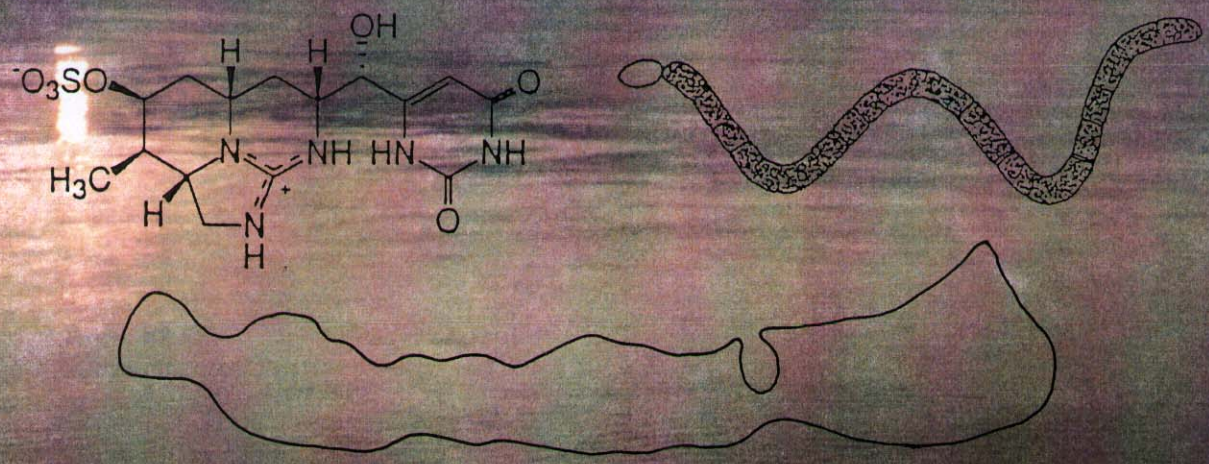
Balaton Limnological Research Institute, Hungary
Norwegian Institute for Water Research, Norway

Lake Balaton
Blue-Green Algae Project

Interim Report
1996



REPORT

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Abstract

The Lake Balaton Blue-green Algae Project is carried out as a research cooperation between Hungary and Norway under the bilateral agreement maintained by The Hungarian Ministry for Environment and The Norwegian Ministry of Environment. The research task is performed as a joint venture made by the Balaton Limnological Research Institute of the Hungarian Academy of Sciences (BLRI) and the Norwegian Institute for Water Research (NIVA). The research project was established in 1996 to provide a better scientific background for understanding the environmental and health problems related to the mass development of toxin producing blue-green algae in Lake Balaton. The year 1996 was used for introductory work and preparations. Field observations in Lake Balaton and Kis-Balaton provided information about the presence and conditions of blue-green algae and the prevailing water quality. The preliminary results from 1996 indicate that the phytoplankton of Lake Balaton contains several potential toxigenic species of blue-greens, besides *Cylindrospermopsis raciborskii*. The production of cyanotoxins in Lake Balaton - as experienced in other comparable geographical areas - will very likely include substances with hepatotoxic, neurotoxic and protracted toxic effects.

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Interim report 1996

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PREFACE

From the beginning (1927) the main mission of the Balaton Limnological Research Institute of the Hungarian Academy of Sciences has been multidisciplinary, long-term, fundamental research on the biogenic determined flow of energy and material, as well as on the structure and functioning of the Lake Balaton ecosystem. Limnological knowledge is the basis for sound use of Lake Balaton's water resources and rational prognostication of the future water quality, and also the best choice of optimal sanitation measures. In this context the Lake Balaton Blue-green Algae Project is subject to the needs of society.

"Well begun is half done". The three year project commenced 1996, and this report describes the task organization and the introductory work performed. Research is done by individuals, and several scientists have been involved in the defining of research tasks and providing a framework for coordination of the efforts. The Norwegian Institute for Water Research (NIVA) and the Balaton Limnological Research Institute (BLRI) will carry out the project as a joint venture.

A continuation of the investigations entered upon 1996 is now the urgent purpose.

December 4th. 1996

Lajos Vörös
BLRI

Bjørn Faafeng
NIVA

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1. STATE OF THE ENTERPRISE

1.1 Magyarul

- A Balatoni Kékalga Projekt a Magyarország és Norvégia közötti kutatási együttmûködés keretében folyik. Az országok közötti kétoldalú egyezmény - amelyet a magyar Környezetvédelmi és Területfejlesztési Minisztérium és a norvég Környezetvédelmi Minisztérium támogat - a projekt hivatalos alapja. A kutatási feladatot a Magyar Tudományos Akadémia Balatoni Limnológiai Kutatóintézete (BLKI) és a Norvég Vízkutató Intézet (NIVA) közösen hajtja végre. Más tudományos intézmények szakértôi kiegészítôleg mûködnek közre, amennyiben szakértelmükre szükség van.

- A kutatási projekt 1996-ban jött létre azzal a céllal, hogy egy jobb tudományos hátteret biztosítson a környezeti- és egészségügyi proplémák megoldásához, amelyek a Balatonban a toxintermelô kékalgák tömeges megjelenésével függenek össze. A sikeres menedzsment stratégia a toxikus algavirágzások hatásainak minimalizálásához függ az ezzel kapcsolatos hidrobiológiai jelenségek releváns ismeretétôl. A kutatás tárgyát ennek alárendelve választották ki és fogalmazták meg a BLKI és a NIVA projektben résztvevô kutatói. A vizsgálatok fô célkitûzése magába foglalja az alga virágzásokat szabályozó tényezôk, a kékalgák és toxintermelésük feltételeinek megismerését, valamint a toxinok vízi környezetben történô detektálási és meghatározási módszereinek kifejlesztését.

- Az 1996-os év a bevezetô munkák és elôkészületek jegyében telt el. A Balatonon és Kis-Balatonon végzett terepvizsgálatok információkat szolgáltattak a kékalgák megjelenésérôl, állapotáról és az uralkodó vízminôségrôl. A BLKI és a NIVA is izolált toxikus kékalga törzseket a Balatonból. Összehasonlító kísérleti vizsgálatokhoz klóntenyészetek szükségesek. A NIVA Alga Tenyészet Gyûjteménye 1996-ban 7 Balatonból származó törzset tartott fenn klóntenyészetben. Közülük 5 a *Cylindrospermopsis raciborskii* fajhoz tartozik, amely a Balaton legjelentôsebb toxintermelô szervezete.

- folyamán elkezdôdött a laboratóriumi munka a cianotoxinok detektásához alkalmas biotesztek kifejlesztésével és tesztelésével, valamint az ezzel kapcsolatos hatásvizsgákatokkal összefüggésben. Kölönbözô teszt rendszereken kezdôdtek el a kísérletek. Az alkalmazott teszt

organizmusok mikroalga, alsórendû rák, rovar, csiga, makrofiton és hal fajok voltak. Egértesztet szûrôvizsgálati és összehasonlító céllal rutinszerûen alkalmaztunk 1996-ban. Általános vagy ellenôrzô vizsgálatok céljára egy törzs vagy egy faj tesztobjektumként való alkalmazása valószínûleg nem kielégitô. A projektben ennek megfelelôen célszerûen válogatott fajok sorát vizsgáltuk, amelyek az anyagcsereutak széles spektrumát ölelték fel, és minôségileg különböztek a cianotoxinokra adott válaszuk môdját tekintve. Biológiai és kémiai módszereket párhuzamosan alkalmaztunk az elôforduló cianotoxinok analíziséhez.

- Az 1996-os év elôzetes eredményei azt jelzik, hogy a Balaton fitoplanktonja számos potenciálisan toxikus kékalga fajt tartalmaz a *Cylindrospermopsis raciborskii*-n kívül . A cianotoxinok produkciója a Balatonban - mint ahogy azt más összevethetô földrajzi térségeken is tapasztalták - igen nagy valószínûséggel olyan anyagokat foglal magába, amelyek hapatotoxikus, neurotoxikus és késleltetett toxikus hatásúak. A *Cylindrospermopsis raciborskii*-ra vonatkozó megfigyelések, amelyek az 1996-ban végzett biotesztekbôl származnak, azt sugallják, hogy ez a szervezet nemcsak az alkaloid tipusú cilindrospermopsint termeli. Lehetséges, hogy a kékalgákból származó toxikus anyagok széles spektruma a másodlagos metabolitok kategóriájába tartozik a Balatonban, amelyek veszélyesek lehetnek más élôlényekre.

1.2 Norsk sammendrag

- Prosjektet: "Lake Balaton Blue-green Algae Project" gjennomføres som et samarbeidsprosjekt mellom Ungarn og Norge. Den bilaterale avtalen om miljøsamarbeid - som er inngått mellom miljøverndepartementene i de to landene - er det formelle grunnlaget for virksomheten. Forskningsprosjektet gjennomføres i felleskap av Balaton Limnological Research Institute i Tihany (BLRI, tilknyttet det Ungarske Vitenskapsakademiet) og Norsk Institutt for vannforskning i Oslo (NIVA). Eksperter fra andre forskningsinstitusjoner i Ungarn og Norge deltar også.

- Forskningprosjektet ble etablert i 1996 for å fremskaffe et bedre faglig grunnlag for å vurdere miljø- og helserelaterte problemer knyttet til masseutvikling av toksinproduserende blågrønn-alger i Balatonsjøen. En god forvaltningsstrategi for å redusere de negative virkningene av slike oppblomstringer må bygge på god kunnskap om de aktuelle fenomene. I henhold til dette har forskere ved BLRI og NIVA satt opp som viktigste målsetting for prosjektet å forstå bedre de faktorer som kontrollerer masseoppblomstring av blågrønnalgene, hvilke organismer og toksiner som er involvert, samt utvikling av metoder for å påvise og kvantifisere mengden av toksinene i innsjøen.

- Prosjektet ble påbegynt i 1996, og innledende studier ble gjennomført som planlagt. Feltobservasjoner i Balatonsjøen og et hovedtilløp, Kis-Balaton, har gitt nyttig informasjon om forekomst av blågrønnalger og den aktuelle miljøtilstanden. Både BLRI og NIVA har isolert toksinproduserende kloner av blågrønnalger fra innsjøen. Slike klonkulturer er forutsetning for å gjennomføre kontrollerte eksperimenter. NIVAs algekultursamling innholder nå 7 stammer av blågrønnalger fra Balatonsjøen; 5 av disse består av arten *Cylindrospermopsis raciborskii,* som er den fremtredende toksinproduserende arten i innsjøen.

- Innledende arbeid med å utvikle biologiske testmetoder (bioassays) for cyanotoksinene er gjennomført i 1996. Ingen enkeltorganisme eller test vil alene kunne gi den nødvendige informasjon om type og mengde av toksiner. I prosjektet gjennomføres derfor utvikling av tester der fem forskjellige organismegrupper inngår. Testorganismene omfatter både mikroalger, insekter, bløtdyr, høyere planter og fisk. Standard musetest og kjemiske analyser har vært utført parallellt for sammenlikning og kontroll.

Foreløpige resultater fra 1996 viser at flere potensielt toksinproduserende arter av planteplankton opptrer i Balatonsjøen i tillegg til *Cylindrospermopsis raciborskii.* Produksjonen av cyanotoksiner i Balatonsjøen vil, ut fra foreliggende erfaringer, kunne omfatte både hepatotoksiner, nevrotoksiner og stoffer med protraherte effekter. Tester med *Cylindrospermopsis raciborskii* indikerer at denne arten ikke bare produserer alkaloidet cylindrospermopsin, men også andre typer toksiner. Sannsynligvis produseres et spektrum av cyanotoksiner i Balatonsjøen som kan medføre ulike effekter på plante- og dyrelivet med negative konsekvenser for vannkvaliteten og innsjøens praktiske anvendelse.

1.3 Abstract in English

- The Lake Balaton Blue-green Algae Project is carried out as a research cooperation between Hungary and Norway. The bilateral agreement between the countries - maintained by The Hungarian Ministry for Environment and The Norwegian Ministry of Environment - is the formal basis for the project. The research task is performed as a joint venture made by the Balaton Limnological Research Institute of the Hungarian Academy of Sciences (BLRI) and the Norwegian Institute for Water Research (NIVA). Experts from other scientific institutions are participating for the supplement with necessary competence.

- The research project was established in 1996 to provide a better scientific background for the understanding of the environmental- and health problems related to the mass development of toxinproducing blue-green algae in Lake Balaton. A successful management strategy for minimizing the impact of nuisance- and toxic algal blooms is depending on relevant knowledge of the hydrobiological phenomena involved. In accordance with this the research objectives have been selected and defined by the scientists at BLRI and NIVA involved in the project. Main targets of the investigation include factors controlling the blue-green algal blooms, the organisms and their conditions for toxin production, and methods for detection and measurement of toxins in the aquatic environment.

- The year 1996 was used for introductory work and preparations. Field observations in Lake Balaton and Kis-Balaton provided information about the presence and conditions of blue-green algae and the prevailing water quality. Isolation of toxigenic strains of blue-green algae from Lake Balaton was made at BLRI and NIVA. Clonal cultures are prerequisites for the comparative experimental studies. The NIVA Culture Collection of Algae is in 1996 maintaining 7 strains of blue-greens from Lake Balaton in clonal cultures. Among these 5 strains belong to the species *Cylindrospermopsis raciborskii*, the prominent toxinproducing organism of Lake Balaton.

- Laboratory work was entered upon during 1996 in connection with the development and testing of bioassays for detection of cyanotoxins and related effect studies. Experiments were started on a variety of test systems and their design. The test organisms used include species of microalgae, crustaceans, insects, gastropods, macrophytes and fish. The mouse

bioassay was routinely carried out during 1996 for screening and comparative purposes. No single strain or species is likely to be ideal for general investigations or satisfying regulatory bodies. Accordingly the project is studying a selection of species sharing a spectrum of metabolic pathways, but with qualitative differences in the way they respond to cyanotoxins. Biological and chemical methods were applied in parallel for the analysis of the cyanotoxins involved.

- The preliminary results from 1996 indicate that the phytoplankton of Lake Balaton contains several potential toxigenic species of blue-greens, besides *Cylindrospermopsis raciborskii*. The production of cyanotoxins in Lake Balaton - as experienced in other comparable geographical areas - will very likely include substances with hepatotoxic, neurotoxic and protracted toxic effects. The observations of *Cylindrospermopsis raciborskii* in the bioassay studies performed in 1996 suggest that not only the alkaloid cylindrospermopsin is produced by this organism. Probably a spectrum of substances from blue-greens in Lake Balaton belong to the category of secondary metabolites with harmful effects on other biota.

2. INTRODUCTION

The basis for this mutual research effort is the bilateral agreement between The Hungarian Ministry for Environment and Regional Policy and The Norwegian Ministry of Environment on Environmental co-operation.

An application for financing the "Lake Balaton Blue-green Algae Project" was made by the Balaton Limnological Research Institute (BLRI) and the Norwegian Institute for Water Research (NIVA) during a workshop in Tihany November 7-8, 1995. This joint project will be performed over a three year period. 1996 was an introductory year, and the results will decide the progression in the following two years.

The partners are collaborating according to special competence and practical conditions, and apply their respective Ministries of Environment for national financing according to "*The Agreement on cooperation between the Government of the Kingdom of Norway and the Government of the Republic of Hungary on the Protection of the Environment*", signed in Budapest, September 27, 1991.

The responsibilities are partitioned in the following way:

- The investigations on relevant phenomena in Lake Balaton will be taken care of by BLRI.
- Algal culture experiments will be performed both at BLRI and NIVA.
- Development of the bioassays and cyanotoxin analysis will be handled by NIVA jointly with specialists from BLRI.

Experts on research fields not adequately covered by BLRI and NIVA will be engaged when necessary for the progress of the project. During 1996 some scientists (see TABLE 2.3.1) at the following institutions (TABLE 2.1) were accordingly participating on selected experimental investigations.

TABLE 2.1 Subcontracting institutions

Hungary	Norway
- Kossuth University of Debrecen, Debrecen - Pannon University of Agricultural Sciences, Mosonmagyaróvár, - 'B. Joan' National Institute of Public Health, Budapest	- National Institute of Public Health, Oslo - The Norwegian College of Veterinary Medicine, Oslo

2.1 Aims of the project

The aims of the joint Hungarian-Norwegian project include:

- to exchange present knowledge on toxinproducing blue-greens, detection and analysis of relevant cyanotoxins
- to promote understanding of the causes and consequences of nuisance algal blooms
- to adapt and improve the necessary tools to manage the risks connected with harmful algal blooms.

The results of this research should serve as professional input to public health and ecotoxicological risk assessment for Lake Balaton. Guidelines for the handling of related problems concerning water quality management will be pursued.

Three main scientific goals include:

- To identify the environmental factors and limnological processes controlling the blue-green algal blooms in the lake.
- To determine the growth requirements and the conditions of toxin production of the predominant blue-green alga (e.g. *Cylindrospermopsis raciborskii*) in laboratory cultures.
- To develop and adapt rapid, simple, selective and ecologically relevant bioassay techniques to detect, identify and quantify toxins produced by blue-greens in lake water and drinking water.

The results will promote a better understanding of the water quality problems related to utilization of Lake Balaton for drinking water and recreational purposes. The methods investigated and

adapted should give relevant information on the development of toxin-producing blue-greens in the lake, and on possible toxins present in the lake itself and in water supply systems. The results will support the understanding of the toxin producing blue-greens, and provide basic knowledge for management actions and information to the public. The results will be of practical value when setting up priorities for pollution control, as well as possible in-lake measures.

2.2 Research needs for a Lake Balaton blue-green algae management strategy

The present joint Hungarian-Norwegian research project is established with the purpose to provide a scientific background to minimize the impact of nuisance- and toxic blue-green algal blooms in Lake Balaton. To fulfil this challenge a relevant reasoning should form the exit of the work. The actual limnological knowledge on eutrophication and the implicated phenomena of blue-green algal blooms constitutes the framework of the research philosophy (Paerl 1988, Carmichael 1992). In short the essentials can be formulated in the following terms, which were discussed among the participating scientists at the Balaton Limnological Research Institute on September 26th 1996.

The occurrence of major blue-green algal blooms in Lake Balaton (see 3.1) - including mass development of toxigenic species - create immediate environmental problems with consequences for water supply and recreational purposes. There is no simple quick-fix solution to difficulties with blue-green algal blooms in the lake. Mass development of blue-green algae have a complexity in cause and effect (Herodek 1988, Vörös & Nagy-Gode 1993). Several environmental factors are involved. The occurrence of toxinproducing blooms creates a wide range of social and environmental impacts (Carmichael & Falconer 1993).

Management measures have to be based on the understanding and appraisal of the prevailing situation in Lake Balaton. The research tasks in this connection target questions like what are the important causes regulating the mass development of blue-green algae, how can major factors affecting bloom formation be controlled and how is it possible to minimize the effects of noxious blue-green algal blooms. The results of the ongoing research should be relevant for making practical actions, and define indicators of success for the accomplishment of a Lake Balaton blue-green algae management strategy. These actions include risk assessment and monitoring programmes which rely on e.g. sensitive and accurate analysis methods for cyanotoxins.

2.3 Accomplishment of cooperation

The scientists participating in the research project are listed in TABLE 2.3.1. Their competence and expertise employed for the investigation are indicated.

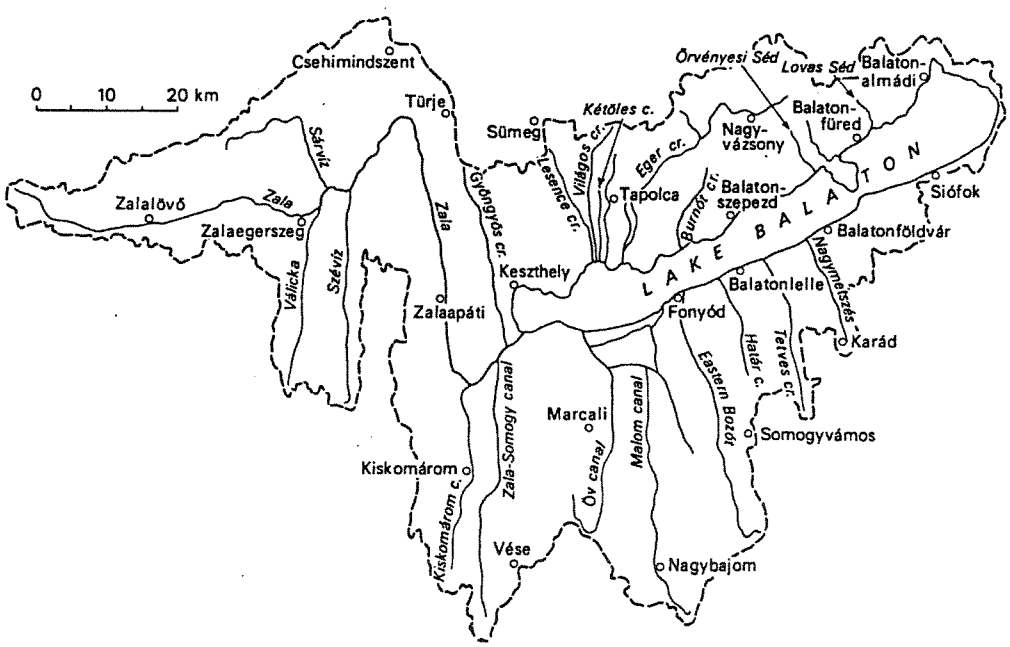
TABLE 2.3.1. Team of scientists - Lake Balaton Blue-green Algae Project

Institution	Scientist	Expertise
HUNGARY		
Balaton Limnological Research Institute	Katalin V.-Balogh	Chemical limnology
	László Hiripi	Toxicology, pharmacology
	Szilveszter Juhos	Ecological modelling
	Tibor Kiss	Neurophysiology of invertebrates
	Attila Kovács	Algal physiology, ecology
	Eszter Kovács	Microbial ecology
	Lajos Nagy	Toxicology, pharmacology, bioassay
	Matyas Presing	Chemical analysis, water quality
	Ágnes Vehovszky	Neurophysiology of invertebrates
	Lajos Vörös	Limnology, phytoplankton ecology
Kossuth University of Debrecen	György Borbély	Cyanobacteriology, virology
Pannon University of Agricultural Sciences	Vince Ördög	Algal physiology, ecotoxicology
'B. Johan' National Institute of Public Health	Andrea Török	Toxicology, bioassay
NORWAY		
Norwegian Institute for Water Research	Bjørn A. Faafeng	Eutrophication, foodchain interactions
	Heike Kiefer	Bioassay
	Tone Jøran Oredalen	Bioassay, limnology
	Olav M. Skulberg	Phycology, cyanotoxins
	Randi Skulberg	Algal cultures, isolation of strains
The Norwegian College of Veterinary Medicine	Bjarne Underdal	Toxicology, bioassay
National Institute of Public Health	Hans Utkilen	Cyanobacteriology, cyanotoxins

Limnological studies. Measurements of environmental factors related to the plant nutrient cycling in the water and bottom sediments. Description of the processes that control the development of the dominant blue-green algae.

Biological studies. Experiments with algal cultures of the toxigenic species. Examination of the biodiversity of toxic waterblooms in Lake Balaton. Establishment of clonal cultures for comparative studies. Autecological studies of *C. raciborskii* characterizing and quantifying the toxin production.

Toxicological studies. Development of rapid and selective bioassay methods to a satisfactory level of an international standard for toxicity assessment of lake water and drinking water. Determine and characterize the toxin(s) involved. Investigate the ecotoxicological effects and evaluate possible public health hazards.



Lake Balaton and its catchment

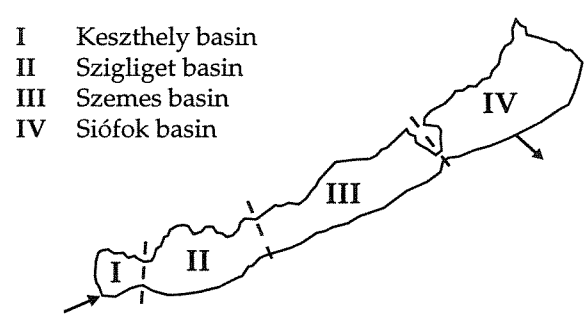


FIGURE 3.1.1 Lake Balaton and its catchment

3. LAKE BALATON

3.1 Lake Balaton - past and present

3.1.1 Morphology, catchment area

Lake Balaton is situated in the western part of Hungary, its surface area is 596 km^2, the mean depth is 3.35 m. The lake has an elongated shape, its length is 78 km while the average width is 7.6 km. The total area of the catchment area including the lake is 5775 km^2 (FIGURE 3.1.1). The main tributary to the lake is the River Zala draining an area of 2622 km^2. The sub catchments of the minor streams on the southern and northern shores are respectively 820 and 1175 km^2. There are 308 municipalities with 420000 permanent residents in the catchment area. During the summer season approximately 2 million tourists visit the lake shorelines, and they spend 20 million visitor-days there. Since 1989 - due to the political and economical changes in Eastern Europe - the importance of Lake Balaton has decreased significantly. However, still Lake Balaton contributes with approximately 25% of the tourist income of Hungary.

3.1.2 Temperature

The climate of Hungary is temperate. In the Lake Balaton region the normal mean air temperature is 10.7 °C. The duration of winter ice cower is appr. two months, the average cover thickness being 20-25 cm. The water temperature in the shallow lake follows with little delay the variations in air temperature. The mean annual water temperature is 12.2 °C. The water temperature is normally above 20 °C from the end of May to early September, and this period is considered suitable for bathing and aquatic sports.

3.1.3 Chemistry of lake water

The geology of the catchment area consists mainly of limestone and dolomite, influencing fundamentally the chemistry of water discharging to and stored in Lake Balaton. The inflowing water carry Ca^{2+}, Mg^{2+} and HCO_3^- in high concentrations. Along the longitudinal axis - from the main inlet to he outlet - the concentrations of Ca^{2+} and HCO_3^- gradually decrease, while concentrations of Mg^{2+}, Na^+, K^+, SO_4^{2+} and Cl^- increase. Continuous lime precipitation (mainly due to the algal photosynthesis) may be offered as an explanation for the reduction of the Ca^{2+}, and HCO_3^- levels, whereas the increasing concentration of the other ions is attributable to

evaporation. The pH of the lakewater is normally 8.4. In summer, in the period of maximum photosynthesis, pH rises generally to 8.6-8.7. Sometimes - during the summer algal blooms - in the western lake areas, pH values above 9.0 are registered.

3.1.4 Phytoplankton

Approximately 2000 species of algae have been identified in Lake Balaton. Two-thirds of them inhabit the littoral and benthic zones. Most of the organic matter is produced over the year by the phytoplankton. The first informative primary production measurements of Lake Balaton phytoplankton using the Steeman-Nielsen method (^{14}C assay) were carried out at the early sixties. Detailed investigations commenced in 1972. Due to the uneven distribution of the nutrient load of the lake, a trophic gradient exists along the longitudinal axis of Lake Balaton. The most eutrophicated parts is the western, so called Keszthely basin, while the less eutrophicated part is the eastern, or Siófok basin (FIGURE 3.1.1).

3.1.5 Eutrophication

In the western basin - during the last two decades - the summer maximum of the *chlorophyll-a* concentration often exceeded the values of 100 µg/l, while in the less eutrophicated eastern basin this value typically was lower than 20 µg/l. However, there were some exceptions, for example during 1994 the maximum *chlorophyll-a* concentration in the western basin was close to 200 µg/l, while in the eastern basin the *chlorophyll-a* concentration exceeded the 70 µg/l (FIGURE 3.1.2). The progressive eutrophication of the Lake Balaton was perceived in the early seventies. The first indications was that the quantity of diatoms and dinoflagellates increased moderately. From 1975 and onwards, the summer mass development of filamentous blue-greens became a regular phenomenon in the western part - and sometimes in the eastern part - of the lake. The eutrophication of Lake Balaton is associated with increasing dominance of blue-greens, a parallel to many temperate and tropic lakes.

There has been a clear tendency towards increased contribution of blue-greens with increasing total phytoplankton biomass in Lake Balaton (FIGURE 3.1.3).

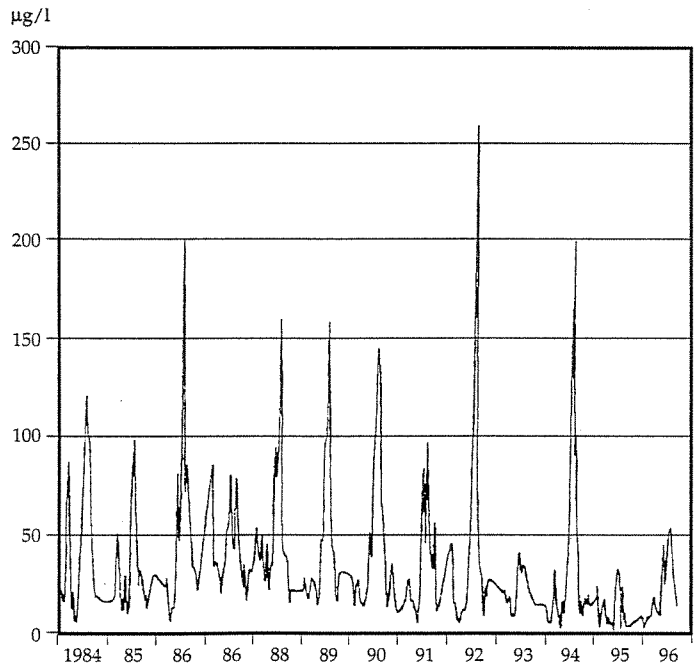


FIGURE 3.1.2 Changes in chlorophyll-a concentration between 1984 and 1996 in the western basin of Lake Balaton

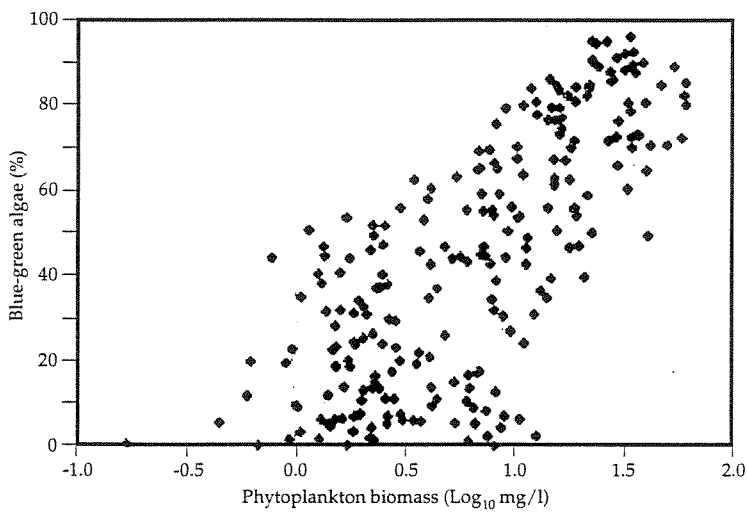


FIGURE 3.1.3 Percentage contribution of blue-green to total phytoplankton biomass as a function of total biomass in Lake Balaton

3.2 *Cylindrospermopsis* in Lake Balaton

The first case of mass development of blue-greens in the whole lake system occured in 1982, caused by *Cylindrospermopsis raciborskii*, an invader species of the lake. A lately mass development took place during 1994 when the water masses of the lake were almost a pure culture of *C. raciborskii*. The summer maximum of chlorophyll concentrations measured in the western and eastern basin during 1995 were 40 and 9 μg/l, respectively.

During 1996 the situation was similar. The maximum *chlorophyll-a* concentration in the western basin reached the 55 μg/l, while in the eastern basin it was 13 μg/l. The maximum value of the photosynthesis measured in the surface of the western basin was 5 g C/m^2/day, while in the eastern basin only 1.4 g C/m^2/day was reached. Also in this year, as in 1995, during the summer months the dominance of blue-green algae was a characteristic phenomenon. But not only the *C. raciborskii* was prevalent among the phytoplankton species. Two species - *Aphanizomenon flos-aquae* and the non N-fixing *Planktothrix agardhii* - contributed significantly to the total phytoplankton biomass.

The relatively moderate development of phytoplankton observed during 1995 and 1996 is not explainable solely by the changes of external nutrient loading. According to the recent measurements and model considerations the summer development of blue-greens is mainly controlled by the direct and indirect effects of the hydrometeorological factors. During the last two years in the geographical area considered, the summer water temperature was lower, and the water discharge was higher than in the previous years.

3.3 Field work operation September 1996

The NIVA-team, consisting of Bjørn A. Faafeng, Olav M. Skulberg and Tone Jøran Oredalen was on a study visit to BLRI in Tihany from the 23rd to the 26th of September 1996.

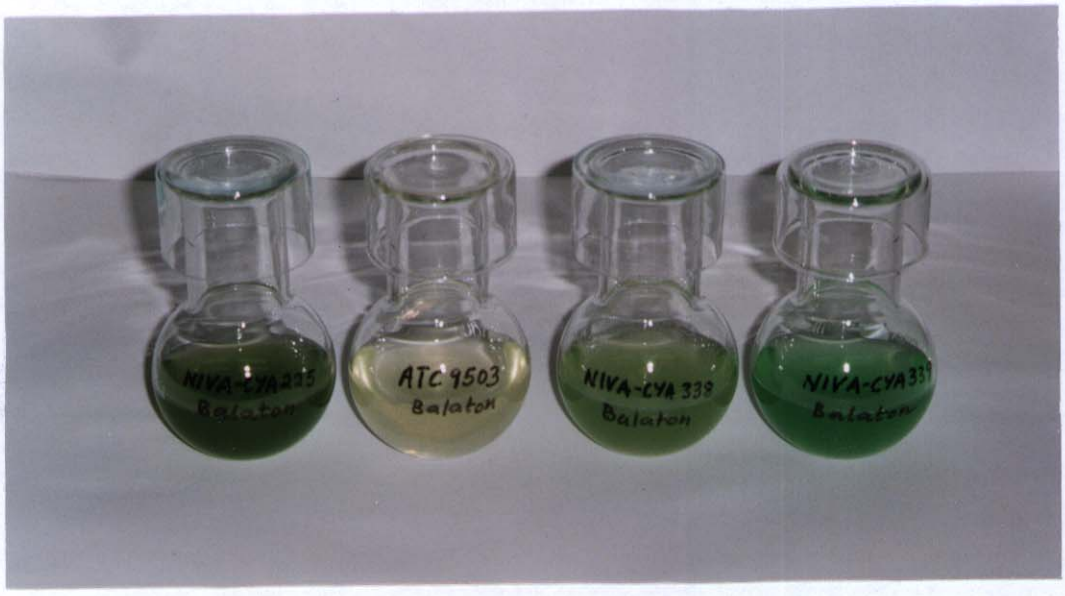
During the stay a joint limnological survey in Lake Balaton and its catchment area was accomplished. The operation was organized from the host institute by Lajos Vörös, Matyás Presing, Attila Kovács and Eszter Kovács. We concentrated the samplings to four stations: Eastern basin (outside the Tihany peninsula), Western- or Keszthely basin, the inlet of River Zala and at Kis-Balaton (outflow of step1). From each of the locations samples were collected for chemical and phytoplankton analysis. Zooplankton-material, both qualitative and quantitative, were taken from the two sampling sites in the lake.

There were daily meetings to discuss the main topics, the time schedule and the project itself. Attention was devoted to the following particulars:

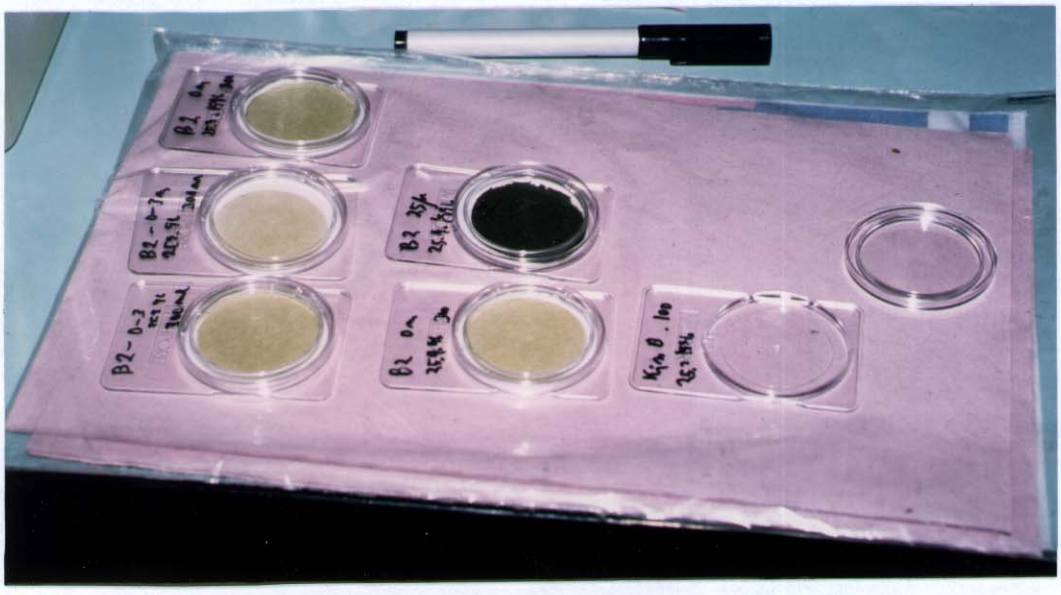
- Presentation of BLRI and NIVA
- Overview of the past and present situation in Lake Balaton.
- Exchange of clone cultures of blue-greens isolated from Lake Balaton.
- Review bioassays under investigation
- Cyanotoxins; development of analysis and new bioassays, culture experiments and risk assessment.
- Discussions of the project aims and course of actions.
- Partitioning of responsibilities for the progress report and practical tasks for the next year.

At the last day of our joint sessions, a workshop was performed with invited scientists from Kossuth University of Debrecen, dept. of Botany, and Pannon University of Agricultural Sciences, in addition to the participants from BLRI and NIVA. The main subjects were mutual information about the specific research interests of the institutions, and the possibilities for continued collaboration.

Summit events 1996



Strains of Lake Balaton cyanophytes deposited
in the NIVA Culture Collection.



Material of *Cylindrospermopsis raciborskii*
secured for assessment and analysis,
Tihany, September 1996

Fruitful joint research work is established
Balaton Limnological Research Institute, September 1996.

Mass development of blue-green algae,
Kis-Balaton, September 1996.

4. COMMENCEMENT OF RESEARCH WORK

4.1 Isolation and culture of algal strains

Samples of sediments and water collected in Lake Balaton have been used as basis for establishing enrichment cultures. The procedure was according to the routine method applied at NIVA (Skulberg & Skulberg 1990). Subsamples were inoculated in inorganic nutrient solution (Z8-medium) and cultivated under various growth conditions (light, temperature etc.). The development of algae was followed by visual observations and microscopic examination.

Numerous species of different algal classes were found growing in abundance. Thirteen species of cyanophytes were identified in the enrichment cultures (TABLE 4.1.1).

TABLE 4.1.1 Species of cyanophytes developing in enrichment cultures

Anabaena affinis Lemm.	*Cylindrospermopsis raciborskii* (Wol.) Seenaya & Subba Raju
Anabaena cf. *flos-aquae* Bréb.	*Planktothrix agardhii* (Gom.) Anagn. & Kom.
Anabaena cf. *spiroides* Kleb.	*Gomphosphaeria* Kütz. sp.
Anabaena planctonica Brunnth.	*Microcystis aeruginosa* Kütz.
Aphanizomenon gracile (Lemm.) Lemm.	*Microcystis* cf. *botrys* Teil.
Aphanizomenon issatschenkoi (Usac.) Prosk.-Lavr.	*Snowella* Elenk. sp.
	Synechococcus Näg. sp.

The enrichment cultures were used for extensive isolations to obtain clonal cultures of strains for comparative experimental studies. Seven strains of blue-greens isolated from Lake Balaton are now (1996) maintained in NIVA's Culture Collection of Algae (TABLE 4.1.2).

The last 4 strains mentioned were isolated at the Balaton Limnological Research Institute by Attila Kovács. The ACT 9502 and ACT 9503 clones originated from a water sample from Lake Balaton (Basin I). The ACT 9504 and ACT 9505 clones originated from a sediment sample from Lake Balaton (Basin IV).

TABLE 4.1.2 Cyanophytes from Lake Balaton in NIVA's Culture Collection

Aphanizomenon gracile	NIVA-CYA 338
Snowella cf. *lacustris*	NIVA-CYA 339
Cylindrospermopsis raciborskii	NIVA-CYA 225
Cylindrospermopsis raciborskii	NIVA-CYA 352 (= ACT 9502)
Cylindrospermopsis raciborskii	NIVA-CYA 353 (= ACT 9503)
Cylindrospermopsis raciborskii	NIVA-CYA 354 (= ACT 9504)
Cylindrospermopsis raciborskii	NIVA-CYA 355 (= ACT 9505)

4.2 Toxigenic strains and toxins

The unialgal cultures of the strains of cyanophytes listed in TABLE 4.1.2 will be used for comparative studies. The biodiversity of indigenous waterblooms should be characterized with respect to components of toxigenic and nontoxigenic strains. Information from both field studies and laboratory experiments using clonal cultures is necessary for the purpose.

The *Cylindrospermopsis*-strains from Lake Balaton will be given priority in the investigation, and their toxigenic properties will be examined. The bioassays so far carried out during 1996 with the Lake Balaton material at BLRI and NIVA indicate that a complex of cyanotoxins are involved in addition to the alkaloid cylindrospermopsin. These results are in accordance with present experience from investigations on other cyanotoxin-producing organisms from different geographical regions (Skulberg et al. 1995, Moore et al. 1993).

The strains of *Cylindrospermosis raciborskii* demonstrates a variety of effects in studies of poisoning. The properties of cylindrospermopsin have recently been elucidated. Some information is pertinent related to the present study. The resume given here is based on literature at hand (see Falconer et al. 1995).

The toxic substance cylindrospermopsin belongs to a new class of alkaloids possessing a cyclic guanidine group. It has a molecular weight of 415 daltons and is thought to be a polypeptide that utilizes an amino acid-derived starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid. The symptoms of cylindrospermopsin poisoning are different from those reported of microcystins, and unlike microcystins it does not inhibit protein phosphates 1, 2A or 3. The LD_{50} (i.p. mouse) is 2.1 mg/kg at 24hr and 0.2 mg/kg at 5-6 days. Experimental animals given a minimum lethal dose of cylindrospermopsin take several days to die, unlike the more rapidly acting microcystins and nodularin. Cylindrospermopsin is a general cytotoxic compound which damages vertebrate organs in a progressive manner, including the liver.

4.3 Methods for extraction and detection of cyanotoxins

The cyanotoxins produced by the blue-green algae in Lake Balaton include substances with hydrophilic and lipophilic properties. Depending on the nature of the sample - water, plankton, fish, foodstuffs etc. - different procedures for pretreatment are necessary.

The samples collected frequently contain a low concentration of either live cells or released toxins. Accumulation of the blue-green algae or toxin may then be required prior to testing. This will bring the potential cyanotoxin concentration up to the sensitivity range which is detectable. The techniques employed during 1996 included freeze drying of water and algae samples, followed by resuspension in physiological saline (Berg et al. 1987).

Laboratory processing procedures are based on understanding of the intra- and extracellular localization of the cyanotoxins. Sample analysis falls into several categories e.g. screening for presence of toxins, identification of toxins present and quantification of toxicity. Biological assays are suitable as screening methods (see 4.4). Screening methods alone are usually not specific for the substance(s) under investigation. Identification and quantification need suitable chemical assays for the purpose. Relevant methods include high-performance liquid chromotography (HPLC), mass spectrometry (MS) and electrophoresis (EPH).

The isolation and identification of the cylindrospermopsin was carried out by the method of Harada et al. (1994). Briefly, the lyophilized cells were homogenized in metanol using a glass potter followed by ultrasonic homogenizor. The homogenate was centrifuged at 6000 g for 15

min. and the supernatant was evaporated to dryness. The residue was dissolved in water and the solution was applied to a column of HP-20 resin. The resin was eluated with water and then 15 % metanol in water. The metanol fraction was evaporated and the residue in 10 ml water was passed through a C_{18} cartridge (Bakerbound SPE 7020-03, 3 ml). After removal of the solvent, the residue was dissolved in 100 µl of water and injected into the HPLC system. The Waters HPLC system consisted of a pump type 510, U6K injector, photodiode array detector type 990, µBondapack 10C-18 reversephase coulomn (4,6 x 300 mm). 5% metanol in water was used as a mobil phase.

The chromatogram of the extract has different peaks (FIGURE 4.3.1). The second peak which has a retention time of 5.2 min., represents the cylindrospermopsin. Its spectra (FIGURE 4.3.2) is identical with that of the autentic toxin published by Harada et al., (1994). This peak has two absorbances at 200 and 262 nm and their ratio is approximately 2. The other peaks of the chromatogram has an absorbance which is different from that of cylindrospermopsin.

The biological and chemical methods mentioned above are often requiring a comparison of the test sample with purified standards. An important progress in 1996 was that a reference standard of cylindrospermopsin was obtained at NIVA for use in the project.

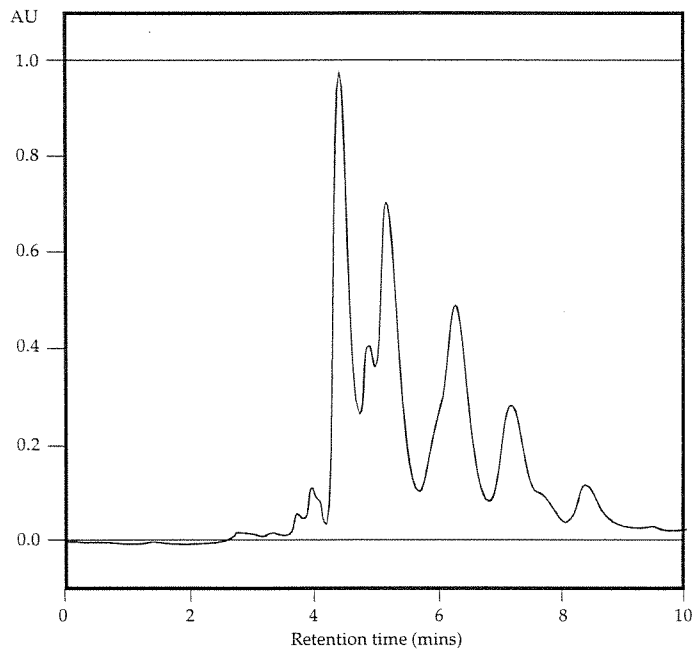


FIGURE 4.3.1 HPLC chromatogram of the extract of *C. raciborskii*. The peak having a retention time 5.2 min. represents the toxin peak.

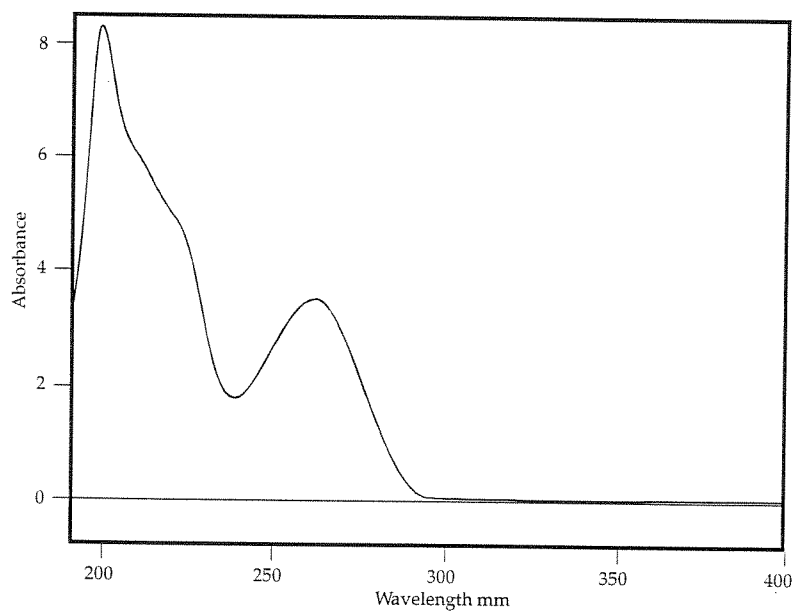


FIGURE 4.3.2 UV spectra of the toxin peak. The peak has two absorbances at 200 and 262 nm, and their ratio is approximately 2.

4.4 Bioassays for effect studies and acute toxicity testing

The use of bioassays for testing water quality in Lake Balaton offers advantages as an aid in analysing autecological as well as synecological questions related to response on cyanotoxins. The results can be used to probe the extent to which an aquatic environment is being contaminated. On the other hand they provide basis for predictions of biological impacts at different levels in the ecological hierarchy (Skulberg 1995). The choice of bioassays and level of observation (molecular, cellular, organismic, community etc.) will depend on the problem considered.

There are several types of bioassays developed for detection and analysis of cyanotoxins (Bell & Codd 1996). The most extensively used and accepted by health authorities is the mouse bioassay for determination of acute toxicity. However this method has several limitations. The low-technology test relies on the use of an in-bred strain of laboratory animals. Specially trained and licensed operators are required. There are growing objections to the use of animal bioassay for human and moral reasons (Roush 1996). Alternative methods that can replace the mouse in a relevant test are important research objectives. The Balaton blue-green algae project provide an incentive to stimulate the development and validation of alternative biological tests for cyanotoxins. In the following some alternatives are described.

4.4.1 *Locusta*-test

(Balaton Limnological Research Institute)

To estimate the toxicity of the algal extract, an insect, the *Locusta migratoria migratorioides* R.F. was used. The animals were taken from a stock culture, reared in the laboratory at 30^0C and a photoperiod of 12 hr. The lyophilized cells were extracted with physiological saline, while the synthetic microcystin-LR was dissolved in physiological saline containing 0.03% Brij-35. The samples of the extract or the authentic toxin was injected (100 µl) into the body cavity. The mortality of the animals was determined after 48 hrs.

The microcystin-LR has a similar toxicity on the locust as in the mouse. The LD_{50} values is 0.2 µg/animal or 133 µg/kg (Fig. 4.4.1). Lyophilized cell extract of two different *Microcystis*-strains cultured respectively at University of Debrecen and at NIVA in Oslo were also investigated. Both *Microcystis* strains were toxic in the locust test (Fig. 4.4.2). The LD_{50} values were LD_{50}=22.6

µg/animal=15.1mg/kg and LD_{50}=15 µg/animal=10mg/kg, respectively. The *Cylindrospermopsis raciborskii* extract proved to be toxic to the locust (Fig. 4.4.3), however its toxicity is weaker than that of the *Microcystis*-strains. The LD_{50} value of the *Cylindrospermopsis raciborskii* extract (ACT-9502 strain, Tihany) is 1159 µg/animal or 767 mg/kg. In the mouse test the LD_{50} values for the autentic microcystin is 30 - 50 µg/kg, while for the authentic cylindrospermopsin is 2100 - 7000 µg/kg. This toxicity ratio was also found for the algal extract, suggesting that the *C.raciborskii* is a less toxic alga than hepatotoxin- or neurotoxin-producing species of blue-green algae.

We started to investigate the toxic effect of the blue-green algae on fish. For this experiment we used the *Rutilus rutilus L.* The *C.raciborskii* extract was injected into the body cavity and the mortality was determined after 48 hr. It was found that the *C.raciborski* extract kill the fish. The LD_{50} value is 1255 mg/kg (Fig. 4.4.4) . The behaviour of the fish injected with *C.raciborskii*-extract suggest that the toxin influence the oxygen consumption of the animal.

Using the locust test we monitored the toxicity of the algae from the Lake Balaton and Kis-Balaton in 1996. We found no toxic blue green alga present in Lake Balaton. The LD_{50} values of the extracts of blue-greens collected from the Kis-Balaton were high (FIGURE 4.4.5), suggesting that the density of the toxic blue-green algae was low. The toxicity and the chlorophyll-*a* concentration did not coincide, the highest concentration of the chlorophyll-*a* was measured in august, while the highest toxicity was found in september.

dose (μg/animal)	*surviving animals (%)*
0.05	94.4
0.1	85.6
0.15	58.3
0.25	39.4
0.5	20.0
0.75	9.2
1.0	0

LD_{50}=0.2 μg/animal=133 μg/kg

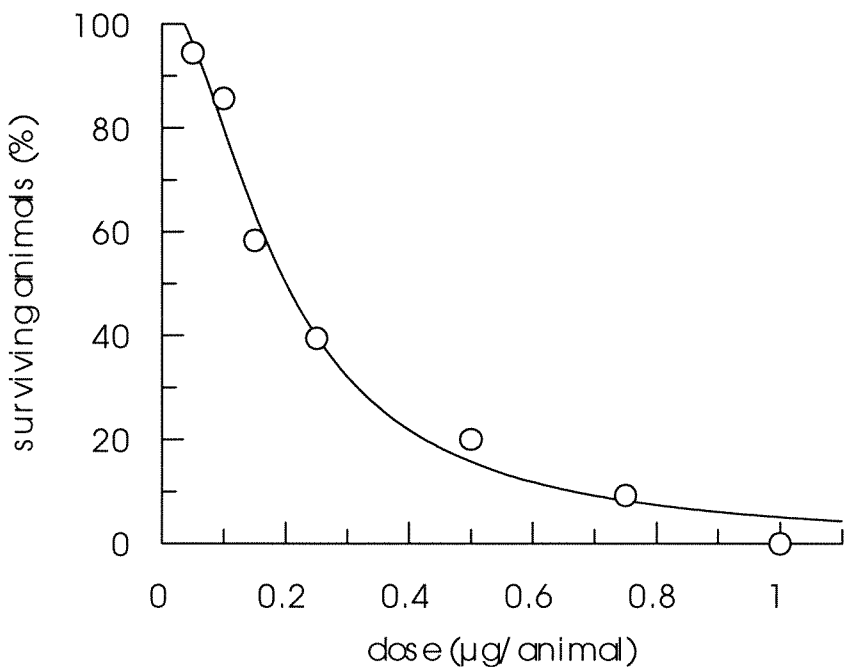


FIGURE 4.4.1 The toxicity of the syntethic microcystin-LR on locust (*Locusta migratoria migratorioides* R.F.).

dose (µg/animal)	*NIVA (%)*	*Debrecen (%)*
2	89.6	100
5	81.9	95.7
10	58.4	72
20	39.8	60
40	26	36
100	7	15
200	3.5	6

LD_{50}15 µg/animal=10mg/kg (NIVA)

LD_{50} =22.6 µg/animal=15.1mg/kg (Debrecen)

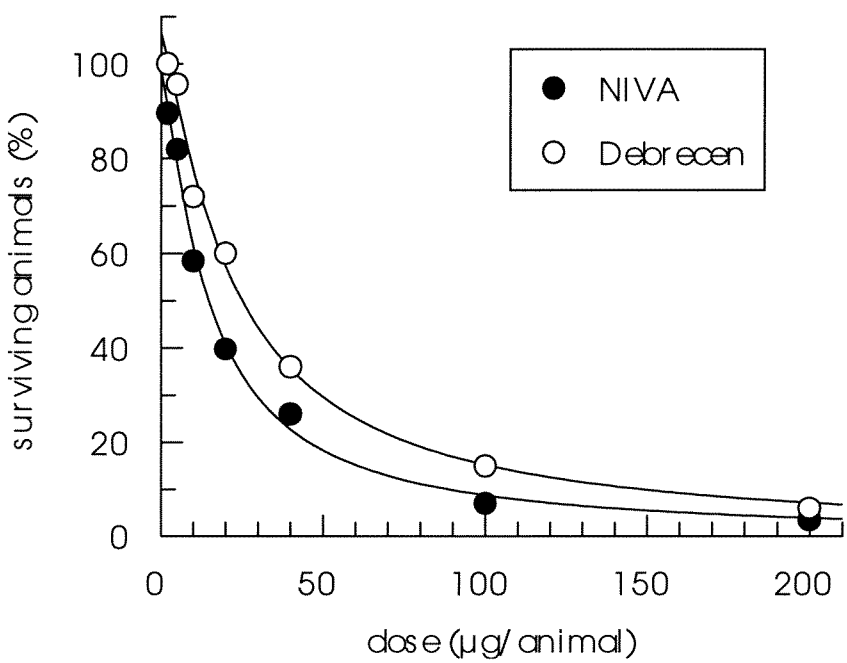


FIGURE 4.4.2 The toxicity of lyophilized cell extract of the *Microcystis aeruginosa* on locust (*Locusta migratoria migratorioides* R.F.).

dose (μg/animal)	*surviving animals (%)*
100	100
200	100
500	87.5
1000	60
1500	28

LD_{50}=1150 μg/animal= 767mg/kg

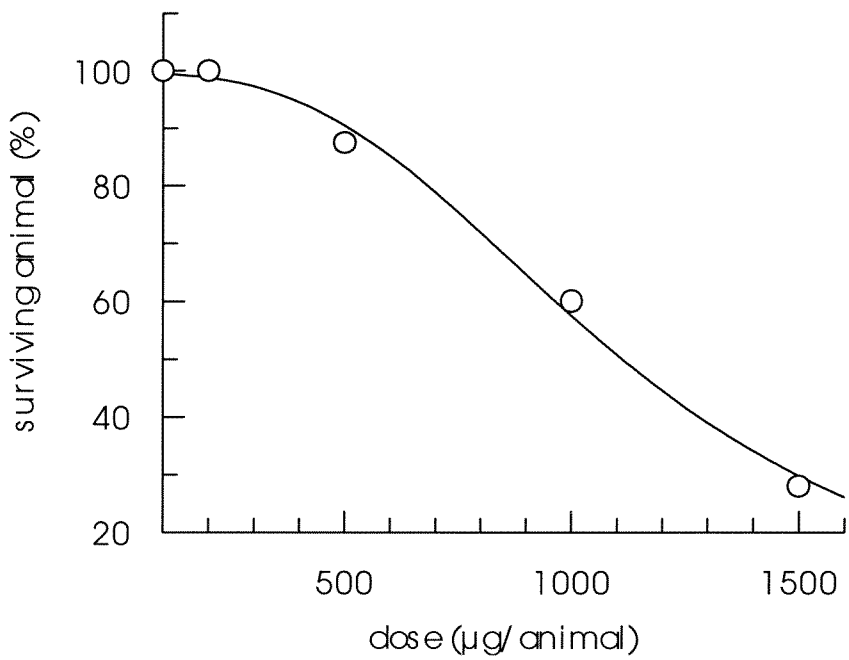


FIGURE 4.4.3 The toxicity of the lyophilized cell extract of the *Cylindrospermopsis raciborskii* on locust.

dose (mg/animal)	*surviving animals (%)*
2	98
10	57.1
20	37.5
30	11.1
40	0

LD_{50}=12.55 mg/animal=1255 mg/kg

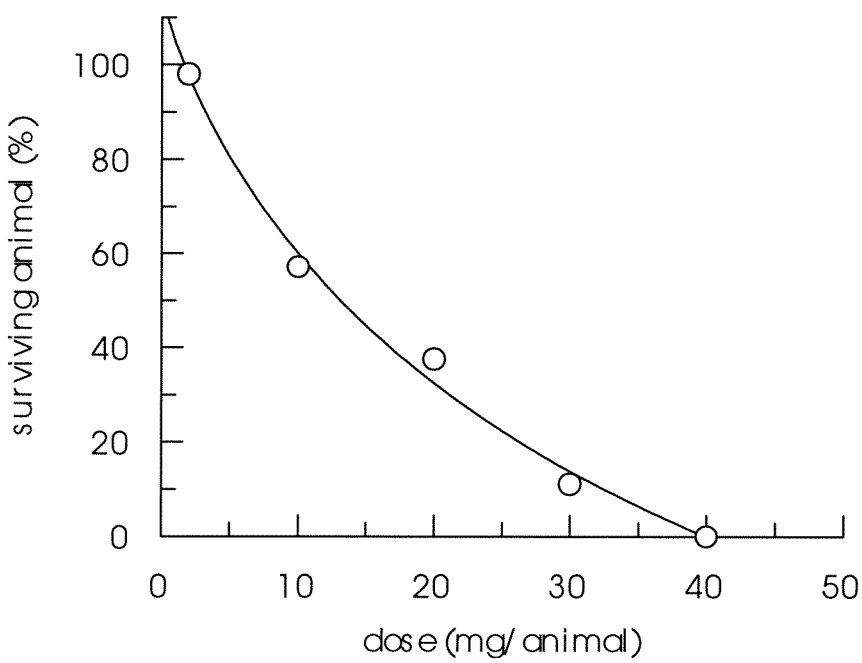


FIGURE 4.4.4 The toxicity of the lyophilized cell extract of the *Cylindrospermopsis raciborskii* on fish (*Rutilus rutilus* L.).

months	*chlorophyll-a (µg/l)*	*LD_{50} (mg/kg)*
June	115	5641
July	166	5660
August	192	1513
September	150	791

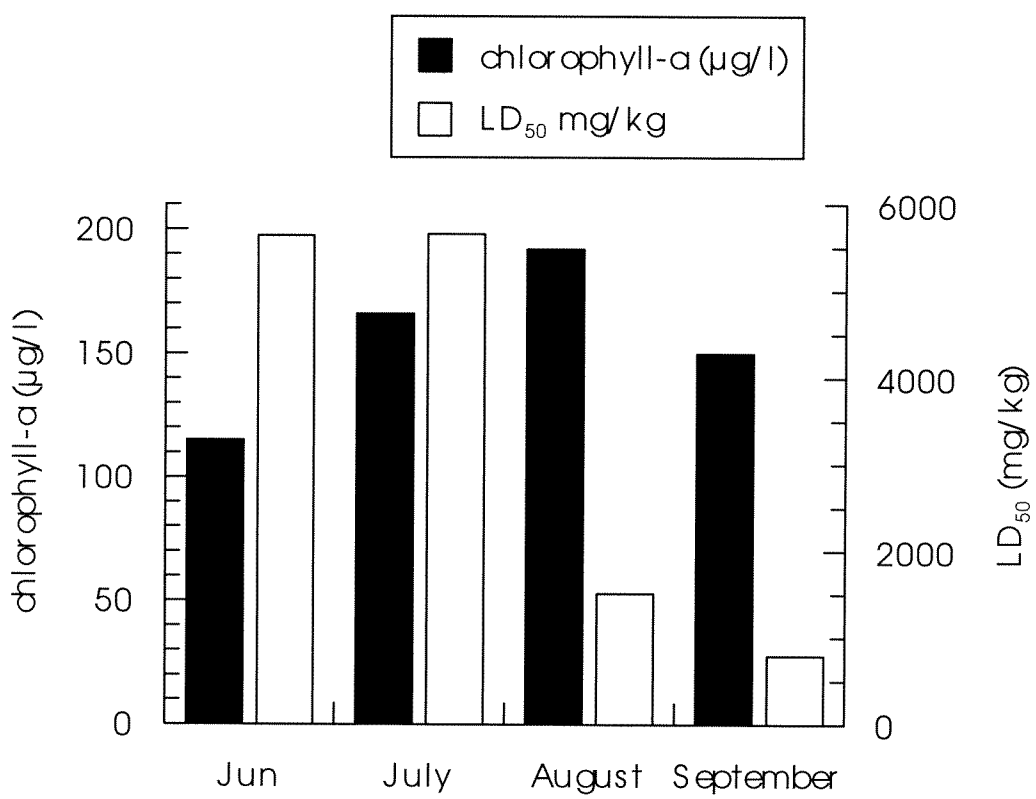


FIGURE 4.4.5 The toxicity of the algae from Kis - Balaton during summer 1996.

4.4.2 Cyanotoxins and plant interactions

(Kosuth University of Debrecen)

Most of the existing data concerning the effect of cyanotoxins on living organisms is based on studies using animal experimental systems, namely the mouse bioassay. *Microcystis aeruginosa* is perhaps the most common nuisance cyanobacterium of temperate surface water bodies since it forms toxic water blooms in eutrophic lakes and reservoirs. Indeed, toxicity assessment of European cyanobacterial blooms revealed a high percentage of those producing toxins (Skulberg et al. 1984). This is true for Hungarian blooms even, since all reported cases have been toxic (Török and Mayer 1988).

Unfortunately the cyanotoxin interaction with plants is a neglected field, though the biochemical mechanism at least for the microcystins is known (protein phosphatase inhibitors). Plant members of freshwater ecosystems are possible subjects of direct effects by cyanotoxins after autolysis of toxin producing cells. Therefore studies are relevant on the interaction of aquatic vegetation and cyanotoxins. As a model system the established plant biotest system at the Kossuth University of Debrecen was used, based on *Sinapis alba*, the mustard plant. Earlier, the Blue-Green *Sinapis alba* Test system (BGST) was developed to determine the possible presence of cyanotoxins (plant interfering ones) in samples of phytoplankton (Kos et al., 1995).

The rationiale of the plant biotest is based on the potent inhibitory effect of microcystin - a cyclic heptapeptide which has protein phosphatase inhibitory activity - that may interfere with the plant growth. It is noteworthy that most species of plants - both dicotyledonous and monocotyledonous - are sensitive to microcystins. Indeed, it is known that a variety of plant cellular processes are regulated by modulation of the phosphorylation state of specific proteins, and protein phosphatases are important in the control of cellular metabolism, growth and differentiation. Microcystin LR prevents the light induced activation of sucrose phosphate synthetase via modulating the inhibition of protein phosphatases. Oilseed-rape (*Brassica napus*), maize (*Zea mays*) and peas (*Pisum sativum*) contain high levels of type 1 and type 2A protein phosphatases with properties similar to those of corresponding enzymes in animal cells.

The *Sinapis*-test (BGST) is performed as follows:

Mustard seeds (*Sinapis alba* L. convar. Budakalászi sárga, from Hermes, Budapest, Hungary) were sterilized with 5% H_2O_2 solution (3x30 min) and washed several times with sterile-distilled water. The seeds were thereafter depositioned onto the surface of 1 ml plant nutrient solution in a cupped test tube solidified with 0,6% agar (Bacto), and supplemented with cyanotoxin containing samples (fraction samples of the purification steps, whole cell's suspension after freezing and cell extracts, etc.). Three seeds were grown per test tube, and at least three parallel tubes were kept at 25 °C ±1) in a growth room (16 h photoperiod at 9). The mean length of seedlings were measured after five to eight days, and standard deviations of data were calculated.

Samples of the purified toxins, crude *Microcystis* sp. - extracts, frozen whole cyanobacterial cells and even bloom material were tested for toxicity using the standardized BGST. Freezing the cells (-20°C) of plankton origin increased the amount of extractable toxin remarkably. We introduced this step into the general extraction procedure, and it was applied even in the *Sinapis*-seedling tests. After centrifugation the collected cell pellets were kept overnight at -20°C (for purification or for obtaining microcystins from whole cells). In all cases, there were a significant concentration-dependent inhibition of the growth of mustard seedlings. The *Sinapis*-test system is sensitive enough, since we observed 50% inhibition of growth (LD_{50}) at 3 μg ml^{-1}. In addition, if we used samples with unknown toxin content and/or freezed whole cells or bloom material BGST was suitable to determine microcystin content of preparations (data not shown).

The planting of sterilized seeds in the BGST is made on the surface of nutrient's solution solidified with agar that contains the toxic materials to be tested. Therefore, during the preparations of cyanotoxin containing nutrient solution in test tubes, there is a possible and significant heat effect on the toxic materials. So, the stability of microcystins under the influence of heat can be an essential and determining factor for the fate of cyanotoxins and the *Sinapis*-test. We determined the heat stability of purified microcystin toxin. 5 and 60 min. heating of toxin in water at 70 and 100°C respectively did not alter significant the toxicity of purified microcystin in BGST. The BGST procedure might be a general, convenient and sensitive procedure for microcystin assessments (similar results were also obtained with cell extracts of *Cylindrospermopsis raciborskii*). Recently we have started to investigate the possible deleterious effects of *Cylindrospermopsis raciborskii*. The last couple of years *Cylindrospermopsis raciborskii* has become one of the most problematic organisms in Lake Balaton. To check the possible toxic effect of this organism on plants was imperative. BGST is suitable to detect the

toxic effect of lyophilized *Cylindrospermopsis raciborskii* in suspension (LD_{50} 2-4 μg ml^{-1}). Experiments indicated that the root system of *Sinapis alba* was more sensitive to cylindrospermopsin than the other parts of the plant (hypocotyl). Since we were not able to obtain germinating reed seeds, instead we used calli of *Phragmites australis* to determine the LD_{50} values.

In preliminary study we have shown that the plant members of water ecosystems (e.g. *Phragmites australis, Typha latifolia, Lemna minor etc.*) are subject to deleterious effects of cyanotoxins (microcystins, cylindrospermopsin). The growth of seedlings of the investigated previously mentioned plants, is inhibited. The ID_{50} value for *Lemna minor* was 20 μg ml^{-1}, and for seedlings of *Typha latifolia* respectively 1-10 μg ml^{-1}.

Using reed callus (*Phragmites australis*), and various plant systems, we obtained data suggesting that cyanotoxins might induce stress related enzymes like ssDN-ase, catalase, peroxidase and superoxide dismutase. The establishment of the reed callus and the regeneration system is in progress. The callus system is well established, but plant regeneration from reed calli requires further studies.

4.4.3 *Nitzschia* - test

(Norwegian Institute for Water Research)

The biotest is based on the *Nitzschia*- clone NIVA-BAC 38 as test organism, a pennate diatom of the family Bacillariaceae. The cells have a gliding motility on a solid surface, and are possessing the typical diatom raphe-structure. Both these properties make the organism especially sensitive to its surroundings. This biotest method was developed at NIVA for experimental studies of cyanotoxins (Kiefer & Oredalen 1996), and is a promising research aid for the Lake Balaton blue-green algae project. The laboratory procedure was introduced and made use of during the limnological investigations in Tihany, September 1996.

The principle of the biotest (NIVA 1996) is to grow the test-organism on the surface of a solid agar in petridishes. The cells are established and evenly distributed on the surface, before a small well is made in the centre of each agar-plate. Into this well the test-material is added (see 4.3). The plates are incubated under defined growth conditions, for 4-7 days. If there are any toxic substances in the test-material, it will diffuse from the well into the agar coming in contact with the cells, and be taken up by the *Nitzschia*-cells together with the nutrients. The cells may react to harmful disturbances in at least three different ways: They may migrate away from the source,

they may alter their behaviour/physiology or die. At low light-irradiation, the cells will be relatively strong pigmented, which make changes in the distribution of algae on the agar surface easy to observe.

The effect of a potentially toxic component in the test-material will gradually decrease with the distance away from the well. If the *Nitzschia*-cells are moving out of the contaminated area or die, a circular zone at a certain distance away from the well will be established, depending on the concentration of the toxic substance(s).

Preliminary experiments with different extracts from blue-green algae were performed during 1996. Algal material from laboratory cultures at NIVA and BLRI, and waterblooms have been applied. Known substances of toxins have served for comparative purposes. The experience and results obtained are promising for the further practical progress of this biotest method.

4.4.4 Other microalgae used for effect studies

(Pannon University of Agricultural Sciences)

The experiments were carried out with a freeze-dried *C. raciborskii* biomass, which was provided by BLRI. The *C. raciborskii* biomass was resuspended in distilled water in a concentration of 10 mg/ml, sonicated for 10 minutes and used for treatment, or mixed with an equal volume of warm (ca 50°C) 2% agar-containing distilled water just before using for treatment. The influence on the growth of *Arthronema africanum, Nostoc commune, Scenedesmus quadricauda, Selenastrum capricornutum, Chlamydomonas reinhardtii, Nitzschia* sp. and *Cyclotella* sp. was investigated by some agar-plate methods.

Different quantities of the investigated strains were (1) sprayed on agar-plate surface or (2) mixed into the agar-plate. The inoculated plates were incubated in inverted position in an algal culture room under 12:12 hours light: dark-cycle and at 25 ±2°C for 4 days before the treatment. Afterwards the agar plates were treated with the prepared biomass of *C. raciborskii* according to the well-known (1) punch-hole method, (2) paper-disc method, and (3) agar-block method. The influence was evaluated by visual observations, and by the measurements of the inhibition or stimulation zones during maximum one week following the treatment.

The results confirmed that the most appropriate method includes the following steps (1) mixing the investigated strain into the agar-plate giving a final biomass concentration of 30 to 50 mg/l, (2)

incubation in inverted position during 4 days before the treatment (3) treatment according to the punch-hole method with *C. raciborskii*, which was solidified by agar, and (4) evaluation of the influence 4-5 days after the treatment.

All kind of experiments proved that none of the investigated strains were inhibited even by a high (5 mg/ml) concentration of the *C. raciborskii* biomass. On the contrary, *Arthronema africanum* was significantly stimulated by the treatment. The stimulation increased with the decreasing density of *A. africanum* mixed into the agar-plate. *Chlamydomonas reinhardtii* and *Nostoc commune* were slightly stimulated by the treatment.

4.4.5 *Thamnocephalus*-test

('B. Johan' National Institute of Public Health and Norwegian Institute for Water Research)

This bioassay was developed by the research team of Prof. Dr. G. Persoone at the State University of Ghent, Belgium. Research scientist Dr. Andrea Török from `B. Johan`National Institute of Public Health in Hungary, has been doing the prelimenary experiments using this test on toxins from blue-green algae. In co-operation with her, during a study visit at NIVA, the *Thamnocephalus*-test was used to screen the effects of three different groups of blue-green algae-extracts. Former results from mouse-tests have shown that these extracts were either neurotoxic, hepatotoxic or protracted toxic (Skulberg 1996). By using extracts from the same algal strains, we wanted to compare the effects on *Thamnocephalus*-larvae with the observed effects on *Nitzcshia* (Kiefer & Oredalen 1996) and other test-organisms.

The principle of this test is to add different concentrations of the test-solution to small (2 mL) microplate test-wells, each inoculated with 10 *Thamnocephalus*-larvae molded to the second or third instar stage. We used four wells for each concentration of the test-material: three parallells with larvae in the test-solution, and one controll with larvae living in standard freshwater. After incubation for 24 hour, we counted survived and dead individuals in all the wells. If mortality exceeds 10 % in the controll, the experiment is rejected. The results are presented as % mortality of *Thamnocephalus*-larvae, for each concentration of the testmaterial.

In the introductory experiments we found 100 % mortality for all three groups of extracts tested, when the concentrations exceeded 3 mg mL^{-1}. For microcystin (hepatotoxic) we observed 100 % mortality down to our lowest concentration; 0.7 mg mL^{-1}. More experiments and comparisons

have to be done, but the method seems promising as a screening tool, both concerning simpleness and sensitivity.

4.4.6 Snail nerve cells used as test system for cyanotoxins

(Balaton Limnological Research Institute)

The central nervous system of gastropods (snails) may provide a suitable model for testing the neurotoxic effect of algal toxins. In our experiments the electrical responses of identified neurons of two snail species (the terrestrial snail *Helix pomatia* and the aquatic snail *Lymnaea stagnalis*) were studied using inctracellular microelectrophysiological methods.

Anatoxin-a evoked specific intracellular responses (depolarizing or hyperpolarizing of the neuronal membrane) on identified nerve cells. The neurotransmitter receptors of the neurons were affected by the neurotoxin. The membrane responses of the neurons were reversibly blocked in the presence of anatoxin-a.

The cylindrospermopsin-containing extract obtained from a pure culture of *Cylindrospermopsis raciborskii* (ACT 9502) had similar neuronal effects on the preparations. Identified neurons of both *Helix* and *Lymnaea* displayed specific membrane responses after application of the algal extract suggesting that snail neurons have receptors or receptor-like structures for the active (neurotoxic) component of the extract. Moreover the cyanophyte extract inhibited the acetylcholine responses of the neurons, similarly to the anatoxin effect described above. The results suggest that although cylindrospermopsin is considered to be a hepatotoxin in vertebrate animals, the cylindrospermopsin-containing extract of *Cylindrospermopsis raciborskii* (ACT 9502) has clear neurotoxic effects on neuronal model preparations.

The hepatotoxin microcystin had no visible effect on the electrical activity of the neurons of the central nervous system of either *Helix pomatia* or *Lymnaea stagnalis*.

4.4.7 Mouse bioassay

(The Norwegian College of Veterinary Medicine and Norwegian Institute for Water Research)

The common technique used for the determination of cyanotoxins is the mouse bioassay (Berg et al. 1987). A variety of procedural differences has been introduced, however the basic concept is the same. An extract of the sample containing the cyanotoxin is injected intraperitoneally (i.p.) into a mouse, which is then observed for symptoms of toxicity. This bioassay has formed the basis for the discovery, study, and health regulation of all relevant cyanotoxins.

Mouse bioassays were performed during 1996 both in the Hungarian and the Norwegian laboratories connected with the investigations of toxinproducing blue-green algae in Lake Balaton. The test material has mostly been freshly sampled phytoplankton blooms with live cells of blue-greens. Concentration has been achieved by centrifuging the algal cells into a pellet. *In vivo* toxicity testing has been performed on the pellet. To do this the pellet is resuspended in physiological saline. Repeated freeze-thawing combined with sonication are necessary to disrupt the cells. For assessment of total cyanotoxin content the cell-free supernatant requires concentration (Sep-Pak cartridge), elution and measurement.

The cyanotoxin cylindrospermopsin (see 4.2) is chemically an alkaloid. On injection into mice this substance attacks a wide range of tissues. The test animals have to be observed during 2-7 days in order to register the progressive organ necrosis.

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