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Cytotoxicity and Nitrogenase Activity of Selected Soil Filamentous Cyanobacteria

Master thesis

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With 4 tables and 15 figures in text

Annotation: Cytotoxicity and nitrogenase activity (acetylene reduction activity) of 16 selected soil filamentous cyanobacteria originating from various habitats were determined. The aim of my experiments was to evaluate potential importance of cyanobacterial toxicity and nitrogenase activity for soil communities and compare measured characteristics of distinct cyanobacterial phenotypes.

I declare, that I have worked up this study myself with assistance of cited literature and people mentioned in acknowledgement.

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Pavel Hrouzek

Poděkování

Na tomto místě bych rád poděkoval všem, kteří mi pomohli dokončit tuto práci, ať již po odborné stránce, nebo mě podpořili kdykoli jsem to potřeboval. Nejprve bych rád poděkoval p. Prof. Jiřímu Komárkovi, Doc. Blahoslavovi Maršálkovi a Prof. Miloslavu Šimkovi za cenné metodické rady, poskytnutí zázemí a přístrojového vybavení a nápady „jak na to“. Můj velký dík patří celé laboratoři Doc. Jana Kopeckého na Parasitologickém ústavu AVČR za poskytnutí zázemí při kultivaci tkáňových linií. Jmenovitě bych velmi rád poděkoval p. Evě Řezníčkové za udržování tkáňových linií a RNDr. Jiřímu Salátovi za nepostradatelnou pomoc při měření a analýzách prováděných na průtokovém cytometru. Doc. Jiřímu Kopeckému a Aleně Lukavské z mikrobiologického ústavu AVČR bych rád poděkoval za provedení testu cytotoxicity na žábřonožkách a za čas věnovaný mi při HPLC-MS analýzách. Srdečný dík patří též Ing. Aleně Lukešové nejen za poskytnutí kmenů použitých v této studii. Největší dík za duševní i všemožnou jinou podporu patří mámě, tátovi a celé naší rodině.

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1. Introduction

1.1. The cyanobacteria

The cyanobacteria are diverse and remarkable group of prokaryotes. Including Prochlorophyta they create distinct phylogenetic cluster inside the eubacterial group. Their photosynthetic apparatus containing chlorophyll *a* consisting of two photosystems (PSI and PSII) is capable to perform oxygenic photoynthesis. According to large amount of fossil records they are suggested to be an ancient group which has played major role in transforming the Earth's atmosphere from its anaerobic to the aerobic state 3500 million years ago (SCHOPF 1993). Recently the cyanobacteria are a very diverse group from morphological and ecological point of view. They are present in a wide range of habitats (plankton, benthos, solid substrates, soils etc...). Traditionally this group was divided into four orders (KOMÁREK & ANAGNOSTIDIS 1989): Chroococales (unicellular and colonial types), Oscillatoriales (filamentous types without heterocytes and akinetes), Nostocales (filamentous cyanobacteria which differentiate akinetes and heterocytes) and Stigonematales (true branched types which differentiate akinetes and heterocytes).

1.2. Nitrogen fixation in heterocytous cyanobacteria

Biological nitrogen fixation is a crucial way of nitrogen supply to biosphere. Although it is relatively widespread in nature, only a few groups of microorganisms developed the ability to assimilate dinitrogen molecules into organic compounds. Filamentous heterocytous cyanobacteria (order Nostocales) represent one of these groups of highly specialized organisms called diazotrophs. Diazotrophs have a unique enzyme system – nitrogenase – which is responsible for the dinitrogen reduction and ammonium formation during nitrogen fixing process. Nitrogenase, however, is extremely sensitive to oxygen. Therefore, the diazotrophs developed various mechanisms to protect the active enzyme against molecular oxygen. In conditions with lack of combined nitrogen, filamentous heterocytous cyanobacteria differentiate special cells with a thick cell wall, heterocytes – old termed heterocysts (FAY *et al.* 1968; FLEMING & HASELKORN 1973; VAN GORKOM & DONZE 1971), in which nitrogenase is effectively protected. Consequently, these photosynthetic microorganisms are able to fix molecular nitrogen in heterocytes during the light

period of a day, while other vegetative cells perform photosynthesis. In dark, the heterocytous cyanobacteria decrease their nitrogen fixation as a response of inability of metabolism to sufficiently generate reductant under these conditions (ERNST & BÖHME 1984). Pattern of heterocytes is variable among distinct genotypes and it is also an important taxonomic marker distinguishing basic groups (KOMÁREK & ANAGNOSTIDIS 1989). Nitrogen fixation of heterocytous cyanobacteria has been widely studied in the last several decades from molecular, biochemical, physiological, and ecological points of view. Differentiation of heterocytes and rate of nitrogen fixation are known to be dependent on many factors including the above mentioned nitrogen compounds, calcium and boron concentrations (OHMORI & HATTORI 1972; ROWELL *et al.* 1977; ZAKI 1999), light regime (MULLINEAUX *et al.* 1981), temperature (KASHYAP *et al.* 1991) and concentration of phytohormones and ecdysteroids (MARŠÁLEK *et al.* 1992; ŠIMEK & MARŠÁLEK 1992). In some cyanobacteria also strong effect of colony morphology to nitrogen fixation was proved (HROUZEK *et al.* 2003).

1.3.1 Nitrogenase was found to reduce also other substrates containing triple bond except nitrogen (e.g. ethylene). Ethylene is in this reaction reduced to acetylene, which can be assayed by gas chromatography. This ability is used to nitrogenase activity measurement (HARDY *et al.* 1973). The assay is called ethylene/acetylene reduction activity (ARA) and is much faster and easier than direct measurement with isotopes of nitrogen. But it has also several limitations connected with recounting of molar amount of reduced ethylene to amount of nitrogen. The value of such converting factor was studied by many authors and was suggested to varied from 2-4.5 mol N₂/C₂H₄ (GIBSON 1980; BODDEY 1987).

Heterocytous cyanobacteria are very important microorganisms of terrestrial ecosystems. Their role as primary producers and their ability to improve soil structure are generally accepted (GOLLERBACH & SHTINA 1969). Moreover, being diazotrophs they play an important role in primary and secondary successions on various substrates (LUKEŠOVÁ 1993) and they improve the fertility of various soils. Especially in tropics and subtropics they are known to be useful in agriculture for fertilizing paddy rice fields (ROGER & WATANABE 1986).

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Kollerbach
& Shtina

1.3. Cyanotoxins

According to ecological conditions the cyanobacteria are known to produce wide range of compounds like **enzymes** (RENSTROM & BERGMAN 1989; WHITON *et al.* 1990), **odors** (SUGIURA & YAGI 1986), **organic acids** (FOGG 1978), **antibiotics** (CARMELI *et al.* 1993) and also **toxic compounds** (e.g. CARMICHAEL 1990). Although the cases of poisoning were known in history, first incident of animal poisoning attributed to toxic cyanobacteria was published in Australian lakes in 1878 (FRANCIS 1878). Progress in analytical methods allowed studying the structures and mechanisms of affecting of cyanotoxins in last decades. Thus the reports became more frequent (CARMICHAEL *et al.* 1985). Toxins of cyanobacteria are grouped into two main categories (biotoxins and cytotoxins) based on mechanism of effect and also on type of bioassay used to screen their activity (CARMICHAEL 1992).

1.3.1. Cyanobacterial biotoxins

Biotoxins are known to have an ability to kill animals and thus they are assayed with mice or aquatic invertebrates. Because of their occurrence in surface waters all around the world, this group was widely studied during last two decades. It is divided into two subgroups – neurotoxins and hepatotoxins.

Neurotoxins are group of five alkaloids or alkaloid related compounds. In general all cyanobacterial neurotoxins are termolabile compounds which are degraded by temperatures higher than 40°C (MARŠÁLEK *et al.* 1996). **Anatoxin-a** (*antx-a*) (fig. 1/a), a secondary amine (DELVIN *et al.* 1899; HUBER 1972), was the first described toxin from freshwater planktic strain of *Anabaena flos-aquae* from Canada (CARMICHAEL *et al.* 1975). This toxin is a potential post-synaptic cholinergic nicotinic agonist, which causes a depolarizing neuromuscular blockage (ARONSTMAN & WITKOP 1981; CARMICHAEL *et al.* 1975, 1979; SPIVAK *et al.* 1980, 1983). This leads mostly to suffocation of the animals. Also other known neurotoxins interfered with acetylcholinesterase or sodium channels. **Anatoxin-a(s)** (fig. 1/d) was shown to be potential inhibitor of cholinesterase (MAHMOOD & CARMICHAEL 1986b, 1987). It is a unique phosphate ester produced by strain of *Anabaena flos-aquae* (MATSUNAGA *et al.* 1989). The last, mostly diversified group of neurotoxins is **saxitoxins** and

neosaxitoxins (PSP – paralytic shellfish poisoning) (fig. 1/c). They are produced by various strains of genus *Anabaena* and *Aphanizomenon* (GENTILE & MALONEY 1969; Humpage *et al.* 1994; MAHMOOD & CARMICHAEL 1986). PSPs block the sodium channel of neuron membranes, causing death in mammals by respiratory arrest (SASNER *et al.* 1984).

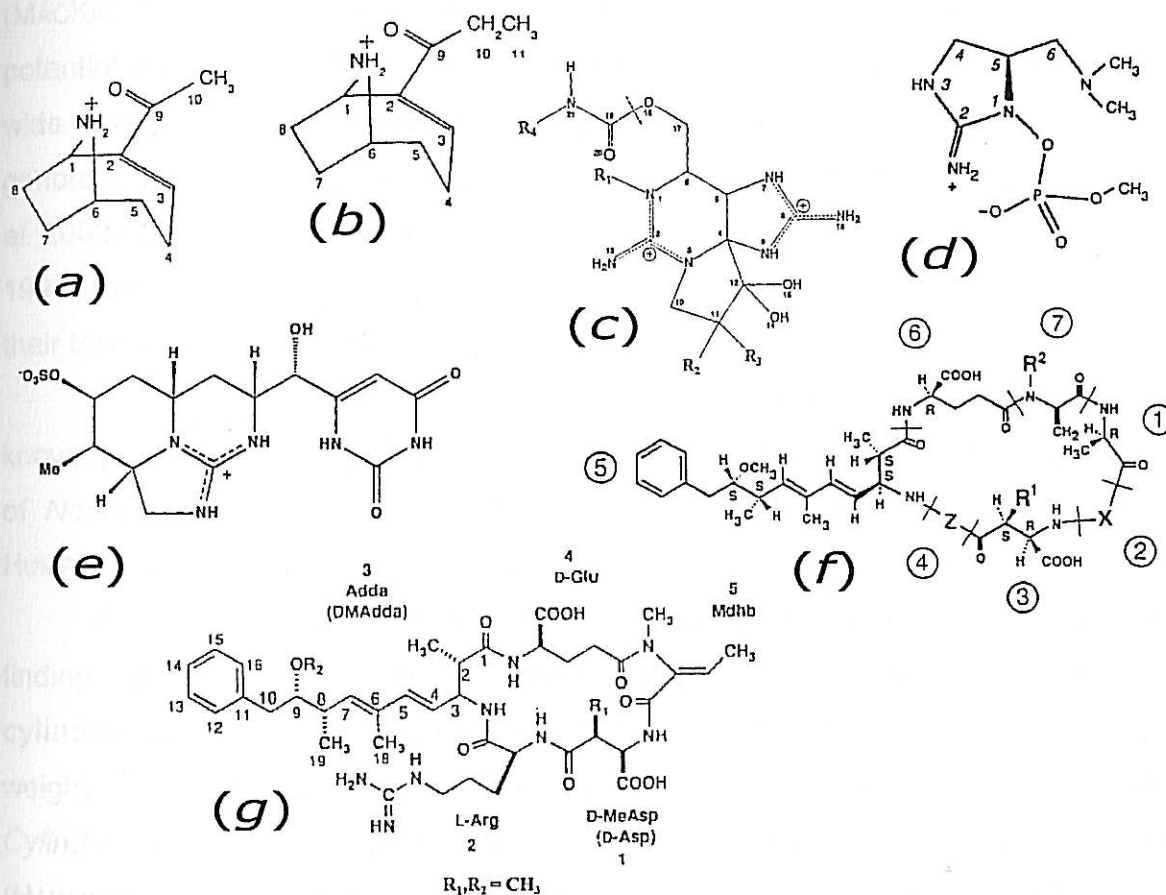


Fig. 1: Main cyanobacterial biotoxins:

(a) – Anatoxin-a, (b) – Homoanatoxin-a, (c) – generalized structure of PSPs, (d) – Anatoxin-a(S), (e) – Cylindrospermopsin, (f) – generalized structure of microcystins (g) – generalized structure of nodularin (from SIVONEN 1996).

Hepatotoxins, the second group of biotoxin, comprise two types of cyclic peptides – microcystins and nodularins and a toxin with alkaloid structure named cylindrospermopsin. Molecules of first mentioned types are stable unique peptides which are synthesized by non-ribosomal biosynthetic apparatus (an integrated peptide-polyketide synthetase system) (KLEINKAUF & DÖHREN 1994; TILLET *et al.* 2000). During the process of synthesis many intermediates and related compounds

with various biological activity like protease inhibition (CANNEL *et al.* 1988) occur (e.g. cyanopeptides, cyanopeptilides, anabaenopeptides, anabaenopeptins, micropeptins and microginins) (FUJII *et al.* 2002).

Microcystins (fig. 1/f) are heptapeptides with molecular weight between 920-1210 produced by many cyanobacterial morphotypes (see Table 1). They were found to affect liver cells through inhibition of protein-phosphatase 1 and 2A (PP1, PP2A) (MACKINTOSCH *et al.* 1990; YOSHIZAWA *et al.* 1990) and they belong to group of potential carcinogens (NISHIWAKI-MATSUSHIMA *et al.* 1992; OHTA *et al.* 1994). But a wide spectrum of microcystins activity like cytotoxicity (ERIKSON *et al.* 1989), genotoxicity (MANKIEWICZ *et al.* 2002) and phytotoxicity were published (WIEGAND *et al.* 2002). Recently app. 60 microcystins types of various toxicity are known (SIVONEN 1996, CHORUS 2003). For complete list of known microcystins, related compounds, their toxic effect and molecular weight see Table 2.

The second group of hepatotoxins – **nodularins** – comprises only three known pentapeptides (fig. 1/g). These were firstly isolated from cyanobacterial strain of *Nodularia spumigena* originating from brackish waters of Australia (BAKER & HUMPAGE 1994; JONES *et al.* 1994).

An outbreak of hepatoenterities at Palm Island in northern Queensland led to finding of the third cyanobacterial toxin with hepatotoxic effects – **cylindrospermopsin** (HAWKINS *et al.* 1985). It is an alkaloid cytotoxin with molecular weight 415 (OHTANI *et al.* 1992) produced by filamentous cyanobacteria *Cylindrospermopsis raciborskii* (fig. 1/e). It affects liver, kidney, thymus and heart (HAWKINS *et al.* 1985) by blocking glutathione synthesis (RUNNEGAR *et al.* 1994, 1995).

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Compound	MW (m/z)	Organism	Ref.	Compound	MW (m/z)	Organism	Ref.
Nostocine A	(152)	<i>No.spongiaeforme</i>	[56]	Cylindrocyclophane B	(626)	<i>Cyl. licheniforme</i>	[95]
Anatoxin-a	165(328)	<i>A. flos-aquae</i>	[60]	Spumigin B1	(627.3)	<i>Nod. spumigena</i>	[45]
Homoanatoxin-a	179(342.1)	<i>O. formosa</i>	[60]	Spumigin B2	(627.3)	<i>Nod. spumigena</i>	[45]
Anatoxin-a(s)	252	<i>A. flos-aquae</i>	[15]	Cryptophycin C	638	<i>N. sp.</i>	[141]
Tubercidin	266.15	<i>Has. byssoidea</i>	[5]	Homo-hydroxydolabelin	641	<i>L. sp.</i>	[82]
Calothrixin B	(282)	<i>Ca. sp.</i>	[120]	Mirabimide D	(649)	<i>S. mirabile</i>	[12]
Tijpanazole I	(291)	<i>T. tjipanasensis</i>	[9]	Cryptophycin A	654	<i>N. sp.</i>	[141]
Calothrixin A	(298)	<i>Ca. sp.</i>	[120]	Aeruginosin 98-B	655	<i>M. aeruginosa</i>	[98]
12-epi-Hapalindole-C-isonitrile	(304)	<i>Hap. langii</i>	[74]	Cylindrocyclophane D	(568)	<i>Cyl. licheniforme</i>	[95]
12-epi-Hapalindol H	(304)	<i>Hap. langii</i>	[74]	Cryptophycin E	686	<i>N. sp.</i>	[141]
12-epi-Hapalindol O isonitrile	(304)	<i>Hap. langii</i>	[74]	Aeruginosin 98-A	689,5	<i>M. aeruginosa</i>	[98]
Hapalindoline B	318	<i>Hap. langii</i>	[74]	Lyngbyabellin A	691	<i>L. majuscula</i>	[83]
Indolo[2,3a]Carbamazol	325	<i>N. sphaericum</i>	[77]	Microginin T2	(698)	plankton sample	[78]
6-cyano-5methoxy-12methylindolo[2,3a]Carbamazol		<i>N. sphaericum</i>	[77]	Microginin 91-C	(704)	<i>M. aeruginosa</i>	[66]
12-epi-Hapalindole-E-isonitrile	(338/340)	<i>Hap. langii</i>	[74]	Aeruginosin 89-A	(716)	<i>M. aeruginosa</i>	[65]
12-epi-Hapalindol G	(338)	<i>Hap. langii</i>	[74]	Aeruginosin 89-B	(716)	<i>M. aeruginosa</i>	[65]
Fisherindole-L	(338)	<i>F.muscicola</i>	[113]	Aeruginosin 101	(723)	<i>M. aeruginosa</i>	[65]
Hapalindoline A	352	<i>Hap. langii</i>	[74]	Lyngbyapeptin B	722	<i>L.sp.</i>	[82]
Nostoclode II	(354.1)	<i>No. sp.</i>	[146]	Microginin FR1	(728)	<i>M. sp.</i>	[103]
Nostoclode I	(388.1)	<i>No. sp.</i>	[146]	Microginin T1	(732.3)	plankton sample	[78]
Tijpanazole J	(396)	<i>T. tjipanasensis</i>	[9]	Aeruginosin 98-C	(733)	<i>M. aeruginosa</i>	[65]
Tijpanazole G	(402)	<i>T. tjipanasensis</i>	[9]	Aeruginosin 102-A	(733)	<i>M. aeruginosa</i>	[65]
Cylindrospermopsin	415	<i>C. raciborskii</i>	[107]	Aeruginosin 102-B	(733)	<i>M. aeruginosa</i>	[65]
Mirabazole A	422	<i>S. mirabile</i>	[12]	Lyngbyapeptin C	736	<i>L. sp.</i>	[82]
Tijpanazole B	(423)	<i>T. tjipanasensis</i>	[9]	Microginin 91-D	(738)	<i>M. aeruginosa</i>	[66]
Mirabazole C	424	<i>S. mirabile</i>	[12]	Nostogin BN741	(742)	<i>N. sp.</i>	[115]
Noscomine	(425)	<i>No.commune</i>	[67]	Microginin SD755	(756.45)	<i>M. aeruginosa</i>	[112]
Tijpanazole D	(425)	<i>T. tjipanasensis</i>	[9]	Microginin 478	(768)	<i>M. aeruginosa</i>	[66]
Palu'imide	429	<i>L. sp.</i>	[82]	Microginin 99-A	772	<i>M. aeruginosa</i>	[64]
Tijpanazole C1-C4	(436)	<i>T. tjipanasensis</i>	[9]	Microginin 99-B	806	<i>M. aeruginosa</i>	[64]
Mirabazole B	438	<i>S. mirabile</i>	[12]	Anabaenopeptin C	(809.5)	<i>An. sp.</i>	[76]
Tijpanazole B	(456)	<i>T. tjipanasensis</i>	[9]	dm-Nodularin-R	811.5	<i>Nod. spumigena</i>	[71]
Aeruginosin 298-B	(462)	<i>M. aeruginosa</i>	[65]	Tolytoxin	815.3	<i>S. mirabile, T. spp.</i>	[14]
Tijpanazole A1-A2	(470)	<i>T. tjipanasensis</i>	[9]	Scytophycin E	818,3	<i>Cyl. muscicola</i>	[70]
Tijpanazole E	(486)	<i>T. tjipanasensis</i>	[9]	Nodularin-R	825.5	<i>Nod. spumigena</i>	[71]
Tanazole A	488,3	<i>S. mirabile</i>	[13]	Anabaenopeptin D	827(828)	<i>An. sp.</i>	[76]
Mirabimide E	492	<i>S. mirabile</i>	[110]	Nodularin	820	<i>Nod. spumigena</i>	[119]
Tanazole B	502	<i>S. mirabile</i>	[13]	Scytophycin B	831	<i>Cyl. muscicola</i>	[70]
Banyascyclamide B	522	<i>N. sp.</i>	[115]	Anabaenopeptin B	(837)	<i>P. agardhii</i>	[99]
Banyascyclamide A	538	<i>N. sp.</i>	[115]	[L-Har ²] Nodularin	839	<i>Nod. sp.</i>	[6]
Cylindrocyclophane A	(549)	<i>Cyl. licheniforme</i>	[95]	Anabaenopeptin A	844(844)	<i>An. sp.</i>	[76]
Cylindrocyclophane E	(552)	<i>Cyl. licheniforme</i>	[95]	6-hydroxy Scytophycin B	847	<i>Cyl. muscicola</i>	[70]
Banyascyclamide C	556	<i>N. sp.</i>	[115]	6-hyd.-7-O-methylScytophycin E	848,3	<i>Cyl. muscicola</i>	[70]
Cylindrocyclophane C	(569)	<i>Cyll. licheniforme</i>	[95]	Anabaenopeptin F	(851)	<i>P. rubescens</i>	[51]
Cryptophycin F	573	<i>N. sp.</i>	[141]	Microginin 299-C	(851)	<i>M. aeruginosa</i>	[64]
Microginin 51-B	(975)	<i>M. aeruginosa</i>	[64]	Microginin 299-D	(856)	<i>M. aeruginosa</i>	[64]
Nostogin BN578	(579)	<i>N. sp.</i>	[115]	Oscillamide	(858)	<i>P. agardhii</i>	[45]
Cryptophycin D	604	<i>N. sp.</i>	[141]	Anabaenopeptin T	(866.5)	plankton sample	[78]
Mirabimide B	(607)	<i>S. mirabile</i>	[12]	Anabaenopeptin T1	866	plankton sample	[78]
Mirabimide C	(607)	<i>S. mirabile</i>	[12]	Planktopeptin BL843	866	<i>P. rubescens</i>	[51]
Lyngbyabellin C	609	<i>L. sp.</i>	[82]	Microginin 299-A	(885)	<i>M. aeruginosa</i>	[64]
Microginin 91-B	(609)	<i>M. aeruginosa</i>	[62]	Nostophycin	888	<i>N. sp.</i>	[76]
Microcin SF608	608	<i>M. sp.</i>	[3]	MCYST-LA	909	<i>M. aeruginosa</i>	[130]
Cylindrocyclophane E	(610)	<i>Cyl. licheniforme</i>	[95]	Micropeptin SF909	(910)	<i>Hap. hibernicus</i>	[111]
Spumigin A	(613.3)	<i>Nod. spumigena</i>	[45]	Microginin 51-A	(917)	<i>M. sp.</i>	[3]
Spumigin C	(613.3)	<i>Nod. spumigena</i>	[45]	Microginin 299-B	(917)	<i>M. aeruginosa</i>	[61]
Cryptophycin B	620	<i>N. sp.</i>	[141]	Nodulapeptin-B	(914.5)	<i>Nod. spumigena</i>	[43]
Mirabimide A	(621)	<i>S. mirabile</i>	[12]	Microginin 299-B	(919)	<i>M. aeruginosa</i>	[64]
				MCYST-Laa	923	<i>M. aeruginosa</i>	[130]
				Anabaenopeptin H	(923)	<i>P.agardhii</i>	[130]
				Nostopeptin BN920	921	<i>N. sp.</i>	[110]
				Cyanopeptolin S	925	<i>M. sp.</i>	[63]

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Compound	MW (m/z)	Organism	Ref.	Compound	MW (m/z)	Organism	Ref.
Anabaenopeptin G	(930)	<i>P. agardhii</i>	[130]	MCYST-RR	1037	<i>M. aeruginosa</i>	[130]
Nodulapeptin-A	(930.5)	<i>Nod. spumigena</i>	[43]	[(6Z)-Adda ⁵]MCYST-RR	1037	<i>M. viridis</i>	[130]
Nostopeptin BN920	(943.5)	<i>N. sp.</i>	[115]	[D-Ser ³ , ADMAdda ⁵]MCYST-LR	1038	<i>N. sp.</i>	[130]
Micropeptin SD944	(945)	<i>M. aeruginosa</i>	[112]	[ADMAdda ⁵ , MeSer ⁷]MCYST-LR	1040	<i>N. sp.</i>	[130]
Anabaenopeptilide 90-A	953(935)	<i>An. sp.</i>	[76]	[L-Ser ⁷]MCYST-RR	1041	<i>M. aeruginosa</i>	[130]
MCYST-LL	951	<i>M. aeruginosa</i>	[130]	[D-Asp ³ , MeSer ⁷]MCYST-RR	1041	<i>P. agardhii</i>	[130]
MCYST-AR	952	<i>M. spp.</i>	[130]	MCYST-YR	1044	<i>M. aeruginosa</i>	[130]
Anabaenopeptilide 90-A	955,4	<i>An. sp.</i>	[46]	[D-Asp ³]MCYST-HtyR	1044	<i>An. flos-aquae</i>	[130]
MCYST-YA	959	<i>M. aeruginosa</i>	[130]	[Dha ⁷]MCYST-HtyR	1044	<i>An. sp.</i>	[130]
[D-Asp ³ , Dha ⁷]MCYST-LR	966	<i>M. aeruginosa</i>	[130]	Oscillapeptilide 97-A	(1046)	<i>P. agardhii</i>	[45]
Anabaenopeptilide 90-B	972(955)	<i>An. sp.</i>	[76]	MCYST-(H ₄)YR	1048	<i>M. spp.</i>	[130]
[D-Asp ³]MCYST-LR	980	<i>An. flos-aquae</i> , <i>M. aeruginosa</i>	[130]	Anabaenopeptilide 202-A	1049(1032)	<i>An. sp.</i>	[76]
[Dha ⁷]MCYST-LR	980	<i>M. viridis</i> , <i>P. agardhii</i> , <i>M. aeruginosa</i> , <i>An. sp.</i>	[130]	Micropeptin T1	1051	plankton sample	[78]
[MAdda ⁵]MCYST-LR	980	<i>M. spp.</i> , <i>N. sp.</i>	[130]	[D-Glu-OC ₂ H ₅ (CH ₂) ₂ H ⁺]MCYST-LR	1052	<i>M. spp.</i>	[130]
Scyptolin A	(981)	<i>S. hofmanii</i>	[87]	Anabaenopeptilide 202-B	1052.5	<i>An. sp.</i>	[46]
MCYST-LF	985	<i>M. aeruginosa</i>	[130]	MCYST-HtyR	1058	<i>A. flos-aquae</i>	[130]
MCYST-LR	994	<i>M. aeruginosa</i> , <i>An. flos-aquae</i>	[130]	Nostopeptolide A1	1059	<i>N. sp.</i>	[50]
[D-Asp ³ , D-Glu(OCH ₂) ₂]MCYST-LR	994	<i>An. flos-aquae</i>	[130]	[L-Ser ⁷]MCYST-HtyR	1062	<i>An. sp.</i>	[130]
[(6Z)-Adda ⁵]MCYST-LR	994	<i>M. viridis</i>	[130]	MCYST-WR	1067	<i>M. spp.</i>	[130]
Micropeptin SF995	(996)	<i>M. sp.</i>	[3]	Nostopeptolide A2	1067	<i>N. sp.</i>	[50]
Oscillacyclin	995	<i>P. agardhii</i>	[45]	Anabaenopeptilide 202-B	1069(1052)	<i>An. sp.</i>	[71]
[L-Ser ⁷]MCYST-LR	998	<i>An. sp.</i>	[130]	Hofmanolin	1073	<i>S. hofmanii</i>	[93]
Micropeptin SD999	(1005)	<i>M. aeruginosa</i>	[117]	Nostofungicine	1081	<i>N. commune</i>	[72]
MCYST-LY	1001	<i>M. aeruginosa</i>	[130]	Nostopeptolide A1	1081	<i>N. sp.</i>	[50]
Micropeptin SD979	(1002)	<i>M. aeruginosa</i>	[117]	Planktopeptin BL1061	1084(1184)	<i>P. rubescens</i>	[51]
MCYST-HiLR	1008	<i>M. spp.</i>	[130]	Microcystilide A	(1095.5)Na	<i>M. aeruginosa</i>	[142]
[D-Asp ³ , ADMAdda ⁵]MCYST-LR	1008	<i>N. sp.</i>	[130]	Oscillapeptin G	(1112)	<i>P. agardhii</i>	[45]
[D-Glu(OCH ₂) ₂]MCYST-RR	1008	<i>An. flos-aquae</i>	[130]	[L-MeLan ⁷]MCYST-LR	1115	<i>M. spp.</i>	[130]
[D-Asp ³ , Dha ⁷]MCYST-RR	1009	<i>P. agardhii</i>	[130]	Scyptolin A - PI-pep	(1122.5)	<i>S. hofmanii</i>	[92]
[L-MeSer ⁷]MCYST-LR	1012	<i>M. spp.</i>	[130]	Aeruginopeptin 228-A	1144	<i>M. sp.</i>	[130]
[Dha ⁷]MCYST-FR	1014	<i>M. sp.</i>	[130]	Aeruginopeptin 95-A	1145(1128)	<i>M. sp.</i>	[130]
Micropeptin T2	1016	plankton sample	[78]	Planktopeptin BL1125	1148	<i>P. rubescens</i>	[51]
[ADMAdda ⁵]MCYST-LR	1022	<i>N. sp.</i>	[130]	Aeruginopeptin 95-B	1149	<i>M. sp.</i>	[130]
[D-Asp ³ , ADMAdda ⁵]MCYST-LHAr	1022	<i>N. sp.</i>	[130]	Aeruginopeptin 228-B	1148	<i>M. sp.</i>	[130]
[D-Asp ³]MCYST-RR	1023	<i>P. agardhii</i>	[130]	Aeruginopeptin 95-B	1149	<i>M. sp.</i>	[130]
[Dha ⁷]MCYST-RR	1023	<i>P. agardhii</i>	[130]	Puwainaphycin-E	(1194)	<i>An. sp.</i>	[52]
Micropeptin SD944	(1025)	<i>M. aeruginosa</i>	[117]	Puwainaphycin-B	(1233)	<i>An. sp.</i>	[52]
MCYST-FR	1028	<i>M. spp.</i>	[130]	Puwainaphycin-A	(1235)	<i>An. sp.</i>	[52]
MCYST-M(O)R	1028	<i>M. spp.</i>	[130]	Kawagushipeptin A	(1421)	<i>M. aeruginosa</i>	[62]
[Dha ⁷]MCYST-HpHR	1028	<i>An. sp.</i>	[130]	Schizotrin A	(1490)	<i>Sch. sp.</i>	[114]
[D-Asp ³ , Dha ⁷]MCYST-HtyR	1030	<i>An. sp.</i>	[130]	Microviridin E	(1665)	<i>P. agardhii</i>	[129]
[Dha ⁷]MCYST-YR	1030	<i>M. aeruginosa</i>	[130]	Microviridin	(1729)	<i>P. agardhii</i>	[100]
[Asp ³]MCYST-YR	1030	<i>M. spp.</i>	[130]	Microviridin I	(1765)	<i>P. agardhii</i>	[45]
Anabaenopeptilide 202-A	(1032.5)	<i>An. sp.</i>	[76]	Microviridin F	(1883)	<i>P. agardhii</i>	[129]
Oscillapeptilide 97-B	(1032)	<i>P. agardhii</i>	[45]	Microviridin D	(1802)	<i>P. agardhii</i>	[129]
MCYST-YM(O)	1035	<i>M. aeruginosa</i>	[130]	Microviridin G	(1806)	<i>N. minutum</i>	[100]
[ADMAdda ⁵]MCYST-LHAr	1036	<i>N. sp.</i>	[130]	Microviridin H	(1838)	<i>N. minutum</i>	[100]

Tab. 1: List of known toxic compounds produced by cyanobacteria. Underlined compound names mark lipophilic character of the compound.

Abbreviations: *An.* - Anabaena, *N.* - Nostoc, *Ca.* - Calothrix, *Nod.* - Nodularia, *Cyl.* - *Cylindrospermum*, *P.* - *Planktothrix*, *C.* - *Cylindrospermopsis*, *S.* - *Scytonema*, *L.* - *Lyngbya*, *Sch.* - *Schizothrix*, *F.* - *Fischerella*, *T.* - *Tolypothrix*, *Hap.* - *Hapalosiphon*, *M.* - *Microcystis*

1.3.2. Cyanobacterial cytotoxins

Except above mentioned cyanobacterial toxins a large group of compounds which does not cause death of vertebrates exists. They are called **cytotoxins**. This group is very heterogenous from chemical and toxicological point of view. Are including from lipophylic alkaloids e.g. **lyngbyabellins** and **hapalindoles** (KLEIN *et al.* 1992; LUESCH *et al.* 2002) to hydrophilic peptides or macrolitic lactones like scytophycins (PATTERSON *et al.* 1992). Many studies were focused on screening for important biotechnological compounds in cyanobacterial extracts. The main interest was given in searching for anti-tumor, antibiotics or antiviral compounds. Several compounds were found to have strong cytostatic activity or ability of selective inhibition of tumors. The most promising was the class of cytotoxic depsipeptides (peptides with ester linkage). **Cryptophycins A-F** isolated from *Nostoc* strains, which are sorted into this group, are lipophilic peptides including monochlorated L-O-methytyrosine (TRIMURTULU *et al.* 1994). However many other types of linear or cyclic depsipeptides were isolated from distinct cyanobacterial morphotypes with various toxic effect. Except above mentioned cryptophycins many studies have reported production of hydrophilic peptides (**cyanopeptides, cyanopeptolins, micropeptins, microginins and aeruginosins**) mainly in *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* strains (BANKER & CARMELI 1999; GOLAKOTI 2000; HARADA *et al.* 1993; ISHIDA *et al.* 1997, 1997, 1999, 2000; RESHEF & CARMELI 2001). For examples of cyanobacterial cyclic peptides see fig. 2.

Such peptides have been reported to have an ability of protease inhibition. Similar compounds were recently reported in branched cyanobacterial strains. Cyclic depsipeptides, **scyptolins A and B**, isolated from *Scytonema hofmannii* were found to have a selective protease inhibition activity (MATERN *et al.* 2001). Most recently **hofmanolin** a cyanopeptolin from the strain of *Scytonema hofmannii* have negligible bioactivity (MATERN *et al.* 2003).

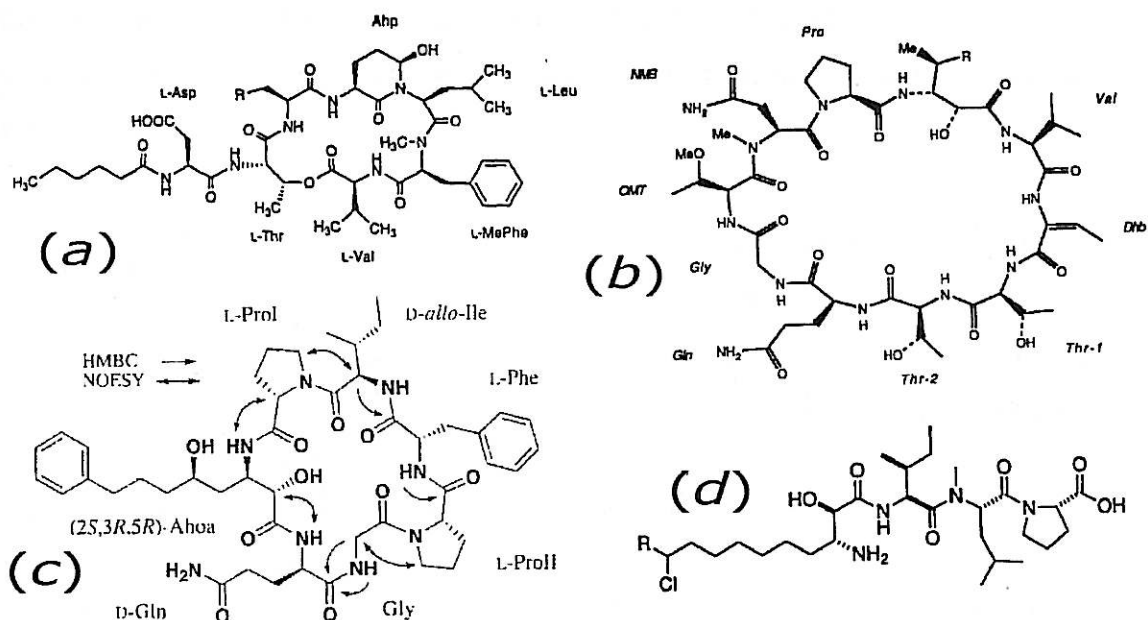


Fig. 2: Examples of cyanobacterial peptides: **(a)** – generalized structure of cyanopeptolin (from NAMIKOSHI & RINEHART 1996), **(b)** – Puwainaphycin (from GREGSON *et al.* 1992), **(c)** – Nostophycin (from FUJII *et al.* 1999, **(d)** – Microginin 91-A (from ISHIDA *et al.* 2000).

Another large group of compounds were found to be associated with *Scytonema* and *Tolypothrix* strains. Four classes of cytotoxins were isolated – **Scytophycins** (MOORE *et al.* 1982), **tanazoles** (CARMELI *et al.* 1993) (fig. 3/f), **mirabimides** (CARMELI *et al.* 1991). Scytophycins, is group of 22 macrolidic compounds, which exhibit significant cytotoxic effect through microfilament depolarizing (Patterson & CARMELI 1992). The best known is **tolytoxin** (fig. 3/d), macrolidic compound with molecular weight 815 firstly isolated from strain of *Tolypothrix conglutinata* (CARMELI *et al.* 1993). Although scytophycins were mostly isolated from branched cyanobacteria, there is a evidence that can be also found in strains belonging to *Nostocaceae* – e.g. *Cylindrospermum muscicola* (JUNG *et al.* 1991). Tanazoles are cytotoxic alkaloids with several heterocycles isolated from *Scytonema mirabile* (CARMELI *et al.* 1991, 1993). Except above cited compounds with relatively high molecular weight also low-molecular cytotoxic compound like **tubercidin** were isolated from *Tolypothrix* strains (BARCHI *et al.* 1983).

Many other compounds with antiviral or fungicide activites like **Indolo[2,3a]Carbamazol** were isolated from *Nostoc* and *Tolypothrix* (KNÜBEL *et al.* 1990; BONJOUKLIAN R. *et al.* 1991). **Calothrixins A and B** (fig. 3/a,b) originating from

scytonema
?
yield to
fungicide

strain of *Calothrix* sp. have efficiently inhibited growth of human HeLa cancer cells, but also functioned as in vitro inhibitors to resistant strain of malaria parasite (RODEY *et al.* 1999).

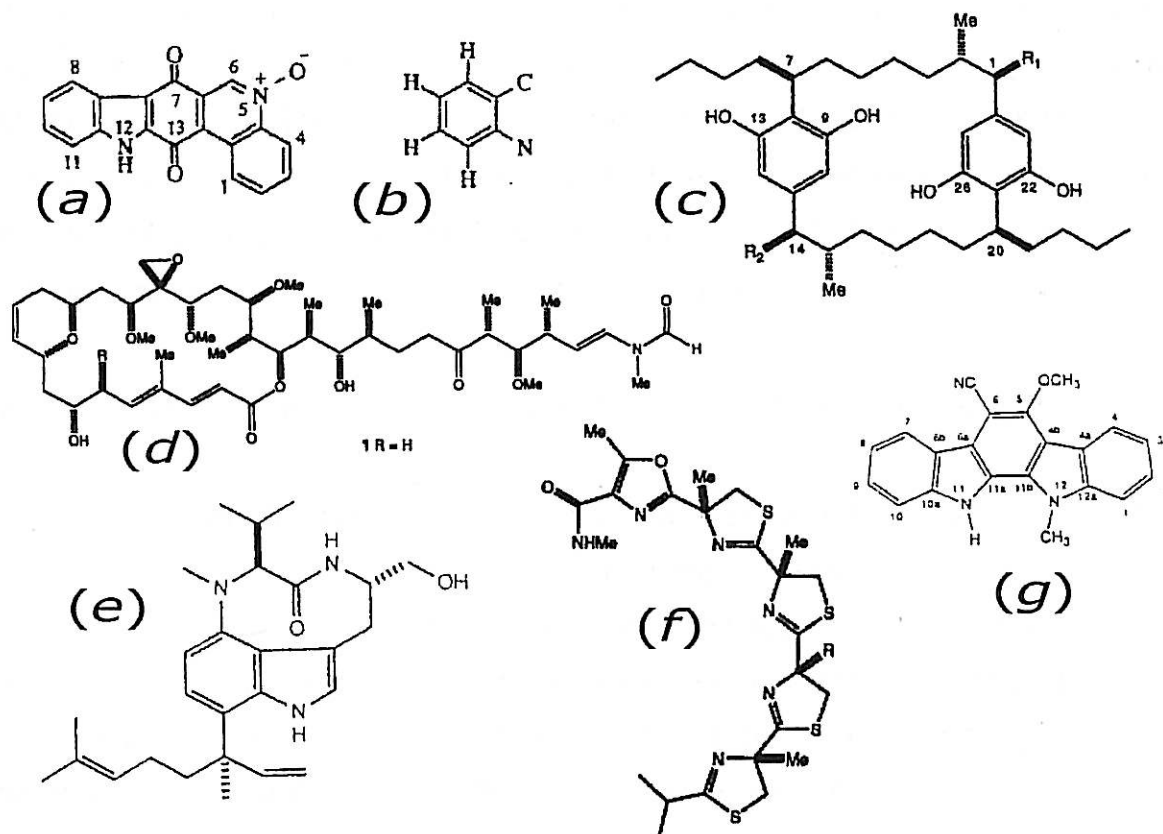


Fig. 3: Examples of other known cyanobacterial secondary metabolites: **(a)** - Calothrixin A, **(b)** - Calothrixin B (RICKARDS *et al.* 1999), **(c)** - generalized structure of cylindrocyclophane (MOORE *et al.* 1992), **(d)** - Scytophycin B (JUNG *et al.* 1991), **(e)** - Lyngbyatoxin (from SIVONEN 1996), **(f)** - Tanazole A (CARMELI *et al.* 1993), **(g)** - Indolocarbamazol (from KNÜBEL *et al.* 1990).

1.4. Methods used in cytotoxicity evaluation

From the fact, that cytotoxins are very variable group of compound, is it clear that a wide range of analytical and toxicological methods is used to screen their activity. The most promising testing system is in vitro cultures of mammal cell lines (eg. LoVo, Hela etc...). Several methods for characterisation of extract influence to the cells have been discovered. One of the most rapid cytotoxicity test is the MTT test (MOSMAN 1983), it is based on detection of dehydrogenase activity. Living cells are able to reduce MTT - methyltetrazolium salt to blue coloured formazan. Absorbance of the cell suspension is measured at 550 and 660 nm. Viability is

expressed as absorbance of test sample to absorbance of control sample. This test can be used as fast and reproducible for large screenings of biological active compounds, however, it has several limitations (ROLLINO *et al.* 1995). Recently, the flow-cytometrical analysis is used in many fields of biological research as the most precise method (ORMEROD 1994). It is based on different affinities of living or necrotic cells to analytic compound (e.g. propidium iodide). Then the population of measured cells can be divided by flow cytometrical analysis very precisely. Also tests using different testing systems like small water invertebrates – *Daphnia* sp. (ARNOLD 1978) or *Artemia salina* (LINCOLN *et al.* 1996) were more or less fruitful.

1.5. Effect of cyanobacterial compounds and extract to mammal cell

Although cytotoxic effect of some cyanobacterial toxins is well documented, mechanisms of this effect are still not properly described. So far, the exact mechanisms of the best known cyanobacterial toxin Microcystin-LR have not been fully explained. It is well known that microcystins and nodularins are able of protein-phosphatase inhibition in mammal cell lines, which lead to increased protein phosphorylation (YOSHIZAWA *et al.* 1990) ended by necrotic cell death.

However, some research groups have reported in recent years that microcystins are capable of initiating apoptosis in hepatocytes (BOTHÁ *et al.* 2004; DING & ONG 2003) characteristic by apoptotic morphological changes including membrane blebbing, cell shrinkage, externalization of membrane phosphatidilserine and chromatin condensation (MCDERMOTT *et al.* 1998; DING *et al.* 2000; FLANDMARK *et al.* 1999). But exact trigger of this process is still not clear. The induction of free radical formation and mitochondrial alternations are two major events found in microcystin-treated cultured rat hepatocytes (DING & ONG 2003, DING *et al.* 1998, DING *et al.* 1998), but if this process stands in the middle of apoptosis triggering is still unclear.

At least two other effects of cyanobacterial secondary metabolites were documented in mammal cells. Most of cyanobacterial depsipeptides are capable of protease inhibition – this can cause various damage to the cell. Also strong interference of some compounds with cytoskeleton structures (tolytoxin, microcystin) was found (ERIKSON *et al.* 1989; PATTERSON & CARMELI 1992).

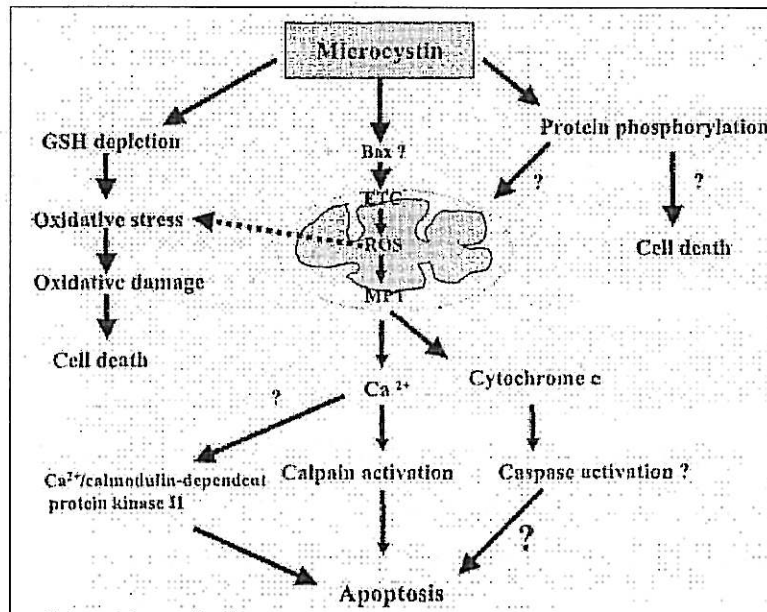


Fig. 4: A proposed model of the cellular events in microcystin-induced cell death in primary rat hepatocytes. Microcystin can cause cell death through at least three pathways. Firstly, microcystin may alter the antioxidant balance through the early GSH depletion, followed by intracellular oxidative stress and oxidative damage and cell death. Secondly, microcystin may disrupt the mitochondrial ETC, followed by ROS production. Thirdly, microcystin cause protein phosphorylation and leads to cell death in a less clear mechanism. (After DING & ONG 2003).

! suggested, or probably mechanism GSH, ETC, ROS

1.6. Aims of my study

- The aim of my experiments was to evaluate potential importance of cyanobacteria in soil communities and compare measured characteristics among distinct cyanobacterial phenotypes.
- Main attention was concentrated to two physiological properties – toxicity and nitrogen fixation. In this wide and complicated fields following question were selected to answer:
 - Cytotoxicity:
 1. Does ^{2 crude?} cruel cyanobacterial extracts cause inhibition of invertebrates and mammalian cell lines?
 2. Is microcystin or any other known compound responsible for this toxic effect?
 - Nitrogenase activity:
 1. Are there differences in light and dark nitrogenase activity of various soil cyanobacterial phenotypes?
 2. What factors are affecting specific nitrogenase activity of cyanobacterial colony?

2. Materials and methods

2.1. Origin of strains and cultivation

- Seventeen different strains of soil cyanobacterial strains (belonging to genera *Anabaena*, *Nodularia*, *Trichormus*, *Nostoc*, *Cylindrospermum*, *Tolypothrix* and *Scytonema*) were taken into this study. Strains have been kindly provided by Dr. Alena Lukešová (Institute Soil Biology – ASCR, České Budějovice) and Culture Collection of Algal Laboratory CCALA - Institute of Botany – ASCR, Třeboň. For list of studied strains see tab. 2. The strains were grown on combined nitrogen-free medium BG-11 (STANIER & COHEN-BAZIRE 1977) solidified with 3% (w/w) agar in Petri dishes for 21 days (nitrogenase activity assay). Mass cultivation for toxin extraction was carried out in liquid BBM (BISCHOFF & BOLD 1963) medium in 250 ml Erlenmayer's flasks percolated by air for 45 days. Cyanobacteria were cultivated under artificial light of the intensity of 35 $\mu\text{mol photons PhAR m}^{-2} \text{s}^{-1}$ at $20 \pm 2^\circ\text{C}$.

Label	Exact name	Isolated in/by	Locality/country	Habitat
1A†	<i>Anabaena torulosa</i>	1994/Lukešová	Novosedly/Czech Rep.	salty meadow
2A*	<i>Anabaena augstumalis</i>	1980/Jahnke	Rostock/Germany	soil
3A†	<i>Anabaena sphaerica</i>	1995/Lukešová	unknown	unknown — <i>neut pravda</i>
4N†	<i>Nodularia</i> sp.	1990/Lukešová	Nadym(Siberia)/Russia	salty sand
5T†	<i>Trichormus variabilis</i>	1988/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
6T†	<i>Trichormus variabilis</i>	1989/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
7T*	<i>Trichormus azolae</i>	1983/Bai	unknown	unknown
8Nc†	<i>Nostoc calcicola</i>	1994/Lukešová	Dobré pole/Czech Rep.	bank of a pond
9Nc†	<i>Nostoc ellipsosporum</i>	1995/Lukešová	Nezamyslice/Czech Rep.	agricultural field
10C†	<i>Cylindrospermum</i> sp.	1998/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
11C†	<i>Cylindrospermum</i> sp.	1995/Lukešová	Manitoba/Canada	forest soil
12T†	<i>Tolypothrix</i> sp.	1989/Lukešová	Č. Budějovice/Czech Rep.	fallow
13T†	<i>Tolypothrix</i> sp.	1995/Lukešová	Sokolov/Czech Rep.	coal-mining dump
14T†	<i>Tolypothrix</i> sp.	1995/Lukešová	Sokolov/Czech Rep.	coal-mining dump
15Ca*	<i>Calothrix parietina</i>	1977/Zehnder222a	Traunstein/Austria	stone wet wall
16Ca†	<i>Calothrix</i> sp.	1995/Lukešová	Sokolov/Czech Rep.	coal-mining dump

*- strain obtained from CCALA collection, Institute of Botany ASCR Třeboň

† - strains obtained from the collection of the Institute of Soil Biology ASCR, České Budějovice

Tab. 2: List of strains under study including data about habitat, place and year of isolation.

2.2. Nitrogenase activity measurement

- *Nitrogenase activity assay.* Nitrogenase activity was determined as acetylene-ethylene reduction activity (HARDY *et al.* 1973) after 21 days of cultivation. It was determined in cultures grown under continuous light regime and in cultures after 12 hours of dark cultivation. Before measurements the block of agar with cyanobacterial culture was taken out of the Petri dish and divided into two strips (approx. size 20 x 90 mm), which were given each in separate 150 ml serum bottle. There were four replicated bottles for each strain. Nitrogenase activity was measured in the bottles with 10 ml of acetylene added. The amount of ethylene in internal atmosphere was determined immediately and after one hour of incubation under laboratory conditions (22±2°C, daylight) using gas chromatography (Hewlett Packard 5890 GC equipped with FID detector) - see ŠIMEK *et al.* (1987) for details on ethylene determination and calculations.

$$n(C_2H_2) = \frac{0,0446 \cdot P(C_2H_2) \cdot V(b) \cdot c(ST)}{P(ST)}$$

$n(C_2H_2)$	- molar amount of C_2H_4 v the serum bottle [nmol]
$c(ST)$	- concentration of C_2H_4 standard [ppm]
$V(b)$	- volume of serum bottle [ml]
$P(ST)$	- integral value of standard peak
$P(C_2H_2)$	- integral value of sample peak

- *Frequency of heterocytes estimation.* Immediately after nitrogenase activity measurements, the bottles were opened and the frequency of heterocytes was estimated. In microscopic view (150 x 150 μm), the amount of heterocytes, vegetative cells and akinetes were counted. Heterocytes frequency was calculated as the ratio of the amount of heterocytes and the amount of all cells. This was done in ten independent samples of each cyanobacterial culture (=bottle).
- *Biomass estimation.* The cyanobacterial culture was scraped off the agar surface and immediately weighed fresh mass (fm). Nitrogenase activity was then expressed in μmol ethylene g⁻¹ (fm) h⁻¹.

2.3. Biomass harvesting and toxin extraction

- Biomass was harvested by centrifugation in 50 ml glass cuvettes (3000 rpm, 15 min.), stored at -40°C and lyophilized. 200 µg of lyophilized biomass was transferred into 10 ml glass test-tubes and 6 ml of pure methanol was added and extracted for 24 hrs. Test tubes were centrifuged (3000 rpm, 15 min), supernatant was transferred and evaporated by vacuous dryer. Solid extract was resuspended in 1 ml of pure methanol.

2.4. Cytotoxicity testing

- *Brine shrimp assay* was done according to LINCOLN 1996. *Artemia* cultures were maintained in seawater prepared by dilution of commercial sea salt AQUA MEDIC (Aqua Medic GmbH, D-49143 Bissendorf, Germany). 0.1 g of *Artemia* eggs were inoculated to 100 ml of sea water and cultivated at 28°C. Before testing 100 µl of extract was evaporated and 1 ml of media with living *Artemia* (containing app. 15-20 individuals) was added. Number of living (moving *Artemia*) and death were counted before extract addition and after 24 hrs. of exposure and percentage inhibition was counted. ✓
- *Mammal cell cultivation*. Two mammal cell lines were selected for cytotoxicity testing – YAC-1 and WEHI. While YAC-1 is a lymphoma cell line (lymfoblasts) which was induced by Moloney leukemia virus, WEHI are semiadherent fibroblasts derived from mouse fibrosarcoma. Cells were cultivated in RPMI 1640 medium (Sigma R-8005) (MARTIN 1994) with addition of 5% foetal calf serum (PAA A15-04), 1% glutamine (Sigma G-5763) and 1% of anitibiotic-antimycotic solution (Sigma A-7292) in plastic tissue culture flask at 37 °C and 3.5% CO₂ concentration. During the cultivation the cell suspensions were controlled with inversion microscope. Before the experiment the viability and abundance of the cells were estimated by coloring suspension with Tripan blue and counting in Bürkers chamber in light microscope. For testing the cell suspension were centrifuged (1000 rpm, 10 min., 4°C) and adequate amount of fresh RPMI checked ✓

Cytotoxicity and Nitrogenase Activity in Selected Soil Cyanobacteria

medium was added to obtain concentration of 1.10^6 cells/ml. Fresh suspension was transferred into tissue culture test plate (TPP panels) and equal amount of RPMI medium with addition of 2% of cyanobacterial extract was added.

- *MTT test.* TPP panels (96-wells) were used for MTT test. 100 μ l of cell suspension in concentration of 1.10^6 cells/ml were transferred into each well. Wells at the margin of panel were filled with 200 μ l of distilled water. Inner 60 wells were used for testing. Four wells of each column were used for exposure of cells to the extract and two cells were used as control (with addition of RPMI with 1% concentration of methanol). Effect of extract concentration (1; 0,1; 0,01; 0,001; 0,0001%) to the cell lines were tested. Panels were incubated at 37 °C and 3.5% CO₂ concentration for 12 hrs. Then 10 μ l of MTT solution (5mg/ml in Hank's solution – see MARTIN 1994) was added and incubated for 4 hrs. After incubation the panels were centrifuged (3000 rpm, 10 min.) and supernatant was removed. 100 μ l of DMSO was added and formazan crystals were dissolved using laboratory shaker Themolyne – Big Bill (300-400 rpm). Test and background absorbance were measured by Elisa reader at 550, 660 respectively. The survival of cell lines was expressed as ratio of test well absorbance to control well absorbance as shown bellow.

$$\text{Cell survival (\%)} = \frac{\text{mean (A}_{550} - \text{A}_{660}) \text{ of tested wells}}{\text{mean (A}_{550} - \text{A}_{660}) \text{ of control wells}}$$

- *Flow cytometry.* For flow cytometry measurement the cells were cultivated in 24-wells TPP panels. Into each well 500 μ l of suspension with 1.10^6 of cells were transferred and 500 μ l of RPMI with needed extract concentration was added. The suspension was transferred into 5 ml glass test tubes and twice washed by 5 ml of PBS and centrifuged (10 min. at 1000 rpm in 4 °C) after 1h. cultivation. Then the supernatant was removed and the pellets were resuspended in 100 μ l Anxin-V-flos incubation buffer (10 mM Hepes, 140 mM NaCl and 5 mM CaCl₂). 2 μ l of anxin-V-Flos (Roche Cat. No. 1 828 618) and 5 μ l of propidium iodide (Sigma P4170) at concentration of 500 μ g/ml was added and incubated for 20

min. After it 1 ml of flow-cytometry buffer (PBS with addition of 1% BOFES) was added. The suspension was measured at EPICS XL-MCL flow-cytometre and 10 000 count were taken into each measurement. In diagrams fluorescence of cells in propidium iodide was plotted against cell fluorescence in annexin-V-flos. Quadrant boundaries were set according fluorescence in three negative samples (control cells treated by propidium iodide, control cells treated by annexin-V-flos, control cells treated by both solutions).

- *Statistical analysis.* Nitrogenase activity data were analysed with Statistica for Windows (for mean, standard deviation and minimum-maximum values computing) and Canoco for Windows was used for RDA analysis with forward selection (Monte Carlo-Permutation test, 499 permutations) (TER BRAAK & ŠMILAUER 1998). CanoDraw software (ŠMILAUER 1992) was used for visualization of RDA model.

2.5. HPLC- MS measurement

- Extracts composition were analyzed with HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap. The extract was subjected to analysis on reversed phase column (Zorbax XBD C8, 46 x 150 mm, 5 μ m) at 35°C using gradient MeOH/H₂O + 0.1 HCOOH (30-100% Me for 30 min, 100% for 5 min) with flow rate 0.6 ml/min.

3. Results

3.1. Nitrogenase activity

Although the values of nitrogenase activity were very variable for each strain we have found significant differences in light nitrogenase activity (NA) among the studied strains as well as differences in response of nitrogenase activity to dark cultivation. It can be clearly seen from our data (fig. 5 and tab. 3), that all strains had significantly lower NA after 12 h cultivation in dark in comparison with NA under light conditions. The degree of reduction, however, differed among various morphotypes. High values of NA in light were detected in *Tolypothrix* strains (ca 6 – 16 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$). These strains also retained comparatively high NA after 12 h of dark cultivation (up to 7 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$). The *Tolypothrix* strains were isolated from fallow or earlier succesional stages of dumps after coal mining and exhibited specific morphology of filaments. Their filaments consisted of compact vegetative cells and basal heterocytes differentiated from end cells of a filament or from the some cells inside of the filament, where the false branching occurred (fig. 6/1). Their frequency of heterocytes was thus quite low (around 3%).

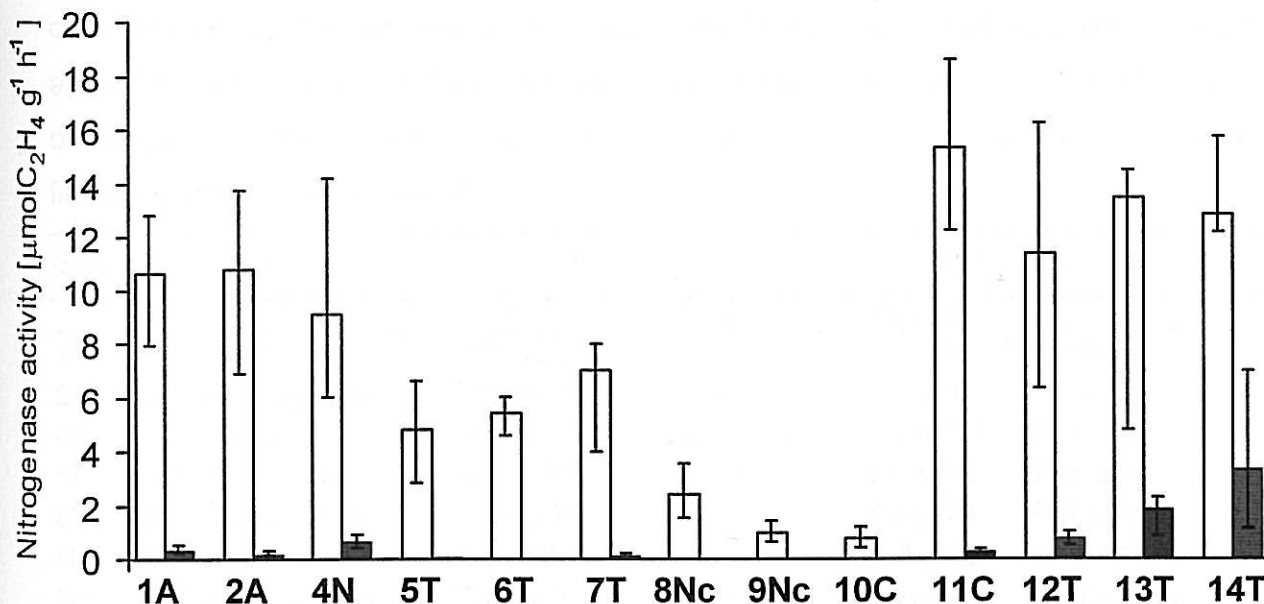


Fig. 5: Graph showing mean values (column) and maximum-minimum span (whiskers) of nitrogenase activity values for tested strains. White columns expresses light NA and dark columns expresses NA after 12 h. dark cultivation.

Also both the strains of *Anabaena* (1A and 2A) and the *Nodularia* strain (4N) had mean values of NA in light around 10 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$ and maintained basal NA after 12 h in dark, too. In comparison with *Tolypothrix* strains, the *Anabaena* and

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Nodularia strains differed largely in the pattern of heterocytes formation and also in the morphology of a filament and colony. *Anabaena* strains (1A and 2A) formed filaments with intercalary heterocytes which grew in a thin film while *Nodularia* (4N) differentiated both terminal and intercalary heterocytes and its filaments aggregated in a thick layer (fig. 6/3, 6/4).

Trichormus 5 and 6T strains (with filaments also aggregated into a thick layer) and *Nostoc* (8,9Nc) strains (which formed typical mucilaginous macrocolonies) had the lowest NA in light from studied strains, and their activity disappeared completely after dark incubation. They both form intercalary and terminal heterocytes (fig. 6/5, 6/6) and with frequency that was very variable but always higher than 5%. Except for the strain 8Nc, these strains were isolated from agricultural fields. Contradictory results were found in *Cylindrospermum* strains. *Cylindrospermum* 10C showed very low specific NA in light and no NA was detected in dark, while strain 11C had the highest NA in light of all studied isolates ($12.29 - 18.61 \mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$) which then decreased to $0.11 - 0.37 \mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$ in dark. Frequency of terminal heterocytes (fig. 6/2) was very similar in these strains: 3.6% and 3.3% respectively, but they substantially differed in the thickness of film. Physiological differences corresponded to the place of origin: strain *Cylindrospermum* 10C was isolated from an agricultural field and its filaments were aggregating into a thick layer similar to that of *Nodularia*, while *Cylindrospermum* 11C was isolated from forest soil and its filaments formed a very thin film.

Strain	Species (Genus)	NA range		f HTC [%]	Colony morphology
		in light	in dark	Mean (SE)	
1A	<i>Anabaena torulosa</i>	7.97 - 12.87	0.13 - 0.51	5.4 (0.58)	thin film
2A	<i>Anabaena augstumalis</i>	6.93 - 13.74	0.15 - 0.17	3.3 (0.28)	thin film
4N	<i>Nodularia</i> sp.	6.03 - 14.19	0.37 - 0.98	11.7 (0.65)	thick layer
5T	<i>Trichormus variabilis</i>	2.85 - 6.61	0	6.5 (0.54)	thick layer
6T	<i>Trichormus variabilis</i>	4.61 - 6.01	0	5.2 (0.49)	thick layer
8Nc	<i>Nostoc calcicola</i>	1.55 - 3.56	0	15.0 (1.12)	macrocolony
9Nc	<i>Nostoc ellipsosporum</i>	0.66 - 1.45	0	5.0 (0.13)	macrocolony
10C	<i>Cylindrospermum</i> sp.	0.58 - 0.95	0	3.6 (0.23)	thick layer
11C	<i>Cylindrospermum</i> sp.	12.29 - 18.61	0.11 - 0.37	3.3 (0.25)	thin film
12T	<i>Tolypothrix</i> sp.	6.36 - 16.25	0.49 - 1.09	4.8 (0.63)	thin film
13T	<i>Tolypothrix</i> sp.	4.83 - 12.02	0.85 - 2.28	2.1 (0.23)	thin film
14T	<i>Tolypothrix</i> sp.	12.21 - 15.67	1.14 - 7.01	3.9 (0.28)	thin film

f HTC - frequency of heterocytes (means and standard errors of means in brackets, n=10)

Tab. 3.: Table showing maximum-minimum values of light and dark nitrogenase activity, mean values of heterocytes frequency and colony morphology.

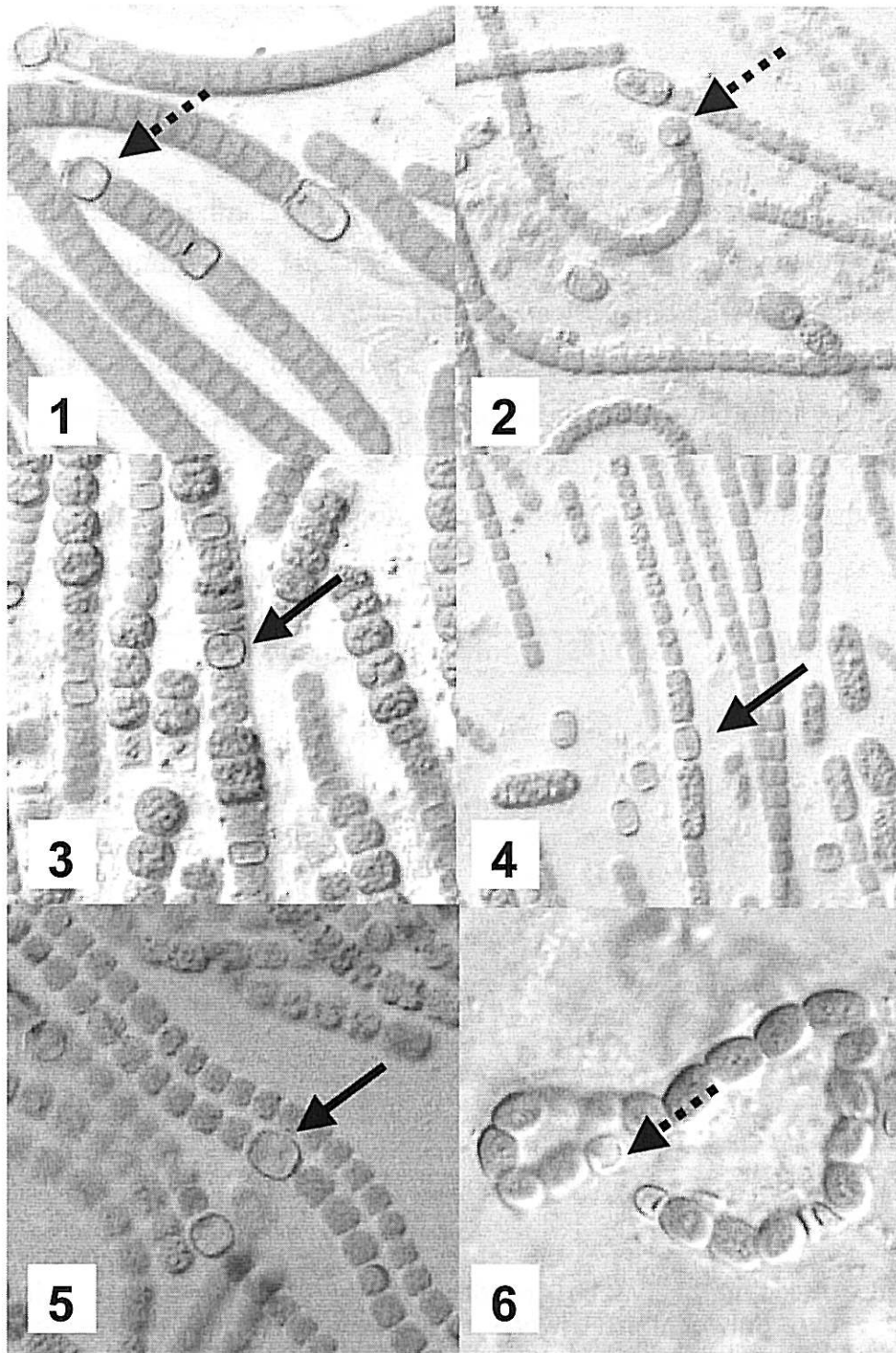


Fig. 6: Microphotographs of filaments of strains under study: **1** - *Tolypothrix* 14T with basal heterocytes, **2** - *Cylandrospermum* 10C with terminal heterocytes, **3** - *Nodularia* 4N filaments with intercalary and terminal heterocytes - akinete differentiation is clearly seen, **4** - *Anabeana* 1A filaments with intercalary heterocytes and akinetes, **5** - vegetative filaments of *Trichormus variabilis* 5T with intercalary heterocytes, **6** - vegetative filaments of *Nostoc* 8Nc with terminal heterocytes and mucilaginous envelope. Black full arrows marks intercalary heterocytes, black dotted arrows marks terminal heterocytes.

3.2. Cytotoxicity

3.2.1. Brine shrimp assay

✓ Two cyanobacterial extract^s out of 10 tested were found to exhibit significant cytotoxicity against *Artemia* sp. These were extracts of *Trichormus variabilis* 5T and *Cylindrospermum* sp. 11C. The level of inhibition was 46% and 55% respectively.

In extracts of *Anabaena torulosa* 1A, *Cylindrospermum* sp. 10C, *Tolypothrix* sp. 14T and *Calothrix* sp. 16Ca one more dead individual was found after 24 hrs after extract addition. In extracts of four strains no inhibition ^{was} found (*Anabaena sphaerica* 3A, *Nodularia* sp. 4N, *Trichormus variabilis* 6T and *Calothrix* sp. 15Ca).

2. Formulation hygiene - in the water be controlled

The data are summarized in tab 4.

strain	(0) living	(0) dead	(24) living	(24) dead	inhibition (%)
1A	9	1	8	2	11
3A	8	1	8	1	0
4N	13	0	13	0	0
5T	13	3	7	9	46
6T	8	2	8	2	0
10C	8	2	7	3	13
11C	11	1	5	7	55
14T	12	1	11	2	8
15Ca	7	0	7	0	0
16Ca	10	1	9	2	10

Tab. 4: Table showing results of Brine shrimp assay. In first two columns culture state before addition of extract is shown. In following two columns culture state after 24 hrs since extract addition is shown.

3.2.2. MTT test

Significant cytotoxic effect was found in 6 of 10 tested cyanobacterial extract (*Anabaena* spp. 1A and 3A, *Trichormus variabilis* 5T and 6T and in both *Cylindrospermum* sp. strains 10 and 11C). Both cell lines differ largely in their response to extract exposure. Data are summarized in figs. 7 and 8.

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Extract of *Anabaena torulosa* 1A has caused mortality over 80% in both cell lines in 1% concentration. However, in ten times diluted solution no decrease of cell viability was found in WEHI cell line and mortality of 20% was found in YAC-1 cell line. Treatment with extract concentration of 0.01% has not resulted in decrease of cell viability. In *Anabaena sphaerica* 3A no cytotoxic effect of 1% concentrated extract was found in WEHI cell line, but 40% mortality was estimated in YAC-1. Other dilution did not exhibit cytotoxic effect.

Both extracts of *Trichormus variabilis* 5T and 6T strains have caused significant mortality to both cell lines. Extract of strain 5T in 1% concentration have caused 90% mortality in WEHI cell line, but only 20% mortality culture of YAC-1. Curiously the situation was opposite in 6T extract where the mortality of 95% was found in YAC-1 cell line and only 15% decrease of cell viability in WEHI were recorded. No cytotoxic effect was found in more diluted extract of both strains.

Extract of *Cylindrospermum* sp. 10C have caused 20% mortality in 1% concentration and 15% mortality in concentration of 0.1% to WEHI cell line. In YAC-1 60% mortality was recorded in 1% concentration and other dilution did not caused cytotoxic effect. Largest damage was observed in WEHI cell line treated by extract of strain *Cylindrospermum* sp. 11C. After exposure of 1% extract no living cell were found. Moreover, cells treated by 0.1% extract concentration exhibited still 50% mortality. No cytotoxic effect of 0.01% extract was found. Also in YAC-1 cell line strong cytotoxic effect was found after treatment with 11C extract (95% mortality) but only in most concentrated variant.

Response of both cell lines in application of *Nodularia* sp. 4N, *Tolypothrix* sp. 14T, *Calothrix* sp. 15 and 16Ca extracts was similar. They did not cause decrease of cell survival to WEHI cell line and only slight cell survival decrease (20-25%) to YAC-1 cell line.

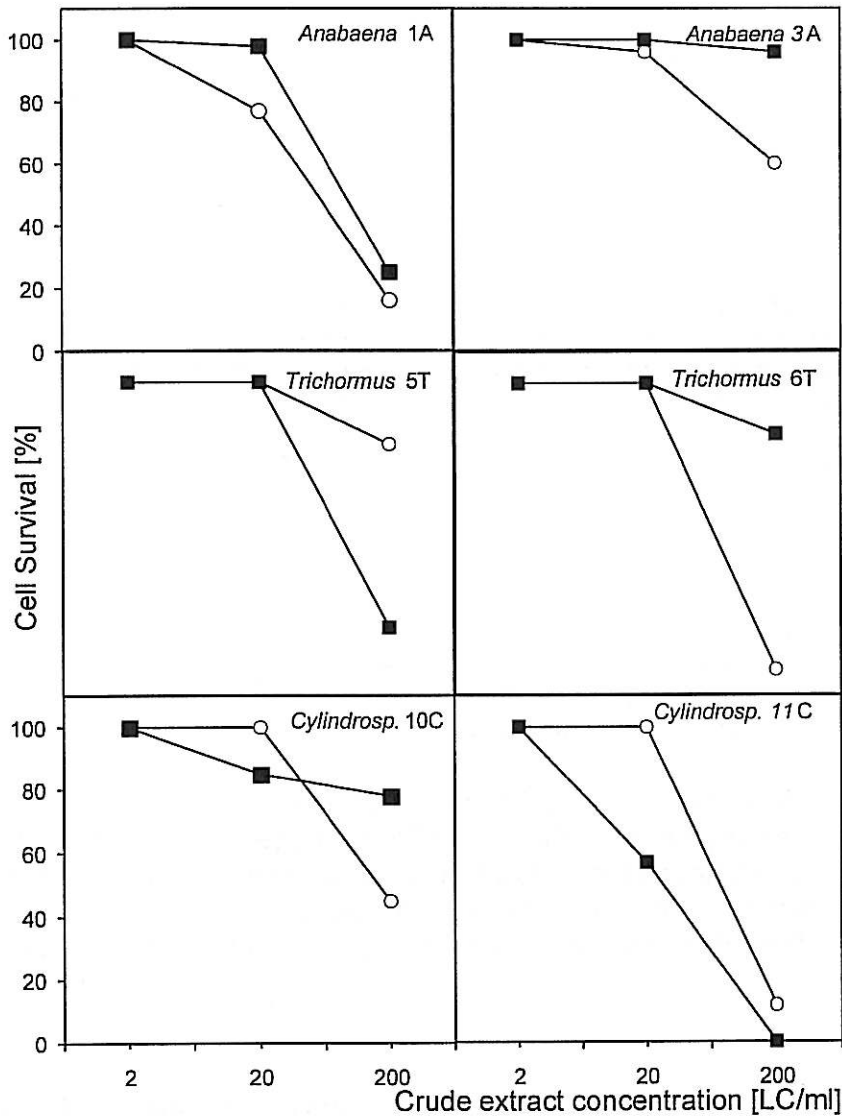


Fig. 7: Survival of two cell lines YAC-1 (*empty circles*) and WEHI (*filled squares*) assayed by MTT test after 12 hrs. exposure of three concentration of cyanobacteria extract. Concentration corresponds to mg of lyophilized cells per ml of crude extract. Crude extract was applied in 1% concentration to cultivation medium RPMI during cell incubation.

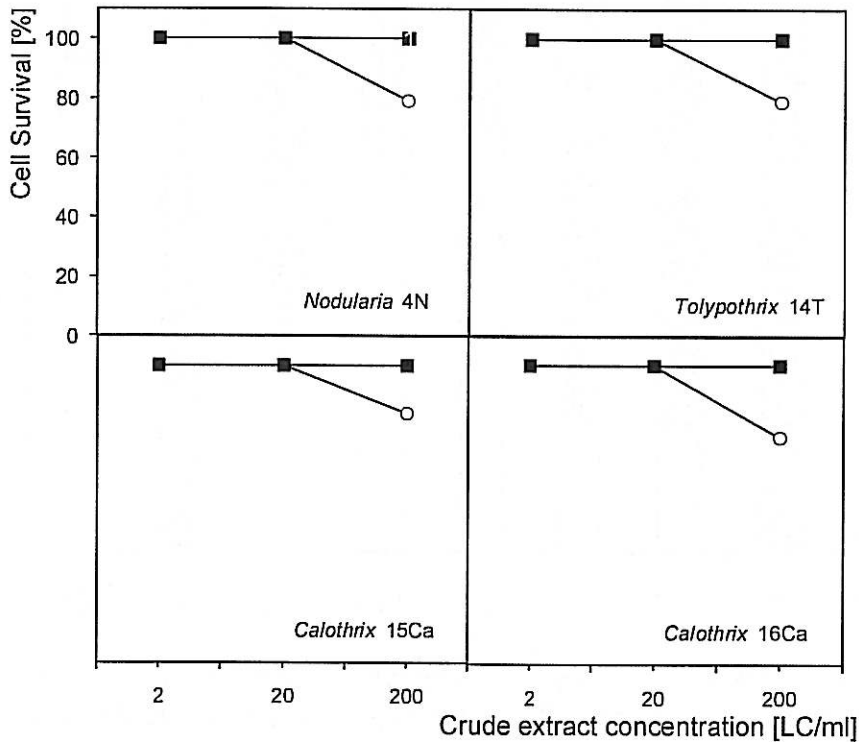


Fig. 8: Survival of two cell lines YAC-1 (empty circles) and WEHI (filled squares) assayed by MTT test after 12 hrs. exposure of three concentration of cyanobacteria extract. Concentration corresponds to mg of lyophilized cells per ml of crude extract. Crude extract was applied in 1% concentration to cultivation medium RPMI during cell incubation.

3.2.3. Flow-cytometrical analysis with propidium iodide and Anexin-V-flos

90 min ^{in table} ~~of~~
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After 90 min. exposure in four treatments mortality of WEHI cell line was higher than 50%. Results of flow-cytometrical analysis are summarized in fig. 9 and 10. These were 1% extracts of *Anabaena torulosa* 1A, *Anabaena sphaerica* 3A, *Cylindrospermum* sp. 10C and 11C.

Exposure of WEHI cell line to 1A extract has caused 99% mortality after 90 min. incubation. Most of the cell population was detected as necrotic cells (see fig. 9 and 10). Also observation of cell morphology under light microscope with coloring of Tripian blue has confirmed that most of the cells were had disrupted cell membrane.

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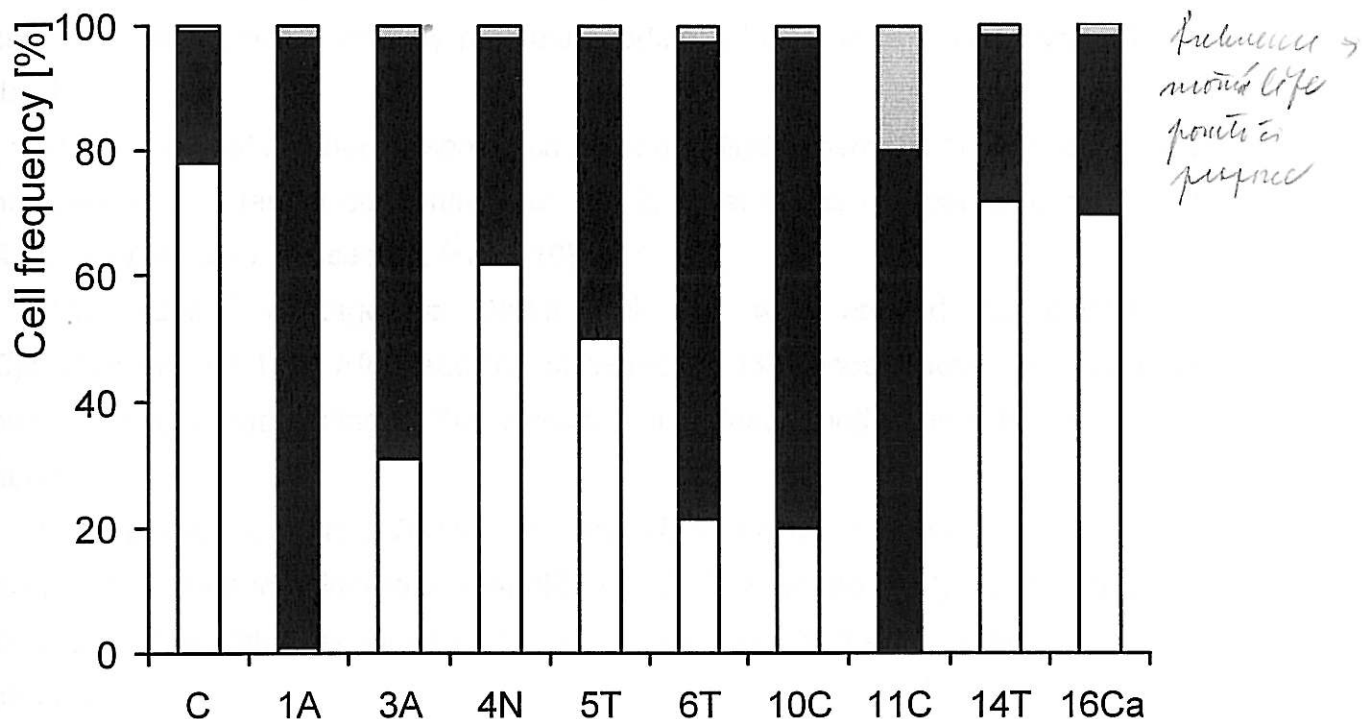


Fig. 9: Graph showing frequency in percent of living (white fields), necrotic (black fields) and apoptotic cells (grey fields) in culture of WEHI cell line treated with cyanobacterial extracts of concentration 1% for 1h.

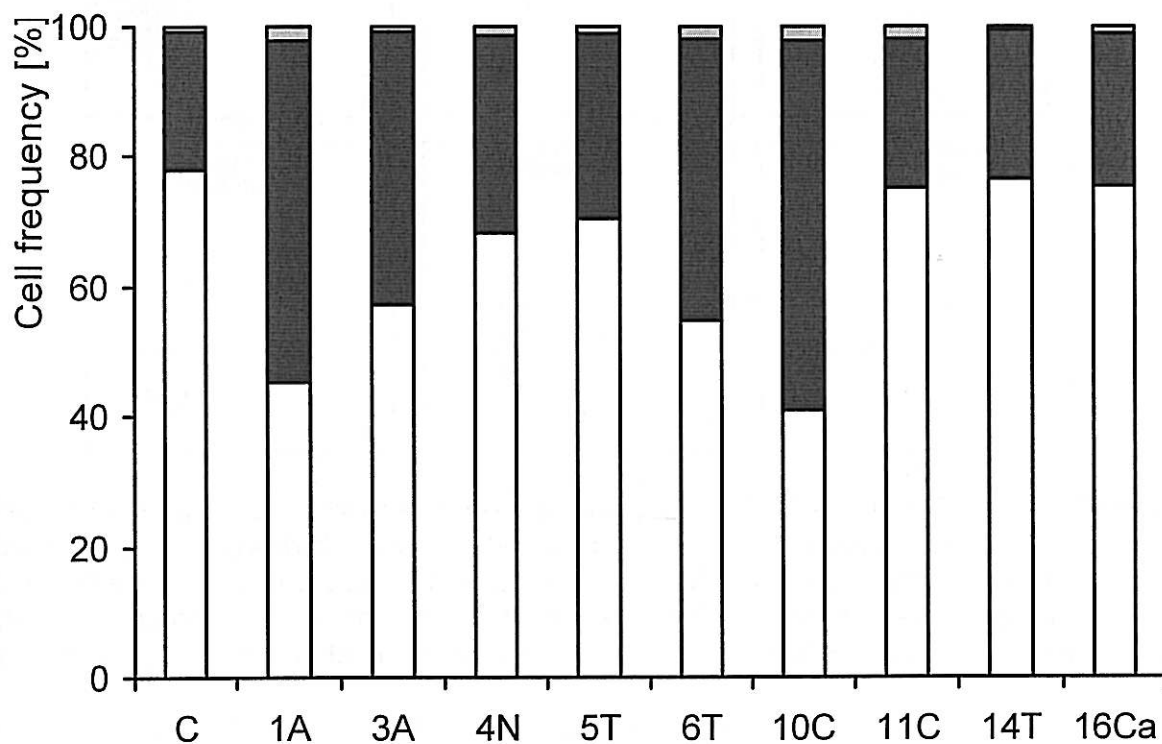


Fig. 10: Graph showing frequency in percent of living (white fields), necrotic (black fields) and apoptotic cells (grey fields) in culture of WEHI cell line treated with cyanobacterial extracts of concentration 0.1% for 1h.

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Extract concentration of 0.1% has caused increase of mortality to 54%. The cells were also colored only by propidium iodide, which marks necrotic way of cell death.

Also extracts of *Anabaena sphaerica* 3A and *Cylindrospermum* 10C have induced necrosis in both tested concentrations, but in lower levels compared to extract of *Anabaena torulosa* 1A (see fig. 9 and 10)

Most severe damage to WEHI cell line was caused by extract of *Cylindrospermum* 11C. After addition of extract in 1% concentration no living cells were detected. Interestingly, the extract has induced both necrosis (80%) and apoptosis (20%).

In *Nodularia* sp. 4N and *Trichormus variabilis* 5T cytotoxic effect was found in 1% extract concentration (increase mortality to 38, 50% respectively), but only slight decrease of viability was found in 0.1 % concentration (increase of cell mortality of about 10%).

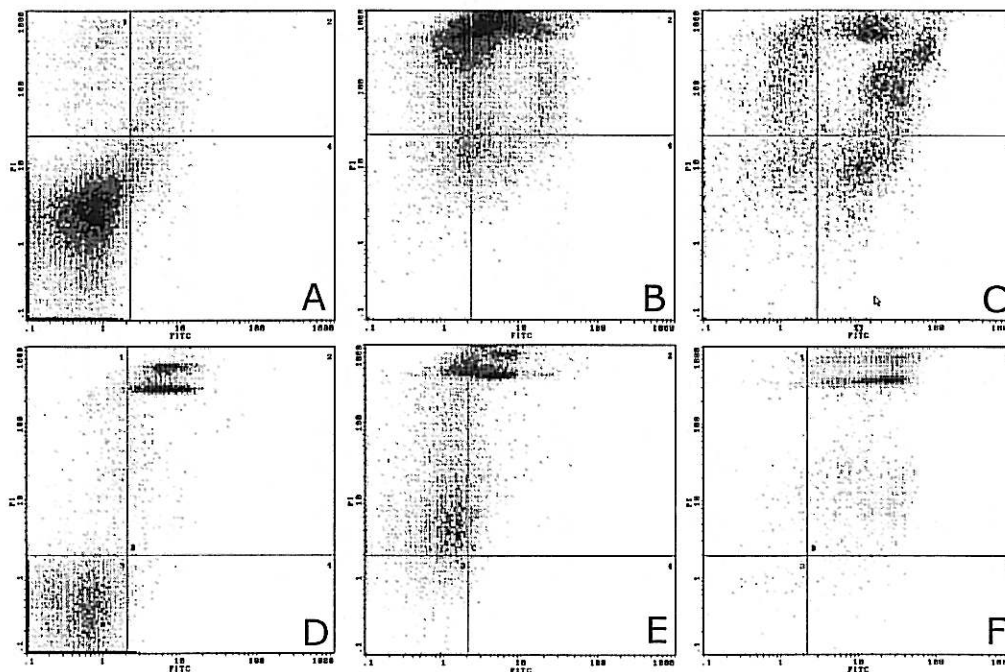


Fig. 11: Results of flow-cytometrical analysis of YAC-1 (A,B,C) and WEHI cell lines (D,E,F) treated with 1% extract of *Anabaena torulosa* 1A and *Cylindrospermum* sp. 11C. Fluorescence of cells in propidium iodide (x-axis) is plotted against cell fluorescence in Annexin-V-flos. Living cells are situated in quadrant 3, necrotic cells in quadrant 1 and 2 and apoptotic cells in quadrant 4. ✓

- A-** Control YAC-1 cells (79% of living cells in quadrant 3)
- B-** YAC-1 cell treated with 1% extract of *Anabaena torulosa* 1A
- C-** YAC-1 cell treated with 1% extract of *Cylindrospermum* sp. 11C
- D-** Control WEHI cells (80% of living cells in quadrant 3)
- E-** WEHI cell treated with 1% extract of *Anabaena torulosa* 1A
- F-** WEHI cell treated with 1% extract of *Cylindrospermum* sp. 11C

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In any of tested concentrations no cytotoxic damage was found in extract of *Calothrix* sp. and *Tolypothrix* sp. strains (for details see figs. 9 and 10).

In three selected strains (*Anabaena torulosa* 1A, *Cylindrospermum* sp. 11C and *Tolypothrix* sp. 14T) 1h. exposure effect to YAC-1 cell line was studied. In general similar results compared to WEHI cell line were obtained (results are summarized in fig. 15). Strong induction of necrosis after treatment with 1A extract was found (88 and 31% for 1% and 10⁻²% concentration respectively - fig.14/B). Extract of *Cylindrospermum* sp. 11C have caused significant cytotoxicity 89,2% in 1% concentration and 42% in 10⁻²% concentration, moreover, similarly to results in WEHI cell line apoptotic population was observed in FC diagram (fig.11/C). In *Tolypothrix* sp. 14T no significant effect was found.

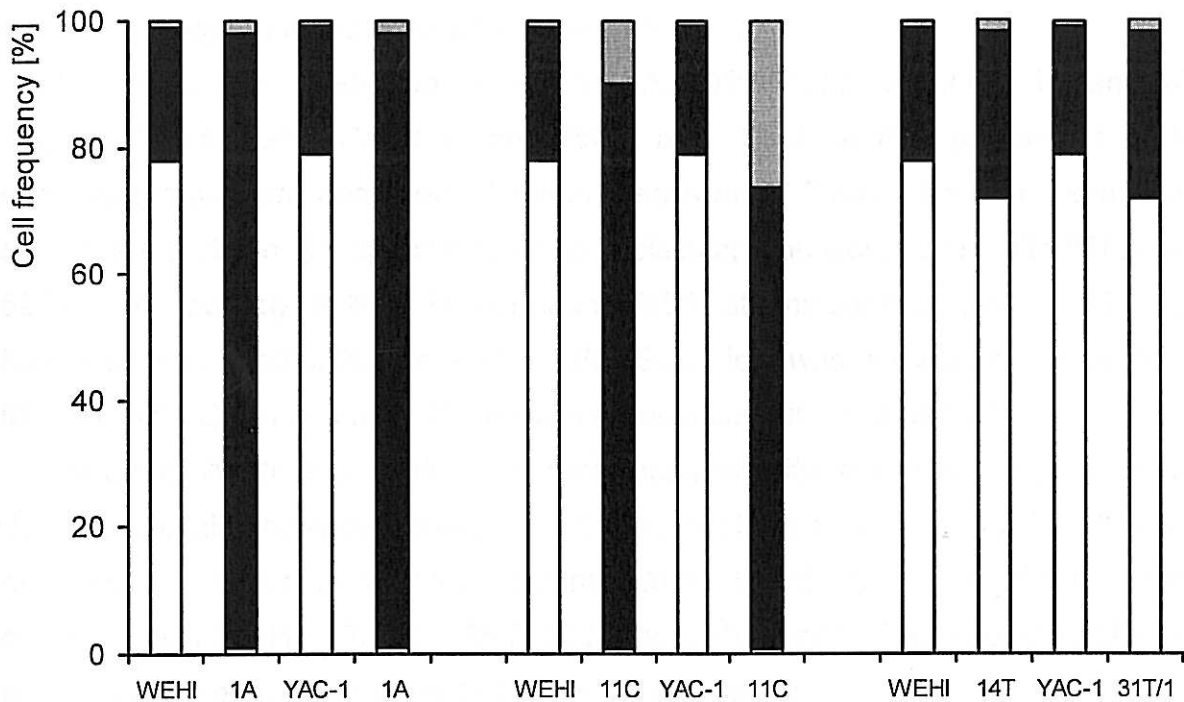


Fig. 12: Graph showing frequency in percent of living (white fields), necrotic (black fields) and apoptotic cells (grey fields) in culture of WEHI and YAC-1 cell line after treatment with 1% extract of *Anabaena torulosa* 1A (left), *Cylindrospermum* sp. 11C (middle) and *Tolypothrix* sp. 4T (right) .

3.3. Analysis of cyanobacteria extracts by HPLC-MS (high performance liquid chromatography with combination of mass spectrometry)

In all studied strains except *Nodularia* sp. 4N HPLC-MS spectra were obtained. Although complete analysis has 30 min. retention, only interval between 18 min. and 27.5 min. was taken into consideration. In earlier times than 15 min. no peaks were observed and in later times than 27.5 min. extract composition was very similar among studied strain with high content of less-polar compound (degradation products of chlorophyll, carotenoids) and contaminations (solvents and phthalates). Complete chromatograms with MS spectra of selected peaks are showed in Apendix B.

Comparison of all obtained HPLC spectra is showed in fig.13. Identification of peaks was done by comparison of peak molecular weights (m/z) with published data (for list of known compounds and their molecular weights see tab 1.). In none of the spectra microcystin-like compounds were found.

In extracts of *Anabaena sphaerica* 3A, *Trichormus variabilis* 5T and 6T, *Tolypothrix* 14T and *Calothrix* sp. 15Ca and 16Ca similar pattern of HPLC chromatograms were observed. Common peaks at 24.7 and 25.6 min. were found (see Apendix B). In MS spectra common molecular ions were found with 578.9 and 620 m/z respectively. In both *Trichormus variabilis* strains common peak at 25.6 min. has contained molecular ion 609.2 m/z . Such ion was previously attributed to Microginin 91-B isolated from *Microcystis aeruginosa* (ISHIDA *et al.* 2000).

Extract of *Anabaena torulosa* 1A have provided different chromatogram, which does not contain any known compound. But molecular ions with lower m/z – 665, 670 and 638 were found. In their MS spectra also double charged molecular ions were observed with $[M+H]^{2+}$ 333.3, 335.3 and 319.3. Presence of such double charged ions can imply peptide structures of unknown compounds.

In strain *Cylindrospermum* sp. 11C five not identified peaks were found with retention times of 24.7, 25.5, 26.1, 26.8 and 27.0 min. First two peaks correspond to previously described peaks in *Trichormus* and *Calothrix* strains. Other 3 peaks contained molecular ions with higher m/z 1122.4, 1092.5 and 1106.5 respectively. For complete chromatogram see Apendix B-6.

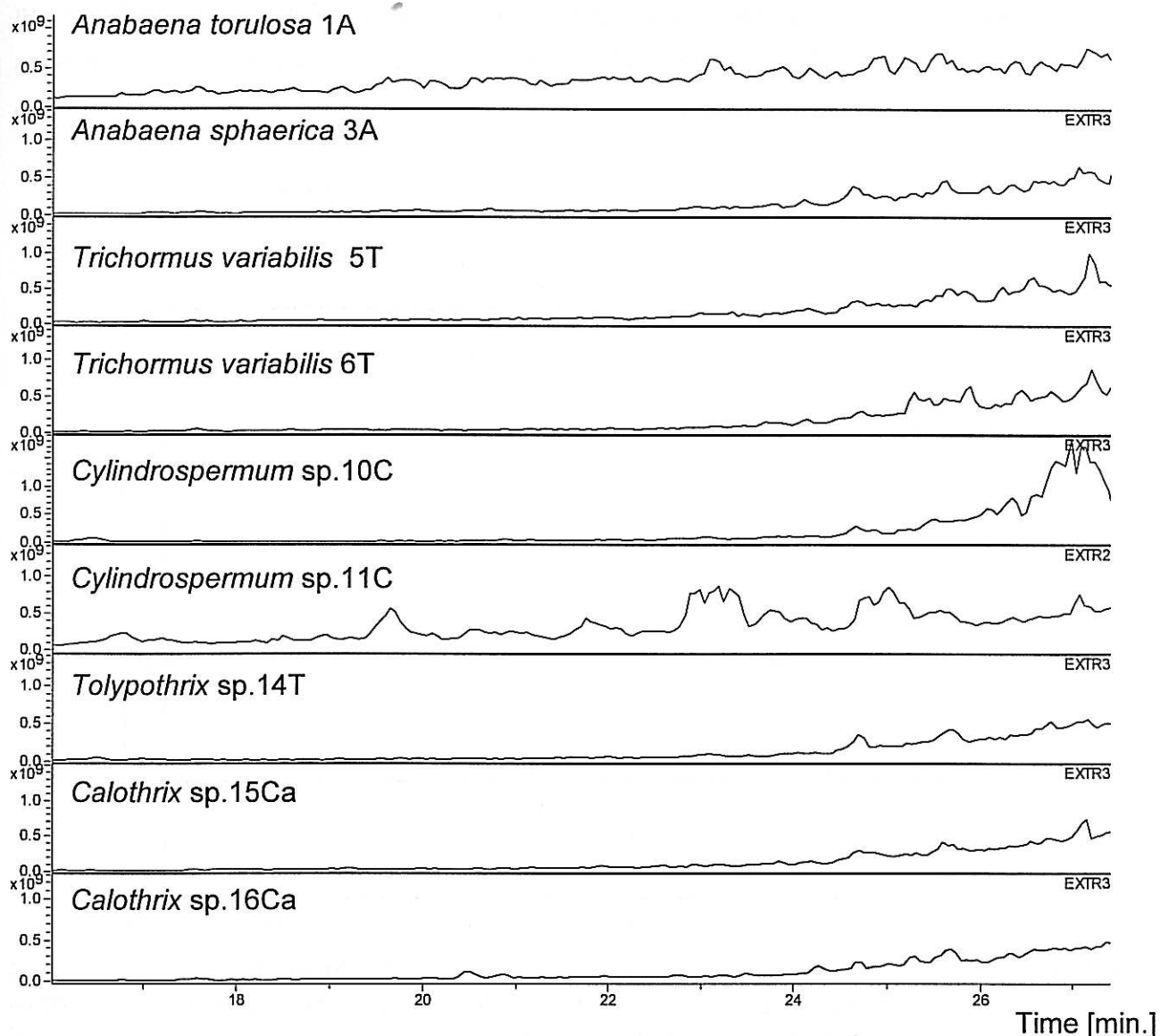


Fig. 13: HPLC-spectra of studied strains. X-axis show retention time, Y-axis show peak intensity (minimum 0, maximum $1 \cdot 10^9$ molecular ions)

Also in the second studied *Cylandrospermum* strain 11C peaks containing molecular ions with relatively high m/z were obtained. In its HPLC-MS spectrum 13 peaks with high intensity (about 10^8 ions) were found. For most of them m/z was around 1100. With retention times and m/z of 1156.2 at 16.8 min., 1182.2 at 19.0 min., 1184.3 at 19.6 min., 1112.2 at 20.5 min., 1174.3 at 21.8 min., 1154.4 at 22.9 min., 1140.2 at 23.3 min., 1202.2 at 23.8 min., 1182.3 at 24.8 min., 1168 at 24.9 min., 1183.2 at 25.1 min., 620.3 at 25.6 min. and 1106.5 at 27.1 min (For complete chromatogram see Appendix B-6). Except peak at 20.5 min. with m/z of 1112.2, which

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corresponds to previously described Oscillapeptin G isolated from *Planktothrix agardii* (Fujii *et al.* 2000), none have correspond to known compound. Moreover, according to the extracted chromatograms, the peaks can be attributed to pure compounds (see fig. 14).

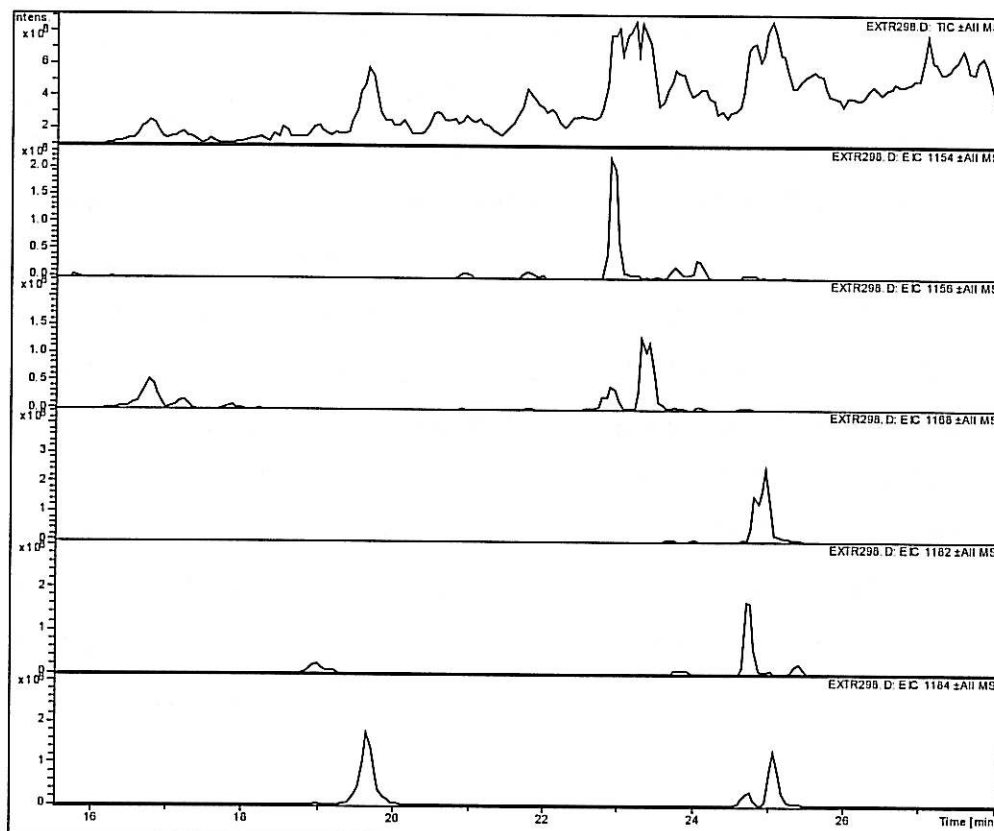


Fig. 14: Figure showing complete HPLC spectrum of 11C extract (1 row), and extracted mass spectra for 1154, 1156, 1168, 1182, 1184 m/z (2-6 rows respectively). X-axis shows retention time in minutes, Y-axis shows peak intensity ($0 - 3 \cdot 10^8$)

4. Discussion

4.1. Nitrogenase activity

Values of light NA were mostly very similar in strains belonging to the same morphotype or genus exhibiting similar morphology of filaments and colonies. This fact indicates that the morphology of the filament and colony can be relevant to nitrogen fixing ability. The only case where strains of the same genera differed substantially in their NA were the *Cylindrospermum* strains, but in this case the difference between their NA could be caused by different colony morphology and may be linked to the place of origin.

Surprisingly, the values of NA did not reflect the frequency of heterocytes. Strains with low and high frequency were randomly dispersed in the group with high NA (9.91 - 15.34 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ FM h}^{-1}$) and in the group with lower NA (see tab. 3). The mutual independence of NA and the frequency of heterocytes formed can be seen from ordination diagram based on RDA analysis (fig. 15). The whole model explained 75% of variability of NA data. The variable frequency of the heterocytes (f HTC) does not explain any variability in NA data and its influence is also not significant ($p=0.37$).

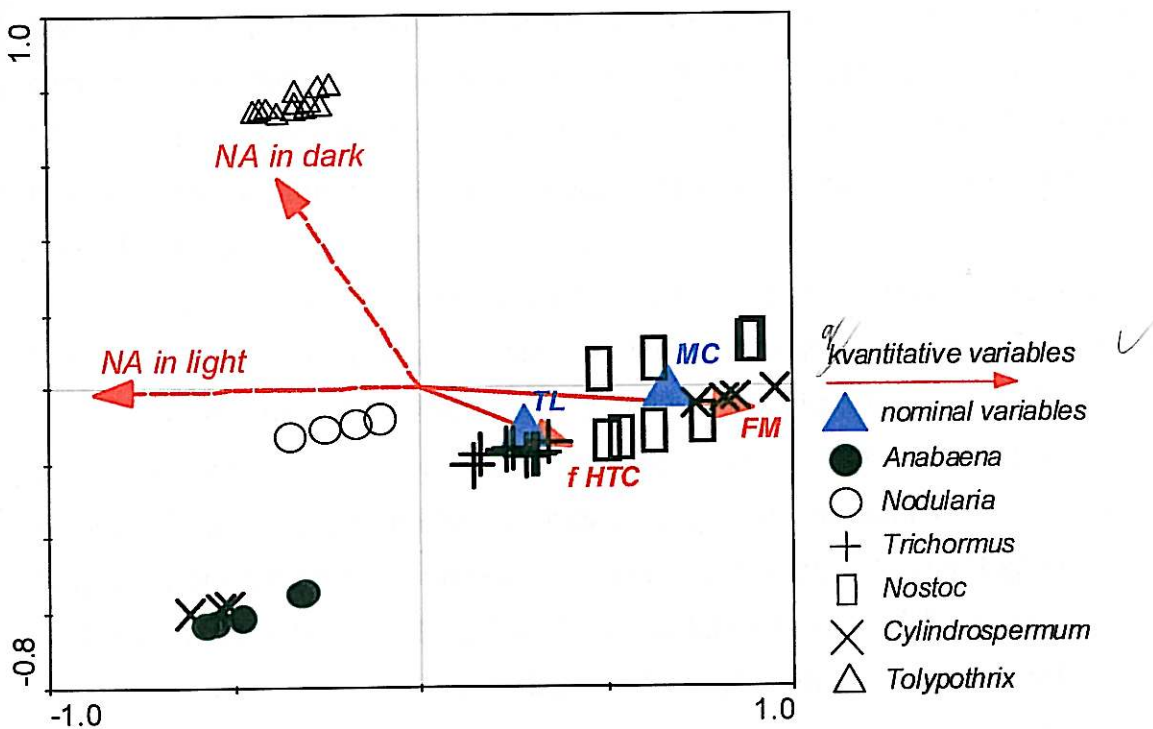


Fig. 15: Ordination diagram based on results of RDA analysis. Abbreviations of variables: TL= thick layer, MC= macrocolony, FM= fresh mass, f HTC= frequency of heterocytes.

Strong negative correlation of biomass and NA of the strains is clearly seen from the diagram (fig. 15). Also the variables film thickness and macrocolony formation seem to be negatively correlated to values of NA. The variable biomass explained 58% of variability in NA values.

The data implies that mucilaginous envelopes and aggregation of filaments to large colonies or thick films can affect diffusion of gases inside the colony. Effect of bacterial mucilage to O₂ diffusion into the colony was shown to be an important factor affecting nitrogen fixation in some aerobic bacteria (BROWN 1970; PAN & VESSEY 2001). DONG (1995) also reported that colony morphology on solid medium and the relative distribution of the bacteria within highly mucilaginous colonies changed with changes in the partial pressure of O₂ surrounding the colonies. In the case of mucilagenous cyanobacteria, diffusion of N₂ into the colony can be significantly affected and thus the specific NA of inner parts of colony is very low. Formation of thick envelopes and cell aggregations could be then more important for total NA than the frequency of heterocytes.

It is conceivable that NA of cyanobacterial isolates could be affected by a nitrogen status of the habitat by which the organism was affected. There is some evidence that there is a significant decrease of NA and also changes in diversity of cyanobacterial community with increasing nitrogen doses used in cultivated fields (DELUCA *et al.* 1996). Also the influence of various organic compounds like pesticides has been studied and its negative effect on NA was demonstrated (ČERŇÁKOVÁ *et al.* 1991; ČERŇÁKOVÁ 1993; ROGER *et al.* 1994).

All strains originated from agricultural fields (4T, 5T, 7Nc and 9C) had very low NA in light conditions and no NA after 12 h of dark cultivation, while the other strains, which were isolated from oligo-mesotrophic habitats, had higher activities. A striking difference can be seen upon comparison of *Trichormus* (4,5T) and *Nodularia* (3N) strains. They differed neither in biomass nor in morphology of the colony or frequency of heterocytes. However, *Nodularia* 3N which exhibited higher mean value of NA both in light and dark has been isolated from a salty meadow while the *Trichormus* strains with low NA in light and no NA in dark has been isolated from an agricultural field. It seems that intensively fixing cyanobacteria are preferred in oligotrophic habitats. The above mentioned hypothesis, as supported by the data, implies that in habitats with higher concentration of combined nitrogen compounds

the nitrogen fixing apparatus is redundant. Therefore, long term growth of nitrogen fixing cyanobacteria in nitrogen rich conditions could reduce nitrogen fixation. The extent of impact of fertilisation on the efficiency of nitrogen fixing apparatus and total nitrogen fixation is, however, not clear.

4.2. Cytotoxicity

4.2.1 Comparison of results obtained by brine shrimp assay, MTT and flow-cytometrical analysis.

In general all three used methods have provided quite similar response in cytotoxicity testing, however, sometimes with different level of cytotoxic effect. As cited before (LINCOLN *et al.* 1996) brine shrimp has provided rough estimation of cytotoxic effect of cyanobacterial extract. In some strains (mainly in *Anabaena torulosa* 1A) only weak cytotoxic effect was estimated in brine shrimp assay after 24 h. exposure (around 10%), but strong damage of this extract was found in cell lines after 1h. exposure (100% inhibition). Similar situation was observed in *Cylindrospermum* sp. 10C extract, where moderate inhibition 40-60% was found in cell lines and only weak inhibition was estimated in *Artemia* sp. Negative effect of *Nodularia*, *Tolypothrix* and *Calothrix* strains extract were identical in both tests. Levels of inhibition in WEHI cell line recorded by MTT spectrophotometric assay and flow cytometric analysis lead to similar results (with tolerance 10%) in strains extract 1A, 2A, 11C, 14T, 15Ca and 16Ca. In 1A, 2A and 24C cytotoxic effect was confirmed by both methods. In 14T, 15Ca and 16Ca both tests lead to negative results. Only in one strain (*Trichormus variabilis* 15T) MTT test lead to more positive results than FC analysis. But also some false positive effect of MTT test has been already reported (ROLLINO *et al.* 1995). Opposite results with negative effect in MTT and some cytotoxicity recorded in FC analysis were found in 10C and 4N extract. In both extracts cytotoxic effect (inhibition of 56, 16% respectively) was found after 1 h. exposure using flow cytometry. But only 20% inhibition was found in 10C extract and negative results for 4N was recorded by MTT assay. Also response of both cell lines differs largely in some extracts. The most ambiguous results were found in *Trichormus* strains, where 80% inhibition of WEHI cell line and only 20% inhibition of YAC-1 cell line were found. But completely contrary results were found in *Trichormus variabilis* 6T.

4.2.2. Cytotoxicity of the strains and composition of extracts

Two out of ten tested strains extract were found to be strongly cytotoxic, 4 have shown moderate cytotoxicity and 4 did not show cytotoxic effect.

Although strains of *Calothrix*, *Tolypothrix* and *Nodularia* have been several times reported to produce wide range of cytotoxic compounds like scytophycin, tolytoxin, calothrixin, nodularin etc. (PATTERSON & CARMELI 1991; RODNEY *et al.* 1999) Studied strains of these genera have not shown significant toxicity and also in their spectra did not contain any of known compound.

✓ In both *Anabaena* strain significant toxicity by induction of necrosis by extract addition was found. The HPLC-MS spectra, containing mainly low-molecular weight peaks, did not implied, that this effect can be attributed to any known compound. MS spectra showing double charged ions refer to peptide structure of the compounds. Also many compounds have been reported from planktic *Anabaena* strains (mainly microcystins and cyclic peptides like anabaenopeptins) (FUJII *et al.* 2002; SIVONEN K. 1996), but there are no reports about secondary metabolites of soil *Anabaena* strains. Moreover, the cytotoxic effect (90% inhibition of growth) of the 1A extract was comparable to results obtained by cells treated with 50 µM microcystin LR (MCLR) (BOTHA *et al.* 2004). If we consider that maximal known MCLR concentration in biomass to be 7300 µg/g DW (ZHANG *et al.* 1991), we should hypothetically (if microcystin is present in *Anabaena torulosa* 1A strain) get extract of 73 µM MCLR concentration (200 µg lyophilized cells were resuspended in 2 ml pure methanol, counted with app. MW 1000 for MCYST). But presence of microcystin in this extract was not proved by HPLC-MS analysis. This implies that other unknown compound with comparable cytotoxicity can be present in *Anabaena* 1A extract.

✓ In *Trichormus variabilis* strains induction of necrosis was found in 5T strain, but in lower level compared to *Anabaena torulosa* 1A extract. In both *Trichormus* strains some inhibition was found in MTT assay. Similarly to *Anabaena* strains HPLC spectra showed only low-molecular peaks with one m/z similar to known Microginin 91-A isolated from planktic *Microcystis aeruginosa* (ISHIDA *et al.* 2000). If such ecologically and phylogenetically unrelated groups can share common synthetic remain a question. But such situation of wide spread synthetic pathway can be seen in case of microcystins. Although, inhibition recorded by addition of 1% 5T and 6T

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extracts was quite high in MTT, FC analysis has revealed only 28% increase of mortality in 1% 5T extract. This indicates that cytotoxic effect of this extract can be rather unspecific.

Only one out of 10 tested cyanobacterial strains (*Cylindrospermum* sp. 11C) have exhibited significant cytotoxic effect in all used methods. It exhibited inhibition larger than 50% against *Artemia* and also against both cell lines (in WEHI also in 0.1% concentration). Moreover, induction of apoptosis (about 20% of cell population) was observed by annexin-V-flores coloring in both cell lines. Recently, there are some studies reporting induction of apoptosis by microcystin LR (BOTHÁ *et al.* 2004, DING & ONG 2003) by unknown way. Also effect of this extract (as mentioned in *Anabaena torulosa* 1A extract) has comparable toxicity to MCYST compounds, but any known molecular ion was not observed in its HPLS-MS spectrum. Several observed peaks of high m/z (about 1100) imply that some peptide structures in the extract can be present. One of peaks correspond to Oscillapeptin G isolated from *Planktothrix agardhii* (ISHIDA *et al.* 2000). Moreover, by high diversity and purity of observed peaks we can assume that this can be intermediates of one biosynthetic pathway.

5. References

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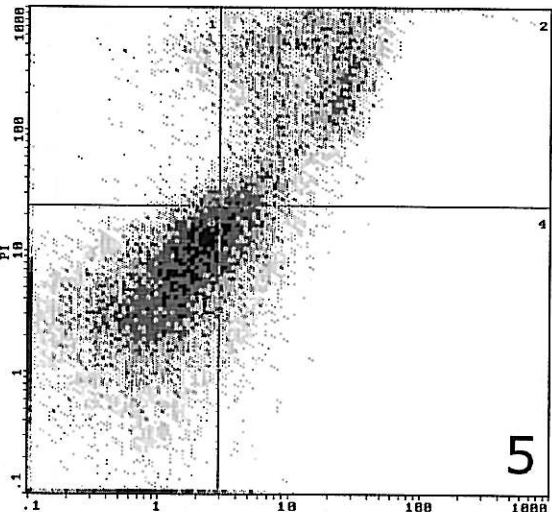
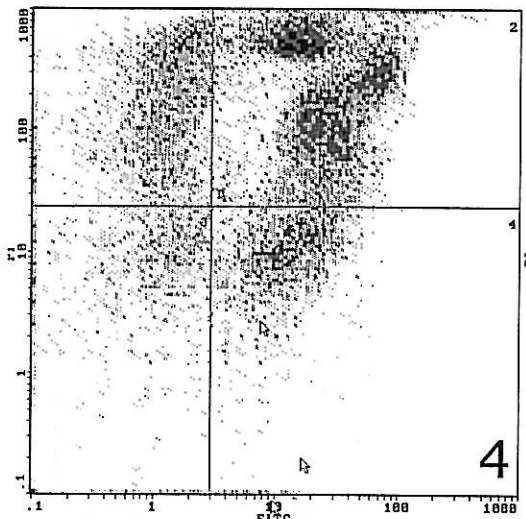
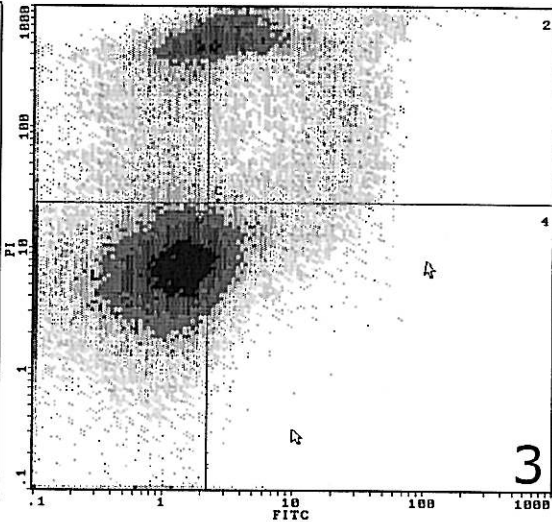
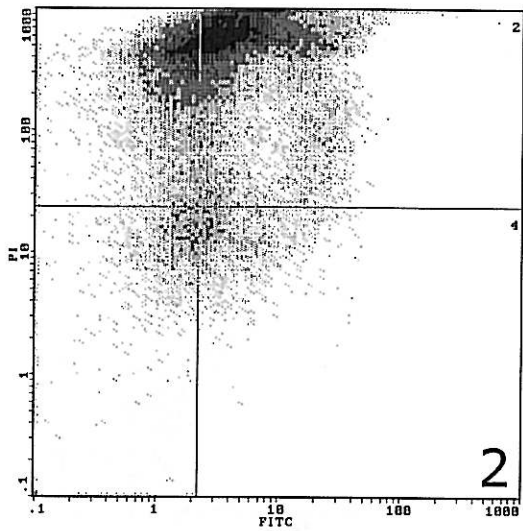
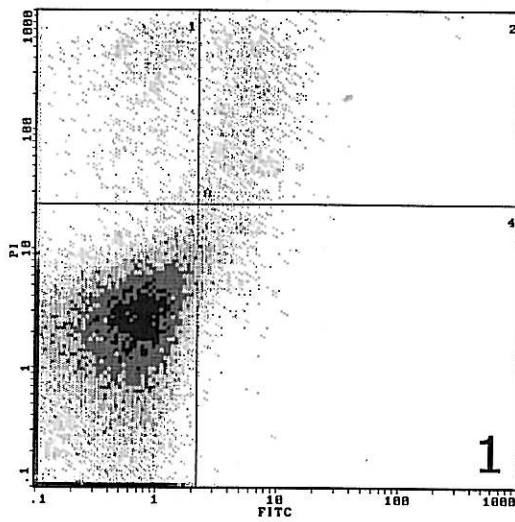
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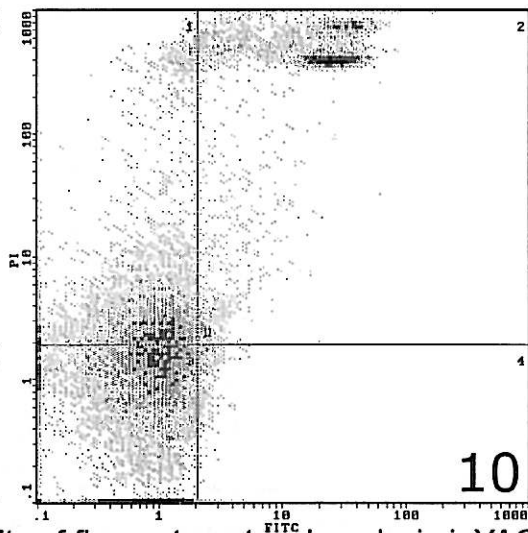
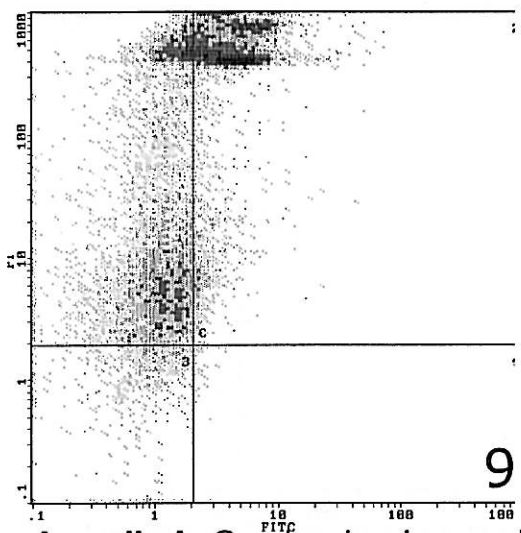
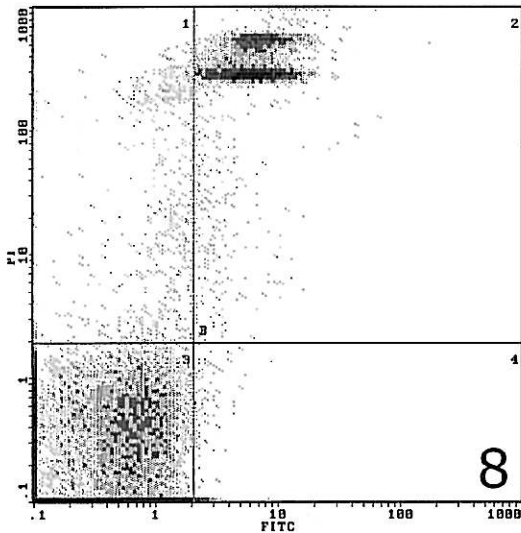
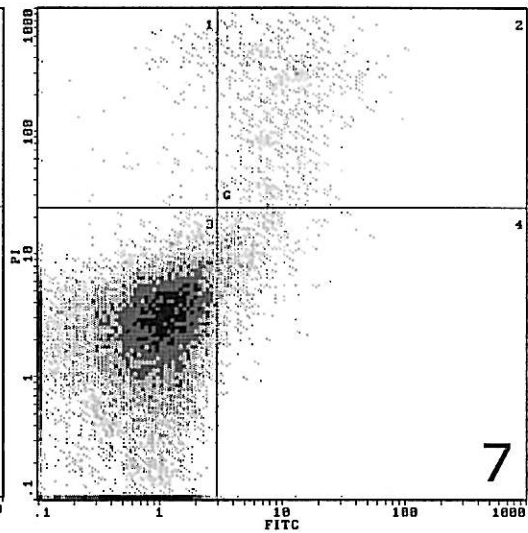
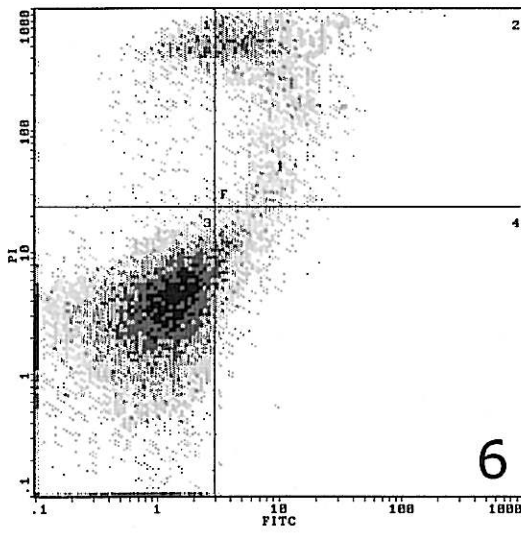
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Appendix A
(Results of FLOW-CYTOMETRY)



Appendix A: Graphs showing results of flow cytometrical analysis in YAC-1 cell line, florescence of cells in propidium is plotted on X-axis, florescence of cells Anxin-V-flos is plotted on Y-axis. *fu*
 1- YAC-1 control sample,
 2 - *Anabaena torulosa* 1A (1%) YAC-1, 3 - *Anabaena torulosa* 1A (0.1%) YAC-1,
 4 - *Cylandrospermum* sp. 11C (1%) YAC-1,
 5 - *Cylandrospermum* sp. 11C (0.1%) YAC-1

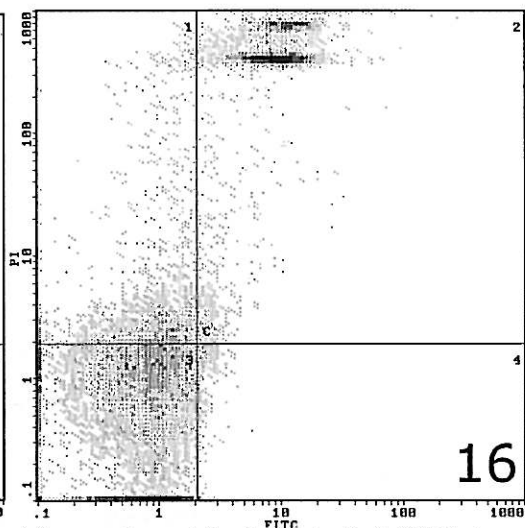
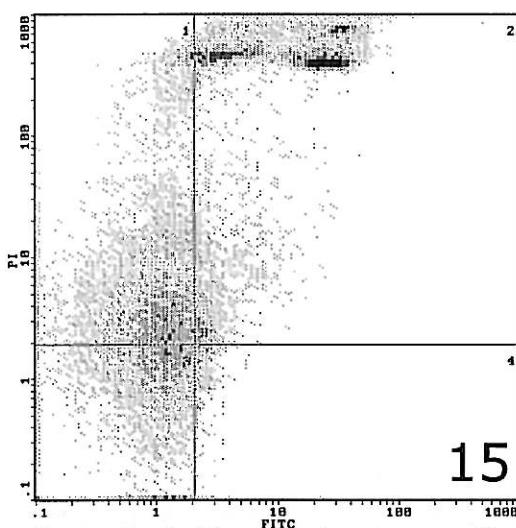
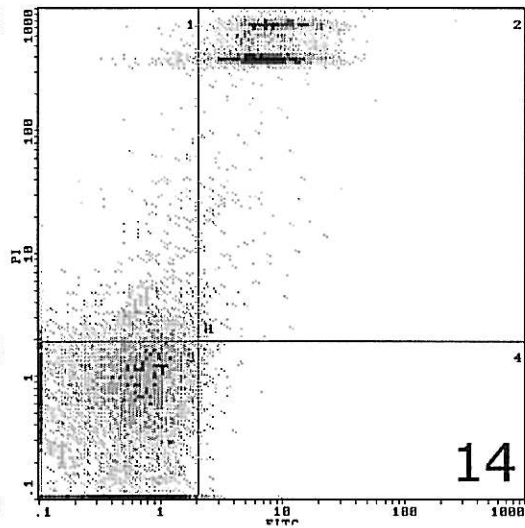
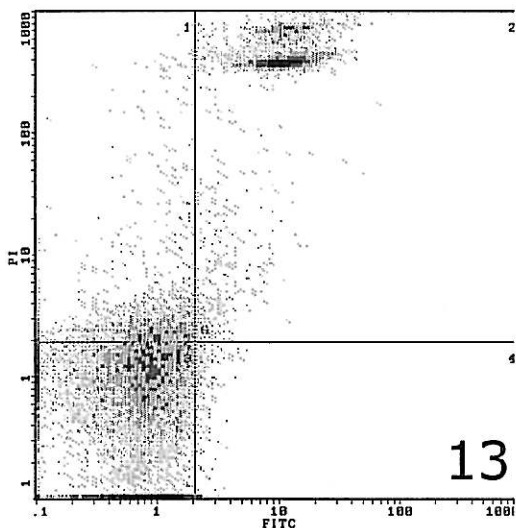
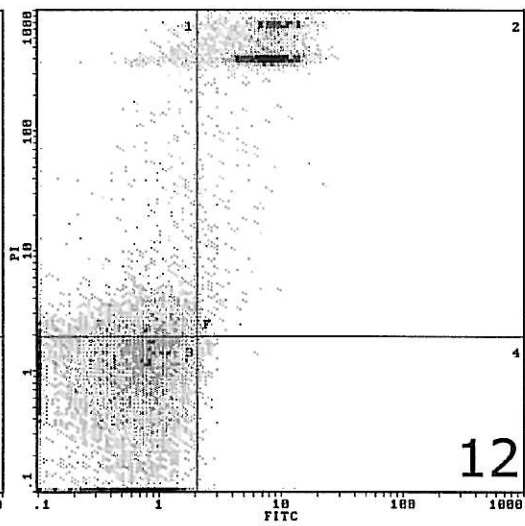
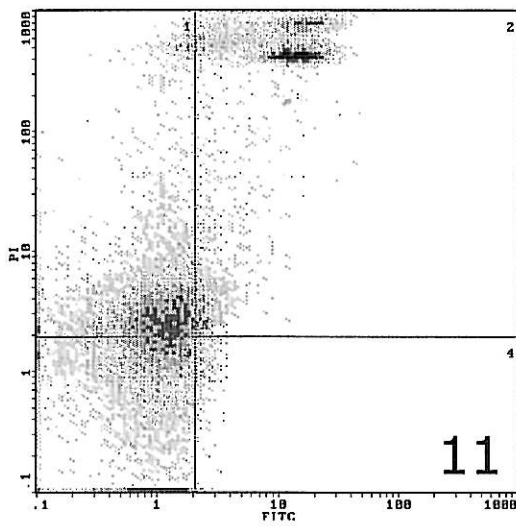


Appendix A: Graphs showing results of flow cytometrical analysis in YAC-1 and WEHI cell line, fluorescence of cells in propidium is plotted on X-axis, fluorescence of cells Annexin-V-fluorescence is plotted on Y-axis.

6- *Trichormus* sp. 14T (1%) YAC-1, **7-** *Trichormus* sp. 14T (0.1%) YAC-1

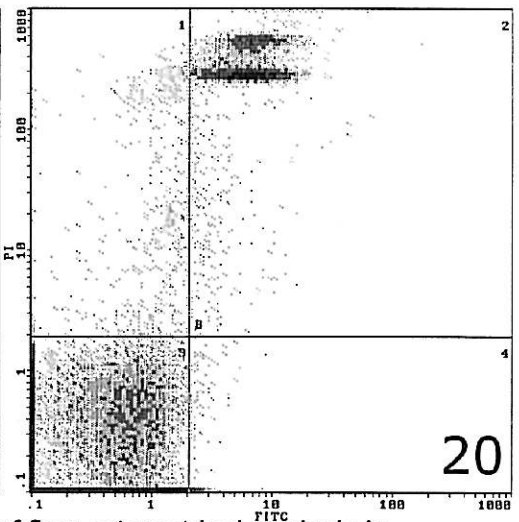
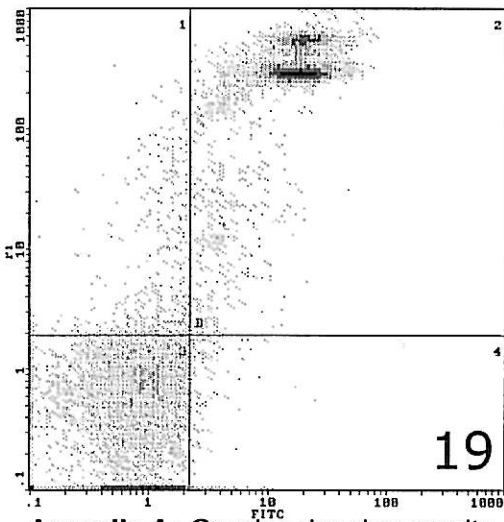
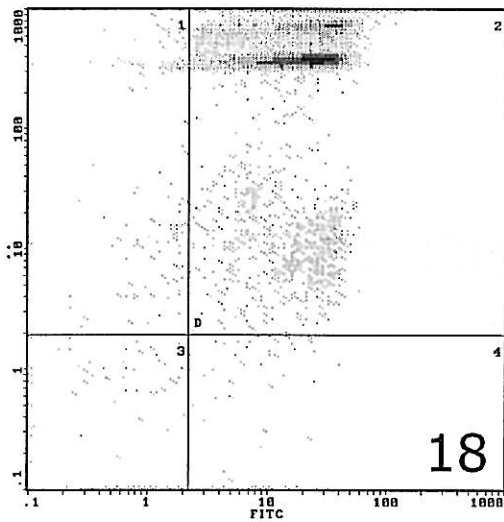
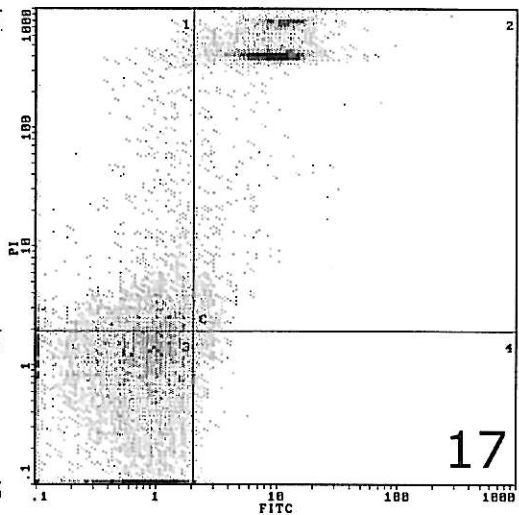
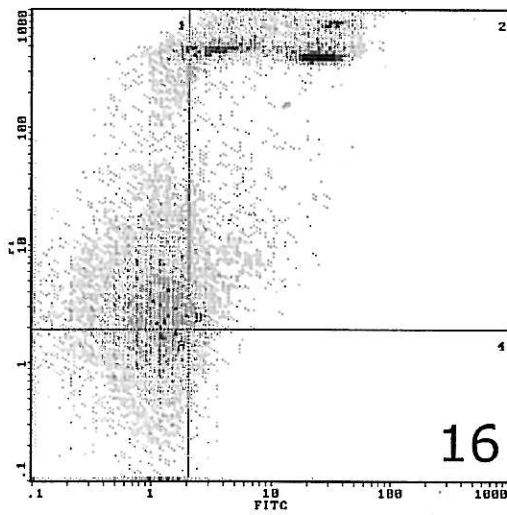
8 - WEHI control sample, **9 -** *Anabaena torulosa* 1A (1%) WEHI,

10 - *Anabaena torulosa* 1A (0.1%) WEHI.



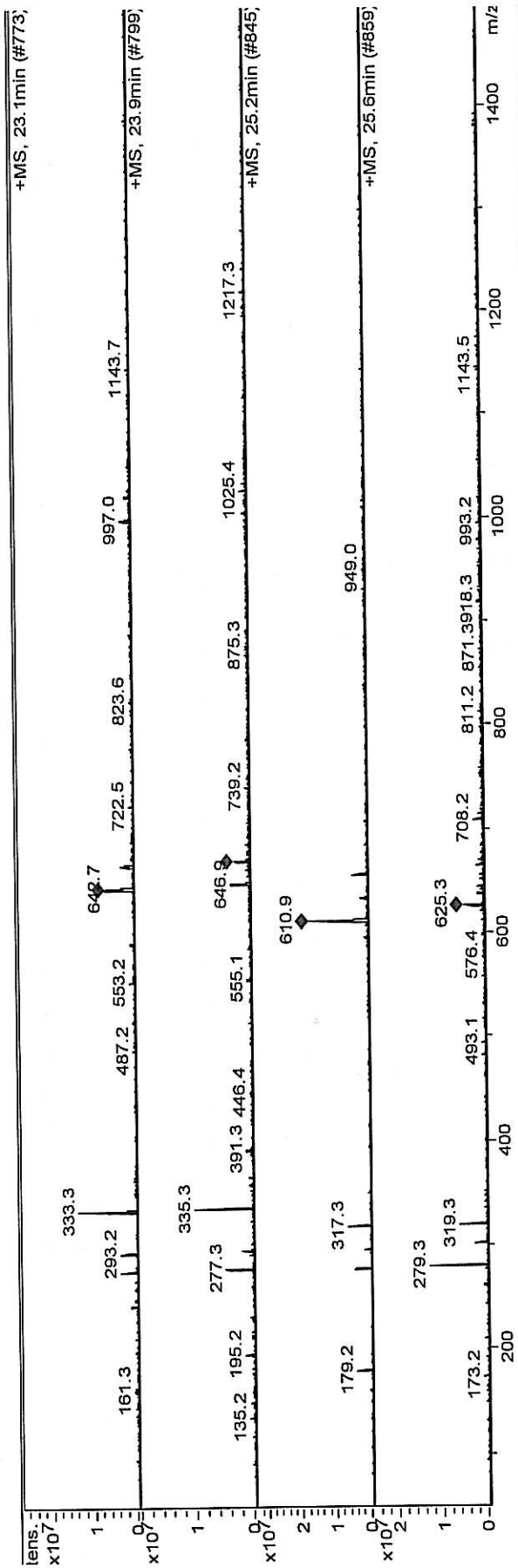
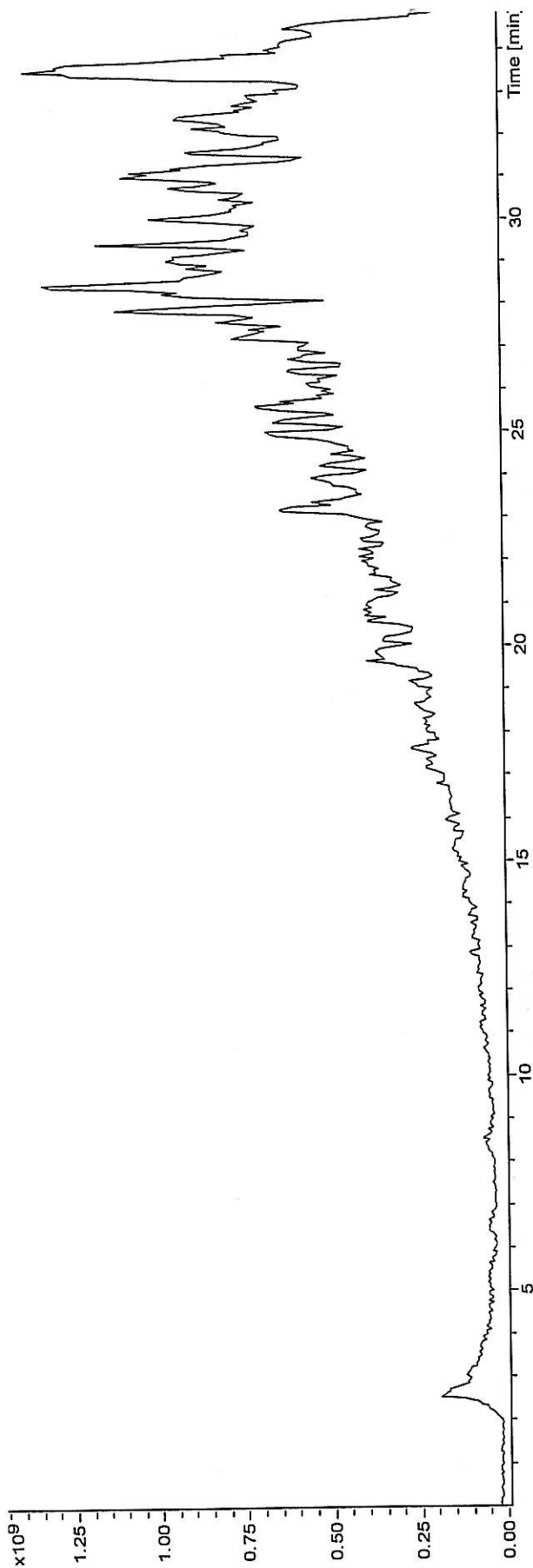
Appendix A: Graphs showing results of flow cytometrical analysis in YAC-1 and WEHI cell line, florescence of cells in propidium is plotted on X-axis, florescence of cells in Anxin-V-flos is plotted on Y-axis.

- 11-** *Anabaena conoidea* 3A (1%)WEHI, **12-** *Anabaena conoidea* 3A(1%)WEHI,
- 13-** *Nodularia* sp.4N (1%)WEHI, **14-** *Nodularia* sp.4N (0.1%)WEHI,
- 15-** *Trichormus variabilis* 5T (1%)WEHI, **16-** *Trichormus variabilis* 5T (0.1%)WEHI

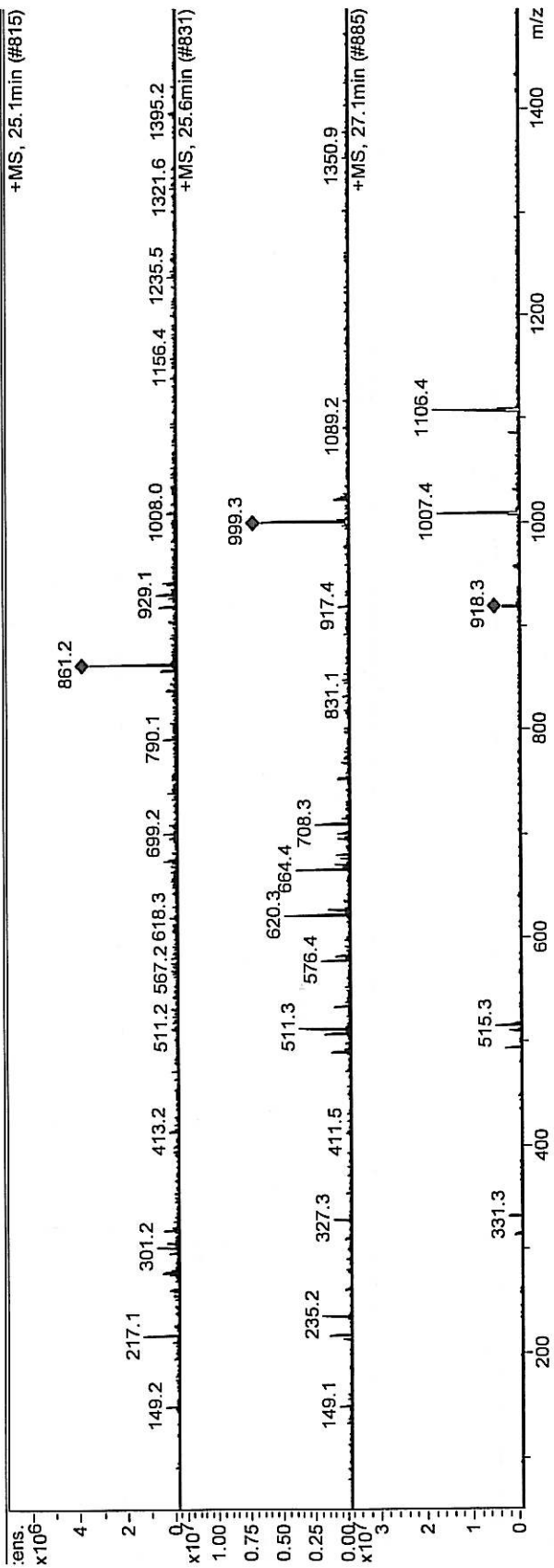
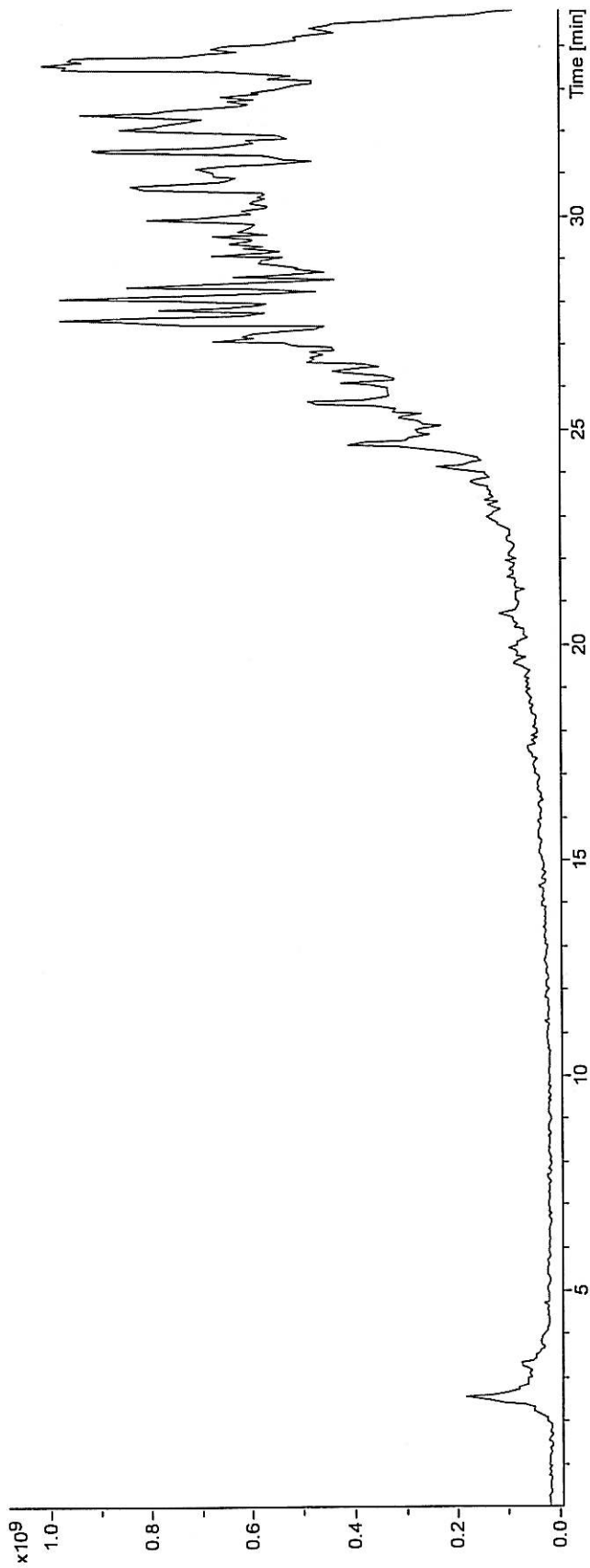


Appendix A: Graphs showing results of flow cytometrical analysis in WEHI cell line, florescence of cells in propidium is plotted on X-axis, florescence of cells in Anxin-V-flos is plotted on Y-axis.
16 - *Cylindrospermum* sp. 10C (1%)WEHI, 17 - *Cylindrospermum* sp. 10C (1%)WEHI, 18 - *Cylindrospermum* sp. 11C (1%)WEHI, 19 - *Calothrix* sp.15 Ca (1%)WEHI, 20 - *Calothrix* sp.15 Ca (1%)WEHI.

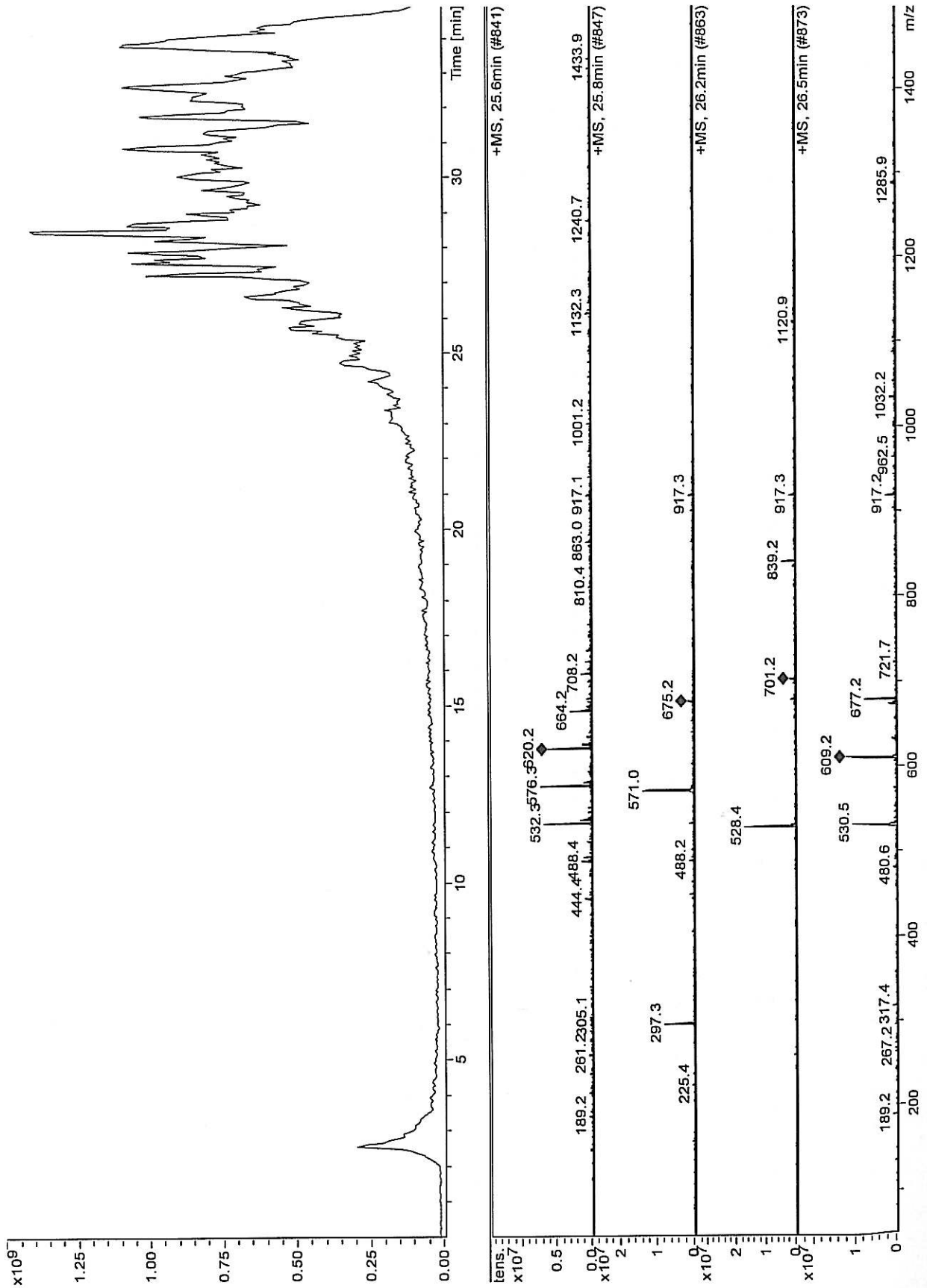
Appendix B
(HPLC-MS Chromatograms)



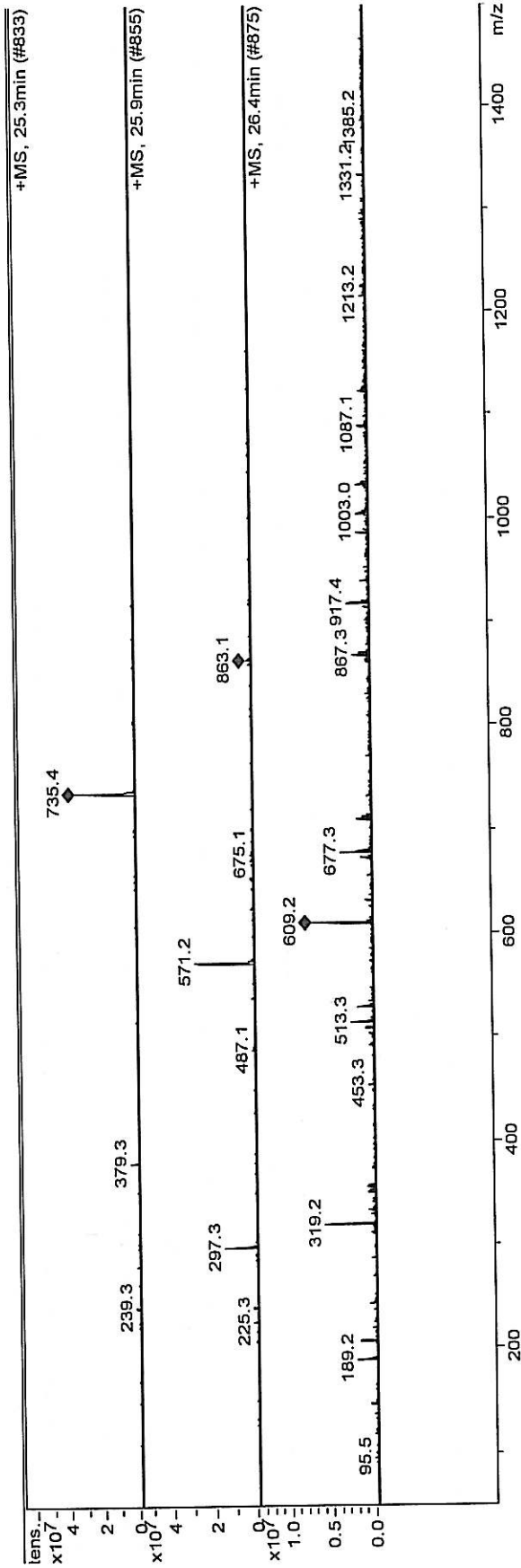
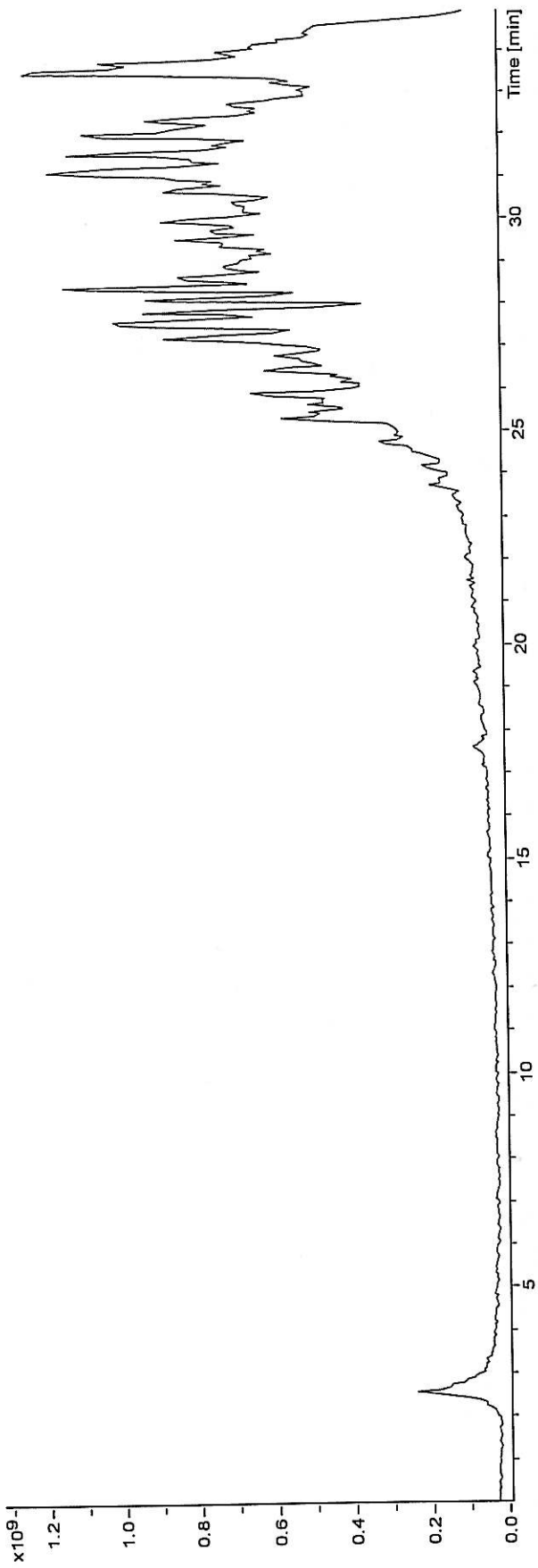
Appendix B-1: HPLC-MS diagram of *Anabaena torulosa* 1A extract.



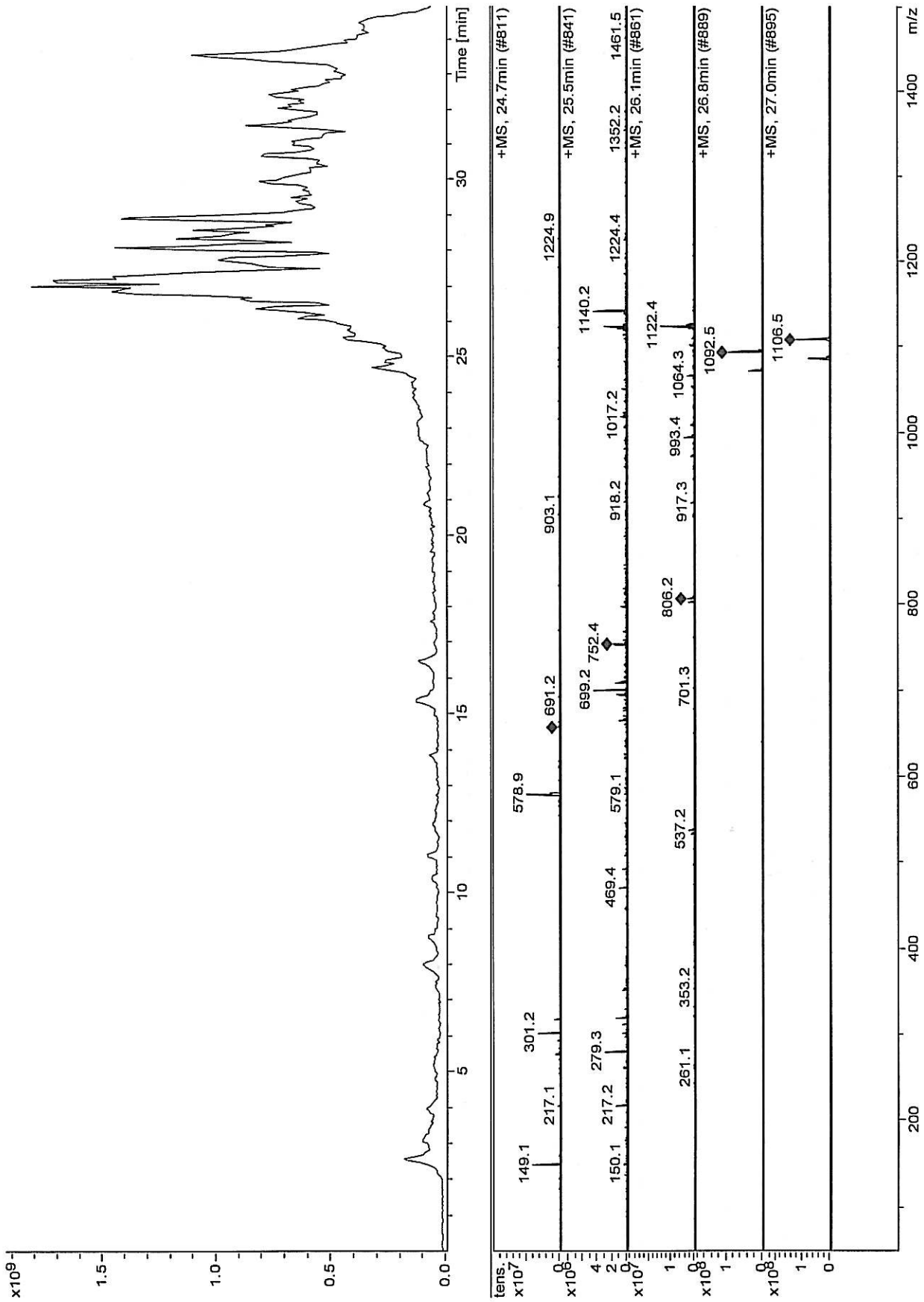
Appendix B-2: HPLC-MS diagram of *Anabaena sphaerica* 2A extract.



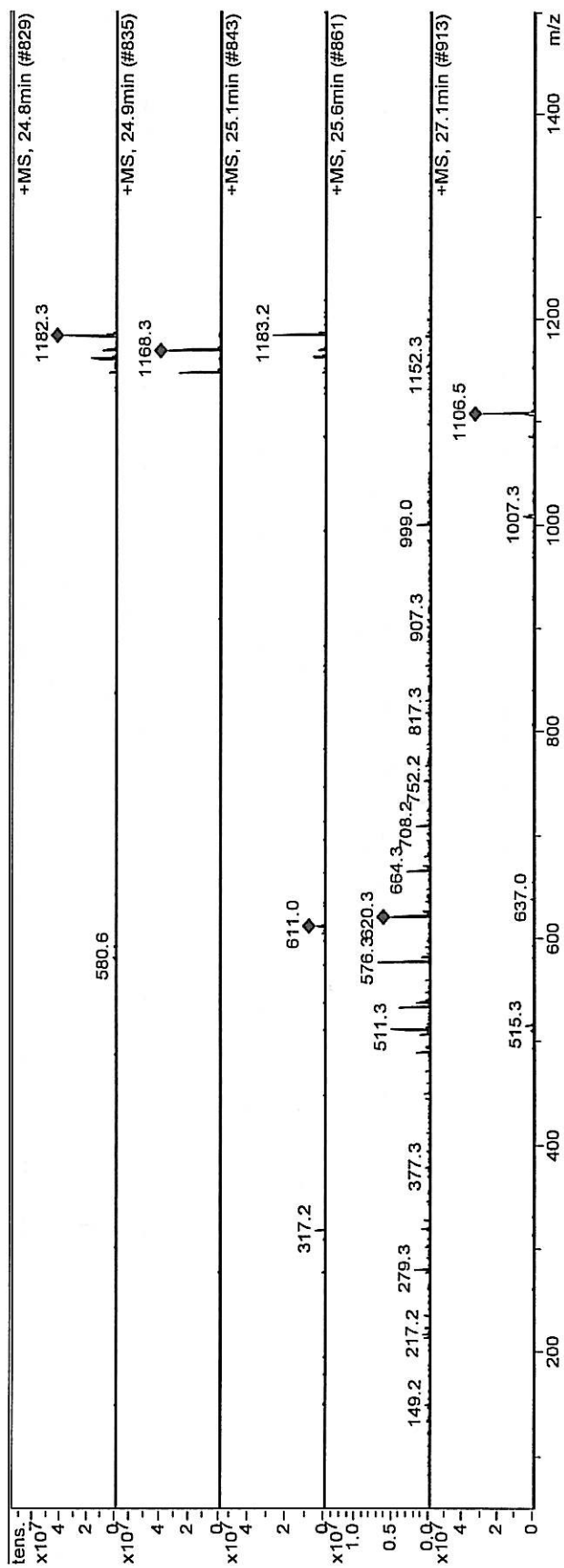
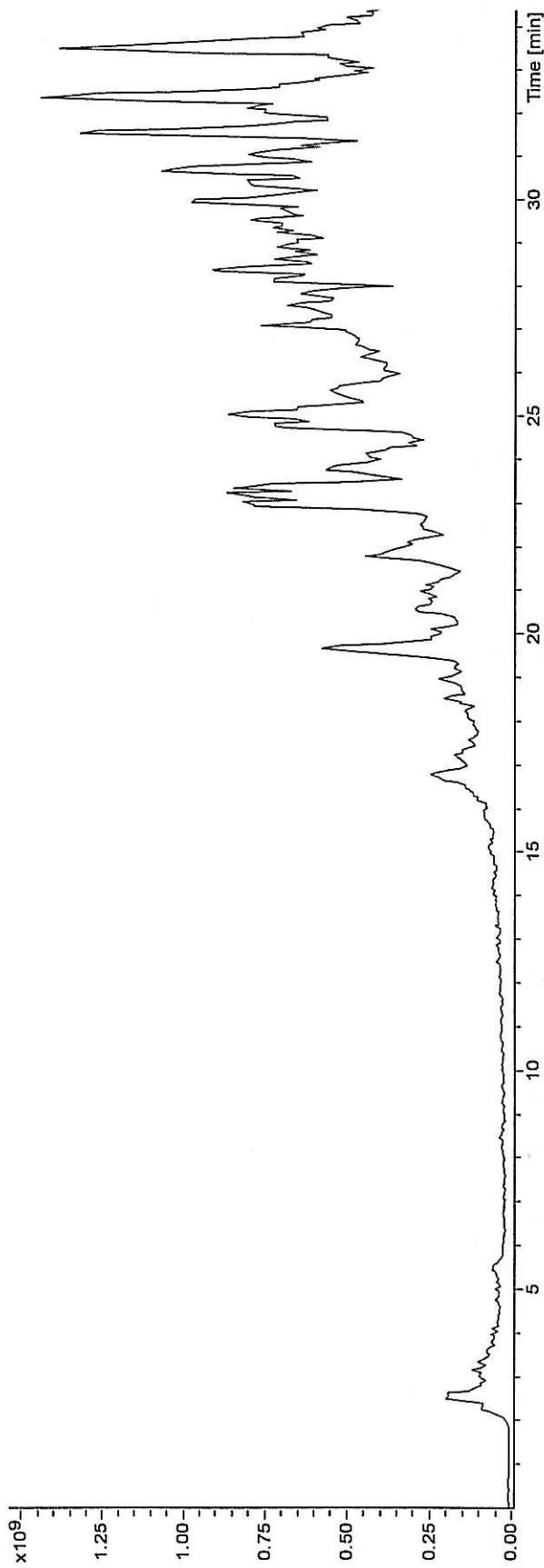
Appendix B-3: HPLC-MS diagram of *Trichormus variabilis* 5T extract.



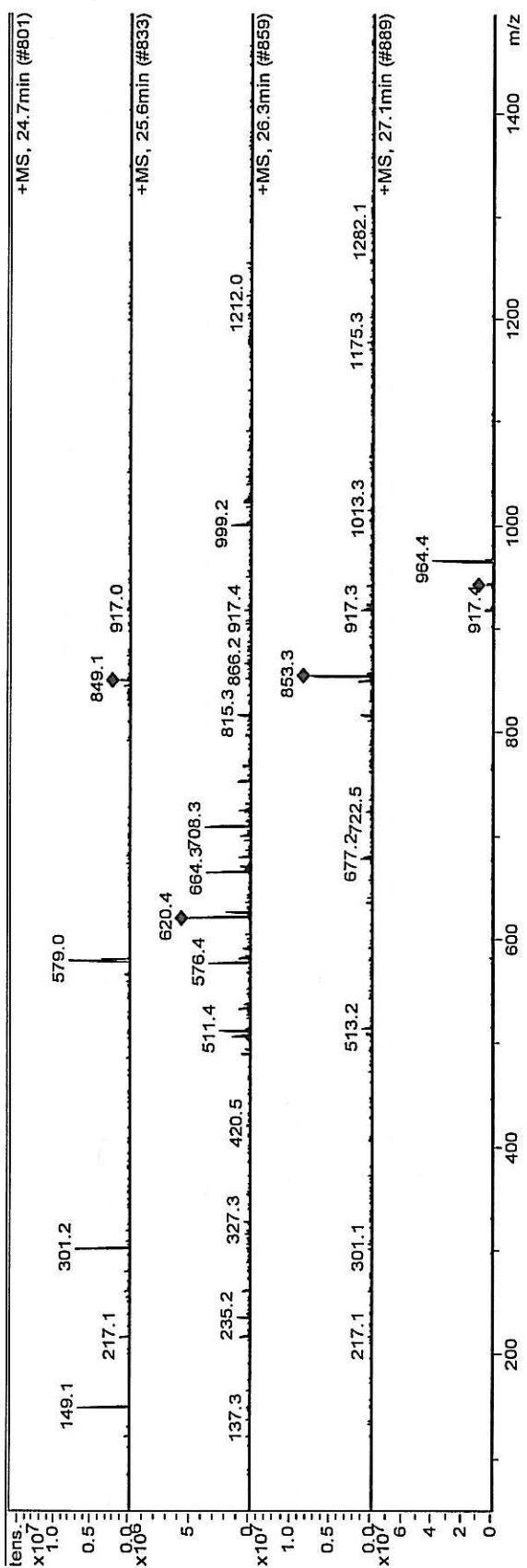
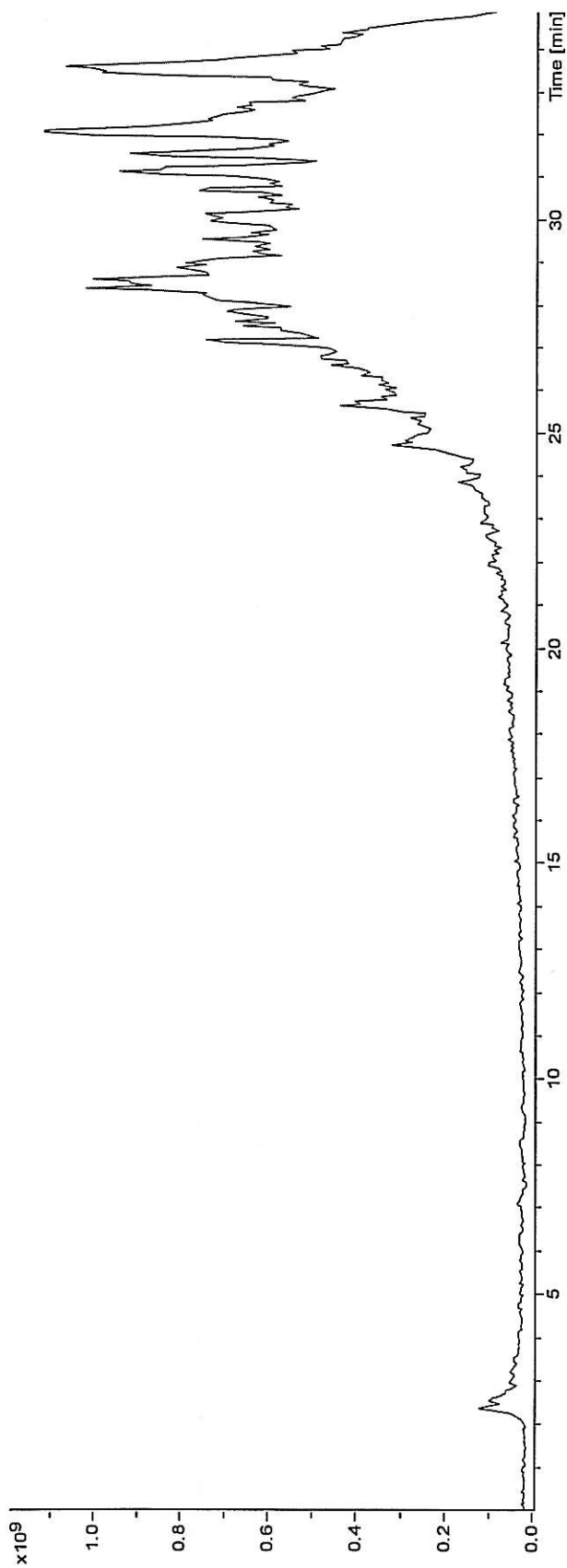
Appendix B-4: HPLC-MS diagram of *Trichormus variabilis* 6T extract.



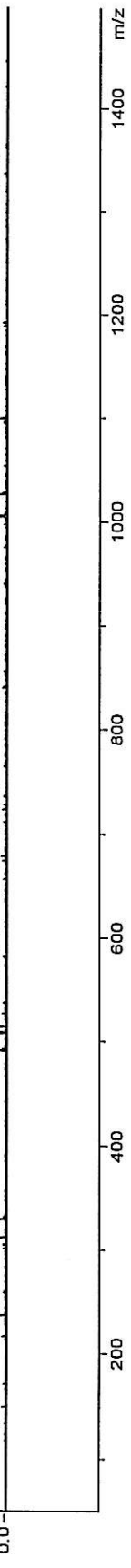
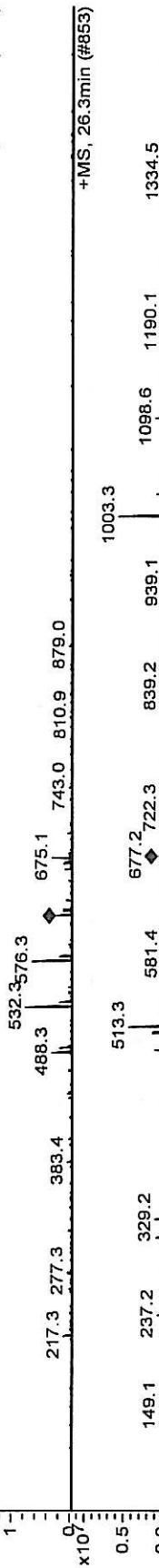
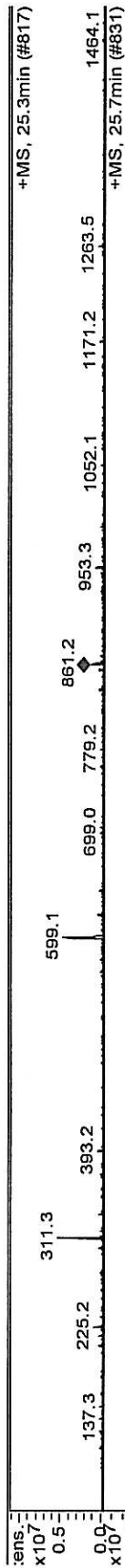
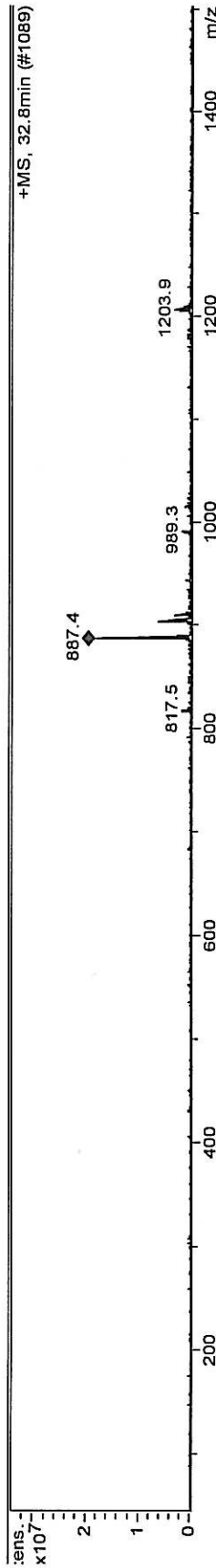
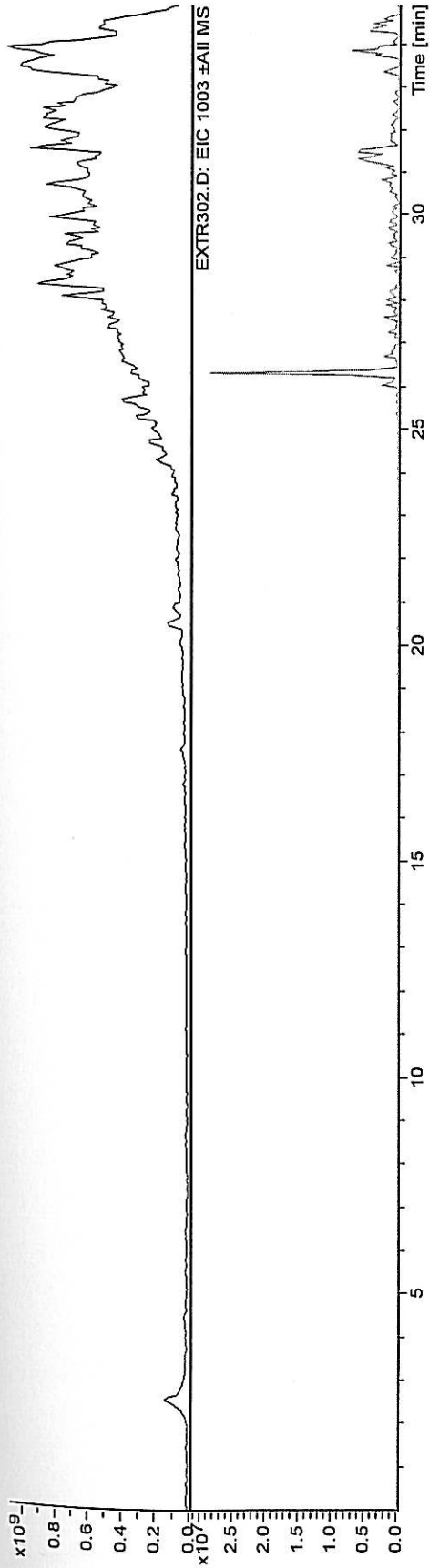
Appendix B-5: HPLC-MS diagram of *Cyindrospermum sp. 10C* extract.



Appendix B-6: HPLC-MS diagram of *Cylindrospermum* sp. 11C extract.



Appendix B-7: HPLC-MS diagram of *Calothrix parietina* 15Ca extract.



Appendix B-8: HPLC-MS diagram of *Calothyrix sp.* 16Ca extract.