Title: Widespread distribution and identification of eight novel microcystins in Antarctic cyanobacterial mats

Running Title: Microcystins in Antarctic cyanobacterial mats

Susanna A. Wood [1, 2], Doug Mountfort [1]*, Andrew I. Selwood [1], Patrick T. Holland [1], Jonathan Puddick [2], S. Craig Cary [2, 3]

[1] Cawthron Institute, Private Bag 2, Nelson, 7042, New Zealand.

[2] Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand.

[3] University of Delaware, College of Marine and Earth Studies, Lewes, DE, 19958, USA.

* Corresponding Author: Tel: 64-03-5482319

Fax: 64-03-5469464

Email: doug.mountfort@cawthron.org.nz

Abstract:

The microcystin content and cyanobacterial community structure of Antarctic microbial mat samples collected from 40 ponds, lakes and hydro-terrestrial environments were investigated. Samples were collected from Bratina Island and four of the Dry Valleys; Wrights, Victoria, Miers and Marshall. Enzyme-linked immunosorbent assays (ELISA), liquid chromatography mass spectrometry (LC-MS), and protein phosphatase inhibition assays (PP-2A) resulted in the identification of low levels (1 - 16 mg/kg dry weight) of microcystins in all samples. A plot of indicative potencies of microcystins (ratio PP-2A:ELISA) versus total microcystins (ELISA) showed a general decrease in potency as total microcystin levels increased and a clustering of values from discrete geographic locations. LC-MS/MS analysis on selected samples identified eight novel microcystin congeners. The low energy collisional activation spectra were consistent with variants of [D-Asp^3] MC-RR and [D-Asp^3] MC-LR containing glycine [Gly^1] rather than alanine and combinations of homoarginine [hAr^2] or acetyldemethyl ADDA [ADMAdda^5] substitutions. *Nostoc* sp. was identified as a microcystin producer using PCR amplification of a region of the 16S rRNA gene and the aminotransferase (AMT) domain of the *mcy*E gene. Automated ribosomal intergenic spacer analysis (ARISA) was undertaken to enable a comparison of cyanobacterial mat community structure from distant geographical locations. Two-dimensional multidimensional scaling ordination analysis of the ARISA data showed that in general samples from the same geographic location tended to clustered together. ARISA also enabled the putative identification of the microcystin producing *Nostoc* sp. from multiple samples.

Introduction

Extreme conditions including relentless katabatic winds, permanently low temperatures and precipitation, and depauparate carbon supply ensure that life in the Dry Valleys of Eastern Antarctica is primarily restricted to soil environments (1, 43). However, in selected above surface habitats e.g. lakes, ponds and on moist soil, cyanobacteria have thrived, forming thick cohesive mats (50). The cyanobacterial species (predominately Nostocales and Oscillatoriales) within these mats are adapted to tolerate harsh physicochemical parameters including high salinities and UV radiation.

Cyanobacteria worldwide produce a range of natural toxins collectively known as cyanotoxins. The mechanisms of toxicity are very diverse, ranging from hepatotoxicity and neurotoxicity, to dermatotoxicity. The most ubiquitous of the cyanotoxins are the hepatotoxic microcystins. Microcystins are cyclic peptides and to date, more than 70 microcystins have been isolated and characterized (55). Microcystins are synthesized non-ribosomally by a large peptide synthetase and polyketide synthase enzyme complex (48). An increasing number of species from both planktonic and benthic habitats are known to produce microcystins (17, 23, 42). Despite considerable research, the biological and functional role of microcystins is poorly understood. Various hypotheses have been proposed including; defence against grazers (27), gene regulation (10), allelopathic interactions (44) and intra-specific regulation (39). Recently, relatively low concentrations (<15 mg/kg microcystin-LR dry weight) of microcystins were identified in cyanobacterial mats from meltwater ponds on McMurdo Ice Shelf in Antarctica (16, 19). The identifications of microcystins in these mats provides evidence to dispute some of their putative roles, for example, defence against grazers (16). To date microcystins have only been identified in Antarctica from meltwater ponds on Bratina Island and the extent of their occurrence in

other locations in Antarctica was unknown. Additionally, definitive identification of specific microcystin producers and information on microcystin variants produced was limited.

In this study samples from 40 ponds, lakes and hydro-terrestrial environments from four Dry Valleys (Wrights, Victoria, Marshall and Miers) in Eastern Antarctica and Bratina Island were investigated for the presence of microcystins. Variations in total microcystin concentration within samples have been reported when different detection methods were used (e.g., 26, 28). Therefore, in our study all samples were analysed for microcystins by at least two of the following methods; liquid chromatography mass spectrometry (LC-MS), protein phosphatase 2A inhibition assay (PP-2A) and enzyme-linked immunosorbent assays (ELISA). The genes involved in microcystin synthesis (*mcy*A-J) have been identified and characterised (10, 31, 48) enabling PCR amplification of them to be used as an indication of microcystin production potential. Sequencing of a region of the *mcy*E gene and 16S rDNA from uni-cyanobacterial material was used to identify one of the cyanobacterial species responsible for microcystin production.

The cyanobacterial community structure of each mat was assessed using automated ribosomal intergenic spacer analysis (ARISA). Subsequent multi-variant analysis of ARISA profiles allowed the investigation of correlations between community structure, microcystin production and geographical locations to be made and enabled the investigation of the influence of water chemistry parameters on cyanobacterial miscellany.

MATERIALS AND METHODS

Samples and sample collection. Benthic microbial mat material was collected from 13 meltwater ponds on the McMurdo Ice Shelf, located south of Bratina Island (78°00’S,

165°30’E; January 2004), 12 ponds in Wright Valley (77^{o} 31’S, 160^{o}45’E; January 2004), six ponds in Victoria Valley (77°22’S, 162°10’E January, 2004) and five locations around Lake Miers (78°6′S, 164°0′E; December 2006). Two samples of hydro-terrestrial mats were collected in Miers Valley from moist areas in front of Adams Glacier (MVAG1) and Miers Glacier (MVMG1; December 2006). One sample was collected from the shoreline of Lake Purgatory (S78°03’S 163°51’E; December 2006, included in the Miers Valley samples for all analyses) and three hydro-terrestrial mats from the upper reaches of Marshall Valley (78°03′S, 136°55′E; December 2006). Samples were scraped from the sediment surface using a stainless steel spatula (swabbed with alcohol between collections) and placed in sterile 50 ml Falcon tubes.

Water chemistry parameters (Cl^{-}, SO_4^{2-}, Ca^{2+}, Na^{+}, pH) were determined for all samples (except Lake Purgatory) as previously described (16, 28). No physicochemical data were collected for hydro-terrestrial mats collected in Mier and Marshall valleys.

Isolation of DNA, ARISA fingerprinting and analysis. Sub-samples of the 40 frozen microbial mats were lyophilized. DNA was extracted from approximately 0.1 g of lyophilized material using the MoBio Power Soil™ kit (Carlsbad, CA, USA) according to the manufacturer's protocol.

ARISA PCR reactions were carried out using cyanobacterial specific primers as described previously (53). ARISA fragments lengths (AFL) were analysed by Genetic Profiler V.2 (GE Healthcare, Auckland, New Zealand) and the data transferred to Microsoft Excel for further processing. All AFL information was transposed to presence/absence data for further analysis. ARISA fragment lengths were aligned using an Excel Macro. ARISA fragments

lengths that differed by less than 3 bp were considered identical (53). If multiple AFL fell within this range then only the AFL with the highest florescence was maintained. ARISA fragment lengths less than 5 times baseline fluorescence in height were removed since they could not be fully distinguished from instrument "noise" (14). ARISA fragment lengths shorter than 300 bp were removed as they were considered to short to be true ITS's (53).

Nonmetric multidimensional scaling (MDS) based on Bray-Curtis similarities was undertaken using the PRIMER 6 software package (PRIMER-E, Ltd., UK). This ordination technique ranks the order of similarity of any two communities as an inverse function of the distance between the points representing the communities on the plot (24). Thus communities with the highest similarity are represented on the plot by points that are plotted closest together. Nonmetric multidimensional scaling was undertaken with 100 random restarts and results plotted in two-dimensions. Plots with a stress value less than 0.20 provide interpretable information (9). Agglomerative, hierarchical clustering of the Bray-Curtis similarities was carried out using CLUSTER function of PRIMER 6 and plotted onto the two-dimensional MDS at a similarity level of 40%.

Analysis of similarities (ANOSIM) was used to test for significant differences in AFL profiles between samples from; Bratina Island, Wright, Victoria, Miers and Marshall valleys. ANOSIM produces a sample statistic, *R* which is a relative measure of separation of the priori-defined groups. The *R* statistic is based both on the difference of mean ranks between groups and within groups. An *R* value of 1 indicates community composition is totally different and 0 no difference. A Monte Carlo randomization was used to test the statistical significances of *R*.

To assess which combination of water chemistry variables accounted for observed biotic patterns the computer program BEST (8) was used. Only samples for which all water chemistry data was available were used in the analysis.

Microcystin analysis. Frozen microbial mat samples were lyophilised and the freeze-dried samples stored at -18^{o}C. Sub-samples (0.2 g) of ground freeze-dried material were placed in 50 ml Falcon tubes and 15 ml of 70% methanol added. The samples were ultrasonicated in a bath (60 min), vortexed and centrifuged at 20,000 x *g* at 4^{o}C (10 min). The extraction was repeated and the supernatants were combined and dried under nitrogen with heating at 35^{o}C. The dried extract was solubilized in 2 ml of 20 % methanol in MilliQ water and stored at -18°C (0.07 g of freeze-dried cyanobacterial material per ml). For LC-MS analysis, samples were filtered through a 0.45 µM filter (Minisart RC 4, Sartorius).

The protein phosphatase inhibition assay was carried out in 96-well plates as described in Mountfort et al. (28). The total ADDA containing microcystin/nodularin content in the reconstituted extracts were quantified with a competitive indirect ELISA using the methods of Fischer et al. (11). The 11 samples collected in 2006 were also analysed using a similar ELISA (AgResearch, Ruakura, New Zealand) that has lower cross-reactivity with free ADDA and nodularin (Lyn Briggs, pers. comm.).

The reconstituted extracts for samples collected in 2006 and selected samples from Bratina Island were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for 13 microcystin variants and nodularin (51). Microcystins were separated by LC (Alliance 2695, Waters Corp., MA) using a 150 × 2 mm 5 µm Luna C18 column (Phenomenex, CA) with water/methanol/acetonitrile gradient containing 0.15% formic acid (0.2 ml min^{-1}, 10 µl

injection). The Quattro Ultima TSQ mass spectrometer (Waters-Micromass, Manchester) was operated in ESI^+ mode with multiple reaction monitoring (MRM) using MS-MS channels set up for microcystin-RR, didesmethyl-RR, demethyl-RR, LR, YR, didesmethyl-LR, desmethyl-LR, FR, WR, AR, LA, LY, LW and LF, and nodularin. The m/z 135 fragment ion from the protonated molecular cation was selected for each toxin ($[MH_2]^{2+}$ for microcystin-RR and variants; MH^+ for the others). The LC-MS responses were calibrated using mixed standard solutions of MC-RR, MC-LR, MC-YR and nodularin (Alexis Corpororation, Lausen, Switzerland). The MRM response factor for MC-RR was used for quantitation of MC-RR variants and the MC-LR factor for MC-LR variants. Full scan and fragment ion spectra were also gathered for samples MVAG1 and MVMG1. Full scan spectra identified molecular species for potential microcystins and parent ion scanning experiments identified the components yielding the ADDA fragment m/z 135 on collisional activation. Daughter ion spectra from the protonated molecular species (collision energy 52 eV for MH^+ or 30 eV for MH_2^{2+}) were gathered for each of the components and examined for microcystin structural fragment ions.

Identification of a microcystin producer. The dominant cyanobacteria species in MVMG1 sample was determined using an Olympus light microscope (BX51, Olympus, Wellington, New Zealand). Species identification were made were made with reference to Komárek and Anagnostidis (22). Uni-cyanobacterial material of the dominant species was isolated from MVMG1 using sterile tweezers. Purity was confirmed using microscopic examination and DNA extracted as described above. PCR amplification of a cyanobacterial partial 16S rRNA gene segment and a region of the *mcy*E gene was carried out as described in Jungblut and Neilan (2006). PCR products were purified using a High Pure PCR product purification kit (Roche Diagnostics) and sequenced Bi-directionally using the BigDye Terminator v3.1

Cycle Sequencing Kit (Applied Biosystems, USA). The phylogenetic relatedness of the 16S rRNA and *mcy*E gene sequence obtained in this study was established using sequences from the NCBI Genbank database. Sequences generated during this work were deposited in NCBI Genbank database under accession numbers EU359045-6. An ARISA profile was obtained from the uni-cyanobacterial DNA material as described above. This profile was then used for the putative identification of this species in each of the cyanobacterial mat community ARISA profiles.

RESULTS

Physical and chemical characteristics of ponds. The chemical characteristics of pond water overlying mats in the various pond systems are shown in Table 1. Sediments underlying the mats from Bratina Island were black in colour producing a sulfidogenic odor. In contrast sediments of the Wright and Victoria valleys ponds ranged from coarse gravel to sand. The partial chemical analysis of pond water revealed elevated salt levels within some ponds from Bratina Island and Wright and Victoria valleys. However the salt profiles for the Bratina Island ponds differed from those in the Wright and Victoria valleys in that sulphate was a major salt constituent while in the latter salt mainly comprised of NaCl. The pH of the pond water trended towards being alkaline with the exception of the surface layers of ponds E4 and Ridge (Wright Valley).

Analysis of ARISA. Analysis of ARISA data for all samples identified a total of 63 distinct ARISA fragment lengths (ALF; i.e., peaks). When AFL where totalled across each of the five locations, the highest diversity was observed in the Bratina Ponds (Σ= 28, $x\bar{x}$ = 6.2), followed by Wrights Valley (Σ= 27, $x\bar{x}$ = 4), Miers Valley (Σ= 26, $x\bar{x}$= 7.1), Victoria Valley (Σ= 18, $x\bar{x}$= 5.6) and Marshall Valley (Σ= 9, $x\bar{x}$= 3).

Multivariate analyses showed that cyanobacterial community structure differed among sampling locations (ANOSIM R = 0.4, P <0.001). Pair-wise comparisons between each sampling location revealed that Bratina and Marshall Valley samples were all significantly different from other locations where as Victoria, Miers and Wright valleys did not vary markedly (Table 2). With the exception of MarV1, MarV3 and Ridge, the two-dimensional multidimensional scaling (MDS) ordination analysis separated the samples into two large groups, united at the similarity level of 40%. Within each of these group samples from the same geographic location tended to clustered together (Fig. 1). One exception to this was the samples from Marshall Valley were all distant from each other.

To elucidate potential water chemistry parameters responsible for differences in community structure among the geographic locations BEST analysis was undertaken. Initial pairwise scatter-plots between all combinations of the water chemistry variables suggested a log(1 +X) transformation of all variables was required (8.) The results from the BEST analysis showed that the highest rank correlation (ρ = 0.158, P <0.008) was due a combination of pH, Ca and Na. This value is low in comparison with other examples (9), indicating that this set of environmental variables has weak explanatory power.

Microcystin detection. Data for microcystins expressed on a μg.kg dry wt^{-1} basis are shown in Tables 2 and 3. Microcystins were detected with at least one of the detection methods in all samples. With the exception of the higher levels of microcystin observed for the Adams and Miers glaciers samples (MVAG1 and MVMG1) no clear differences could be seen between microcystin concentrations that could be attributable to geographical location. However, when indicative potencies of microcystins (ratio PP-2A: ELISA-ADDA; 27) were

plotted against total microcystins (ELISA-ADDA; Fig. 2) several trends became evident: (i) generally potency decreased as total microcystin levels increased (this was particularly evident for samples from Miers and Marshall valleys) and (ii), values for sites for discrete locations tended to cluster (particularly evident for samples from Bratina and Victoria valleys).

LC-MS analysis of selected samples from Bratina Island, did not show the presence of microcystins. Analysis of samples from the Miers and Marshall valleys by LC-MS identified seven microcystin congeners. LC-MS peaks for parent MC-RR and MC-LR congeners were present although the retention times did not exactly match the standards. The most prevalent congeners observed using MRM were a desmethyl MC-LR, a didesmethyl MC-LR, a desmethyl MC-RR and a desdimethyl MC-RR. More detailed analysis of samples MVMG1 and MVAG1 using parent ion scanning for components yielding the m/z 135 ADDA fragment did not reveal other microcystin congeners. However, the relative retention time data for the MC-RR and MC-LR congeners found were slightly different to those for the parents or known demethylated analogues of these microcystins. Furthermore full scan LC-MS revealed the presence of a further four components with MH^+ 1038 and 1052 (retention region for MC-RR; strong $[MH_2]^{2+}$) and MH^+ 995 and 1009 (retention region for MC-LR) which did not yield significant m/z 135 on collisional activation. Full daughter ion spectra were obtained for these four components and the four major microcystins in the extracts of samples MVAG1 and MVMG1. The spectra were consistent these samples containing eight novel variants of [D-Asp^3] MC-RR and [D-Asp^3] MC-LR containing glycine [Gly^1] rather than alanine and combinations of homoarginine [hAr^2] or acetyldemethyl ADDA [$ADMAdda^5$] substitutions (Fig. 3). MS alone cannot distinguish between the isobaric N-methyl dehydroanaline (Mdha) and dehydrobutyrine (Dhb) which is another

potential substitution (38). The details of these structural assignments will be published elsewhere but are consistent with the analyses of low energy collisionally activated ion spectra for similar microcystin variants identified from *Nostoc* species and *Planktothrix agardhii* (25, 32, 54). The [Gly^1] substitution is novel but is supported by several peptide fragment ion series (54) and in analogy to those observed for [D-Leu^1] MC-LR (34).

The microcystin content of samples collected in 2006 was determined using four different methods allowing a comparison of results from these methods to be undertaken. There was a strong correlation between the LC-MS results and the two ELISA methods (R^2 = 0.9997, ELISA-ADDA and R^2 = 0.999, ELISA-MC) and between the two ELISA methods (R^2 = 0.974). However, the correlation was weak when the PP-2A assay results were compared to all other methods (R^2 = 0.168, LC-MS; R^2 = 0.160, ELISA-ADDA and R^2 = 0.187 ELISA–MC).

Confirmation of a microcystin producer. Based on morphology the dominant species in sample MVMG1 was identified as *Nostoc* sp. with the following features: long and irregularly trichomes surrounded by a diffuse mucilaginous envelope; vegetative cells sub-spherical, 4 ± 2 μm wide and 2.8 ± 1.2 μm long; heterocytes 5 ± 1 μm wide and 6.4 ± 1.4 μm long. Segments of the 16S rRNA and the *mcy*E gene were successfully amplified from the purified *Nostoc* sp. material. The 685 bp 16S rRNA gene sequence (Genbank EU359045) was submitted to BlastN (2) and matched at greater than 99% sequence homology to *Nostoc* sp. ANT.LH52B.8 (Genbank AY493593). The 364 bp segment of the *mcy*E gene (Genbank EU359046) and had a high (93%) sequence homology with *Nostoc* sp. 152 (Genbank AY817163). ARISA analysis from the purified *Nostoc* sp. material identified two distinct AFL at lengths of 471 and 733 bp. At least one of these peaks was identified in ARISA

profiles from samples; LMM1, LMM2, MVAG1, MVMG1 (Miers Valley) and MarVM3 (Marshall Valley).

DISCUSSION

Microcystin production. In this study we have demonstrated that microcystin production by cyanobacteria in Antarctica is not confined to the meltwater ponds on McMurdo Ice Shelf (16, 18). Using a combination of ELISA, PP-2A and LC-MS, microcystins were detected in cyanobacterial mats from four distant geographic locations within the Dry Valleys of Eastern Antarctica. Previous studies (16, 18) detected only low levels of microcystins and this was also the case in our study were total microcystin levels by ELISA were all <16 $mg.kg^{-1}$ dry weight. These levels are significantly lower than those reported from planktonic cyanobacterial blooms (7). Interestingly, the highest concentrations of microcystins were not detected in the samples from the lake and pond mats, but from the hydro-terrestrial mats adjacent to the bases of Miers and Adams Glaciers. This is the first report of microcystins from hydro-terrestrial mats in Antarctica. Jungblut et al. (18) postulated that the low levels of microcystins could be due to either; the low abundance of the microcystin producer within the mat community or low levels of biosynthesis by the producer. *Nostoc* sp. made up a large portion of the biomass of sample MVMG1 (sample with highest microcystin concentrations), thus we suggest that the former is the most plausible explanation.

Previous studies have reported only two microcystin congeners; [D-Asp^3]-microcystin-LR and microcystin-LR in Antarctic cyanobacterial mat samples (16, 18). The quantitative measurements by LC-MS using MRM initially indicated the presence of MC-LR, MC-LR and MC-FR with higher proportions of four additional congeners; a desmethyl microcystin-RR, a desdimethyl microcystin-RR, a desmethyl microcystin-LR and a desdimethyl

microcystin-RR (Table 4). However, full scan LC-MS followed by detailed MS/MS daughter ion analysis revealed that there were eight major microcystin components which had novel structures based on variants of MC-LR and MC-RR and these included four ADMAdda variants. The latter were not detected by the MRM or parent ion scan experiments because the ADDA fragment at m/z 135 is not significant when the 9-acetoxy substitution is present (Fig. 3, 54). ADMAddda microcystin analogues have reported in benthic *Nostoc* strains from Finland (32, 40, 41) and *Planktothrix agardhii* (planktonic) from Denmark (25). This is the first reporting of such variants from Southern Hemisphere cyanobacteria. Substitution of hAr for Arg was also relatively common in microcystins from Finnish *Nostoc* sp. The [D-Ala^1] in microcystins is generally highly conserved, although substitutions by serine or leucine in MC-LR have been reported (34, 41). Therefore the novel finding in these Antarctic mats of eight variants of MC-RR and MC-LR all containing [Gly^1] is remarkable.

Hitzfeld et al. (16) and Jungblut et al. (18) hypothesized on potential microcystin producing genera; *Oscillatoria, Phormidium* and *Nostoc* were all given as likely candidates. The ability of *Nostoc* sp. (in sample MVMG1) to produce microcystins was confirmed by PCR amplification and sequencing of a region of the aminotransferase (AMT) domain of the *mcy*E gene. *Nostoc* species have previously been shown to produce microcystins (40, 41, 52). Wood et al. (52) detected high levels of microcystin-RR and a desmethyl microcystin-RR in benthic *Nostoc commune* mats collected from a New Zealand lake. The diagnostic ARISA fragment lengths (AFL) for this species was observed in only five samples from the Miers and Marshall valley regions. Interestingly, four of these samples had the highest total microcystin concentrations recorded in this study. The absence of the *Nostoc* sp. AFL from

other samples indicates that there are other yet to be identified microcystin producers within these mat communities.

Several studies have demonstrated variability in microcystin concentrations when applying different detection methods (e.g., 3, 28, 52). The ADDA-ELISAs used during the present study measure the total amount of ADDA-containing compounds in the sample, with the second ELISA having lower cross-reactivity with free ADDA and nodularin (Lyn Briggs, pers. comm.). As both ELISAs used antibodies raised against the ADDA moiety it is very likely that cross-reactivity to ADMAdda variants was low (25) and therefore the total microcystins in these samples were underestimated. Similarly the LC-MS (MRM) analyses targeting 13 common ADDA-containing microcystins did not determine the ADMAdda variants. This explains the high correlations between results for both ELISA's and the LC-MS (MRM) method. Based on the scanning LC-MS data for samples MVAG1 and MVMG1, it is estimated that including the four major ADMAdda variants would approximately double the total microcystin concentrations reported in Table 4. The correlations were weak (< R^2 = 0.19) between the PP-2A assay and the ELISA or the LC-MS methods with concentrations by PP-2A being consistently lower for samples containing >20 $\mu g.kg^{-1}$ microcystins (Table 3). The response of the PP-2A assay varies depending on the toxicity of microcystin congeners present in a sample (28) which will explain some of the inconsistencies observed when comparing results obtained via these methods.

Mountfort et al. (28) suggested that for samples containing mixtures of microcystin congeners the response ratios (ratio of the amount determined by PP-2A equivalent to microcystin LR: to that determined by ELISA-ADDA) assigns an indicative toxicity to a sample as well as toxin equivalence. In our study when indicative potencies of microcystins

were plotted against total microcystins (as measured by ELISA-ADDA; Fig. 2) samples from discrete locations tended to cluster together. Microcystins were not detected by LC-MS for the samples from Bratina Island or Victoria and Wrights valleys, but it was presumed that the microcystin congener composition for from samples in the same geographic location were similar. The two samples with the lowest PP-2A/ELISA ratio (MVMG1 and MVAG1 from Miers and Marshall valleys) were the samples with the highest total microcystin levels and therefore it is likely that the novel MC-LR and MC-RR variants identified in these samples by LC-MS have significantly lower PP-2A activities than MC-LR [Asp^3] variants have been reported to be of lower intraperitoneal (IP) toxicity and while ADDMAdda variants were toxic by IP (37, 42), somewhat lower PP-2A activities have been reported (25). The effects on toxicity of [Gly^1] or [hAr^4] substitution have not been determined. None or only low levels of the target 13 ADDA-microcystins were detected by LC-MS in the three Miers and Marshall valley samples with the highest PP-2A/ELISA ratio (LMM3, LMM4 and MarV2). This potentially indicates the presence of other toxins with high inhibitory potential for PP2A.

ARISA and microcystin production. Morphological surveys (e.g., 5, 6) and more recently polyphasic approaches using 16S rRNA clone libraries (e.g., 18, 45, 46, 47) have helped establish an inventory of Antarctic cyanobacteria and allowed investigations into endemism and biogeographical distributions. However, these identification methods are often protracted and therefore not applicable for analysis of large sample numbers. Recently a sensitive and high-throughput finger-printing method known as automated rRNA intergenic spacer analysis (ARISA) has been developed (12). This PCR-based method exploits the length heterogeneity of the intergenic spacer region (ITS) between the 16S and 23S ribosomal genes. Total community DNA is amplified with a fluorescently labelled

oligonucleotide, allowing the electrophoretic step to be performed with an automated system in which a laser detects the fluorescent DNA fragments. In this study ARISA was used to assess cyanobacterial community structure in 40 samples from five distant locations. This enabled us to investigate; (i) the influenced of community structure on microcystin production, (ii) biogeographical distribution and (iii) the effect of selected water chemistry parameters on cyanobacterial community structure.

The nonmetric multidimensional scaling (MDS) analysis of the ARISA data showed community structure appears to have little effect on microcystin concentration. MVAG1 and MVMG1 plotted close to each other, however, MarV3 which also had a high concentration of microcystins, was distant. Rather than community structure, we postulate that it is the presence and abundance of one or more toxin producing genotypes that influences the amount of microcystin in a sample. Numerous studies have shown that the presence of microcystin genes (i.e., toxic genotypes) correlates with detection of microcystins (e.g., 15, 49).

The study of microcystin production in extreme environments may help in understanding their functional role. Within these mats, especially the hydro-terrestrial mats, there are minimal grazers and few other phytoplankton (16), thus microcystin production to prevent grazing or allelopathy seem unlikely explanations. The low levels of microcystins found in Antarctic mats to date suggest minimal biosynthesis is occurring. Although no studies have investigated Antarctic cyanobacterial growth in the field it is likely that given the extreme cold and dark conditions for many months of the year growth is minimal. Orr and Jones (33) in a study on cultured *Microcystis* sp. showed that microcystin production was limited to the phase of growth when cell concentration was increasing and suggest that microcystin plays

an important (perhaps essential) role in the cellular metabolism of toxigenic strains. If this hypothesis is correct then given the presumably slow growth rate of cyanobacteria in Antarctica then this may explain the low microcystin levels. Microcystins are extremely stable and resistant to chemical hydrolysis or oxidation at near neutral pH (42). In the inherently cold and often dark Antarctic environment it seems likely that these toxins may persist for many months or years. Investigations on Antarctic isolates and on microcystin gene expression during different phases of the year are planned to further explore this.

ARISA and community structure. Taton et al. (47) carried out a detailed analysis of cyanobacterial diversity in sample from four different ponds on Bratina Island and identified between four to 12 operational taxonomic units (OTU's; based on 16S rRNA gene sequences) per pond. In an analogous study, Jungblut et al. (18) identified five to 15 OTU's from three ponds on Bratina Island. A similar diversity was observed in our samples with the number of AFL ranging from one to 14. One caveat when interpreting ARISA data is that interoperonic differences in spacer length occur within the genomes of microorganisms (29), such that a single species may contribute more than one peak to an ARISA profile. Previous studies (e.g., 13, 53) indicate that species of the order Nostocales commonly have two types of intergenic spacer regions (i.e., two AFL), whereas Chroococcales and Oscillatoriales have only one. Thus it is highly likely that the number of AFL is greater than the actual number of OTU's.

Morphological studies (e.g., 6, 21, 36) suggest that many cyanobacterial species are widespread across the continent. In contrast, recent molecular studies have shown that the communities of four lake mats where distinct with 71.4% of OTU's found only in one sample (47). This may however be due to artefacts (e.g., produced during DNA extraction,

PCR and cloning; 47) or may reflect the small number of samples used in this study. The MDS analyses of our ARISA profiles suggests that cyanobacterial community structure within a geographic location generally does not vary markedly, with most samples showing greater than 40% similarity. Interestingly, there were two clusters on the MDS plot. One containing mainly the Wrights and Victoria samples and the other primarily consisting of the Bratina Island samples. This result was also shown in the ANOSIM analysis where the Bratina samples were significantly different from the samples from Wrights and Victoria valleys. Wrights and Victoria valleys sit adjacent to edge other approximately 150 km north of Miers Valley and Bratina Island. It seems plausible that the close proximity of these valleys has enabled similar cyanobacterial communities to develop. It has been suggested that wind is an important dispersal agent for biomass in Antarctica (4, 35) and this may have also played a role in structuring the similarity of these communities. The Marshall Valley samples did not cluster close to one another and were usually quite distant from the other samples. These samples were hydro-terrestrial mats. Unfortunately no physico-chemical data was collected for these sites, which may have helped in investigating explanations for the community composition differences.

Jungblut et al. (18) suggest that salinity may influence community structure. The ponds and lakes in our study spanned a wide range of salinities. The results of the BEST analysis indicated that differences in the water chemistry parameters (Cl^-, SO_4^{2-}, Ca^{2+}, Na^+, pH) were unlikely to be contributing to the community structure. Thus rather than physiochemical parameters dictating which cyanobacteria species are present in these ponds we suggest some species have adapted to tolerate a wide range of conditions. A similar conclusion was found by Taton et al. (47). Following a comparison of their cyanobacterial OTU's with

sequence databases, they suggest that given the ubiquity of several OTU's, cyanobacteria must have the ability to tolerate a range of harsh environmental conditions.

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Table 1. Chemical analysis of pond water in ponds from Bratina Island and Wright, Victoria, Miers valleys. No data is available for the Marshal Valley, Adams and Miers glacier and Lake Purgatory sites.

		concentration (g m^{-3})				
Geographical location	**Pond**[a]	**Cl^-**	**SO_4^{2-}**	**Ca^{2+}**	**Na^+**	**pH**
Bratina Island	Bambi	550	180	30	310	9.6
	Brack	1300	8200	100	3800	10.1
	Caston	270	310	11	300	9.7
	Heart	840	130	11	480	8.8
	Moist	ND[b]	ND	ND	ND	ND
	No Name	430	130	30	210	9.3
	P70	1400	230	24	720	9.7
	Pancreas	1100	220	29	700	8.9
	Retro	550	390	46	340	9.6
	Salt	5100	33000	200	14000	9.7
	Skua	120	140	8.9	140	9.7
	Vent	1200	220	30	720	9.1
	Weather Stn	1400	230	24	740	9.7
Victoria Valley	Basalt	5300	960	1100	1800	8.5
	River Gauge	4.5	3.2	3.1	1.8	8.1
	TP Lower	8800	1100	1900	2800	8.9
	TP Upper	ND	370	870	1200	8.3
	Upper Victoria	2000	ND	ND	ND	ND
Wright Valley	E3	5.3	12	1.6	6.7	7.9
	E4	46	120	33	45	8.6

	L01	110	160	39	81	9.3
	L15	49	70	23	35	9.4
	L16	380	140	28	190	9.2
	L26	2800	580	91	1300	8.5
	L3	8.2	18	6.2	7.4	9.3
	L4	11000	2000	520	6000	8.3
	L9	750	280	40	460	9
	Puddle	430	210	43	250	9.5
	Ridge	31	37	6.1	41	7.1
Miers Valley	Lake Miers - LMM1	3.9	3	ND	ND	ND
	Lake Miers - LMM2	3.9	3	ND	ND	ND
	Lake Miers - LMM3	3.9	3	ND	ND	ND
	Lake Miers - LMM4	3.9	3	ND	ND	ND
	Lake Miers - LMM5	2.0	3.9	ND	ND	ND

[a] Unofficial names of study ponds/sites

[b] Not determined

Table 2. ANOSIM statistics for tests involving a comparison of all five sampling locations.

Comparison		*R* Statistic	*P* value
Location 1	**Location 2**		
Bratina	Victoria	0.689	0.001
Bratina	Wright	0.508	0.001
Bratina	Miers	0.401	0.002
Bratina	Marshall	0.788	0.002
Victoria	Wright	-0.106	0.800
Victoria	Miers	0.190	0.066
Victoria	Marshall	0.569	0.018
Wright	Miers	0.177	0.027
Wright	Marshall	0.387	0.011
Miers	Marshall	0.504	0.012

Table 3. Determination of microcystins in cyanobacterial mat samples taken at various sites in the vicinity of Bratina Island, and the Wright, Victoria, Miers and Marshall valley regions.

Geographical location / Date	Sample[a]	Total microcystin concentration (μg.kg dry wt^{-1})			
		PP-2A	ELISA-ADDA	ELISA-MC	LC-MS[b]
Bratina Island	Bambi	12.4	26.1	-	-
December 2004	Brack	8.8	74.6	-	ND
	Casten	1.1	21.2	-	-
	Heart	4.5	19.6	-	-
	Moist	13.1	12.0	-	-
	No Name	18.7	31.6	-	ND
	P70	5.7	35.6	-	-
	Pancreas	18.4	13.7	-	ND
	Retro	11.7	15.8	-	-
	Salt	30.5	132	-	-
	Skua	11.0	38.2	-	-
	Vent	9.2	25.8	-	ND
	Weather Stn	2.0	8.0	-	ND
Victoria Valley	Basalt	2.5	2.4	-	-
December 2004	River Gauge	1.7	1.0	-	-
	TP Lower	1.1	1.8	-	-
	TP Upper	2.6	1.9	-	-
	Upper Victoria	11.9	5.9	-	-
Wright Valley	E3	26.0	8.6	-	-
December 2004	E4	5.1	2.8	-	-
	L01	13.4	7.9	-	-
	LI5	10.8	2.4	-	-

	L16	12.3	9.5	-	-
	L26	57.7	27.3	-	-
	L3	57.6	19.4	-	-
	L4	11.2	0.2	-	-
	L9	11.7	40.0	-	-
	Puddle	0.4	1.3	-	-
	Ridge	1.7	0.0	-	-
Miers Valley	LMM1	697	360	223	143
December 2006	LMM2	92.1	8.2	4.3	3.1
	LMM3	254	2.1	2.1	2.9
	LMM4	116	0.7	0.7	<1
	LMM5	408	89.5	64.2	40.1
	MVAG1	710	2960	1550	1153
	MVMG1	1510	15900	7490	6609
	LPM1	1020	8.3	8.3	<1
Marshall Valley	MarV1	374	187	158	46.6
December 2006	MarV2	98.3	0.9	0.9	<1
	MarV3	2610	1070	812	515

[a] Unofficial names of study ponds/sites
[b] ADDA containing microcystins for LC-MS
- = not analysed
ND = not determined

Table 4. Concentrations of ADDA microcystin congeners (μg. kg dry wt^{-1}) in microbial mat samples from Miers and Marshall valleys determined by liquid chromatography mass spectrometry using multiple reaction monitoring.

	LMM1	LMM2	LMM3	LMM4	LMM5	MVAG1	MVMG1	LPM1	MarV1	MarV2	MarV3
Desdimethyl microcystin-RR (MC-2)	36.4	0.5	<1	<1	9.3	216	568	<1	1.8	<1	89.4
Desmethyl microcystin-RR (MC-4)	45.0	0.8	<1	<1	8.6	344	1226	<1	2.9	<1	128
Microcystin-RR	<1	<1	<1	<1	<1	10.8	29.7	<1	1.3	<1	4.7
Desdimethyl microcystin-LR (MC-1)	11.5	<1	<1	<1	5.5	137	1253	<1	6.9	<1	76.2
Desmethyl microcystin-LR (MC-3)	40.0	1.8	2.9	<1	14.8	371	3226	<1	24.5	<1	200
Microcystin-LR	9.3	<1	<1	<1	1.5	38.6	219	<1	9.2	<1	17.0
Microcystin-FR	<1	<1	<1	<1	1.5	35.9	87.7	<1	<1	<1	<1
Total ADDA microcystins	**143**	**3.1**	**2.9**	**<1**	**40.1**	**1153**	**6609**	**<1**	**46.6**	**<1**	**515**

Figure legends

Figure 1. Two-dimensional non-metric multidimensional scaling ordination (stress = 0.12) based on Bray-Curtis similarities of ARISA fingerprints of cyanobacterial communities from various locations in Eastern Antarctica. Points enclosed by dashed line cluster at 40% similarity. △ Bratina Island, ● Marshall Valley, ◆ Miers Valley, ◘ Wrights Valley, ▼ Victoria Valley.

Figure 2. Plot of ratio PP-2A: ELISA versus ELISA concentrations for microcystins in cyanobacterial mats taken from different sites in a range of geographical locations in Eastern Antarctica. △ Bratina, ● Marshall Valley, ◆ Miers Valley, + Miers (glacier base samples), ◘ Wrights Valley, ▲ Victoria Valley.

Figure 3. Structures of microcystins RR and LR and the eight novel variants from Antarctic cyanobacterial mats MVAG1 and MVMG1 (Miers Valley).

Figure 1.

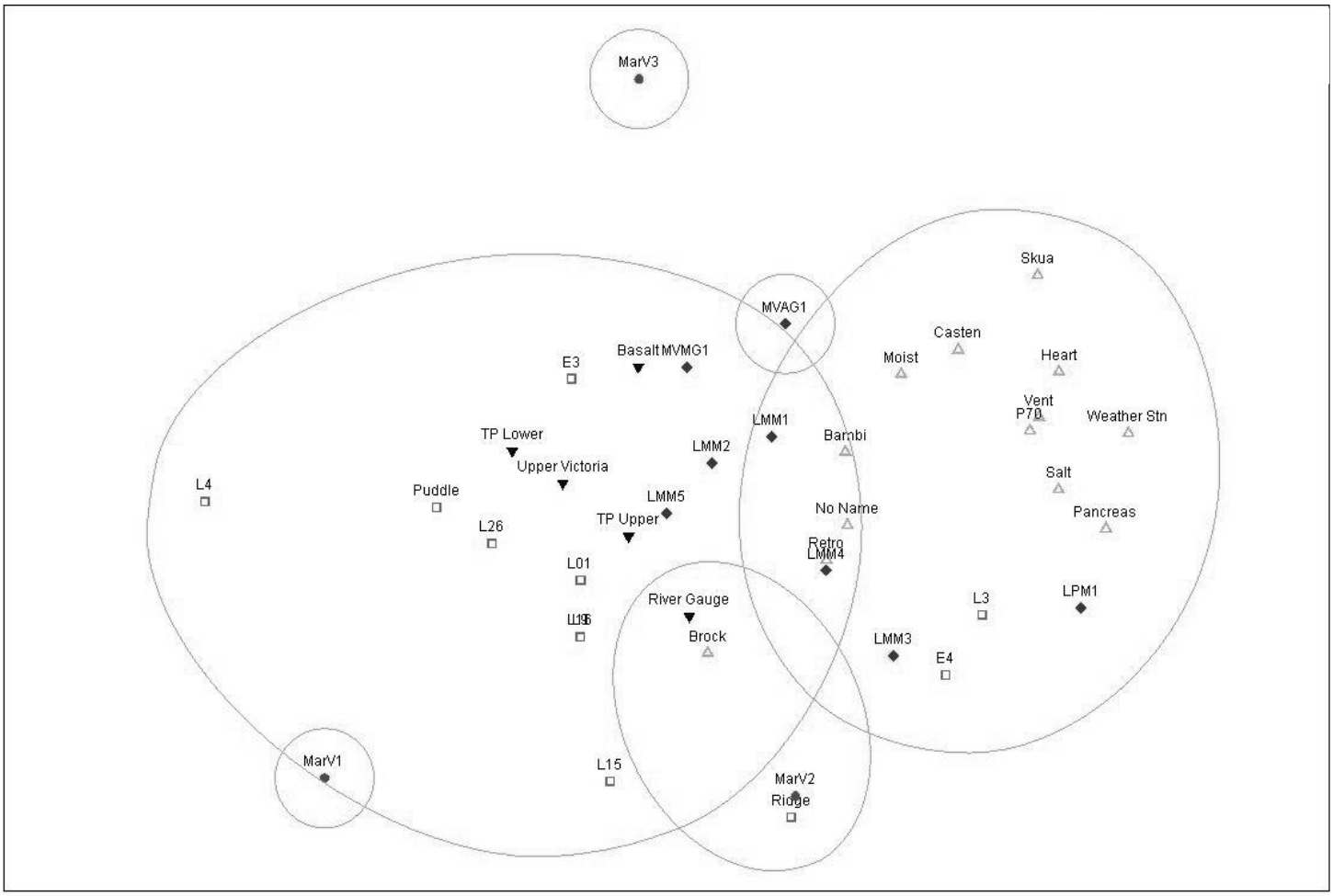

MarV3
Skua
MVAG1
Casten
Moist
Heart
E3
Basalt MVMG1
Vent
P70
Weather Stn
LMM1
TP Lower
Bambi
LMM2
Upper Victoria
Salt
L4
Puddle
LMM5
No Name
Pancreas
TP Upper
L26
Retro
LMM4
L01
River Gauge
L3
LPM1
Brock
LMM3
E4
MarV1
L15
MarV2
Ridge

Figure 2.

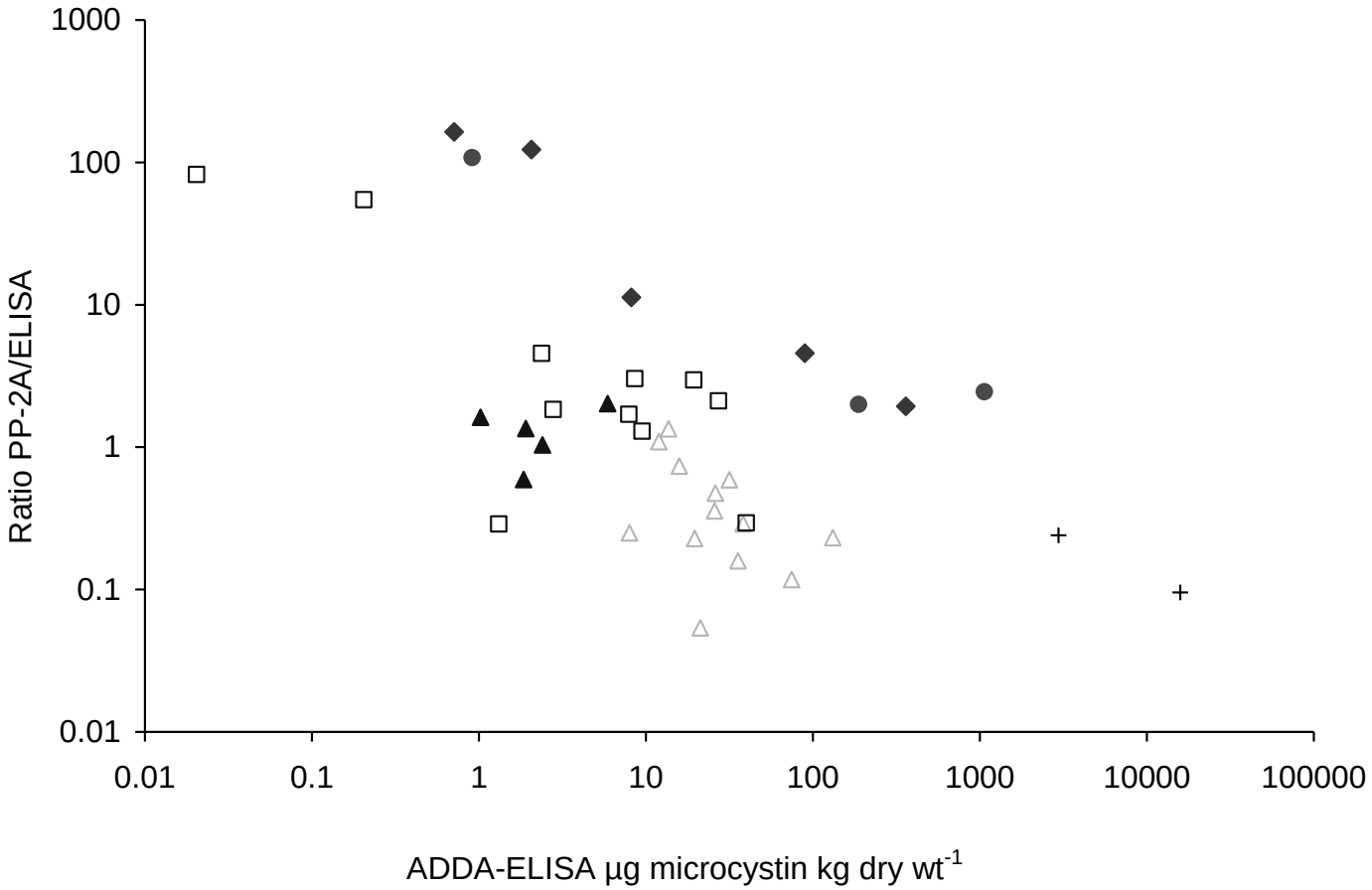
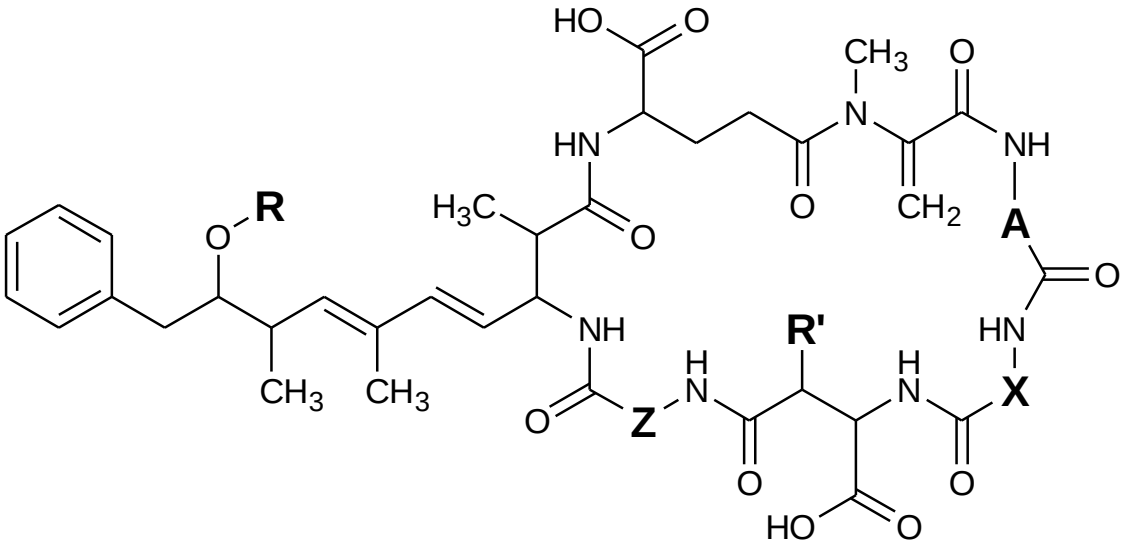

1000
100
10
1
0.1
0.01
Ratio PP-2A/ELISA
0.01
0.1
1
10
100
1000
10000
100000
ADDA-ELISA µg microcystin kg dry wt-1

Figure 3.



	M+H	RRT[a]	A	X	Z	R	R’
MC-LR	995.6	1.000	D-Ala	L-Leu	L-Arg	$-CH_3$	$-CH_3$
MC-1	967.6	0.981	Gly	L-Leu	L-Arg	$-CH_3$	-H
MC-3	981.6	0.995	Gly	L-Leu	L-hAr	$-CH_3$	-H
MC-5	995.6	0.989	Gly	L-Leu	L-Arg	$-COCH_3$	-H
MC-7	1009.6	1.000	Gly	L-Leu	L-hAr	$-COCH_3$	-H
MC-RR	1038.7	1.000	D-Ala	L-Arg	L-Arg	$-CH_3$	$-CH_3$
MC-2	1010.7	0.972	Gly	L-Arg	L-Arg	$-CH_3$	-H
MC-4	1024.7	0.993	Gly	L-Arg	L-hAr	$-CH_3$	-H
MC-6	1038.7	0.991	Gly	L-Arg	L-Arg	$-COCH_3$	-H
MC-8	1052.7	1.009	Gly	L-Arg	L-hAr	$-COCH_3$	-H

[a] Relative Retention Time : Rt MC-LR 10.24 min.; Rt MC-RR 8.55 min.