The effects of environmental conditions on growth and protease inhibitor production of the cyanobacterium *Microcystis aeruginosa*

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“It's been a long road, getting from there to here.

It's been a long time, but my time is finally near.

And I can feel a change in the wind right now. Nothing's in my way.

And they're not gonna hold me down no more.

No they're not gonna hold me down.”

Russell Watson (2001) - Faith of the Heart.

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Zusammenfassung

Cyanobakterien sind photoautotrophe Organismen, die sich unter günstigen Bedingungen massenhaft in Seen, Teichen und Flussmündungen vermehren. Aufgrund der starken Nährstoffbelastung vieler Gewässer, sowie steigender Temperaturen und CO_2 Konzentrationen hat die Häufigkeit von cyanobakteriellen Massenvermehrungen („Blüten“) in den letzten Jahrzehnten stark zugenommen, mit weitreichenden ökologischen und ökonomischen Folgen. Daher bilden Cyanobakterien seit Jahrzehnten einen Schwerpunkt der limnologischen Forschung. Dabei liegt der Fokus auf zwei Fragen: Welche Bedingungen kontrollieren die Entstehung von cyanobakteriellen Blüten und welche Rolle spielt die Vielfalt an Sekundärmetaboliten, welche von Cyanobakterien produzieren werden, für den Erfolg von Cyanobakterien?

Einige dieser Metabolite wirken sich nachgewiesenermaßen toxisch auf andere Wasserorganismen aus, können aber auch massive gesundheitliche Probleme bei Menschen hervorrufen. Ein großer Teil der Forschung konzentrierte sich daher auf Microcystine, eine der toxischsten Gruppen von cyanobakteriellen Metaboliten. Andere Sekundärmetabolite, wie z. B. die Gruppe der Proteaseinhibitoren, wurden hingegen eher vernachlässigt. Proteaseinhibitoren, wie beispielsweise Nostopeptin 920 und Cyanopeptolin 954, inhibieren spezifisch Serinproteasen, z. B. im Darm vom Wasserfloh *Daphnia*, was unter anderem zu einem Mangel an Aminosäuren und daraus folgend zu weiteren negativen Konsequenzen führt (z. B. reduziertem Wachstum). Dies hat wiederum weitreichende Folgen für das gesamte Ökosystem, da Daphnien als Hauptkonsumenten von Phytoplankton (u. a. auch Cyanobakterien) und als Nahrung für eine Vielzahl an Räubern essentiell für den Energie- und Nährstofftransfer von der Ebene der Primärproduzenten hin zu den höheren trophischen Ebenen sind. Die Erforschung von Proteaseinhibitoren ist daher notwendig, um das Entstehen und die Auswirkungen von Cyanobakterien-Blüten vollständig zu verstehen. Dabei sind besonders Kenntnisse über den Einfluss verschiedener Umweltfaktoren auf Cyanobakterien, deren Wachstum und die Produktion von Proteaseinhibitoren erforderlich.

Im Rahmen dieser Dissertation sollte daher der Effekt von drei wesentlichen Umweltfaktoren auf das Cyanobakterium *Microcystis aeruginosa*, einem der bedeutendsten blütenbildenden Cyanobakterien, untersucht werden. Die grundlegende Fragestellung war: Wie beeinflussen verschiedene Konzentrationen von Stickstoff und Phosphor, aber auch verschiedene Lichtintensitäten, das Wachstum und vor allem die Produktion und den Gehalt von Proteaseinhibitoren in *M. aeruginosa*? Zur Beantwortung dieser Fragen wurden Wachstumsexperimente (Batch-Kulturen) mit *M. aeruginosa* NIVA Cya 43 durchgeführt, bei denen die Startkonzentrationen von Stickstoff und Phosphor variiert wurden, bzw. bei denen

das Cyanobakterium verschiedenen Lichtintensitäten ausgesetzt wurde. Die Kulturen wurden regelmäßig beprobt, um das Wachstum des Cyanobakteriums, dessen Stöchiometrie und den Gehalt der zwei Proteaseinhibitoren (Nostopeptin 920 und Cyanopeptolin 954) zu bestimmen. Bei der Auswertung der Proben nutzte ich eine Kombination von klassischen (z. B. mikroskopische Zellzählungen) und modernsten analytischen Methoden (z. B. Hochdruck-flüssigkeitschromatographie und hochauflösende Massenspektrometrie). Darüber hinaus nutzte ich mathematische Modelle, darunter ein selbst entwickeltes, dynamisches, prozessbasiertes Modell, um das Wachstum aber auch die Dynamiken der Proteaseinhibitoren zu beschreiben und auszuwerten.

In dieser Dissertation konnte ich zeigen, dass *M. aeruginosa* unter einer Vielzahl von experimentellen Bedingungen wachsen kann und dass besonders erhöhte Nährstoffkonzentrationen und geringe Lichtintensitäten das Wachstum fördern. Außerdem zeigt diese Arbeit, dass die beiden untersuchten Inhibitoren permanent produziert werden, der Gehalt sich aber stark mit der Wachstumsphase und den getesteten Versuchsbedingungen verändern kann. Dementsprechend habe ich auch Zusammenhänge zwischen dem Inhibitorgehalt und der Wachstumsrate bzw. der Stöchiometrie des Cyanobakteriums finden können. Außerdem konnte ich zeigen, dass die Proteaseinhibitoren besonders unter nährstoffreichen Bedingungen in den frühen Wachstumsphasen gebildet werden, aber mit Abnahme der externen Nährstoffe und gleichzeitig steigender Nährstofflimitierung in den späteren Wachstumsphasen auch wieder abgebaut werden. Diese Dynamiken deuten darauf hin, dass die zwei untersuchten Inhibitoren an intrazellulären Prozessen beteiligt sind. Eine mögliche Beteiligung der Proteaseinhibitoren an der Speicherung von Stickstoff scheint dabei aufgrund meiner Ergebnisse wahrscheinlich und sollte zukünftig weiter untersucht werden.

Die Ergebnisse dieser Dissertation unterstützen das bereits vorhandene Wissen, dass *M. aeruginosa* vom anthropogenen Nährstoffeinträgen profitiert, und liefern zudem neue Erkenntnisse über den Einfluss von Umweltbedingungen auf Cyanobakterien, insbesondere mit Hinblick auf Proteaseinhibitoren. Diese Dissertation gibt einen Einblick in die funktionelle Rolle von Proteaseinhibitoren in Cyanobakterien, diskutiert aber auch die potentiellen ökologischen Auswirkungen, die mit Schwankungen des Gehalts von Proteaseinhibitoren einhergehen. Dabei könnten besonders nährstoffreiche Bedingungen zu einem Anstieg der cyanobakteriellen Biomasse und zugleich erhöhten Inhibitorgehalten führen, was eine potentielle Kontrolle von Cyanobakterien durch Daphnien verhindern und das Entstehen von Blüten begünstigen könnte. Daher sollten zum einen die Bemühungen zur Reduktion von Nährstoffen (Stickstoff, Phosphor) in Seen zukünftig weiter erhöht werden und zum anderen mehr Fokus auf die Gesamtheit der von Cyanobakterien produzierten Metabolite gelegt werden, um so die ökologischen Auswirkungen von cyanobakteriellen Blüten in der Zukunft zu minimieren.

Abstract

Cyanobacteria are photoautotrophic organisms that proliferate in lakes, ponds and estuaries under favorable conditions. The nutrient pollution of waterbodies, together with increasing temperatures and CO_2 levels, has led to an increased frequency of cyanobacterial mass developments ('blooms') in the last decades, with severe economic and ecological consequences. Therefore, cyanobacteria became an important topic within limnological research. Special emphasis lies on two central question: Which conditions control the development of cyanobacterial blooms and what is the role of the variety of secondary metabolites produced by cyanobacteria in the success of cyanobacteria?

Some of those metabolites have proven to be toxic for other aquatic organisms, but have also detrimental effects on human health. Much research was focused on microcystins, one of the most toxic groups of cyanobacterial metabolites. Other secondary metabolites, such as protease inhibitors, have received less attention. Protease inhibitors, like nostpeption 920 and cyanopeptolin 954, specifically inhibit serine proteases, for example in the gut of the water flea *Daphnia,* which results in a limitation by amino acids and leads subsequently to other negative consequences (e.g. reduced growth). This in turn has detrimental effects on the whole ecosystem, because *Daphnia* are the main consumers of phytoplankton (including cyanobacteria) and serve additionally as food for a number of predators and are therefore essential for the transfer of nutrients and energy from the level of primary production to higher trophic levels. The investigation of protease inhibitors is therefore necessary to understand the development and consequences of cyanobacterial blooms. In particular, knowledge about the influence of environmental factors on cyanobacteria, their growth and the production of protease inhibitors is required.

This dissertation investigated the effect of three major environmental factors on the cyanobacterium *Microcystis aeruginosa,* one of the most common bloom-forming cyanobacteria. The fundamental question was: How do different concentrations of nitrogen and phosphorus as well as different light intensities affect the growth and more interestingly the production and content of protease inhibitors in *M. aeruginosa*? To answer this question, I performed growth experiments (batch cultures) with *M. aeruginosa* NIVA Cya 43 in which the cyanobacterium was grown at different initial nitrogen and phosphorus concentrations as well as under different light intensities. The cultures were sampled regularly to measure the growth, stoichiometry and the content of two protease inhibitors (nostopeptin 920 and cyanopeptolin 954) of *M. aeruginosa*. The samples were analyzed using a combination of classical (e.g. microscopic cell counting) and modern analytic methods (e.g. high-pressure liquid chromatography and high resolution mass spectrometry). Additionally, I used mathematical

models, including a new developed dynamic process-based model, to describe and analyze the growth and the dynamics of the protease inhibitor content.

In this dissertation, I showed that *M. aeruginosa* can grow under various experimental conditions and that in particular high nutrient concentrations and low light intensities support cyanobacterial growth. This thesis, furthermore, shows that the protease inhibitors are constitutively produced, but that the inhibitor content changes with the growth phase and the tested experimental conditions. Accordingly, I found coherences between the inhibitor content and the growth rate, respectively the stoichiometry, of the cyanobacterium. Additionally, I showed that protease inhibitors are especially produced under nutrient-rich conditions during the early growth phases, while they are degraded in the late growth phase, when the external nutrients are depleted and the nutrient limitation increases. These dynamics indicate that both investigated inhibitors are involved in intracellular processes. A potential involvement of protease inhibitors in the storage of nitrogen is, according to my results, plausible and should be investigated in the future.

The results of this dissertation support the already existing knowledge that *M. aeruginosa* benefits from the anthropogenic nutrient input and adds new insights about the influence of environmental factors on cyanobacteria, in particular on protease inhibitors. This dissertation also provides insights into the functional role of protease inhibitors in cyanobacteria, but also discusses the ecological consequences of changes in the protease inhibitor content. Especially nutrient-rich conditions will most likely result in an increase of the cyanobacterial biomass and a simultaneously increase in the inhibitor content, which will reduce the potential control of cyanobacteria by *Daphnia* and could promote the development of cyanobacterial blooms. Hence, we should increase the efforts to reduce the nutrient input (nitrogen, phosphorus) into lakes and should additionally put more focus on the variety of metabolites produced by cyanobacteria to mitigate the ecological consequences of cyanobacterial blooms in the future.

General Introduction

Cyanobacteria – A success story

Cyanobacteria, also called blue-green algae, are photoautotrophic prokaryotes that have inhabited Earth for at least 2.4 billion years (Demoulin et al. 2019). During this time, they adapted to various habitats from oceans and freshwater lakes to even the most inhospitable habitats, like hot springs or hypersaline ponds (Whitton 2012). The conquest of these habitats was possible because cyanobacteria possess many special traits, such as the ability to regulate their buoyancy (Reynolds et al. 1987), a wide range of photosynthetic pigments, including the special phycobiliproteins (Saini et al. 2018), or the capability of some cyanobacteria to fix atmospheric N_2 (Latysheva et al. 2012; Huisman et al. 2018). Additionally, some cyanobacteria are well protected against grazers, e.g. through the formation of colonies (Xiao et al. 2018) or the production of toxic metabolites (Hansson et al. 2007). Altogether, this led to the long standing success of cyanobacteria and permanent changes for the entire world. Cyanobacteria were involved in the transformation of Earth´s atmosphere from an anaerobic to an aerobic state (Demoulin et al. 2019), are the progenitor of chloroplasts in all eukaryotic algae and higher plants (Cavalier-Smith 2002; Archibald 2009) and are an essential component in the global carbon and nitrogen cycle (Flores and Herrero 2005; Bergman et al. 2013; Fuchsman et al. 2019).

Cyanobacterial blooms – The role of humans and nutrients

Under favorable conditions, cyanobacteria and other algae groups mass-proliferate and form dense algae blooms. Globally the magnitude, frequency and duration of such blooms has increased in the last decades due to anthropogenic nutrient pollution, rising water temperatures and increasing CO_2-levels (Kosten et al. 2012; O'Neil et al. 2012; Huisman et al. 2018; Glibert 2019). A prominent example is Lake Taihu, where the frequency and duration of blooms formed by the cyanobacterium *Microcystis aeruginosa* has increased dramatically in the last decades (Duan et al. 2009). In particular nutrient-rich manure and fertilizers, but also untreated sewage water and atmospheric N deposition, substantially contribute to the eutrophication of lakes, rivers and estuaries (Ebeling et al. 2002; Dumont et al. 2005; Harrison et al. 2005; Erisman et al. 2007; Robertson and Saad 2011). Due to an increasing world population, the usage of fertilizers has dramatically increased over the past century (Erisman et al. 2007; Glibert and Burford 2017) and is assumed to further increase, in particular for fertilizers containing urea (Glibert et al. 2014; Glibert et al. 2018). In some parts of the world, regulations of fertilizer usage and wastewater management will likely improve the situation (Glibert 2019), but the global demand for food, fuel and water is still on the rise, so that the anthropogenic impact on aquatic ecosystems will continue and presumably further contribute

to the expansion of cyanobacterial blooms. As a consequence, ecological problems caused by cyanobacterial blooms, such as the suppression of submerged vegetation through shading or the killing of fish through hypoxia during the decay of blooms (Havens 2008; Huisman et al. 2018), will also continue and create tremendous economic costs as fishery, tourism and other important sectors are affected (Steffensen 2008; Sanseverino et al. 2016).

The productivity of aquatic systems and therefore the proliferation of cyanobacteria is usually limited by the availability of either nitrogen (N) or phosphorus (P). Both are essential to all organisms as they are required for the production of all major biomolecules (Conley et al. 2009; Tetu et al. 2009; Stal 2015) and a limitation by one of the nutrients consequently leads to the inhibition of essential processes, such as photosynthesis (Dong et al. 2019). Traditionally, P is seen as the limiting factor in freshwater systems (Schindler and Fee 1974; Schindler et al. 2008) and N as the limiting factor in marine ecosystems (Nixon 1995; Beman et al. 2005). But such generalizations are not valid for all systems, as studies show that P-rich lakes can be limited by N and that P limitation is common in open ocean areas (e.g. Tetu et al. 2009; Chaffin et al. 2013; Paerl et al. 2016). However, when massive amounts of nutrients are introduced into the system the natural limitation of cyanobacteria vanishes, resulting in their mass proliferation. This is further supported by traits that help cyanobacteria outcompete other algae. Cyanobacteria can for example use most N sources, including nitrate, urea, amino acids, and in some cases atmospheric N_2 (Flores and Herrero 2005), and have a particular high affinity for reduced N forms, which are commonly used in fertilizers (Erisman et al. 2007; Donald et al. 2011). Furthermore, nutrient pollution does not only increase the absolute nutrient concentrations, but shifts the stoichiometry towards lower N/P ratios (Elser et al. 2009; Glibert 2019). This is beneficial for cyanobacteria, as they prefer low N/P ratios and outcompete other algae under those conditions (Elser 1999). Additionally, cyanobacteria can take up more N and P than actually required for growth, a process called luxury consumption (Sterner and Elser 2008), and store those nutrients in the two storage compounds cyanophycin (Berg et al. 2000; Flores et al. 2019) and polyphosphates (Jacobson and Halmann 1982; Solovchenko et al. 2019). This reduces the nutrient availability to other organisms, while allowing cyanobacteria to grow even when external nutrients are depleted. However, a major contributor for the dominance of cyanobacteria is their ability to produce a wide range of secondary metabolites.

Cyanobacterial Metabolites

Cyanobacteria produce several hundred known compounds (Welker and Von Döhren 2006; Gademann and Portmann 2008), for which cellular or ecological function are often unknown or under discussion. Some metabolites are known for their anti-grazer properties (DeMott et al. 1991; Agrawal et al. 2001), while other increase the fitness of cyanobacteria under oxidative stress (Zilliges et al. 2011; Schuurmans et al. 2018) or work allopathic by suppressing the growth of competing phytoplankton species (Leão et al. 2009; Pei et al. 2018). The huge repertory of compounds generates special interest as those compounds could be utilized as potential drugs (Nagarajan et al. 2013). However, many cyanobacterial compounds have been proven toxic to animals (McBarron and May 1966; Backer et al. 2013; Davis et al. 2019) and humans (Jochimsen et al. 1998). With an increasing number of cyanobacterial blooms, the risk of exposure to such toxins increases as well, in particular when contaminated water is used for drinking or recreational purposes (Codd et al. 1999; Sanseverino et al. 2016). Symptoms range from skin irritations to allergic reactions to organ failures (Jochimsen et al. 1998; Codd et al. 1999). Some diseases, like ALS or Alzheimer´s (Dunlop and Guillemin 2019), and certain forms of cancer (Fleming et al. 2002; Martínez Hernández et al. 2009) have also been related to cyanobacterial toxin exposure.

One of the most toxic metabolite groups are microcystins (MCs). MCs are cyclic heptapeptides, that are synthesized by a non-ribosomal enzyme complex (Tillett et al. 2000) and specifically inhibit protein phosphatases type 1 and 2A, leading to acute liver necrosis and consequently death (MacKintosh et al. 1990; Jochimsen et al. 1998). By now, more than 250 MCs congeners are known, of which MC-LR (Fig. 1 A) is the most common variant (Díez-Quijada et al. 2019). Due to their medical implications, their wide distribution among cyanobacterial taxa (Sivonen 2009) and their presence in cyanobacterial blooms worldwide (e.g. Vegman and Carmeli 2013; Bortoli et al. 2014; Beversdorf et al. 2017), MCs became the best studied group of cyanobacterial toxins. However, cyanobacteria produce several other metabolite groups, including protease inhibitors (PIs). Like MCs, PIs are non-ribosomal synthesized (Köcher et al. 2019), widespread among cyanobacteria (Rohrlack et al. 2005; Gademann and Portmann 2008; Janssen 2019) and can be found in significant concentrations in surface waters (Beversdorf et al. 2017; Beversdorf et al. 2018). According to their structure, PIs are divided into several groups, of which cyclic depsipeptides with a 3-amino-6-hydroxy-2-piperidione (Ahp)-moiety are the most common (Namikoshi and Rinehart 1996; Köcher et al. 2019). This class of PIs includes more than 200 known compounds, including cyanopeptolins, most of which specifically inhibit serine proteases. Two cyanopeptolins are cyanopeptolin 954 (CP954, Fig. 1 B) and nostopeptin 920 (BN920). The latter was first isolated and structurally described from the cyanobacterium *Nostoc* sp. (Ploutno and Carmeli 2002), but was later also found in the cyanobacterium *M. aeruginosa* NIVA Cya 43 together with its chlorinated

congener CP954 (Von Elert et al. 2005). Both PIs are structurally identical, except that CP954 contains a chloride whereas BN920 does not (Fig. 1 B). Even though PIs are getting more attention in recent years (Janssen 2019), they were often considered as non-toxic (Neumann et al. 2000) and were therefore not in the focus of cyanobacterial research, so that many aspects concerning PIs are still unresolved.

An important aspect, that has been insufficiently studied, is the involvement of PIs in the interaction between cyanobacteria and zooplankton. Zooplankton, in particular *Daphnia*, is the major consumer of cyanobacterial biomass in most lakes and ponds and is capable of controlling cyanobacterial blooms (Elser 1999; Chislock et al. 2013), although cyanobacteria are in general considered a bad food source. Cyanobacteria contain only small amounts of nutritional molecules, such as polyunsaturated fatty acids and sterols (Ahlgren et al. 1992; Müller-Navarra et al. 2000; Von Elert et al. 2003), and often form colonies and filaments that interfere with the filter apparatus of potential grazers (DeMott et al. 2001; Wejnerowski et al. 2017; Xiao et al. 2018). However, the production of potentially toxic metabolites, including PIs, makes cyanobacteria a particularly bad food source. Due to their detrimental effects on natural grazers, PIs might even be considered as a chemical defense of cyanobacteria. Despite the benefit of reducing the grazing pressure, the production of defensive metabolites is probably costly in terms of resources and energy, as assumed by the optimal defense theory (ODT, Rhoades (1979)), and might lead to a trade-off between defense and other processes, such as growth. This trade-off is potentially disadvantageous, in particular when energy (light) and resources (nutrients) are limiting, and might, for example, lead to growth reduction (Agrawal 1998; Briand et al. 2012). Thus a constitutive production of defensive PIs would be unfavorable and it would be beneficial for cyanobacteria to regulate their PI content depending on the actual grazing pressure. Such a inducible defense mechanism is supported by findings that actively grazing zooplankton (e.g. *Daphnia*) as well as chemical cues released during grazing both induce morphological and physiological changes in algae and cyanobacteria, including increased toxin contents (e.g. Hansson et al. 2007; Jang et al. 2007; Bergkvist et al. 2008).

Overall, the interaction between cyanobacteria and zooplankton is essential for the whole ecosystem, because the interface between phytoplankton and zooplankton is responsible for the transfer of carbon and energy from the level of primary producers to higher trophic levels (Müller-Navarra et al. 2000; Von Elert et al. 2003). The disturbance of this link due to the detrimental effects of cyanobacterial an their metabolites on zooplankton consequently affects the productivity of the rest of the ecosystem (Müller-Navarra et al. 2000; Von Elert et al. 2003; García-Comas et al. 2016).

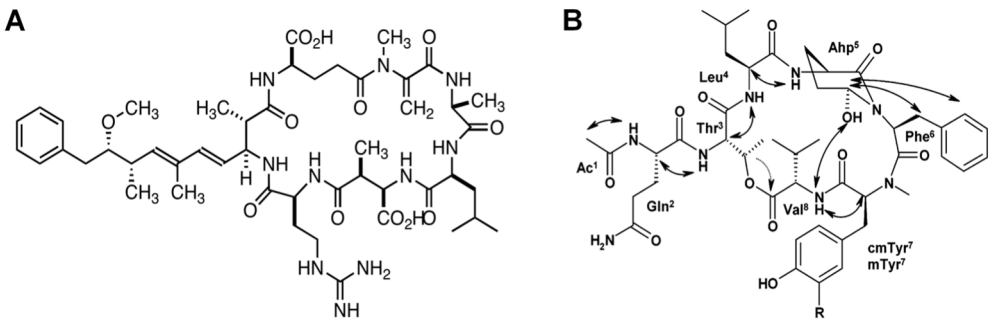

Figure 1. Chemical structure of microcystin-LR (**A**, Gademann and Portmann 2008) and the two protease inhibitors (**B,** Von Elert et al. 2005) nostopeptin 920 (BN920, R = H) and cyanopeptolin 954 (CP954, R = Cl).

Aim of this thesis

As described above, the massive inflow of nutrients into many aquatic systems promotes cyanobacteria and is therefore detrimental for the balance between phytoplankton and zooplankton. Additionally, studies have shown that the nutrient availability affects the production and content of MCs and other metabolites (e.g. Amé and Wunderlin 2005; Sevilla et al. 2010; Van de Waal et al. 2014), which is probably also the case for PIs and might therefore directly affect the toxicity of cyanobacteria. The carbon-nutrient balance hypothesis (CNBH, Bryant et al. 1983) assumes that the concentration of defensive secondary metabolites in plant tissue is primarily determined by the availability of carbon, light and nutrients and less by the grazing risk. This might equally be true for cyanobacteria, as one study already showed that cyanobacterial MC production followed the presumption of the CNBH (Van de Waal et al. 2009). However, the effects of major nutrients and other environmental factors on PI production and content in cyanobacteria are mostly unknown, because only a few studies (e.g. Tonk et al. 2009; Schwarzenberger et al. 2013) have targeted this topic. In this thesis, I therefore investigated three major environmental factors for their effect on the cyanobacterium *Microcystis aeruginosa*, which is the most common bloom-forming cyanobacterium in lakes and ponds (Sanseverino et al. 2016; Svirčev et al. 2019).

In chapter 1, the cyanobacterium *M. aeruginosa* NIVA Cya 43 was grown in batch-cultures at varying nitrate (NO_3^-) concentrations with the goal to understand how N availability affects growth, stoichiometry and PI content of *M. aeruginosa*. This particular cyanobacterial strain does not produce any MCs, but the two PIs BN920 and CP954 (Von Elert et al. 2005), which makes it an optimal candidate to investigate PIs without the influence of MCs. The N availability is an important factor for the synthesis of PIs, because PIs can be considered N-rich and their production therefore requires N. It was hypothesized that higher NO_3^- concentrations will promote the growth of the cyanobacterium and simultaneously lead to increased PI contents, while N limitation might result in growth inhibition and a decreased PI content. Throughout the experiment, the cultures were constantly sampled and analyzed using a combination of

classical methods (e.g. microscopic cell counting) and modern analytical techniques (e.g. liquid chromatography - mass spectrometry (LC-MS)). This allowed me to not only measure PI contents at single time points, but to follow the dynamical changes in the PI content during the growth of *M. aeruginosa*. A logistical growth model was used to model cyanobacterial growth, what also allowed me to analyze the relationship between growth rate, estimated from the growth model, and PI content. Furthermore, stochiometric ratios of the cyanobacterial biomass were used as indication for possible nutrient limitation and tested for their effect on the PI content. Altogether, the results in this chapter indicate that increasing NO_3^- concentrations foster cyanobacterial blooms by supporting the biomass production and increasing the content of defensive PIs. Additionally, chapter 1 highlights the potential intracellular role of PIs in the storage of N.

Chapter 2 focused on another important nutrient that contributes to the eutrophication of freshwater systems: phosphorous. P promotes cyanobacterial blooms, but PIs do not contain P and their content might therefore be unaffected by the P availability. However, under P limitation, growth might become inhibited and N might be reallocated to the production of PIs, resulting in increasing PI contents. This hypothesis was tested in chapter 2, using the same experimental setup as described in chapter 1, with the exception that this time a range of phosphate (PO_4^{3-}) concentrations was tested. Additionally to the analyses performed in chapter 1, the PI content was not only compared between treatments but also between different growth phases (early exponential, mid-exponential and late growth), which showed that the effect of the P availability depends on the growth phase of *M. aeruginosa*. Overall, the results of chapter 2 indicate that P limitation supports higher PI contents and therefore increases the toxicity towards potential grazers.

Following the experimental studies in chapter 1 and 2, chapter 3 describes a new dynamic process-based model that was developed to describe how PI production and degradation were affected by the N and P availability. Different from the logistical growth models used in chapter 1 and 2, the new model does not only describe cyanobacterial growth but also the turnover in the PI concentration in dependence of the supplied nutrients. The model assumes that *M. aeruginosa* takes up N to produce either PIs or other N-containing compounds, e.g. proteins or storage compounds, until the extracellular N is depleted. Similar, P is also taken up and used for biomass production and other processes. At some point, either N or P starts limiting growth (Liebig's minimum principle), so that biomass production stops. The assumption that PIs may serve as N storage was also implemented into the model. According to the model, *Microcystis* degrades the PIs when the extracellular N is depleted and reallocates the stored N to further growth. Chapter 3 highlights that mathematical models are a useful tool to analyze data from experimental studies and gain a deeper knowledge of the underlying processes. The results from this chapter support the assumption made in chapter 1, that PIs might be involved

in the storage of N and that both nutrient species and nutrient concentration affected the production, degradation and therefore the dynamics of PI found during the experiments.

In the final chapter, chapter 4, another environmental factor was investigated. Light provides the energy required for carbon fixation and therefore the production of all organic molecules, including PIs. Batch cultures of *M. aeruginosa* were grown at a range of light intensities, by covering the culture flasks with neutral density foil (ND-foil), which reduces the light intensity without affecting the light quality. According to literature, cyanobacteria are better adapted to low light intensities (e.g. Muhetaer et al. 2020), so that *M. aeruginosa* was expected to grow better at low light intensities. Accordingly, it was expected that the PI content will be higher at low light intensities. Chapter 4 shows that high light intensities can have detrimental effects on the growth of *M. aeruginosa* and that the light intensity affects not only the PI content but also the PI composition of the cyanobacterium. Furthermore, I discuss my results regarding their potential implications for the rest of the ecosystem.

In summary, this dissertation combined laboratory experiments with modern analytical methods and modeling approaches to broaden our understanding of how major environmental factors affect cyanobacteria, with emphasis on protease inhibitors. This thesis shows how the content of PIs is affected by tested conditions, provides insights into potential roles of PIs in intracellular processes and discusses the implications that changing PI contents might have on the entire ecosystem. In a world that is constantly changing due to human activities, understanding the principles that allow cyanobacteria to become dominant is essential for all efforts aiming to mitigate cyanobacterial blooms.

Chapter 1

Nitrate determines growth and protease inhibitor content of the cyanobacterium *Microcystis aeruginosa*

corresponds to the publication:

Burberg, C; Ilić, M; Petzoldt, T. & Von Elert, E. 2018. Nitrate determines growth and protease inhibitor content of the cyanobacterium Microcystis aeruginosa. J Appl Phycol 464, 37. DOI: 10.1007/s10811-018-1674-0.

1.1. Abstract

The eutrophication of lakes and ponds through anthropogenic nutrient input has resulted in higher frequencies of cyanobacterial blooms worldwide. The increased availability of nitrate (NO_3^-) plays an important role for the development of such blooms and also affects the content of secondary metabolites in cyanobacteria. Cyanobacterial protease inhibitors (PIs) are widespread, nitrogen (N)-rich metabolites that can be considered potential defense molecules, as they have detrimental effects on herbivorous zooplankton, the major consumer of cyanobacteria. In batch culture experiments, we investigated the effect of varying NO_3^- concentrations on the growth, stoichiometry, and PI content of the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43, which synthesizes the two PIs nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954). The dynamics of particulate organic nitrogen (PON) and the concentration of the two PIs indicate that BN920 and CP954 serve as temporary N storage compounds and are suggested to be degraded under N limitation. When related to the cyanobacterial biomass, the inhibitor content varied by more than 80% with NO_3^- concentration and time. The PI content increased with growth rate and N content of the cyanobacterium, which indicates that increased N availability supports higher cyanobacterial biomass with a higher content of defensive PIs. Therefore, increased NO_3^- concentrations foster cyanobacterial blooms directly by providing more nutrients and indirectly by increasing the negative interference of cyanobacteria with their consumers due to an increased content of PIs.

Keywords: Cyanobacteria – Eutrophication – Nitrogen – Nutrients – Secondary metabolites

1.2. Introduction

Over the last decades the number and frequency of toxic cyanobacterial blooms in freshwater systems has increased worldwide (Harke et al. 2016b). Under favorable conditions cyanobacterial genera, like *Planktothrix, Anabaena* or *Microcystis,* can form blooms with severe ecological and economic consequences, especially with regard to drinking water production (Steffensen 2008). Cyanobacterial blooms are an issue of concern because they dramatically change the ecosystem, e.g. by suppressing macrophytes or by fish kills (Paerl and Otten 2013). Additionally, many cyanobacteria produce a wide range of secondary metabolites of which many are toxic to other organism, including livestock and humans (Dittmann and Wiegand 2006; Gademann and Portmann 2008). Besides rising temperatures and CO_2-levels, the main cause of cyanobacterial blooms is the eutrophication of water bodies due to anthropogenic nutrient input (Huisman et al. 2018). Traditionally, phosphorus (P) is considered the most limiting nutrient in freshwater systems, whereas nitrogen (N) is normally limiting the productivity of marine ecosystems (Schindler 1974; Xu et al. 2010). However, N limitation has also been reported for lakes, mainly when the availability of P is high (Dolman et al. 2012; Paerl et al. 2016).

Research on toxic cyanobacterial metabolites has mainly focused on microcystins (MCs), a group of cyclic heptapeptides that are produced by different cyanobacterial genera and which specifically inhibit protein phosphates type 1 and 2A, which renders them toxic to mammals and humans (MacKintosh et al. 1990; Runnegar et al. 1993). Several studies have shown that the production and the cellular content of MCs are affected by environmental factors, such as light, CO_2, iron, temperature and sulphur (Wiedner et al. 2003; Amé and Wunderlin 2005; Jähnichen et al. 2011). Nutrients, most importantly N and P, as well as their ratio are considered crucial factors determining the MC production (Downing et al. 2005; Van de Waal et al. 2009; Van de Waal et al. 2014). Van de Waal et al. (2009) found that the total cellular MC content increased with increasing nitrogen to carbon (C) ratio. This finding is in accordance with the carbon nutrient balance hypothesis (CNBH, Bryant et al. 1983), which predicts that C-rich metabolites are favored under nutrient limitation, whereas in nutrient-replete or C-poor environments more N-based metabolites are produced. Even though MCs are important from the perspective of human health, it was shown that *Microcystis aeruginosa* NIVA Cya 43 inhibited the growth of *Daphnia*, a key genus in freshwater systems, although NIVA Cya 43 does not contain MCs (Lürling 2003). Instead, this strain contains other secondary metabolites, like the two serine protease inhibitors (PIs) nostopeptin 920 (BN920) and cyanopetolin 954 (CP954), which chemically belong to the group of cyanopeptolines (Von Elert et al. 2005). PIs are depsipeptides that are present in many cyanobacterial taxa and have been found in high

frequencies in blooms worldwide (Weckesser et al. 1996; Agrawal et al. 2001; Gademann and Portmann 2008).

Representatives of the genus *Daphnia* are the most important herbivores in freshwater plankton and play an important role in controlling cyanobacterial biomass (Chislock et al. 2013; Urrutia-Cordero et al. 2015). In a study by Chislock et al. (2013), *Daphnia* reduced the biomass of a phytoplankton community dominated by cyanobacteria by over 70% even with high initial MC concentrations. In case of cyanobacterial PIs it was shown that they effectively inhibit trypsin and chymotrypsin, two classes of digestive proteases in the gut of *Daphnia* (Von Elert et al. 2004; Agrawal et al. 2005). The inhibition of proteases resulted in a reduced growth of *Daphnia* and was shown to increase the mortality of zooplankter in general and of *Daphnia* in particular (Agrawal et al. 2001; Rohrlack et al. 2004; Gademann et al. 2010; Von Elert et al. 2012). These negative effects on *Daphnia* suggest that PIs might reduce the control of *Daphnia* over the cyanobacterial biomass and thus may foster the development of cyanobacterial blooms.

The high prevalence and the negative environmental impact of cyanobacterial PIs makes it necessary to understand how the availability of nutrients affects the cyanobacterial production of PIs. Furthermore, the above mentioned effects of dissolved N on the MC content of cyanobacteria do not necessarily hold for cyanopeptolines, which are synthesized by a different synthetase complex than MCs (Tooming-Klunderud 2007). In this study we performed batch culture experiments with *M. aeruginosa* strain NIVA Cya 43, which produces the two PIs BN920 and CP954, to analyze the effect of different initial NO_3^- concentrations on growth, carbon to nitrogen (C/N) ratios and PI content of the cyanobacterium. The two PIs are relatively rich in N, an element that can be limiting for cyanobacteria. We hypothesized that a higher initial NO_3^- concentration will result in a higher growth rate, an elevated cyanobacterial biomass and a higher content of PIs in the biomass of *Microcystis.*

1.3. Material and Methods

1.3.1. Culturing conditions

The cyanobacterium *Microcystis aeruginosa* NIVA Cya 43 (NORCCA, Norwegian Institute for Water Research) does not produce any microcystins but contains two N-rich protease inhibitors (PIs): cyanopetolin 954 (CP954) and nostopeptin 920 (BN920, Von Elert et al. 2005). The two PIs are structurally identical, except for that CP954 contains a chloride whereas BN920 does not. *M. aeruginosa* was grown in pre-cultures for 9 days in 1 L Erlenmeyer flasks filled with 400 mL modified WC medium (Guillard 1975) under constant light (45 ± 3 µE m^{-2} s^{-1}) at 20 ± 1°C on a horizontal shaker (90 rpm) with 500 µM initial nitrate (NO_3^-).

Experiments were performed with 500, 1000, 1500 and 2000 µM initial NO_3^- with four replicates each. 1 L flasks were filled with 400 mL of the respective medium, autoclaved and inoculated under sterile conditions with 1.5 $\cdot 10^5$ cell mL^{-1} of *M. aeruginosa* from the pre-culture, which is similar to inocula used in similar studies before (Long 2001; Jähnichen et al. 2011). The flasks were cultivated for 28 days and daily randomized to compensate for inhomogeneities in the light regime. Until day 22, samples (0.5 mL) were taken every 1 – 2 days to microscopically determine the cell density using a Neubauer improved counting chamber, and every 2 – 4 days volumes between 10 and 200 mL were taken for the determination of particulate organic carbon (POC) and nitrogen (PON) as well as for the extraction and quantification of the two PIs. An additional sample was taken after 28 days. The volume needed for the analyses was estimated from the cell density.

During the experiment the cultures were also checked for heterotrophic bacteria. The biovolume of the heterotrophic bacteria reached between 5.27 ± 0.98 % and 6.07 ± 0.98 % of the cyanobacterial biovolume. For the determination of the biovolume, samples were taken from the cultures, fixed with formaldehyde (2%) and filtered onto polycarbonate filters (Whatman). After staining the samples with 4′,6-diamidino-2-phenylindole (DAPI), heterotrophic bacteria and *Microcystis* cells were counted and their cell size was measured using a fluorescence microscope (Axio Lab.A1, Zeiss). Based on the cell abundance and the measured cell sizes the total biovolume of *Microcystis* and the heterotrophic bacteria in the cultures was calculated.

1.3.2. Determination of POC and PON

For the determination of POC and PON a sample volume equivalent to approx. 0.5 mg POC was filtered on pre-combusted GF/F filters (Macherey & Nagel), which subsequently were dried at 60°C for at least 24 hours and then packed into tin capsules for the measurements using a Flash 2000 elemental analyzer (Thermo Fisher). The results were used to calculate the concentration of POC and PON in the cultures and to determine the molar C/N ratio of the cyanobacterial biomass.

1.3.3. Extraction and quantification of PIs

For the extraction of the two PIs from the cyanobacterial cells, samples (approx. 0.25 mg C) were centrifuged for 5 minutes at 15557 g or 4500 g respectively depending on the sample volume. The supernatant was discarded and 10 mL methanol (MeOH, 80%) were added to the cell pellet. The re-suspended pellets were sonicated for 7 minutes and then centrifuged for 3 minutes (4500 g). The supernatant was transferred into test tubes, evaporated to dryness using a vacuum centrifuge (RVC 2-25, Christ) and re-dissolved in 1 mL MeOH (100%). The samples were dried again, taken up in 100 µL MeOH, centrifuged for 2 minutes (20000 g). The supernatant was transferred into HPLC vials.

PIs were quantified using an ultra-high-pressure liquid chromatography system (UHPLC, Accela, ThermoFisher Scientific) coupled with an Exactive Orbitrap mass spectrometer (MS, ThermoFisher Scientific). The chromatographic separations was carried out on a C_{18}-column (Nucleosil, 125/2, 100-3, Macherey and Nagel) as stationary phase and acetonitrile (A) and ultra-pure water (B), each containing 0.05% trifluoracetic acid (TFA), as mobile phase with the following gradient: 0 min: 20% A, 14 min: 100% A, 16 min: 100% A, 16.5 min: 20% A, 18 min: 20% A. The column temperature was set to 30°C, the flow rate was 300 µL min^{-1} and the injection volume of the sample was always 10 µL.

The MS was operated in positive scan mode using an electrospray ionization (ESI) source. The source parameters were set as follows: spray voltage 4.5 kV, capillary temperature 325°C, capillary voltage 60 V, flow rate of sheath (N_2) 40 a.u. and aux gas (N_2) 15 a. u. Under these conditions BN920 and CP954 form two positively charged adduct ions ($[M+H-H_2O]^+$; $[M+Na]^+$) with m/z = 903.46108 and 943.45331 (BN920 adducts) and m/z = 937.42211 and 977.41394 (CP954 adducts). The adducts of each PI were summed up to ´BN920´ and ´CP954´. The internal standard microcystin LR (MC-LR, Enzo Life Sciences), which was added at a final concentration of 1 µg mL^{-1} prior to the extraction, was measured at m/z = 995.55604 ($[M+H]^+$). All measurements were carried out at mass range of m/z = 120 – 1500 with a granted mass deviation of 10 ppm. Peak intensities were extracted from the chromatograms using the R-package 'enviMass' (Loos 2018) and converted to concentrations via previously established

calibration curves. Subsequently, the PI concentrations in the samples were normalized to the volume of the cultures or to extracted POC, as proxy for the cyanobacterial biomass.

1.3.4. Modeling and statistical analyses

All analyses were carried out using R (R Core Team 2018) and RStudio (RStudio Team 2016). *Microcystis* was expected to show logistic growth, which is characterized by an initial exponential growth phase followed by a leveling off as the growth becomes limited by one or more resources, e.g. N, resulting overall in a sigmoidal (S shaped) growth curve. A logistic growth model was fitted to the POC or the cell abundance data using the R-package 'growthrates' (Petzoldt 2017), to test if *Microcystis* grew logistically. The model equation was

$$N_t = (K' \cdot N_0) / (N_0 + (K' - N_0) \cdot e^{-\mu_{max} \cdot t})$$

with N_0 as the initial biomass or cell abundance, N_t as the biomass or cell abundance at time (t) and the two model parameters carrying capacity (K') and maximal growth rate (μ_{max}). K' and μ_{max} were tested for effects of the initial NO_3^- concentrations using one-way analysis of variances (ANOVAs) followed by Tukey HSD *post hoc* tests. Further, one-way ANOVAs were used to test for differences in the PON and PI concentration between the treatments and a two-way repeated measures ANOVAs was used to determine the effects of time and initial NO_3^- concentration on the PI content of the cyanobacterium. The assumptions of the ANOVA, approximate normal distribution and variance homogeneity, were checked using Shapiro-Wilk´s and Levene´s test, respectively.

Instantaneous growth rates for each sampling day were extrapolated from the model and correlated to the PI content to analyze the effect of growth rate on inhibitor content. A linear model was applied in case of BN920, while the content of CP954 was better described with a logistic model.

1.4. Results

Batch culture experiments were performed to investigate the effects of different initial nitrate (NO_3^-) concentrations in the medium on the growth performance, stoichiometry and content of the two N-rich secondary metabolites CP954 and BN920 in a strain of *Microcystis aeruginosa* that does not produce microcystins.

1.4.1. Growth of *M. aeruginosa* NIVA Cya 43 under different nitrate conditions

M. aeruginosa showed logistic growth under all tested NO_3^- concentrations, regardless whether growth was determined by cell abundances or particulate organic carbon (POC; Fig. 1 A & B). The carrying capacity (*K´*) was reached between days 10 and 21, depending on the treatment. *K´* and maximal growth rate (μ_{max}) were both affected by the initial NO_3^- concentration (Tab. 1 & 2). *K´* increased with increasing NO_3^- concentration, whereas μ_{max} in the two lower NO_3^- treatments exceeded those in the two higher NO_3^- concentrations.

The initial NO_3^- concentration significantly affected the particulate organic nitrogen (PON) concentration in the cultures at the end of the experiment (Fig. 1 C, one-way ANOVA: $F_{3,12} = 332.6$, $p < 0.001$). After 28 days, the highest PON was found with the highest initial NO_3^- concentration, whereas the lowest PON was found in the lowest NO_3^- condition. However, PON did not increase in any of the treatments after day 10. The nitrogen content per cell showed similar dynamics across all treatments, such that it peaked on day 4 and reached identical values towards the end of the experiments (Fig. 1 D). Cellular nitrogen quota decreased after day 4 in line with the increase of cell numbers (Fig. 1 A).

1.4.2. Intracellular inhibitor content of *M. aeruginosa*

M. aeruginosa NIVA Cya 43 produces the two PIs BN920 and CP954, which were measured in intervals of 2 – 4 days.

The dynamics of the concentrations of each PI differed from those of the cyanobacterial biomass. Both PIs were present in the batch cultures at all time points with maximal mean concentrations at day 10 of the experiments (Fig. 2). For BN920 the maximal mean concentrations ranged between 1.3 and 2.1 µg mL^{-1}. The concentrations of CP954 were 4 – 5 times higher than those of BN920 with maxima between 4.8 – 10.8 µg mL^{-1}. Remarkably, differences between the four treatments occurred only from day 10 onwards. On day 10, concentrations of both inhibitors were significantly different between the treatments (one-way ANOVA, BN920: $F_{3,12} = 5.754$, $p = 0.0112$; CP954: $F_{3,12} = 14.04$, $p < 0.001$): PI concentrations were significantly higher in the 1500 and 2000 µM treatment compared to the lowest NO_3^- treatments (Tukey HSD *post hoc* test, $p < 0.05$).

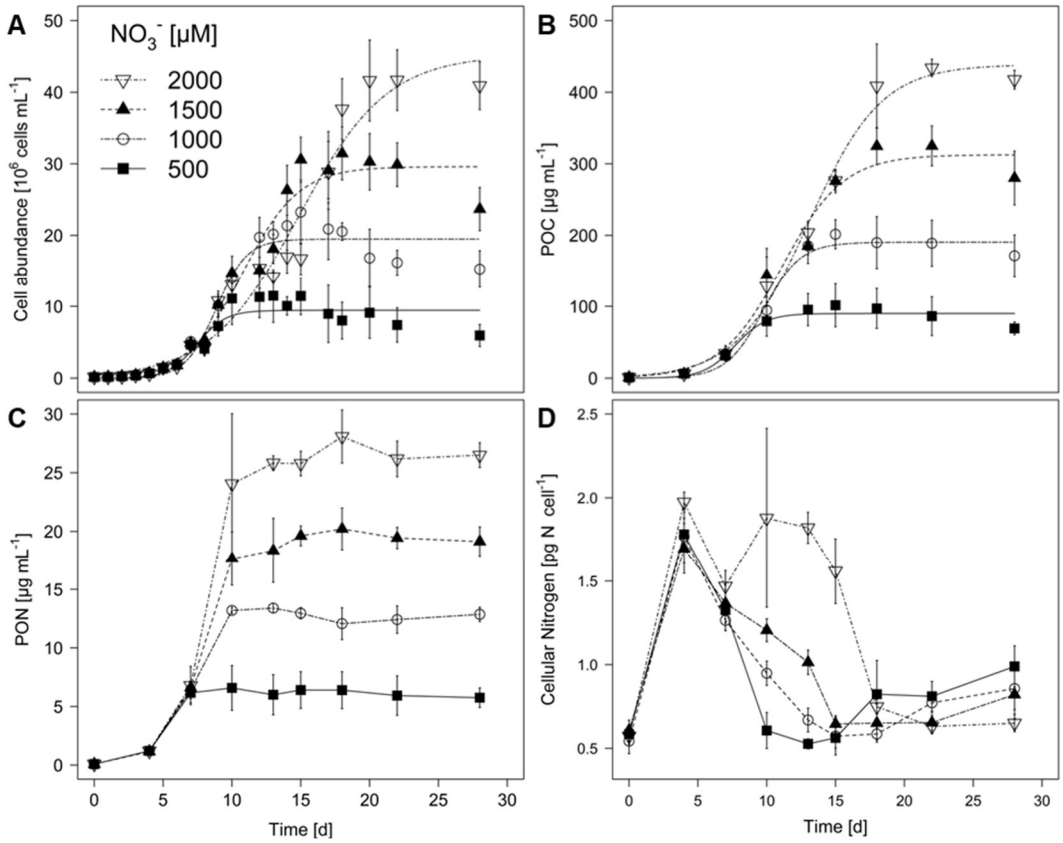

Figure 1. Cell abundance (**A**), particulate organic carbon (POC, **B**), particulate organic nitrogen (PON, **C**) and cellular nitrogen (**D**) in batch culture growth experiments of the cyanobacterium *M. aeruginosa* NIVA Cya 43 grown with 500 µM, 1000 µM, 1500 µM and 2000 µM initial nitrate (NO_3^-). Mean ± SD, n = 4. Curves in plot A and B were fitted using a logistic growth model. Equations and parameters are given in Tab. 1 and 2.

Table 1. The cyanobacterium *M. aeruginosa* NIVA Cya 43 was grown in batch cultures with 500 µM, 1000 µM, 1500 µM and 2000 µM initial nitrate (NO_3^-). The cell abundance was determined (Fig. 1 A) and used to model the growth of the cyanobacterium using the R-package 'growthrates'. Equation and R^2 are given for curve fits based on the mean cell abundance (n = 4). The growth parameters N_0 (initial cell abundance), μ_{max} (maximal growth rate) and K' (carrying capacity) were determined for all replicates separately. Mean values (± SD) were calculated and the parameters μ_{max} and K' were tested for differences between treatments (Tukey HSD *post hoc* test after one-way ANOVA, p = 0.05). Capital letters indicate significant differences between treatments.

NO_3^- [µM]	Equation [a]	R^2	N_0 [10^4 cells mL^{-1}]	μ_{max} [d^{-1}]	K' [10^4 cells mL^{-1}]
500	$N_t = \frac{6.77 \cdot 10^{10}}{7.2 \cdot 10^3 + 9.4 \cdot 10^6 \, e^{(-0.96t)}}$	0.74	0.74 ± 0.52	1.00 ± 0.13 ^{A}	945 ± 267 ^{A}
1000	$N_t = \frac{1.32 \cdot 10^{11}}{6.8 \cdot 10^3 + 1.9 \cdot 10^7 \, e^{(-0.9t)}}$	0.91	1.02 ± 0.98	0.92 ± 0.19 ^{A}	1954 ± 124 ^{B}
1500	$N_t = \frac{4.38 \cdot 10^{12}}{1.5 \cdot 10^5 + 2.9 \cdot 10^7 \, e^{(-0.49t)}}$	0.93	13.19 ± 9.34	0.52 ± 0.08 ^{B}	2963 ± 333 ^{C}
2000	$N_t = \frac{2.03 \cdot 10^{13}}{4.5 \cdot 10^5 + 4.5 \cdot 10^7 \, e^{(-0.31t)}}$	0.95	44.32 ± 13.07	0.31 ± 0.02 ^{B}	4508 ± 437 ^{D}

[a] General equation: $N_t = (K' \cdot N_0) / (N_0 + (K' - N_0) \cdot e^{-\mu max \cdot t})$ with t = time [d], N_t = cell abundance at time t [cells mL^{-1}].

In order to determine the PI content of the cyanobacterium, values were normalized to the cyanobacterial biomass, measured as POC. The mean content of both PIs increased until day 4 (BN920) or day 7 (CP954) and declined thereafter to relatively low values for the rest of the experiment (Fig. 3). These low values were more than 95% (BN920) and 85% (CP954) lower than the maximal inhibitor content. Both, sampling date and the treatment affected the content of BN920 and CP954, and the significant interaction of treatment with sampling date indicating that the dynamics of PI content over time differed with initial NO_3^- concentration (Tab. 3). Because there was a linear correlation between the biomass and cell abundance of *Microcystis* (R^2 = 0.9322), PI content per cell showed a similar pattern as the content per cyanobacterial biomass (data not shown). The cellular PI quota ranged from 16 to 527 pg cell^{-1} for BN920 and from 72 to 850 pg cell^{-1} for CP954.

Table 2. The cyanobacterium *M. aeruginosa* NIVA Cya 43 was grown in batch cultures with 500 µM, 1000 µM, 1500 µM and 2000 µM initial nitrate (NO_3^-). POC was measured as proxy for the cyanobacterial biomass (Fig. 1 B) and used to model the growth of the cyanobacterium using the R-package 'growthrates'. Equation and R^2 are given for curve fits based on the mean POC (n = 4). The growth parameters N_0 (initial concentration of POC), μ_{max} (maximal growth rate) and K' (carrying capacity) were determined for all replicates separately. Mean values (± SD) were calculated and the parameters μ_{max} and K' were tested for differences between treatments (Tukey HSD *post hoc* test after one-way ANOVA, p = 0.05). Capital letters indicate significant differences between treatments.

NO_3^- [µM]	Equation [a]	R^2	N_0 [µg C mL^{-1}]	μ_{max} [d^{-1}]	K' [µg C mL^{-1}]
500	$N_t = \frac{9.02}{0.1 + 89.8\ e^{(-0.89t)}}$	0.79	0.12 ± 0.09	0.88 ± 0.09^A	89.9 ± 23.1^A
1000	$N_t = \frac{24.49}{0.13 + 190.3\ e^{(-0.75t)}}$	0.93	0.13 ± 0.11	0.83 ± 0.21^A	191.1 ± 27.2^B
1500	$N_t = \frac{661.88}{2.1 + 310.5\ e^{(-0.45t)}}$	0.95	1.88 ± 1.15	0.48 ± 0.08^B	312.1 ± 26.9^C
2000	$N_t = \frac{1287}{2.9 + 435.6\ e^{(-0.38t)}}$	0.97	3.53 ± 3.35	0.39 ± 0.06^B	439.6 ± 12.9^D

[a] General equation: $N_t = (K' \cdot N_0) / (N_0 + (K' - N_0) \cdot e^{-\mu max \cdot t})$ with t = time [d], N_t = POC at time t [µg mL^{-1}].

Table 3. Two-way ANOVAs with repeated measures performed for the content of nostopeptin 920 (BN920, in µg mg C^{-1}) and cyanopeptolin 954 (CP954, in µg mg C^{-1}). DF: Degrees of freedom, SS: Sum squares, MS: Mean squares, Test statistics: F- and p-value.

Inhibitor	Source	DF	SS	MS	F	p [a]	
BN920	Treatment	3	0.9178	0.30593	5.538	0.0197	*
	Day	8	215.67	26.959	181.7	< 0.001	***
	Treatment : Day	24	2.156	0.08982	2.144	0.00697	**
	Residual	72	3.016	0.04189			
CP954	Treatment	3	0.6756	0.22518	4.351	0.0374	*
	Day	8	105.66	13.207	257.1	< 0.001	***
	Treatment : Day	24	1.874	0.07810	2.388	0.00244	**
	Residual	72	2.355	0.03271			

[a] Significance levels: * p < 0.05, ** p < 0.01, *** p < 0.001.

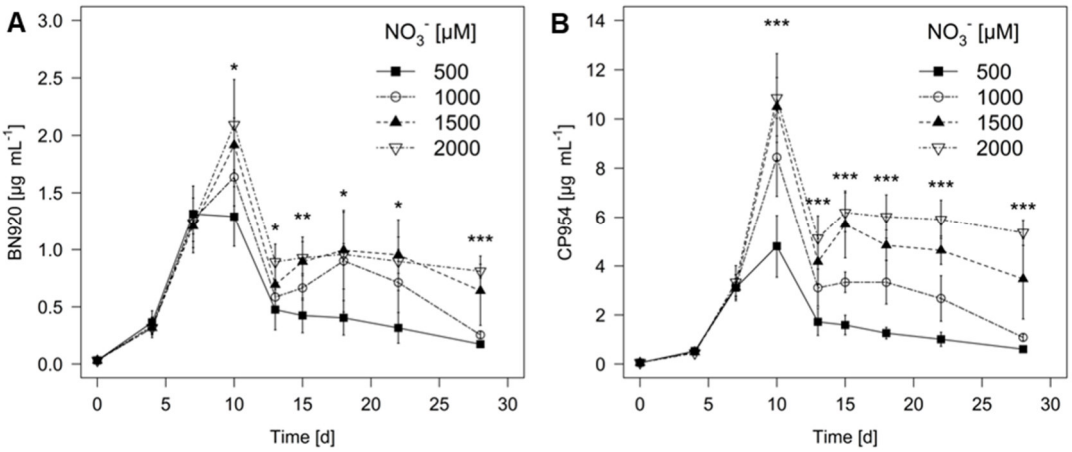

Figure 2. Concentration of the protease inhibitors nostopeptin 920 (BN920, **A**) and cyanopeptolin 954 (CP954, **B**) in batch culture growth experiments of the cyanobacterium *M. aeruginosa* NIVA Cya 43 grown with 500 µM, 1000 µM, 1500 µM and 2000 µM initial nitrate (NO_3^-). Mean ± SD, n = 4. Asterisks denote significant differences in inhibitor concentration among treatments for the indicated day based on one-way ANOVAs (*p < 0.05, **p < 0.01, ***p < 0.001).

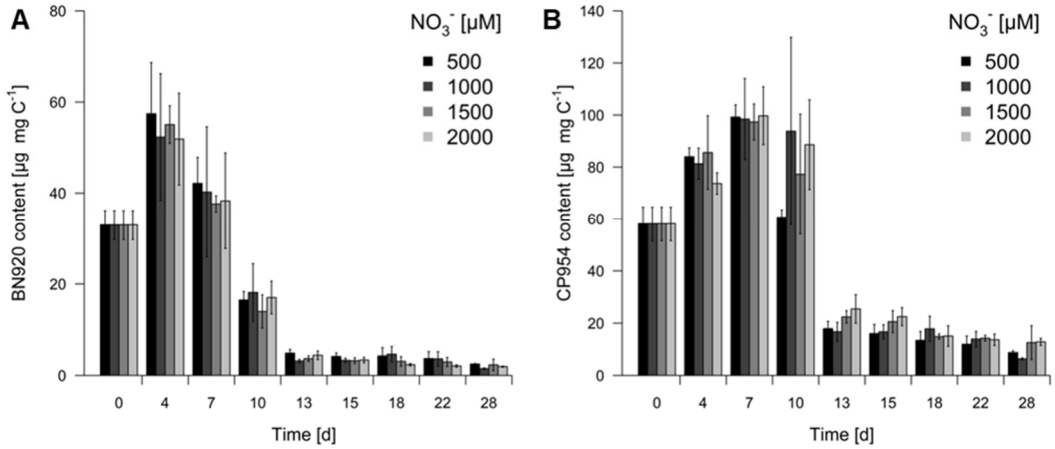

Figure 3. Content of the protease inhibitors nostopeptin 920 (BN920, **A**) and cyanopeptolin 954 (CP954, **B**) in batch culture growth experiments of the cyanobacterium *M. aeruginosa* NIVA Cya 43. The cyanobacterium was grown with 500 µM, 1000 µM, 1500 µM and 2000 µM initial nitrate (NO_3^-). Inhibitor content was normalized to the particulate organic carbon (mg C), as a proxy for the cyanobacterial biomass. Mean ± SD, n = 4.

1.4.3. Inhibitor content versus stoichiometry and growth rate

When the PI content of the cyanobacterial biomass was plotted as a function of the carbon to nitrogen (C/N) ratio of the cyanobacterium, the PI content was inversely related to the C/N ratio (Fig. 4), indicating that N depletion of the cyanobacterium resulted in decreased PI contents.

An inherent feature of logistic growth is the change of the instantaneous specific growth rate over time. Here the specific growth rates had been obtained from the logistic growth model based on the POC data (Fig. 1 B). In order to test for effects of growth, the content of the two cyanopeptolines in the cyanobacterium was plotted versus the respective growth rate. The content of BN920 increased significantly with the growth rate (Fig. 5 A, linear regression,

$F_{1,142}$ = 318.6, p < 0.001). The content of CP954 as well increased with growth rate; however, the relation was better described by a logistic than by a linear model (Fig. 5 B, $F_{1,142}$ = 188.71, p < 0.001). The CP954 content increased linearly with growth and reached a plateau from growth rates of 0.4 d^{-1} onwards.

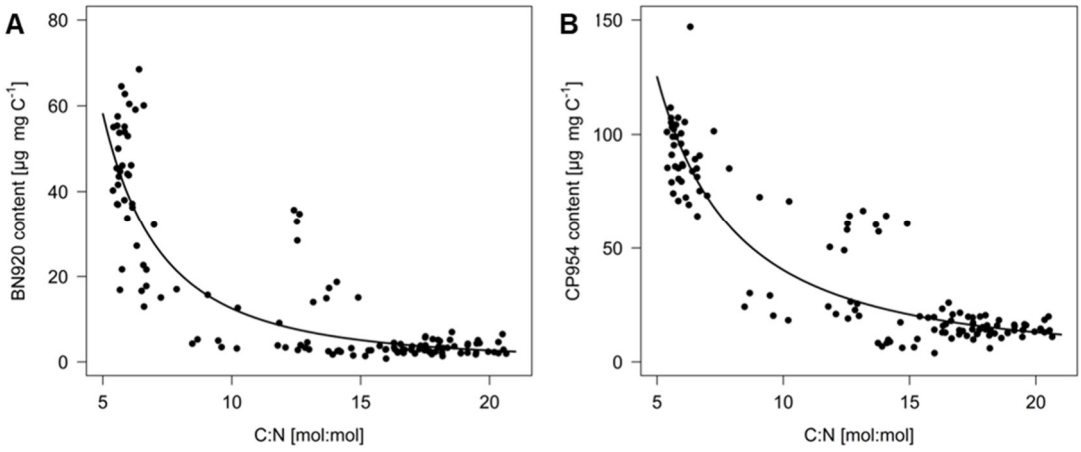

Figure 4. Content of the protease inhibitors (**A**) nostopeptin 920 (BN920, y = 2045$x^{-2.21}$, R^2 = 0.65, for log transformed data) and (**B**) cyanopeptolin 954 (CP954, y = 1733$x^{-1,63}$, R^2 = 0.71, for log transformed data) in the cyanobacterial biomass as a function of the molar ratio of carbon to nitrogen (C/N) of the cyanobacterium *M. aeruginosa* NIVA Cya 43. Each point represents the inhibitor content in a single replicate at a single sampling day and the corresponding stoichiometric ratio. Lines represent the applied regression models.

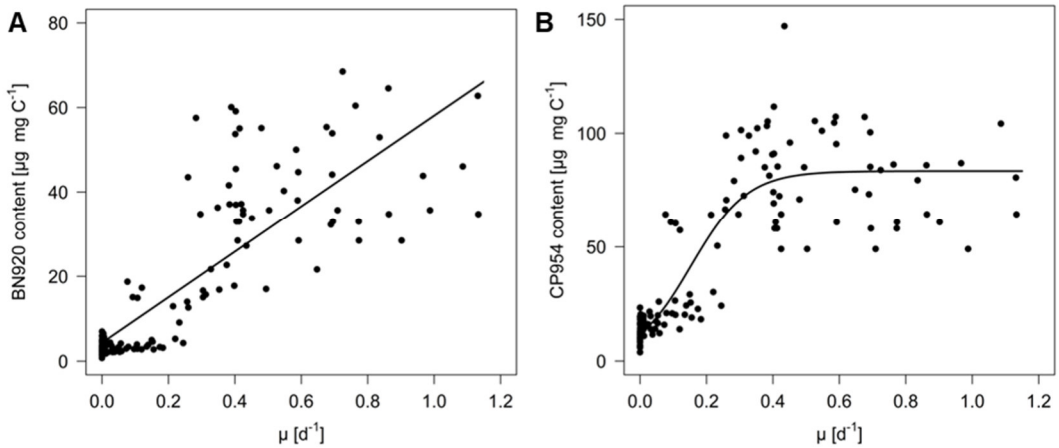

Figure 5. Content of the protease inhibitors (**A**) nostopeptin 920 (BN920; y = 53.72x – 4.36, R^2 = 0.69) and (**B**) cyanopeptolin 954 (CP954, y = 71.7 / [11.9 + (11.9 $e^{-11.7x}$)], R^2 = 0.79) as a function of the specific growth rate μ of the cyanobacterium *M. aeruginosa* NIVA Cya 43. Growth rates were extrapolated from the logistic growth model based on the POC data (see Fig. 1 B). Each point represents the measured inhibitor content in a single replicate at a single sampling day and the corresponding specific growth rate. Lines represent the applied regression models.

1.4. Discussion

This study focuses on the effect of nitrate (NO_3^-), the most abundant oxidized form of nitrogen (N) in freshwater and marine ecosystems (Glibert et al. 2016), on the cyanobacterium *Microcystis aeruginosa*, a globally occurring bloom-forming cyanobacterium. We hypothesized that a higher initial NO_3^- concentration results in an elevated biomass, a higher maximal growth rate (μ_{max}) and a higher content of protease inhibitors (PIs) of *M. aeruginosa* NIVA Cya 43.

As expected, the cell abundance and biomass of *Microcystis* increased with the initial NO_3^- concentration, which is in accordance with other studies (Orr and Jones 1998; Sevilla et al. 2010; Kim et al. 2017). Even though phosphorus (P) is traditionally considered the limiting factor for biomass production of freshwater phytoplankton (Schindler 1974), N limitation can restrict the productivity of lakes, especially when P availability is high (Dolman et al. 2012; Paerl et al. 2016). The ongoing input of N into water bodies through the use of fertilizers, untreated sewage water or increased atmospheric N deposition is therefore fostering the dominance of cyanobacteria and the formation of cyanobacterial blooms, which often result in dramatic changes of the aquatic environment (Paerl and Otten 2013; Glibert and Burford 2017). Here we ran replicated batch cultures with identical inocula of *Microcystis* and four treatments with different initial NO_3^- concentrations. Batch cultures were expected to show logistic growth with per-capita growth rates being high in the beginning and decreasing over time until they become zero in the stationary phase (Lampert and Sommer 2010). Due to the *Microcystis* biomass required for the quantification of PIs via UHPLC-MS, we used fairly high *Microcystis* inocula and NO_3^- starting conditions. However, all treatments showed similar logistic growth curves with a gradient of per-capita growth rates and C/N ratios over time, which indicates that despite different NO_3^- starting conditions all treatments resulted in increased N limitation over time. Our experimental starting conditions did not affect the principle effects of N limitation on growth and PI content of *Microcystis*.

However, the maximal growth rate of *M. aeruginosa* was expected to increase with the initial NO_3^- concentration, like it was shown for NO_3^- concentrations varying from 1 to 500 µM (Kim et al. 2017). Contradictory to Kim et al. (2017), the maximal growth rate decreased with initial NO_3^- concentration. Chen et al. (2009) reported similar observations for 1.7 to 10.7 fold higher NO_3^- concentrations than the maximum concentration in this study and explained this by an increase of the intracellular nitrite concentration. This suggests that extreme concentrations of NO_3^- can negatively affect cyanobacteria by reducing their growth and biomass production. This is also in accordance with results from field studies, which show that high ratios of TN:TP can suppress cyanobacteria and might shift communities away from dominant, bloom-forming cyanobacteria towards chlorophytes (Smith 1983; Harris et al. 2014).

Such a shift might in consequence result in a reduction of cyanobacterial toxins, e.g. microcystin (Orihel et al. 2012; Harris et al. 2014).

The uptake and incorporation of NO_3^-, as the only N source provided in the experiment, into the biomass is essential for *Microcystis,* as it cannot fix atmospheric N. The fact that in all treatments the particulate organic nitrogen (PON) increased only until day 10 indicates that no N was taken up thereafter. Until day 10, the molar C/N ratios of *M. aeruginosa* (data not shown) remained below 6.6, which provides evidence for non-limiting N supply during these 10 days (Redfield 1958). The Redfield ratio can be considered a good indicator for nutrients stress, because phytoplankton that is not limited by N or P displays often a molar C:N:P ratio of 106:16:1 (Goldman et al. 1979; Sommer 1990). However, after day 10, C/N ratios increased in all treatments up to 20, which indicated N depletion of the cyanobacterium. In conclusion, stagnation of PON and increasing C/N ratios point at N limitation of the cyanobacterium after day 10. Nevertheless, biomass and cell abundances still increased, which indicates a reallocation of N to growth either by shut down or modification of N consuming pathways or by scavenging N through reduction of pigments (Saha et al. 2003) or storage compounds that had been produced under high N availability (first 10 days).

Cyanobacteria can store N in form of cyanophycin, a protein consisting of the two amino acids arginine and aspartic acid, which is synthesized under N repleted conditions and degraded under N limitation (Richter et al. 1999; Berg et al. 2000). The concentration of the two PIs BN920 and CP954 in the cultures reached its maximum on day 10, which indicates a high synthesis of these peptides under N replete conditions. The decrease of PI concentrations with increasing C/N ratios denotes degradation of the two PIs under increasing N depletion. These dynamics of the two PIs corroborate the well-established carbon nutrient balance hypothesis (CNBH), according to which primary producers synthesize secondary metabolites dependent on the availability of nutrients (Bryant et al. 1983) or more precisely, the concentration of N-rich inhibitors should increase with increasing N availability.

The observed dynamics of both PIs in response to N availability also raises the question if BN920 and CP954 might serve as temporary N storage**.** The PIs consist of 8 amino acids (Von Elert et al. 2005) and have a C/N ratio of 5.75 so that they can be considered N-rich (Van de Waal et al. 2014). It can be assumed that the amino acids and with them the N from the PIs are repurposed by *Microcystis*, e.g. for growth, when the PIs are degraded under N depletion, which would make them a temporary storage. However, despite ongoing N limitation, PIs remained at low concentrations and did not disappear, which indicates that *Microcystis* maintains a minimal PI content that is not used for resource allocation. In line with the theory that PIs might be storage compounds, PIs of plants have also been suggested to serve as N

storage because of their homology and close evolutionary relationship to storage proteins (Odani et al. 1983; Mosolov and Valueva 2005).

As mentioned above, the observed changes in PI concentration in response to N availability are in good agreement with the CNBH. In line with these findings, Schwarzenberger et al. (2013) showed in *M. aeruginosa* that the content of three micropeptins, a class of N-rich chymotrypsin inhibitors, was reduced under N limitation but increased when phosphorous (P) was limiting. Our findings and those of Schwarzenberger et al. (2013) are supported by Harke et al. (2016a), who found an up-regulation of PI synthetase genes (aer, mcn) in *Microcystis* supplementation of ammonium and urea. Similar to PIs, the content of the N-rich toxin MC increases with increasing N availability (Sivonen 1990; Van de Waal et al. 2009; Horst et al. 2014), and MCs synthetase genes (mcy) have been found to be down regulated under N limitation (Harke and Gobler 2013). Contradictory, other studies report no changes or even increased expressions of mcy-genes under N limitation (Sevilla et al. 2010; Pimentel and Giani 2014), and it has been suggested that an increase in the cellular MC content with increasing N availability might be rather indirectly mediated through changes in growth and biomass than directly due to changes in the transcription of the mcy-genes (Sevilla et al. 2010). Furthermore, the N availability changes the composition of MC congeners according to the CNBH (Van de Waal et al. 2014; Puddick et al. 2016). As N-rich and N-poor MC congeners differ in toxicity, the availability of N is an important parameter to be considered in lake management of toxic cyanobacterial blooms (Puddick et al. 2016).

Even though our results show that NO_3^- supports good growth and affects the PI content, it has to be mentioned that cyanobacteria can utilize multiple N sources including reduced N species like ammonium or urea as well as dissolved organic nitrogen like certain amino acids (Yan et al. 2004; Li et al. 2016). The chemical form of N is of high importance as different N species differ in their effects on the growth (Li et al. 2016) and secondary metabolite content (Yan et al. 2004; Donald et al. 2011) of cyanobacteria as well as on the species composition of cyanobacteria in lakes (Monchamp et al. 2014). Like NO_3^-, urea supports cyanobacterial growth, while ammonium (NH_4^+) can support growth at low concentrations as well as it can inhibit the growth of cyanobacteria at higher concentrations (Glibert et al. 2016; Li J et al. 2016; Erratt et al. 2018). The N species also affect the content of secondary metabolites in cyanobacteria. Yan et al. (2004) showed that NO_3^- can support higher MC content than urea and NH_4^+, which is supported by findings that NH_4^+ can actually reduce the MC content (Amé and Wunderlin 2005). However, in general it is assumed that cyanobacteria prefer reduced N species due to lower energetic cost for uptake and assimilation (Glibert et al. 2016; Harris et al. 2016). Additionally, cyanobacteria are superior competitors under high availabilities of reduced N species (Donald et al. 2011), which is alarming as the loads of reduced N species into aquatic systems are increasing, e.g. through increased usage of reduced N in fertilizers

(Glibert et al. 2014; Glibert et al. 2016). Harris et al. (2016) showed that it is not only the concentration of N that controls the biomass of cyanobacteria, but that the ratio of oxidized and reduced N might be essential and a shift towards reduced N might favor strains that produce toxic secondary metabolites. Altogether, investigations about the effects of different N species on the PI content of *Microcystis* are of interest for future studies. In our study, we focused on NO_3^-, because NO_3^- contributes to the dramatic eutrophication of aquatic systems (Glibert et al. 2016), can support equal or even better growth of *Microcystis* than reduced N species (Yan et al. 2004; Erratt et al. 2018), and does not suppress algae or cyanobacterial growth like ammonium does at high concentrations (Glibert et al. 2016).

The mechanisms that affect the production and content of secondary metabolites in cyanobacteria are, however, even more complex as other environmental variables like light, temperature, iron, CO_2 or sulphur can also alter the content of microcystin and other metabolites (Wiedner et al. 2003; Amé and Wunderlin 2005; Van de Waal et al. 2009; Jähnichen et al. 2011). Some of these variables might have changed with increasing cell density in our batch culture experiment. Light is a resource that is known to affect the content of secondary metabolites in cyanobacteria (Wiedner et al. 2003; Tonk et al. 2005) and that might have become a limiting factor in our growth experiment as the cultures became more dense. However, a light intensity of 45 µE m^{-2} s^{-1} is suitable to allow optimal growth (Hesse et al. 2001), and under light limitation the final cyanobacterial biomasses should not have differed. As this was clearly not the case, light limitation seems rather improbable. Potentially, P might have become limiting during the course of the growth experiments. At the end of the experiments, an increase of the C/P ratios of the cyanobacterial biomass was noted with values up to 300 in the high NO_3^- treatments (data not shown). Although this points at moderate P depletion towards the end of the experiments, effects of P limitation are fairly unlikely. According to the CNBH and to Schwarzenberger et al. (2013) P limitation should lead to an increased PI content, which clearly was not the case at the end of the experiments. Our findings that $K´$ as well as the time at which $K´/2$ was reached are significantly correlated with the initial NO_3^- concentration further points against effects of other resources than nitrogen.

The genus *Daphnia* is a major consumer of phytoplankton biomass in lakes and ponds and plays an important role in controlling cyanobacterial blooms (Chislock et al. 2013; Urrutia-Cordero et al. 2015). Cyanobacterial PIs specifically inhibit digestive proteases in the gut of *Daphnia* (Von Elert et al. 2004) and have detrimental effects on *Daphnia* reaching from growth reduction (Agrawal et al. 2005; Schwarzenberger et al. 2010; Schwarzenberger et al. 2013) to increased mortality (Rohrlack et al. 2004). Thus, PIs can be considered defensive molecules of cyanobacteria and their production might not only depend on the nutrient availability, as it is suggested by the CNBH, but also on the presence of herbivores, as is described by the optimal defense theory (ODT, Rhoades 1979). However, a recent study showed that the transcription

of genes of three PIs classes (aeruginosin, cyanopeptolin, microviridin) were not affected by zooplankton (Harke et al. 2017), indicating that the defensive potential of cyanobacteria due to PIs might be more properly described by the CNBH than by the ODT.

As PIs might function as a cyanobacterial defense against herbivores, we analyzed the PI content per cyanobacterial biomass and found that it varied by more than 80% with maxima on day 4 (BN920) and 7 (CP954), respectively, with C/N ratios (data not shown) indicating N replete conditions. This suggests that the defense potential of *Microcystis* against *Daphnia* is high under N replete conditions but decreases when N becomes depleted. Additionally, the high PI content was positively correlated to the growth rates of *Microcystis*, which is in accordance with strong positive correlations between the cellular content of microcystin or cyanopeptolins and the cyanobacterial growth rate (Orr and Jones 1998; Long 2001; Downing et al. 2005; Tonk et al. 2009) and which is backing the observation that the content of certain toxins is higher under favorable growth conditions (Sivonen 2009).

1.5. Conclusion

The frequency of cyanobacterial blooms in lakes and ponds will further increase due to a high input of N and P (Glibert and Burford 2017; Huisman et al. 2018). Because PIs are frequent in cyanobacterial blooms and have detrimental effects on *Daphnia*, the impact of nutrient availability on the PI content is essential for understanding the development of cyanobacterial blooms. We propose that PIs might serve as temporary storage for N and that increasing NO_3^- concentrations can contribute to the development of *Microcystis* blooms directly by providing more resources for growth and indirectly by increasing the negative interference of *Microcystis* with their consumers due to an increased content of PIs.

1.6. References

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Chapter 2

Phosphate limitation increases content of protease inhibitors in the cyanobacterium *Microcystis aeruginosa*

corresponds to the peer-reviewed publication:

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2.1. Abstract

Increased anthropogenic nutrient input has led to the eutrophication of lakes and ponds, resulting in more frequent and severe cyanobacterial blooms worldwide. In particular, enhanced availability of phosphorus (P) can promote cyanobacterial mass developments and may affect the content of secondary metabolites, such as protease inhibitors (PIs), in cyanobacteria. PIs are common among cyanobacteria and have been shown to negatively affect herbivorous zooplankton. Here, we test the hypothesis that P limitation reduces the growth of *Microcystis*, but increases the content of PIs. In batch culture experiments with eight different initial phosphate concentrations (5 – 75 µM) we determined growth, stoichiometry, and PI content of *Microcystis aeruginosa* NIVA Cya 43. This strain produces the protease inhibitor BN920 that is converted by chlorination to CP954, which constitutes the major PI in this strain. C:N:P ratios of the biomass indicated variations of P limitation with treatment and time. When normalized to biomass, the PI content varied up to nearly nineteen-fold with treatment and time and was highest in the low P treatments, especially during the mid-exponential growth phase. However, these effects were alleviated under nitrogen co-limitation. The content of CP954 showed an inverse u-shaped response to growth rate and C/N ratio of the cyanobacterial biomass, whereas it increased with cyanobacterial C/P. The results indicate that P limitation supports a higher content of defensive PIs and may indirectly foster cyanobacterial blooms by increasing the negative interference of cyanobacteria with their consumers.

Keywords: Eutrophication – Freshwater – Protease inhibitors – Toxin production – Cyanotoxins

2.2. Introduction

The frequency of cyanobacterial blooms in lakes and ponds has increased due to eutrophication and rising water temperatures (Taranu et al. 2015; Lürling et al. 2017). Many bloom-forming cyanobacteria, such as *Microcystis*, produce a wide range of toxic secondary metabolites that pose an eminent threat to the public health (Jochimsen et al. 1998; Lenz et al. 2018) and the environment by impacting the food web and ecosystem functioning (Paerl and Otten 2013; Rigosi et al. 2014; Burford et al. 2019). This results in substantial economic costs as cyanobacterial blooms reduce lake services like drinking water quality, recreational usage or fisheries (Steffensen 2008).

Nutrient loading has been considered a major cause for the increasing number of cyanobacterial blooms. In freshwater systems, phosphorus (P) is traditionally seen as the limiting factor for productivity (Schindler et al. 2008), whereas nitrogen (N) is regarded as the limiting factor in marine ecosystems (Nixon 1995). Phosphate (PO_4^{3-}) is the most common P form on earth (Sterner and Elser 2008), but it is often immobilized and has therefore a low bioavailability (Reynolds and Davies 2001). Through the extensive use of P containing washing agents and fertilizers in agriculture, P is washed into ponds and lakes, where it promotes the development of cyanobacterial blooms. A prominent example is Lake Erie, where P input through the Maumee River has repeatedly lead to blooms of *Microcystis* in the western basin of the lake (Wilson et al. 2018). Reduction of P is therefore a widely used approach to reduce eutrophication and thus lower the probability of cyanobacterial blooms (Schindler and Fee 1974; Schindler et al. 2008). Despite attempts to reduce the input of P into freshwater systems, the effects of high P loads are still visible in many aquatic systems, and climate change may even increase the P input in the future (Forber et al. 2018; Wilson et al. 2018). However, recent research demonstrated that P is not always the only factor that determines the formation of cyanobacterial blooms but that N and the N/P ratio are also important, and at least in some cases, a reduction of N and P seems necessary to suppress cyanobacterial bloom formation (Xu et al. 2010; Glibert 2017; Newell et al. 2019).

Increasing nutrient concentrations do not only promote the development of blooms, they may also affect the toxicity of such blooms. A number of studies have investigated the effects of environmental factors such as pH, light, iron and major nutrients like N and P on the production and content of secondary metabolites (Sivonen 1990; Amé and Wunderlin 2005; Van de Waal et al. 2009; Van de Waal et al. 2014; Gobler et al. 2016; Burberg et al. 2018). Most of these studies focused on microcystin (MC) and MC-producing cyanobacteria. MCs are the most prominent group of cyanobacterial metabolites and are highly toxic, even for humans (Jochimsen et al. 1998). However, besides MCs there are a variety of other cyanobacterial

secondary metabolites, including the huge group of protease inhibitors (PIs, Köcher et al. 2019).

PIs are widespread among cyanobacterial taxa and were isolated from many cyanobacterial blooms (Agrawal et al. 2001; Patel et al. 2014). In drinking water treatment plants, PIs can reach loadings that are comparable to that of MCs, and these loadings are strongly correlated with the abundance of cyanobacteria (Beversdorf et al. 2018). This underlines the importance to analyze these compounds. Cyanopeptolines are a group of potent PIs, of which for example cyanopeptolin 1020 has been shown to inhibit human kallikrein (Gademann et al. 2010). Ahp-cyclodepsipeptides, including cyanopeptolines, have been reported in field samples (Adiv and Carmeli 2013; Vegman and Carmeli 2013; Beversdorf et al. 2017; Bartlett et al. 2018; Beversdorf et al. 2018), and more than 200 different Ahp-cyclodepsipeptides have been identified in cyanobacterial strains covering several genera (Köcher et al. 2019), which underlines that they are widespread.

However, PIs might not only pose a threat to human health, but as well affect trophic interactions in freshwater food chains. In lakes and ponds the microcrustacean *Daphnia* is a major consumer of planktonic primary production and serves as an important prey for planktivorous fish. Hence, the trophic transfer efficiency from primary producers to *Daphnia* is key to the trophic transfer of mass and energy in pelagic foodwebs in freshwaters (Von Elert et al. 2003). Cyanobacterial PIs reduce this trophic transfer by specifically inhibiting digestive proteases in the gut of *Daphnia* and thereby decreasing the growth of this herbivore (Lürling 2003; Schwarzenberger et al. 2010; Von Elert et al. 2012). This is in accordance with findings of Von Elert et al. (2004), who demonstrated that chymotrypsin and trypsin account for 80% of the total digestive protease activity in the gut of *Daphnia*.

More detailed investigations of the interaction of cyanobacterial PIs with *Daphnia* have been performed with a strain of *Microcystis aeruginosa* that produces two cyanopeptolins that specifically target chymotrypsins in the gut of *Daphnia*; these inhibitors have been identified as cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920, Von Elert et al. 2005). Although many aspects of the interference of these two cyanopeptolin PIs with *Daphnia* physiology are understood, effects of nutrient concentrations on the cellular content of these two inhibitors have not been investigated yet (except for Burberg et al. 2018), although this strain is ideally suited for such approaches, as it does not produce MCs (Von Elert et al. 2005).

Changes in resource availability may affect cyanobacterial blooms directly by providing more nutrients, but also indirectly by changing their cellular toxin content and thus altering the negative interference of cyanobacteria with their grazers (Van de Waal et al. 2009; Burberg et al. 2018). In this study, we evaluated the effect of P availability on the growth and the content of the two cyanopeptolin PIs CP954 and BN920 in *M. aeruginosa*. In batch culture growth

experiments with eight different initial P concentrations we quantified cell numbers and particulate organic carbon over time and used a logistic growth model to determine growth rates at all time points. We further determined the C:N:P stoichiometry of the cyanobacterial biomass and quantified the content of CP954 and BN920 using UHPLC coupled to a high-resolution mass-spectrometer. The two PIs CP954 and BN920 are N-rich secondary metabolites that do not contain P. We hypothesized that P limitation of the cyanobacterium would result in higher availability of N for synthesis and hence a higher biomass content of CP954 and BN920.

2.3. Material and Methods

2.3.1. Culturing conditions

Microcystis aeruginosa NIVA Cya 43 (NORCCA, Norwegian Institute for Water Research) was grown in pre-cultures for 9 days in 1 L Erlenmeyer flasks filled with 400 mL modified WC medium (Guillard 1975). The culture was kept under constant light (45 ± 3 µmol photons m^{-2} s^{1}) and temperature (20 ± 1°C) on a horizontal shaker (90 rpm). *M. aeruginosa* was pre-cultured at 10 µM initial phosphate (PO_4^{3-}) to deplete the internal P reserves of the cyanobacterium prior to the experiment. Unlike some other *Microcystis* strains NIVA, Cya 43 does not produce colonies or microcystins, but it does produce cyanopetolin 954 (CP954) and nostopeptin 920 (BN920, Von Elert et al. 2005), which are two nitrogen-rich protease inhibitors (PIs). The cultures were not axenic, but heterotrophic bacteria made up just a small percentage compared to the cyanobacterial biomass (Burberg et al. 2018).

In the experiment eight initial PO_4^{3-} concentrations in a range from 5 to 75 µM with 3 – 4 replicates each were tested. Therefore, 400 mL of the respective medium were filled into 1 L flasks, autoclaved and inoculated with *M. aeruginosa* ($1.5 \cdot 10^5$ cells mL^{-1}), which is similar to the inocula used by Long (2001). The experiment lasted for 28 days, and the flasks were randomized daily to compensate for potential heterogeneities in the light regime. Every 1 – 2 days, samples (0.5 mL) were taken and if necessary diluted for cell counts using a Neubauer improved counting chamber. Per sample, at least 100 – 150 cells (at low densities) or 3 large squares (at high densities) were counted to ensure appropriate accuracy. In intervals of 2 – 4 days, between 10 and 200 mL were taken to measure the particulate organic carbon (POC), nitrogen (PON) and phosphorus (P_{Part}) as well as the two PIs. The volume needed for the analyses was roughly estimated based on the cell density.

2.3.2. Determination of POC, PON and P_{Part}

POC and PON were measured by filtering a sample volume equivalent to approx. 0.25 mg POC on pre-combusted GF/F filters (Macherey & Nagel), which were subsequently dried at 60°C for at least 24 hours. The filters were packed into tin capsules and analyzed using a Flash EA2000 Analyzer (Thermo Fisher). For the analysis of P_{Part}, 0.5 mg C was filtered on GF/F filters, transferred into 10 mL of a potassium peroxodisulphate and sodium hydroxide and autoclaved for 1 hour at 120°C; soluble reactive P was subsequently analyzed with the molybdate-ascorbic acid method (Greenberg et al. 1985) with a DR5000 UV-Vis spectrometer (Hach). The obtained values were used to calculate the molar stoichiometric ratios (C/N, C/P & N/P) of the cyanobacterial biomass.

2.3.3. Extraction and quantification of PIs

PIs were extracted and measured according to Burberg et al. (2018). Briefly, samples (approx. 0.25 mg C) were centrifuged (5 minutes). The supernatant was discarded and 10 mL methanol (80%) as well as 10 µL of the internal standard (microcystin LR, 10 µg mL^{-1}, Enzo Life Sciences) were added to the cell pellet. The samples were re-suspended, sonicated and again centrifuged for (3 minutes, 4500 g). The supernatant was evaporated to dryness, re-dissolved in 1 mL methanol (100%) and again dried and taken up in methanol (100%, 100 µl). Finally, the samples were centrifuged (2 minutes, 20000 g), and the supernatant was transferred into HPLC vials.

PIs were quantified using an ultra-high-pressure liquid chromatography system (UHPLC, Accela, ThermoFisher Scientific) coupled with an Exactive Orbitrap mass spectrometer (MS, ThermoFisher Scientific). The chromatographic separations were carried out on a C_{18}-column (Nucleosil, 125/2, 100-3, Macherey and Nagel) as stationary phase with acetonitrile (A) and ultra-pure water (B), each containing 0.05% trifluoracetic acid (TFA) as mobile phase with the following gradient: 0 min: 20% A, 14 min: 100% A, 16 min: 100% A, 16.5 min: 20% A, 18 min: 20% A. The column temperature was set to 30°C, the flow rate was 300 µL min^{-1} and the injection volume of the sample was 10 µL.

The MS was operated according to Burberg et al. (2018). Under the applied conditions the two PIs form two positively charged adduct ions ($[M+H-H_2O]^+$; $[M+Na]^+$) with m/z = 903.46108 and 943.45331 (BN920 adducts) and m/z = 937.42211 and 977.41394 (CP954 adducts). For further calculations and analyses the single adducts of each PI were summed up to ´BN920´ and ´CP954´. MC-LR ($[M+H]^+$) was measured at m/z = 995.55604. Peak intensities were extracted from the chromatograms using the R-package ´enviMass´ (Loos 2018) and converted to concentrations via previous established calibration curves. Subsequently, the PI concentrations in the samples were normalized to the culture volume or to the extracted POC, as proxy for the cyanobacterial biomass.

2.3.4. Modeling and statistical analyses

All statistical analyses were performed using R (R Core Team 2018) and RStudio (RStudio Team 2016). Our expectation was that *Microcystis* shows logistic growth. A logistic (sigmoidal) growth curve starts with an initial exponential growth phase followed by a mid-exponential and then a stationary phase when one or more resources, e.g. P, become limiting. The R–package ‘growthrates‘ (Petzoldt 2017) was used to fit logistic growth models based on POC and *Microcystis* cell abundance. The model equation was

$$N_t = (K' \cdot N_0) / (N_0 + (K' - N_0) \cdot e^{-\mu_{max} \cdot t}) \quad (1)$$

with N_0 as the initial biomass or cell abundance, N_t as the biomass or cell abundance at time (t) and the two model parameters carrying capacity (K') and maximal growth rate (μ_{max}). Effects of the initial PO_4^{3-} concentration on K' and μ_{max} were tested using one-way analysis of variance (ANOVAs) followed by Tukey HSD *post hoc* tests. Residuals were tested for normal distribution (Shapiro-Wilk's test) and variance homogeneity (Levene's test). Growth rates were taken from the logistic growth model for POC, and are therefore based on changes in POC. Thus, they may be referred to as C net production rate. The growth rates were correlated to the PI content, which allowed us to analyze how the inhibitor content was affected by the growth rate. The effects of sampling day, initial PO_4^{3-} concentration and sampling day : PO_4^{3-} concentration on P_{Part}, stoichiometry and PI content were tested applying linear mixed effects models (LME) using the R-package 'lme4' (Bates et al. 2015).

LME models were also used to analyze the relationship between PIs and growth rate as well as between PIs and elemental molar ratios. Natural logarithm (ln) respectively square root transformation was used for stabilization of variance and normality of residuals so that the transformed linear regressions can be written as: $\ln y = a + b \sqrt{x}$ for the growth rate and $\ln y = a + b \ln x$ for elemental ratios. To account for a potential nonlinear relationship, a quadratic term was employed (Bates et al. 2015):

$$ln\ y = a + b\ ln\ x + c\ (ln\ x)^2 \quad (2)$$

in case of a log transformed independent variable (x) and

$$ln\ y = a + b\sqrt{x} + c\ (\sqrt{x})^2 = a + b\sqrt{x} + cx \quad (3)$$

for square root transformed variables. To account for pseudo-replication within trials, combinations between treatment and replicates were treated as random effect (individual slopes for all 28 combinations of treatment and replicate), while intercept (a), slope (b) and quadratic term (c) describe dependency on the fixed-effect variable ln x respectively $\sqrt{x}$. Models with increasing complexity were fitted, a null model with only the random effects, a linear model, and a quadratic model (Tab. 5). *AIC* (Akaike information criterion) values are given as an indicator of model adequacy. *P*-values were estimated by likelihood ratio tests (Chi^2) between consecutive models (i.e., between null model and linear model resp. between quadratic and linear model). The coefficient of determination (R^2) accounts for the explanatory contribution of the fixed effect of the models only. In contrast, the Pearson correlation coefficient R_P measures direct linear dependency of the transformed data. The effectivity of the transformations was checked graphically: normality of residuals with quantile-quantile plots and variance homogeneity by plotting residuals versus fitted values.

For a comparison of PI content and cellular nutrient quota within growth phases and between P regimes (high > 30 µM, medium = 15 – 30 µM, low < 15 µM), data from each combination of growth period and phosphorus treatment were pooled while retaining individual experimental units as replicates. Logistic growth was subdivided into three phases (early exponential, mid-exponential and late growth) at time points where the modeled POC approached 25% resp. 75% of the carrying capacity (*K'*). The calculation was done for each treatment and replicate separately. Pairwise differences between combinations of growth phase and P regime were tested *post hoc* using generalized linear hypothesis tests of linear models (Hothorn et al. 2008), with P-regime, growth period and their interaction as explanation variables. A letter-based representation was used to indicate significant and non-significant pairwise comparisons (Piepho 2004) at a significance level of 0.05.

2.4. Results

In batch culture experiments different initial phosphate (PO_4^{3-}) concentrations were tested for their effects on the growth performance, stoichiometry and content of two nitrogen (N)-rich protease inhibitors (PIs), nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954), in *Microcystis aeruginosa* strain NIVA Cya 43.

2.4.1. Growth performance of *M. aeruginosa* under different phosphate conditions

Under all tested PO_4^{3-} concentrations, *M. aeruginosa* showed logistic growth, regardless whether growth was measured as cell abundance or particular organic carbon (POC; Fig. 1 A & B). The carrying capacity (*K'*; Tab. 1 & 2) was lowest at 5 µM phosphorus (P) and reached only 20% of the biomass that was obtained with the highest P concentration. This indicates a growth limitation at very low P concentrations. The strength of the inhibition seems to decrease with increasing P concentration, as both *K'* and maximal growth rate (μ_{max}) increased with initial P concentration, when growth was measured as POC (Tab. 2). When growth was modeled using the cell abundance, *K'* was significantly lower at 5 and 10 µM P, while μ_{max} showed no clear tendency in response to the P concentration (Tab. 1). Except for the 30 µM P treatment, the biomass, determined as POC, remained stable towards the end of the experiment.

In all treatments, the particulate phosphorus (P_{Part}) increased during the experiment (Fig. 1 C). The linear mixed model showed that especially the sampling day as well as the treatment : day interaction had an impact on P_{Part}, while the treatment alone had no significant effect on P_{Part} (Tab. 3). However, at the end of the experiment (day 28) P_{Part} was highest at the highest P concentration and lowest at 5 µM P (Fig. 1 C, Tukey HSD *post hoc* test after one-way ANOVA, $F = 28.78$, $p < 0.001$).

Table 1. Modeled growth parameters of *M. aeruginosa* grown with 8 different initial phosphate (PO_4^{3-}) concentrations. The model was based on cell abundance (Figure 1 A). Mean values (± SD, n = 3 – 4) of the initial cell abundance (N_0), carrying capacity (*K'*) and maximal growth rate (μ_{max}) were determined and tested for differences between treatments (Tukey HSD *post hoc* test after one-way ANOVA, $p = 0.05$). Capital letters indicate significant differences between treatments.

PO_4^{3-} [µM]	N_0 [10^4 cells mL^{-1}]	K' [10^4 cells mL^{-1}]	μ_{max} [d^{-1}]	R^2 [a]
5	24.7 ± 3.3	1022 ± 63 A	0.34 ± 0.03 AB	0.82
10	14.8 ± 4.5	1272 ± 172 A	0.33 ± 0.02 AB	0.87
15	18.2 ± 18.7	4718 ± 362 C	0.45 ± 0.09 A	0.96
20	7.28 ± 7.24	4284 ± 247 BC	0.44 ± 0.06 A	0.97
30	51.7 ± 14.8	4328 ± 381 BC	0.27 ± 0.04 B	0.94
40	62.9 ± 32.6	3729 ± 516 B	0.25 ± 0.04 B	0.92
50	7.9 ± 6.5	4129 ± 484 BC	0.43 ± 0.1 A	0.93
75	23.9 ± 19.9	4312 ± 239 BC	0.33 ± 0.06 AB	0.94

[a] General fit equation: $N_t = (K' \cdot N_0) / (N_0 + (K' - N_0) \cdot e^{-\mu_{max} \cdot t})$ with t = time [d], N_t = Cell abundance at time t [cells mL^{-1}].

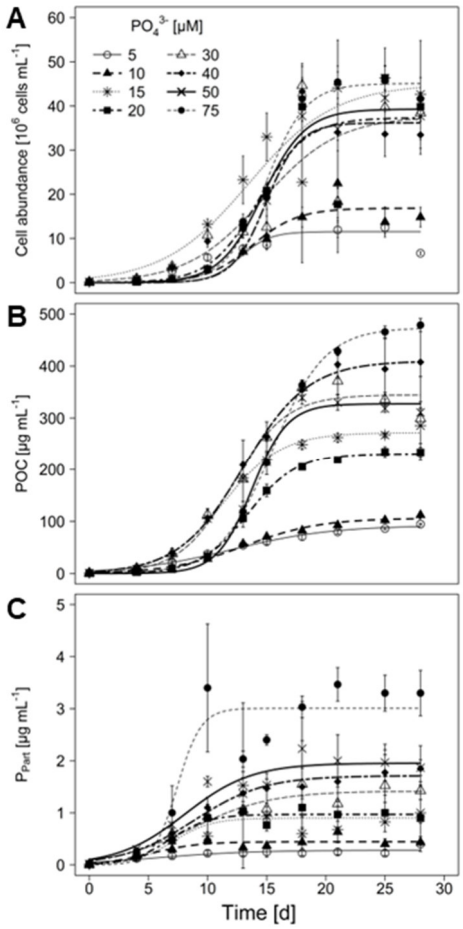

Figure 1. Cell abundance (**A**), particulate organic carbon (POC, **B**), and particulate phosphorus (P_{Part}, **C**) in batch culture growth experiments of *M. aeruginosa* with 8 different initial PO_4^{3-} concentrations. Mean ± SD, n = 3 – 4. Displayed curves were fitted using a logistic growth model. Model parameters for A and B are given in Tab. 1 and 2.

Table 2. Modeled growth parameters of *M. aeruginosa* grown with 8 different initial PO_4^{3-} concentrations. The model was based on POC (Fig. 1 B). Mean values (± SD, n = 3 – 4) of the initial POC concentration (N_0), carrying capacity (K') and maximal growth rate (μ_{max}) were determined and tested for differences between treatments (Tukey HSD *post hoc* test after one-way ANOVA, p = 0.05). Capital letters indicate significant differences between treatments.

PO_4^{3-} [µM]	N_0 [µg C mL^{-1}]	K' [µg C mL^{-1}]		μ_{max} [d^{-1}]		R^2 [a]
5	5.02 ± 1.17	93.1 ± 3.6	A	0.24 ± 0.03	A	0.98
10	1.17 ± 0.13	106.2 ± 4.6	AB	0.32 ± 0.004	AB	0.98
15	2.92 ± 1.62	272.9 ± 9.2	CD	0.41 ± 0.04	BCD	0.99
20	0.32 ± 0.18	230.8 ± 5.5	BC	0.5 ± 0.03	D	0.99
30	1.07 ± 0.84	345.1 ± 20.4	E	0.49 ± 0.05	D	0.92
40	3.82 ± 2.33	415 ± 67.3	F	0.37 ± 0.07	BC	0.97
50	0.03 ± 0.008	327.5 ± 6.8	DE	0.67 ± 0.02	E	0.99
75	0.5 ± 0.28	473.4 ± 9.6	F	0.45 ± 0.04	CD	0.99

[a] General fit equation: $N_t = (K' \cdot N_0) \,/\, (N_0 + (K' - N_0)\, e^{-\mu max \cdot t})$ with t = time [d], N_t = Cell abundance at time t [cells mL^{-1}].

Table 3. Linear mixed-effects model for the particulate phosphorus (P_{Part}, Fig. 1 C) and biomass stoichiometry (C/N, C/P & N/P; Fig. 2) of *M. aeruginosa* during a 28 days growth experiment. Mean Sq: Mean square, NumDF: Numerator degrees of freedom, DenDF: Denominator DF, Test statistics: *F*- and *p*-value.

	Mean Sq	NumDF	DenDF	*F*	*p* [a]	
P_{Part}						
Treatment	1.18e-07	7	261.25	0.6286	0.7321	
Day	5.27e-05	1	260.22	280.27	<2e-16	***
Treatment : Day	3.53e-06	7	260.22	18.74	<2e-16	***
C/N ratio						
Treatment	138.742	7	197.87	67.057	< 2.2e-16	***
Day	250.839	9	197.07	121.265	< 2.2e-16	***
Treatment : Day	26.879	63	197.07	12.994	< 2.2e-16	***
C/P ratio						
Treatment	614724	7	197.02	54.6476	< 2.2e-16	***
Day	1364784	9	197.22	121.3772	< 2.2e-16	***
Treatment : Day	32240	63	197.22	2.8673	1.323e-08	***
N/P ratio						
Treatment	12892.4	7	197.97	110.690	< 2.2e-16	***
Day	8209.8	9	197.06	70.502	< 2.2e-16	***
Treatment : Day	724.3	63	197.06	6.220	< 2.2e-16	***

[a] Significance levels: *** < 0.001

2.4.2. Stoichiometry of *M. aeruginosa* under different phosphate conditions

The elemental molar ratios (C/N, C/P & N/P) of the cyanobacterial biomass showed high flexibility throughout the experiment and were affected by the treatment (P concentration), the sampling day and the interaction of treatment and sampling day (Tab. 3). The C/P ratio increased independent from the initial P concentration up to ratios of 300 and more (Fig. 2 B), indicating an ongoing P limitation in all treatments. The highest C/P ratios were reached with 5 and 15 µM initial P, while the lowest were found in the 75 µM treatment. The C/N ratio increased with initial P concentrations greater than 15 µM (Fig. 2 A), while below 15 µM P the C/N ratio remained around the Redfield ratio (C/N = 6.6). This indicates N limitation (C/N > 20) at the highest and no N limitation at the low P concentrations. The N/P ratio increased especially if the cyanobacterium was grown at low P availability, but was around the Redfield ratio at the highest P concentration (Fig. 2 C). This points at an excess of N, which might be available for the production of secondary metabolites, under low P.

Cellular carbon (C) was grouped according to growth phase and P regime (low, intermediate and high), to estimate if changes in cell size of *Microcystis* occurred (Fig. 3 A). Even though not significant, cellular C changed slightly, depending on P regime and growth phase. Especially during the mid-exponential phase, cellular C was significantly higher in the high P treatments compared to the low P treatments, indicating that during this growth phase cell size increased with increasing P concentrations. Additionally, cellular C decreased from the mid-exponential to the late growth phase in the high P treatments, which might suggest

that *Microcystis* became light-limited. Cellular N decreased significantly in high and medium P treatments during growth (Fig. 3 B) and was nearly 1.5 fold higher in the low P treatments (< 15 µM) than in the high P treatments (> 30 µM) during the late growth phase. Cellular P increased with initial P during the early exponential phase (Fig. 3 C) and decreased later on in all treatments pointing at initial P storage.

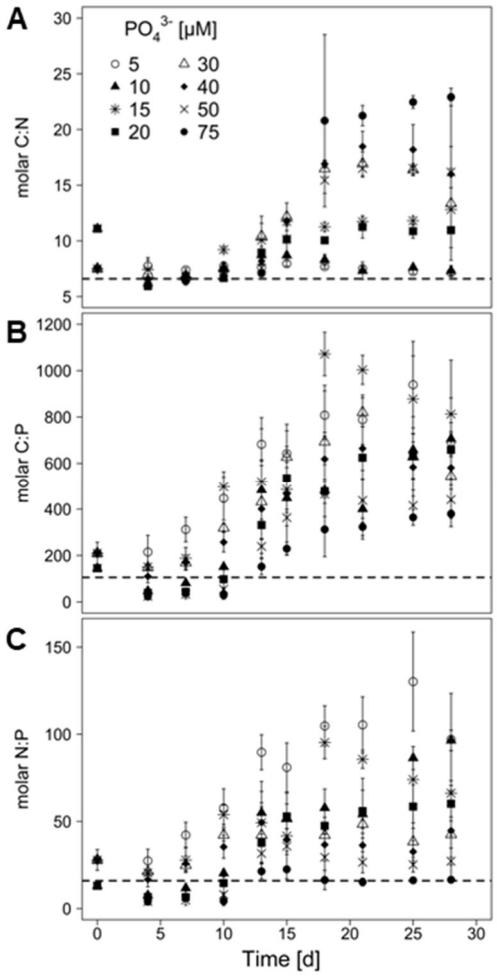

Figure 2. Elemental molar ratios C/N (**A**), C/P (**B**) and N/P (**C**) of the biomass of *M. aeruginosa* grown on 8 different initial of PO_4^{3-} concentrations. Mean ± SD, n = 3 – 4. The dashed horizontal lines indicate the respective Redfield ratio.

2.4.3. Inhibitor content of *M. aeruginosa* under different phosphate conditions

M. aeruginosa constitutively produced the two PIs BN920 and CP954, which accounted for 2.5 to 10% of the total cyanobacterial carbon during the experiment, depending on the day and the treatment. This indicates the high biological relevance of PIs for this *M. aeruginosa* strain.

The PI content of the cyanobacterium was determined by normalizing the measured PI amount to POC, which served as a measure of cyanobacterial biomass. The PI content of *M. aeruginosa* changed during the experimental time, with the highest contents between day 10 and 15 (Fig. 4). The patterns for the single inhibitors were similar, even though changes in

the content of BN920 came slightly ahead of changes in the CP954 content. The content of BN920 varied up to elevenfold and that of CP954 by nearly nineteen-fold with initial P concentration, time, and their combined effect (Tab. 4) showing that the dynamics in the PI content over time differed with initial P concentration. In particular, the content of CP954, the higher concentrated PI, was highest in the low-P treatments on most days, indicating that low P concentrations can lead to a higher PI content. When these data were grouped according to growth phase and P regime (Fig. 5), the content of both PIs was maximal in the low P treatments (< 15 µM) during mid-exponential growth phase. Additionally, there was a tendency for the PI content to decrease from low to high P treatments during mid-exponential and late growth, which was not the case during the early exponential phase.

The PI content per cell showed a similar pattern as the content per cyanobacterial biomass (data not shown), ranging from 14.3 to 199.5 pg cell^{-1} for BN920 and from 52 to 725 pg cell^{-1} for CP954.

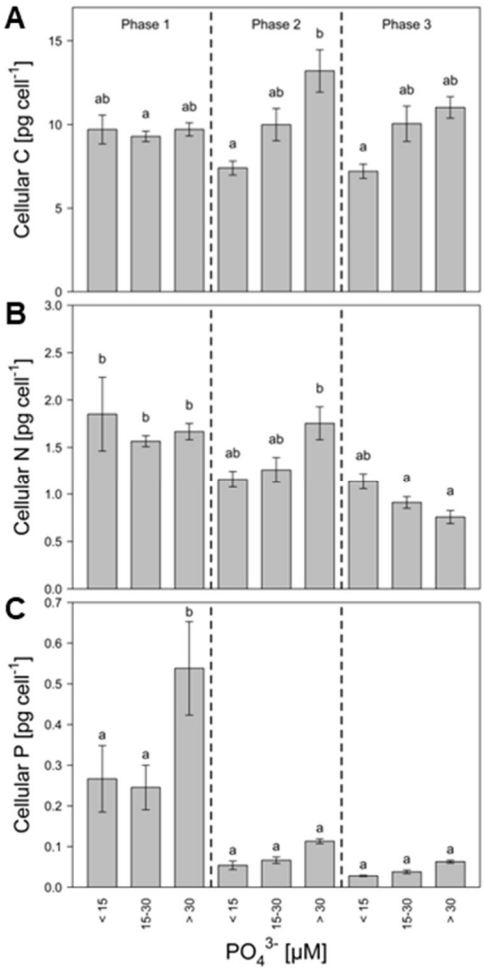

Figure 3. Cellular quota of carbon (**A**), nitrogen (**B**) and phosphorus (**C**) of *M. aeruginosa* during early exponential (phase 1), mid-exponential (phase 2), and late growth (phase 3) in treatments with low (< 15 µM), medium (15-30 µM) and high (> 30 µM) initial P concentrations. Bars denoted by the same letter are not significantly different (α = 0.05), whiskers indicate standard errors.

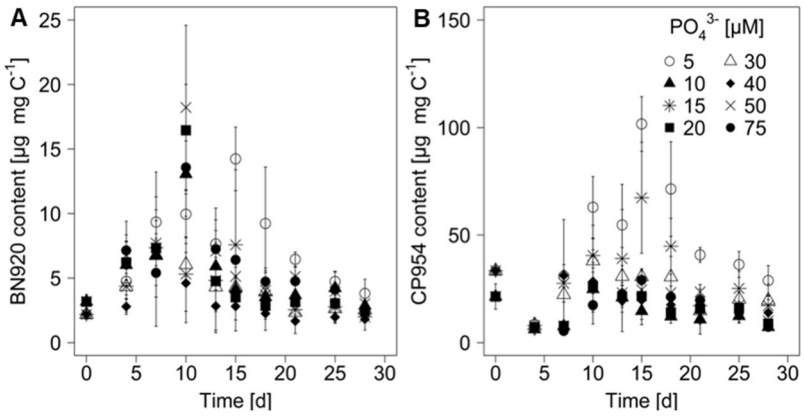

Figure 4. Protease inhibitor content of nostopeptin 920 (BN920, **A**) and cyanopeptolin 954 (CP954, **B**) in the biomass of *M. aeruginosa.* The inhibitor content was normalized to the particulate organic carbon (mg C), which served as a proxy for cyanobacterial biomass. Mean ± SD, n = 3 – 4.

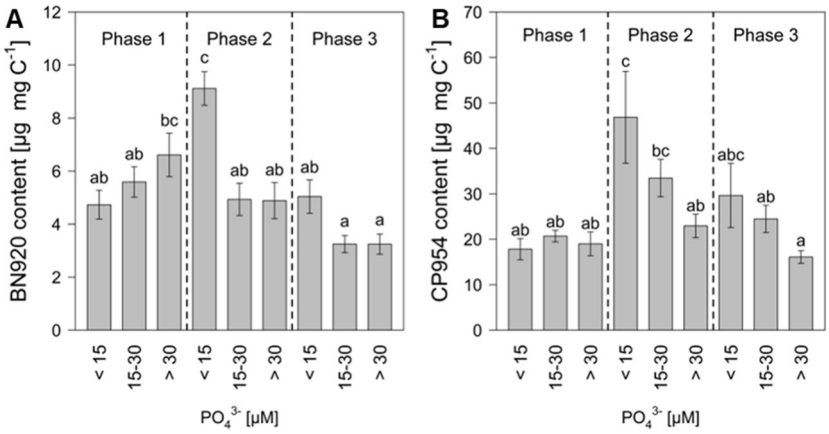

Figure 5. Protease inhibitor content of nostopeptin 920 (BN920, **A**) and cyanopeptolin 954 (CP954, **B**) of *M. aeruginosa* during early exponential (phase 1), mid-exponential (phase 2), and late growth (phase 3) in treatments with low (< 15 µM), medium (15-30 µM) and high (> 30 µM) initial P-concentrations. Bars denoted by the same letter are not significantly different (α = 0.05), whiskers indicate standard errors.

Table 4. Linear mixed-effects model for effects of treatment and sampling day on the protease inhibitor content of BN920 and CP954 in *M. aeruginosa*. Mean Sq: Mean square, NumDF: Numerator degrees of freedom, DenDF: Denominator DF, Test statistics: *F*- and *p*-value.

	Mean Sq	NumDF	DenDF	*F*	*p* [a]	
BN920						
Treatment	71.715	7	197.36	18.2154	< 2.2e-16	***
Day	172.494	9	197.14	43.8287	< 2.2e-16	***
Treatment x Day	14.333	63	197.14	3.6419	2.744e-12	***
CP954						
Treatment	4408.5	7	197.47	53.8364	< 2.2e-16	***
Day	2508.0	9	197.12	30.6383	< 2.2e-16	***
Treatment x Day	342.5	63	197.12	4.1837	8.755e-15	***

[a] Significance levels: *** < 0.001

2.4.4. Inhibitor content as a function of growth rate and stoichiometry

It is characteristic for logistic growth that the instantaneous specific growth rate changes over time. In this experiment, specific growth rates were obtained by applying a logistic growth model to the POC data (Fig. 1 B) and therefore might be referred to as C net production rate. Growth rates were then used to test how the content of each PI behaved as a function of the growth rate of *Microcystis* (Fig. 6). For this purpose, mixed effects models with linear and quadratic terms were fitted to the data. In case of BN920, the quadratic model was slightly better than the linear model in describing the relationship of BN920 and growth rate, as indicated by a smaller *AIC* and a minimal increase of R^2 (Tab. 5). For CP954 the linear model was not significant (p = 0.83). Instead, the quadratic model was substantially better suited as indicated by a lower *AIC* and a higher R^2. Accordingly, the CP954 content showed a reversed U–shaped (optimum) relationship with the growth rate, reaching a maximum at growth rates of 0.15. However, though significant, the models explained only a low degree of variation within our data (see R^2, Tab. 5).

The PI content was also plotted as a function of the stoichiometric ratios of the cyanobacterial biomass to test how the stoichiometric ratios affected the PI content of *M. aeruginosa.* Linear models significantly described the relationship between BN920 and the elemental stoichiometric ratios (Tab. 5), even though the quadratic model was slightly better suited in case of C/N (lower *AIC*, higher R^2). In case of CP954, all applied models were significant, except for the linear model with C/N. The lower *AIC* values indicate that the quadratic model described the relationship of CP954 and C/N much better, while the linear model was sufficient to describe the relationship between CP954 and N/P. More specific, the content of BN920 decreased with increasing C/N ratios (Fig. 7 A, Tab. 5); the CP954 content however increased with the C/N ratio until a C/N ratio of around 11 and declined at higher C/N ratios (reversed u-shaped curve; Fig. 7 B, Tab. 5). This indicates that N depletion of the cyanobacterium resulted in decreased PI contents. When plotted against the C/P (Fig. 7 C & D, Tab. 5) and N/P ratios (Fig. 7 E & F, Tab. 5) we found that the contents of BN920 and CP954 were affected differently. While the BN920 content decreased with increasing C/P and N/P ratios, the CP954 content increased. As CP954 makes up a larger proportion of the total PI content, a lower P regime might promote the biomass content of PIs.

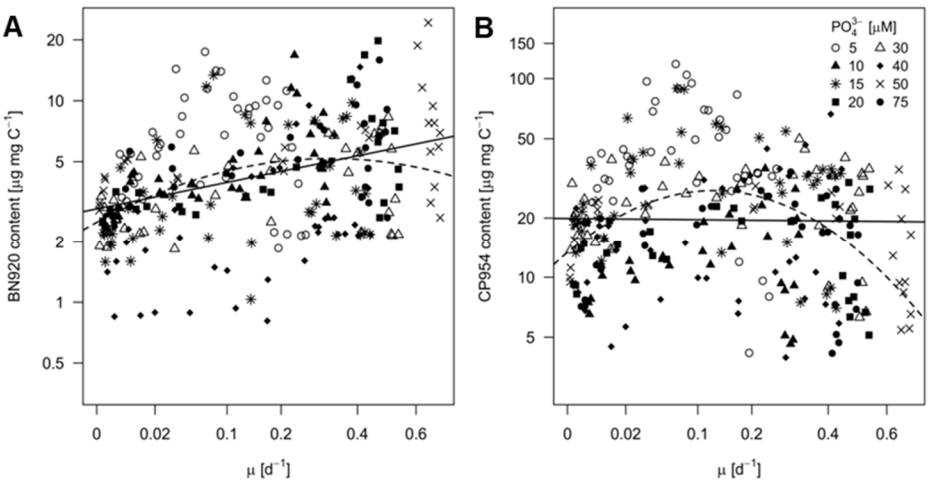

Figure 6. Protease inhibitor content of nostopeptin 920 (BN920, **A**) and cyanopeptolin 954 (CP954, **B**) as a function of the growth rate (μ) of *M. aeruginosa*. Growth rates were taken from the logistic growth model for POC (see Fig. 1 B). Each point represents the measured inhibitor content in a single replicate at a single sampling day and the corresponding specific growth rate. Different symbols represent the different treatments. The x-axis was square root and the y-axis natural logarithmic transformed. Linear (solid lines) and squared (dashed lines) regression models were applied. Equations, R^2- and *p*-values are shown in Tab. 5.

Table 5. Equations, parameters and significance tests for the regression models applied to the data shown in Fig. 4 and 5. Data were natural logarithmic (ln) resp. square root transformed and then analyzed using linear mixed-effects models with a single explanation variable (x) as fixed effect (intercept, slope and quadratic term) and individual slopes for the 28 treatment : replicate combinations. The null model (–) contains the same random effects but omitted the explanation variable. P-values indicate significant likelihood ratio (Chi^2) of consecutive models (i.e. between linear and null model resp. between quadratic and linear model). R_p: Pearson correlation coefficient, calculated from the transformed x and y data. R^2: coefficient of determination of the mixed model (fixed effects only). *AIC*: Akaike-Information-Criterion.

	Equation	R_p	R^2	*AIC*	*p* [a]	
BN920						
–	ln y = 1.576			497.1		–
μ_{max}	ln y = 1.072 + 0.9555 $\sqrt{x}$	0.37	0.13	493.1	1.00e-06	***
μ_{max}	ln y = 0.9178 + 2.578 $\sqrt{x}$ - 2.28 x		0.14	485.3	0.0036	**
C/N ratio	ln y = 3.098 - 0.7396 ln x	-0.43	0.18	442.3	1.20e-14	***
C/N ratio	ln y = 6.426 - 3.612 ln x + 0.6036 (ln x)^2		0.21	439.7	0.018	*
C/P ratio	ln y = 2.916 - 0.2622 ln x	-0.38	0.14	449.0	2.00e-13	***
C/P ratio	ln y = 3.179 - 0.3684 ln x + 0.01027 (ln x)^2		0.14	456.3	0.7	
N/P ratio	ln y = 2.301 - 0.2586 ln x	-0.24	0.05	473.6	4.60e-08	***
N/P ratio	ln y = 2.838 - 0.6473 ln x + 0.06414 (ln x)^2		0.09	477.5	0.081	
CP954						
–	ln y = 2.768			549.1		–
μ_{max}	ln y = 2.989 - 0.03925 $\sqrt{x}$	-0.07	0.002	597.5	0.83	
μ_{max}	ln y = 2.591 + 4.081 $\sqrt{x}$ - 5.75 x		0.11	557.9	2.40e-10	***
C/N ratio	ln y = 2.602 + 0.1626 ln x	-0.009	-0.01	565.0	0.16	
C/N ratio	ln y = -7.357 + 8.758 ln x - 1.807 (ln x)^2		0.10	533.8	5.60e-09	***
C/P ratio	ln y = 1.736 + 0.2134 ln x	0.37	0.13	533.5	1.50e-07	***
C/P ratio	ln y = -1.478 + 1.515 ln x - 0.126 (ln x)^2		0.14	524.6	6.10e-05	***
N/P ratio	ln y = 1.896 + 0.3076 ln x	0.42	0.18	519.5	1.70e-09	***
N/P ratio	ln y = 1.135 + 0.8565 ln x - 0.09022 (ln x)^2		0.17	521.1	0.026	*

[a] Significance levels: '***' < 0.001, '**' < 0.01, '*' < 0.05.

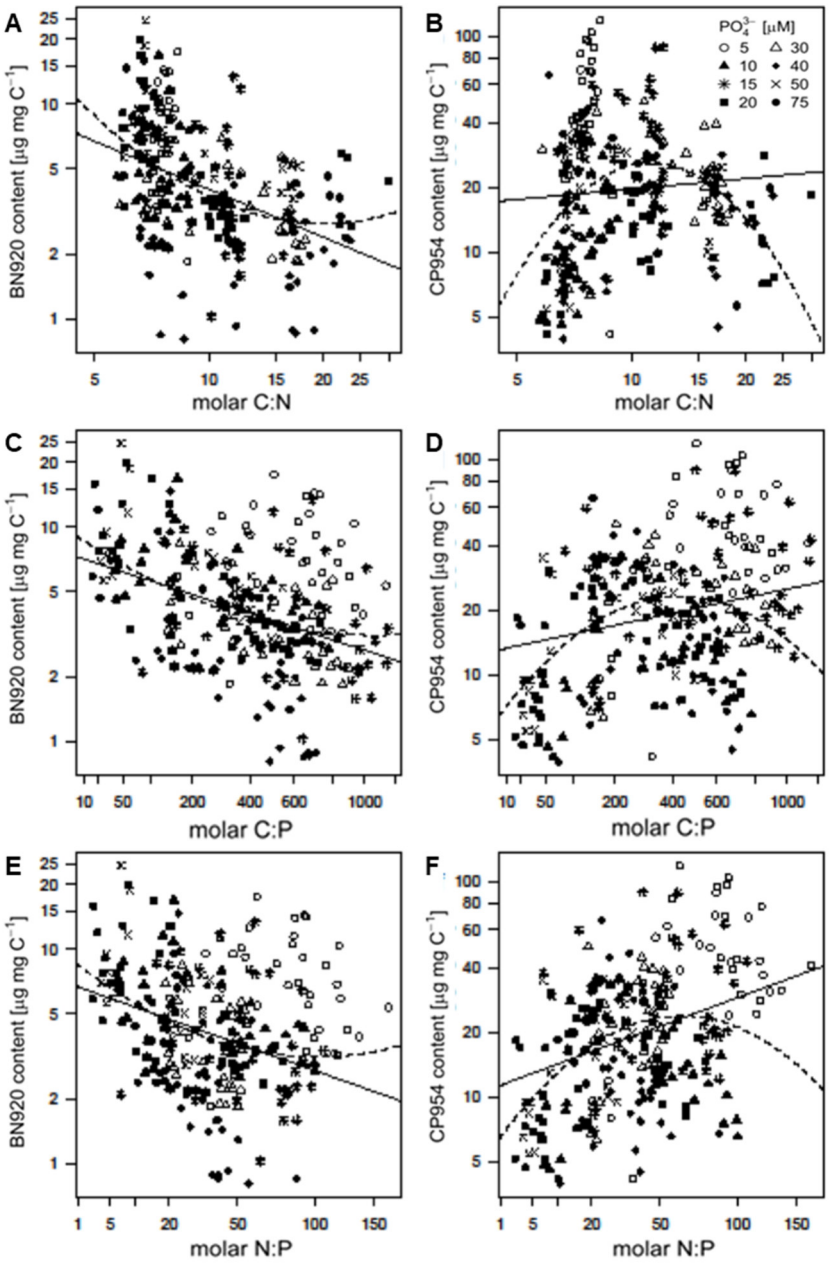

Figure 7. Protease inhibitor content of nostopeptin 920 (BN920; **A, C, E**) and cyanopeptolin 954 (CP954; **B, D, F**) as a function of the molar ratios between carbon, nitrogen and phosphorus of *M. aeruginosa*. Each point represents the measured inhibitor content in a single replicate at a single sampling day and the corresponding stoichiometric ratio. Different symbols represent the different treatments. Both axes were natural logarithmic transformed. Linear (solid lines) and squared (dashed lines) regression models were applied. Equations, R^2- and *p*-values are shown in Tab. 5.

2.5. Discussion

The eutrophication of freshwater systems with nitrogen (N) and phosphorus (P) is the main factor promoting cyanobacterial biomass and biovolume (Patel et al. 2014; Rigosi et al. 2014). In particular, P loading plays an essential role in lakes and ponds, as many of these systems are limited by P availability (Schindler et al. 2008). It has been suggested that global warming will increase bloom frequencies directly by promoting cyanobacterial growth (Huisman et al. 2018) and indirectly via enhanced release of P from sediments (Jeppesen et al. 2009). However, it remains controversial to which degree this putatively increased P availability will shift primary production from P to N limitation in freshwater systems. Increased P availability may be counteracted by regional N deposition from the atmosphere, which has been shown to shift more lakes to P limitation (Elser et al. 2009); on the other hand, internal processes like denitrification may compensate for additional N input (Paerl et al. 2016). The frequencies of N or P limitation in freshwater systems are similar, and quite often co-limitation by N and P is controlling cyanobacterial growth (Elser et al. 2007; Paerl et al. 2016).

Here we tested 5 – 75 µM initial P concentrations in batch culture experiments, in which, due to the logistic growth, the degree of resource-limitation changes with time. Our results demonstrate that *Microcystis aeruginosa* strain NIVA Cya 43 reached its maximum biomass at the highest P concentration with biomasses decreasing with decreasing initial P, which indicates a strong P limitation. This is supported by the very low cellular P contents that were reached in the late growth phase. Except for the lowest P treatments (5 – 10 µM), particulate phosphorus (P_{Part}) had already reached its maximum when biomass was still further increasing. This decoupled increase of biomass points at the well-known rapid uptake of available P and its internal storage as polyphosphate with a subsequent re-mobilization of polyphosphate for further biomass synthesis and growth in cyanobacteria (Jacobson and Halmann 1982; Huisman et al. 2018).

In line with this, we observed increasing carbon to phosphorus (C/P) ratios of biomass over time. The inoculum for the batch culture growth experiment had been pre-cultivated under P limiting conditions. The initial very low P_{Part} content per carbon (0.015 mg P / mg C), which is equivalent to 1.5% per dry weight) matches values reported for P limited *M. aeruginosa* (Jacobson and Halmann 1982), and the initial C/P values of 145 and 210 (Fig. 2 B) corroborate that internal polyphosphate pools had been depleted prior to the experiment.

In all treatments C/P increased over time which corroborated the well-known stoichiometric plasticity of photoautrophs (Sterner and Elser 2008). Initial C/P ratios roughly matched the Redfield ratio (C:N:P = 106:16:1, Redfield 1958), which is an indicator for non-limiting growth conditions. However, over time, C/P ratios increased and reached maxima

ranging from 300 (initially high P) to 1100 (initially low P), which indicates that in all treatments *M. aeruginosa* was P-limited, though to differing degrees. This is further supported by the cellular P quota, which were lower at low P concentrations. The only exceptions to this increase over time are C/P ratios in early samples of high-P treatments, in which C/P ratios dropped initially. This points at initial luxury consumption of P and a temporary internal P storage, probably as polyphosphate. Cellular N and P quota were also much higher in the early exponential growth phase, underlining that maybe not only P but also N was stored temporary, for example as cyanophycin (Simon 1971).

If growth would be limited by P only, other resources should be non-limiting and hence the C/N ratio should not be deviating from the Redfield ratio (C/N = 6.6). This was the case in the two lowest P treatments, which indicates that they were only P limited and that C did not accumulate under severe P limitation. However, in batch cultures other factors (e.g. N, light) might become (co-) limiting, especially towards the end of the experiment. The fact that final C/N ratios increased with increasing initial P might in theory be caused by increased C assimilation. However, as final cellular C quota were not enhanced in high P treatments, increased final C/N ratios point at increasing limitation by N. This is supported by the low cellular N content in the high P treatments. However, in high P treatments also the cellular C quota declined, compared to the mid-exponential phase, which points at reduced cell volumes, which suggests light limitation due to self-shading towards the end of the experiment. This indicates that in high P treatments in the end a co-limitation of N and light might have occurred. Between these two extremes of final strong P limitation (low initial P) and final light and N limitation (high initial P) putatively increasing N co-limitation has occurred with increasing P concentration, as is suggested by increasing final C/N ratios.

The production of cyanobacterial secondary metabolites (i.e., toxins and inhibitors) may be linked to the nutrient status of cyanobacteria. If toxins contain the limiting nutrient, cyanobacteria should reduce the production of these metabolites (Van de Waal et al. 2014) except that these metabolites contribute to nutrient storage (e.g. Burberg et al. 2018). Accordingly, the content of microcystins (MCs), N-rich cyanobacterial metabolites, was reduced upon N limitation (Horst et al. 2014), and MC production was highest under conditions where N was least limiting (Van de Waal et al. 2009).

The ecological role of secondary metabolites for cyanobacteria is still not clear (Holland and Kinnear 2013). Several functions have been proposed, but many are still lacking real evidence. This is different for the huge group of protease inhibitors (PIs, Köcher et al. 2019), which tend to be at least as frequent as MCs in natural cyanobacterial blooms (Agrawal et al. 2001; Patel et al. 2014) and which serve as anti-grazer defense against *Daphnia* (Von Elert et al. 2004; Schwarzenberger et al. 2010; Von Elert et al. 2012; Lange et al. 2018). Here the two

PIs nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954) were investigated, for which IC_{50} values of 31 nM and 45 nM for the inhibition of bovine chymotrypsin have been reported (Von Elert et al. 2005), and IC_{50} values for the inhibition of chymotrypsin activity in the gut of *Daphnia* were 5.4 nM and 7.4 nM respectively (Von Elert et al. 2012), which classifies BN920 and CP954 as the most potent inhibitors containing 3-amino-6- hydroxy-2-piperidone (Ahp).

BN920 and CP954 consist of 8 amino acids (Von Elert et al. 2005), do not contain P and have a C/N ratio of 5.75, so that they can be considered N-rich (Van de Waal et al. 2014). Both inhibitors were constitutively present in this strain, which confirms findings for other cyanopeptolins (Tonk et al. 2009). The fact that a similar ratio of both inhibitors was observed when the cyanobacterium was grown under different degrees of N limitation (Burberg et al. 2018) suggests a joint biosynthetic pathway with CP954 as the chlorine-containing adduct of BN920.

In our experiment, the PI content was highest in the low-P treatments. Likewise, P limitation increased the content of N-rich micropeptin PIs in *Microcystis* sp. (Schwarzenberger et al. 2013). Similar effects were reported for the N-rich paralytic shellfish poisoning (PSP) toxin in dinoflagellates (Van de Waal et al. 2013; Van de Waal et al. 2014). This increase in PSP-toxin has been interpreted as evidence for resource-driven toxin synthesis, as P limitation releases the autotroph from potential N limitation so that N becomes non-limiting. As a consequence, P limitation causes a resource-driven increase of N-rich compounds that do not contain P. The same explanation is valid here for a cyanobacterium, where the low P treatments rendered N non-limiting. Such an increase of the content of BN920 and CP954 with increasing N availability has been reported earlier (Burberg et al. 2018). With ≥ 20 µM initial P, increasing N co-limitation was observed towards the end of the experiment, which explains the concomitant decrease in PI content: the carbon nutrient balance hypothesis (CNBH, Bryant et al. 1983) predicts that C-rich metabolites are favored under nutrient limitation, whereas in nutrient-replete (here N-replete) environments, more N-based metabolites are produced. Even though, in some cases, the CNBH fails to explain the relationship between secondary metabolite concentrations and nutrient availability (discussed in Hamilton et al. 2001 & Koricheva 2002), our results are in accordance with the CNBH.

For MCs, the effects of P limitation are less clear than those reported here for BN920 and CP954 (Van de Waal et al. 2014). This can probably be attributed to the fact that in the latter study, among others, N fixing cyanobacteria have been considered, in which P limitation might have reduced N fixation and thus have caused a decline in MC content with P limitation (pers. comm. Van de Waal). For the cyanobacterium *Aphanizomenon* it was shown that the content of cylindrospermopsin increased with increasing P concentration, but decreased under P

limitation (Bácsi et al. 2006). However, for *Nostoc* sp. and *Microcystis* the highest concentration of MC was found under P reduced conditions (Lee et al. 2000; Oh et al. 2000; Kurmayer 2011).

In our experiments, the PI content showed pronounced temporal variability. In the treatments with ≥ 20 µM initial P, the content of CP954, the major inhibitor, decreased from day 15 onwards. The concurrently increasing N co-limitation (i.e. increasing C/N ratios) provides a very plausible explanation for the decrease in PI content after day 15. In treatments with < 20 µM initial P, the CP954 content declined as well after day 15, but in a more pronounced way. Here the only explanation is that by day 18 C/P ratios in these low-P treatments had reached fairly extreme C/P values ranging from 800 to 1000, and that this strong P limitation might have caused a degradation of CP954 by 50 to 70%. However, we cannot exclude that, depending on culture conditions, release of PIs into the medium has occurred. For MCs, which are known as endotoxins, it is known that they can be found in the surrounding environment due to cell lysis as it occurs during collapse of cyanobacterial blooms (Omidi et al. 2019). Cell lysis would be associated with the release of cell content into the medium and should thus lead to lower POC values. Such a decline in POC was not observed in our treatments, which suggests that no significant cell lysis has occurred. An exception is the 30 µM P treatment, in which POC declines during the last two samplings. However, this drop in POC is not associated with a decline in inhibitor content at the last two samplings (Fig. 3). In conclusion, it is rather improbable that cell lysis has contributed to declines in PI content of *Microcystis* biomass in our experiments.

For P limitation, effects of growth rate on cyanobacterial secondary metabolites are controversial, sometimes even within the same study. Tonk et al. (2009) found that with increasing growth rates, the content of MC and anabaenopeptin increased while the content of cyanopeptolins declined. This was partly corroborated by Long (2001), who reported that content and production rate of MC and cyanopeptolins increased with the specific growth rate under P limitation. As well for P limitation, but not corroborating these findings, Oh et al. (2000) found in chemostat experiments that the MC content of *M. aeruginosa* was highest when the growth rate was lowest. These divergent results indicate that growth rate alone is insufficient to explain the content of secondary metabolites in cyanobacteria, but that it rather depends on which factor controls the growth (e.g. N, P, light). Similar results were also found for the marine dinoflagellate *Alexandrium* (Van de Waal et al. 2013), suggesting that this dependency on the kind of growth-limitation might not be restricted to cyanobacteria.

In our study, growth rates are based on changes in POC and not on cell numbers. Even though cellular C showed small differences (Fig. 3 A), POC serves as a good proxy for the cyanobacterial biomass, and therefore growth rates based on POC are well suitable to describe changes in biomass. We found that BN920 significantly increased with growth rate,

while for CP954 the content increased with growth rate until 0.15 and declined at higher growth rates (reversed u-shape). This difference is remarkable, as the high chemical identity of both PIs strongly suggests that they share the same biosynthetic pathways with an additional chlorination of BN920, which yields CP954. The fact that the CP954 content exceeds that of BN920 by one order of magnitude strongly suggests that BN920 serves as an intermediate that subsequently undergoes chlorination, probably by a halogenase. The occurrence of chlorinated secondary products is widespread in cyanobacteria (Huang and Zimba 2019), and in several cases halogenases have been shown to be involved in their synthesis. Among such cyanobacterial metabolites are, in addition to CP954, also other chlorinated protease inhibitors (Al-Awadhi et al. 2017). However, both regressions explain only a small degree of data variability (R^2), which most probably can be attributed to the fact, that the growth rates represent not only treatments with P limitation but as well other treatments with N co-limitation. These two resource-regimes have opposite effects on the content of CP954 and BN920, and it may be hypothesized that they are as well differently related to growth rate, so that no conclusions about growth rate effects can be drawn here.

We had hypothesized that P limitation reduces the growth of *Microcystis,* but increases the content of PIs. Our experiments confirm growth reduction with P limitation in the *M. aeruginosa* strain NIVA Cya 43 and demonstrate that only under strong P limitation a nutrient-driven increase of PI content in cyanobacteria is to be expected, which could lead to enhanced negative effects on grazers like *Daphnia.* Cyanobacteria can be good competitors at low P_i due to several alkaline phosphatases and the capability to store P (Gobler et al. 2016). Still, not all cyanobacteria are good competitors under strong P limitation (Reynolds 1984), and thus cyanobacteria might not be dominating phytoplankton communities under strong P limitation. More moderate P limitation would be associated with N co-limitation that would reduce PI content in cyanobacteria. However, the resulting interference with grazers like *Daphnia* will largely depend on tolerance traits in the grazer community. Standing populations of *Daphnia* harbor pronounced clonal variability with respect to tolerance of cyanobacteria (Jiang et al. 2013b) that allows for the reported microevolutionary adaptation of *Daphnia* populations both in time and space (Hairston et al. 1999; Sarnelle and Wilson 2005). Additionally, phenotypic plasticity constitutes another mechanism for acquired tolerance to toxic cyanobacteria. For example, in some cladocerans transgenerational adaptation has been shown to increase tolerance in zooplankton to toxic dietary cyanobacteria (Guo and Xie 2006; Jiang et al. 2013a). Although the molecular mechanisms driving these maternal effects have received some attention (Schwarzenberger et al. 2012; Schwarzenberger and Von Elert 2013; Schwarzenberger et al. 2014; Lyu et al. 2015), the overall effects of evolution and plasticity of grazers in mitigating resource-driven changes in cyanobacterial toxin content remains to be understood.

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Chapter 3

A dynamic process-based modeling approach to understand the effects of nutrients on protease inhibitors in cyanobacteria

3.1. Abstract

The enrichment of lakes and ponds with nitrogen (N) and phosphorus (P) has led to a worldwide increase of cyanobacterial blooms. Many bloom-forming cyanobacteria produce N-rich metabolites, e.g. protease inhibitors (PIs), that are potentially toxic to humans and animals and in particular detrimental to *Daphnia*, which is a major grazer of cyanobacteria. Understanding the effects of nutrient availability on the production and degradation of PIs in cyanobacteria is therefore essential. In this study, a dynamic process-based model was used to understand the effects of N and P on the production and degradation of two PIs in the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43. Production and degradation rates of the two PIs declined with increasing N concentrations. These dynamics of the PI concentrations indicate their involvement in N storage of the cyanobacterium. The initial P concentration did not affect the production rates and showed a negative relation with the degradation rates, indicating that the relationship between PIs and P concentration is more complex. Altogether, nutrient species and concentration affected the PI dynamics and therefore the toxicity of the cyanobacterium. Analyzing the dynamics of PIs, microcystins and other metabolites using dynamic process-based models could be important to understand and manage cyanobacterial blooms in the future.

Keywords: *Microcystis* – Eutrophication – Nitrogen – Phosphorus – Protease inhibitors – Dynamic Model

3.2. Introduction

Cyanobacterial mass developments, so called blooms, are a major problem in many freshwater systems worldwide (Xiao et al. 2020). In the last decades, the number, frequency and duration of cyanobacterial blooms has increased and is predicted to further increase due to nutrient pollution and rising temperatures (Carey et al. 2012; Kosten et al. 2012; Glibert 2017). Cyanobacterial blooms result in a decline of water quality causing dramatic ecological and economical costs (Wurtsbaugh et al. 2019). In the US alone, the annual cost of eutrophication and the resulting consequences, including cyanobacterial blooms, are estimated to be over $2 billion per year (Dodds et al. 2009).

A major cause for the detrimental effect of cyanobacteria is the production of numerous bioactive secondary metabolites that are toxic to animals (Davis et al. 2019; Moustaka-Gouni and Sommer 2020) and humans (Cao et al. 2019; Dunlop and Guillemin 2019). Among those metabolites, microcystins (MCs) are well known for their toxicity (Svirčev et al. 2017) and have been directly linked to the death of humans (Jochimsen et al. 1998). Therefore a lot of research has been done to understand how nutrients and other environmental conditions affect the content and production of MCs in cyanobacteria (Amé and Wunderlin 2005; Van de Waal et al. 2009; Chaffin et al. 2018). However, other secondary metabolites received less attention, even though they appear as frequently and in similar concentrations as MCs and have been proven to be harmful to other organisms (Beversdorf et al. 2017; Beversdorf et al. 2018; Janssen 2019). This includes the large group of protease inhibitors (PIs) that are widespread among cyanobacteria and can be found in many lakes and reservoirs worldwide (Adiv and Carmeli 2013; Beversdorf et al. 2017). For zooplankter, like *Daphnia,* it was shown that PIs can limit growth, reproduction, and even cause increased mortality (Rohrlack et al. 2004; Agrawal et al. 2005; Kuster et al. 2013; Schwarzenberger et al. 2013). Other studies report negative effects of PIs on zebrafish embryos (Faltermann et al. 2014) and even on human hormones, i.e. kallikrein (Gademann et al. 2010).

An important component, in understanding the relevance of PIs, is to understand how the production of PIs is affected by environmental factors, in particular by the availability of nitrogen (N) and phosphorus (P). According to the carbon-nutrient balance hypothesis (Bryant et al. 1983), the production of N-rich PIs should increase with increasing N availability. This is in accordance with findings that PI synthetase genes are up-regulated when *Microcystis* is supplied with ammonium and urea (Harke et al. 2016) and in agreement with findings that the content of micropeptins, a group of N-rich chymotrypsin inhibitors, is reduced under N limitation and increased under P limitation in *Microcystis* (Schwarzenberger et al., 2013). However, there is a need to better understand the dynamics of PIs and the influence of nutrients species and concentration as PIs contribute significantly to the overall toxicity of cyanobacterial blooms.

Besides growth and production of secondary metabolites, the uptake and storage of essential nutrients is important for cyanobacteria especially when resources are scarce and interspecific competition is high. During times of high nutrient availability, cyanobacteria take up more N and P than required for immediate growth ('luxury uptake') and store them in form of cyanophycin (Berg et al. 2000; Watzer and Forchhammer 2018) and polyphosphate respectively (Jacobson and Halmann 1982; Solovchenko et al. 2019). When nutrient availability declines, those storage compounds are degraded and the resources are used to further maintain growth (Jacobson and Halmann 1982; Zhang and Yang 2019).

Due to their toxicity and potential ecological impacts, understanding growth and PI dynamics is crucial to predict cyanobacterial blooms, their potential toxicity and thus the risk for human health and the economy (Burford 2019). Mathematical models play an important role in this process and have become a widely used tool that improves the understanding of underlying processes and allows to check the consistency of results, develop new hypothesis and make accurate predictions (Jørgensen 1994; Jachner et al. 2007). In this study, a dynamic process-based model was developed and used to gain more insights into the connection between nutrient concentration (N, P), biomass production and PI concentration using the cyanobacterium *Microcystis aeruginosa*. The aim was to understand how growth parameters (e. g. growth rate), production and degradation rates of PIs are affected by different initial concentrations of N and P and ultimately which nutrient conditions might favor the production of cyanobacterial PIs. It was hypothesized already in previous studies that N containing PIs have a dual function as potential defensive protease inhibitor and as N storage (Burberg et al. 2018; Burberg et al. 2020). Therefore, PIs should be produced in the early stages of growth, when nutrients are not limiting, and should be degraded and recycled intracellularly in the late growth phase.

3.3. Material and Methods

The dynamic process-based model was applied to data sets from two previously published papers (Burberg et al. 2018; Burberg et al. 2020). Experimental conditions and performed analyses are briefly outlined below with reference to more detailed information in the corresponding papers.

3.3.1. Culturing conditions

The experiments were performed using the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43, a strain that produces the two nitrogen–rich protease inhibitors (PIs) cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920), but no microcystins (Von Elert et al. 2005). The cyanobacterium was cultivated for 28 days either under a variation of nitrogen (N, 4 different initial concentrations) or phosphorus (P, 8 different initial concentrations) respectively. WC medium (Guillard 1975) was used as culture medium, light (45 ± 3 µmol photons m^{-2} s^{-1}) and temperature (20 ± 1°C) were kept constant.

3.3.2. Sampling and sample processing

Every 2 – 4 days samples were taken from the cultures to quantify particulate organic carbon (POC, proxy for cyanobacterial biomass), particulate organic nitrogen (PON) and the PI concentration. POC and PON samples were filtered onto pre-combusted GF/F filters, dried for at least 24 hours, packed into tin capsules and analyzed using a Flash EA2000 Analyzer (Thermo Scientific). The results were used to determine the concentrations of POC and PON in the cultures.

PIs were extracted by first centrifuging the samples, adding 10 mL methanol (80%) as well as 10 µL of the internal standard (Microcystin LR) to the cell pellet and re-suspending the pellets by ultrasonification. After another centrifugation step, the supernatant was transferred into test tubes, evaporated to dryness and re-dissolved in 1 mL methanol (100 %). After a second drying step, samples were taken up in 100 µL methanol (100 %), centrifuged and transferred into HPLC vials. Subsequently, PIs were quantified using an ultra-high-pressure liquid chromatography system (UHPLC, Accela, ThermoFisher Scientific) coupled with an Exactive Orbitrap mass spectrometer (ThermoFisher Scientific). The chromatographic separation was carried out on a C_{18}-column (Nucleosil, 125/2, 100-3, Macherey and Nagel) with acetonitrile (A) and ultra-pure water (B), each containing 0.05% trifluoracetic acid, as mobile phase. For further details see Burberg et al. (2018). Under the applied conditions BN920 and CP954 form two positively charged adduct ions. The adducts of each PI were summed up to ´BN920´ and ´CP954´. Peak intensities were extracted from the chromatograms

using the R–package ‘enviMass’ (Loos 2018) and converted to concentrations via previous established calibration curves.

3.3.3. Modeling and statistical analysis

3.3.3.1. Model description

A dynamic process-based model was developed to describe cyanobacterial growth and the turnover of the PI concentration in dependence of the supplied nutrient. In general such models assume, that a resource declines (e.g. through growth) until the resource is depleted and all downstream processes become limited by this resource. According to the model (Fig. 1), N taken up by *Microcystis* is used to produce either PIs or other N containing compounds (PON) resulting in a decline of the extracellular N. The model assumes that PON compounds as well as inorganic P are required for C fixation, growth and biomass production (measured as POC). Both resources decline over time, until one of them starts, according to Liebig´s minimum principle, to limit growth (Fig. 1, control path), so that POC production eventually stops. However, following the results of Burberg et al. (2018), it was hypothesized that PIs serve as a temporary N storage. When extracellular N becomes scarce, *Microcystis* will start to degrade the PIs and recycle the stored N, which then goes into the PON pool allowing further growth.

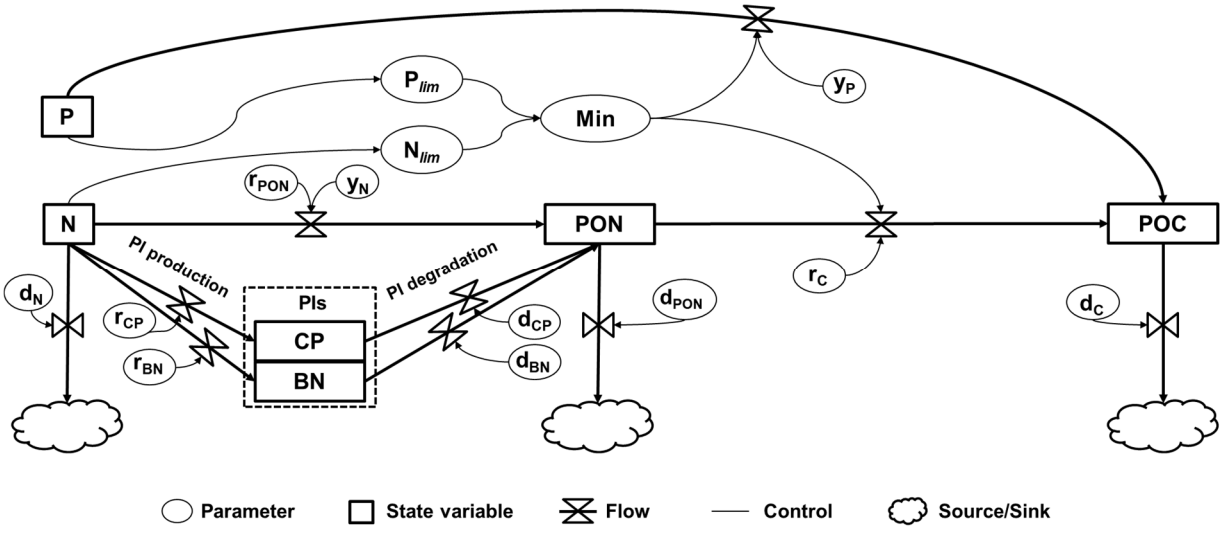

Figure 1. Flow diagram of the modeling approach. Abbreviations are explained in Tab. 1 and 2.

Table 1. List of state variables. For the simulation, all state variables were expressed as mg L^{-1} to ensure consistent dimensions.

State Variable	Identifier	Unit
POC	Particulate organic carbon	mg L^{-1}
PON	Particulate organic nitrogen	mg L^{-1}
P	Initial dissolved phosphorus concentration	mg L^{-1}
N	Initial dissolved nitrogen concentration	mg L^{-1}
BN	Particulate BN920 concentration	mg L^{-1}
CP	Particulate CP954 concentration	mg L^{-1}

3.3.3.2. Model equations

The model consists of 6 ordinary differential and 3 supporting equations with a total of 6 state values and 16 parameters (see Tab. 1 & 2). The 6 ordinary differential equations describe the changes in the external nutrient pools (eq. 1 & 2), the intracellular nitrogen pool (PON, eq. 3), the cyanobacterial biomass (POC, eq. 4) and the intracellular BN920 and CP954 content (eq. 5 & 6) over time.

$$\frac{dN}{dt} = -\frac{1}{y_N} \cdot r_{PON} \cdot \frac{N}{k_N + N} \cdot PON - d_N \cdot N \quad (1)$$

$$\frac{dP}{dt} = -\frac{1}{y_P} \cdot r_C \cdot min(P_{lim}, N_{lim}) \cdot POC \quad (2)$$

$$\frac{dPON}{dt} = r_{PON} \cdot \frac{N}{k_N + N} \cdot PON - d_{PON} \cdot PON \quad (3)$$

$$\frac{dPOC}{dt} = r_C \cdot min(P_{lim}, N_{lim}) \cdot POC - d_C \cdot POC \quad (4)$$

$$\frac{dBN}{dt} = r_{BN} \cdot \frac{N}{k_{N\_BN} + N} \cdot POC - d_{BN} \cdot BN \quad (5)$$

$$\frac{dCP}{dt} = r_{CP} \cdot \frac{N}{k_{N\_CP} + N} \cdot POC - d_{CP} \cdot CP \quad (6)$$

Table 2. List of model parameters. Due to over-parametrization of the model not all of the 16 parameters could be determined simultaneously. After a qualitative sensitivity analysis, 9 calibration parameters were selected (indicated by asterisks). The remaining 7 parameters were set to fixed values. The Monod constants (*k*) are uncritical and can be considered as 'safeguard values' to avoid negative states.

	Parameter	Identifier	Unit	Start	Minimum	Maximum
*	y_N	yield coefficient for N	dimensionless	1	0.1	5
*	y_P	yield coefficient for P	dimensionless	200	20	1000
*	r_C	growth rate	d^{-1}	0.6	0.06	3
*	r_{PON}	production rate of PON	d^{-1}	0.7	0.07	3.5
*	r_{BN}	production rate of BN920	d^{-1}	0.07	0.007	0.35
*	r_{CP}	production rate of CP954	d^{-1}	0.2	0.02	1
*	d_C	degradation (-rate) of POC	d^{-1}	0.02	0.00	0.1
*	d_{BN}	degradation rate of BN920	d^{-1}	0.06	0.006	0.3
*	d_{CP}	degradation rate of CP954	d^{-1}	0.1	0.01	0.5
	d_N	degradation rate of N	d^{-1}	0.01		
	d_{PON}	degradation rate of PON	d^{-1}	0		
	k_N	half-saturation constant for N	mg L^{-1}	5		
	k_P	half-saturation constant for P	mg L^{-1}	0.2		
	k_{PON}	half-saturation constant for PON/POC quota	mg L^{-1}	0.02		
	k_{N_BN}	nitrogen half-saturation constant of BN920	mg L^{-1}	150		
	k_{N_CP}	nitrogen half-saturation constant of CP954	mg L^{-1}	55		

The three supporting equations (eq. 7 – 9) describe the Monod-kinetics for P and N respectively (eq. 7 & 8), and eq. 9 calculates the C/N ratio of the cyanobacterial biomass. According to the model assumptions, the growth of biomass depends on the internal N (PON) so that the decrease of N (N_{lim}) in return depends on the biomass that is produced (POC). Therefore, the PON/POC quota was introduced into eq. 2.

$$P_{lim} = \frac{P}{k_P + P} \quad (7) \qquad N_{lim} = \frac{\frac{PON}{POC}}{k_{PON} + \frac{PON}{POC}} \quad (8) \qquad NC = \frac{POC}{PON} \quad (9)$$

3.3.3.3. Modeling process and validation

In a first step, the model was adjusted manually to single replicates of each dataset. In the second step, the non-linear least squares method was used to fit the model to both datasets (N- and P-experiment). Weights of the state variables were set equal to the reciprocal of the standard deviation of the observed data. Due to dependencies between the parameters (especially production rates r´s and half-saturations constants k´s), 9 out of the 16 parameters were selected and the remaining 7 were assumed as fixed (see Tab. 2). The calibration parameters were selected after a qualitative sensitivity analysis, considering the main processes of the model. All state variables were expressed as mg L^{-1} (Tab. 1) or as dimensionless ratio to ensure consistency of the dimensions and numerical stability of the differential equation solver. The initial values of the sate variables were also estimated from the data during calibration.

For model validation it is necessary to accept a model, determine the quality and identify its applicability (Mayer and Butler 1993; Elliott et al. 2000). After an initial qualitative plausibility check by visual comparison of modeled and measured values, the quantitative model evaluation criteria root mean squared error (*RMSE*), coefficient of determination (R^2) and Nash-Sutcliffe efficiency factor (*NSE*) were calculated (Jachner et al. 2007). Among the three used measures, the *NSE* considers both systematic and random error and is the strictest criterion.

The fitted model parameters were then plotted against each other and against the treatment to visualize how they depend on each other and how they were affected by initial nutrient concentrations. Linear and exponential regressions were applied to each pair and used to further describe the relationships. Additionally, Spearman´s rank correlations (R_S) were calculated as a further indicator for the interactions between the parameters.

Dynamic modeling, visualization and statistical analyses were done using R (R Core Team 2020) and RStudio (RStudio Team 2020). Numerical integration of the ordinary differential equations was performed using the *lsoda* algorithm (Hindmarsh 1983; Petzold 1983) available from the R-package ‘deSolve’ (Soetaert et al. 2010). Parameter identification was performed

with the R-package ‘FME’ (Soetaert and Petzoldt 2010) and the *Levenberg-Marquardt* algorithm from the R-package ‘minpack.lm’ (Elzhov et al. 2016).

3.4. Results

In this study a dynamic process-based model was applied to describe and analyze how the availability of nitrogen (N) and phosphorus (P) affects the growth of the cyanobacterium *Microcystis aeruginosa* and the concentration of two protease inhibitors.

3.4.1. Data dynamics and model quality

The plausibility of the model was validated visually and by the use of common similarity measures (Fig. 2, Tab. 3). The visual validation considered individual fits for each replicate. A series of replicates was depicted in Figure 2 to show exemplarily the general dynamics of the protease inhibitors (PIs), particulate organic nitrogen (PON), particulate organic carbon (POC) and the C/N ratio. The concentration of the two PIs nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954) increased until it reached a maximum around day 10 – 15 and declined thereafter, but stayed on a level above the initial concentration. Similar, POC and PON increased according to a logistic growth model until reaching stationary phase. During the stationary phase, POC and PON did not remain constant in all replicates (Fig. 2); while PON for example varied in some replicates around the maximum, POC declined in a few replicates after reaching maximal biomass. Depending on the initial nutrient concentrations, the C/N ratio varied between 5 and 20, except for the initial value in some of the P treatments. In many replicates, there was an initial decrease in the C/N ratio followed by an increase towards the end of the experiment indicating an initial uptake of N followed by an increase of C through C fixation. Judged on the basis of the individual graphs for each replicate, the model fitted the data well.

Table 3. Model validation statistics for two independent data sets. Data sets were obtained from performing batch culture growth experiments with different initial concentrations of phosphorus (P-experiment) or nitrogen (N-experiment) respectively. Given are the *RSME* (root mean square error), the R^2 (coefficient of determination) and the *NSE* (Nash-Sutcliffe model efficiency coefficient) for the two protease inhibitors (BN920, CP954), particulate organic nitrogen (PON), particulate organic carbon (POC) and the C/N ratio.

	Variable	n	*RMSE*	R^2	*NSE*
N-Experiment	**BN920**	144	0.26	0.78	0.78
	CP954	144	1.21	0.82	0.82
	PON	144	1.34	0.98	0.98
	POC	144	24.46	0.97	0.96
	C/N ratio	144	1.17	0.93	0.93
P-Experiment	**BN920**	280	0.18	0.88	0.88
	CP954	280	1.26	0.87	0.87
	PON	280	2.59	0.93	0.93
	POC	280	29.01	0.96	0.96
	C/N ratio	280	1.46	0.88	0.88

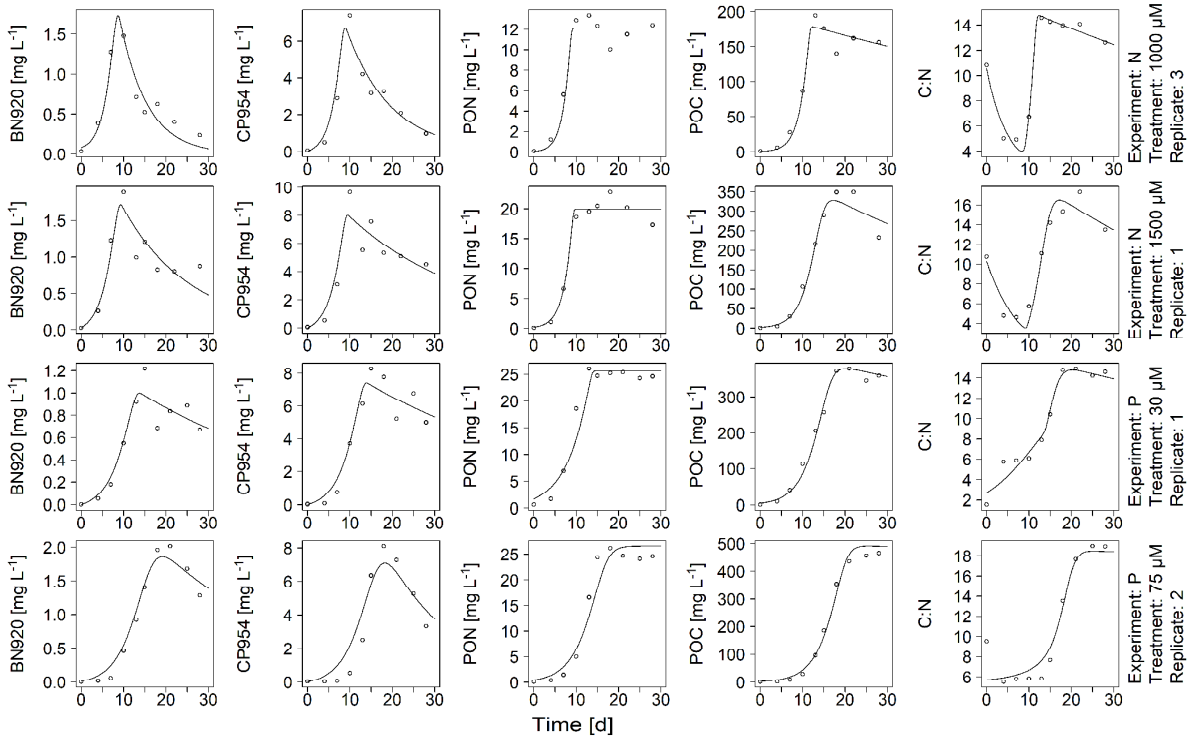

Figure 2. Dynamics of the two protease inhibitors BN920 and CP954, particulate organic nitrogen (PON), particulate organic carbon (POC) and the C/N ratio. Depicted are two exemplary replicates of the two experiments (points) and the calibrated model simulations (lines).

The quantitative model evaluation criteria were calculated for each state variable for the two data sets (Tab. 3) and individually for each replicate and treatment. Both, the coefficient of determination (R^2) and the Nash-Sutcliffe model efficiency coefficient (*NSE*), were in all cases 0.78 or higher, which indicates a good model performance. Within the experiments, only four replicates scored low (< 0.5) R^2 and *NSE* values for individual fits. In particular, two replicates of the 10 µM treatment in the P-experiment had *NSE* and R^2 values around 0.27 for the C/N ratio fit, which were the lowest values for both data sets. Except for those replicates the validation measures confirm that the model worked fairly well for the rest of the two data sets and indicates general applicability.

3.4.2. Effect of N concentration on model parameters

The initial N concentration affected the model parameters differently (Fig. 3). While the yield coefficients of N (y_N) and P (y_P) were unaffected by the treatment ($p > 0.05$), all other parameters significantly declined with an increasing initial N concentration (all with $p < 0.05$, $R^2 > 0.36$, $R_S < -0.56$). While the relationship between the N concentration and the growth rate (r_C) and the production rate of BN920 (r_{BN}) were better described by a negative exponential regression, the other parameters decrease linearly with increasing N concentration.

The parameters did not only depend on the initial N concentration, but showed relations among each other (Fig. 3). The two PI production rates (r_{BN}, r_{CP}) showed a positive relation with each other (R^2 = 0.93, p < 0.001, R_s = 0.96), which can be attributed to the biosynthetic pathway with BN920 being the precursor for the synthesis of CP954. The relationship between PI production and degradation is also interesting, as both processes are likely related to each other. The degradation rate of CP954 (d_{CP}) was strongly related to both r_{BN} (R^2 = 0.67, p < 0.001, R_S = 0.84) and r_{CP} (R^2 = 0.81, p < 0.001, R_S = 0.89), showing that under conditions which favored inhibitor production (reduced N), the resources are repurposed from CP954, e.g. for growth. With R^2 of 0.56 and 0.41, the relationships between the degradation rate of BN920 (d_{BN}) and the production rates were weaker but still significant. Other parameters also affected the PI production and degradation. r_{BN} and r_{CP} increased for example with increasing r_{PON} (production rate of PON), but remained unaffected by y_N and y_P (p > 0.05). In case of r_C, we expected the PI production rates to increase with increasing growth rate. According to our model, r_{BN} and r_{CP} indeed increased with increasing r_C. This shows that lower N concentration can lead to faster growth and a faster production of PIs.

3.4.3. Effect of P concentration on model parameters

Most of the model parameters (y_N, y_P, r_{BN}, r_{CP}, r_{PON}, d_C) were unaffected by the initial P concentration (Fig. 4). Only r_C (R^2 = 0.35, p < 0.001, R_S = -0.38), d_{BN} (R^2 = 0.16, p = 0.03, R_S = -0.32) and d_{CP} (R^2 = 0.17, p = 0.03, R_S = -0.37) declined significantly with increasing initial P concentration. The finding that r_C declines with increasing P concentration corresponds to the results of the N-experiment, were r_C decreased with increasing N concentration. This indicates that high nutrient concentration supported higher biomasses (Burberg et al. 2018; Burberg et al. 2020), but reduced the growth rate of *Microcystis*.

In accordance with the results of the N-experiment, r_{BN} and r_{CP} correlated positively with each other (R^2 = 0.86, p < 0.001, R_S = 0.88) and were both unaffected by y_P (Fig. 4). Like the production rates, the PI degradation rates (d_{BN}, d_{CP}) showed a positive linear relationship with each other (R^2 = 0.83, p < 0.001, R_S = 0.82). Differently from the N-experiments, PI production and degradation rates were not correlated with each other and a higher r_C was not accompanied by higher PI production rates. All together this indicates, that production and repurposing of PIs depends on the type of nutrient and the absolute nutrient concentration.

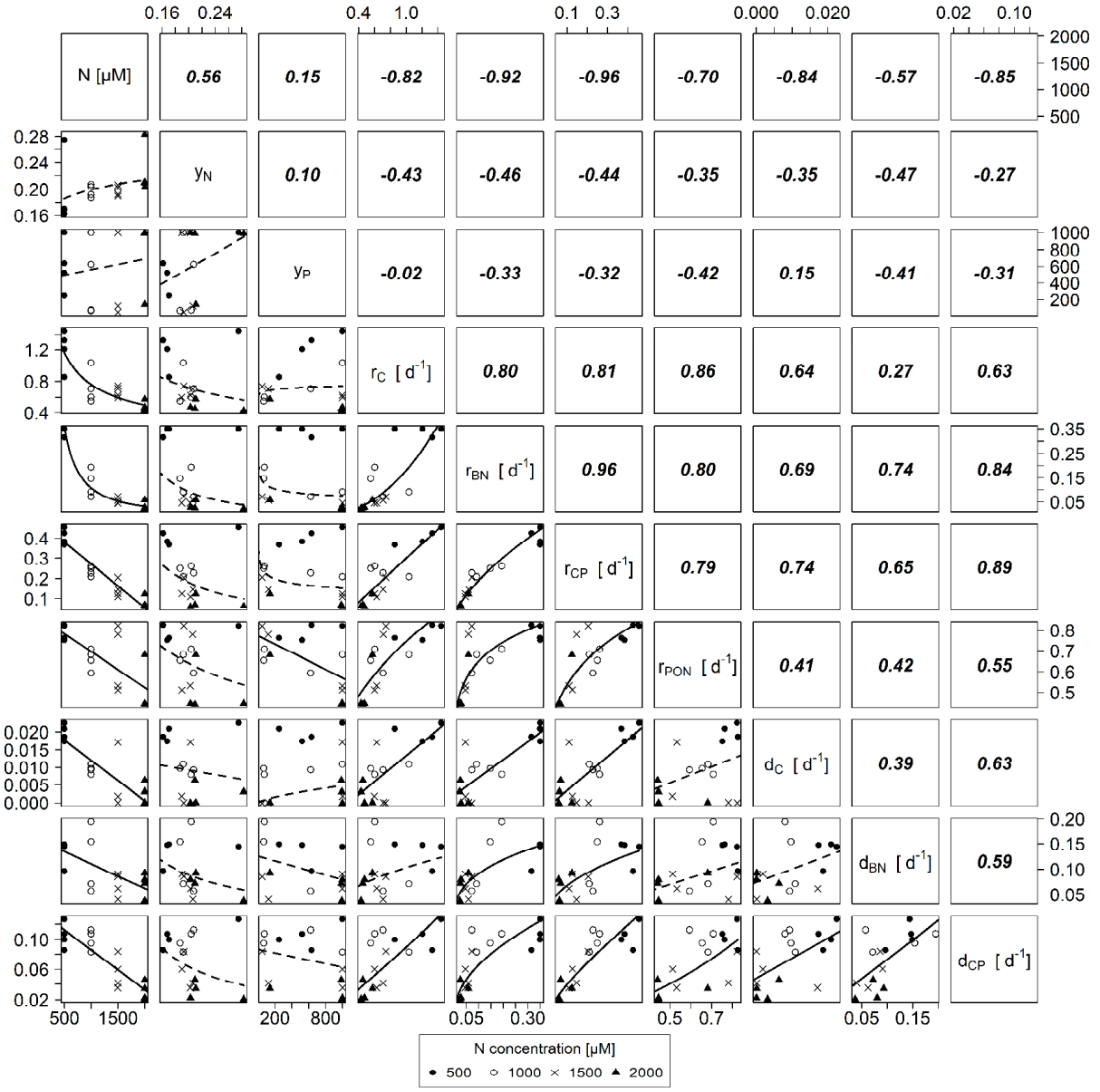

Figure 3. Correlation matrix for model parameters based on growth experiments with different initial nitrogen concentrations (N-experiment). Different symbols indicate the different N concentrations. Solid lines represent significant ($p < 0.05$) linear or exponential regressions, respectively; dashed lines represent non-significant regressions ($p \geq 0.05$). Numbers in the upper panels represent Spearman´s rank correlation coefficients (R_S) of the corresponding plots. Parameter explanations are given in Tab. 2.

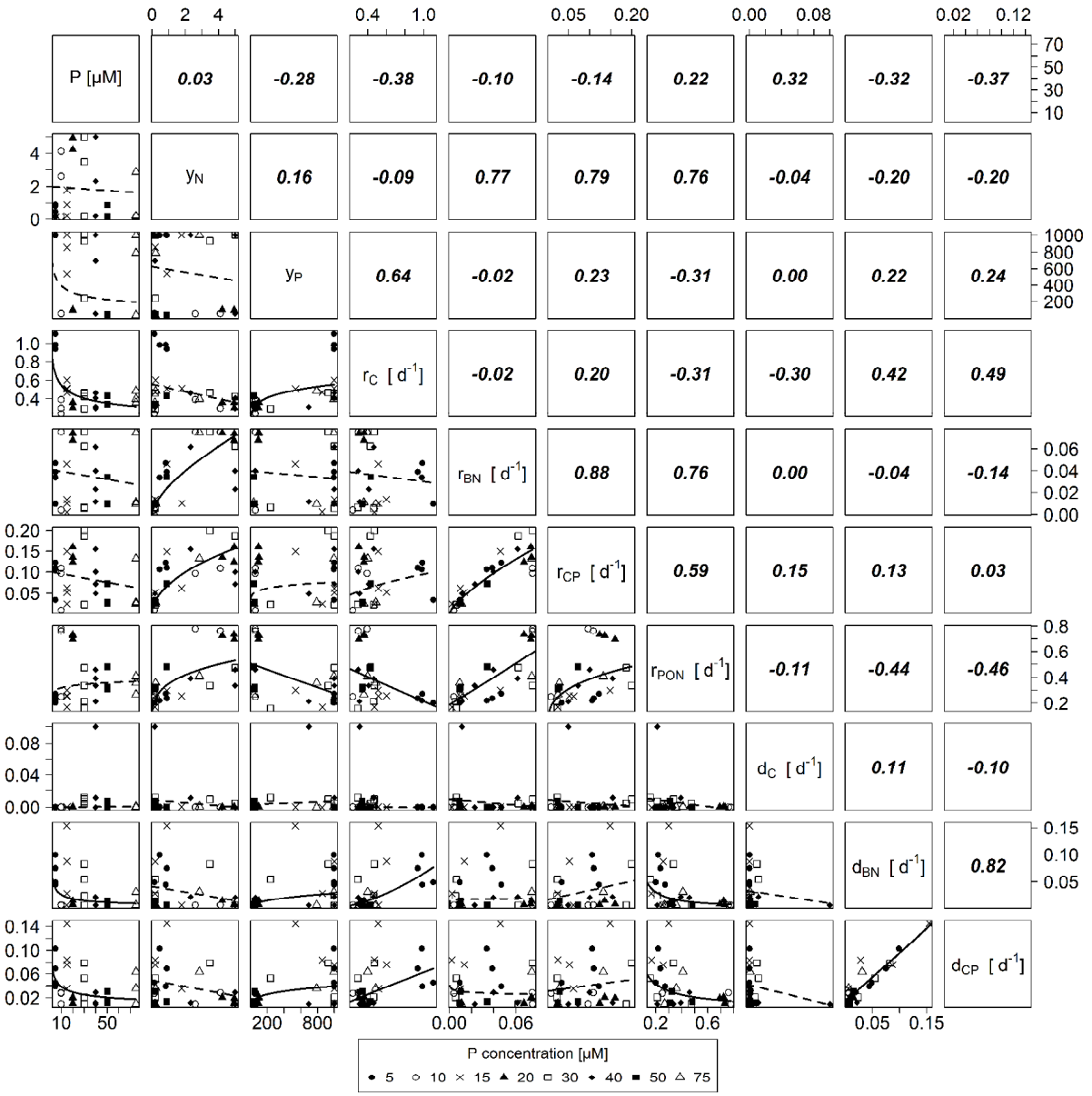

Figure 4. Correlation matrix for model parameters based on growth experiments with different initial phosphorus concentrations (P-experiment). Different symbols indicate the different P concentrations. Solid lines represent significant ($p < 0.05$) linear or exponential regressions, respectively; dashed lines represent non-significant ($p \geq 0.05$) regressions. Numbers in the upper panels represent Spearman´s rank correlation coefficients (R_S) for the corresponding plots. Parameter explanations are given in Tab. 2.

3.5. Discussion

The eutrophication of freshwater systems worldwide results in cyanobacterial blooms that cause serious environmental and ecological problems (O'Neil et al. 2012; Wurtsbaugh et al. 2019). Prediction and management of cyanobacterial blooms requires to understand the major drivers of population dynamics (Burford et al. 2019). In this study a dynamic process-based model was used to analyze how the initial availability of nitrogen (N) and phosphorus (P) affects the growth and volumetric concentration of two protease inhibitors (PIs), nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954), of the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43. Therefore, two data sets from batch culture growth experiments were used, in which different initial N and P concentrations were tested (Burberg et al. 2018; Burberg et al. 2020).

The ongoing input of N and P into lakes and ponds repeals the natural limitation of cyanobacteria and results in their mass proliferation. A long standing tenet was that P controls the productivity in freshwater systems (Schindler and Fee 1974; Downing et al. 2001). However, N limitation can also constrain the productivity of lakes and the development of cyanobacterial blooms (Conley et al. 2009; Dolman and Wiedner 2015; Gobler et al. 2016; Paerl et al. 2016). Our data support that increased N and P concentrations both boost the cellular abundance and biomass of *Microcystis* (Burberg et al. 2018; Burberg et al. 2020). Diverging from our expectation, the growth rate (r_C) declined with rising concentrations. Increasing N and P concentrations have been shown to increase the growth rate of *M. aeruginosa* (Saxton et al. 2012; Kim H et al. 2017). However, depending on the strain, growth rates reach a maximum at certain concentrations and show at higher concentrations either no further increase or even a decline (Saxton et al. 2012; Kim H et al. 2017). Other studies also report that the growth rate of *Microcystis* can decrease with increasing nutrient concentrations (Chen et al. 2009; Ghaffar et al. 2017). One explanation is, that high nutrient concentrations can cause stress to the cyanobacterium, e.g. by increasing the intracellular nitrite concentration (Chen et al. 2009). Admittedly, the tested nutrient concentrations were rather high and are in nature only reached after extreme events, like heavy rainfalls (Chaffin et al. 2013; Davis et al. 2015). This might have caused stress to *Microcystis*, especially in the beginning of the experiment, which led to the lower growth rates under high nutrient concentrations. The growth rates is an essential parameter in most phytoplankton growth models (Xiao et al. 2020) and understanding the growth kinetics of cyanobacteria is important for managing blooms (Tsukada et al. 2006; Giannuzzi 2019). Testing lower, more natural nutrient concentration could help to verify the effect of nutrient concentration on the growth rate and determine at which concentrations *M. aeruginosa* NIVA Cya 43 might reach its optimal growth.

Cyanobacteria produce a wide range of N-rich secondary metabolites, that are detrimental to many water organisms and potentially toxic for humans (Jochimsen et al. 1998; Gademann and Portmann 2008). PIs, for example, inhibit gut proteases in the genus *Daphnia,* a major consumer of phytoplankton and a keystone species in many lakes and ponds (Sarnelle 2005), resulting in a reduced growth of *Daphnia* (Von Elert et al. 2004; Agrawal et al. 2005). Cyanobacterial PIs can even increase the mortality of *Daphnia* directly by interfering with the molting process (Rohrlack et al. 2004). Therefore, it is necessary to understand how nutrient availability (N, P) impacts the content and production of bioactive secondary metabolites. Many studies have addressed this issue, in particular for microcystins (MCs), and found partly contradictory results. While there is the general tendency that N limitation reduces the content of N-rich toxins, the effect depends on the cyanobacterial taxa, the respective toxin and other environmental factors (Jähnichen et al. 2008; Schwarzenberger et al. 2013; Brandenburg et al. 2020). P limitation on the other hand is often associated with an increase in the toxin content (Brandenburg et al. 2020), as relatively more N is available for the production of N-rich compounds. However, many other abiotic and biotic factors, e.g. light, temperature and grazer presence (Wiedner et al. 2003; Amé and Wunderlin 2005; Jang et al. 2008), have been shown to affect the production and content of toxins in cyanobacteria. This complex network of factors explains why study results can be diverging. The focus on a single parameter, i.e. the concentration of a nutrient, might therefore be insufficient to predict the toxicity of cyanobacterial blooms. Nevertheless, N and P are the two major nutrients in freshwater systems and their influence on PI content and production has been studied insufficiently. The occurrence of PIs is widespread, and despite the fact that PIs frequently reach considerably concentrations (Agrawal et al. 2001; Adiv and Carmeli 2013; Beversdorf et al. 2017; Beversdorf et al. 2018) and are known to be detrimental to *Daphnia* and other organisms, it is still unresolved how different concentrations of N and P affect PI production and thereby the overall toxicity of cyanobacterial blooms.

The production and degradation of metabolites is best described by volumetric concentrations. Volumetric PI concentrations reflect changes in the intracellular PI pool, which can be caused by either production (increase) or degradation/loss (decrease). Many studies analyzed the effects of environmental factors on the production of cyanobacterial toxins by using gravimetric concentrations, which means that they normalized the toxin concentrations to the cyanobacterial biomass (Orr et al. 2018). Even though gravimetric concentrations (‘content’) are a good proxy for the relative toxicity of the cyanobacterial biomass, which is important for potential effects on grazers, they are not suitable to track the production or degradation of PIs (Orr et al. 2018). This is due to the fact that a change in the biomass content of a PI might reflect not a change of the actual PI concentration but of the cyanobacterial biomass. Changes of the PI concentrations are therefore better described such that they are

not normalized to another variable (Lyck 2004), and such a normalization is avoided by using volumetric concentrations (Orr et al. 2018). Admittedly, in this study extracellular PI concentrations were not determined and therefore degradation and loss (e.g. through cell lysis) cannot be distinguished with certainty. However, PI release through cell lysis is at least unlikely as POC did not decline simultaneously with declining PI concentrations.

Here, volumetric concentrations of two PIs, BN920 and CP954, were measured during batch culture growth experiments. A general feature of batch cultures is that each culture undergoes a sequence from unlimited through moderately limited growth to the stationary phase, which usually reflects full limitation by one or more resources. Interestingly, the volumetric PI concentration of the cyanobacterium showed a high temporal dynamic such that, independent from the type of the varied nutrient and its initial concentration, the PI concentration increased until day 10 – 15 and declined thereafter. This suggested that those PIs serve as temporal storage for N (Burberg et al. 2018). Cyanobacteria can store N in form of cyanophycin (Berg et al. 2000; Flores et al. 2019), while P is stored as polyphosphates (Jacobson and Halmann 1982). An inherent feature of storage compounds is that they are synthesized under nutrient-replete conditions and degraded under nutrient deprivation to reallocate stored nutrients to more essential processes. The fact that the two PIs are N-rich compounds (Burberg et al. 2018), that accounted for up to 13% of the total PON, makes them a good N storage compound. And as on average the concentration of BN920 reached only 30% of that of CP954, the latter clearly is the dominant storage compound in the cyanobacterial strain investigated here. The observed dynamics of the PI concentrations clearly point at a role of these PIs in N storage. However, the PI concentration remained in all treatment above the initial PI concentration. This indicates that N, that had been stored in PIs, is partly reused during the stationary phase; this would be unlikely if *Microcystis* would be solely limited by N and rather points at another limiting nutrient. This reasoning is supported by the fact that the difference between initial and final PI concentration increased with increasing N concentration, showing that the recycling was less important under high N levels. It is more likely that *Microcystis* was in several treatments co-limited by N and another factor, probably P. The C/P ratios increased in all treatments above the Redfield ratio (Redfield et al. 1958), which indicates ongoing P limitation. The degree of P limitation, however, varied with the initial nutrient concentrations. Especially during late stages of batch culture experiments, co-limitation of nutrients is common and applies to many eutrophic freshwater systems, in which phytoplankton and particularly cyanobacteria are co-limited by N and P (Elser et al. 2007; Elser et al. 2009; Paerl et al. 2011; Paerl et al. 2016). However, a co-limitation aggravates the clear identification of causal effects of single nutrients on the PI concentration.

Nevertheless, N storage and N recycling are more likely to depend rather on N than on P availability and so does probably the rate of PI production. Like for the growth rate, PI production rates (r_{BN}, r_{CP}) declined with increasing N concentrations, indicating that higher N concentrations slow down PI production. This finding is in favor of a role of PIs as N storage products, as storing N is more important under lower N availability and therefore requires a faster PI synthesis under these conditions. The observed upregulation of PI synthetase genes with addition of ammonium and urea (Harke et al. 2016) does not necessarily contradict our findings, but rather supports the notion that cyanobacteria generally prefer uptake of reduced forms of N (urea, ammonium) over that of nitrate and indicates that PIs serve as N storage compounds not only for nitrate but for all types of inorganic N. Interestingly, the initial P concentration did not affect the PI production rates, even though a lower P availability can shift internal nutrient ratios towards N, providing relatively more N for the synthesis of N-rich PIs (Brandenburg et al. 2020). As BN920 serves as a precursor of CP954 biosynthesis, it was additionally hypothesized that r_{BN} and r_{CP} are directly linked to each other and that there is a time delay between the production of BN920 and CP954. While the production rates indeed were in both experiments positively correlated, there was no time delay between the two PIs (analysis not shown). This indicates that the production of both PIs is either happening without a delay or that the temporal resolution of our experiments (sampling every 3 – 4 days) was too low.

After day 10 – 15 the PI concentrations started to decrease, while C/N ratios, cell abundance and cyanobacterial biomass still increased (Burberg et al. 2018; Burberg et al. 2020). This indicates that PIs are degraded under N depletion and the stored nutrients are allocated to growth. The release of N from internal storages is presumably of higher importance when *Microcystis* faces N scarcity, which is the case when N availability is low or P availability is high. The C/N ratios indicate that especially high P concentration resulted in a strong N limitation during the experiments (Burberg et al. 2018; Burberg et al. 2020). Therefore, it was expected that PI degradation rates (d_{BN}, d_{CP}) increase with decreasing N and increasing P. Interestingly, the d_{BN} and d_{CP} declined with increasing N and P concentration, which supports the hypothesis that lower N concentrations increase the need for degrading PIs but contradicts that high P concentrations have the same effect. As P is not a constituent of the PIs, its effect on PI degradation might be more complex than for N and could be mediated by other processes, like growth.

Beside direct effects on the PI production, nutrients might indirectly affect the production of secondary metabolites through growth. It has been suggested before that conditions that favor growth might also favor the production of toxins (Tonk et al. 2009) and it can also be expected that N and P affect the interaction differently, as N is an essential constituent of PIs while P is not. The N-experiment shows that the PI production rates increased with increasing growth rates, which is in accordance with studies that showed a positive linear correlation between growth rate and the production of MCs, anabaenopeptins and microviridins (Orr and Jones 1998; Long et al. 2001; Rohrlack and Utkilen 2007; Jähnichen et al. 2008). However, in the P-experiment there was no correlation between PI production rates and growth rates. The interaction of growth rate and metabolite production under P limitation is controversial (Long 2001; Tonk et al. 2009). Interestingly, the highest growth rates together with the highest PI production rates were reached in the lowest N and P concentrations. It would be plausible to assume, that reduced resources would result in a stronger trade-off between growth and PI production and therefore a negative correlation between these two rates. However, this seemed not to be the case here. Perhaps, the high initial nutrient concentrations have in general been a stress factor (see above) that inhibited intracellular processes like growth and PI production.

In conclusion, the usage of models provides valuable insight beyond measurable PI concentration. Models support our understanding of how different factors contribute to the success of cyanobacteria and can help predict cyanobacterial blooms, which is important as the threat by cyanobacterial blooms is increasing (O'Neil et al. 2012; Ostfeld et al. 2015; Glibert 2019). While many studies show that nutrient availability can alter the metabolite content in cyanobacteria, they often lack temporal information about the rate at which changes occur. In this study, a dynamic processed-based model was applied to close that knowledge gap about how PI dynamics are affected by nutrient concentrations. The use of rates to describe temporal changes in cyanobacterial metabolites concentrations is not new. Orr and Jones (1998) for example calculated cyanotoxin production rates (μ_{tox}) for MCs to analyze the relationship with the cell division rates and Jähnichen et al. (2008) modelled the production coefficients and rates of production and depletion for MC in *Microcystis*. However, similar studies concerning PIs are scarce. The results of this study show that the PI dynamics in *Microcystis aeruginosa,* one of the major bloom-forming cyanobacterium, are highly affected by the nutrient species and its availability, and it is likely that this is also the case for other non-N-fixing cyanobacteria. Therefore, PIs deserve far more attention and should be considered in the discussion about the toxicity of cyanobacterial blooms and the mitigation of cyanobacterial blooms through nutrient manipulation.

3.6. References

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Chapter 4

The effect of light intensity on growth and protease inhibitor content of *Microcystis aeruginosa*

4.1. Abstract

Cyanobacteria are bloom-forming photoautotrophs that produce a wide range of potential toxic metabolites, including protease inhibitors (PIs). As the metabolite production requires energy and resources, many environmental factors potentially affect the content of PIs. One important factor could be the availability of light, which provides the energy necessary for carbon fixation and the subsequent production of organic substances, such as PIs. In batch-culture experiments it was therefore tested how different light intensities (26, 65, 130 & 200 µmol photons m^{-2} s^{-1}) affect growth, stoichiometry and PI content of the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43. This strain does not contain any microcystins, but produces two PIs: nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954). I hypothesized that lower light intensities would promote the growth of *M. aeruginosa* and concurrently result in higher PI contents. Indeed, *M. aeruginosa* reached the highest biomass and cell abundance at the lowest light intensity. An increase in the light intensity resulted in growth reduction, most likely caused by oxidative stress. The effect of light on the PI content, however, was not conclusive. While the biomass BN920 content was positively correlated with the light intensity, the biomass CP954 content decreased with increasing light intensity, which resulted in a change of the PI composition depending on the light intensity. Additionally, the PI content positively correlated with the growth rate and decreased with increasing stoichiometric (C/N, C/P) ratios, showing that the growth and nutrient availability also affected the PI content under varying light conditions. Altogether, the results indicate that the light intensity has pronounced effects on the growth and the PI content of *M. aeruginosa* and could change the toxicity of cyanobacterial blooms by directly affecting the PI content and by changing the composition of PIs.

Keywords: Cyanobacteria – Light – Protease Inhibitor – Growth – Stoichiometry

4.2. Introduction

Cyanobacteria are photoautotrophic organisms that, under favorable environmental conditions, proliferate in freshwater systems worldwide. In the last decades, the number, frequency and severity of so-called cyanobacterial blooms have increased dramatically (Duan et al. 2009; Harke et al. 2016; Paerl and Barnard 2020), causing problems ranging from unpleasant odors to the death of wild and domestic animals (McBarron and May 1966; Smith et al. 2008; Hilborn and Beasley 2015; Davis et al. 2019) and potentially health hazards for humans (Jochimsen et al. 1998). Consequently, economic sectors like fisheries, tourism or drinking water production are affected, resulting in billions of dollars in additional costs (Steffensen 2008; Dodds et al. 2009; Sanseverino et al. 2016). The expansion of cyanobacterial blooms is primarily caused by anthropogenic eutrophication, warm water temperatures and rising CO_2 levels (Kosten et al. 2012; Paerl and Otten 2013; Verspagen et al. 2014; Paerl and Barnard 2020). Even though regulations, e.g. the control of fertilizer usage, might reduce the risk of cyanobacterial blooms in some regions, the global expansion of cyanobacterial blooms is likely to continue and with it the economic and ecological problems (Paerl and Huisman 2009; Glibert 2019).

One characteristic of cyanobacteria is the production of toxic secondary metabolites, including microcystins (MCs). MCs are the best studied group of cyanobacterial metabolites due to their toxicity for humans (Jochimsen et al. 1998), their wide distribution upon many bloom-forming cyanobacterial genera, e.g. *Anabaena* (Rapala et al. 1997), *Oscillatoria* (Sivonen 1990) and *Microcystis* (Jähnichen et al. 2011), and their presence in ponds and lakes worldwide (Svirčev et al. 2019). Especially major consumers of cyanobacterial biomass, like the water flea *Daphnia* (Chislock et al. 2013), are harmed upon exposure to MCs (Rohrlack et al. 1999; Lürling 2003). However, cyanobacterial strains that do not produce MCs have also been shown to be harmful to *Daphnia*, due to feeding inhibition caused by other secondary metabolites (Lürling 2003), in particular protease inhibitors (PIs). PIs are a structurally diverse family that inhibit serine proteases (Namikoshi and Rinehart 1996), in particular trypsins and chymotrypsins, which are the major proteases in the gut of *Daphnia* (Von Elert et al. 2004), resulting in e.g. reduced growth (Schwarzenberger et al. 2012; Kuster et al. 2013). Some PIs, e.g. microviridin J, can even increase the mortality of daphnids by interfering with the molting process of *Daphnia* (Rohrlack et al. 2003; Rohrlack et al. 2004). Even though PI concentrations in lake surface waters reach similar concentration as MCs (Beversdorf et al. 2018) and are known to inhibit human proteases, like kallikrein (Gademann et al. 2010), the potential risk for human health is still unknown and many aspects about PIs are still uncertain.

One important aspect that needs further investigation, is the effect of environmental conditions on the PI production and content in cyanobacteria. It is known that the concentration of nitrogen (N) and phosphorus (P) affects the PI content of *M. aeruginosa* (Schwarzenberger et al. 2013; Burberg et al. 2018; Burberg et al. 2020). Other studies report that additional factors, e.g. trace metals or temperature (Amé and Wunderlin 2005; Facey et al. 2019), have an effect on the production of secondary metabolites in cyanobacteria. Of special interest is the factor light and in particular the light intensity. The light intensity is among the factors that determine the amount of carbon assimilated by cyanobacteria, which is subsequently available for the production of PIs and other organic molecules (Tilzer 1987; Gregory 1990; Sujatha 2015). It is long known that the light intensity affects the production of cyanobacterial metabolites and therefore the toxicity of *Microcystis* (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Utkilen and Gjølme 1992). However, the reported results are sometimes contradictory and therefore not clear. The response to the light intensity depends on the tested species and strains (Pineda-Mendoza et al. 2016) as well as on the light quality (Kaebernick et al. 2000; Carneiro et al. 2009), the tested light intensity range (Utkilen and Gjølme 1992) and the interaction with other factors, such as temperature (Song et al. 1998) or nutrient availability (Chaffin et al. 2018). A few studies have described the effect of light intensity on different groups of PIs. One study analyzed microviridins in the cyanobacterium *Planktothrix* and found an increase in the cell-bound microviridin content with declining light availability (Rohrlack and Utkilen 2007), while another study found that *Anabaena* strain 90 had the highest concentration of anabaenopeptins at a rather low light intensity of 23 µmol photons m^{-2} s^{-1} (Repka et al. 2004). Tonk et al. (2009) confirmed the results for anabaenopeptins in *Anabaena* and found additionally a decrease of cyanopeptolins in *M. aeruginosa* when grown at light intensities of 150 instead of 40 µmol photons m^{-2} s^{-1}.

However, there is still a lack of knowledge about the effect of light intensity on the production and content of cyanobacterial PIs. It was therefore tested how a range of light intensities affect growth, stoichiometry and PI content of the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43, a strain that produces two N-rich PIs, but no MCs (Von Elert et al. 2005). As *M. aeruginosa* normally shows good growth at light intensities below 50 µmol photons m^{-2} s^{-1} (e.g. Hesse et al. 2001; Muhetaer et al. 2020), it was expected that *M. aeruginosa* will grow better at a low light intensity and that high light intensities will inhibit growth. In case of PIs, it was hypothesized that, in accordance with Tonk et al. (2009), better growth conditions (low light intensity) will promote higher PI contents, while high light intensities might diminish the PI content.

4.3. Material and Methods

4.3.1. Culturing conditions

The experiments were performed with the cyanobacterium *M. aeruginosa* NIVA Cya 43 (NORCCA, Norwegian Institute for Water Research), which produces the two PIs cyanopetolin 954 (CP954) and nostopeptin 920 (BN920) but no MCs (Von Elert et al. 2005). *M. aeruginosa* was pre-cultured in 1 L Erlenmeyer flasks filled with 400 mL modified WC medium (Guillard 1975) for 11 days. The flask were kept on a horizontal shaker at constant temperature (20 ± 1°C). Nitrogen (as nitrate NO_3^-) and phosphorus (as phosphate PO_4^{3-}) were set to initial concentrations of 2000 µM and 50 µM respectively.

For the experiment, twelve 1 L Erlenmeyer flasks were filled with 400 mL medium, autoclaved and inoculated with $1.3 \cdot 10^5$ cell mL^{-1} of *M. aeruginosa* from the pre-culture. The cultures were cultivated under constant illumination with a mixture of warm (Osram, Lumilux T8, L 36W/840) and day light (Osram, Lumilux De LUXE T8, L 36W/954). The light intensity gradient was achieved by covering the culture flasks with 0, 1, 3 or 6 layers of neutral-density (ND) filter foil (Nr. 298, ND 0.15, Chris James Lighting Filters, London, UK), which reduces the light intensity by approx. 30% per layer without affecting the light spectrum ('color') and light quality. This resulted in light intensities of roughly 200, 130, 65 and 26 µmol photons m^{-2} s^{-1}, which are hereafter referred to as 'very high (VL)', 'high (HL)', 'intermediate (IL)' and 'low (LL)'. All treatments were set up in triplicates. The cultures were cultivated for 27 days and randomized daily. Every 1 – 2 days, samples (0.5 mL) from all cultures were taken under sterile conditions to determine the cell density using a Neubauer improved counting chamber. Volumes between 10 and 200 mL were used every 3 days to measure the particulate organic carbon (POC, as proxy for cyanobacterial biomass), particulate organic nitrogen (PON) and particulate phosphorus (P_{Part}) as well as the PI content. The necessary volumes were estimated from the cell density.

4.3.2. Determination of POC, PON and P_{Part}

Sample volumes equivalent to 0.25 mg POC were filtered onto pre-combusted GF/F filters (Macherey & Nagel) to determine POC and PON. The filters were dried at 60 °C for 24 h and packed into tin capsules for the subsequent analysis using a Flash EA2000 Analyzer (Thermo Fisher). Another sample equivalent to 0.5 mg C was filtered on GF/F filters for the analysis of P_{Part}. The filters were transferred into 10 mL of a potassium peroxodisulphate and sodium hydroxide solution and autoclaved for 1 hour at 120 °C. The soluble reactive P was subsequently analyzed following the molybdate-ascorbic acid method (Greenberg et al. 1985) using a DR5000 UV-Vis spectrometer (Hach). The measurements were used to calculate the

concentration of POC, PON and P_{Part} in the cultures and the elemental stoichiometry of the cyanobacterial biomass (C/N, C/P & N/P ratios).

4.3.3. Extraction and quantification of PIs

PIs were extracted according to Burberg et al. (2018). In short, culture samples (equivalent to approx. 0.25 mg C) were centrifuged for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in 10 mL methanol (80%) and 10 µL of microcystin LR (internal standard, 10 µg mL^{-1}, Enzo Life Sciences). The samples were sonicated for several minutes and centrifuged again. The supernatant was transferred into test tubes, evaporated to dryness using a vacuum centrifuge (RVC 2-25, Christ) and re-dissolved in 1 mL methanol (100%). The samples were dried again, taken up in 100 µL methanol (100%) and centrifuged for another 2 minutes. Finally, the supernatant was transferred into HPLC vials and measured using an ultra-high-pressure liquid chromatography system (UHPLC, Accela, Thermo Fisher Scientific) coupled with an Exactive Orbitrap mass spectrometer (MS, Thermo Fisher Scientific). The chromatographic separations were carried out on a C_{18}-column (Nucleosil, 125/2, 100-3, Macherey and Nagel) with a gradient of acetonitrile and ultra-pure water, each containing 0.05% trifluoracetic acid, as mobile phase. A detailed description of the used gradient and the applied device settings is given in Burberg et al. (2018). Under the applied conditions, the PIs form two positively charged adduct ions ($[M+H-H_2O]^+$; $[M+Na]^+$), which were measured at m/z = 903.46108 and m/z = 943.45331 (BN920 adducts) and m/z = 937.42211 and m/Z = 977.41394 (CP954 adducts). The adducts of each PI were summed up to ´BN920´ and ´CP954´. Using previous established calibration curves, PI concentrations for each sample were calculated and normalized to the cell abundance (cellular PI content) and the POC (biomass PI content).

4.3.4. Data analysis

All analyses were carried out using R (R Core Team 2020) and RStudio (RStudio Team 2020). In batch cultures, *M. aeruginosa* typically shows logistic growth, characterized by an initial lag phase, followed by exponential growth and a leveling off, as growth becomes limited by one or more resources (stationary phase). Logistic growth models were fitted to the POC and cell abundance data using the R-package ‘growthrates’ and the function *all_growthmodels* (Petzoldt 2017). The applied model equation was

$$N_t = (K´ \cdot N_0) / (N_0 + (K´ - N_0) \cdot e^{-\mu max \cdot t})$$

with N_t and N_0 as cyanobacterial biomass or cell abundance at time t or t_0 respectively, and the two model parameters $K´$ and μ_{max} as carrying capacity and maximal growth rate. For each model parameter (N_0, $K´$, μ_{max}), averages for each treatment were calculated based on individual fits for each replicate and tested for differences using one-way analysis of variance

(ANOVAs) and Tukey HSD *post hoc* tests. ANOVAs and Tukey HSD tests were also used to compare treatments on individual days.

To verify the effect of the light intensity on the PI content, the PI content measured on the individual sampling days was summed up for each individual replicate and normalized by the number of sampling days, which resulted in a mean PI content per sampling day for each replicate. These means were then used to calculate an average for each treatment, which were subsequently compared using ANOVAs and Tukey HSD *post hoc* tests. Prior to analysis, data were tested for normal distribution (Shapiro-Wilk's test) and for variance homogeneity (Levene's test).

Linear mixed effect models (R-package 'lme4', Bates et al. 2015) were used to analyze the effects of light intensity and sampling day (fixed effects) on the PI content. Replicates were set as random factor. Data were transformed using the natural logarithm prior to analysis, in order to achieve normal distribution and variance homogeneity of model residuals. The effectivity of the transformation was checked graphically by generating quantile-quantile plots (to check the normality) and plotting (standardized) residuals versus fitted values (to check the variance homogeneity and homoscedasticity). The normality and the variance homogeneity were additionally tested using Shapiro-Wilk's and Levene's tests.

The correlations between biomass PI content and growth rate and between PI content and elemental stoichiometry, were analyzed using regression models. Linear ($y = ax + b$), exponential ($y = e^a x^b$) and quadratic ($y = ax^2 + bx + c$) models were fitted to the data. The best model fit was chosen depending on the Spearman correlation coefficient (R_S), the coefficient of determination (R^2) and the *p*-value of the regression models together with a visual data exploration.

4.4. Results

The effect of different light intensities on the growth, stoichiometry and protease inhibitor (PI) content of *Microcystis aeruginosa* NIVA Cya 43 was tested in batch culture experiments at 4 different light intensities over 27 days.

4.4.1. Growth performance of *M. aeruginosa* under different light intensities

The growth of *M. aeruginosa* was measured by changes in cell abundance and particulate organic carbon (POC), which served as proxy for cyanobacterial biomass. *M. aeruginosa* showed logistic growth in all treatments, with a slow increase in the beginning, followed by an exponential and finally a stationary phase (Fig. 1 A & B, Tab. 1). By the end of the experiment (at day 27), *Microcystis* reached a higher cell abundance and biomass when grown at low light intensities compared to high light intensities, as shown by the carrying capacity (*K´*, Tab. 1). *K´* was more than 40 % higher at the lowest light intensity (26 µmol photons m^{-2} s^{-1}) compared to the highest intensity (200 µmol photons m^{-2} s^{-1}) for both cell abundance and biomass, indicating that the tested strain of *M. aeruginosa* is better adapted to low light intensities. However, the maximal growth rate (μ_{max}) was unaffected by the light intensity (one-way ANOVA, biomass: $F_{3,8} = 2.307$, $p = 0.153$; cellular: $F_{3,8} = 0.388$, $p = 0.765$). μ_{max} was around 0.3 d^{-1}, when growth was measured as POC change, and in the range of 0.3 to 0.44 d^{-1}, when measured using the cell abundance.

Growth does not only require carbon (C), but also nitrogen (N) and phosphorus (P). N and P are taken up from the medium and are incorporated into the biomass. These N and P fractions were measured as particulate organic nitrogen (PON, Fig. 1 C) and phosphorus (P_{Part}, Fig. 1 D). The analysis showed that PON and P_{Part} were both affected by the interaction of light intensity and time (sampling day) as well as by time and treatment alone (Tab. 2). PON increased in all treatments, in particular between day 6 and 9, and remained around 25 – 30 µg mL^{-1} thereafter. Simultaneously, the cellular N content (Supplementary Fig. S1 B) increased until day 9, but decreased thereafter. This indicates that N was taken up and used to synthesize N containing compounds, such as proteins or PIs, during early to mid-exponential growth phase. While cellular growth continued (Fig. 1 A), the external N was likely depleted, which resulted in the decline of the cellular N content. Compared to PON, P_{Part} increased slower and reached maximal values (0.068 – 0.075 µg mL^{-1}) from day 15 onwards, while the cellular P content constantly decreased from day 0 on (Supplementary Fig. S1 C). By the end of the experiment (day 27), P_{Part} and PON were the highest at the lowest light intensity, even though P_{Part} and PON both increased slower at the lowest light intensity. This difference was, however, only significant for PON ($F_{3,8} = 4.583$, $p < 0.05$), but not for P_{Part} ($F_{3,8} = 3.084$, $p = 0.09$).

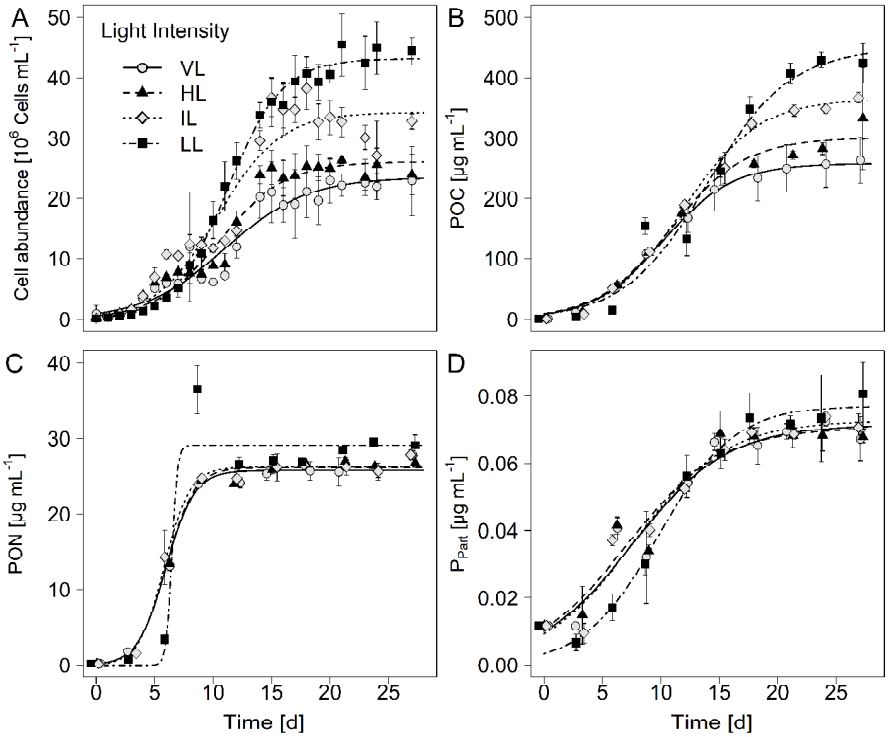

Figure 1. Cell abundance (**A**), particulate organic carbon (POC, **B**), particulate organic nitrogen (PON, **C**) and particulate phosphorus (P_{Part}, **D**) in batch cultures of *M. aeruginosa* grown at light intensities of 26 ('Low, LL'), 65 ('Medium, ML'), 130 ('High, HL') and 200 µmol photons m^{-2} s^{-1} ('Very High, VL'). Displayed are mean values (± SD, n = 3). Curves were fitted using a logistic growth model. Equations and growth parameters for **A** and **B** are given in Tab. 1. The readability of the figures was increased by jittering the data points around the respective x-value (sampling day).

4.4.2. Elemental stoichiometry of *M. aeruginosa*

The stoichiometry of the cyanobacterial biomass was measured every 3 days to find possible light or nutrient limitations. The stoichiometric ratios were significantly affected by the light intensity, the sampling day and the interaction of day and light intensity (Tab. 2), with the exception that light intensity alone had no significant effect on the N/P ratio. Until day 9, the C/N ratios (Fig. 2 A) stayed mostly below the Redfield ratio (C/N = 6.6), but increased thereafter to ratios between 11 and 17. The Redfield ratio of 106:16:1 (C:N:P, Redfield 1958) indicates growth under unlimited nutrient conditions. Values exceeding these ratios, however, point towards nutrient limitation. In particular, at the lowest light intensity the C/N ratios increased to values of up to 17, which indicates that *M. aeruginosa* was limited by N. The level of N limitation seemed to decrease with increasing light intensity, as indicated by the C/N ratios that were significantly lower at the two highest light intensities compared to the two lowest light intensities, in particular from day 18 onwards (Tukey HSD *post hoc* tests following one-way ANOVAs for days 18 – 27, $p < 0.05$). The C/P ratios (Fig. 2 B) reached their minima (50 to 85) on day 3 at all light intensities and increased thereafter to values between 300 and 500, indicating P limitation at all light intensities.

Similar to the C/N ratio, C/P ratios were tendentially the highest at the lowest light intensity, even though this trend was only significant on some days (i.e. day 18 & 21, $p < 0.05$). Nevertheless, this indicates that the growth of *M. aeruginosa* was limited by both N and P, in particular when grown at the lowest light intensity. The N/P ratio did not differ between the light intensity on most days (Fig. 2 C, Tab. 2), except for the days 6 and 9 were N/P ratios were significantly different between the treatments (Tukey HSD *post hoc* tests following one-way ANOVAs, $p < 0.05$). However, the N/P ratios dropped in the beginning (day 3) to their lowest values and spiked on day 9, indicating that the stoichiometric balance was disturbed by the fast uptake of N, as shown by the PON (Fig. 1 B). The N/P ratio of the cyanobacterial biomass stabilized after day 15 at values of 25 – 29, with no significant differences between the light intensities (Tukey HSD *post hoc* tests following one-way ANOVAs for each day, $p > 0.05$).

Table 1. Equations, model parameters and coefficients of determination (R^2) for the logistic growth models fitted to cell abundance (Fig. 1 A, 10^6 cells mL^{-1}) or POC respectively (Fig. 1 B, µg mL^{-1}) of *M. aeruginosa* grown at different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). For each light intensity, mean values for the initial cell abundance or POC concentration (N_0), the maximal growth rate (μ_{max}) and the carrying capacity ($K´$) were calculated based on individual fits for each replicate (n = 3), and tested for differences between the light intensities (Tukey HSD *post hoc* test following one-way ANOVA). Significant differences were only found for $K´$ (indicated by capital letters).

Cell abundance

	Equation [a]	R^2	N_0 [10^6 cells mL^{-1}]	μ_{max} [d^{-1}]	$K´$ [10^6 cells mL^{-1}]	
VL	$N_t = \frac{22.49}{0.96 + 22.49\ e^{(-0.29t)}}$	0.86	1.10 ± 0.87	0.30 ± 0.12	24.11 ± 3.38	C
HL	$N_t = \frac{15.13}{0.58 + 25.48\ e^{(-0.36t)}}$	0.93	0.58 ± 0.24	0.36 ± 0.04	26.01 ± 2.22	C
IL	$N_t = \frac{23.38}{0.68 + 33.51\ e^{(-0.37t)}}$	0.90	0.69 ± 0.11	0.37 ± 0.02	34.23 ± 0.88	B
LL	$N_t = \frac{15.30}{0.43 + 42.81\ e^{(-0.43t)}}$	0.98	0.32 ± 0.10	0.44 ± 0.06	43.29 ± 3.42	A

POC

	Equation [a]	R^2	N_0 [µg mL^{-1}]	μ_{max} [d^{-1}]	$K´$ [µg mL^{-1}]	
VL	$N_t = \frac{2175.8}{8.4 + 249.3\ e^{(-0.33t)}}$	0.95	8.49 ± 0.87	0.33 ± 0.12	257.7 ± 3.4	C
HL	$N_t = \frac{2877.6}{9.6 + 291.2\ e^{(-0.31t)}}$	0.95	12.28 ± 0.24	0.31 ± 0.04	318.9 ± 2.2	BC
IL	$N_t = \frac{3185.2}{8.8 + 354.9\ e^{(-0.31t)}}$	0.99	8.69 ± 0.11	0.31 ± 0.02	364.3 ± 0.9	AB
LL	$N_t = \frac{3804.6}{8.5 + 440\ e^{(-0.28t)}}$	0.97	8.52 ± 0.10	0.28 ± 0.06	452.1 ± 3.4	A

[a] Growth equation: $N_t = (K´ \cdot N_0) / (N_0 + (K´ - N_0) \cdot e(-\mu_{max} \cdot t))$, with t = time [d], N_t = cell abundance [cells mL^{-1}] or POC [µg mL^{-1}] at time t.

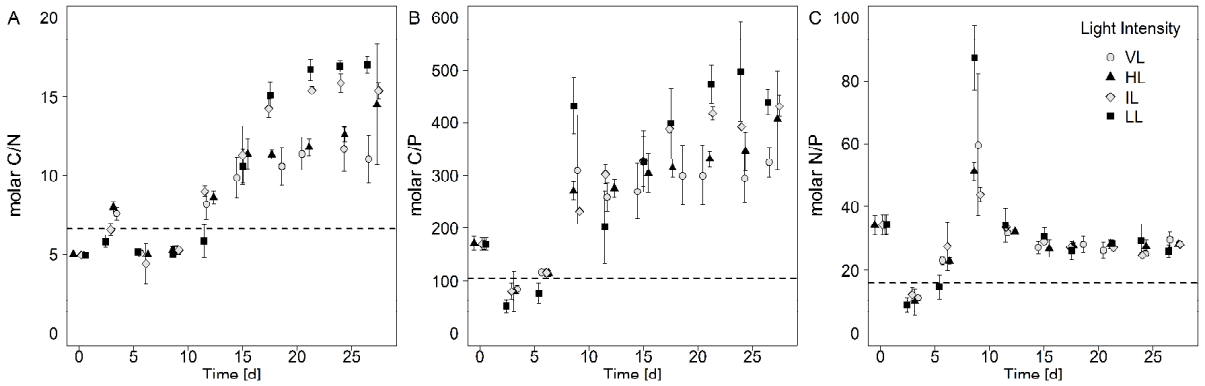

Figure 2. Molar C/N (**A**), C/P (**B**) and N/P (**C**) ratios of *M. aeruginosa* grown at different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Displayed are mean values (± SD, n = 3). Dashed lines represent the Redfield ratios (C/N = 6.6, C/P = 106, N/P = 16; Redfield 1958). The readability of the figures was increased by jittering the data points around the respective x-value (sampling day).

Table 2. Linear mixed-effects models for particulate organic carbon (POC, Fig. 1 B), particulate organic nitrogen (PON, Fig. 1 C), particulate phosphorus (P_{Part}, Fig. 1 D) and elemental stoichiometry (C/N, C/P, N/P ratio; Fig. 2 A – C) of *M. aeruginosa* grown for 27 days at four light intensities. Prior to the analysis, data were log transformed. Mean Sq: Mean of squares, NumDF: Numerator degrees of freedom, DenDF: Denominator DF, Test statistics: *F*- and *p*-values.

POC	**Mean Sq**	**NumDF**	**DenDF**	***F***	***p***
Light intensity	0.148	3	78	17.933	< 0.001
Day	48.958	9	78	5941.470	< 0.001
Light intensity : Day	0.300	27	78	36.422	< 0.001
PON					
Light intensity	0.132	3	78	37.773	< 0.001
Day	35.548	9	78	10139.877	< 0.001
Light intensity : Day	0.216	27	78	61.682	< 0.001
P_{Part}					
Light intensity	0.128	3	78	5.739	< 0.01
Day	6.963	9	78	311.217	< 0.001
Light intensity : Day	0.094	27	78	4.189	< 0.001
C/N ratio					
Light intensity	0.079	3	78	9.898	< 0.001
Day	2.458	9	78	306.375	< 0.001
Light intensity : Day	0.061	27	78	7.578	< 0.001
C/P ratio					
Light intensity	0.083	3	78	2.899	< 0.05
Day	4.306	9	78	150.791	< 0.001
Light intensity : Day	0.108	27	78	3.791	< 0.001
N/P ratio					
Light intensity	0.004	3	78	0.159	0.923
Day	2.253	9	78	98.598	< 0.001
Light intensity : Day	0.067	27	78	2.933	< 0.001

[a] Significance levels: < 0.05; < 0.01; < 0.001

4.4.3. Inhibitor content of *M. aeruginosa*

The light intensity might not only affect the growth of *M. aeruginosa*, but also its toxicity. Therefore, the content of two N-rich PIs, BN920 and CP954, was measured and analyzed for potential light intensity effects. Both PIs were produced constitutively, but the PI content changed while *M. aeruginosa* grew. The statistical analysis showed that the light intensity significantly affected both the cellular and biomass PI content (Tab. 3). The biomass content of BN920 (Fig. 3 A) ranged from 1 to 56 µg mg C^{-1}, while the cellular content (Fig. 3 B) was between 10 and 436 fg $cell^{-1}$. In both cases, the BN920 content was the highest at the highest light intensity throughout the experiment and decreased with decreasing light intensity. Additionally, the BN920 content varied over time (Tab. 3). Interestingly, the BN920 content decreased from day 0 to day 10 – 13 at the two lowest light intensities and remained at low levels thereafter. At higher light intensities, the BN920 content increased initially and declined thereafter, but remained above the content observed at the lower light intensities on day 27 (Tukey HSD test following *post hoc* one-way ANOVA, cellular: $F_{3,8} = 87.37$, $p > 0.001$; biomass: $F_{3,8} = 30.88$, $p > 0.001$).

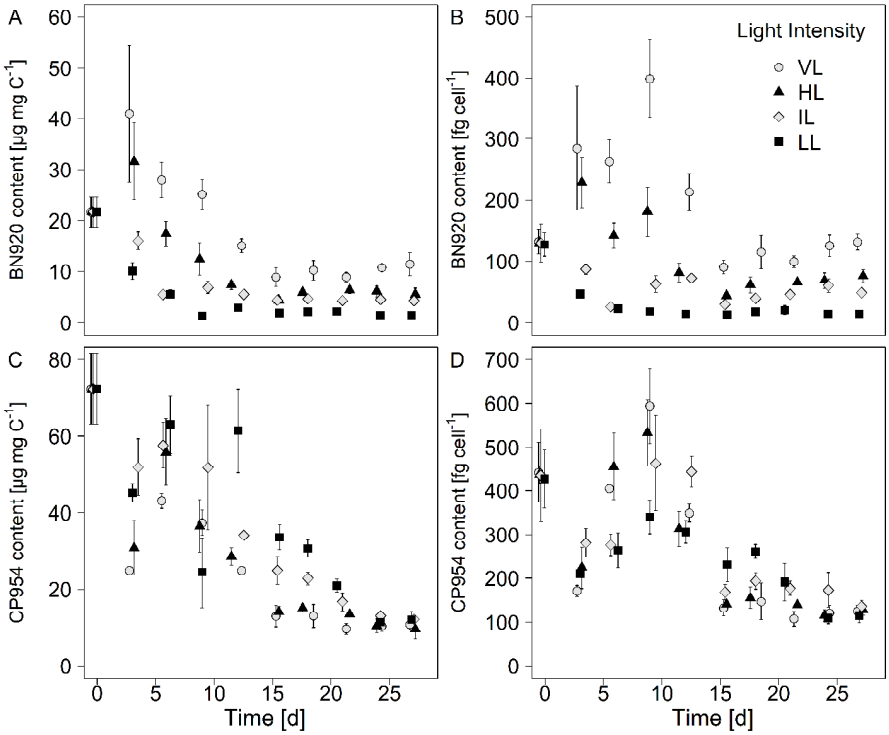

Figure 3. Biomass (**A**, **C**) and cellular (**B**, **D**) BN920 and CP954 content of *M. aeruginosa* grown at different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Displayed are mean values (± SD, n = 3). The readability of the figures was increased by jittering the data points around the respective x-value (sampling day).

A different picture was found for CP954 (Fig. 3 C & D). Like for BN920, the light intensity, sampling day and their interaction significantly affected the CP954 content (Tab. 3). The biomass and cellular content decreased at all light intensities during early growth (days 0 – 3), followed by a short increase between days 6 to 13 (exponential growth) and another decrease thereafter (late growth phase). On most days, the biomass CP954 content was negatively correlated with the light intensity. Towards the end of the experiment the difference between light intensities narrowed down and was no longer present on day 27 (cellular: $F_{3,8}$ = 1.172, p = 0.38; biomass: $F_{3,8}$ = 1.313, p = 0.34). Overall, the CP954 content was between 6 and 80 µg mg C^{-1}, respectively 89 and 655 fg $cell^{-1}$, and tended to be higher than the BN920 content.

The different behavior of the two PIs was confirmed by comparing the summed up PI content, normalized to the number of sampling days, between the tested light intensities (Fig. 4). Both the biomass and cellular BN920 content increased with increasing light intensity (Tukey HSD *post hoc* test following one-way ANOVA; biomass: $F_{3,8}$ = 71.72, p < 0.001; cellular: $F_{3,8}$ = 248.6, p < 0.001). The biomass CP954 content, on the other hand, decreased with increasing light intensities ($F_{3,8}$ = 32.1, p < 0.001), while the cellular CP954 content was significantly higher at the intermediate light intensity compared to the lowest light intensity ($F_{3,8}$ = 4.47, p < 0.05). The changes in the BN920 and CP954 content resulted in a total biomass PI content that was unaffected by the light intensity ($F_{3,8}$ = 1.383, p = 0.316), but a significant increase of the total cellular PI content with increasing light intensity ($F_{3,8}$ = 87.07, p < 0.001). This shows that *M. aeruginosa* maintains its overall biomass PI content with increasing light intensity, but shifts proportionally towards CP954. This is also supported by the ratios of the two PIs; on average, the BN920 content was only 12% of the CP954 content at the lowest light intensity, but over 80% at the highest light intensity.

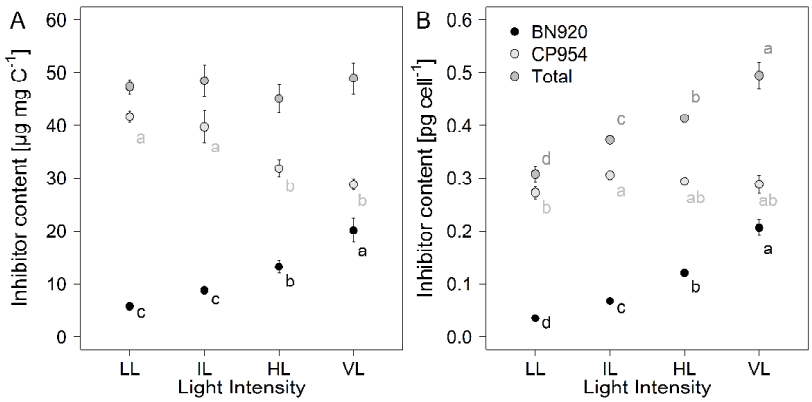

Figure 4. Biomass (**A**) and cellular (**B**) protease inhibitor content of *M. aeruginosa* grown at different light intensities ('Low, LL'; 'Medium, ML'; 'High, HL'; 'Very High, VL'). Displayed are mean values (± SD, n = 3) for each treatment calculated from the summed up inhibitor content of each replicate, normalized by the number of sampling day. Different letters indicate differences between the light intensities (Tukey HSD *post hoc* test following one-way ANOVA, p = 0.05).

Table 3. Linear mixed-effects models for the biomass and cellular content BN920 and CP954 (Fig. 3 A – D) of *M. aeruginosa* grown for 27 days at four light intensities. Prior to the analysis, data were log transformed. Mean Sq: Mean of squares, NumDF: Numerator degrees of freedom, DenDF: Denominator DF, Test statistics: *F*- and *p*-values.

cellular BN920	**Mean Sq**	**NumDF**	**DenDF**	***F***	***p***
Light intensity	21.153	3	78	750.941	< 0.001
Day	2.272	9	78	80.667	< 0.001
Light intensity : Day	0.499	27	78	17.705	< 0.001
cellular CP954					
Light intensity	0.125	3	78	6.382	< 0.001
Day	3.079	9	78	157.008	< 0.001
Light intensity : Day	0.136	27	78	6.937	< 0.001
biomass BN920					
Light intensity	13.712	3	78	573.444	< 0.001
Day	4.714	9	78	197.138	< 0.001
Light intensity : Day	0.365	27	78	15.251	< 0.001
biomass CP954					
Light intensity	1.288	3	78	58.008	< 0.001
Day	5.098	9	78	229.646	< 0.001
Light intensity : Day	0.158	27	78	7.127	< 0.001

4.4.4. Effects of stoichiometry and growth on the PI content

The biomass PI content was also analyzed as a function of the modeled growth rates (Fig. 5) and elemental ratios (Fig. 6). The Spearman correlation coefficient (R_S) and the applied regression models (Tab. 4, Fig. 5) showed that the content of both PIs was positively correlated with the growth rate. This correlation was more pronounced for the CP954 content than for the BN920 content, as indicated by the higher R_S and R^2. The elemental ratios had the reversed effect on the PI content. The PI content decreased exponentially with increasing C/N and C/P ratios (Fig. 6 A – D; Tab. 4), which indicates that condition that favored growth (low C/N and C/P ratios) also lead to increased biomass PI contents. The correlation between N/P ratio and the PI content was only weak (R_S < 0.2, Tab. 4 and Tab. S1). Albeit the decline of the BN920 content with increasing N/P ratio was significant (Tab. 4), the regression models only described a small percentage of the data, as indicated by the low R^2 values. This was due to data points clustering around N/P ratios of 25 – 30 and simultaneously low PI contents, which reflects the late growth phases (day 15 – 27, Fig. 2 C, Fig. 3). Altogether, the results show that potential nutrient limitations, in particular in the late growth phases, might not only reduce growth but also the PI content of *M. aeruginosa*.

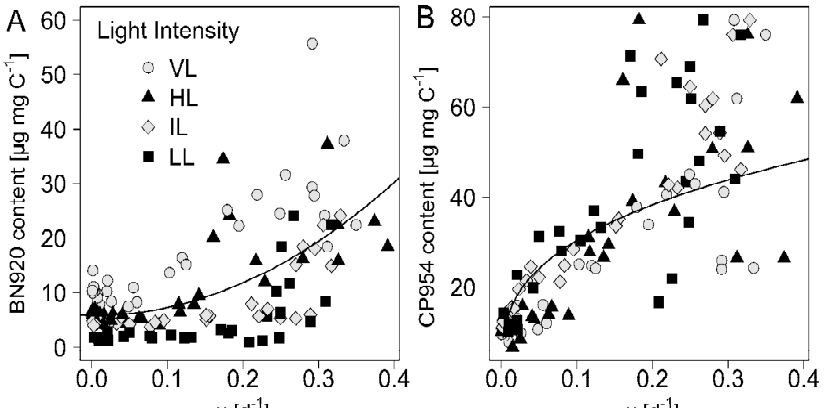

Figure 5. Biomass content of the two protease inhibitors BN920 (**A**) and CP954 (**B**) as a function of the growth rate (μ) of *M. aeruginosa*. Each points represents the measured inhibitor content in a single replicate at a single sampling day and the corresponding growth rate, which was taken from the logistic growth model for POC (see Fig. 1 B). Different symbols indicate the different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Solid lines represent regression models applied to the data (Tab. 4).

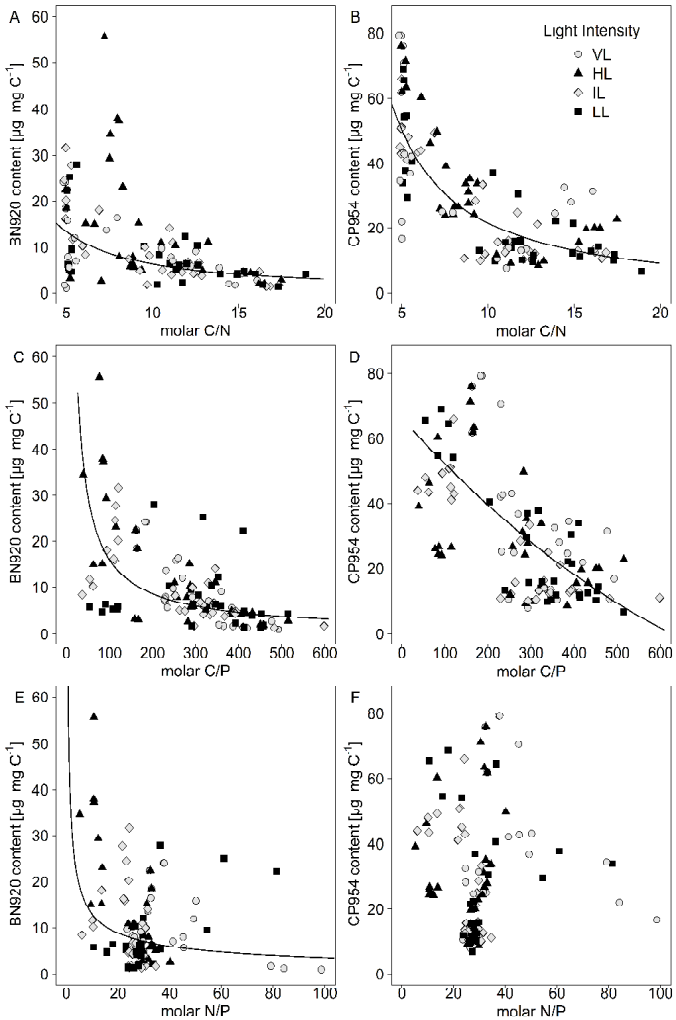

Figure 6. Biomass content of the two protease inhibitors BN920 (**A**, **C**, **E**) and CP954 (**B**, **D**, **F**) as a function of elemental ratios (C/N, C/P & N/P) of *M. aeruginosa*. Each points represents the measured inhibitor content in a single replicate at a single sampling day and the corresponding elemental ratios. Different symbols indicate the different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Solid lines represent regression models applied to the data (Tab. 4).

Table 4. Spearman correlation coefficient (R_S), model equations and the corresponding model coefficient of determination (R^2) and *p*-values for regression models applied to the biomass PI content as response variable (y) and growth rate (μ, Fig. 5) or elemental ratios (Fig. 6) as single explanatory variables (x).

BN920	R_S	**Equation** [a]	R^2	*p*
μ	0.49	$y = 154.7\ x^2 - 0.89\ x + 5.8$	0.37	< 0.001
C/N	-0.6	$y = e^{4.29} \cdot x^{-1.06}$	0.29	< 0.001
C/P	-0.7	$y = e^{6.9} \cdot x^{-0.897}$	0.37	< 0.001
N/P	-0.07	$y = e^{3.86} \cdot x^{-0.056}$	0.07	< 0.01
CP954				
μ	0.86	$y = e^{4.18} \cdot x^{0.33}$	0.68	< 0.001
C/N	-0.79	$y = e^{5.88} \cdot x^{-1.22}$	0.66	< 0.001
C/P	-0.66	$y = 6.5 \cdot 10^{-5}\ x^2 - 1.467\ x + 66.16$	0.44	< 0.001

[a] Regression models: exponential, $y = e^a \cdot x^b$; quadratic, $y = ax^2 + bx + c$

4.5. Discussion

Light is an essential factor for all photoautotrophic organisms, as it provides energy and is required for carbon assimilation and thus the production of all organic compounds. In this study, a gradient of light intensities was tested for its effects on the growth and the PI content of the cyanobacterium *M. aeruginosa* NIVA Cya 43.

4.5.1. Light and cyanobacterial growth

Cyanobacterial growth depends on several factors, such as nitrogen (N, Burberg et al. 2018), phosphorous (P, Burberg et al. 2020), carbon dioxide (CO_2, Verspagen et al. 2014) and temperature (Imai et al. 2009a). However, the energy required for carbon fixation and therefore growth of photosynthetic organisms depends on the availability of light (Tilzer 1987; Sujatha 2015). In this study, *M. aeruginosa* grew at all tested light intensities, no matter if growth was measured as changes in cell abundance or particulate organic carbon (POC, used as proxy for cyanobacterial biomass). The highest biomass and cell density were reached at the lowest light intensity (approx. 26 µmol photons $m^{-2}s^{-1}$). Accordingly, the carrying capacity (*K'*) decreased with increasing light intensity and was the lowest at the highest light intensity (approx. 200 µmol photons m^{-2} s^{-1}). This verified the expectation that low light intensities are beneficial for the growth of the tested *M. aeruginosa* strain and that higher light intensities can inhibit cyanobacterial growth. This is also in accordance with studies that found an increase in the cell density of *M. aeruginosa* with decreasing light intensity, in particular at light intensities below 50 µmol photons m^{-2} s^{-1} (Briand et al. 2012; Pineda-Mendoza et al. 2016). However, Imai et al. (2009b) found higher cell densities of *Microcystis* with increasing light intensity at a similar light intensity range (0 – 60 µmol photons m^{-2} s^{-1}). Nevertheless, the results confirm that *M. aeruginosa* is capable of growing at a wide range of light intensities. This is in accordance with studies that measured growth at light intensities between ≤ 10 and 600 µmol photons m^{-2} s^{-1} (Krüger and Eloff 1977; Islam and Beardall 2017; Muhetaer et al. 2020), even though low light intensities were the most beneficial conditions for growth.

This observation is also reflected in the growth rate, which is an important and widely used parameter to describe and compare cyanobacterial growth in laboratory studies. In this study, the maximum growth rates (μ_{max}) were between 0.28 and 0.44 d^{-1}, which is comparable to growth rates reported for other strains of *M. aeruginosa* (Hesse et al. 2001; Imai et al. 2009a; Tonk et al. 2009; LeBlanc Renaud et al. 2011). However, *M. aeruginosa* can sometimes reach growth rates of 0.7 and higher (Krüger and Eloff 1977; Watanabe and Oishi 1985; Oh and Rhee 1991). The light intensity had no effect on μ_{max}, even though μ_{max} showed a tendency to increase with decreasing light intensity when growth was measured as changes in POC. This tendency is contradictory to studies that found an increase in the growth rates of *M. aeruginosa*

with increasing light intensity, in particular for light intensities below 40 µmol photons m^{-2} s^{-1} (Krüger and Eloff 1977; Watanabe and Oishi 1985; Briand et al. 2012). Some studies, which tested a broader range of light intensities, reported a growth rate increase until a certain threshold is reached beyond which growth rates stagnated or declined (Hesse et al. 2001; Wiedner et al. 2003; Islam and Beardall 2017; Muhetaer et al. 2020). This light intensity threshold depends on the strain (Krüger and Eloff 1977; Islam and Beardall 2017), but might even vary for the same strain depending on the experimental conditions (Hesse et al. 2001; Wiedner et al. 2003). The measured growth rates also depended on the method used to determine growth, as shown by the differences between biomass μ_{max} and cellular μ_{max} in this study. Differences between methods have also been found by LeBlanc Renaud et al. (2011), which indicates that comparisons of growth rates between studies should be done with caution.

An inherent feature of batch-cultures is that exponential growth is followed by a leveling off due to limitations, e.g. by nutrients or light, which is reflected by the stoichiometry of the cyanobacterial biomass. The C/N and C/P ratio increased at all tested light intensities and exceeded the Redfield ratio of 106:16:1 (C:N:P, Redfield 1958). This indicates N and P depletion and a possible (co-) limitation by the two nutrients, in particular at the lowest light intensity at which *M. aeruginosa* also reached the highest biomass and cell abundance. This is supported by the particulate organic nitrogen (PON) and particulate phosphorus (P_{Part}) as well as the cellular N and P content, which indicate that *M. aeruginosa* took up and assimilated nutrients during early to mid-exponential phase. Even though C/N and C/P ratios also increased at the highest light intensity, it is unlikely that the reduced growth was solely caused by nutrient limitation. Light limitation can also be excluded, as *M. aeruginosa* grew better at the lowest light intensity. However, light induced oxidative stress might explain the poor growth performance. At light intensities of 200 µmol photons m^{-2} s^{-1} and above, *Microcystis* produces more reactive oxygen species (ROS) during photosynthesis that damage cellular components, e.g. DNA and lipids (He et al. 2002; Latifi et al. 2009), cause photosystem II photoinhibition (Whitelam and Codd 1983; Muramatsu and Hihara 2012) and result in growth reduction (Islam and Beardall 2017; Muhetaer et al. 2020) and potentially photooxidative death (Abeliovich and Shilo 1972; Eloff et al. 1976).

In nature, the light intensity is highly variable and depends on multiple factors, such as latitude, season, daytime and cloud coverage (Wu et al. 2011; Huryn and Benstead 2019). At lakes that regularly face *Microcystis* blooms, such as Lake Taihu (China) and Lake Erie (USA), light intensities at the water surface can reach up to 2000 µmol photons m^{-2} s^{-1} during mid-day at full sunlight (Wu et al. 2011; Chaffin et al. 2018). However, the light intensity declines rapidly with depth, cyanobacterial biomass and turbidity (Brown 1984; Utkilen and Gjølme 1992; Kardinaal et al. 2007). The results show that *Microcystis* is well adapted to grow under low light intensities, but *Microcystis* can also cope with high light intensities. Due to their capability to

control their buoyancy via gas vacuoles (Reynolds et al. 1987; Brookes and Ganf 2001), *Microcystis* can descend to lower water layers and avoid high light intensity at the water surface. Additionally, factors like colony formation, self-shading, pigment composition and the activity of antioxidant enzymes contribute to the protection against light stress (Eloff et al. 1976; Hesse et al. 2001; Wu et al. 2011; Hernando et al. 2018; Saini et al. 2018). In this study, the maximal light intensity was 200 µmol photons m^{-2} s^{-1}, which is only one tenth of the maximal light intensity reported by Chaffin et al. (2018), but it already showed the impact that high light intensity can have on the growth performance of *Microcystis*. The growth is, however, only one aspect that is influenced by the light. Another aspect is the production and therefore content of potentially toxic metabolites and the implications this might have for the overall toxicity of the cyanobacterium.

4.5.2. The effects of light on the toxicity of *Microcystis*

Like most cyanobacteria, *M. aeruginosa* produces a wide range of secondary metabolites. This includes protease inhibitors (PIs) that are known to negatively affect zooplankter, like *Daphnia* (Rohrlack et al. 2004; Schwarzenberger et al. 2012), and other water organisms, such as zebrafish (Faltermann et al. 2014). Cyanobacterial PIs are even known to inhibit human enzymes (Gademann et al. 2010). Understanding the effect of environmental factors, such as light, on PIs is therefore relevant to fully estimate the risk of cyanobacteria for humans and other organism.

In accordance with previous studies (Burberg et al. 2018; Burberg et al. 2020), the two investigated PIs nostopeptin 920 (BN920) and cyanopeptolin 954 (CP954) were produced constitutively throughout the experiment. A constitutive production indicates that the PIs are required for intracellular processes, such as the storage of N as proposed in Burberg et al. (2018). Cyanopeptolins, the class of PIs to which BN920 and CP954 belong, have also been suggested to be involved in the light response of cyanobacteria (Hesse et al. 2001). This suggestion was based on the structural similarity of cyanopeptolins to microcystins (MC, Hesse et al. 2001) and the finding that the transcription of MCs is light regulated and that MCs are localized on the thylakoid membrane (Shi et al. 1995; Kaebernick et al. 2000). Although the involvement of PIs in the light response has not been proven yet, the involvement in intracellular processes in general requires not only a constant production but also a flexible response in the metabolite content to adapt to environmental changes, e.g. fluctuations in the light intensity.

The PI content of *M. aeruginosa* was indeed highly flexible and changed drastically within days, no matter if the content was normalized to biomass (POC) or cell abundance. In general, the PI content reached the maximum between days 0 and 9, but declined thereafter. This is in accordance with previous studies that found similar patterns for BN920 and CP954 when N and P concentrations were varied (Burberg et al. 2018; Burberg et al. 2020). The production and content of MCs have also been shown to be the highest in the beginning and during the mid-exponential growth phase, but to decline during later growth phases (Song et al. 1998), which indicates that this might be a general pattern for cyanobacterial metabolites. Accordingly, the content of both PIs increased with increasing growth rates. A positive correlation between cyanobacterial metabolite content, respectively production, and the growth rate have been reported before (Orr and Jones 1998; Wiedner et al. 2003; Rohrlack and Utkilen 2007), showing that conditions that favor growth also favor the production of cyanobacterial metabolites. At the start of the experiment, the availability of N and P is high and supports growth and simultaneously the production of PIs. During growth, *M. aeruginosa* took up N and P from the medium, as shown by PON and P_{Part}. This led to nutrient depletion and a possible limitation, which is shown by increasing C/N and C/P ratios. Consequently, the decrease in the growth rates coincided with a decrease of the PI contents.

Besides nutrients and growth, the analysis showed that the PI content was also affected by the light intensity. The BN920 content was on most days the highest at the highest light intensity. To point out the differences between the treatments, the PI contents measured at the 9 sampling days were summed up for each replicate individually, normalized by the number of sampling days and used to calculate an average for each light intensity. The comparison of those averages confirmed that, diverging from expectations, the BN920 content was in general the lowest at the lowest light intensity and increased with increasing light intensity, despite *M. aeruginosa* growing better at low light. This could indicate a trade-off between growth and BN920 production, as both processes might compete for resources. This trade-off might have been the strongest at the lowest light intensity due to the potential limitation by N and P, in particular in the late growth phase. Under higher light intensities the growth of *M. aeruginosa* declined, so that nutrients became available for the production of BN920, which might have led to the higher BN920 content. However, the cellular CP954 content did barely change with the light intensity and the biomass CP954 content even declined with increasing light intensity. The difference between the PIs is surprising, as both PIs are structurally nearly identically (Von Elert et al. 2005), and might indicate different intracellular roles of the two PIs in response to the light intensities.

Another aspect affected by the light intensity is the change in the relative composition of the two PIs. Depending on the light intensity, the total PI content shifted either towards CP954 (low light intensity) or BN920 (high light intensity). Similar changes in the metabolite compositions in response to varying light conditions have also been reported for other strains of *Microcystis* (Tonk et al. 2009; Liu et al. 2017), *Anabaena* (Rapala et al. 1997) and *Planktothrix* (Tonk et al. 2005), suggesting that such metabolic changes are common in cyanobacteria. Those shifts in the metabolite composition might even have a broader ecological impact. Different metabolites, even though structurally nearly identical, differ in their toxicity. This has been shown for different MC variants (Ikehara et al. 2009; Díez-Quijada et al. 2019), but also for BN920 and CP954, which differ in their level of chymotrypsin inhibition, as shown by the IC_{50} values (BN920: 31 nM, CP954: 45 nM) reported by Von Elert et al. (2005). Chymotrypsins are important proteases in the gut of *Daphnia* (Von Elert et al. 2004; Agrawal et al. 2005), and a change in PI composition would change the toxicity of cyanobacteria to *Daphnia* and other grazers, even though the total content might remain unchanged. This implicates that the monitoring of lakes and ponds should not only focus on measuring the concentration of a single toxin, but should consider the diversity of toxins.

A decline of cyanopeptolines with increasing light intensity, as found for the biomass content of CP954, was also found by Tonk et al. (2009). Tonk et al. (2009) analyzed the effects of light on cyanopeptolins (CyaA, CyaC, Cya970) in *Microcystis* and found that the cyanopeptolin content decreased when *Microcystis* was grown at a light intensity of 150 instead of 40 µmol photons m^{-2} s^{-1}. In another study, Rohrlack and Utkilen (2007) found the cellular-bound content of microviridin, another class of PIs, in the cyanobacterium *Planktothrix* to increase with decreasing light intensity, and Repka et al. (2004) reported that the concentration of anabaenopeptins in *Anabaena* was highest at rather low light intensity of 23 µmol photons m^{-2} s^{-1}. Such pattern have not only been found for PIs but also for the content of MC (Song et al. 1998; Tonk et al. 2009). However, the results are quite controversial. Several studies described an increase in the toxicity and the toxin content of *Microcystis* with increasing light intensities (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Utkilen and Gjølme 1992; Wiedner et al. 2003). Such an increase was more pronounced at light intensities below 40 µmol photons m^{-2} s^{-1}, because beyond 40 µmol photons m^{-2} s^{-1} some of those studies report no further increase (Watanabe and Oishi 1985) or even a decrease (Utkilen and Gjølme 1992). Either way, changes in the metabolite content seem to be directly mediated by changes in the transcription of genes involved in the metabolite synthesis, as shown for the transcription of MC genes (mcy). Salvador et al. (2016) showed an increased transcription of the mycA-gene in *Microcystis* with increasing light intensity in a range of 4 to 30 µmol photons m^{-2} s^{-1} and Kaebernick et al. (2000) reported that the transcription of *mcyB* and *mcyD* increased with increasing light intensities between 16 and 68 µmol photons m^{-2} s^{-1},

but found no further increase at light intensities of up to 400 µmol photons m^{-2} s^{-1}. Similar patterns have also been reported for the transcription of the mcyA-gene in *Planktothrix* (Tonk et al. 2005), indicating that this might be a general process among cyanobacteria in respond to light.

However, the response of *M. aeruginosa* to light depends on multiple other factors. Studies showed that not only light intensity, but also the light quality has an effect on the toxin content and the gene expression (Utkilen and Gjølme 1992; Kaebernick et al. 2000; Carneiro et al. 2009). Other studies report that temperature (Song et al. 1998), CO_2 level (Geada et al. 2017) and nutrients (Kurmayer 2011; Chaffin et al. 2018) affect the response of cyanobacteria to different light intensities. And still others showed species and strain specific responses to light variations (Sivonen 1990; Repka et al. 2004; Tonk et al. 2009; Salvador et al. 2016), which in turn might contribute to the natural succession of strains in cyanobacterial populations during blooms (Kardinaal et al. 2007). Laboratory studies showed that light contributes to the shift of a population from toxic to non-toxic strains and vice versa (Briand et al. 2012), which is supported by findings that non-MC-producing strains outcompete MC-producing strains under low light conditions (< 25 µmol photons m^{-2} s^{-1}) within two weeks (Kardinaal et al. 2007). Shifts in the strain compositions have been attributed to costs of producing toxins (Kardinaal et al. 2007; Briand et al. 2012) and potential roles of metabolites in the light response (Hesse et al. 2001; Xu et al. 2019). Even though some studies suggest otherwise (LeBlanc Renaud et al. 2011), the production of toxic metabolites requires energy and nutrients and presumably stands in concurrence with growth and other cellular processes. Under certain conditions, such as low light or low N, the cost of toxin production might outweigh the benefits, resulting in the selection of non-toxic strains over toxic strains and a potential reduction of bloom toxicity (Kardinaal et al. 2007; Chaffin et al. 2018).

4.6. Conclusion

In conclusion, this study showed that the light intensity is a major factor that affects the growth as well as the PI content in the cyanobacterium *Microcystis aeruginosa*. *Microcystis,* which is one of the most common bloom-forming cyanobacteria, is often considered a good competitor for light (Huisman et al. 1999; Ji et al. 2017) and the results confirm that *Microcystis* grows well at low light intensities, but struggled at higher light intensities most likely due to oxidative stress. Although, surface water light intensities can be much higher in nature (Chaffin et al. 2018), the adaptation to low light intensities is beneficial for bloom-forming cyanobacteria, as light intensities rapidly decrease with cyanobacterial biomass and depth during cyanobacterial blooms (Utkilen and Gjølme 1992; Kardinaal et al. 2007). Even though the cyanobacterial biomass might be reduced at high light intensities, the toxicity of the cyanobacterium could simultaneously increase due to an increased cellular PI content. Furthermore, changes in the light intensity might change the PI composition and could also lead to shifts in the species/strain composition. Altogether, this might affect the impact of cyanobacterial blooms on the natural communities and organisms directly feeding on cyanobacteria, but it might additionally increase the health risk for humans, in particular because high light intensities favor the release of toxic metabolites from cyanobacterial cells and increase so the exposure risk for humans (Rapala et al. 1997; Liu et al. 2017). With the rise of cyanobacteria blooms worldwide, understanding the complex interactions between light and cyanobacteria will be necessary to successfully mitigate the ecological and economic impacts of cyanobacterial blooms.

4.7. References

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4.8. Supplementary

Table S1. Spearman correlation coefficient (R_S), model equations and the corresponding coefficient of determination (R^2) and *p*-values for regression models applied to the biomass PI content as response variable (y) and growth rate (μ, Fig. 5) or elemental ratios (Fig. 6) as single explanatory variables (x). Three different models (linear, exponential and quadratic) were used and based on the R^2 and visual exploration, the best fitted regression model was chosen (in bold, see Tab. 4) and displayed in Fig. 5 and 6.

BN920	R_S	**Equation** [a]	R^2	*p*
μ	0.49	$y = 47.98\ x + 4.19$	0.35	< 0.001
μ		$y = e^{2.5} \cdot x^{0.18}$	0.11	< 0.001
μ		**$y = 154.7\ x^2 - 0.89\ x + 5.8$**	**0.37**	**< 0.001**
C/N	-0.6	$y = 22.1x - 1.2$	0.26	< 0.001
C/N		**$y = e^{4.29} \cdot x^{-1.06}$**	**0.29**	**< 0.001**
C/N		$y = -0.02\ x^2 - 0.82\ x + 20.54$	0.26	< 0.001
C/P	-0.7	$y = -0.05\ x + 23.56$	0.38	< 0.001
C/P		**$y = e^{6.9} \cdot x^{-0.897}$**	**0.37**	**< 0.001**
C/P		$y = 3.7 \cdot 10^{-5}\ x^2 - 0.07\ x + 25.69$	0.38	< 0.001
N/P	-0.07	$y = -0.13\ x + 14.48$	0.02	< 0.05
N/P		**$y = e^{3.86} \cdot x^{-0.056}$**	**0.07**	**< 0.01**
N/P		$y = 0.005\ x^2 - 0.55\ x + 22.06$	0.06	< 0.05
CP954				
μ	0.86	$y = 143.8\ x + 12.37$	0.65	< 0.001
μ		**$y = e^{4.18} \cdot x^{0.33}$**	**0.68**	**< 0.001**
μ		$y = -296.3\ x^2 - 237.4\ x + 9.3$	0.67	< 0.001
C/N	-0.79	$y = -3.81\ x + 68.55$	0.57	< 0.001
C/N		**$y = e^{5.88} \cdot x^{-1.22}$**	**0.66**	**< 0.001**
C/N		$y = 0.52\ x^2 - 14.6\ x + 115.34$	0.7	< 0.001
C/P	-0.66	$y = -0.11\ x + 62.38$	0.44	< 0.001
C/P		$y = e^{7.1} \cdot x^{-0.71}$	0.39	< 0.001
C/P		**$y = 6.5 \cdot 10^{-5}\ x^2 - 1.467x + 66.16$**	**0.44**	**< 0.001**
N/P	0.19	$y = 0.05\ x + 30.33$	0.01	0.70
N/P		$y = e^{3.39} \cdot x^{-0.044}$	0.01	0.75
N/P		$y = -0.002\ x^2 + 0.23\ x + 27.23$	0.01	0.85

[a] Regression model equations: linear, $y = ax + b$; exponential, $y = e^a \cdot x^b$; quadratic, $y = ax^2 + bx + c$

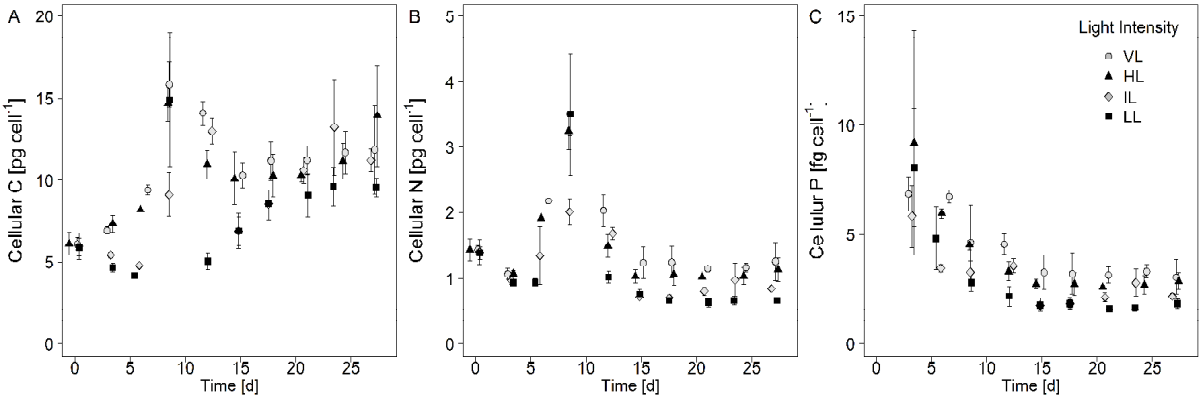

Figure S1. Cellular carbon (**A**), nitrogen (**B**) and phosphorous (**C**) content of *M. aeruginosa* grown at different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Displayed are mean values (± SD, n = 3). The readability of the figures was increased by jittering the data points around the respective x-value (sampling day).

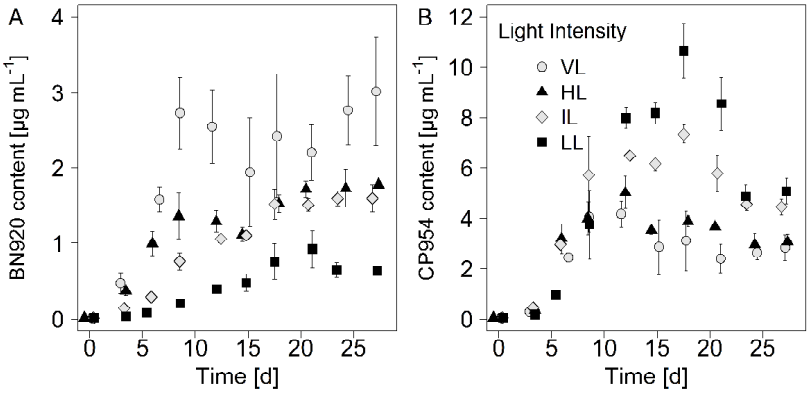

Figure S2. Volumetric concentration of BN920 (**A**) and CP954 (**B**) in cultures of *M. aeruginosa* grown at different light intensities ('Very High, VL'; 'High, HL'; 'Medium, ML'; 'Low, LL'). Displayed are mean values (± SD, n = 3). The readability of the figures was increased by jittering the data points around the respective x-value (sampling day).

General Discussion and concluding remarks

In the last centuries, humans have changed their environment dramatically. One example is the eutrophication of surface waters caused primarily by the anthropogenic input of nutrients (Conley et al. 2009; Robertson and Saad 2011). In limnic systems, the introduced nutrients contribute to the mass proliferation of cyanobacteria, like *Microcystis aeruginosa*, resulting in so called cyanobacterial blooms (e.g. Duan et al. 2009). Blooming events are often associated with severe ecological consequences, such as a reduced transfer of carbon and energy from primary production to higher trophic levels caused by the negative interference of cyanobacteria with their planktonic grazers, in particular *Daphnia* (Müller-Navarra et al. 2000; Von Elert et al. 2003). Cyanobacteria are considered a bad food source for zooplankton (e.g. Von Elert et al. 2003; Wejnerowski et al. 2017), in particular because cyanobacteria produce a wide range of bioactive secondary metabolites (Gademann and Portmann 2008; Huang and Zimba 2019). Cyanopeptolins are a class of cyanobacterial serine protease inhibitors (PIs) with dozens of known compounds, including the two in this dissertation investigated PIs cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920). Cyanopeptolins are among the most frequent groups of cyanobacterial metabolites and are known to be toxic to small crustaceans and other aquatic organisms (Gademann et al. 2010; Schwarzenberger et al. 2010; Faltermann et al. 2014; Mazur-Marzec et al. 2018). But still, the investigation of cyanobacterial metabolites has primarily focused on microcystins (MCs), due to their toxicity for humans (Jochimsen et al. 1998), and neglected PIs, which were often considered as non-toxic for humans (Neumann et al. 2000). Hence, many aspects concerning PIs were at the beginning of this thesis still unknown. This dissertation, therefore, aimed to gain deeper insights into how cyanobacteria, in particular cyanobacterial growth, and the production of PIs, are affected by three major environmental factors: nitrogen (N, chapters 1+3), phosphorus (P, chapters 2+3) and light (chapter 4).

M. aeruginosa under varying environmental conditions: growth performance

The growth of cyanobacteria depends on a number of environmental factors, among which N, P and light are likely the most important ones as they regularly limit the growth and productivity of freshwater phytoplankton (Elser et al. 2007; Karlsson et al. 2009; Kolzau et al. 2014). The effect of these three factors was investigated in batch culture growth experiments using the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43. On a regular basis, the cultures were sampled to determine parameters, such as the stoichiometry (elemental ratios) and PI content (BN920, CP954) of the cyanobacterial biomass. Cyanobacterial growth in the cultures was estimated using the cell abundance and biomass (measured as particulate organic carbon, POC) of *M. aeruginosa*. During batch culture experiments, *M. aeruginosa*

typically shows logistic growth, which allowed me to model the growth using a logistic growth model (chapters 1, 2 & 4). The growth of *M. aeruginosa* under varied N and P concentrations (chapters 1 & 2) was additionally modeled using a dynamic process-based model (chapter 3), which had the advantage that it was more dynamic and anticipated for example declines in the biomass at later growth phases (i.e. in some treatments of the P-experiment), while the logistical model did not. Additionally, the new developed model allowed me to model the PI dynamics and get so a deeper insight into the production and degradation of the two PIs, which is discussed later on. Nevertheless, both models allowed me not only to describe the growth of *M. aeruginosa*, but also to determine growth parameters, such as the cyanobacterial growth rates. Prior to the experiments, it was expect that increasing nutrient concentrations and low light intensities will promote the growth of *M. aeruginosa*, resulting in higher cell abundances, biomasses and growth rates.

The massive input of N, in particular of nitrate (NO_3^-), has supported the eutrophication of freshwater systems worldwide and is recognized as one of the factors that controls cyanobacterial blooms (Beaulieu et al. 2013; Glibert et al. 2016). In the first chapter, I therefore tested a range of NO_3^- concentrations and clearly showed that increasing NO_3^- concentrations boost the cell abundance and biomass of *M. aeruginosa*. Even though this has been shown before (e.g. Orr and Jones 1998; Sevilla et al. 2010), it highlights once again the importance of NO_3^- and N in general for the growth of the bloom-forming cyanobacterium *M. aeruginosa* and shows that the anthropogenic input of NO_3^- into lakes supports the development of cyanobacterial blooms. In particular *Microcystis* and other cyanobacteria, which cannot fix atmospheric N_2, depend on the free availability of N in their environment and benefit so especially from N inflow. The importance of N for *Microcystis* is additionally shown by the number of different chemical forms that can be used by this cyanobacterium. In all experiments described in this dissertation, NO_3^- was used as N source, because it supports good growth, is the most common oxidized form of N and substantially contributes to the eutrophication of aquatic systems (Glibert et al. 2016; Li et al. 2016). However, *M. aeruginosa* utilizes multiple other N sources, including urea (CH_4N_2O), ammonium (NH_4^+), nitrite (NO_2^-) and amino acids (Yan et al. 2004; Li et al. 2016). Even though *M. aeruginosa* has a high affinity for NO_3^-, reduced nitrogen sources, in particular urea, can support even better growth (Yan et al. 2004; Chen et al. 2019). This is problematic, as an increasing atmospheric N deposition and the increased use of urea in fertilizers is currently shifting some systems towards reduced N forms, which will likely promote *Microcystis* blooms even further (Glibert et al. 2014; Glibert et al. 2016). Considering different nutrient forms is therefore necessary to fully understand the response of *M. aeruginosa* and other cyanobacteria towards changing N conditions.

However, even though N is essential for cyanobacterial growth, a long standing tenet in freshwater ecology is that P is the limiting factor for phytoplankton growth in lakes and ponds (Schindler and Fee 1974; Schindler et al. 2008). Hence, it was often hypothesized that the reduction of P should reduce the eutrophication of waterbodies and prevent the development of cyanobacterial blooms (e.g. Schindler et al. 2008). In chapter 2, I therefore analyzed the effect of a range of phosphate concentrations (PO_4^{3-}, 5 – 75 µM) on the growth of *M. aeruginosa*. As expected, the biomass and cell abundance of *M. aeruginosa* increased with increasing PO_4^{3-} concentrations and was severely limited at the lowest tested PO_4^{3-} concentrations (5 & 10 µM), in which the cyanobacterial biomass only reached ~20% of the biomasses measured at the highest concentration (75 µM). The degree of P limitation was also reflected in the stoichiometry of the cyanobacterial biomass. The carbon to phosphorus (C/P) ratio reached maximal values between 380 and 1150 depending on the initial P concentration and exceeded the well-established Redfield ratio (C:N:P = 106:16:1, Redfield 1958), which is a widely used reference for non-limited phytoplankton growth. These results indicate that the growth of *M. aeruginosa* was severely limited by P, in particular at the lowest P concentrations, and that the reduction of P could be quite efficient to limit the ability of *Microcystis* to produce cyanobacterial blooms.

Last, but not least, light is an essential factor for the growth of the photoautotrophic cyanobacteria, as it delivers the energy required for carbon fixation and therefore growth (Sujatha 2015). Hence, I tested the effect of four different light intensities (26, 65, 130 & 200 µmol photons m^{-2} s^{-1}) on the growth of *M. aeruginosa* (chapter 4). The results of this study show that even the lowest light intensity was still sufficient to allow for good growth of *M. aeruginosa*, while the growth drastically decreased with increasing light intensities. *M. aeruginosa* is known for growing under a range of light intensities (10 – 600 µmol photons m^{-2} s^{-1}), but prefers low light intensities between 15 and 50 µmol photons m^{-2} s^{-1} (Krüger and Eloff 1977; Islam and Beardall 2017; Muhetaer et al. 2020). The adaptation to low light intensities might be an advantage for *M. aeruginosa*, as light intensities can be rather low during cyanobacterial blooms due to (self-) shading (Mischke 2003; Kardinaal et al. 2007). Additionally, cyanobacterial blooms suppress other phytoplankton species and macrophytes by reducing the light availability (Huisman et al. 2004; Kosten et al. 2012; Paerl and Otten 2013), which further supports cyanobacterial dominance when light intensities are low. However, the growth of *M. aeruginosa* was drastically reduced on the other end of the tested light intensity range (200 µmol photons m^{-2} s^{-1}), most likely due to photooxidative stress. At high light intensities, *Microcystis* produces more reactive oxygen species (ROS) during photosynthesis, which damages cellular components, reduces cyanobacterial growth and can potentially cause photooxidative death in cyanobacteria (Eloff et al. 1976; Muhetaer et al. 2020). Even though the here tested light intensities were low enough to not cause

photooxidative death, my findings clearly show that *M. aeruginosa* is very sensitive to high light intensities and the associated photooxidative stress. In recent years, some studies showed that the sensitivity of cyanobacteria towards oxidative stress might even be actively exploited to control cyanobacterial blooms in lakes (Schuurmans et al. 2018). The addition of hydrogen peroxide (H_2O_2), one form of ROS, has reportedly been used to collapse cyanobacterial blooms without affecting eukaryotic phytoplankton, zooplankton or macrofauna (Matthijs et al. 2012; Schuurmans et al. 2018).

The use of mathematical models to describe cyanobacterial growth also allowed me to determine the growth rate of *M. aeruginosa*, which is an essential parameter in most growth models (Xiao et al. 2020) and is often used to describe and compare the growth of phytoplankton under varying environmental conditions. The maximal growth rates determined by the logistic growth model were over all in the range of 0.24 up to 1.00 d^{-1} (chapters 1, 2 & 4), while the growth rates derived from the process-based model covered a slightly broader range (0.23 – 1.44 d^{-1}, chapter 3). Nevertheless, the determined growth rates were reasonable and comparable to growth rates found for *M. aeruginosa* in other studies (e.g. Krüger and Eloff 1977; Watanabe and Oishi 1985; Tonk et al. 2009; Islam and Beardall 2017). The growth rates reported here and in the literature depended on the one hand on the method that is used to measure growth, as shown by the differences found between growth rates that were determined based on the biomass compared to the ones determined from the cell abundance. On the other hand, growth rates are strongly affected by the applied experimental conditions. Prior to the experiments, I expected the growth rate to increase with an increasing N and P concentration and decreasing light intensities, because I assumed that these conditions will support good and therefore fast growth. Despite the expectation, maximal growth rates determined by the logistic growth model increased with decreasing N, showed no clear response to the P concentration and showed no significant effect of the light intensity. The results from the process-based model confirmed a decrease of the growth rate with increasing N concentrations, but found also that the growth rate decreased with increasing P concentrations. Decreases in the growth rates have been associated to nutrient stress before (Chen et al. 2009) and it is possible that this might have happen in my experiments as well. Nevertheless, the results highlight two things: first, environmental factors do not affect growth rates the same way as the biomass respectively cell abundance and secondly, that growth rates should be used with caution when comparing different (cyanobacterial) strains or species, in particular between studies that used different methodologies (e.g. different growth model, different growth proxies).

The results discussed so far point out the individual effects of the investigated factors on the growth of *M. aeruginosa*. However, this might be over-simplified. Regardless of the testes experimental conditions, the stoichiometric ratios (C/N, C/P & N/P) of the cyanobacterial biomass changed dramatically during the growth of *Microcystis*. In particular, the C/N and C/P ratios often decreased initially (early growth phase) due to nutrient uptake, but decreased towards the end of the experiments, often exceeding the Redfield ratios, especially during the late growth phase. This indicates that *M. aeruginosa* was in many cases not limited by N or P alone, but by both nutrients together. Only in a few treatments, such as the low P concentrations (5 & 10 µM) in chapter 2, the stoichiometry indicated a clear limitation by a single factor. Co-limitations are common in the late growth phases of batch culture experiments and make it difficult to assign effects found in the studies unambiguously to a single factor. Nevertheless, due to co-limitations, the batch culture experiments might mimic the natural conditions in freshwater systems even better, because cyanobacteria are quite often co-limited by N and P (Elser et al. 2007; Dolman et al. 2012; Paerl et al. 2016).

In summary, the results show that *M. aeruginosa* is capable of growing under a range of environmental conditions and that increasing N and P concentrations (chapters 1 – 3) as well as low light intensities (chapter 4) support high cell densities and biomasses of *M. aeruginosa*, one of the most common bloom-forming cyanobacteria. This underlines that the anthropogenic input of nutrients into freshwater systems is a major cause for the increasing frequency and severity of cyanobacterial blooms in the last decades. As long as the nutrient pollution continues, the number of cyanobacteria blooms will further rise. Reducing the inflow of nutrients into freshwater systems is therefore necessary to mitigate cyanobacterial blooms. In the last two decades, some success has been achieved in case of P, as stricter regulations (e.g. for fertilizers and detergents) and improved procedures for sewage water treatment reduced regionally the P pollution of surface waters (Glibert 2019). However, the reduction of nutrient concentrations alone is not always successful in reducing cyanobacterial blooms and the nutrient ratios as well as the nutrient forms will need more consideration in management strategies (Harris et al. 2014; Glibert et al. 2016; Gobler et al. 2016; Glibert 2017). The successful control of the nutrient input will become even more important in the future, as rising temperatures in the wake of climate change are predicted to support the cyanobacterial dominance due to the relatively high temperature optima of cyanobacteria and other effects of higher temperature, such as a stronger vertical stratification of lakes (Paerl and Huisman 2008, 2009; Kosten et al. 2012). A variety of other abiotic and biotic factors is also known to affect the success of cyanobacteria, which increases the complexity of this issue even further. In particular the variety of cyanobacterial metabolites play a central role as they affect the interaction with grazers that could potentially prevent cyanobacterial blooms.

M. aeruginosa under different environmental conditions: protease inhibitors

The effect of environmental factors on the production and content of cyanobacterial metabolites has been studied before, in particular for MCs. A number of factors, like iron, temperature, sulphate or CO_2, have been shown to influence the production and content of MCs (Amé and Wunderlin 2005; Jähnichen et al. 2011; Van de Waal et al. 2011). However, the knowledge about the effect of environmental conditions on PIs is scarce. Hence, I used the performed batch culture growth experiments to analyze the two N-rich cyanopeptolins (BN920 and CP954) in *M. aeruginosa* using liquid chromatography and high-resolution mass spectrometry. The measured PI concentrations were normalized to the culture volume (volumetric PI concentrations), cell abundance or biomass (cellular/biomass PI content).

Overall, the experimental results indicate that the two analyzed PIs are of high biological relevance for *M. aeruginosa* NIVA Cya 43. This is indicated by the constitutive production of the two PIs. Regardless of the tested experimental conditions, the two PIs were measurable at all times, even in the late growth phase when *M. aeruginosa* was in most cases limited by N and/or P. The PIs reached considerable biomass contents of 0.8 – 68.5 µg mg C^{-1} (BN920) and 3.9 – 147 µg mg C^{-1} (CP954), with CP954 being the predominant PI in this strain of *M. aeruginosa*. Together, the two PIs accounted for up to 15% of the total cyanobacterial biomass (measured as POC), depending on the respective test conditions and the sampling day. Along with the fact that the production of PIs requires relatively high amounts of N as well as a non-ribosomal peptide synthetase complex (NRPS, Köcher et al. 2019), it is unlikely that PIs are produced without fulfilling a biological purpose (discussed below).

Remarkable is also the temporal dynamics found for the PI content. In general, the PI content increased initially (early to mid-exponential growth phase) until it reached a maximum (around day 3 – 15) after which it declined gradually to constant, low levels (late growth phases). This resulted in variations of the inhibitor content by up to 80%. In batch cultures, it is typical that the concentration of nutrients is initially high and declines over the course of the experiment due to the uptake and incorporation of nutrients (N, P) by the cyanobacterium. Thus, high PI contents coincided with high concentrations of nutrients in the beginning of the experiment, regardless of the applied nutrients concentrations (treatments). The dynamics of the two PIs corroborates the well-established carbon-nutrient balance hypothesis (CNBH, Bryant et al. 1983), according to which primary producers synthesize secondary metabolites in dependence of the availability of nutrients. This means that N-rich PIs are produced when N is available and that the concentration, respectively content, of PIs should increase with increasing N availability. This was further supported by the finding that the PI content decreased in most cases with increasing C/N ratios. Overall, the dynamics indicate that conditions that favor growth, in particular a high N availability, also favor the production of PIs.

Ergo, I found positive relationships between the PI content and the cyanobacterial growth rate in most experiments, which is in accordance with findings for MCs (e.g. Orr and Jones 1998). The here described dynamics might actually reflect natural systems quite well, as nutrient pulses, e.g. after heavy rainfalls, transport high amounts of nutrients into aquatic systems and trigger so an increase in cyanobacterial biomass (Lürling et al. 2018 and reference therein). According to my results, such an increase of the biomass could be accompanied by an increase in the PI content of the cyanobacteria, which renders the cyanobacterium more toxic, e.g. for grazers. However, this effect is reversed when nutrients are depleted and start to limit the cyanobacterium.

Moreover, the temporal dynamics were accompanied by effects caused by the applied experimental conditions. In chapter 1, I found that the volumetric PI concentration increased with an increasing NO_3^- concentration, in particular from day 10 onwards. This tendency was also present in the PI content, even though not as clear as for the PI concentration. In combination with the C/N ratios, these results showed that the stronger the N limitation the lower the PI concentration and confirm so the findings described in the previous paragraph. This corroborates once more the CNBH and is in accordance with studies that showed an increase in the metabolite content of *M. aeruginosa* with increasing N availability (e.g. Sivonen 1990; Van de Waal et al. 2009). It is also in line with Schwarzenberger et al. (2013), who found that the content of micropeptins, a class of cyanobacterial PIs, is reduced under N limitation. Schwarzenberger et al. (2013) also showed that the content of micropeptins is reduced under P limitation, what has also been shown for other toxic metabolites (e.g. Van de Waal et al. 2014). This is in line with the results presented in chapter 2. I found that the biomass PI content was higher at the lowest tested P concentrations (5 & 10 µM) compared to higher P concentrations (> 30 µM), in particular during the mid-exponential growth phase. This indicates that P limitation can induce an increase in the PI content of *M. aeruginosa*, but that the effect might be mediated by cyanobacterial growth. P limitation is often associated with high contents of toxic metabolites in phytoplankton (Brandenburg et al. 2020), as relatively more N is made available for the production of N-rich metabolites.

Besides N and P, I tested the effect of different light intensities and found rather inconclusive results. While the content of BN920 increased with increasing light intensities, the CP954 content was unaffected by the light intensity when normalized to the cell abundance and decreased when normalized to the biomass. The same effect was present in the volumetric PI concentrations (chapter 4, Supplementary Fig. S2). The BN920 concentration was constantly higher at the highest light intensity, while the CP954 concentration was the highest at the lowest light intensity. The difference between the PIs is interesting, as both PIs are structural nearly identical (Von Elert et al. 2005), and might point either towards different roles of the two PIs depending on the light intensity or towards the light inhibition of the halogenation

process by which BN920 is transformed into CP954, resulting in the accumulation of BN920. Considering that *M. aeruginosa* was likely under oxidative stress at high light intensities, the inhibition of enzymes (such as halogenases) seems plausible, but requires further investigation. Either way, the different responses of the two PIs resulted in a change in the relative PI proportion; the total PI content shifted towards BN920 at high light intensity, while it shifted towards CP954 when light intensities were low. Such changes in the metabolite composition in response to light are common among cyanobacteria (e.g. Tonk et al. 2005; Tonk et al. 2009) and might alter the overall toxicity of *M. aeruginosa*, as different metabolites differ in their toxicity, even when structurally nearly identical (Von Elert et al. 2005; Ikehara et al. 2009).

Furthermore, is the basis of all changes in the concentration and content of the two PIs a change in the production and degradation of PIs by the cyanobacterium. In chapter 3, I used a modeling approach to investigate the effect of N and P on the production and degradation of PIs, using the data from chapter 1 and 2. As mentioned above, the dynamic resource-based model allowed to model growth and simultaneously the dynamics of the volumetric PI concentrations. Based on the fits, model parameters, such as production and degradations rates for the two PIs, were determined. According to the results, the PI production and degradation rates declined with increasing N concentration, which shows that low N concentrations promote a faster PI turnover and indicates that the two PIs might have a special role at lower N concentration, e.g. in the storage of N (discussed below). Increasing P concentrations on the other hand did not affect the production rates and resulted in a decline in the degradation rates. It is reasonable that the PI production is more affected by the N concentration than by the P concentration, as N is an essential constituent of PIs while P is not. Originally it was assumed that a high P concentration could increase the PI degradation, as the relative N availability decreases with increasing P availability so that the reallocation of resources stored in PIs could be increased at high P concentrations. This was, however, not the case and shows that the effects of P might be more complex and could be mediated by other processes, such as growth. This underlines that further investigations are needed to understand the effect of nutrients and other factors on the production and degradation of PIs. Mathematical models could support these investigations, as they allow not only to describe the data, but to get a deeper understanding of the underlying processes.

In summary, this dissertations shows the effects of N, P and light on PIs in *M.* aeruginosa. I was able to show that the production and degradation of PIs and consequently the PI content, respectively concentration, is dramatically affected by the experimental conditions. In particular, my results show that high N and low P concentrations resulted in an increased PI content, while varying light intensities caused either an increase or a decrease in the PI content depending on the respective PI, which might result in shifts in the PI composition. Noteworthy

is also that both PIs were produced constitutively and showed a high temporal variability, regardless of the tested experimental conditions. The findings indicate that the two PIs are highly relevant in this strain of *M.* aeruginosa and open up the question about their potential roles.

Protease inhibitors: Potential nitrogen storage compounds?

As described in the previous paragraph, the content of the two PIs showed a typical pattern: An initial increase during times of high nutrient (i.e. N) availability, followed by a decrease in the content at later growth phases, when the nutrients are depleted. This kind of dynamics led to the hypothesis that the two PIs are involved in the N storage (chapter 1) and was supported by the results in chapter 3, which showed that the turnover of PIs is increased under decreasing N concentrations (higher production and degradation rates), as N storing became more important at low N availability. PIs can be considered good candidates for N storage compounds, because they are N-rich (C/N = 5.75; Van de Waal et al. 2014) and consist of eight amino acids (Von Elert et al. 2005), which could easily be repurposed for growth and other processes once PIs are degraded. Furthermore, cyanophycin, the primary N storage compound in cyanobacteria (Watzer and Forchhammer 2018; Flores et al. 2019), displays dynamics in response to varying N availabilities that are similar to the found PI dynamics. Cyanophycin is a reserve polymer, which contains only the two amino acids arginine and aspartic acid, that is synthesized under N replete conditions, but gets degraded by the enzyme cyanophycinase when N becomes limiting (Richter et al. 1999; Berg et al. 2000). The degradation of cyanophycin and presumably PIs releases the stored N (in form of amino acids) and allows so further growth, even though external N sources are depleted. Further supports comes from PIs of higher plants that have also been suggested to play a role in the storage of N due to their homology and close evolutionary relationship with storage proteins (Odani et al. 1983; Mosolov and Valueva 2005). Altogether, this indicates that PIs and structurally similar N-rich metabolites could be involved in the storage of N in cyanobacteria. Hence, this potential intracellular function should be investigated in future studies.

However, it should be assumed that if the two PIs serve only as N storage that they would be completely degraded under N limitation to reallocate the stored nutrients to growth or other processes. This was not the case, as *M. aeruginosa* maintained a low PI content even under strong N limitation (high C/N ratios). This could be explained by the possible co-limitation of *M. aeruginosa*, which could reduce the necessity of reallocating N from the PIs. However, the involvement of PIs in other processes is also plausible. In chapter 3 the PI content and PI composition of *Microcystis* changed depending on the light intensity, which might hint at a role of PIs in the response to light and/or oxidative stress. Similar suggestions have been made for MCs, whose transcription is regulated by light (Hesse et al. 2001) and that have been shown

to bind covalently to redox-sensitive proteins preventing so their proteolytic degradation (Zilliges et al. 2011). Multiple other functions of cyanobacterial metabolites have been proposed, such as the involvement in iron scavenging or cell signaling (Holland and Kinnear 2013 and references therein), and could potentially be valid for PIs as well. In particular, the involvement of secondary metabolites in the chemical defense of cyanobacteria against natural grazers has been suggested multiple times (e.g. DeMott et al. 1991; Jang et al. 2003) and seems plausible for PIs, which are toxic to other aquatic organisms.

Protease inhibitors: A potential anti-grazer defense?

In nature, cyanobacteria are under a constant grazing pressure. In particular, the cladoceran genus *Daphnia* is a major consumer of phytoplankton biomass and is capable of controlling cyanobacterial blooms (Chislock et al. 2013; Urrutia-Cordero et al. 2015). *Daphnia* is likewise a major food source for higher trophic levels and contributes thus to the transfer of carbon and energy from the level of primary producers to higher trophic levels. This transfer is, however, diminished by PIs, which have been shown to negatively affect *Daphnia* (e.g. Jakobi et al. 1996; Rohrlack et al. 2004; Schwarzenberger et al. 2010). This includes BN920 and CP954 that inhibit specifically chymotrypsins, but no trypsins (Ploutno and Carmeli 2002). Chymotrypsin and trypsin are the major digestive proteases in the gut of *Daphnia* and account for up to 80% of the total protease activity (Von Elert et al. 2004). BN920 and CP954 have been shown to inhibit the chymotrypsin activity in the gut of *Daphnia* at rather low IC_{50} values of 5.4 nM and 7.4 nM respectively (Von Elert et al. 2012), which classifies BN920 and CP954 as two of the most potent serine protease inhibitors in the group of cyanopeptolins.

Furthermore, PIs are probably among the first metabolites that unfold their negative effects on *Daphnia*. After ingestion, cyanobacterial cells are broken down and PIs are released into the gut of *Daphnia,* where the PIs directly inhibit the proteases. Proteases degrade proteins and thus their inhibition results in a limitation by amino acids, which subsequently reduces the growth of *Daphnia* (e.g. Von Elert et al. 2012; Schwarzenberger et al. 2013). Digestive proteases, however, might not be the only proteases affected by the cyanobacterial PIs. Studies have shown that other classes of proteases, such as elastases (Lange et al. 2018) and thrombins (Jakobi et al. 1996), are also inhibited by cyanopeptolines, which could increase the negative impact of PIs on *Daphnia* even further. Altogether, the negative interference of PIs and *Daphnia* makes PIs a perfect chemical defense against grazers.

As described before, the PI content is composed of a constitutive and a flexible (inducible) part. The constitutive production of PIs would provide a constant protection against grazing, but is costly in terms of resources (i.e. N) and energy. This circumstance is described by the optimal defense theory (ODT, Rhoades 1979), which predicts that the production of defensive molecules is regulated by the actual risk and the negative fitness consequences of grazing.

The chemical defense is thereby associated with costs (e.g. resources consumption) and is only possible at the expense of other processes, such as growth. The active regulation of the defense level (e.g. the PI content) in response to the actual grazing risk is therefore one way of optimizing the resource allocation. This type of defense is known as an inducible defense and PIs are optimal candidates for an inducible, chemical defense of cyanobacteria against grazers. In particular, the highly flexible PI content and the negative interference with grazers support this idea. Inducible, chemical defenses are not uncommon in the plankton community. It has for example been shown that the content of the paralytic shellfish toxin (PST) in dinoflagellates increases upon exposure to grazers (Selander et al. 2006; Bergkvist et al. 2008). Similarly, the content of MCs in *M. aeruginosa* has been shown to increase in the presence of grazers and grazer related cues (Jang et al. 2003; Jang et al. 2007). Even though the induction of PI by grazers as not been shown for PIs yet and results of Harke et al. (2017) rather indicate that the transcription of multiple PI genes (including cyanopeptolins) is unaffected by the presence of *Daphnia*, such a mechanisms is in principle still possible. Nevertheless, even if the primary function of PIs is not the deterrence of grazers, a sudden increase in the PI content in response to changing nutrient or light conditions could be detrimental to *Daphnia* and all other organisms that directly feed on the cyanobacteria.

Conclusion

In the near future, the number, frequency and severity of cyanobacterial blooms will further increase. Especially the still common use of high amounts of nutrient-rich fertilizers in agriculture that leads to the massive inflow of nutrients into surface waters combined with the ongoing global warming will provide optimal conditions for bloom-forming cyanobacteria, such as *Microcystis.* Drastic changes in our thinking and our actions will be necessary to reduce cyanobacterial bloom frequency and restore the functionality of many freshwater systems. This dissertation provided insight into the factors that control cyanobacterial growth and affect the production of protease inhibitors, an important group of cyanobacterial metabolites. In the last decades, cyanobacterial metabolite research was focused on microcystins, but this thesis emphasizes that protease inhibitors and other cyanobacterial metabolites might have essential functions and should receive more attention in the future. There are still unanswered questions (e.g. about the intracellular function of PIs) and further laboratory and field studies combined with the use of modern analytical methods and mathematical models will be necessary to help understand and manage cyanobacterial blooms and restore the health of freshwater ecosystems.

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Authors contribution

Chapter 1: Nitrate determines growth and protease inhibitor content of the cyanobacterium *Microcystis aeruginosa*

The experiment was planned and performed by me. Data visualization and analysis were performed by me in cooperation with Thomas Petzoldt (TU Dresden) and Maja Ilić. Eric von Elert was involved in technical discussions. The manuscript was written by me and was critically read by Thomas Petzoldt, Maja Ilić and Eric von Elert.

Chapter 2: Phosphate limitation increases content of protease inhibitors in the cyanobacterium *Microcystis aeruginosa*

The experiment was planned and performed by me. Data visualization and analysis were performed by me in cooperation with Thomas Petzoldt (TU Dresden). Eric von Elert was involved in technical discussions. The manuscript was written by me and was critically read by Thomas Petzoldt and Eric von Elert.

Chapter 3: A dynamic process-based modeling approach to understand the effects of nutrients on protease inhibitors in cyanobacteria.

The experiment was planned and performed by me. Data visualization and analysis/modeling were performed by me in cooperation with Thomas Petzoldt (TU Dresden). Eric von Elert was involved in technical discussions. The manuscript was written by me and was critically read by Thomas Petzoldt and Eric von Elert.

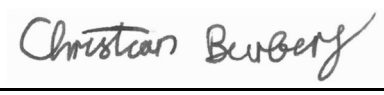
Chapter 4: The effect of light intensity on growth and protease inhibitor content of *Microcystis aeruginosa*

The experiment was planned and performed by me. Data visualization and analysis were performed by me. Eric von Elert was involved in technical discussions. The chapter was written exclusively by me and was critically read by Maja Ilić.

Eidesstattliche Erklärung (gemäß §7 Absatz 8)

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Köln, den 28.03.2021



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[1] corresponds to chapter 1

[2] corresponds to chapter 2

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