The Journal of Experimental Biology 216, 3649-3655

doi:10.1242/jeb.088849

RESEARCH ARTICLE

Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of *Daphnia magna*

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SUMMARY

Herbivore–plant interactions have been well studied in both terrestrial and aquatic ecosystems as they are crucial for the trophic transfer of energy and matter. In nutrient-rich freshwater ecosystems, the interaction between primary producers and herbivores is to a large extent represented by *Daphnia* and cyanobacteria. The occurrence of cyanobacterial blooms in lakes and ponds has, at least partly, been attributed to cyanotoxins, which negatively affect the major grazer of planktonic cyanobacteria, i.e. *Daphnia*. Among these cyanotoxins are the widespread protease inhibitors. These inhibitors have been shown (both *in vitro* and *in situ*) to inhibit the most important group of digestive proteases in the gut of *Daphnia*, i.e. trypsins and chymotrypsins, and to reduce *Daphnia* growth. In this study we grew cultures of the cyanobacterium *Microcystis* sp. strain BM25 on nutrient-replete, N-depleted or P-depleted medium. We identified three different micropeptins to be the cause for the inhibitory activity of BM25 against chymotrypsins. The micropeptin content depended on nutrient availability: whereas N limitation led to a lower concentration of micropeptins per biomass, P limitation resulted in a higher production of these chymotrypsin inhibitors. The altered micropeptin content of BM25 was accompanied by changed effects on the fitness of *Daphnia magna*: a higher content of micropeptins led to lower IC_{50} values for *D. magna* gut proteases and *vice versa*. Following expectations, the lower micropeptin content in the N-depleted BM25 caused higher somatic growth of *D. magna*. Therefore, protease inhibitors can be regarded as a nutrient-dependent defence against grazers. Interestingly, although the P limitation of the cyanobacterium led to a higher micropeptin content, high growth of *D. magna* was observed when they were fed with P-depleted BM25. This might be due to reduced digestibility of P-depleted cells with putatively thick mucilaginous sheaths. These findings indicate that both the grazer and the cyanobacterium benefit from P reduction in terms of digestibility and growth inhibition, which is an interesting starting point for further studies.

Key words: *Microcystis* sp., cyanotoxins, *Daphnia*, protease inhibitors, nutrient limitation, grazer defence.

Received 27 March 2013; Accepted 10 June 2013

INTRODUCTION

Herbivore–plant interactions play an important role in the trophic transfer of carbon and energy from primary producers to the consumer level in both terrestrial (e.g. Mello and Silva-Filho, 2002) and aquatic ecosystems (e.g. Liess and Hillebrand, 2004). In this transfer, food quantity (e.g. Lampert, 1977) and quality (e.g. Dale, 1988; Ferrão-Filho et al., 2007) are determining factors for animal growth.

In limnetic systems, the transfer of carbon and energy to the consumer level is to a large extent due to the interaction between the herbivore *Daphnia* and single-celled phytoplankton and cyanobacteria in the pelagic environment. For *Daphnia*, cyanobacteria are of especially low food quality. This could be caused by several factors, among them: (1) cyanobacteria are deficient in fatty acids and sterols, which are essential for *Daphnia* (Martin-Creuzburg et al., 2005; Von Elert et al., 2003), and (2) cyanobacteria often contain a wide array of toxic secondary metabolites (Gademann and Portmann, 2008). Another food quality constraint is the stoichiometric mismatch between consumer and nitrogen (N)- or phosphorus (P)-depleted autotrophs. These stoichiometric constraints on herbivore growth appear to be qualitatively similar and widespread in both aquatic and terrestrial environments (Elser et al., 2000).

In eutrophic lakes, phytoplankton show a pronounced seasonality of succession (Sommer et al., 1986). In spring, the stratification of lakes is re-established because of warmer weather, and the higher availability of mineral resources and light lead to an increase in fast-growing phytoplankton. However, in early summer, easily ingestible phytoplankton biomass decreases while grazing-resistant phytoplankton taxa, among them cyanobacteria, increase in relative abundance. During the last few decades, cyanobacterial mass developments, so-called ‘blooms’, have become widespread. This increase in bloom frequency is due to anthropogenic phosphorus input, which frequently results in N limitation of phytoplankton (Paerl, 1988; Paerl et al., 2001; Trimbee and Prepas, 1987; Downing et al., 2001).

Phytoplankton that grows in the absence of nutrient limitation is known to have a stoichiometric molar ratio of carbon:nitrogen:phosphorus (C:N:P) of approximately 106:16:1, which is known as the Redfield ratio (Goldman et al., 1979). However, the C:N:P ratio of phytoplankton sharply deviates from the Redfield ratio when nutrients are limiting. Nutrient limitations of phytoplankton have been shown not only to reduce primary productivity and growth, but also to affect biochemical composition of the phytoplankton (Harrison et al., 1990). For example, P limitation interacts with fatty acid composition (Ahlgren et al., 1997;

Spijkerman and Wacker, 2011), sterol content (Piepho et al., 2010) and, in the case of cyanobacteria, a decrease in phycobiliprotein content and carotenoid composition has been observed (Biswal et al., 1994). In limnetic ecosystems, phytoplankton growth is not only limited by phosphorous but also possibly co-limited by other macronutrients, e.g. nitrogen (Sterner, 2008), which has been shown to be a limiting factor for growth of *Microcystis aeruginosa* (Gerloff and Skoog, 1957). Only recently has the effect of N limitation on gene expression of a cyanobacterium been described; genes that are involved in nitrogen uptake and assimilation were upregulated (Aguirre von Wobeser et al., 2011).

Deviations from the Redfield ratio in phytoplankton have been shown to lead to reduced food quality for herbivorous zooplankton (Sterner et al., 1993). This reduction in food quality may be due to the following factors: (1) direct effects because of stoichiometric mismatches between diet and requirement of the herbivore, and (2) indirect effects that result from secondary changes in the phytoplankton under nutrient limitation. The latter comprise morphological defences in phytoplankton, e.g. thicker cell walls that resist digestion (DeMott and Van Donk, 2013; Van Donk and Hessen, 1993; Van Donk et al., 1997) or altered cellular content of secondary metabolites that are deleterious for herbivorous zooplankton. In the case of the diatom *Pseudo-nitzschia multiseries*, it has been shown that the toxin domoic acid, which is deleterious to marine copepods (Shaw et al., 1997), was produced at three times the normal concentration under P limitation (Pan et al., 1996). In the case of cyanobacteria, Rohrlack and Utkilen (Rohrlack and Utkilen, 2007) showed that the amounts of cell-bound anabaenopeptins and microviridin I [the latter being a toxin that leads to a lethal moulting disruption in *Daphnia* (Rohrlack et al., 2004)] per cyanobacterial biovolume were associated with availability of nitrogen and phosphorus. Similarly, Kurmayer (Kurmayer, 2011) found that P limitation led to higher microcystin content; microcystin is a peptide that negatively affects growth and reproduction in *Daphnia* (Rohrlack et al., 1999; Lürling, 2003).

Protease inhibitors are another group of cyanobacterial secondary metabolites that interfere with growth and reproduction of herbivorous zooplankton. These protease inhibitors have been found in many cyanobacterial blooms (Jakobi et al., 1996; Jakobi et al., 1995; Agrawal et al., 2001; Czarnecki et al., 2006; Kuster et al., 2012) and have been shown to inhibit digestive serine proteases of *D. magna* both *in vitro* (Agrawal et al., 2005) and *in situ* (Schwarzenberger et al., 2010). These serine proteases represent the most important digestive enzymes in the gut of *D. magna* (Von Elert et al., 2004). When ingested with food particles, protease inhibitors negatively affect *Daphnia* by decreasing protease activity and reducing somatic growth (Lürling, 2003; Rohrlack et al., 1999; Von Elert et al., 2012; Schwarzenberger et al., 2012). The inhibition of digestive enzymes by these protease inhibitors should result in lower availability of amino acids. A reduction in *D. magna* growth was also observed when protease inhibitors encapsulated in liposomes were added to high-quality food (Von Elert et al., 2012), indicating that the inhibitors (and not some other cyanobacterial factor) were responsible for the lower growth. Cyanobacterial protease inhibitors are produced constitutively. However, it is not known whether the intracellular concentration of cyanobacterial protease inhibitors is affected by nutrient availability.

Here we use a strain of *M. aeruginosa* (BM25) that produces chymotrypsin inhibitors to investigate whether nutrient limitation affects the content of these protease inhibitors. For this purpose we grew this cyanobacterial strain on nutrient-rich, P-depleted or N-depleted medium. We determined the chymotrypsin inhibitors of this *M. aeruginosa* strain and quantified their concentration *via* LC-MS in the three different nutrient regimes. In order to test for the adaptive value of changes in the content of protease inhibitor in the cyanobacterium, we measured the somatic growth of one clone of *D. magna* grown on the cyanobacterium cultured under the different nutrient regimes. By adding an inhibitor-free green alga as food source, we ensured that *D. magna* themselves were not nutrient-limited in the growth experiment. In a subsequent *in vitro* study we further determined the inhibitory effect of methanolic extracts of these cyanobacterial cultures on digestive proteases of *D. magna*.

MATERIALS AND METHODS

The green alga *Chlamydomonas klinobasis* Skuja 1956 (strain 56, culture collection of the Limnological Institute, University of Konstanz, Konstanz, Germany) was cultivated semi-continuously in cyanophycean medium (Von Elert and Jüttner, 1997) at 20°C at 130 $\mu E\,m^{-2}\,s^{-1}$, with 20% of the medium exchanged daily. The cyanobacterium *Microcystis* sp. strain BM25 originates from Lake Bysjön in Southern Skania, Sweden (kindly provided by Ineke van Gremberghe, Ghent University, Ghent, Belgium). BM25 has been shown to inhibit *D. magna* chymotrypsins (but not trypsins) *in vitro* (Schwarzenberger et al., 2013). The cyanobacterium was cultivated semi-continuously at 20°C and constant light (50 $\mu E\,m^{-2}\,s^{-1}$) in three different 100 ml batch cultures on cyanophycean medium (Von Elert and Jüttner, 1997). The P-depleted medium was modified by decreasing the $K_2HPO_4 \times 3H_2O$ content to 6.25% of the original medium, while adding 74.55 $g\,mol^{-1}$ KCl to keep the osmolarity of the medium constant. The N-depleted medium contained only 5% of the $NaNO_3$ concentration of the nutrient-rich medium. One quarter of the medium was exchanged weekly, which should have been sufficient to keep the slow-growing cyanobacteria in an overall similar growth phase. Carbon concentrations of the autotrophic food suspensions were estimated from previously determined photometric light extinction (470 nm) and carbon extinction equations. One hundred millilitres of each BM25 culture were frozen at −80°C and freeze-dried (Christ LOC-1m freeze dryer, ALPHA 1-4, Merrington, Shrewsbury, Shropshire, UK). The freeze-dried seston was pestled, and 50 mg of the powder was dissolved in 1 ml of 100% methanol, sonicated for 10 min and centrifuged at 14,000 ***g*** for 3 min. The supernatant was used to inhibit the chymotrypsin activity of the *Daphnia* homogenate (see below).

Aliquots of the *C. klinobasis* culture, the three different BM25 cultures and the food mixtures were filtered onto pre-combusted glass fibre filters (Whatman GF/F). Immediately afterwards, the filters were dried and then analyzed with the Flash 2000 organic elemental analyzer (ThermoFisher, Schwerte, Germany) for particulate organic carbon and particulate organic nitrogen. To determine carbon concentrations, we also used 20 mg dry weight from each of the lyophilized cultures of BM25. For determination of particulate phosphorus, aliquots of the three cyanobacterial and the *C. klinobasis* cultures were collected on pre-combusted glass fibre filters and digested with a solution of 10% potassium peroxodisulfate and 1.5% sodium hydroxide in an autoclave for 60 min; soluble reactive phosphorus was determined using the molybdate-ascorbic acid method (Greenberg et al., 1985).

Daphnia magna Straus 1820 clone B originated from Lake Binnensee, Germany (Lampert and Rothhaupt, 1991) and was cultivated for several generations at 20°C in membrane-filtered (0.2 μm), aged tap water with *C. klinobasis* as food alga. From a cohort of newborn *D. magna*, four to five animals each were transferred to 200 ml of aged tap water with a food concentration of 2 $mg\,C\,l^{-1}$. The animals were fed either the green alga *C.*

klinobasis as a control for food without chymotrypsin inhibitors, or 65% *C. klinobasis* and 35% of one of the three different nutrient-depleted BM25 cultures. The high addition of *C. klinobasis* in the food mixtures was chosen in order to ensure that *D. magna* were not nutrient limited in the experiment, and that all effects on growth rate only resulted from differences in micropeptin content. The medium and the food were exchanged daily. The experiment was performed under low light conditions at 20°C and lasted for 6 days. All food treatments were run in triplicate, and somatic growth rates of *D. magna* were determined from dry weight of animals collected at the start and on day six of the experiment, as according to Von Elert (Von Elert, 2002). As a measure of tolerance to dietary protease inhibitors, the relative growth rate reduction (RGR; %) was calculated using the formula RGR=$(1–g_S/g_M)\times100$, for which g_M is the growth rate of the single replicates of *D. magna* grown on 35% BM25, and g_S is the arithmetic mean of the growth rates of *D. magna* grown on 100% *C. klinobasis*.

For the enzyme activity studies, 15 third clutch neonates from one mother were grown in 1 litre aged tap water for 6 days with a food concentration of 2 mg C l^{-1} of *C. klinobasis*. These animals were homogenized and centrifuged for 3 min at 14,000 ***g***. The protein concentration of the supernatant (i.e. the *Daphnia* homogenate) was analyzed using a Qubit fluorometer and the Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Chymotrypsin activity of the *Daphnia* homogenate was measured photometrically using the artificial substrate *N*-succinyl-alanine-alanine-proline-phenylalanine-*para*-nitroanilide (Sigma-Aldrich, St Louis, MO, USA) according to Von Elert et al. (Von Elert et al., 2004). Ten microlitres of *Daphnia* homogenate were mixed with 990 µl 0.1 mol l^{-1} potassium phosphate buffer, pH 7.5. The buffer contained 125 µmol l^{-1} S(Ala)$_2$ProPhepNA and 1% DMSO. The change in absorption was measured at a wavelength of 390 nm at 30°C continuously over 10 min. Five or six different concentrations of each of the methanolic extracts from *M. aeruginosa* BM25 grown under the three different conditions (i.e. nutrient rich, P depleted or N depleted) were tested for inhibition of *D. magna* chymotrypsin activity. The concentration at which 50% of protease activity was inhibited (IC_{50}) was calculated fitting a sigmoidal dose–response curve using the software GraphPad Prism, version 4.0 (GraphPad Software, La Jolla, CA, USA). The resulting IC_{50} values were related to the carbon concentration of each lyophilized powder.

Determination of inhibitors and inhibitor concentrations of BM25

Eighty micrograms of lyophilized powder of the three *M. aeruginosa* BM25 cultures were suspended in 2 ml 80% methanol, sonicated for 5 min and centrifuged for 10 min at 16,000 ***g***. One millilitre of the supernatant, i.e. the cyanobacterial extract, was mixed with 20 µl of a solution of the internal standard Met-Arg-Phe-Ala acetate (166.7 pmol MRFA μl^{-1} 50% methanol), dried in a speed-vac and re-dissolved in 200 µl methanol. From this solution, 5 µl (which corresponded to 1 mg lyophilized powder) was analyzed on a UPLC (Accelera, ThermoFisher) coupled with a mass spectrometer (Exactive, ThermoFisher). Chromatography was performed on a Nucleosil C18 column (2×125 mm length, pore size 100 Å, particle size 3 µm; Macherey and Nagel, Düren, Germany) at 30°C with the following gradient: 0 to 15 min, 10% acetonitrile in water with 0.05% TFA to 100% acetonitrile, three additional minutes at 100% acetonitrile and 1.5 min at 10% acetonitrile in water. The flow rate was 300 µl min^{-1}. The mass spectrometry parameters were as follows: positive ionization took place at 325°C with a capillary voltage of 60 V with a constant N_2 flow (sheath gas 45, aux gas 15). The scan range was 150 to 1500 *m*/*z*. The peak area (PA) of the internal standard MRFA was related to the PA of each of the inhibitors and normalized against the carbon concentration of each lyophilized powder.

Statistics

Statistical analyses were conducted with the program Statistica 6.0 (StatSoft, Hamburg, Germany). The data were analyzed using one-way ANOVA (growth rate reductions, C:N and C:P ratios) and a *post hoc* analysis [Tukey's honestly significant difference (HSD)]. A Levene's test was conducted to confirm homogeneity of variance. When needed, the data were transformed *via* x^3 or $\sqrt{x}$. The level of significance was $P<0.05$. IC_{50} values with non-overlapping 95% confidence intervals were regarded as being significantly different.

RESULTS

Elemental ratios

The nutrient-rich cyanophycean medium led to a C:N:P ratio of 91:24:1 in BM25, which was close to the Redfield ratio of 106:16:1. When grown on the P-limited medium, the cyanobacterium showed a significantly higher C:P ratio than the nutrient-rich-grown culture (Tukey's HSD after one-way ANOVA, $F_{3,4}=508.31$, $P<0.01$; Table 1). This high C:P ratio was well above the Redfield ratio. This deviation from Redfield also resulted in a significantly higher C:P ratio of the food mixture with 35% P-depleted BM25 than found for the other food mixtures (Tukey's HSD after one-way ANOVA, $F_{3,4}=65.2$, $P<0.05$). However, because of the addition of 65% *C. klinobasis*, all food mixtures resulted in C:P values between 42 and 74. These are very low ratios in comparison to the mean C:P ratio of 300 for lakes demonstrated by Elser et al. (Elser et al., 2000). Gächter and Bloesch (Gächter and Bloesch, 1985) reported ratios below 100 for some mesotrophic and eutrophic lakes. Plath and Boersma (Plath and Boersma, 2001) demonstrated that low C:P ratios decreased the feeding activity of *Daphnia*. However, because all the food mixtures used in our experiments had similar C:P ratios, we were able to exclude C:P-induced effects on growth rate. All effects on growth should therefore have resulted from differences in micropeptin content.

When grown on the N-limited medium, the pure cyanobacterial culture showed a significantly higher C:N ratio than all other cultures of BM25 (Tukey's HSD after one-way ANOVA, $F_{3,4}=26.13$, $P=0.004$; Table 1), and with an N:P ratio of 2.02 it was far below the Redfield ratio. However, all food mixtures with or without BM25 had C:N ratios between 6.85 and 8.48, and did not differ from each

Table 1. Molar ratios of C:P and C:N of *Chlamydomonas klinobasis* in the three nutrient-rich (BM25), P-limited (BM25-P) or N-limited (BM25-N) cultures of *Microcystis* sp. BM25, and the four different food mixtures (100% *C. klinobasis* or 65% *C. klinobasis* and 35% of any of the three different BM25 cultures)

	C:P	C:N
BM25	90.98±0.44	3.79±0.34
BM25-P	233.84±11.91	4.59±0.08
BM25-N	25.87±0.57	13.03±2.35
100% *C. klinobasis*	51.30±0.00	7.62±1.06
35% BM25	57.28±4.02	7.23±1.39
35% BM25-P	73.49±2.26	6.85±0.42
35% BM25-N	41.82±0.66	8.48±0.19

Data are means ± s.d.

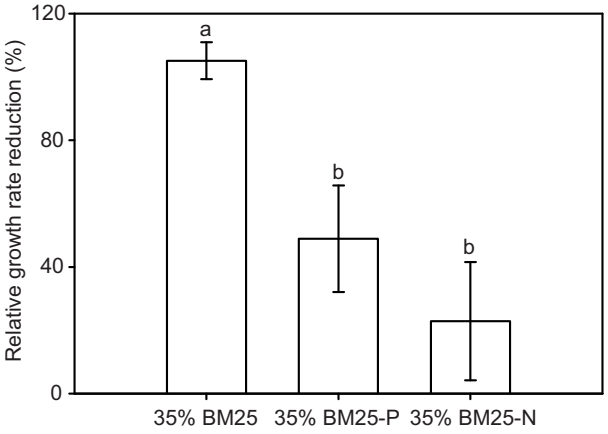

Fig. 1. Relative growth rate reductions (means ± s.d., *N*=3) of *Daphnia magna* grown on a mixture of *C. klinobasis* with 35% *Microcystis* sp. BM25 in relation to growth on 100% *Chlamydomonas klinobasis*. *Microcystis* sp. BM25 was cultured under nutrient-rich (BM25), P-depleted (BM25-P) or N-depleted (BM25-N) conditions. Different letters indicate a significant difference (Tukey's HSD after one-way ANOVA, $P<0.05$) between food treatments.

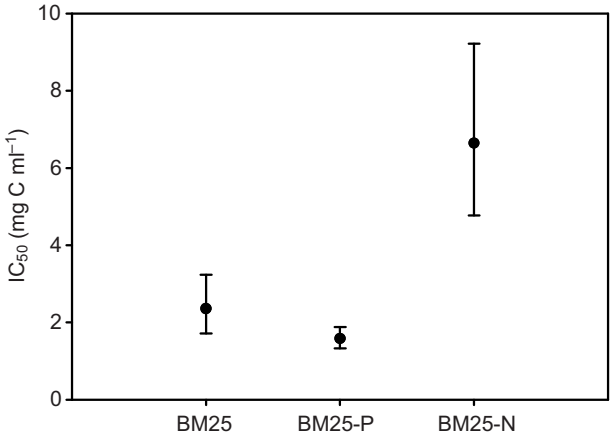

Fig. 2. IC_{50} values (mg C ml^{-1}; means ± 95% confidence intervals) obtained with the *Daphnia magna* homogenate inhibited with the three different methanolic extracts from the *Microcystis* sp. BM25 cultures, which were cultured under nutrient-rich (BM25), P-depleted (BM25-P) or N-depleted (BM25-N) conditions.

other significantly (Tukey's HSD after one-way ANOVA, $F_{3,4}$=1.19, P=0.42).

Relative growth rate reductions

Daphnia magna grown on 100% *C. klinobasis* had a high growth rate of 0.42±0.009 day^{-1}. When grown on 35% of the nutrient-rich culture of BM25, *D. magna* showed zero growth (−0.021±0.025 day^{-1}). The relative growth rate reduction of *D. magna* grown on 35% of the nutrient-rich culture of BM25 was therefore higher than that of *D. magna* grown on 35% of the nutrient-depleted cyanobacteria (Tukey's HSD after one-way ANOVA, $F_{2,3}$=48.17, $P<0.05$; Fig. 1). The weaker effects of P- and N-limited *M. aeruginosa* BM25 on *D. magna* growth did not differ significantly (Fig. 1).

IC_{50} and protease inhibitor (PI) content of the BM25 cultures

When methanolic extracts of BM25 were assayed for their inhibitory effects on *D. magna* chymotrypsins, the IC_{50} value obtained for the extract of the N-depleted culture was significantly higher than the value obtained for the nutrient-rich and the P-depleted cultures. This indicates a reduced inhibitory content of the N-depleted culture (Fig. 2). Using high-resolution mass spectrometry, no microcystins and no known trypsin inhibitors were found in the raw extract of BM25. However, molecular masses could be assigned to three known chymotrypsin inhibitors: the micropeptins DR1006, DR1056 (Adiv et al., 2010) and MM978 (Zafrir-Ilan and Carmeli, 2010). The concentration of each of these inhibitors differed significantly between cultures (one-way ANOVA, DR1006: $F_{2,6}$=604.92, $P<0.01$; DR1055: $F_{2,6}$=210.45, $P<0.01$; MM978: $F_{2,6}$=834.8, $P<0.01$; Fig. 3). For each of the three micropeptins, the highest content was observed in the P-depleted culture. The contents were lowest in the N-depleted culture except for DR1056, which was intermediate. In comparison with the nutrient-rich BM25, the concentrations of DR1006 and MM978 were significantly lower in the N-depleted culture and higher in the P-depleted culture (DR1006: Tukey's HSD after one-way ANOVA, $F_{2,6}$=604.92, $P<0.01$; MM978: Tukey's HSD after one-way ANOVA, $F_{2,6}$=834.8, $P<0.01$). For DR1056, the content was higher in the N-depleted culture and even higher in the P-limited culture. The fact that all three chymotrypsin inhibitors belong to the class of micropeptins makes it reasonable to assume that their recovery rates should be very similar. Based on this reasoning we calculated the total PA for all three inhibitors as a proxy for the total content of protease inhibitors in each of the three cyanobacterial cultures. The overall concentration of chymotrypsin inhibitors was highest in the P-depleted culture (5.79±0.22 rel. PA mg^{-1} C), lowest in the N-depleted BM25 (1.97±0.08 rel. PA mg^{-1} C) and intermediate in the nutrient-rich culture (3.37±0.02 rel. PA mg^{-1} C; Tukey's HSD after one-way ANOVA, $F_{2,6}$=789.84, $P<0.01$).

DISCUSSION

Over the last few decades, cyanobacterial blooms in limnetic systems have become a common phenomenon and are very frequently associated with a biomass decline of the major herbivore, *Daphnia* (de Bernardi and Giussani, 1990; Gilbert, 1990). A reason for this might be the low food quality of cyanobacteria for *Daphnia*, for which different causes have been identified: filamentous cyanobacteria interfere with the filtering apparatus of *Daphnia* (DeMott et al., 2001; Gliwicz and Lampert, 1990), cyanobacteria are lacking in many essential lipids, i.e. polyunsaturated fatty acids (PUFAs) and sterols (Von Elert, 2002; Von Elert et al., 2003; Martin-Creuzburg et al., 2008), and cyanobacteria often contain deleterious secondary metabolites (Gademann and Portmann, 2008). The *M. aeruginosa* strain used here was single-celled and small enough to be readily ingested. A reduction in growth because of the lack of PUFAs or sterols could be excluded because 65% of dietary carbon was of eukaryotic origin, i.e. *C. klinobasis* (Martin-Creuzburg and Von Elert, 2009). While the C:N:P ratio of phytoplankton can fluctuate with changing availability of nutrients, *Daphnia* maintain a much less variable elemental ratio (Andersen and Hessen, 1991; Urabe and Watanabe, 1992).

Here, because of the high N and P content of *C. klinobasis* (which comprised 65% of their diet), *D. magna* were not P or N limited in our experiments, although the cyanobacterial cultures grown under nutrient-depleted conditions were actually nutrient depleted (Table 1). *Daphnia* are not N limited when the dietary C:N ratio is below 15 (Urabe and Watanabe, 1992), which was the case here

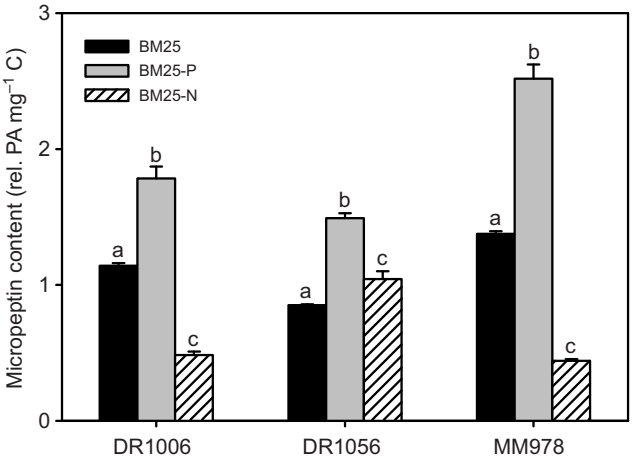

Fig. 3. Relative content of the chymotrypsin inhibitor micropeptins DR1006, DR1056 and MM978 in the three different methanolic extracts from the *Microcystis* sp. BM25 cultures, which were cultured under nutrient-rich (BM25), P-depleted (BM25-P) or N-depleted (BM25-N) conditions. The peak areas (PA) of the micropeptins were related to the PA of the internal standard and were normalized to the extracted biomass measured as particulate organic carbon. Different letters indicate a significant difference (Tukey's HSD after one-way ANOVA, $P<0.05$) between the cyanobacterial cultures.

for all food mixtures. *Daphnia magna* were also not P limited in any of the food mixtures, all of which showed a C:P ratio of 42 to 73 (Table 1), as only a dietary C:P >300 is generally assumed to lead to P-limited growth of *Daphnia* (Urabe and Watanabe, 1992). We thus concluded that the difference in *D. magna* growth must have been due to other causes, e.g. the content of cyanotoxins.

The most extensively studied group of toxins from cyanobacteria are microcystins, none of which were detected in *Microcystis* sp. BM25. However, this cyanobacterium has been shown to inhibit chymotrypsins (Schwarzenberger et al., 2013), and actually three different chymotrypsin inhibitors were identified in our study: micropeptin DR1056, micropeptin DR1006 and micropeptin MM978. Such protease inhibitors have been found in many blooms worldwide (Jakobi et al., 1996; Jakobi et al., 1995; Agrawal et al., 2001; Czarnecki et al., 2006), suggesting that they are among the most frequent cyanobacterial secondary metabolites. Only recently has the seasonal succession of chymotrypsin inhibition been studied (Kuster et al., 2012).

Here, each of the inhibitors of BM25 proved to differ in content between the nutrient-rich and nutrient-depleted cultures. The N-depleted BM25 had a lower content of micropeptins than the nutrient-rich culture, which corresponded to a higher IC_{50} value of the methanolic extracts for the inhibition of the *D. magna* gut proteases. In line with this, N-depleted BM25 caused a smaller reduction of growth of *D. magna* than nutrient-rich BM25, which suggests that inhibitory effects on growth are caused by the protease inhibitors produced by this cyanobacterium. However, this conclusion did not hold for the P-depleted culture of BM25. Similar to the increase of other toxins under P limitation (Watanabe and Oishi, 1985; Pan et al., 1996; Boyer et al., 1987), we also found a higher micropeptin content in the P-depleted BM25, which could have translated into the slightly although not significantly lower IC_{50} value than found for the nutrient-rich culture (Fig. 2).

However, although the P-limited culture of BM25 had a higher micropeptin content and a higher inhibitory potential against *D. magna* chymotrypsins, than the nutrient-rich culture, growth of *D. magna* was less affected, a finding contrary to our expectations. This finding, that the content of chymotrypsin inhibitors should have resulted in a stronger reduction of *D. magna* growth than was experimentally observed, strongly suggests that the detrimental effects of the micropeptins have been alleviated by a factor associated with P-depleted growth of the cyanobacterium. *Microcystis*, a member of the Chroococcaceae, is characterized as having a mucilaginous sheath. Such sheaths have been suggested to reduce vulnerability to grazing (Lampert, 1982; Porter, 1973), and for another member of Chroococcaceae, *Chroococcus*, it has been demonstrated that cyanobacteria with mucilaginous sheaths are able to survive *Daphnia* gut passage (Kerfoot et al., 1988). Van Donk and Hessen (Van Donk and Hessen, 1993) showed that green algae produced thicker cell walls under P limitation and were thus more resistant to digestion when grown in P-depleted conditions, whereas a similar effect due to N limitation is unknown. Such an increased resistance to digestion under P depletion is also probable for Chroococcaceae with mucilaginous sheaths. Therefore, it seems likely that under P depletion the grazing resistance of BM25 increased because of enhanced mucilage production, and that the resultant reduced digestibility led to smaller effects by the protease inhibitors than expected. Thus, the growth rate reduction was a combined effect of the non-digestible and therefore non-inhibiting cyanobacterium and the lower concentration of high-quality food than in the other food treatments. Nevertheless, the possibility that both the cyanobacterium and the grazer might benefit from reduced digestibility of the P-depleted cells should be given further study.

To our knowledge, this is the first study that investigates the effects of nutrient depletion on the production of cyanobacterial chymotrypsin inhibitors and their effects on herbivorous zooplankton. We previously identified the micropeptins underlying the inhibition of chymotrypsins by BM25 (Schwarzenberger et al., 2013) and demonstrated that cyanobacteria produce more (P depletion) or less (N depletion) of these protease inhibitors under nutrient depletion. These findings are in full agreement with the carbon-nutrient balance hypothesis according to which the relative availability of carbon and nutrients affects the production of secondary metabolites (Stamp, 2003): because micropeptins are cyclic peptides that contain nitrogen but no phosphorus (Adiv et al., 2010), N-depleted growth of the cyanobacterium led to reduced synthesis of micropeptins, while P depletion resulted in non-limiting availability of carbon and nitrogen, which then fuelled the increased synthesis of the protease inhibitors.

The changes in micropeptin content of the cyanobacterium were the reason for differences in inhibition of *D. magna* gut proteases *in vitro*: in the case of N depletion, the lower content of protease inhibitors was most probably the cause of a lower growth rate reduction of *D. magna*. Therefore, protease inhibitors seem to be a 'nutrient-dependent defence'. Although protease inhibitors affect grazers, it has been shown that *Daphnia* are not helpless victims of dietary inhibitors: Von Elert et al. (Von Elert et al., 2012) have demonstrated that the gut proteases in one *D. magna* genotype are adjusted to be more resistant to inhibition after feeding on dietary protease inhibitors. It has also been shown that different *D. magna* genotypes differ in their tolerance to dietary protease inhibitors (Schwarzenberger et al., 2012), and even local adaptation of *Daphnia* to cyanobacterial protease inhibitors has been demonstrated *in vitro* (Blom et al., 2006). Therefore, it might be interesting to investigate whether the ability of *Daphnia* to tolerate protease inhibitors is linked to the nutrient status of dietary cyanobacteria.

ACKNOWLEDGEMENTS

The authors thank Alexander Wacker for measurement of carbon and nitrogen. Thanks also to Patrick Fink, Jens Schröder, Ina Jantsch and Katja Preuß for their help in conducting the experiments, and Ineke van Gremberghe from Ghent University, Belgium, for the provision of the cyanobacterial strain. The authors acknowledge the significant contributions by the Biocenter-MS facility, University of Cologne.

AUTHOR CONTRIBUTIONS

A.S. designed and coordinated the study and wrote the manuscript. A.S. conducted the growth experiments, the measurement of IC_{50} values and of the nutrient ratios under the supervision of E.V.E. T.S. determined the chymotrypsin inhibitors in the cyanobacterium and conducted the measurement of inhibitor content *via* LC-MS. All authors contributed to, read and approved the final manuscript.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This study was supported by a grant within the Collaborative Research Centre 680 ‘Molecular Basis of Evolutionary Innovations’.

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