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**MOLECULAR MARKERS FOR THE
IDENTIFICATION OF THE CYANOTOXIN-
PRODUCING STRAINS OF GENUS *MICROCYSTIS***

- PhD thesis abstract -

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1. Introduction

In many places in the world, surface waters are used as drinking water resource and the quality of these waters is very variable. Cyanobacteria represent an important part of the phytoplankton in surface waters, toxic species being frequently encountered.

Toxins from cyanobacteria (cyanotoxins) are resistant to boiling, are too small to be retained by filters and are resistant also to conventional treatments. The importance of studying cyanotoxins in drinking water supplies, as well as in pisciculture was recently outlined after adopting the so-called „guide values” by the World Health Organization for microcystin LR and for cylindrospermopsins, these being the most frequent cyanotoxins encountered. Several countries have adopted these standards, developing certain methods for water monitoring alongside with analytical methods for qualitative and quantitative testing of cyanotoxins.

The need of strict monitoring of cyanobacterial toxins was highlighted after a series of tragic cases in which a great number of people have lost their lives after intoxication with these metabolites. There are two aspects of cyanobacterial toxicity which need a special attention: the possible long-term effects on the population exposed to small quantities of toxins and the effects of intermittent exposure to an increased concentration of toxins.

There are 3 types of cyanotoxins: hepatotoxins, neurotoxins and dermatotoxins. Cyanobacterial hepatotoxins (microcystins and nodularins) are produced by a great number of species, included in four distinct orders: Chroococcales, Oscillatoriales, Stigonematales și Nostocales. Microcystins are protein-phosphatase 1 and 2A inhibitors, affecting primarily the liver of superior organisms, often leading to severe liver diseases or even death (Carmichael, 1994, Carmichael, 1997, Pouria et al., 1998). Subchronic exposure to microcystins is associated with accelerated tumor development, the toxins being a co-factor in cancer tissue proliferation (Ito et al., 1997).

Due to all of these aspects, it is essential to be able to identify toxic blooms from their early stages so that their unwanted effects can be avoided. For this, a series of methods were developed to detect and quantify the microcystins, which differ by the work procedure and necessary equipment, as well as by the costs involved. Some of these

methods are: intraperitoneal injection of lab mice, HPLC (High Performance Liquid Chromatography), MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization – Time Of Flight - Mass Spectrometry), ELISA (Enzyme-Linked Immunosorbent Assay), identification of microcystins using the Immuno Gold technique or the protein-phosphatase inhibition tests (PPIA – Protein Phosphatase Inhibition Assay). These are more or less accurate, the cost of reagents and equipment being the primary impediment in using them. This was one of the reasons which lead to the attempt of evaluating the toxic potential of cyanobacterial strains by analysing the gene cluster responsible for microcystin synthesis (Neilan et al., 1999, Kaebernick et al., 2000, Hisbergues et al., 2003, Jungblut and Neilan, 2006).

The first part of this PhD thesis presents the information written in the literature regarding the cyanobacterial species with toxic potential, the chemistry and toxicity of the most relevant cyanotoxins, as well as the possible molecular markers which can be used for easy and rapid discrimination between toxic and non-toxic strains. The second part presents the results of the phylogenetic analysis carried out on 24 cyanobacterial strains belonging to the *Microcystis* genus belonging to the Algae and Cyanobacteria Culture Collection from the Institute for Biological Research, Cluj-Napoca. This part also presents the strains' morphologic analysis by light and electron microscopy, as well as the taxonomic studies based on repeated genomic sequences. The last part is focuses on the results of the toxic potential study of the 24 strains. The analyses performed allowed the precise identification of microcystin producing strains, as well as the types of toxins that confer their toxic potential. The most important result of this paper consists in the possibility of precise identification of toxic cyanobacteria directly from the field samples, the usefulness of this consisting in avoiding the intoxication of humans and animals which get in contact with cyanobacterial blooms.

2. Objectives

The goals of this thesis:

- The morphologic study of the 25 *Microcystis* strains from the AICB Collection (ICB Cluj)
- The taxonomic and phylogenetic analyses based on repeated genomic sequences and on the sequence for the 16S rRNA gene
- The estimation of the toxic potential of the strains
- Design of a method for the precise and quick detection of toxic cyanobacteria from cultures and from environment

3. Material and methods

3.1 Taxonomic and phylogenetic studies

In this work 25 cyanobacterial strains belonging to genus *Microcystis* were used: AICB 34, AICB 35, AICB 36, AICB 318, AICB 619, AICB 620, AICB 644, AICB 679, AICB 680, AICB 681, AICB 682, AICB 688, AICB 689, AICB 695, AICB 697, AICB 702, AICB 746, AICB 747, AICB 748, AICB 822, AICB 823, AICB 826, AICB 827, AICB 832 and AICB 833. All these strains are kept in test tubes containing liquid BG11 growth medium (Dragoş et al., 1997). The strains were isolated from single colonies, using a micromanipulator.

Morphologic investigation of the *Microcystis* strains

The molecular studies were preceded by some morphologic analyses, based on the cyanobacterial cells observation in light and electronic microscopy.

For light microscopy the cells were analyzed with a Nikon TE 2000 U inverted microscope. The pictures were taken with a Nikon D 200 digital camera, with 12 Mpx resolution.

For the observation of the details inaccessible for the light microscope I have used the Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). SEM allows the precise analysis of the surfaces, whereas TEM permits the observation of cells structural details.

DNA extraction

The genetic material was isolated by two methods: a classical one, involving various buffers, and a rapid one, based on the use of commercial kits provided by certain suppliers. The classical method is based on using phenol, chloroform and isoamyl alcohol for DNA purification. After the spectrophotometric measurement of DNA, the concentration was adjusted to 50 ng/ μ l.

Analysis of genomic repeated sequences

The amplification of genomic repeated sequences was accomplished with a Beckman thermocycler. In order to amplify the HIP1 sequences I used specific primers, with two nucleotides added to the 3'-OH end. The distance between the electrophoretic bands was measured (LuciaNet 1.20), and with these measurements I have obtained a dendrogram which has grouped the *Microcystis* strains according to electrophoretic

patterns. The dendrogram was obtained with the STATISTICA 6 software, using the UPGMA algorithm („Unweighted Pair Group Method with Arithmetic Mean”), which links the strains according to the presence/absence of the bands.

Molecular biology studies

These studies were represented by the bioinformatics analysis of the 16S rRNA gene and of ITS (Internal Transcribed Spacer), the fragment between the 16S rRNA and the 23S rRNA genes. The amplified fragments were cloned in *E. coli* cells, in the pGEM-T vector (Promega), and the colonies containing recombinant plasmids were isolated through blue/white selection. The sequencing was accomplished with a Beckman CEQ 8800 Genetic Analysis System, at the Institute for Interdisciplinary Experimental Research of the “Babeş-Bolyai” University. The quality of resulted sequences was estimated by analyzing the chromatograms (VectorNTI software). Thus, the genic fragments were concatenated in order to obtain the whole genes sequences. Then I have multiple align (MEGA4 software), for the obtaining of the conservation regions which were used for the reconstruction of the phylogenetic tree. This was generated using all the 16S rRNA gene sequences of the AICB strains together with other similar sequences from the NCBI database. As an outgroup I used the sequence of an *Escherichia coli* strain.

3.2 The study of the toxic potential

The intraperitoneal injection of laboratory mice

In this study the intraperitoneal injection of laboratory mice with cyanobacterial material was the first test for the estimation of the hepatotoxic potential of the AICB strains. For this, 24 strains were grown in BG11 culture medium, under continuous agitation. The material injected to mice was quantified through the Lowry method. The mice which have died 5-8 hours after the injection were dissected for the analysis of the liver and kidneys, while the other mice were dissected with the same purpose. I looked on the general appearance of the organs, their color being a sign of the intoxication. The organs were weighted, their mass being related to the mice weight before being injected.

The MALDI-TOF test

In order to accomplish the MALDI-TOF MS test I obtained a series of cyanobacterial cultures similar to those needed for the intraperitoneal injections. The centrifuged cyanobacterial material was lyophilized for 48 hours, at -60°C. The MALDI-TOF MS analysis was carried on at the Technical University of Berlin, with a Shimadzu AXIMA Confidence device. As a matrix a mix of 2,5-dihydroxybenzoic acid (75 mg / ml of acetonitrile:ethanol:water 1:1:1 mix acidified with 1% trifluoroacetic acid).

The PCR amplification of certain genic regions involved in toxicity

For the astimation through PCR of the AICB strains toxic potential I have used certain primers from the literature (Neilan et al., 1999; Kaebernick et al., 2000; Jungblut and Neilan, 2006; Hisbergues et al., 2003; Tillet et al., 2001). These primers are specific for 5 of the 10 genes with a role in the microcystins biosynthesis. According to the primers annealing temperature I have used adequate temperature cycles.

Amplicons analysis by DGGE and their sequencing

The purpose of the DGGE analysis of the PCR amplicons consisted in trying to identify a connection between the DGGE profiles and *Microcystis* strains toxicity, as long as the PCR has generated electrophoretic bands both in the toxic strains, but also in some of the non-toxic ones. For this I have used the mcyDF2-mcyDR2 primer pair, with a 28-nucleotide GC fragment added to the 5' end of the forward primer. For the gel I used a 30%-70% gradient, the DNA fragments being migrated for 16 hours, at 100 V. Eventually the gels were stained with ethidium bromide, and captured with an Olympus C3040 digital camera. The fragments sequencing was accomplished with a Beckman CEQ 8800 Genetic Analysis System, at the Institute for Interdisciplinary Experimental Research of the "Babeş-Bolyai" University.

PCR reaction with the new primers

The melting (T_m) and the annealing (T_a) temperature were estimated for each primer. In the end I have designed a PCR program, which was supposed to generate amplicons in the toxic strains only. The amplicons were migrated in 1% agarose gel , stained with ethidium bromide (5 mg/ml) and captured in UV light with an Olympus C3040 digital camera.

Identification of the microcystin-producing cyanobacteria from environmental samples

For the capitalization of the new primers, it is important for them to be useful in the identification of toxic cyanobacteria not only from cultures, but also from environmental samples. For this, a series of samples were taken from certain locations in Mures county, four of these containing *Microcystis* species. The genomic DNA was amplified by PCR with the new primers, in order to detect the presence of a *mcyD* gene fragment, this being a gene involved in the microcystins biosynthesis. In order to confirm the PCR results, the four samples were lyophilized and analyzed by the MALDI-TOS MS technique.

4. Results and discussions

4.1 Identification of fimbriae by electron microscopy

The strains analysis with the scanning electron microscope has shown the presence of fimbriae on cell surface. The negative staining has allow the calculation of their thickness.

A first observation is that fimbriae cover the cells on the entire surface (fig. 2). In some strains I identified two different types of fimbriae (thin and thick), like in *Synechocystis sp.* (Bhaya et al., 2000). The thickness of the thin fimbriae is about 2-3 nm, while the thick ones have 6-10 nm (fig. 4). But unlike the fimbriae from *Synechocystis sp.*, which are about 5 μm long (Ranta et al., 1983), the ones from *Microcystis* exceed 20 μm (fig. 2).

In some experiments it has been shown that sometimes fimbriae attach one to each other and form characteristic bundles (Nakasugi and Neilan, 2005; Ranta at al., 1984). In this study I have observed the existence of these bundles, but not in all strains (fig. 1). Even if fimbriae attach one to each other, they do this only if they belong to the same cell, and not between different cells. Even so, it can be presumed that fimbriae have a role in the colonies stabilization.

The observations with the transmission electron microscope indicate that some strains have more fimbriae than others. Moreover, in some strains the fimbriae were absent.

With SEM I have detected some extracellular structures which seem to connect the cells (fig. 3), and these could be bundles of fimbriae and mucus.

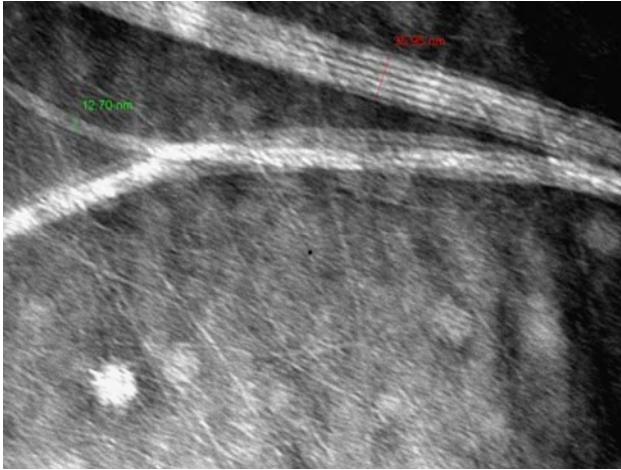


Fig. 1 *Microcystis* sp AICB 695, TEM.

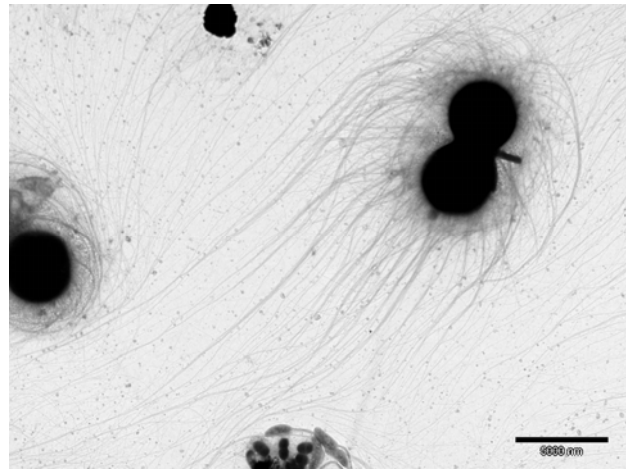


Fig. 2 *Microcystis* sp AICB 754, TEM. Bara = 5 μ m

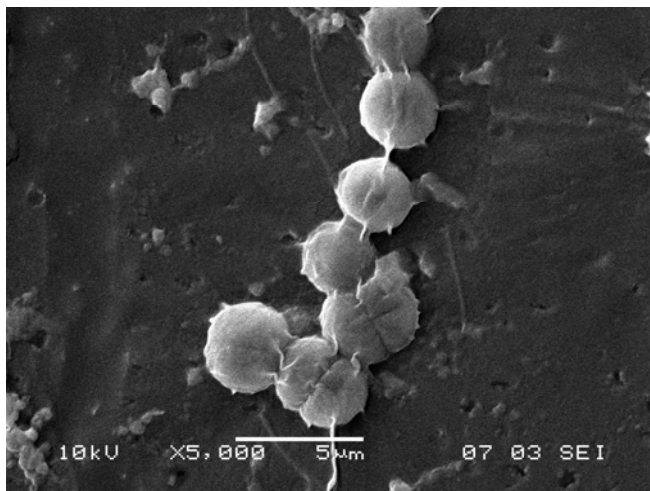


Fig. 3 *Microcystis* sp AICB 318, SEM. Bara = 5 μ m.

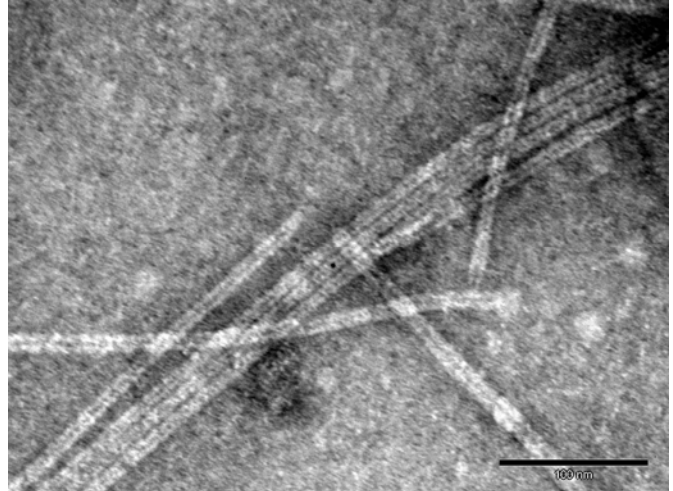


Fig. 4 *Microcystis* sp AICB 318, TEM. Bara = 100 nm

4.2 Analysis of the repeated genomic sequences

The study of the repeated sequences was accomplished by analyzing the electrophoretic patterns obtained subsequent the PCR amplification with specific primers. The electrophoresis gels were captured with an Olympus C3040 digital camera, and the length of the obtained fragments was measured related to the marker bands (FastRuler Middle Range - Fermentas). The bands were very well individualized (fig. 5). All the strains have generated a common band, of 630 bp. The strain AICB 689 has 4 characteristic bands, of 325 bp, 940 bp, 1142 bp and 1224 bp. The strain AICB 748 has

generated a 325 bp fragment, while strains AICB 34, AICB 318 and AICB 689 gave a 940 bp band.

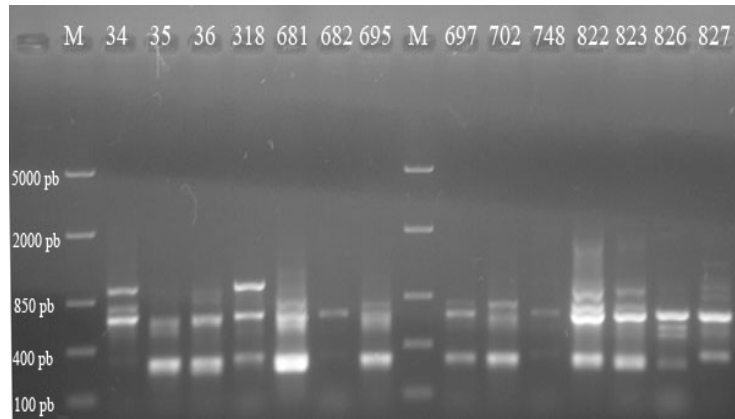


Fig. 5 The electrophoretic pattern obtained subsequent to the amplification of HIP1 genomic repeated sequences with the HIP-AT primer in 14 *Microcystis* strains. M = FastRuler Middle Range marker.

On the basis of resulted measurements I have obtained a dendrogram, using the UPGMA algorithm (fig. 6). Thus, the presence or the absence of electrophoretic bands has lead to an association of the strains in clusters, emphasizing their similarity. The dendrogram does not reflect the evolutionary relations between the strains, as long as the genomic repeated sequences are not phylogenetic markers.

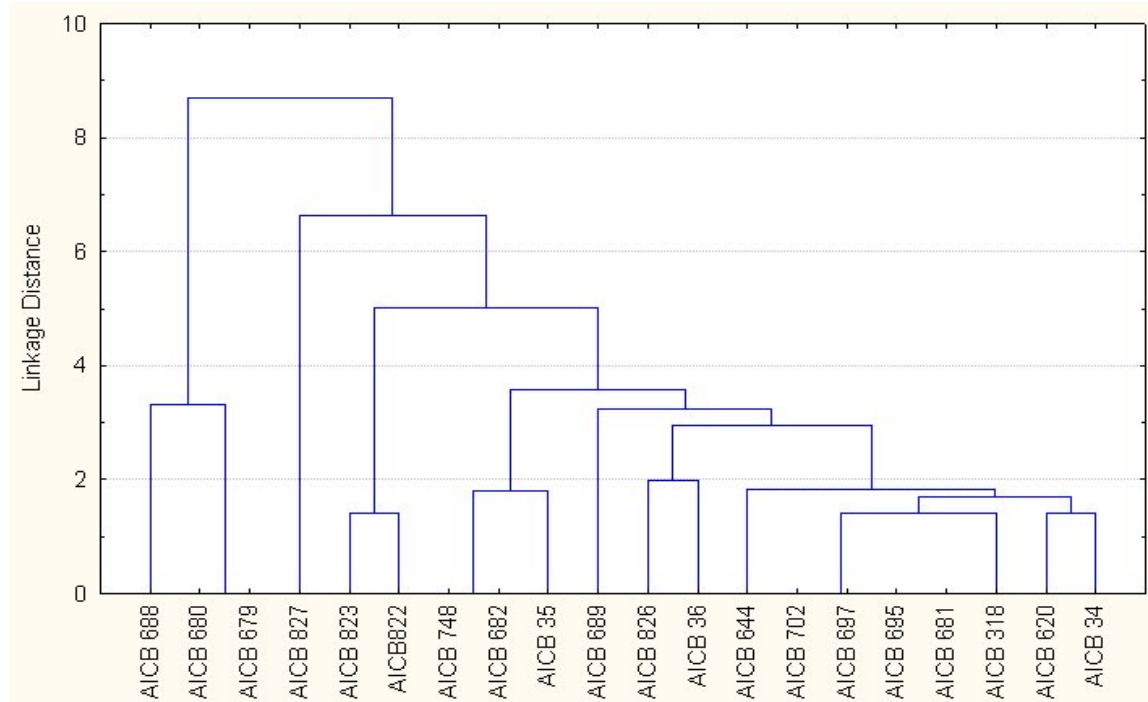


Fig. 6 Dendrogram generated based on the electrophoretic patterns obtained subsequent to the PCR amplification of genomic repeated sequences with the HIP-AT primer. The scale shows the similarity in relative units

In the dendrogram it is obvious the grouping of certain strains which were collected from the same place. For example, strains AICB 679, AICB 680 and AICB 688 are clustered together, they being sampled in 2001 from a lake in Zau de Câmpie, Mureș County. Strains AICB 822 and AICB 823 are grouped together, being collected in 2006 from a lake in Geaca, Cluj County. On the other hand, the dendrogram also clusters some strains originally collected from different locations, in different periods of time: strains AICB 682 and AICB 748, collected in 2001 from Zau de Câmpie, and in 2003, from Lacu, respectively. Moreover, the two strains are clustered together with strain AICB 35, collected from Cătina, Cluj County, in 1980.

With this dendrogram it is again confirmed the fact that the genomic repeated sequences can discriminate between different cyanobacterial strains, even if they are collected from the same location, in the same period of time. It is also obvious that these sequences do not offer information about the evolutionary relations between the analyzed strains, because they are not phylogenetic markers. Thus, in this dendrogram the strains AICB 34 and AICB 620 appear grouped together, as well as well as AICB 702 which is clustered with AICB 681, AICB 695 and AICB 697, while from the phylogenetic analysis it seems that strain AICB 702 belongs to species *Microcystis wesenbergii*, unlike AICB 697.

4.3 Phylogenetic analysis based on the 16S rRNA gene and the ITS fragment

Amplificarea secvenței genei pentru ARNr 16S / ITS cu amorsele 27F și ITER a condus la obținerea unor fragmente de ADN cu mărimea de aproximativ 2000 pb (fig. 7).

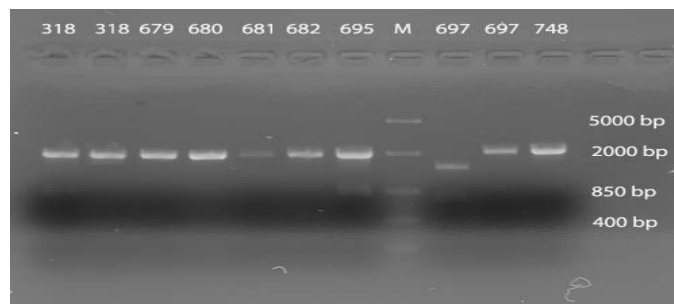


Fig. 7 Electrophoregram of 16S rRNA gene/ITS PCR products amplified with specific primers from 8 *Microcystis* strains. M= FastRuler Middle Range marker..

Until now, 16S rRNA/ITS genes were sequenced from 18 strains belonging to the Algae and Cyanobacteria Culture Collection from the Institute of Biological Research, Cluj-Napoca: AICB 34, AICB 35, AICB 36, AICB 318, AICB 618, AICB 619, AICB 620, AICB 644, AICB 680, AICB 681, AICB 682, AICB 688, AICB 689, AICB 695, AICB 697, AICB 748, AICB 822 și AICB 826. The sequences obtained after amplification with internal primers were assembled with the VectorNTI program. The length of the sequences obtained with different primers varied between 335 and 857 bp, the longest one being amplified from the AICB 619 strain with the HR primer. Initially, I have assembled a great number of sequences obtained with different primers. The number of primers used to amplify the whole sequence of 16S rRNA gene was reduced afterwards. Sometimes, fragments with a common region can be assembled only if an intermediate sequence is present. This is due to the fact that the VectorNTI program assembles sequences only if they have a corresponding region of at least 20 nucleotides.

In some cases (AICB 697 and AICB 822 strains), it was possible to sequence the whole 16S rRNA gene using only 3 primers: HR, FR and CYA 784R (fig. 8). This fact is important due to the costs of reagents necessary in the sequencing reaction, because not before long the amplification of the entire gene was possible only using 5 primers.

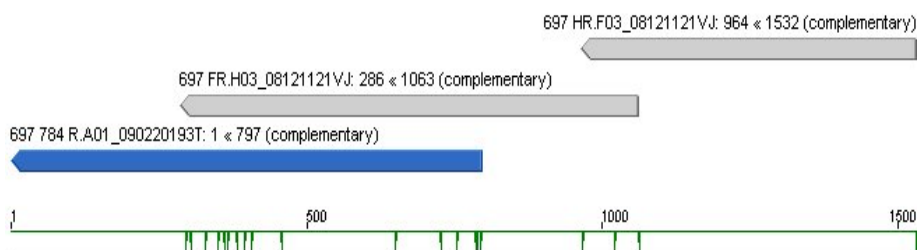


Fig. 8 The 16S rRNA gene sequence from the AICB 697 strain, having 1532 bp, obtained after assembling the fragments obtained after DNA amplification with HR, FR and CYA 784R primers (ContigExpress – VectorNTI program).

Multiple alignment of sequences was done using the MEGA4 program. In the alignment, were used also sequences downloaded from the GeneBank database: 5 strains of *Microcystis* sp. (*M. wesenbergii*, *M. ichtioblabe*, *M. viridis*, *M. aeruginosa* și *M. novacekii*), 1 *Synechocystis* sp. strain, 2 *Arthrospira* sp. strains, 3 *Anabaena* sp. strains, 2

Oscillatoria sp. strains and 2 *Nodularia* sp. strains. The 16S rRNA gene sequence from a strain of *Escherichia coli* was used as outgroup. The Phylogenetic tree was constructed using the MEGA4 program, based on the “Minimum Evolution” algorithm (fig. 9).

The phylogenetic tree shows that all the 18 cyanobacterial strains investigated belong to the *Microcystis* genus. These form a distinct group among the other cyanobacteria, with a bootstrap value of 93. Because of the sequence similarity, validating the inclusion of the strains in a certain specie is difficult. Anyway, we can say that the AICB 702 strain belongs to the *Microcystis wesenbergii* specie, after grouping with a strain of this specie.

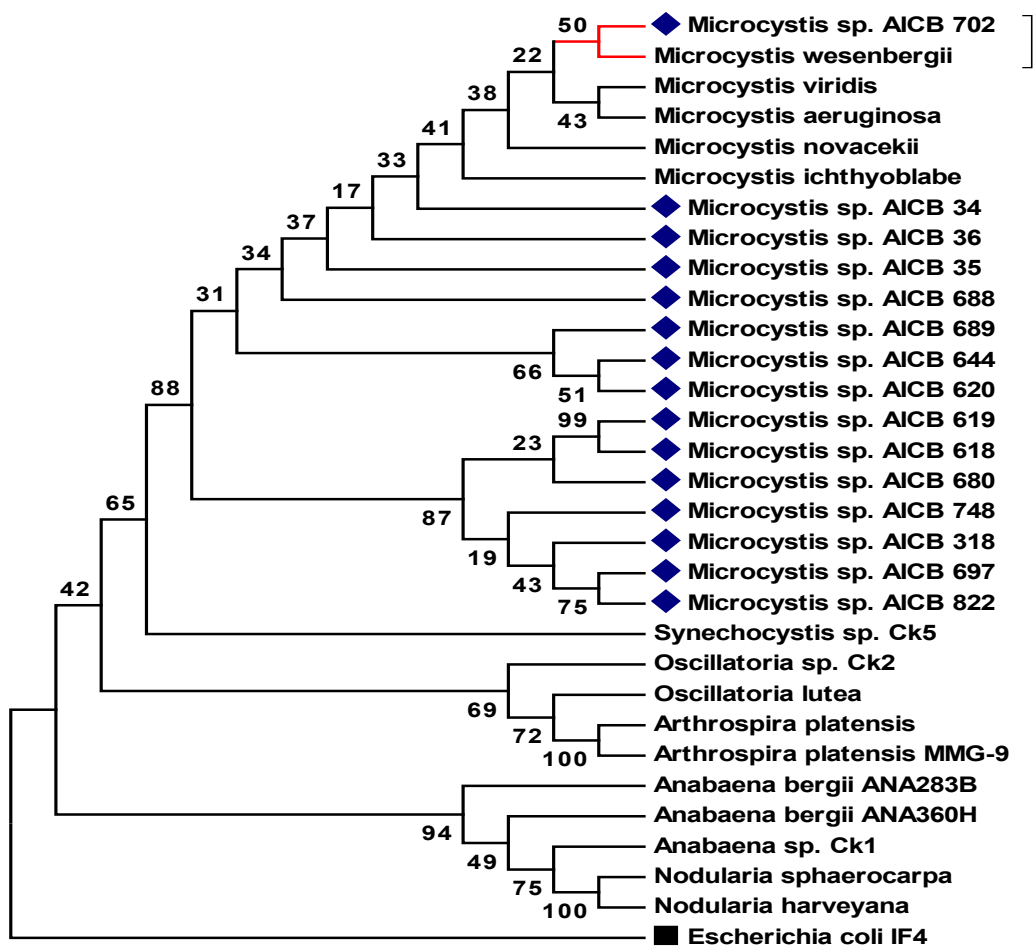


Fig. 9 The phylogenetic tree constructed based on the 16S rRNA/ITS sequences, with the “Minimum Evolution” algorithm. Numbers on the branches show the bootstrap values for 100 replicates. The strains from the AICB collection are marked with blue.

The *Synechocystis* sp., *Arthrospira* sp., *Anabaena* sp., *Oscillatoria* sp. and *Nodularia* sp. strains form distinct groups, proving the fact that the tree is true and expresses accurately the Phylogenetic relationships among the taxons investigated.

4.4 The toxicity tests on mice

Based on the carotene and chlorophyll *a* concentration from the 5 dilutions of cyanobacterial material, two calibration curves were drawn (fig. 10, 11). The related second grade equations allow the estimation of the concentration of these cellular components based on the spectrophotometric measurement of cyanobacterial suspension at 600 nm. The extinction measurement in three samples of each dilution has permitted the obtaining of standard deviation. In carotenes and chlorophyll *a* the R² value exceeded 0.99, certifying the value of the results. In soluble proteins the R² value was only of 0.93.

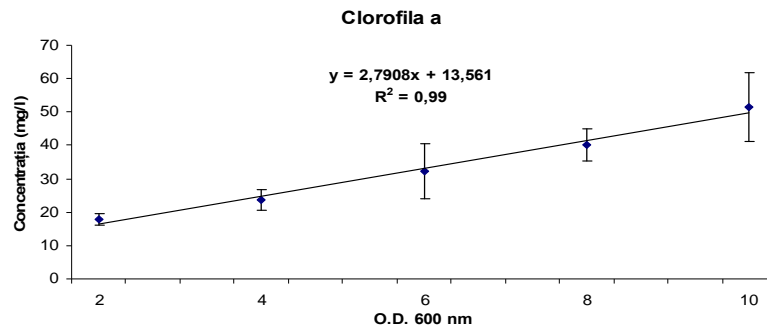


Fig. 10 Calibration curve obtained based on the chlorophyll *a* concentration in 5 different cyanobacterial suspension dilutions.

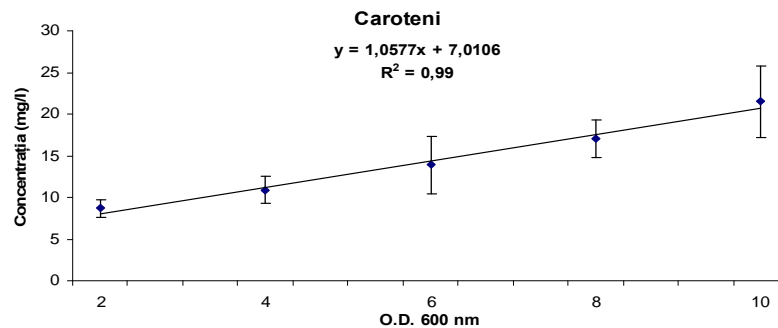


Fig. 11 Calibration curve obtained based on the carotenes concentration in 5 different cyanobacterial suspension dilutions

The intraperitoneal injection of mice with cyanobacterial material concentrated by centrifugation has lead to the death of mice injected with 5 of the 24 strains. These strains

are: AICB 318, AICB 682, AICB 689, AICB 697 and AICB 702. The death has occurred 5-8 from injection. The dead mice were dissected, and their livers and kidney were sampled. The unaffected mice were sacrificed for organs sampling. In both cases, the organs were weighted, and their weight was related to the weight of the each mouse before injection.

The general appearance of the mice affected by microcystins (fig. 12) was obviously different of the one of healthy organs (fig. 13).



Fig. 12 The liver of two mice affected by microcystins, being injected with cyanobacteria (strains AICB 697 and AICB 702)

The color of the affected organs was dark-red, developing colorless swellings. The weight of the livers related to the body weight is greater with 15% than the healthy organs. These modifications of the liver are due to the microcystins mechanism of action, which lead to the apparition of spaces between hepatocytes, these spaces being eventually filled with blood. All these lead to liver failure, and eventually to death.

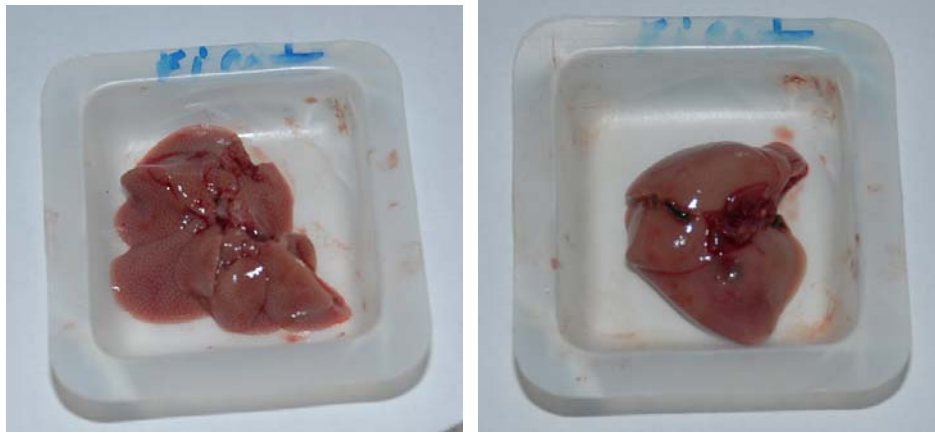


Fig. 13 The liver of two mice injected with non-toxic cyanobacteria (AICB 696 and AICB 823)

The unaffected livers have a normal pink color, and they did not develop the swellings. The kidneys also were not affected by the presence of microcystins. I did not notice significant differences between the weight of kidneys sampled from intoxicated mice and the weight of the kidneys sampled from unaffected mice.

The obtained data were used for the obtaining of diagrams which would reflect the differences between the affected and unaffected organs (fig. 14, 15). It is obvious that the ratio between the weight of the liver and the weight of the body in different in the mice affected by microcystins and the other ones.

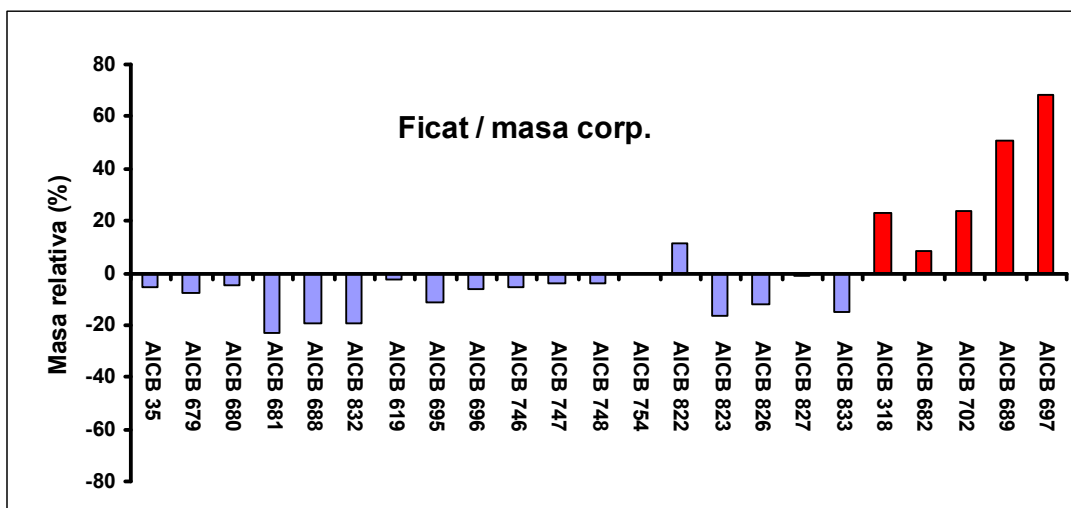


Fig. 14 The graphic representation of the differences between the relative weight of the affected (red) and non-affected liver (blue). Between the two groups there are obvious differences, the relative weight of the affected liver being greater than the one of non-affected organs.

As for kidneys there are no differences to clearly discriminate the group of the affected mice and non-affected ones.

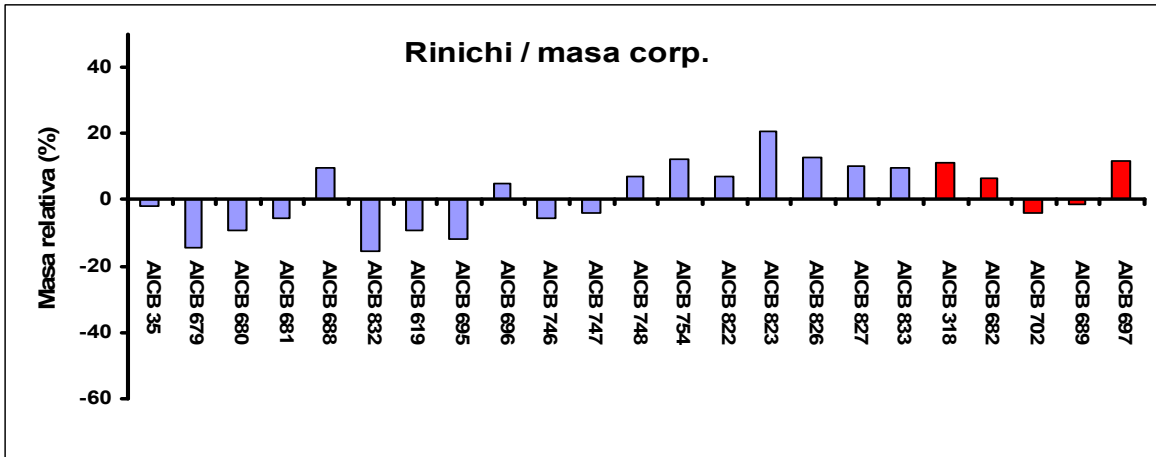


Fig. 15 The graphic representation of the weight of affected (red) and non-affected (blue) mice. There are no obvious differences between the two groups.

The results obtained are even better represented in a graph which displays the differences between the group of the affected and non-affected mice. It is noticed that the liver is the organ most affected by microcystins (fig. 16). As for the liver, the result is statistically significant, the P value obtained with the T Test, being 0.015, while the maximal accepted value is 0.05. On the other hand, as for the kidneys, the P value is 0.38.

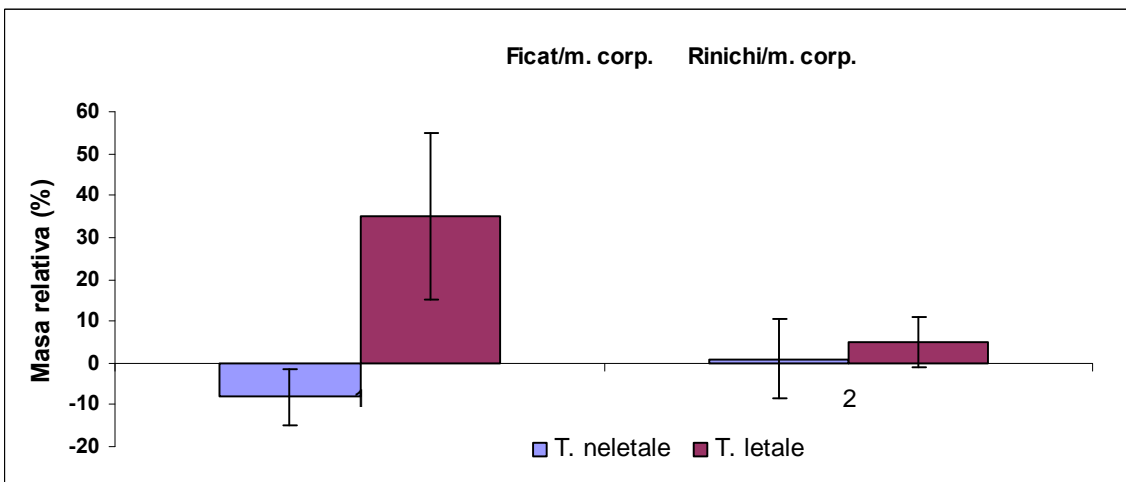


Fig. 16 The difference between the weight of the liver and kidneys of the mice from two groups (affected/non-affected by microcystins), related to the body weight.

The results of this test certify that the intraperitoneal injection of mice with cyanobacteria represents a proper way to identify the toxic potential of the analyzed samples. The appearance of the liver indicates without doubts the hepatotoxic effect of microcystins, excluding the possibility that the mice have died from another cause than the intoxication with these toxins. These results were also confirmed by the second injection test, on 5 mice.

The blood accumulation in the affected liver is emphasized by the weight of these organs related to the body weight. Thus, in the case of mice from the control group and of those unaffected by intraperitoneal injection, the liver represents 5-6% of body weight, while in the mice intoxicated with microcystins the liver is 7.3-12.58 of body weight.

4.5 MALDI-TOF MS spectra

The analysis of lyophilized cyanobacterial samples highlighted the presence of microcystins in 5 of 24 *Microcystis* strains investigated. These are identical to those which proved to be toxic after intraperitoneal injection of lab mice AICB 318, AICB 682, AICB 689, AICB 697 and AICB 702). Two types of microcystins were pointed out: MC-LR, in AICB 682, AICB 689, AICB 697 strains, and MC-RR, in AICB 318 and AICB 702 strains (fig. 17-19).

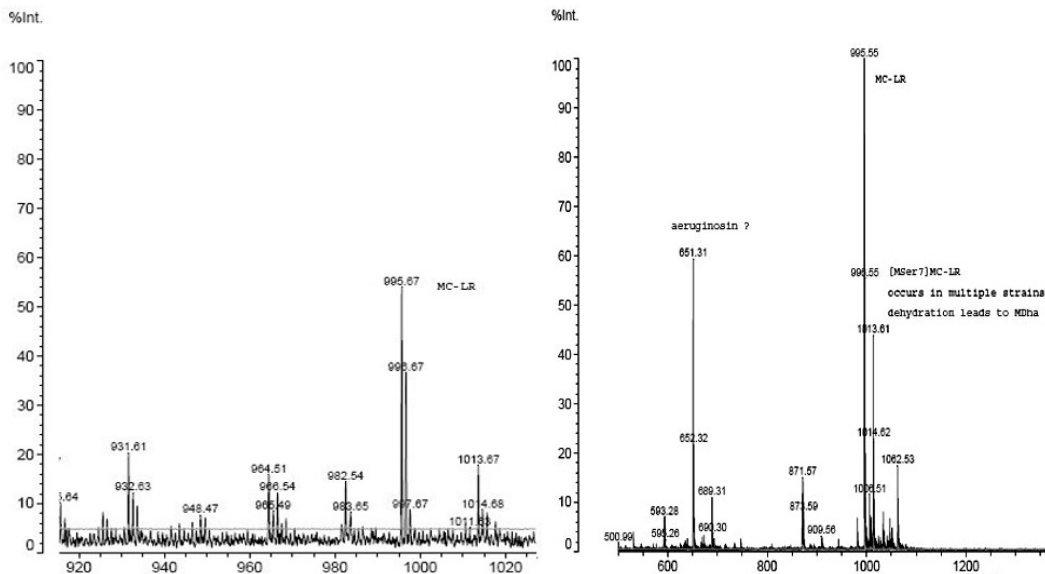


Fig. 17 MALDI-TOF-MS spectra obtained after analyzing the lyophilized cyanobacterial material from the AICB 682 and AICB 689 strains.

Microcystin MC-LR is found in the 995 m/z area, while the MC-RR microcystin generates a response in the 1038 m/z area.

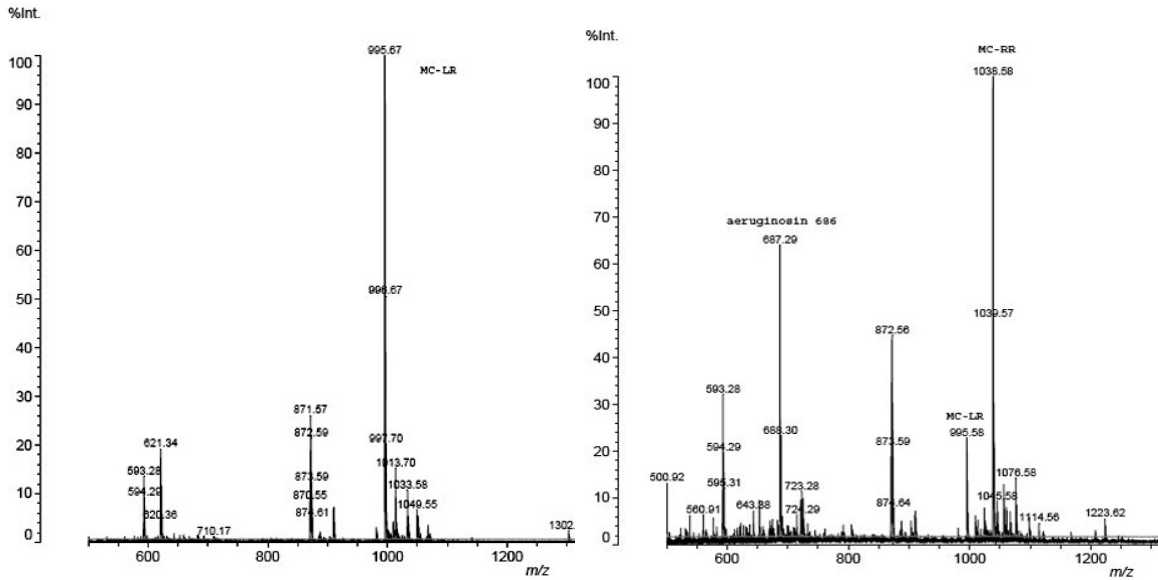


Fig. 18 MALDI-TOF-MS spectra obtained after analyzing the lyophilized cyanobacterial material from the AICB 697 and AICB 318 strains.

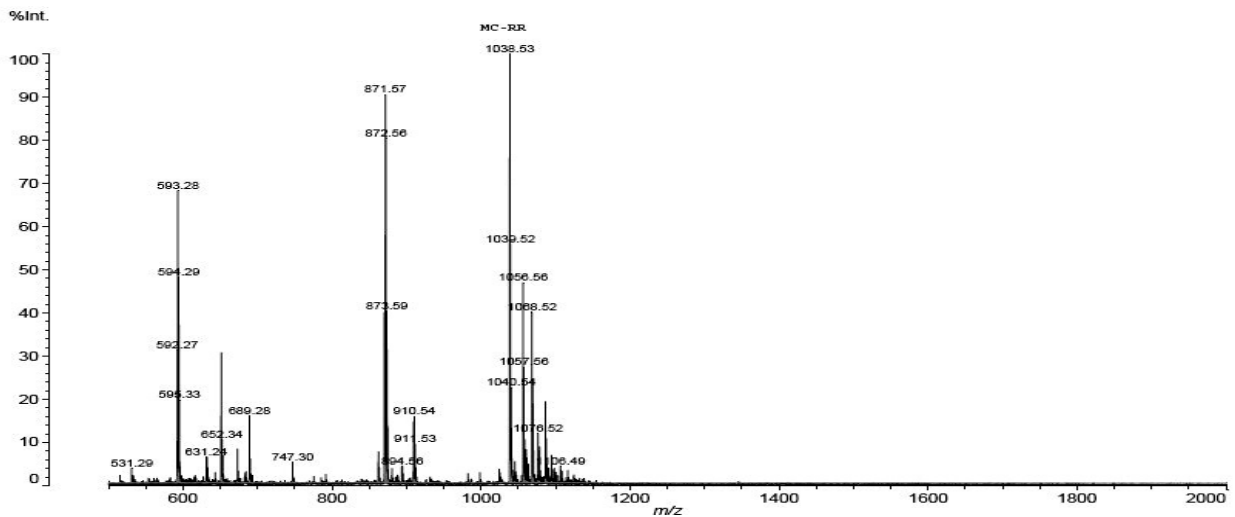


Fig. 19 MALDI-TOF-MS spectrum obtained after analyzing the lyophilized cyanobacterial material from the AICB 702 strain.

The analysis of *Microcystis* strains from the AICB collection confirmed the results obtained after intraperitoneal injection of lab mice with these cyanobacteria.

4.6 PCR amplification using primers taken from literature

The amplification of some toxicity involved DNA fragments with primers taken from scientific literature produced more or less specific and consistent amplification

product. The primer pairs *mcyA*-Cd 1F – *mcyA* – Cd 1R and FAA –RAA did not generate distinct lanes, whereas the TOX2+-TOX2 – pair yielded overloaded electrophoretic profiles with multiple lanes. *McyDF2*-*mcyDR2* and HEPF-HEPR were the most suitable primer pairs for obtaining the most definite electrophoretic lanes for the AICB strains (fig. 20). The generated electrophoretic patterns were similar as for the presence or absence of the lanes, the only difference being the length of amplified fragments.

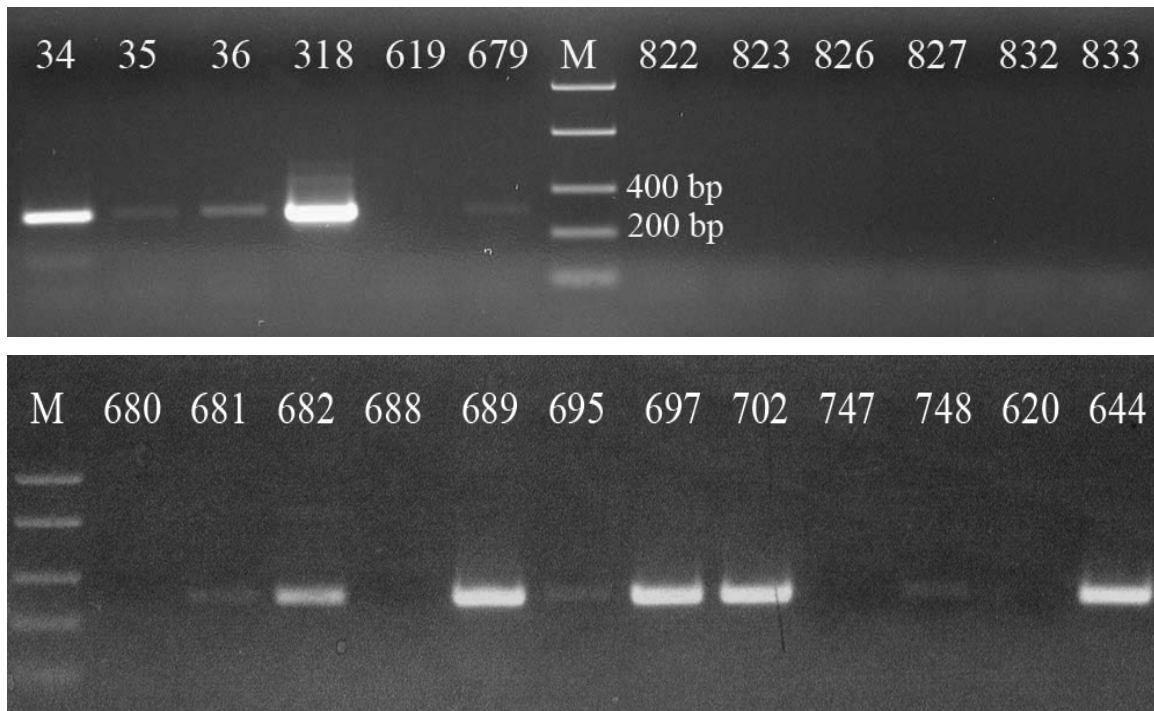


Fig. 20 The electrophoregram of the amplified products obtained by PCR with *mcyDF2*-*mcyDR2* primers, using the genomic cyanobacterial DNA as template.

Using genomic DNA extracted from AICB 34, 318, 682, 689, 697, 702 and 644 strains produced the most intense electrophoretic lanes. Among these, the AICB 644 strains could not be analyzed because it is no longer deposited in the AICB Collection.

It is remarkable the fact that from the 6 strains remained in the AICB Collection that generated obvious amplicons in the electrophoregram (AICB 644 being lost), 5 of them possessed toxic potential (AICB 318, 682, 689, 697 and 702). Although, a very intense lane is observed for the AICB 34, meaning that this strain should be a microcystins producer, the intraperitoneal injection and MALDI-TOF-MS tests

contradicted this presumption. Also, the existence of some thin lanes for other strains (AICB 35, 36, 681 or 702) raised questions regarding the specificity of these primers for the strains with toxic potential. This was the main premises for which I tried, in the first place, to discriminate between PCR amplicons by DGGE, in an attempt to identify a relation between the amplicons position in agarose gel and the toxic potential of *Microcystis* strains.

4.7 Migration of DGGE amplification products

The PCR products obtained with mcyDF2-mcyDR2, have been separated through DGGE, being stopped in certain positions along the gel, depending of the GC percentage of each fragment (fig. 21).

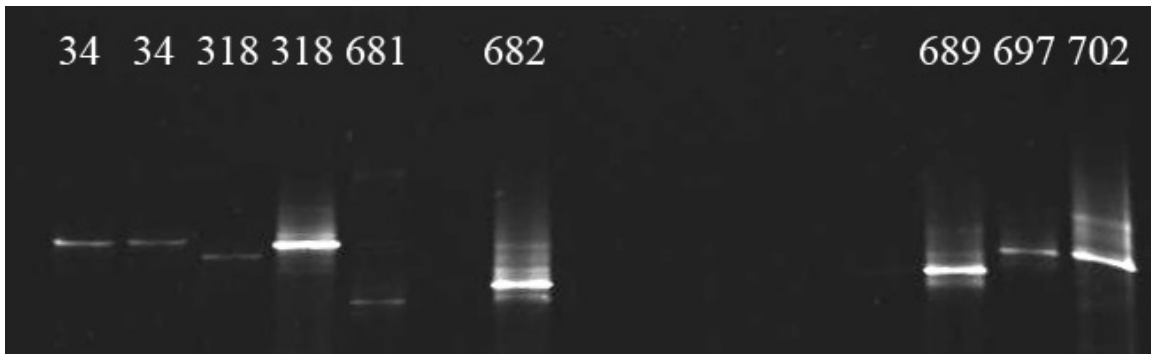


Fig. 21 The DGGE pattern of PCR products obtained with mcyDF2-mcyDR2 primers, using the cyanobacterial DNA as template.

Based on the electrophoretic pattern we could not make an accurate distinction between the toxic and nontoxic strains (AICB 34 AND 681). The migration distance of the amplicons did not permit the observation of a certain correlation between their position and the toxic/nontoxic character of the strains.

4.8 PCR amplification with the new set of primers

The new primers, obtained using the PerlPrimer software (tab. 1) were combined, so that the genetic material provided by each strain was amplified with both possible pairs (MCYD-F1 – MCYD-R1 and MCYD-F1 – MCYD-R2).

Tab. 1

Cele 3 noi amorse obținute pentru amprentarea tulpinilor cu potențial toxic

Gene	Primer	Primes sequence
<i>mcyD</i>	MCYD-F1	5' TCA ATA TCG AGA ACT ATC CC 3'
<i>mcyD</i>	MCYD-R1	5' AGC AAC GGC AAA GTT TAG 3'
<i>mcyD</i>	MCYD-R2	5' CAG CAA CGG CAA AGT TTA 3'

The PCR products electrophoresis in agarose gel produced electrophoretic lanes for all the 5 toxic strains, but showed no evidence of this kind for the nontoxic strains AICB 34 and 681 (fig. 22), as was happened in amplification with primers found in scientific literature (fig.20).

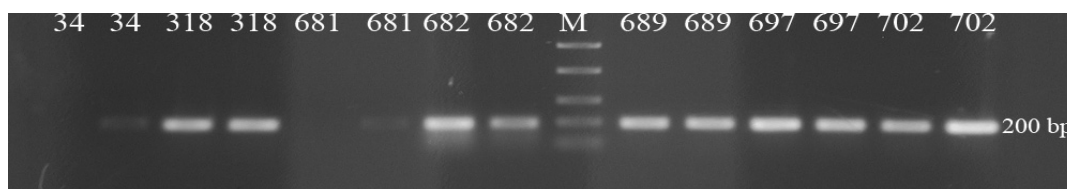


Fig. 22 The amplicons obtained for the gene *mcyD* using the new set of primers. The DNA from each strain was amplified with both primer pairs: F1-R1 and F1-R2.

We could observe the fragment sizes of approximately 200 bp, from the electrophoretic pattern. As for the AICB 34 and 861 strains a weak electrophoretic lane of approximately 200 bp was obtained with the MCYD-F1 – MCYD-R2 pair. Instead of it, no amplicon was obtained in the PCR reaction with the MCYD-F1 – MCYD-R1 primers. Thus, this primer pair proved to be the most suitable for the characterization of microcystins producing strains belonging to the *Microcystis* genus.

4.9 Identification of toxic cyanobacteria from lakes and ponds

The PCR amplification of a *mcyD* fragment with MCYD-F1 - MCYD-R1 primers using as template genomic DNA derived from samples generated amplification products in each of the four cases (fig. 23).



Fig. 23. The PCR amplification pattern of a fragment approximately of 200 bp from cyanobacterial samples collected from the environment: M=marker; T-Z=Tăul Zaului; Z-L=Zau de Câmpie, Lake; Z-B=Zau de Câmpie, fishpond.

The PCR reaction produced fragments of approximately 200 bp long, similar to the amplification pattern obtained with toxic AICB strains (fig. 22), which theoretically means that all the four samples contained toxic, microcystins producing cyanobacteria.

For the toxicity confirmation, the four samples were analyzed by MALDI-TOF MS technique, the results being shown in fig. 24.

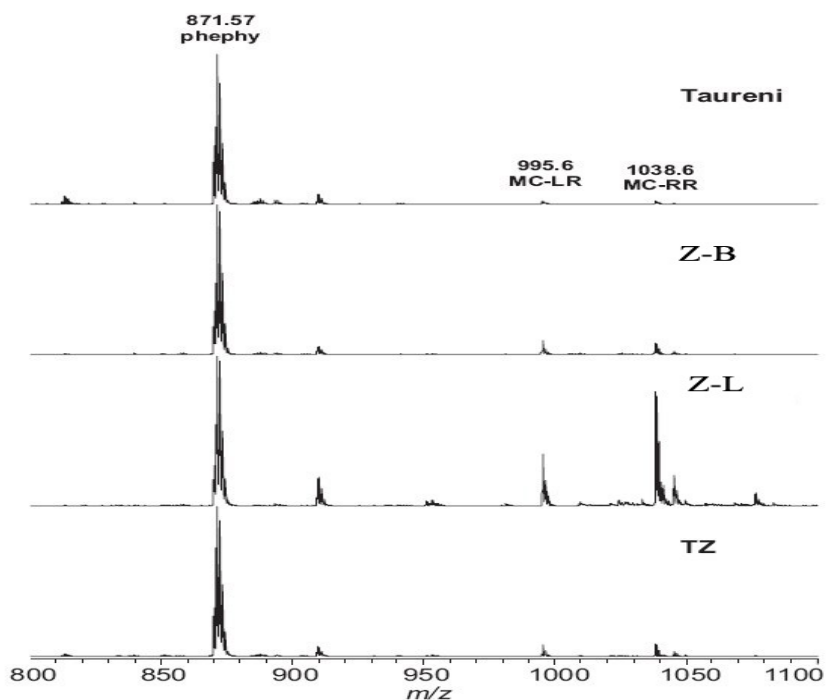


Fig. 24 The MALDI-TOF MS spectra obtained for the cyanobacterial samples collected from four different locations: M=marker; T-Z=Tăul Zaului; Z-L=Zau de Câmpie, Lake; Z-B=Zau de Câmpie, fishpond.

All the four MALDI-TOF MS spectra certify the microcystins presence in the samples collected from the environment. The LR-microcystins corresponds to an m/z ratio of 995.6, as for the RR-microcystins, this corresponds to 1038.6. According to these spectra, the samples from Tăureni, Tăul Zaului and Zau de Câmpie fishpond contained a much smaller amount of microcystins, unlike the higher microcystins amount identified in the Zau de Câmpie Lake. The ratio of the percentage of microcystins present in water and the pheophytin quantity was determined in hope for better representation of the reality (tab. 2). This compound is a variant of chlorophyll-a and it has a m/z ratio of 871.6 and its quantity is constant in all the analyzed samples.

Tab. 2

Cantitatea de microcistine raportată la conținutul în feofitină-a în cazul celor 4 probe prelevate din natură

	Tăureni		Zau de C.-pond		Zau de C. - Lake		Tăul Zaului	
	m/z	MC%/Feo	m/z	MC%/Feo	m/z	MC%/Feo	m/z	MC%/Feo
Pheophytin-								
a	871,57		871,57		871,57		871,57	
MC-LR	995,65	8,46	995,56	2,46	995,66	37,88	995,65	6,11
MC-RR	1038,66	7,35	1038,56	2,55	1038,67	74,94	1038,69	6,34

The table shows clearly the same conclusion as the spectra, namely, just for the cyanobacterial sample collected from Zau de Câmpie Lake we can presume a significant microcystins amount. As for the RR microcystins, this is comparable with that of pheophytin, reaching up to 75% of the last.

In the case of the other three locations, the microcystins percentage is much smaller. Nevertheless, the small amount of microcystins is sufficient to induce deadly effects on fishes (fig. 25). The MALDI-TOF MS analyses showed that the microcystins LR and RR were the only compounds with toxic potential present in these habitats.



Fig. 25. Toxic cyanobacterial bloom in the Tăureni Lake. The dead fished prove for the toxic character.

4.10 Strain AICB 702

Maybe the most interesting strains from the AICB Collection from the toxicity point of view is the AICB 702 strain, which according to MALDI-TOF-MS spectrum

contains a significant amount of MC-RR microcystin (fig. 19). According to the phylogenetic analysis based on the 16S rRNA and ITS fragments, presented above (fig. 9), this strain belongs to the *Microcystis wesenbergii* species, which is considered a nontoxic strain. Also, the cell sizes allow the classification of this strain within the *M. wesenbergii* species. The most important morphologic feature for the identification of this strain is the strongly refractive mucilaginous matrix surrounding the colonies. Sadly, in the case of cyanobacterial cultures from the AICB Collection the mucilaginous matrix is not preserved and cannot be used for the validation of the strain pertaining to a certain species.

This constitutes the main impediment in the confirmation – on morphologic grounds – of the AICB 702 as a strain belonging to *M. wesenbergii* species. As the molecular data clearly indicated this fact, some additional analyses became necessary in order to confirm that other cyanobacteria was not present in the culture.

In this way, the 16S rRNA and ITS fragments were amplified by PCR with the 27F-ITER primers and subsequently cloned into pGEM-T plasmid. 24 colonies were chosen by blue-white selection and the ITS fragment was sequenced, using the ITEF primer. Finally, 15 sequences were obtained. These were used for multiple sequence alignment with some fragments derived from other *Microcystis* strains (toxic and nontoxic) from AICB Collection (AICB 318, 619, 679, 680, 748 and 826). Two phylogenetic trees were generated based on the alignment, using two Distance methods: Neighbour Joining and Minimum Evolution (fig. 26, 27).

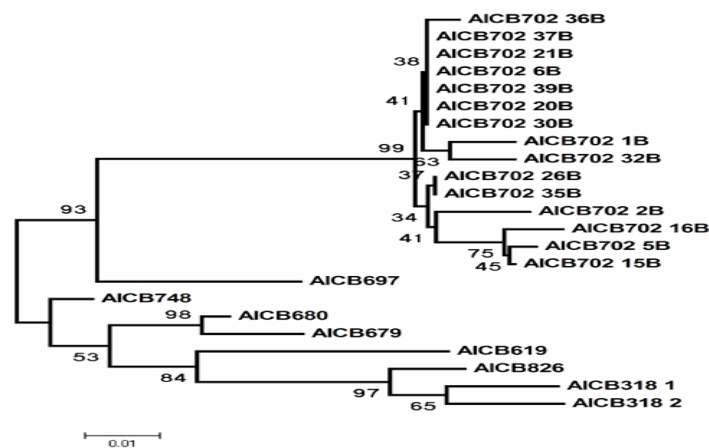


Fig. 26 The Distance tree constructed with Neighbour Joining algorithm using 15 ITS sequences from AICB 702 strain and some fragments from different strains of *Microcystis* deposited in the AICB Collection. The Bootstrap was done for 500 replicates (Mega 4-software).

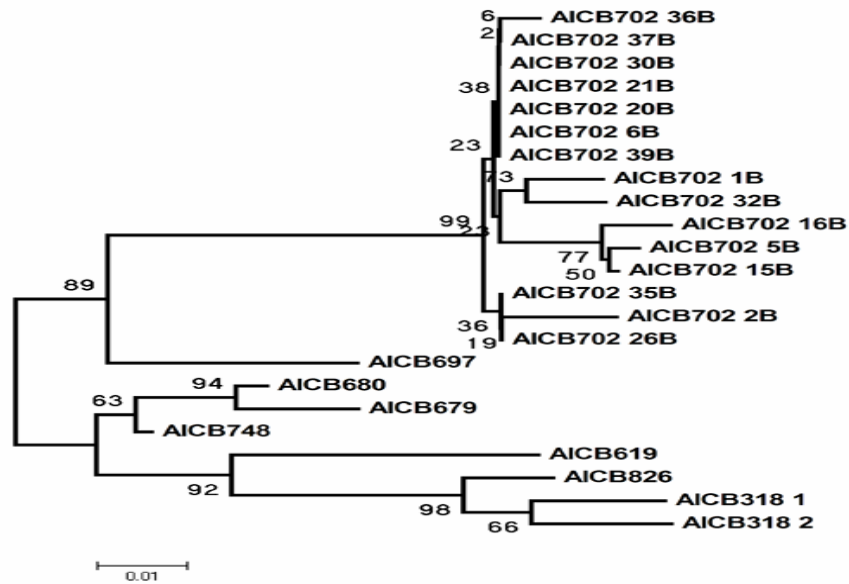


Fig. 27 Distance tree constructed with Minimum Evolution algorithm using 15 ITS sequences from AICB 702 strain and some fragments from different strains of *Microcystis* deposited in the AICB Collection. The Bootstrap was done for 500 replicates (Mega 4-software).

Both trees certify the fact that AICB 702 sequences are very much alike and they formed a tight group in each tree, isolated from the rest of the sequences. In both cases, the cluster which gather the 15 AICB 702 sequences is validated with a 99 Bootstrap value, which means that this group was present in 99% of the generated trees, the 2 consensus trees being reported here. In return, within this cluster the 15 sequences presented very small Bootstrap values, ranges between 2 and 77. This means that these sequences are very similar, thus almost every grouping among them is possible. A simple search, using the BLAST service from the NCBI public database confirmed that all the 15 sequences belonged to the AICB 702 strain.

The rest of the *Microcystis* strains gathered distinctively from the AICB 702 cluster and the higher Bootstrap values indicate that these strains are different.

5. Conclusions

- The 16S rRNA and ITS sequences and the phylogenetic trees demonstrate that all the studied strains belonged to *Microcystis* genus and the analyses of the repetitive sequences HIP type permitted the discrimination between the *Microcystis* strains from AICB Collection.
- A new set of primers were found useful for sequencing the 16S rRNA gene, entirely, unlike the 5 primers generally used.
- 5 strains of LR-microcystins and RR-microcystins producing strains belonging to the *Microcystis* genus are deposited in the AICB Collection.
- The new set of primers (original) permit a very easy identification of the microcystins producing strains (*Microcystis*) from the AICB Collection but also, of the toxic cyanobacteria from samples.
- The AICB 702 strain (most probably belonging to the *Microcystis wesenbergii*) produces RR-microcystin, until now this species being considered nontoxic.

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Key words

Microcystis, microcystins, MALDI-TOF MS, PCR