Peptides In Cyanobacteria Under Different Environmental Conditions

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„Rien ne crée, rien se perd, tout se transforme“

„Na natureza nada se cria, nada se perde, tudo se transforma“

“Es geht also weder Masse verloren, noch kommt welche hinzu.”

Antoine Laurent de Lavoisier (1743-1794)

Kurzfassung

Die Hauptzielsetzungen dieser Arbeit waren unbekannte Peptide aus Cyanobakterien zu identifizieren und zu charakterisieren und den Einfluss von unterschiedlichen Umweltbedingungen (Licht, Temperatur, Nährstoff-komposition) auf die Peptidbildung zu analysieren.

Drei *Microcystis*-Stämme und zwei *Aphanizomenon-Stämme* wurden auf ihre Peptidzusammensetzung untersucht. In allen Stämmen wurden schon beschriebenen Cyanopeptide gefunden. In den *Aphanizomenon* Stämmen wurden 2 neue noch nicht beschriebene Cyanopeptide gefunden. Diese Cyanopeptide wurden isoliert und deren chemische Struktur aufgeklärt. Zur Charakterisierung dieser Peptide wurden einige Biotests durchgeführt. Die *in vitro* Zelltoxizitätstests ergaben, dass die Peptide nicht cytotoxisch, mutagen oder tumorfördernd sind. Andererseits zeigten *in vitro* Experimente mit *Daphnia magna*, dass diese Peptide Trypsininhibitoren sind.

Die meisten Untersuchungen, die bislang zum Einfluss der Umweltbedingungen auf die Peptidbildung durchgeführt wurden, basieren auf Strukturvarianten der gut untersuchten Cyanopeptidgruppe der Microcystine. Die Untersuchungen an Microcystinen ergaben, dass die Umweltbedingungen nur wenig Einfluss auf die Peptidbildung haben. In dieser Arbeit wurde überprüft, ob diese Aussage auch auf andere Cyanopeptide zutrifft.

Acht Peptide aus fünf Cyanobakterienstämmen wurden in dieser Arbeit untersucht, Microcystin [Leu1]-desmethylated-LR, Aeruginosin 102A, Microviridin, Microginin, Anabaenopeptin F, Oscillamide Y, Aphapeptin F1 (eines der neu isolierten Cyanopeptide) und ein weiteres unbekanntes Cyanopeptid, P677.

Mit diesen fünf Stämmen bzw. acht Peptiden wurden Experimente in Batch-Kultur mit unterschiedlichen Temperaturen, Lichtintensitäten und Nährstoffkonzentrationen durchgeführt.

Das zentrale Resultat dieser Arbeit ist die sehr große Variabilität der Peptidmenge pro Zellbiovolumen in Reaktion auf die Umweltbedingungen für diese acht Cyanopeptide. Eine ähnlich große Variabilität wurde auch in den Fällen beobachtet, in denen die gleichen Peptide in verschiedenen Stämmen

auftraten. Die Ergebnisse zeigen, dass die Peptidmenge häufig durch die Umweltbedingungen Temperatur und Lichtintensität beeinflusst war. Eine Erhöhung der Temperatur oder der Lichtintensität führte in den meisten Fällen zu einer Zunahme der Peptidmenge pro Zellbiovolumen. Unterschiede in der Peptidmenge pro Zellbiovolumen zwischen verschiedenen Wachstumsbedingungen betrugen selten mehr als einen Faktor von 5. Nur in 3 Ausnahmefällen bei gleichzeitig stark reduziertem Wachstum der Kulturen wurden bei -Stickstoffmangel zwei Peptide gar nicht mehr nachgewiesen.

Ein anderes interessantes Resultat ist die Erkenntnis, dass die Regulierung der Bildung dieser verschiedenen Cyanopeptide divers ist und nicht dem Muster folgt, das für Microcystin beschrieben ist.

Ähnlich wie Microcystin liegen 7 dieser 8 Peptide unter den meisten Wachstumsbedingungen zu über 90% zellgebunden vor, und der im Wasser gelöste Anteil ist gering. Geringfügige Anstiege des gelösten Anteils werden für Oscillamide Y insb. im stickstoff- oder phosphatfreien Medium beobachtet. Eine Ausnahme bildet lediglich das Aeruginosin 102A, das unter fast allen Wachstumsbedingungen etwa zur hälfte im Wasser gelöst auftrat.

Abstract

The main objectives of this work were to identify and characterize unknown cyanobacterial peptides and analyze the influence of different environmental conditions (temperature, light intensity, and nutrient composition) on cyanopeptide concentration.

Three *Microcystis* strains and two *Aphanizomenon* strains were screened for their peptide composition. All strains contained previously described cyanopeptides, and the *Aphanizomenon* strains contained 2 unknown cyanopeptides. These cyanopeptides were isolated and their chemical structure was elucidated. For characterization of these peptides, some biotests were done. The *in vitro* cytotoxicity tests indicated that these new peptides are not cytotoxic, mutagenic or tumor promoter. However, the *in vitro* experiments with *Daphnia magna* showed that these new cyanopeptides are trypsin inhibitors.

Most studies of the influence of environmental conditions on cyanopeptide concentration so far have addressed the well examined cyanopeptide group of microcystins, and they showed that environmental conditions have little influence on the microcystin concentration. The thesis presented here examined whether this statement also applies to other cyanopeptides.

Eight cyanopeptide from five cyanobacterial strains were examined, i.e. microcystin [Leu1]-desmethylated-LR, aeruginosin 102A, microviridin, microginin, anabaenopeptin F, oscillamide Y, aphapeptin F1 (the new isolated cyanopeptide) and another unknown cyanopeptide P677.

With these five cyanobacterial strains, i.e. eight cyanopeptides, batch culture experiments were done with different temperatures, light intensities and nutrient composition.

The central result is the pronounced variability of the peptide concentration per cell biovolume in reaction to the environmental conditions for these eight cyanopeptides. A similarly large variability was found also in the cases where the same peptides were produced in different strains. The results show that the peptide concentrations were frequently affected by temperature and light intensity. In most cases an increase of temperature and of light intensity led,

to an increase of the peptide concentration per biovolume. Differences in the peptide concentration per cell biovolume between different growth conditions rarely amount to more than a factor of 5. The only exception is a strain in which 3 peptides were no longer detected under nitrogen-deplete conditions.

Another interesting result is the observation that the regulation of these different cyanopeptides is diverse and does not follow the pattern described for microcystin.

Similarly as for microcystin, for 7 of these 8 peptides under most growth conditions more than 90% of the total pool is cell bound, and the portion dissolved in the water is small. Slight increases of the extracellular share occurred with oscillamide Y, especially in medium free of nitrogen and phosphate. The only exception is aeruginosin 102A, of which under nearly all growth conditions approximately half was found dissolved in the water.

Abbreviations

2D-NMR	Two dimensional – Nuclear Magnetic Resonance
7-AAD	7-amino-actinomysin
A	Adenylation
Ac	Acelaldehyde
ACE	Angiontensin-converting enzyme AMES II Mutagenicity Assay
ACP	Acyl carrier protein
Adda	2S,3S,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4Edienoic acid
Ahda	3-amino-2-hydroxy-decanoic acid
ALA	Alanine
Annexin V	Cause the antiphospholipid syndrome with abnormal blood clotting
ANOVA	Analysis of Variance
APM	Aminopeptidase
Arg	Arginine
Argol	Argininol; H_2N-CH(CH_2OH)-CH_2-CH_2-CH_2-NH-CNH(NH_2)
ASM-1	Culture medium
Asp	Aspartic acid
AT	Acyltransferase
BAPNA	N-α-benzol-DL-arginine-*p*-nitroanilide
C	Condensation
CACO-2	Intestinal epithelial cells
Choi	2-carboxy-6-hydroxy-octahydroindole
CPA	Cyrboxypeptidase A
DA	Dalton
DNA	Dexoxyribonucleic acid
FBS	Fetal Bovine Serum
FURG	Fundação Universidade do Rio Grande
Glu	Glutamate
HEP-G2	Human liver cells
HIS	Histidine
HL	High light intensity
Hpla	4-Hydroxylphenylacetic acid
HPLC	High Performance Liquid Chromatography

HSD	Honestly Significantly Difference
Hty	Homotyrosine
IC_{50}	half maximal inhibitory concentration
Ile	Isoleucine
KS	Ketoacyl synthase
LAP	Leucine aminopeptidase
Leu	Leucine
LDH	Lactase Dehydrogenase
LL	Low light intensity
Lys	Lesine
MA	Million of years
MAla	Methylalanine
MALDI-TOF	Matrix Assisted Laser Desorptio/Ionisation – Time of Flight
MANOVA	Multivariante Analysis of Variance
Mdha	N-methyldehydroalanine
Mdhb	Methyl dehydro Alanine
MeAsp	D-erythro-β-methylaspartic acid
MeOH	Methanol
Mhty	Methylhomotyrosine
Milli-Q	Double distilled water
MLeu	Methylleucine
MPT	Mitochondrial permeability transition
MTT	3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
m/z	Mass to charge ratio
M+H	Molecular mass of the neutral amino acid residue + hydrogen
NAD	Nicotinamide Adenine Dinucleotide
NRPS	Non-ribosomal peptide synthetases
OD	Optical Density
ODS	Octadecyl Silane C_{18} column
PCP	Peptidyl carrier protein
PI	Propidium iodine
Phe	Phenylalanine
PKS	Polyketide synthetases
PP1	Protein phosphatase type 1
PP2A	Protein phosphatase type 2A

Pro	Proline
PS	Phospholipid Phosphatidylserine
PSD	Post Source Decay
PSP	Paralytic Shellfish Toxins
RPMI	Roswell Park Memorial Institute medium
ROS	Reactive oxygen species
Ser	Serine
TCP	Thiolation carrier protein
TFA	Trifluoroaceticacid
Thr	Threonine
TUB	Technische Universität Berlin
Tyr	Tyrosine
UFRJ	Universidade Federal do Rio de Janeiro
vol/vol	Volume/volume

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1 Introduction

The cyanobacteria or “blue-green” algae have caused lethal animal intoxications worldwide since many decades and several human illnesses have been attributed to them (Carmichael, 1986, Kuiper-Goodman *et al.*, 1999, Chorus, 2001).

Cyanobacterial mass developments have been recorded from early history (Reynolds and Walsby, 1975). In the second half of the 20th century, reports of these mass developments became more and more frequent, probably caused by both the increase of research on cyanobacterial toxins and the acceleration of eutrophication in many water bodies around the world (Carmichael *et al.*, 1985, Chorus, 2001).

Surveys of water blooms and mass developments of planktic and benthic cyanobacteria in different countries have shown that a medium to high percentage (25 to 90%) of the blooms are toxic (Carmichael, 1988a; Baker and Humpage, 1994; Codd, 1995; Sivonen, 1996; Codd, 2000; Chorus 2001).

The toxins produced by cyanobacteria, also called cyanotoxins, are a diverse group of cellular metabolites and their natural function is still unclear. Cyanobacteria produce a large number of bioactive peptides (Moore, 1996; Moore *et al.*, 1996; Namikoshi and Rinehart, 1996; Weckesser *et al.*, 1996; Burja *et al.*, 2001). The best known are the cyclic toxins microcystins and nodularins, which are potent inhibitors of protein phosphatases 1, 2A (MacKintosh *et al.*, 1990; Yoshizawa *et al.*, 1990; Honkanen *et al.*, 1990, 1991),

Among the known cyanopeptides, microcystins have been found to be the most frequently occurring substances in most surveys conducted so far (Sivonen and Jones, 1999). In the last decade several other cyanopeptides have been described, such as aeruginosins (Murakami *et al.*, 1993) which are linear peptides, the microginins several of which are serine protease inhibitors (Okino *et al.*, 1993, Namikoshi and Rinehart, 1996) and the cyclic depsipeptides with an Ahp moiety such as the cyanopeptolins and the anabaenopeptolides (Martin *et al.*, 1993, Rouhiainen *et al.*, 2000). There are also some cyclic peptides with an ureido linkage, like the anabaenopeptins,

isolated from *Anabaena flos-aquae* (Harada *et al.*, 1995) and the ones with a β amino acid, like the nostophycins (Fujii *et al.*, 1999). Depsipeptides with a tricyclic ring system are named microviridins and were first isolated from *Microcystis viridis* (Ishitsuka *et al.*, 1990).

In the last decade, the amount of data on cyanobacterial peptides and their biosythentical pathways has increased, but many are still unknown. The ecological and physiological functions of these metabolites to this day are still not understood.

Different hypotheses exit involving the function of cyanopeptides in the physiology and ecology of the cyanobacteria, most of them involve grazing protection (Carmichael, 1992; Sivonen & Jones, 1999; Welker and von Döhren, *in press*) and allelopathy (von Elert & Jütter, 1997; Schagerl *et al.*, 2001; Suikkanen *et al.*, 2004, Welker and von Döhren, *in press*) or signaling between cells (Dittman *et al.*, 2001, Kehr *et al.*, 2006).

A single cyanobacterial strain may produce many peptides and consequently the isolation and purification of these peptides, which is required for structural determination, is a time consuming multistep process. Until this moment few experimental studies have been published about the ecological role of cyanobacterial oligopeptides and the published ones almost exclusively addressed microcystins, the most studied cyanopeptide group, with demonstrated toxicity from zooplankton to mammals.

Environmental factors could have an impact on cyanopeptide occurrence through a number of mechanisms which potentially include:

- Differences in growth rates of strains containing different peptides or different concentrations of the same peptides, including the other not yet well described peptides,
- Differences in bioactivity to zooplankton which feeds on cyanobacteria, potentially leading to differences in loss rates,
- Differences in cyanopeptide production and thus cellular and/or extracellular peptide concentrations, triggered physiologically by specific growth conditions, e.g. light or nutrient limitation, or temperature.

In order to better understand the cyanopeptides' biological role more information about them is needed. This includes a more comprehensive understanding of the variety of cyanopeptide structures, and the isolation and elucidation of new variants may promote the understanding of them as a group.

This thesis addresses two of these issues, i.e. isolation and structural elucidation of new cyanopeptides and the impact of environmental conditions on peptide production. Before discussing these objectives in more detail, in the following an introduction to the scientific background and state of knowledge will be given.

1.1 Scientific background

The cyanobacteria are an ancient group of prokaryotic organisms that are found all over the world in environments as diverse as Antarctic soils and volcanic hot springs and often where no other vegetation can exist (Bold & Wynne, 1985, Ressom *et al.*, 1994). They are of great antiquity being found in fossil stromatolites in northwestern Western Australia dated to at least 3500 Million of years (MA) ago (Schopf, 2000). Cyanobacteria have the capability to carry out oxygen-producing photosynthesis, using H_2O as an electron donor for CO_2 reduction, distinguishing them from all other prokaryotes (Schopf, 2000). This ability explains the cyanobacteria dominance in the early Earth history, and they are considered to have been the organisms responsible for the early accumulation of oxygen in the earth's atmosphere (Harlin and Darley, 1988, Schopf, 1994).

Their long evolutionary history is considered a reason for the success of cyanobacteria in many habitats and their wide ecological tolerance (Whitton and Potts, 2000). Nowadays cyanobacteria are found in freshwater, marine and terrestrial environments.

Cyanobacterial photosynthetic pigments are located in thylakoids and the light reactions of photosynthesis involve photosystems I and II as in higher plants (van den Hoek *et al.*, 1995). However, the cellular arrangement of photosynthetic pigments is in thylakoids occurring freely in the cytoplasm, and

the lack of membrane-bound organelles clearly defines the cyanobacteria as prokaryotic organisms (van den Hoek *et al*., 1995). Among the chlorophylls, most cyanobacteria contain only chlorophyll *a* (some species were shown to be able to produce chlorophyll *b* and *d*, Roche *et al.*, 1996, Chen *et al.*, 2005b). The cyanobacteria or “blue-green algae” are so called from the first species to be recognized and named. These species were blue-green in color, and although most species are in fact blue-green, many diverse other ranges of pigments, like olive-green, grey-green, yellow-brown or purplish to red are observed (Echlin, 1966, Prescott, 1968). This typical color is due to the presence of pigments called phycobilins. These pigments are associated with proteins and arranged in the phycobilisomes (Glazer, 1982, Oliver and Ganf, 2000). These phycobilisomes are the most abundant structures within cyanobacterial cells (Allen, 1984). They are accessory pigments in photosynthesis and are used as light harvesting complex (van den Hoek *et al.*, 1995). The phycobilisomes absorb light over a wider range of wavelengths than the antennae of other phytoplankton, making this a fundamental difference between cyanobacteria and the eukaryotic micro-algae (Oliver and Ganf, 2000). In addition, aquatic cyanobacteria can grow at very low irradiance compared with most of their eukaryotic counterparts, and are capable of continued growth even in face of pronounced shading by other phytoplankton (van Liere and Walsby, 1982).Therefore, in waters with high turbidity the cyanobacteria have a better chance of surviving and even multiplying than other species. Thus, even their slow growth under low irradiance can lead to cyanobacterial dominance (Mur *et al.*, 1978, 1999). This, coupled with the ability of cyanobacteria to regulate their buoyancy, also permits cyanobacteria to compete successfully with other phytoplankton in highly turbid environments (Long, 2001).

Most cyanobacterial cells also contain cyanophycin granules (multi-L-arginyl-poly-[L-aspartic acid]), polyphosphate and polyglucose bodies, all of which are utilized as nutrient storage compounds (Allen, 1984). These storage compounds are accumulated when particular nutrients occur in excess in the environment. When nutrient limitation occurs the cyanobacteria make use of these stores accumulated earlier.

An important feature of the cyanobacteria is the ability of some species to fix elemental nitrogen dissolved in water, and even without nitrogen fixation many species are capable of living in water with low levels of nitrogen. Among the genera able to fix nitrogen are e.g. *Anabaena* and *Aphanizomenon*. In the genus *Microcystis* this ability is missing, since they do not develop heterocysts (large differentiated cells, which are capable of fixing nitrogen) (Kumar *et al.*, 1982, Ressom *et al*., 1994, Long, 2001).

A common feature of many water bodies around the world and one of the major water quality problems is eutrophication, or the enrichment of aquatic systems with nutrients. The principal elements involved in this process are phosphorus, nitrogen (especially the inorganic forms, such as phosphate, nitrate and ammonia) and more rarely (e.g. under strongly acidified conditions) carbon (Rosenberg and Freeman, 1991, Svrcek and Smith, 2004).

Phosphorus is a vital cellular constituent of all living organisms and is involved particularly in energy dynamics and protein synthesis. It is a natural part of all ecosystems deriving ultimately from the weathering of rock and subsequently by recycling in the biosphere. It finds its way into water bodies primarily via diffuse runoff from catchment soils or from point sources. Both inputs have been heavily influenced by human activity. Soils runoffs have increased over the natural levels by the widespread application of artificial fertilizers and from livestock manure, sewage outfalls. Urban wastewater and waste from industries also contribute significantly (Ressom *et al.*, 1994, Svrcek and Smith, 2004).

Nitrogen is an essential component of all living cells and it is involved primarily in the synthesis of amino acids and proteins. It moves freely into and out of aquatic ecosystems in the molecular state as dinitrogen gas with the atmosphere being the ultimate source. Nitrogen can also enter water bodies in catchment runoff as breakdown products of organic substances in the form of ammonia, urea or the inorganic ions nitrate and nitrite. Most phytoplankton are able to assimilate and utilize nitrogen in the form of ammonium, nitrate, nitrite or urea, while some cyanobacteria have the additional ability to assimilate molecular nitrogen (Bold and Wynne, 1985).

Many cyanobacteria can consume and absorb far more nutrients than they need when these are freely available, storing them for times when they are depleted in the water body (Kronkamp, 1987, Long, 2001). Compared to most other phytoplankton, the cyanobacterial species have a higher affinity for the uptake of these nutrients, giving them a distinct advantage under conditions where these nutrients are available in low concentrations. The eukaryotic algae have an N:P optimum ratio of 16:23, and for cyanobacteria this optimum ratio is lower, i.e. 10:16, since some species have the capability of fixing atmospheric nitrogen, i.e. the Nostocales (Rhee and Gotham, 1980; Mur *et al.*, 1999). Storage allows growth to proceed for several cell divisions in the complete absence of extracellular sources of these nutrients as the reserve materials are utilized (Allen 1984; Kromkamp *et al.*, 1989).

Planktonic cyanobacterial contain aggregates of gas-filled structures, known as gas vesicles, which are hollow chambers with a hydrophilic outer surface and a hydrophobic inner surface (Walsby, 1978, Allen, 1984). These gas vesicles can help aquatic species to control their depth in the water column by adjusting their buoyancy (Walsby 1994).

Compared to many algal species, cyanobacteria usually have a lower maximal growth rate. This slow growth rate requires a long water retention time to form a substantial population or mass development (Reynolds, 1984, Mur *et al.*, 1999). Although all phytoplankton are capable of taking advantage of eutrophic waters, characteristics such as buoyancy control, production of nutrient storage compounds and their persistence at relatively slow growth rates, regardless of the prevailing growth conditions, often mean that cyanobacteria dominate the phytoplankton in the long term (Mur *et al.*, 1999).

Cyanobacteria are integral parts of many ecosystems and in limited numbers they are present in most surface waters at all times. However, concerns arise when they dominate in high cellular densities or cyanobacterial blooms (Svrcek and Smith, 2004).

Cyanobacterial blooms, normally described as a significantly higher phytoplankton biomass than the lakes' average, have been recorded from early history (Reynolds and Walsby, 1975). Especially under eutrophic and hypertrophic conditions, mass developments are observed worldwide (Paerl,

1996). They can be differentiated between high biomass density and turbity as compared to surface scums, with the term “bloom” often being used synonymously to “scum”, but sometimes also for homogeneously distributed mass developments. Commonly produced by gas-vesicle containing cyanobacterial taxa that can vary in form and size from small filaments to large globular colonies, scums occur mainly under conditions of high water temperatures and reduced turbulence (Reynolds and Walsby, 1975; Robarts and Zohary, 1987; Paerl, 1996). Scum forming cyanobacteria mainly belong to the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, and *Microcystis* and *Planktothrix* (Reynolds and Walsby, 1975; Oliver and Ganf, 2000).

Mass occurrences of cyanobacteria may cause severe problems in reservoirs and lakes used as drinking water suppliers, recreational areas and watering places for livestock. These mass developments may be dominated by a single species or several different species, some of which may be toxic (Sivonen and Jones, 1999).

In temperate regions water blooms develop frequently during the warmer summer and autumn. According to Robarts & Zohary (1987), the maximum growth rates of many bloom-forming cyanobacteria are achieved at temperatures greater than 25℃. With decreasing lat itude towards the subtropics and tropics the changes in the daily solar input and temperature become less variable. Therefore, in the tropics cyanobacterial blooms can occur at almost any time of the year, due to the relatively constant annual air temperature and solar radiation (Oliver and Ganf, 2000). Water systems polluted with nutrients (P and N) usually also have increased concentrations of other nutrients essential for cyanobacterial growth (e.g. S, Ca, Mg and Fe) so that blooms can be easily formed once a cyanobacterial species has become dominant.

1.2 Cyanobacterial toxins

Cyanobacteria produce a variety of secondary metabolites, including toxins. These are compounds that have a harmful effect on other tissues, cells or organisms (Svrcek and Smith, 2004).

Cyanobacteria have been known to cause lethal intoxications in farm animals and wildlife for many decades (Carmichael, 1986; Kuiper-Goodman *et al.*, 1999), and a number of cases of human illness can be attributed to cyanotoxin exposure rather clearly (Kuiper-Goodman *et al.*, 1999). The tragical deaths of 70 of 131 patients exposed to cyanobacterial toxins (microcystins) through renal dialysis in Brazil are the only well substantiated human fatalities due to cyanotoxins (Jochimsen *et al.*, 1998), although some illnesses reported previously were life-threatening (Hawkins *et al.*, 1985, Ressom *et al.*, 1994, Chorus and Bartram, 1999).

Geographically, toxic cyanobacteria have a wide distribution (Hoffmann, 1996). Toxicity attributable to cyanobacteria has now been identified and described on all continents (Skulberg *et al.*, 1984; Galey *et al.*, 1987; Jones, 1994; Yunes *et al.*, 1998; Hitzfeld *et al.*, 2000), making public awareness and management of toxic blooms a matter of widespread concern (Chorus and Bartram, 1999). Many cyanobacterial blooms tend to involve a single cyanobacterial species. However, some blooms involve several genera and within a toxic bloom, both toxic and non-toxic strains of a single cyanobacterial species can often be found (Carmichael and Gorham, 1981). This restricts the options for prediction of its toxicity from cyanobacterial biomass to worst case scenarios, assuming the entire biomass to contain the maximal known amount of toxin. Generally, most of the toxic cyanobacterial blooms analyzed contain the liver damaging hepatotoxins, i.e. microcystins and/or nodularins (Sivonen and Jones, 1999). In Danish, German, Czech and Korean surveys between 80-90% of all samples dominated by *Microcystis* spp. were found to contain microcystins or to show hepatotoxicity. Among samples dominated by *Planktothrix agardhii,* between 80 and 100% of those tested showed hepatotoxicity or contained microcystins (Chorus, 2001).

In the last decades the cyanobacterial blooms have called attention to the apparently extensive degradation of surface water and its recreational value, causing oxygen depletion of the water that may lead to fish kills, increasing the water treatment problems such as filter blockages, taste and odor problems and, with a number of widespread species, the presence of toxins.

1.2.1 Hepatotoxins

The cyanotoxin mostly found worldwide, in freshwater and brackish waters, are the hepatotoxins from the microcystin and nodularin family (Sivonen and Jones, 1999). Hepatotoxins are also the most common of the cyanobacterial toxins and mostly involved in acute toxicoses (Ressom *et al.*, 1994).

Hepatotoxins are strong inhibitors of type 1 and 2A serine protein phosphatases (PP1 and PP2A) (MacKintosh *et al.*, 1990). These enzymes are vital to various cellular processes such as cell growth and tumor suppression and therefore these toxins are possible potent cancer promoters (MacKintosh *et al.*, 1990; Carmichael, 1992, 1994, Luukkainen *et al.*, 1993, 1994; Runnegar *et al.*, 1995). Laboratory research has indicated that microcystin-LR is an extremely potent tumor promoter in animals (Nishiwaki-Matsushima *et al.*, 1992) and is the most potent liver carcinogen yet characterized.

The occurrence of these toxins in surface waters makes the production of safe drinking waters a challenge. Their presence in potable water may present a serious health hazard to humans. If these peptide toxins are consumed over a long period of time, they may contribute to chronic liver illnesses, such as liver tumors or necrosis (Falconer, 1991; Falconer *et al.*, 1998, Sivonen and Jones, 1999).

1.2.1.1 *Microcystin*

One of the most widespread bloom-forming cyanobacteria is the genus *Microcystis*, a well known producer of the hepatotoxic peptide microcystin. This toxin was first isolated from one *Microcystis aeruginosa* and named after this genus (Carmichael *et al.*, 1988a). Soon it became evident that a large number of structural variants exist. Their chemical structure was identified in the early 1980s and the identification of variants increased during the last two decades (Sivonen and Jones, 1999).

Microcystins are a group of closely related cyclic heptapeptides sharing the common structure cyclo (D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), in which MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine, Adda is 2S,3S,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6Edienoic acid, and X and Z are variable L-amino

acids, e.g., microcystin-LR (MC-LR) contains leucine (L) and arginine (R). So far more than 90 derivatives of microcystins have been identified, varying largely by the degree of methylation, peptide sequence, and toxicity (Fastner *et al.*, 2001). One of the most important fragments from the microcystin is the Adda amino acid fragment, which is characteristic to microcystins and to date has not been found in any other group of substances (Carmichael *et al.*, 1988a).

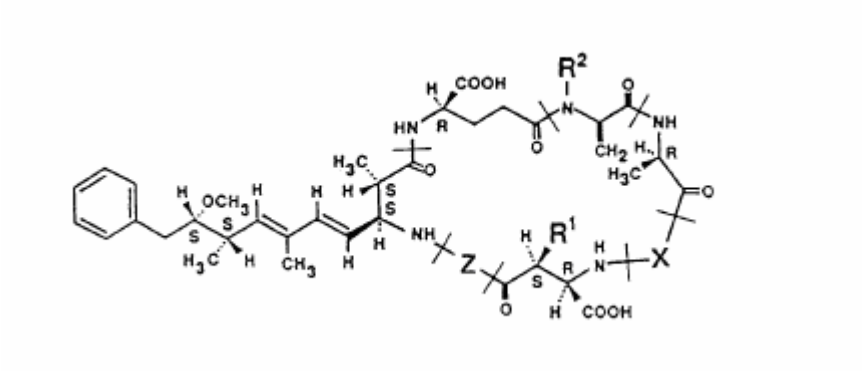


Figure 1: General structure of microcystin (Carmichael *et al.*, 1988a).

The hepatotoxicity of microcystins is based on their inhibition of protein phosphatases 1 and 2A in combination with transport into hepatocytes via the bile acid carrier. At sufficiently high dose this leads to acute liver failure due to the disruption of hepatocyte cytoskeletal components. Furthermore, liver damage through chronic exposure has been demonstrated in several animal experiments, most prominently by Falconer (1994) and Fawell *et al.* (1994), and microcystins have been shown to promote tumor growth, though by themselves, they probably are not carcinogenic (Falconer *et al.,* 1988).The widespread occurrence and acute toxicity of microcystins and their tumor-promoting properties imply the need for identification and prediction of toxic blooms (Fastner *et al.*, 2001).

Microcystins are synthesized non-ribosomally via peptide synthetases (von Döhren *et al.*, 1997; Marahiel *et al.*, 1997). Dittmann *et al.* (1997) cloned the microcystin synthetase genes, *mcy*A and *mcy*B from the *M. aeruginosa* strain, PCC7806. Nishizawa *et al.* (1999) also identified three genes (*mcy*A, *mcy*B, *mcy*C) encoding microcystin synthetases from toxic *M. aeruginosa* K-139, which produced desmethylmicrocystin-LR and microcystin-LR (Harada *et al.*,

1991). Depending on whether or not they contain these genes, *Microcystis aeruginosa* strains can be divided into toxic and non toxic genotypes.

Since microcystins proved harmful to the health of a wide range of animals and humans, there was pronounced interest in determining which factors may influence or control the production of these compounds (Long, 2001). Studies were carried out preponderantly in the 90's, particularly with laboratory cultures but also analyzing data from field samples, concerning the factors that affect the toxicity in microcystin-producing cyanobacterial strains and the environmental conditions leading to an increase in toxin concentration. These studies focused on the factors also determining cyanobacterial growth rates such as light, temperature, nitrogen, phosphorus, micronutrients and pH.

Physiological investigations of microcystin production in batch cultures showed that the toxin concentration in the culture generally follows a pattern similar to the cell concentration, i.e. microcystins are produced at the same rate as other cellular consitutents or cellular biomass in general. Long *et al.* (2001) showed that variation of the microcystin concentration of cells was most pronounced when comparing the two extremes of growth rate, i.e. the exponential and stationary growth phases. This is important in the context of an ongoing discussion about the need to use continuous cultures which argues that as batch cultures grow, conditions change continuously with respect to more than one parameter at a time, e.g. as a nutrient concentration is depleted by uptake and cell growth, light intensity in the culture also changes in response to cell growth, and thus physiological responses such as changes in microcystin concentration cannot be clearly attributed to a single cause. Long and Jones, however, point out that the exponential and stationary growth phases should be exceptions: in the exponential phase at a given temperature, growth should be maximal and only be limited by light, whereas in the stationary phase growth is minimal and limited only by the minimum factor determined by the experimental design, i.e. light or a nutrient. Consequently, the batch culture approach should be useful for staking out the extremes, i.e. the range of variation in concentration of a metabolite when a given environmental factor is severely limiting as related to unlimiting conditions. Thus, to characterize the range of variation of the concentration of a given peptide, sampling of batch cultures should focus on these two phases

and do not need to be sampled at regular intervals, e.g. every other day, as is done in most batch culture study designs.

Investigations under different culture conditions suggest that resource limitation causes some variation of microcystin concentration, but only by a factor of 2-4 (Sivonen and Jones, 1999, Hesse and Kohl, 2001). Currently, it is unclear to which extend this pattern of variability also applies to concentrations of other peptides in cyanobacteria.

Temperature and Light

Temperature and light are important factors in cyanobacterial bloom events. Several studies have examined the temperature and light influence on microcystin production in different cyanobacterial species.

An evaluation of the published data from culture experiments addressing the effect of temperature or of light shows some trend, but with a number of contradictory observations:

For most of the temperature effects studies, toxicity or microcystin concentration are higher between 20° and 25℃ than the other temperature tested (Table 1). Microcystin toxicity always refers to the effect of this peptide on a whole organism such as mouse, or human or to a substructure such as a liver. The microcystin concentration refers to the amount of this peptide per cell, independent of its toxicity.

Contradicting most of the other studies Song *et al.* (1998) and Ohtake *et al.* (1989) found no effect of temperature of microcystin concentration or, respectively, of toxicity in relation to cellular dry weight (Table 1).

Results published by different authors on the effect of light are yet more contradictory than those on temperature effects. There is a small tendency that the toxicity of microcystin increases as irradiance increases, as in van der Westhuizen & Eloff (1985), Watanabe & Oishi (1985), Rapala & Sivonen (1998), Kaebernick *et al.* (2000) and Böttcher *et al.* (2001). However, other studies also showed that for some strains irradiance do not affect the toxicity (Codd & Poon, 1988; Rapala *et al.*, 1997). A few authors found also the

decrease of toxicity as irradiance was increased (Sivonen, 1990; Hesse & Kohl, 2001) (Table 2).

The interrelationship of temperature and light is an important regulatory factor in the physiology and behavior of cyanobacteria. To obtain a better comprehension of these effects, some studies have addressed this problem analyzing the combined effect of temperature and light. Song *et al.* (1998) found no significant effect of irradiance on microcystin per dry weight at 15℃ but at 25℃ microcystin/dry weight decreased with increasing irradiance. Rapala & Sivonen (1988) described the combined effect of light and temperature on two *Anabaena* strains, in which for microcystin-LR light was the regulating factor, but for microcystin-RR temperature was the relevant factor.

Table 1: Influence of temperature on microcystin concentration, summary of studies.

Temperature range studied	Cyanobacterial strain	Culture type and toxin/toxicity determination	Results	Reference
20°-35°C	*M. aeruginosa* NC-1	Batch/semi continuous Mouse bioassay	Optimum growth at 32°C. At 25° five-fold increase in toxicity than at 20° or 30°C	Gorham, 1964
18°-34°C	*M. aeruginosa*	Batch culture Mouse bioassay	A four-fold increase in toxicity at 18° compared to 29°C	Runnergar *et al.*, 1983
16°-36°C	*M. aeruginosa* UV-006	Batch culture Mouse bioassay	Growth rate increase with temperature, but toxicity greater at 20°C and decreased in 3,75 folds as the temperature increased	Van der Westhuizen & Eloff, 1985)
18°-32°C	*M. aeruginosa* M228	Batch culture Mouse bioassay	Optimum growth at 32°C. At 18°C the greatest toxicity, and it decreases in 1,4 folds as temperature increases	Watanabe & Oishi, 1985
16°-36°C	*M. aeruginosa* UV-006	Batch culture Mouse bioassay	Highest toxicity at 20°C	Van der Westhuizen *et al.*, 1986
10°-34°C	*M. aeruginosa* 7813	Batch culture Mouse bioassay	Optimum growth at 25°C. Higher toxicity at 25°C than at 10° and 34°C	Codd & Poon, 1988
22 and 30°C	*M. aeruginosa* K-139	Batch culture Mouse bioassay	No effect on toxicity	Ohtake *et al.*, 1989
15°-30°C	*Planktothrix agardhii* 97 and CYA 128	Batch culture Microcystin analysis (HPLC)	Optimum growth at 25°C. 97: higher microcystin concentration at 25°C (1.4 fold increase) and decreased as temperature further increased; CYA 128: the same microcystin concentration at 15° and 25°C (2 to 3 fold increase) but lower at 30°C.	Sivonen, 1990
12,5°-30°C	*Anabaena* 90, 202A1	Batch culture Microcystin analysis (HPLC)	Highest microcystin values at 25°C, 90: increases at 12,5 °C and 25°C, but decreases at 20°C and 30°C 202A1 : increases as temperature increases, only until 25°C afterwards decreases with the temperature increase.	Rapala *et al.*, 1997
15°-30°C	*M. viridis*	Batch culture Microcystin analysis (HPLC)	No effect of temperature on microcystin concentration	Song *et al.*, 1998

Table 2: Influence of light intensity on microcystin concentration, summary of studies.

Light range	Cyanobacterial strains	Culture type and toxin/toxicity determination	Results	Reference
21-205 µmol $m^{-2}s^{-1}$	*M. aeruginosa* UV-006	Batch culture Mouse bioassay	Toxicity increased in 1,4 folds with the increase of irradiance	Van der Westhuizen & Eloff, 1985
21-205 µmol $m^{-2}s^{-1}$	*M. aeruginosa* UV-006	Batch culture Mouse bioassay	Highest toxicity at 145 µmol $m^{-2}s^{-1}$	Van der Westhuizen *et al.*, 1986
7.5-75 µ$Em^{-2}s^{-1}$	*M. aeruginosa* M228	Batch culture Mouse bioassay	Optimum growth at highest irradiance, high toxicity at high irradiance and low toxicity at low irradiance, 3,7 folds less than at high irradiance	Watanabe & Oishi, 1985
5-50 µ$Em^{-2}s^{-1}$	*M. aeruginosa* 7813	Batch culture Mouse bioassay	No effect on toxicity	Codd & Poon, 1988
12-95 µ$Em^{-2}s^{-1}$	*P. agardhii* 97 and CYA 128	Batch culture Microcystin analysis (HPLC)	97:microcystin concentration decreased as irradiance increased; CYA 128: highest microcystin concentration at 12 and 24 µ$Em^{-2}s^{-1}$, but lower concentration at high irradiance	Sivonen, 1990
20-75 µ$Em^{-2}s^{-1}$	*M. aeruginosa* CYA 228/1	Chemostat Microcystin analysis (HPLC)	Microcystin/dry weight increased with irradiance up to 40 µ$Em^{-2}s^{-1}$, then decreased with further increased of irradiance: Microcystin per protein increased with irradiance up to 40 µ$Em^{-2}s^{-1}$, then remained constant	Utkilen & Gjølme, 1992
2-100 µmol $m^{-2}s^{-1}$	*Anabaena* 90, 202A1	Batch culture Microcystin analysis (HPLC)	Highest microcystin at 25 µmol $m^{-2}s^{-1}$ for *Anabaena* 90 but no effect on 202A1	Rapala *et al.*, 1997
7-42 µ$Em^{-2}s^{-1}$	*Anabaena* 90, 2002A1	Turbidostat Microcystin analysis (HPLC)	Growth rate and microcystin/dry weight correlate positively with increase of irradiance	Rapala & Sivonen, 1998
15-100 µmol $m^{-2}s^{-1}$	*M. viridis*	Batch culture Microcystin analysis (HPLC)	No effect of irradiance at 15℃. At 25℃ the microcystin concentration decreased with the increased of irradiance	Song *et al.*, 1998
16-68 µmol $m^{-2}s^{-1}$	*M. aeruginosa* PCC7806	Batch culture *mcy* gene transcripts	High transcript levels at high irradiance and low transcripts levels at low irradiance[2]	Kaebernick *et al.*, 2000
5-150 µmol $m^{-2}s^{-1}$	*M. aeruginosa* HUB 5-2-4, W334, W368	Semi-continuous Microcystin analysis (HPLC)	Microcystin concentration increased with irradiance in HUB 5-2-4 and W368, but decreased with the increased of irradiance in W334	Hesse & Kohl, 2001
2.4-73 µmol $m^{-2}s^{-1}$	*M. aeruginosa* HUB 5-2-4, *P. agardhii* HUB 076	Turbidostat Microcystin analysis (HPLC)	Microcystin/dry weight increased with increased of irradiance	Böttcher *et al.*, 2001

[2] elevated gene transcript levels suggest elevated production of the peptide.

Nitrogen

Nitrogen (N) is an essential element of amino and nucleic acids, making it indispensable to all living beings. Even for nitrogen-fixing cyanobacteria, as for all phytoplankton its abundance or limitation can influence growth rates and thus biomass of cyanobacteria and in consequence also bloom formation and toxin levels. Moreover, for peptide toxins such as microcystins, a direct impact of nitrogen on their cellular levels is conceivable: under severe limitation, the production of non-essential cellular metabolites would be expected to be down regulated, and thus the response of cellular levels of such metabolites to nitrogen stress would indicate whether or not they are essential for cell growth and multiplication.

As for temperature and light, several studies were done concerning the effect of nitrogen on microcystin concentration of cultures. These results are not as contradictory as the results found for other parameters. Most studies done since the 1980's concurred on the fact that a higher nitrogen concentration leads to a greater toxicity or microcystin concentration in relation to cellular dry weight (Table 3). Rapala *et al.* (1997) describes a greater concentration of microcystin/dry weight in N free medium, expect for *Anabaena* strains, which posses heterocyst and are able to fix N.

Song *et al.* (1998), Long *et al.* (2001) and Hesse & Kohl (2001) presented results distinctly different from the all other studies. Both studies were done using *Microcystis* strains, which are not capable of fixing N, but they describe higher microcystin/dry weight concentration in N limited and free medium (Table 3).

Table 3: Influence of nitrogen concentration on microcystin concentration, summary of studies.

Nitrogen range	Cyanobacterial strains	Culture type and toxin/toxicity determination	Results	Reference
0.1 to 2.0 mM	*M. aeruginosa* M228	Batch culture Mouse assay	Higher toxicity at highest N level	Watanabe & Oishi, 1985
0 and 0.9 mM	*M. aeruginosa* 7813	Batch culture Mouse assay	10 fold higher toxicity at 0.9mM/l	Codd & Poon, 1988
0.03-6 mM	*P. agardhii* 97 and CYA 128	Batch culture Microcystin analysis (HPLC)	Microcystin/dry weight increased as N increased in both strains	Sivonen, 1990
0.35-5.8 mM/	*M. aeruginosa* CYA 228/1	Chemostat	Microcystin/dry weight increased as N level increased. Microcystin/protein not affected by N level	Utkilen & Gjølme, 1995
0-3.6 mM/	*Anabaena* 90, 202A1	Batch culture	microcystin/dry weight highest in N free medium	Rapala *et al.*, 1997
0,2 mM	*M. viridis*	Batch culture	Higher microcystin/dry weight in medium without N	Song *et al.*, 1998
0.0118-1.18 mM	*M. aeruginosa* MASH-01	Batch culture	Microcystin/dry weight 3 fold higher during non-limiting conditions for all concentrations tested	Orr & Jones, 1998
6.5-650 µM (P fixed at 6.5 µM/l)	*M. aeruginosa* UTEX 2388	Batch culture	No effect on growing, high microcystin/dry weight at medium N (104 and 325 µM)	Lee *et al.*, 2000
	M. aeruginosa MASH-01A19	N-limited chemostat	5.48 fold increase of microcystin/dry weight as compared to unlimited growth	Long *et al.*, 2001
	M. aeruginosa HUB 5-2-4, W368 and W334	Semi continuous, chemostat principle	N-limitation: HUB 5-2-4: 24% lower microcystin/biovolume W368: 20% higher microcystin/biovolume W334: 49% lower microcystin/biovolume	Hesse & Kohl, 2001

Phosphorus

Phosphorus (P) is a major nutrient used by all living organisms for incorporation into macromolecules, such as DNA and phospholipids. Uptake rates of dissolved P vary tremendously depending on the organism and the growth conditions.

Comparable to the data available for temperature and light, the influence of phosphorus on microcystin production is difficult to interpret from the experimental studies reported to date. For example, Watanabe & Oishi (1985) found that in full medium (sufficient P available) batch cultures of *M. aeruginosa* were more toxic than P limited cultures, whereas the opposite was the case in a study by Codd & Poon (1988).

Oh *et al.* (2000) recently showed that in P-limited chemostats, microcystin-LR and RR decreased in relation to both dry weight and protein as the growth rate increased. This contradicts the relationship proposed by Orr & Jones (1998) (Table 4).

Table 4: Influence of phosphorus concentration on microcystin concentration, summary of studies.

Phosphorus range	Cyanobacterial strains	Culture type and toxin/toxicity determination	Results	Reference
23-460 µM	*M. aeruginosa* M228	Batch culture Mouse bioassay	Higher toxicity at highest P level	Watanabe & Oishi, 1985
0 and 225 µM	*M. aeruginosa* 7813	Batch culture Mouse bioassay	Higher toxicity in P free medium	Codd & Poon, 1988
3,2-177 µM	*P. agardhii* 97 and CYA 128	Batch culture Microcystin analysis (HPLC)	97: microcystin/dry weight increased with P; 128: microcystin/dry weight at medium concentration. No production at the lowest P level.	Sivonen, 1990
14.4 and 143.5 µM	*M. aeruginosa* CYA 228/1	Chemostat Microcystin analysis (HPLC)	Microcystin/dry weight greater at higher P level. Microcystin/protein higher at lower P level	Utkilen & Gjølme, 1995
1,6-177 µM	*Anabaena* 90, 202A1	Batch culture Microcystin analysis (HPLC)	Microcystin/dry weight increased with P level	Rapala *et al.*, 1997
0, 460µM	*M. viridis*	Batch culture Microcystin analysis (HPLC)	Highest microcystin/dry weight without P	Song *et al.*, 1998
P-limited	*M. aeruginosa* UTEX 2388	Chemostat Microcystin analysis (HPLC)	Microcystin/dry weight decreased as growth rate increased, i.e. at less P-limitation	Oh *et al.*, 2000
0.7-71 µM (N fixed at 71.4µM)	*M. aeruginosa* UTEX 2388	Batch culture Microcystin analysis (HPLC)	Microcystin/dry weight tend to decrease with reduced P level	Lee *et al.*, 2000
5-150 µM	*M. aeruginosa* CYA 228/1	Chemostat Microcystin analysis (HPLC)	Microcystin/protein increased as P and thus growth rate decreased	Bickel *et al.*, 2000
0-2 mM	*M. aeruginosa* MASH-01A19	Batch cultures Microcystin analysis (HPLC)	Microcystin/dry weight decreased as P increased.	Long *et al.*, 2001
P-limited	*M. aeruginosa* HUB 5-2-4, W368 and W334	Semi continuous, chemostat principle Microcystin analysis (HPLC)	P-limitation: HUB 5-2-4: 21% lower microcystin/biovolume; W368: 2 fold higher microcystin/biovolume W334: 14% lower microcystin/biovolume	Hesse & Kohl, 2001

1.2.1.2 *Nodularin*

A cyclic pentapeptide isolated for the first time from *Nodularina spumigena*, has a very similar chemical structure and similar activities as the microcystin.

This peptide is named nodularin in reference to the organism in which this substance was first isolated.

The pentapeptide hepatotoxin nodularin has been found in brackish waters in Australia, New Zealand and the Baltic Sea, where blooms have caused numerous cases of animal poisonings (Carmichael *et al.*, 1988b).

The chemical structure of nodularin is cyclo-(D-MeAsp-L-arginine-Adda-D-glutamate-Mdhb). As for microcystin, the toxicity of this toxin results from its ability to inhibit the serine, threonine protein phosphatases, PP1 and PP2 (Otha *et al.*, 1994). It is a potent tumor promoter and a possible carcinogen. There are no published reports on the chemical antidotes or therapeutic measures against nodularin poisoning (Rao *et al.*, 2002).

1.2.1.3 *Cylindrospermopsin*

An outbreak of hepatoenteritis at Palm Island in northern Queensland, Australia, led to the finding of a new cyanobacterial toxin, cylindrospermopsin.

Cylindrospermopsin is produced by *Cylindrospermopsis raciborskii* (Woloszynska), and recently characterized from a Japanese *Umezakia natans* (Harada *et al.*, 1994), *Aphanizomenon ovalisporium* (Banker *et al.*, 1997) and *Aphanizomenon flos-aquae* (Preußel *et al.*, 2006).

It is an alkaloid cytotoxin with molecular weight 415 and it affects the liver, kidneys, thymus and heart (Hawkins *et al.*, 1985). Studies using cultured rat hepatocytes have shown that cylindrospermopsin inhibits glutathione synthesis (Terao *et al.*, 1994).

1.2.2 Neurotoxins

Reports on animal poisonings caused by neurotoxic cyanobacteria have been published from several continents, e.g. North America, Europe, Australia and Brazil (Sivonen and Jones, 1999, Lagos *et al.*, 1999).

Neurotoxins are produced by several genera e.g. *Anabaena*, *Aphanizomenon*, *Planktothrix* and *Trichodesmium* (Keevil, 1991; Carmichael

1992, 1994). The major neurotoxins are anatoxin-a, homoanatoxin-a, anatoxin-a(s) and paralytic shellfish toxins (PSP) (Dow and Swoboda, 2000).

1.2.3 Other bioactive peptides

Major shares of the cyanobacterial secondary metabolites are peptides or possess peptidic structures. Numerous further bioactive oligopeptides and other substances are being discovered in cyanobacteria, in part due to substantial advances in chemical methods of structural elucidation and in part due to pharmacological research addressing the cytostatic, enzyme-inhibiting, antibiotic and anti-fungal substances potentially contained in these organisms (Moore, 1996, Welker and von Döhren, *in press*). Pronounced toxic effects have been observed, particularly in fish embryo tests and in cellular bioassays (Heinze, 1997, Oberemm, 1999) – effects which are supplementary to those which can be attributed to known substances at the moment (Bury *et al.*, 1998; Feuillade *et al.*, 1996; Haney *et al.*, 1995; Jungmann, 1992; Reinikainen *et al.*, 1995). Peptides, particularly cyclic peptides and depsipeptides, have been considered likely candidates for some of these effects as they frequently show bioactivity *in vitro*.

Most cyanobacterial oligopeptides are assumed to be synthesized by non-ribosomal peptide synthetases (NRPS) or non-ribosomal peptide synthetases/polyketide synthetases (NRPS/PKS) hybrid pathways (Welker and von Döhren, *in press*).

As summarized in the review by Ansari and colleagues (2004), non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) are multi-enzymatic, multi-domain megasynthases involved in the biosynthesis of non-ribosomal peptides and polyketides. Non-ribosomal peptides are biosynthesized by sequential condensation of amino acid monomers, whereas polyketides are made from repetitive addition of two carbon ketide units derived from thioesters of acetate or other short carboxylic acids.

NRPSs and modular PKSs are comprised of so-called modules, which are sets of distinct active sites for catalyzing each condensation and chain elongation step. Each module in an NRPS or PKS consists of certain

obligatory or core domains for addition of each peptide or ketide unit and a variable number of optional domains responsible for modification of the peptide/ketide backbone. The minimal core module in the case of an NRPS consists of an adenylation (A) domain for selection and activation of amino acid monomers, a condensation (C) domain for catalyzing the formation of peptide bonds and a thiolation or peptidyl carrier protein (T or PCP) domain with a swinging phosphopantetheine group for transferring the monomers/growing chain to various catalytic sites. Similarly an acyltransferase (AT) domain for extender unit selection and transfer, an acyl carrier protein (ACP) with a phosphopantetheine swinging arm for extender unit loading and a ketoacyl synthase (KS) domain for decarboxylative condensations constitute the core domains of PKS modules.

According to Hutchinson (2003) the polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) constitute a class of multifunctional proteins that regulate complex enzymes activities. These enzymes represent some of the largest proteins known, a single protein can catalyze dozens of discrete biochemical reactions, and many of them have become important drugs.

Numerous cyanopeptides demonstrate bioactivities in tests using mammalian (or other vertebrate) cells and these are frequently similar to the effects observed in invertebrate animals, which are potential cyanobacteria feeders. In the interest of pharmaceutical applications of cyanopeptides, many of them have been studied and several cases of protease inhibitory activity were found. In terrestrial plants the protease inhibition is known to act as a grazing protection (Welker and von Döhren, *in press*).

Some cyanopeptides have further been shown to exert allelopathic effects which reduce the photosynthetic activity in other planktonic autotrophs, thus leading to the dominance of the cyanobacteria (von Elert & Jütter, 1997; Schagerl *et al.*, 2001; Suikkanen *et al.*, 2004, Welker and von Döhren, *in press*).

1.2.3.1 *Aeruginosin*

In general the aeruginosins are characterized by the presence of an octahydroindole carboxylic acid subunit, which is attached to other amine and carboxylic acid residues by peptidic linkages. The ensemble of the peripheral groups provides some of the requisite pharmacophoric sites for binding to the appropriate enzyme (Hanessian *et al.*, 2003).

They are linear tetrapeptides, which were first isolated from the toxic strain *Microcystis aeruginosa* NIES-298 (Murakami *et al.*, 1994), but are also present in different *Microcystis*, *Planktothrix* and *Anabaena* strains (Matsuda *et al.*, 1996, Shin *et al.*, 1997, Welker *et al.*, 2004). The 27 known variants of aeruginosin isolated from *Microcystis aeruginosa*, *Microcystis viridis* and *Planktothrix agardhii* are potent inhibitors of trypsin, thrombin, plasmin and serine protease (Murakami *et al.*, 1994, 1995; Shin *et al.*, 1997; Kodani *et al.*, 1998, Fukuta *et al.*, 2004; Hanessian *et al.*, 2003). They also have similarities to dysinosins, linear tetrapeptides from a dysideid sponge and to suomilide and bynyasides, peptides from *Nodularia* and *Nostoc*, respectively (Welker and von Döhren, *in press*).

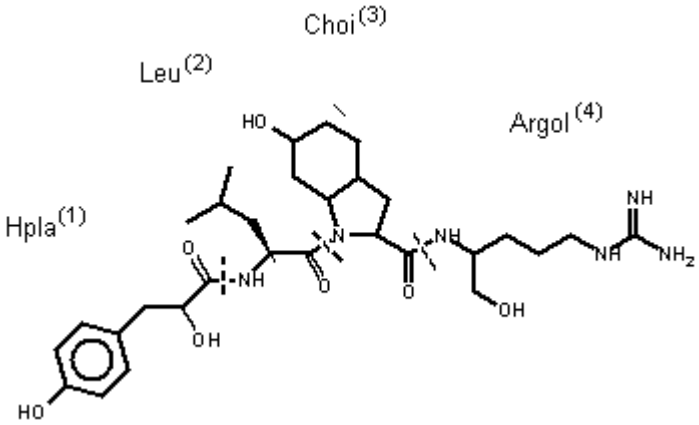


Figure 2: The aeruginosin 102A chemical structure (Fukuta *et al.*, 2004).

Figure 2 shows the chemical structure of the aeruginosin 102A, reported to have the highest activity within the aeruginosins, being a thrombin inhibitor (IC_{50} 0.04µg/ml) (Matsuda *et al.*, 1996). Mass spectral analyses of strains and bloom samples indicate a higher number of structural variants yet to be isolated and further elucidated.

1.2.3.2 *Anabaenopeptin*

Anabaenopeptins have been reported from cyanobacteria isolated from a variety of habitats: terrestrial, freshwater and brackish water and also from marine sponges (Welker and von Döhren, *in press*). Anabaenopeptins were first isolated from the cyanobacterium *Anabaena flos-aquae* NRC 525-17 and proved to be protease inhibitors (Shin *et al.*, 1997). Oscillamide Y is a cyclic peptide very similar to anabaenopeptin F and belongs to the anabaenopeptin type class.

Anabaenopeptin are cyclic peptides with 6 amino acids. Anabaenopeptides characteristically have a lysine in position 5 and the formation of the ring by an N-6-peptide bond between the ureido bond in position 6. The general structure of anabaenopeptins was shown to be cyclo [(X-α-NH-CO-α-NH)-Lys-Y-Hty-Z-Phe-ϵ-NH-(Lys)], here X,Y and Z may vary (Sano *et al.*, 2001) (Figure 3).

Different anabaenopeptin variants have been found in *Anabaena* sp. (Harada *et al.*, 1995, Repka *et al.*, 2004), *Planktothrix* sp. (Murakami *et al.*, 1997, Erhard *et al.*, 1999), *Microcystis* sp. (Williams *et al.*, 1996, Erhard *et al.*, 1997, Fastner *et al.*, 2001), *Nodularia* sp. (Fujii *et al.*, 1997) and *Aphanizomenon* sp. (Murakami *et al.*, 2000). Already 29 currently known anabaenopeptin variants have been elucidated from different strains of *Microcystis*, *Planktothrix* and *Anabaena* (Welker and von Döhren, *in press*).

Anabaenopeptins have different inhibiting properties, e.g. some cause relaxation of norepinepherine-induced contraction (Harada *et al.*, 1995), carboxypeptidase A inhibition (Shin *et al.*, 1997, Itou *et al.*, 1999, Murakami *et al.*, 2000), protein phosphatases 1 and 2A (Sano *et al.*, 2001) and chymotrypsin inhibition (Sano and Kaya, 1995).

The anabaenopeptins A and B cause vasodilatations to norepinephrine-induced constriction of rat aortic preparations and the variant oscillamide Y inhibits the chymotrypsin activities (Harada *et al.*, 1995, Namikoshi and Rinehart, 1996, Shin *et al.*, 1997, Bickel *et al.*, 2001). Anabaenopeptin F is a cyclic peptide having an unusual ureido bond and 7-Mhty or Hty residue and inhibitor of protein phosphates PP1 and PP2A (Shin *et al.*, 1997, Sano *et al.*, 2001) and to cyrboxypeptidase A (CPA) (Itou *et al.*, 1999). The oscillamide B

and C were isolated from *Planktothrix agardhii* and *Pl. rubescens* and show inhibitory activities against protein serine/threonine phosphatases PP1 and PP2A (Sano *et al.*, 2001).

In anabaenopeptin F, the X is arginine and considerable inhibitory activity against PP1 and PP2A was observed, but in the oscillamide Y tyrosine is present and chymotrypsin inhibition was observed (Sano and Kaya, 1995, Sano *et al.*, 2001).

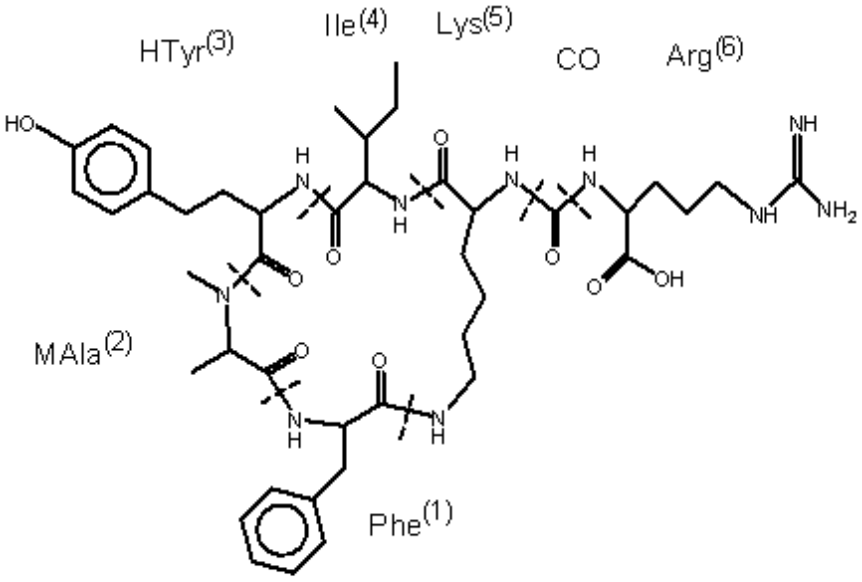


Figure 3: The chemical structure of an anabaenopeptin F (Shin *et al.*, 1997).

1.2.3.3 *Microviridin*

Microviridins are the largest known cyanobacterial oligopeptides. They are characterized by a structure arranged in one to three rings and two side chains of variable length (Rohrlack *et al.*, 2003, Welker and von Döhren, *in press*). They were first isolated from *Microcystis viridis* and *Microcystis aeruginosa* (Ishitsuka *et al.*, 1990, Okino *et al.*, 1995, Rohrlack *et al.*, 2004).

The general sequence of microviridins is Ac-Y-(Y*)-Y-Thr-Y-Lys-Tyr-Pro-Ser-Asp-X-Glu-Y-X in which X is always an aromatic amino acid residue and Y being variable molecule (Figure 4).

All amino acids in microviridins are in L-configuration and the only non-proteinogenic unit is the N-terminal acetic acid. This combination makes it possible that microviridins, different from the others cyanopeptides, may be synthesized ribosomally (Welker and von Döhren, *in press*).

Most variants show no bioactivity, but a recent isolated variant, microviridin J, is toxic to the planktonic crustacean *Daphnia* (Rohrlack *et al*, 2004).

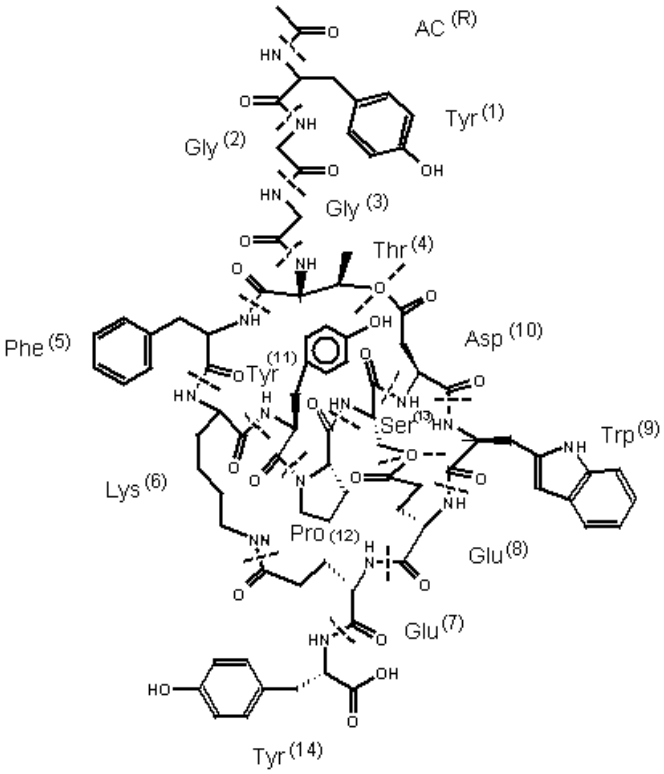


Figure 4: The chemical structure of a microviridin (Erhard *et al.*, 1999).

1.2.3.4 *Microginin*

Microginins are linear pentapeptides characterized by a decanoic acid derivative, 3-amino-2-hydroxy-decanoic acid (Ahda) and a predominance of two tyrosine units (Figure 5).

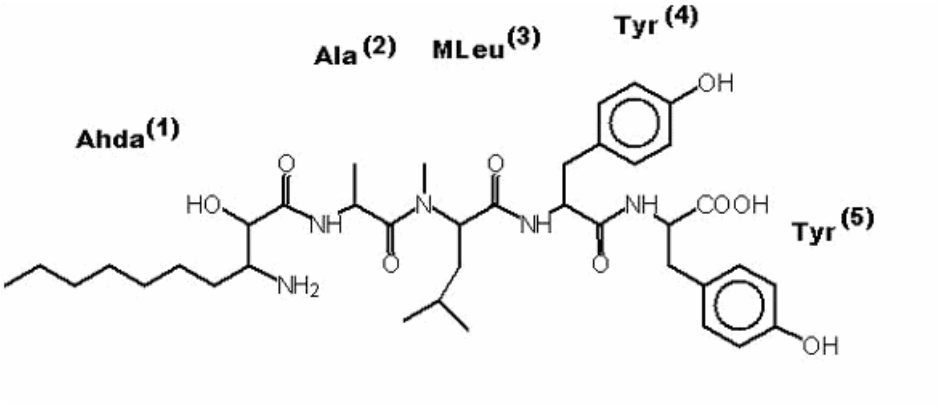


Figure 5: The chemical structure of a microginin (Okino *et al.*, 1993).

This peptide was originally isolated from *Microcystis aeruginosa* (Okino *et al.*, 1993) and until known approximately 30 structure variants were found in *Microcystis* sp. and *Planktothrix* sp. (Welker and von Döhren (*in press*).

The different variants of microginins are angiotensin-converting enzyme (ACE), aminopeptidase (APM) and leucine aminopeptidase (LAP) inhibitors (Okino *et al.*, 1993, Ishida *et al.*, 1997, 1998, 2000, Neumann *et al.*, 1997, Kodani *et al.*, 1999).

1.3 Objectives

The objectives of the work presented here include the following:

- Description and characterization of unknown peptides
  - Isolation and structural elucidation of unknown cyanopeptides from two strains
  - Production of these cyanopeptides for testing their bioactivity
- Assessing the influence of different growth conditions, i.e. temperature, light and nutrient composition on cyanopeptide production by clarifying the following questions:
  - Do strains react differently to these factors when growing at maximal rate, i.e. in the exponential phase as compared to minimal growth rate, i.e. in the stationary phase?
  - How do the different conditions affect total cyanopeptide production?
  - Do they affect intra and extracellular cyanopeptide concentration differently?

These objectives will test the following working hypothesis:

Regulation of all cyanopeptides is similar and follows the pattern already described for microcystins, i.e. the environmental conditions and growth phase have fairly little influence and differences in the ratio of peptide per cell are in the range of not more than a factor of 2-3 (Hesse and Kohl, 2001; Böttcher *et al*., 2001).

2 Material and Methods

2.1 Cyanobacterial strains

For the isolation and production of the unknown peptides two *Aphanizomenon flos-aquae* (X008a and X0023) strains were used, kindly provided by Dr. Martin Welker from Technische Universität Berlin (TUB).

For the experiments regarding the influence of different growth conditions on cyanopeptide production the following strains were chosen:

- two Brazilian *Microcystin* spp. strains (RST9501 and NPRG-2), generously provided by Dr. João Sarkis Yunes from Rio Grande Federal University (FURG) and Dr. Sandra Azevedo from Rio de Janeiro Federal University (UFRJ) respectively,

- one German *Microcystis* spp. strain (BM 10), kindly provided by Dr. Barbara Meyer (Max Planck Institut für Limnologie, Plön) ,

- the above mentioned German *Aphanizomenon flos-aquae* (X008a), which was also used for the isolation of the unknown and production.

2.2 Isolation and analyses of peptides

2.2.1 Determination of the peptide profiles of culture strains

To investigate the peptide composition of all strains (*Aphanizomenon flos-aquae* X008a and X0023, *Microcystis* RST9501, BM 10 and NPRG-2), culture material was filtered and/ or lyophilized.

From each one of the strains a 10 ml culture was filtered (Schleicher & Schuell, RC 55 membrane filter regenerated cellulose, 0.45 µm) or the cells were lyophilized (Fa. Christ., Germany), then frozen and stored at -20℃. The filters and the dried cell material were extracted as described in Fastner *et al.* (1999) with 1.5 ml 75% MeOH, afterwards sonicated for 10 min (Bandelin Sonorex super RK255H), shaken for 30 min (Edmund Bühler Swip KL-2) and

centrifuged for 10 min at 13 000 rpm (Biofuge vito, Heraeus, Germany). This procedure was repeated three times. The supernatants after centrifugation were pooled and either blown to dryness with nitrogen or dried by vacuum centrifugation (Concentrator 5301, Eppendorf, Germany).

2.2.2 Peptide isolation for preliminary structure elucidation

The supernatants were resuspended in 50% aqueous MeOH and analyzed by HPLC-photodiode-array detection (High Performance Liquid Chromatography) with a Waters 616 solvent delivery system, a 717 WISP auto sampler and a 991 photo diode array detector (Waters, Germany). Extracts were separated on a LiChrospher® 100, ODS, 5 µm, LiChroCART® 250-4 cartridge system (Merck, Germany) using a gradient of aqueous acetonitrile (with 0.05% TFA) according to Lawton *et al.* (1994) at a flow rate of 1 ml/min. All chemicals were of chromatographic grade (Merck, Germany). UV-spectra were recorded from 200-300 nm.

Peaks showing absorption at 214 nm indicating peptide bonds as well as peaks showing the characteristic spectra of microcystins were collected by hand, dried and stored frozen until MALDI-TOF MS analysis (Matrix Assisted Laser Desorptio/Ionisation – Time of Flight).

2.2.3 Peptide identification

The dried isolated peaks were dissolved in 50% aqueous methanol, sonicated for 10 min (Bandelin Sonorex super RK255H) and left for 15 min. From this solution 1 µl was mixed with 1 µl of matrix (10 mg of 0.5-dihydrobenzoic acid per ml in water-acetonitrile [1:1] with 0.03% TFA). Positive ion mass spectra were recorded, by a MALDI-TOF mass spectrometer (Voyager DE-PRO; PerSeptive BioSystems, Framingham, Mass.) equipped with a reflectron (Fastner *et al.*, 2001).

In this MALDI-TOF technique, biomolecules are ionized, transformed in gas, accelerated in a magnet field, from which they enter a detector. The molecule mass is determined according to their *time of flight*. The lighter

molecules arrive at the detector faster than the heavier molecules. The advantage of this method is that the amount of sample needed is very small, some µg of raw material, ng scale for pure compounds. Up to 400 samples can be measured using one template.

The mass signals of unknown compounds with sufficient intensities were further analyzed by recording post-source decay fragment spectra. Fragment patterns were compared to a database containing fragment patterns of known and partly characterized cyanobacterial peptides (Welker *et al.*, 2004).

The cyanopeptides, for which the structures have been already elucidated, were used for the quantitative experiments. Since standards for all of the investigated peptides were not available, quantification of the peptides was not possible. For comparison of peptide concentration per cell, the relative change of the peptide concentrations was determinate by using the detected peptide peak area at 214 nm in relation to the measured sampled biovolume (peak area/mm³ biovolume). These experiments are described in section 2.3.

Peptides for which fragment patterns describe them as a peptide, but which are not available in the database, were considered as unknown cyanopeptides. The unknown cyanopeptides were only found in the *Aphanizomenon* strains. In order to describe and elucidate their structures, several steps were necessary and they are described in the following sections.

2.2.4 Mass cultivation for the isolation of the unknown cyanopeptides

Cyanobacteria mass cultivation was performed for *Aphanizomenon* X008a and X0023, since there was a needed for the isolation of the unknown peptides, in mg quantities, for structure elucidation and bioactivity tests. The inoculua were added to a 100 ml medium (Table 5) in Erlenmeyer flask and grown for a week. These cultures were then transferred to a 5 Liter balloon, supplied with new medium and continuous aeration. After some days, the culture was again transferred to a 20 Liters balloon with new medium, supplied with continuous aeration. Upon reaching the ends of the exponential

growth phase, these mass cultures were harvested by flow-through centrifugation and the cells were lyophilized (Fa. Christ., Germany).

Table 5: The medium used for the mass cultivation from *Aphanizomenon* (according to Martin Welker, *pers. comm.*)

Macronutrient		**g/300 ml Stock Solution**	**ml Stock Solution in 1 l Medium**
$NaNO_3$		50,4	5,0
K_2HPO_4		10,4	5,0
$MgSO_4 \times 7H_2O$		51,8	1,0
$CaCl_2 \times 2H_2O$		8,8	1,0
Na_2CO_3	Autoclave separately	6,0	1,0
Na_4EDTA		11,4	1,0
Citric acid	Combine in 300 ml	5,8	1,0
$FeCl_3 \times 6H_2O$		1,6	1,0
Trace elements (after Gaffon)		**mg/l Stock Solution**	
H_3BO_4		2677	1,0
$MnSO_4 \times H_2O$		1546	
$ZnCl_2$		129	
$Na_2MoO_4 \times 2H_2O$		389	
$CuSO_4 \times 6H_20$		80,2	
$CoCl_2 \times 6H_20$		40,9	
$Na_2SeO_3 \times 5H_2O$		34,9	
$NiCl_2$		12,96	
NH_4VO_3		1,80	
$Na_2WO_4 \times 2H_2O$		4,95	
Vitamins	0,2 µm filtrated	**mg/l Stock Solution**	1,0
Thiamine HCl		100	
Cyanocobalamin (B_{12})b		0,5	
biotin		0,5	

2.2.5 Peptide isolation

2.2.5.1 Extraction

Around 30 Liters of pure *Aphanizomenon* culture were lyophilized, as described in section 2.2.2, and 2 g of dried cell material was obtained.

One g of this dried cell material was extracted with 10 ml of 75% aqueous MeOH, afterwards shaken for 30 min (Edmund Bühler Swip KL-2, Germany) and centrifuged for 20 min at 11 000 rpm (Biofuge stratos, Heraeus, Germany). After the centrifugation, the supernatants were pooled and separated.

2.2.5.2 Purification

The supernatants were diluted in Milli-Q water until reaching a concentration of only 10% aqueous MeOH in the sample. They were pre-cleaned using a Solid phase extraction, using a C_{18} cartridge (Waters® Sek-Pak 10 g). The C18 cartridge was activated with 100 ml of 100% MeOH and 100 ml of 100% Milli-Q water and loaded with the sample (dried material + 10% 75% aqueous MeOH). Afterwards the cartridge was washed with 100 ml of 100% Milli-Q water. The elute was obtained using 100 ml of 90% aqueous MeOH and blown to dryness with nitrogen.

2.2.5.3 Semi-preparative HPLC

The dried elute was resuspended in 300µl acetonitrile and sonicated for 10 min (Bandelin Sonorex super RK255H), afterwards 300 µl Milli-Q water was added and centrifuged for 10 min at 13 000 rpm (Biofuge vito, Heraeus, Germany).

The 50% aqueous MeOH sample was injected in a HPLC-photodiode-array detection with a Waters 616 solvent delivery system, a 717 WISP auto sampler and a 991 photo diode array detector (Waters, Germany). Extracts were separated on a Bondapak® 100, ODS, 10 µm, Waters® 125 Å cartridge system (Waters, Germany). The mobile phase were Milli-Q water (solvent A) and acetonitrile (solvent B), both with 0.05% TFA. The cyanopeptides were separated an isocratic run with 30 to 35% of solvent B in 20 min at a flow rate of 3 ml/min. Absortion was done at 214 nm.

The desired fractions were collected in a fraction collector (Waters) for several runs. These isolated fractions were dried by vacuum centrifugation (Concentrator 5301, Eppendorf, Germany).

2.2.5.4 Analytical HPLC

The dried isolated fractions were treated as described in section 2.2.5.2, but with one difference. The fractions were separated on a LiChrospher® 100,

ODS, 5 µm, LiChroCART® 250-4 cartridge system (Merck, Germany) using Milli-Q water (solvent A) and acetonitrile (solvent B) was mobile phase (with 0.05% TFA). This last step on the peptide cleaning was done an isocratic run with 30% to 35% of solvent B in 20 min at a flow rate of 1 ml/min. Absortion was done at 214 nm.

These were again collected using a fraction collector (Waters), dried by vacuum centrifugation (Concentrator 5301, Eppendorf, Germany) and lyophilized.

These lyophilized peptide fractions were resuspended with 1 ml 100% MeOH and diluted in 10 ml Milli-Q water. To avoid any TFA and acetonitrile, they were once again cleaned using a C_{18} cartridge.

The 5 g C_{18} cartridge was activated with 100 ml of 100% MeOH and 100 ml of 100% Milli-Q water and loaded with the sample (dried material + 10ml Milli-Q water). Afterwards the cartridge was washed with 300 ml of 100% Milli-Q water. The elute was obtained using 100 ml of 90% aqueous MeOH, blown to dryness with nitrogen and stored frozen.

2.2.6 Structure elucidation of the unknown peptides

As described in section 2.2.1, after the preliminary structure elucidation through the HPLC and further MALDI-TOF MS analysis to confirm the peptide mass and the purity of the fraction, the fragment patterns which did not match any known cyanopeptides were selected for further investigation.

After the unknown peptides isolation and purification, as described in section 2.2.5, the chemical structures of these cyanopeptides were elucidated by the firma AnalytiCon Discovery using two-dimensional - nuclear magnetic resonance spectroscopy (2D-NMR).

Nuclear magnetic resonance spectroscopy is the use of the NMR phenomenon to study physical, chemical, and biological properties of matter. Nuclear magnetic resonance spectroscopy finds application in several areas of science and has been routinely used by chemists to study chemical structure (Hornak, J.P, 2004).

2.2.7 Bioactivity tests

Meanwhile these cyanopeptides fractions were tested for bioactivity. These were performed by Dr. Stephan Höger and Prof. Dan Dietrich at the University of Constance, Germany and Dr. Kirsten Christoffersen at the University of Copenhagen, Denmark in the context of collaboration in the EU-Project PEPCY (PEPtides in CYanobacteria).

At the University of Constance, Germany, three different kinds of bioactivity were tested: the cytotoxicity test with intestinal and/or hepatic cell lines; the Apoptosis assays and the Ames mutagenicity assay.

At the University of Copenhagen *in vitroo* experiments were performed with *Daphnia magna*, which are among the planktonic cyanobacteria grazers.

The cytotoxicity test uses liver cell, also known HEP-G2 Human cell line assay to examine the release of lactate dehydrogenase (LDH). And another cytotoxicity test with hepatic cell lines, commonly known as CACO-2 cell line assay, to examine the reduction of 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).

LDH is released to the medium by damaged cells, and increased LDH-levels thus indicate potential damage to the plasma membrane and thus cytotoxicity. The LDH catalyses the NAD^+ - dependent reaction from lactate to pyruvate. In order for this reaction to be measured photometrically the enzyme diaphorase transfers H/H+ from NADH/H+ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolim chloride) which is reduced to formazan (red color).

MTT enters the cells via diffusion and is reduced to an alcohol soluble dark blue formazan (Mosmann, 1983), for which cell membranes are largely impermeable. This reaction is catalyzed by NADH-dependent enzymes of the endoplasmatic reticulum and – to a lesser extent – by mitochondrial succinate dehydrogenase. Solubilisation of cells by addition of a detergent results in the release of the solubilised formazan. The number of surviving cells is directly proportional to the concentration of formazan (Höger, S.J. and Dietrich, D.R., 2005).

Both assays were done using Human cell lines HEP-G2 (DSM ACC 180) and CACO-2 (DSM ACC 169) provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The cells were grown in culture flasks with RPMI (PAA Laboratories, E15-840) (10% Fetal bovine serum (FBS); PAA Laboratories, A15-649) as culture medium at 37℃. At the exponential growth phase the cultured cell densities were determined and cells were seeded with a density of 2-3 x 10^5 cells/ml in RPMI (10% FBS) in 96 well plates (200 µl/well). After 3-4 h the medium was decanted and 200 µl RPMI with 1% (FBS) per well were added.

The solution to be tested was prepared in 10% MeOH, mixing 405 µl RPMI (1% FBS) with 45 µl of the test substance (1:10) followed by sterile filtration.

In the first row of the 96 well plates a negative control (culture cells with RPMI medium) was added, in the second row 100 µl of the test substance was added, and further serial dilutions followed. As a positive control, RPMI (1% FBS) with 1% Triton X-100 was added. Then the plate was incubated for the desired period of time (see below).

For the LDH assay a Cytotoxicity Detection Kit (Cat. No. 1644793, Roche) was used. After 20, 48 or 72 h in the incubator 150 µl of the medium was transferred into rounded bottom 96 well plates and centrifuged for 10 min at 250 x g. Bottle 1 and bottle 2 of the LDH kit were mixed 1:45. Hundred µl from the centrifuged round bottom plates were transferred to flat-bottom 96 well plates. 100 µl of LDH kit mix were added to each well, vortex and the plate was stored in a dark place. The plate was measured at 490 nm against a reference wavelength of 750 nm after 9 min.

For the MTT assay a MTT 5 mg/ml PBS (Sigma-Aldrich, Art.-Nr.: M-2128) was used. Fifty µl of RPMI (1% FBS) were added to the incubated cells after that 10 µl of the MTT solution was added and the plate was incubated for 1.5 h at 37℃. After this period, the supernatant was r emoved, 100 µl of the solubilisation buffer (formic acid 5% (vol/vol), isopropanol 95% (vol/vol).was added, mixed in a vortex for 10 min and measured at 550 nm.

Apoptosis is one of the main types of programmed cell death. Contrary to necrosis, which is a form of cell death that results from acute cellular injury,

apoptosis is carried out in an ordered process. Apoptosis can occur, for instance, when a cell is damaged beyond repair, or infected. If a cell's capability of apoptosis is damaged, or if the initiation of apoptosis is blocked, a damaged cell can continue dividing without restrictions, developing into cancer.

Apoptosis is one mechanism used by cells and tissues in response to various toxins; it is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, plasma membrane blebbing, oligonucleosomal DNA fragmentation and finally the breakdown of the cell into smaller units (apoptotic bodies).

One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, a 35-36 kDa, Ca^{2+}-dependent, phospholipid binding protein with a high affinity for PS. To detect apoptotic cells the Annexin-V-fluorescent binding assay (Roche) was used.

Because PS translocation also occurs during necrosis, Annexin V is not an absolute marker of apoptosis. Therefore it is often used in conjunction with vital dyes such as 7-amino-actinomysin (7-AAD) or propidium iodide (PI), which binds to nucleic acids but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis.

For the vital dyes, cells were then re-suspended in 200 ml binding buffer (0.1M Hepes, pH 7.4, 14M NaCl, 25mM $CaCl_2$) at a concentration of $20x10^6$. 7-amino-actinomycin D (7-AAD) subsequently added to the cell solution. Cells were then incubated in the dark for 30 min. At room temperature and diluted with 400 µl of binding buffer for flow cytometric analysis. The analysis of 10,000 cells in each group was performed on a FACS Calibur (Becton Dickinson, USA) equipped with a single laser emitting excitation light at 488 nm (Chen *et al.*, 2005a).

Cells that are negative for both Annexin V and the vital dye have no indications of apoptosis: PS translocation has not occurred and the plasma membrane is still intact.

The Ames test, named after its developer Bruce Ames, is used to determine if a chemical is a mutant. This test is based on the assumption that any substance that is mutagenic may also turn out to be a carcinogen. This is an easy and low cost test for screening environmental substances for possible carcinogenicity even though some substances that cause cancer in laboratory animals do not give a positive result in the Ames test.

The bacterium used in this test is a mutant strain of *Salmonella typhimurium* which is unable to synthesize the amino acid histidine (*His*) resulting in *his*-organisms that cannot grow unless histine is supplied. When a mutagenic event occurs base substitutions within the *His* gene cause the reversion of the *his*-Ames II™ strains to *His*. A chemical's mutagenic potential is assessed by exposing these *his*-organisms to varying concentrations of chemicals and selecting for the reversion event.

Cell line cytotoxicity does not necessarily reflect whole animal toxicity because *in vitro* systems reflect only a small part of the mechanisms regulating toxicity highly integrated organism functions (Ferro and Doyle, 2001). However, studies with a large variety of reference chemicals have shown that *in vitro* cytotoxicity results usually correlate with rodent toxicity or human lethal doses (Ekwall, 1999; Evans *et al.*, 2001). It was suggested that the most accurate approach to determining toxicity would be an integrated use of *in vitro* and *in vivo* studies (Ferro and Doyle, 2001). *In vitro* cytotoxicity assays are a good starting point for toxicity studies because they enable the screening of a large number of samples and because the results of cytotoxicity assays help in the estimation of doses used in animal studies and in that way reduce the number of animals used in whole animal toxicity assays.

Determination of the *in vitro* cytotoxicity was carried out with enzyme inhibition assays (Rohrlack *et al.*, 2003) with a serine protease origination from *Daphnia*, a cladoceran genus playing a key role in freshwater pelagic food webs (Lampert and Sommer, 1997). Von Elert *et al.* (2004) recently showed

evidence for the presence of trypsin, an important proteolytic enzyme in the digestive physiology of *Daphnia*.

The enzyme inhibition assays with *Daphnia* extract and porcine trypsin where run as described by Rohrlack *et al.* (2003). To obtain the *Daphnia* extract, a *Daphnia magna* clone, isolated from Lake Langedam (Denmark), was maintained as mass culture (3 l) on synthetic zooplankton medium (Klüttgen *et al.*, 1994). The daphnids were fed daily with *Scenedesmus acutus* (1 mg C/l) as sole food. The temperature was kept at 19℃. The trypsin-like proteases were extracted as described by Hasler (1935). Circa 300 adults were dehydrated in 40 ml acetone, washed with 40 ml acetone and thereafter washed four times with 40 ml *n*-hexane, and dried in constant flow of air. Dried daphnids were homogenized and dissolved in 15 ml 50% (vol/vol) glycerol. After 24 h at 7℃, the extract was centrifuged (10, 000 x *g*, 10 min). The supernatant, containing the required proteases, was collected and stored at -20℃ until further use. The total protein concentra tion (1.42 g/l protein) was determined according to the methods of Bradford (1976) and Zor and Selinger (1996). The protein concentration used in the assays (0.5 g/l) was obtained by dilution of the crude extract with 50% (vol/vol) glycerol.

The enzyme inhibition assays were conducted with *Daphnia* extract and with porcine trypsin (Sigma-Aldrich, Trypsin type II-S). The activity was measured with 1 mM aqueous solution of N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as trypsin-specific substrate (Erlanger *et al.*, 1961) and 225 mg/l porcine trypsin or 0.5 g/l *Daphnia* extract, both dissolved in 50% (vol/vol) glycerol. The activity of the enzymes was measured by mixing (1) 1 ml 100 mM Tris-HCl (pH 7.2), (2) 100 µl enzyme solution and (3) a defined amount of the cyanobacterial peptide (1, 5 10 or 20 µl) dissolved in 100 µl 50% (vol/vol) MeOH. Control measurements were carried out with 100 µl 50% (vol/vol) MeOH without cyanobacterial peptide. The reaction components were carefully mixed in disposable cuvettes, and incubated for 30 min at 23℃. To initiate the enzymatic reactions, 300 µl 1mM BAPNA was added directly to the cuvettes. The production of *p*-nitroanilide was monitored photometrically at 395 nm for 10 min, and the inhibitory effect of the cyanobacterial peptide on the enzyme activity was determined by linear regression as *p*-nitroanilide production rate. The system was calibrated using pure *p*-nitroanilide. Based

on the measurements with different peptide concentrations, the concentration which caused 50% inhibition of the enzyme activity (IC_{50}) was determined.

2.3 Culture experiments

2.3.1 Culture conditions

The experiments with the two Brazilian *Microcystis*, RST9501 and NPRG-2, one German *Microcystis*, BM 10, and one German *Aphanizomenon*, X008a, strains to study the influence of different growth conditions on cyanopeptide production were done in batch cultures. In these, cell physiology changes continuously over time. Following Long (2001), the extremes of toxin concentration will occur in the exponential phase when growth is maximal (in the case of microcystin the concentration was highest in this phase), and in the stationary phase when growth is close to zero, as these two conditions characterize the extremes of the physiological states. Thus, to characterize the range of variation of the concentration of a given peptide, sampling of batch cultures should focus on these two phases. It is not necessary to sample at regular intervals, e.g. every other day, as is done in most batch culture study designs, as the information gleaned between the exponential and stationary phase is at best spurious, and as growth conditions are not clearly defined.

Growth conditions chosen for testing in batch cultures were temperature, light, nitrogen and phosphorus limitation. Two different temperatures, 20℃ and 25℃ were tested, and at each temperature the cultures were grown under two light intensities, 20 and 60 $\mu Em^{-2}s^{-1}$. The medium used was the ASM-1 according to Gorham *et al.* (1964), see table 6.

Table 6: ASM-1 medium composition according to Gorham *et al.* (1964).

Stock Solution A	g/1000 ml
$NaNO_3$	8,5
$MgCl_2.6H_2O$	2,05
$CaCl_2.2H_2O$	1,45
$MgSO_4.7H_2O$	2,45
$CaCl_2$	1,1
Stock Solution B	
$K_2H_2PO_4.3H_2O$	8,7
$NaH_2PO_4.7H_2O$	12,3
Stock Solution C	
H_3BO_3	24,8
$MnCl_2.4H_2O$	13,9
$FeCl_3.6H_2O$	10,8
$ZnSO_4.7H_2O$	7,07
$CoCl_2.6H_2O$	0,19
$CuCl.2H_2O$	0,013
Stock Solution D	
$EDTA.Na_2$	18,6

For each ASM-1 liter add 20 ml Stock Solution A, 2 ml Stock Solution B, 0.1 ml Stock Solution C and 0.4 ml Stock Solution D.

For each temperature and light intensity the cultures were grown in four different medium compositions:

- one the full ASM-1 medium described above (indicated as ASM-1),
- ASM-1 free of nitrogen (indicated as N^-),
- ASM-1 free of phosphorus (indicated as P^-), and
- ASM-1 free of nitrogen and phosphorus (indicated as NP^-).

For the culture experiments, a pre-culture was grown for a week in full (ASM-1) medium at the temperature and light intensity to be tested. This pre-culture was diluted to the initial cell density with the full medium (ASM-1). The inocula for the N^-, P^- and NP^- experiments were washed, suspended in medium free of the nutrient to be tested and only then diluted to the initial cell density. All experiments were done in triplicates.

The experiments were started with the same concentration of biomass, measured as optical density (OD) at 750 nm with a photometer (Hitachi model U 2000). The triplicate cultures were grown in a 12:12 hours light:dark cycle. Table 7 describes the structure of the batch experiments.

Table 7: The structure of the experiments done with three *Microcystis* strains, RST9501, NPRG-2, BM 10 and one *Aphanizomenon* strain, X008a.

Medium	20℃		25℃	
	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$
ASM-1	triplicate	triplicate	triplicate	triplicate
N^-	triplicate	triplicate	triplicate	triplicate
P^-	triplicate	triplicate	triplicate	triplicate
NP^-	triplicate	triplicate	triplicate	triplicate

2.3.2 Sampling

The batch cultures' growth was observed by daily measurements of the optical density (OD) at 750 nm with the photometer (Hitachi model U 2000).

Samples for peptide analysis and biovolume determination were taken on two occasions during culture growth, at the beginning of the exponential growth phase and at the beginning of the stationary growth phase. At each occasion, cultures were sampled twice on subsequent days.

For each sample, 10 ml of each culture was filtered through a membrane filter (Schleicher & Schuell RC 55 regenerated cellulose membranes 0.45 µm) for the intracellular peptide analysis and the filtrate used for the extracellular peptide analysis.

Simultaneously, 1 ml from each culture was taken and fixed with Lugol' solution as sample to determine the biovolume. The biovolumes were determinated using a cell counter (Casy, Schärfer, Germany) for the *Microcystis* strains and determined by measurements of the filament length and width, according to Utermöhl (1958), using an inverted microscope (Zeiss, MC80, Germany) for the *Aphanizomenon* strains.

2.3.2.1 Determination of intra and extracellular peptide concentration

For the determination of the intracellular peptide concentration, the filters were extracted as described in Fastner *et al.* (1999), and as explained in section 2.2.1.

For the determination of the extracellular peptide concentrations, the filtrates were concentrated on a C_{18} cartridge (Waters Sep-Pak 500 mg). The C_{18} cartridges were activated with 10 ml of 100% MeOH followed by 10 ml of 100% Milli-Q water, afterwards 10 ml of the sample filtrate and finally washed with 10 ml of 100% Milli-Q water. The elute was obtained using 90% aqueous MeOH and blown to dryness with nitrogen.

As for the intracellular, the extracellular peptide concentration were resolved in 50% aqueous methanol and analyzed using a HPLC-photodiode-array, as described in section 2.2.2.

2.4 Statistic methods

Data was analyzed using the procedure of analysis of variance (ANOVA/MANOVA) with the statistical software STATISTICA. The ANOVA (analysis of variance) has the purpose of testing the significant differences between means.

The multivariate analysis of variance (MANOVA) is an ANOVA with several dependent variables. One advantage over ANOVA is the measurement of several variables in a single experiment, increasing the chance of discovering which factor is truly important (StatSoft, Inc., 2006). As the test results F_{ratios} are given, but their significance tells only that the aggregate difference among the means of the several samples is significantly greater than zero. The result does not tell whether any particular sample mean significantly differs from any particular one.

To find out significant differences between any samples mean from another, a post-hoc test should be done. The “Honestly Significantly Difference” (HSD) test proposed by the statistician John Tukey can be used to

determine the significant differences between group means, or to test the individual test condition against each other (StatSoft, Inc., 2006).

For the *Daphnia magna* assay, all IC_{50} value calculations and regression analyses were performed using the Microsoft Excel 2003. All statistical tests or calculations were performed at the 95% level of significance.

3 Results and Discussion

3.1 Description of the new peptides

The following part of this work was done in cooperation with the firma AnalytiCon Discovery (Dr. Jakupovic and Dr. Karsten Siems), responsible for the peptides elucidation and with the University of Constance (Dr. Daniel Dietrich and Dr. Stefan Höger) and the University of Copenhagen (Dr. Kirsten Cristoffersen and Dr. Gabi Mulderij) responsible for the bioassays.

3.1.1 Identification of the newly isolated peptides

The lyophilized material obtained from the mass cultivation from the *Aphanizomenon* strains (X008a and X0023), as described in section 2.2.4, were isolated and then purified, according to section 2.2.5.2.

After this purification, a pre-cleaning process, the peptide fractions were purified using a semi-preparative HPLC, in an isocratic run with 30 to 35% of acetonitril in 20 min at a flow rate of 3 ml/min, as described in section 2.2.5.3 (Figure 6 and 7).

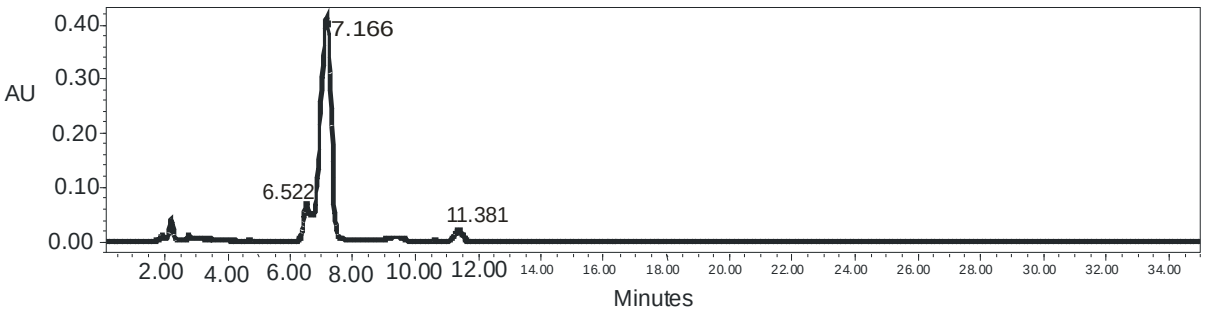


Figure 6: Peptide profile from the *Aphanizomenon* X008a obtained by the semi-preparative HPLC. A 50% aqueous MeOH sample was injected and the extract separated on a Bondapak®110, ODS, 10 µm Waters®. The mobile pphase were Milli-Q water (solvent A) and acetonitril (solvent B), both with 0.05% TFA. The gradient was 30% to 35% of solvent B ata flow rate of 3 ml/min. Absorption at 214 nm.

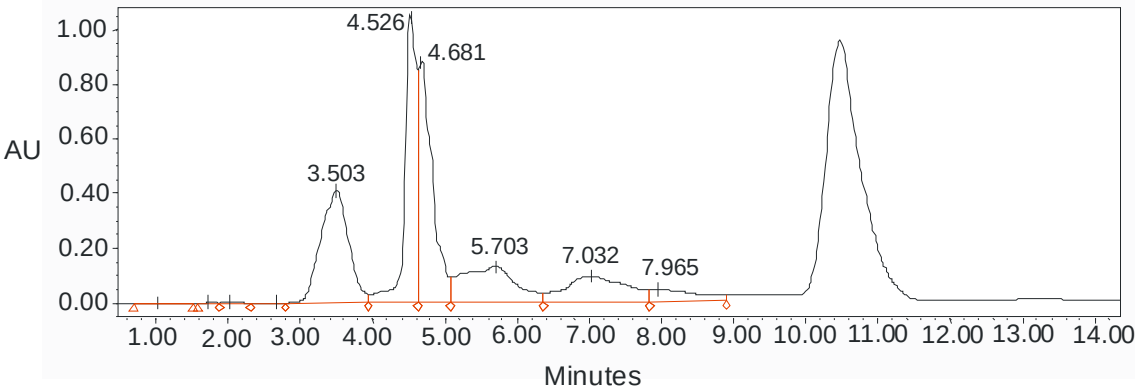


Figure 7: Peptide profile from the *Aphanizomenon* X0023 obtained by the semi-preparative HPLC. A 50% aqueous MeOH sample was injected and the extract separated on a Bondapak®110, ODS, 10 µm Waters®. The mobile pphase were Milli-Q water (solvent A) and acetonitril (solvent B), both with 0.05% TFA. The gradient was 30% to 35% of solvent B ata flow rate of 3 ml/min. Absorption at 214 nm.

For the final purification process an analytical HPLC was used. The fractions cleaned by the semi-preparative HPLC were in 50% aqueous MeOH separated on a LiChrospher® 100, ODS, 5 µm LiChroCART® 250-4 using Milli-Q water and acetonitril as mobile phase. This final cleaning was done in an isocratic run with 30% to 35% of acetonitril in 20 min at a low rate of 1 ml/min as described in section 2.2.5.4 (Figure 8 and 9)

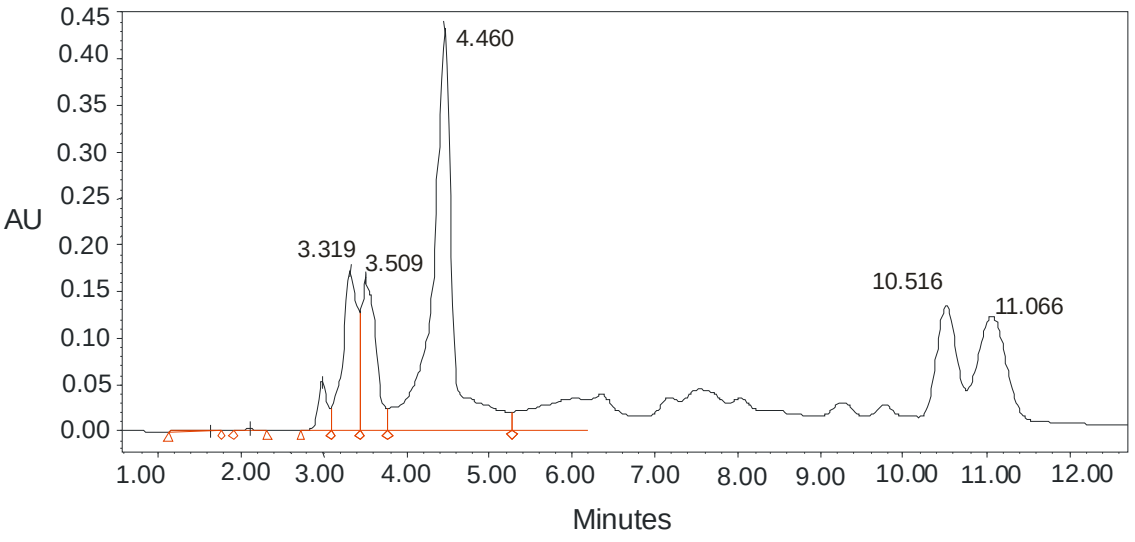


Figure 8: Peptide profile from the *Aphanizomenon* X008a obtained by the analytical HPLC. A 50% aqueous MeOH sample was injected and the extract separated on a LiChrospher® 100, ODS, 5 µm, LiChroCART® 250-4.The mobile pphase were Milli-Q water (solvent A) and acetonitril (solvent B), both with 0.05% TFA. The gradient was 30% to 35% of solvent B ata flow rate of 3 ml/min. Absorption at 214 nm.

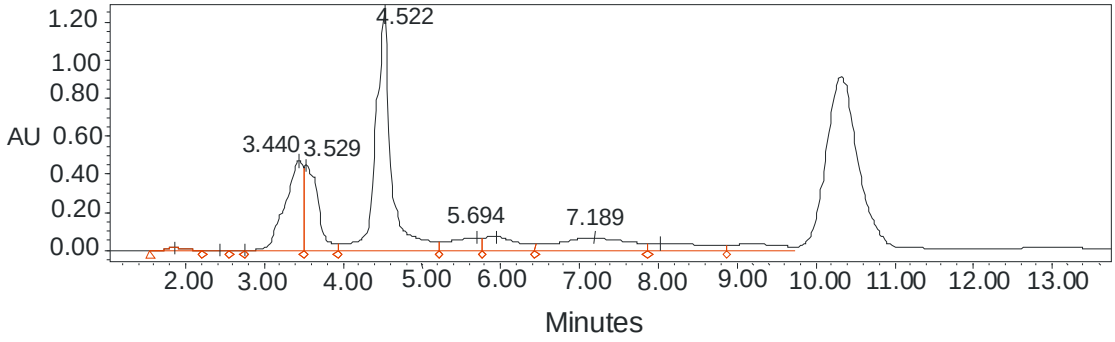


Figure 9: Peptide profile from the *Aphanizomenon* X0023 obtained by the analytical HPLC. A 50% aqueous MeOH sample was injected and the extract separated on a LiChrospher® 100, ODS, 5 µm, LiChroCART® 250-4.The mobile pphase were Milli-Q water (solvent A) and acetonitril (solvent B), both with 0.05% TFA. The gradient was 30% to 35% of solvent B ata flow rate of 3 ml/min. Absorption at 214 nm.

A MALDI-TOF MS analysis of the peptide factions were done to confirm the peptide mass and the purity of the fraction. The PSD spectra show the fragment patterns for the new cyanopeptides (Figure 10 and 11 and Table 8 and 9).

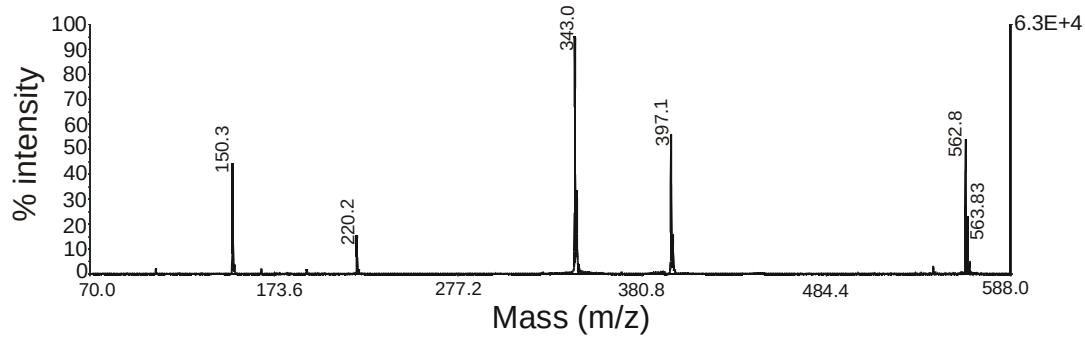


Figure 10: The PSD spectrum of the mass signal $M+H^+$ = 562, produced by the *Aphanizomenon* X008a.

Table 8: MALDI-TOF MS fragment patterns from M+H+=562, produced by *Aphanizomenon* X008a.

Sequence	m/z
Htyr+Htyr+Htyr+H	562
Htyr+Htyr+2CH_2O+2Na	397
Htyr+Htyr	343
Htyr+2H+CH_2O+Na+H	220
Htyr+2H	166
Htyr	150
Phe-Immoniumion	120
Eth+H	44

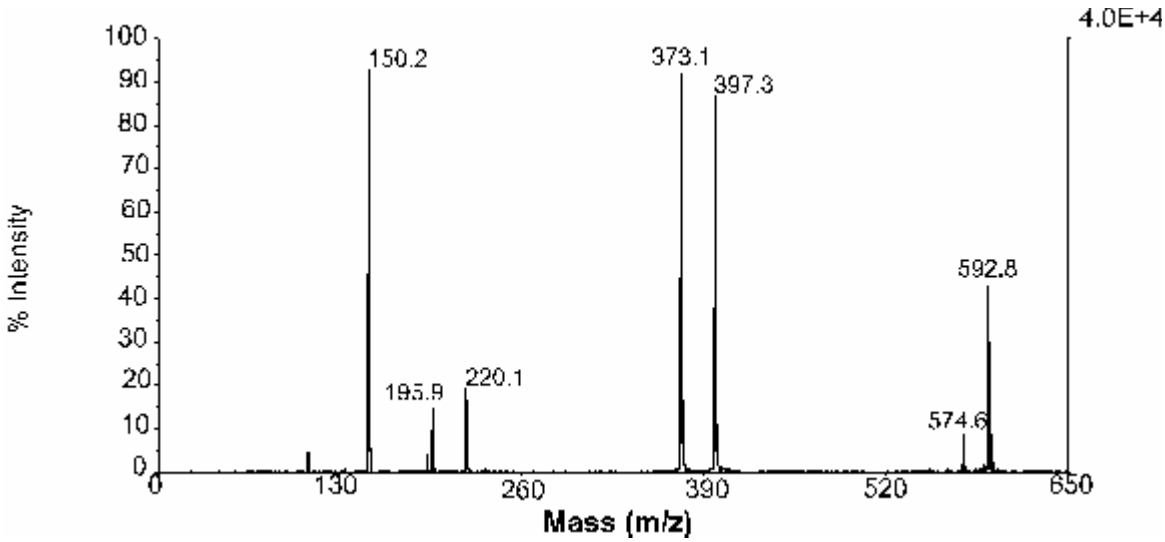


Figure 11: The PSD spectrum of the mass signal $M+H^+$ = 592, produced by the *Aphanizomenon* X0023.

Table 9: MALDI-TOF MS fragment patterns from M+H+=592, produced by *Aphanizomenon* X0023.

Sequence	m/z
Htyr+Htyr+Phe+H	592
Htyr+Phe+2CH_2O	397
Htyr+Phe	373
Htyr	150

The firma AnalytiCon Discovery elucidated the chemical structure of these fractions using a two-dimendional-nuclear magnetic resonance spectroscopy (2D-NMR). The results proposed the structures shown in Figure 12, 13, 14 and 15.

From the *Aphanizomenon* X008a, this new peptide was named aphapeptin F1 and the chemical formula is $C_{19}H_{22}O_4$ (Figure 12 and 13). From the *Aphanizomenon* X0023, the new peptide was named aphapeptin F2 with a chemical formula of $C_{25}H_{26}O_5$ (Figure 14 and 15).

The new peptide isolated from the strain *Aphanizomenon* X008a, named aphapeptin F1 is a very unsual cyanopeptide. This peptide is formed by a sequence of three homotyrosine (Figure 12 and 13).

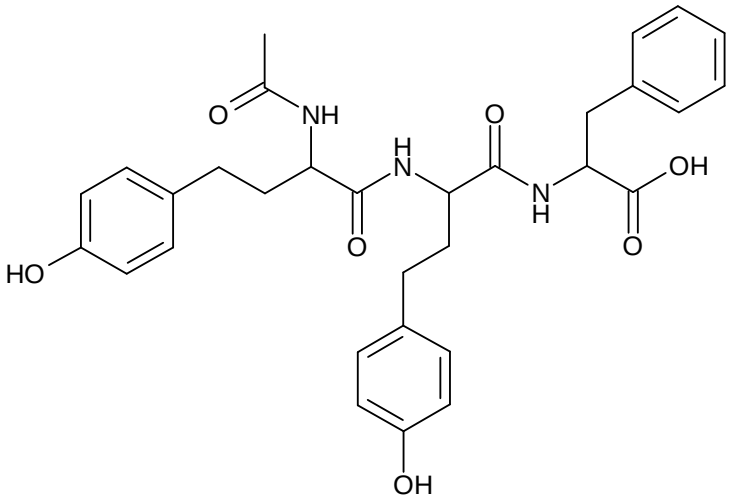


Figure 12: The chemical structure of the aphapeptin F1 (m/z 562 $[M+H]^+$) isolated from the strain *Aphanizomenon* X008a.

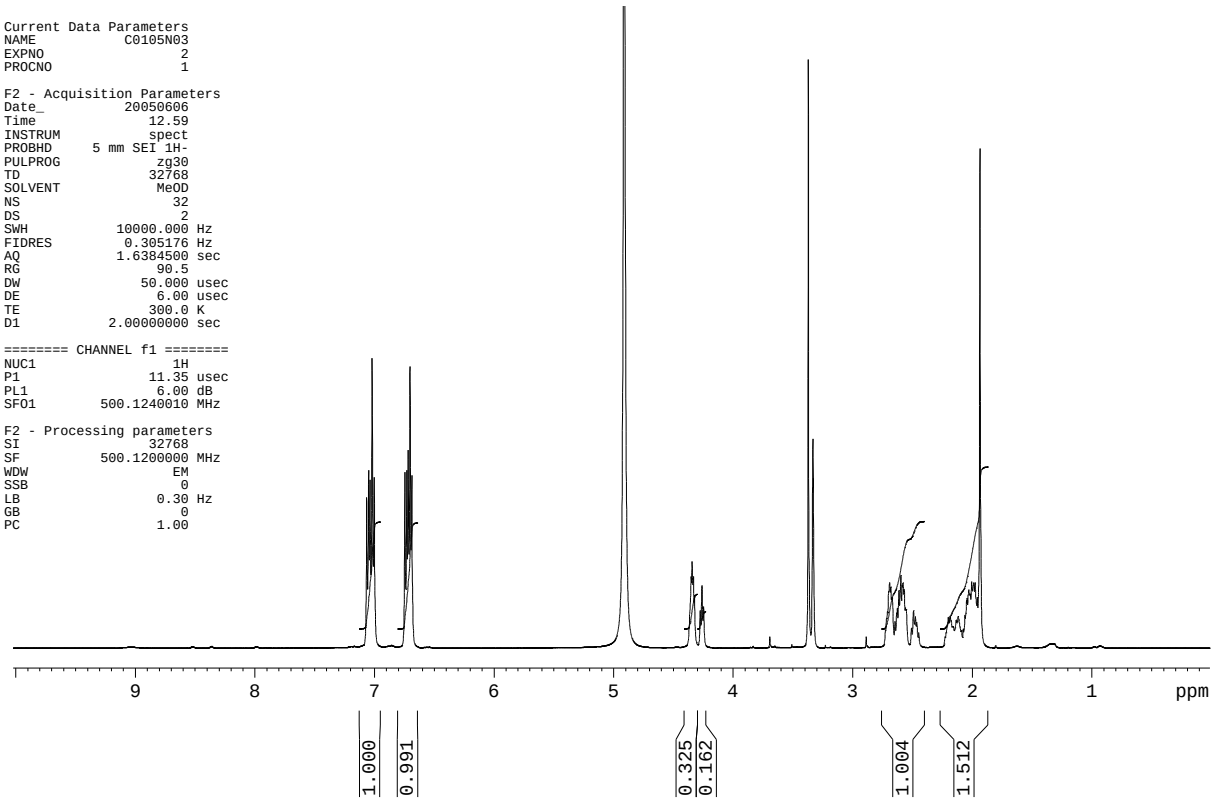


Figure 13: The 2D-NMR spectrum from the new aphapeptin F1, isolated from the *Aphanizomenon* X008a.

The new peptide isolated from the strain *Aphanizomenon* X0023, named aphapeptin F2, is very similar to the previous peptide. This peptide has also a very unsual aminoacids sequence, as the aphapeptin F1. This peptide is formed by two homotyrosine and a phenylalanine (Figure 14 and 15).

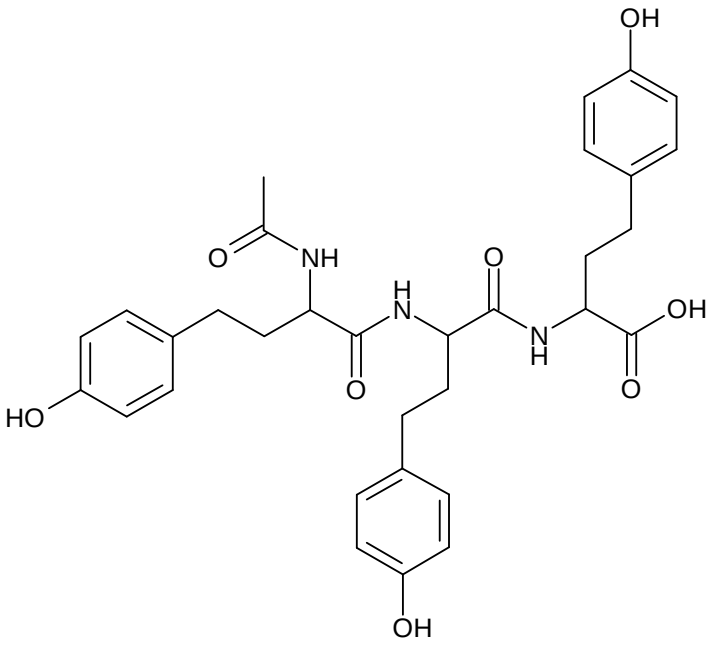


Figure 14: The chemical structure of the aphapeptin F2 (m/z 592 $[M+H]^+$) isolated from the strain *Aphanizomenon* X0023.

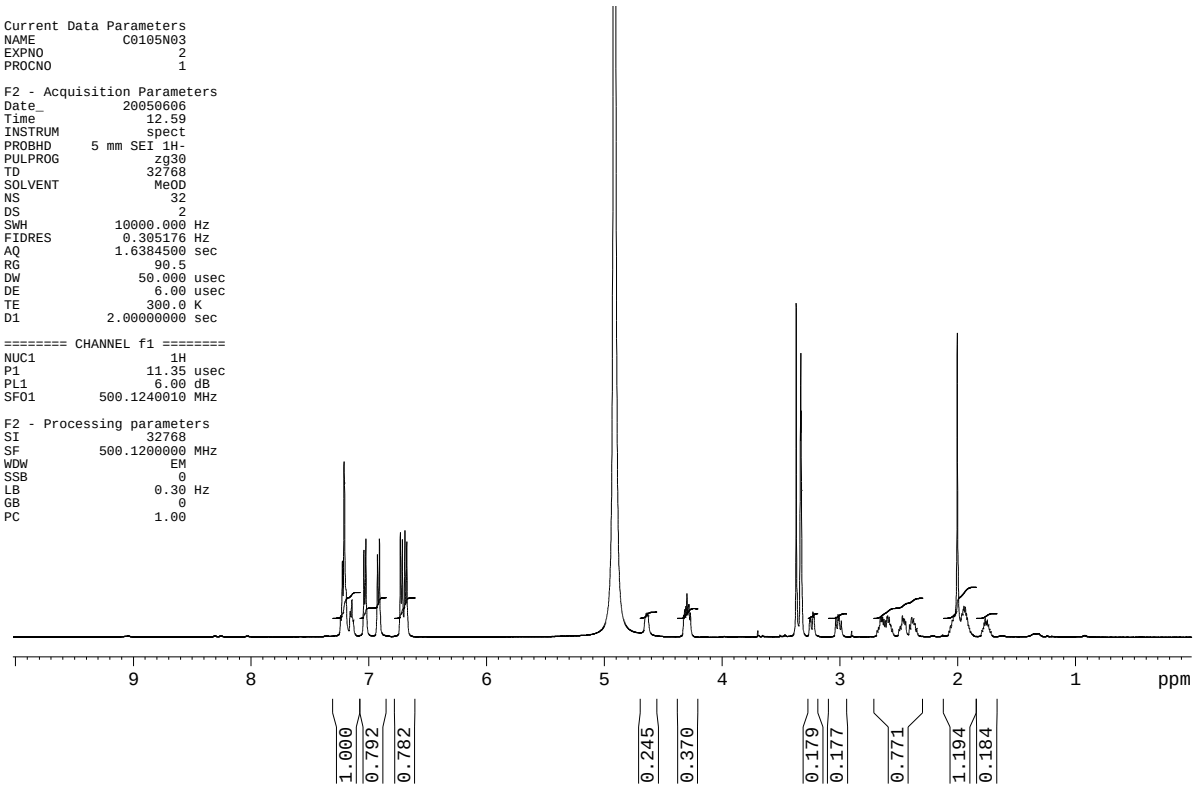


Figure 15: The 2D-NMR spectrum from the new aphapeptin F2, isolated from the *Aphanizomenon* X0023.

The similarity of both newly isolated peptides is great, leading to characterizations of them as belonging to the same peptide class. However, they do not have any of the characteristic chemical structure that would classify them as a variant of any known peptide class well-known from

cyanobacteria. They have a very unsual sequence of double and triple equal peptides.

Since one of the main aspects of cyanobacterial peptide occurrence is the production of different peptides classes and within those of multiple variants by individual strains across a range of cyanobacterial taxa, these peptides already isolated in *Aphanizomenon* are likely present in strains of other cyanobacterial taxa as well.

3.1.2 The bioactivities of the two new *Aphanizomenon* peptides

To test the cytotoxicity of these two new isolated peptides around 5 mg of dried material was sent to the University of Constance, Germany for testing by Dr. Stefan Höger with the Human cell lines HEP-G2 and CACO-2, Apoptosis assay and the Ames assay as described in section 2.2.5.

The aphapeptin F1 (m/z 562 $[M+H]^+$) isolated from the strain *Aphanizomenon* X008a showed no effect in the LDH and MTT assays up to a concentration of 83.3 µg/ml after 72 h, nor did the newly isolated peptide, aphapeptin F2, from the strain *Aphanizomenon* X0023 (m/z 592 $[M+H]^+$) up to a concentration of 93 µg/ml.

This result is different from those reported for microcystins or for cyanobacterial extracts potentially containing unknown bioactive substances. According to Moreno *et al.* (2003), the LDH in cells treated with microcystin-LR was 3.8 fold higher than the control. Ding *et al.* (2000) reported an increase of the release of LDH of 70% compared to the control, caused by a microcystin containing cyanobacterial extract. In *Daphnia*, the increased LDH activity was also seen as response to the stress of microcystin exposure (Chen *et al.*, 2005b). Surakka *et al.* (2005) found that 8 of the 21 Baltic Sea *Anabaena*, *Nodularia*, and *Nostoc* strains, which did not produce microcystins or nodularins, to be cytotoxic to the LDH assay. Fastner *et al.* (2003) found most isolates of *Cylindrospermopsis raciborskii*, which did not produce cylindrospermopsin or microcystin, were toxic to primary rat hepatocytes and to the human derived cell lines HEP-G2 and CACO-2, though it remained

unclear whether the cellular components causing these effects were peptides or other substances.

Ernst *et al.* (2001) tested isolates from *Planktothrix* for cytotoxicity using the standard MTT assay. The assay measures the activity of the mitochondrial succinate-tetrazolium reductase system, which is active only in viable cells. The extract caused a significant reduction in hepatocyte viability up to 69% compared to controls.

As neither for the aphapeptin F1 nor for the aphapeptin F2 were detected apoptosis or necroses, in concentrations extending to 100 µg/ml, these peptides apparently are not the substances responsible for toxicity seen from crude extracts. This is not surprising since these assays are designed to indicate cytotoxicity to mammals, and newest results on cyanopeptide toxicity obtained in the PEPCY project (Höger *et al.*, 2004) indicate that so far the peptides tested scarcely proved to be cytotoxic in these assays.

The apoptotic effects of microcystins have become a focal point of research and many of the effects on cells and tissues triggered by microcystins have been documented. In 1990, Repavich *et al.* found that in human lymphocytes, microcystins had a dose-dependent clastogenic effect that was connected with chromosomal breakage. Microcystins are also capable of causing apoptosis in rat hepatocytes and DNA damage in human hepatoma cells. Ding and Org (2003) found that reactive oxygen species (ROS) and mitochondrial permeability transition (MPT) played an important role in apoptosis induced by microcystin-LR, though very little is known about the exact apoptotic mechanism of microcystins.

In this study, the Ames II™ showed no effect in concentrations tested up to 2.77 mg/ml for any of the isolated peptides, and therefore none of these tests indicate that the aphapeptin F1 and the aphapeptin F2 to be cytotoxic, mutagenic or cancer promoters.

For microcystins, the best studied cyanobacterial peptide, the Ames assay also showed no mutagenicity, according to Kubo *et al.* (2002) and to Runnegar & Falconer (1982). Then again, Ding *et al.* (1999) reported that a microcystic cyanobacterial extract (extract prepared was derived from >90%

Microcystis aeruginosa) showed a strong mutagenic response in the Ames test (strains TA97, TA98, TA100 and TA102; with or without S9 activation), induced significant DNA damage in primary cultured rat hepatocytes (comet assay) and produced bone marrow micronucleated polychromatic erythrocytes in mice. Thus, peptide mutagenicity needs further research.

The newly isolated peptide, aphapeptin F1, was found to be a significant ($p < 0.01$) inhibitor of both enzymes (*Daphnia* trypsin and porcine trypsin). The IC_{50} for the enzymes in *D. magna* extract was 8.48 µg/g protein and the enzyme activity showed a significant linear correlation to the peptide concentration ($r^2 = 0.99$). The IC_{50} value for porcine trypsin was 33.12 µg/g protein and there was a significant linear correlation between the enzyme activity and the peptide concentration ($r^2 = 0.84$).

Aphapeptin F2 was also tested on both proteases (*Daphnia* and porcine trypsin), but it was only found to inhibit the enzyme activity in *Daphnia* extract. The IC_{50} value was 13.35 µg/g protein in *D. magna* extract and there was a significant ($p < 0.05$) linear correlation between the enzyme activity and the peptide concentration ($r^2 = 0.88$).

Trypsin is one of most produced digestive enzymes by *Daphnia* (von Elert *et al.*, 2004), and the inhibition of the enzyme by cyanobacterial peptides may cause a complete collapse of the zooplankton digestion (Rohrlack *et al.*, 2005).

A continuous absence of this essential amino acid may induce starvation, molting problems, reduced growth, lower reproductive activity and early death (Jongsma and Bolter, 1997).

Since protease inhibitors are found in several laboratory strains and field populations of *Microcystis*, *Planktothrix* and other cyanobacterial genera, all herbivorous that feed on cyanobacteria can suffer by such a protease inhibitors (Fastner *et al.*, 2001, Rohrlack *et al.*, 2005).

The detection of new peptides, their chemical structure and biochemical impact on other organisms, may improve the comprehension of the cyanobacterial biosynthetical potential and the distribution of these on taxonomical and geographical scales (Welker and von Döhren, *in press*). The

development of new drugs may also result from detecting new cyanobacterial peptides and understanding their bioactivity.

3.2 The influence of different growth conditions on the intra and extracellular peptide concentration

In the experiments done during this work, three different strains of *Microcystis* and one *Aphanizomenon* strain were used, which together produce 8 different peptides in concentrations sufficient for determination. These strains were grown in batch cultures under two different temperature conditions, 20℃ and 25℃, two light intensities, 2 0 and 60 $\mu Em^{-2}s^{-1}$, and four different medium composition, full medium (ASM-1), media free of N (N^-), free of P (P^-) and free of N and P (NP^-).

To understand the influence of different growth conditions on peptide concentrations in the cells and their surrounding medium, of the peptides found in the strains investigated, eight peptides occurring in significant amounts were studied from four different cyanobacteria strains (Table 10).

Table 10: The strains and peptides used to understand the influence of different growth conditions.

	Strain			
Peptide	*Microcystis* RST9501	*Microcystis* NPRG-2	*Microcystis* BM 10	*Aphanizomenon* X008a
microcystin [Leu1]-desmethylated–LR	X			
aeruginosin 102A	X			
microviridin	X			
microginin		X		
anabaenopeptin F			X	X
oscillamide Y		X	X	
aphapeptin F1				X
P677	X		X	

3.2.1 Difference of growth in response to different nutrient composition

The use of growth cultures enables to study and observe the effect of environmental factors on peptide concentration, during specific growth conditions (Long, 2001). The understanding how environmental factors may affect the peptides production in cyanobacteria has been a challenge for almost 40 years (Orr and Jones, 1998).

Although, it is important to observe the physiological state of the cells during the growth experiments, since the effect of any environmental parameter on the peptide concentration of a culture population is influenced through overall effects on the rate of increase or decline of the culture or strain. Not by individual and specific influence on the peptide biosynthetic or catabolic pathways (Orr and Jones, 1998).

Therefore to a better understanding of the environmental effects on the peptides, there is a need of comprehending the growth responses to the different temperature and light conditions and nutrient composition before any conclusion about the peptides themselves.

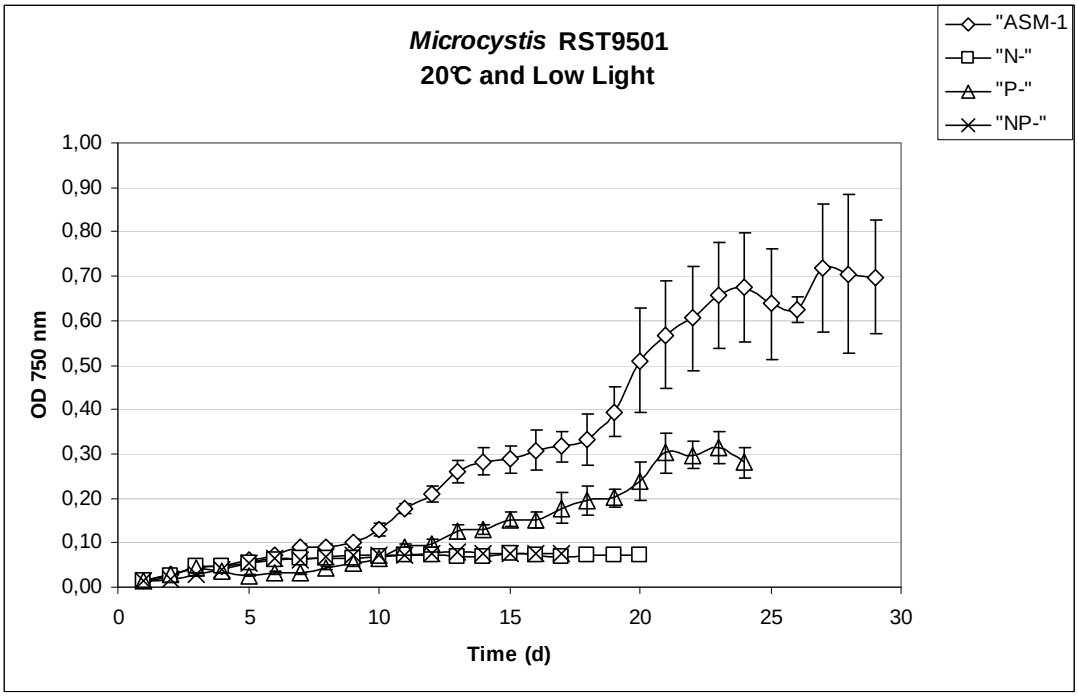


Figure 16: *Microcystis* RST9501 batch culture growth curves using full medium (ASM-1), N^-, P^- and NP^- media at 20℃ and low light (measured as increase of "OD" at 750 nm). The bars show the standard deviation for 3 measurements.

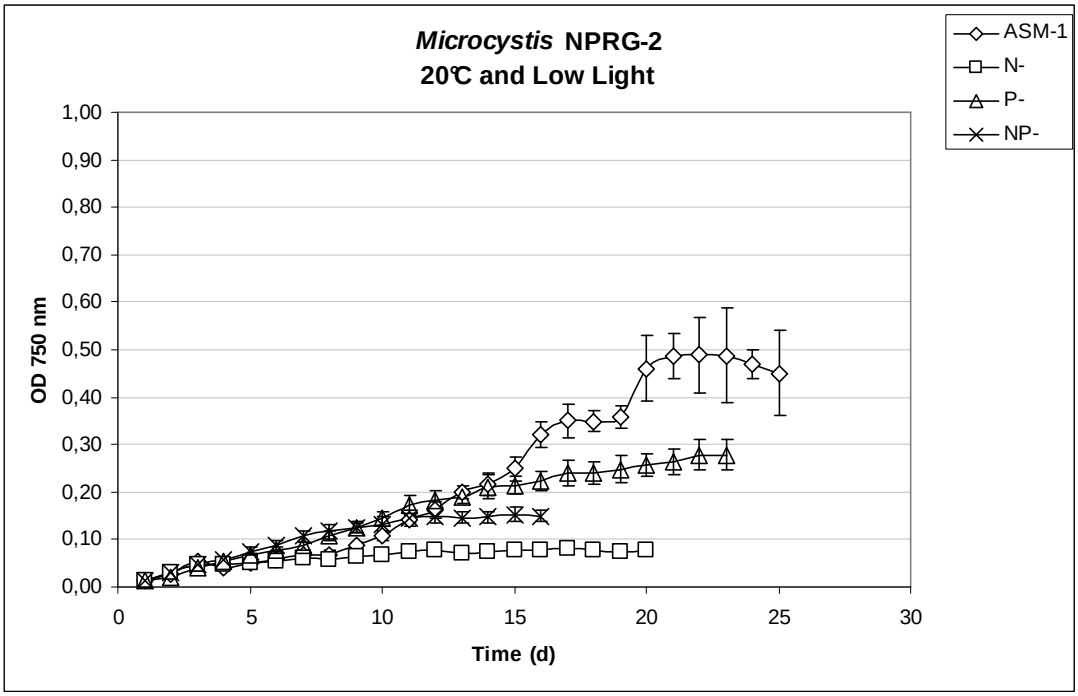


Figure 17: *Microcystis* NPRG-2 batch culture growth curves using full medium (ASM-1), N^-, P^- and NP^- media at 20℃ and low light (measured as increase of "OD" at 750 nm). The bars show the standard deviation for 3 measurements.

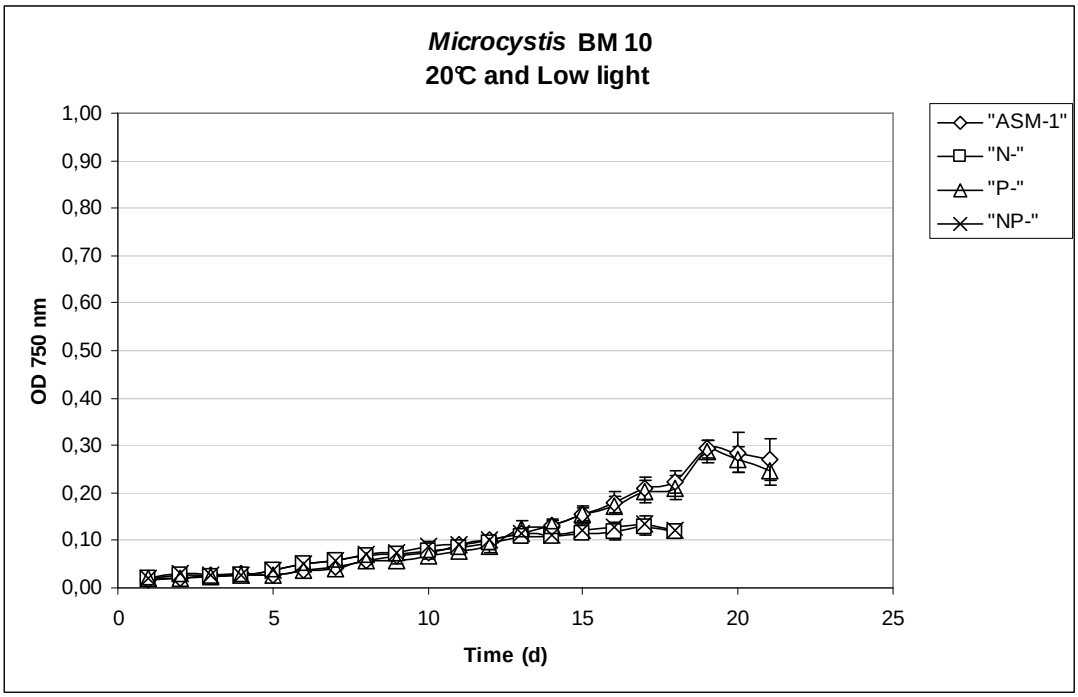


Figure 18: *Microcystis* BM 10 batch culture growth curves using full medium (ASM-1), N^-, P^- and NP^- media at 20℃ and low light (measured as increase of "OD" at 750 nm). The bars show the standard deviation for 3 measurements.

As expected for *Microcystis*, the exponential growth phase reflects maximum growth in full medium (ASM-1), i.e. conditions limited only by light. In the N^-, P^- and NP^- conditions, as the cells divide, they mobilize the nitrogen reserves and use the stored phosphorus. As the nitrogen reserves are

modest, in the N^- and NP^-, the cell division is more strongly reduced than in the phosphorus limited experiments. This internal reserve allows continued growth for some time even with no dissolved nitrogen or phosphorus in the medium. The decrease of growth rates begins when cell-internal nutrient concentrations start to become limiting.

Figure 16, 17 and 18 represent the different *Microcystis* strains grown as batch culture under different nutrient compositions and at 20℃ and low light intensity (20 $\mu Em^{-2}s^{-1}$). All three *Microcystis* strains had a similar exponential growth rate, especially for full medium (ASM-1) (Table 9). The *Microcystis* RST9501 has the highest growth rates and reaches the stationary phase later than the others *Microcystis* strains, in this study, particularly in full medium (ASM-1) and in medium free of P (P^-). The *Microcystis* NPRG-2 has the slowest growth rates but reaches the stationary phase relatively later, in full medium (ASM-1) and in medium free of P (P^-). The *Microcystis* BM 10 has a similar exponential phase for all four media composition. For this strain the presence of N is very important. The full medium (ASM-1) and the medium free of P (P-) have a similar growth and reach the stationary phase together. The media free of N (N-) and free of N and P (NP-) reach the stationary phase at the same time and react very alike to each other during all growth.

Comparing the growth rates of the 3 *Microcystis* strains under the two different light intensities tested, it can be seen that the 3 fold increase on light intensity caused an average increase of 1.5 folds. As expected was the strain NPRG-2, which presented a 2.42 fold increase at 20°C. When comparing the growth rates between 20℃ and 25℃, or an increase of 1.2 fold in temperature, a very similar increase pattern can be obtained. The average growth rate increases in 1.3 fold with the 1.2 fold increase of temperature (Table 11). So, according to these results, the temperature is more important for the strain growth than the light intensity.

Table 11: The *Microcystis* strains' growth rates (μ±SD (d^{-1})) in full medium at the different combinations of temperature and light.

Strain	20℃		25℃	
	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$
RST9501	0,151±0,002	0,237±0,002	0,253±0,001	0,277±0,002
NPRG-2	0,121±0,002	0,293±0,003	0,193±0,001	0,327±0,002
BM 10	0,140±0,001	0,226±0,0013	0,179±0,001	0,326±0,002

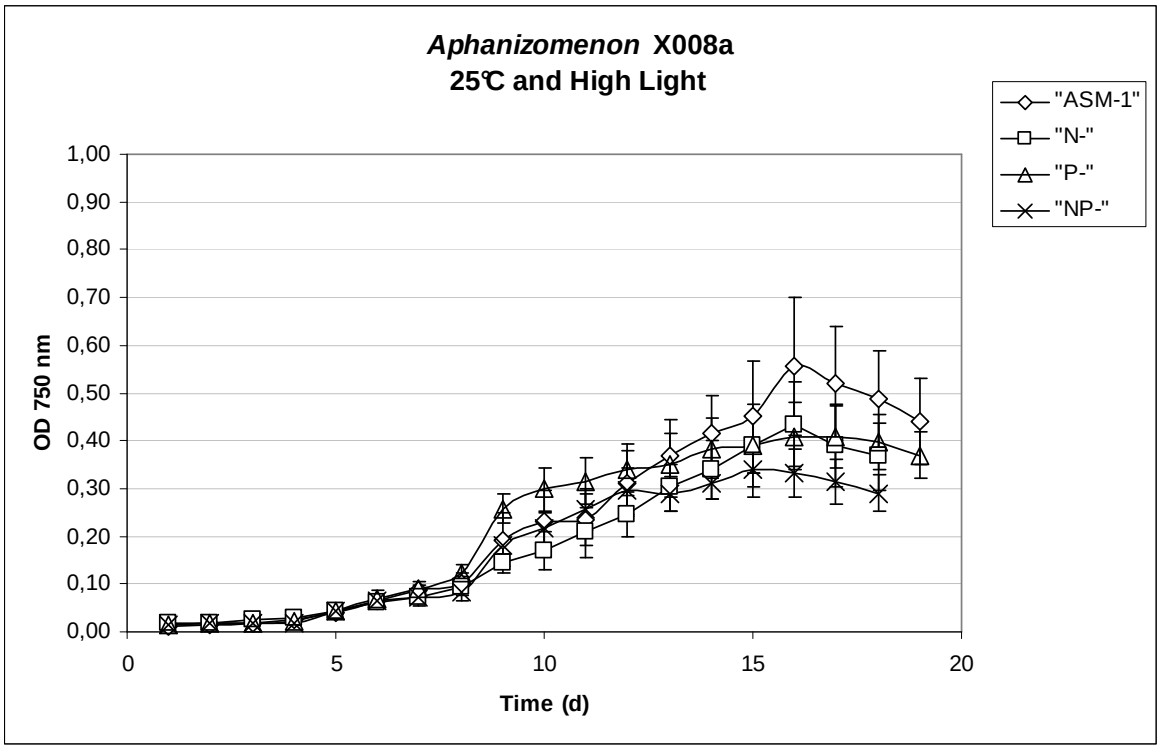


Figure 19: *Aphanizomenon* X008a batch culture growth curves using full medium (ASM-1), N^-, P^- and NP^- media at 25℃ and high light (measured as increase of "OD" at 750 nm). The bars show the standard deviation for 3 measurements.

Aphanizomenon spp. is a genus of cyanobacteria with specialized cells, i.e. heterocysts, capable of fixing atmospheric nitrogen dissolved in the water, and thus potentially balancing N-depletion in the culture medium. Correspondingly and different from the results for the 3 *Microcystis* strains, N^- alone reduced growth less than P^- or combined NP^- (Figure 19).

The increases of 3 fold on light intensity causes in growth from the *Aphanizomenon* X008a only an increase of 1.2 fold. The 1.2 fold increase on temperature stimulates the *Aphanizomenon* growth in 1.7 fold. As for the *Microcystis* strains, the variation on temperature is more important than the variation in light intensity.

As well as at 20℃ and 25℃, there was a similar in crease of over 1 fold, on the growth rate from the low light intensity to the high light intensity. The comparison the growth rates between the temperatures under the same light intensity, also describes a similar increase on growth rate at 25℃ (Table 12).

Table 12: *Aphanizomenon* strain growth rate (μ±SD (d^{-1}) in full medium at the different combinations of temperature and light.

Strain	20℃		25℃	
	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$	20 $\mu Em^{-2}s^{-1}$	60 $\mu Em^{-2}s^{-1}$
X008a	0,141 ± 0,03	0,176 ± 0,015	0,248 ± 0,02	0,278 ± 0,02

3.2.2 Differences in peptide concentrations between the exponential and stationary phase

As discussed above a number of literature studies are available on the effect of nutrient limitation on microcystin production and nodularin (Lehtimäki *et al.*, 1997), but not on other peptides also produced by cyanobacteria. The literature shows rather minor variability of cellular microcystin concentrations by a factor of up to 2-4. Therefore, the objective of the work presented in the following was to test the hypothesis that all these peptides are regulated similarly, i.e. that nutrient limitation, light intensity and temperature should not have a major impact on cellular peptide concentration.

According to Orr and Jones (1998) and Long (2001), the two extremes of growth states can be characterized by sampling during the exponential and stationary growth phases. The exponential phase, when growth is maximal and the cultures are not yet limited by nutrients or light, was found the highest microcystin concentration (Long, 2001) and any variation on peptide concentration should reflect changes on the ecophysiological conditions. The stationary phase reflects severe limitation, in which no cell multiplication happens, either because light is limiting or because nutrients have become limiting, and often because of a mixture of both.

Figure 20 gives an example of the results of one of the experiments with the strain *Microcystis* RST9501 under 20℃ at low photon flux density [1]. For all four peptides the amounts of total peptide per biovolume decreased as the cultures entered the stationary phase.

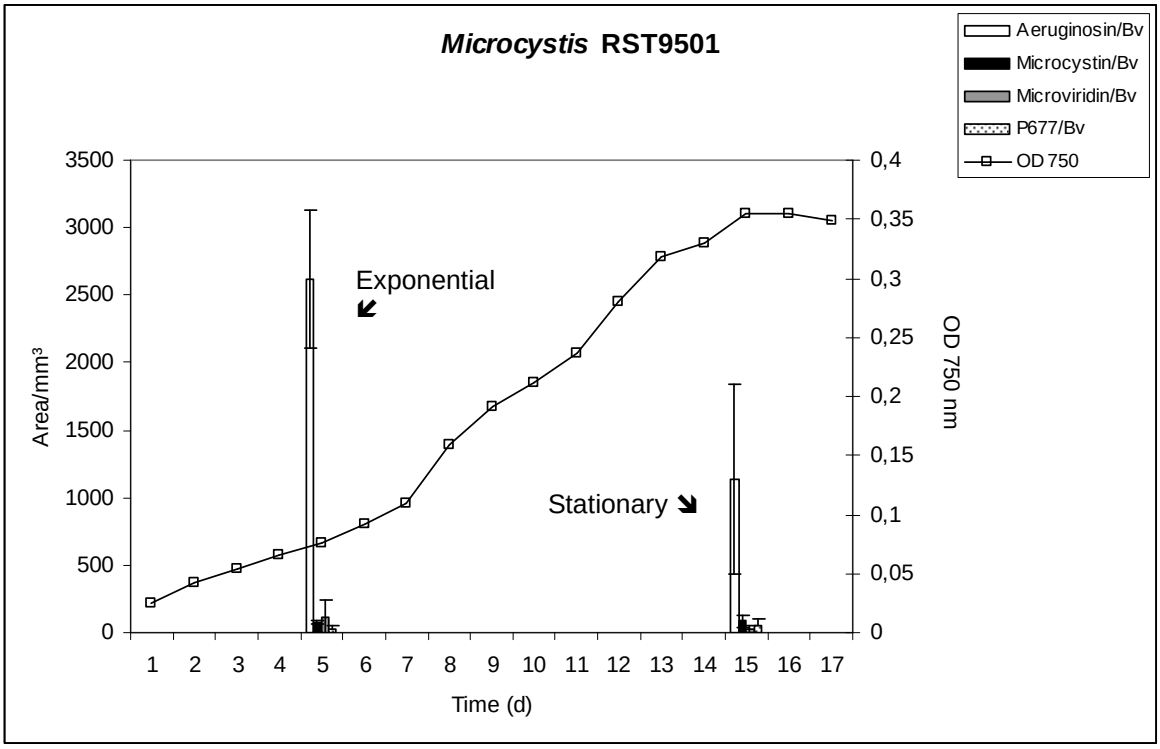


Figure 20: The amount of aeruginosin, microcystin, microviridin and the peptide P677 (given as peak area per unit biovolume due to lack of standards) during the exponential and stationary phases of *Microcystis* RST9501 grown in ASM-1 medium at 20℃ and high lig ht (60 $\mu Em^{-2}s^{-1}$).

However, a variance analysis between the exponential and stationary phase showed that the difference in peptide per biovolume was not significant ($p < 0.01$) in almost all cases. Only five out of 176 (hundred seventy-six) cases studied for these strains showed exceptions. All the exceptions occurred with the *Microcystis* strain RST9501, which strain had the highest growth rate and reached later the stationary phase. In this strain in four cases studied of these peptide concentrations in the stationary phase were substantially lower (by a factor of 0.3) than those in the exponential phase. Only the microcystin in this strain showed a 3 fold increase in the stationary phase as compared to the exponential one (Table 13).

[1] "low light" and "high light" is used for the photon flux density of 20 and 60 $\mu Em^{-2}s^{-1}$, respectively

Comparing the results of the exponential and the stationary phase, it is possible to conclude that peptide concentration shows little change in response to the age of the culture and thus to increasing severity of resource limitation.

Table 13: The results of the significant test under all different conditions and the significance ($p < 0.01$) between exponential and stationary phase.

Strain	Peptide	Medium	20℃		25℃	
			Low Light	High Light	Low Light	High Light
RST9501	aeruginosin 102A	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	[Leu1]-desmethylated microcystin-LR	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	*	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	microviridin	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	*	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	*	n. s.	n. s.	n. s.
	677	ASM-1	*	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	*	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
NPRG-2	microginin	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	oscillamide Y	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
BM 10	anabaenopeptin F	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	oscillamide Y	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	677	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
X008a	anabaenopeptin F	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.
	aphapeptin F1	ASM-1	n. s.	n. s.	n. s.	n. s.
		N^-	n. s.	n. s.	n. s.	n. s.
		P^-	n. s.	n. s.	n. s.	n. s.
		NP^-	n. s.	n. s.	n. s.	n. s.

n. s. = not significant, *= significant

3.2.3 Results of the variability of peptides concentration in the exponential phase

The following analysis was therefore done based on peptide concentration determined in the exponential phase, which should reflect the effect of the different conditions tested on the peptide under the different conditions tested. The total peptide concentration is the sum of the intracellular and the extracellular peptide concentration.

3.2.3.1 Microcystin

The major microcystin variant produced in the strain RST9501 was [Leu1]-desmethylated–LR (1037 m/z), as determined by its peptide fragment pattern with post source decay (PSD) in MALDI-TOF MS (Figure 21). This microcystin was isolated from a Brazilian strain, and it appears to be the most widespread microcystin in Brazil (Alexandre Matthiesen, *personal communication*). Among the strains studied, this peptide, is only produced by the *Microcystis* strain RST9501.

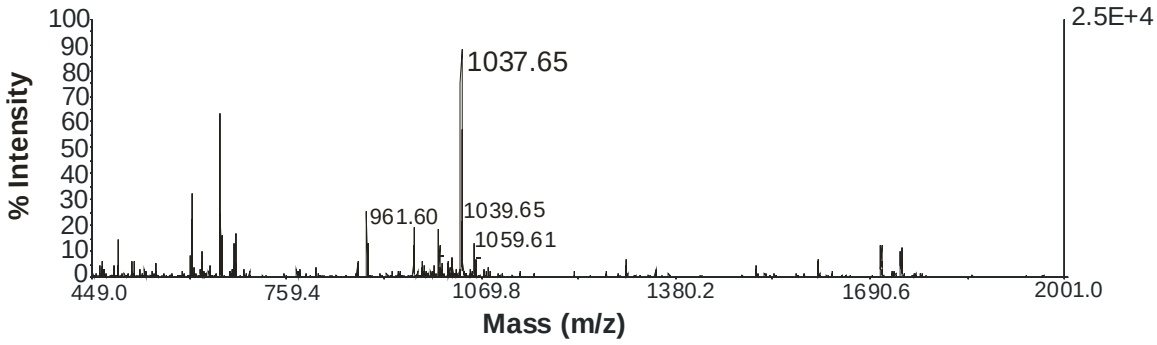


Figure 21: The PSD spectrum of the mass signal $M+H^+$ = 1037, [Leu1]-desmethylated-LR, produced by *the Microcystis* strain RST9501.

Microcystin concentrations in relation to biovolume

As discussed in section 2.2.3 in the following, changes in the production of peptides under different growth conditions are expressed in terms of concentration in relation to biovolume. For the intracellular fraction, this expresses cellular concentration. For the total fraction, i.e. intra plus

extracellular, it reflects production. However, changes in growth conditions may influence production of different fractions of cellular biomass in different ways. For example, an increase in peptide per unit biovolume may either be due to an increase of peptide production in relation to the production of other cellular matter, or to a decrease of the latter. Thus, concentrations in relation to biovolume are not a precise measure of changes in production, but do reflect these roughly.

The cyclic peptide [Leu1]-desmethylated microcystin-LR is the only peptide in this study for which an increase of peptide concentration per mm³ biovolume was seen as the culture reached the stationary phase (see Table 13).

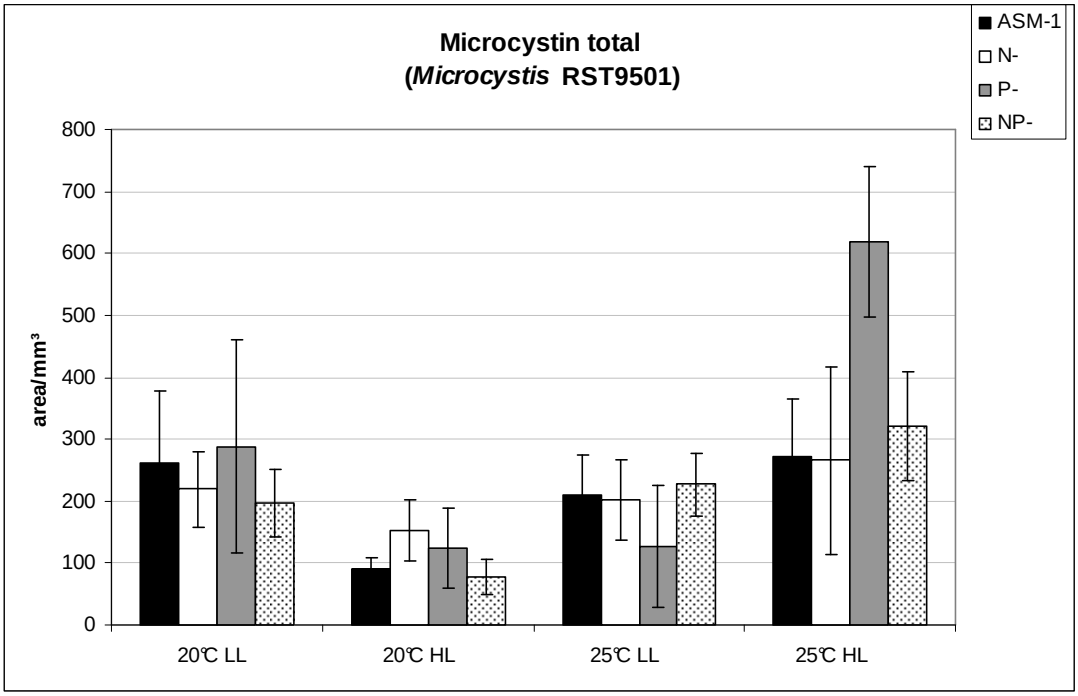


Figure 22: The total (i.e. intra plus extracellular) concentration of microcystin [Leu1]-desmethylated–LR under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

The concentration of the total pool of this microcystin (i.e. intra and extracellular) in relation to the biovolume of the culture reacts distinctly to variation of the temperature and light conditions and the different nutrient compositions (Figure 22). At 20° C, the concentration of this peptide was greater at the low light intensity than at the high light intensity. At this temperature, the concentrations at the different nutrient limitations were very similar, some slight differences were observed but none of them were

statistically significant. Under low light intensity, the peptide amount in the medium free of P (P^-) was slightly higher than in the other media, but it was similar to the amount in the full medium (ASM-1). The media free of N (N^-) and free of N and P (NP^-) showed similar microcystin concentrations, slightly lower than in the other two media. At the high light intensity and 20℃, the concentration of this peptide was highest in medium free of N (N^-), followed by the medium free of P (P^-). The production was almost the same between full medium (ASM-1) and medium free of both N and P (NP^-).

In contrast to the results at 20℃, at 25℃ microcy stin production was higher at the higher light intensity. Under low light intensity microcystin concentrations in full medium (ASM-1) and the media free of N (N^-) and free of N and P (NP^-) were fairly equal and approximately less than 0,7 fold the amount produced at high light intensity. Light intensity switched the direction in which P absence affected microcystin concentration: Under low light intensity the absence of P (P^-) reduced the microcystin concentration to half of that found in the other media, but under high light intensity in increased more than 2 fold increase in the medium free of P (P^-) as compared to all other combinations of temperature, light or nutrient composition.

The Tukey HSD post-hoc test was carried out to determine which individual test conditions were significantly different from each of the others, as described in section 2.4. The results of this test (Table 15) showed that differences were not significant between almost all the different combinations of temperature and light conditions and the different nutrient concentrations, with significantly higher microcystin concentrations only under conditions of high light intensity, 25℃ and medium free of P (P^-), as also conspicuous in Figure 22.

As described in section 2.4, to determine the significance of the differences between peptide concentration in relation to the three variables (temperature, light intensity and medium composition), the multiple analysis of variance (MANOVA) proved to be the most adequate statistical test, as it tests the significant differences between means of peptide concentrations between all experiments conducted in relation to one variable (e.g. all results gained at

20℃ against the results obtained at 25℃), and thi s increases the chance to discover which factor was really important.

Table 14 shows the results of the MANOVA test for the total (intra plus extracellular) microcystin concentration. No statistical significance of the effect of light intensity as single variable was found, but effects of temperature as single variable and medium composition as single variable proved statistically significant. Also, the variables light intensity and temperature and the variables light intensity and medium together had a statistically significant impact on microcystin concentrations, while the variables temperature and medium together did not. Nevertheless, the impact of the three factors together was statistically significant.

As described in section 2.4, to determine the significance of the differences between peptide concentration in relation to the three variables (temperature, light and medium composition), the multiple analysis of variance (MANOVA) proved to be the most adequate statistical test, as it tests the significant differences between means of peptide concentrations between all experiments conducted in relation to one variable (e.g. all results gained at 20℃ against the results obtained at 25℃), and thi s increases the chance to discover which factor is really important.

Table 14: The multiple analysis of variance (MANOVA) had done for the intra and extracellular microcystin concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	2,82	0,09650
Temperature	44,46	0,00000
Medium	8,80	0,00004
Light + Temperature	43,11	0,00000
Light + Medium	5,31	0,00215
Temperature + Medium	2,71	0,050
Light + Temperature + Medium	9,09	0,00003

* Statistically significant differences are highlighted in gray.

Table 15: Results of the Tukey HSD test for intra and extracellular microcystin concentration.

Microcystin intra and extracellular			20℃								25℃							
			20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}				20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}			
			ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$
20℃	20 µEm^{-2}s^{-1}	ASM-1																
		N$^-$	ns															
		P$^-$	ns	ns														
		NP$^-$	ns	ns	ns													
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns												
		N$^-$	ns	ns	ns	ns	ns											
		P$^-$	ns	ns	ns	ns	ns	ns										
		NP$^-$	ns	ns	ns	ns	ns	ns	ns									
25℃	20 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns							
		P$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
		P$^-$	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	

n. s. = not significant. * indicates significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular microcystin

Between 65% and 98% of total peptide was found intracellularly under all conditions. Analyzing all combinations of temperature and light intensity, the full medium (ASM-1) leaded to a higher intracellular concentration than the media in which one or two essential nutrients were missing (Figure 23). This result corresponds to observations reported by Preußel *et al.* (2004) showing elevated extracellular shares of microcystin in nutrient limited cultures of *Planktothrix agardhii*.

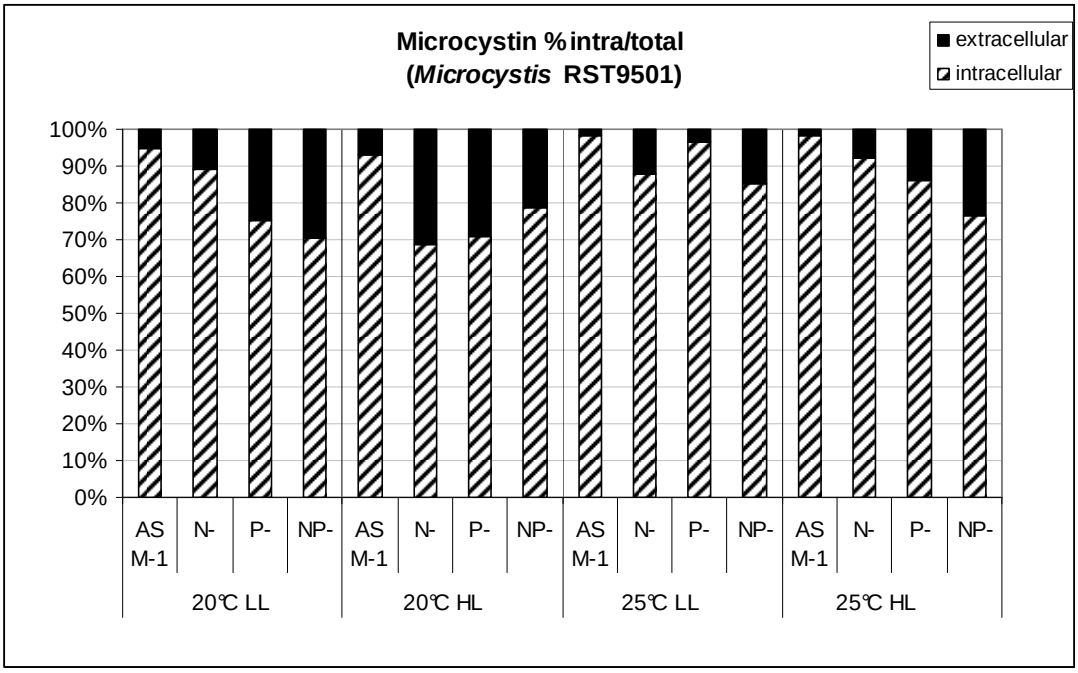


Figure 23: The percentage of the intracellular microcystin in relation to the total peptide concentration under the different temperature and light conditions and the different nutrient compositions. (LL= low light; HL=high light).

A similar result was also found by Sivonen (1990) and Rapala *et al.* (1997) who described that most of the microcystin produced remains in the cells. Since the greater amount of this microcystin was located intracellularly, strong similarity in results between those for the total and those for the intracellular peptide concentration was inevitable. As for the total concentration of this peptide, for the intracellular fraction the highest ratio of microcystin to biovolume was also at 25℃ and high light intensity in medium free of P (P^-). Therefore, this condition was also significantly different from all others situations (data not shown).

The intracellular microcystin concentration in full medium (ASM-1), was relatively constant at 90%, regardless of temperature and light intensity. On the other hand, microcystin concentrations under nutrient limitation in the media free of N (N^-), free of P (P^-) and free of N and P (NP^-) were sometimes higher (see Fig. 23), but from the higher extracellular shares we may conclude that a higher share of this increase of concentration is also exported out of the cells.

Combining the observation of increased microcystin production at medium free of P (P^-) in the exponential phase under high light intensity and elevated temperature with the observation of the general increase of extracellular

shares of microcystin under nutrient limited conditions, we may speculate that pronounced nutrient stress might enhance peptide export out of the cells. This may relate to the signaling hypothesis proposed by Dittmann *et al.* (2001) and Kehr *et al.* (2006), as increased signaling might be relevant for the cells under stress.

Specific growth conditions might therefore induce both an increase of microcystin production and an increase of extracellular toxin. If this is responsible for signaling, or for inhibitory and/or allelopathic effects on other organisms (as discussed in the introduction), the results obtained by this study would imply that such impacts were strongest at high temperature (25℃) and the absence of nutrients in the medium (e.g. N, P or NP).

3.2.3.2 Aeruginosin 102A

This aeruginosin was identified by its PSD pattern (Figure 24) and also only found in only one of the four strains analyzed, in the *Microcystis* RST9501.

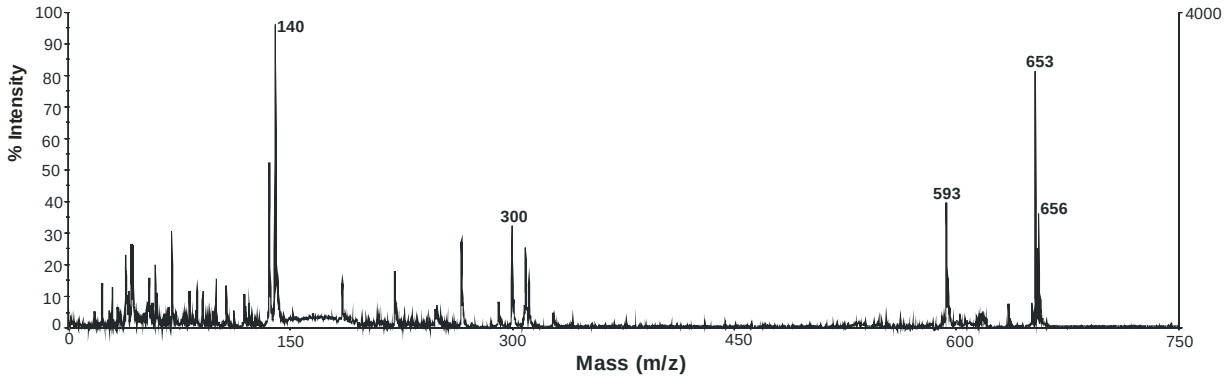


Figure 24: The PSD spectrum of the mass signal $M+H^+$ = 653, aeruginosin 102A, produced by *the Microcystis* strain RST9501.

Aeruginosin 102A concentrations in relation to biovolume

The production of this peptide was extremely greater than the other peptides produced by this *Microcystis* strain, i.e. it reached up to 30 times more peak area per biovolume than the amount of microcystin produced. Although signal intensity may vary between peptides and no standards are available (see section 2.2.3), this 30 times higher peak per biovolume suggests that the concentration of this aeruginosin is substantially higher than that typically reached for other peptides in this strain, e.g. microcystins.

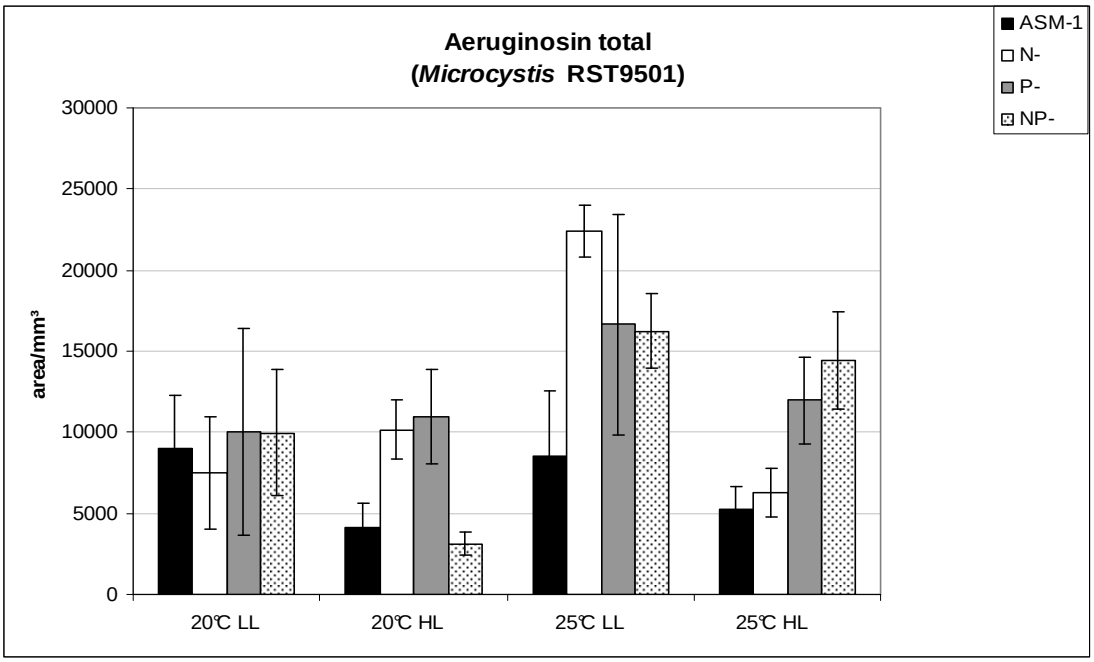


Figure 25: The total (intra plus extra) concentration of aeruginosin 102A under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

The total (i.e. intra plus extracellular) aeruginosin concentration reacted in a different way to the variation of temperature and light conditions and different nutrient composition, as seen in Figure 25. At 20℃ , for the full medium (ASM-1) and the medium free of both N and P (NP^-), the concentration of this peptide was lower at the higher light intensities. In the media free of N (N^-) and free of P (P^-), at low light intensity the aeruginosin concentration was similar to that in the other two media, but at the higher light intensity it increased slightly, though this increase was not statistically significant (Table 17) .

The total concentration of aeruginosin 102A was higher at 25℃ than at 20℃ in most media, especially under low light intensity and particularly in the nutrient deficient media, and the effect of temperature proved to be statistically significant by the MANOVA (Table 16). Elevated peptide concentration at higher temperatures had been also described for microcystin (Gorham, 1964; Codd & Poon, 1988; Sivonen, 1990 and Rapala *et al.*, 1997).

Medium composition as a single factor also caused statistically significantly different aeruginosin concentrations (Table 16). This was most pronounced for medium free of N (N^-), but statistically significant also for medium free of P (P^-) and those media free of both N and P (NP^-) (Table 17). The medium free of N (N^-) at 25℃ together with low light intensity resulte d in a concentration of

aeruginosin per unit biovolume 4 fold higher than at high light intensity and 2 fold higher than at 20℃. For the P^- and NP^- free media, the higher temperature enhanced the concentration of this peptide in both light conditions as compared to the concentration at 20℃. For light intensity as a single effect a statistically significant influence was found by the MANOVA, as well as for all three variables light intensity, nutrients and temperature together (Table 16). The Tukey HSD post-hoc test, performed as described in section 2.4, showed that the increase of the aeruginosin concentration at 25℃ and low light intensity in the media free of N (N^-) and free of P (P^-) were statistically significantly different from almost all aeruginosin concentrations under the other combinations of temperature, light conditions and nutrient composition.

The interrelationship of temperature and light intensity is an important regulatory factor in the physiology of cyanobacteria. As discussed in the introduction, previous studies showed that different microcystin variants could be regulated by temperature, or by light intensity. Similar trends could also be observed with the aeruginosin studied here. Analyzing both factors together showed that the higher temperature (25℃) and the lower irradiance (20 $\mu Em^{-2}s^{-1}$) favor the aeruginosin production; at the lower temperature (20℃) this effect could not be observed (Figure 25 and Table 17).

Table 16: Results of the multiple analysis of variance (MANOVA) for the total aeruginosin concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	23,48	0,0000
Temperature	32,72	0,0000
Medium	7,02	0,0009
Light + Temperature	8,36	0,0068
Light + Medium	0,22	0,87
Temperature + Medium	2,17	0,11
Light + Temperature + Medium	6,56	0,0013

* Statistically significant differences are highlighted in gray.

Table 17: Results of the Tukey HSD test for the total aeruginosin 102A concentration.

Aeruginosin 102A total			20℃								25℃							
			20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}				20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}			
			ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$
20℃	20 µEm^{-2}s^{-1}	ASM-1																
		N$^-$	ns															
		P$^-$	ns	ns														
		NP$^-$	ns	ns	ns													
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns												
		N$^-$	ns	ns	ns	ns	ns											
		P$^-$	ns	ns	ns	ns	ns	ns										
		NP$^-$	ns	ns	ns	ns	ns	ns	ns									
25℃	20 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N$^-$	*	*	*	*	*	*	*	*	*							
		P$^-$	*	*	ns	*	*	*	*	*	ns	ns						
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	*	ns	ns						
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*				
		N$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns			
		P$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * indicates significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular aeruginosin

Aeruginosin 102A was the only one of the 8 peptides studied that showed a high extracellular fraction, with the total concentration of aeruginosin 102A distributed almost 50% to 50% between intracellular and extracellular occurrence (Figure 26). The share of intracellular peptide concentration in relation to the total pool varied between 45% of the total peptide concentration at 25℃ and low light intensity in the medium free of N (N$^-$) reached up to 80% in full medium (ASM-1) and the same temperature and light regime.

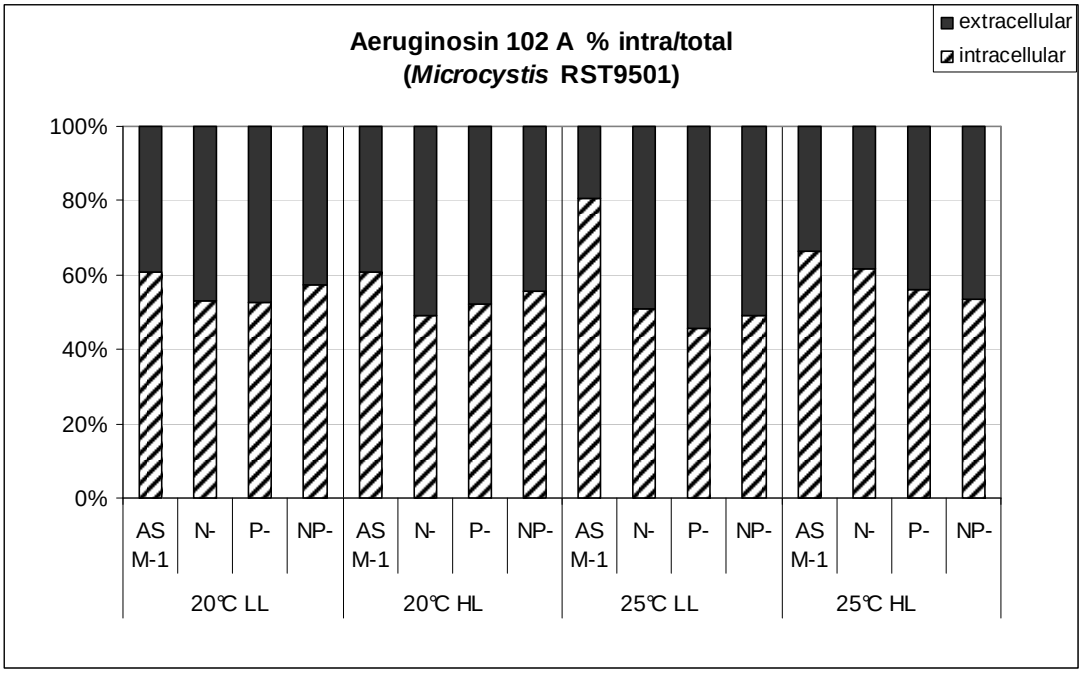


Figure 26: The percentage of the intracellular aeruginosin 102A in relation to the total peptide concentration under the different temperature and light conditions and the different nutrient compositions (LL= low light; HL=high light).

This aeruginosin 102A is a relatively small linear peptide, with 4 amino acids, and quite polar, as seen from a retention time of 2.8 minutes in the isocratic HPLC run, and these two properties may render the peptide more amenable to transport out of the cells, which would explain the balanced distribution of this peptide intracellularly and extracellularly.

Figure 26 shows that as for microcystin, at all four combinations of temperature and light, the share of intracellular aeruginosin was highest in the full medium (ASM-1) and lower in the media free of N (N^-), free of P (P^-) and free of both (NP^-). This effect was most pronounced at low light and 25℃.

Intracellular aeruginosin 102A

While extracellular occurrence may also be affected by biodegradation, intracellular concentration reflects cellular regulation of production and export from cells. As the ratio of intra to extracellular aeruginosin 102A showed some variability and represented only about half of the total pool, intracellular concentration was analyzed separately in order to assess whether it showed any patterns different from those for the total pool. The results indicated that patterns of intracellular concentration showed similar responses to different

nutrient composition, changes in light intensity and temperature as those observed for the total composition.

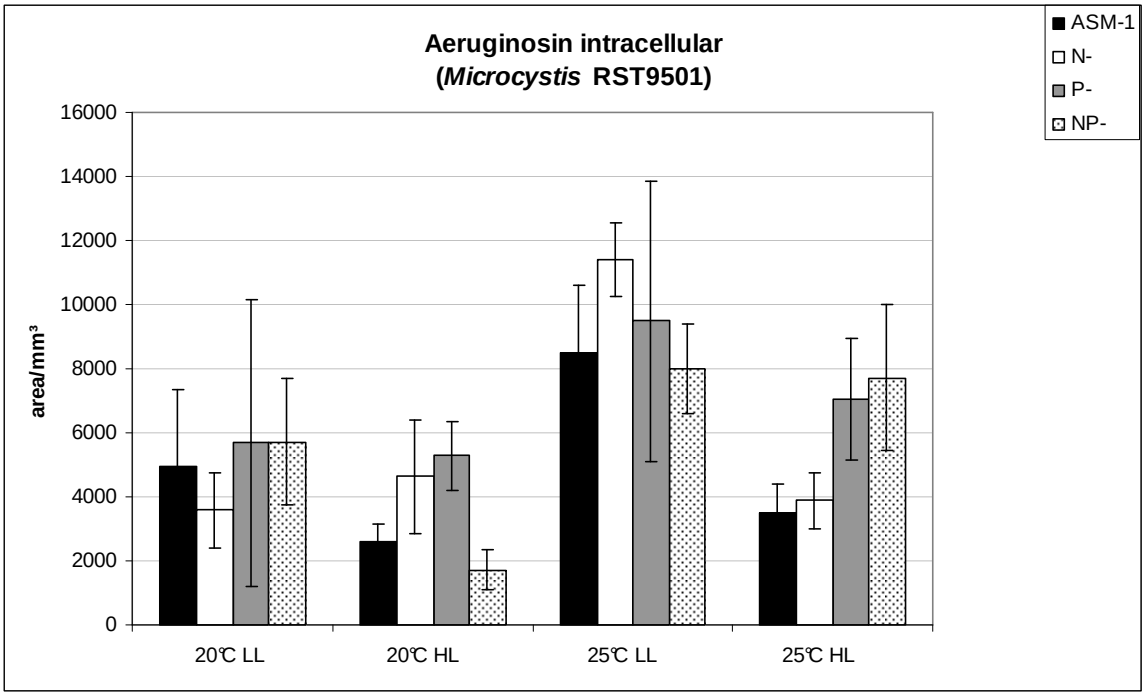


Figure 27: The intracellular aeruginosin 102A concentration under the different combination of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

The greatest difference between intra and extracellular concentration was the amount of aeruginosin: the maximal intracellular concentration was almost 2 fold lower than the total concentration of this peptide (Fig. 25 and 27). Patterns in relation to light intensity, temperature and nutrient concentration of the media were very similar. The intracellular peptide fraction, just as the total peptide concentration, was elevated at 25℃ (Figure 27).

As for the total aeruginosin concentration, this temperature and light condition enhanced the levels of this peptide especially in the media free of N (N^-) or N and P (NP^-), but at low light intensity also for in medium free of P (P^-) and for the full medium. For the medium free of N (N^-), at 25℃ and low light intensity the cellular concentration of this peptide was 3 fold higher than at high light intensity and 1.5 fold that at 20℃. The higher temperature, for the media free of P (P^-) and free of both (NP^-), increased cellular peptide concentration 1.5 fold compared to that at 20℃ und er both light intensities.

The intracellular concentration, as compared to the total aeruginosin 102A concentration, showed a higher internal variance, a greater standard

deviation, and not such an outstanding difference at 25℃ and low light intensity in the medium free of N (N^-) (Figure 27 and Table 19). In consequence, the Tukey HSD post-hoc test (Table 19) showed that less of the observations described above were statistically significant than for total aeruginosin 102A, but similar to total aeruginosin, significant differences were limited to peptide concentration at 25℃ as compare d to 20℃, particularly in the media free of N (N^-) and free of P (P^-) and at low light intensity.

The pronounced effect of light and temperature was confirmed by the results of the multiple analysis of variance (MANOVA), as described in section 2.4. For the intracellular aeruginosin concentration it showed statistical significance of the single effect of light intensity, the single effect of temperature and no significance of nutrient composition. No statistical significance was additionally found by the combined action of two components, but once all three components are taken together, a statistical significance can be seen (Table 18). These results were different to the obtained for total aeruginosin concentration, where also medium composition as a single factor and the interaction of light and temperature showed statistically significant impacts on total aeruginosin concentration.

Table 18: Results of the multiple analysis of variance (MANOVA) for the intracellular aeruginosin.

	$F_{(0,01,32)}$	p (<0.01)
Light	23,25	0,0000
Temperature	37,87	0,0000
Medium	2,37	0,088
Light + Temperature	5,92	0,020
Light + Medium	0,85	0,47
Temperature + Medium	0,72	0,54
Light + Temperature + Medium	6,22	0,0018

* Statistically significant differences are highlighted in gray.

Table 19: Results of the Tukey HSD test for intracellular aeruginosin 102A concentration.

Aeruginosin 102A intracellular			20℃								25℃							
			20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}				20 µEm^{-2}s^{-1}				60 µEm^{-2}s^{-1}			
			ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$	ASM-1	N$^-$	P$^-$	NP$^-$
20℃	20 µEm^{-2}s^{-1}	ASM-1																
		N$^-$	ns															
		P$^-$	ns	ns														
		NP$^-$	ns	ns	ns													
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns												
		N$^-$	ns	ns	ns	ns	ns											
		P$^-$	ns	ns	ns	ns	ns	ns										
		NP$^-$	ns	ns	ns	ns	ns	ns	ns									
25℃	20 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	*								
		N$^-$	ns	*	ns	ns	*	*	ns	*	ns							
		P$^-$	ns	*	ns	ns	*	ns	ns	*	ns	ns						
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns					
	60 µEm^{-2}s^{-1}	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns			
		P$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns		
		NP$^-$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

Conclusions for aeruginosin 102A

To conclude, intracellular aeruginosin concentration is enhanced by a higher temperature (here 25℃) and low light intens ity, independent of the media composition. Aeruginosin concentrations were higher in 10 of the 12 experiments lacking one or both of the nutrients (i.e. N$^-$, P$^-$ and NP$^-$), but this enhanced production only partially increased the intracellular pool, while a higher share was released extracellularly.

3.2.3.3 Anabaenopeptin

3.2.3.3.1 *Anabaenopeptin F*

Anabaenopeptin F was produced by the *Microcystis* BM 10 and the *Aphanizomenon* X008a. In this *Microcystis* strain, anabaenopeptin F was the major peptide. This peptide is rarely found together with microcystin in the environment (Fastner *et al.*, 2001, Welker *et al.*, 2004), and also in both strains studied, microcystin was not produced. Figure 28 shows the PSD spectrum of this peptide.

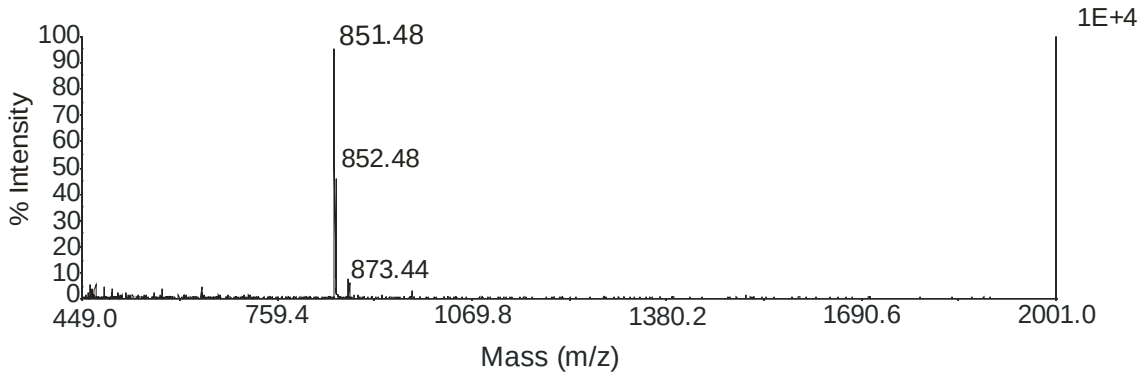


Figure 28: The PSD spectrum of the mass signal $M+H^+$ = 851, anabaenopeptin F, produced by the *Microcystis* strain BM 10 and the *Aphanizomenon* strain X008a.

Anabaenopeptin F concentrations in relation to biovolume

***Microcystis* BM 10**

The anabaenopeptin F present in the *Microcystis* strain showed the highest concentration when grown at 20℃ in the full medium (ASM-1) and at low light intensity or in medium free of P (P^-) and high light intensity (Figure 29).

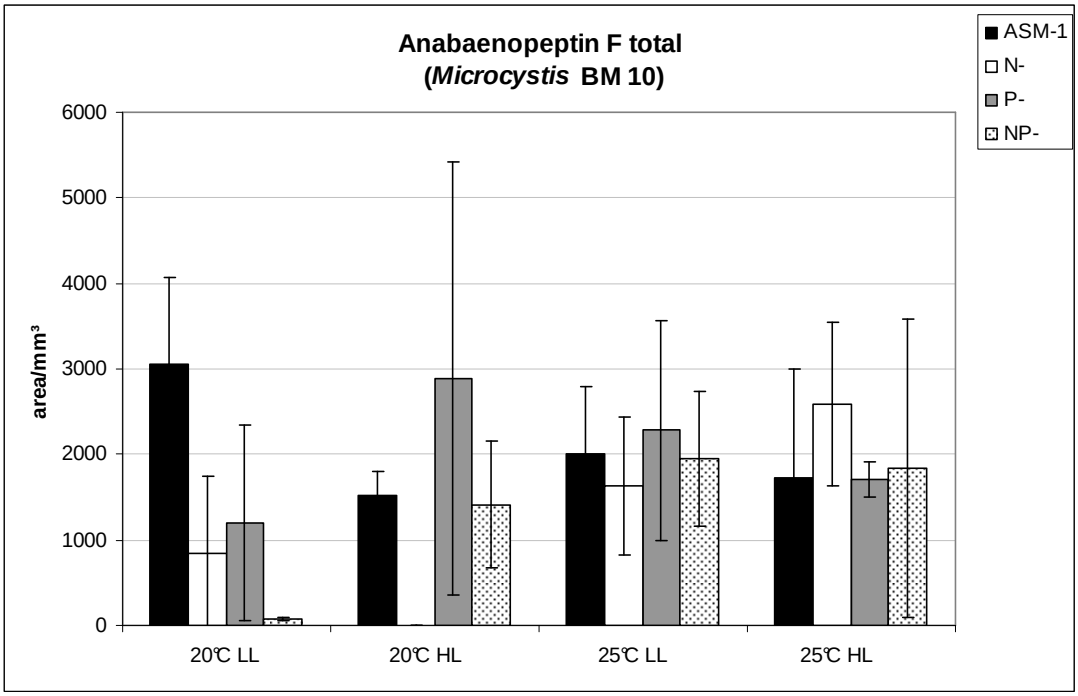


Figure 29: The total anabaenopeptin F, produced by *Microcystis* BM 10, concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

At 20℃ growth of this strain was very poor under two conditions, i.e. in the medium free of N (N^-) at high light and in the medium free of N and P (NP^-) at low light, resulting in negligible or not detectable levels of anabaenopeptin F. At a higher temperature, 25℃, these difficulties were overcome (Figure 29).

In general, as observed in figure 29, this peptide concentration was quite similar under the different combinations of temperature, light intensity and media composition. The MANOVA and the Tukey HSD test showed differences to be statistically significant only for the two situations in which this strain showed very little growth and correspondingly poor peptide production, i.e. 20℃ and low light intensity in medium free of both (NP^-) and 20℃ and high light intensity in medium free of N (N^-) (Table 20 and 21).

Temperature did not show a statistically significant impact on anabaenopeptin F concentration, but at 20℃, nutrient deficiency affected the anabaenopeptin F concentration per unit biovolume. Under low light intensity was the peptide concentration almost not detectable once both nutrients were absent (NP^-), but the growth rate was similar to the full medium, suggesting that energy available was used rather in growth than in peptide production. Under the higher light intensity, the absence of N made impossible for this

strain to grow, it survived 5 to 6 days with an optical density similar to the initial one and afterwards the culture died.

Table 20: Results of the multiple analysis of variance (MANOVA) for the total anabaenopeptin F (BM 10) concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	0,09	0,7594
Temperature	7,24	0,0112
Medium	3,70	0,0213
Light + Temperature	0,10	0,7441
Light + Medium	2,58	0,0707
Temperature + Medium	4,76	0,0074
Light + Temperature + Medium	5,05	0,0056

* Statistically significant differences are highlighted in gray.

Table 21: Results of Tukey HSD test for the total anabaenopeptin F (BM 10) concentration.

Anabaenopeptin F (BM 10) total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	*	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	*	ns	ns	ns	ns											
		P^-	ns	ns	ns	*	ns	*										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

***Aphanizomenon* X008a**

In general, in relation to biovolume the anabaenopeptin F concentration of *Aphanizomenon* X008a was at least five times higher than that of *Microcystis* BM 10 (see section 3.2.3.2).

The anabaenopeptin F present in this *Aphanizomenon* strain, similar to this peptide in the *Microcystis* strain, showed a high concentration in full medium (ASM-1) and medium free of P (P^-) (Figure 30). A consistent result under all combinations of light intensity and temperature was that when grown in the media free of N (N^-) or of both N and P (NP^-), the concentration of anabaenopeptin F was lower, indicating that the absence of N reduced the production of this peptide.

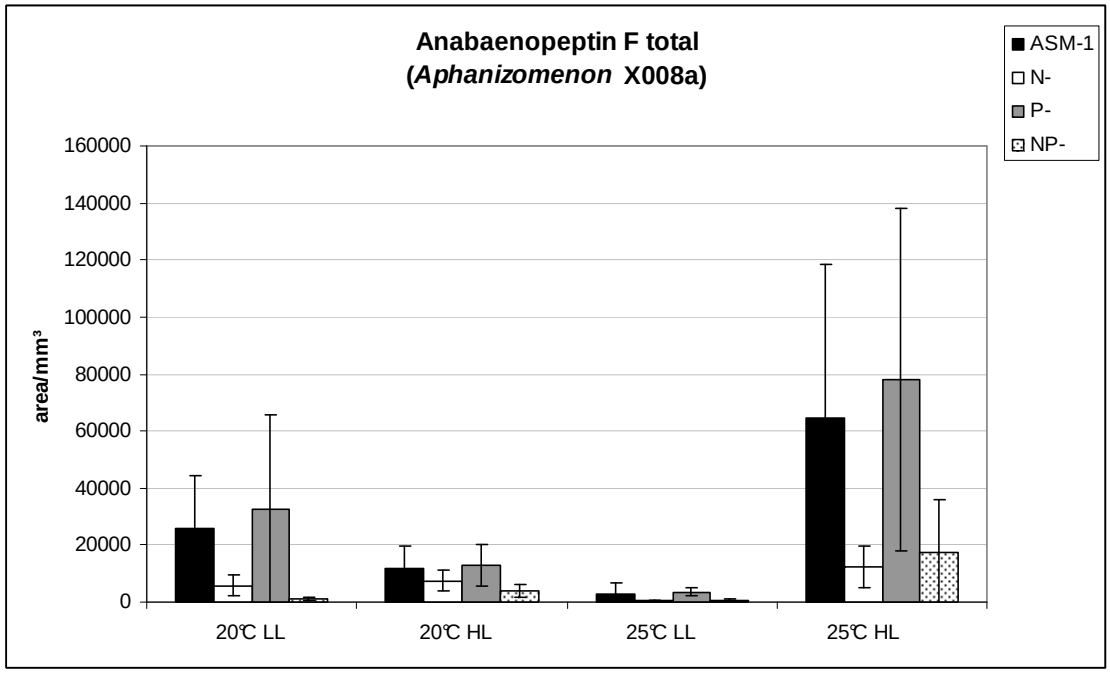


Figure 30: The total anabaenopeptin F, produced by *Aphanizomenon* X008a, concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

Aphanizomenon is a strain forming heterocysts for nitrogen fixation under nitrogen deficient conditions, and this could lead to differences in peptide concentration under N deficient conditions, as compared to the other anabaenopeptin F producer, the non-nitrogen fixing *Microcystis* strain. However, the results shown in Fig. 30 indicate that this ability to fix N does not seem to enhance the production of anabaenopeptin F in N deficient medium. Rather, the results suggested that in the absence of N, this strain invests energy into growth and survival rather than into producing a secondary metabolite.

A conspicuous result was that concentrations of anabaenopeptin F showed patterns depending on light intensity and temperature. They could be grouped in a sequence, with the lowest concentration observed at 25℃ and low light intensity, followed by those at 20℃ first at high and then at low light intensity, and by far the highest observation at the high temperature and the high light intensity. The MANOVA (Table 22) showed that the impact of light intensity and temperature together was significant, as was the action of these two factors and medium composition. At 25℃ and high light intensity, anabaenopeptin F production reached more than four times the amount produced under the same conditions at 20℃.

Even though figure 30 gave the impression that the full medium (ASM-1) and the medium free of P (P^-) at 25℃ and high light intensity were statistically significantly different from all other combinations, this was only true for the medium free of P (P^-), according to the Tukey HSD test carried out as described in section 2.4 (Table 23).

Once N was available, the anabaenopeptin F concentrations were elevated, indifferent of the combination of temperature and light intensity. Meaning that as long as N was available for growth and survival, anabaenopeptin F production increased.

Table 22: Results of the multiple analysis of variance (MANOVA) for the total anabaenopeptin F (X008a) concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	11,137	0,0021
Temperature	2,843	0,1014
Medium	8,626	0,0002
Light + Temperature	25,540	0,0000
Light + Medium	1,153	0,3426
Temperature + Medium	0,8392	0,4825
Light + Temperature + Medium	5,3890	0,0041

* Statistically significant differences are highlighted in gray.

Table 23: Results of Tukey HSD test for the total anabaenopeptin F (X008a) concentration.

Anabaenopeptin F (X008a) total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	*	ns	*										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	*	ns	*	*	*	*	*	*	*	*	*	ns	*		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular anabaenopeptin F

Microcystis BM 10

In the *Microcystis* BM 10, over 90% of the produced anabaenopeptin F remained in the cells, with the exception of anabaenopeptin produced at 20℃ and low light intensity in the medium free of N and P (NP^-). In this case only 40% of the total amount remained in the cells, but as described below (see Fig. 31), altogether very little of this peptide was produced. This indicates that in a case of extreme stress this peptide may be released.

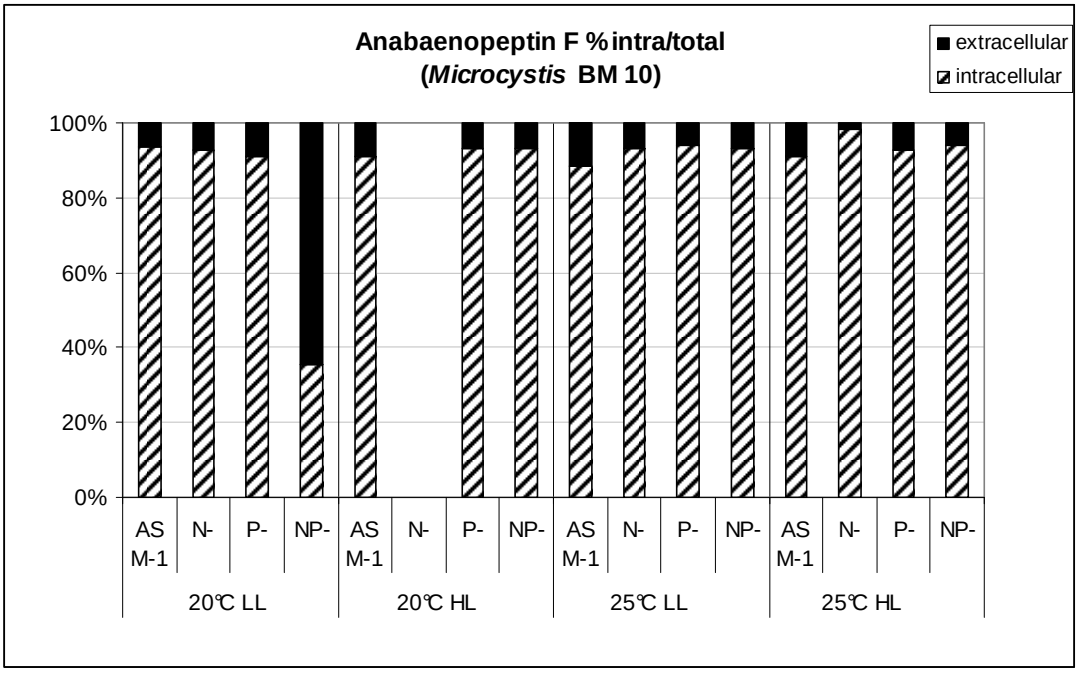


Figure 31: The percentage of the intracellular anabaenopeptin F, produced by *Microcystis* BM 10 in relation to the total peptide concentration under the different temperature and light conditions and the different nutrient concentrations. (LL= low light; HL=high light).

***Aphanizomenon* X008a**

For *Aphanizomenon* X008a, the pattern was somewhat different: although always more than 60% and in some cases up to 98% of the anabaenopeptin F remained in the cells, there was more release at 25℃ and low light intensity. Within this temperature and light condition, the pattern in relation to nutrient deficiency was similar to the one seen for both microcystin and aeruginosin, i.e. less export in full (ASM-1) medium than in medium deficient of one or both of these essential nutrients (Figure 32). This indicates a combined stress situation due to enhanced metabolism at the higher temperature, but low energy input at the low light intensity and additionally nutrient limitation. Noticeably, in this situation with an enhanced peptide export, the production of anabaenopeptin F was lower than at the same temperature, but higher light intensity.

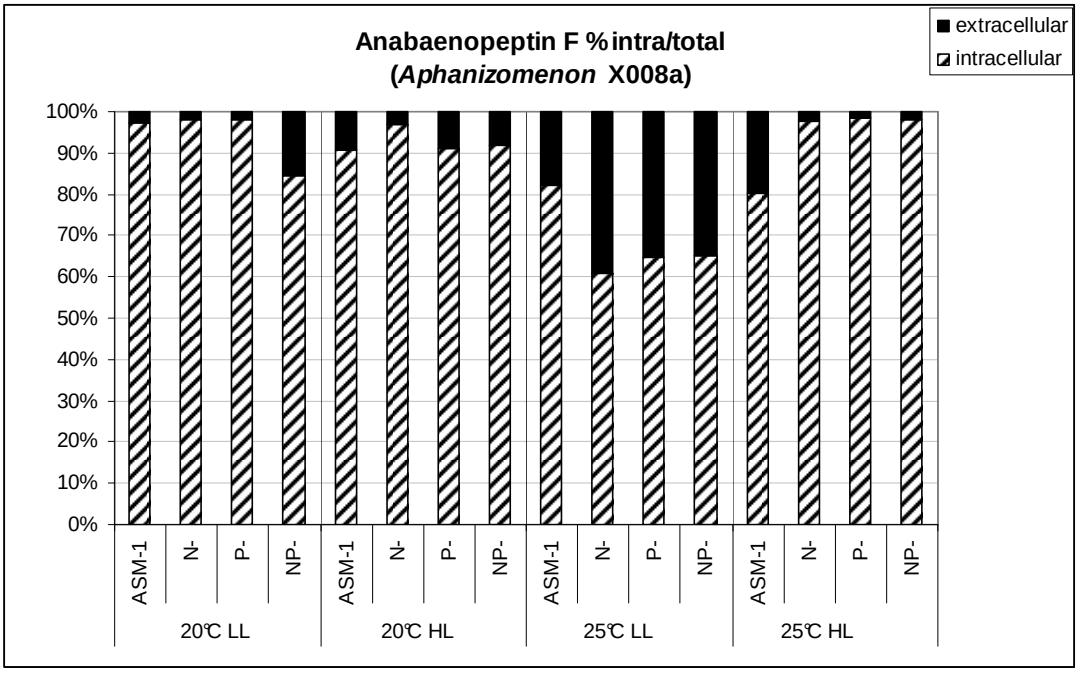


Figure 32: The percentage of the intracellular anabaenopeptin F, produced by *Aphanizomenon* X008a in relation to the total peptide concentration under the different temperature and light conditions and the different nutrient concentrations. (LL= low light; HL=high light).

Differently from the microcystin and the aeruginosin, in most cases (i.e. with the exception of low light intensity and the higher temperature for *Aphanizomenon* X008a), nutrient deficient medium did not enhance peptide export. Overall, both strains appeared to maintain most of the anabaenopeptin F pool within the cells, largely independent of the medium composition or temperature and light condition under which they grow. Only in case of extreme stress, i.e. high temperature and low light availability for the *Aphanizomenon* strain and deficiency of both nutrients at low light availability for the *Microcystis* strain, was a larger fraction of anabaenopeptin F released out of the cells.

3.2.3.3.2 *Oscillamide Y*

Oscillamide Y was produced by two *Microcystis* strains studied, i.e. the Brazilian NPRG-2 and the German BM 10. The figure below shows the PSD spectrum of this peptide (Figure 33).

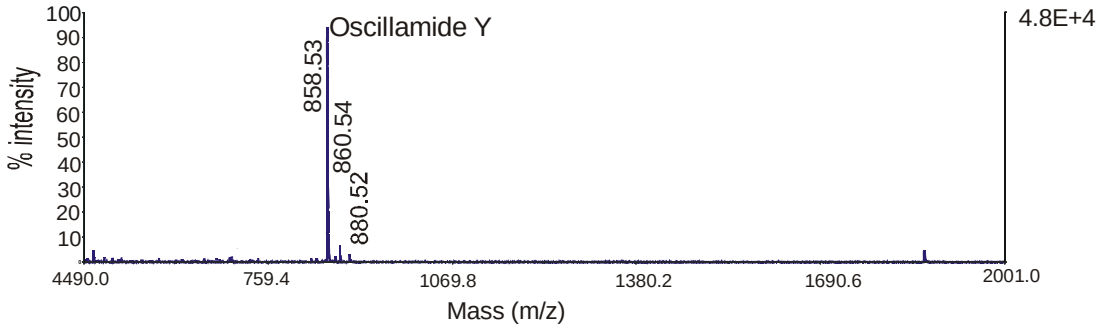


Figure 33: The PSD spectrum of the mass signal M+H = 858, oscillamide Y, produced by the *Microcystis* NPRG-2 and BM 10 strains.

Oscillamide Y concentrations in relation to biovolume

Microcystis BM 10

In the *Microcystis* BM 10 strain, estimating from peak areas the amount of oscillamide Y per cell biovolume was relatively small when compared to the amount of the other peptides in this strain (Figure 29 and 49). This seemed to be strain specific, since in contrast, oscillamide Y was the major peptide in the *Microcystis* strain NPRG-2. At 20℃ in high light intensity in medium free of N (N^-), there was no growth of this strain, so as well as for the anabaenopeptin F, no oscillamide Y was produced.

Figure 34 shows the total oscillamide Y concentration was by far the highest at 25℃ in high light intensity and full me dium (ASM-1).

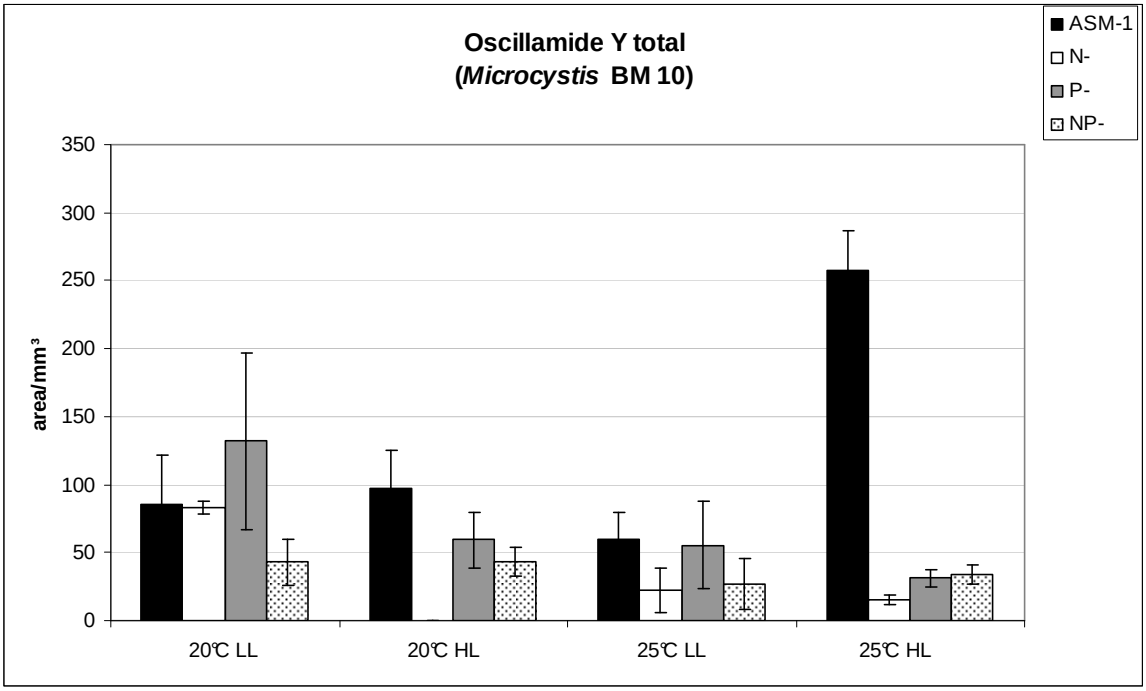


Figure 34: The total oscillamide Y concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

At 20℃ the total (intra plus extracellular) oscillamide Y production varied less than 2 fold between light intensities. The full medium results in slightly increased concentration at the high light intensity, the medium free of P (P^-) medium decreases concentration about 2 fold at the higher light intensity and this decrease on peptide concentration was statistically significant (see Table 25). For the medium free of N and P (NP^-) the concentration seemed not to be affected by light intensity.

At 25° the increase on light intensity caused a 4 fold increase of peptide production in full medium (ASM-1) compared to the low light intensity. In the media free of N (N^-) and free of P (P^-) there was a little decrease of peptide concentration at high light intensity. In the medium free of N and P (NP^-) the intensity of light was irrelevant, when compared to 20 ℃ a decrease of concentration was observed with the increase of temperature.

The differences in peptide concentration at the different temperature and light intensities taken alone were not statistically significant as tested by MANOVA, only medium composition taken alone proved to be significant (Table 24).

Considering the different media compositions, peptide concentration is higher in full medium (ASM-1), though this did not prove to be statistically by the Tukey test (Table 25). At 20℃ and low light intensity the amount of oscillamide Y produced in the full medium (ASM-1) is lower than the production at medium free of P (P^-) condition, but only in this combination of conditions. The absence of N caused almost no difference to the peptide production at 20℃ and low light intensity, but analyzing the peptide concentration under the high light intensity or at 25℃, this decreased to at least half of that in the full medium. Interestingly, in the medium free of N and P (NP^-), in both light intensities at 25℃ more oscillamide Y was produced as in the absence of only N.

For the medium free of P (P^-) at 20℃ and low light intensity oscillamide Y concentrations proved to be statistically significant different from medium free of both (NP-) at 20℃ and from all combination of difference media composition at 25℃. The full medium (ASM-1) at 25° C and high light intensity was found to induce statistically significantly different concentrations as compared to all combinations of media composition, independently of temperature or light intensity (Table 24). This information showed that the observation from Figure 34 was statistically significant (Table 24).

Table 24: Results of the multiple analysis of variance (MANOVA) for the total oscillamide Y concentration in the *Microcystis* BM 10.

	$F_{(0,01,32)}$	p (<0.01)
Light	0,36	0,5492
Temperature	0,74	0,3959
Medium	51,17	0,0000
Light + Temperature	43,07	0,0000
Light + Medium	34,54	0,0000
Temperature + Medium	17,75	0,0000
Light + Temperature + Medium	10,10	0,0000

* Statistically significant differences are highlighted in gray.

Table 25: Results of Tukey HSD test for total oscillamide Y concentration in the *Microcystis* BM 10.

Oscillamide Y total BM 10			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	*													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	*	*	*	ns	*											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	*	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	*	ns	*	ns	ns	ns	ns							
		P^-	ns	ns	*	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	*	*	*	*	*	*	*	*	*	*	*	*				
		N^-	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns	ns	*			
		P^-	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns		
		NP^-	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

***Microcystis* NPRG-2**

The total oscillamide Y presented in the *Microcystis* strain NPRG-2 (Figure 35) showed the same enhanced production in the full medium (ASM-1) and high light intensity as for the strain *Microcystis* BM 10, but in this strain at lower temperature (20℃).

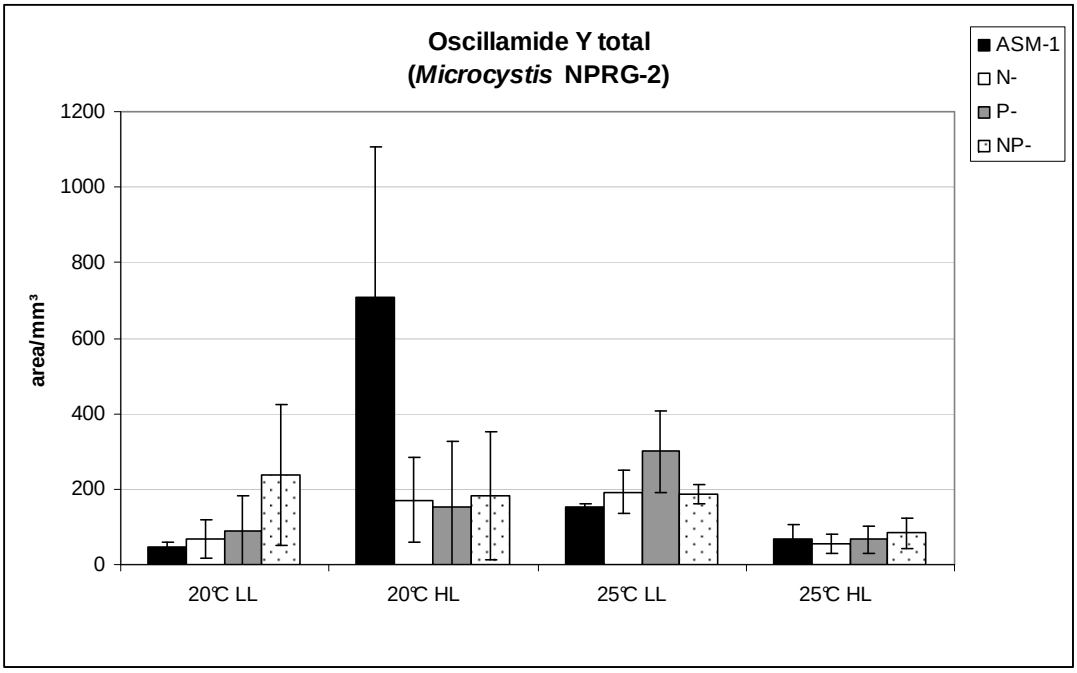


Figure 35: The total oscillamide Y concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

In the strain *Microcystis* NPRG-2, the oscillamide Y concentration at 20℃ and low light intensity was very modest in the full medium (ASM-1), as well as in the media free of N (N^-) and free of P (P^-), but interestingly in the medium free of both N and P (NP^-) it was almost 2 fold that in the full medium (ASM-1), although in consequence of high standard deviations of the results this difference was not statistically (see Table 27). At the higher light intensity and in full medium (ASM-1) concentrations of this peptide were almost 10 fold higher. In the media free of N (N^-) and free of P (P^-), concentrations were slightly higher than at low light, but this was again, not statistically significant (see Table 27). In the medium free of N and P (NP^-), the concentrations of oscillamide Y were similar to those in low light intensity.

At 25℃ light intensity affected the peptide concen tration in a different way. The concentrations of oscillamide Y were higher at low light intensity than at high light intensity, but this difference did not prove to be statistically significant (Table 27). The differences between concentrations of oscillamide Y in full medium (ASM-1) and medium free of N and P (NP^-) were in the range of a factor of 1.5 factor but no statistical significantly differences could be found (Table 27). For the medium free of N (N^-) and free of P (P^-) this factor

reached 3 fold the production under low light intensity and here also no statistically significant differences were found.

The Tukey HSD post-hoc test confirmed the observation in figure 35. According to this test only one combination of conditions seemed to induce relevantly different peptide concentrations: The association of full medium (ASM-1), 20℃ and high light intensity caused an in crease of peptide concentrations which was statistically significantly different from all other combinations of temperature, light intensity and media composition (Table 27).

With the exception of the light as a single factor affecting peptide production, the MANOVA showed all other factors to cause statistically significant differences in oscillamide Y concentrations. The combined action of two or all three factors caused always statistically significant differences in peptide concentration (Table 26).

Even though no effect of light as single factor showed to be significant different, as described by previous studies, the interrelationship of temperature and light is an important regulatory factor for cyanobacteria. This explain the difference on oscillamide Y production in the *Microcystis* strain NPRG-2, in which according to the temperature tested there was an increase or decrease on peptide concentration in high light intensity (Figure 35).

Table 26: Results of the multiple analysis of variance (MANOVA) for the total oscillamide Y concentration in the *Microcystis* NPRG-2.

	$F_{(0,01,32)}$	p (<0.01)
Light	1,32	0,2589
Temperature	8,35	0,0068
Medium	4,58	0,0088
Light + Temperature	37,76	0,0000
Light + Medium	10,99	0,0000
Temperature + Medium	6,77	0,0011
Light + Temperature + Medium	8,64	0,0002

* Statistically significant differences are highlighted in gray.

Table 27: Results of Tukey HSD test for total oscillamide Y concentration in the *Microcystis* NPRG-2.

Oscillamide Y total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	*	*	*	*												
		N^-	ns	ns	ns	ns	*											
		P^-	ns	ns	ns	ns	*	ns										
		NP^-	ns	ns	ns	ns	*	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	*	ns	ns	ns								
		N^-	ns	ns	ns	ns	*	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular oscillamide Y

Microcystis BM 10

For the oscillamide Y present in the strain *Microcystis* BM 10, the intracellular peptide concentration varied between 60% of the total peptide concentration, at 25℃ and high light intensity in medium free of P (P^-), and 100% of total peptide concentration. There was one outlier, as seen in Figure 34, in the full medium (ASM-1) at 25℃ and high lig ht intensity. In this case less than 20% of the total peptide was located intracellularly. Notably, this was also the one condition under which the total concentration of oscillamide Y was several fold higher than under the others (compare Fig. 36).

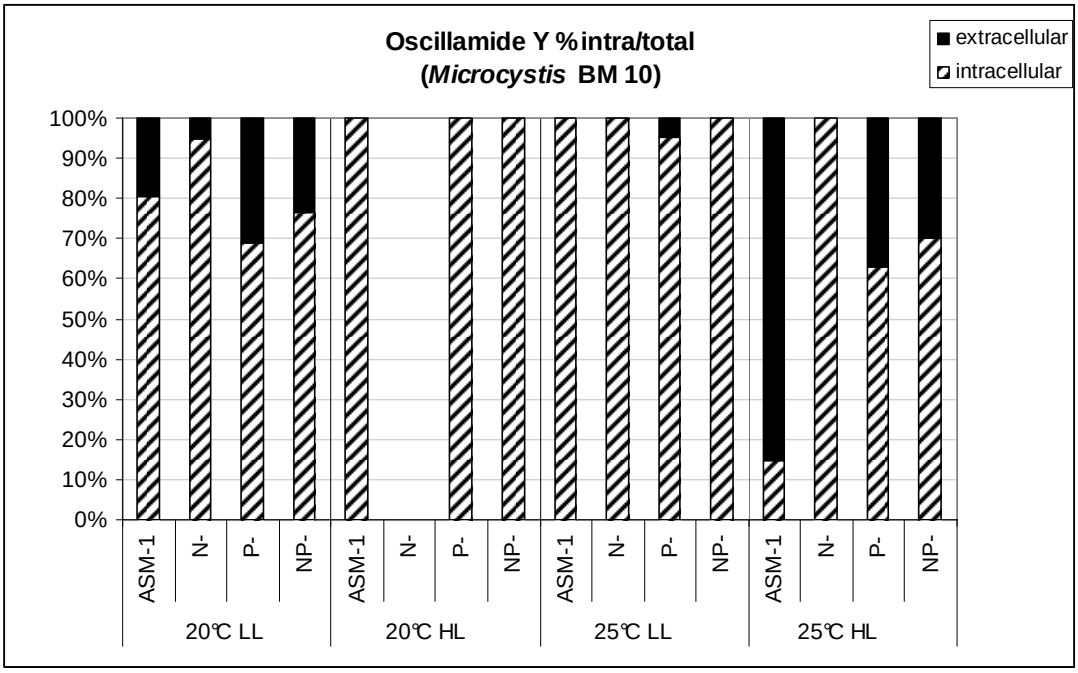


Figure 36: The percentage of the intracellular oscillamide Y (BM 10) in relation to the total peptide concentration under different temperature and light conditions and the different nutrient compositions. (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

***Microcystis* NPRG-2**

The strain *Microcystis* NPRG-2 showed a clear temperature effect on the ratio between intra and extracellular oscillamide Y. At 20℃, 70-95% remained intracellular (Figure 37). At a higher temperature, at 25℃, only between 40 and 60% of this peptide was found in the cells.

At 25℃ and high light intensity, once again there was less export of this peptide in full medium (ASM-1) than in all three nutrient deficient media. As for the anabaenopeptin F, this was observed only under one condition, here at 25℃ and high light intensity, whereas for anabaeno peptin F this pattern was seen at 25℃ and low light intensity.

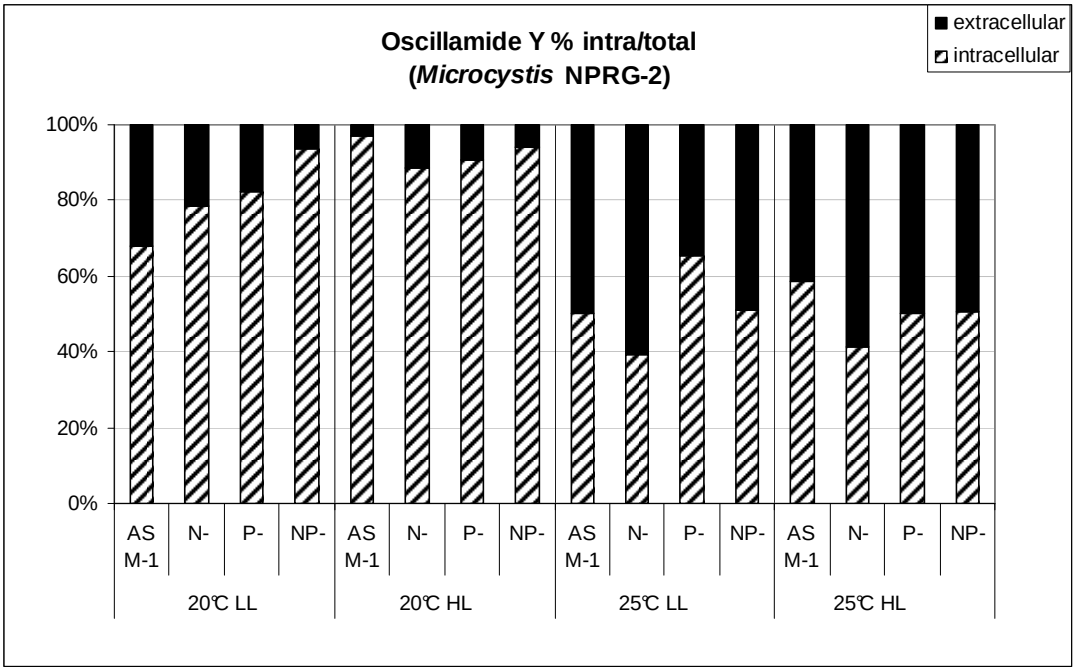


Figure 37: The percentage of the intracellular oscillamide Y (NPRG-2) in relation to the total peptide concentration under different temperature and light conditions and the different nutrient compositions. (LL= low light; HL=high light).

Intracellular oscillamide Y in *Microcystis* BM 10

Figure 38 shows the intracellular oscillamide Y in the strain *Microcystis* BM 10. This pattern observed here was very similar to that for the total peptide concentration (compare Figure 34), but without the great concentration in the full medium (ASM-1) at 25℃ and high light intensity, reflecting the major fraction of the extracellular fraction under this special condition as shown in Fig. 38. This result provided the opportunity to revisit the statistical significance of differences in the concentration of this peptide without statistical significance being strongly influenced by one extreme result.

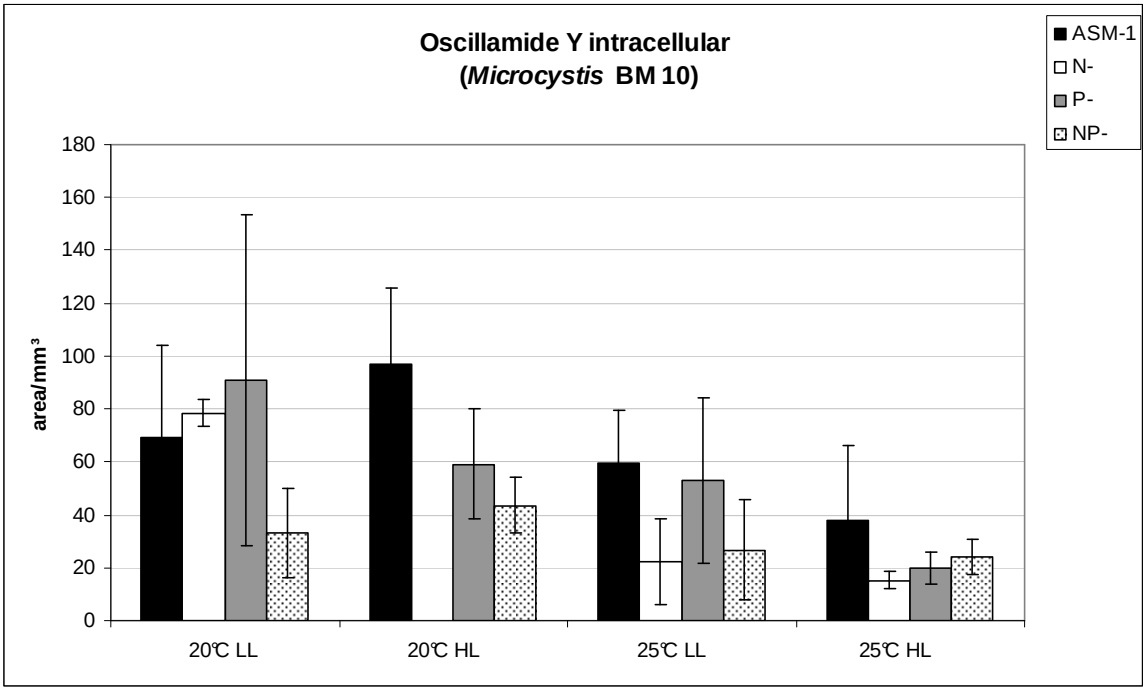


Figure 38: The intracellular oscillamide Y concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

The intracellular oscillamide Y concentration in the *Microcystis* BM 10 appeared to be favored by 20℃ as compared to the h igher temperature, and the MANOVA test showed temperature to have a significant impact (see Table 28). Further, the oscillamide Y concentration in medium free of P (P^-) in low light intensity was significantly different (see Table 29) from that observed in most of the other nutrient deficient media (N^- and NP^-) conditions. The same observation was made in full medium (ASM-1) at 20℃ and high light intensity. Though the results seen in Fig. 38 indicate some further differences in peptide concentrations, these proved not to be statistically significant (Table 29), due to the large standard deviation between the results.

In the same way as the aeruginosin 102A, the significance of intracellular patterns for this peptide differs from that for the total peptide concentration. Table 28 shows the multiple analysis of variance (MANOVA). Opposite to the total peptide, here all three factors analyzed separately had a statistically significant impact on peptide concentration. The combined action of light intensity plus temperature and temperature plus medium was not significant, but the interaction of light intensity, temperature and medium was statistically significant.

The difference to the total oscillamide Y could also be seen in the Tukey HSD test. In this case the peptide concentrations in medium free of P (P^-) at 20℃ and low light intensity and the full medium (ASM-1) at 20℃ and high light intensity were higher, and this result was statistically significantly different from the peptide concentration in medium free of N (N^-) at 20℃ and high light intensity, and at 25℃ in both light intensities. The peptide concentration was also higher and statistically significant different from medium free of P (P^-) and medium free of both (NP^-) at 25℃ and high light intensity (Table 29).

Table 28: Results of the multiple analysis of variance (MANOVA) for the intracellular oscillamide Y concentration in the *Microcystis* BM 10.

	$F_{(0.01,32)}$	p (<0.01)
Light	10,28	0,0030
Temperature	24,99	0,0000
Medium	11,47	0,0000
Light + Temperature	0,02	0,8724
Light + Medium	5,07	0,0055
Temperature + Medium	1,26	0,3042
Light + Temperature + Medium	5,71	0,0030

* Statistically significant differences are highlighted in gray.

Table 29: Results of Tukey HSD test for intracellular oscillamide Y concentration.

Oscillamide Y intracellular			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	*	*	*	ns	*											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	*	ns	*	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	*	ns	*		ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP^-	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

3.2.3.4 Microviridin

The peptide microviridin was only present in one of the three *Microcystis* strains, *Microcystis* RST9501.

Microviridin concentrations in relation to biovolume

Figure 39 shows a pronounced variation of this *Microcystis* strain's total microviridin concentration. Concentrations of total microviridin in relation to cell biovolume appeared particularly elevated in both media free of N (N^-) and free of P (P^-).

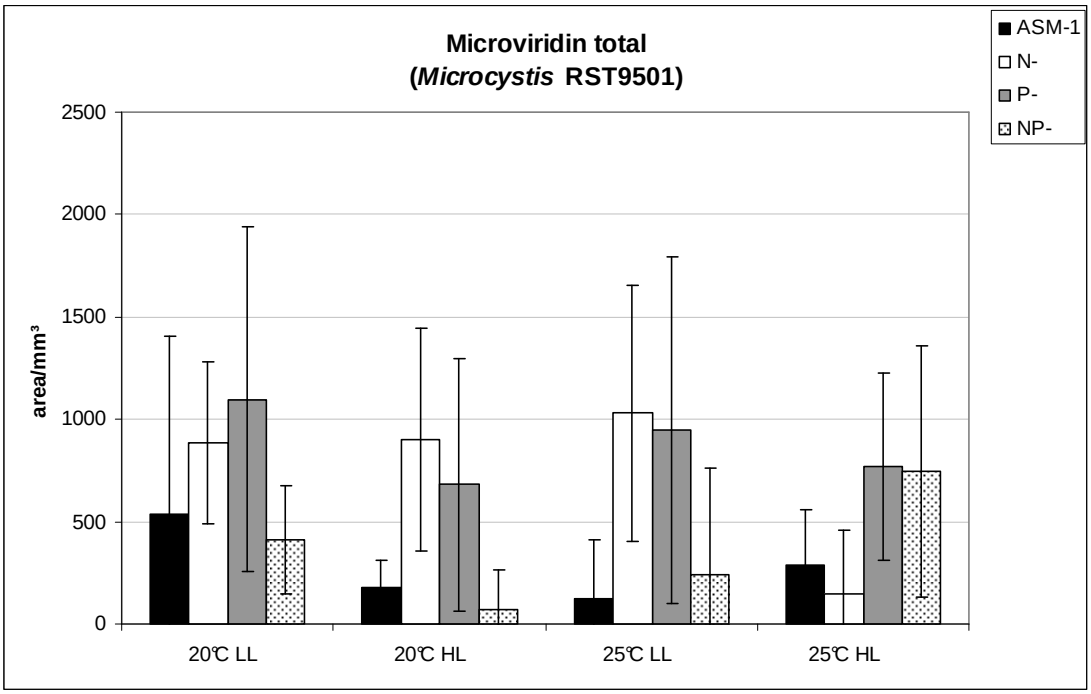


Figure 39: The total microviridin concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

However, variability between the triplicate experiments was also very pronounced as shown by the standard deviation. In consequence, the Tukey HSD post-hoc test (Table 31) indicated none of these differences in peptide concentration to be statistically significant. Likewise, multiple variance analysis showed none of the growth parameters to have a statistically significant impact on the concentration of microviridin, neither singly nor for any combined action of temperature, light intensity or medium composition (Table 30).

Table 30: Results of the multiple analysis of variance (MANOVA) for the total microviridin concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	4,28	0,047
Temperature	1,52	0,226
Medium	3,00	0,044
Light + Temperature	0,27	0,606
Light + Medium	0,56	0,642
Temperature + Medium	0,659	0,582
Light + Temperature + Medium	1,80	0,166

Table 31: Results of Tukey HSD test for the total microviridin concentration.

Microviridin total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

n. s. = not significant. * indicates the significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular microviridin

The intracellular fraction in most cases amounted to over 80% of the total microviridin pool, with an exception at 20℃ and low light intensity in medium free of N and P (NP^-). In this situation the intracellular peptide concentration decreased to 65% of the total pool (Figure 40).

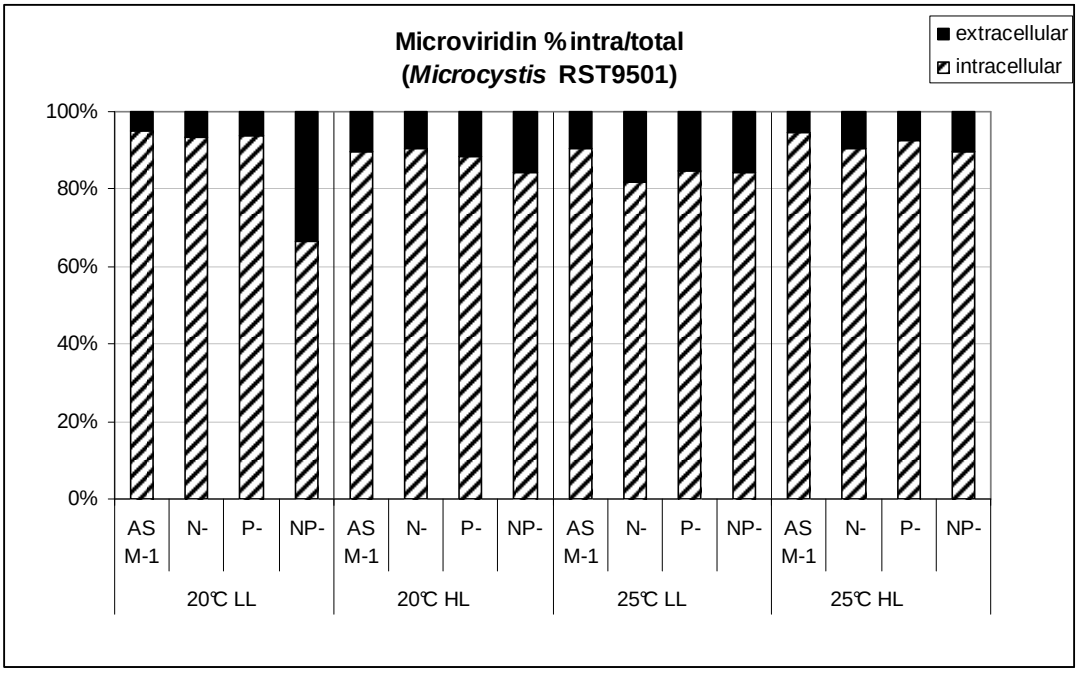


Figure 40: The percentage of the intracellular microviridin in relation to the total peptide concentration under the different temperature and light condition and the different nutrient composition. (LL= low light; HL=high light).

As the percentage of the intracellular microviridin concentration in relation to the total microviridin concentration was so high, consequently no difference of the effect of temperature, light intensity and nutrient concentration was found between total and intracellular microviridin concentration.

The only conclusion possible for this peptide was that this strain produced very variable amounts of microviridin and while nutrient limitation appeared to enhance concentration as compared to full medium (ASM-1), temperature and light intensity showed no impact. For any further conclusion, new experiments using a different strain and a different microviridin variant may clarify if any environmental conditions may have an influence on microvidirin concentration.

3.2.3.5 Microginin

This peptide was only produced by *Microcystis* NPRG-2. The figure below shows the PSD spectrum of this peptide (Figure 41).

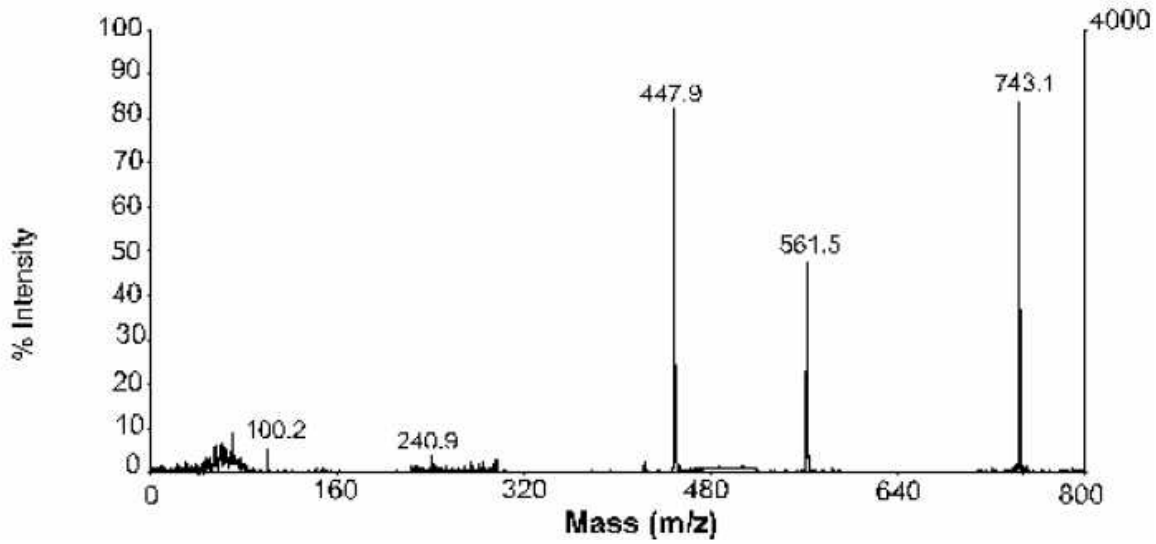


Figure 41: The PSD spectrum of the mass signal M+H = 742, microginin, produced by the *Microcystis* NPRG-2.

Microginin concentrations in relation to biovolume

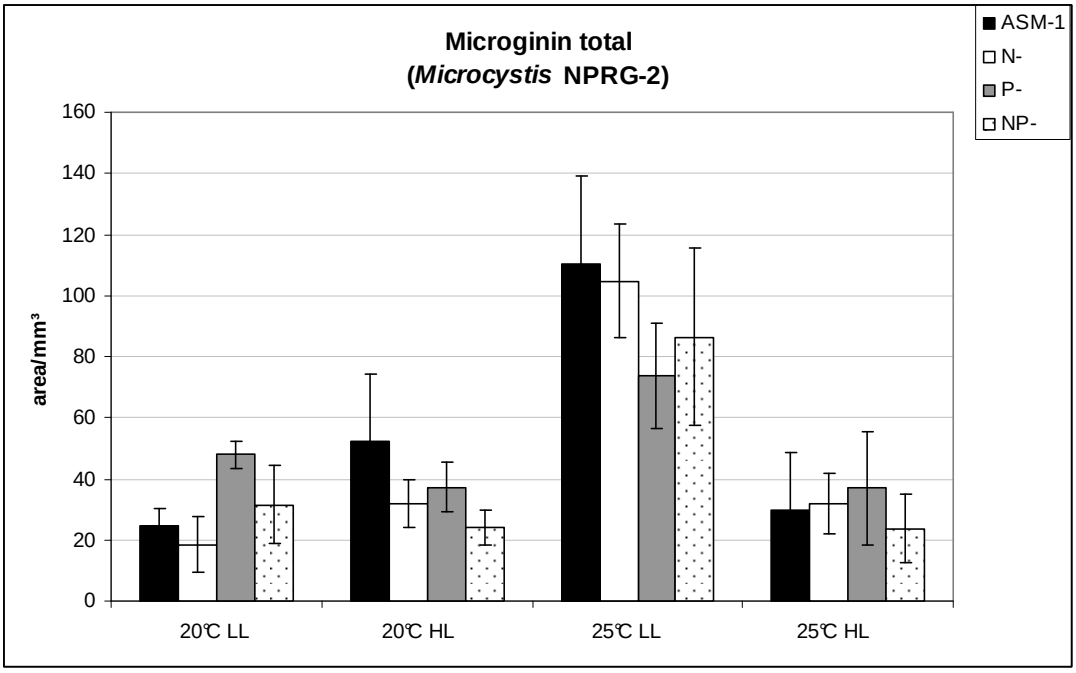


Figure 42: The total microginin concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

The most prominent result for microginin concentration was that it was significantly higher in all media at the higher temperature, 25℃, and the low light intensity (Figure 42). In full medium, this amounted to a 5.5 fold higher level than that at 20℃ and low light intensity and this increase of peptide production was statistically significant (Table 33). Under the higher light intensity, the microginin concentrations at 25℃ were in the range found at 20℃.

At 20℃ and low light intensity, the concentrations of microginin found in full medium (ASM-1) and the medium free of N (N^-) were similar and almost half of those found in the media free of P (P^-) and free of N and P (NP^-). At the higher light intensity, however, patterns reversed and concentrations increased about 2.5 fold in full medium (ASM-1), and this increase was statistically significant (Table 33). In the media free of P (P^-) and free of N and P (NP^-) at a higher light intensity, concentrations of this peptide were lower, but this proved not to be statistically significant (Table 33).

The comparison, carried out using the Tukey HSD post-hoc test, confirmed that the results for full medium (ASM-1) and medium free of N (N^-) at 25℃ and low light intensity were statistically significantly different from those found at 20℃ and both light intensities in all media compositions, as well as from those observed at 25℃ and high light intensity. The microginin concentrations in media free of P (P^-) and free of N and P (NP^-) at 25℃ and low light intensity were also statistically different from those at 20°C and low light intensity and 25℃ and high light intensity (Table 32).

The multiple analysis of variance (MANOVA) for the total microginin concentration demonstrated that the effects of temperature alone, light intensity alone and nutrient composition alone were statistically significant. The combined action of temperature and light intensity and therefore the interaction of all three components confirmed the observations from Figure 42 (Table 33).

Table 32: Results of the multiple analysis of variance (MANOVA) for the total microginin concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	35,52	0,0000
Temperature	31,59	0,0000
Medium	6,53	0,0014
Light + Temperature	77,72	0,0000
Light + Medium	1,09	0,3635
Temperature + Medium	1,54	0,2227
Light + Temperature + Medium	10,93	0,0000

* Statistically significant differences are highlighted in gray.

Table 33: Results of Tukey HSD test for the total microginin concentration.

Microginin total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	*	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	*	*	*	*	*	*	*	*								
		N^-	*	*	ns	*	ns	ns	ns	*	ns							
		P^-	*	*	ns	ns	ns	ns	ns	*	*	ns						
		NP^-	*	*	ns	ns	ns	ns	ns	*	*	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	*	ns	ns	ns	ns	ns	ns	*	*	ns	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns	ns			
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	ns	ns	ns	

n. s. = not significant. * indicates the significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular microginin

For this microginin, the intracellular fraction represented more than 70% of the total peptide pool. In most cases only 10 to 20% was found extracellularly. Only at 25℃ and low light intensity, i.e. the condition with the highest concentrations of this peptide, was there a higher percentage of extracellular peptide, ranging from 20 to 30% (Figure 43).

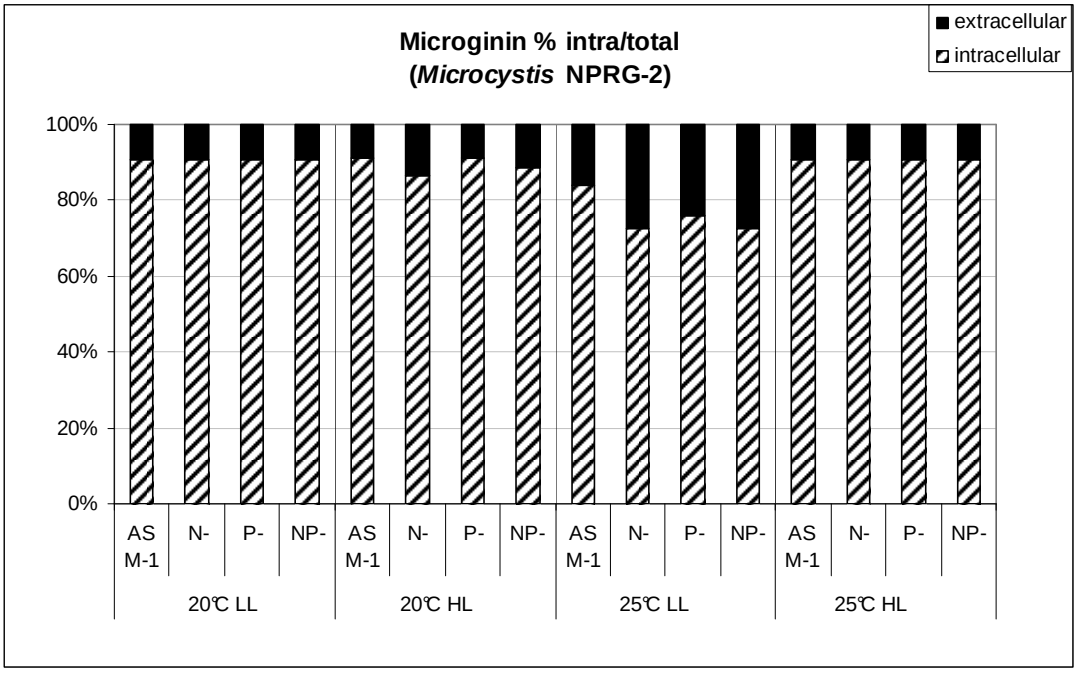


Figure 43: The percentage of the intracellular microginin in relation to the total peptide concentration under the different temperature and light condition and the different nutrient composition. (LL= low light; HL=high light).

3.2.3.6 Aphapeptin F1

This newly described cyanopeptide, done during this study, was isolated from an *Aphanizomenon* strain as explained in section 2.2, and the chemical structure of this peptide was identified (see section 3.1.1). It proved not to be bioactive to any cell line activity test, but to inhibit trypsin in *D. magna* (see section 3.1.2). Figure 44 shows the peptide fragment patterns.

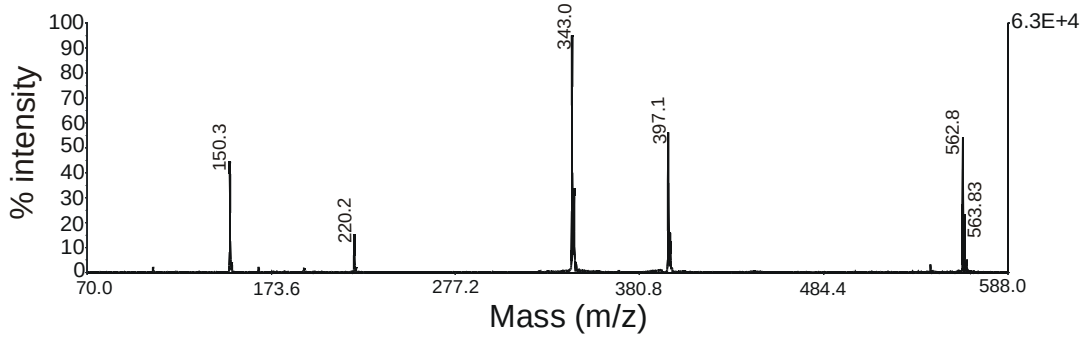


Figure 44: The PSD spectrum of the mass signal $M+H^+$ = 562, produced by the *Aphanizomenon* X008a.

Aphapeptin F1 concentrations in relation to biovolume

The higher temperature, and higher light intensity enhanced the peptide production for all nutrient conditions, but especially for full medium (ASM-1) and the medium free of P (P^-). The peptide production at 25℃, high light intensity and medium free of P (P^-) reached more than 10 fold the production at 20℃. The low light intensity did not affect the concentration of this peptide under full medium (ASM-1), free of N (N^-) or free of N and P (NP^-), the production remained similar to the amount produced at 20℃. Only for the medium free of P (P^-) there was an increase of peptide concentration, which was also statistically significant (Figure 45 and Table 35).

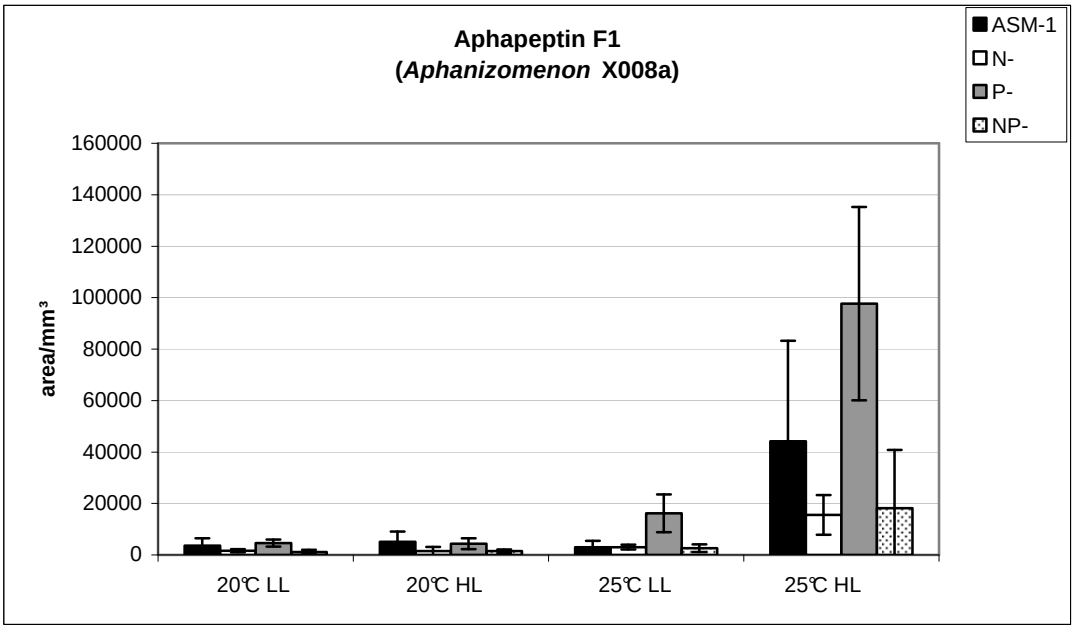


Figure 45: The total aphapeptin F1 concentration under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

The lower concentration observed at 20℃ for this peptide corresponded to a very similar observation for the other peptide found in this strain, i.e. anabaenopeptin F. At both temperatures and both light intensities, the full medium (ASM-1) and the medium free of P (P^-) were the two conditions in which most of this peptide was observed. At 20℃, the media free of N (N^-) and free of N and P (NP^-) also did not show any difference of light intensity, they were less produced than the other two media composition, but in the same range in both light intensities.

The interaction of high temperature (25℃) and high light intensity seemed to be fundamental to an enhanced production of this peptide.

The Tukey HSD post-hoc test (Table 35) showed that there was a statistically significant difference between the concentration of this peptide in the full medium (ASM-1) and the medium free of P (P^-) at 25℃ and high light intensity, as compared to all others combinations of temperature, light intensity and nutrient composition, thus supporting the observations in Figure 45.

The results of the multiple analysis of variance (MANOVA) showed that each single factor induces statistically significant differences in the

concentration of this peptide, as did two components or all three conditions together (Table 34).

Table 34: Results of the multiple analysis of variance (MANOVA) for the total aphapeptin F1 (X008a) concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	112,154	0,0000
Temperature	154,939	0,0000
Medium	50,390	0,0000
Light + Temperature	107,808	0,0000
Light + Medium	22,388	0,0000
Temperature + Medium	40,153	0,0000
Light + Temperature + Medium	23,119	0,0006

* Statistically significant differences are highlighted in gray.

Table 35: Results of Tukey HSD test for total aphapeptin F1 (X008a) concentration.

Aphapeptin F1 (X008a) total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns								
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns							
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	*	*	*	*	*	*	*	*	*	*	ns	*				
		N^-	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns			
		P^-	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	

n. s. = not significant. * are the significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular aphapeptin F1

In most situations, over 80% of the total concentration of this peptide remained intracellular, alike most of other peptides analyzed by this study. Only one exception was observed, at 20℃ and high light intensity and

medium free of N (N^-), only around 50% of the total peptide concentration was found in the cells. However, for this peptide there were no clear patterns indicating specific stress situation to lead to a higher extracellular fraction (Figure 46).

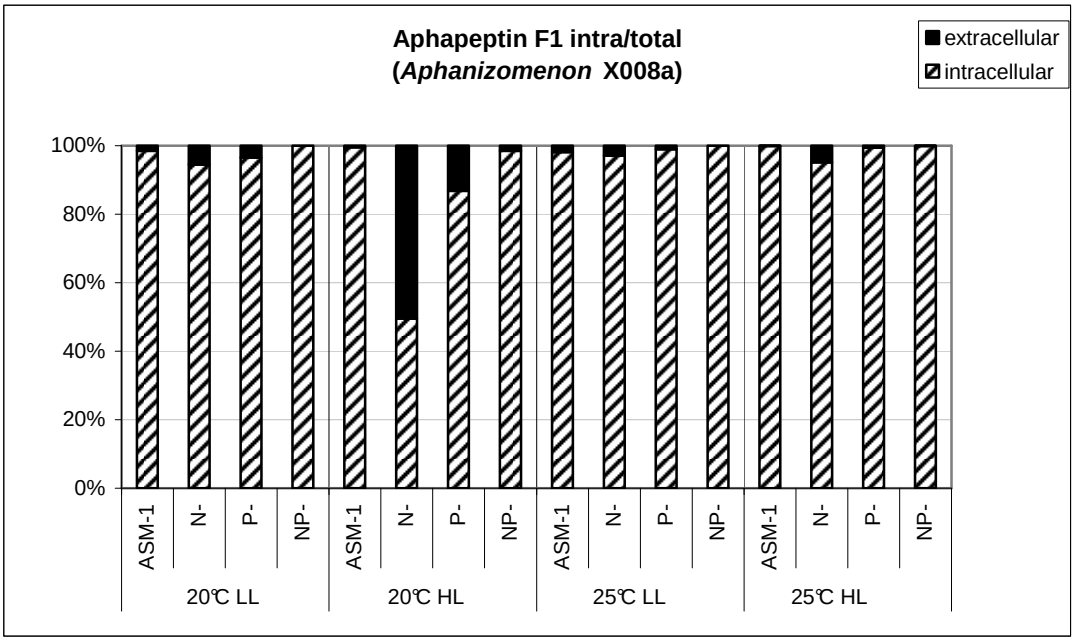


Figure 46: The percentage of the intracellular aphapeptin F1 in relation to the total peptide concentration under the different temperature and light conditions and the different nutrient compositions. (LL= low light; HL=high light).

3.2.3.7 P677

P677 is a frequent peptide in *Microcystis* strains (Dr. Martin Welker, *personal communication*), but not yet isolated or described. The understanding gained here of conditions leading to higher cellular concentration will be useful to facilitate isolation of this peptide in sufficient amounts for structural elucidation. It is provisionally named P677, relating to its molecular mass identified in MALDI-TOF MS.

The intracellular unknown peptide P677 was present in two *Microcystis* strains, in the RST9501 and BM 10. This peptide is rather apolar, having a retention time of 34.6 minutes in an isocratic HPLC run. Figure 37 shows its PSD spectrum.

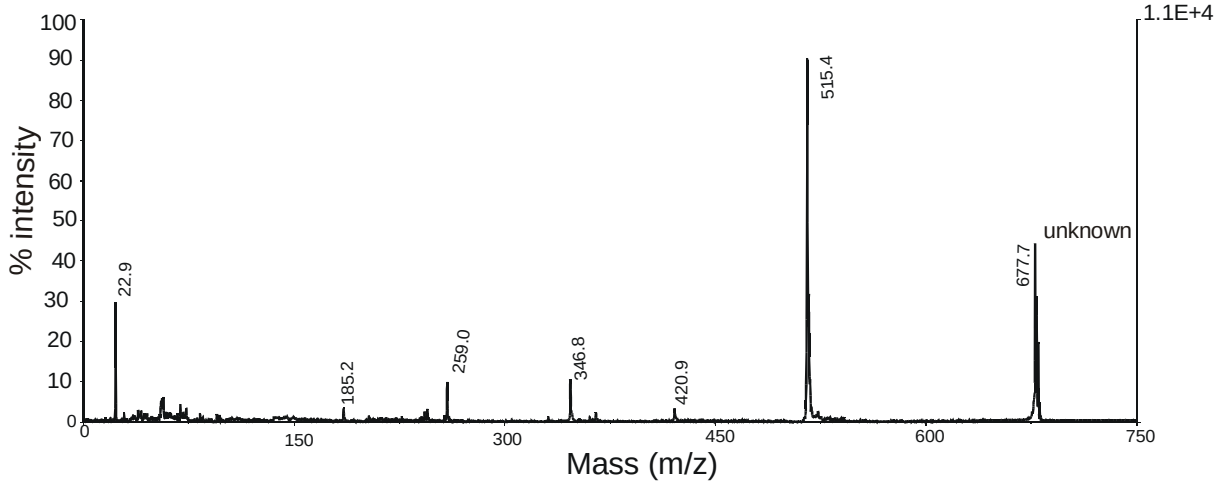


Figure 47: The PSD spectrum of the mass signal $M+H^+$ = 677, the unknown peptide produced by the *Microcystis* RST9501 and BM 10 strains.

P677 concentrations in relation to biovolume

Microcystis RST9501

In the *Microcystis* RST9501, a significant difference was found between the exponential and the stationary phase, but in both cases this difference is due to a greater decrease of concentration towards the stationary phase (data not shown).

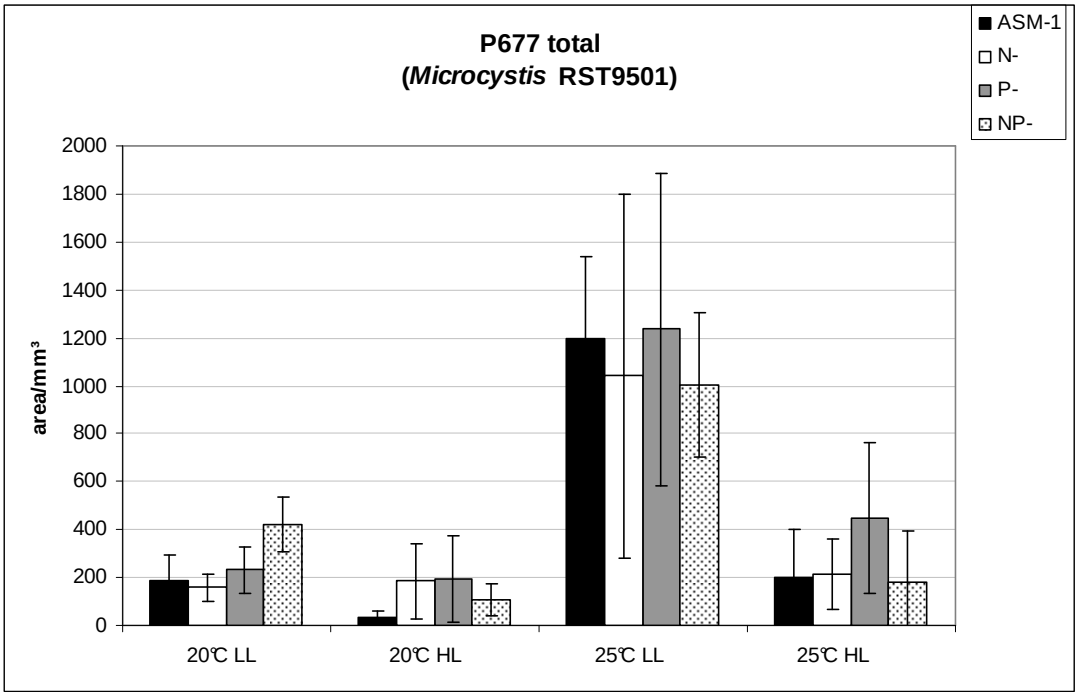


Figure 48: The total concentration of unknown peptide P677 (RST9501) under the different combinations of temperature and light conditions and the different nutrient compositions in the exponential phase (LL= low light; HL=high light).

Figure 48 shows that the total concentrations per unit cell biovolume of the unknown peptide P677 tended to be elevated at low light intensity, particularly at 25℃, and the results Tukey HSD post-hoc test co nfirmed the peptide concentrations under low light intensity and at 25 ℃ to be statistically significantly different from almost all other combination tested (Table 37). This result was similar to the observations for aeruginosin and microginin. At 20℃ and low light intensity, the concentrations of this peptide in the different nutrient concentrations were similar to one another, whereas at high light intensity, variability was higher and concentrations in the full medium were conspicuously (but according to the Tukey test not significantly; see Table 37) lower than in nutrient deficient media. At 25℃ var iance of results was generally higher, but no patterns between the different media were conspicuous.

Overall, regarding the differences in peptide concentration, the Tukey HSD post-hoc test confirmed their statistical significance for full medium (ASM-1) and medium free of P (P^-) at 25℃ and low light intensity in relation to al most all other combination tested (Table 37).

The MANOVA showed significance for the effects of temperature alone, light intensity alone and the interaction of both conditions, but no significance for the combined action of all three conditions (Table 36). This statistical analysis confirmed that there was a light intensity and a temperature influence but differently from the other peptides tested, the interaction of temperature, light intensity and medium was not significant for the concentration of this peptide.

Table 36: Results of the multiple analysis of variance (MANOVA) for the total P677 (RST9501) concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	34,33	0,0000
Temperature	36,57	0,0000
Medium	0,54	0,6573
Light + Temperature	20,06	0,0000
Light + Medium	0,36	0,7787
Temperature + Medium	0,66	0,5775
Light + Temperature + Medium	0,24	0,8618

* Statistically significant differences are highlighted in gray.

Table 37: Results of Tukey HSD test for the P677 (RST9501) total concentration.

P677 total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	ns	*	ns	ns	*	ns	ns	*								
		N^-	ns	*	ns	ns	ns	ns	ns	ns	ns							
		P^-	*	*	ns	ns	*	*	*	*	ns	ns						
		NP^-	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
		P^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	

n. s. = not significant. * indicates significant difference between experiment condition ($p < 0.01$).

***Microcystis* BM 10**

In the strain *Microcystis* BM 10, the total concentrations per unit cell biovolume of this unknown peptide P677 also tended to be elevated at low light intensity, particularly at 25℃ (Figure 49), similarly as for the RST9501 strain.

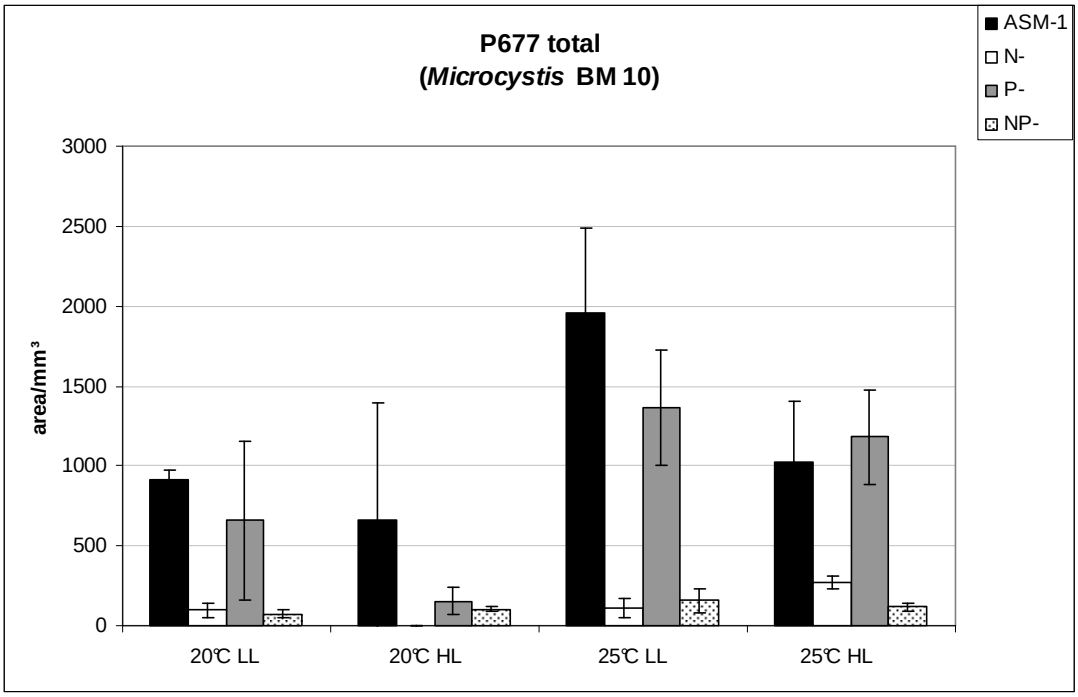


Figure 49: The total concentration of the unknown peptide P677 (BM 10) under the different combinations of temperature and light conditions and the different nutrient concentrations in the exponential phase (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

In contrast to the strain *Microcystis* RST9501, however, the strain BM 10 showed a great difference in peptide amount under the different nutrient compositions. Very low concentrations in medium free of N (N^-) and free of both N and P (NP-) reflected very low cellular growth (data not shown) under this deficiency. This result was consistent throughout all 4 combinations of light intensity and temperature, and its statistical significance.

In contrast to the strain RST9501, the multiple variance analysis (MANOVA) described the effects of temperature, light intensity and medium composition alone to have a significant impact on the concentration of this peptide in strain BM 10. The combined action of the components was not significant for the concentration this peptide, as was found for the RST9501 strain (Table 38).

The Tukey HSD post-hoc test showed that the peptide concentration in full medium (ASM-1) and in the medium free of P (P^-) at 25℃ and low light intensity were statistically significantly different from almost all other conditions. This result was very similar to the one obtained for this peptide with the other *Microcystis* strain, for which concentration was likewise statistically significantly different at 25℃, high light and in the medium free of P (P^-) from that observed under the other conditions (Table 39).

Table 38: Results of the multiple analysis of variance (MANOVA) for the total P677 (BM 10) concentration.

	$F_{(0,01,32)}$	p (<0.01)
Light	8,68	0,0059
Temperature	32,27	0,0000
Medium	45,03	0,0000
Light + Temperature	0,07	0,7809
Light + Medium	3,71	0,0212
Temperature + Medium	6,82	0,0011
Light + Temperature + Medium	2,22	0,1048

* Statistically significant differences are highlighted in gray.

Table 39: Results of Tukey HSD test for the total unknown P677 (BM 10) concentration.

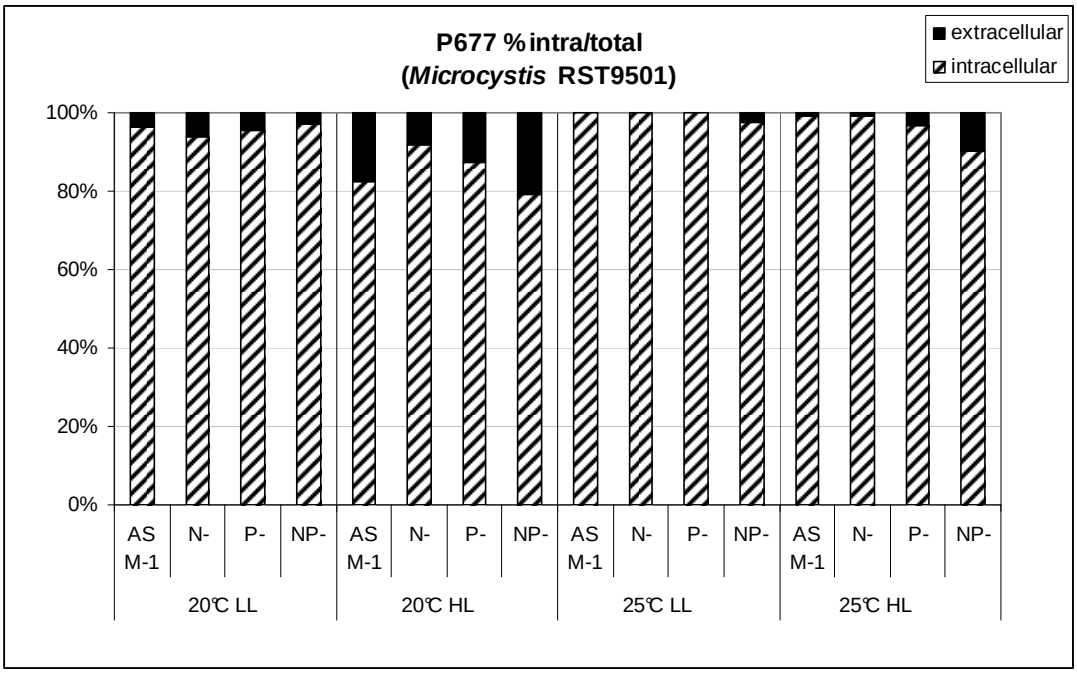
P677 (BM 10) total			20℃								25℃							
			20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$				20 $\mu Em^{-2}s^{-1}$				60 $\mu Em^{-2}s^{-1}$			
			ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-	ASM-1	N^-	P^-	NP^-
20℃	20 $\mu Em^{-2}s^{-1}$	ASM-1																
		N^-	ns															
		P^-	ns	ns														
		NP^-	ns	ns	ns													
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns												
		N^-	ns	ns	ns	ns	ns											
		P^-	ns	ns	ns	ns	ns	ns										
		NP^-	ns	ns	ns	ns	ns	ns	ns									
25℃	20 $\mu Em^{-2}s^{-1}$	ASM-1	*	*	*	*	*	*	*	*								
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	*							
		P^-	ns	*	ns	*	ns	*	*	*	ns	*						
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*					
	60 $\mu Em^{-2}s^{-1}$	ASM-1	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns				
		N^-	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns			
		P^-	ns	*	ns	*	ns	*	*	*	ns	*	ns	*	ns	ns		
		NP^-	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns	*	

n. s. = not significant. * indicates significant differences between experimental conditions ($p < 0.01$).

Ratio of intra to extracellular P677

This peptide was exclusively found intracellularly, in the strain *Microcystis* BM 10 (Figure 50b). For the *Microcystis* RST9501, intracellular concentration varied between 75% and 100% of the total concentration, with the highest extracellular shares found at 20 ℃ and high light intensity. For this peptide, the extracellular fraction was not enhanced by nutrient deficiency, as for some of the other peptides discussed above.

a)



b)

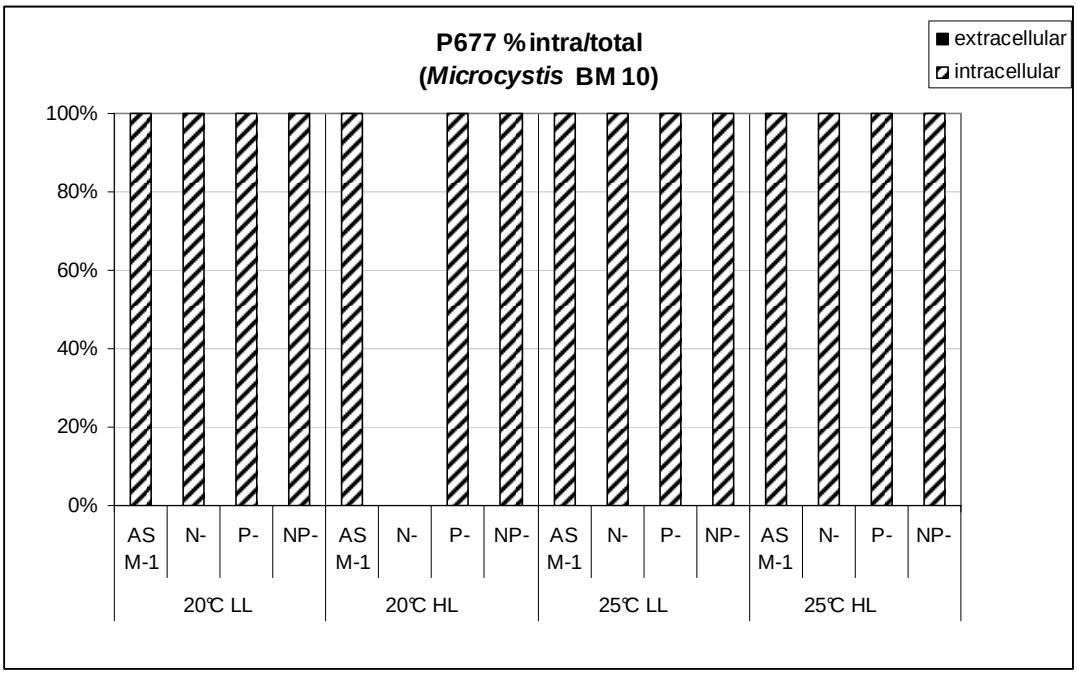


Figure 50: The percentage of the intracellular unknown P677 from RST9501 (a) and the unknown P677 from BM 10 (b) in relation to the total peptide concentration under different temperature and light conditions and the different nutrient compositions. (LL= low light; HL=high light). Note: at 20℃ HL and medium free of N (N^-), this strain did not grow, therefore not peptide was produced.

3.2.4 Discussion of the variability of peptides concentration in the exponential phase

Most of the studies done up to this moment were made mostly involving laboratory experiments of microcystin producers culture work (Gorham, 1964, Watanabe & Oishi, 1985, Codd & Poon, 1998, Sivonen, 1990, Song *et al.*, 1998, Orr & Jones, 1998, Hesse and Kohl, 2001, Long *et al.*, 2001, Böttcher *et al.*, 2001), nodularin (Lehtimäki *et al.*, 1997) or cylindrospermopsin producers (Hawkins *et al.*, 2001; Chonudomkul *et al.*, 2004) but little is known about the other cyanopeptides and their development during laboratory growth experiments. Therefore, the discussion about the results on the cyanopeptides concentration will be compared to the knowledge obtained for microcystin.

The increase of microcystin concentration, produced by the *Microcystis* RST9501, seen at 25℃ corresponds to results reported by Gorham (1964), Codd & Poon (1988), Sivonen (1990) and Rapala *et al.* (1997), who also showed increased microcystin concentrations at elevated temperatures, by factors ranging from 1.5 to 5 in relation to the lower temperatures investigated.

The insignificance of the effect of the increase of irradiance on the concentration of the microcystin (Table 15) differs from observations reported by other authors who describe an increase of microcystin production as the irradiance increases (van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985; Rapala & Sivonen, 1998; Kaebernick *et al.*, 2000; Hesse & Kohl, 2001 and Böttcher *et al.*, 2001). In contrast, the results presented here for [Leu1]-desmethylated–LR showed significance only together with temperature, and this could largely be attributed to the impact of temperature alone.

Microcystin concentrations in media with different medium composition (i.e. P, N, or both) proved to be very similar, when comparing the same temperature and light condition. The only exception was, as already mentioned, the medium free of P (P^-) at 25℃ and high light (Fig. 22, Table 14). The combination of high temperature and high light intensity with the absence of P appears to promote production of this microcystin, or to reduce biovolume (as detected by the CASY counter) more strongly than microcystin. This is a possibility, as rates of photosynthesis and biomass production will be high at elevated irradiance and temperature, but storage of – e.g. –

polyphosphate granules will be strongly limited. This result converges with those found by Codd & Poon (1988), Song *et al.* (1998), Bickel *et al.* (2000) and Hesse & Kohl (2001), which all found an increase of microcystin in P-free or P-depleted medium.

The aeruginosin 102A results, as also observed for microcystin, indicated that pronounced nutrient stress might enhance peptide export out of the cells, as proposed by Dittman *et al* (2001) and Kehr *et al.* (2006) for microcystins in the context of a signaling hypothesis, in which under stress there would be an increased signaling between cells.

Conditions causing the highest aeruginosin concentration, both intracellular and in total, also appear to favor its export out of cells. If this peptide is responsible for signaling, inhibitory and/or allelopathic effects to other organisms (as discussed in the introduction) similar to microcystin, the results obtained by this study would imply that such impacts are strongest at high temperature (25℃) and low light intensity (20 $\mu Em^{-2}s^{-1}$).

The production of anabaenopeptin F by the *Microcystis* BM 10, like the observations done for microcystin by Codd & Ponn (1988), Song *et al.*, (1998) and Hesse & Kohl (2001), increased in medium free of P (P^-), especially at 20℃ and high light intensity and 25℃ and low ligh t intensity.

For the anabaenopeptin F produced by the *Aphanizomenon* X008a, the temperature alone does not affect the peptide production, as also found by Song *et al.* (1998) for the microcystin concentration. Then again, light is significant for the anabaenopeptin F produced by this *Aphanizomenon* stain, especially when combained impact with temperature. At 25℃ and high light intensity, anabaenopeptin F production reached more than four times the amount produced under the same conditions at 20℃.

The oscillamide Y production in the *Microcystis* BM 10 case, as observed in other *Microcystis* strains, by Runnegar *et al.*, (1983), Van der Westhuizen & Eloff (1985), Van der Westhuizen *et al.* (1986), even if at 25℃ some strains have their optimum growth, at 20℃ more peptide con centration was produced. By this strain, up to four fold more oscillamide Y was produced at

20℃ than at 25℃, when considering the full medium (ASM-1) at 25℃ and high light intensity as an outlier.

The dominance of the intracellular fraction of the microginin produced by *Microcystis* NPRG-2 is similar to the results described for anabaenopeptin F, microviridin and microcystin. For these peptides in most situations nutrient deficiency of the medium had very little impact on the ratio between extra and intracellular peptide. However, under some situations such as 25℃ and low light intensity here for microginin, a higher extracellular share can be observed, usually in the nutrient deficient media. This may indicate a stress situation for the cells.

4 General Conclusion

Regarding previously unknown cyanopeptides this study achieved:

- Isolation of two new cyanopeptides,
- Elucidation of their chemical structure through collaboration with AnalytiCon Discovery. These two new cyanopeptides do not belong to any previously described class of cyanopeptides,
- Preliminary characterization of both new cyanopeptides with assays reflecting their cytotoxicity to mammalian cells through collaboration with the University of Constance. None of the three different assays applied showed any cytotoxicity. *In vitro* experiments were in collaboration with the University of Copenhagen showed both new peptides, aphapeptin F1 and aphapeptin F2 to be trypsin inhibitors.

For the influence of different growth conditions on cyanopeptide production this study showed:

- Statistically significant differences of cyanopeptide concentration between the exponential and stationary growth phase were only found for microcystin. Upon reaching the stationary phase, the cellular concentration of this peptide increased up to 3 fold in relation to the exponential phase (with extracellular concentration being low and scarcely influencing this relationship). This increase of microcystin concentration happened only under one condition, i.e. at 25℃ and low light intensity in the medium free of N (N^-). Total cellular concentration (i.e. intra plus extracellular) of the other seven peptides did not increase or show any statistically significant difference between the exponential and stationary growth phases for any of the growth conditions tested;
- The effect of temperature on the total peptide concentration (i.e. intra plus extracellular) was found to be significant for five of the eight peptides studied. For these five peptides in most cases an increase in temperature induced an increase of the total peptide concentration;

- For six of the eight peptides studied, total peptide concentration responded to differences in light intensity. For four of these peptides, there was a greater total production in low light intensity and for two of them in high light intensity;

- Changes in temperature and light often acted together, and for seven different peptides, this effect is statistically significant. For the microcystin, concentrations were significantly higher at the higher temperature and light intensity. In the 6 other cases, the peptide concentration increased at the higher temperature but lower light intensity. A consequence at a larger time scale could be peptide concentrations responding to water turbidity, with more microcystin under less turbid conditions and more other peptides at elevated turbidity;

- Nutrient concentrations in the medium had a statistically significant impact on the concentrations of six of the eight peptides studied. In the absence of N, peptide concentration was observed to be dramatically reduced in one strain (BM 10) especially at a lower temperature and low light; none of this strain's three peptides were produced under this condition. Overall, very different responses to the absence of N were obtained between strains. For two peptides there was an increase of peptide production in absence of N, in some cases the production maintained on the same level as the production in the full medium, and for two peptides the production level was similar to the production once both nutrients were absent (NP^-);

- The absence of the nutrient P seems not to decrease peptide production. In fact, in most cases P-deficiency of the medium rather enhanced than impaired peptide levels, reaching, sometimes 2 fold the peptide concentration per biovolume observed in the full medium. These results are in agreement with those found by few authors also for microcystin, the concentration of which increased under P-limitation;

- Some surprising results were obtained from the medium in which both N and P were absent. It was expected that under this extreme condition peptide production would be reduced, but in some cases this medium resulted in the highest peptide levels. Generally under this condition the peptide concentration reaches half the concentration observed in full

medium and in some cases levels were higher than or very similar to those under the absence of only N. Even though the culture may not prevail for much longer, the peptide concentration can be high, sometimes higher than under any other condition;

- Analyzing the amount of produced peptide found intra and extracellularly it was found that with a very few exceptions, over 60% of the peptide production remains intracellular independently of the condition tested. Sometimes 100% of the produced peptide was found intracellularly;

- For five of the eight peptides analyzed, the intracellular peptide concentration found in full medium was relatively invariable, between 80 and 90%, disregarding temperature and light intensity. Nevertheless, the total peptide concentration found in the media free of N (N^-), free of P (P^-) and free of both (NP^-) was higher or similar to the peptide concentration in full medium, but this higher shares on peptide concentration were also exported out of the cells;

- The working hypothesis for this thesis was that all cyanopeptides are regulated similarly, following the pattern described for microcystis, i.e. the environmental conditions have an influence of no more than a factor of 2-3 on peptide per cell. This was confirmed only in as far as peptide production very rarely ceased altogether. However, the results obtained showed that alterations of the environmental condition can indeed the influence on the peptide concentration per cell substantially. For at least five out of the eight peptide studied, a factor of 4 or higher could be found when comparing the peptide concentration produced at a certain combination of temperature, light intensity and media composition to any other combination, e.g. the oscillamide Y concentration in full medium (ASM-1) at 25℃ and high light intensity is 4 times higher than the concentration of this peptide in the same medium composition, same temperature but in a lower light intensity. All peptides showed at least 2 fold differences in concentration between specific growth conditions. Overall, the results show that not just temperature, light intensity or media composition (i.e. nutrients available) individually have an influence on the

peptide concentration per cell, but in combination these factors may indeed increase or decrease peptide concentration per cell considerably.

5. Outlook

This work had the intention of developing an initial understanding of the effects of light intensity, temperature and nutrient composition on the production of a selected variety of cyanopeptides. This information may be used as a tool to understand the occurrence of these peptides in nature and which key environmental conditions may influence their occurrence. The working hypothesis that these growth conditions would have fairly little impact on peptide content – i.e. by less than a factor of 5 – was confirmed only for some of the peptides and conditions studied. Open questions remaining or raised be these results are:

- Further research should address the causes of the pronounced variability of the concentrations of some of the peptides, preferably with continuous cultures to ensure more tightly defined growth conditions, and by varying single parameters over a gradient. Candidate peptides would particularly be microviridin, anabaenopeptin F and oscillamide Y.

- The extracellular fraction of the total pool of most peptides was clearly smallest under non-limiting growth conditions, i.e. in full medium. This raises the question whether this increased release under stress is due merely to cells becoming more ‘leaky’ under stress, or whether this is an active process serving some function for the cell. Aeruginosin 102A, with its generally high extracellular share would be an interesting peptide for such further analysis. For example, in a continuous culture with N or P being limiting could be observed for how long the extracellular peptide pool concentration would increase, as indicated by the preliminary batch culture results presented here.

- The peptide isolation done in both *Aphanizomenon* strains showed that very unusual peptides (i.e. with sequences of identical amino acids) can be produced in different cyanobacteria groups and new peptides classes may be found in these groups. However, to date this genus has scarcely

been investigated for its peptide patterns. Further isolation and elucidation of peptides from Aphanizomenon would be interesting in order to clarify whether these are in fact “normal” for this genus.

- The role of the cyanopeptides could also be better understood by the use of mutant cyanobacterial strains. In such mutants one known cyanopeptide was knocked out, but the other are still being synthesized. Culture studies addressing levels of the different peptides still produced by such knock-out mutants in comparison to levels in the wild type would show if the peptides still present in the mutant respond to shifts growth conditions in the same way as the wild type. If not, this would indicate that internal cell regulation compensates for the missing peptide in some way, which would be a step towards understanding peptide functions for the cell. .

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