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Quorum sensing em cianobactérias

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Quorum sensing em cianobactérias

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SUMÁRIO

Resumo

O termo "*quorum sensing"* refere-se a um fenômeno de comunicação celular existente em bactérias, onde ao se atingir uma certa densidade populacional mudanças no padrão de expressão gênica, e consequentemente da fisiologia das células são disparadas. No que diz respeito a cianobactérias, poucos trabalhos com enfoque em *quorum sensing* foram realizados. Alguns estudos mostram que substâncias indutoras de *quorum sensing* podem alterar algumas características fisiológicas de algumas cepas de cianobactérias. Evidências indiretas mostraram que a diferença na densidade celular pode afetar a produção de microcistinas. A capacidade de produzir florações em determinadas condições é outro aspecto importante das cianobactérias. Nestas situações a densidade populacional das espécies dominantes atinge valores muito altos, podendo constituir uma situação ideal para a ocorrência do *quorum sensing*. Existem diversos estudos sobre florações, no entanto pouco se sabe sobre o que ocorre com as populações de cianobactérias em nivel molecular e também fisiológico. Este trabalho teve o objetivo de caracterizar quatro cepas de cianobactérias quanto à produção de peptídeos e utilizá-las em experimentos com a finalidade de encontrar evidências da existência de *quorum sensing* em cianobactérias e sua relação com a produção de oligopeptídeos. Para cumprir estes objetivos foram utilizadas técnicas de bioquímica e biologia molecular. Os resultados encontrados demonstraram que a produção de oligopeptídeos é afetada pela densidade celular, sendo, na maioria dos casos, maior nas situações com alto número de células. Foi verificado através de PCR em tempo real que auto indutores do tipo acil-homoserina lactonas (AHLs), responsáveis pela ativação do *quorum sensing* em diversos grupos de bactérias gram-negativas, afetam a transcrição de genes ligados a produção dos

peptídeos microcistina, cianopeptolina e microviridina. Através de testes de ELISA foi visto também que as AHLs afetam a produção de microcistinas da mesma forma que influenciam a transcrição dos genes liagados à sua síntese. Com este trabalho foi visto que densidade celular e, possivelmente, *quorum sensing* são fatores importantes na síntese de metabólitos secundários em cinobactérias, que devem ser levados em consideração ao se estudar estes compostos e suas relações com o ambiente.

Palavras chave: Quorum sensing, cianobacterias, microcistina, densidade celular

Abstract

The terminology *"quorum sensing"* is used to identify a cellular communication phenomenon in the bacterial domain, which happens when a bacteria population reaches a defined cellular density. During the activation of the phenomenon changes in the expression of several genes and consequently in the physiology of the cell are triggered. Concerning cyanobacteria, there is a lack of information about quorum sensing. Some studies show that quorum sensing inducer compounds may alter physiological characteristics of certain cyanobacteria strains. Besides that, indirect evidences have shown that cellular density may influence microcystin production. The capacity to form blooms in certain conditions is also an important characteristic found in the cyanobacteria group. In these conditions the cellular density of a population may reach elevated numbers, consisting of an ideal scenario for quorum sensing. There are a remarkably number of studies regarding cyanobacterial blooms, but little is known about what happens with a population at molecular and physiological levels. The aim of this study was to characterize four strains of cyanobacteria regarding the production of peptides and to use these strains in experiments with the objective of investigating evidences of quorum sensing system in cyanobacteria and its connection with the synthesis of oligopeptides. In order to fulfill these objectives biochemical and molecular techniques were used. The results obtained showed that the production of oligopeptides is affected by cellular density, being, in most cases, higher in situations with an elevated number of cells. It was demonstrated by real time PCR that acylhomoserine lactone autoinducers (AHLs), which are responsible for the activation of the quorum sensing in various groups of gram-negative bacteria, affect the transcription of genes linked to the production of

microcystins, cyanopeptolins and microviridins. Through ELISA assays it was also seen that AHLs affect the microcystin production in the same pattern in which they affect the transcription of genes connected to its synthesis. With this work it was observed that cellular density and possibly quorum sensing are key factors in secondary metabolite synthesis in cyanobacteria and should be considered when studying these compounds and their connection with the environment.

Keywords: Quorum sensing, cyanobacteria, microcystin, cellular density

1 – Introdução Geral

1.1 – Quorum sensing

Em populações bacterianas, organismos sem sistema nervoso e hormônios, onde cada indivíduo é constituído por uma célula que se reproduz por fissão binária e precisa competir por recursos com outros da mesma espécie, seria esperado que não existissem mecanismos de reconhecimento e cooperação entre os indivíduos isolados. No entanto, existem diversas situações nas quais o comportamento cooperativo numa população pode ser extremente vantajoso, como: conjugação, simbiose, adaptação de nicho, produção de metabólitos secundários, combate à sistemas de defesa de organismos superiores e até migrações populacionais com intenção de fugir de locais com condições desfavoráveis (Williams *et al.,* 2007).

Para que os indivíduos que formam uma população bacteriana expressem o comportamento cooperativo é necessário que exista alguma forma de comunicação química entre as células através de moléculas de sinalização. O primeiro trabalho a identificar este tipo de comunicação foi realizado por Tomasz (1965), que demonstrou que a bactéria gram-positiva *Streptococcus pneumoniae* controla fatores de competência genética através de substâncias produzidas pelo próprio organismo, seguido do trabalho de Nealson *et al.* (1970) que mostrou a existência do controle de bioluminescência na bactéria gram-negativa *Vibrio fischeri* através de uma substância chamada de auto-indutor. Os auto-indutores são assim chamados pois parte de sua função é estimular a própria produção (Williams *et al.,* 2007). Além disso, também permitem que as populações bacterianas determinem sua densidade numérica, pois, à medida em que a

população cresce, os sinalizadores acumulam no meio e, ao atingir um limiar na concentracão da substância em consequência ao tamanho populacional, mudanças coordenadas no comportamento bacteriano são disparadas (Fuqua & Greenberg, 2002).

Apesar dos primeiros trabalhos relacionados à comunicação celular em bactérias terem surgido há mais de 40 anos, foi apenas no começo da década de 1990 que a pesquisa nessa área se desenvolveu e que o termo "*quorum sensing*" foi introduzido por Fuqua *et al.,* (1994) para descrever este fenômeno. Desde então, diversos trabalhos sobre o funcionamento do *quorum sensing* em diversos grupos de bactérias foram publicados e acredita-se que a comunicação celular seja algo comum em bactérias (Miller & Bassler, 2001). No entanto é importante ressaltar que o termo *quorum sensing* não descreve adequadamente todas as formas de comunicação celular onde bactérias utilizam sinais químicos. O tamanho do "*quorum*" não é fixo e depende da taxa de produção e degradação dos indutores, fatores que são afetados pelo ambiente no qual os organismos estão inseridos (Williams *et al.,* 2007). Células solitárias podem tambem mudar do estado de não *quorum sensing* para *quorum sensing*, como observado por Qazi *et al.* (2001) em células de *Staphylococcus aureus* aderidas a endossomos de células endoteliais. Nestes casos, mesmo em baixa densidade, ocorre acúmulo do auto-indutor em quantidade suficiente para disparar o fenômeno. Nestas situações o *quorum sensing* poderia ser na verdade descrito como "diffusion sensing" ou "compartment sensing", já que a informação levada aos indivíduos diz respeito mais ao ambiente onde os indivíduos estão inseridos do que ao tamanho populacional (Redfield, 2002; Winzer *at al.,* 2002). Outro aspecto que deve ser levado em consideração é o fato de que o *quorum sensing* é apenas um dos componentes ligados à regulação gênica global e que existem vários outros sinais

ambientais (ex: temperatura, pH, osmolaridade, concentração de nutrientes) percebidos por uma população bacteriana, influenciando sua estratégia de sobrevivência e adaptação à situações de stress (Withers *et al.*, 2001).

Dentre os diversos auto-indutores responsáveis pela comunicação no *quorum sensing*, podem-se destacar as acilhomoserina lactonas (AHLs) (figura 1). Estes compostos são caracterizados por uma homoserina lactona com um grupo acyl de cadeia carbônica na posição α. A cadeia de carbono pode ser de tamanho variável (entre C4 e C18), pode também variar no grau de saturação e oxidação (Williams *et al.*, 2007). O funcionamento do sistema à partir de AHLs é baseado em duas famílias de proteínas, a LuxI é a estrutura responsável por sintetizar os AHLs enquanto a LuxR é o receptor dos AHLs (Fuqua *et al.*, 2001; Swift *et al.*, 2001). O sistema funciona a partir do momento em que o AHL se liga e ativa a proteína LuxR. O complexo formado pelo AHL e a LuxR é então responsável pela ativação e repressão de diverssos genes (Fuqua *et al.*, 2001; Swift *et al.*, 2001). Os AHLs também ativam o gene responsável pela síntese da proteína LuxI, gerando um "feedback" positivo. A figura 2 apresenta o esquema básico de funcionamento do *quorum sensing*.

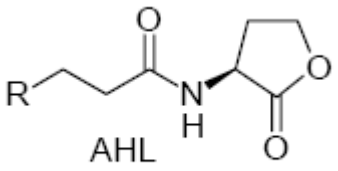

Figura 1 – Estrutura básica de uma molécula de AHL. R= grupo alquila linear. Fonte Williams *et al.*, 2007.

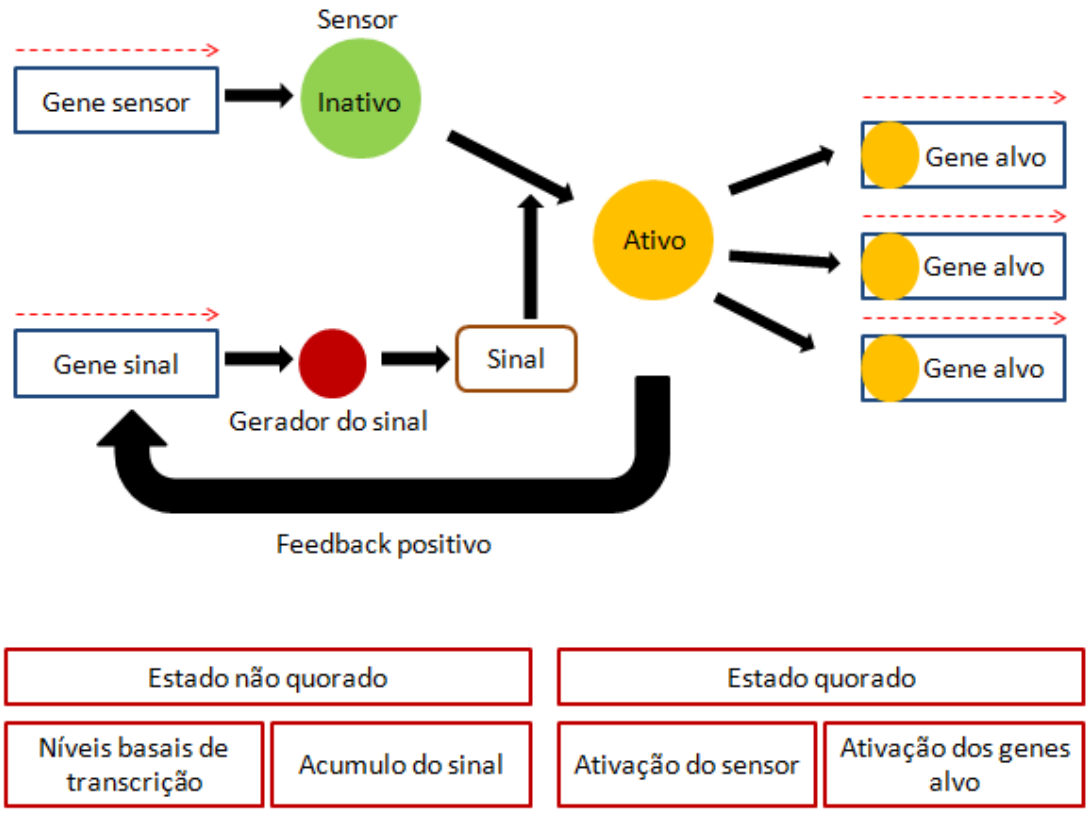

Figura 2 – Esquema básico do funcionamento do *quorum sensing*.

1.2 – Cianobactérias

Cianobactérias são organismos fotoautotróficos, que podem ser encontrados em uma grande variedade de habitats aquáticos, e até terrestres, e são um dos principais produtores primários em grande parte destes ambientes. Recentemente o aumento do aporte de nutrientes, levou a formação de grandes biomassas de algas nos ambientes aquáticos (florações), onde frequentemente as cianobactérias predominam. As florações geralmente ocorrem em águas férteis e com condições favoráveis ao crescimento destes microrganismos (Paerl, 1996). O sucesso destes organismos não pode ser explicado apenas por uma característica, mas por vários fatores intrínsecos e ambientais em

conjunto, como por exemplo, o fato de serem os únicos organismos do fitoplâncton capazes de fixar nitrogênio, controlar a flutuabilidade, acumular grandes quantidades de fósforo entre outros (Paerl, 1996; Schindler *et al.,* 2008). Durante as floracões é possivel observar uma queda da diversidade de toda a comunidade aquática, pela dominância de uma ou poucas espécies (Romo & Miracle, 1995; Giani *et al.,* 2005).

Diversas espécies de cianobactérias podem produzir diferentes tipos de peptídeos bioativos e as vezes tóxicos (figuras 3, 4, 5, 6, 7 e 8) dentre os quais podem-se destacar as aeruginosinas (Murakami *et al.,* 1994), anabaenopeptinas (Harada *et al.,* 1995), cianopeptolinas (Martin *et al.,* 1993), microviridinas (Ishitsuka *et al.,* 1990), microgininas (Okino *et al.,* 1993) e microcistinas (Carmichael, 1992). A formação destas moléculas ocorre através de complexos enzimáticos formados por vários módulos, conhecidos como NRPSs (non-ribossomal peptide synthetases) e PKS (polyketide synthase) (Weber & Marahiel, 2001; Finking & Marahiel, 2004). Cada módulo é composto de domínios catalíticos, sendo que um módulo mínimo é composto de um domínio de adenilação, responsável pela ativação dos aminoácidos, um módulo de tiolação, responsável por transferir os intermediários ativados, e um modulo de condensação (von Döhren *et al.*, 1997; Stachelhaus *et al.,* 1998). Com exceção das microviridinas, todos os compostos são sintetizados por vias não ribossomais, o que pode explicar a sua alta variabilidade (Welker & von Döhren, 2006).

As funções fisiológicas e ecológicas destes compostos ainda não foram esclarecidas, mas é sabido que diferentes cepas de uma espécie podem ser ou não produtoras no que diz respeito a cada tipo de peptídeo e classe (Fastner *et al.,* 2001; Rohrlack *et al.,* 2001;

Welker *et al.*, 2004). O entendimento dos fatores que afetam a produção destes metabólitos secundários pode ajudar no entendimento de suas funções fisiológicas, de suas relações com o meio ambiente e pode ser também uma importante ferramenta a ser usada no tratamento de água e na prevenção de producão de cianotoxinas.

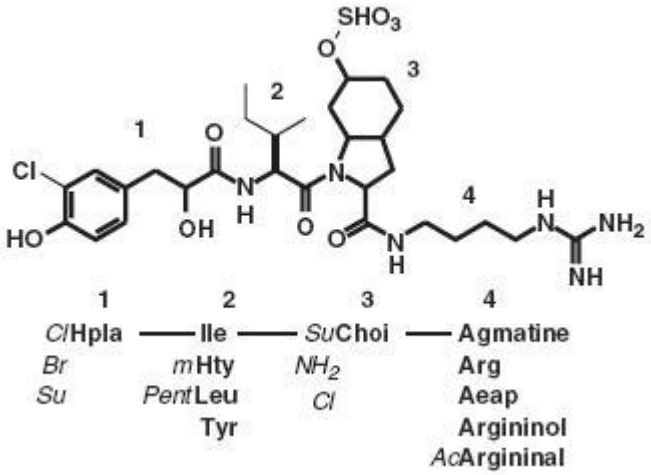

Figura 3 – Estrutura da aeruginosina 98-A e esquema geral da estrutura das aeruginosinas. Fonte: Welker & von Döhren (2006).

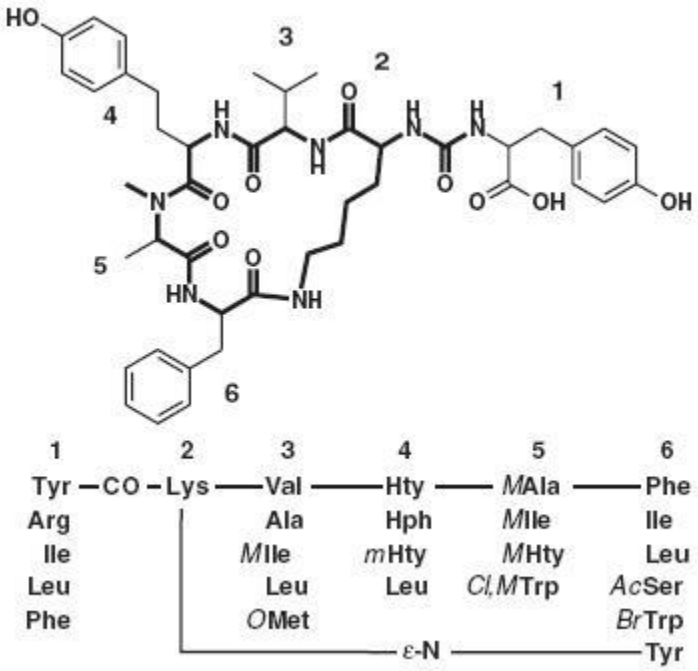

Figura 4 – Estrutura da anabaenopeptina A e esquema geral da estrutura das anabaenopeptinas. Fonte: Welker & von Döhren (2006).

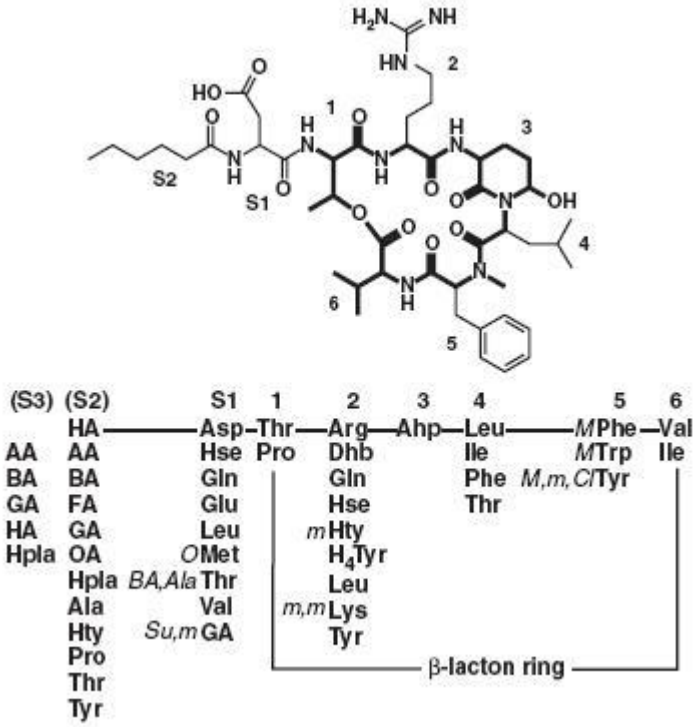

Figura 5 – Estrutura da cianopeptolina A e esquema geral da estrutura das cianopeptolinas. Fonte: Welker & von Döhren (2006).

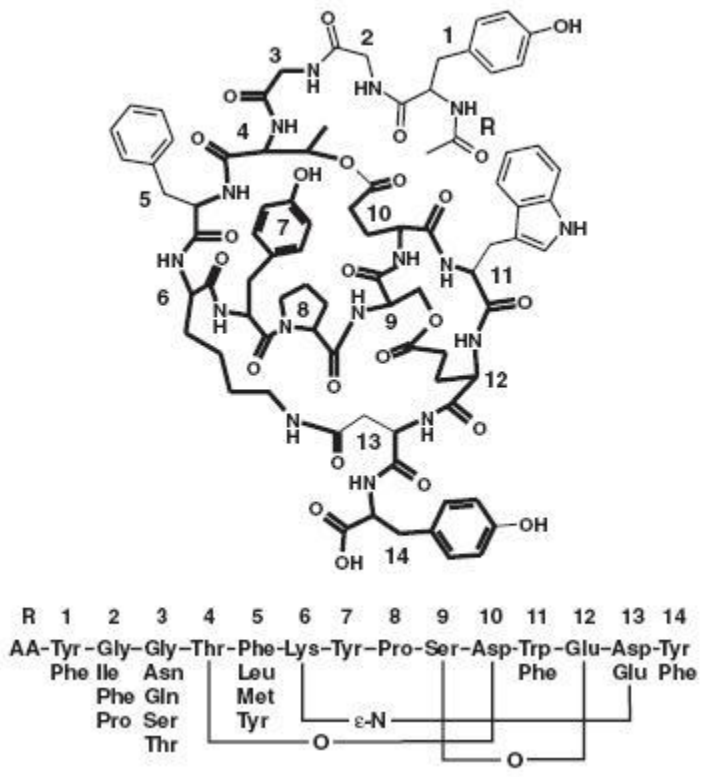

Figura 6 – Estrutura da microviridina A e esquema geral da estrutura das microviridinas.

Fonte: Welker & von Döhren (2006).

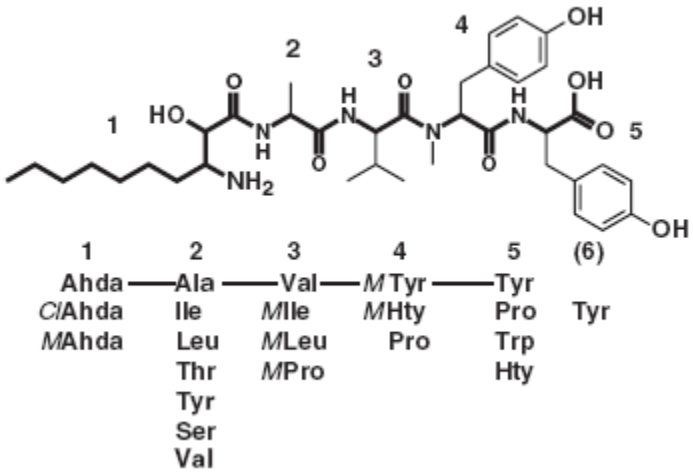

Figura 7 – Estrutura da microginina e esquema geral da estrutura das microgininas. Fonte: Welker & von Döhren (2006).

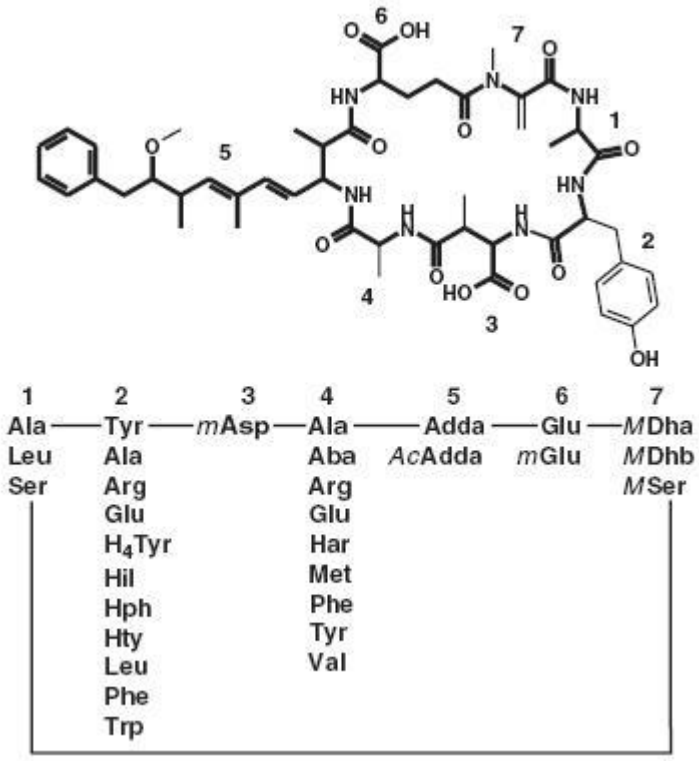

Figura 8 – Estrutura da microcistina-LA e esquema geral da estrutura das microcistinas.

Fonte: Welker & von Döhren (2006).

1.3 – *Quorum sensing* em cianobactérias

Apesar das pesquisas com *quorum sensing* terem se intensificado a partir da década de 1990, muito pouco foi estudado no grupo das cianobactérias. Existem estudos que relatam a produção de auto indutores do tipo acil homoserina lactonas (AHLs) em culturas axênicas de cianobactérias do gênero *Gloeothece* (Sharif *et al.*, 2008). Trabalhos realizados com o gênero *Anabaena* demonstraram que estes organismos podem produzir enzimas do tipo AHL-acilase que degradam os AHLs (Romero *et al.*, 2008) e que a fixação de nitrogênio é inibida pela presença de diferentes AHLs com cadeias laterais de variados tamanhos e graus de saturação (Romero *et al.*, 2011). Van Mooy *et al.* (2012) demonstrou que a aquisição de fósforo em cianobactérias marinhas do gênero *Trichodesmium* é estimulada na presença de auto-indutores.

Além dos trabalhos já citados, existem evidências indiretas de *quorum sensing* em cianobactérias. O trabalho de Gobler *et al.* (2007), estudando a dinâmica populacional e a toxicidade de uma floração de cianobactérias em lago eutrófico de Nova Iorque relata um aumento na expressão do gene *mcyE*, envolvido na produção de microcistina, em meses onde a densidade celular era maior. Em estudos realizados em um pequeno lago eutrófico na Nova Zelândia, Wood *et al.* (2011) mostraram que a expressão do gene *mcyE* não é constitutiva, podendo ser ativada ou não ao longo do dia. O mesmo estudo mostrou que a expressão do gene *mcyE* é maior em momentos onde a densidade celular é elevada. Outro estudo comparando situações de alta e baixa densidade celular mostrou, em experimentos de mesocosmos, que a quantidade de microcistina por célula era maior em situações onde a densidade celular era mais elevada (Wood *et al.*, 2012).

2 – Hipóteses e justificativa

- Hipóteses:

As cianobactérias, por fazerem parte do grupo das bactérias possuem alguma forma de comunicação celular através de *quorum sensing*.

A densidade celular em cianobactérias influencia a produção de metabólitos secundários, como microcistinas, aeruginosinas, cianopeptolinas, microviridinas e outros.

- Justificativa:

O aumento do processo de eutrofização cultural, associado à alta proliferação de cianobactérias potencialmente tóxicas tem causado diversos problemas relacionados ao uso de ambientes de água doce. Apesar de existirem diversos estudos sobre as toxinas produzidas pelas cianobactérias, a maioria dos trabalhos sobre sua ocorrência no ambiente é descritiva ou é voltada principalmente para aspectos bioquímicos e moleculares. Poucos estudos foram feitos relacionando aspectos da ecologia das cianobactérias com a produção de metabólitos secundários. Pouco se sabe também sobre a fisiologia das cianobactérias durante situações de floração e como estes episódios podem afetar a produção de toxinas e outros compostos. O fato das florações apresentarem alta densidade celular sugere, por exemplo, uma situação favorável ao aparecimento de fenômenos como o *quorum sensing*, bastante estudado em diversos grupos de bactérias, porém, com poucos estudos em cianobactérias.

Os trabalhos apresentados nesta tese fornecem dados que permitam uma melhor compreensão do *quorum sensing* em cianobactérias e sua relação com alguns metabólitos secundários, amplamente produzidos por estes organismos.

A tese está divida em três capítulos, que estão apresentados no formato de artigos científicos. O capitulo 2 foi aceito para publicação na revista FEMS Microbial Ecology (doi: 10.1111/1574-6941.12281).

3 – Objetivo

3.1 – Objetivo geral

Encontrar evidências da existência de *quorum sensing* em cianobactérias e verificar sua relação com a produção de oligopeptídeos.

3.2 – Objetivos específicos

– Caracterizar, de forma bioquímica e molecular, quatro cepas de cianobactérias com relação à produção de diferentes peptídeos.

– Avaliar os efeitos de diferentes densidades celulares em algumas cepas de cianobactérias na produção de peptídeos tais como microcistinas, aeruginosinas, cianopeptolinas e microviridinas.

– Estudar os efeitos de substâncias conhecidas como promotoras de *quorum sensing* (acil homoserina lactonas, AHLs) na expressão de genes ligados à produção de microcistinas, cianopeptolinas e microviridinas, e também na concentração final de microcistina, em cepas de cianobactérias.

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Capítulo 1

Peptide profile of four cyanobacteria strains

Abstract

Cyanobacteria are known to produce a broad variety of peptides as secondary metabolites. These oligopeptides are usually synthesized by non-ribosomal units. A characteristic of these systems is the capacity to combine proteinogenic amino acids with non-proteinogenic amino acids, carbohydrates, fatty acids and other building blocks, being this characteristic one of the reasons for the great variability found in these peptides and creating structures that cannot be achieved by ribosomal synthesis. Aeruginosins, cyanopeptolins, microcystins and microviridins are some of the main peptides classes produced by cyanobacteria. The objective of this work was to determine a peptidic profile based on biochemical methods for four strains of cyanobacteria (two *Radiocystis fernandoii*, one *Microcystis aeruginosa* and one *Microcystis panniformis)*. The strains were grown for approximately 10 days, then extracted with methanol 75% and the extract was purified with SPE cartridges. The purified material was analysed in a HPLC followed by and identification of the peptides in a MALDI-TOF system. A total of 17 peptides were found, among aeruginosins, cyanopeptolins, microcystins and microviridins. The *Microcystis* strains produced aeruginosins, cyanopeptolins and microcystins while the *Radiocystis* strains produced cyanopeptolins, microcystins and microviridins. A hierarchical analysis showed that the two *Radiocystis* strains and the two *Microcystis* species form two separate groups according to their peptidic profile. The technique used in this study showed to be efficient for isolation and identification of peptides. Besides the compounds already described by other authors, we found some oligopeptides that are not yet described in the literature.

1 – Introduction

Cyanobacterial secondary metabolites are represented by a great variety of structures and were isolated from several taxa coming from different geographic regions (Welker & von Dohren, 2006). The majority of these secondary metabolites are small peptides or have peptidic substructures. Most of these peptides are assumed to be synthesized by NRPS (non-ribosomal peptide synthetase) or NRPS/PKS (polyketide synthetase), creating structures that cannot be achieved by ribosomal synthesis (Welker & von Dohren, 2006). The NRPS system operates at the protein level without the use of nucleic acids (Finking & Marahiel, 2004). The condensation of amino acids and carboxyl compounds is driven by protein templates and usually each step requires a different protein module (Weber & Marahiel, 2001; Schwarzer *et al.,* 2003). The modules are composed of catalytic domains being formed at least by an adenylation domain for amino acid activation, a thiolation domain for transfer of activated intermediates, and a condensation domain (von Dohren *et al.*, 1997; Marahiel *et al.*, 1997; Stachelhaus *et al.*, 1998). An interesting characteristic of NRPS systems is the capacity to combine proteinogenic amino acids with non-proteinogenic amino acids, carbohydrates, fatty acids and other building blocks (Welker & von Dohren, 2006). The ability to synthesize structures with several different types of building blocks allows the production of a great variety of secondary metabolites, which are grouped in classes according to their structures.

Aeruginosins (Murakami *et al.,* 1995) are characterized by a linear structure with a derivative of hydroxy-phenyl lactic acid (Hpla) at the N-terminus, the amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi) and an arginine derivative at the C-terminus.

The amino acids presented in position 2 are usually in D-configuration, although amino acids in L-configuration can also be found in position 2 (Ishida et al 1999). Chlorination (*Cl*) and sulphation (*Su*) can occur at the Choi (Shin *et al.*, 1997) or Hpla (Ishida *et al.*, 1999). One aeruginosin variant (aeruginosin 98-C) was also reported to contain a brominated Hpla (Ishida *et al.*, 1999).

Cyanopeptolins (Martin *et al.*, 1993) are characterized by a cyclic structure formed by an ester bond between the β-hydroxy group of threonine with the carboxy group of the terminal amino acid and by the presence of the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) in position 3. A side chain of variable length is usually connected to amino group of a threonine in position 1. The side chain can be formed by one or two amino acids with an aliphatic fatty acid (Okino *et al.*, 1993) or by a glyceric acid unit at the N-terminus (Jakobi *et al.*, 1995).

Microcystins (Botes *et al.*, 1984) are characterized by the amino acid Adda ((2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at position 5. Positions 2 and 4 show high variability while other positions have only minor variability. Position 3 is occupied by an aspartate derivative and position 6 is occupied by a glutamate derivative or by a glutamate in D-configuration. Position 7 is usually occupied by dehydro alanine (Dha) or by a methyl-dehydro alanine (mDha), in some variants this position is occupied by a serine (Namikoshi *et al.*, 1992).

Microviridins (Ishitsuka *et al.*, 1990) are characterized by a multicyclic structure formed by peptide and ester bonds, the peptide also have a side chain of variable length. They are the largest known cyanobacterial peptides, with molecular weight around 1700 Daltons.

Their amino acids are all in L-configuration and the only non-proteinogenic unit is the N-terminal acetic acid. Variation in this group of peptides occurs primarily due to modifications in the side chain and in position 5 in the ring (Welker & von Dohren, 2006). In a different way microviridins are synthesized ribossomaly and not trough NRPS/PKS systems (Philmus *et al.,* 2008).

The structural variety of cyanobacterial peptides can also be explained by the diversity of NRPS and PKS gene clusters in cyanobacteria. Christiansen *et al.,* (2001) found NRPS genes in 75% of 146 axenic cultures of all subsections cyanobacteria. NRPS pathways have been described for aeruginosins (Ishida *et al.,* 2007), cyanopeptolins (Rouhiainen *et al.,* 2000; Tooming-Klunderud *et al.,* 2007; Rounge *et al.,* 2007), microcystins (Tillet *et al.,* 2000; Moffitt & Neilan, 2001; Rouhiainen *et al.,* 2004) and other peptides (Welker & von Dohren, 2006).

In this work, strains of cyanobacteria were characterized according to their peptide production pattern. The objective was to create a peptide profile of each strain using biochemical techniques.

2 – Methodology

2.1 – Strains

Four strains were used in the experiments, two *Radiocystis fernandoii* strains (R28 and R86), one *Microcystis panniformis* strain (Mp9) and one *Microcystis aeruginosa* strain (Ma26). *Microcystis* is a well-known bloom forming genera (Oliver & Ganf, 2002), which is also notorious for its ability to produce secondary metabolites (Welker *et al.*,

2006). *Radiocystis* is a genus commonly found in tropical regions (Sant´Anna *et al.*, 2008), which also produces peptides (Vieira *et al.*, 2003; Lombardo *et al.*, 2006; Pereira *et al.*, 2012). All strains are maintained in the culture collection of the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais (Brazil).

2.2 – Biochemical analysis

Cultures were grown in 500 ml of WC media (Guillard & Lorenzen) for approximately 10 days. Growth conditions were 12h light: 12h dark photoperiod at 20°C and 60 $\mu mol.m^{-2}.s^{-1}$ of light intensity (PAR). After growth, cultures were freeze-dried and the obtained powder used for biochemical analysis. Secondary metabolites were extracted with methanol 75%, the procedure was undertaken with the addition of the solvent, followed by sonication on ice and centrifugation (12000 RPM), and for better results the process was repeated three times. The obtained extract was purified according to Lawton & Edwards (2001), by reverse phase chromatography using SPE-C18 cartridges (Waters, Sep-Pak Vac 3cc – 500 mg). The purified material was concentrated with the aid of a speed-vac system and used for the HPLC analysis.

Chromatography was done in a HPLC (Waters Alliance 2695) coupled with a photodiode array detector (PDA - Waters 2996) and measurements were made at 225 nm and 238 nm. The column used was a Waters Symmetry C18 (4,6 X 250 mm I.D., 5 µm ODS). Mobile phase A was acetonitrile, containing 0.1% (v/v) trifluoroacetic acid (TFA), and mobile phase B was water, containing 0.1% (v/v) trifluoroacetic acid (TFA). The chromatographic run consisted of a linear gradient from 30% A to 34% in 33.5 minutes then 40% for 6.5 minutes with a flow-rate of 1ml/min.

To identify the peptides produced by each strain, the HPLC fractions were collected and analysed in a mass spectrometer system. The equipment used was a MALDI-TOF-TOF Autoflex III (Bruker Daltonics, Billerica, USA). The products were mixed with an α-cyano-4-hydroxycinnamic acid matrix solution (1:1, v/v) and left to dry at room temperature in a MALDI target plate Anchorchip 600 (Bruker Daltonics, Billerica, USA). The peptide masses were obtained using a reflector mode and compared with known cyanobacterial metabolites. All peptides were then fragmented using the LIFT fragmentation mode (MS/MS), and the fragment patterns were analysed according to Erhard *et al.* (1999) and Welker *et al.* (2006).

2.3 – Statistical analysis

A dendogram based on hierarchical analysis was done using the software JMP, version 7. For the construction of the dendogram, it was considered the production of each individual peptide and class of peptide by each strain.

3 – Results

3.1 – Peptide pattern

A total of 17 different oligopeptides were identified, the strain R28 produced six different compounds while each of the other three strains produced five different peptides. The identified compounds include aeruginosins, cyanopeptolins, microcystins and microviridins. Aeruginosins were detected only in the *Microcystis* strains, while microviridins were found only in the *Radiocystis* strains. Microcystins were produced by both genera and by all strains tested, Strain R86 produced only one microcystin (MC-

RR), both *Microcystis* strains produced two microcystins (MC-LR and MC-976) and strain R28 produced four microcystins (MC-RR, MC-YR, MC-FR and MC-WR). Cyanopeptolins were produced by both *Radiocystis* strains and by one of the *Microcystis* strains. Strain Ma 26 was the only one that did not produce any kind of cyanopeptolin.

Peptides and their respective producer strains are listed on table 1. A hierarchical analysis divided the two *Microcystis* species and the two *Radiocystis* strains into two separate groups (Figure 1). Figures 2, 3, 4 and 5 present, respectively, the chromatographic profiles for strains R28, Mp9, Ma26 and R86. The chromatograms are shown in two different wavelengths, 225nm and 238nm. The first wavelength is used to detect proteins and peptides in general and the 238nm is the optimum wavelength to detect microcystins. Figures 6, 7, 8 and 9 present the fragmentation pattern obtained from the MALDI-TOF mass spectrometer used to identify each class of peptides studied.

Table 1 - Peptides produced by each strain

Peptide	Mass (M+H)	Strain
Aeruginosin 98B	575.3	Ma26
Aeruginosin 101	643.3	Ma26; Mp9
Aeruginosin 608	609.3	Ma26
Aeruginosin 684	685.3	Mp9
Cyanopeptolin 980	981.5	R86
Cyanopeptolin 1014	1015.5	R86
Cyanopeptolin 1043	1044.5	R28; Mp9
Cyanopeptolin 1071	1072.5	R28
Microcystin 976	976.5	Ma26; Mp9
Microcystin-LR	995.6	Ma26; Mp9
Microcystin-RR	1038.6	R28; R86
Microcystin-YR	1045.6	R28
Microcystin-FR	1029.6	R28
Microcystin-WR	1068.6	R28
Microviridin 1707	1707.7	R86
Microviridin 1709	1709.7	R28
Microviridin 1739	1039.7	R86

Masses (M+H) are given in Daltons

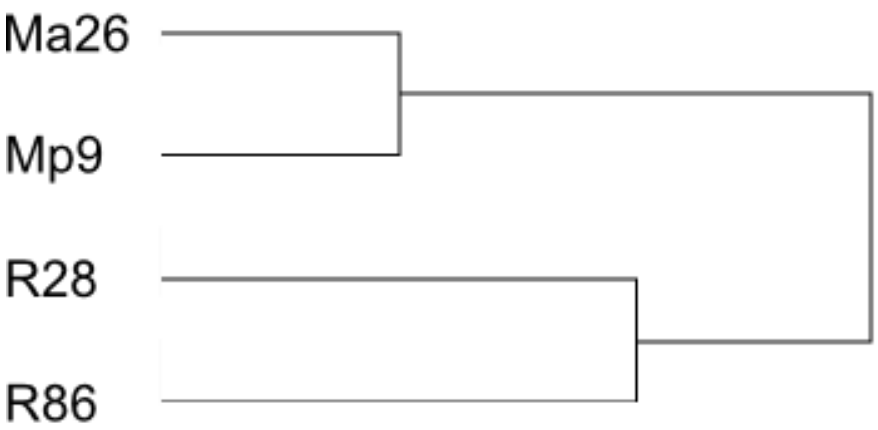

Figure 1 – Dendogram showing the groups formed by the four strains tested, according to their peptide profile.

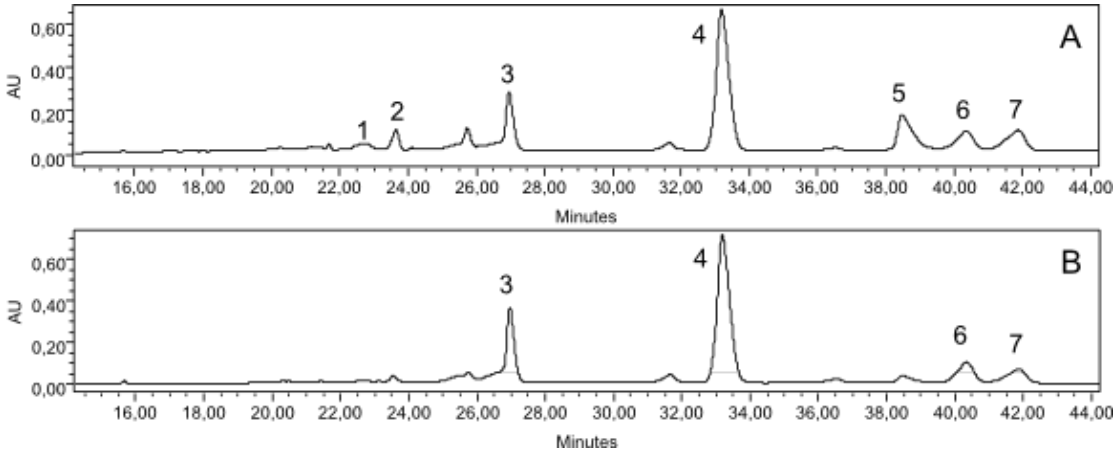

Figure 2 – Chromatograms obtained for strain R28. A – 225nm; B – 238nm. Peaks legend: 1-Mv1709, 2-Cy1043, 3-Mc-RR, 4-Mc-YR, 5-Cy1071, 6-Mc-FR, 7-Mc-WR.

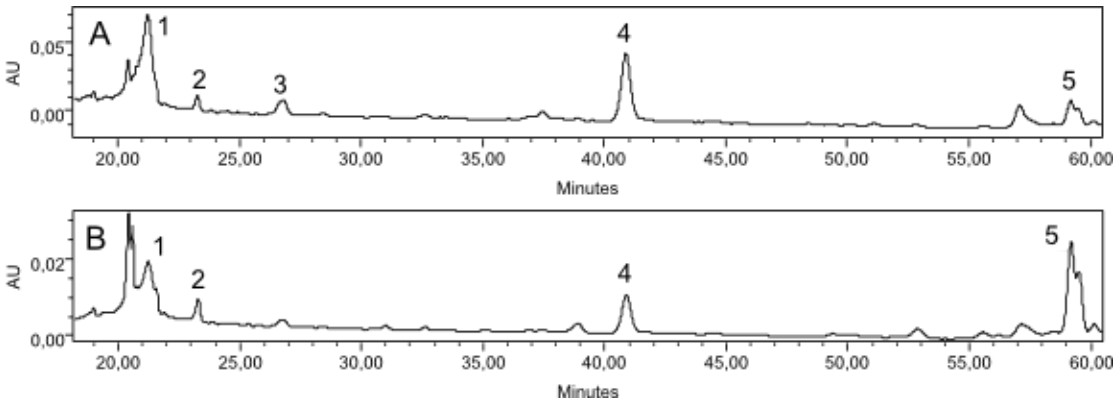

Figure 3 – Chromatograms obtained for strain Mp9. A – 225nm; B – 238nm. Peaks legend: 1-Aer101, 2-Cy1043, 3-Aer684, 4-Mc-LR, 5-Mc-976.

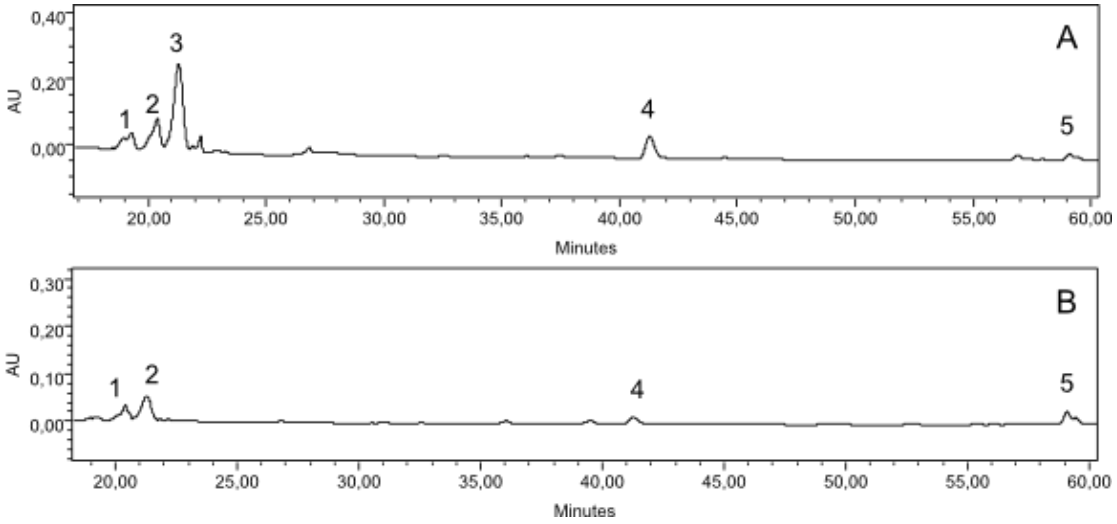

Figure 4 – Chromatograms obtained for strain Ma26. A – 225nm; B – 238nm. Peaks legend: 1-Aer98B, 2-Aer608, 3-Aer101, 4-Mc-LR, 5-Mc-976.

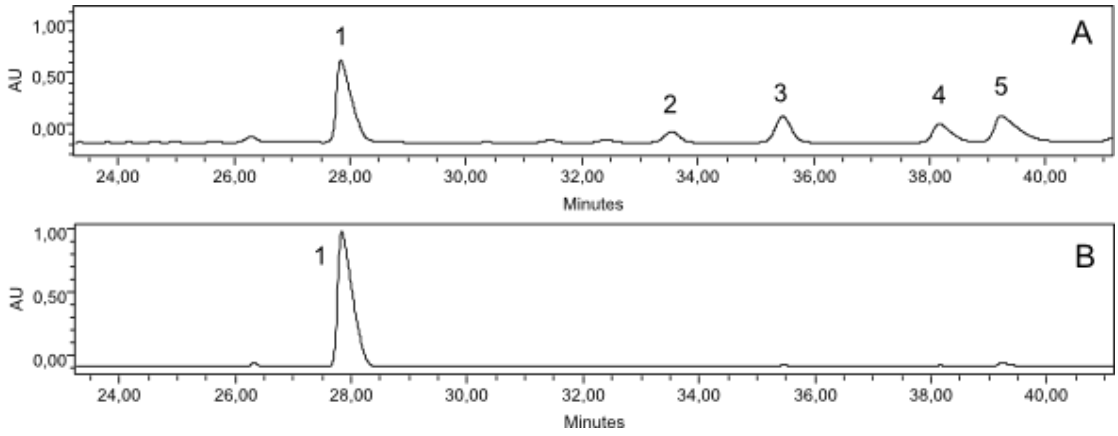

Figure 5 – Chromatograms obtained for strain R86. A – 225nm; B – 238nm. Peaks legend: 1-Mc-RR, 2-Cy980, 3-Cy1014, 4-Mv1707, 5-Mv1739.

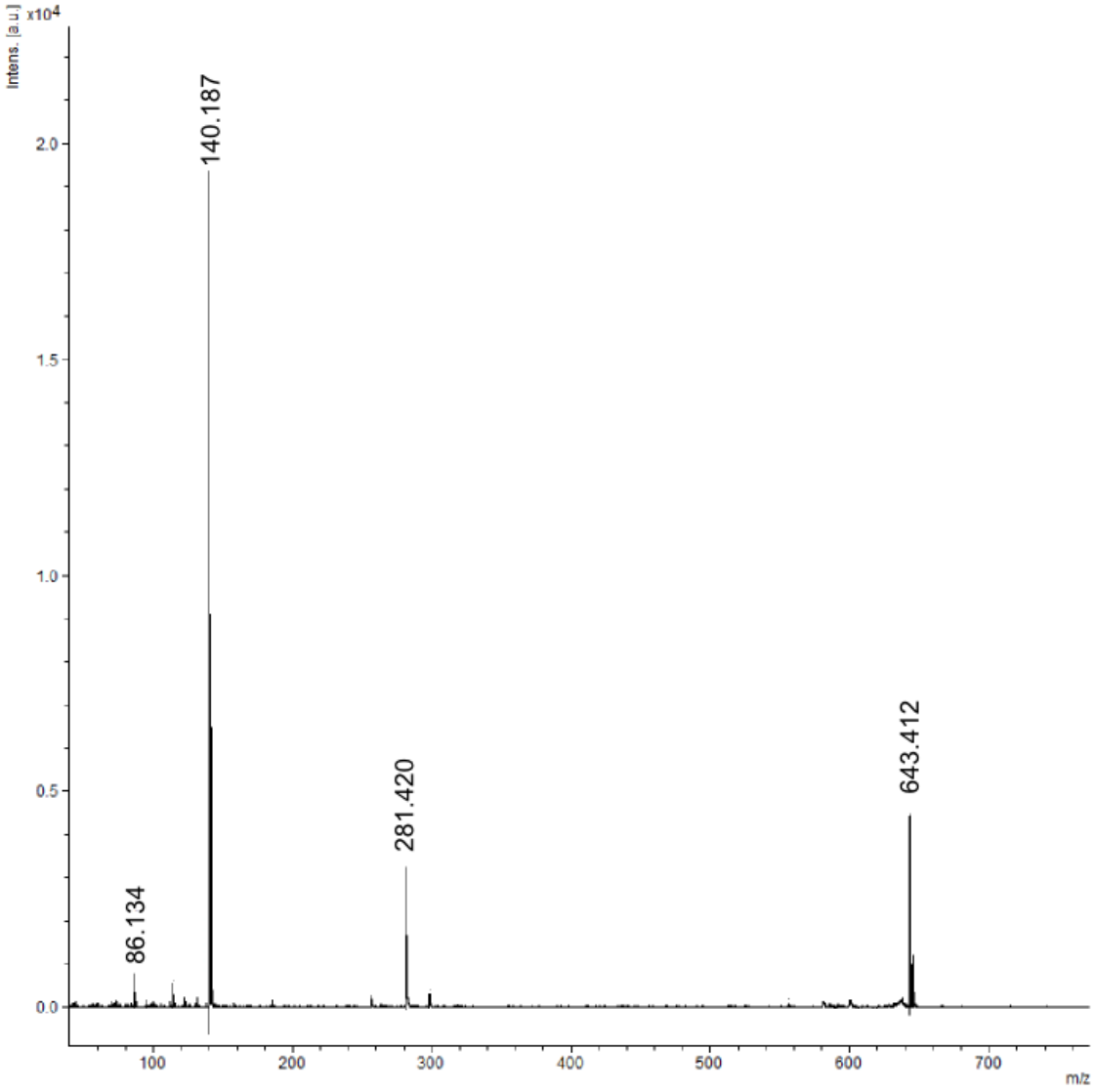

Figure 6 – Fragmentation pattern obtained for the ion m/z 643 (aeruginosin 101) in the MALDI-TOF mass spectrometer.

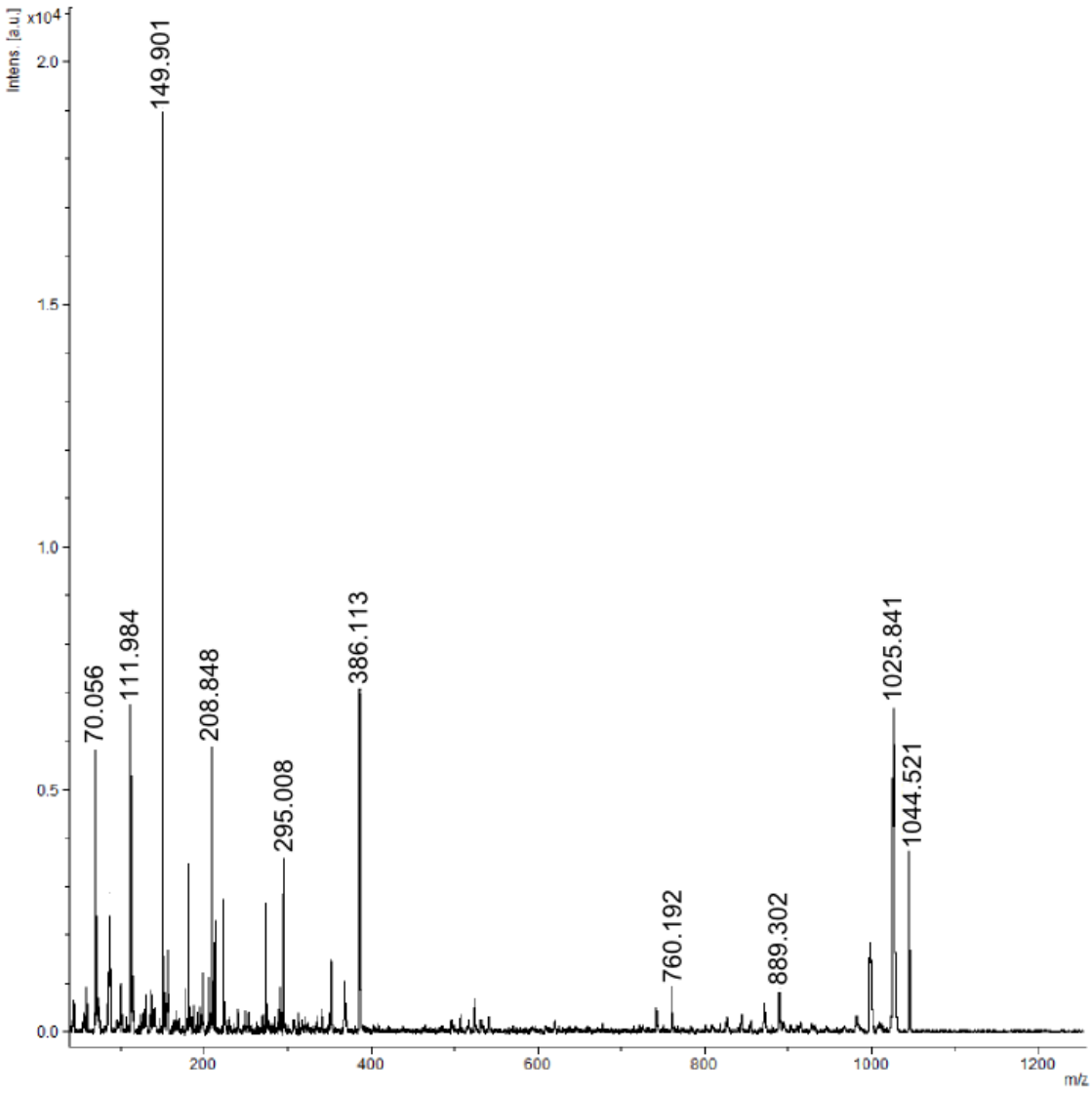

Figure 7 – Fragmentation pattern obtained for the ion m/z 1044 (cyanopeptolin 1043) in the MALDI-TOF mass spectrometer.

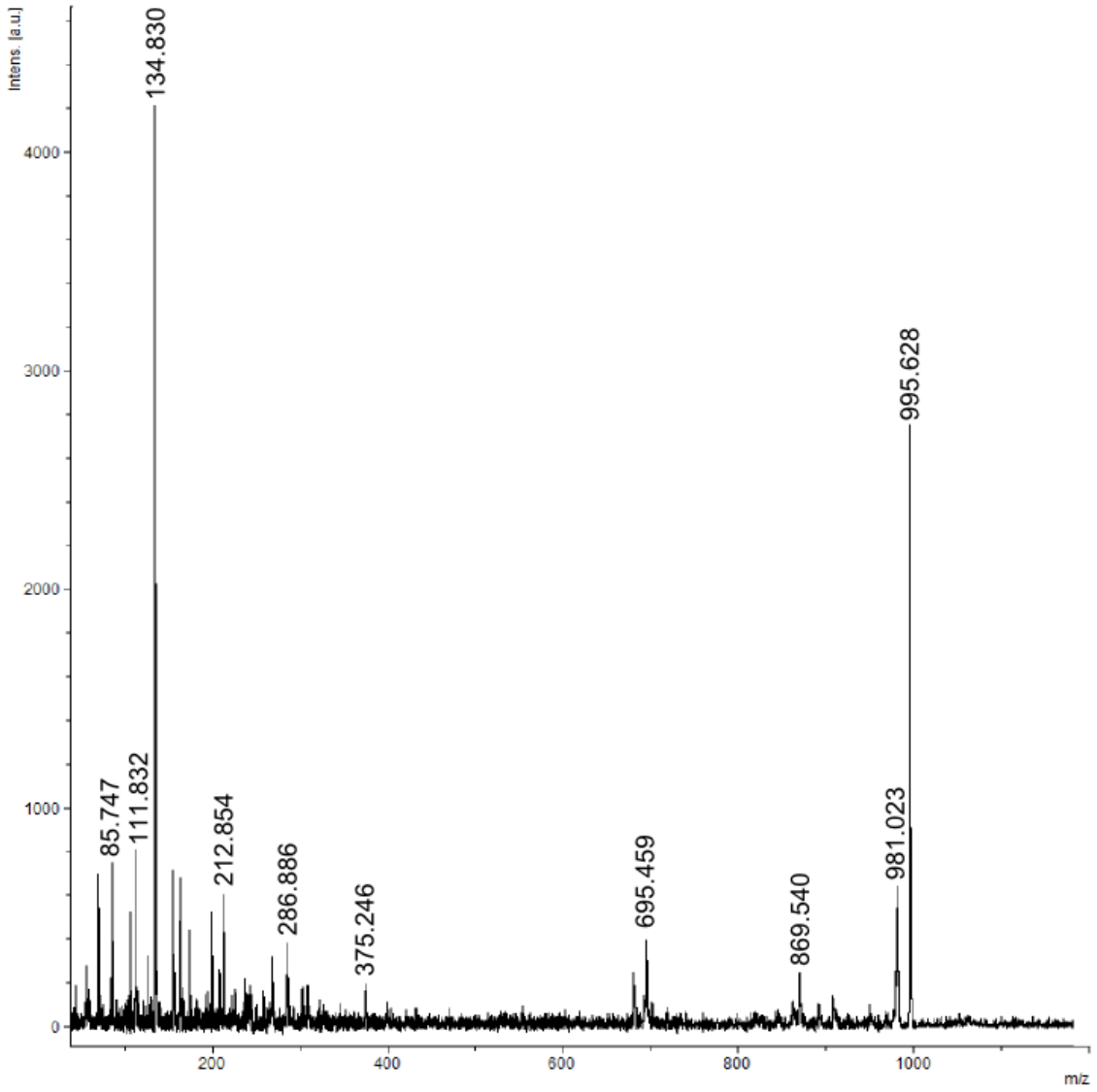

Figure 8 – Fragmentation pattern obtained for the ion m/z 995 (microcystin-LR) in the MALDI-TOF mass spectrometer.

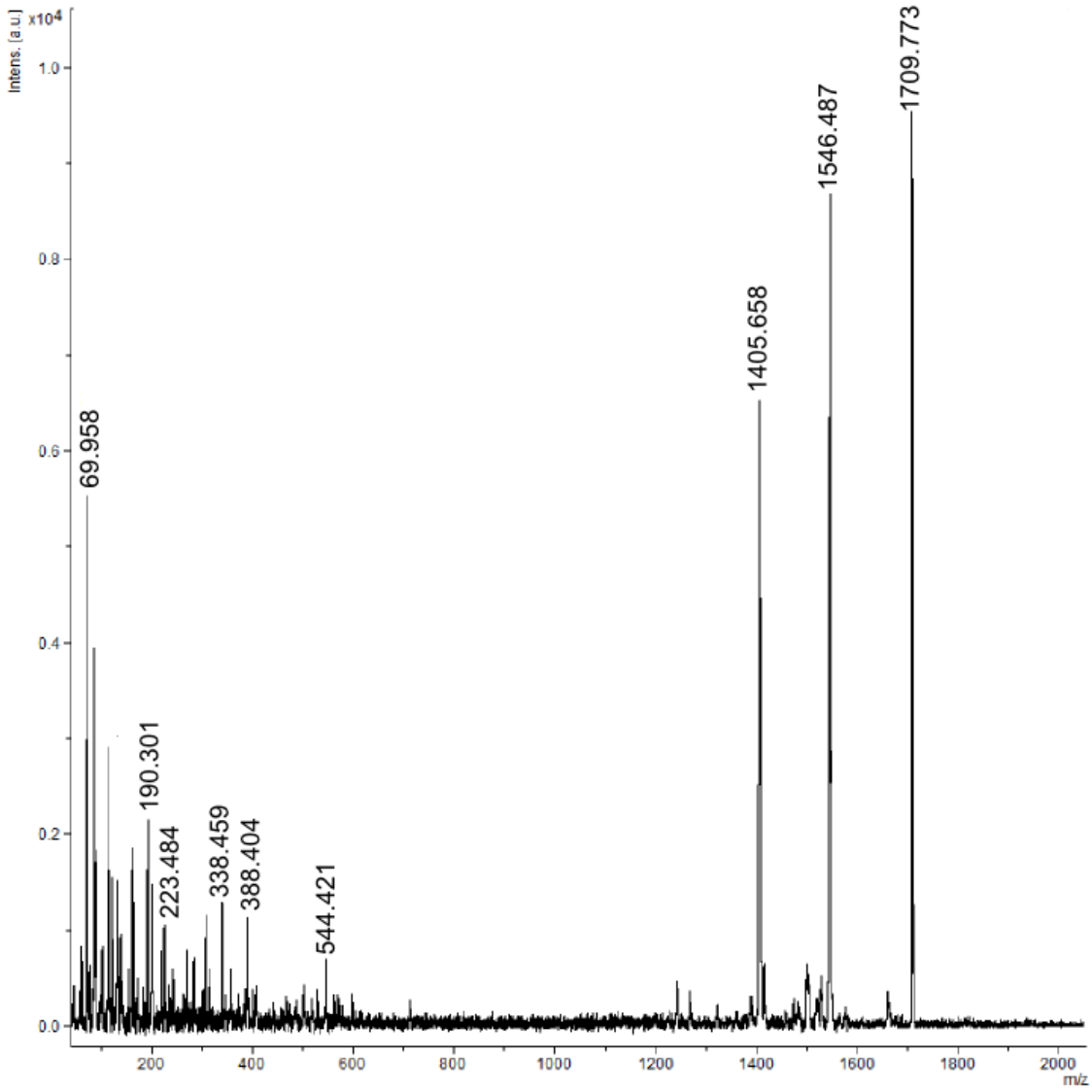

Figure 9 – Fragmentation pattern obtained for the ion m/z 1709 (microviridin 1709) in the MALDI-TOF mass spectrometer.

4 – Discussion

As research progresses new compounds produced by cyanobacteria are discovered, and some of these substances are already being proved as toxic to humans and other mammals (Welker & von Döhren, 2006). Other important characteristic of cyanobacteria is the ability to form blooms in some situations, especially in water bodies suffering from nutrient enrichment (Paerl *et al.,* 2001). The production of toxic compounds coupled with the capacity of forming blooms is a growing concern worldwide not only for human health but also for the entire aquatic trophic chain.

The *Microcystis* genus is known for its capacity to grow in eutrophic environments, usually forming blooms (Oliver & Ganf, 2002). Several studies were done concerning the production of secondary metabolites in this genus (Fastner *et al.*, 2001; Welker *et al.*, 2004a; Welker *et al.,* 2006), and these findings showed that *Microcystis* species are able to produce several kinds of peptides of different classes and that a single strain may produce several peptides at the same time. *Radiocystis* (Komárek & Komárková-Legenerová, 1993) is a genus known to form blooms in tropical regions (Sant´Anna *et al.,* 2008). It is not as common as *Microcystis* and as a consequence, studies about this genus are less frequent. Regarding the production of secondary metabolites, it is known that it is able to synthesize microcystins and other peptides (Vieira *et al.,* 2003; Lombardo *et al.,* 2006; Pereira *et al.,* 2012). In this study all strains produced from five to seven peptides of three different classes: aeruginosins, cyanopeptolins and microcystins for the *Microcystis* strains and cyanopeptolins, microcystins and microviridins for the *Radiocystis* strains. It is known that cyanobacterial oligopeptides can be found in all

sections of cyanobacteria and have a patchy distribution, which is expected for true secondary metabolites (Christiansen *et al.,* 2001). In this work no aeruginosins were found in the *Radiocystis* strains, while no microviridins were found in the *Microcystis* strains and cyanopeptolins could not be found in the *Microcystis aeruginosa* strain. The lack of certain classes of peptides in some strains might be explained by gene deletion events. For example, the microcystin gene cluster is a very ancient unit that was probably present in the common ancestor of modern *Anabaena, Microcystis, Nostoc* and *Planktothrix* (Rantala *et al.,* 2004), and the gene deletion events already proved for microcystins (Christiansen *et al.,* 2008) might also be expected to occur for other non-ribossomal peptides.

Some authors (Fastner *et al.,* 2001; Welker *et al.,* 2006) discussed the use of the peptidic pattern as distinctive chemotypes and the possibility to associate them with known morphospecies of *Microcystis*. Because of the wide variety of peptides produced by a single strain this approach appears to be very complicated. The number of strains used in this work is not suited for a correlation between chemotypes and morphospecies, but a simple dendogram based on hierarchical analysis done with the peptide classes and the individual peptides produced by each strain showed that both species of *Microcystis* and the two strains of *Radiocystis fernandoii* form two separate groups. While the *Microcystis* species are the only ones that produce aeruginosins and share the same microcystins, the *Radiocystis* are the only ones that produce microviridins and both of them produce the microcystin-RR. This result suggests that in a study with a wider range of species and genera it might be possible to correlate the chemotypes with different genera.

In the other hand, it may be possible to correlate chemotypes with ecotypes, as suggested by Welker *et al.,* (2004b), since chemotypes can also be regarded as evolutionary units, and their interactions would resemble competitive interactions among species more than co-operative interactions between clonal cells, thus justifying the term "community" to define their individual colonies in a sample. Therefore, if a strain can be characterized by its chemical profile as a chemotype and if an ecotype is defined as a group of microorganisms that shares similar ecological characteristics, these two *Radiocystis* strains and *Microcystis* species would be chemotypes representing different ecotypes, each one probably adapted to certain environmental conditions.

The majority of studies that focus on identifying cyanobacterial peptides usually use mass spectrometry. Fastner *et al.* (2001) and Welker *et al.* (2004b) findings were obtained from the use of this approach. Using a MALDI-TOF system, they were able to analyze several samples of colonies and filaments of cyanobacteria. Although this method allows a quick examination of a great number of samples it has some disadvantages. The use of mass spectrometry alone shows only the major peptides produced by each sample, while compounds that are produced in lower quantities may be confused with noise signals because of the peaks produced by the major peptides. This work used liquid chromatography (HPLC) technique coupled with a MALDI-TOF mass spectrometer, and even if this form of analysis is more laborious and needs a great amount of material for each sample, it allows a previous separation of the compounds before the analysis in the MS system. The compound separation permits the isolation and characterization of minor compounds that would be "invisible" in a situation with no

previous separation. The use of HPLC also allows the quantification of the compounds, which might be very difficult to accomplish with mass spectrometry alone.

So far, more than 600 different peptides were isolated and identified in cyanobacteria (Welker *et al.*, 2006). Most of these 600 compounds were identified in strains and environmental samples from North America and Europe, thus there is a lack of information for other parts of the globe and there are probably a lot of different compounds still to be found. In this study of only four strains, for example, we found two aeruginosins (Aer-608 and Aer-684), one cyanopeptolin (Cy-1043) and even one microcystin (Mc-976) that are not yet been described in the literature.

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Capítulo 2

CELL DENSITY DEPENDENT OLIGOPEPTIDE PRODUCTION IN CYANOBACTERIAL STRAINS

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Abstract

Cyanobacteria can form blooms and in these situations they dominate the phytoplanktonic community, reaching extremely high densities. In the domain Bacteria, high population densities can stimulate a phenomenon known as quorum sensing, which may produce several modifications in the cell physiology. Very little is known about quorum sensing in cyanobacteria. Because of their planktonic way of life, quorum sensing should be more evident during a bloom event. In this work, we tested whether cell density could shape the production of bioactive compounds produced by cyanobacteria. The experiments consisted of two treatments, where cultures of cyanobacteria were maintained at low and high cellular densities through a semi-continuous set-up. Analyses were performed by HPLC-PDA and MALDI-TOF MS. Seventeen peptides were detected and 14 identified, including microcystins, aeruginosins, cyanopeptolins and microviridins. The results showed that cellular density seems to have a significant effect on the peptides production. Most of the compounds had significantly higher cellular quotas in the higher density treatment, although microviridins and an unknown peptide were produced only at low density. These results may hint at a possible role for quorum sensing in triggering the production of several cyanobacterial peptides.

1-Introduction

Cyanobacteria can form blooms in aquatic environments such as lakes, reservoirs, and oceans; these situations consist of dominance of the phytoplankton community by one or a few cyanobacteria species and an exponential increase in the biomass of the dominant group (Oliver & Ganf, 2002). Blooms have received considerable worldwide attention, because they are natural events in which populations of cyanobacteria can reach extremely high densities. Since most blooms are toxic, they represent a severe problem. It is acknowledged that nutrient enrichment of aquatic ecosystems promotes their appearance and persistence (Paerl *et al.,* 2001). Phosphorous has been considered the primary nutrient source responsible for algal biomass accumulation in freshwater systems (Schindler *et al.,* 2008) and together with nitrogen it may support the development of blooms when present in excess (Downing *et al.,* 2001). Besides nutrient-rich conditions, other factors favour intensive cyanobacterial growth, like temperatures above 20°C, persistent vertical stratification, calm surface waters and low flushing rates (Reynolds, 1987; Paerl, 1988; Shapiro, 1990). After its establishment, a bloom can persist for several months and in tropical regions they can in some extreme situations become permanent (McGregor & Fabbro, 2000; Figueredo & Giani, 2009).

Cyanobacteria can produce several kinds of oligopeptides whose synthesis is usually achieved by non ribosomal peptide synthetase (NRPS) systems (for a full review see Welker & von Döhren, 2006). The major classes of these peptides are aeruginosins (Murakami *et al.,* 1994), cyanopeptolins (Martin *et al.,* 1993), microginins (Okino *et al.,* 1993), microviridins (Ishitsuka *et al.,* 1990), anabaenopeptins (Harada *et al.,* 1995) and microcystins (Carmichael, 1992). The functions of these compounds are still unclear;

however it is known that some peptides are toxic to zooplankton (Agrawal *et al.*, 2001; Czarnecki *et al.*, 2006) and that microcystins might be related to physiological processes such as photosynthesis (Long *et al.*, 2001; Young *et al.*, 2005) and iron metabolism (Martin-Luna *et al.*, 2006). Microcystins are also toxic to humans and other mammals (Sivonen & Jones, 1999). The increase of cyanobacterial blooms and the toxic nature of some of their peptides create major public health and water treatment problems (Watson *et al.*, 2000; Watson, 2004).

There is a substantial amount of information and research on the factors controlling and affecting the appearance and persistence of cyanobacterial blooms. In addition, the knowledge about the production of microcystins and other cyanobacterial peptides is increasing. However, there is not much information about possible changes in the physiology of the cyanobacterial cells during a bloom event and its potential connection with the production of secondary metabolites. Some authors have previously observed changes in the relative amount of toxic genotypes in a bloom (Briand *et al.*, 2008; Okello *et al.*, 2010; Sabart *et al.*, 2010; Pimentel & Giani, 2013). Furthermore Wood *et al.* (2011), found that the expression of the *mcyE* gene during a bloom event was not constitutive, but was influenced by cell concentration and it could produce increases of up to 28 fold in microcystin levels. In mesocosm experiments, Wood *et al.* (2012) also observed that microcystin cell quota increased significantly with cell density.

Several bacterial species have the ability to release signalling compounds in their environment (Waters & Bassler, 2005) and when these molecules reach a threshold, they trigger a coordinated response able to change the gene expression pattern of the affected

cells and, as a consequence, the metabolism and physiology of the bacterial population. The term quorum sensing is used to describe this density-dependent phenomenon (Fuqua *et al.,* 1994). It is known that quorum sensing can control different biological functions as motility, aggregation, swarming, conjugation, luminescence, virulence, symbiosis, biofilm differentiation, antibiotics biosynthesis and others (Swift *et al.,* 2001; Waters & Bassler 2005; Williams *et al.,* 2007). Up to date a wide variety of molecules responsible for quorum sensing in bacteria have been isolated (for a review, see Williams *et al.,* 2007). The phenomenon of quorum sensing is believed to be widespread in the bacteria domain (Miller & Bassler, 2001), but concerning cyanobacteria there is still a lack of information.

In this work, we examined whether low and high cellular densities could affect peptide concentrations under semi-continuous experimental conditions. The idea was that experimentally-maintained high density cultures would mimic a situation similar to a bloom event. The experiments were run using three different species of cyanobacteria, isolated from Brazilian freshwater systems.

2-Methodology

2.1 – Strains

The strains used in these experiments were *Microcystis panniformis* (strain Mp9), *Microcystis aeruginosa* (strain Ma26) and a *Radiocystis fernandoii* (strain R28). *Microcystis* is one of the most studied genera and is known for its ability to form blooms (Oliver & Ganf, 2002) and produce secondary metabolites such as microcystins and others peptides (Welker *et al.,* 2006). *Radiocystis* (Komárek & Komárková- Legenerová,

1993) is less common and less studied than *Microcystis*, but it is known to form blooms in tropical regions (Sant´Anna *et al.,* 2008) and produce microcystins and other peptides (Vieira *et al.,* 2003; Lombardo *et al.,* 2006; Pereira *et al.,* 2012). All strains are maintained in the culture collection of the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais and were isolated from Furnas reservoir ($20^{o}40'S$; 46^{o} 19'W), located in the south-eastern region of Brazil.

2.2-Experiments

Experiments were performed in two treatments, low cell density and high cell density. Each treatment was prepared in triplicate and received a different amount of inoculum, varying from 20 to 100 mL, in order to create two different cell densities, and the final volume was completed to 250 mL with WC medium (Guillard & Lorenzen, 1972). Growth conditions were 12h light: 12h dark photoperiod, temperature 20±1 °C and 65 $\mu mol.m^{-2}.s^{-1}$ of irradiance. To avoid differences between experiments due to faster nutrient consumption in the higher density treatment, experiments were conducted in semi-continuous cultures. Semi-continuous cultures allow the maintenance of a constant level of biomass, and nutrient-replete growth under controlled conditions. Every other day, a constant volume of the experimental culture was removed and the same amount of fresh medium was added, to maintain the low or high cell density conditions within fixed limits. Under these settings, the experiments ran for 6 days, and cultures were kept at low and high cell density and exhibited similar growth rates. Samples were taken every two days to follow growth and a minimum of 400 cells were counted in a Fuchs-Rosenthal hemocytometer. A 20 mL sample was filtered on the last day and used to

measure chlorophyll, according to the methodology described by Nusch (1980). The rest of the culture was freeze-dried for further biochemical analyses.

2.3-Biochemical analysis

The dry material was extracted three times with methanol 75% (v/v). The procedure was undertaken with sonication on ice followed by centrifugation (15 min, 8900 g). The extract was purified by reverse phase chromatography using SPE-C18 cartridges (Waters, Sep-Pak Vac 3cc – 500 mg) as described by Lawton & Edwards (2001). The purified extract was dried in Speed-Vac system and diluted in a known volume of methanol 75% (v/v). The analyses were done using HPLC (Waters Alliance 2695) with a photodiode array detector (PDA - Waters 2996) at 225 nm and 238 nm wavelength. The column used was a Waters Symmetry C18 (4.6 X 250 mm I.D., 5 µm ODS). Mobile phase A was acetonitrile, containing 0.1% (v/v) trifluoroacetic acid (TFA), and mobile phase B was water, containing 0.1% (v/v) trifluoroacetic acid (TFA). The chromatographic run consisted of a linear gradient from 30% A to 34% in 33.5 minutes then 40% for 6.5 minutes with a flow-rate of 1 mL. min^{-1}. The peptide quantification was done dividing the peak area by the dry weight of the lyophilized material, obtaining a measurement of the relative change in the peptide concentration; this method was chosen because of the lack of standards for most peptides. Dry weight was used to standardize the measurements, since in previous experiments it showed high correlation with cellular biovolume (Pereira *et al.*, 2012). This relationship biovolume/dry weight was tested statistically by regression analysis and results showed highly significant correlation for all strains ($R^2 \geq 0.9$).

Peptides were identified by collecting the HPLC fractions and submitting them to analysis in a MALDI-TOF-TOF Autoflex III mass spectrometer (Bruker Daltonics, Billerica, USA). The products were mixed with α-cyano-4-hydroxycinnamic acid matrix solution (1:1, v/v) and left to dry at room temperature in a MALDI target plate Anchorchip 600 (Bruker Daltonics, Billerica, USA). The peptide masses were obtained using a reflector mode and compared with known cyanobacterial metabolites. Known and unknown peptides were then fragmented using the LIFT fragmentation mode (MS/MS), and the fragment patterns were analysed according to Erhard *et al.* (1999) and Welker *et al.* (2006).

2.4-Statistics

ANOVA tests were used to compare the means of the two treatments (high and low cell densities). The parameters analysed were: peptide concentration (area.mg^{-1}), cell density (cell. mL^{-1}), chlorophyll (µg.mg^{-1}) and growth rate (µ.day^{-1}). The statistical analyses were performed with JMP version 7 software.

3-Results

Fourteen peptides were identified according to their fragmentation pattern and UV absorbance: 4 aeruginosins, 2 cyanopeptolins, 7 microcystins and 1 microviridin. Three metabolites could not yet be identified. Some peptides were produced at both high and low cell density, while others appeared in only in one of the treatments (Table 1). Figures 1 A and B show the chromatographic pattern found in low and high cell density

treatments for strain R28, Figures 1 C and D present results for strain Mp9, and Figures 1 E and F for strain Ma26.

Table 1 - Peptides produced by the studied strains

M+H	Name	Characteristic Ions - M+H	Strain	Scenario	Ratio HD/LD
575.3	Aeruginosin 98B	140	Ma26	Both	2.12
609.3	Aeruginosin 608	140	Ma26	Both	3.66
643.3	Aeruginosin 101	140	Ma26	Both	4.30
643.3	Aeruginosin 101	140	Mp9	High	-
685.3	Aeruginosin 684	140	Mp9	High	-
1044.5	Cyanopeptolin 1043	150	R28	Both	1.72
1044.5	Cyanopeptolin 1043	150	Mp9	High	-
1072.5	Cyanopeptolin 1071	150	R28	Both	1.44
976.5	Microcystin 976	135	Ma26	Both	4.08
976.5	Microcystin 976	135	Mp9	Both	3.69
995.6	Microcystin-LR	135	Ma26	Both	1.89
995.6	Microcystin-LR	135	Mp9	Both	8.33
1029.6	Microcystin-FR	135	R28	Both	1.49
1038.6	Microcystin-RR	135	R28	Both	2.03
1045.6	Microcystin-YR	135	R28	Both	1.41
1058.6	Microcystin-YR*	135	Ma26	Both	89.66
1068.6	Microcystin-WR	135	R28	Both	1.33
1709.7	Microviridin 1709	-	R28	Low	-
563.3	Unknown (P 562)	-	Ma26	Low	-
563.3	Unknown (P 562)	-	Mp9	Low	-
1058.5	Unknown (P 1057)	-	Ma26	Both	2.91
1058.5	Unknown (P 1057)	-	Mp9	High	-
1103.2	Unknown (P 1102)	-	Mp9	High	-

Masses (M+H) are given in daltons; * homo variant of the aminoacid tyrosine in position 2

Strain R28 produced four microcystins. The variants RR and YR were the major constituents, while the variants FR and WR were produced in lower concentration. Strains Ma26 and Mp9 produced the well-studied microcystin-LR and a non-identified variant with a molecular weight of 976 daltons. There is no evidence in the literature of a microcystin molecule with 976 daltons, but the peptide we found presented the same fragmentation pattern and UV absorbance spectra that is characteristic of microcystins

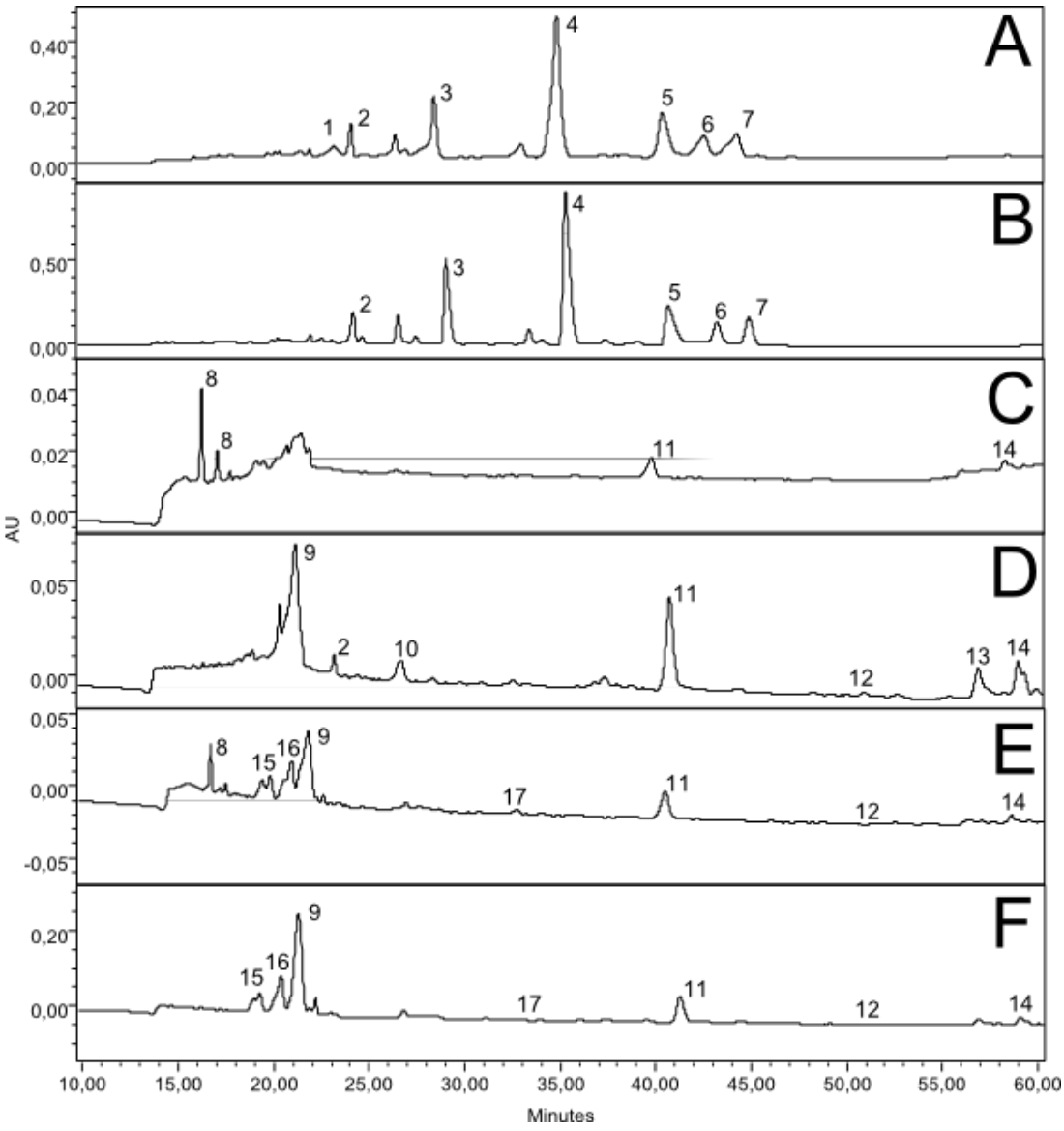

Figure 1 - Chromatograms obtained for low and high cellular densities in the three strains tested. (a) R28, low cell density; (b) R28, high cell density; (c) Mp9, low cell density; (d) Mp9, high cell density; (e) Ma26, low cell density; (f) Ma26, high cell density. Peaks legend: 1-Mv-1709, 2-Cy-1043, 3-Mc-RR, 4-Mc-YR, 5-Cy-1071, 6-Mc-FR, 7-Mc-WR, 8-P562, 9- Aer101, 10-Aer984, 11-Mc-LR, 12-P-1057, 13-P-1102, 14-Mc-976, 15-Aer98B, 16- Aer608, 17-Mc-YR*.

(see supplementary material, supplement 1, Fig. 1). Strain Ma26 also produced a different microcystin-YR, which seems to be a homovariant showing the amino acid tyrosine in position 2. This compound had a molecular mass of 1058 daltons, which is the same mass of a non indentified peptide (peptide 1057) produced by strains Ma26 and Mp9. However, the microcystin presented the fragmentation pattern and UV absorbance spectra that are typically found in microcystins, while the peptide 1057 had different fragmentation pattern and UV absorbance spectra. Furthermore, retention time was different for each one of the two compounds, characterizing them as different peptides.

Among aeruginosins, we found a total of four variants, aeruginosin 98B, aeruginosin 101 and aeruginosin 608 were produced by strain Ma26 and aeruginosin 684 and aeruginosin 101 were produced by strain Mp9. Aeruginosins were the main peptide class produced by strain Ma26, with aeruginosin 101 being the major compound, followed by aeruginosin 608. Only two cyanopeptolins were found in the strains used in these experiments, cyanopeptolin 1071 produced exclusively by R28 strain and cyanopeptolin 1044 produced by strains R28 and Mp9. For strain R28, both cyanopeptolins were produced at low and high cell densities, but the production was significantly higher in the high density treatment for cyanopeptolin 1071. In this strain, the production of cyanopeptolin 1043 was higher in the high density treatment, but not significant. However, for the strain Mp9, the cyanopeptolin 1043 was only found in the high density treatment. It is possible that the production of this peptide in strain Mp9, growing at low cell density, is kept at basal levels that could not be measured by the HPLC technique for lack of sensitivity of the method. Only one microviridin was found in the experiments, the microviridin 1709, produced by strain R28: no other strains produced microviridins.

Three non-identified peptides were detected in the experiments. Their fragmentation pattern could not be associated with any class of cyanobacterial peptides and additional biochemical studies are needed to establish whether they belong to an existing class of peptides or not. Among these peptides there are two compounds (P 1102 and P 1057) that had increased production at high cellular densities and one compound that was seen only at low cell density (P 562).

Figure 2 shows the peptide concentrations in all strains and treatments. The amount of all substances produced by strain 28 was significantly higher in the high density treatment, with the exception of cyanopeptolin 1043 which showed no significant difference between treatments and the microviridin 1709 that was detected only in the low density treatment (Figure 2-A). The amounts of microcystin-LR and microcystin 976 produced by strain Mp9 were significantly higher in the high density treatment, the other compounds were only detected in the high density treatment and peptide 562 was detected in two different peaks only in the low density treatment (Figure 2-B). For all compounds observed in strain Ma26, a significantly greater amount was produced in the high density treatment, with the exception of the peptide 562 which was detected only in the low density treatment (Figure 2-C and D).

The difference between cell density (cell.mL^{-1}), and chlorophyll concentration (µg.mg^{-1}) in the low and high cell density treatments was statistically significant (Table 2). Growth rates showed no significant difference between treatments, since cultures were maintained stable in a semi-continuous set up. For strain Ma 26 growth rate was 0.14 (low density) and 0.16 day^{-1} (high density), for Mp9, 0.16 (LD) and 0.15 day^{-1} (HD), and for R28 growth rate was 0.06 (LD) and 0.05 day^{-1} (HD).

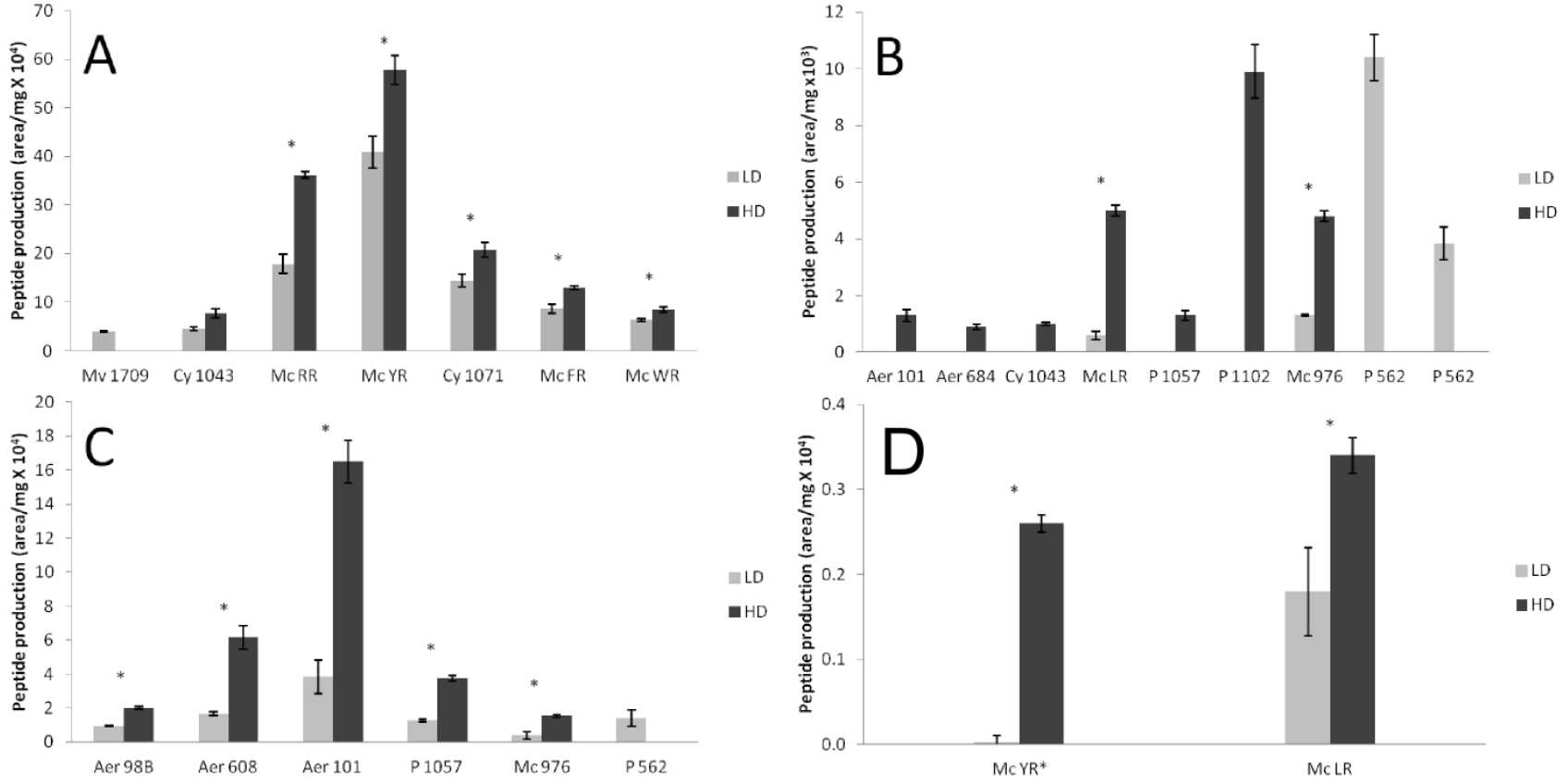

Figure 2 - Peptides concentration in all strains tested under low and high cellular densities. (a) *Radiocystis fernandoii* 28; (b) *Microcystis panniformis* 9; (c) *M. aeruginosa* 26; (d) *M. aeruginosa* 26. Detail of Mc-YR* and Mc-LR. Error bars denote standard deviations. The symbol * denotes significant differences between means ($P < 0.001$).

Table 2. Cell density, chlorophyll a concentration and growth rate in the three strains studied at low and high cell density treatments

	Cell.ml-1	Chl-µg.mg-1	µ.day-1
Strain R28			
LD	1027000 ± (46000)	1.61 ± (0.09)	0.146 ± (0.054)
HD	1928000 ± (62000)	3.33 ± (0.31)	0.087 ± (0.009)
P	0.0036*	0.0054*	0.274
Strain Ma26			
LD	606000 ± (94000)	0.15 ± (0.005)	0.097 ± (0.037)
HD	3137000 ± (174000)	0.99 ± (0.021)	0.100 ± (0.040)
P	0.0001*	0.0001*	0.814
Strain Mp9			
LD	891000 ± (127000)	0.30 ± (0.030)	0.085 ± (0.015)
HD	2596000 ± (333000)	0.77 ± (0.004)	0.064 ± (0.024)
P	0.0012	0.0021*	0.299

* significant difference

4-Discussion

Cyanobacteria are known to produce several types of bioactive oligopeptides (Welker & von Döhren, 2006) and they also form blooms where cell density is ten to hundred times higher than in normal phytoplanktonic populations (Oliver & Ganf, 2002). In this research we investigated whether cell density could affect the production of some of these peptides. Our results showed that the production of peptides was significantly different at low cell and high cell densities, suggesting the existence of a quorum sensing phenomenon in planktonic cyanobacteria: the higher cell density may have modified the communication patterns among cells and, as a result, peptide concentrations changed.

During a bloom event, the elevated cellular density of cyanobacterial populations creates a favourable environment for quorum sensing. In this kind of situation, the high cellular density would intensify the accumulation of signaling molecules in the environment, thus

optimizing the conditions for the occurrence of quorum sensing. Gobler *et al.* (2007) investigated the dynamics and toxicity of cyanobacteria in a eutrophic lake in New York and found that the expression of the *mcyE* gene, which is part of the microcystin operon, was higher during periods of high cellular density and declined during months of lower cell density. Even though the authors did not link their results with quorum sensing, their findings may hint to a potential correlation between the existence of this phenomenon and the *mcyE* gene expression. Similar results were also found by Wood *et al.* (2011) who observed that higher levels of microcystin per cell (up to 28 fold) occurred when cell concentration increased from 70,000 to 4,000,000 cells. mL^{-1} and corresponded to changes in the *mcyE* gene expression. In mesocom experiments, Wood *et al.* (2012) also detected a significant up-regulation of microcystin cell quota upon increasing the concentration of *Microcystis* cells. Of course caution has to be used when correlating laboratory experiments with natural environments, since experiments represent conditions that are simpler than natural scenarios; nevertheless they allow the isolation and testing of a specific factor and the understanding of single phenomena. In our experiments, the high cell density cultures represent a bloom situation, and the results obtained suggest significant differences in the production of secondary metabolites between bloom and non-bloom events.

The experiments were made in semi-continuous cultures, which is an important procedure that guarantees that a low cell density culture does not attain high cellular density and that a high cell density culture does not reach stationary phase. This method assured that the strains maintained their cell density almost stable during the experimental

period, which is evidenced by the non-significantly different growth rates measured in both situations for all strains tested.

How the quorum sensing modulates metabolic responses in cyanobacteria is still poorly understood, but it is known that acylhomoserine lactones (AHLs), which is a class of autoinducers produced by several gram-negative bacteria, are produced and can influence the physiology of the organisms. Sharif *et al.* (2008), for example, showed that cyanobacteria from the genus *Gloeothece* produce an *N*-octanoyl-homoserine lactone, the accumulation of which is able to change the expression of 43 different proteins. Romero *et al.* (2011) also showed that AHLs with different side chains can inhibit nitrogen fixation in *Anabaena*, while Van Mooy *et al.* (2012) found that phosphorous acquisition is increased in marine *Trichodesmium* consortia in the presence of AHLs.

Our results are an indication of a potential link between quorum sensing and production of secondary metabolites in cyanobacteria as shown by the different peptide pattern at low and high cell densities. For most of the compounds measured, the production was stimulated in the high cell density treatment, with a significant increase in concentration usually from 1.5 up to 4 times at the high density, and in some cases even reaching 8.3 (Figure 2) and 89.6 times higher values, respectively for microcystin-LR in Mp9 (Figure 2B) and microcystin-YR* in Ma26 strain (Figure 2D). Interestingly, some peptides were present only in the low density treatment, having their production suppressed when the cellular density of the culture was higher, as observed for microviridin 1709 in R28 strain and peptide 562 in Mp9 and Ma26 strains. In a mesocosm experiment, Wood *et al.* (2012) found an increase in microcystin quota in situations where the cell number per mL reached 2.9 million and 7 million. In our experiments the number of cells in the high

density treatment varied from 1.9 to 3.1 million per mL and even though the experiments had different designs and conditions, the results were similar, which corroborates the idea that microcystin production is stimulated in situations of high cellular density.

Although there is a lot of new information on cyanobacterial peptides, it is still difficult to understand their functions in the physiology and ecology of cyanobacteria; some of them can inhibit proteases from zooplanktonic organisms, characteristic of protection against grazing (Agrawal *et al.,* 2001; Rohrlack *et al.,* 2003; von Elert *et al.,* 2004; Czarnecki *et al.,* 2006). There are also indications that microcystins may be involved in light-related photosynthetic processes (Long *et al.,* 2001; Young *et al.,* 2005). It is known that no specific group of oligopeptide is essential for the growth of cyanobacteria, as natural populations are composed of producing and non-producing strains, for each peptide and peptide class (Fastner *et al.,* 2001; Rohrlack *et al.,* 2001; Welker *et al.,* 2004) and no clear advantage has yet been seen for producing over non producing strains (Kaebernick *et al.,* 2001). In our experiments we saw that the peptide pattern changes not only in concentration, but also, in peptide composition according to the cellular density of the culture. All strains had some peptides that appeared only in the low cell density treatment and strain Mp9 had several peptides that exclusively appeared in high cellular density, which is in accordance with the idea that these compounds are not essential for survival, at least individually, although they may offer some advantage to the individual strains in particular situations.

Microcystins are characterized by the amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda, position 5) (Carmichael, 1992). In this study, we found seven different variants of these compounds. Our experiments

showed that all microcystin variants were produced at both cell densities, nevertheless their production was stimulated when the cell density was higher. Because of their toxicity to humans and other mammals, microcystins are cause of concern for human health and water treatment plants (Watson, 2004) and, as a consequence, a wide range of information is already available. Our findings add new knowledge to the ecology and physiology of this compound, because the fact that quorum sensing may stimulate the production of microcystins is of importance to the management of lakes, reservoirs and water treatment plants.

Aeruginosins stand as a class of compounds formed by linear peptides characterized by a hydroxy-phenyl lactic acid (Hpla) at the N-terminus, the amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi) and an arginine derivative at the C-terminus (Murakami *et al.*, 1995). In strain Ma26, aeruginosins were the main peptide class. The production pattern for aeruginosins was the same as for microcystins, with significantly higher concentrations observed at the high density treatment. Only two cyanopeptolins were found in the strains used in these experiments. This class of peptides is characterized by the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) and the cyclization of the peptide ring by an ester bond of the b-hydroxy group of threonine with the carboxy group of the terminal amino acid (Martin *et al.*, 1993). Cyanopeptolins also presented a production response similar to the observed for microcystins and aeruginosins.

The resembling patterns found for microcystin, aeruginosin and cyanopeptolin may reflect their biosynthesis route, which is based on non ribosomal peptide synthetase systems. We observed similar responses for the three classes of peptides and each strain had a major peptide belonging to a different class (microcystin for R28, an unknown

peptide for Mp9 and aeruginosin for Ma26). Such results point to the fact that these compounds may have interchangeable functions. These observations, added to the knowledge that cyanobacterial natural populations consist of a mix of producing and nonproducing strains for each class of peptide (Fastner *et al.,* 2001; Welker *et al.,* 2004), are good indicators of the multiplicity of profiles found in nature with potential similar cellular function.

Microviridin is the largest known cyanobacterial oligopeptide (Ishitsuka *et al.*,1990), and this class is characterized by a multicyclic structure established by secondary peptide and ester bonds and a side chain of variable length; its amino acids are all in L-configuration and the only non-proteinogenic unit is the N-terminal acetyl group. In our experiments only one variant was found in strain R28 and this peptide was produced only at low cell density, opposite to the observed for the other classes of peptides. As previously noted by some authors (Welker & von Döhren, 2006; Philmus *et al.,* 2008) microviridins are synthesized ribosomally and their structure is finalized by post-translational modifications. The different form of synthesis might be one explanation for the opposite behaviour of this microviridin, and also an indication that its function might be different when compared to the peptides synthesized by NRPS.

There are still many open questions concerning the complex connections between cyanobacteria, blooms, quorum sensing and production of secondary metabolites. Genetic studies and field experiments are needed to expand the knowledge on these interactions. Our results suggest that quorum sensing may be an important mechanism in regulating the physiology of bloom forming cyanobacteria and that this phenomenon can play a significant role in the production of toxic and non-toxic peptides in these organisms.

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Supporting information

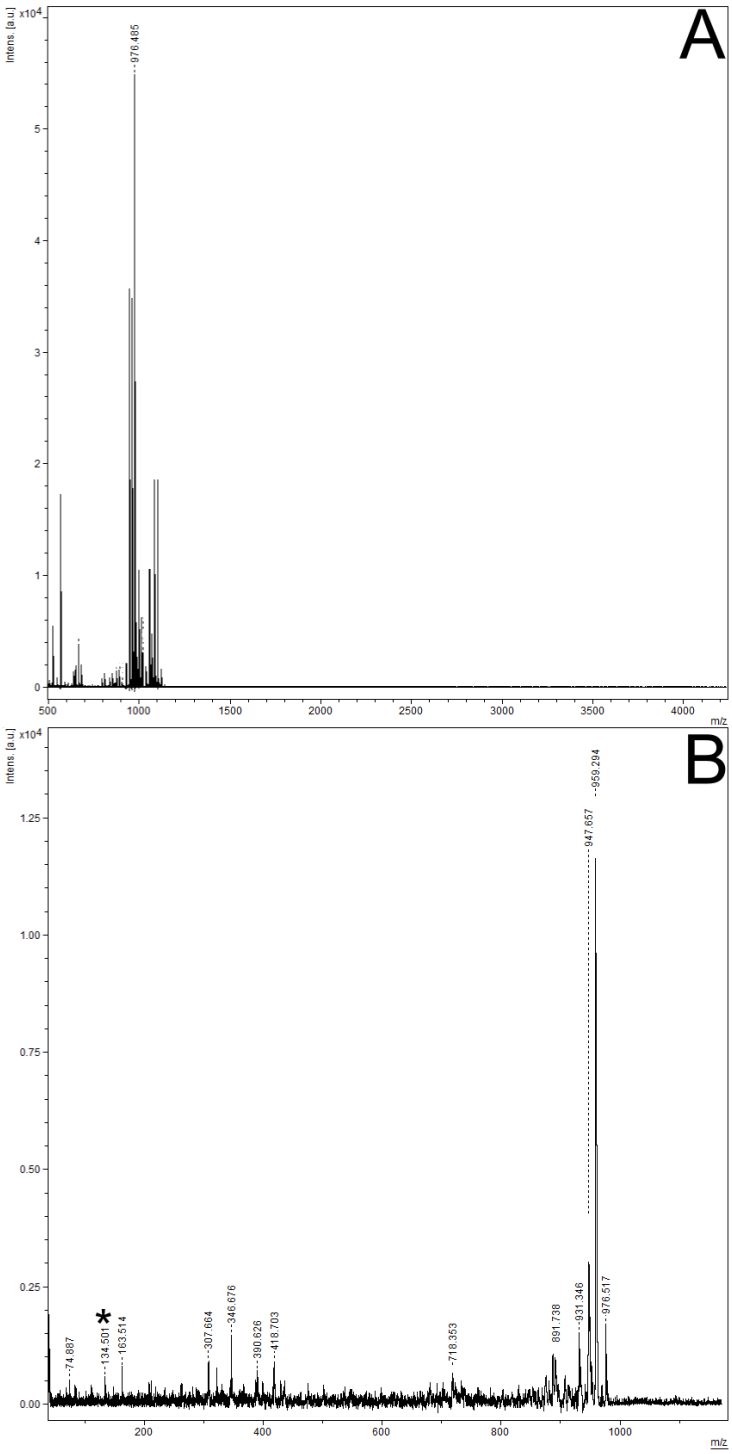

Figure S1. A - Peptide profile of the Mc-976 as identified by MALDI-TOF-TOF mass spectrometry.

B – Fragmentation pattern of the peptide, * denotes the ADDA fragment

Table S1 – Sequences of the peptides produced by the studied strains

M+H	Name	Sequence	References
575.3	Aeruginosin 98B	Hpla-Leu-Choi-Agm	Murakami et al., 1995
609.3	Aeruginosin 608	-	
643.3	Aeruginosin 101	Cl2Hpla–Leu–Choi–Agm	Ishida et al., 1999
685.3	Aeruginosin 684	-	
1044.5	Cyanopeptolin 1043	-	
1072.5	Cyanopeptolin 1071	-	Pereira et al., 2012
976.5	Microcystin 976	-	
995.6	Microcystin-LR	[Ala–Leu–meAsp–Arg–Adda–Glu–MDha]	Botes et al., 1985
1029.6	Microcystin-FR	[Ala–Phe–meAsp–Arg–Adda–Glu–MeDha]	Namikoshi et al., 1992
1038.6	Microcystin-RR	[Ala–Arg–meAsp–Arg–Adda–Glu–MeDha]	Kusumi et al., 1987
1045.6	Microcystin-YR	[Ala–Tyr–meAsp–Arg–Adda–Glu–MeDha]	Botes et al., 1985
1058.6	Microcystin-YR*	[Ala–Tyr–meAsp–Arg–Adda–Glu–MeDha]	Lawton & Edwards, 2001
1068.6	Microcystin-WR	[Ala–Trp–meAsp–Arg–Adda–Glu–MeDha]	Namikoshi et al., 1992
1709.7	Microviridin 1709	-	Pereira et al., 2012

Masses (M+H) are given in daltons; * homo variant of the amino acid tyrosine in position 2

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Capítulo 3

Quorum sensing signaling affects peptide production in the cyanobacteria *Radiocystis fernandoii*

Abstract

Quorum sensing is a bacterial phenomenon that changes gene expression, metabolism and physiology of bacterial populations according to their density. It occurs when the bacterial population reaches a certain density and it is controlled by chemical communication achieved by substances called autoinducers. Acylhomoserine lactones (AHLs) are a group of autoinducers found in gram-negative bacteria. Concerning cyanobacteria, there is a lack of information about how the quorum sensing works in this group. It is known that the genus *Gloeothece* produces one kind of AHL and that nitrogen fixation is affected by AHLs in *Anabaena* strains. It is also known that several species of cyanobacteria produce a wide variety of peptides as secondary metabolites, including the toxic and well studied microcystin. In this study, the effects of two AHLs (C8-HSL and C12-HSL) on the production of cyanobacterial peptides (cyanopeptolins, microcystins and microviridins) were tested through gene expression (RTqPCR) and ELISA assays (only for microcystins). The experiments were done with two strains of the cyanobacteria *Radiocystis fernandoii.* For the gene expression experiments the C8-HSL compound showed significant results for microcystins (inhibition) and microviridins (stimulation) while C12-HSL showed significant results for cyanopeptolins (inhibition), microcystins and microviridns (stimulation). The ELISA assay showed significant results for both substances tested and exhibited the same pattern of inhibition or stimulation seen in the qPCR analysis. The results obtained with these experiments suggest the existence of a quorum sensing system in *Radiocystis fernanadoii* that may play an important role in the production of cyanobacterial peptides.

1 – Introduction

Quorum sensing is the term used to describe communication between bacterial cells through the release of signalling molecules in the environment (Fuqua *et al.,* 1994). This phenomenon is density dependent and only happens when the bacterial population reaches a threshold and, as a consequence of the quorum sensing, synchronized responses induce changes in gene expression, metabolism and physiology of the bacterial population. It is known that quorum sensing can control different biological functions as motility, aggregation, swarming, conjugation, luminescence, virulence, symbiosis, biofilm differentiation, antibiotics biosynthesis and others (Swift *et al.,* 2001; Waters & Bassler 2005; Williams *et al.,* 2007). Although most of the research concerning quorum sensing began in 1990 decade and the term appeared in 1994, the idea of bacterial cells being able to communicate and release "pheromone like" substances is older (Tomasz 1965; Nealson *et al.,* 1970). Until now a wide variety of compounds responsible for quorum sensing in bacteria were isolated. These substances are called autoinducers and can be acylhomoserine lactones (AHLs), furanones, fatty acids, peptides and others (for a review see Williams *et al.,* 2007). In the case of the AHLs, the system is based on two protein families, LuxI, which is the AHL synthetase and LuxR which is the AHL receptor (Fuqua *et al.,* 2001; Swift *et al.,* 2001). AHLs diffuse and accumulate in the surrounding media and after a determined AHL concentration is reached they bind and activate the LuxR proteins. The complex formed by the AHL and the LuxR protein is then responsible for the activation and suppression of various target genes (Fuqua *et al.,* 2001; Swift *et al.,* 2001). The AHLs also activate the *LuxI* gene, generating a positive autoinduction where the AHL controls its own synthesis (Williams *et al.,* 2007).

The quorum sensing occurrence is believed to be widespread in the bacteria domain and the AHL system is commonly found in gram-negative bacteria (Miller & Bassler, 2001). Concerning cyanobacteria there is still a lack of information about the phenomenon. It is known that AHLs are produced by the cyanobacteria genus *Gloeothece* (Sharif *et al.*, 2008), that *Anabaena* strains can produce an AHL-acylase enzyme (Romero *et al.*, 2008) and that nitrogen fixation in *Anabaena* strains is affected by presence of different forms of AHLs (Romero *et al.*, 2011).

Some species of cyanobacteria have the ability to produce several types of secondary metabolites, including different kinds of peptides (Welker & von Döhren 2006). The most studied group of cyanobacterial peptides are the microcystins, an heptapeptide characterized by the amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda, position 5) (Carmichael, 1992). Besides microcystins, there are also cyanopeptolins, characterized by the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) and the cyclization of the peptide ring by an ester bond of the b-hydroxy group of threonine with the carboxy group of the terminal amino acid (Martin *et al.*, 1993) and microviridins the largest known cyanobacterial oligopeptides, characterized by a multicyclic structure established by secondary peptide and ester bonds and a side chain of variable length (Ishitsuka *et al.*, 1990).

Although some of these peptides are toxic to humans (Sivonen & Jones 1999), their role in the cyanobacterial physiology is still unclear. Their possible functions include defence against grazing (Agrawal *et al.*, 2001, 2005; Czarnecki *et al.*, 2006), protection against oxidative stress (Zilliges *et al.* 2011), involvement in iron metabolism (Martin-Luna *et*

al., 2006) and photosynthesis (Long *et al.*, 2001; Young *et al.,* 2005). Their ecological roles are also not well understood. It is known that cyanobacterial populations are usually formed by strains that produce and do not produce these metabolites (Welker *et al.*, 2004), but the ecological advantage for producing over non-producing strains is still under discussion (Kaebernick *et al.,* 2001; Neilan *et al*., 2013).

Another characteristic of certain cyanobacteria species is the ability to form blooms in determined environmental conditions. These blooms are situations where the cyanobacteria dominate the phytoplankton community and increase their biomass, reaching extremely high densities (Oliver & Ganf, 2002). A cyanobacterial bloom is a scenario where the quorum sensing phenomenon would be active. So far, only studies with indirect evidences have shown correlations between conditions with high cellular density and production of microcystins (Wood *et al.*, 2011, Wood *et al.*, 2012) and other peptides (Pereira & Giani 2014).

The aim of this work was to evaluate the effects of exogenous AHLs, molecules known to be involved in the bacterial quorum sensing signaling, in the expression of genes related to the production of secondary metabolites (peptides) and in the total amount of microcystin in different strains of cyanobacteria.

2 – Methodology

2.1 – Strains

The study was done using two strains of the cyanobacteria *Radiocystis fernandoii* (strains 28 and 86) that are maintained in the culture collection of the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais. *Radiocystis* (Komárek & Komárková-Legenerová, 1993) is a genus known to form blooms in tropical regions (Sant´Anna *et al.,* 2008) and produce several types of peptides (Vieira *et al.*, 2003, Lombardo *et al.,* 2006, Pereira *et al.,* 2012). Strain 28 was isolated from Furnas reservoir ($20^{o}40$'S; 46^{o} 19'W), which is a large oligo- to mesotrophic reservoir located in the south-eastern region of Brazil and receives inputs of nutrients from agricultural activities and domestic sewages commonly presenting cyanobacterial blooms. Strain 86 was isolated from Pampulha reservoir ($19^{o}55$'S; $43^{o}56$'W), which is an eutrophic urban reservoir located in the city of Belo Horizonte, Brazil, that suffers heavy impact of pollution from domestic and industrial sewages and shows permanent cyanobacterial blooms.

2.2 – Experiments

The AHLs used in the experiments were: *N*-Octanoyl-DL-homoserine lactone (C8-HSL) and *N*-Dodecanoyl-DL-homoserine lactone (C12-HSL) both purchased from Sigma-Aldrich. All substances were diluted in acetonitrile (AHL solvent) to 1mg/ml stock solution and added to the culture media in a final concentration of 100 µM. Experiments were run in triplicates, in a final volume of 25 ml of WC medium (Guillard & Lorenzen, 1972), growth conditions were: 12h light: 12h dark photoperiod at 20°C and 65 µmol.m^{-}

$^{2}.s^{-1}$of irradiance. Two treatments were used, one with the addition of AHL and another with addition of acetonitrile without AHL. After the addition of the substances, the growth period was of 48 hours. In the end of the experiments two 10 ml samples were taken and filtered for molecular analysis and ELISA assay, 2 ml samples were taken for cell number establishment, counting a minimum of 400 cells in a Fuchs-Rosenthal hemocytometer.

2.3 – Gene expression analysis

The gene expression analysis was done according to Pimentel, 2013. Briefly, RNA was obtained after cell lysis by mechanical maceration followed by trizol extraction according to the manufacturer's instructions (Invitrogen). RNA was then treated with Dnase (Promega) for removal of DNA. Approximately 500 ng of RNA was used to generate cDNA by reverse transcription (RT-PCR), with the aid of a High Capacity Kit (Applied Biosystems) and RT random primers. Manufacturer's recommendations were used in the RT-PCR cycle.

Real time PCR (RTqPCR) was performed using a StepOne™ System (Applied Biosystems) with 1 µl of cDNA sample, 0.3 µl of each primer (10pmol/µL), 5 µl of Power SYBR Green I (Applied Biosystems) and sterile milli-Q water for a final volume of 10 µl. The analysis was done in duplicates and the qPCR cycle followed the manufacturer's recommendations. The 16S ribosomal RNA gene was used as a housekeeping gene. The genes tested in the experiments were *mcyD* (microcystin), *cnp* (cyanopeptolin) and *mvd* (microviridin). Data analysis was done with the StepOne™ Software, version 2.0. The primers used in this work are listed in table 1. Previous studies

identified the production of microcystins, cyanopeptolins and microviridins in both strains used in the experiments.

Table 1 - Primers used in this study

Primer	Description	Sequence 5' - 3'	Reference
16s F	Reference gene	TGCGTAGAGATTGGGAAGAACATC	Sevilla *et al.,* 2008
16s R	Reference gene	GCTTTCGTCCCTGAGTGTCA	Sevilla *et al.,* 2008
qmcyD F	Microcystin gene	GCATCTTCTAAAGAAAAGACTCC	Pimentel & Giani, 2013
qmcyd R	Microcystin gene	AAATTATGGCAATCTTGGGGAATA	Pimentel & Giani, 2013
cnp F	Cyanopeptolin gene	GCTAGAAATTCACAGCCATCA	Pimentel, 2013
cnp R	Cyanopeptolin gene	ACCCCCATTGACCAACCATC	Pimentel, 2013
mdn F	Microviridin gene	CAGGGGTTATTGCAGGTGGT	Pimentel, 2013
mvn R	Microviridin gene	TAGGCTCAGGGGAAGGAGAC	Pimentel, 2013

2.4 – Microcystin analysis

Filtered material was extracted three times with methanol 75% (v/v). The procedure was done with sonication on ice, followed by centrifugation (15 min at 12000 rpm). The microcystin quantification was done using a Beacon® ELISA kit according to the manufacturer's instructions.

2.5 – Statistic

ANOVA tests were used to compare all parameters measured in the two treatments (with and without AHLs). The analyses were performed with the R software using GLM models suited for each type of data.

3 – Results

3.1 – Cultures growth

The results for cell number per ml at the end of all experiments are presented in table 2. All cultures were maintained in low density and there was no significant difference between treatments with and without the tested substances.

3.2 – Gene expression analysis

The results for the experiments with the *mcyD* gene showed a decrease in the gene expression when in the treatment with the C8 autoinducer and an increase when in the treatment with the C12 autoinducer (Figure 1). For the experiments with the *cnp* gene there was no significant difference in the gene expression when in the treatment with the C8 autoinducer and a decrease when in the treatment with the C12 autoinducer (Figure 2). For the experiments with the *mvd* gene there was an increase in the gene expression when in the treatment with both autoinducers (Figure 3). All experiments presented statistically significant results with a P value lower than 0,05 except when stated.

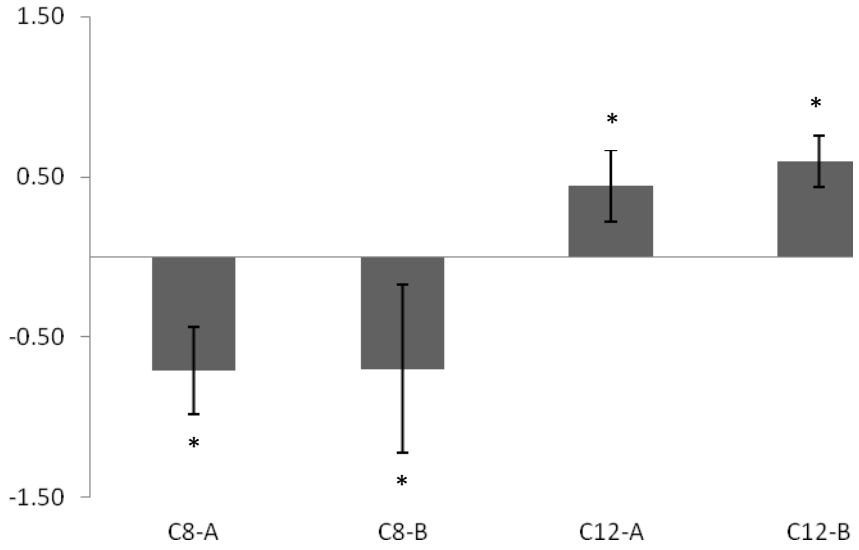

Figure 1 – Relative quantification of *mcyD* gene expression for the experiments with the autoinducers C8-HSL and C12-HSL. Letter A indicates strain R86; letter B indicates strain R28. * Denotes significative difference, P<0,05.

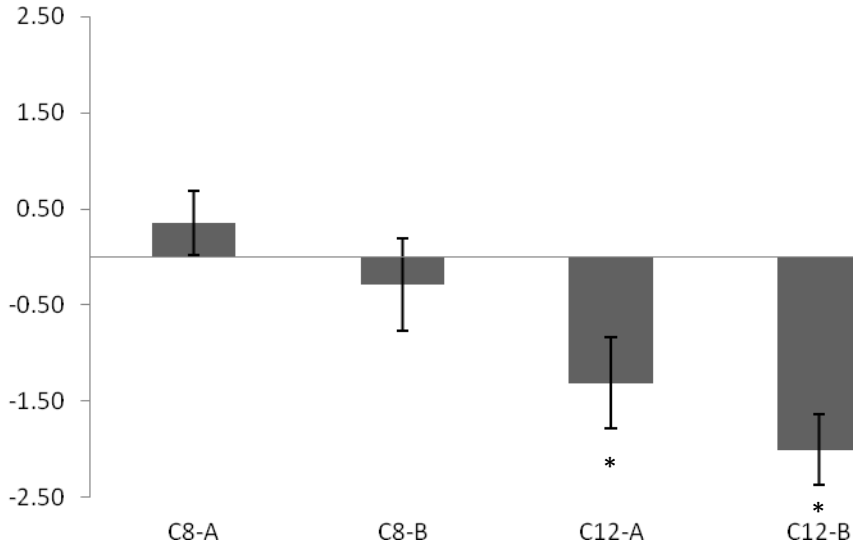

Figure 2 – Relative quantification of *cnp* gene expression for the experiments with the autoinducers C8-HSL and C12-HSL. Letter A indicates strain R86; letter B indicates strain R28. * Denotes significative difference, P<0,05.

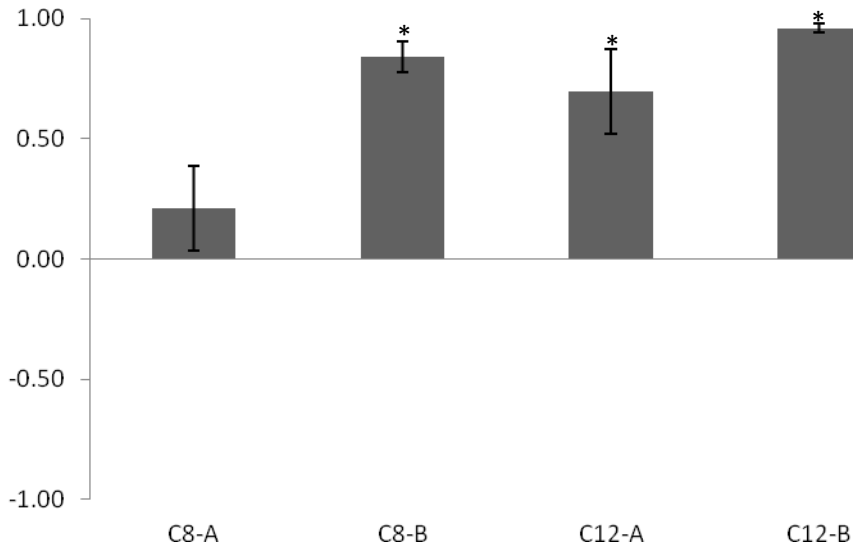

Figure 3 – Relative quantification of *mvd* gene expression for the experiments with the autoinducers C8-HSL and C12-HSL. Letter A indicates strain R86; letter B indicates strain R28. * Denotes significative difference, P<0,05.

Table 2 - Number of cells (cell.mL^{-1}) at the end of each experiment

	C8-HSL		C12-HSL	
	Present	Absent	Present	Absent
R86	34010 ±(2386)	31188 ±(1459)	23560 ±(5411)	16562 ±(1894)
R28	428155 ±(106239)	378187 ±(82405)	674712 ±(100575)	548779 ±(52265)

Differences between present and absent treatments were not significant in all experiments

Table 3 -Total amount of microcystin (pg. cell $^{-1}$) measured by ELISA assay in each strain and experiment

	C8-HSL		C12-HSL	
	Present	Absent	Present	Absent
R86	0.07 ± (0.01)	0.15 ± (0.03)	0.38 ± (0.08)	0.21 ± (0.02)
R28	0.13 ± (0.02)	0.25 ± (0.04)	0.47 ± (0.06)	0.31 ± (0.04)

Differences between present and absent treatments were significant ($P<0.05$) for all experiments

Differences between absent treatments were not significant for both strains

3.3 – ELISA assay

The amount of microcystin per cell in the experiments with the C8-HSL were significantly lower in the treatments where the AHL was present; 54% lower in the experiment with strain 86 and 48% with strain 28. For the procedures with the C12-HSL, the amount of microcystin was significantly higher in the treatment where the AHL was present, 45% higher in the experiment with strain 86 and 33% with strain 28. The comparison between the treatments with no AHL showed no significant difference for both strains. The results of the ELISA analysis are represented in table 3.

4 – Discussion

In gram-negative bacteria, quorum sensing was first described in *Vibrio fischeri* (Nealson & Hastings, 1979) as based on autoinducer systems. The molecules are able to diffuse inside and outside the bacterial cells and their concentration increase with increasing cell density (Kaplan & Greenberg, 1985), when these molecules reach a threshold concentration the transcription of several genes is activated. In our experiments, AHLs were used to induce a quorum sensing response in strains of cyanobacteria. The comparison between cell number in the treatments (AHL addition) and controls (no AHL addition) showed no significant difference for all strains, which demonstrates that the AHLs used in the experiments did not cause any inhibition or stimulation in the growth of the cyanobacteria. Considering the lack of significant difference in the strains growth, it is possible to assume that the results found in the RTqPCR analysis and in the ELISA assays are not a consequence of cell number and are in fact a direct effect of the AHLs. The cellular density used in the experiments was chosen according to the growth curves

obtained in previous experiments which used both strains (Pereira *et al.*, 2012), the different values in cell number between strains 28 and 86 are a reflect of specific characteristics that each one of them has.

The microcystin production was measured by ELISA assays and by qPCR through the expression of the *mcyD* gene. The cyanopeptolin and microviridn production were measured only by qPCR through the expression of genes related to their production. Unfortunately, there are no ELISA kits available for cyanopeptolins, microviridins and other cyanobacterial peptides, making impossible the direct measurement of these peptides in the experiment done in this study. The quantification of these peptides could have been done by HPLC, but this would demand a large scale experiment with a bigger volume of cultures in order to have more material to make the HPLC measurements possible. Considering that the AHLs are very expensive and that the amount of each compound necessary to make a large scale experiment would be very large, a small scale approach with measurements by qPCR and ELISA was chosen.

Concerning the production of microcystin, strain R28 had a higher amount of toxin per cell, approximately 0,09 $pg.cell^{-1}$ more than strain R86. The ELISA results for strain R86 were more intense than the results found in the experiments with the strain R28, but the differences between the two strains were not statistically significant (for both substances tested). This shows that the substances effects in inhibition (C8-HSL) and stimulation (C12-HSL) are the same, independently of the strain and the amount of microcystin produced by each of them. For the qPCR results, the same pattern found in the ELISA measurements was verified. The contradictory results for the C8-HSL and C12-HSL may

look strange at first, but the fact that the same patterns were found for two different strains, with different peptide profiles, confirms that they are reliable. Another point that supports our results is that the similar results were obtained with two different techniques, measuring two different products. While the qPCR measures the gene expression (RNA production) the ELISA measures the final product in the synthesis pathway (the microcystin amount). It also reveals that during the microcystin synthesis the inhibition/stimulation occurs at the genetic level and not after the RNA transcription, because if that was the case there would be no difference between controls and treatments in the qPCR analysis, but a significant difference in the ELISA measurements would still be observed.

The explanation for the opposite results observed for microcystin production under the effect of two different AHL compounds might be connected to how the quorum sensing system works in the *Radiocystis*. It is known that some species of bacteria have more than one quorum sensing system and that these systems can work in parallel, in series or in a competitive way (Waters & Bassler, 2005). It is possible that the *Radiocystis*, as other bacteria, has more than one quorum sensing system, which could be activated at different cellular densities. This would also make sense when considering the development of a bloom: for example, at the beginning of the phenomenon, at a particular cell density, the first system would be activated, triggering a series of physiological responses. In a second moment, after reaching another cellular density, another series of physiological responses would be activated. It is possible that the C8-HSL and the C12-HSL stimulate two different quorum sensing systems and this would explain the different responses produced.

For the cyanopeptolins, only C12-HSL had a significant effect, suppressing the gene expression, while the microviridin production was significantly stimulated in three experiments out of four. The experiment with the C8-HSL and the strain R86 showed an increase in the microviridin gene expression but had no significant results while the other experiments showed an increase in the gene expression with significant results. It is possible to say that both substances affect the production of microviridins, with the C12-HSL having a more intense effect. For the cyanopeptolins, it appears that C8-HSL has no effect on their production.

When comparing the gene expression related to three studied peptides, opposite patterns are seen for microcystins compared to microviridins for the experiments with the C8-HSL and microcystins and microviridins compared to cyanopeptolins for the experiments with the C12-HSL. If the *Radiocystis fernandoii* has any sort of quorum sensing system based on AHLs, it is possible to assume that it does not act in the same way with all peptides, which would not be a surprise considering that the auto inducers usually stimulate and inhibit several genes at the same time (Williams *et al.,* 2007).

We used two AHLs with different side chain length in the experiments, the C8-HSL which was shown to be produced by axenic cultures of the genera *Gloeothece* (Sharif *et al.,* 2008) and the C12-HSL which was one of the AHLs used by Romero *et al.,* (2011) and was shown to be related with the inhibition of the enzyme nitrogenase, consequently reducing nitrogen fixation in a *Anabaena* strain. The specificity of AHLs receptors is connected with the side chain length, saturation and substitutions (Gould *et al.,* 2004), being one of the reasons for the use of AHLs with medium (C8-HSL) and long (C12-

HSL) side chains. The different length in their side chains could also be one of the explanations for the different results found for each one of the AHLs, since C8-HSL and C12-HSL may not activate the same quorum sensing receptors and, consequently, promote different physiological changes.

Environmental factors, such as light, temperature, nitrogen, phosphorous and iron were extensively tested on the production of microcystins and other peptides (Utkilen & Gjolme 1999; 2000; Wiedner *et al.*, 2003; Repka *et al.*, 2004; Tonk *et al.*, 2005; Sevilla *et al.*, 2008; Tonk *et al.*, 2009; Alexova *et al.*, 2011; Pereira *et al.*, 2012), but to date, there are no studies linking quorum sensing signaling molecules and the control of peptide production on cyanobacteria. The regulatory mechanisms behind peptide production in cyanobacteria might be an intricate system and in natural scenarios the secondary metabolites production is probably regulated by a series of features from physical, chemical and biological origin, which interact together in order to create a production pattern for these compounds. It is very difficult to study and make statements in situations with a series of variable factors, which makes laboratory experiments a very useful tool to understand the role that each parameter has in the peptide production. With the results obtained in this work, it is possible to conclude that aspects such as quorum sensing and cellular density may have an important role in the production of cyanobacterial secondary metabolites.

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5. Conclusões finais

As análises realizadas nesta tese apontaram para uma conexão entre *quorum sensing* e produção de peptídeos em cianobactérias. Os experimentos realizados sobre o efeito da densidade celular permitiram concluir que este parâmetro tem uma influência sobre a produção da maioria dos peptídeos encontrados nas cepas estudadas. Estes experimentos também mostraram que em situações de floração existe a possibilidade de que a densidade celular seja um dos fatores que controlam a produção de metabólitos secundários.

Os testes feitos com as homoserinas lactonas, substâncias produzidas por bactérias como sinalizadores celulares intra-populacionais, mostraram que estes compostos afetam a produção de microcistinas, cianopeptolinas e microviridinas e levantam a possibilidade da existência de um ou mais sistemas de *quorum sensing* na cianobactéria *Radiocystis fernandoii*.

Os métodos utilizados nos experimentos se mostraram eficientes nas medições. As técnicas de cromatografia e espectrometria de massa foram adequadas para o isolamento e identificação dos peptídeos. Os testes de ELISA e a PCR em tempo real permitiram uma medição eficiente e precisa, mesmo em amostras com baixa concentração de material. A PCR em tempo real (RTqPCR) permitiu a mensuração da expressão gênica dos peptídeos alvo, mostrando-se suficientemente sensível para detectar as diferenças entre os tratamentos realizados.

Os dados apresentados nesta tese abrem novas perspectivas na investigação das relações entre células de populações de cianobactérias, abrindo-se com isso novas possibilidades

para o desenvolvimento de mecanismos de controle de florações em ambientes eutrofizados.