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Profiles of toxic and non-toxic oligopeptides of *Radiocystis fernandoii* (Cyanobacteria) exposed to three different light intensities

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ABSTRACT

Cyanobacteria produce a high variety of bioactive oligopeptides, which function, ecological, physiological roles and responses to environmental changes are still unclear. The influence of light intensity on the cell quota and the diversity of oligopeptides of two strains of the cyanobacterium *Radiocystis fernandoii* were experimentally tested. The peptides were quantified by HPLC and identified by a MALDI-TOF–TOF. Microcystins (MC) were generally more abundant in the treatment with low light. A compensatory mechanism was observed for the different variants of microcystin, whereby MC-RR responses were contrary to those observed for the other three variants and showed higher concentration in the treatment with intermediate light. Two microviridins were also produced at higher amounts at intermediate irradiance. For cyanopeptolins and a third microviridin no significant difference among treatments was found. The absence of a similar response for all peptides suggests that these compounds may have unique cellular functions, which better understanding could help explaining changes in toxicity. Finally, we observed that each chemical profile reflected in physiological differences between strains, strengthening the idea that chemotypes may act as distinct ecotypes in nature.

1. Introduction

Cyanobacteria are an important component of the phytoplankton community, but their relative importance and dominance in aquatic systems has augmented in the last years, as a consequence of increasing eutrophication and nutrient input. They are morphologically simple but chemically diverse, and are acknowledged to produce several bioactive peptides, such as aeruginosins (Murakami et al. 1994), cyanopeptolins (Martin et al. 2003), microginins (Okino et al. 1993), microviridins (Ishitsuka et al. 1990), anabaenopeptins (Harada et al. 1995) and the well studied microcystins (Carmichael 1992) known for their toxic effects on mammals and humans that cause public health concerns around the world. According to Welker and von Döhren (2006), at least 600 peptides have been already described in cyanobacteria. The high variability of compounds found in these organisms can be explained by their biosynthethic pathways, which are independent from ribosomes and RNA and consist of multifunctional enzyme complexes composed of non-ribosomal peptide synthetases (NRPSs) and polyketide synthase (PKS) modules (Börner and Dittmann 2005; Welker and von Döhren 2006).

Despite their ubiquity and variety, the ecological and physiological functions of these peptides are still not well understood. Some studies have suggested allelopathic effects (Pflugmacher 2002; Schatz et al. 2005; Smith and Doan 1999), others have proposed a bacterial quorum-sensing hypothesis (Kaebernick et al. 2000) or a possible relation with internal metabolism (Lyck 2004). A few peptides were shown to be inhibitors of proteases of the cladoceran *Daphnia* (Agrawal et al. 2001, 2005; Czarnecki et al. 2006; Rohrlack et al. 2003; Von Elert et al. 2004), suggesting protection against grazing. Additionally, natural populations of cyanobacteria are composed of producers and non-producers strains, for each peptide or peptide class (Fastner et al. 2001; Rohrlack et al. 2001; Welker et al. 2004), and up to now, non-producing strains have not been shown to have any clear advantage or disadvantage when compared with producing strains (Hesse et al. 2001; Kaebernick et al. 2001).

An understanding of the factors affecting the production of the cyanobacterial peptides may contribute to the comprehension of the functions of these compounds and their relationship with the environment. It can also be an important tool for the assessment of the cyanotoxin risk and water treatment policies. To date, several studies have investigated the effect of light, temperature, phosphorus and nitrogen on cyanobacterial peptides production (Oh et al. 2000; Repka et al. 2004; Rohrlack and Utkilen 2007; Sivonen 1990; Tonk et al. 2005; Tonk et al. 2009; Utkilen and Gjolme 1995; Wiedner et al. 2003; Zilliges et al. 2011). Because of its toxicity to

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humans, microcystin has been, in general, the main focus in these studies and just few have considered other peptides. However, understanding the interaction among several peptides could help bringing new knowledge about the function of these compounds.

There are indications that that MC may be involved in photosynthetic light-related processes (Long et al. 2001; Young et al. 2005). Some studies showed that toxin production and growth can be stimulated over a certain range of light intensities, but at saturating light intensities, toxin production decreased (Hesse and Kohl 2001; Utkilen and Gjolme 1992; Wiedner et al. 2003), even when no inhibitory effect was observed on growth rates (Wiedner et al. 2003).

Many cyanobacteria prefer growing at lower irradiance ($30\ \mu mol\ m^{-2}\ s^{-1}$) and can be sensitive to photoinhibition (Walsh et al. 1997). They can adapt to varying irradiance by changing the proportion of light-harvesting complexes in the thylakoid membrane and the pigment ratios. Fluctuations in light intensity may also have a significant impact in species interactions (Lichtman et al. 2004), slowing rates of competitive exclusion or reversing the identity of the superior competitor, and finally altering the structure of phytoplanktonic communities.

In this study, we tested the influence of light intensity in the production of the oligopeptides cyanopeptolins, microviridins and microcystins. Two strains of the cyanobacterium *Radiocystis fernandoii* (Komárek and Komárková-Legenerová, 1993) isolated from two Brazilian reservoirs were used in the experiments. This genus is morphologically similar to *Microcystis* except for its characteristic radially oriented cell disposition and by the crosswise cell division in one plane. *Radiocystis* is found in lakes in the northern hemisphere, but also in tropical waters. Komárek described the species *R. fernandoi* as occurring in tropical regions of central America (Komárek 2003). In Brazil, it is quite common in tropical and subtropical regions where it is one of the main genera responsible for toxin production (Sant'Anna et al. 2008). Borges et al. (2008) observed blooms in subtropical Brazil where this species represented up to 70% of the total biomass. Anjos et al. (2006) and Fonseca et al. (2011) observed the presence of toxic genes (mcy B and mcy A, respectively) in natural blooms of *R. fernandoi*. The level of toxicity and MC-LR production can be quite elevated in this species (Vieira et al. 2003), nevertheless still not much information concerning its ecology, physiology and toxicity is available. A recent study reported the presence in this species of MC-LR, one micropeptin and two other compounds identified as belonging to the aeruginosin class (Lombardo et al. 2006). Our research however is the first one, to our knowledge, describing the presence and behaviour of different oligopeptides in two strains of *R. fernandoii*, when submitted to different growth conditions. These two strains were selected from our cultures initially because of their fast growth rates, which allowed easier experiments' development. Second, we also wished to compare strains with different peptide profiles, looking for possible peptide effects on the strain fitness. The individuality of strain responses can eventually turn to be important in the management of toxic blooms.

2. Material and methods

2.1. Strains

The *R. fernandoii* 28 strain was isolated from Furnas reservoir (20°40′S; 46°19′W), located in the south-eastern region of Brazil. Furnas is a large oligo- to mesotrophic reservoir that receives inputs of nutrients from agricultural activities and domestic sewages and frequently presents cyanobacterial blooms in the upper reaches. The *R. fernandoii* 86 strain was isolated from Pampulha reservoir (19°55′S; 43°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. Both strains are maintained in the culture collection of the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais.

2.2. Experiments

The experiments were performed in triplicates, in batch cultures of 500 ml of WC medium (Guillard and Lorenzen 1972). Growth conditions were a 12 h light: 12 h dark photoperiod at 20 °C. Experiments were carried out at three different irradiances: $25\ \mu mol\ m^{-2}\ s^{-1}$, $65\ \mu mol\ m^{-2}\ s^{-1}$ and $95\ \mu mol\ m^{-2}\ s^{-1}$. These light intensities were selected as low, medium and high, based on literature data (see for example Hesse et al. 2001; Tonk et al. 2005; Preussel et al. 2009). In all treatments, experiments were interrupted when cultures growth was still exponential and always before reaching the stationary phase (approximately 7–10 days). At the end of the experimental period, the content of the entire flask of each replicate was freeze dried and the dry material was accurately weighed and then used for further biochemical analyses.

During the growing period, cell growth was daily assessed by measuring culture's optical density (OD) at 750 nm and growth rates were calculated. At the end of each experiment, chlorophyll content was estimated from spectrophotometric measurements after extraction in 90% hot ethanol (Nusch 1980). In the same samples, cell numbers were estimated by counting a minimum of 400 cells in a Fuchs-Rosenthal hemocytometer.

2.3. Oligopeptide analysis

The dry material was extracted three times using methanol 75% (v/v), by sonication on ice followed by centrifugation, and the final extract was applied to SPE C18 cartridges (Waters, Sep-Pak Vac 3cc – 500 mg) for purification by reverse phase chromatography, as described by Lawton and Edwards (2001). The dry extracts were resuspended in methanol 75% (v/v) and analysed by HPLC (Waters Alliance 2695) with a photodiode array detector (Waters 2996) at 225 nm and a Waters symmetry C18 Column (4,6 × 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 min then 40% for 6.5 min. The flow-rate was 1 ml/min. Because of the lack of standards for most peptides, their quantification was done by dividing the peak area of each compound by the dry weight of the culture, obtaining a measurement of the relative change in the peptide concentration. For the identification of the peptides, fractions were collected and analysed 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 Welker et al., 2006. Chromatographic profiles of both *Radiocystis* strains and the MS/MS spectra of three identified and fragmented fractions, corresponding to each one of the observed classes of peptides, can be seen in Appendix A.

We used dry weight to standardize the measurement of peptides, since it is a parameter that has a high correlation with cell number and cellular biovolume. This relationship was tested

Table 1
List of the peptides identified in the two strains of *Radiocystis fernandoii* with their corresponding mass.

M+H[a]	Name	Strain
1029.5	Microcystin-FR	28
1031.5	[Asp^3]Microcystin-YR	28
1038.5	Microcystin-RR	28, 86
1045.5	Microcystin-YR	28
1068.5	Microcystin-WR	28
981.5	Cyanopeptolin-980	86
1015.5	Cyanopeptolin-1014	86
1072.5	Cyanopeptolin-1071	28
1707.7	Microviridin-1707	86
1709.6	Microviridin-1709	28
1739.7	Microviridin-1739	86

[a] Masses (M+H) are given in Da.

statistically by regression analysis and results showed highly significant correlation for both strains ($R^2 = 0.9$).

2.4. Statistical analysis

The influence of light intensity on oligopeptides content was tested by ANOVA (one way) followed by Tukey–Kramer test for post hoc pairwise comparisons. Data represented in Tables 2 and 3 were also compared performed by the same statistical method. All analyses were done with the JMP 7 software.

3. Results

A total of twelve peptides were identified in the two strains. The *Radiocystis* 28 strain produced four microcystins (MC-RR, MC-YR, MC-FR and MC-WR), a demethylated variant of microcystin YR (dMC-YR), one microviridin (MV-1709) and one cyanopeptolin (CY-1071). The *R. fernandoii* 86 strain produced one microcystin (MC-RR), two microviridins (MV-1707 and MV1739), two cyanopeptolins (CY-980 and CY-1014) and one unidentified peptide (Pep-7). The microcystin RR was the only peptide produced by both strains (Table 1).

Fig. 1 shows the relative amount of the different peptides in the strain 28. We observed three significantly different response patterns of the peptides to the light intensity: MC-YR, MC-FR and MC-WR showed lower concentration at 65 μmol m^{-2} s^{-1}, opposite to MC-RR and MV-1709 that were higher at the same light intensity ($P < 0.001$). The production of CY-1071 and the dMC-YR showed no significant differences among the treatments.

Table 2
Ratios among microcystin variants measured in *Radiocystis fernandoi* strain 28 at the three different irradiances (μmol photons m^{-2} s^{-1}). Values represent means ($n = 3$; SD in parenthesis). Different letters indicate significant differences for $P < 0.001$.

Irradiance	25	65	95
RR:YR	0.69[a] (0.02)	1.64[b] (0.12)	0.76[a] (0.04)
RR:FR	3.05[a] (0.17)	6.18[b] (1.00)	3.65[a] (0.16)
RR:WR	2.57[a] (0.04)	6.79[b] (0.70)	3.45[a] (0.19)
RR:dYR	5.73[a] (0.16)	7.05[b] (0.30)	5.34[a] (0.47)
YR:RR	1.46[a] (0.04)	0.61[b] (0.04)	1.33[a] (0.07)
YR:FR	4.44[a] (0.17)	3.76[b] (0.37)	4.83[a] (0.05)
YR:WR	0.45[a] (0.01)	0.96[b] (0.06)	0.65[c] (0.05)
YR:dYR	8.36[a] (0.16)	4.32[b] (0.15)	7.08[c] (0.64)

This difference in production of single MC variants at different irradiance reflected changing their respective ratios. Table 2 shows that all ratios were affected by light intensity, especially at medium irradiance.

The results of the same experiments for strain 86 are presented in Fig. 2. Concentration of MC-RR was higher at 25 μmol m^{-2} s^{-1} and lower in the other treatments ($P < 0.001$). One microviridin (MV-1739) was only detected at 65 μmol m^{-2} s^{-1}. The other peptides were unaffected by changes in light intensity.

When peptides were grouped into their respective class, for strain 28 the sum of all microcystins was significantly higher in the cultures with low light (25 μmol m^{-2} s^{-1}) ($P < 0.001$) (Fig. 3A). The same occurred for the single microcystin produced by strain 86 ($P < 0.001$) (Fig. 3B). Microviridin was higher at medium light intensity (65 μmol m^{-2} s^{-1}) ($P < 0.001$). No significant difference in the content of cyanopeptolins among treatments was registered.

Table 3 represents cell specific growth rate (μ, day^{-1}), chlorophyll content (pg/cell), cell biovolume (μm^3) and microcystin RR and YR content (fg/cell) for each strain and light treatment. For strain 28, under medium irradiance (65 μmol m^{-2} s^{-1}), significant differences were observed in growth rate ($P < 0.005$) and microcystin content (MC-RR, $P = 0.0001$; MC-YR, $P = 0.005$), showing higher growth rate, higher MC RR and lower MC YR content than at the other light conditions. Higher chlorophyll content and lower cell biovolume were registered at low light, but differences among treatments were not statistically significant.

Results were different for strain 86 that presented significantly lower growth rate ($P < 0.05$), higher chlorophyll content ($P < 0.05$), higher biovolume ($P < 0.05$) and higher MC content ($P < 0.0005$) at

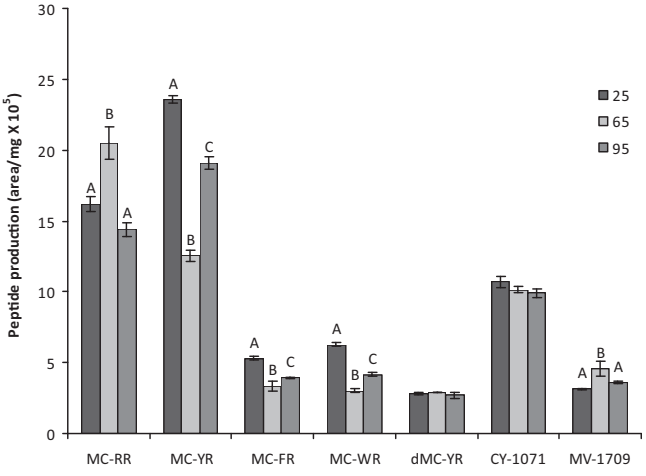

Fig. 1. Peptides produced by the *Radiocystis fernandoii* 28 under three different light intensities (low, medium and high). Error bars show standard deviation. Significant differences between means were tested by ANOVA ($P < 0.001$, except for dMC-YR and CY-1071 that were not significant). The letters (A–C) indicate significant differences. For all figures, MC: microcystin, CY: cyanopeptolin, MV: microviridin.

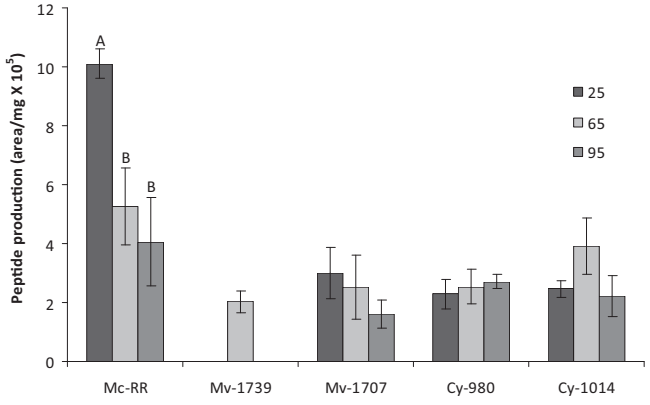

Fig. 2. Peptides produced by the *Radiocystis fernandoii* 86 under three different light intensities (low, medium and high). Error bars show standard deviation. Significant differences between means were tested by ANOVA ($P < 0.001$, except for MV-1707, CY-980 and CY-1014 that were not significant). The letters (A–C) indicate significant differences. Legend: see Fig. 1.

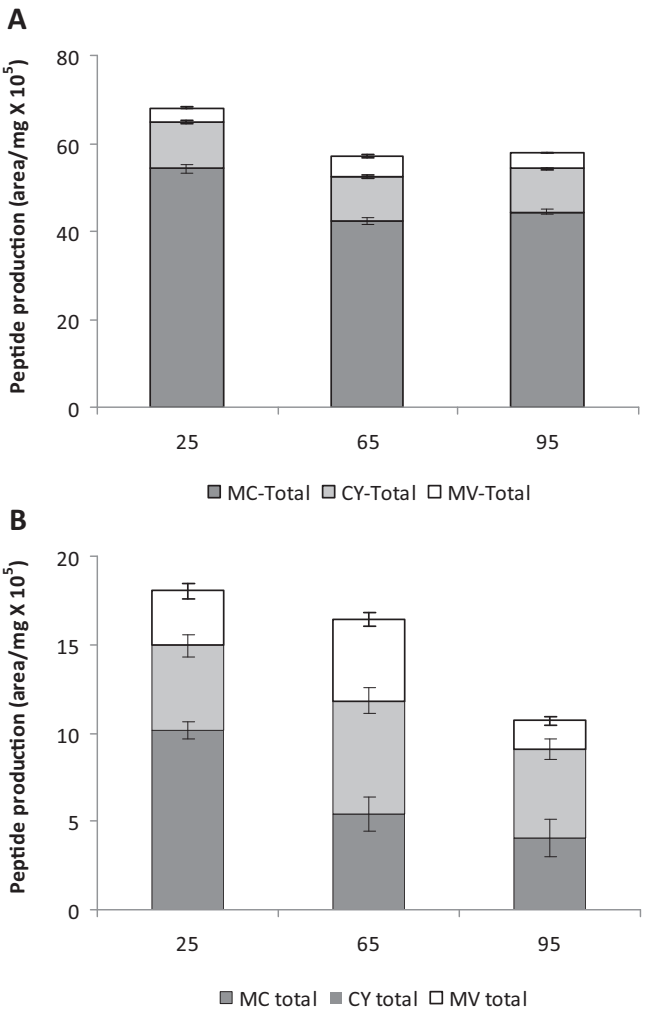

Fig. 3. Classes of peptides (total concentration) produced by the *Radiocystis fernandoiii* strain 28 (A) and strain 86 (B) under three different light intensities (25, 65 and 95 μmol m^{-2} s^{-1}). Significant differences between means were tested by ANOVA ($P < 0.001$ except for CY-Total that was not significant).

the lowest irradiance (25 μmol m^{-2} s^{-1}). However, differently from strain 28, strain 86 has just one variant of microcystin.

4. Discussion

As research progresses new compounds produced by cyanobacteria are discovered, and because some are already being proved as toxic (see for example Welker and von Döhren 2006), their existence is a growing concern worldwide not only for human health but also for the entire aquatic trophic chain. The majority of these secondary metabolites are peptides and several studies showed that microcystin, the best known of them, is probably produced constitutively, since cells maintain its production even under changing environmental conditions (reviewed by Kardinaal and Visser 2005). The presence of microcystin at all light intensities observed in our experiments seems to well support this idea.

Among microcystins, we found five different variants in strain 28 (MC-RR, MC-YR, MC-FR, MC-WR and dMC-YR) and one in strain 86 (MC-RR). The high variability of amino acid positions 2 and 4 in the molecule structure is the main responsible for the high variety of isoforms within the microcystin class, which at present are known to be around 90 (Welker and von Döhren 2006). In general however, in isolated strains and in field blooms, only a few variants are found to be dominant and the others are generally present just at low concentrations (Welker et al. 2004). In our study, for example, in strain 28 the dominance of MC-RR and MC-YR was observed and just lower amount of the remaining three variants was measured (Fig. 1). As for MC-YR and MC-RR, where the amino acids in position 2 are tyrosine and arginine, respectively, they were described as hepatotoxic (Gupta et al. 2003). Hepatotoxicity was also confirmed for MC-FR and MC-WR, where position 2 amino acids are, respectively, phenylalanine and tryptophane (Chen et al. 2006).

Interestingly, Chen et al. (2006) observed that with the substitutions of leucine in MC-LR by arginine (R), phenylalanine (F) or tryptophan (W) at the X amino acid (position 2), the inhibitory effects on phosphatases PP-1 and -2A were reduced. The authors also found that the least toxic of the four studied microcystins was MC-RR and its derivatives. They pointed out that the replacement of the amino acid at the second position had the greatest influence on phosphatase inhibitions and their acute animal toxicity. Coincidentally in our study, MC-RR was the variant that showed an opposite response to light changes when compared to all other MCs (MC-YR, MC-FR and MC-WR) (Tables 2 and 3, Fig. 1), suggesting that this difference in the molecular structure may as well be quite important to regulate the production of each MC isoform when growth conditions are varying.

It is also important to observe that growth conditions, in changing the ratios among isoforms, may have a strong influence on the final toxicity of the strains. For example, the compensatory mechanism of MC production in strain 28, regulated by light intensity, generated different ratios among microcystin variants (Table 2). Similar results were recorded by Tonk et al. (2005), who showed a comparable regulatory mechanism connecting light intensity and production of demethylated variants of MC-RR and MC-LR, which also resulted in different ratios. As discussed above, the explanation for this phenomenon is probably linked to the conformational structure of the enzymatic complex responsible for the synthesis of each microcystin isoform (Tonk et al. 2005).

In our experiments, cyanopeptolins were also present at all light intensities (Figs. 1 and 2), a possible sign that, similar to microcystins, they can be produced constitutively. Like microcystins,

Table 3
Cell specific growth rate (μ, day^{-1}), chlorophyll content (pg/cell), cell biovolume (μm^3) and microcystin RR and YR (fg/cell) for each *Radiocystis* strain at different irradiances (μmol photons m^{-2} s^{-1}).

Irradiance	Growth rate (μ)	Chlorophyll cellular content	Cell biovolume	MC-RR cellular content	MC-YR cellular content
Strain 28					
25	0.33 (0.01)	0.264 (0.007)	39.9 (13.1)	117 (3)	171 (2)
65	0.40 (0.01)	0.226 (0.032)	48.6 (16.4)	262 (21)	150 (11)
95	0.31 (0.00)	0.228 (0.019)	46.6 (16.1)	140 (5)	186 (6)
Strain 86					
25	0.16 (0.03)	0.909 (0.038)	51.1 (16.3)	200 (1)	–
65	0.31 (0.02)	0.627 (0.135)	41.5 (14.2)	62 (10)	–
95	0.25 (0.04)	0.653 (0.045)	38.3 (11.7)	49 (3)	–

Data represent means (SD); $N = 3$ for μ and MC, $N = 6$ for chlorophyll, $N = 20$ for biovolume; (–) not present.

cyanopeptolins are highly variable peptides, with different molecular masses. Cyanopeptolins are even more variable, because of the presence of high number of non-proteinogenic amino acids (Welker and von Döhren 2006). As for their effects, some cyanopeptolin seem to have an inhibitory activity against serine-threonine proteases (Matern et al. 2003), trypsin and human kallikrein (Gademann et al. 2010). Gademann et al. (2010) also found that cyanopeptolin was toxic against a crustacean, at the same toxicity range of microcystin, suggesting that these peptides could be considered a second class of critical toxins in the environment in addition to microcystins. In our strains, cyanopeptolins were the second most important peptides after microcystins.

Unlike the other two classes of compounds, microviridins were not present at all growth conditions (Fig. 2). Microviridins are the largest cyanobacterial oligopeptides: their amino acids are all in L-configuration and the only non-proteinogenic unit is the N-terminal acetyl group. Most of the 13 variants known at present show inhibitory activities against serine-type proteases (Ziemert et al. 2010). As already suggested by some authors (Philmus et al. 2008; Welker and von Döhren 2006) and observed in other prokaryotic peptides (Blond et al. 1999), it seems that microviridins are synthesized ribosomally and that their structure is finalized by post-translational modifications. Their differential biosynthesis pathway could help explaining the suppression of microviridin MV-1739 production in two of our experimental treatments. A switch to higher production of the other congener (MV-1707) was not observed. Even in the eventuality that MV-1739 was present but at a concentration below detection limits, the observed response underlines a difference among the three treatments.

For total microcystins, the results showed that the total content of these peptides was higher at the treatment with lowest light (25 μmol m^{-2} s^{-1}) and decreased as the amount of light increased (Fig. 3). The results were the same for *R. fernandoii* 28, which produced 5 kinds of microcystins, and for *R. fernandoii* 86, which had only one microcystin. These observations agree with previous studies, which have shown an effect of light in the transcription of the mcy gene complex (Kaebernick et al. 2000) and a reduction of the amount of microcystin under higher light intensity (Tonk et al. 2009; Wiedner et al. 2003). One interesting hypothesis for the reduction in microcystin concentration under high light intensities was presented by Zilliges et al. (2011) who showed that under very high light intensities microcystins bind covalently to other proteins. It is important to notice however that the Zilliges et al. worked at light intensities as high as 700 μmol m^{-2} s^{-1} while our highest irradiance was around 100 μmol m^{-2} s^{-1} and we cannot necessarily assume that the same process occurred in our experiments. In field studies, increased microcystin production in *Microcystis* blooms has been associated with low light conditions (Kotak et al. 2000; White et al. 2003) and decreased microcystin content in cell exposed to higher light intensities may arise through photoinhibition.

There was no significant difference in the amount of the three cyanopeptolins detected in the experiments. These results suggest that light has no effect on this class of peptides. In experiments performed by Tonk et al. (2009), however, light affected production of some cyanopeptolins. Based on their observations and on our results, we may assume that peptides of the same class not always show similar responses to changes of a single factor.

We observed that higher growth rates were not always related to higher toxin content (Table 3). Tonk et al. (2009) also showed that the production of some peptides can be higher under less favourable growth conditions. The production of microcystins and anabaenopeptins in several strains of the genus *Planktothrix* (Kosol et al., 2009) were also found to be independent from growth rates.

As previously discussed by Kaebernick et al. (2000), the substrate availability and gene regulation are most likely the main factors controlling the peptide production under changing environmental conditions. Beside production, these external changes could modify the amino acid composition available for incorporation during microcystin synthesis (Tonk et al. 2008) and a conformational change of the substrate-binding pocket at the first module of the McyB enzyme could lead to a change in the substrate specificity of the module (Tonk et al. 2005). In the complex molecules of cyanobacterial peptides, each step of their biosynthesis pathway could be affected by external changes, controlling the activation reactions for the production of each respective congener. Belshaw et al. (1999) showed, for example, that the speed of further peptides synthesis was held back by the incorporation of the 'wrong' amino acid up to the point where the production of corresponding variants would be unlikely. Thus, different light intensities could also affect the substrate availability and consequently change the composition of microcystin variants in the cell.

The results observed in these two different strains of the same species suggest that none of these peptides or peptide class is probably required for growth, since strain 86 was able to grow under the same environmental conditions even with lower peptide diversity. Thus, the lack of genes for the ability of producing certain peptides is apparently not a disadvantage for the individual survival (Kaebernick et al. 2001). Interestingly however, strain 86, which showed lower peptides diversity, also presented lower growth rates and higher chlorophyll content per cell, especially at low light conditions, even if cell size (biovolume) was very similar in both clones. Because of these lower growth rates, strain 86 would possibly lose competition in a low irradiance environment. Its sensibility to light changes is also confirmed by the significant increase in cellular chlorophyll at 25 μmol photons m^{-2} s^{-1}.

However, the amount of peptides produced by both strains changed in a more or less consistent pattern (Fig. 3), and except for microviridin (Figs. 2 and 3), they were present in all three light treatments. Welker and von Döhren (2006) pointed out that many strains may grow in the laboratory for decades without losing their ability to produce their typical peptides and observed that, when individual peptides are considered rather than peptide classes, the number of possible chemotypes seems endless and exceeds by far the number of morphotypes.

Thus, if a strain can be characterized by its chemical profile as a chemotype and if an ecotype is defined as a clade of microorganisms that shares similar ecological characteristics, these two *Radiocystis* strains would be chemotypes representing two different ecotypes, each one adapted to certain environmental conditions. According to Welker et al. (2004), 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.

Finally, because of the complex connections between peptides and environment, their diversity and their strain specific character, our results indicate that the simultaneous study of several classes of compounds and their potentially complementary responses may be needed if we aim the understanding of their individual function, as well as other important aspects of the metabolism of cyanobacteria in general. Increasing knowledge of these mechanisms and responses may also help explaining changes in toxicity in field samples.

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Appendix A.

See Figs. A1–A4.

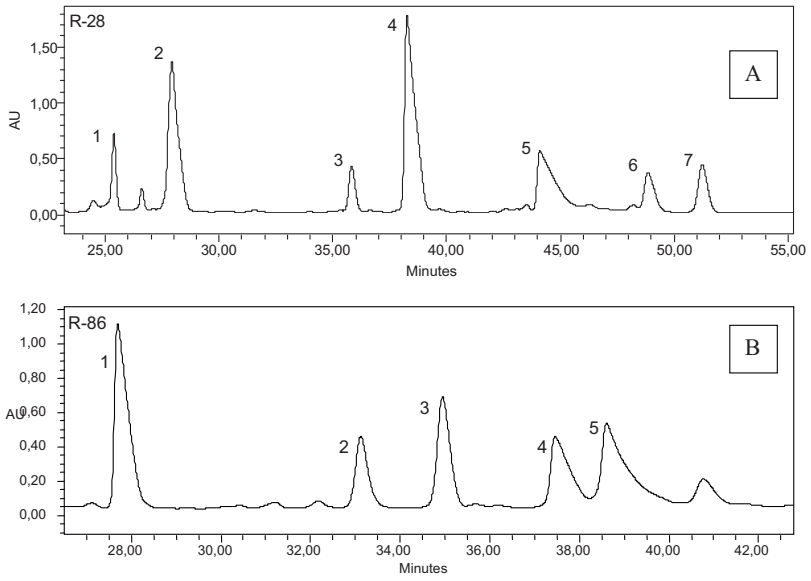

Fig. A1. Reversed-phase HPLC-PDA profiles of extracts of *Radiocystis fernandoi* (strains 86 and 28). Numbered peaks were identified by MALDI-TOF–TOF mass spectrometry analyses (see text). Peptides legend is: (A – R28) 1-MV-1709, 2-MC-RR, 3-dMC-YR, 4-MC-YR, 5-CY-1071, 6-MC-FR, 7-MC-WR; (B – R86) 1-MC-RR, 2-CY-980, 3-CY-1014, 4-MV-1707, 5-MV-1739.

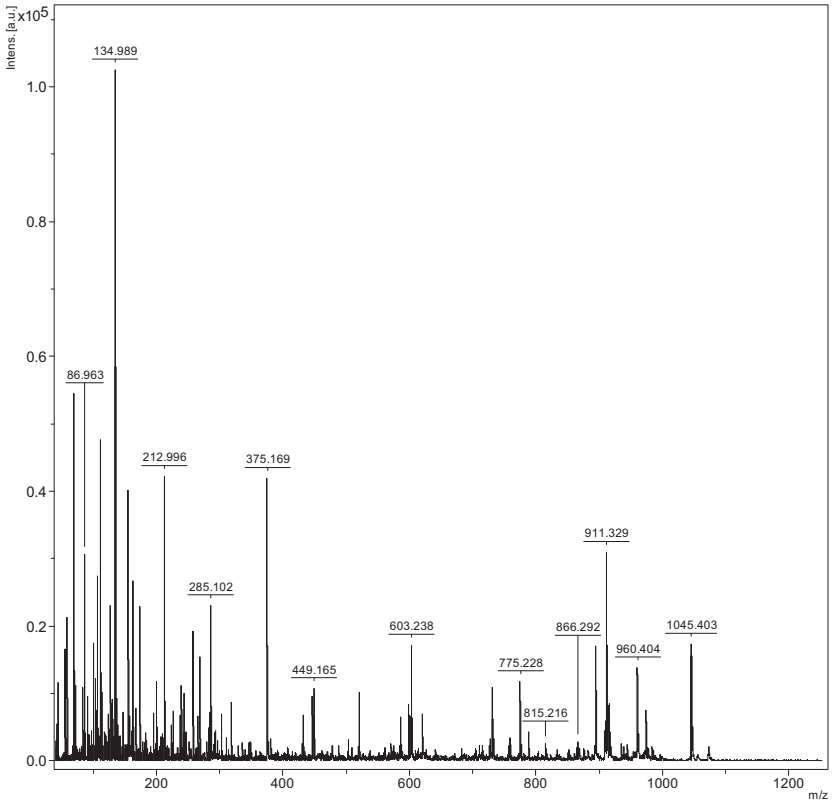

Fig. A2. MS/MS spectrum of *m*/*z* 1045.4 (microcystin YR) parent ion. Fragmentation was achieved in MALDI-TOF–TOF mass spectrometer (Bruker Autoflex III). The parent ion was from *R. fernandoi* 28 fraction 4 collected from RP-HPLC (Rf-F4, Fig. A1(A) – Appendix A).

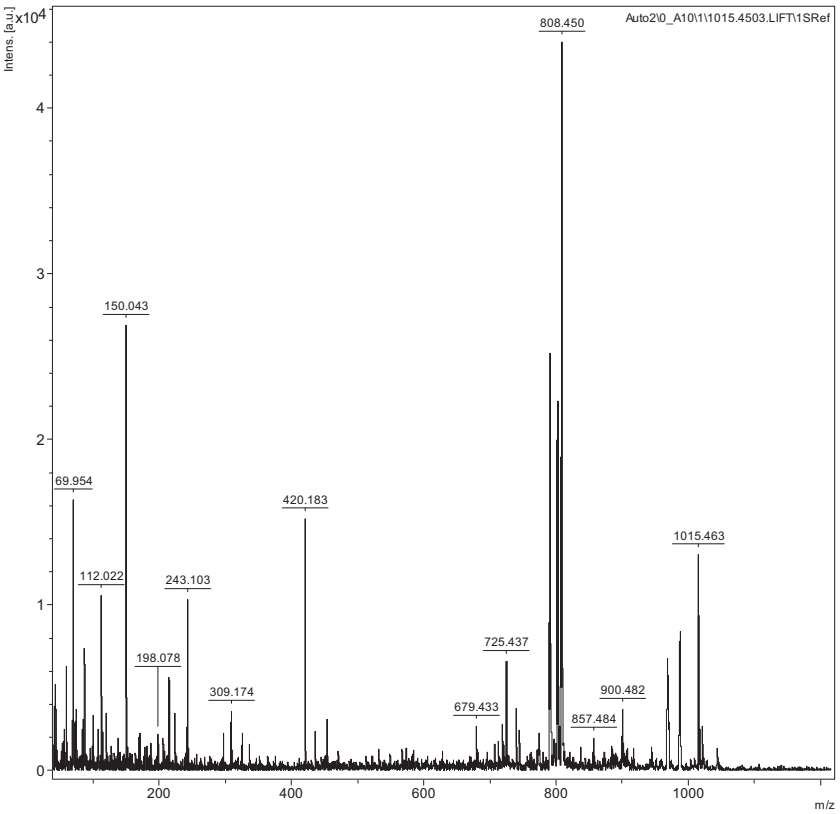

Fig. A3. MS/MS spectrum of *m*/*z* 1015.5 (cyanopeptolin) parent ion. Fragmentation was achieved in MALDI-TOF–TOF mass spectrometer (Bruker Autoflex III). The parent ion was from *R. fernandoi* 86 fraction 3 collected from RP-HPLC (Rf-F4, Fig. A1(B) – Appendix A).

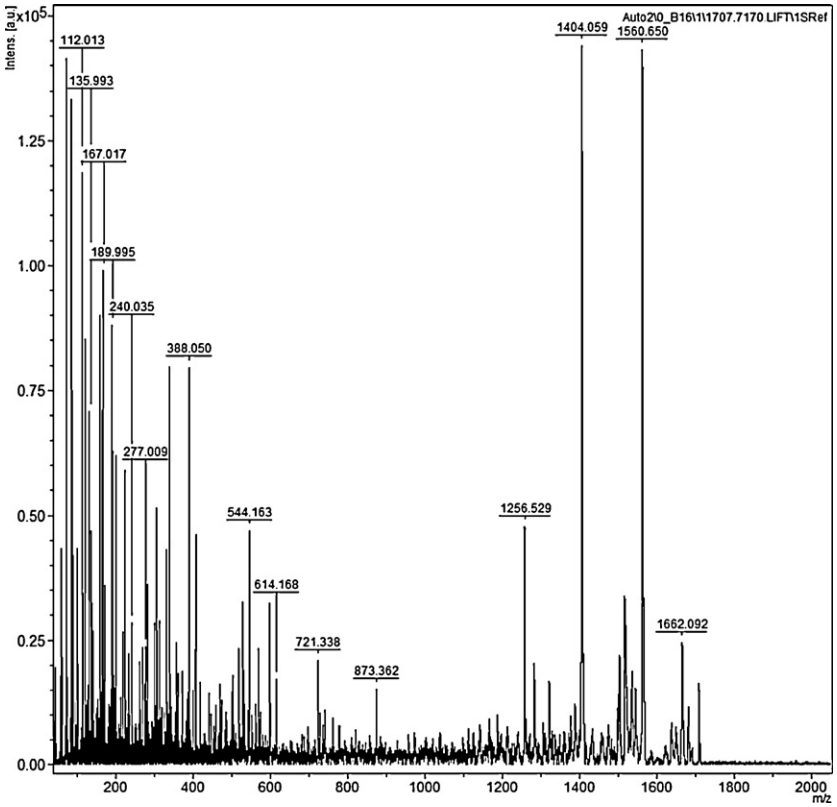

Fig. A4. MS/MS spectrum of *m*/*z* 1707.7 (microviridin) parent ion. Fragmentation was achieved in MALDI-TOF–TOF mass spectrometer (Bruker Autoflex III). The parent ion was from *R. fernandoi* 86 fraction 4 collected from RP-HPLC (Rf-F4, Fig. A1(B) – Appendix A).

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