R&D Project 349

Field validation of algal toxin test kit

University of Dundee

December 1994

R&D Draft Final Report 349/7/A
(R&D Note)

FIELD VALIDATION OF ALGAL TOXIN TEST KIT

S BELL

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R&D Draft Final Report 349/7/A

QUALITY REVIEW INVITATION

PM3/cp

Project Reference: NRD 349 Function: Water Quality Region/H.O. Dept Anglian	Prepared by: G Brighty Date: 24 November 1994

Project Title	Stage *(if applicable)*
Validation of field test kit for blue-green algal toxins	Draft Final Report

You are invited to attend a Quality Review of the following Product(s):

Product Code(s)	Product Name(s)
349/7/A	Draft Final Report - Field validation of algal toxin test kit

Venue (if postal enter "POSTAL")	Postal	Date Time Duration	19/12/ 94 **closes: 10/1/95**
Chairman	Paul Williams	Project Manager	M Pearson
Reviewers	P Williams (SW) M Bramley (HO) J Seager (HO) T Long (HO)	M Pearson (A) A Ferguson (A) G Brighty (A)	

Product Description(s) attached

Draft Final Report summarising the work undertaken on R&D project 349. This project was set up to produce toxin for test kit development, assess and inspect the work of the contractor for project 348, Biocode, validate the test kit to be produced from that project and to develop field protocols for the test kit's use, including algal sample preparation.

As the overall project objective for Project 348 was not achieved, clearly the objectives for project 349 were similarly frustrated. However, despite this, the Draft Final Report does present results of significance and interest to the Authority.

Fully detailed Interim Reports (6 no) already presented and are bound as a Project Record. The DFR presents the work undertaken and presents algal cell lysis procedures and validation work performed on the antibodoies produced by Biocode.

The Draft Final Report and final version, an R&D Note, should remain Externally Restricted due to a confidentiality agreement between NRA and Biocode.

Product(s) attached (where applicable)

Review criteria: The project did not achieve its objective. However, in order for NRA to benefit from the project work, please consider the following review criteria.

1 Does the report present the full detail required for a summary document from the project ?

2 Of the information presented, is it clear to the NRA audience what was undertaken and achieved ?
What would you add/remove ?

3 Was the technical quality input by University of Dundee good/average/poor ?

Qualify your comment by stating what Dundee's strengths were, or the aspects you would have changed.

4 What recommendations, if any, would you like to be added ?

5 Recommend dissemination of final R&D Note and Project Record

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Dissemination Status

Internal: Limited Release
External: Restricted

Statement of Use

This report is the Draft Final Report (R&D Note) from R&D Project 349. It is to be used for assessment and review, December 1994 to January 1995.

Dissemination List

1 copy of Draft Final Report 349/7/A to:

P Williams (SW)
M Bramley (HO)
J Seager (HO)
T Long (HO)
M Pearson (A)
A Ferguson (A)
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NRA Project Leader
The NRA's Project Leader for R&D Project 349 is:

M Pearson - Anglian Region

CONTENTS

1. SUMMARY

Work has been carried out in three principle areas:

A. Purification and supply of cyanobacterial peptide toxins to Biocode Ltd.

- 89.2 mg of purified microcystin-LR were sent to Biocode Ltd. for their use in producing and screening anti-microcystin antibodies.

- Other microcystin variants (microcystin-LW and 3-desmethyl microcystin-RR) and the closely related cyanobacterial toxin nodularin were also purified and sent to Biocode Ltd. for screening purposes.

B. Development of lysis procedures for the convenient release of cyanobacterial microcystins from cells, necessary for application to antibody-based test procedures.

- Microcystin-LR was successfully released from *Microcystis aeruginosa* cells by the addition of 0.3% (v/v) toluene. An incubation time of 15 minutes was suitable for the toluene-mediated cell-lysis, releasing almost 100% of the intra-cellular microcystin-LR into the cell-free medium. Filter-entrapment on GF/C glass-fibre filters and subsequent toluene-lysis was achieved. Toluene-mediated cell lysis was found to be temperature-dependent at temperatures below 20°C, the lower the temperature the lower the amount of microcystin-LR released from the cells.

- Methanol was found to be a suitable reagent for extracting microcystin-LR from *Microcystis aeruginosa*. Almost all of the microcystin-LR was released from the cells within 10 minutes of incubation with methanol, and the procedure was successfully applied to a range of cyanobacterial species known to contain microcystins. Filter-entrapment and subsequent methanol cell lysis was achieved. Concentrations of methanol below 100% did not recover sufficient microcystin-LR, and 100% methanol was considered too flammable for inclusion in a field-test kit.

- The addition of 20% (v/v) methanol to the 0.3% (v/v) toluene reagent increased the solubility of microcystin-LR phosphate buffered saline-based solutions, and greatly reduced the temperature-dependency of the cell-lysis release of microcystin-LR. This was considered to be the most appropriate reagent for use in a field-test kit.

- Non-ionic detergents appeared to be unsuccessful for the release of intracellular microcystin-LR into the cell-free medium.

- A protocol for use as a cell-lysis method in the field is presented.

C. Assessment of anti-microcystin-LR IgM antibodies produced by Biocode Ltd.

- An enzyme-linked immunosorbent assay (ELISA) format appeared to detect microcystin variants and nodularin using the anti-microcystin-LR IgM antibodies, but non-specific binding of these antibodies to coated test plates was implicated. A number of methods, including various blocking reagents and different assay conditions were applied in

attempts to reduce the non-specific binding, but were unsuccessful.

- A dot-blot assay whereby microcystin-LR was spotted onto a nitrocellulose membrane, blocked, the anti-microcystin-LR antibody applied, followed by antibody conjugate, then visualised by a luminescent reagent/X-ray film method was investigated. Ambiguous weak detection of the microcystin-LR spot was achieved, but attempts at optimisation of the assay were not successful.

- Incubation of microcystin-LR with the anti-microcystin-LR IgM antibody, followed by ammonium sulphate-precipitation, centrifugation, and HPLC-analysis of the supernatants, was performed. This resulted in a removal of microcystin-LR from solution when antibodies were present. This provided unequivocal evidence that the IgM antibodies bound to microcystin-LR in solution.

- Incubation of microcystin-LR with the anti-microcystin-LR IgM antibody, and ammonium-sulphate precipitation, lead to a reduction in toxicity of the precipitate-free solution when administered to laboratory mice.

- Attempts at assessing the anti-microcystin-LR IgM antibodies by radio-immuno assay were unsuccessful.

- Attempts at assessing the anti-microcystin-LR IgM antibodies by a gel-diffusion immuno assay were also unsuccessful.

2. OBJECTIVES

2.1 Overall project objectives

To validate for the NRA the development and performance of a field test kit for microcystin-LR (MC-LR) to be developed by Biocode Ltd, and to develop field procedures for its use by NRA staff.

2.2 Specific objectives

The specific objectives for the project as laid down in the "Memorandum of agreement for research contract" ref NRD040 were as follows:

2.2.1. To liaise with and provide technical advice to Biocode Ltd throughout the contract.

2.2.2. To purify and supply MC-LR to Biocode Ltd for the production and screening of monoclonal antibodies (MAbs), and supply information on purity and possible contaminants.

2.2.3. To inspect and assess the results of the performance of antisera, produced by Biocode, in Biocode's laboratory tests at all stages of MAb production.

2.2.4. To receive results and advise NRA on selection of most appropriate MAb(s) for further trials.

2.2.5. To purify cyanobacterial peptide toxins related to MC-LR (other microcystins and nodularin) for Biocode to test against the Biocode MC-LR antibodies for cross-reactivity and sensitivity.

2.2.6. To advise the NRA on the selection of final MAb(s) for test kit production, based on Biocode's laboratory assessment procedures.

2.2.7. To perform supplementary tests as necessary, but agreed by NRA, to confirm the selection of the most appropriate MAb(s).

2.2.8. To assess the performance of the test columns, assessing objectives 2.2.9 to 2.2.14.

2.2.9. To determine whether the Biocode MC-LR test prototypes would only detect cyanobacterial peptide toxins(s) in free/released form in water samples. [Assuming this was the case, then procedures were to be developed to disrupt/lyse cyanobacterial cells so as to release the toxins and facilitate detection by the MAb(s).]

2.2.10. To evaluate Biocode MC-LR test kit(s) using laboratory cultures of cyanobacteria.

2.2.11. To evaluate Biocode MC-LR test kits for the detection of cyanobacterial peptide toxins in natural cyanobacterial blooms.

2.2.12. To confirm the ability of the test kits to detect and distinguish between extracellular and intracellular peptide toxins, and therefore enable a total estimate of MC-LR and related

toxins in water, bloom and scum samples.

2.2.13. To define detection limits for the test kit for MC-LR and related cyanobacterial toxins and to compare these with other current methods.

2.2.14. To produce a Validation Report detailing experimental approaches and findings to NRA.

3. FACTORS AFFECTING COMPLETION OF THE WORK PROGRAMME

The project commenced with the appointment of Dr Steven G. Bell as a full-time Post-doctoral Research Assistant on 1 November 1991.

To enable Biocode to begin their project (0348) without delay, University staff were temporarily assigned to the cultivation of toxic *Microcystis* cells and purification of the toxin for some weeks before the contract for Project 0349 was signed. This enabled purified MC-LR to be supplied to Biocode on 22 October 1991 at the commencement of the project.

Due to technical difficulties unforeseen by Biocode, the production and subsequent supply to Dundee University of MAbs raised against MC-LR was delayed, resulting in a delay to Project 0349 at Dundee. When anti-MC-LR (αMC-LR) MAbs were produced and selected after screening by Biocode they were subsequently found to be unsuitable in an affinity column format (when the MAbs were bound to the column matrix they were unable to bind purified MC-LR passed through the column). The decision was taken by the NRA and Biocode not to proceed with these antibodies in a column test kit format. Instead, Biocode were to proceed with the development of different MAbs raised against synthesized ADDA, an integral part of the microcystin molecule. As a result of this decision, we were not required to meet objectives 2.2.6 to 2.2.14 (above) in this project.

Objectives 2.2.6 to 2.2.14 were replaced by an objective to further assess the αMC-LR antibodies produced by Biocode in a number of procedures and to produce a final report for internal use by the NRA.

4. RESEARCH PROGRAMME

4.1 Purification and supply of microcystin variants and nodularin

The microcystins are hepatotoxic cyclic peptides whose structure is shown in Figure 4.1.1. The structure of microcystin is a 7-amino acid ring, the two amino acid regions X and Y being variable between the different microcystin variants. The structure of the closely related toxin nodularin is presented in Figure 4.1.2. This is a penta-peptide ring that differs from the microcystins in that the alanine and X amino acids are absent and the N-methyl-dehydroalanine is substituted by N-methyldehydroaminobutyric acid. The ADDA residue was regarded as unique to the microcystins and nodularin, and its presence is required for these molecules to be toxic.

The microcystin variant microcystin-LR (MC-LR) contains leucine (Leu) at position X and arginine (Arg) at position Y. It is the most commonly occurring microcystin variant found in British freshwaters, and it is present as the major variant in our laboratory cultures of *M.aeruginosa* PCC 7813. This variant was therefore chosen as the variant against which attempts to raise monoclonal antibodies would be made.

The MC-LR was purified by extraction from freeze-dried cyanobacterial cells in 5% acetic acid, followed by solid-phase extraction through Sep-Pak C18 cartridges and elution with an increasing step-wise methanol gradient. Final analysis of the purity was made by HPLC on a reverse-phase C18 column eluted with 35 - 47% acetonitrile (in water containing 0.05% TFA) over 20 minutes then 47 - 100% acetonitrile over 5 minutes. Figure 4.1.3 illustrates a typical HPLC chromatogram of purified MC-LR.

A total of 89.2 mg of MC-LR were sent to Biocode throughout the course of the project, this ranging in purity from 91% in the early stages of the project to typically 98 - 99% over the majority of the project (Table 4.1.1).

Other microcystin variants and nodularin were purified to enable Biocode to carry out screening of the monoclonal antibodies with these. These variants have also been used in our laboratory for antibody assessment purposes. The variants purified are; microcystin 3-desmethyl RR, where X and Y (Figure 4.1.1) are both arginine and the Masp is demethylated to form aspartic acid; microcystin-LW, where X is leucine and Y is tryptophan; and nodularin.

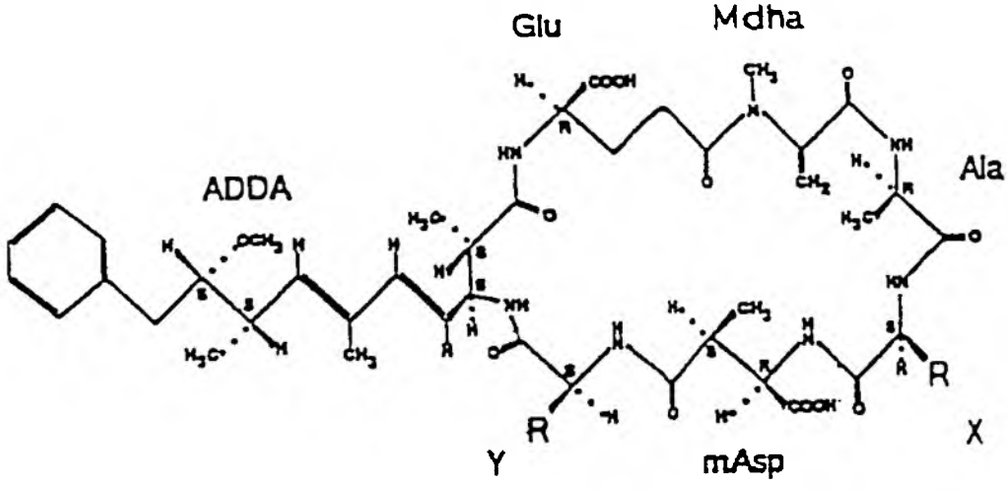


Figure 4.1.1 Structure of microcystin

Abbreviations:

mAsp β-methylaspartic acid
Ala alanine
Mdha N-methyldehydroalanine
Glu glutamic acid
ADDA 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca4,6-dienoic acid
X variable amino acid
Y variable amino acid

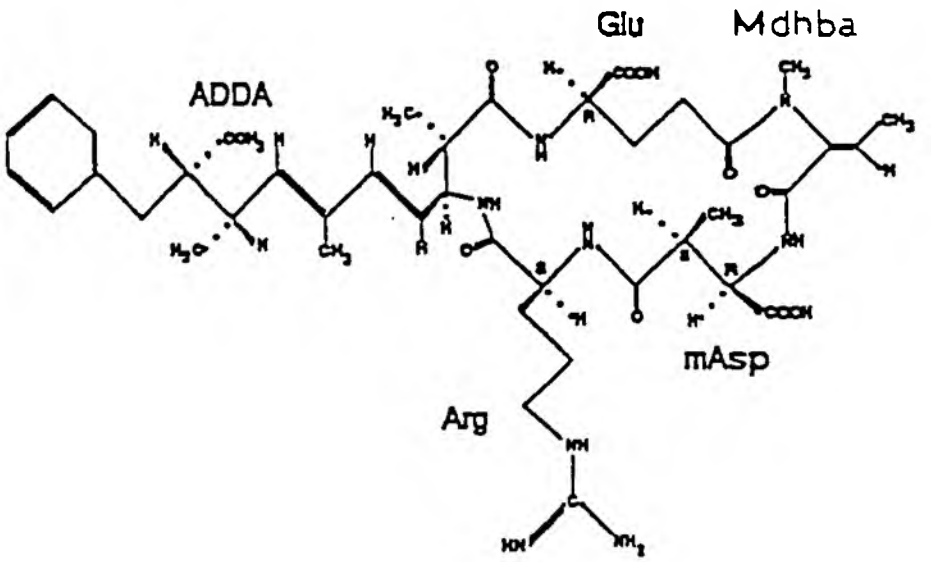


Figure 4.1.2 Structure of nodularin

Abbreviations:

mAsp β-methylaspartic acid
Mdhba N-methyldehydroaminobutyric acid
Glu glutamic acid
ADDA 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Arg arginine

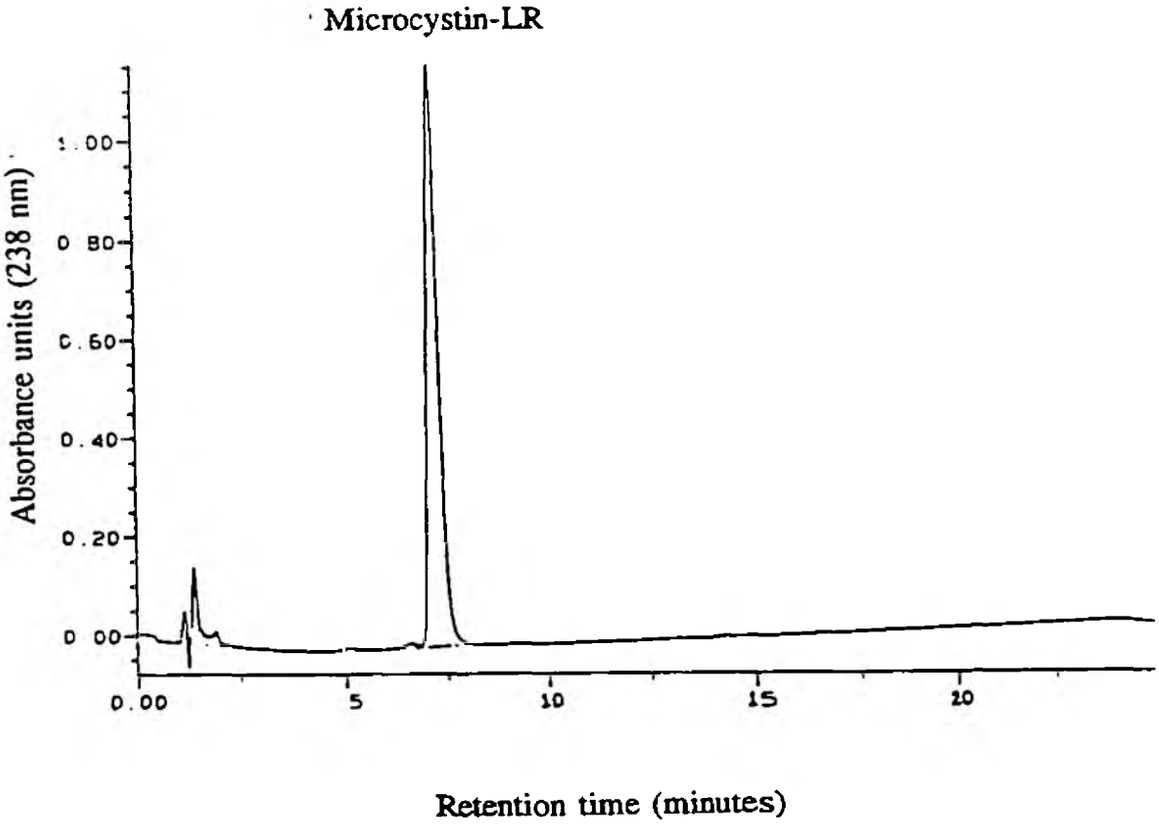


Figure 4.1.3 Typical HPLC chromatogram of purified MC-LR

(using a reverse-phase C18 column and eluted with 35 - 47% acetonitrile in water containing 0.05% TFA (20 min) then 47 - 100% acetonitrile (5 minutes)).

Table 4.1.1 Consignments of purified MC-LR supplied to Biocode Ltd.

MC-LR Lot No.	Purity[a]	Amount dispatched	Date dispatched
Initial supply	> 90%	10.0 mg	22.10.91
SGB251191	93%	5.0 mg	26.11.91
SGB251191.2	97%	4.5 mg	02.12.91
SGB281191	91%	4.5 mg	02.12.91
SGB221191	98%	3.6 mg	04.12.91
SGB021291	91%	4.0 mg	08.01.92
SGB291191	98%	4.7 mg	13.02.92
SGB111291	98%	5.3 mg	13.02.92
SGB210292	97%	10.0 mg	24.02.92
SGB280292	98%	10.0 mg	10.03.92
SGB050392	98%	5.0 mg	24.04.92
SGB291093	99%	10.0 mg	01.11.93
SGB051193	99%	12.6 mg	10.11.93
Total		89.2 mg	

a, estimated by HPLC analysis at 238nm as detailed in footnote to Figure 4.1.3

4.2 Lysis of cyanobacterial cells

4.2.1 Introduction

Objective 2.2.9 in section 2 states the need to develop a procedure whereby cyanobacterial cells can be lysed or disrupted at the field test site. This would be necessary since the peptide toxins are retained within intact cells, and the availability of a procedure would facilitate estimation of the intracellular toxin content by releasing toxin followed by subsequent detection by the MAb-containing test kit. The lysis procedure should therefore release the intracellular toxin, and should be sufficiently rapid so as to allow this at the field-site.

In order to release intracellular toxin from intact cyanobacterial cells, two approaches may be followed: addition of lysis reagent(s) to a relatively large volume of cells in the test sample, incubate to allow lysis to occur, then passage of the entire volume of sample through the test kit to retain/detect the released toxin; or concentration/entrapment of the cyanobacterial cells in the test sample by filtration, addition of the entrapped cells on the filter to a much reduced volume of lysis reagent(s), then passage of the smaller volume of released toxin in the lysis reagent(s) through the test kit. The second method would have the advantage of transportation of reduced volumes of lysis reagent(s) to the field, and would facilitate the measurement/distinction of intracellular toxin from free toxin in the water by providing a cell-free water sample as well as entrapped cyanobacterial cells. Attempts to develop a filtration method were therefore made.

In order to develop a suitable lysis method, different reagents were investigated using cultures of cyanobacteria in the laboratory. These included acid-, alkali-, solvent- and detergent-based procedures. Efficiency of the more suitable procedures was investigated under various incubation conditions and using cyanobacterial species, and optimisation of the procedures was carried out.

4.2.2 Entrapment of cyanobacterial cells

The type of filter used routinely in this laboratory to remove intact cyanobacterial cells from culture medium and natural water samples is the Whatman GF/C glass-fibre filter. It was therefore decided to employ this filter to entrap cyanobacteria prior to cell lysis. Initial investigations involved an estimation of the loading capacity for *Microcystis aeruginosa* cells as determined by the chlorophyll *a* (Chl *a*) measurement of the filtered cells.

A laboratory culture of *M. aeruginosa* PCC 7813 was measured for Chl *a* content by centrifuging four 1 ml aliquots at 14,200 x g for 5 minutes. The supernatants were aspirated and discarded and the pellets were each resuspended in 1 ml of methanol. The methanol suspensions were then stored at 2 - 8°C for 24 hours before measuring the absorbance in a spectrophotometer at 663 nm against a blank of methanol. The Chl *a* concentration was then calculated using the formula:

Chl *a* concentration (μg/ml) = A_{663} x 12.63

Using a 60 ml syringe, measured volumes of the culture were passed through three 2.5 cm diameter GF/C filters until they became blocked. The loading capacity of the GF/C filter for *M. aeruginosa* PCC 7813 was then calculated as follows:

mean volume of *M. aeruginosa* PCC 7813 loaded = 10.0 (± 1.0) ml

Chl *a* concentration of *M. aeruginosa* PCC 7813 = 2.37 (± 0.1) μg/ml

∴ amount of Chl *a* loaded = 23.7 μg

surface area of 2.5 cm diameter filter = 4.9 cm^2

∴ Chl *a* loading capacity of GF/C filter = 4.84 $\mu g/cm^2$

Natural blooms of *Microcystis* have been collected by this laboratory for which the Chl *a* concentrations have ranged from 0.002 μg/ml to 0.25 μg/ml. If the above loading capacity was used then 11.5 l (lowest Chl *a* concentration) and 0.092 l (highest Chl *a* concentration) would be required to block a 2.5 cm diameter GF/C filter. If a 4.7 cm diameter filter was used then these values would be 41.9 l and 0.335 l respectively. These values were calculated assuming that only *Microcystis* cells were present in the water.

An investigation of the suitability of the GF/C filter for entrapment of and subsequent lysis of *M. aeruginosa* was carried out. A suspension of *M. aeruginosa* PCC 7813 was entrapped on a 2.5 cm diameter GF/C filter and subsequently lysed using a solution of toluene. The filter was found to be completely disrupted after a few seconds of vigorous shaking in this solution. After removal of the lysed cell material, the cell-free solution was analysed by HPLC for MC-LR content. The results were compared with free cells lysed by the toluene solution and the filtration method was shown to be as effective for releasing MC-LR into the surrounding medium. The details and results of this investigation will be presented in section 4.2.3. It was thus concluded that use of a Whatman GF/C filter was a suitable method for entrapment before subsequent lysis of *Microcystis* cells.

4.2.3 Toluene lysis of cyanobacterial cells

Three reagents were initially added to cultures of *M. aeruginosa* PCC 7813 to investigate lysis of cells. The lysis of cells was indicated by the presence of the intracellular pigment phycocyanin in the cell culture medium:

A culture of *M. aeruginosa* PCC 7813 was centrifuged at 2,500 x g for 10 minutes, then the pellet washed in an equal volume of growth medium before recentrifuging under the same conditions and final resuspension in an equal volume of growth medium. Three aliquots were taken and acetic acid, sodium hydroxide, and toluene were added to final concentrations of 10%. After 5, 10, 15, 30, 60, 120 minutes, and 48 hours, the suspensions were examined under the light microscope for cell appearance. 1 ml of each suspension was centrifuged at 14,200 x g for 5 minutes and the absorbance of the supernatants were measured in a

spectrophotometer at 620 nm against a growth medium blank, in order to estimate the amount of phycocyanin present in the growth medium. A control suspension containing no added reagent was investigated in the same way. After 48 hours, the acetic acid-treated cells appeared unaffected under the light microscope. The sodium hydroxide-treated cells appeared to be unaffected after 2 hours incubation, but their structures appeared to be partially disrupted after 48 hours. The toluene-treated cells appeared to be totally disrupted after only 5 minutes incubation. The results in Table 4.2.1 substantiate these observations in that only the toluene-treated cells appeared to release phycocyanin into the growth medium. Thus only toluene of the three reagents tested appeared to be suitable for lysing *M. aeruginosa* cells.

Table 4.2.1 The release of phycocyanin (absorbance at 620 nm) from *Microcystis aeruginosa* PCC 7813 cells by various agents

Addition	Final concentration	Incubation time	A_{620} cell-free supernatant[a]
None	-	48 h	0.000
Acetic acid	10% (v/v)	48 h	0.000
Sodium hydroxide	10% (w/v)	48 h	0.000
Toluene	10% (v/v)	5 min	0.144 ($\pm$0.01)

a, cell suspension in water incubated with the reagent for the time stated then centrifuged at 14,200 x g for 5 minutes in a microcentrifuge

The concentration of toluene was then reduced from 10% to 0.1% - 0.5% (v/v) and the experimental protocol described previously was performed. In this case the concentration of phycocyanin in the growth medium was measured more accurately by measuring the absorbance of the cell-free supernatants at 615 and 652 nm. The results in Table 4.2.2 indicate that release of phycocyanin from *M. aeruginosa* PCC 7813 cells was maximally achieved at a final toluene concentration of 0.2 - 0.3% (v/v).

Table 4.2.2 The release of phycocyanin from *Microcystis aeruginosa* PCC 7813 cells using toluene

Final concentration of toluene (v/v)[a]	Concentration of cell-free phycocyanin (μg/ml)[b]
0.0%	0.00
0.1%	0.00
0.2%	23.15 ($\pm$0.23)
0.3%	23.38 ($\pm$0.22)
0.4%	23.22 ($\pm$0.38)
0.5%	21.97 ($\pm$1.77)

a, toluene was added to the final concentration noted and the mixture shaken intermittently for 20 minutes

b, after toluene-treatment, the cell suspension was centrifuged at 14,200 x g for 5 minutes in a microcentrifuge and absorbances measured in the cell-free supernatant at 615 nm and 652 nm. The phycocyanin concentration was measured as followed:

$$\text{conc. of phycocyanin } (\mu g/ml) = \frac{A_{615} - (0.474 \times A_{652})}{0.00534}$$

To investigate the time-course of the toluene-mediated release of phycocyanin from cyanobacterial cells, toluene was added to washed *M. aeruginosa* PCC 7813 at a final concentration of 0.3% (v/v). 1 ml aliquots were taken in triplicate from the suspension after 5, 10, and 15 minutes, then centrifuged at 14, 200 x g for 5 minutes. The absorbance of the supernatants was measured at 615 nm and 652 nm and the concentration of phycocyanin calculated. The results in Table 4.2.3 indicate that all the phycocyanin was released from the cells after only 5 minutes incubation with 0.3% (v/v) toluene.

Table 4.2.3 Time-course of the release of phycocyanin from *Microcystis aeruginosa* PCC 7813 during treatment with 0.3%(v/v) toluene

Incubation time (minutes)	Concentration of cell-free phycocyanin (μg/ml)[a]
0	0.197 ($\pm$0.000)
5	17.331 ($\pm$0.491)
10	16.731 ($\pm$0.881)
15	13.198 ($\pm$0.652)

a, see footnote b to Table 4.2.2

At this stage it was decided to examine the cell-free medium from toluene-treated cells for the presence of microcystin-LR, as until now the presence of phycocyanin in the growth medium had been used as an indicator for cell lysis. A time-course study was carried out as detailed previously, but was allowed to run for 30 minutes instead of 15 minutes, the microcystin-LR concentration of the cell-free supernatants was estimated by HPLC analysis. The results presented in Figure 4.2.1 indicate that microcystin-LR was not released from the *M. aeruginosa* PCC 7813 cells as rapidly as the phycocyanin. There appeared to be a lag period of 5 minutes before the toxin was released, the majority (approximately 90%) of which was released within 15 minutes of incubation with toluene.

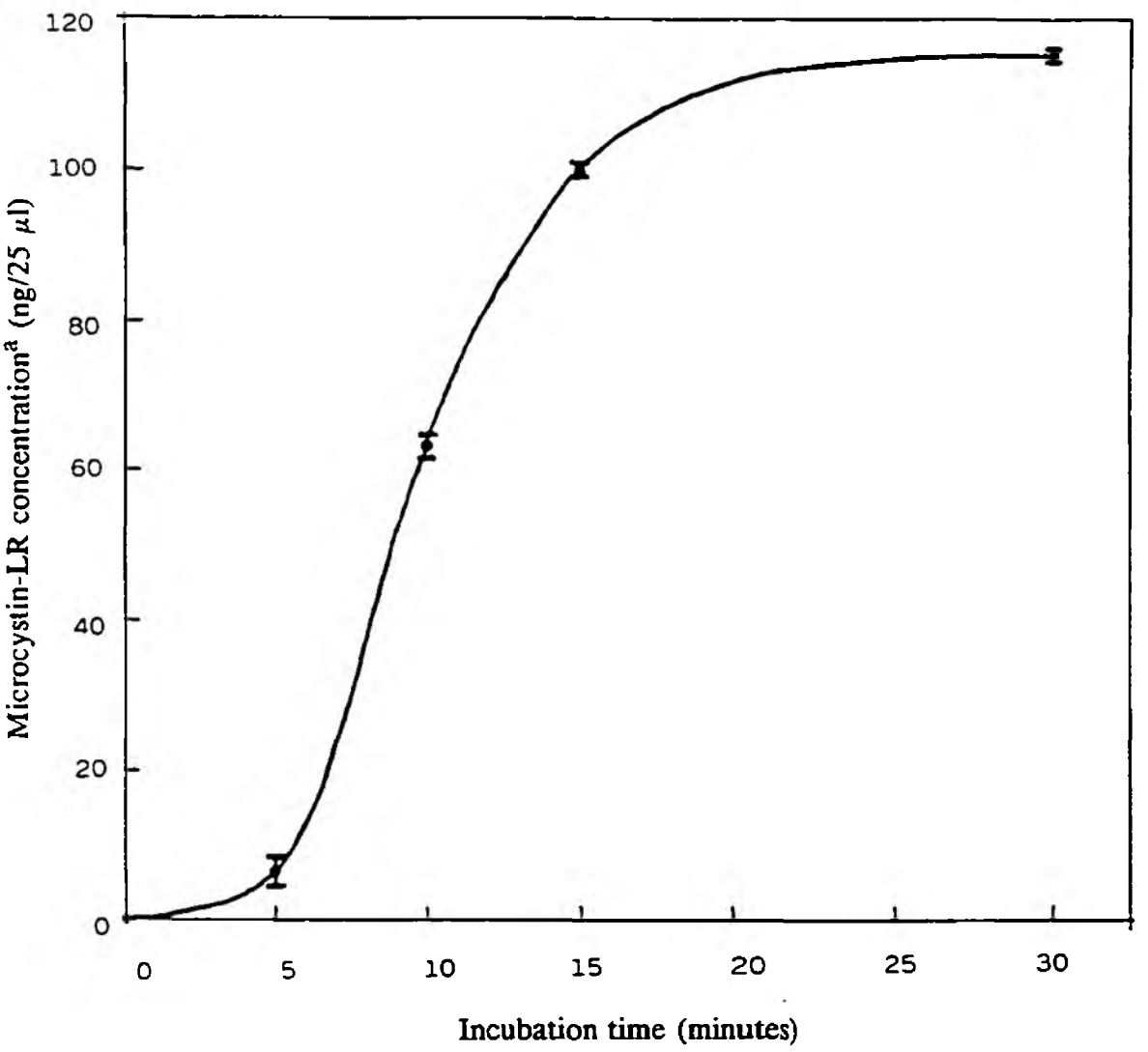


Figure 4.2.1 Release of microcystin-LR from *Microcystis aeruginosa* 7813 during incubation with 0.3% (v/v) toluene.

a, measured by HPLC, using a reversed-phase C18 column, and eluted with 35% - 47% acetonitrile in water containing 0.05% trifluoro-acetic acid (TFA) over 20 minutes.

To evaluate the efficiency of the toluene-mediated release of MC-LR from the cyanobacterial cells two approaches were used; comparison with the acetic acid extraction procedure routinely used in the purification of MC-LR in this laboratory, and an estimation of the intra- and extra-cellular toxin concentrations before and after toluene-lysis.

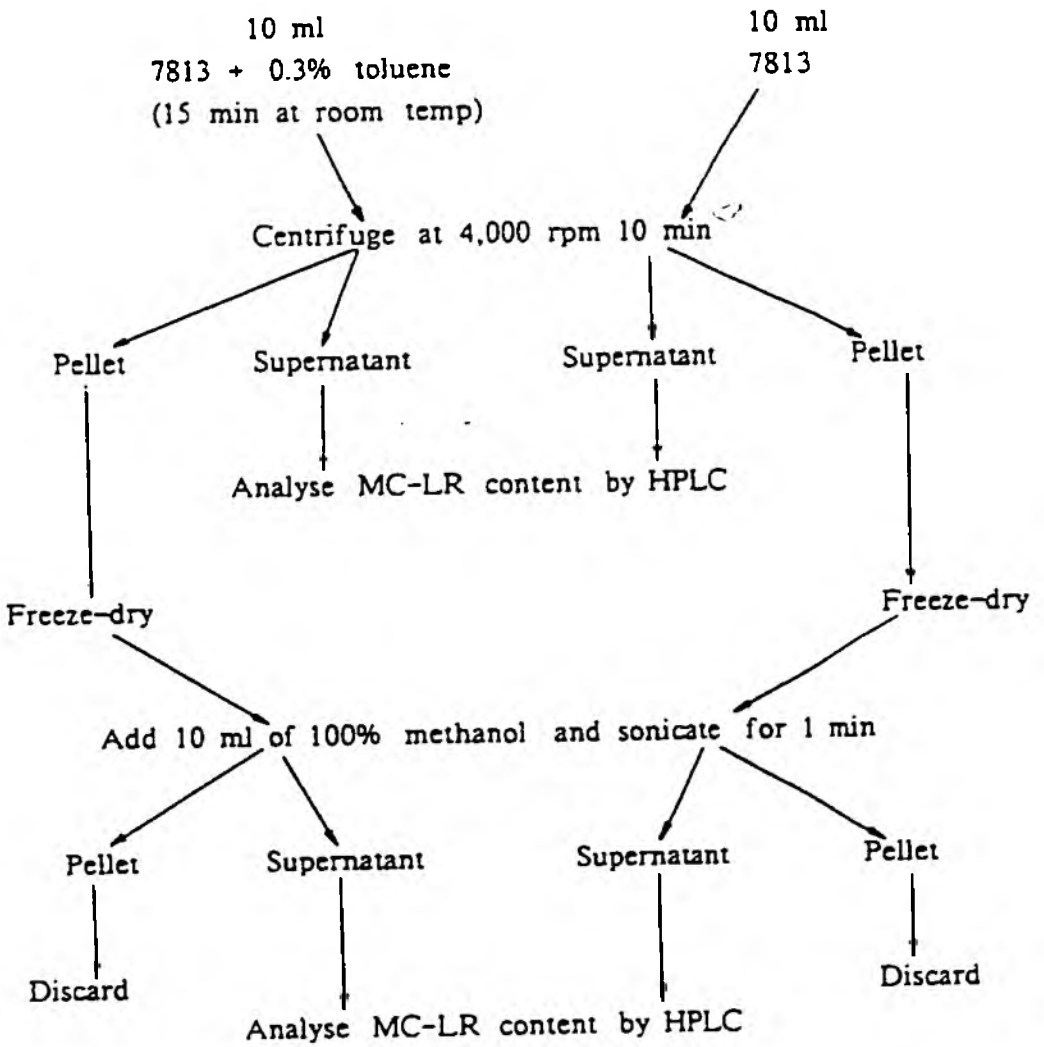
A washed suspension of *M. aeruginosa* PCC 7813 was divided into 2 aliquots. One was treated with 0.3% toluene for 15 minutes as detailed previously before being centrifuged, then loaded onto a Sep-Pak C18 reversed-phase cartridge. The cartridge was washed with 20% (v/v) methanol before the MC-LR was eluted with 60% (v/v) methanol. The second aliquot of *M. aeruginosa* PCC 7813 was freeze-dried, before extracting twice in 5% (v/v) acetic acid for 15 minutes at room temperature. After centrifugation, the supernatant was loaded onto a Sep-Pak cartridge and eluted in the same way as described above. The 60% methanol fractions were then analysed by HPLC and the MC-LR concentrations calculated. The results presented in Table 4.2.4 indicate that the toluene-mediated release of MC-LR from *M. aeruginosa* was at least as efficient as the routine procedure for extraction/purification used in our laboratory.

Table 4.2.4 Comparison of toluene-mediated release of MC-LR from *Microcystis aeruginosa* PCC 7813 into the surrounding medium, versus MC-LR release from an equivalent amount of freeze-dried cells using acetic acid extraction.

Source and extraction procedure	Total yield of MC-LR[a]
Aqueous cell suspension; 0.3% (v/v) toluene	53.49 μg
Freeze-dried cells;	49.63 μg

a, analysed by HPLC as detailed in footnote a, of Figure 4.2.1

In order to compare the intra- and extracellular MC-LR, before and after treatment of *M. aeruginosa* PCC 7813 with toluene, the following procedure was performed:



After analysis by HPLC the concentration of MC-LR was used to calculate the distribution of this toxin between cells and extra-cellular medium. These results, presented in Table 4.2.5, demonstrate the efficiency of the toluene-lysis procedure. In untreated cells all the MC-LR was present in the cells with no detectable extra-cellular toxin in the surrounding medium. However, when cells were treated with 0.3% (v/v) toluene almost all the detectable toxin was released to the surrounding medium, less than 1% remaining within the cells.

Table 4.2.5 Distribution of MC-LR between cell material and cell-free medium from untreated and toluene-incubated *Microcystis aeruginosa* PCC 7813

	0.3% (v/v) toluene-incubated cells[a]	untreated cells
MC-LR content[b] of cell-free medium	3.148 (±0.031) μg/ml	0.000 (±0.000) μg/ml
MC-LR content of cell material	0.013 (±0.013) μg/ml	3.628 (±0.060) μg/ml
% total MC-LR in cell-free medium	99.6%	0.0%
% total MC-LR in cell material	0.4%	100.0%

a, cells were incubated in 0.3% (v/v) toluene for 15 minutes at room temperature

b, 25 μl of sample was analysed by HPLC as detailed in footnote a, of Figure 4.2.1

If toluene were to be used as the reagent for lysing cyanobacterial cells in a field-test kit, it would have to be effective against all microcystin-containing cyanobacteria. The effectiveness of the toluene reagent against the following strains of cyanobacteria was investigated:

- *Microcystis aeruginosa* AK1
- *Microcystis aeruginosa* CYA 43
- *Microcystis aeruginosa* PCC 7820
- *Microcystis aeruginosa* RID 1
- *Oscillatoria agardhii* CYA 29

Aliquots of each culture were centrifuged at 2,500 x g for 10 minutes and the pellets were washed in an equal volume of fresh growth medium. After further centrifugation under the same conditions the pellets were resuspended in an equal volume of fresh medium. An aliquot of each washed culture was extracted in methanol to analyse the chlorophyll *a* concentration as detailed earlier. A further aliquot was centrifuged at 14,200 x g for 5 minutes and the supernatants retained for analysis in the spectrophotometer. Toluene was added to the remaining cells to a final concentration of 0.3% (v/v) and incubated at room temperature for 15 minutes. After this period the suspensions were centrifuged at 14,200 xg

for 5 minutes and the supernatants analysed in the spectrophotometer. Absorbance was measured at 615 nm and 652 nm, and the phycocyanin concentrations were calculated. Table 4.2.6 presents the results of this investigation, which demonstrate that the toluene lyses all the cultures investigated, releasing phycocyanin into the growth medium.

Table 4.2.6 Release of phycocyanin from cyanobacteria during incubation with 0.3% (v/v) toluene[a].

Cyanobacterial species and strain	chlorophyll *a* concentration (μg/ml)[b]	Concentration of phycocyanin released[c]	
		untreated	toluene-treated
M. aeruginosa AK1	10.0 (±0.9)	2.1 (±0.3)	79.1 (±2.0)
M. aeruginosa CYA 43	30.7 (±0.3)	10.6 (±1.4)	366.5 (±7.4)
M. aeruginosa PCC 7820	24.8 (±0.2)	7.5 (±0.6)	297.1 (±6.7)
M. aeruginosa RID 1	33.9 (±0.5)	8.3 (±0.4)	333.8 (±2.9)
O. agardhii CYA 29	14.8 (±1.4)	4.5 (±0.5)	183.0 (±1.0)

a, incubated 15 minutes at room temperature

b, aliquots extracted with methanol for 24 h at 2 - 8°C. Chl *a* = 12.63 x A_{663}

c, μg/ml, determined as described in footnote a, Figure 4.1.1

As described in Section 4.2.2 the release of MC-LR from *M. aeruginosa* cells in liquid suspension using toluene was compared with the release of the toxin from cells entrapped on a glass-fibre filter, again using toluene. A washed suspension of *M. aeruginosa* PCC 7813 was divided into 2 aliquots, one of which was subjected to toluene lysis for 15 minutes as described earlier. The remaining aliquot was filtered through three 2.5 cm diameter GF/C glass-fibre filters to entrap the cells. The filters were then added to 20 ml of 0.3% (v/v) toluene solution shaken intermittently for 15 minutes, during which time the filters disintegrated. After this, the slurry of cells and filter débris were passed through a 50 ml plastic syringe plugged with glass-wool and the cell-free solution was collected. The cell-free

solution and the earlier toluene-treated cell suspension (unfiltered) were centrifuged at 2,500 x g for 10 minutes and the supernatants passed through Sep-Paks as described earlier. 60% (v/v) methanol was used to elute the toxin from the cartridges, and the concentration of MC-LR was estimated by HPLC analysis (Table 4.2.7). It can be seen that the amount of MC-LR released from the filter-entrapped cells was equivalent to that released from the free-cell suspension.

Table 4.2.7 Release of MC-LR from filter-entrapped *Microcystis aeruginosa* PCC 7813 cells compared with that released by free cells of the same culture, mediated by 0.3% (v/v) toluene[a].

Source of cells	Total MC-LR yield[b]
liquid suspension	5.98 (±0.37) μg
filter-entrapped	5.81 (±0.11) μg

a, incubated with toluene for 15 minutes at room temperature

b, MC-LR concentration estimated by HPLC under the conditions described in footnote a, Figure 4.2.1

With a view to using the lysis procedure at the side of a waterbody, the temperature at which the toluene-mediated lysis of *M. aeruginosa* cells could be performed was then investigated. A washed suspension of *M. aeruginosa* PCC 7813 was divided into five aliquots and one of these incubated at 10°C, 15°C, 20°C, 25°C and 25°C for at least 10 minutes. Toluene was added to each suspension to a final concentration of 0.3% (v/v) and incubated at the same temperature for 15 minutes. The suspensions were then centrifuged at 14,200 x g for 5 minutes and the supernatants analysed by HPLC for MC-LR concentration. This was then plotted against incubation temperature (Figure 4.2.2).

Figure 4.2.2 illustrates the fact that release of MC-LR from *M. aeruginosa* PCC 7813 in the presence of 0.3% (v/v) toluene increased with temperature up to 20°C. At temperatures equal to or greater than 20°C the release of MC-LR reached a maximum. Thus below 20°C toluene-mediated lysis of *M. aeruginosa* cells was temperature-dependent.

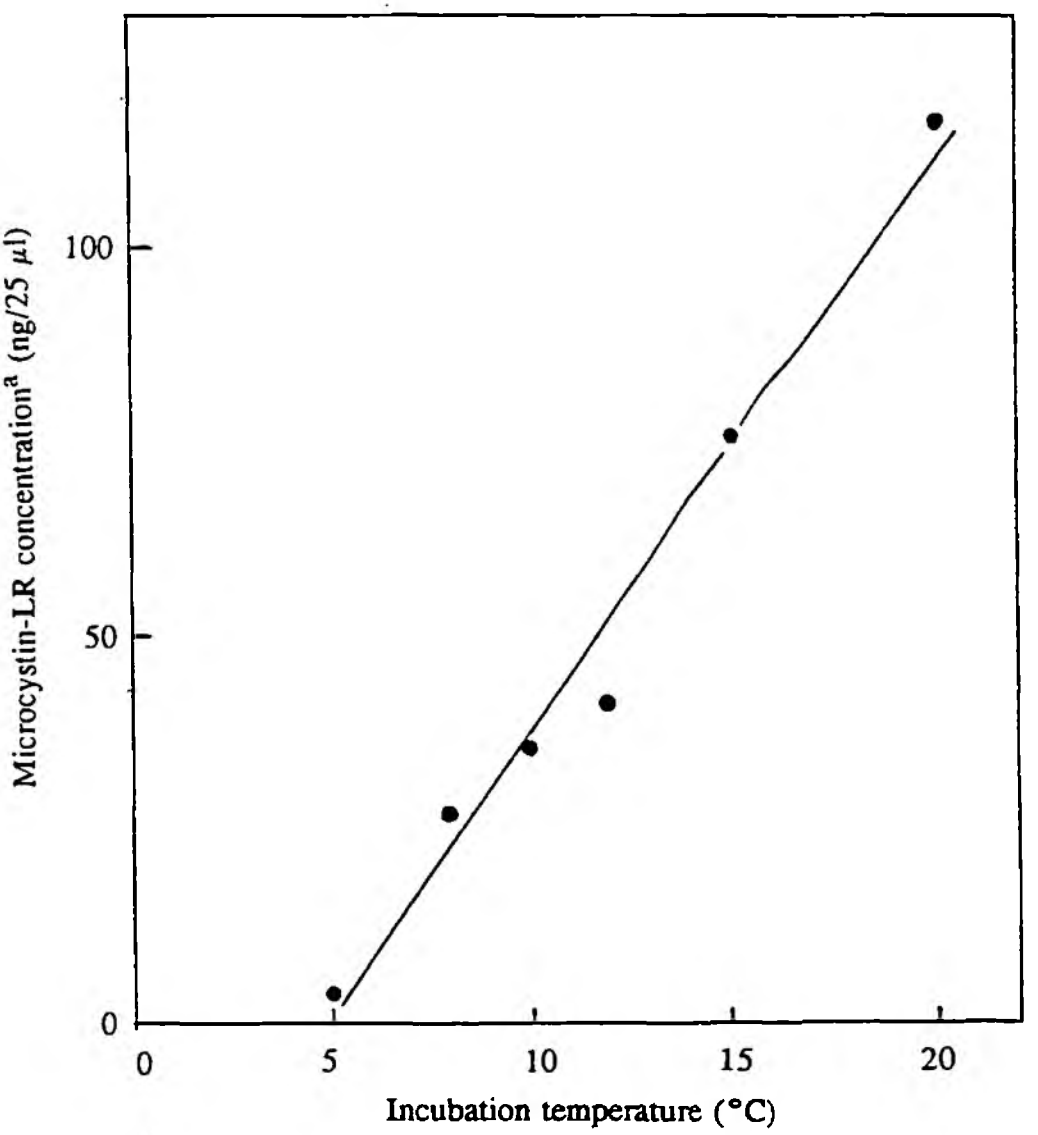


Figure 4.2.2 Release of MC-LR from *Microcystis aeruginosa* PCC 7813 during incubation with 0.3% (v/v) toluene versus incubation temperature.

a, MC-LR analysed by HPLC under the conditions described in footnote a, Figure 4.2.1

4.2.4 Methanol extraction of microcystin-LR from cyanobacterial cells

As a contingency to the use of toluene as the lysis reagent for cyanobacterial cells, the use of methanol was investigated.

The pigment chlorophyll *a* (Chl *a*) is extracted from cyanobacterial cells when incubated with methanol. It was decided to investigate the time course of this extraction in order to assess the feasibility of employing this procedure as a method for extracting MC-LR. A suspension of *M. aeruginosa* PCC 7813 was centrifuged at 14,200 x g for 5 minutes and the supernatant was measured at 663 nm in the spectrophotometer. The pellet was resuspended in an equal volume of methanol and the centrifugation/spectrophotometer stage repeated with aliquots taken after 5 min, 30 min, 1 h, 2 h, 4 h and 24 h at room temperature. The extracted Chl *a* concentrations were then calculated using the formula presented in Section 4.2.2, and these were plotted against incubation time (Figure 4.2.3). It can be seen that almost all the Chl *a* was released from the cyanobacterial cells within the first 5 minutes of incubation in methanol, and so the extraction of this pigment was rapid.

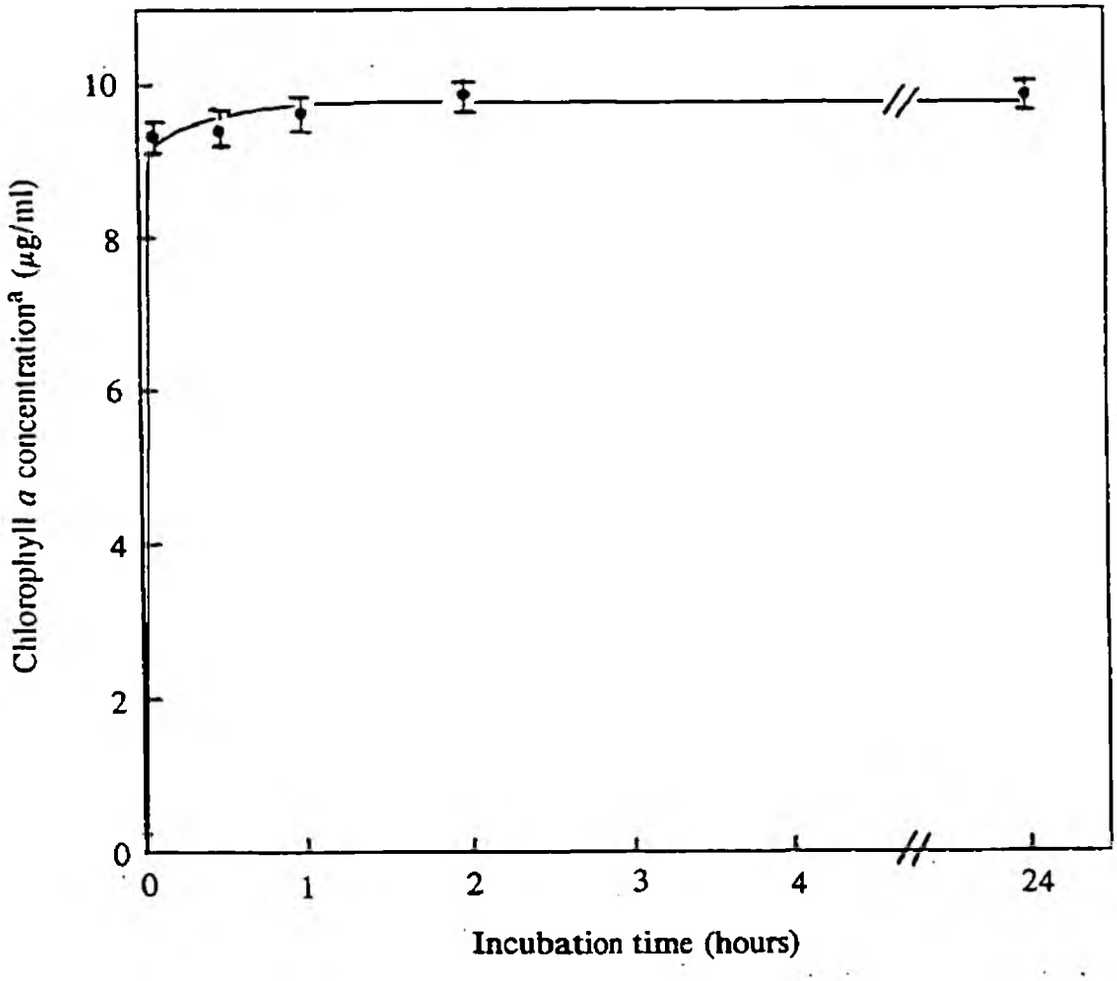


Figure 4.2.3 Chlorophyll *a* extraction from *Microcystis aeruginosa* PCC 7813 with methanol versus incubation time.

a, Chl *a* (μg/ml) = 12.63 x A_{663}

A further suspension of *M. aeruginosa* PCC 7813 was centrifuged at 14,200 x g for 5 minutes and the supernatant was retained for HPLC analysis. The pellet was resuspended in an equal volume of methanol and the centrifugation step repeated with aliquots removed from the suspension after 5 min, 10 min, 15 min, 30 min, 1 h and 2 h at room temperature. The supernatants were analysed, along with the supernatant of the original suspension, by HPLC in order to estimate the MC-LR concentration of each. These values were then plotted against incubation time in Figure 4.1.4. As with Chl *a*, the extraction of MC-LR was rapid, the majority of the toxin being released into the extracellular medium within 5 minutes of incubation with methanol.

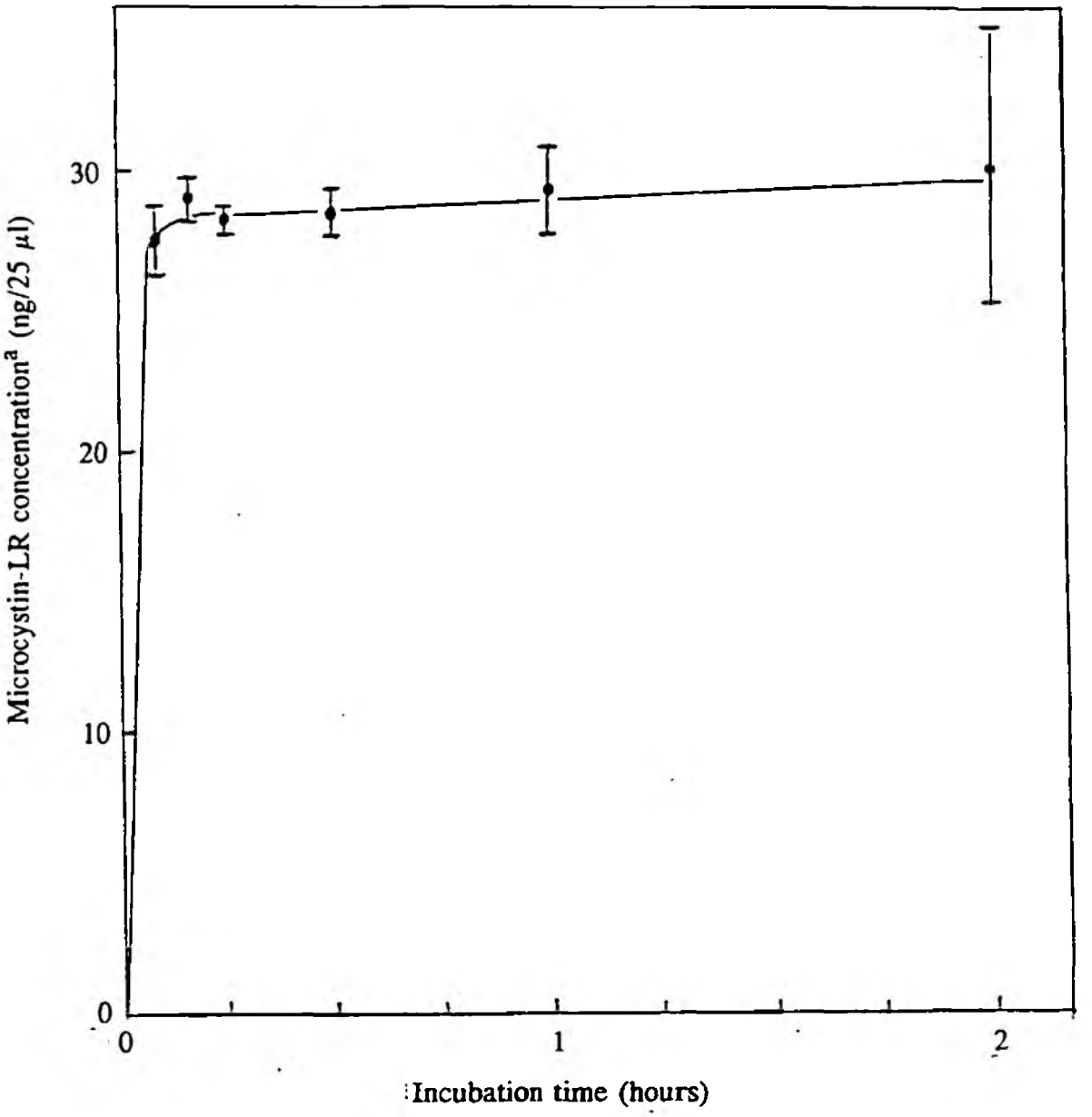


Figure 4.2.4 Extraction of MC-LR from *Microcystis aeruginosa* PCC 7813 with methanol versus incubation time.

a, MC-LR concentration was estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

To estimate the efficiency of the methanol-extraction procedure, the distribution of intra- and extra-cellular MC-LR before and after methanol-extraction was investigated. A suspension of *M. aeruginosa* PCC 7813 was divided into two aliquots and centrifuged at 14,200 x g for 5 minutes. The supernatant of one aliquot ("untreated supernatant") was retained for HPLC analysis. The supernatant of the other aliquot was discarded and the pellet resuspended in an equal volume of methanol. After 15 minutes at room temperature the methanol suspension was once more centrifuged and the supernatant retained for HPLC analysis ("methanol-treated supernatant"). The untreated pellet and the methanol-treated pellet were freeze-dried for 24 h then resuspended in a volume of methanol equal to the original suspensions. After ultrasonication for 2 minutes the suspensions were centrifuged as before and the supernatants (labelled "untreated cells" and "methanol-treated cells") were retained for HPLC analysis. HPLC analysis was carried out on the four samples in order to estimate the MC-LR concentration of each. The results are presented in Table 4.2.8, along with the percentage distribution of intra- and extra-cellular MC-LR.

Table 4.2.8 Distribution of MC-LR between cell material and cell-free medium from untreated and methanol-incubated *Microcystis aeruginosa* PCC 7813

	methanol-incubated cells[a]	untreated cells
MC-LR content[b] of cell-free medium	3.915 (±0.245) μg/ml	0.000 (±0.000) μg/ml
MC-LR content of cell material	0.025 (±0.025) μg/ml	3.845 (±0.205) μg/ml
% total MC-LR in cell-free medium	99.4%	0.0%
% total MC-LR in cell material	0.6%	100.0%

a, cells were incubated in methanol for 15 minutes at room temperature

b, 25 μl of sample was analysed by HPLC as detailed in footnote a, of Figure 4.2.1

The effect of reducing the concentration of methanol in the extraction reagent was investigated. A suspension of *M. aeruginosa* PCC 7813 was washed in phosphate-buffered saline (PBS) pH 7.4 then 1 ml aliquots were centrifuged at 14,200 x g for 5 minutes. The pellets were resuspended in 1 ml of 70%, 80%, 90% and 100% (v/v) methanol in PBS. The cells were incubated in the methanol solutions for 10 minutes at room temperature and then centrifuged as before. The supernatants were analysed in 10 μl aliquots in the HPLC to estimate the cell-free MC-LR concentration (Table 4.2.9). The results presented demonstrate that at concentrations below 100% methanol the extraction of MC-LR was not efficient in that less than 90% of the MC-LR released by 100% methanol was observed in these cell-free solutions.

Table 4.2.9 Release of MC-LR from *Microcystis aeruginosa* PCC 7813 by various concentrations of methanol in phosphate-buffered saline.

Concentration of methanol (v/v)[a]	MC-LR content (μg/ml)[b]	% MC-LR released
70%	3.49 ($\pm$0.70)	15
80%	13.97 ($\pm$3.44)	61
90%	19.40 ($\pm$1.27)	85
100%	22.81 ($\pm$1.14)	100

a, cells incubated in methanol solution for 10 min at room temperature

b, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

As with the toluene-mediated lysis of cyanobacteria, a range of cyanobacteria were investigated for release of cell contents during incubation with methanol. As some of the strains investigated were non-toxic the extraction of Chl *a* was measured. The species and strains investigated were:

- *Nodularia* sp. PCC 7804
- *Oscillatoria agardhii* CYA 29

- *Anabaena flos-aquae* CCAP 1403/21
- *Aphanizomenon* sp. PCC 7905

Aliquots of each were centrifuged and resuspended in an equal volume of methanol. After 10 minutes and 64 hours in the dark at room temperature, the suspensions were centrifuged and the supernatants measured in the spectrophotometer at 663 nm against a blank of methanol. Chl *a* concentrations were then calculated and presented in Table 4.2.10. The results clearly demonstrate that between 10 minutes and 64 h no further Chl *a* was extracted from the cyanobacteria upon storage in methanol. The difference in Chl *a* values between species was a factor of the difference in cell counts.

Table 4.2.10 Chl *a* concentrations of methanol extracts of cyanobacterial cultures.

Culture strain No.	Incubation time	Chl *a* concentration[a] (μg/ml)
Nodularia sp. PCC 7804	10 min	17.80 ($\pm$0.38)
Nodularia sp. PCC 7804	64 h	16.97 ($\pm$1.50)
Oscillatoria agardhii CYA 29	10 min	5.73 ($\pm$0.58)
Oscillatoria agardhii CYA 29	64 h	5.76 ($\pm$0.58)
Anabaena flos-aquae 1403/21	10 min	3.84 ($\pm$0.28)
Anabaena flos-aquae 1403/21	64 h	3.83 ($\pm$0.28)
Aphanizomenon sp. PCC 7905	10 min	15.11 ($\pm$0.77)
Aphanizomenon sp. PCC 7905	64 h	15.42 ($\pm$0.23)

a, $[\text{Chl } a] = 12.63 \times A_{663}$

The next stage carried out, concerning the optimisation of the methanol mediated MC-LR release from cyanobacterial cells, was an investigation of the incubation time using filter-entrapped cells. The use of glass fibre filters to entrap cyanobacterial cells, and their

subsequent lysis with toluene, has been discussed in Section 4.2.3. A suspension of *M. aeruginosa* PCC 7813 was passed through a 2.5 cm diameter Whatman GF/C glass-fibre filter until the filter was blocked with cells, and a duplicate procedure was performed. The filters were then placed in 5 ml of 100% methanol at room temperature, shaken vigorously to disintegrate the filter, and 0.5 ml aliquots taken from the suspension after 5, 10, 15, 30, and 60 minutes. The aliquots were centrifuged and the supernatants analysed by HPLC for MC-LR content (Figure 4.2.5). The data demonstrate that most of the MC-LR was released between 5 and 10 minutes of incubation of the filter-entrapped cells in methanol.

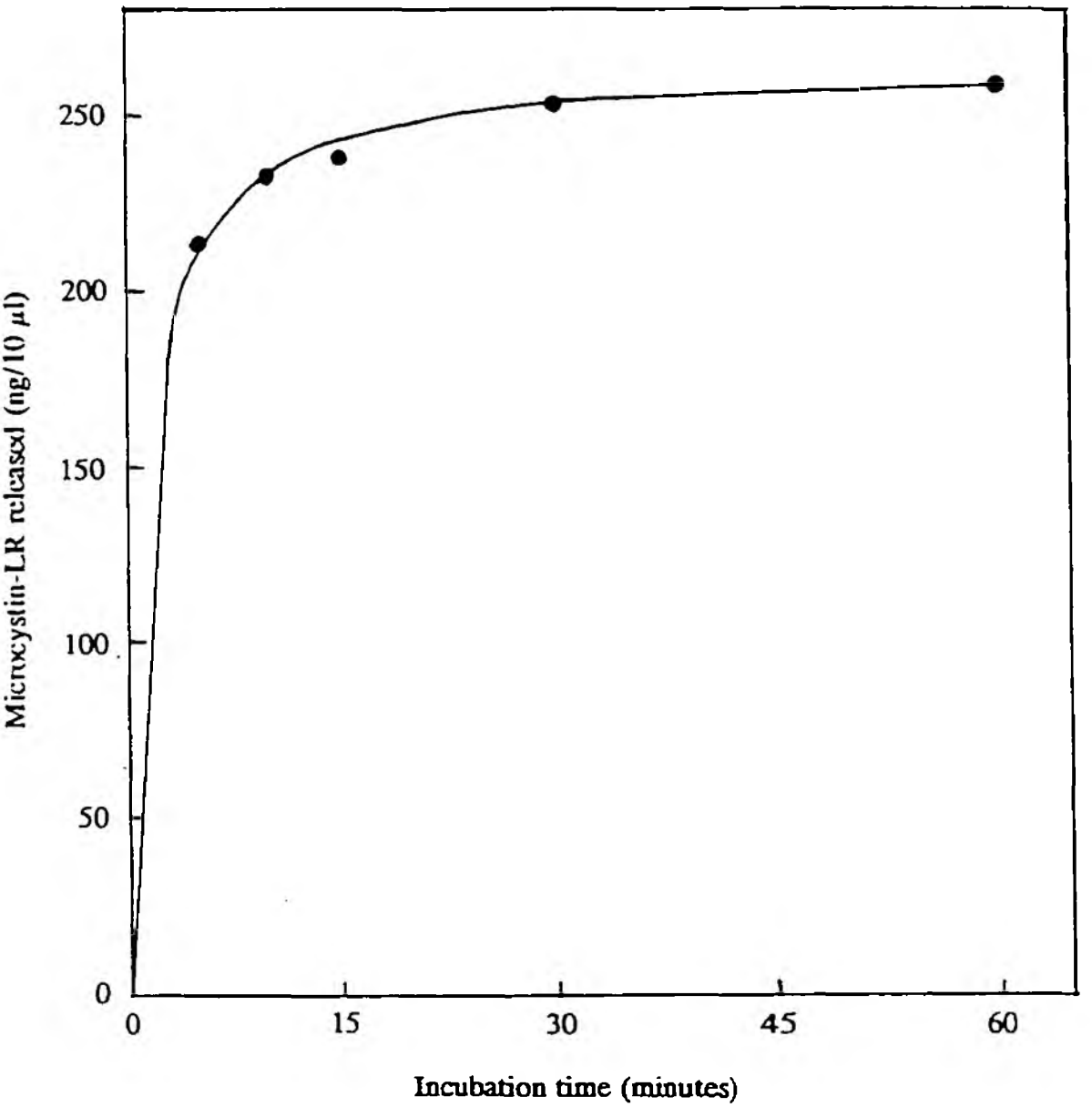


Figure 4.2.5 Release of MC-LR[a], from filter entrapped *Microcystis aeruginosa* PCC 7813 by 5 ml of 100% methanol[b], versus time.

a, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

b, 2.5 cm diameter filter containing cells incubated in methanol at room temperature.

The toluene-mediated release of MC-LR from *M. aeruginosa* was shown to be temperature-dependent in Section 4.2.3, therefore this effect was investigated for the methanol-extraction of the toxin from the same species. Aliquots of *M. aeruginosa* PCC 7813 were centrifuged at 14,200 x g for 5 minutes and resuspended in an equal volume of methanol which had been pre-incubated at 10°C, 15°C, 20°C and 25°C. The suspensions were then incubated at the same temperatures for a further 10 minutes before being centrifuged under the same conditions. The supernatants were then analysed by HPLC and the concentration of MC-LR estimated (Figure 4.2.6). The data presented in Figure 4.2.7 clearly demonstrate that extraction of MC-LR from *M. aeruginosa* PCC 7813 by methanol was constant between 10°C and 25°C.

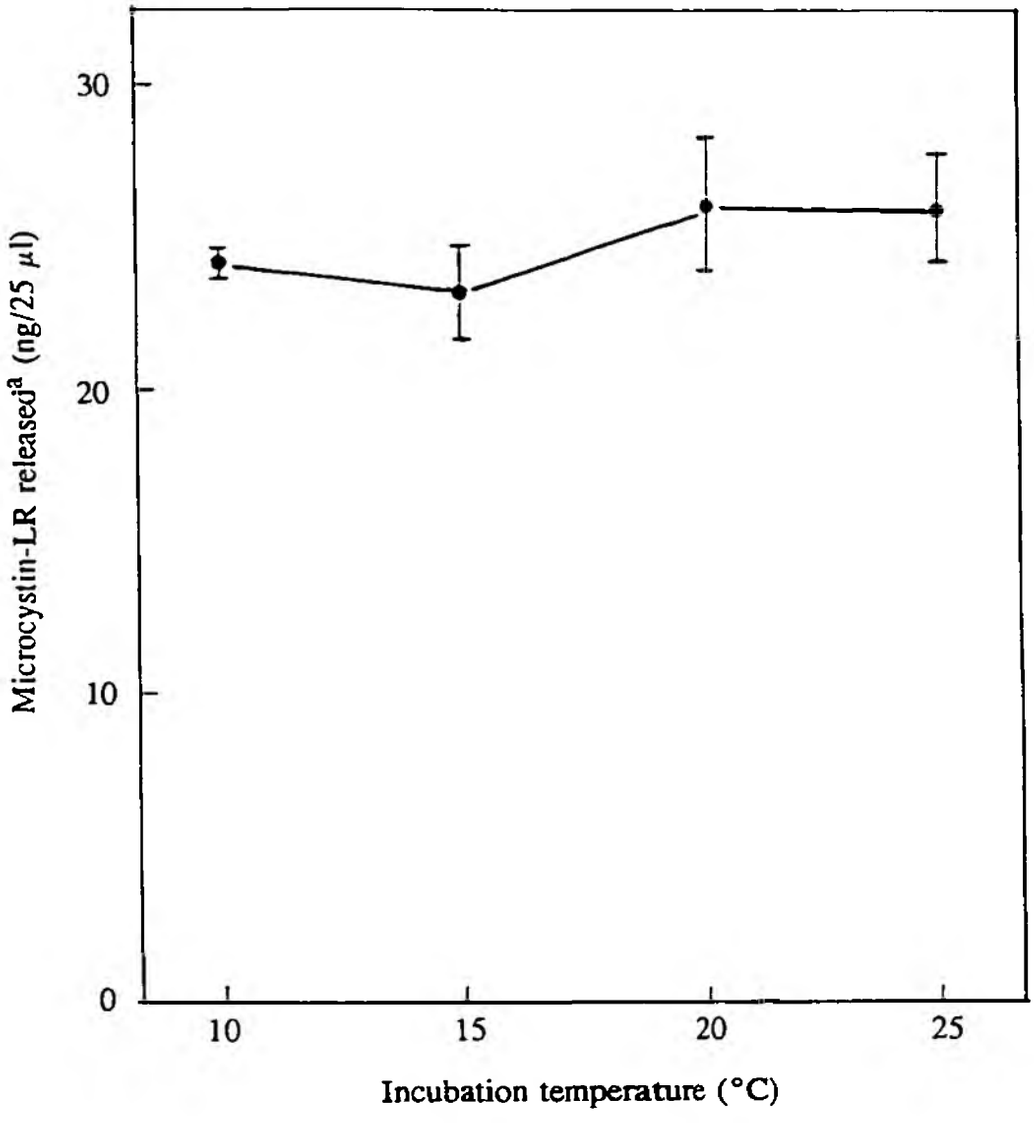


Figure 4.2.6 Extraction of MC-LR from *Microcystis aeruginosa* PCC 7813 with methanol at various incubation temperatures

a, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

b, cells were incubated with methanol for 10 minutes at the appropriate temperature

4.2.5 Toluene/methanol extraction of microcystin-LR from cyanobacterial cells

For a field-test kit, the cell-lysis reagent should ideally be present in a suitable buffer such as PBS pH 7.4. The toluene-mediated lysis of cyanobacterial cells was therefore investigated in the presence of this buffer. *M. aeruginosa* PCC 7813 was centrifuged and washed in 0.01M phosphate buffer pH 7.4. The suspension was then centrifuged in aliquots of 5 ml and resuspended in equal volumes of deionised water, phosphate buffer or PBS, in duplicate. Toluene was added to one of each pair of suspensions to a final concentration of 0.3% (v/v). After 15 minutes at room temperature aliquots from each suspension (± toluene) were centrifuged and supernatants were analysed by HPLC. The concentration of cell-free MC-LR was estimated for each suspension (Table 4.2.11). The data presented show that the amount of MC-LR released into the cell-free medium was reduced in the presence of PBS.

Table 4.2.11 Release of MC-LR from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of deionised water, phosphate buffer and PBS.

Incubation medium[a]	Toluene status[b]	MC-LR concentration[c] (μg/ml)
Deionised water	-	0.000 (±0.000)
Deionised water	+	4.810 (±0.282)
Phosphate buffer	-	0.101 (±0.003)
Phosphate buffer	+	5.247 (±0.289)
PBS	-	0.026 (±0.026)
PBS	+	3.406 (±0.146)

a, cells incubated in the appropriate medium for 15 minutes at room temperature

b, - = absent, + = present

c, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

In an attempt to increase the extraction of MC-LR from cyanobacterial cells in toluene/PBS solution, methanol was added to the suspension. A suspension of *M. aeruginosa* PCC 7813 was washed in phosphate buffer as in the previous investigation, and then resuspended in aliquots of phosphate buffer, PBS, PBS containing 10% (v/v) methanol, PBS containing 25% (v/v) methanol, and PBS containing 50% (v/v) methanol. Toluene was added to a final concentration of 0.3% (v/v) to half the aliquots containing methanol and to the phosphate buffer and PBS aliquots. After 15 minutes at room temperature the suspensions were centrifuged and analysed for cell-free MC-LR by HPLC as before (Table 4.2.12). It can be seen that adding as little as 10% (v/v) methanol to the toluene/PBS reagent increased the amount of MC-LR extracted from *M. aeruginosa* PCC 7813 cells to levels approximately equal to those where PBS was absent.

Table 4.2.12 Release of MC-LR from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of PBS containing methanol.

Incubation medium[a]	Toluene status[b]	MC-LR concentration[c] (μg/ml)
Phosphate buffer	+	4.035 (±0.205)
PBS	+	3.480 (±0.310)
PBS + 10% (v/v) MeOH	-	0.120 (±0.120)
PBS + 10% (v/v) MeOH	+	3.965 (±0.525)
PBS + 25% (v/v) MeOH	-	0.830 (±0.130)
PBS + 25% (v/v) MeOH	+	3.740 (±0.250)
PBS + 50% (v/v) MeOH	-	2.525 (±0.195)
PBS + 50% (v/v) MeOH	+	3.875 (±0.345)

a, cells were incubated in the appropriate medium for 15 minutes at room temperature

b, - = absent, + = present

c, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

In Section 4.2.3 the toluene-mediated cell lysis was shown to be temperature-dependent below 20°C, but the methanol-extraction of MC-LR was constant between temperatures of 10°C and 25°C (Section 4.2.4). However, due to the high flammability of 100% methanol compared with 0.3% (v/v) toluene, the toluene-mediated procedure would be preferable. In an attempt to overcome the temperature-dependency, methanol was added to the toluene at various concentrations. A culture of *M. aeruginosa* PCC 7813 was harvested and washed in 0.01M phosphate buffer. 1 ml aliquots were then centrifuged and the pellets resuspended in 1 ml of 10%, 20% or 50% (v/v) methanol in PBS preincubated at 10°C, 15°C, 20°C, and 25°C. Toluene (0.3% v/v) was then added to triplicate aliquots, which were then incubated at each of the above temperatures for 15 minutes. After this time the suspensions were centrifuged and the supernatants were analysed by HPLC for cell-free MC-LR concentration (Figure 4.2.7). The results infer that at 10% (v/v) methanol the release of MC-LR was temperature dependent below 15°C, in the presence of 0.3% (v/v) toluene. This was also true for the 20% (v/v) methanol solution, but in this case the amount of MC-LR released was only fractionally less then that released at the higher temperatures. At 50% (v/v) methanol, MC-LR release was reduced at all the temperatures investigated, and appeared to be temperature dependent.

Upon examination of a Biocode Standard Operating Procedure for the formulation of PBS, it was observed that the recipe for this was different from that previously used by this laboratory. Our buffer contained sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate, whereas the Biocode buffer contained sodium chloride, potassium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate. In both cases the chloride salt concentration was 0.85% (w/v). It was therefore decided to compare the Biocode buffer with that used previously, in the investigation into methanol concentration and incubation temperature and their effect on MC-LR release. The previous protocol was repeated using the Biocode formulation of PBS instead of our formulation, and the results are plotted in Figure 4.2.8. The data presented infer that the release of MC-LR from *M. aeruginosa* PCC 7813 by toluene/methanol/PBS (Biocode formulation) was different from that released by the formulation previously used. The release of MC-LR appeared to be temperature-dependent up to 20°C in the Biocode formulation of PBS, compared with 15°C in the previous formulation. The amount of MC-LR released appeared to be generally lower in the Biocode formulation.

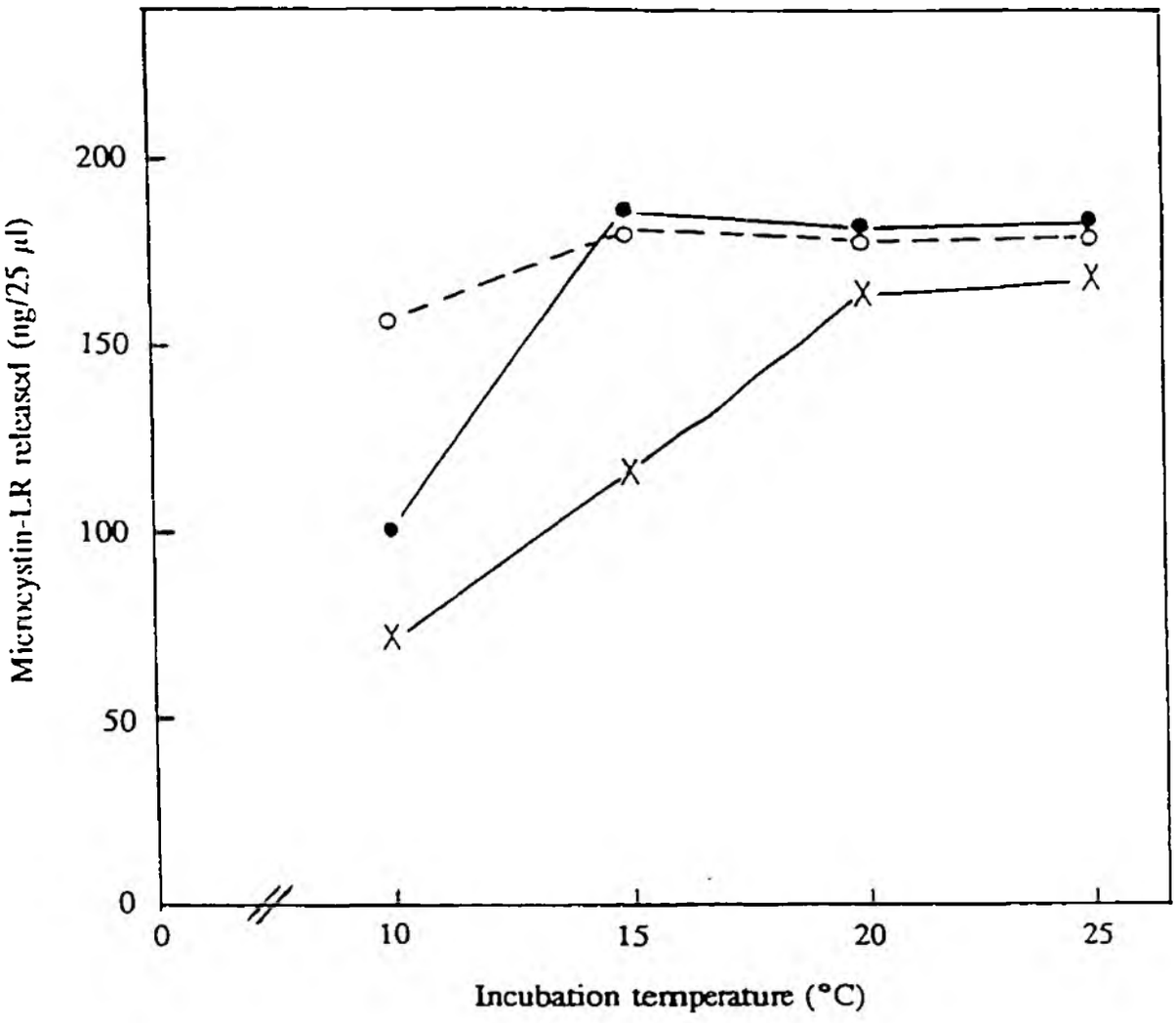


Figure 4.2.7 Release of MC-LR[a] from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene/methanol/PBS[b], at various concentrations of methanol and incubation temperatures.

a, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

b, suspensions incubated for 15 minutes

●——● 10% (v/v) methanol/toluene/PBS

○---○ 20% (v/v) methanol/toluene/PBS

X——X 50% (v/v) methanol/toluene/PBS

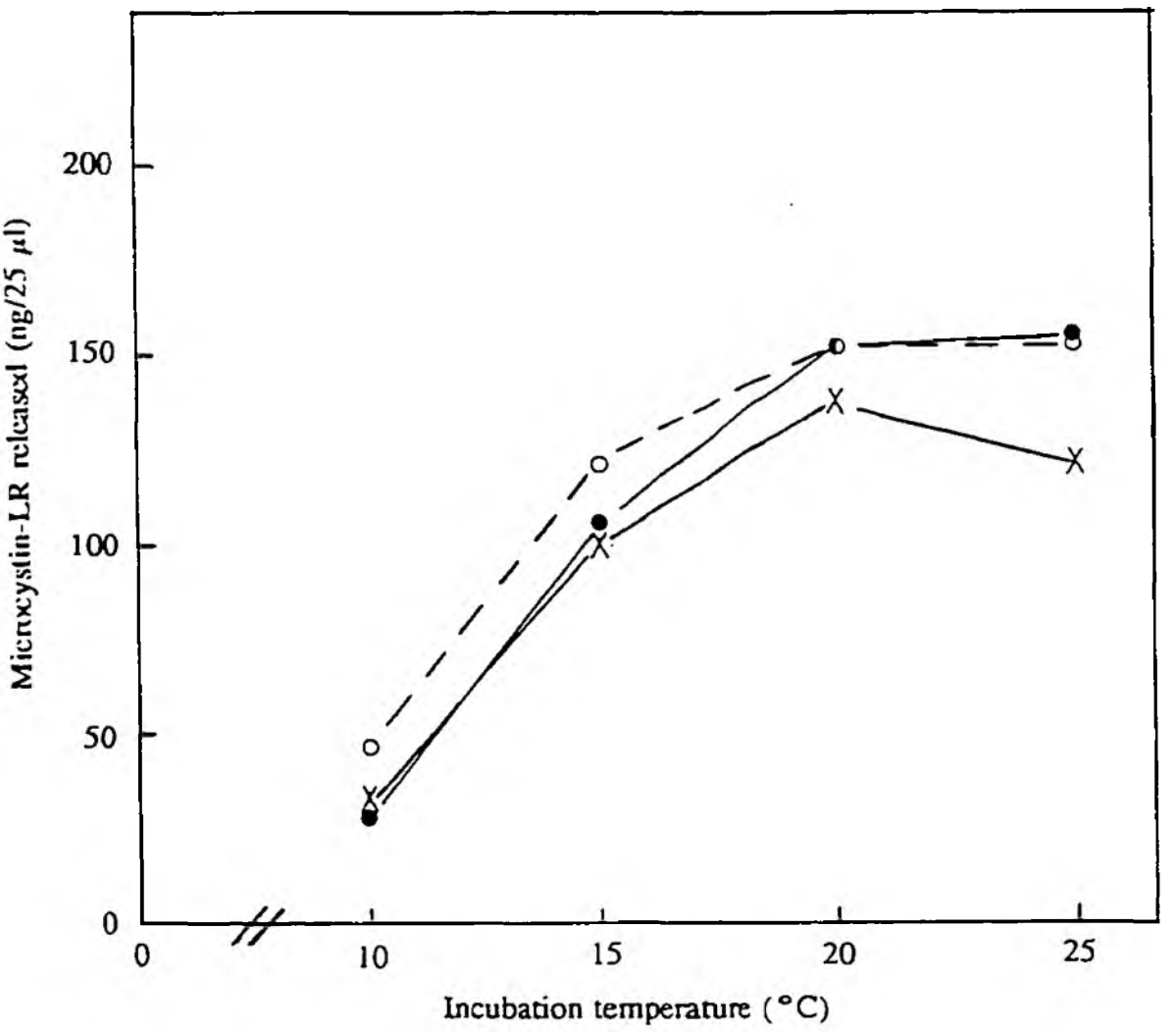


Figure 4.2.8 Release of MC-LR[a] from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene/methanol/PBS[b] (Biocode formulation), at various concentrations of methanol and incubation temperatures.

a, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

b, suspensions incubated for 15 minutes

●——● 10% (v/v) methanol/toluene/PBS

○- - -○ 20% (v/v) methanol/toluene/PBS

X——X 50% (v/v) methanol/toluene/PBS

4.2.6 Lysis of cyanobacterial cells with non-ionic detergents

As an alternative to the use of the flammable/toxic mixture of toluene/methanol the use of non-ionic detergents to lyse cyanobacterial cells was investigated. Five detergents were investigated in the first instance:

- Triton X-100 (Sigma)
- Sarkosyl (IBI)
- Tween 20 (Sigma)
- Nonidet P40 (Sigma)
- Span 20 (Sigma)

A suspension of *M. aeruginosa* PCC 7813 was divided into 11 equal aliquots. The five detergents were added to five aliquots to a final concentration of 0.1% (v/v), and to a further five aliquots to a final concentration of 0.01% (v/v). After 2 hours at room temperature, with intermittent shaking, the suspensions were centrifuged at 14,200 x g for 5 minutes. Toluene was added to the 11th aliquot to a final concentration of 0.3% (v/v), and after 15 minutes at room temperature the suspension was centrifuged in the same way as the other aliquots. Each supernatant was then passed through a Sep-Pak C18 cartridge, which was in turn eluted with 60% (v/v) methanol. The eluted fractions were then analysed by HPLC and the concentration of cell-free MC-LR estimated (Table 4.1.13). The five detergents investigated did not release MC-LR from the *M. aeruginosa* cells, and were thus considered unsuitable for this purpose.

Table 4.2.13 MC-LR release from *Microcystis aeruginosa* PCC 7813 incubated with non-ionic detergents.

Detergent added	Concentration of MC-LR[a] (μg/ml) 0.01% (v/v) detergent	0.1% (v/v) detergent
Triton X-100	0.0 (±0.0)	0.0 (±0.0)
Sarkosyl	0.0 (±0.0)	0.0 (±0.0)
Tween 20	0.0 (±0.0)	0.0 (±0.0)
Nonidet P40	0.0 (±0.0)	0.0 (±0.0)
Span 20	0.0 (±0.0)	0.0 (±0.0)
0.3% (v/v) toluene	3.67 (±0.34)	

a, estimated by HPLC analysis under the conditions described in footnote a, Figure 4.2.1

Two further non-ionic detergents were investigated for use as a lytic agent for cyanobacterial cells, n-dodecyl β-D maltoside and n-octyl β-D glucopyranoside (both Sigma). These were added to *M. aeruginosa* PCC 7813 (to final concentrations of 0.01% and 0.1% v/v) in the presence and absence of lysozyme (0.1% and 1.0% w/v), added to digest the cell wall of the cyanobacterial cell. Absorbance at 615 nm and 652 nm was measured in the spectrophotometer after 5 min, 30 min, 1 h, 2 h and 24 h at room temperature, and the concentration of cell-free phycocyanin was calculated. No phycocyanin was detected in any of the cell-free solutions and so it was concluded that neither of the two detergents were suitable for lysing cyanobacterial cells.

4.3 Assessment of Biocode anti-microcystin-LR antibodies

4.3.1 Introduction

A number of the specific objectives of Project 0349 (objectives 2.2.4 - 2.2.7) required the assessment of antibodies produced by Biocode Ltd. This entailed discussion of the performance of the antibodies with Biocode by telephone, assessment of results presented in Biocode's interim reports, assessment of Biocode's results and procedures during a visit to Biocode's facilities by Dr Bell in January 1993, and direct assessment of the antibodies by procedures carried out by Dundee University.

The direct assessment by Dundee University initially entailed assessment by enzyme-linked immunosorbent assay (ELISA) methods used by Biocode. A number of supplementary methods were also performed due to the ambiguity of results yielded by the ELISA procedure (see Section 4.3.2). The further procedures carried out were:

- Dot-blot assays
- HPLC analysis
- Mouse bioassays
- Radio-immunoassays
- Gel-diffusion assays

4.3.2 ELISA evaluation

Monoclonal antibodies raised against microcystin-LR (αMC-LR), cell-line number A005-1D 13C6, batch number 006, were received from Biocode Ltd and were assessed by ELISA.

The ELISA method employed was the poly-L-lysine coated plate method developed by Biocode Ltd, and their Standard Operating Procedures were followed. In summary, poly-L-lysine hydrobromide was coated at a concentration of 1 mg per ml onto PVC 96-well microassay plates at 50 μl per well, and left at room temperature for 15 minutes. After washing the plates, microcystin variants and nodularin were then added at 100 ng per well in glycine/NaOH pH 8.8 buffer (50 μl) and left at 37°C for 3 hours, then at room temperature for 24 hours. After washing the plates, 100 μl of 3% bovine serum albumen (BSA) was added to each well and left at room temperature for 20 min to 2 hours. Again plates were washed and the anti-microcystin-LR MAbs were added to each well (50 μl). After incubating at room temperature for 1 hour, the plates were washed and 50 μl of

enzyme-conjugated rabbit anti-mouse IgM antibodies were added to each well. After a further 1 hour at room temperature, on an orbital shaker, the plates were washed and 50 μl of enzyme substrate were added to each well. Colouration was allowed to develop for up to 15 minutes and then the reaction was stopped by adding 50 μl of 20% (v/v) sulphuric acid to each well. The amount of yellow colour formation was then measured in a Dynotech MR5000 plate reader at 410 nm. It should be noted that the optimum wavelength for measuring colour development is 450 nm, but such a filter was not available to our laboratory. However, although the values obtained were lower than would be expected using a 450 nm filter, they can be used as direct comparison with each other in order to assess the cross-reactivity of the antibodies.

Table 4.3.1 presents data obtained from ELISA plates on which microcystin-LR, microcystin-LW, and nodularin, were coated as detailed previously. The values presented are mean values of 12 wells. The blank value was obtained from wells coated with the microcystin and nodularin variants, but no anti-microcystin-LR antibodies added, and again these values are presented as mean values of 12 wells.

The data presented infer that the A005-1D 13C6 batch 006 monoclonal antibodies cross-reacted with both microcystin variants and the nodularin. The mean absorbance values at 410 nm for the three toxins were approximately equal.

Table 4.3.1 Cross-reactivity of A005-1D 13C6 batch 006 αMC-LR monoclonal antibodies with microcystin variants and nodularin in ELISA.

Variant investigated[a]	Absorbance 410 nm[b]
microcystin-LR	0.134 ($\pm$0.017)
microcystin-LR blank[c]	0.016 ($\pm$0.008)
microcystin-LW	0.127 ($\pm$0.018)
microcystin-LW blank	0.014 ($\pm$0.007)
nodularin	0.151 ($\pm$0.019)
nodularin blank	0.013 ($\pm$0.007)

a, each toxin was coated at 100ng per well onto poly-L-lysine precoated plates

b, plates were read in a Dynatech MR5000 plate reader, and values presented are mean values of 12 wells

c, toxin blanks contained toxin minus antibodies

The next stage of the assessment of αMC-LR antibodies was an attempt to quantify the amount of MC-LR coated onto the ELISA plates. MC-LR was coated onto poly-L-lysine-treated plates at 1, 5, 10, 25, 50, and 100 ng per well, and assayed with the αMC-LR batch 006 antibodies as described previously. True blanks of glycine-NaOH buffer containing no MC-LR (the "blanks" in the previous investigation were wells containing MC-LR but no αMC-LR antibody) were assayed concomitantly. The results of this assay were a uniform colour development in every well, including the buffer-only blanks (the colour developed was above the measurable range of the plate-reader). These observations indicated that non-specific binding (NSB) of the αMC-LR antibodies occurred in the wells of the plate.

The observed NSB of αMC-LR MAb could have been due to insufficient blocking of the wells with BSA. That is, in wells containing lower than 100 ng of MC-L, poly-L-lysine binding sites may have been available for binding the antibodies, and these may not have been blocked by 3% BSA in the incubation time allowed. To investigate this, various blocking reagents and conditions were employed.

MC-LR was coated at 100 ng per well onto a poly-L-lysine-treated plate, along with MC-LR-free buffer blanks, as before, but after 17 hours incubation at room temperature the contents of half the wells containing each reagent were removed. Glutaraldehyde was added to PBS pH 7.4 to a final concentration of 0.2% (v/v) and 50 μl were added to each evacuated well. The plates were then incubated at room temperature for a further 6.5 hours before washing. 3% BSA was added to the wells which had not been treated with glutaraldehyde and incubated for a further 1.5 hours at room temperature. The assay was then continued as before. The results presented in Table 4.3.2 demonstrate an increased colour development in wells which had been treated with glutaraldehyde, an equal colour development in wells containing MC-LR and MC-LR-free buffer, and a decreased colour development in wells containing no MC-LR or glycine/NaOH buffer. These results again indicate that NSB of αMC-LR MAbs occurred, and that this was elevated by the presence of glutaraldehyde, not decreased. It may also be inferred that the presence of glycine/NaOH buffer also increased NSB.

The above investigation was repeated, and in addition PBS pH 7.4 was used in place of the glycine/NaOH as the coating buffer. In this case 0.2% (v/v) glutaraldehyde was compared with 1% (w/v) BSA in PBS as the blocking reagent (Table 4.3.3).

The results in Table 4.3.3 again indicate that NSB occurred, MC-LR-free assays resulting in equal A_{410} values as the equivalent assays containing MC-LR. Once more glutaraldehyde appeared to increase NSB slightly, the presence of BSA appearing to have little or no effect on the outcome of the assay. There appeared to be little difference between glycine/NaOH and PBS for use as a coating buffer.

Further investigations were carried out where the assay was repeated in the presence and absence of MC-LR in either glycine/NaOH or PBS buffers, blocking in 1 % (w/v) gelatine, 1% (v/v) heparin, 3% (w/v) BSA, 3% (w/v) milk powder, 2% (w/v) newborn-calf serum (NCS), or 5% NCS. In all cases MC-LR-free assays resulted in A_{410} values equal to those of assays with MC-LR present. Thus NSB of αMC-LR antibodies to the poly-L-lysine-treated plates could not be prevented with any of the reagents used.

Table 4.3.2 ELISA colour development in the presence of MC-LR and glutaraldehyde.

Reagents added	Absorbance 410 nm[a]
BSA only[b]	0.189 (±0.072)
Glutaraldehyde only[c]	0.270 (±0.056)
Gly/NaOH + BSA	0.278 (±0.042)
Gly/NaOH + glutaraldehyde	0.606 (±0.048)
MC-LR + gly/NaOH + BSA	0.268 (±0.054)
MC-LR + gly/NaOH + glutaraldehyde	0.616 (±0.062)

a, absorbance was measured in a plate reader

b, 3% (w/v) BSA was added as a blocking reagent to wells coated with poly-L-lysine

c, 0.2% (v/v) glutaraldehyde was added to wells coated with poly-L-lysine

Table 4.3.3 ELISA colour development in the presence of glycine/NaOH pH 8.8 and PBS pH 7.4 coating buffers.

Reagents added	Absorbance 410 nm[a]
Gly/NaOH only[b]	0.359 (±0.060)
PBS only[b]	0.397 (±0.032)
Gly/NaOH + glutaraldehyde[c]	0.482 (±0.060)
PBS + glutaraldehyde[c]	0.432 (±0.042)
Gly/NaOH + BSA[c]	0.368 (±0.069)
PBS + BSA[c]	0.391 (±0.068)
Gly/NaOH + BSA + glutaraldehyde[d]	0.494 (±0.050)
PBS + BSA + glutaraldehyde[d]	0.434 (±0.060)
MC-LR + gly/NaOH + BSA[c]	0.374 (±0.078)
MC-LR + PBS + BSA[c]	0.417 (±0.092)
MC-LR + gly/NaOH + BSA + glutaraldehyde[d]	0.485 (±0.023)
MC-LR + PBS + BSA + glutaraldehyde[d]	0.452 (±0.043)

a, absorbance was measured in a plate reader

b, reagents were added to wells coated with poly-L-lysine, and no blocking solutions were added

c, 1% (w/v) BSA was added to block the plate for 16 h at room temperature or 0.2% (v/v) glutaraldehyde was added for 2 h at 37°C then 6 h at room temperature.

d, the wells were blocked first in glutaraldehyde, then in BSA as in footnote c

4.3.3 Dot-blot assay

As one of a number of further methods to the ELISA format to assess the cross-reactivity of the αMC-LR MAbs a dot-blot assay was evaluated. The rationale here was that if binding occurred between the toxin spotted onto a filter and antibody subsequently added, then it should be possible to detect antibody binding as a dark spot on exposed X-ray film.

The procedure for this assay was as follows:

- Spot MC-LR onto a nitro-cellulose membrane and allow to dry.

- Block binding sites on the membrane with an appropriate reagent such as 5% (w/v) milk powder in PBS containing 0.2% (v/v) Tween 20, incubated at room temperature for 1 - 2 hours.

- Wash the membrane in deionised water then four changes of PBS containing 0.05% (v/v) Tween 20 (PBST), 5 minutes in each change.

- Add the αMC-LR antibodies diluted appropriately, including 1/50 in 0.5 M NaCl containing 0.01% Tween 20 (NaClT), and incubate for instance at room temperature for 1 hour.

- Wash the membrane as before.

- Add goat anti-mouse-IgM horse-radish peroxidase conjugated antibody (HRP-conjugate) diluted appropriately, such as 1/2000 in NaClT, incubated at room temperature for 1 hour on a shaking incubator.

- Wash the membrane as before.

- Prepare ECL luminescent Western-blotting detection solution by mixing reagents A and B in the dark. Incubate the membrane in the ECL reagent for 1 minute then expose to X-ray film in the dark for 1.5 minutes.

- Develop the film and examine for the appearance of darkened areas around the point of MC-LR application.

MC-LR (60 μg/ml in 40% v/v methanol) was spotted onto two nitro-cellulose membranes by application with a capillary-tube. Six applications were made to the same point of the membrane by touching the membrane with the capillary-tube. The spot of MC-LR was allowed to dry before the next spot was applied. It was estimated that approximately 1 μl (0.06 μg of MC-LR) was applied to the membrane by this procedure. As a negative control, 40% (v/v) methanol containing no MC-LR was applied in the same way to two more membranes. Each membrane was allowed to air-dry for at least 30 minutes, then the procedure described above was performed. Blocking the membrane with 5% (w/v) milk-powder was carried out at room temperature for 1.5 hours.

This procedure resulted in very weakly darkened areas around the point of application of MC-LR, but no darkening on the membranes applied with the negative control.

Attempts to optimise the assay (i.e. increase the darkened dot signal without increasing the background NSB signal) were then carried out. These attempts followed the basic procedure described previously, applying the same amount of MC-LR in 40% (v/v) methanol as before. The alterations to the procedure and the results achieved are presented in Table 4.3.4.

Table 4.3.4 Optimisation of the dot-blot assay of MC-LR in 40% (v/v) methanol

	Assay conditions	Results of assay[a]
1.	1 wash in PBST after blocking	MC-LR f; CON f; background pale
2.	block in i. 5% milk-powder/PBS	MC-LR f; CON ?; background pale
	ii. 10% milk-powder/PBS	MC-LR f; CON ?; background pale
	iii. 5% BSA/PBS	background very dark[b]
	block as in i. dilute αMC-LR MAb in PBS (0.25 M NaCl - PB2S)	MC-LR f; CON -; background pale
3.	block as in 2.ii, dilute αMC-LR in:	
	i. NaClT + 1% milk (NaClTM)	MC-LR f; CON f; background pale
	ii. PB2S + Tween + 1% milk (PB2STM)	MC-LR ?; CON ?; background pale
	iii. NaClM (1% milk)	MC-LR f; CON f; background pale
	iv. 0.5 M NaCl	MC-LR ?; CON -; background dark

a, CON = negative control; f = faint signal; ? = very faint signal; - = no signal

b, the darkness of the background masked any dot signals

In spite of numerous attempts under varying conditions, a discernible and reproducible signal from membranes applied with MC-LR could not be achieved. Non-specific binding of the αMC-LR antibodies to the membrane occurred.

It is possible that the ambiguous results achieved in these investigations could be attributed to the fact that the MC-LR was applied to the membrane in 40% (v/v) methanol. It was therefore decided to apply the MC-LR to the membrane in a lower concentration of methanol.

0.2 μg of MC-LR were applied to the nitro-cellulose membrane in 4% (v/v) methanol using a capillary-tube. 4% methanol was also used as the negative control. The basic procedure for the dot-blot assay described previously was carried out, but 10% milk-powder in PBS was used to block the binding sites of the membrane, and the αMC-LR antibody solution was

diluted 1/50 in NaClTM. After developing the X-ray film no darkened areas were observed. Further attempts to optimise the assay were therefore carried out (Table 4.3.5.).

The results again showed that either a very weak ambiguous signal from MC-LR could be obtained or that non-specific binding of the αMC-LR antibody to the membrane occurred.

Table 4.3.5 Further attempts to optimise a dot-blot assay for MC-LR in 4% (v/v) methanol

	Assay conditions	Results of assay[a]
1.	0.5 μg of MC-LR applied to membrane and αMC-LR antibodies diluted in:	
	i. PBS	background very dark[b]
	ii. PBST	background very dark[b]
	iii. PBS + 1% milk powder	MC-LR f; CON -; background dark
	iv. 0.5 M NaCl	MC-LR ?; CON -; background pale
	v. NaCl + 1% milk powder	MC-LR -; CON -; background pale
	vi. NaClM + Tween	MC-LR -; CON -; background pale
2.	Block in PBS + 10% milk powder for 17 h at 4°C. αMC-LR MAb diluted in following for 3 h:	
	i. PBS + 2% milk powder 1/50[c]	MC-LR ?; CON -; background dark
	ii. 0.5 M NaCl 1/50	MC-LR ?; CON -; background dark
	iii. PBS + 5% milk powder 1/50	MC-LR ?; CON -; background pale
	iv. as i 1/10	background very dark[b]
	v. as ii 1/10	background very dark[b]
	vi. as iii 1/10	MC-LR ?; CON -; background dark
3.	Block as in 2. Dilute αMC-LR MAb 1/50 in PBS + 5% milk powder, incubated as follows:	
	i. room temperature 3 hours	MC-LR ?; CON -; background pale
	ii. 37°C 1.5 hours	MC-LR f; CON -; background dark

a, CON = negative control; f = faint signal; ? = very faint signal; - = no signal

b, the darkness of the background masked any dot signals

c, the αMC-LR antibodies were diluted 1/50 or 1/10

4.3.4 HPLC analysis

The rationale in this case was as follows: if a solution of MC-LR is presented with ∝MC-LR antibodies and binding takes place, then this may result in immunoprecipitation with a resulting decrease in the amount of free toxin in solution. The level of MC-LR in solution was determined by HPLC.

First, 10 μg of MC-LR in 100 μl of PBS was incubated with 100 μl of ∝MC-LR antibody at 2 - 8°C for 16 hours. The solution was then centrifuged at 14,200 x g for 5 minutes in order to sediment any immune complex formed. The supernatant was removed and analysed by HPLC for MC-LR content. The results were compared with a MC-LR control (100μl + 100μl of PBS). The antibody-incubated toxin solution showed no decrease in MC-LR content as compared with the antibody-free control (Table 4.3.6). Indeed even if binding between the toxin and an IgM antibody had occurred, this may not have resulted in a pelletable immunoprecipitate as there is only one antibody-binding site on each toxin molecule.

Table 4.3.6 Concentration of MC-LR solution incubated with αMC-LR MAbs

Solution analysed	MC-LR concentration[a] (μg/ml)
MC-LR in PBS	49.35 (±0.52)
MC-LR + αMC-LR MAb	49.41 (±0.43)

a, run through a reverse-phase C18 column and eluted with 35 - 47% acetonitrile in water containing 0.05% TFA (20 min) then 47 - 100% acetonitrile (5 minutes)

The next approach was to incubate toxin in solution with ∝MC-LR antibody to allow binding to occur then to precipitate the antibody using ammonium sulphate. If binding between the antibodies and toxin molecules had occurred, this should result in a decline in toxin levels in the supernatant after centrifugation to sediment the salted-out antibody molecules.

MC-LR was incubated with ∝MC-LR antibody (0.1 mg:0.05 mg) for 16 hours at 2 - 8°C stirring gently. An antibody-free MC-LR control was also incubated under these conditions. Ammonium sulphate was then added to a final concentration of 32% (w/v), to precipitate antibody and also any immune complex. After stirring over ice for a further 15 minutes, the solutions were centrifuged at 14,200 x g for 5 minutes and the supernatants were analysed by HPLC for MC-LR content. The results (Table 4.3.7) indicate a 60% reduction in the MC-LR content of the solution incubated with antibody as calculated by the reduction in peak height of the HPLC trace (Figure 4.3.1).

Table 4.3.7 Concentration of MC-LR solution incubated with αMC-LR MAbs followed by precipitation with ammonium sulphate

Solution analysed	MC-LR concentration[a]
MC-LR/$(NH_4)_2SO_4$	71.50 μg/ml
MC-LR/αMC-LR/$(NH_4)_2SO_4$	28.00 μg/ml

a, estimated by HPLC as in footnote a, Table 4.3.6

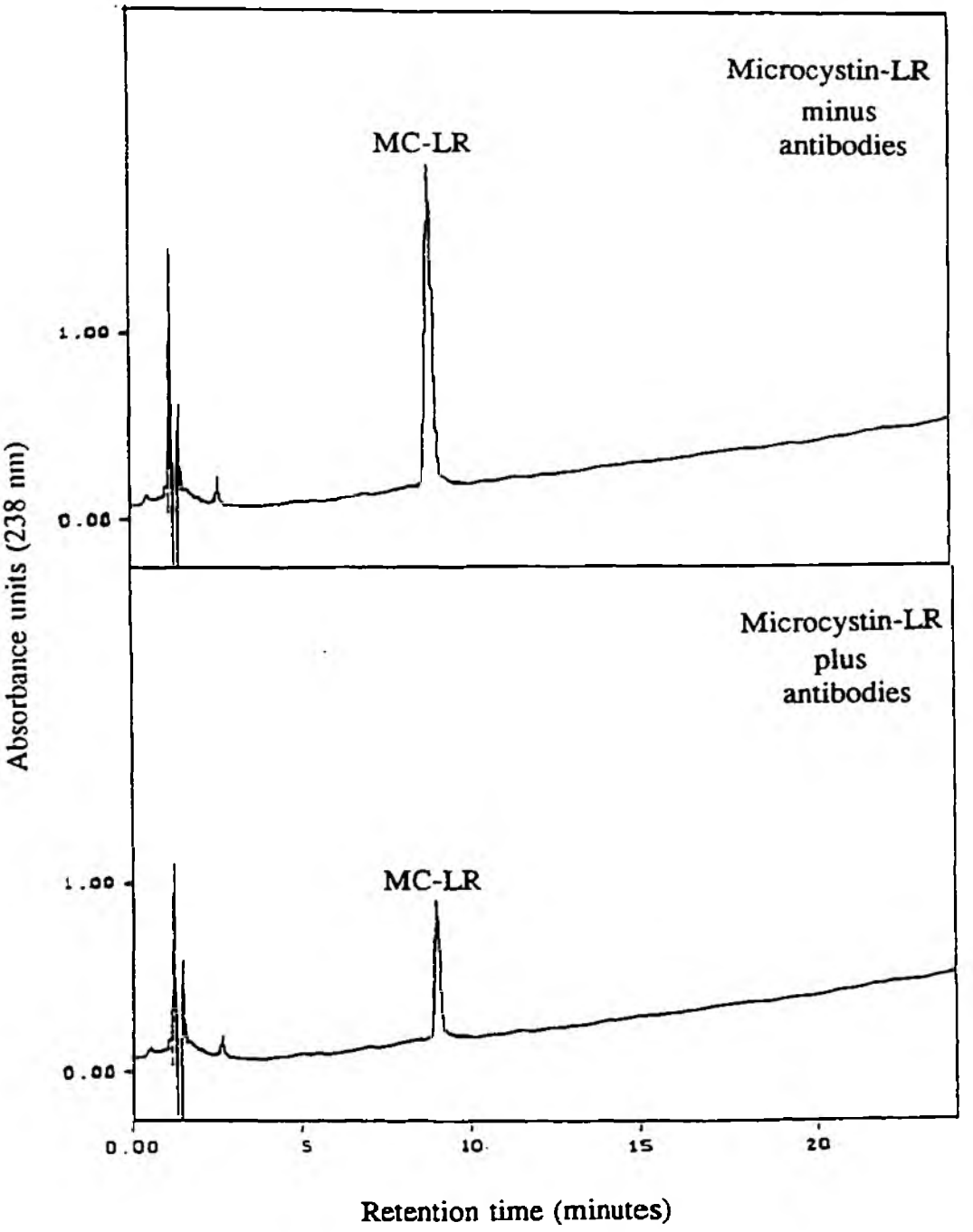


Figure 4.3.1 Reduction of MC-LR HPLC[a] peak height after incubation with αMC-LR antibodies[b] followed by precipitation with 32% (w/v) ammonium sulphate[c].

a, analysed under the conditions described in footnote a, Table 4.3.6

b, incubated at 2 - 8°C for 16 hours

c, incubated with ammonium sulphate over ice for 15 minutes followed by centrifugation at 14,200 x g for 5 minutes

Further work where the incubation temperatures and time were varied between 16 hours at 2 - 8°C, 3 hours at room temperature, or 1 hour at 37°C, resulted in losses of MC-LR from the supernatant in the order of 70 - 30%, the lowest losses being obtained with MC-LR:antibody ratios of 10 μg:5 μg (2:1), the highest losses with a 10 μg:50 μg (1:5) ratio (Table 4.3.8). Rabbit null-serum (N-S) was incubated with MC-LR at a ratio of 10 μg:100 μg (MC-LR:null-serum), and this resulted in a zero % loss of MC-LR from solution.

Table 4.3.8 Investigation of incubation conditions for αMC-LR-mediated reduction of MC-LR HPLC peak height[a]

Sample	MC-LR:MAb weight ratio	Incubation temperature °C	Incubation time h	MC-LR concentration μg/ml
MC-LR/$(NH_4)_2SO_4$[b]	-	4	17	2.832[c]
MC-LR/$(NH_4)_2SO_4$/MAb	2:1	4	17	3.758
MC-LR/$(NH_4)_2SO_4$/MAb	1:5	4	17	2.185
MC-LR/$(NH_4)_2SO_4$	-	room temperature	3	5.205
MC-LR/$(NH_4)_2SO_4$/MAb	2:1	room temperature	3	3.034
MC-LR/$(NH_4)_2SO_4$/MAb	1:5	room temperature	3	0.000
MC-LR/$(NH_4)_2SO_4$	-	37	1	4.875
MC-LR/$(NH_4)_2SO_4$/MAb	2:1	37	1	3.244
MC-LR/$(NH_4)_2SO_4$/MAb	1:5	37	1	1.678
MC-LR/N-S/$(NH_4)_2SO_4$	1:10	room temperature	3	5.497

a, estimated under the conditions described in footnote a, Table 4.3.6

b, incubated as described in footnote c, Figure 4.3.1

c, the value obtained was assumed to be an underestimate of the true value as compared with the two values for MC-LR incubated at the other two temperatures

In further attempts to investigate the cross-reactivity of the αMC-LR antibodies, HPLC analysis of microcystin variants, before and after incubation with the antibodies, was carried out. An acetic acid extract of *M. aeruginosa* PCC 7813, purified on a Sep-Pak C18 cartridge then eluted with methanol, was rotary evaporated to dryness then resuspended in approximately 5% (v/v) methanol. The toxin variants present were MC-LR (leucine-arginine), MC-LY (leucine-tyrosine), MC-LM (leucine-methionine), MC-LW (leucine-tryptophan) and MC-LF (leucine-phenylalanine). This was then incubated with an equal volume of the A005-1 13C6 αMC-LR antibody, batch R248, for 3 hours at room temperature (gently stirring). A control containing the microcystin variants solution and equal volume of water was incubated under the same conditions. After the incubation period an equal volume of 10% (v/v) trichloro acetic acid (TCA) was added to precipitate the antibodies and any immuno-complex formed (ammonium sulphate was not suitable for precipitating the antibodies as this was found to precipitate the MC-LW and MC-LF variants from solution). The suspensions were then centrifuged at 14,200 x g for 5 minutes and the supernatants were analysed by HPLC (Figure 4.3.2.).

The areas of the HPLC peaks of the microcystins were reduced after incubating with the antibody, and the extent of this reduction is presented in Table 4.3.9. These results clearly indicate that the αMC-LR antibodies cross-reacted with MC-LR and the other microcystin variants.

Table 4.3.9 HPLC data of microcystin variants from *Microcystis aeruginosa* PCC 7813 incubated alone and in the presence of αMC-LR antibodies.

Microcystin	Peak area		Reduction
	incubated alone	incubated with antibodies[a]	in peak area
MC-LR	0.010332	0.009316	10%
MC-LY	0.003207	0.002188	32%
MC-LM	0.000395	0.000333	16%
MC-LW	0.000410	below the detection limits	not known
MC-LF	0.000943	0.000503	47%

a, incubated at room temperature for 3 hours

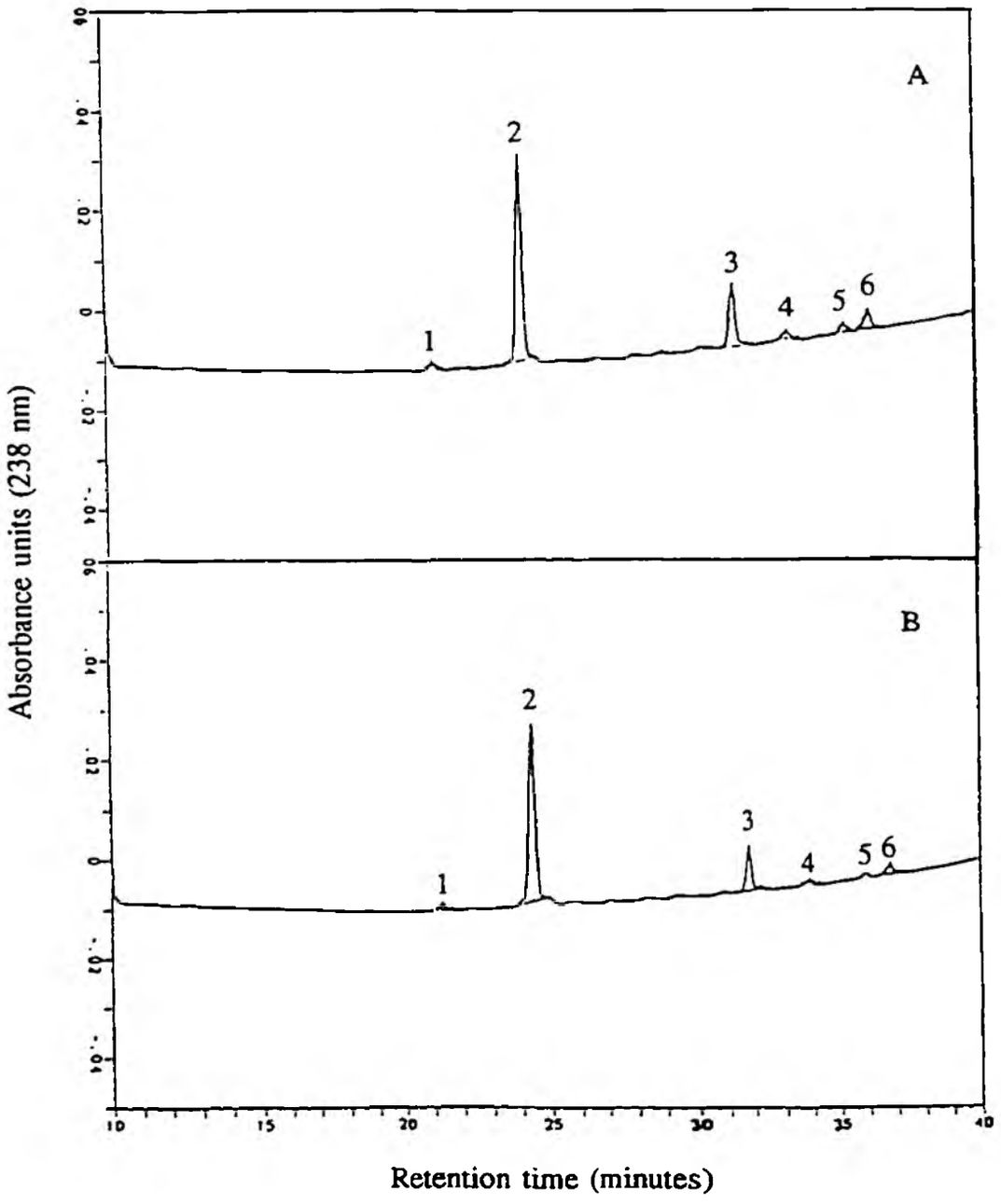


Figure 4.3.2 HPLC analysis of microcystin variants from *Microcystis aeruginosa* PCC 7813 incubated[a] alone (A) and in the presence of αMC-LR antibodies (B).

a, incubated at room temperature for 3 hours.
1, non-microcystin
2, MC-LR
3, MC-LY
4, MC-LM
5, MC-LW
6, MC-LF

4.3.5 Mouse bioassays

The rationale here was as follows: if binding between toxin and ∝MC-LR antibodies occurred then this may have affected the toxicity of the toxin which could be detected by mouse bioassay.

In the first trials, the MC-LR and αMC-LR MAbs were incubated in a 1:1 ratio for 3 hours at room temperature. The solution plus controls were then tested by mouse bioassay, via the intraperitoneal route. Controls were toxin minus antibody and toxin plus null serum, all at the same initial concentration, just above the LD50 concentration, i.e. lethal to a 20g mouse. The results (Table 4.3.10) demonstrate that all treatments were lethal by bioassay but survival times in mice administered with toxin preincubated with ∝MC-LR were slightly increased.

Table 4.3.10 Mouse bioassay: Toxin/∝MC-LR antibody incubation, immunocomplex left *in situ*

solution administered[a]	mean mouse survival time[b]
MC-LR control	66 min (± 13)
MC-LR/null serum	62 min (± 2)
MC-LR/∝MC-LR (1:1)	58 min (± 11)
MC-LR/∝MC-LR (1:2)	67 min (± 4)
MC-LR/∝MC-LR (1:4)	81 min (± 3)

a, solutions were gently stirred at 22 - 30°C for 3 hours before administration to mice

b, mean value of 3 test animals

In a second approach, ammonium sulphate precipitation and centrifugation, as in Section 4.3.4, were carried out after incubation of toxin with ∝MC-LR antibodies. The supernatant was then tested by bioassay. Table 4.3.11 demonstrates that controls of toxin plus null serum, after ammonium sulphate treatment, were lethal by bioassay whereas the supernatant from the toxin plus antibody was not. It can therefore be inferred that the αMC-LR antibodies reduced the toxicity of the MC-LR solution by binding to the toxin molecules, and then subsequently removing them from solution upon ammonium sulphate precipitation.

Table 4.3.11 Mouse bioassay: Toxin/∝MC-LR incubation followed by ammonium sulphate addition and removal of immunoprecipitate[a]

solution administered	mouse weight	survival time
MC-LR/null serum	26.6 g	120 min
" " "	26.9 g	107 min
" " "	16.4 g	209 min
MC-LR/∝MC-LR (1:1)	23.9 g	survived[b]
" " "	28.9 g	survived[b]
" " "	16.0 g	survived[b]

a, solutions were incubated at 22 - 30°C for 3 hours before addition of 32% (w/v) ammonium sulphate. After 15 min on ice, solutions were centrifuged and supernatants administered to mice.

b, mice were sacrificed after 240 minutes.

4.3.6 Radio-immuno assays (RIA)

The rationale here was that if binding occurred between radioactive toxin in solution and ∝MC-LR antibodies and the toxin-antibody complexes were subsequently precipitated from solution by ammonium sulphate, this should result in a decrease in radioactivity in the supernatant.

100 ng of tritiated toxin was incubated with 1, 10, 20, 50, and 100 μg of ∝MC-LR antibody solution. 500 μg null serum/MC-LR controls were also employed, along with MC-LR-only controls. After incubation at room temperature for 3 hours, ammonium sulphate precipitation was performed by adding 32% (w/v) for 15 minutes over ice. Centrifugation was carried out at 14,200 x g for 5 minutes, and the radioactivity of supernatants measured in a scintillation counter. The results in Table 4.3.12 indicate that no reduction in radioactivity was obtained in the supernatants from antibody-incubated solutions, but rather there appeared to be slight increases.

Table 4.3.12 Radio-immuno assay of tritiated MC-LR with αMC-LR MAbs employing ammonium sulphate precipitation[a].

Reagents present	Weight of αMC-LR MAb added[b] (μg)	H^3 DPM[c]
MC-LR	-	3299.1 (±96.7)
MC-LR + null-serum	-	3884.5 (±187.1)
MC-LR + MAb	1	4663.8 (±188.6)
MC-LR + MAb	10	4478.9 (±47.7)
MC-LR + MAb	20	3843.7 (±51.5)
MC-LR + MAb	50	4507.3 (±183.2)
MC-LR + MAb	100	4722.3 (±218.6)

a, incubated as described in footnote c, Figure 4.3.1

b, MAbs were added and the solution was incubated at room temperature for 3 h before adding ammonium sulphate

c, DPM (disintegrations per minute) counted in a scintillation counter

The above protocol was repeated but the ^{3}H-MC-LR/MAb and control solutions were incubated at 37°C for 2 hours gently shaking. Two alternatives to ammonium sulphate precipitation were used; precipitation with an equal volume of 10% (v/v) trichloro acetic acid (TCA) for 60 minutes at room temperature, and filtration through a micro-partition cartridge (Amicon Centrifree) which passes the solution through a membrane upon centrifugation at 2,000 x g, retaining molecules greater than 10,000 Daltons molecular weight (including IgM antibodies and their immuno-complexes) but allowing smaller molecules (including tritiated MC-LR) to pass through into the filtrate. In both cases almost identical results to those with ammonium sulphate precipitation were obtained.

4.3.7 Gel-diffusion assay

The final method of assessment of MC-LR antibodies supplied was the gel-diffusion assay. The rationale behind this investigation was that αMC-LR antibodies added to wells in agarose gel would diffuse through the gel. The antibodies could then bind to MC-LR contained within the gel and form an immuno-precipitate. The gel would then be stained to reveal the presence of immuno-precipitin lines.

100 μl of MC-LR at a concentration of 0.1 mg/ml was added to 2.5 ml of molten 1% (w/v) agarose, i.e. to a final concentration of 0.04 mg/ml. The agarose solution was then poured onto a 76 x 26 mm glass microscope slide and allowed to solidify. 5 holes were bored into the set gel at 1 cm intervals. αMC-LR antibodies at a concentration of 0.5 mg/ml were serially diluted ten-fold to 1/10,000 in PBS. Each antibody solution was then added to one of the wells in a volume of 5 μl. The slide was then put in a sealed chamber containing a dampened pad, and incubated at room temperature for 24 hours. After this period the slide was fixed in 1 M NaCl for 30 minutes then washed in deionised water. The slide was then pressed under an absorbent pad and then placed on a hot plate to dry. The dried gel on the slide was placed in a stain containing 0.5% (w/v) Coomassie Blue R-250 dye, 45% (v/v) methanol and 10% (v/v) acetic acid. After 30 minutes at room temperature the slide was removed from the stain and destained in 45% (v/v) methanol/10% (v/v) acetic acid. The gel was then examined for the presence of blue immuno-precipitin lines.

Examination of the gel revealed no precipitin lines, indicating one of the following:

- the antibodies did not diffuse through the agarose gel.
- the antibodies diffused through the gel but did not bind to the MC-LR.
- the antibodies diffused through the gel and bound to the MC-LR but did not form a immuno-precipitate.

5. DISCUSSION

5.1 Purification and supply of microcystin variants and nodularin

Throughout the course of this project a substantial amount of MC-LR (89.2 mg) was purified and sent to Biocode Ltd for their use in raising and screening antibodies. This has involved a large amount of work in culturing cyanobacteria in the laboratory, followed by subsequent freeze-drying and toxin-purification.

The purity of this toxin was always greater than 90% by HPLC analysis, and after initial batches, the purity of the supplied toxin was routinely greater than 97% by HPLC.

Other microcystin variants, 3-desmethyl MC-RR and MC-LW, and the closely related toxin, nodularin, were purified from different laboratory cultures of cyanobacteria, and have been supplied to Biocode for screening purposes.

5.2 Lysis of cyanobacterial cells for the release of peptide toxins

The entrapment of cyanobacterial cells on a glass-fibre filter, prior to treatment of the cells to release the intracellular microcystin(s), was successfully demonstrated in Section 4.2.2. The strategy of trapping the cyanobacterial cells from a natural water-bloom would permit the lysis procedure to be carried out in a small volume of lysis-reagent, and would therefore make the addition of large volumes of the reagent to unconcentrated water samples unnecessary. This would aid the adaptation of the lysis procedure to a kit format, in reducing the bulk of the kit and reducing the volume of potentially hazardous lysis reagent.

It is proposed that either 2.5 cm or 4.7 cm diameter GF/C filters, available from Whatman, should be used for the purpose of cell-entrapment. These would be placed in a suitable filter funnel, also available from Whatman, Gelman Sciences and Fisons. The funnel and filter would be sealed into a side-arm flask, which would be connected via tubing to a vacuum pump. A portable hand-held vacuum pump is available from Nalgene. Using this system water samples containing cyanobacterial cells could be drawn through the filter. In Section 4.2.2. the volumes of water sample containing *M. aeruginosa*, at a range of chlorophyll *a* concentrations typically observed in the field, required to block 2.5 cm and 4.7 cm diameter filters were estimated. These values were calculated assuming that only the cyanobacterial cells were present in the water sample. However it is also assumed that there would be occasions when particulates in the water reduced the amount of cyanobacterial cells trapped on the filter by competing for the available surface area of the filter. In these cases it would be advisable to use the larger 4.7 cm diameter filter. The filter disc would then be added to a suitable lysis reagent. These filters were disrupted when shaken for a few seconds in toluene, and would thus aid the extraction of the intracellular toxin by presenting more cells into the reagent solution.

Toluene added to a final concentration of 0.3% (v/v) caused the release of microcystin-LR (MC-LR) from cyanobacterial cells within 15 minutes of incubation at room temperature. Although the toluene-mediated lysis of the cyanobacterial cells was shown to be almost 100% effective at releasing the MC-LR, by comparison with a standard MC-LR purification method

and by measurement of intra- and extra-cellular MC-LR concentrations before and after cell-lysis, this procedure was shown to be temperature-dependent below 20°C (the amount of MC-LR released from cells decreased with decreasing temperature). As the cell-lysis procedure would be used in the field, the temperature-dependency of the toluene method might pose problems when air- and sample-temperatures are below 20°C. It was therefore decided that the toluene-mediated procedure would not be suitable for use in the field in the existing format.

As a contingency to the use of toluene, methanol was investigated as a reagent for the extraction of MC-LR from cyanobacterial cells. Methanol is routinely used as a method for extracting chlorophyll *a* from cyanobacterial cells in laboratories. The chlorophyll was shown to be extracted within the first 5 minutes of incubation with methanol at room temperature, as indeed was MC-LR. As with toluene, the methanol-lysis procedure was shown to be efficient, releasing 100% MC-LR from the cells, and was shown to be effective against a number of species of cyanobacteria from which microcystins or nodularin have been isolated. The filter-entrapment method on glass-fibre discs was again shown to be feasible, and as with the toluene, the filter was disrupted after a few second shaking in methanol. The methanol method of extraction of MC-LR from the cyanobacterial cells appears to have an advantage over the toluene method, in that with methanol, release of MC-LR appears to be constant with temperature down to 10°C. Unfortunately, 100% methanol is required to release all of the intracellular MC-LR from the cells. This method was not considered suitable for a field-test kit format due to the high flammability of 100% methanol, after consultation with NRA and Biocode staff.

The most appropriate method for the extraction of microcystin(s) from cyanobacterial cells in the field, that we have investigated, is by a reagent containing 0.3% (v/v) toluene and 20% (v/v) methanol. The presence of these two reagents permits the use of phosphate-buffered saline pH 7.4 (PBS) as the diluent buffer in this reagent, as they negate the reduced solubility of MC-LR in this buffer. However, the PBS should be prepared with sodium salts only, and should not contain potassium salts, as the presence of these reduces the amount of MC-LR released from the cyanobacterial cells. The presence of 20% (v/v) methanol appears to reduce the temperature-dependency observed with the toluene method in that the amount of MC-LR released is constant with temperatures down to 15°C, and at 10°C the amount of MC-LR released is only slightly reduced. This reagent is also less flammable than a 100% methanol reagent and is therefore more acceptable for use in a field-test kit.

A recommended procedure for extracting microcystin(s) from water samples in the field is presented in Section 6.

5.3 Assessment of Biocode anti-microcystin-LR antibodies

The enzyme-linked immuno-sorbent assay (ELISA) of MC-LR with αMC-LR IgM antibodies produced by Biocode, performed by this laboratory, was found to exhibit non-specific binding of the antibodies to the assay plate. This was thought to be due to binding of the αMC-LR antibodies to the poly-L-lysine on the plate, which was insufficiently blocked by the conditions investigated. This meant that the result whereby MC-LR, MC-LW and nodularin appeared to produce a positive colour reaction in the ELISA assay, could not be

attributed to binding of these toxins with the antibodies.

Several attempts to overcome the non-specific binding of the antibodies were made by changing the blocking conditions or the coating buffer. None of these, including the use of glutaraldehyde which binds strongly to poly-L-lysine, but which in this case increased the non-specific binding of the antibody, were successful. The ELISA may have been optimised by further changes in conditions, or a different ELISA format could have been investigated. However, further attempts to develop a successful ELISA format would have taken a considerable time beyond the scope of this contract. It was decided to assess the αMC-LR IgM antibodies by several additional methods.

A dot-blot assay, whereby MC-LR spotted onto a nitrocellulose membrane then assayed by the αMC-LR antibodies and indirectly visualised by a luminescent reagent, was investigated. After initial ambiguous results, probably caused by interference by the 40% (v/v) methanol in which the MC-LR was applied to the membrane, an ambiguous weakly positive result was obtained. Again optimisation of the assay was attempted, but this resulted in either an unamplified positive signal or high non-specific binding of the αMC-LR antibody to the membrane, which masked any signal from the MC-LR spot. Again, time could have been spent further optimising this assay, but again it was decided to use this time to investigate further alternative methods, to provide clearer indications of whether the antibodies did cross-react with MC-LR and the other toxins, or not.

The previous two assay methods involved binding MC-LR onto a solid support as the initial stage. It was decided to assess the binding of MC-LR to αMC-LR IgM antibodies in free solution. This was subsequently followed by addition of ammonium sulphate to precipitate any immunocomplex formed, then analysis of the MC-LR remaining in solution by HPLC methods. This gave the first unequivocal evidence that MC-LR was binding to the αMC-LR antibody, in that after incubating the two together and removing the immunoprecipitate, less MC-LR was observed in solution than when no antibody was present. Although there was not a total reduction of MC-LR in solution, this was probably due to the weight:weight ratio of antibody:MC-LR. That is, the highest ratio was 5:1 (antibody:MC-LR), whereas the molecular weight ratio is 970:1 (antibody:MC-LR). Thus, if five molecules of MC-LR can bind to one molecule of IgM (which has five binding sites per molecule) the optimum weight ratio for binding is 194:1 (antibody:MC-LR), much higher than the ratios employed in our investigations. Unfortunately, due to the relatively low concentrations of the antibody solutions held, to achieve the desired ratio would have meant diluting the MC-LR to such an extent that it would be below the detectable level of the HPLC. If further work could be carried out employing this method a more concentrated antibody solution should be investigated.

The immunoprecipitation procedure described above was also the first, and to-date the only method, which has demonstrated the cross-reactivity of the A005-1 13C6 IG6 2B2 αMC-LR antibodies. It cross-reacted with the variants MC-LR, MC-LY, MC-LW, MC-LM and MC-LF, reducing the HPLC peak heights. Although the MC-LR peak height was only reduced by 10% in this case, compared with 30% or greater reduction previously, this was probably due to competition for antibody binding sites from the other microcystin variants (previous studies were carried out with only MC-LR present). In terms of % reduction, the αMC-LR antibody appears to have a lower affinity for MC-LR than the other microcystins. However,

if the actual weight reduction of microcystin in solution is compared, i.e. MC-LR 20 ng, MC-LY 20 ng, MC-LM 1.2 ng, MC-LW not known (estimated at 50% or 4 ng reduction), MC-LF 9 ng, then it can be seen that the comparative affinity of the αMC-LR antibodies for each microcystin can be estimated as follows:

MC-LR = MC-LY > MC-LF > MC-LW > MC-LM (only 7.7 ng of MC-LM were present before antibody addition).

The mouse bioassay results confirmed the observations made by HPLC analysis, in that after incubating MC-LR with antibody, followed by ammonium-sulphate precipitation, then removal of the immuno-precipitate by centrifugation, toxicity of the supernatant containing MC-LR was reduced, indicating a removal of MC-LR in solution. When MC-LR and antibody were incubated together, but no ammonium-sulphate precipitation was performed, mouse survival times were only slightly increased. In this case toxin was still present in an unaltered concentration, in the solution administered to mice, even though some of this may have been bound to the antibody molecules. It could be argued that if toxin was bound to antibody molecules the toxicity would be expected to be reduced due either inhibition of toxin active site (the ADDA region in this case) by bound antibody, or by the fact that when the IgM molecule is bound to MC-LR the toxin/antibody complex is much larger than the toxin-only molecules, and therefore should be prevented from crossing tissue membranes in the animal. The reason that toxicity was only slightly reduced in this case may be a reflection of the affinity for MC-LR by the antibody. That is, if the antibody has a low affinity for MC-LR then even though it binds to the MC-LR molecules in buffered solution, once the environment is changed by injection into the animal, the antibody/MC-LR complex could dissociate to release free MC-LR molecules available once more.

Attempts at assessing MC-LR/antibody binding by means of a radio-immuno assay (RIA), employing tritiated MC-LR, were unsuccessful. This cannot be readily explained because the strategy employed was very similar to the HPLC and mouse bioassay procedures whereby the toxin and antibody were incubated in free solution, then the immunocomplex removed by precipitation with ammonium-sulphate or TCA, or by membrane size-exclusion. In theory, as results from the other two methods suggest, this should have resulted in a reduction in the amount of the radio-labelled MC-LR in solution. The fact that this did not happen points to the possibility that the tritium label was in some way dissociating from the MC-LR molecule and giving misleading results. Another possibility is that addition of a precipitating reagent was interfering with the counting of the radio-label, but this is unlikely since the third method involved no addition of further reagents but still lead to the same results.

The final method of assessing the αMC-LR antibodies, the gel-diffusion immunoassay, was unsuccessful for any one of a number of reasons; 1, the antibody molecules may not have diffused through the agarose gel and would not have encountered the MC-LR molecules (in this method the antisera is normally added to the molten gel and the antigen molecules are allowed to diffuse through the gel, the opposite was performed in this case because the MC-LR molecule is so small that it would diffuse through the gel too rapidly and would be unable to form an immunoprecipitate with the antibody) This possibility is not considered likely however: we have used gel-diffusion immunoassays successfully in this laboratory for more than 10 years involving the diffusion of proteins of high molecular weight (>500,000

Daltons) through agarose; 2, the antibody diffused through the gel but did not bind to the MC-LR; or 3, the antibody diffused through the gel, bound to the antibody but may not have formed a immunoprecipitate because of the MC-LR having only one binding site for the antibody (in this case a net-like structure of antibody-antigen-antibody-antigen etc molecules could not form).

5.4 Recommendations for the use of the αMC-LR antibodies

The binding of the A005-1 (13C6 IG6-2B2) antibody to MC-LR has been indicated, namely by the incubation of antibodies and toxin in solution followed by HPLC analysis of MC-LR or mouse bioassay.

This antibody has also been shown to cross-react with the other microcystin variants i.e. MC-LY, MC-LM, MC-LW, and MC-LF, having a slightly higher comparative affinity for the MC-LR and MC-LY variants.

The antibody may therefore be of use for laboratory research when the antibodies and the microcystin are both in solution prior to detection methods.

However, the affinity of this antibody for MC-LR and related toxins appears to be low as suggested by the ambiguous results in various assays performed in this laboratory, along with the fact that this antibody would not bind MC-LR when coupled to a gel-matrix, as found by Biocode. This is possibly not surprising when the production of IgM by mammals is taken into account. The IgM molecule is usually associated with a primary response to antigenic challenge, i.e the IgM-type antibody is produced first, followed by secondary production of IgG-type antibodies. The IgM antibodies produced in this case usually have low affinity for the antigen, whereas the IgG antibodies usually have a much higher affinity.

It may be possible to develop a immuno-detection method for microcystins using these antibodies, but attachment of the antibodies to a solid matrix has led to inhibition of MC-LR binding (as demonstrated by Biocode Ltd using an affinity-column format), and problems have been demonstrated in this project when MC-LR was attached to a solid matrix such as used in the ELISA, dot-blot and gel-diffusion formats. It would therefore be prudent to attempt microcystin-detection methods where the antibody-microcystin binding is carried out in solution first, followed by the elucidation procedure.

Thus, if an ELISA method was to be developed, then it would seem logical to develop a competitive format where the antibody and microcystin-containing test sample are incubated in solution first, then added to the ELISA plate coated with microcystin (BUT NOT TREATED WITH POLY-L-LYSINE). The assay would then proceed with the addition of enzyme-conjugated anti-mouse IgM antibody then the enzyme-substrate. A reduction of colour development would indicate the presence of microcystin(s) in the test solution, as this would bind to the free antibody, reducing the amount available for binding to the MC-LR on the plate. However, a suitable MC-LR plate-coating procedure would have to be developed to replace the attempted poly-L-lysine method. Rigorous optimisation of the method, including affinity and cross-reactivity studies, would have to be carried out.

The above principles may also be applied to a membrane assay format, again an initial antibody-microcystin step being carried out in solution.

In principle a radio-immunoassay method may be developed, as this would involve binding of free antibodies with microcystin in solution. However, due to the difficulties observed various different methods of radio-labelling microcystin would have to be investigated, as would the immunocomplex precipitation/separation stage. Again a competitive format may prove more feasible. Unlike the previous two formats, this format could not be applied in the field, and would be a laboratory-based procedure. It may however prove to be more sensitive than the previous two formats.

Finally it should be taken into consideration that the affinity of the IgM antibody for microcystins appears to be low, and that, due to the large size of the IgM molecule, the application of the antibody for *in situ*-detection of microcystin(s), such as immuno-gold labelling, may prove difficult. Therefore, if different antibodies, with a stronger affinity for microcystin(s) and of the IgG class, were to be developed, then the present IgM antibodies would be virtually made redundant.

If however, the present antibodies bind to the microcystin molecules at a different site to any future antibodies (the binding site of the present antibodies is not known) then they may be used in a sandwich-type ELISA, where one of the antibodies is coated to the ELISA plate and is used to capture microcystin(s), and the second antibody is bound to the captured microcystin(s). The assay would then proceed with the addition of conjugated antibodies against the second anti-microcystin antibodies,followed by the addition of enzyme-substrate. Again, the performance of the present antibodies in such a format would have to be thoroughly investigated as this involves antibody binding on a solid phase.

6. RECOMMENDED CYANOBACTERIAL CELL-LYSIS PROCEDURE

6.1 Equipment required

water sampling vessel
measuring cylinder (500 - 1000 ml)
2.5 cm or 4.7 cm diameter GF/C glass-fibre filters (Whatman)
appropriate filter funnel fitted with rubber bung or appropriate seal
side-arm flask
tubing
hand-operated vacuum pump
20 ml glass bottle
spoon spatula with a known capacity (1 - 2 ml is sufficient)
sodium dihydrogen orthophosphate dihydride (analytical grade)
disodium hydrogen orthophosphate dihydride (analytical grade)
sodium chloride (analytical grade)
toluene (analytical grade)
methanol (analytical grade)
deionised water
thermometer

6.2 Procedure

6.2.1 Preparation

- Weigh out reagents as follows:

disodium hydrogen orthophosphate dihydride	1.6 g/l
sodium dihydrogen orthophosphate dihydride	0.157 g/l
sodium chloride	8.5 g/l

- Add the reagents to deionised water, stir to dissolve the reagents, and make the volume up to 1.0 l. Adjust the pH to 7.4 with hydrochloric acid or sodium hydroxide.

- Prepare 5 ml and 10 ml aliquots of the pH 7.4 buffer containing 0.3% (v/v) toluene and 20% (v/v) methanol, in 20 ml bottles.

- Cap the bottles and allow contents to remain at room temperature until leaving the laboratory. Keep in an insulated box at $>15°C$ until used at the field test-site.

6.2.2 Field-procedure: Water samples

- Connect the filter-funnel and fitted seal to the side-arm flask. Connect the tubing from the side-arm to the hand-operated pump. Fit the appropriate filter-disc into the filter-funnel.

- Collect the test-water (containing cyanobacteria) in the sampling-vessel and pour into the measuring cylinder.

- Add the water to the filter-funnel and operate the vacuum pump. Filter the water until the filter disc becomes blocked (or flow rate has almost stopped), or until the filter is dark green (if the 2.5 cm filter becomes blocked but colouration is light, the operation should be repeated with a 4.7 cm filter). Record the volume of water filtered.

- Remove the filter (bearing cyanobacterial cells) and add this to the appropriate volume of lysis reagent (5 ml for the 2.5 cm filter, 10 ml for the 4.7 cm filter).

- Cap the bottle and shake the contents for a few seconds until the filter disintegrates. Keep the bottle at 15°C or above, if possible, for at least 15 minutes.

- The contents of the bottle should then be centrifuged or filtered, before analysing the supernatant or filtrate for microcystin content. The concentration of microcystin(s) in the cells (μg per μg chlorophyll *a*) can be calculated by measuring the chlorophyll *a* concentration of the water in the laboratory, and using the recorded volume of filtered water to calculate the amount of chlorophyll *a* on the filter.

6.2.3 Field-procedure: Scum samples

- The above lysis procedure can be performed using scum material by collection of a spatula-load (known volume).

- Add the collected scum material to 10 ml of the lysis reagent and perform the lysis procedure as above.

- Measure the chlorophyll *a* concentration of the scum in the laboratory and calculate the microcystin concentration as above.

6.2.4 Limitations of the procedure

- The lysis procedure will not be as efficient at temperatures below 15°C, and at these temperatures the filter/cyanobacterial cells should be held in the lysis-reagent for at least 30 minutes.

- The filtration procedure may not be appropriate at very high non-cyanobacterial particulate loading of the water, as the filter may become blocked before a sufficient amount of cyanobacteria is trapped for microcystin-analysis.