Ct) value were set automatically.

Results

Oatp inhibitors

In the first 24 h exposure experiment we tested the effect of three different Oatp inhibitors (naringin, taurocholate and dipyridamole), which were dissolved in the medium, on the uptake

or release of dietary MCLR by *D. magna*. We therefore determined the concentration of MCLR per mg *Daphnia* fresh weight (FW) after a 24 h feeding period with *M. aeruginosa* PCC7806 and normalized this value to *Microcystis* ingestion to obtain the microcystin tissue level (MTL). The MTL values ranged from 1.61 ng MCLR per mg *Daphnia* FW in the control to 1.89 ng MCLR per mg *Daphnia* FW in the dipyridamole treatment. We found values in the same range for the naringin (1.76 ng MCLR per mg *Daphnia* FW) and the taurocholate treatment (1.84 ng MCLR per mg *Daphnia* FW) (Fig.1a). However, none of the three substances had a significant effect on the MCLR concentration in the *Daphnia* tissue (one way ANOVA, $F = 1.245$, $P = 0.356$) (Fig.1a) Since effects of Oatp inhibitors in the first 24 h feeding experiment might be hidden by high MCLR amounts due to *Microcystis* cells in the *Daphnia* gut, we performed a similar 24 h feeding experiment, this time followed by an additional 6 h chase period with the nutritious green alga *C. klinobasis* again in the presence of Oatp inhibitors. Prior studies could show that a 6 h feeding duration is sufficient for *D. magna* to entirely clear the gut of cyanobacteria (Sadler and von Elert 2014). After this 6 h feeding period, we in general found lower MTL values compared to the first experiment (Fig.1a and b). Within this second experiment, we detected similar MTL values in the control (0.68 ng MCLR per mg *Daphnia* FW), the naringin (0.74 ng MCLR per mg *Daphnia* FW) and the dipyridamole treatment (0.82 ng MCLR per mg *Daphnia* FW) (Fig.1b). However, the MTL value in the taurocholate treatment (1.19 ng MCLR per mg *Daphnia* FW) was significantly elevated by 75 % compared to the control (Tukey test $P = 0.01$ after one way ANOVA, $F = 7.728$, $P = 0.01$) (Fig.1b).

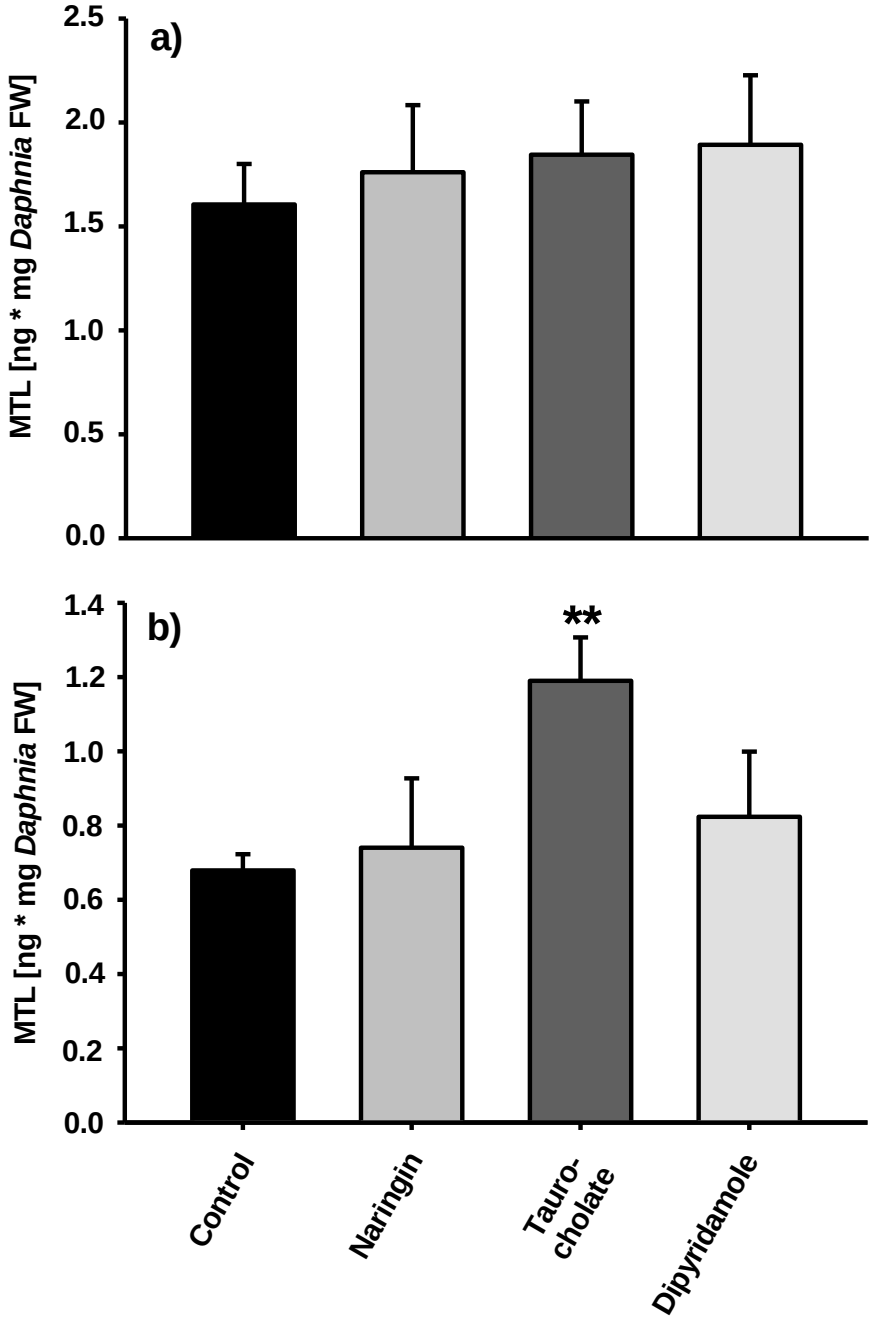

Fig.1 Microcystin tissue level (MTL) given as microcystin amount [ng] per mg *Daphnia* fresh weight (FW) normalized to *Microcystis* ingestion for a) a 24 h feeding period on *Microcystis aeruginosa* PCC7806 in the absence (control) or presence of Oatp inhibitors (final concentrations: naringin and dipyridamole 10 µg mL^{-1}; taurocholate 50 µg mL^{-1}) and b) a 24 h feeding period on *M. aeruginosa* PCC7806 followed by a 6 h chase period with both periods in the absence (control) or presence of the same Oatp inhibitors at the same concentrations as in a). Means + SD, n = 3, Tukey test ** $P < 0.01$ after one way ANOVA.

P-gp inhibitors

In order to investigate the role of P-gp with regard to MCLR uptake or release in *Daphnia* we performed an experiment with four different putative P-gp inhibitors (erythromycin, lidocaine, cyclosporin A and hydrocortisol). The experimental setup was similar to the experiment for Oatp inhibition (Fig.1b) including the 6 h chase period in the presence of the nutritious green alga *C. klinobasis*. We detected similar low MTL values in the control (0.25 ng MCLR per mg *Daphnia* FW), the erythromycin (0.27 ng MCLR per mg *Daphnia* FW), the lidocaine (0.28 ng MCLR per mg *Daphnia* FW) and the hydrocortisol treatments (0.28 ng MCLR per mg *Daphnia* FW) (Fig.2). Notwithstanding the other putative P-gp inhibitors, Cyclosporin A caused a significant increase of the MTL value by 106 % (0.52 ng MCLR per mg *Daphnia* FW) compared to the control (Tukey test: P = 0.002 after one way ANOVA, F = 10.421, P = 0.002).

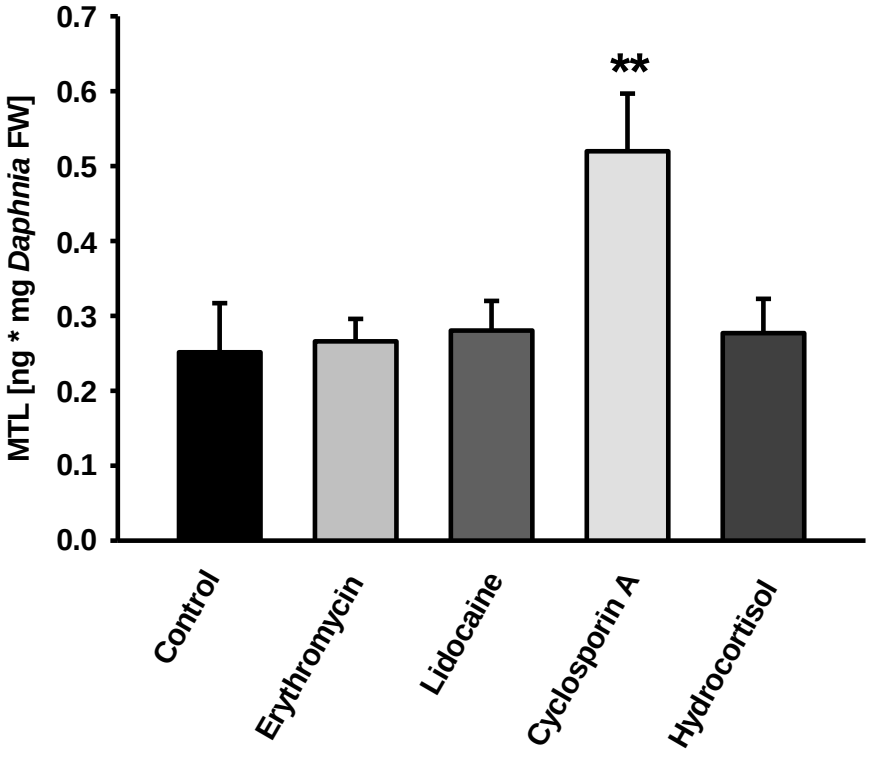

Fig.2 Microcystin tissue level (MTL) given as microcystin amount [ng] per mg *Daphnia* fresh weight (FW) normalized to *Microcystis* ingestion for a 24 h feeding period on *Microcystis aeruginosa* PCC7806 followed by a 6 h chase period on *Chlamydomonas klinobasis* with both periods in the absence (control) or presence of P-glycoprotein inhibitors (final concentrations: erythromycin and lidocaine 1 µg mL^{-1}; cyclosporin A 10 µg mL^{-1}; hydrocortisol 15 µg mL^{-1}). Means + SD, n = 3 (for cyclosporin A n = 2), Tukey test ** P < 0.01 after one way ANOVA.

qPCR analysis with transporter genes

The results from the experiments with different Oatp and P-gp inhibitors suggested that both transporter types might be involved in the export of MCLR out of *Daphnia* tissue. Therefore, the up-regulation of these specific transporter genes could constitute a defense mechanism against MCLR containing diets. In order to test this hypothesis we performed a qPCR analysis with primers for four different *D. magna* sequences. Two of these sequences could be identified as P-gp related, whereas the other two revealed similarities with Oatp transporter sequences (Tab.2). Prior to RNA extraction, the daphnids were fed for 24 h with a 100 % diet of *M. aeruginosa* PCC7806 wildtype (WT) or the mutant form (mcy-) of that strain, which lacks the ability to produce microcystins. As controls we used animals that were fed on 100 % *C. klinobasis* diet and a 24 h starvation control.

For none of the putative Oatp related sequences we found any differential gene expression, due to the different diets or the starvation control (Oatp 1: one way ANOVA, $F = 1.972$, $P = 0.197$; Oatp 2: one way ANOVA, $F = 1.532$, $P = 0.279$) (Fig.3).

With regard to the P-gp related sequences we found significantly different gene expression levels for both of the sequences between the different treatments (P-gp 1: one way ANOVA, $F = 8.757$, $P = 0.007$; P-gp 2: one way ANOVA, $F = 4.438$, $P = 0.041$) (Fig.3). P-gp 1 expression was significantly increased due to all different diets in comparison to the starvation control (*C. klinobasis*: Tukey-test $P = 0.007$ after one way ANOVA; *M. aeruginosa* PCC7806 WT: Tukey-test $P = 0.014$ after one way ANOVA; *M. aeruginosa* PCC7806 mcy-: Tukey-test $P = 0.043$ after one way ANOVA). However, there were no differences between the respective diets (Fig.3). P-gp 2 expression was also increased due to a *C. klinobasis* diet (Tukey-test $P = 0.031$ after one way ANOVA), whereas the comparison between the cyanobacterial diets and the starvation control did not reveal any differences (*M. aeruginosa* PCC7806 WT: Tukey-test $P = 0.212$ after one way ANOVA, *M. aeruginosa* PCC7806 mcy-: Tukey-test $P = 0.579$ after one way ANOVA) (Fig.3). The same is true for the comparison between the cyanobacterial diets (*M. aeruginosa* PCC7806 WT: Tukey-test $P = 0.543$ after one way ANOVA, *M. aeruginosa* PCC7806 mcy-: Tukey-test $P = 0.194$ after one way ANOVA) (Fig.3).

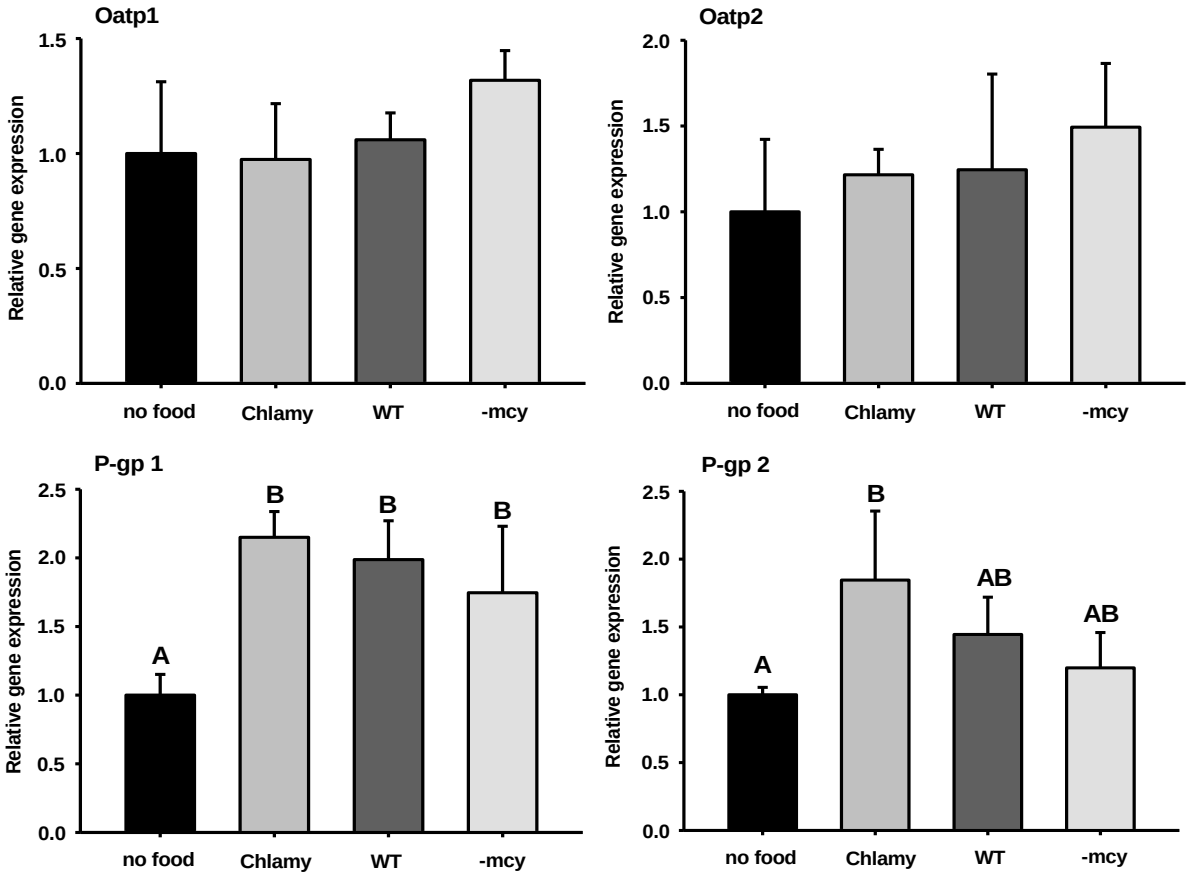

Fig.3 Relative gene expression in *D. magna* for two putative Oatp genes (Oatp1 and Oatp 2) and two putative P-gp genes (P-gp 1 and P-gp 2). Prior to RNA-extraction the daphnids fed for 24 h on a 100 % diet of *Microcystis aeruginosa* PCC7806 wildtype (WT), the microcystin-free mutant form of the strain (mcy-), the nutritious green alga *Chlamydomonas klinobasis* (Chlamy) or were kept in the absence of food (no food). Means + SD, n = 3. Different letters indicate significantly different treatments. Tukey test $P < 0.05$ after one way ANOVA.

Discussion

Transporter inhibition

The data presented here provide clear and concise evidence for the involvement of trans membrane transporters, namely multi xenobiotic resistance proteins (MXR, P-gp) and organic anion transporting polypeptides (Oatps) in the export of dietary unconjugated cyanotoxin MCLR out of *Daphnia* tissue. The application of specific transporter inhibitors acting against these two transporter types each revealed one inhibitor, leading to an increased microcystin tissue level (MTL). Since this MTL value was normalized to the *Daphnia* fresh weight and the amount of ingested cyanobacteria, any differences must be interpreted as differences in the efficiency of cyanotoxin uptake or release.

The highly increased MTL values in response to taurocholate (+75 %) and Cs A (+106 %) (Figs.1 and 2) strongly point at an involvement of both transporter types, namely P-gp and Oatp in the export of dietary MCLR out of *Daphnia* tissue.

With regard to MCLR detoxification in *Daphnia* but also in other aquatic organisms it has been assumed that the enzyme glutathione-S-transferase (GST) is of major importance (He et al. 2012; Li et al. 2014; Pflugmacher et al. 1998), due to its ability to conjugate microcystins (MCs) with glutathione (GSH) (Buratti et al. 2011; Takenaka 2001). In line with that Pflugmacher et al. (1998) have demonstrated that *Daphnia* crude extracts lead to the formation of microcystin glutathione (MC-GSH) conjugates *in vitro*. However, Sadler and von Elert (2014) demonstrated that this process seems to be of minor relevance when it comes to dietary MCs in *Daphnia* and pointed out the importance of transport mechanisms capable of exporting non-metabolized MCs. Although only very little is known about MC transport mechanisms in aquatic organisms, Contardo-Jara et al. (2008) proposed that the presence of multi xenobiotic resistance proteins (MXR, P-gp) might deliver an explanation for the insensitivity of bivalves towards cyanobacterial toxins such as MCs. In agreement with this, the application of dissolved MCLR led to an up-regulated expression of a P-gp gene in the fish *Jenynsia multidentata* (Ame et al. 2009). In general, the MXR system is of major importance for aquatic organisms in order to deal with complex mixtures of water pollutants (Kurelec 1992), and MXR related gene sequences could be identified in *D. pulex* and *D. magna* (Campos et al. 2014; Sturm et al. 2009). Moreover, Campos et al. (2014) provided convincing evidence for the importance of the MXR mechanism in *D. magna* with regard to detoxification of xenobiotics, i.e. an increased concentration of dye substrates due to the application of MXR transporter inhibitors. Among those inhibitors was the cyclic peptide Cs A, which impacted tissue efflux resulting in higher concentrations of dye substrate within the *Daphnia*. In line with these findings, our results provide evidence for the importance of the MXR system not only with regard to the export of toxic water pollutants, but also for the export of dietary cyanotoxins such as MCLR.

The MXR mechanism observed in different aquatic organisms is similar to the human multi drug resistance (MDR) mechanism, which is associated with tumor cells becoming refractory to chemotherapeutic agents (Kurelec 1992). In a similar way, Oatp mediated MCLR uptake in mammals, as has been observed in the liver tissue (Fischer et al. 2005) or in the blood-brain barrier (Feurstein et al. 2009; Fischer et al. 2005), might teach a lesson for the transportation of xenobiotics in aquatic organisms. Oatps represent a transporter type that is widely

distributed among the animal kingdom. Respective gene sequences could be identified in a variety of bilaterians belonging to the clade of prostomia (arthropods and nematodes) or deuterostomia (vertebrates and echinoderms) (Hagenbuch and Meier 2004; Meier-Abt et al. 2005). It seems reasonable to assume that Oatps are also present in crustaceans, such as *Daphnia*, although this has not been proven yet. However, in the fully available *D. pulex* genome (wFlea base) and were able to identify respective Oatp associated gene sequences. Homologues of these sequences could also be found in the *D. magna* expressed sequence tag (EST) database (Tab.2). Nevertheless, research on function of Oatp transporters has so far focused solely on mammals, and only little is known about their function in other taxa. The bile acid taurocholate acts as a potent inhibitor of human OATPs (Karlgren et al. 2012), and to our knowledge there is no evidence for an inhibitory effect of taurocholate with regard to P-gp. Therefore, the effect of taurocholate on the MTL provides a strong argument for the involvement of *Daphnia* Oatps in MCLR export.

Cs A is a cyclic peptide consisting of eleven amino acids, which has been shown to efficiently inhibit human P-gp transporters (Hunter and Hirst 1997; Liu et al. 2003; Varma et al. 2003). The strong elevation of *Daphnia's* MTL value therefore provides good evidence for the function of *Daphnia* P-gp not only in the export of pollutants dissolved in the water, as reported by Campos et al. (2014), but also for dietary MCLR. However, although traditionally regarded as inhibitor for P-gps, Cs A also has an inhibitory effect on human OATPs (Giacomini et al. 2010). Therefore, we cannot deliver final proof for the involvement of P-gp in the export of dietary MCLR out of *Daphnia* tissue, since the observed Cs A effect could be caused by Oatp inhibition, similar to taurocholate. However, even if in *Daphnia* Cs A should as well inhibit Oatps, the stronger effect of Cs A (106 %) in comparison to the effect of taurocholate (75 %) on the MTL value strongly suggests that Cs A in addition to Oatps is inhibiting P-gps. Furthermore, there is good evidence for the role of MXR (P-gp) mechanisms in aquatic organisms in general (Ame et al. 2009; Contardo-Jara et al. 2008; Kurelec 1992) and also specifically in *Daphnia* (Campos et al. 2014) with regard to the export of xenobiotics. However, our data provide the first hints for the function of Oatps as important trans membrane transporters with regard to the export of dietary xenobiotics. Accordingly we argue that our results most probably point at an interference of Cs A with the well established MXR system. In summary, we provide strong evidence for the involvement of Oatps in the export of dietary MCLR due to the effect of taurocholate on the MTL value. In addition we

argue that most probably P-gp is as well involved in this process due to the effect of Cs A on the MTL value.

The comparison of the Figs.1a and b shows, that an additional chase period of 6 h on a non-cyanobacterial diet (*C. klinobasis*) is necessary in order to detect effects of transporter inhibitors on the MTL within our experimental approach. Obviously, high amounts of MC containing cyanobacteria in the *Daphnia* gut mask inhibitor effects on the MTL, thus we do not see any differences after 24 h. Accordingly, the MTL value decreased during the chase period by 65 % (control and naringin), 68 % (dipyridamole) and 42 % (taurocholate) (Figs.1a and b). This shows that on one hand significant amounts of the MCLR after 24 h must be attributed to the *Microcystis* cells in the gut. On the other hand significant amounts of MCLR had obviously been taken up into the *Daphnia* tissue, which emphasizes the importance of transport mechanisms for daphnids.

MTL values in the P-gp inhibition experiment (Fig.2) are in general lower in comparison to the MTL values in the Oatp inhibition experiment (Fig1b). However, the experiments were not performed at the same time, and we cannot rule out that the MC content within the cyanobacteria varied a little over time. For that reason the daphnids in the experiment might have ingested similar amounts of cyanobacterial biomass (5 mg POC L^{-1}), but less amounts of MCLR. Therefore the MTL values are consistent within the respective experiments, but differ between different experiments not carried out simultaneously.

Since we have only very little knowledge about the structural properties of Oatps and P-gps present in *Daphnia*, we cannot draw conclusions from the fact that two of the applied Oatp inhibitors and three of the applied P-gp inhibitors did not affect the MTL value. A possible explanation might be the different concentrations that we used. Taurocholate (50 µg mL^{-1}) was five times higher concentrated than the other two putative Oatp inhibitors (naringin and dipyridamole 10 µg mL^{-1}). In case of the P-gp inhibitors, Cs A (10 µg mL^{-1}) was ten times higher concentrated than erythromycin and lidocaine (1 µg mL^{-1} each). However, hydrocortisol (15 µg mL^{-1}) was used at a higher concentration than Cs A, but had no effect on the MTL value. Another explanation for differing or lacking effects of putative inhibitors on the MTL value within our experiment might be caused by structural differences of the respective compounds. In general, the applied putative inhibitors exhibit very different structural properties. Among the Oatp inhibitors the bile acid taurocholate is a sulphur containing steroid, naringin is a polyphenol derived from the grapefruit, and dipyridamole is a

complex molecule consisting of piperidine and pyrimidine units. Three human OATP transporters (OATP1B1, OATP1B3 and OATP2B1) are of major importance with regard to the transport of xenobiotics in liver tissue (Karlgren et al. 2012). Arabic numbers thereby designate the respective transporter family, which is defined by ≥ 40 % amino acid similarity; additional letters designate subfamilies (≥ 60 % amino acid similarity) (Hagenbuch and Meier 2004).Within the well investigated human system dipyridamole as well as taurocholate were demonstrated to be highly efficient against all three of these human OATPs (OATP1B1, OATP1B3 and OATP2B1), despite their immense structural differences, whereas naringin only displays a very low efficiency (Karlgren et al. 2012). However, little is known about the effects of these inhibitors on other Oatp families. Therefore, it is hardly possible to draw conclusions about the structure of the Oatp transporters in *Daphnia*.

The applied P-gp inhibitors are structurally diverse as well. Erythromycin is a rather large and complex glycoside, lidocaine is a small aromatic acetamide, hydrocortisol is a steroid, and Cs A is a polypeptide consisting of eleven amino acids. Possibly the function of these inhibitors with regard to *Daphnia* trans membrane transporter inhibition are strongly depending upon their structure. Likewise to Oatps there is no resilient knowledge about the effects of these transporters on different P-gp subfamilies. Accordingly, we cannot draw any conclusions about the structural P-gp properties within *Daphnia*. Inhibition efficiency in general depends upon the structure of the respective substrate. Therefore, the huge structural differences within the applied inhibitors could well explain the different effects on the MTL value.

Transporter gene expression

The expression levels of four different *D. magna* trans-membrane transporter genes (two putative P-gp gene sequences and two putative Oatp gene sequences (Tab.2)) were not influenced by a MC containing cyanobacterial diet (*M. aeruginosa* WT) in comparison to diets that did not contain any MCs. Therefore, we cannot support the initial hypothesis that the up-regulation of trans-membrane transporters constitutes an inducible defense mechanism in daphnids, in order to cope with dietary MCLR.

With regard to Oatp sequences we did not find any differential gene expression in response to cyanobacteria, a green alga or starvation (Fig.3), which suggests that Oatp expression levels are not affected by differences in food quality.

For P-gp related *D. magna* sequences we do find differential gene expression for the different *Daphnia* diets in comparison to the starvation control. P-gp 1 is significantly up-regulated due to the presence of food in general, although it does not differ with the type of food (Fig.3). This suggests that P-gp 1 expression is affected by food quantity. With regard to P-gp 2 we find an up-regulation of the respective gene expression for daphnids feeding on *C. klinobasis* in comparison to the starvation control, but not when feeding on cyanobacteria (WT and mcy-). Hence P-gp 2 is affected by food quantity and quality. These findings indicate a role of P-gp transporters in food processing, since feeding activity in general causes an elevated gene expression level.

Daphnids pre-exposed to toxic cyanobacteria develop an increased tolerance against these cyanobacteria within an individual's lifetime, which can then be passed on to the next generation via maternal effects (Gustafsson and Hansson 2004; Gustafsson et al. 2005; Jiang et al. 2013a; Schwarzenberger and Von Elert 2013; Von Elert et al. 2012). In addition to that, the temporal and spatial adaption of daphnids in natural environments has been demonstrated convincingly (Hairston et al. 1999; Sarnelle and Wilson 2005).

An increased export efficiency of detrimental cyanobacterial compounds caused by an up-regulated transporter gene expression would constitute a good physiological explanation for such adaption processes. However, our data do not support the idea of a fast inducible defense reaction based on the MXR mechanism or Oatp transporters within the first 24 h of exposure. Nevertheless, different *Daphnia* genotypes display a remarkable variability with regard to their tolerance to cyanobacterial diets (Jiang et al. 2013b). It seems reasonable to investigate if among different *Daphnia* genotypes increased tolerance to MCs goes along with a constitutively higher activity of P-gp or Oatp-based transporters. Further studies are necessary to explore this idea and compare gene expression levels of suitable transporter genes and transporter activities between different *Daphnia* genotypes that differ in their tolerance to toxic cyanobacteria.

Conclusion

The data presented here provide strong evidence for the involvement of the trans membrane transporters Oatp and P-gp with regard to the export of dietary MCLR out of *Daphnia* tissue. The application of specific transporter inhibitors (taurocholate and Cs A) led to increased MTL values within the *Daphnia*. Although gene expression levels were not affected by

dietary MCs in a 24 h feeding experiment, differing amino acid sequences and expression levels of these transporters might provide a good explanation for the remarkable variability among different *Daphnia* genotypes in tolerance to cyanobacterial toxins. Therefore further studies exploring this aspect on different *Daphnia* genotypes are needed in order to shed more light on the role of these transporters with regard to daphnids confronted with toxic cyanobacterial diets.

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Chapter 4

Seasonal dynamics of chemotypes

in a freshwater phytoplankton community

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A metabolomic approach

Abstract

Cyanobacteria are known to produce a huge variety of secondary metabolites. Many of these metabolites are toxic to zooplankton, fish, birds and mammals. Therefore, the toxicity of cyanobacterial blooms is strongly dependent on the cyanobacterial strain composition. These strains produce distinct bouquets of secondary metabolites, and thus constitute different chemotypes. Some of the cyanobacterial metabolites are potent inhibitors of gut proteases of the filter-feeder *Daphnia*.

Here, we investigate the seasonal dynamics of secondary metabolites in a phytoplankton community from a hypertrophic pond, making use of a new metabolomic approach. Using liquid chromatography coupled with high-resolution mass spectrometry (LC-MS), we obtained mass spectra of phytoplankton samples taken on different dates throughout the summer season. By applying multivariate statistics, we combined these data with the protease inhibition capacity of the same samples. This led to the identification of metabolites with cyanobacterial origin and as well of distinct cyanobacterial chemotypes being dominant on different dates.

The protease inhibition capacity varied strongly with season, and only one out of 73 known cyanobacterial protease inhibitors could be confirmed in the natural samples. Instead, several so far unknown, putative protease inhibitors were detected.

In conclusion, the creation of time series of mass spectral data of a natural phytoplankton community proved to be useful for elucidating seasonal chemotype succession in a cyanobacterial community. Additionally, correlating mass spectral data with a biological assay provides a promising tool for facilitating the search for new harmful metabolites prior to structure elucidation.

Introduction

Cyanobacteria play an important role in aquatic ecosystems worldwide (Dokulil and Teubner 2000; O'Neil et al. 2012). Cyanobacterial blooms occur frequently, particularly in eutrophic ponds and lakes, and have detrimental effects on the water quality with respect to domestic, industrial and recreational usage of water bodies and also strongly influence zooplankton communities (Dokulil and Teubner 2000; Ghadouani et al. 2003; O'Neil et al. 2012). Cyanobacteria are well known to negatively affect herbivorous zooplankton, e.g. the unselective filter-feeder *Daphnia* (Ghadouani et al. 2003; Wilson et al. 2006). Besides the fact that cyanobacteria provide low quality food for zooplankton (Porter and Mcdonough 1984; Von Elert et al. 2003), the presence of cyanotoxins such as microcystins is a major issue (Rohrlack et al. 2001). A negative effect of microcystins on somatic growth and reproduction of *Daphnia* has been shown. Nevertheless, cyanobacterial strains lacking microcystins also lead to reduced fitness parameters of *Daphnia* (Lürling and van der Grinten 2003; Tillmanns et al. 2008). Among other putative mechanisms, this reduction of fitness might be attributed to the presence of protease inhibitors, which are widespread in cyanobacteria, where they function as anti-herbivore defense (Agrawal et al. 2005; Gademann and Portmann 2008; Von Elert et al. 2010; Schwarzenberger et al. 2010). Many of these protease inhibitors efficiently inhibit chymotrypsins or trypsins (Gademann and Portmann 2008), which together are responsible for 80 % of the proteolytic activity in the *Daphnia* gut (Von Elert et al. 2004). Natural lake phytoplankton frequently contains protease inhibitors (Czarnecki et al. 2006; Kuster et al. 2013); accordingly protease inhibitors could not only be obtained from pure cyanobacterial cultures (Bister et al. 2004; Shin et al. 1996; Von Elert et al. 2005), but also from cyanobacterial blooms (Agrawal et al. 2001; Gesner-Apter and Carmeli 2009; Grach-Pogrebinsky et al. 2003; Lifshits et al. 2011).

The ability to produce high amounts of highly variable secondary metabolites is one of the major features of cyanobacteria (Carmichael 1992; Dittmann and Wiegand 2006; Ferrao and Kozlowsky-Suzuki 2011). Therefore, cyanobacteria are a widely explored source for natural products (Gademann and Portmann 2008) and new pharmaceutical compounds (Chlipala et al. 2011; Silva-Stenico et al. 2012). For this reason the search for bioactive compounds has been extended from cyanobacterial cultures to biomass obtained from natural cyanobacterial blooms (Agrawal et al. 2001; Banker and Carmeli 1999; Jakobi et al. 1995; Reshef and Carmeli 2001). In particular protease inhibitors are of interest with respect to their ecological

relevance due to effects on zooplankton communities and their putative pharmaceutical relevance. In the present study, we address both aspects using a metabolomic approach.

The term “Metabolomics” in general refers to the scientific field concerned with the study of naturally occurring, low molecular weight organic metabolites within cells, tissues or biofluids (Bundy et al. 2009). Especially in chemical ecology this approach has increasingly attracted attention. Metabolomic data are useful for the elucidation of chemically mediated interactions between organisms, and many substances with pharmacological properties were discovered, while investigating such chemical interactions (Prince and Pohnert 2010). Recent studies have shown, that the search for biologically active metabolites benefits from combining metabolomic profiles with data on biological activity, which led to the identification of single compounds (Gillard et al. 2013) or even the elucidation of pathways (Nylund et al. 2011). Thus, metabolomic profiling has proven to facilitate the traditional bioassay-guided isolation of bioactive compounds by identifying a limited number of target molecules via multivariate statistics prior to the isolation and structural elucidation of compounds (Prince and Pohnert 2010).

The aim of this study was to analyze several consecutive natural freshwater phytoplankton samples, in order to test whether or not repeated metabolomic profiling is a promising approach for the identification of known and unknown protease inhibitors. For this purpose we applied extracts from natural phytoplankton samples from a hypertrophic pond to liquid chromatography coupled with high resolution mass spectrometry (LC-MS). We then used multivariate statistics to analyze the obtained metabolic profiles and combined these data with the previously determined potential of the phytoplankton to inhibit digestive chymotrypsins of *Daphnia* (Kuster et al. 2013). In addition to that, we measured protease inhibition potentials from fractions of LC-runs to attribute biological activity to selected metabolites.

A second goal of this study was the exploitation of the richness of cyanobacteria in secondary metabolites by using the same method to unravel seasonal succession patterns among cyanobacteria in terms of differing cyanobacterial secondary metabolite profiles, also referred to as chemotypes, present on different sampling dates. Cyanobacterial biomass often peaks in late summer and early fall (Sommer et al. 1986) due to high temperatures in the epilimnion (Jöhnk et al. 2008), and the cyanobacterial composition may vary strongly with season. Kuster et al. (2013) have recently shown that the inhibitory potential of natural phytoplankton varies seasonally in a similar pattern. However, so far no data with regard to the chemical

identity of the secondary metabolites and the cyanobacterial chemotypes that might account for the differing inhibition potentials have been obtained. To our knowledge, the data presented here provide the first metabolomic analysis in terms of seasonal succession in freshwater phytoplankton samples.

Materials and Methods

Phytoplankton samples

In order to screen for putative protease inhibitors in phytoplankton samples obtained from the Aachener Weiher (AaW; N50°56´2.40´´, E6°55´40.81´´), an urban pond that can be regarded as hypertrophic due to its Secchi depth of 1.6 m (Carlson 1977), eight samples were taken between the 26th of July 2007 and the 9th of October 2007 (26/06; 13/07; 26/07; 10/08; 21/08; 28/08; 12/09; 09/10). When investigating a hypertrophic pond with filamentous and colonial cyanobacteria, Müller-Navarra (2000) found that the ingestible fraction of phytoplankton displayed similar detrimental effects on *Daphnia* growth rates as the unfiltered phytoplankton samples, which indicated that even large-sized cyanobacteria are represented in the ingestible fraction. At each sampling date volumes of 10 L of surface water were collected from different areas of the pond. These samples were subsequently mixed to account for any spatial heterogeneity in the pond, and 50 L to 80 L of this mixed surface water was screened with a 55 µm mesh to obtain the fraction, that is ingestible for daphnids.

The filtrate was then gently concentrated by hollow-fiber filtration and freeze-dried (Kuster et al. 2013). Lyophilized phytoplankton was homogenized using a mortar and pestle. We obtained the ash-free dry mass (AFDM) of the samples by heating a known amount of each sample in a muffle furnace at 550 °C for eight hours and measuring the weight before and afterwards. The mass loss was regarded as the AFDM and varied between 20 % and 30 %. For the LC-MS measurements known amounts of dry mass were resuspended in 25 µl of 80 % methanol (MeOH, HPLC-grade) per mg sample, sonicated (5 minutes) and then centrifuged (10 minutes, 4,500 g). For each sample 5 ml of the supernatant were transferred into new test tubes. Afterwards, the peptide Met-Arg-Phe-Ala (MRFA, 20 µl, 16.6 µM) was added as internal standard. Samples were then evaporated to dryness in a rotary evaporator (40 °C), re-dissolved in 1 ml 100 % MeOH and subsequently evaporated in a vacuum centrifuge. Finally, the samples were redissolved in 200 µl 100 % MeOH, centrifuged (2 minutes, 11,500 g), and the supernatant transferred into glass vials.

Microcystis aeruginosa PCC7806 and BM25

M. aeruginosa strains PCC7806 (Dittmann et al. 1997) and BM25 (Schwarzenberger et al. 2013) were cultured in cyanophycean medium (Von Elert and Jüttner 1997) in a chemostat under constant light conditions (50 µE m^{-2} s^{-1}) at 20 °C, with a dilution rate of 0.23 per day. In the case of strain BM25 sample preparation was carried out as described for the phytoplankton samples using freeze-dried powder of a pure *M. aeruginosa* BM25 culture. Extracts of strain PCC7806 were obtained by re-suspending 150 mg of freeze-dried PCC7806 cells in 1.5 ml 60 % MeOH. After vortexing and sonicating the sample for ten minutes, the sample was centrifuged (2 minutes; 4,500 g). The supernatant was transferred into a new test tube, evaporated to dryness in a vacuum centrifuge and dissolved in 200 µl 100 % MeOH.

Protease inhibition

The half maximal inhibitory concentration (IC_{50}) values for chymotrypsins and trypsins of methanolic extracts of freeze-dried phytoplankton samples have already been published (Kuster et al. 2013). Extracts were normalized to dry mass in that study, whereas here we determined the AFDM and normalized the IC_{50} values (Kuster et al. 2013) to the AFDM to rule out effects of inorganic material in the samples. For the sample from 12/09, no more material was available to determine the AFDM and thus we used the average of all other samples to calculate the IC_{50} value normalized to AFDM.

Liquid chromatography – mass spectrometry

Measurements of the relative amounts of protease inhibitors and other metabolites were carried out using an ultra-high pressure liquid chromatography (U-HPLC) system (Accela, Thermo Fisher) consisting of an Accela 1250 psi pump, an Accela autosampler (AS) and an Accela photo diode array (PDA) coupled with an orbitrap mass spectrometer (Exactive, Thermo Fisher) equipped with an electrospray ionization module (ESI). The orbitrap is a special type of ion trap measuring unit, which allows determining the monoisotopic masses of molecules in the range of 75 Da to 1500 Da with a very high accuracy of 3 parts per million (ppm) mass deviation. Chromatography was performed on a C_{18} column (Nucleosil, 2 mm x 125 mm length, 100 - 3 µm, Macherey and Nagel) with mobile phases acetonitrile (ACN, HPLC-grade) and ultrapure water, each containing 0.05 % trifluoroacetic acid (TFA), a flow rate of 300 µl min^{-1} with a gradient: 0-2 minutes: 3 % ACN; 15 minutes: 100 % ACN; 18 minutes: 100 % ACN; 20 minutes: 3 % ACN. Mass spectrometry measurements were carried

out during the entire 20 minutes at a rate of one scan per second and a mass range of between 75 and 1500 Da (spray voltage 4.5 kV, capillary temperature 325°C, sheath and aux gas: nitrogen set to 45 and 15). We used alternating scan events with negative or positive ionization mode. All measurements were carried out in triplicates, with the exception of the samples 10/08 and 28/08, for which only material for two replicates was available. For each run, 10 µl of the sample were injected. For primary data analysis we used the programs Xcalibur (2.1.0.1140) and Exactive Tune (1.1 SP2).

Selection and analysis of putative protease inhibitors

For the analysis of putative protease inhibitors, we used a list of 37 chymotrypsin inhibitors and 36 trypsin inhibitors (Adiv et al. 2010; Banker and Carmeli 1999; Bister et al. 2004; Gademann et al. 2010; Gesner-Apter and Carmeli 2009; Grach-Pogrebinsky et al. 2003; Grewe 2013; Ishida et al. 1995; Jakobi et al. 1995; Jakobi et al. 1996; Kodani et al. 1999; Kisugi and Okino 2009; Lifshits et al. 2011; Murakami et al. 1997; Okano et al. 1999; Ploutno et al. 2002; Reshef and Carmeli 2001; Sano and Kaya 1995; Shin et al. 1996; Von Elert et al. 2005; Yamaki et al. 2005; Zafrir and Carmeli 2010; Zafrir-Ilan and Carmeli 2010). LC-MS runs were analyzed over the entire time for the presence of known chymotrypsin or trypsin inhibitors. We used the mass range function of the Qual Browser, which is an integral part of the Xcalibur software, granting a mass deviation of 3 ppm. For quantitative comparisons between the different samples, we used the peak area ratios between the metabolite and the internal standard MRFA. All investigated compounds were additionally subjected to database inquiry using an internet database (SciFinder).

Selection and analysis of other prominent metabolites

In addition to protease inhibitors we also screened for a list of 20 microcystins (the most prominent group of cyanobacterial toxins) to find evidence for the presence of cyanobacteria in the phytoplankton samples. Screening and quantitative analyses were carried out as described above for protease inhibitors. We further analyzed the dynamics of other prominent masses in our samples. For this we only considered masses with retention times between 5 and 13 minutes and within the mass range of 500 Da to 1500 Da. In addition, the masses had to appear as a single, clearly shaped peak, and the peak area ratio between the metabolite and MRFA in one of the samples should equal or exceed a ratio of 5 (positive ionization) or 20 (negative ionization). Relative amounts of these molecular species were determined in the

same way as described above for protease inhibitors and microcystins, respectively. LC-MS analyses of methanolic extracts of *M. aeruginosa* strains were performed as described above in order to investigate whether unknown masses detected in the natural samples could be attributed to cyanobacteria.

Fractionation

In order to test the different metabolites in the extracts of phytoplankton samples for the inhibition of chymotrypsins, we collected fractions of one minute intervals each in an exemplary LC-run (21/08/07) in the retention time range between 5 and 12 minutes. This time range covers the retention time window in which all of the investigated metabolites appeared. Fractionation was carried out using a splitter which divided the flow from the LC pump (300 µl) into 180 µl that were collected in an automatic fraction collector and 120 µl that were used for mass spectrometry.

Chymotrypsin inhibition assay

The chymotrypsin activity of *D. magna* clone B was measured using the method of von Elert et al. (2004) and Kuster et al. (2013). Twenty 6-day-old individuals were transferred into 1.5 ml tubes on ice. After that 200 µl ultrapure water were added. Individuals were then homogenized with a teflon pestle, the homogenate was centrifuged (3 minutes; 14,000 g), and the supernatant was used immediately in the enzyme assay. As a substrate for chymotrypsins we used SuccPNA (N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide, Sigma, 125 µM). The enzyme assays were performed in a potassium phosphate buffer (0.1 M, pH 7.5). The absorption was measured continuously for six minutes at time intervals of 20 s at 30 °C and 390 nm with a Cary 50 photometer (Varian, Palo Alto, USA) and the increase of absorption was checked for linearity. Five to 50 µl of different concentrations of LC fractions or the unfractionated crude extract were used for the assays. Thus concentrations of extracted phytoplankton ranged from $7*10^{-3}$ µg (AFDM) per ml to $3.5*10^{3}$ µg (AFDM) per ml. Controls consisting of 50 % ACN and 0.05 % TFA had no effects on enzyme activity. The resulting protease activities were plotted as a function of AFDM. By fitting a sigmoidal dose response curve, the extracted biomass that resulted in a 50 % inhibition of *Daphnia* protease activity (IC_{50}) was calculated. 95 % confidence intervals were calculated, and values were regarded as significantly different when the confidence intervals did not overlap. With results that could not be plotted in a sigmoidal dose response curve due to a lack of inhibition even at

high concentrations, no IC_{50} value could be calculated. Per definition we set those IC_{50} values to zero.

Statistics

Data transformation

To check for putative correlations between a known (or unknown) metabolite and the chymotrypsin inhibition, we used log-transformed IC_{50} values to calculate the chymotrypsin inhibitory potential (CIP) as according to formula 1.

(1) $$(IC_{50\ high} + IC_{50\ low}) - x = CIP$$

$IC_{50\ high}$: log-transformed highest IC_{50} value

$IC_{50\ low}$: log-transformed lowest IC_{50} value

x: log-transformed IC_{50} value

CIP chymotrypsin inhibitory potential

Low IC_{50} values indicate a high inhibitory potential. Application of (1) results in values of CIP, which increase with inhibitory potential and thus facilitate data interpretation.

For the analysis of masses (known and unknown) we were primarily interested in the seasonal dynamic of the abundances. Mass abundances were given as metabolite peak areas normalized to the internal standard (MRFA) peak area. The data were further normalized by calculating standard scores (z-score). For this purpose we calculated the average abundance for each metabolite over all the sampling time points. Then we subtracted the average from each actual value (centralization); these centralized values were then divided by the standard deviation over the sampling period (standardization). Since especially in the case of unknown masses we do not know anything about, for instance, the ionizability, we cannot draw absolute quantitative conclusions from the data, and therefore the quantitative comparison of different masses at one sampling date must not be performed. Standardization allowed us to

sum up the different metabolites without creating false results due to artificially caused different absolute abundances of metabolites. The CIP was standardized in the same way and could therefore be plotted on the same scale as the metabolites. If not stated otherwise, all data shown are standard scores.

Regression analysis

We performed logarithmic regressions with the CIP as the dependent variable (y-axis) and the relative abundance of a single unknown compound or known metabolite as the independent variable (x-axis) using Sigma Plot (11.0 Build 11.2.0.5). A logarithmic regression was necessary, since we used log-transformed data for the chymotrypsin inhibitory potential (CIP).

Principal component analysis (PCA)

We performed a principal component analysis (PCA) using all parameters including the relative abundances of all known and unknown compounds (eight positively and thirteen negatively ionized metabolites) and the CIP, as well as the log-transformed IC_{50} values, which resulted in 24 different variables (note that IC_{50} and CIP are not distinct, independent variables, since they are per definition 100 % negatively correlated). Sample dates were used as cases in the analysis. The PCA was carried out using the program Statistica (6.0.437.0)

Results

Protease inhibitors and microcystins

In order to detect chymotrypsin and trypsin inhibitors in the eight phytoplankton samples covering the time from 26th June until 9th October, we screened for the exact masses of 73 different trypsin and chymotrypsin inhibitors. We found no matching mass for a trypsin inhibitor and only one for a chymotrypsin inhibitor. This metabolite ($M+H^+$; m/z: 851.47682 with z = 1) (Fig.1C) matched anabaenopeptin MM850 (Zafrir-Ilan and Carmeli 2010) and was found in all phytoplankton samples.

We also screened for a list of 20 microcystins; the only quantifiable compound was microcystin-LR (MCLR), based on an accurate mass of m/z 995.55386 (positive ionization mode) (Fig.1D). The identity of MCLR was confirmed by identical mass data and retention

time obtained with an extract of the MCLR containing strain *Microcystis aeruginosa* PCC7806 (Sevilla et al. 2010) (data not shown).

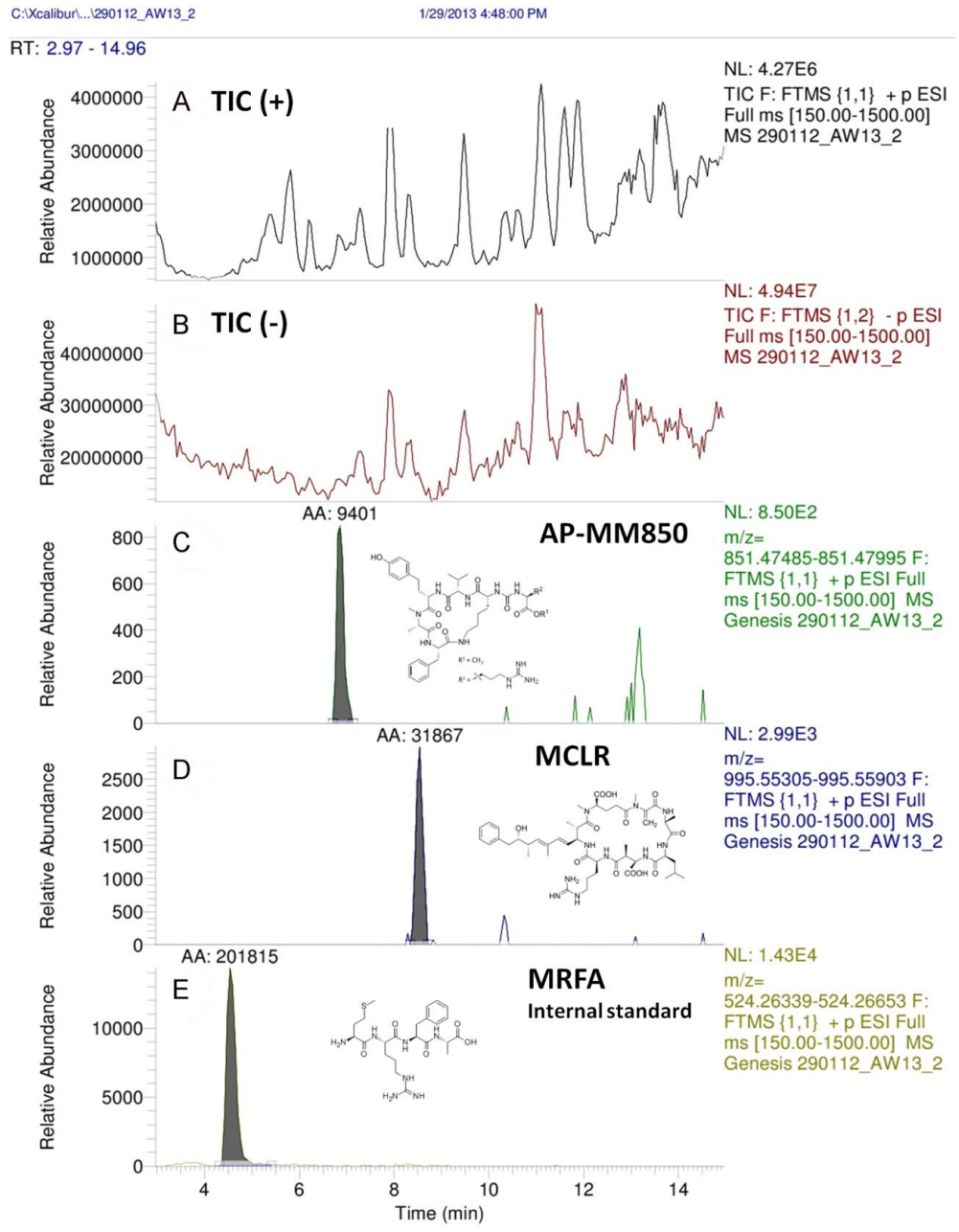

Fig.1 Exemplary LC-MS run (sample from 21st of August). Total ion chromatogram (TIC) in A) positive and B) negative ionization mode. Extracted ion chromatograms for C) anabaenopeptin MM850 (AP-MM850; m/z = 851.47682 with z = 1), D) microcystin-LR (MCLR; m/z = 995.55604 with z = 1), and E) internal standard Met-Arg-Phe-Ala (MRFA; m/z = 524.26496 with z = 1), each with a granted mass deviation of ≤ 3 ppm.

The content of AP-MM850 in the phytoplankton varied substantially over time (Fig.2C). We found the lowest standard scores of AP-MM850 on the first and last dates of the sampling season (26/06 and 09/10). After 26/06, the content increased to its maximum (13/07) followed by a decrease (26/07) and a subsequent increase that lead to a second peak (10/08). Subsequently the content of AP-MM850 decreased to its lowest value on the last sampling date (09/10). The temporal dynamics of MCLR showed a pattern that was similar insofar that a low content in the phytoplankton occurred at the beginning and at the end of the sampling season (Fig.2C). In contrast to AP-MM850, MCLR increased until its maximum on 21/08, followed by a sharp decrease between 21/08 and 28/08. A linear regression of the logarithmic content of AP-MM850 or MCLR in the phytoplankton versus the CIP revealed no significant correlation with MCLR, and only a rather weak correlation of AP-MM850 with the CIP (Tab.1). In addition to that, the principal component analysis revealed that MCLR and AP-MM850 were displayed orthogonally to each other and therefore did not correlate with each other at all. The correlation with the CIP was also not strong and did not differ much between MCLR and AP-MM850 (Fig.3A).

Metabolomics

In search for other cyanobacterial metabolites, we detected eight masses in the positive ionization mode, and thirteen masses in the negative ionization mode, which fulfilled the criteria defined beforehand (see Material and Methods). Two of these masses (555.3 Da and 767.4 Da) could be directly attributed to a cyanobacterial origin, as they occurred in extracts of *M. aeruginosa* strains BM25 and PCC7806 with the same retention time (data not shown).

Positive ionization

All positively ionized metabolites showed a very similar seasonal pattern, with the exception of the masses 715.3 Da and, with constrictions, 909.5 Da (Fig.2A). At the beginning and the end of the sampling season (26/06 and 09/10), the content in the phytoplankton was generally very low. After the 26th of June, values increased to a first peak on 13/07, followed by a decrease and relatively low amounts on the next two sampling dates, with a subsequent increase giving rise to a second peak on 21/08. Thereafter, the contents decreased close to detection limits (Fig.2A). The summed relative abundances of all positively charged masses resembled the seasonal pattern, obtained for the CIP, very closely (Fig.2D).

Negative ionization

The seasonal dynamics of the negatively ionized masses were similar to those of the positively ionized molecules with regard to the two maxima of the concentration in the phytoplankton (13/07 and 21/08) and to fairly low concentrations between these two maxima and at the beginning and at the end of the sampling season (Fig.2B). The general pattern of abundance of the negative ions resembled the CIP pattern very well, which is consistent with the data of the positively ionized metabolites (Fig.2D).

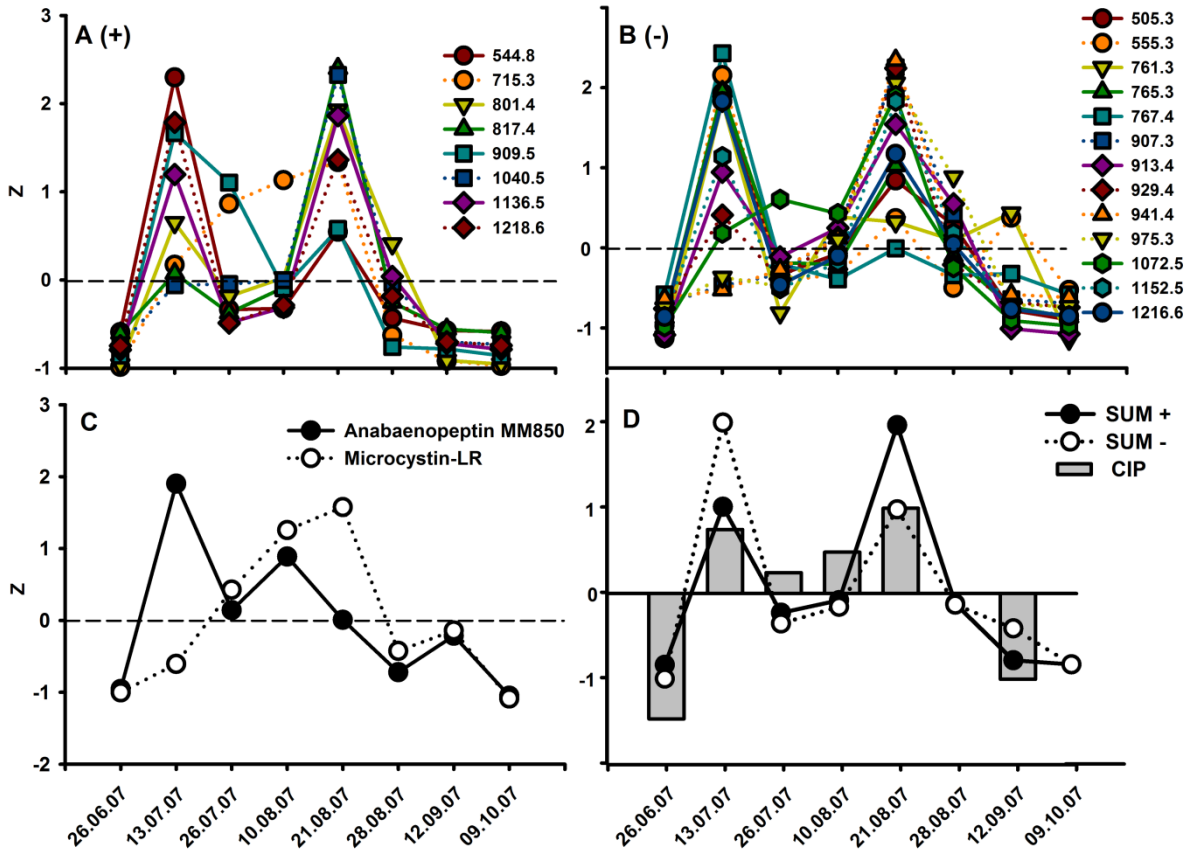

Fig.2 The chymotrypsin inhibitory potential (CIP) and different masses detected in the different phytoplankton samples depicted as standard scores (z). Z-scores were obtained from mass peak areas normalized to the peak area of the internal standard Met-Arg-Phe-Ala (MRFA) (three technical replicates) and the CIP, respectively. A z-score of 0 indicates the average of each value over the sampling period. Therefore positive z-scores indicate an above average value, whereas negative z-scores indicate a value below average. The x-axis represents the different sampling time points. All the investigated unknown compounds are displayed in A) for positive and B) for negative ionization mode. C) Z-scores of anabaenopeptin MM850 and microcystin-LR. D) Z-scores of the total sum of different unknown compounds and the CIP (bars) in the different phytoplankton samples. Depicted is the sum of the phytoplankton content of eight positively charged ions (SUM +) and the sum of 13 negatively charged ions (SUM -).

Regression analysis

Linear regressions of the logarithmic abundance of each analyzed metabolite with the CIP exhibited significant correlations for all positively ionized metabolites and ten out of thirteen negatively ionized metabolites on a half-logarithmic scale, explaining between 78 % and 97 % of the variance (Tab.1). Only the metabolites with the masses 555.3 Da, 761.3 Da and 767.4 Da did not reveal significant correlations with the CIP. Interestingly, two of these metabolites (555.3 Da and 767.4 Da) are those that could be directly attributed to a cyanobacterial origin. The summed positively and negatively ionized metabolites each showed a significant correlation with the CIP (Tab.1).

Principal component analysis (PCA)

The PCA led to a parameter reduction from 24 variables to five factors, which explained 100 % of the results. The first two factors already explained 90.55 % of the results, with a contribution of factor 1 of 67.31 % and factor 2 of 23.24 %, (Fig.3). All of the metabolites exhibited a strong negative correlation with the IC_{50}-value. The vector plotting of the different variables resulted in the formation of four distinct groups (A-D). Group A consists of nine variables with the masses 715.3 Da, 817.4 Da, 907.3 Da, 929.4 Da, 941.4 Da, 975.3 Da, 1040.5 Da, 1072.5 Da and MCLR. Group B is constituted by four variables with masses of 913.4 Da, 1136.5 Da, 1152.5 Da and the CIP. Group C consists of five variables with the masses 505.3 Da, 765.3 Da, 909.5 Da, 1216.6 Da and 1218.6 Da and group D comprises the variables with the masses 544.8 Da, 555.3 Da, 761.3 Da, 767.4 Da and AP-MM850. The metabolite with the mass 801.4 Da could not unambigiously be attributed to a group and lies in the middle between groups A and B. When comparing the distinct groups, one finds that group C and D, although distinct, are still very closely positively correlated. Taken together, groups C and D are displayed almost orthogonally to group A, which means that these groups are not correlated at all. Groups A, C and D show a correlation, albeit weak, with group B, which includes the CIP.

The plotting of the sampling dates in the same factorial frame revealed the contribution of these different sampling dates to the formation of the factors (Fig.3B). The lowest contribution was provided by the cases 10/08 and 26/07. The cases 26/06 and 12/09 contribute slightly to factor 1 but hardly to factor 2. In general the sampling dates 13/07 and 21/08 appear to contribute to both factors very strongly.

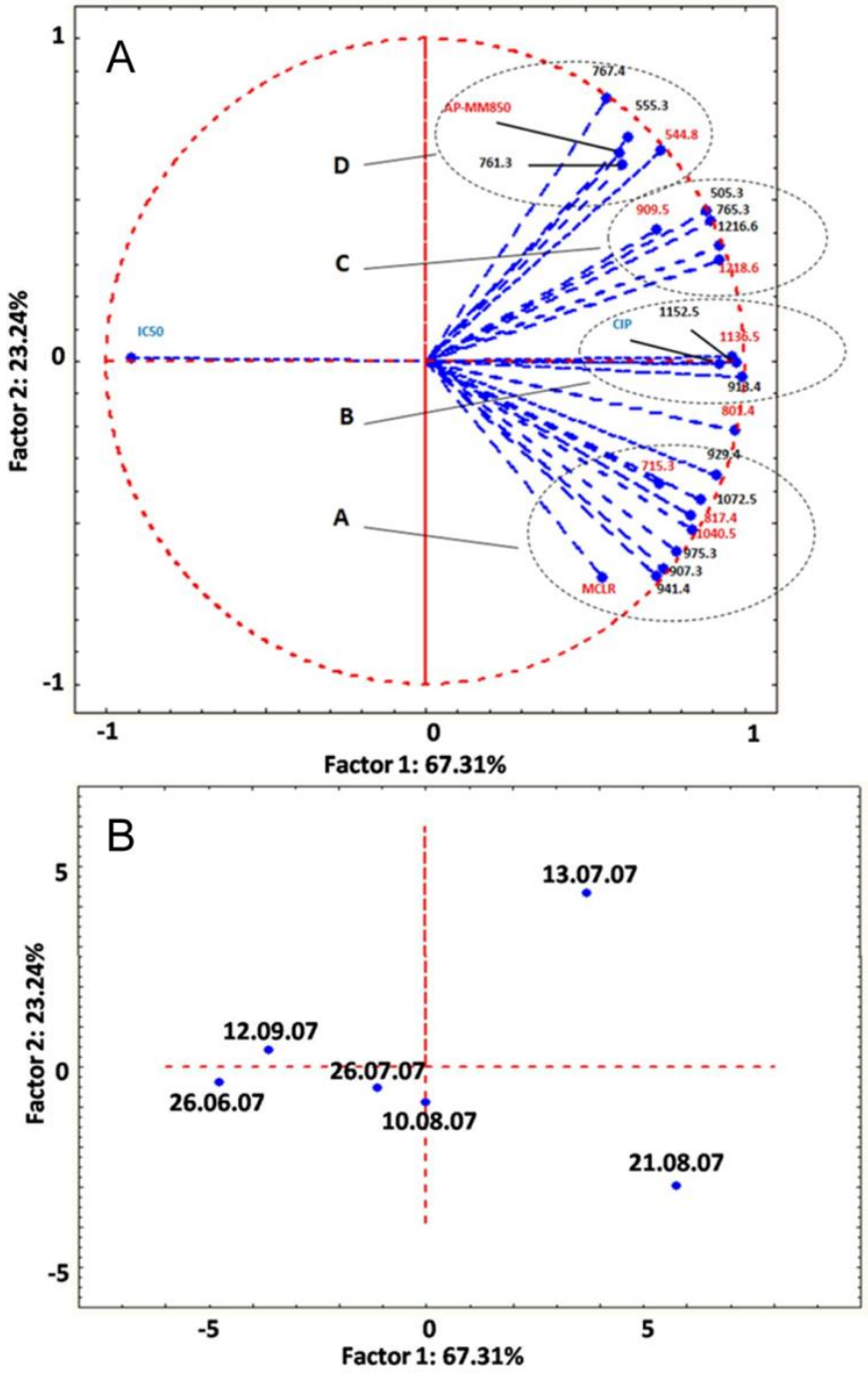

Fig.3 Principal component analysis (PCA) carried out for six phytoplankton samples obtained from a hypertrophic pond at different sampling dates. A) Projection of all variables on the plane of factor 1 x factor 2. The variables comprise the masses of all positive ions including anabaenopeptin MM850 (AP-MM850) and microcystin-LR (MCLR) and all negative ions as well as the IC_{50}-value of a phytoplankton extract and the chymotrypsin inhibitory potential (CIP). Four groups (A-D) are clustering in the factor plane (1x2). B) Projection of all cases (whole datasets of each sampling date including chymotrypsin inhibitory potential (CIP), IC_{50} and all analyzed masses) on the factor plane (1x2).

Chymotrypsin inhibitory potential (CIP) of LC-MS fractions

In order to determine which of the investigated masses might be responsible for the CIP, we measured the potential of a phytoplankton extract (21/08) to inhibit chymotrypsin activity. In addition, we measured the CIP of fractions obtained from a LC-MS-run carried out with the same extract. The whole extract showed a higher CIP than each single fraction (Fig.4): Fractions 5 and 11 did not show any inhibitory potential, whereas fractions 6, 7, 9 and 10 showed rather low CIPs; their CIPs were (with exception of fraction 7) significantly lower than that of fraction 8, which exhibited the highest CIP-value.

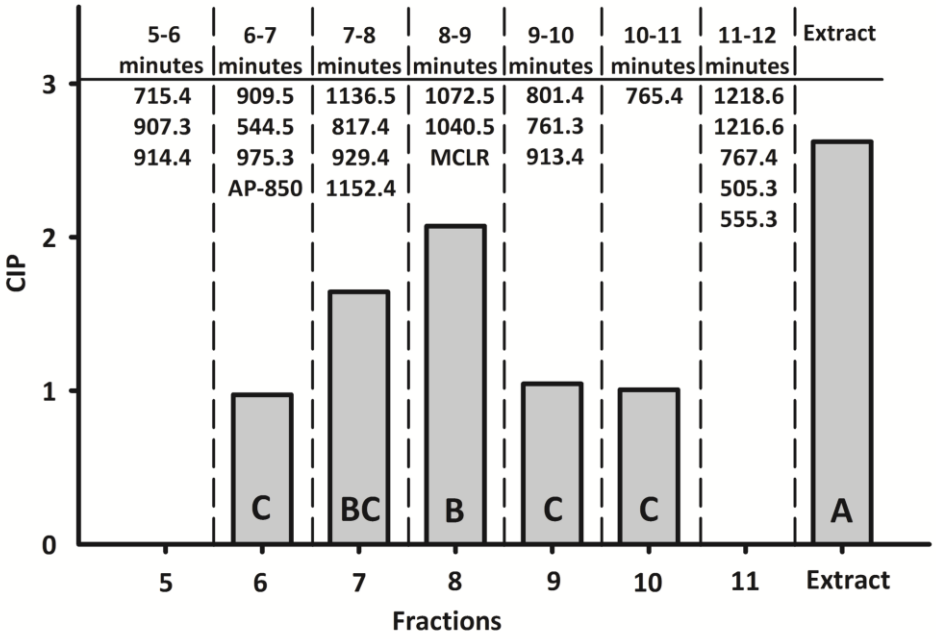

Fig.4 Chymotrypsin inhibitory potential (CIP) of one-minute LC-MS fractions as well as CIP of the whole phytoplankton extract on 21/08/07 in the retention time range between 5 and 12 minutes. Given above each bar are the retention time of the fraction and a list of the compounds detected in it. Bars are regarded as significantly different, in case of non-overlapping 95 % confidence intervals of the original IC_{50}-values.

Tab.1 R^2- and p-values of linear regressions of the log scale of relative peak areas of selected metabolites versus the chymotrypsin inhibitory potential (CIP) of the natural phytoplankton. Depicted are the results for anabaenopeptin MM850, microcystin-LR, all the positive and negative ionized unknown compounds investigated, and the summed up peak areas of masses in both ionization modes. The content of all the metabolites was determined as the ratio of the peak area of the given accurate mass of $M+H^+$ and that of the internal standard.

Metabolite	$M+H^+$ (experimental)	R^2-value	p-value
Anabaenopeptin MM850	851.477	0.664	0.048
Microcystin-LR	995.554	0.49	0.124
unknown masses - positive ionization	544.8	0.907	0.003
	715.3	0.869	0.007
	801.4	0.92	0.003
	817.4	0.907	0.003
	909.5	0.888	0.005
	1136.5	0.921	0.002
	1040.5	0.974	0.001
	1218.6	0.937	0.002
Σ positive ions	SUM+	0.962	< 0.001
unknown masses - negative ionization	505.3	0.924	0.002
	765.3	0.932	0.002
	907.3	0.781	0.02
	913.4	0.892	0.005
	929.4	0.944	0.001
	941.4	0.974	0.001
	975.3	0.874	0.006
	1072.5	0.9	0.004
	1152.5	0.91	0.003
	1216.6	0.974	0.001
	555.3	0.427	0.159
	761.3	0.398	0.179
	767.4	0.545	0.094
Σ negative ions	SUM-	0.77	0.021

Discussion

Protease inhibitors

The data presented here suggest the presence of additional so far unknown trypsin and chymotrypsin inhibitors within the investigated phytoplankton samples. Apparently, naturally occurring protease inhibitors display a very high structural variability, of which only a small fraction has been described until now.

A screening for 73 protease inhibitors within several consecutive natural phytoplankton samples throughout a summer season revealed the presence of only one known chymotrypsin inhibitor, i.e. AP-MM850 (Zafrir-Ilan and Carmeli 2010) and no known trypsin inhibitor, although the same phytoplankton samples exhibited a remarkable potential to inhibit gut proteases of *Daphnia*, e.g. trypsins and chymotrypsins in a prior study (Kuster et al. 2013). Furthermore, the abundance of AP-MM850 was only weakly correlated with the chymotrypsin inhibitory potential (CIP) (Tab.1), and its IC_{50}-value for the inhibition of chymotrypsin is rather high (Zafrir-Ilan and Carmeli 2010). Therefore AP-MM850 cannot explain the CIP pattern of the respective phytoplankton samples. Even the pure substance AP-MM850 does not reach the IC_{50}-value of the phytoplankton sample with the highest CIP (2.3 $\mu g\ ml^{-1}$). These findings strongly suggest the presence of further, so far unknown chymotrypsin inhibitors (CIs) in the phytoplankton. Regarding trypsin inhibition, this indication is even more obvious, since inhibition takes place but no inhibitor could be identified.

Presence of cyanobacteria

Besides the detection of AP-M850 our mass spectral data provided very strong evidence for the so far unconfirmed presence of cyanobacteria within the phytoplankton samples. We detected the cyanotoxin MCLR in the phytoplankton (Figs.1 and 2C). Since both metabolites (AP-MM850 and MCLR) are exclusively produced by cyanobacteria (Dittmann and Wiegand 2006; Moore 1996), their detection clearly indicates the occurrence of cyanobacteria in the phytoplankton. In addition, we found two metabolites with the masses 555.3 Da and 767.4 Da (Fig.2B), that could also be detected in two *M. aeruginosa* strains (PCC7806 and BM25) with the same retention time (data not shown).

Seasonal succession

The data presented here demonstrate, that the combination of a metabolomic analysis of consecutive phytoplankton samples throughout a summer season combined with a biological activity of these samples provides a powerful tool to elucidate seasonal succession patterns of distinct cyanobacterial chemotypes.

Cyanobacterial proportion

All investigated metabolites revealed a very similar seasonal pattern with a few exceptions only (Figs.2A and B). This pattern is as well apparent in the CIP data, and the similarity between the summed up metabolite abundances and the CIP is striking (Fig.2D). Due to the fact that we normalized all phytoplankton samples to the AFDM, all observed seasonal patterns reveal relative changes in the phytoplankton composition and do not reflect changes in overall phytoplankton biomass.

We therefore argue that both data sets, the CIP as well as the overall mass abundance, indicate high proportions of cyanobacteria in the phytoplankton. The strong correlation of the CIP with the single and the summed masses as revealed by the regression analysis (Tab.1) underlines this assumption and the reasoning that the different molecular species most likely are cyanobacterial in origin.

Rohrlack et al. (2005) found metabolites that were inhibitory to proteases in 70 % of 89 different *Planktothrix* strains. Additionally, many cyanobacterial strains have been shown to contain a whole variety of protease inhibitors (Bister et al. 2004; Gunasekera et al. 2009; Murakami et al. 1994; Murakami et al. 1997; Von Elert et al. 2005), and the occurrence of protease inhibitors in cyanobacterial blooms has been demonstrated several times (Agrawal et al. 2001; Banker and Carmeli 1999; Gesner-Apter and Carmeli 2009; Grach-Pogrebinsky et al. 2003; Lifshits et al. 2011; Reshef and Carmeli 2001). Thus, protease inhibition is widely distributed not only among cyanobacterial strains or genera, but among cyanobacterial blooms as well. Variations in the CIP of different phytoplankton samples can therefore be expected to reflect varying relative cyanobacterial abundances in those samples. Since cyanobacteria are known to produce a wide variety of secondary metabolites (Ferrao and Kozlowsky-Suzuki 2011; Leao et al. 2012), a higher relative abundance of cyanobacteria in the phytoplankton not only increases the CIP but also the number and the abundance of secondary metabolites.

Cyanobacterial chemotype composition

However, while the CIP provides quantitative information about the cyanobacterial share in the phytoplankton, the mass distribution patterns additionally reveal chemotypes and their relative abundances on different sampling dates, thus providing additional qualitative information. Obviously, changes within the cyanobacterial community with regard to the chemotype composition happen very rapidly within few days only; a fact which might explain the necessity for the recently proposed inducible physiological response mechanisms in *Daphnia* due to an exposure to cyanobacteria (Schwarzenberger et al. 2010; Von Elert et al. 2012).

Chemotypes are specific assemblies of secondary metabolites produced by a certain cyanobacterial genotype, and there is solid evidence that these remain stable over decades (Repka et al. 2004; Tillett et al. 2000; Welker et al. 2007). This is also true for oligopeptide patterns, which may remain stable for several consecutive seasons within a given lake (Baumann and Jüttner 2008).

It is unclear whether the seasonal variation of the CIP can be attributed to a change of the composition of cyanobacterial genotypes, or alternatively, to a change in the cellular content of CIs within a genotype or to a combination of the two (Kuster et al. 2013). Nevertheless, variations in the content of specific secondary metabolites due to abiotic factors such as light or nutrient availability (Long et al. 2001; Wiedner et al. 2003) or to biotic factors such as chemical cues from grazers (Jang et al. 2003) have been demonstrated, but did not exceed five-fold differences. On the other hand, the microcystin content can vary by several orders of magnitude between strains (Carrillo et al. 2003). Here, the CIP varies seasonally by up to 100-fold, which therefore is a clear indication for a change in the cyanobacterial composition rather than in the metabolite production within the species or strains.

In line with the regression analysis (Tab.1), all mass vectors in the PCA point to a certain degree in the same direction as the CIP vector, thus indicating a positive correlation (Tab.1). Therefore the PCA data provide additional evidence for our assumption that overall mass abundance as well as CIP act as indicators for relative cyanobacterial abundance in the phytoplankton.

Additionally, the directions of the different mass vectors and the CIP form four distinguishable groups (A-D; Fig.3A). These groups could be interpreted as distinct

chemotypes, since assemblies of metabolites co-occurring in one cyanobacterial strain should show a high degree of correlation among each other. Besides the occurrence of different chemotypes, the PCA also reveals their seasonal variation. A comparison of Figs.3A and 3B shows an interesting similarity between the mass clustering and the sampling dates. If regarded as chemotypes, it seems obvious that chemotype A is closely related to 21st of August, whereas chemotypes C and D are related to 13th of July. This assumption can be confirmed by comparing these data with the seasonal mass distribution (Figs.2A and 2B). Without exceptions, masses contained in groups C or D have their maximum on the 13th of July, whereas all masses contained in group A have their maximum on the 21st of August. This is also true for AP-MM85 (maximum on 13th July, contained in group D) and MCLR (maximum on 21st August, contained in group A) (Fig.2C). We summed up the masses of group A and of group C+D and calculated their standard scores: it became obvious that a seasonal chemotype change actually took place, since group A and group C+D peaked at different time points (Fig.5).

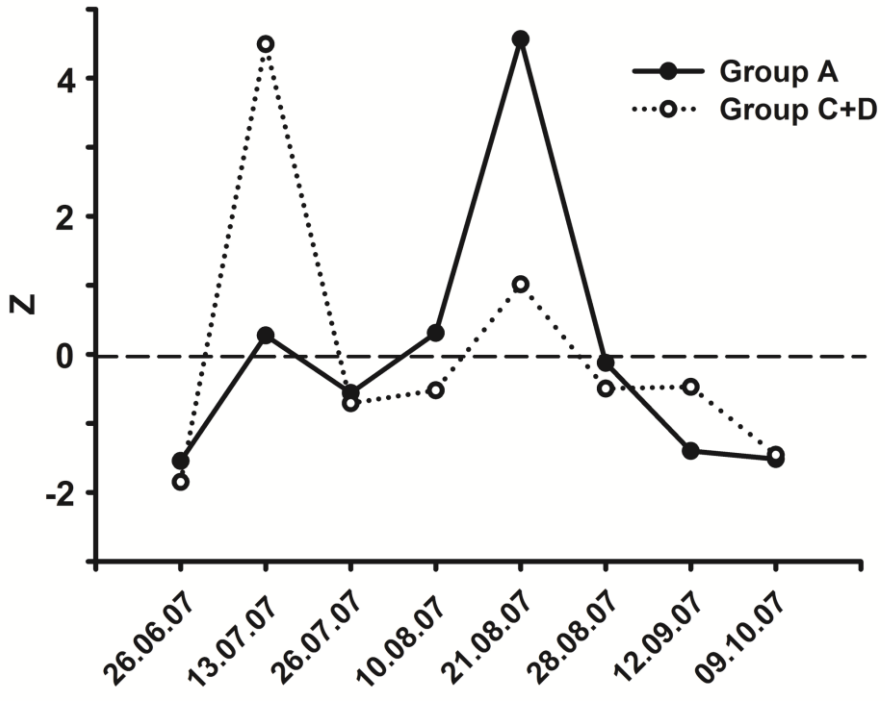

Fig.5 Standard scores (z) of the total sum of group A (nine masses) and group C+D (ten masses) according to Figure 3a. Z-scores were obtained from the sums of the mean values of the peak area from a metabolite normalized to the peak area obtained for MRFA (three technical replicates). A z-score of 0 indicates the average of all values of all samples. Therefore positive z-scores indicate an above average value, whereas negative z-scores indicate a value below average.

Changes within the phytoplankton composition happen very quickly. For example between August 21st and 28th, a massive change in the phytoplankton is observable, confirmed by mass abundances as well as the CIP: The relative abundance of cyanobacteria seemed to be very high on 21st August and to drop drastically within the next week. In general, most metabolites and the CIP formed two maxima. Between those two maxima a change in the cyanobacterial composition took place, resulting in a different dominant chemotype after the minimum (group A), compared to the first maximum, when chemotypes C and D were dominant (Figs.2 and 5).

Daphnia populations are known to adapt to toxic cyanobacteria (Sarnelle and Wilson 2005). According to this, Gustafsson et al. (2004) could demonstrate that *Daphnia* populations develop a tolerance to cyanobacteria within a pre-exposure period of only four weeks. Our results, however, suggest the necessity for even faster inducible defense mechanisms towards toxic cyanobacteria, due to the very rapid change of chemotype composition within the phytoplankton.

Several such inducible physiological responses by *Daphnia* have been reported. Schwarzenberger et al. (2010) found an up-regulation of protease genes in *Daphnia magna* of up to 25 fold, when exposed to cyanobacteria that contained protease inhibitors. Further studies confirmed the notion, that the reorganization of *Daphnia*'s gut proteases is an important adaption mechanism (Schwarzenberger et al. 2012; Von Elert et al. 2012). Also an increase of glutathione-S-transferase activity, an enzyme that is assumed to be involved in the detoxification of cyanobacterial secondary metabolites, has been reported several times in response to cyanobacterial diets (Dao et al. 2013; Guo and Xie 2011; Sadler and von Elert 2014). The fast changing chemotype composition of a natural phytoplankton community observed in this study therefore provides a plausible explanation for the presence of inducible rather than constitutive physiological responses in *Daphnia*.

Welker et al. (2007) similarly observed a seasonal change of cyanobacterial chemotypes, which could be related to a change in genotype composition. Although no genotype data were available in our case, we assume that the observed seasonal change might have been due to a genotype shift as result of a species or clonal succession.

New putative protease inhibitors

The combination of metabolomic data with multivariate statistics allows for a pre-selection of metabolites with putative biological properties. It therefore facilitates the search for new natural products within complex phytoplankton samples. However, at the same time the high variability of metabolites in natural phytoplankton hampers their isolation.

The PCA analysis revealed a clustering of four distinct groups (A, B, C, and D). With regard to the existence of putative new protease inhibitors, group B is the most interesting one, since it shows the highest degree of correlation with the CIP (Fig.3A). The clustering of group B could be explained in two different ways. Possibly metabolites contained in group B occur ubiquitously in cyanobacteria. Such metabolites would not cluster with one specific sampling date, but rather in between. Another possibility would be the existence of an actual chemotype B being abundant on the 13th of July and on the 21st of August. Although the PCA results do not allow distinguishing between these possibilities, metabolites contained in group B clearly are the best candidates for being new chymotrypsin inhibitors. However, different chymotrypsin inhibitors might be present at different sampling times. An inhibitor only present on 13th of July or 21st of August would be weakly correlated with the CIP, but still might have a high inhibitory potential. In this case, however, more than one metabolite would be necessary to explain the CIP pattern in our samples. This appears to be true, since five out of seven sample fractions revealed a potential to inhibit chymotrypsins to a certain extent (Fig.4). However, the CIP of each single fraction was always significantly lower than the CIP of the unfractionated crude extract (Fig.4). This indicates that the CIP of natural phytoplankton samples is caused by a whole variety of metabolites with chymotrypsin inhibitory potentials.

The IC_{50} value of the crude extract was well in concordance with the IC_{50} value obtained by Kuster et al. (2013) (2.3 µg ml^{-1}, present study: 3.3 µg ml^{-1}). Unfortunately, we were not able to isolate single molecular species due to similar retention times. Moreover, we have no knowledge about putative synergistic or antagonistic effects caused by the great variability of metabolites in the phytoplankton. The high variability of metabolites in complex phytoplankton samples therefore hampers the isolation of single protease inhibitors.

Conclusions

The combination of metabolomic profiles with a biological property, such as the potential to inhibit proteases, allows conclusions about the proportion and composition of cyanobacterial chemotypes within natural phytoplankton communities. In this respect, cyanobacteria turn out to be very suitable organisms for metabolomic approaches, due to their richness in secondary metabolites. The high correlation of these metabolites with the CIP, a biological activity that can be clearly attributed to cyanobacteria, supports this notion. On 13th of July the phytoplankton was dominated by two putative chemotypes (groups C and D), whereas on 21st of August a different chemotype (group A) was more dominant. In addition, the same approach can be used for identifying natural products in the phytoplankton, in our case i.e. protease inhibitors. While using chemically diverse natural phytoplankton samples instead of single clonal cultures increases the probability of detecting new natural products, the isolation of these natural products from the far more complex natural background is challenging. Nevertheless, the approach performed here might help identifying new natural products with different biological activities by crossing metabolomics with different bioassays, while at the same time unraveling seasonal succession patterns.

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Concluding remarks and perspectives

The transfer of energy, carbon and nutrients across food webs is a central function in all ecosystems (Persson et al. 2007a). In this respect, first-order consumers, such as *Daphnia* play a critical role (Cebrian 2004) since they mediate the energy transfer across the plant-herbivore interface (Müller-Navarra and Lampert 1996; Persson et al. 2007b).

Especially in highly eutrophic lakes or ponds, this transfer is usually constrained by food quality rather than food quantity which is at least partly attributable to high abundances of cyanobacteria (Persson et al. 2007a; Watson et al. 1997). Cyanobacteria provide a low quality resource for daphnids for several reasons. Among those reasons is the mechanical interference of colony forming cyanobacteria with the filter apparatus of *Daphnia* (Porter and Mcdonough 1984) but also the lack of several dietary components, which are essential for daphnids (Martin-Creuzburg et al. 2005; Martin-Creuzburg et al. 2008; Von Elert 2002; Von Elert et al. 2003). Moreover, cyanobacteria frequently contain secondary metabolites, which exert negative effects on *Daphnia* fitness (Lürling and van der Grinten 2003; Rohrlack et al. 2001; Von Elert et al. 2012). In line with this numerous studies could demonstrate the suppression of *Daphnia* populations by cyanobacteria (Ghadouani et al. 2003; Tillmanns et al. 2008; Wilson et al. 2006). However, other studies found the opposite situation, namely the suppression of cyanobacterial bloom formation by *Daphnia* (Chislock et al. 2013a; Sarnelle 2007). These contradictory findings can be explained by the fact that different *Daphnia* species but also different genotypes of the same species display a remarkable variety with regard to their sensitivity towards cyanobacterial diets (Jiang et al. 2013; Sarnelle and Wilson 2005; Tillmanns et al. 2008). One of the physiological mechanisms underlying this phenomenon might be the ability to cope with cyanobacterial toxins, such as microcystins (MCs).

With regard to the detoxification of microcystin-LR (MCLR) in *Daphnia*, the enzyme glutathione-S-transferase is assumed to act as a first step of detoxification (Pflugmacher et al. 1998). This hypothesis is plausible for several reasons: First of all, MCs do provide suitable substrates for GSTs since they contain a α, β-unsaturated carbonyl group (Michael-acceptor) which conjugates easily to the cystein residue of glutathione (GSH) in a Michael-reaction (Kondo et al. 1992). In addition, numerous studies could show the importance of the GST detoxification pathway in several other aquatic organisms (Campos and Vasconcelos 2010;

He et al. 2012; Li et al. 2014; Zhang et al. 2009). Finally, the formation of an enzymatically formed MCLR-GSH conjugate could be shown *in vitro* by adding MCLR and GSH to *Daphnia* crude extracts (Pflugmacher et al. 1998).

Notwithstanding this, the results in **chapter 1** showed that this detoxification process does not take place in *Daphnia in vivo*.

Exposure experiments with *Daphnia magna* and the MC containing cyanobacterium *Microcystis aeruginosa* demonstrated that the overall MCLR amount within an enclosed experimental setup was not decreased due to the presence of daphnids. Biotransformation via GST, but also any other biotransformation processes inevitably have to result in such decreased overall MCLR amounts. Therefore, I could rule out that biotransformation or biodegradation processes are of relevance for *Daphnia in vivo*. In line with this, the presence of *Daphnia* does not lead to an overall increase of MCLR-GSH conjugation product. Finally, GST enzyme activities were not different in daphnids that fed on cyanobacteria with or without MCs.

Instead, the data suggested an alternative, so far unconsidered hypothesis, i.e. the involvement of transport mechanisms capable of exporting unconjugated MCLR out of *Daphnia* tissue. *Daphnia* feeding activity led to a reduction of cyanobacterial biomass of 35 %. In accordance with this, the MCLR amount within the cyanobacterial cell fraction decreased similarly. However, this reduction of MCLR in the cell fraction was reflected by a comparable increase of MCLR in the surrounding medium, which suggests that ingestion of MCLR by *Daphnia* was followed by a fast and efficient MCLR export.

Summarized, these results reject the widespread assumption of biotransformation via GST and point at the possibility of efficient export mechanisms for MCLR in *Daphnia*.

Cyanobacteria that do not produce any MCs nevertheless cause detrimental effects in *Daphnia* (Lürling 2003; Schwarzenberger et al. 2009; Tillmanns et al. 2008). Therefore it is reasonable not only to focus on one cyanobacterial toxin, but to take other compounds into account as well.

In a second study (**chapter 2**), I present evidence that this finding does not represent a special case only for dietary MCLR in *Daphnia*. Instead I demonstrate that other cyanobacterial compounds are similarly not subjected to biotransformation but exported out of the *Daphnia* tissue.

Hence, I re-examined the mass spectral dataset obtained from the exposure experiment performed in the first study (**chapter 1**) this time analyzing seven further cyanobacterial metabolites (**chapter 2**). The compounds consisted of three cyanopeptolines (CP A, B and C) and four cyclamides (microcyclamide 7806A and aerucyclamides B, C and D). CPs are depsipeptides and inhibit the serine proteases trypsin and chymotrypsin (Gademann and Portmann 2008; Martin et al. 1993), which account for more than 80% of the proteolytic activity in the *Daphnia* gut (Von Elert et al. 2004). The function of CPs as anti-herbivore defense has been demonstrated convincingly (Agrawal et al. 2005; Gademann and Portmann 2008; Schwarzenberger et al. 2010; Von Elert et al. 2010). Less is known about the effects exerted by cyclamides on daphnids, although it could be shown that some cyclamides display toxic properties with regard to other crustaceans (Ishida et al. 2000). CPs and cyclamides differ substantially with regard to their production mode as well as their general structure. Nevertheless, none of the compounds overall amounts were decreased within the exposure experiment due to the presence of daphnids. This rules out any forms of biotransformation or biodegradation, similar as it had been shown for MCLR in **chapter 1**. Instead the data at least partly suggest the presence of export mechanisms for unconjugated compounds. Apparently, transport mechanisms are of general relevance with regard to dietary cyanobacterial compounds in *Daphnia* and appear to cover a wide range of structurally diverse metabolites.

While these transport processes appear to be defense mechanisms against toxic cyanobacteria in daphnids, the cyanobacteria in return have been suggested to possess defense mechanisms against grazing *Daphnia*. Prior studies could show that some cyanobacteria react to grazing pressure with an increase in MC production (Jang et al. 2003; Jang et al. 2004; Jang et al. 2007; Jang et al. 2008). This was confirmed by findings in **chapter 1** where the amount of MCLR per cyanobacterial cell was increased due to the presence of grazing *Daphnia*. In **chapter 2,** I provide first evidence for an elevated production of aerucyclamides due to the presence of *Daphnia*, which points at an involvement of aerucyclamides in grazer defense. When calculating the intracellular compound concentrations within the cyanobacteria two aerucyclamides were significantly higher concentrated when daphnids were present. For microcyclamide 7806A (MC 7806A), I also found elevated metabolite production due to the presence of daphnids. However, this was not indicated by an increased intracellular concentration of this compound but by a strongly increased overall amount of MC 7806A in the presence of daphnids. This increase of the overall amount is mostly caused by additional MC 7806A in the surrounding medium, which is not explainable by *Daphnia* feeding activity

alone. Accordingly, the metabolite must have been excreted by the cyanobacteria themselves. Thus, I could for the first time provide evidence for a putative cyanobacterial defense mechanism which includes the export of a detrimental compound into the surrounding medium.

Finally, **chapter 2** contains first results with regard to the properties of trans membrane transport mechanisms in *Daphnia*. The long term chase experiment (**chapters 1** and **2**) was carried out in the absence of cyanobacteria and demonstrated that MCLR is excreted out of the *Daphnia* tissue with a higher efficiency in comparison to the cyanopeptolines. The data do not reveal whether this difference is due to different specific transporters for MCs and CPs or due to different efficiencies of the same transporter for the different substrates.

However, results presented in **chapter 3** provide evidence for the involvement of two different transporter types with regard to MCLR export in *Daphnia*: The multi xenobiotic resistance mechanism (MXR, P-gp) and organic anion transporting polypeptides (Oatps).

The MXR mechanism was proposed to be a reason for the insensitivity of likewise unselective filter-feeding bivalves towards cyanobacterial toxins (Contardo-Jara et al. 2008). In addition, an up-regulation of the respective gene was demonstrated in the freshwater fish *Jenynsia multidentata*, when confronted with dissolved MCLR in the water (Ame et al. 2009). The MXR system in daphnids and other aquatic animals is generally involved in the export of complex mixtures of water pollutants and is therefore regarded as a rather unspecific transporter with a broad range of substrates (Campos et al. 2014; Kurelec 1992). Therefore, I hypothesized that this transporter type might also be involved in the export of dietary MCLR out of *Daphnia* tissue.

Oatps are another class of transporters that is widely distributed within the animal kingdom (Hagenbuch and Meier 2003; Hagenbuch and Meier 2004). Oatps have been repeatedly reported to transport MCLR, although these studies are confined to humans or other mammals (Feurstein et al. 2009; Fischer et al. 2005). **Chapter 3** represents the first study that considered *Daphnia* Oatps as putative transporters involved in the export of MCLR.

Daphnids that fed on the MC-containing cyanobacterium *M. aeruginosa* in the presence of an Oatp or P-gp inhibitor contained more MCLR in their tissues in comparison to those that fed on *Microcystis* in the absence of any inhibitors. According to these findings, I hypothesize that the inhibitors interfered with the respective trans membrane transporters. As a

consequence, this impaired the export of MCLR which subsequently resulted in higher MCLR amounts within the *Daphnia* tissue. The presented data therefore provide first evidence for the involvement of P-gp and Oatp transporters in the export of MCLR from *Daphnia magna* tissue.

The ability of *Daphnia* to adapt to cyanobacteria spatially (Sarnelle and Wilson 2005) as well as temporally (Hairston et al. 1999) has been demonstrated repeatedly. Accordingly, the ability and extent to which daphnids are able to excrete detrimental cyanobacterial toxins might explain the high variability of *Daphnia*'s tolerance against dietary cyanobacteria (Chislock et al. 2013b; Jiang et al. 2013; Sarnelle and Wilson 2005). The improvement of these transport mechanisms might constitute an adaptation process which leads to better adapted daphnids with regard to dietary cyanobacteria. In addition to such microevolutionary processes, the acquirement of resistance against cyanobacteria in shorter time spans was reported as well (Gustafsson and Hansson 2004; Gustafsson et al. 2005). The data presented in **chapter 3**, however, rather point at an involvement of transport mechanisms in long term microevolutionary adaptation processes, since an up-regulation of respective transporter genes after a 24 h exposure to cyanobacteria was not observed. I hypothesize that the different degrees of adaptation towards cyanobacterial diets among *Daphnia* clones and species will be resembled by similarly different capabilities to export detrimental cyanobacterial compounds. The identification of specific *Daphnia* trans membrane transporters responsible for MCLR export will aid further studies, which are necessary in order to test this hypothesis.

The necessity for daphnids to react to cyanobacterial metabolites on shorter time spans as reported by Gustaffson et al. (2005) is emphasized by results reported in **chapter 4**. Here I could show that the distinct chemical compositions of natural phytoplankton samples can change very quickly within a given lake or pond.

In this study, I used high resolution LC-MS technology to investigate the seasonal dynamic of a freshwater phytoplankton community by analyzing several consecutive phytoplankton samples from an urban pond. In order to do so I developed a new method by combining metabolomic data with the protease inhibition potential of natural lake phytoplankton samples. As outlined above, cyanobacteria frequently contain potent inhibitors of *Daphnia* gut trypsins and chymotrypsins (Martin et al. 1993). Accordingly, phytoplankton samples that contain such cyanobacteria obtain the ability to inhibit gut proteases as well (Czarnecki et al. 2006). In a previous study, it could be shown that the potential to inhibit gut proteases varies

seasonally in a similar pattern as it would be expected for the cyanobacterial proportion within a complex phytoplankton sample (Kuster et al. 2013; Sommer et al. 1986). Hence, I compiled metabolomic fingerprints of phytoplankton samples and used multivariate statistics to combine the metabolomic data with the protease inhibition potential. This way, I could determine whether any metabolites detected within these phytoplankton samples were of cyanobacterial origin. Simultaneously, I could identify specific cyanobacterial chemotypes that were differently abundant at different time points. Chemotypes are specific assemblies of secondary metabolites produced by a cyanobacterial genotype and there is solid evidence that these chemotypes remain stable over longer time periods (Repka et al. 2004; Tillett et al. 2000; Welker et al. 2006). A change in the cyanobacterial chemotype composition can therefore be interpreted as a change in the genotype composition. The data revealed rapid changes within the phytoplankton community with regard to the cyanobacterial chemotype composition. This finding constitutes the main reason for the fast changes of the chemical composition of the different phytoplankton samples. Such fast changes within the cyanobacterial community can in return be assumed to strongly impact *Daphnia* populations.

In summary, the present dissertation investigated important aspects of the physiology of the *Daphnia* - cyanobacteria interaction via high-resolution LC-MS technology. The application of this technique led to several new insights into this interaction that is crucial for freshwater ecosystem structure. I was able to reject the widespread hypothesis of biotransformation of the most common cyanotoxin MCLR via GST in *Daphnia*. Instead, I provide evidence for the involvement of transport processes capable of transporting unconjugated microcystins. Moreover, I could show that structurally diverse cyanobacterial compounds, other than microcystins, are also not subjected to biotransformation but excreted from the *Daphnia* tissue. Two types of transporters could be identified which are involved in the excretion of MCLR and might therefore be involved in the excretion of other metabolites as well. Finally, with the usage of LC-MS technology I was able to develop a new method to investigate seasonal succession patterns of cyanobacterial chemotypes by combining the potential to inhibit grazer proteases with metabolomic fingerprinting. This approach revealed rapid changes within a cyanobacterial community which emphasizes the need for adaptation processes in *Daphnia* with regard to dietary cyanobacteria.

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Abstract

In most freshwater ecosystems the unselectively filter feeding cladoceran *Daphnia* is of major importance for the transfer of energy, carbon and nutrients across the plant - herbivore interface. Especially in freshwaters with a high abundance of cyanobacteria this transfer is often constrained by food quality rather than food quantity, since cyanobacteria are considered as low quality food for daphnids. Cyanobacteria lack several nutritional components that are important for daphnids, they can interfere mechanically with the filter apparatus of daphnids due to colony formation and they contain numerous cyanotoxins. The importance of the *Daphnia* - cyanobacteria interaction is additionally emphasized by an increasing anthropogenic influence on ecosystems, which includes artificial nutrient input into lakes and ponds as well as increasing temperatures due to global warming. These human impacts contribute to the extent and frequency of cyanobacterial blooms, which in return constitute a serious hazard for public health and ecosystem services. Studies concerned with this crucial interaction reveal contradictory results, in which sometimes cyanobacteria suppress *Daphnia* populations whereas in other cases the opposite situation can be observed, namely the suppression of cyanobacteria by daphnids. *Daphnia* species display an enormous ability to adapt to cyanobacteria. As a result, huge differences among *Daphnia* species and genotypes with regard to their sensitivity towards cyanobacterial diets can be found. Despite the importance of the *Daphnia* - cyanobacteria interaction for the structure of freshwater ecosystems, the knowledge about the physiological basis of this interaction is scarce.

In order to elucidate important aspects of the physiology that underlies the *Daphnia* - cyanobacteria interaction the present study focused mainly on toxic cyanobacterial secondary metabolites. To detect and quantify cyanobacterial compounds in exposure experiments with daphnids and toxin containing cyanobacteria, high resolution LC-MS technology was applied. This approach, in combination with other techniques, such as enzyme activity measurements and molecular biological methods, revealed several new insights into the physiology of the interaction of *Daphnia* with cyanobacteria.

It is argued that one major reason explaining the huge differences among daphnids with regard to their sensitivity towards cyanobacteria might lie in their ability to cope with cyanotoxins such as microcystin-LR (MCLR). The widely accepted hypothesis regarding

dietary MCLR in *Daphnia* includes the involvement of glutathione-S-transferase acting as a first step of detoxification by conjugating the toxin with glutathione (GSH).

I was able to reject this hypothesis by measuring MCLR amounts in all parts of an exposure experiment (incubation medium, cyanobacteria and *Daphnia* tissue) with *Daphnia magna* and the MCLR containing cyanobacterium *Microcystis aeruginosa*. Since the presence of daphnids neither led to a decrease of overall MCLR nor to an increase of the MCLR-GSH conjugate significant processes of biotransformation of any kind could be ruled out. Instead the data revealed that MCLR is exported out of *Daphnia* tissue with a high efficiency. These transport processes had not been considered with regard to dietary MCLR in *Daphnia* yet. Furthermore, the study reveals that these transport mechanisms should not be considered as a special case for MCLR alone but that transport is of general importance with regard to dietary cyanotoxins in *Daphnia*, since a whole set of other structurally diverse cyanobacterial components were similarly exported out of *Daphnia* and not subjected to any forms of biotransformation.

Additionally, I could identify two types of trans membrane transporters, namely multi xenobiotic resistance (MXR) proteins and organic anion transporting polypeptides (Oatps) being responsible for the export of MCLR out of *Daphnia* tissue. The application of specific inhibitors of these transporters resulted in significantly increased MCLR amounts within the *Daphnia* tissue. Although a qPCR analysis demonstrated that a 24 h exposure of daphnids to a MCLR containing diet is not enough to cause significant up-regulation of respective transporter genes, the results in this dissertation strongly emphasize the importance of transport mechanisms for the understanding of the *Daphnia* - cyanobacteria interaction.

In the last part of my thesis I successfully developed a new method in order to unravel seasonal succession patterns of cyanobacterial chemotypes within a pond. Metabolomic fingerprints of a time series of phytoplankton samples were combined with the potential of these samples to inhibit grazer proteases by using multivariate statistics. This new approach revealed rapid changes within the cyanobacterial chemotype composition in a lake, which in return strongly reflect on *Daphnia* populations and emphasize the need for fast adaptation processes on the *Daphnia* side.

In summary, the results of this dissertation reveal several new insights in the physiology of the *Daphnia* - cyanobacteria interaction, which elucidate the importance, necessity and nature of transport processes for detrimental cyanotoxins in *Daphnia*.

Zusammenfassung

In den meisten stehenden Süßwasser Ökosystemen sind unselektive Filtrierer der Gattung *Daphnia* von größter Wichtigkeit für den Transfer von Energie, Kohlenstoff und Nährstoffen von der Ebene der Primärproduzenten auf höhere trophische Ebenen. Insbesondere in Gewässern in denen Cyanobakterien in großer Menge vorkommen ist dieser Transfer in der Regel nicht mehr durch Futterquantität, sondern durch Futterqualität bestimmt. Cyanobakterien stellen für Daphnien eine Nahrungsquelle von sehr schlechter Qualität dar. Zum einen fehlen Ihnen wichtige Nährstoffe, die für Daphnien essentiell sind, zum anderen kommt es bei Cyanobakterien häufig zur Koloniebildung. Diese häufig fädigen Kolonien können den Filtrierapparat der Daphnien empfindlich stören oder sogar verstopfen. Zu guter Letzt enthalten Cyanobakterien häufig eine große Zahl verschiedener Toxine welche sich auch negativ auf Daphnien auswirken können. Entsprechend ist die Interaktion von Daphnien und Cyanobakterien von großer Wichtigkeit für limnische Ökosysteme. Dieser Befund wird noch weiter verstärkt durch den zunehmenden anthropogenen Einfluss auf solche Ökosysteme, welcher vor allem den künstlichen Eintrag von Nährstoffen, aber auch generell erhöhte Temperaturen aufgrund von globaler Erwärmung beinhaltet. Diese Einflüsse wirken sich positiv auf die Häufigkeit und auch das Ausmaß cyanobakterieller Massenaufkommen, sogenannter Blüten, aus. Solche cyanobakteriellen Blüten wiederum stellen eine große Gefahr für die öffentliche Gesundheit aber auch für die Nutzbarkeit von Ökosystemen dar. Studien die sich mit dem Zusammenspiel von Daphnien und Cyanobakterien auseinandersetzen haben sehr widersprüchliche Resultate geliefert. Manchmal wird von Cyanobakterien berichtet, welche einen starken Rückgang von Daphnien Populationen bewirken. Andere Studien zeigen das Gegenteil, nämlich den Rückgang von Cyanobakterien durch die Anwesenheit von Daphnien.

Im Hinblick auf Cyanobakterien verfügen Daphnien über ein enormes Adaptationspotential. Dementsprechend findet man sehr große Unterschiede bzgl. der Sensitivität von verschiedenen Arten oder auch Genotypen derselben Art gegenüber Cyanobakterien. Trotz der Wichtigkeit der Interaktion von Daphnien mit Cyanobakterien für die Struktur von limnischen Ökosystemen weiß man nur wenig über die zugrundeliegende Physiologie.

Der Hauptfokus der vorliegenden Dissertation bestand darin wesentliche physiologische Aspekte dieser Interaktion zu beleuchten. Dabei habe ich mich besonders auf toxische

cyanobakterielle Sekundärmetabolite konzentriert. Insbesondere stand die Frage im Vordergrund, wie Daphnien mit diesen toxischen Komponenten umgehen. Um diese Komponenten, z.B. in Fraßexperimenten mit Daphnien und toxischen Cyanobakterien, zu detektieren und zu quantifizieren wurde hochauflösende Massenspektrometrie verwendet. Diese Methodik in Kombination mit weiteren Techniken, wie beispielsweise Enzymaktivitätsmessungen oder molekularbiologischen Methoden ergab eine Reihe neuer Erkenntnisse in die Physiologie der Interaktion von Daphnien mit Cyanobakterien.

Ein Hauptgrund für die unterschiedlichen Sensitivitäten der Daphnien könnte darin bestehen, dass verschiedene Klonlinien unterschiedlich stark ausgeprägte Fähigkeiten besitzen mit solchen cyanobakteriellen Komponenten umzugehen.

Bezüglich eines der häufigsten und am besten untersuchten Cyanotoxine, Microcystin-LR, geht eine weithin akzeptierte Theorie davon aus, dass das Enzym Glutathion-S-transferase von entscheidender Bedeutung für die Detoxifizierung ist, indem es Microcystin in einem erstem Schritt an das Tripeptid Glutathion konjugiert.

Diese Theorie konnte von mir klar widerlegt werden. In einem Fütterungsexperiment mit *Daphnia magna* und dem Microcystin enthaltenden Cyanobakterium *Microcystis aeruginosa* wurden alle experimentellen Komponenten (Umgebungsmedium, Cyanobakterien und Daphniengewebe) bezüglich ihres Microcystingehalts untersucht. Da sich der Gesamtgehalt an Microcystin innerhalb des Experiments nicht verringert, unabhängig davon ob Daphnien präsent sind oder nicht, kann ausgeschlossen werden, dass es zu signifikanten Biotransformationprozessen von Microcystin gekommen ist. Es wurde passend dazu auch kein Anstieg des entsprechenden Konjugates von Microcystin und Glutathion gemessen. Stattdessen zeigten die Daten, dass Daphnien in der Lage sind, nichtkonjugierte Microcystine mit hoher Effizienz aus ihrem Gewebe zu transportieren. Solche Transportprozesse wurden für Daphnien in Bezug auf Cyanotoxine, die über die Nahrung aufgenommen werden, bisher nicht in Betracht gezogen. Darüber hinaus konnte ich zeigen, dass es sich bei diesem Befund nicht um einen Spezialfall für Microcystin handelt, sondern dass auch andere strukturell sehr unterschiedliche cyanobakterielle Metabolite in vergleichbarer Weise aus Daphniengewebe exportiert werden. Genau wie bei Microcystin kam es auch bei anderen Metaboliten nicht zu Biotransformationsprozessen irgendeiner Art.

Zusätzlich konnte ich zwei Klassen von Transportern identifizieren die für den Export von Microcystin aus dem Daphniengewebe verantwortlich sind. Dabei handelt sich um „multi

xenobiotic resistance“ (MXR) Proteine sowie „organic anion transporting polypeptides“ (Oatps). Die Anwendung spezifischer Inhibitoren für beide Transportertypen resultierte in erhöhten Microcystin Messwerten innerhalb der Daphniengewebe. Obwohl eine qPCR Analyse erbrachte, dass eine 24 stündiger Fraßperiode von microcystinhaltigen Cyanobakterien nicht ausreichen ist, um eine Hochregulierung entsprechender Transportergene in Daphnien zu bewirken, zeigen die Ergebnisse dennoch eindeutig, dass Transportprozesse von entscheidender Bedeutung für die Interaktion von Daphnien mit Cyanobakterien sind.

Im letzten Teil meiner Dissertation habe ich unter Benutzung von hochauflösender Massenspektrometrie eine neue Methode entwickelt, um saisonale Sukzessionsmuster cyanobakterieller Chemotypen aufzudecken. Chemotypen bezeichnen die Summe der Sekundärmetabolite die von einem cyanobakteriellen Genotypen produziert werden. Hierfür wurden zunächst metabolomische Fingerabdrücke einer zeitlichen Serie von Phytoplanktonproben erstellt. Diese wurden anschließend unter Benutzung von multivariater Statistik mit dem Potential dieser Proben, Proteasen von Daphnien zu inhibieren, kombiniert. Dabei stellte sich heraus, dass die Chemotypen Zusammensetzung innerhalb des Phytoplanktons sehr starken und schnellen Schwankungen unterworfen ist. Dieser Befund wiederum wirkt sich massiv auf Daphnien Populationen aus und unterstreicht die Wichtigkeit von schnellen Anpassungsmechanismen.

Zusammengefasst erbrachten die Ergebnisse dieser Dissertation mehrere neue Einsichten in die Physiologie der Interaktion von Daphnien und Cyanobakterien, welche die Wichtigkeit, die Notwendigkeit und die Eigenschaften von Transportprozessen für über die Nahrung aufgenommene Cyanotoxine in Daphnien aufzeigen.

Record of achievement

Chapter 1: Dietary exposure of *Daphnia* to microcystins: No *in vivo* relevance of biotransformation

Results in this chapter were exclusively performed by me or under my direct supervision.

Chapter 2: Physiological interaction of *Daphnia* and *Microcystis* with regard to cyanobacterial secondary metabolites

Results in this chapter were exclusively performed by me or under my direct supervision.

Chapter 3: Dietary microcystins in *Daphnia magna* – The role of multi xenobiotic resistance (MXR) proteins and organic anion transporting polypeptides (Oatps)

Results in this chapter were exclusively performed by me or under my direct supervision.

Chapter 4: Seasonal dynamics of chemotypes in a freshwater phytoplankton community – A metabolomic approach

Results in this chapter were exclusively performed by me or under my direct supervision. C. Kuster helped with the literature survey in order to compile the list of protease inhibitors.

Köln, den 11.08.2014

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen und Abbildungen -, die anderen Werken in Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Eric von Elert betreut worden.

Thomas Sadler

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List of peer-reviewed publications

[1] **Sadler, T.** and von Elert, E. (2014) Dietary exposure of *Daphnia* to microcystins: No *in vivo* relevance of biotransformation. Aquatic Toxicology 150: 73-82. (doi: 10.1016/j.aquatox.2014.02.017)

[2] **Sadler, T.** and von Elert, E. (2014) Physiological interaction of *Daphnia* and *Microcystis* with regard to cyanobacterial secondary metabolites. Aquatic Toxicology, *in press*.

[3] **Sadler, T.** and von Elert, E. (2014) Dietary microcystins in *Daphnia magna* – The role of multi xenobiotic resistance (MXR) proteins and organic anion transporting polypeptides (Oatps). Aquatic Toxicology, *under review*.

[4] **Sadler, T.**, Kuster, C. and von Elert, E. (2014) Seasonal dynamics of chemotypes in a freshwater phytoplankton community – A metabolomic approach. Harmful algae 39: 102 – 111. (doi: 10.1016/j.hal.2014.07.006)

Schwarzenberger A., **Sadler, T**. and von Elert, E. (2013) Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of *Daphnia magna*. Journal of Experimental Biology 216: 3649 – 3655. (doi: 10.1242/jeb.088849)

[1] corresponds to chapter 1

[2] corresponds to chapter 2

[3] corresponds to chapter 3

[4] corresponds to chapter 4