R&D Project 349

Field validation of algal toxin test kit

University of Dundee

December 1994

Contains Appendices – Interim reports.

R&D Draft Final Report 349/8/A

(R&D Project Record)

FIELD VALIDATION OF ALGAL TOXIN TEST KIT

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R&D Draft Final Report 349/8/A

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Appendices

Appendix 1 Interim Report 349/1/A

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SUMMARY

1. Microcystin-LR purification has been established and the purified toxin has been supplied to Biocode to date.

2. Toxin has been supplied at two levels of purity:

   a 91-93%. This was understtod to be of sufficient purity for the purposes for which it was required.
   b 97-98%. This is the routine level of purity attained.

3. According to the Biocode estimate of 5 November 1991 of the amounts of toxin required and of the timing of requirements, toxin provision has been prompt and sufficient.

4. Unscheduled requests for toxin have been received although it has been possible to supply material between 1 and 6 days after receiving requests.

5. A laboratory procedure has been developed for the release of microcystin-LR from intact cells of *Microcystis aeruginosa* using small quantities of toluene. Optimization studies are in progress.

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

The specific objectives to commence in the present reporting period were as follows:

- Liaise with and provide technical advice to Biocode Ltd.
- Purify microcystin-LR (MC-LR) and supply Biocode Ltd for the production and screening of monoclonal antibodies (MAbs).
- Inspect and assess work undertaken by Biocode Ltd.
- Develop methods for cyanobacterial cell lysis in water.

2. ESTABLISHMENT OF PROJECT AND APPOINTMENT

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 Ltd to be supplied with purified MC-LR 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 was signed. This enabled 10 mg of MC-LR to be supplied to Biocode on 22 October 1991 at the start of the Project.

3. RESEARCH PROGRAMME

3.1 Technical progress

Work has progressed as per the schedule agreed with the NRA in the Memorandum of Agreement for Research Contract NRD 040.

Since the initial 10 mg of purified MC-LR were dispatched, a further 21.6 mg of the toxin have been purified and sent to Biocode Ltd following individual requests

(Table 3.1.1). The structure of MC-LR and a typical HPLC chromatogram of the purified toxin before dispatch are presented in Figures 3.1.1 and 3.1.2.

The purity of the toxin batches can be grouped into 2 categories:

a. 91-93%. Early batches were supplied at this level since it was indicated by Biocode that this level of purity would be sufficient for the procedures to be performed.

b. 97-98%. Microcystin-LR has otherwise been supplied at 97% and 98% purity. This level of purity can be achieved routinely.

Cyanobacterial cultures have been established for the production of other microcystin variants for later testing.

3.2 Lysis of cyanobacterial cells

In order to use microcystin antibodies for the optimal detection (and quantification) of the toxin(s) in water samples, it is necessary for the toxin to be released from cyanobacterial cells, if it remains in an intracellular form in the samples, to be tested. Procedures for the lysis of cyanobacterial cells for the release of toxin are being investigated.

Three treatments were initially examined using cultures of *M. aeruginosa* strain 7813. As an indicator of cell leakage, the appearance of the high molecular weight, water-soluble blue pigment phycocyanin in the water after cell removal has been initially monitored. Pigment release is measured as absorbance at 620 nm. As shown in Table 3.2.1, only toluene treatment was found to be potentially useful. In this case, toluene addition to a final concentration of 10% (v/v) to the *Microcystis* cell suspension in water, with mixing, resulted in the appearance of phycocyanin in the water.

The leakage of *M. aeruginosa* cells was subsequently investigated at a range of lower concentrations of toluene. As shown in Table 3.2.2, phycocyanin release was maximally achieved at a final concentration of 0.2 - 0.3% (v/v) toluene.

When 0.3% (v/v) toluene-treated cells were examined under the microscope about 75% of the cells were disrupted after 5 min, 95% after 10 min and the 100% after 15 min incubation. However, after only 5 min incubation, the cell-free phycocyanin concentration was as its maximum (Table 3.2.3), indicating that permeabilization and pigment (protein) release occurred before all of the cells appeared to be disrupted.

A cell-free supernatant of *M. aeruginosa* 7813 incubated with 0.3% toluene for 15 min was analysed by HPLC for MC-LR content. This was compared with an acetic

Figure 3.1.1 Structure of microcystin-LR

Abbreviations:

Masp	ß-methylaspartic acid
Leu	leucine
Ala	alanine
Mdha	N-methyldehydroalanine
Glu	glutamic acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Arg	arginine

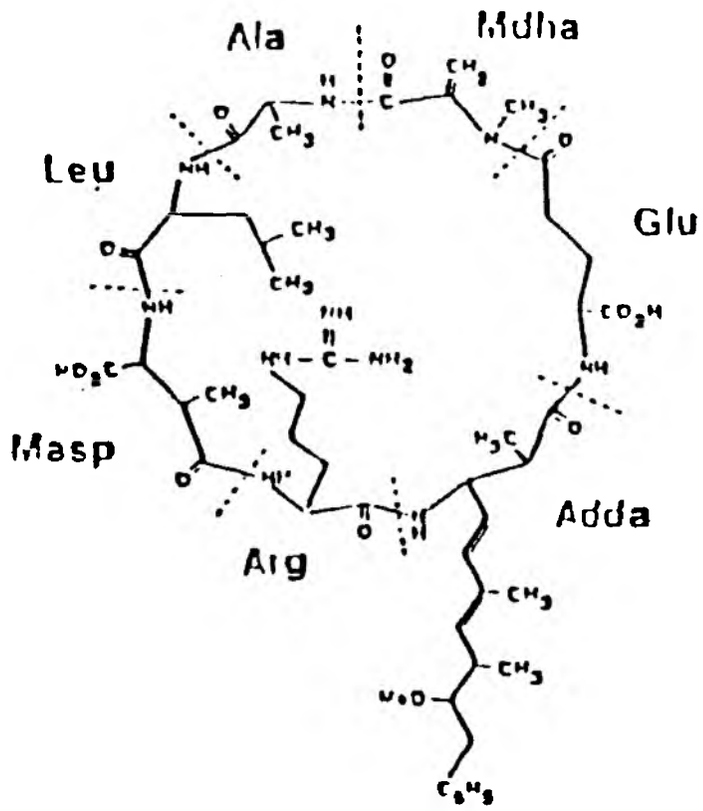

Figure 3.1.1 Structure of microcystin-LR

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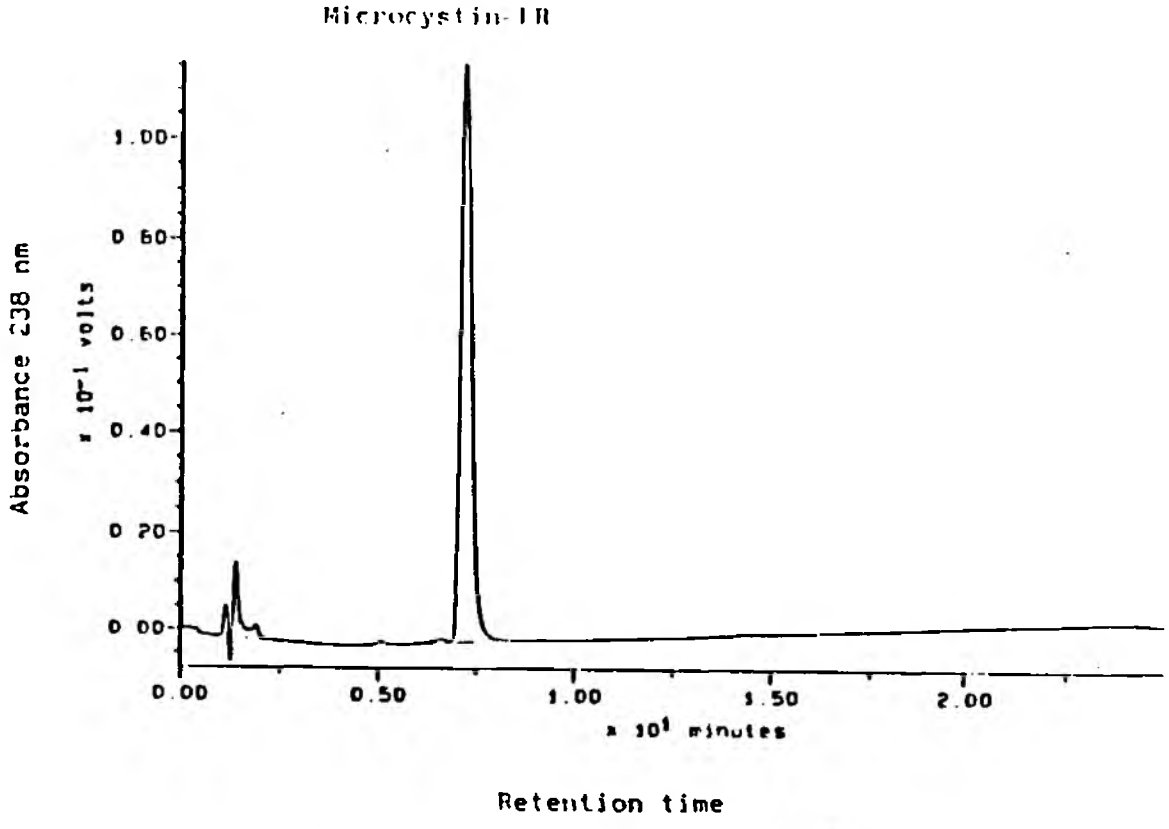

Figure 3.1.2 Typical HPLC chromatogram of purified microcystin-LR supplied to Biocode Ltd as in Table 3.1.1.

Table 3.1.1 Consignments of purified microcystin-LR dispatched to Biocode Ltd

MC-LR Lot No.	Amount Dispatched	Date Dispatched
Initial supply	10.0 mg	22.10.91
SGB 251191	5.0 mg	26.11.91
SGB 251191.2	4.5 mg	02.12.91
SGB 281191	4.5 mg	02.12.91
SGB 221191	3.6 mg	04.12.91
SGB 021291	4.0 mg	08.01.92
Total	31.6 mg	

Table 3.2.1 The release of phycocyanin (absorbance at 620 nm) from *Microcystis aeruginosa* cells by various agents

Addition	Final concentration (v/v)	Incubation time	Abs 620 nm cell-free supernatant[1]
None	-	-	0.000
Acetic acid	10%	48 hr	0.000
Sodium-hydroxide	10%	48 hr	0.000
Toluene	10%	5 min	0.144

1, cell suspension in water incubated with the reagent for the time stated then centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge.

Table 3.2.2 The release of phycocyanin from *Microcystis aeruginosa* cells in water using toluene

Final concentration of toluene (v/v)[1]	Concentration of cell-free phycocyanin (μg per ml)[2]
0.0%	0.00
0.1%	0.00
0.2%	23.15 ± 0.23
0.3%	23.28 ± 0.22
0.4%	23.22 ± 0.38
0.5%	21.97 ± 1.77

1, toluene was added to the final concentration stated and the mixture shaken intermittently for 20 min.

2, after toluene-treatment, the cell suspension was centrifuged for 5 min at 14,000 rpm in a microcentrifuge and absorbances measured in the cell-free supernatant at 615 nm and 652 nm. The phycocyanin concentration was calculated as follows:

conc. of phycocyanin (μg per ml)

$$= \frac{Abs_{615} - (0.474 \times Abs_{652})}{0.00534}$$

Table 3.2.3 Time-course of the release of phycocyanin from *Microcystis aeruginosa* 7813 during treatment with toluene (0.3% v/v)

Incubation time (min)	Concentration of cell-free phycocyanin (μg per ml)[1]
0	0.197 (± 0.000)
5	17.331 (± 0.491)
10	16.731 (± 0.881)
15	13.198 (± 0.652)

1, see legend to Table 3.2.2

Table 3.2.4 Comparison of the release of MC-LR from *Microcystis aeruginosa* 7813 into water by toluene treatment, versus MC-LR from an equivalent amount of freeze-dried cells using acetic acid extraction.

Source and extraction procedure	Total yield of MC-LR[1]
Aqueous cell suspension; 0.3% toluene	53.49 µg
Freeze-dried cells; 5.0% acetic acid	49.63 µg

1, analysis was carried out using a C_{18} reversed phase HPLC column and water/acetonitrile/trifluoroacetic acid solvents.

acid extract of an equal amount of freeze-dried cells from the same culture (part of our procedure for the extraction and purification of MC-LR from dry cells). The results (Table 3.2.4) indicated that at least an equivalent amount of MC-LR was released from toluene-treated cells into water, compared to the amount of toxin extracted from an equivalent quantity of acid-treated freeze-dried cells.

It has been subsequently shown that *M. aeruginosa* cells trapped by passage of an aqueous cell suspension through a glass fibre-filter (Whatman GF/C), then incubated with 0.3% (v/v) toluene, release a similar amount of MC-LR as freeze-dried, acid-treated cells. The filter plus entrapped cells were placed in the toluene/water suspension and mixed intermittently for 15 min. Within this incubation period, the filter disintegrated, thus easing the release of the permeabilized cells into suspension. The resulting slurry of glass fibre and treated cell suspension was passed through a syringe plugged with glass wool, the filter material and majority of cell particulates being retained within the syringe. The cell-free solution passed through the syringe and was analysed by HPLC for MC-LR content.

3.3 Future programme

The specific objectives for the reporting period February 1992 - April 1992 are to continue the work objectives begun during the present reporting period (see section 1.2).

4. DISCUSSION

The work has begun and is progressing well. The supply of MC-LR to Biocode has progressed without delay even though the quantities required (total to date, 31.6 mg) have been considerably greater than was originally budgeted for when the contract was drawn up. These large quantities of purified toxin have required large-scale culture of *M. aeruginosa* as well as substantially more time on toxin purification than initially envisaged. A schedule of quantities and dates of microcystin-LR requirements was received from Dr D. Baron on 5 November 1991. According to these estimates, toxin supply has been both prompt and sufficient. Since the amounts of toxin required have been higher than anticipated this may lead to spending over budget.

It has been possible to meet unscheduled requests for toxin within 1 to 6 days of receiving a telephone request. Although unscheduled requests for toxin have not affected progress on other aspects of the work, the latter may be adversely affected if further unscheduled increases in the need for toxin occur. Culture of cyanobacteria which produce MC-LR-related toxins has begun.

Studies on the lysis of cyanobacterial cells to increase the availability of microcystin in various water samples for binding by antibodies in the prospective test are progressing well. A method involving the use of toluene to permeabilize cells is under investigation. Filtration of the cells from water samples by glass fibre filters and the subsequent permeabilization of cells with toluene has been achieved. This may have favourable implications for the separate measurement of microcystin(s) in the water and in the cyanobacterial cells.

It is understood from Biocode that toluene at the low levels used (e.g. 0.3% v/v) should not adversely affect the binding of toxin to antibodies in the columns to be developed. However, if this were the case, a remedy would be possible: the filter supporting the cells may be placed in a small volume of 0.3% toluene which would then be diluted after the cells had been permeabilized, thus reducing the toluene concentration. Work in future will include investigations of filter loading with cyanobacterial cells, optimum volume of toluene suspension and the use of cyanobacteria from diverse groups which can produce microcystins. Further lysis procedures will also be investigated.

5. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

Toxin purification according to a schedule of times and quantities is necessary to allow the other essential work on cyanobacterial cell lysis for toxin release, comparative studies and the production of additional toxin variants to proceed on time. If unforeseen increases occur in the amounts of toxin needed, then these may delay the completion of the development of the lysis and assay procedures.

6. COST OF WORK CARRIED OUT IN THE PERIOD NOVEMBER 1991 - JANUARY 1992

The cost of the work carried out in the first three months of the project (1 November 1991 - 31 January 1992) is summarised below alongside the 1991/1992 and total project budgets:

	This period (£)	1991/1992 Total (£)	Project Total (£)
Staff salaries and overheads	8,982	27,500	57,938
Travel and subsistence	99	1,000	1,800
Laboratory consumables	250	4,000	6,000
Total	£9,331	£32,700	£66,738

7. ESTIMATE OF COST OF WORK FOR THE PERIOD FEBRUARY 1992 - APRIL 1992

It is estimated that the work to be carried out between 1 February 1992 and 30 April 1992 will cost the following:

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	1,000
Reports	166
Total	£11,466

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SUMMARY

1. 35 mg of purified microcystin-LR (MC-LR) has been supplied to Biocode since the beginning of the present reporting period; 66.6 mg since the project began. Purity has been 97% or greater.

2. Attempts to lyse/permeabilise *Microcystis aeruginosa* PCC 7813 with five non-ionic detergents proved unsuccessful.

3. Permeabilisation of *M. aeruginosa* PCC 7813 with 0.3% (v/v) toluene resulted in almost 100% release of MC-LR from the cells. Release of MC-LR was at a maximum 15-30 min after adding toluene. Other species and strains of toxigenic cyanobacteria released phycocyanin during incubation with 0.3% (v/v) toluene for 15 min.

4. The loading capacity of glass-fibre filters for the recovery of toxic cells of *M. aeruginosa* 7813 has been established.

5. Variants of microcystin have been purified or partially purified from laboratory cultures of cyanobacteria for future comparative purposes.

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

The specific objectives to be carried out in the present reporting period were as follows:

- Purify further batches of MC-LR and supply Biocode Ltd for the production and screening of monoclonal antibodies (MAbs).
- Develop methods for cyanobacterial cell lysis in water.
- Liaise with and provide technical advice to Biocode Ltd.
- Inspect and assess work undertaken by Biocode Ltd.
- Purify cyanobacterial peptide toxins related to MC-LR (other mycrocystins and nodularin) for Biocode to test against the Biocode MC-LR antibodies for cross-reactivity and sensitivity (toxins to be named when supplied).

2. RESEARCH PROGRAMME

2.1 Technical progress

Work is still progressing as per the schedule agreed with the NRA (Memorandum of Agreement of Research Contract NRD 040) including the increased requirement for purified MC-LR by Biocode Ltd. This has required a substantial increase in the amount of time needed for the mass culturing of cyanobacteria, harvesting and freeze-drying cells, and extraction and purification of the toxin.

In the present reporting period a further 35 mg of purified MC-LR has been supplied to Biocode (see Table 2.1.1), and this has been at least 97% pure. We have now supplied the entire amount of purified MC-LR requested by Biocode on 13.2.92, to the purity and date required.

Table 2.1.1 Further consignments of purified microcystin-LR supplied to Biocode Ltd

MC-LR Lot No.	Purity	Amount Dispatched	Date Dispatched
SGB 291191	98%	4.7 mg	13.2.92
SGB 111291	98%	5.3 mg	13.2.92
SGB 210292	97%	10.0 mg	24.2.92
SGB 280292	98%	10.0 mg	10.3.92
SGB 050392	98%	5.0 mg	20.4.92
Total		35.0 mg	

2.2 Lysis of cyanobacterial cells: non-ionic detergents

Following discussions at the last reporting meeting, five non-ionic detergents were incubated with *Microcystis aeruginosa* PCC 7813 in attempts to release phycocyanin and MC-LR from the cyanobacterial cells. The five detergents were as follows:

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

1% (w/v or v/v) solutions of each detergent were prepared in deionised water and these were added to suspensions of *M. aeruginosa* 7813 to final concentrations of 0.01% and 0.1% (v/v) detergent. The suspensions were kept at room temperature for 2 hours with intermittent shaking. After this time, aliquots were removed and centrifuged at 14,000 rpm for 5 minutes. Supernatants were decanted and measured at 615 nm and 652 nm in a spectrophotometer against a blank of deionised water. Phycocyanin concentrations were then calculated (Table 2.2.1).

The above procedure was repeated, but instead of phycocyanin measurement the entire suspension was centrifuged and supernatants were passed through C18 Sep-Pak cartridges. These were eluted with 60% methanol and the eluant was analysed by HPLC for MC-LR content (Table 2.2.2).

The results indicate that little or no phycocyanin, and no MC-LR was released, upon incubation with the non-ionic detergents investigated, thus indicating the unsuitability of these for lysing cyanobacteria in the present programme.

Table 2.2.1 Phycocyanin release from *Microcystis aeruginosa* 7813 incubated with non-ionic detergents.

Detergent added	Cell-free phycocyanin concentration[a] (µg/ml) 0.01% (v/v) detergent	0.1% (v/v) detergent
Triton X-100	0.00 (± 0.2)	0.93 (± 0.00)
Sarkosyl	0.00 (± 0.2)	0.13 (± 0.11)
Tween 20	0.00 (± 0.2)	0.20 (± 0.37)
Nonidet P-40	0.00 (± 0.2)	1.02 (± 0.00)
Span 20	0.00 (± 0.2)	18.80[b] (± 2.22)
Untreated cells	0.00 (± 0.00)	

a, phycocyanin content (µg/ml) = $\frac{A615 - (0.474 \times A652)}{0.00534}$

b, No blue colour was observed. Detergent solution and cell-free supernatants were cloudy.

Table 2.2.2 MC-LR release from *Microcystis aeruginosa* 7813 incubated with non-ionic detergents.

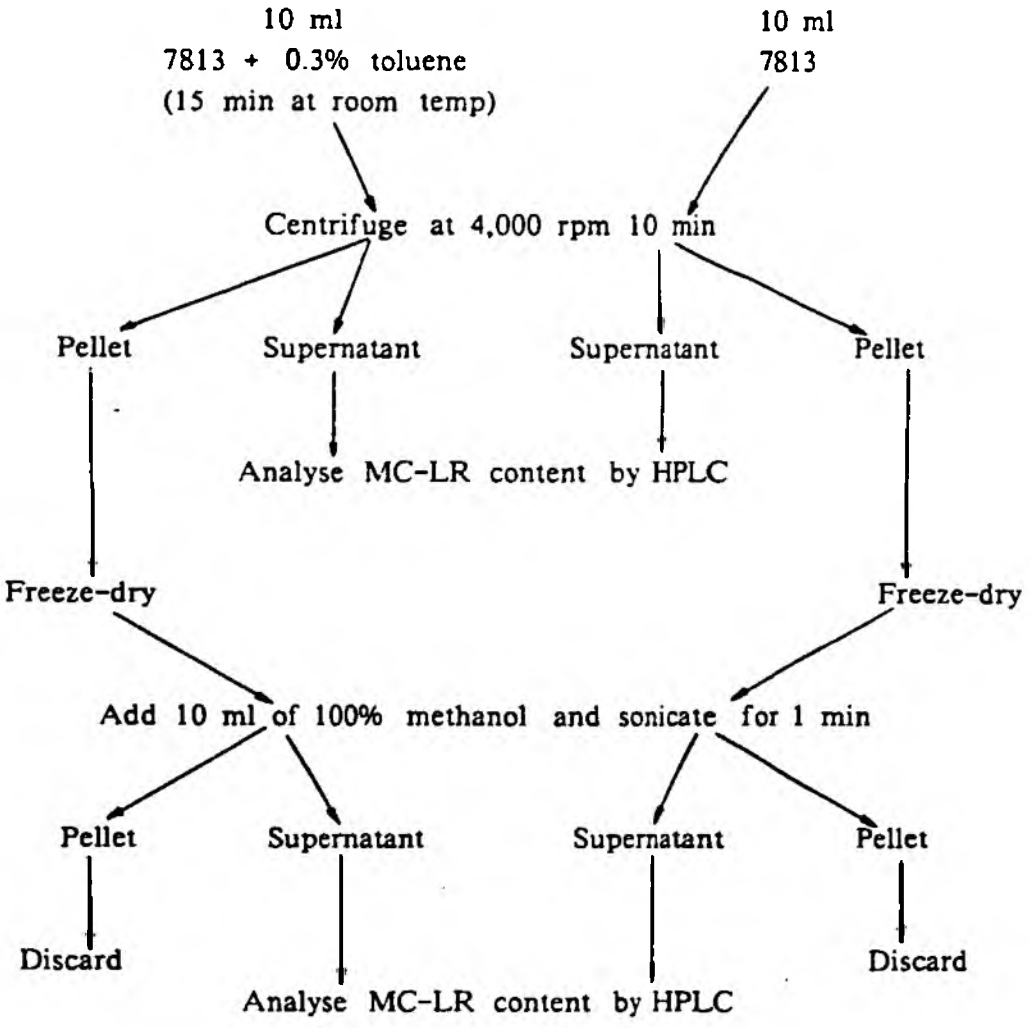
Detergent added	Amount of MC-LR per 25 µl sample [a]. 0.01% (v/v) detergent	0.1% (v/v) detergent
Triton X-100	0.0 (± 0.0)	0.0 (± 0.0)
Sarkosyl	0.0 (± 0.0)	0.0 (± 0.0)
Tween 20	0.0 (± 0.0)	0.0 (± 0.0)
Nonidet P-40	0.0 (± 0.0)	0.0 (± 0.0)
Span 20	0.0 (± 0.0)	0.0 (± 0.0)
0.3% (v/v)[b] Toluene	92.2 (± 8.6) ng	

a, 25 µl of sample loaded onto a Novapak RCM reverse phase column and eluted with 65% Milli Q water (0.05% TFA)/35% acetonitrile (0.05% TFA) to 53% Milli Q water (TFA)/47% acetonitrile (TFA) over 20 minutes.

b, 0.3% (v/v) toluene was used as a positive control (see previous report) for release of MC-LR. The *M. aeruginosa* 7813/toluene suspension was incubated at room temperature for 15 min then centrifuged and extracted through a C18 Sep-Pak in the same manner as the detergent suspensions.

2.3 Lysis of cyanobacterial cells using toluene

To investigate the efficiency of toluene-mediated release of MC-LR from *M. aeruginosa* 7813, the following procedure was performed:



The percentage of total MC-LR in the cell pellet and the medium supernatant, after toluene incubation and untreated, was then calculated (Table 2.3.1).

Some optimisation of the incubation time with 0.3% (v/v) toluene was then carried out. 3 ml were removed from a suspension of *M. aeruginosa* 7813 and toluene was added to the remainder of the suspension to a final concentration of 0.3% (v/v). After 5, 10, 15 and 30 min, 3 ml were removed from the suspension. Immediately after removal, the aliquots were centrifuged at 14,000 rpm for 5 minutes and the supernatants were decanted and retained. 25 µl aliquots were then analysed by HPLC in triplicate in order to estimate the MC-LR content of each supernatant, (Figure 2.3.1).

Table 2.3.1 Distribution of MC-LR between cell material and cell-free medium from toluene incubated and untreated *Microcystis aeruginosa* 7813.

	0.3% toluene-incubated cells	untreated cells
MC-LR content[a] of cell-free medium	78.71 (± 0.78) ng	0.00 (± 0.00) ng
MC-LR content of cell material	0.32 (± 0.32) ng	90.70 (± 1.51) ng
% total MC-LR in cell-free medium	99.6%	0.0%
% total MC-LR in cell material	0.4%	100.0%

a, 25 µl of sample loaded onto the HPLC and run as in footnote a, Table 2.2.2.

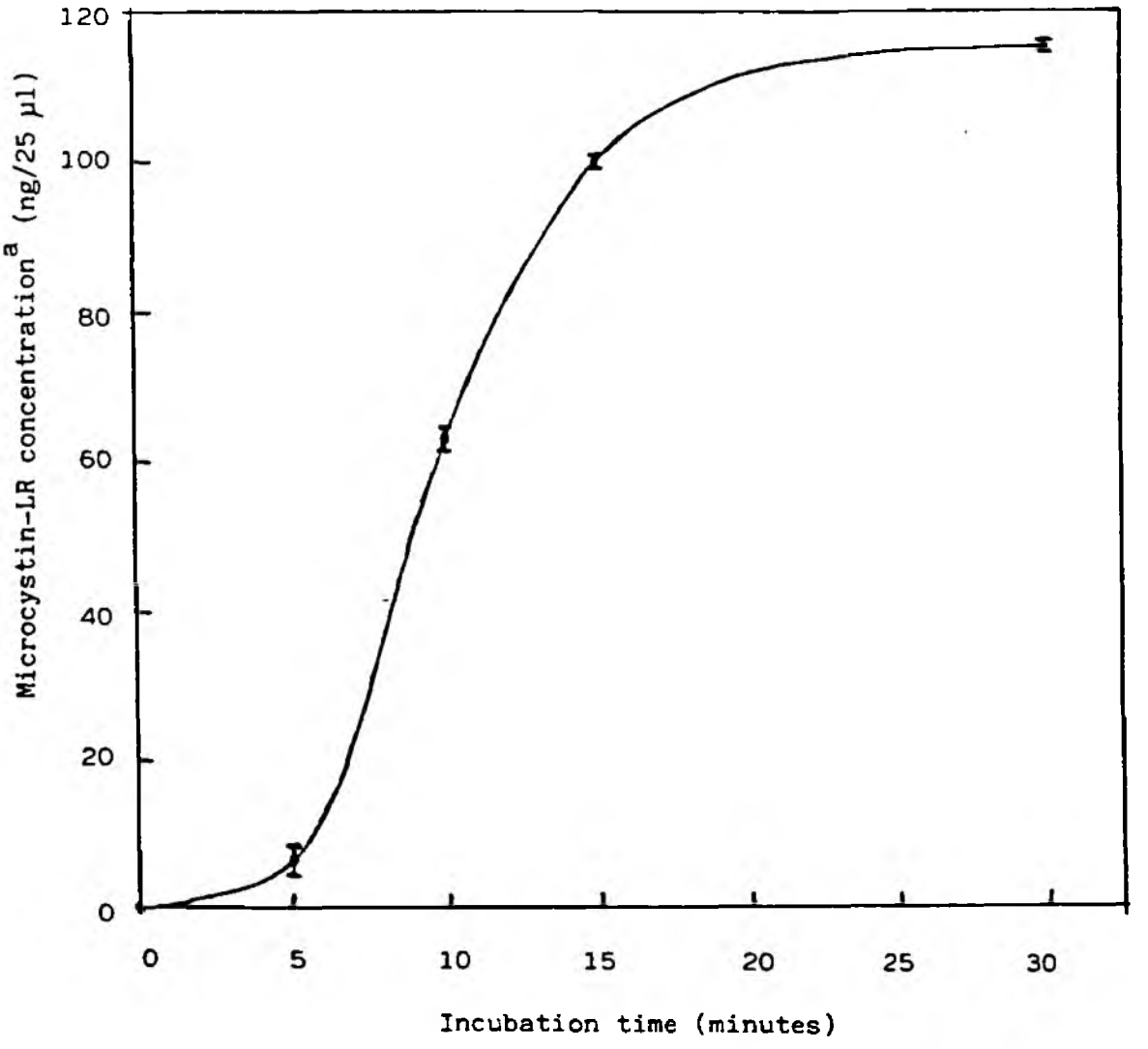


Figure 2.3.1 Release of microcystin-LR from *Microcystis aeruginosa* 7813 during incubation with 0.3% (v/v) toluene.

a, Measured by HPLC analysis.

In order to estimate the effectiveness of toluene to permeabilise other strains and species of cyanobacteria, the following laboratory cultures were investigated:

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

Aliquots of each culture were washed by centrifuging at 4,000 rpm for 10 min and the pellet resuspended in an equal volume of fresh culture medium. An aliquot of the washed cells was centrifuged at 14,000 rpm for 5 min and the supernatants were decanted and retained. Toluene was added to the remaining washed cells to a final concentration of 0.3% (v/v). After 15 min at room temperature, aliquots were centrifuged at 14,000 rpm for 5 min and the supernatants decanted and retained. Supernatants of the untreated and toluene-treated cultures were then measured at 615 nm and 652 nm in the spectrophotometer in order to estimate phycocyanin release (Table 2.3.2).

It appears that 0.3% (v/v) toluene is suitable for lysing the species of toxigenic cyanobacteria examined, in that all cultures released substantial amounts of phycocyanin into the aqueous medium during incubation with the solvent.

2.4 Filter loading with *Microcystis aeruginosa*

If filters are to be employed in the field test kit for determining the toxin in whole cyanobacterial cells, an estimate of the loading capacity of the filters should be obtained. In the previous report (Progress Report 1, 0349) the use of Whatman GF/C filters to entrap *M. aeruginosa* 7813 was described. In an attempt to estimate the loading capacity of this type of filter, a suspension of *M. aeruginosa* 7813 of a known chlorophyll a concentration was passed through a 2.5 cm diameter filter until it became blocked. The volume of suspension passed through the filter was

Table 2.3.2 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 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 (± 1.2)	4.5 (± 0.5)	183.0 (± 1.0)

a, 15 min at room temperature

b, aliquots extracted with 100% methanol. Chl *a* = 12.63 x A663.

c, μg/ml, determined as in footnote to Table 2.2.1

measured, and the loading capacity (µg chlorophyll a per cm^2) was calculated as follows:

chlorophyll a concentration = 2.37 (± 0.1) µg/ml.

Volume of *M. aeruginosa* 7813 loaded = 10.0 (± 1.0) ml.

∴ amount of chlorophyll a loaded = 23.7 µg.

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

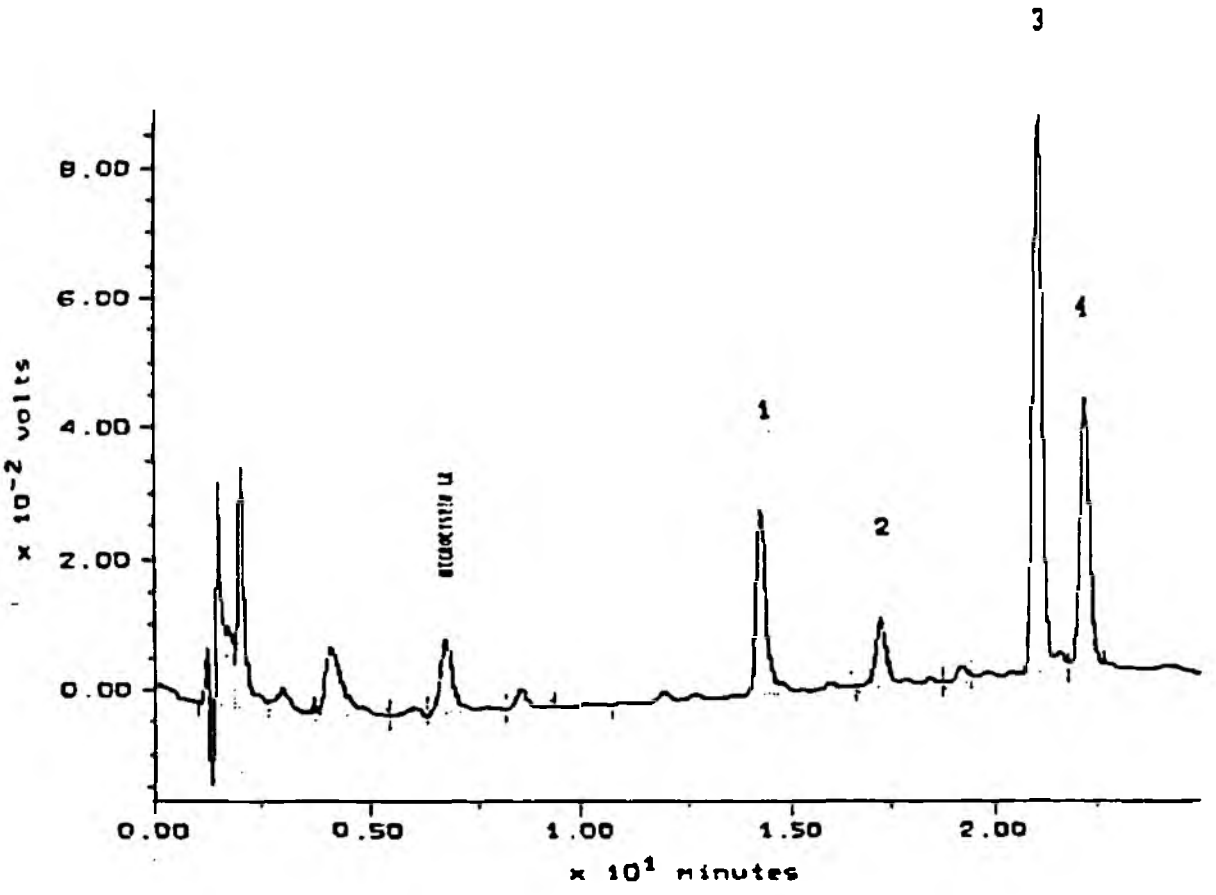
∴ chlorophyll a loading capacity of filter (GF/C) = 4.84 µg/cm^2.

2.5 Purification of microcystin variants

Figure 2.5.1 illustrates a HPLC chromatogram of an extract taken from *M. aeruginosa* 7813. During purification of microcystin-LR from this strain, an acetic acid extract is passed through Sep-Pak C18 cartridges and then eluted with an increasing step-wise concentration (10% increase per step) of methanol in water. The chromatogram illustrated represents a pool of 60% methanol cuts taken from *M. aeruginosa* 7813 extractions.

Peaks 1 to 4 are thought to be microcystin variants and these have been purified or partially purified. Toxicity (BALB/C mice) of each peak will be tested, and toxin characterizations carried out.

Further cultures of cyanobacteria containing microcystin variants are being grown and freeze-dried. Crude toxin extracts have been performed and these will be analysed by HPLC for toxin content before further purification.



Detection time

Figure 2.5.1 HPLC chromatogram[a] of microcystin variants[b].

a, An acetic acid - Sep-Pak/60% methanol extraction was loaded onto a Novapak RCM reverse phase column and eluted with Water/TFA:acetonitrile/TFA.

b, Microcystin variants = peaks 1-4.

2.6 Future programme

- Continue optimisation of toluene-mediated cell lysis.
- Investigate further detergents as an approach to cell lysis as an alternative and contingency to toluene.
- Continue purification of microcystin variants.
- Inspect and assess Biocode monoclonal antibody production, and preliminary column validation.

3. DISCUSSION

The increased requirement for microcystin-LR by Biocode has been met to date. Each batch of toxin has been of high purity (at least 97% pure by reverse phase HPLC) and has been supplied to the required quantity. A subsequent 10 mg of purified MC-LR will be prepared and held in reserve for Biocode. This will bring the total of purified MC-LR supplied or held in reserve for Biocode to 45 mg since the first progress report, and 76.6 mg since the project began.

The five non-ionic detergents investigated have been found to be unsuitable as tools for the lysis/permeabilisation of *Microcystis aeruginosa*. However, further detergents will be investigated as to the possible role of less hazardous permeabilisation agents as a substitute for toluene.

As no information has been received to the contrary, toluene has continued to be investigated as the permeabilisation agent to be supplied in the test kit. Upon permeabilisation with 0.3% (v/v) toluene for 15 min at room temperature, *M. aeruginosa* PCC 7813 releases almost all its MC-LR (99.6%) into the surrounding aqueous medium. This study, and a further investigation into the incubation time of 0.3% (v/v) toluene in the cyanobacterial suspension, were carried out with a dense culture of *M. aeruginosa* 7813, far in excess of the chlorophyll a levels found in natural blooms (30 µg/ml cf < 1 µg/ml). Thus it would be expected that a natural bloom of *Microcystis* would release its entire intracellular MC-LR under the conditions of toluene incubation. Further strains of *M. aeruginosa* and other toxigenic cyanobacterial species have been successfully permeabilised by 0.3% (v/v) toluene. Further investigations into permeabilisation of cyanobacteria with toluene, will include temperature of incubation, and further investigation of filter-entrapped cyanobacterial permeabilisation.

A 2.5 cm diameter GF/C filter has been shown to entrap approximately 23 µg of *M. aeruginosa* 7813 chlorophyll a. Natural blooms of *Microcystis* have been collected by this laboratory for which the chlorophyll a contents range from 0.002 µg/ml to 0.25 µg/ml. If these were applied to the filter mentioned above, then approximately 92 ml to 11.5 l of the water containing the bloom would be required to load the filter to capacity. It should be noted however that this is assuming a 100% pure *Microcystis* content. Other components of natural water could cause the filters to be blocked with lower volumes than those calculated above.

Microcystin variants have been purified or partially purified from *M. aeruginosa* 7813 and other cyanobacterial cultures. Future work will involve further purification and identification of the toxin variants.

4. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

As in the previous reporting period, any unforseen requirements for further purified microcystin-LR may delay the completion of other work such as lysis studies.

The supply of microcystin variants may also cause a delay in the work. The cultures of cyanobacteria presently being held contain relatively small amounts of these variants in comparison to MC-LR. The greater the requirement for each of these variants the greater the period required to culture the cyanobacteria and purify the toxins.

5. COST OF WORK CARRIED OUT IN THE PERIOD FEBRUARY 1992 - APRIL 1992

	This period (£)	Total to date (£)	Project total (£)
Staff salaries and overheads	8,982	17,964	57,938
Travel and subsistence	340	440	1,800
Laboratory consumables	1,700	1,950	6,000
Reports	160	160	1,000
Total	11,182	20,514	66,738

6. ESTIMATE OF COST OF WORK FOR THE PERIOD MAY 1992 - JULY 1992

It is estimated that the work to be carried out between 1 May 1992 and 31 July 1992 will cost the following:

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	1,000
Reports	166
Total	£11,466

SUMMARY

1. Further attempts to lyse *Microcystis aeruginosa* PCC 7813 with non-ionic detergents, in the presence and absence of lysozyme, proved unsuccessful.

2. Lysis of *M. aeruginosa* 7813 with toluene resulted in maximum levels of microcystin-LR being released after 15 minutes' incubation or greater in 10-20 ml of reaction agent, and at 20°C or greater.

3. Initial investigations into the use of methanol have revealed that this solvent may be at least as effective as toluene at releasing microcysti-LR from *M. aeruginosa* 7813.

4. Microcystin-3-desmethyl-RR, nodularin, and a further toxin variant provisionally named PK-3 have been purified and dispatched to Biocode Ltd.

CONTENTS

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

The specific objectives to be carried out in the present reporting period were as follows:

- Continue optimisation of toluene-mediated cell lysis
- Investigate further detergents as an approach to cell lysis as an alternative and contingency to toluene
- Continue purification of microcystin variants
- Liaise with and provide technical advice to Biocode Ltd
- Inspect and assess Biocode monoclonal antibody production

2. RESEARCH PROGRAMME

2.1 Technical progress

We are continuing to work to the schedule in Memorandum of Agreement of Research Contract NRD 040, all technical work in our laboratory is progressing on schedule.

The extra 10 mg of purified MC-LR requested by Biocode as a contingency has been produced.

Three further toxin variants have been dispatched to Biocode Ltd.

2.2 Lysis of cyanobacterial cells: non-ionic detergents

In the previous progress report (February 1992 - April 1992) the results of attempts to release phycocyanin and MC-LR from *Microcystis aeruginosa* PCC 7813 with non-ionic detergents were presented. Due to the failure of any of these surfactants to release either component it was decided to investigate more non-ionic detergents for the same effects. The non-ionic detergents investigated were as follows:

- n-dodecyl β-D maltoside (Sigma)
- n-octyl β-D glucopyranoside (Sigma)

1% (w/v) suspensions of each detergent were prepared and added to suspensions of *M. aeruginosa* 7813 to final concentrations of 0.001%, 0.01% and 0.1% (v/v) detergent. The suspensions were centrifuged at 14,000 rpm for 5 min and supernatants measured at 615 nm and 652 nm, for phycocyanin concentration estimation, after 5 min, 30 min, 1 h, 2 h, and 24 h, at room temperature (Table 2.2.1).

As no phycocyanin was released from the cyanobacterial cells with the detergents, it was decided to investigate the effect of adding lysozyme to the cell/detergent suspension. This would theoretically digest the cyanobacterial cell wall and expose the cell membrane for detergent permeabilisation.

Suspensions of *M. aeruginosa* 7813/detergent were prepared to the same concentrations as stated previously and lysozyme was added to a final concentration of 0.1% and 1.0% (w/v). Again phycocyanin concentrations were measured in the cell-free supernatants after 5 min, 30 min, 1 h, 2 h and 24 h, at room temperature (Table 2.2.2).

Once again, the results indicate that no phycocyanin was released from the cyanobacterial cells upon incubation with either detergent, in the absence or presence of lysozyme. It was therefore assumed that no, or insubstantial amounts of MC-LR would be released from the *M. aeruginosa* 7813 under the same treatment.

2.3 Lysis of cyanobacterial cells using toluene

In the previous progress report results were presented which indicated that 0.3% (v/v) toluene-mediated release of MC-LR from *M. aeruginosa* 7813 in a cell suspension was at a maximum between 15 and 30 minutes after addition of the toluene. Further work was carried out to investigate the effect of incubation time with toluene upon the release of MC-LR from filter entrapped *M. aeruginosa* 7813 cells.

M. aeruginosa 7813 cells were entrapped on a 2.5 cm diameter GF/C glass fibre filter then added to 10 ml of 0.3% (v/v) toluene. After shaking to disrupt the filter 1 ml aliquots were removed, centrifuged at 14,000 rpm for 5 minutes, then analysed by HPLC for MC-LR content, 5 min, 10 min, 15 min, and 30 min after adding to the toluene suspension (Figure 2.3.1).

These results confirm the findings in the previous report that maximum release of MC-LR from *M. aeruginosa* 7813 occurs 15 to 30 minutes after adding to 0.3% (v/v) toluene. In this case the cyanobacterial cells were entrapped on a glass fibre filter before adding to the toluene suspension.

Table 2.2.1 Phycocyanin release from *Microcystis aeruginosa* 7813 incubated with n-dodecyl β-D maltoside or n-octyl β-D glucopyranoside.

	Cell-free phycocyanin concentration[a] (μg/ml)		
Incubation time	0.001% (v/v) detergent	0.01% (v/v) detergent	0.1% (v/v) detergent
5 min			
30 min			
1 h		0.00 in all cases	
2 h			
24 h			

a, phycocyanin content (μg/ml) = $\dfrac{A_{615} - (0.474 \times A_{652})}{0.00534}$

Further optimisation of the toluene-mediated lysis method involved analysis of the incubation volume. *M. aeruginosa* 7813 was entrapped on 2.5 cm GF/C filters as before, in triplicate. One filter was added to 5 ml of 0.3% (v/v) toluene (20 ml Universal bottle), the second filter was added to 10 ml of 0.3% (v/v) toluene (20 ml Universal bottle) and the third filter was added to 20 ml of 0.3% (v/v) toluene (100 ml bottle). All 3 vessels were shaken to disrupt the filters, and after 15 minutes at room temperature the contents were centrifuged at 14,000 rpm for 5 minutes. Supernatants were analysed by HPLC for MC-LR content (Table 2.3.1.), after SepPak concentration.

Although it appears that the amount of MC-LR released from *M. aeruginosa* 7813 increases with increasing volume of toluene suspension, up to 20 ml, the difference is comparatively small. It may be sufficient to use 10 ml of 0.3% (v/v) toluene in the procedure described.

The next stage of optimisation of the release of MC-LR from toluene treated *M. aeruginosa* 7813 was the investigation of incubation temperature.

A suspension of *M. aeruginosa* was incubated at each of the following temperature for at least 10 minutes; 5°C, 8°C, 10°C, 12°C, 15°C and 20°C. Toluene was added to a final concentration of 0.3% (v/v). and the suspension was shaken and incubated at the same temperature for a further 15 minutes. It was then centrifuged at 14,000 rpm for 5 minutes and the supernatant analysed by HPLC for MC-LR content (Figure 2.3.2).

The result indicate that release of MC-LR from *M. aeruginosa* 7813 with 0.3% (v/v) toluene increases with temperature to 20°C. To investigate if this effect reaches a maximum, the experiment was repeated at temperatures of 10°C, 15°C, 20°C, 25°C and 30°C (Figure 2.3.3). It appears that the release of MC-LR from *M. aeruginosa* 7813 with 0.3% (v/v) toluene is at a maximum at 20°C or greater.

2.4 Release of microcystin-LR using methanol

The pigment chlorophyll a (Chl a) is extracted from cyanobacterial cells when incubated with methanol. It was decided to investigate this effect in order to determine if microcystin-LR is also released from *Microcystis* cells under the same conditions.

A suspension of *M. aeruginosa* 7813 was centrifuged at 14,000 rpm for 5 min, and the supernatant was measured at 663 nm in the spectrophotometer. The pellet was resuspended in methanol (same volume as supernatant) and after 5 min, 30 min, 1 h, 2 h, 4 h, and 24 h at room temperature an aliquot was centrifuged as before and the supernatant measured at 663 nm in the spectrophotometer. Chl a contents were then calculated and plotted against incubation time (Figure 2.4.1).

A further suspension of *M. aeruginosa* 7813 was centrifuged and the supernatant retained for HPLC analysis. The pellet was resuspended in an equal volume of methanol and after 5 min, 10 min, 15 min, 30 min, 1 h and 2 h at room temperature, aliquots were removed and centrifuged. The supernatants were analysed by HPLC for MC-LR

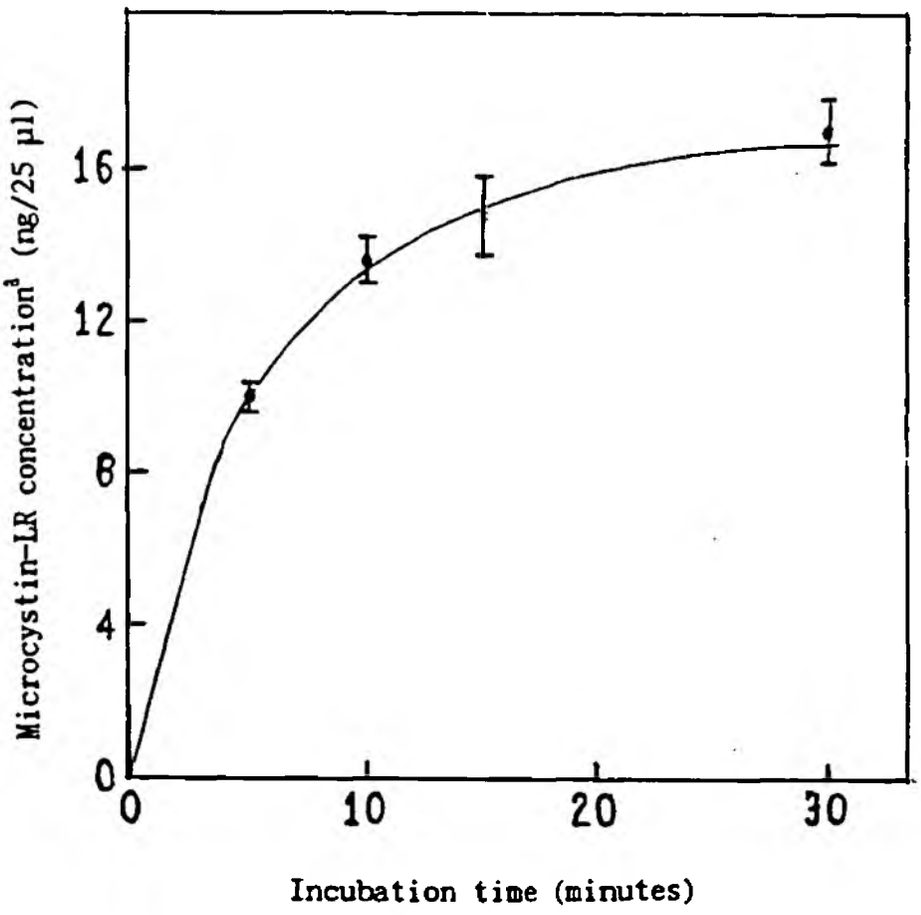


Figure 2.3.1 Release of microcystin-LR from *Microcystis aeruginosa* 7813 entrapped on a glass fibre filter and incubated in 0.3% (v/v) toluene compared with incubation time.

a, measured by HPLC analysis

Table 2.3.1 Optimisation of the volume of 0.3% (v/v) toluene for release of microcystin-LR from *Microcystis aeruginosa* 7813.

Volume of toluene suspension	Amount of MC-LR released[a] (ng/25 μl)
5 ml	4.02 (± 1.67)
10 ml	5.19 (± 0.76)
20 ml	5.76 (± 1.13)

a, measured by HPLC analysis

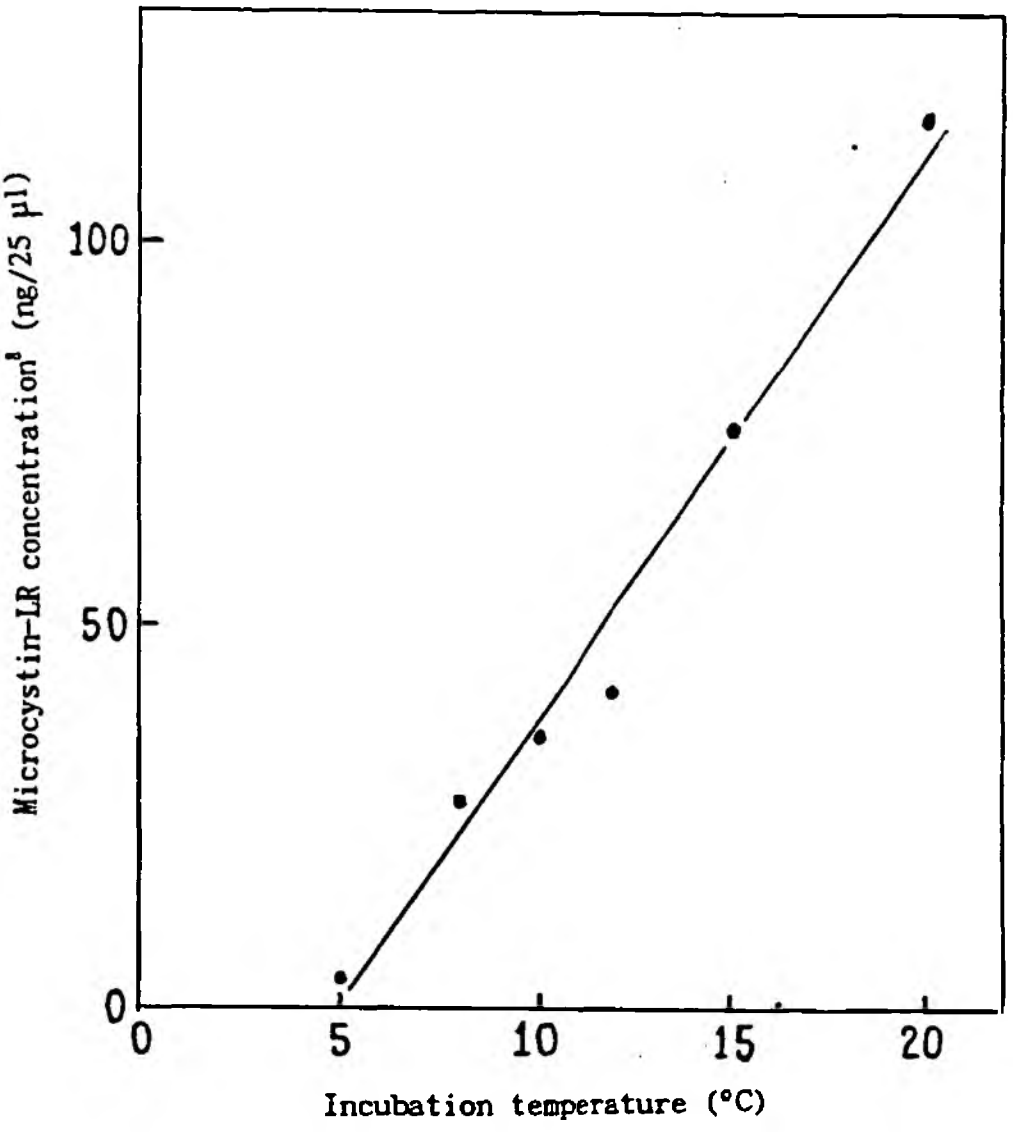


Figure 2.3.2 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with 0.3% (v/v) toluene compared with incubation temperatures to 20°C.

a, measured by HPLC analysis

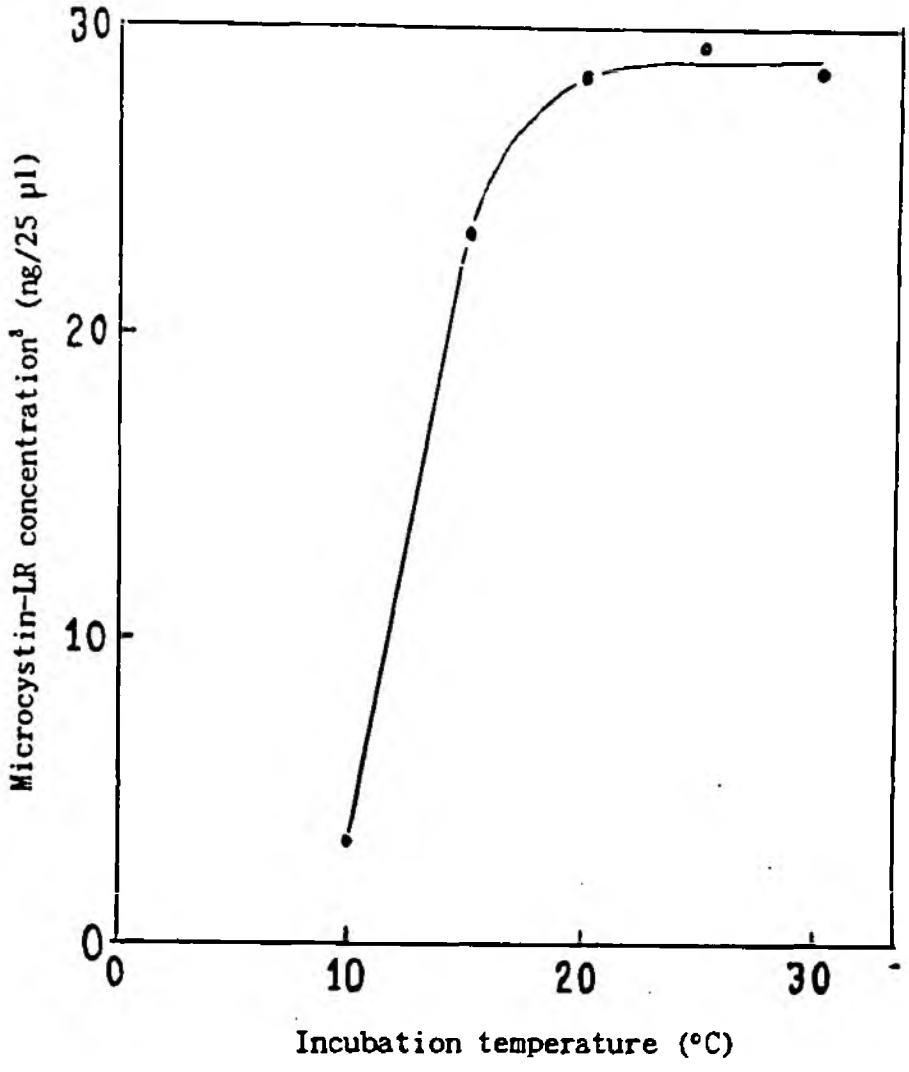


Figure 2.3.3 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with 0.3% (v/v) toluene compared with incubation temperatures to 30°C.

a, measured by HPLC analysis

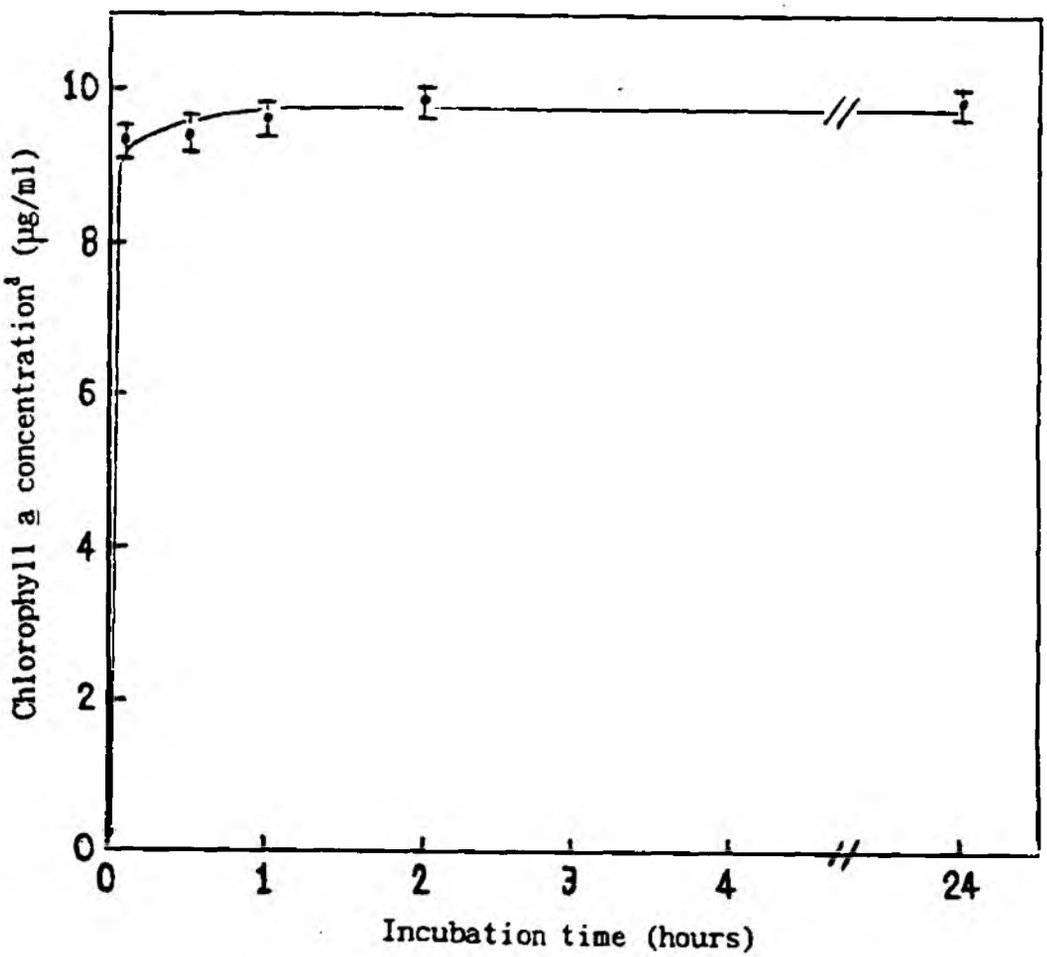


Figure 2.4.1 Chlorophyll a extraction from *Microcystis aeruginosa* 7813 with methanol compared with incubation time.

a, [Chl a] μg/ml = 12.63 x A663

content. The content of MC-LR was then plotted against incubation time (Figure 2.4.2).

From the results it appears that almost all the MC-LR is released from *M. aeruginosa* 7813 in the first 5 to 10 minutes (Table 2.4.1).

2.5 Purification of microcystin variants

Three microcystin variants have been purified, freeze-dried and dispatched to Biocode Ltd (Table 2.5.1).

Microcystin-3-desmethyl-RR was purified from *Oscillatoria agardhii* CYA29, employing the standard method used for microcystin LR. It was shown to be 96% pure by HPLC (Figure 2.5.1 A) and has the structure illustrated in Figure 2.5.2 A.

Nodularin was purified from *Nodularia spumigena* T2 employing two 5% acetic acid extractions, 50% acetone precipitation, and clean-up with SepPak C18 reverse phase cartridges eluted with a step wise increasing gradient of acetonitrile. This was shown to have a purity of 99% by HPLC (Figure 2.5.1 B) and has the structure illustrated in Figure 2.5.2 B.

The third variant purified (Figure 2.5.1 C) was that represented by peak 3 of the HPLC chromatogram of an extract of *M. aeruginosa* 7813, presented in the previous progress report. Although purified to 94% purity, only a relatively small yield was obtained, sufficient to allow a mouse toxicity test and 0.2 mg to be sent to Biocode Ltd. This was hepatotoxic to BALB/C mice. At present insufficient quantities have allowed characterization or identification of the variant and it has been given the preliminary name PK-3. It is hoped further purification will yield sufficient quantities for characterization at a future date.

2.6 Future programme

- Continue optimisation of toluene-mediated cell lysis
- Continue to investigate and develop alternative approaches and contingencies to toluene-cell lysis
- Continue microcystin variant purification/characterization
- Obtain and store natural blooms/water samples for future testing of MAbs or columns
- Evaluate MAbs, validate preliminary column, and assess MAb(S) for large scale production by Biocode Ltd

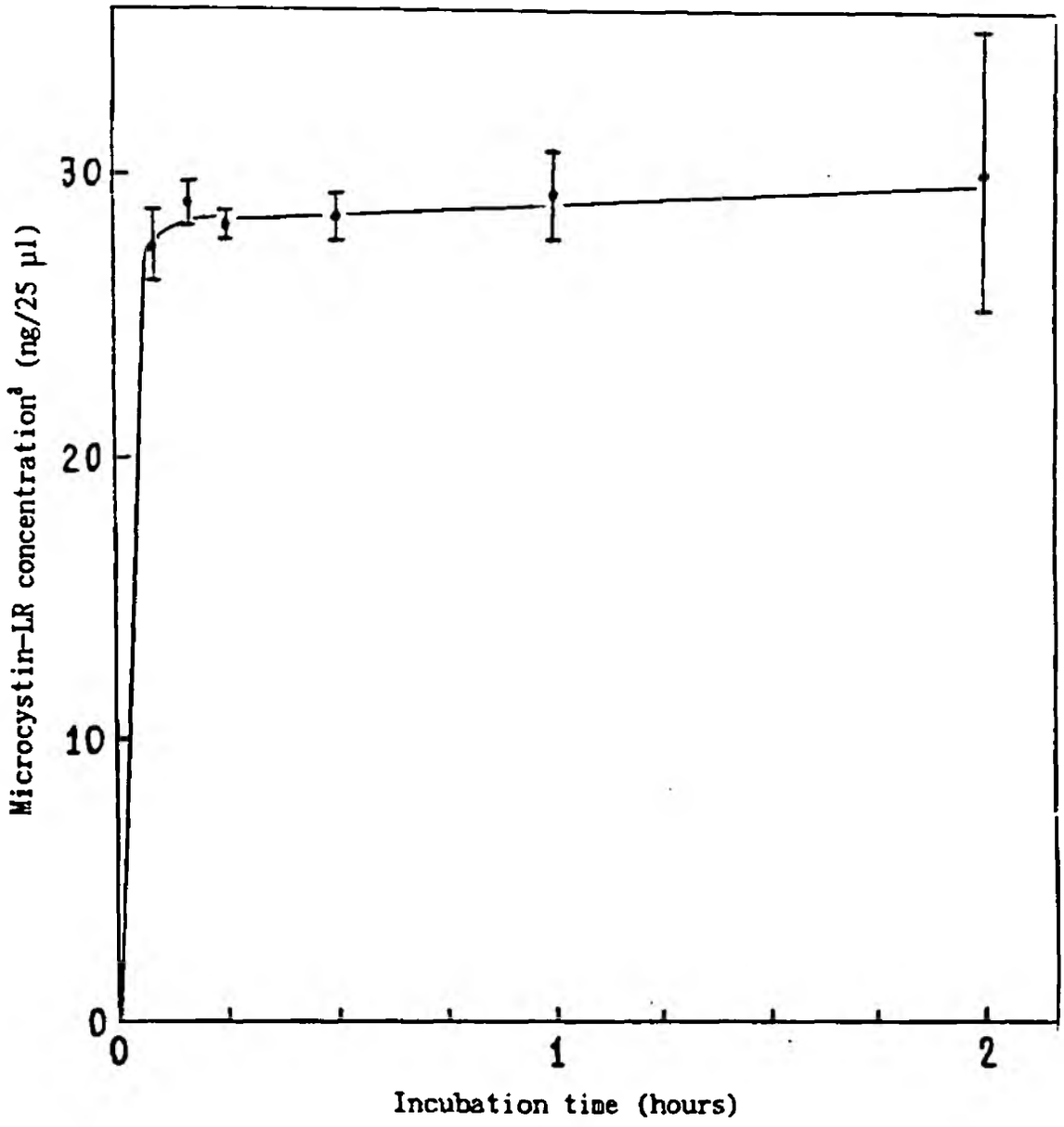


Figure 2.4.2 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with methanol compared with incubation time.

a, measured by HPLC analysis

Table 2.4.1 Percent release of microcystin-LR from *Microcystis aeruginosa* 7813 with methanol compared with incubation time.

Incubation time	Percent release[a] of MC-LR
0 min	0.9%
5 min	92%
10 min	96%
15 min	94%
30 min	95%
1 h	98%
2 h	100%

a, expressed as a percentage of the amount of MC-LR released after 2 h

Table 2.5.1 Consignments of cyanobacterial toxins supplied to Biocode Ltd.

Lot No.	Identification	Purity	Amount Dispatched	Date Dispatched
SGB260592	3-desmethyl-RR	96%	1.0 mg	2.7.92
SGB110692	nodularin	99%	0.5 mg	2.7.92
SGB090792	PK-3[a]	94%	0.2 mg	13.7.92

a, identification to be confirmed

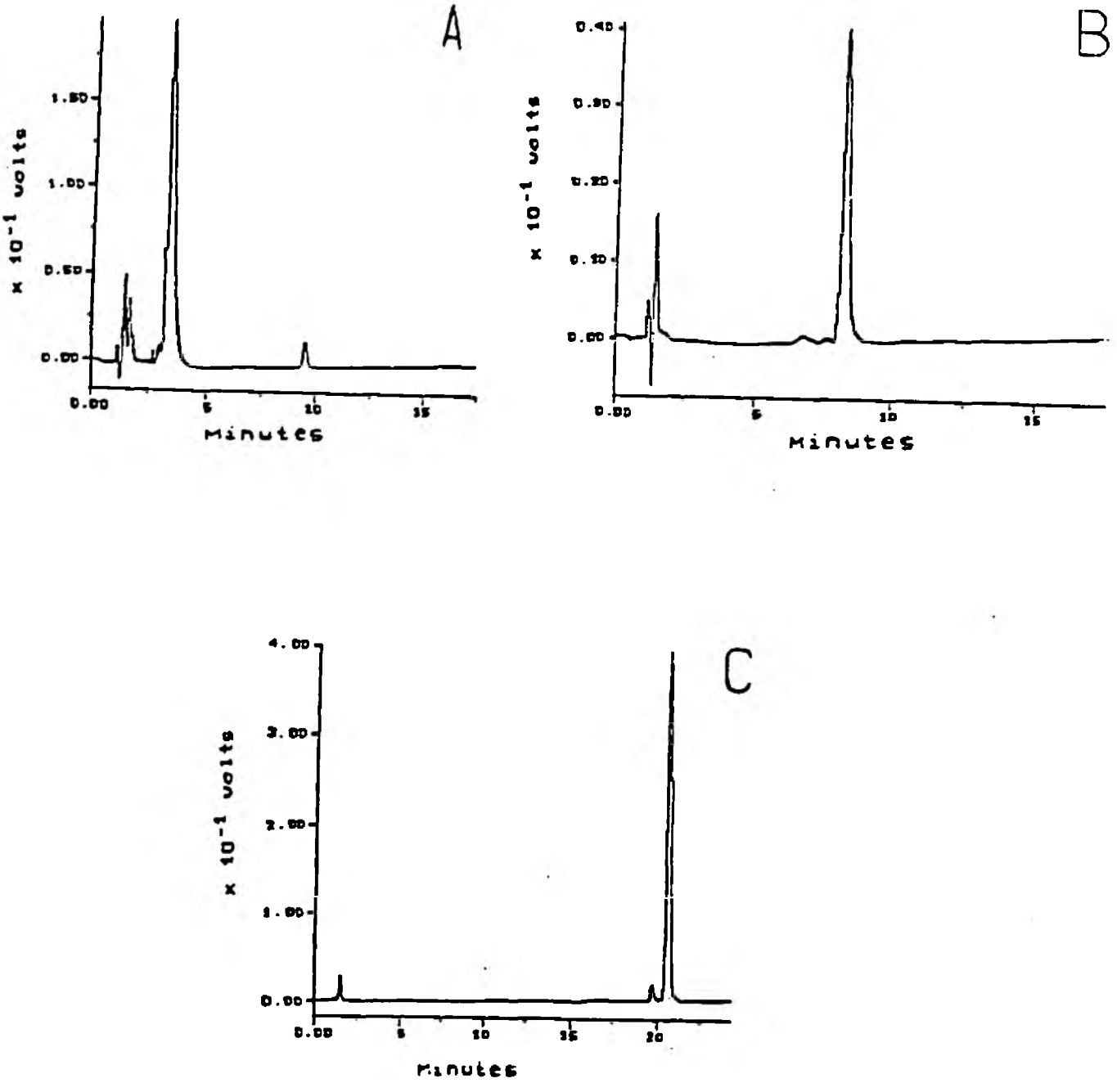


Figure 2.5.1 HPLC chromatograms of cyanobacterial toxin variants

A microcystin-3-desmethyl-RR

B nodularin

C PK-3

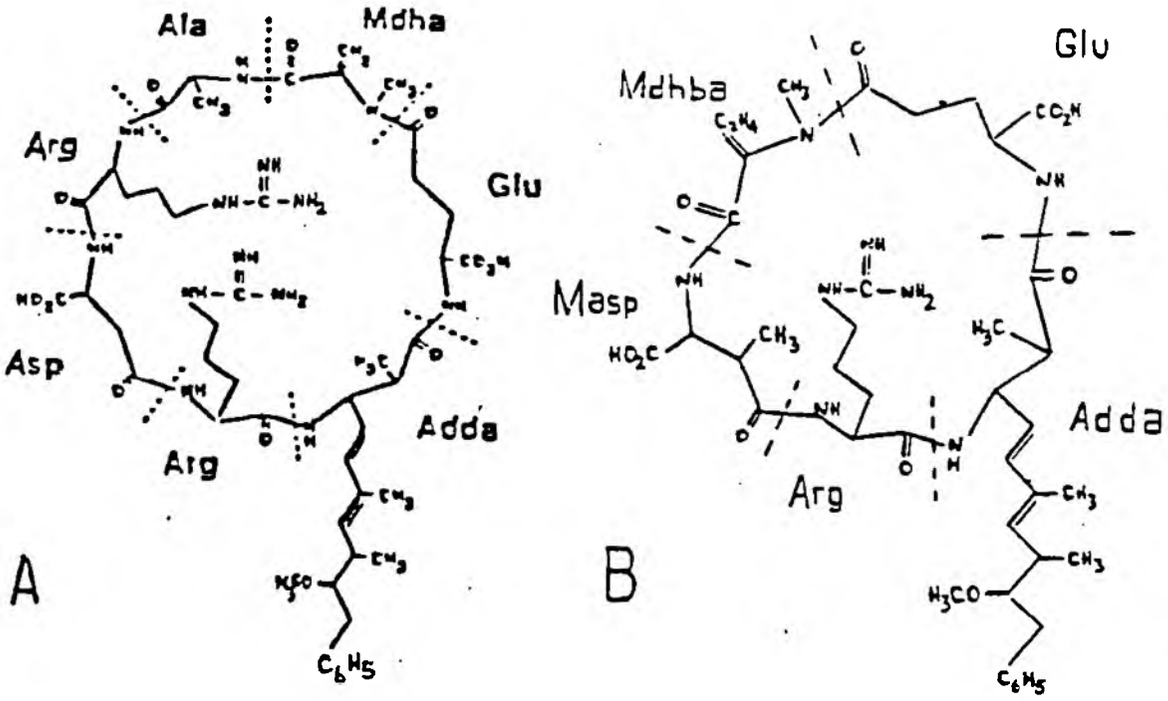


Figure 2.5.2 Structure of cyanobacterial toxin variants

A microcystin-3-desmethyl-RR

B nodularin

Abbreviations:

Glu	glutamic acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Arg	arginine
Asp	aspartic acid
Masp	β-methylaspartic acid
Ala	alanine
Mdha	N-methyldehydroalanine
Mdhba	N-methyldehydroaminobutyric acid

3. DISCUSSION

Further investigations into the suitability of further non-ionic detergents or surfactants for releasing phycocyanin from *M. aeruginosa* 7813 have proved unsuccessful. The two surfactants investigated, n-dodecyl β-D maltoside and n-octyl β-D glucopyranoside, have been used by other workers to solubilize and characterize photosystem particles from cyanobacteria (references 1 and 2, section 5). It was therefore decided to assess their suitability as lysis agents. However, when these proved unsuitable, it was thought the reason for this was that they act as membrane solubilizers and may not have been able to attack the membrane due to the presence of the cell wall. When lysozyme, a cell wall digester, was employed however, the two surfactants still proved unsuitable for release of phycocyanin.

A further alternative to toluene, as a cell lysis agent, methanol, has been investigated. This was found to be at least as effective as toluene ($\sim$ 29 ng MC-LR/25 μl released with methanol c.f. $\sim$ 23 ng MC-LR/25 μl released with toluene) and was found to be effective after only 10 minutes incubation. However, further investigations into this solvent should be carried out, as should discussions into its suitability in kit form due to its high flammability in absolute form.

Development of toluene-mediated cell lysis methods has continued. Lysis appears to be most effective after at least 15 minutes incubation at room temperature, in 10-20 ml reaction volume, and equal to or greater than 20°C. The last factor of the three mentioned may be the most important as natural bloom or water samples may quite often be less than 20°C. However, if the filter entrapment method for lysing the cells is used, it should be possible to keep the toluene suspension prewarmed at 20°C or greater, before transferring the filter plus cells into it.

Purification of microcystin-3-desmethyl-RR and nodularin proved straightforward, but purification and characterization of variants present in the extract of *M. aeruginosa* 7813 proved more difficult due to the relatively low yields. Only sufficient of the peak 3 variant (PK-3) allowed mouse toxicity tests to be performed, and subsequently 0.2 mg were supplied to Biocode Ltd. Further purification of PK-3 variant will be required to allow characterization to be carried out.

4. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

The relatively low yield of microcystin variants from *M. aeruginosa* 7813 may cause a delay in the work. The variant already purified and dispatched to Biocode Ltd, PK 3, will need to be characterized, and any further requirement for this by Biocode will mean an increased amount of time spent purifying this variant. Similarly, any requirement for other variants will require time culturing and purification.

5. REFERENCES

1. Dekker, J.P., Boekema, E.J., Witt, H.T. and Roegner, M. (1988). Refined purification and further characterization of oxygen-evolving and Tris-treated photosystem II particles from the thermophilic cyanobacterium *Synechococcus* sp. Biochim. Biophys. Acta 936, 307-318.

2. Sukenik, A., Falkowski, P.G. and Bennett, J. (1989). Energy transfer in the light-harvesting complex II of *Dunaliella tertiolecta* is unusually sensitive to Triton X-100. Photosynth. Res. 21, 37-44.

6. COST OF WORK CARRIED OUT IN THE PERIOD MAY 1992 - JULY 1992

	This period (£)	Total to date (£)	Project total (£)
Staff salaries and overheads	8,982	26,946	57,938
Travel and subsistence	517	957	1,800
Laboratory consumables	2,393	4,343	6,000
Reports	160	320	1,000
Total	12,052	32,566	66,738

7. ESTIMATE OF COST OF WORK FOR THE PERIOD AUGUST 1992 - OCTOBER 1992

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	1,000
Reports	166
Total	11,466

R & D Project 0349
Field Validation of Algal Toxin Test Kit

Progress Report for Period
August 1992 - October 1992

University of Dundee
October 1992

CONTENTS

SUMMARY

1. Release of microcystin-LR from *Microcystis aeruginosa* PCC 7813 by toluene is reduced in the presence of phosphate-buffered saline. The reduction in solubility is overcome by adding as little as 10% (v/v) methanol to the reaction mixture.

2. Methanol releases its entire microcystin-LR content from *M. aeruginosa* PCC 7813 after 10-15 minutes incubation. The effect appears to be temperature-independent, and can be achieved using 5 ml of 100% methanol or 10 ml of 75% (v/v) methanol. Chlorophyll a is released from other toxigenic genera of cyanobacteria after 10 minutes incubation with methanol. Methanol therefore appears to be a more suitable reagent for extracting microcystin from cyanobacterial cells than toluene.

3. Microcystin variants have continued to be purified as an aid to future work.

4. Natural blooms of cyanobacteria and water samples have been collected or received, and are being kept stored at reduced temperatures until required for future column validation work.

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

During the present reporting period, the specific objectives to be carried out were as follows:

- Continue optimisation of toluene-mediated cell lysis
- Continue to investigate and develop alternative approaches and contingencies to toluene-cell lysis
- Obtain and store natural blooms/water samples for future testing of monoclonal antibodies (MAbs) or columns
- Evaluate MAbs, validate preliminary column and assess MAb(s) for large scale production by Biocode Ltd

2. RESEARCH PROGRAMME

2.1 Technical progress

Technical work has progressed well and is continuing to meet the schedule in the Memorandum of Agreement of Research Contract NRD 040.

Although no MAbs or columns have been received by us from Biocode, discussions have been held between us, NRA and Biocode at the previous progress meeting (17 September 1992, Dundee) and informally by telephone with Dr Baron of Biocode regarding the screening of MAbs and their suitability for large scale production.

2.2 Lysis of cyanobacterial cells using toluene

After a previous request by Biocode that the test sample applied to the field test column should be in phosphate buffered saline (in order to allow an efficient antibody/antigen reaction to occur), the suitability of toluene lysis of *Microcystis* cells in the presence of phosphate buffered saline (PBS) was investigated.

M. aeruginosa PCC 7813 was harvested from a growing culture and washed in 0.01M phosphate buffer (pH 7.4). 5ml aliquots were then centrifuged and the cells resuspended in 5ml of deionised water, phosphate buffer, or PBS (pH 7.4), all in duplicate. To one of each duplicate was added toluene to a final concentration of 0.3% (v/v). After 15

minutes at room temperature, aliquots from each suspension (± toluene) were centrifuged. Supernatants were removed and analysed by HPLC for MC-LR content. Results are presented in Table 2.2.1.

The results indicate that although microcystin-LR is released from *M. aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of PBS, the amount released is approximately 70% of that released in the presence of deionised water or phosphate buffer.

In an attempt to clarify the observed effects, the effect of varying salt concentration in phosphate buffer upon release of MC-LR from *Microcystis* by toluene was investigated.

The above procedure was repeated, but centrifuged cells were resuspended in two aliquots of phosphate buffer and one aliquot each of 0.1% (w/v) NaCl, 0.25% NaCl, 0.5% NaCl and 0.85% NaCl, in phosphate buffer. Toluene was added to one aliquot of the phosphate buffer suspensions, and to each suspension containing NaCl, to a final concentration of 0.3% (v/v). After 15 minutes at room temperature the suspensions were centrifuged, and the supernatants analysed by HPLC for MC-LR content (Table 2.2.2).

The inference to be taken from the results is that at concentrations of sodium chloride greater than 0.1% (w/v), solubility of MC-LR in the experimental buffer was reduced. In an attempt to overcome this effect, methanol was added to PBS at varying concentrations.

The suspension of cells employed in the previous investigation was centrifuged and the cells resuspended in one aliquot of phosphate buffer, 2 aliquots each of phosphate buffered saline (0.85% w/v) plus 10% (v/v) methanol, PBS plus 25% (v/v) methanol, PBS plus 50% (v/v) methanol, and one aliquot of PBS. Toluene was added (final concentration 0.3% v/v) to the phosphate buffer and PBS aliquots, and to one of each duplicate of PBS plus methanol. After 15 minutes at room temperature the aliquots were centrifuged, and the supernatants were analysed by HPLC for MC-LR content (Table 2.2.3).

The resulting data indicate that adding as little as 10% methanol to PBS (0.85% w/v NaCl) negates the reduced solubility effect of the sodium chloride upon MC-LR. Although MC-LR is released by methanol in the absence of toluene, this is substantially less than when toluene is employed, even in 50% (v/v) methanol.

2.3 Extraction of MC-LR using methanol

In the previous Progress Report (May 1992 - July 1992) the release of microcystin-LR from *Microcystis* with methanol was described. It was indicated that the majority of the microcystin-LR was released by methanol in the first 10-15 minutes of incubation. To investigate this further, two aliquots of a *M. aeruginosa* PCC 7813 culture were centrifuged, and one supernatant was retained for HPLC analysis (untreated supernatant). The second supernatant was discarded and the pellet was resuspended in an equal volume of methanol. After 15 minutes at room temperature the suspension was once more centrifuged and the supernatant retained for HPLC analysis (Methanol-

Table 2.2.1 Release of microcystin-LR[a] from *M. aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of deionised water, phosphate buffer, or PBS[b].

Medium	Toluene status[c]	MC-LR content (ng/10 μl)
H_2O	-	0.00
H_2O	+	48.10 ($\pm$ 2.82)
Phosphate buffer	-	1.01 ($\pm$ 0.03)
Phosphate buffer	+	52.47 ($\pm$ 2.89)
PBS	-	0.26 ($\pm$ 0.26)
PBS	+	34.06 ($\pm$ 1.46)

a, analysed by HPLC

b, incubated for 15 minutes at room temperature (23°C)

c, - = absent, + = present

Table 2.2.2 Release of microcystin-LR[a] from *M. aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of phosphate buffer containing varying sodium chloride concentrations[b]

Medium	Toluene status[c]	MC-LR content (ng/10 μl)
Phosphate buffer	-	0.25 ($\pm$ 0.25)
Phosphate buffer	+	40.35 ($\pm$ 2.05)
Phosphate buffer + 0.1% (w/v) NaCl	+	40.45 ($\pm$ 3.75)
Phosphate buffer + 0.25% (w/v) NaCl	+	35.95 ($\pm$ 5.05)
Phosphate buffer + 0.5% (w/v) NaCl	+	36.00 ($\pm$ 2.00)
Phosphate buffer + 0.85% (w/v) NaCl	+	34.80 ($\pm$ 3.10)

a, analysed by HPLC

b, incubated for 15 minutes at room temperature (22°C)

c, - = absent, + = present

Table 2.2.3 Release of microcystin-LR[a] from *M. aeruginosa* PCC 7813 by 0.3% (v/v) toluene in the presence of PBS containing methanol[b]

Medium	Toluene status[c]	MC-LR content (ng/10 μl)
Phosphate buffer	+	40.35 ($\pm$ 2.05)
PBS	+	34.80 ($\pm$ 3.10)
PBS + 10% (v/v) MeOH	-	1.20 ($\pm$ 1.20)
PBS + 10% (v/v) MeOH	+	39.65 ($\pm$ 5.25)
PBS + 25% (v/v) MeOH	-	8.30 ($\pm$ 1.30)
PBS + 25% (v/v) MeOH	+	37.4 ($\pm$ 2.50)
PBS + 50% (v/v) MeOH	-	25.25 ($\pm$ 1.95)
PBS + 50% (v/v) MeOH	+	38.75 ($\pm$ 3.45)

a, analysed by HPLC

b, PBS (0.85% w/v NaCl), incubated for 15 minutes at room temperature (22°C)

c, - = absent; + = present

treated supernatant). The resulting pellet and the earlier pellet from the untreated cells were freeze-dried for 24h, then resuspended in a volume of methanol equal to the original aliquots. These suspensions were ultrasonicated over ice for 2 minutes and then allowed to stand for 15 minutes at room temperature. These were finally centrifuged before supernatants were analysed for MC-LR content, along with those supernatants retained previously. The results presented in Table 2.3.1 confirm previous findings that all microcystin-LR is extracted from *M. aeruginosa* PCC 7813 within 15 minutes of incubation with methanol.

The above investigation reveals the effectiveness of methanol against *Microcystis* for extracting microcystin-LR. In addition, because of the high hydrophobic conditions of this solvent extraction, other microcystin variants are also extracted by the methanol (see Figure 2.3.1). The identity of these variants will be discussed later in this report.

Further work has involved an investigation into the effect of methanol upon other species of cyanobacteria. The following species and strains of cyanobacteria were investigated.

Nodularia sp. PCC 7804

Oscillatoria agardhii CYA 29

Anabaena flos-aquae CCAP 1403/21

Aphanizomenon sp. PCC 7905

Due to the unavailability of toxic strains of the above toxigenic genera, it was decided to measure the chlorophyll a upon extraction by methanol.

Multiple aliquots of the above cultures were centrifuged and the supernatants discarded. The pellets were resuspended in equal volumes of methanol. After 10 minutes triplicate aliquots were recentrifuged, and supernatants were measured at 663 nm in the spectrophotometer, before calculating the chlorophyll a concentration. Three further aliquots were treated in the same way after 64 hours incubation with methanol in the dark. Results are presented in Table 2.3.2.

From the results it can be seen that methanol extracts virtually all the chlorophyll a from the cyanobacterial cells, after 10 minutes incubation. After 64 h incubation no further chlorophyll a was extracted.

The temperature dependency of methanol extraction of MC-LR was then investigated. Aliquots of *M. aeruginosa* PCC 7813 were centrifuged and the supernatants discarded. Triplicate pellets were resuspended in methanol which had been preincubated at 10°C, 15°C, 20°C and 25°C. These suspensions were then incubated for 10 minutes at the same temperature before centrifuging. The supernatants were analysed by HPLC for MC-LR content (Figure 2.3.2).

Table 2.3.1 Intra- and extracellular concentration of MC-LR from methanol-treated[a] and untreated *M. aeruginosa* PCC 7813

Sample	MC-LR content[b] (ng/10μl)
Untreated cells	38.45 ($\pm$ 2.05)
Untreated culture supernatant	0.00
Methanol-treated cells	0.25 ($\pm$ 0.25)
Methanol-treated culture supernatant	39.15 ($\pm$ 2.45)

a, incubated for 15 minutes at room temperature (23°C)

b, analysed by HPLC

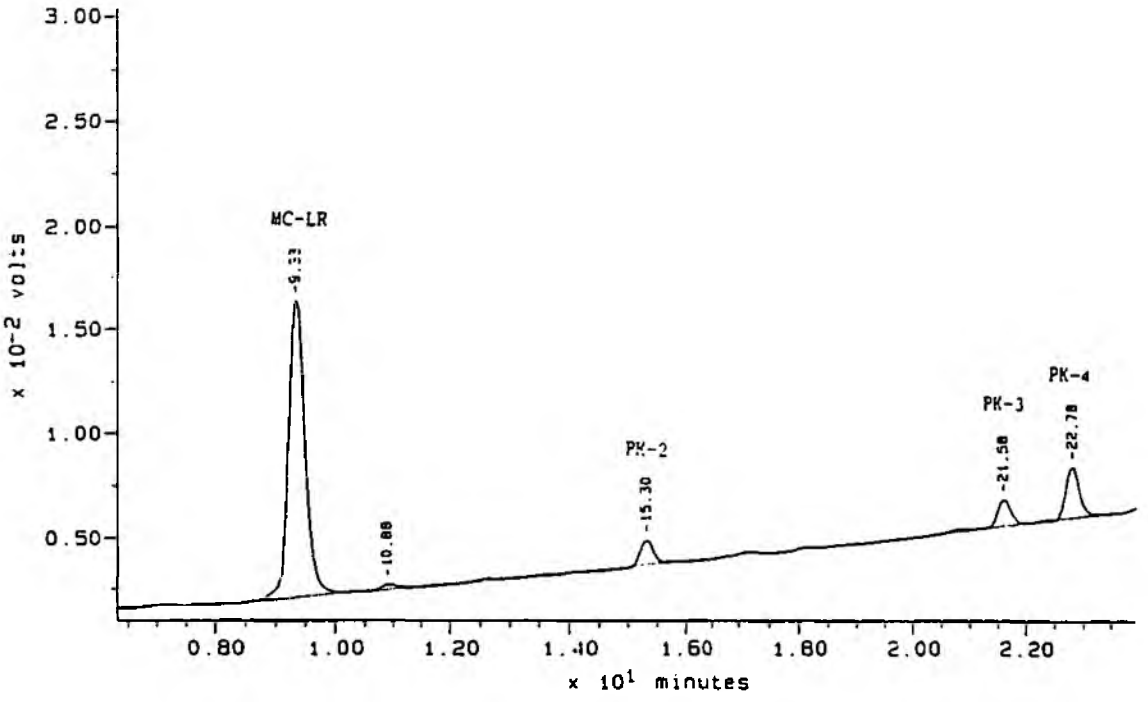


Figure 2.3.1 HPLC chromatogram of methanol extract of *M. aeruginosa* PCC 7813

MC-LR = microcystin-LR

PK-2, PK-3, PK-4 = microcystin variants

Table 2.3.2 Chlorophyll a concentrations of methanol extracts of cyanobacterial cultures

Culture strain No.	Incubation time	Chlorophyll 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, [Chl a] = 12.63 x A 633 nm

The results infer that methanol extraction of microcystin-LR from *M. aeruginosa* PCC 7813 is independent of incubation temperature.

The concentration of the methanol extraction reagent was the next factor to be investigated. *M. aeruginosa* PCC 7813 was entrapped on 2.5 cm diameter Whatman GF/C filters (glass fibre) and the filters placed in 50% (v/v) methanol, 75% (v/v) methanol, or 100% methanol, for 10 minutes at room temperature. After the incubation period, the suspensions were centrifuged. The supernatants were then rotary evaporated to dryness and resuspended in 1 ml of methanol, in order to concentrate them. The concentrated solutions were then analysed by HPLC for MC-LR content (Table 2.3.3).

When examining the data obtained it appears that the methanol concentration used to extract microcystin-LR from *M. aeruginosa* PCC 7813 may be reduced to 75% (v/v) but not to 50%. These assumptions are made performing the extraction at room temperature (approximately 20°C), and in a volume of 10 ml of the methanol solution.

An attempt was made to examine the effect of methanol extraction volume upon the amount of MC-LR released by *M. aeruginosa* PCC 7813. The cyanobacterial cells were once more entrapped on GF/C filters, and then triplicate filters were placed in 5 ml methanol, 10 ml methanol, or 20 ml methanol. After 10 minutes at room temperature the suspensions were centrifuged and the supernatants were rotary evaporated to dryness. After resuspension in 1 ml of methanol, the solutions were analysed by HPLC for MC-LR content (Table 2.3.4).

The results infer that as little as 5 ml of methanol solution may be used to extract microcystin-LR from *M. aeruginosa* PCC 7813 entrapped on a 2.5cm filter disc. Again this is assuming that the extraction is carried out at room temperature (approximately 20°C), and that 100% methanol is used.

2.4 Purification/characterization of microcystin variants

The microcystin variants described in the previous report (May 1992 - July 1992) have been further purified/partially purified. These are being held for screening of antibodies and/or prototype columns from Biocode.

The "PK 3" variant has been characterised by fellow workers (Dr L. A. Lawton, personal communication) along with the other variants from *M. aeruginosa* PCC 7813 (see Figure 2.3.1), and details of this will be presented in the next Progress Report for Project 0271.

2.5 Collection of natural blooms/water samples

A number of natural blooms/scums of cyanobacteria have been collected from local waterbodies or have been received from outside bodies. These are being stored in either a frozen or freeze-dried state. In addition, a number of water samples have been collected or received, and are being kept frozen or in cold storage.

These will be used in attempts to screen the prototype columns from Biocode when available.

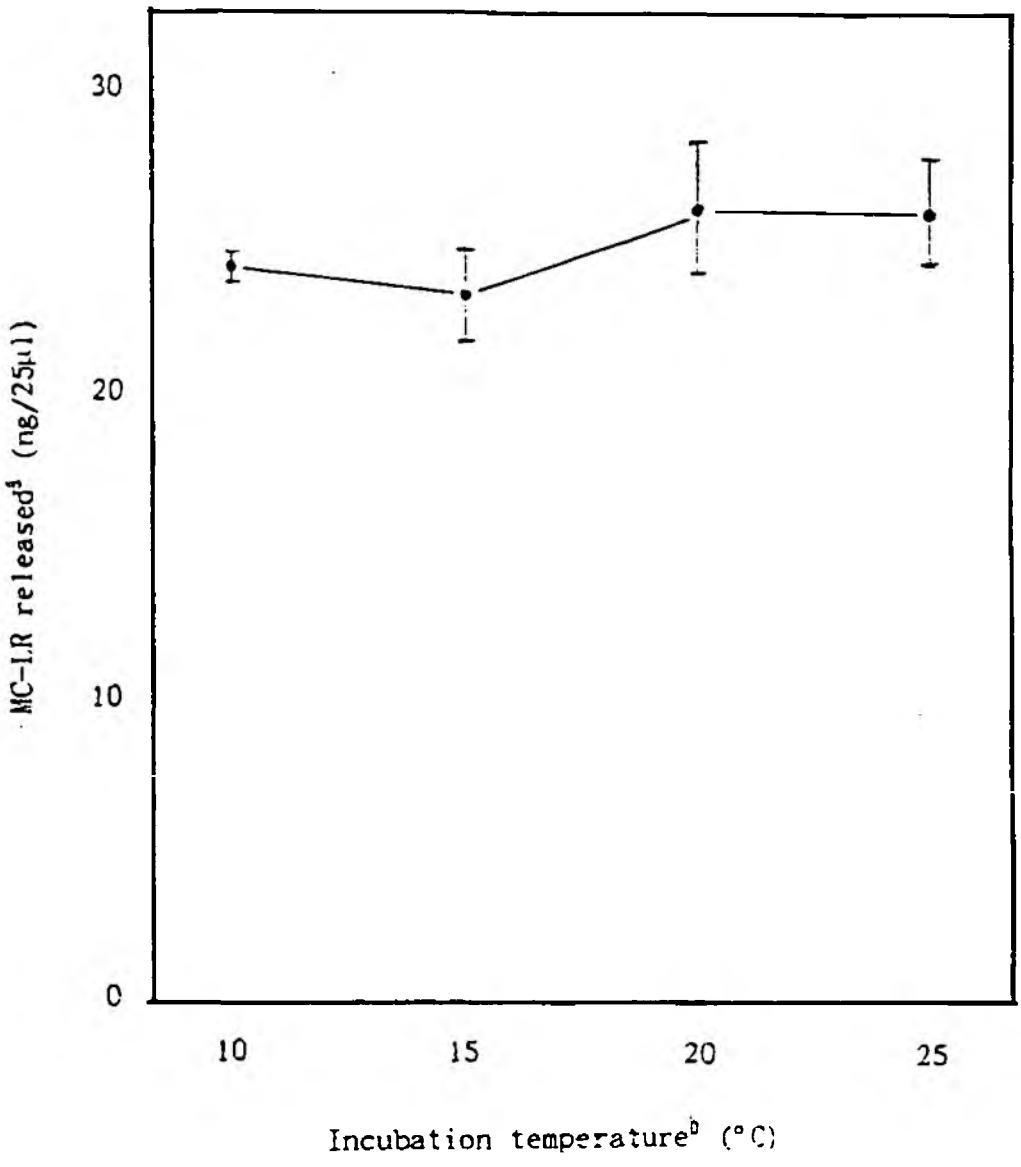


Figure 2.3.2 Extraction of microcystin-LR from *M. aeruginosa* PCC 7813 with methanol versus incubation temperature.

Table 2.3.3 Extraction of microcystin-LR from *M. aeruginosa* PCC 7813 by various concentrations of methanol[a]

Extraction reagent	Microcystin-LR content[b] (ng/10μl)
50% methanol	22.35 ($\pm$ 0.63)
75% methanol	118.44 ($\pm$ 11.02)
100% methanol	116.92 ($\pm$ 7.95)

a, cyanobacterial cells filter-entrapped and then incubated in the methanol solutions for 10 minutes at room temperature

b, analysed by HPLC

Table 2.3.4 Extraction of microcystin-LR from *M. aeruginosa* PCC 7813 with various volumes of methanol[a]

Volume of methanol	Microcystin-LR content[b] (ng/10μl)
5 ml	125.05 ($\pm$ 1.67)
10 ml	116.92 ($\pm$ 7.95)
20 ml	133.94 ($\pm$ 4.21)

a, see footnote a, Table 2.3.3

b, analysed by HPLC

2.6 Future programme

- Continue optimisation of methanol method of microcystin extraction
- Continue purification of microcystin variants
- Continue collection of natural bloom/water samples
- Evaluate MAbs, validate preliminary column, and assess MAb(s) for large scale production by Biocode Ltd
- Assess the linkage on MAb(s) to column support materials
- Compare Biocode test kits for specificity, recovery capacity, and detection limits for microcystins from aquatic environments with standard laboratory methods

3. DISCUSSION

The work presented in this report has focused on the further refinement of methods of extracting microcystin-LR from cyanobacterial cells.

It appears that in the presence of phosphate-buffered saline, there is a solubility problem for microcystin-LR (MC-LR) when released from cyanobacterial cells by the addition of 0.3% (v/v) toluene. The resulting decrease in the amount of MC-LR released by the cells appears to be due to the concentration of sodium chloride present in the buffer, concentrations greater than 1%-2.5% reducing the solubility. However, the addition of as little as 10% (v/v) methanol to the buffer appears to negate the reduced solubility effect.

Work has progressed well using methanol as the agent for releasing MC-LR from cyanobacterial cells. It has been shown that 10-15 minutes incubation of *Microcystis* cells with methanol releases all the MC-LR into the extracellular medium. Microcystin variants are also released. Maximum chlorophyll a pigment is also released from other species of cyanobacteria with as little as 10 minutes incubation with methanol. Not all the cultures of cyanobacteria were toxic, but those that were will be tested for toxin release, measured by HPLC, at a future date.

It appears that 10 ml of 75% (v/v) methanol or 5 ml of 100% methanol, may be used to extract microcystin-LR from *Microcystis* cells. Future work will be carried out on "fine tuning" the volume and concentration of the methanol extraction reagent to be used.

Due to the problems faced when employing the toluene method of lysis in the presence of phosphate-buffered saline, and due to its temperature dependency (see Progress Report 3), the methanol method of extraction is more likely to be adopted for the field test kit. The latter method appears to be temperature-independent, and once extracted into methanol the microcystin solution may be diluted in PBS to a final methanol concentration of 10%. Methanol is also less toxic to operators than toluene.

Natural bloom/scum/water samples have been collected and more will be collected in order to evaluate MAbs/prototype columns from Biocode. It should however be noted that because the cyanobacterial bloom season is likely to be over when these MAbs/columns are received by us, the majority if not all these test samples will not be fresh but will have been stored at reduced temperatures when tested. Microcystin variants are presently being purified for evaluation in the columns.

4. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

We look forward to receiving antibody samples/prototype antibody columns from Biocode colleagues. Most of our work in the next period (November 1992 - January 1993) depends upon receiving these materials. Their prompt availability will affect the completion of the work programme on time.

5. COST OF WORK CARRIED OUT IN THE PERIOD AUGUST 1992 - OCTOBER 1992

	This period (£)	Total to date (£)	Project total (£)
Staff salaries and overheads	8,982	35,928	57,938
Travel and subsistence	262	1,219	1,800
Laboratory consumables	385	4,728	6,000
Reports	160	480	1,000
Total	9,789	42,355	66,738

6. ESTIMATE OF COST OF WORK FOR THE PERIOD NOVEMBER 1992 - JANUARY 1993

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	1,000
Reports	166
Total	11,466

R & D Project 0349
Field Validation of Algal Toxin Test Kit

Progress Report for Period
November 1992 - January 1993

University of Dundee
February 1993

CONTENTS

SUMMARY

1. 100% methanol is required for acceptable release of microcystin-LR (MC-LR) from *Microcystis aeruginosa* PCC 7813. 90% or less methanol (v/v) in deionised water or phosphate buffered saline (PBS) results in less than 90% release of MC-LR.

2. The addition of 10% or 20% (v/v) methanol to toluene reagent, results in a reduction of the temperature-sensitivity of MC-LR release to 15°C or less. However, the presence of potassium salts reduces the amount of MC-LR released.

3. Equipment for field filtration of cyanobacteria/toxin-containing water samples, and suppliers, have been identified.

4. Microcystin variants have continued to be purified as an aid to future validation of antibodies and columns.

5. ELISA reagents and materials have been obtained and are in place ready for the assessment of the antibodies, received from Biocode, to begin.

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

The specific objectives to be carried out during the present reporting period were as follows:

- Continue optimisation of cell lysis procedure
- Continue purification of microcystin variants
- Continue collection of field samples of cyanobacteria/water
- Evaluate MAbs, validate preliminary columns, and assess MAb(s) for large scale production by Biocode Ltd
- Assess the linkage on MAb(s) to column support materials
- Compare Biocode test kits for specificity, recovery capacity, and detection limits for microcystins from aquatic environments with standard laboratory methods

2. RESEARCH PROGRAMME

2.1 Technical progress

Due to the unforseen slippage of the project resulting from ELISA difficulties and the unusual IgM antibodies, we have not received any prototype columns/kits from Biocode. We have therefore not carried out work on specific objectives involving the columns/kits.

However, we have had discussions with Biocode by telephone, and during a visit by Dr Bell to the Biocode facilities at York, concerning the assessment of MAbs for large scale production.

We have also received MAbs from Biocode, and we now have in place reagents and equipment which will allow us to begin ELISA assessment of these antibodies.

Other work has progressed well and to schedule.

2.2 Lysis of cyanobacterial cells

In a previous report (Progress Report 4, 0349) results were presented indicating that 75% methanol may be sufficient to release MC-LR from cells of *M. aeruginosa* PCC 7813 into

the surrounding medium. In an attempt to optimise this further, 50%-100% methanol, in 10% increments, was investigated.

A culture of *M. aeruginosa* PCC 7813 was harvested and washed in phosphate buffer pH 7.4. 1 ml aliquots were centrifuged and resuspended in 1 ml of 50%, 60%, 70%, 80%, 90%, and 100% methanol in deionised water, in triplicate. After 10 minutes at room temperature (21°C) the aliquots were again centrifuged and the supernatants were analysed by HPLC for MC-LR content (Table 2.2.1).

The results indicate that, assuming 100% MC-LR release by 100% methanol, greater than 80% release of MC-LR only occurs at 80% methanol or above, the 70% methanol solution resulting in approximately 40% release. Except for the 100% methanol, all solutions released less than 90% MC-LR.

If a diluted form of methanol were to be used as the lysing reagent in the field test kit manufactured by Biocode, this would probably be in phosphate buffered saline (PBS). The previous experiment was therefore repeated using methanol diluted in PBS pH 7.4 rather than in deionised water. The concentrations of methanol used were 70%-100%, in 10% increments, as 70% methanol or below proved ineffective previously. Again lysis was carried out in triplicate samples, and, after centrifugation, supernatants were analysed for MC-LR content by HPLC (Table 2.2.2).

The data demonstrates that 70% methanol results in less than 50% release of MC-LR, again assuming a 100% release in 100% methanol. Once more, all solutions other than 100% methanol resulted in less than 90% release of MC-LR, the 90% methanol solution inflicting a 85% release.

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 previous reports. In the last report (Progress Report 4, 0349) the use of 5 ml of 100% methanol to release MC-LR from filter-entrapped *M. aeruginosa* PCC 7813, after 10 minutes incubation, was demonstrated. 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, quickly shaken, 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 2.2.1).

The data in Figure 2.2.1 and the results presented as percentage release of MC-LR in Table 2.2.3 demonstrate that greater than 80% MC-LR is released after only 5 minutes incubation in 100% methanol, and at least 90% is released after 10 minutes incubation. These figures were calculated assuming 100% release of MC-LR after 60 minutes incubation in 5 ml of 100% methanol.

Table 2.2.1 Release of microcystin-LR[a] from *Microcystis aeruginosa* PCC 7813 by various concentrations of methanol in deionised water[b].

Concentration of methanol (v/v)	MC-LR content (ng/10 μl)	% MC-LR released
50%	3.5 (±0.15)	6.6
60%	7.5 (±1.53)	14.1
70%	21.1 (±3.34)	39.4
80%	45.3 (±6.90)	84.7
90%	40.1 (±2.23)	75.0
100%	53.4 (±4.16)	100.0

a, analysed by HPLC

b, incubated for 10 minutes at room temperature (21°C)

Table 2.2.2 Release of microcystin-LR[a] from *Microcystis aeruginosa* PCC 7813 by various concentrations of methanol in phosphate buffered saline[b].

Concentration of methanol (v/v)	MC-LR content (ng/10 μl)	% MC-LR released
70%	34.9 ($\pm$ 7.0)	15
80%	139.7 ($\pm$34.4)	61
90%	194.0 ($\pm$12.7)	85
100%	228.1 ($\pm$11.4)	100

a, analysed by HPLC

b, incubated for 10 minutes at room temperature (21°C)

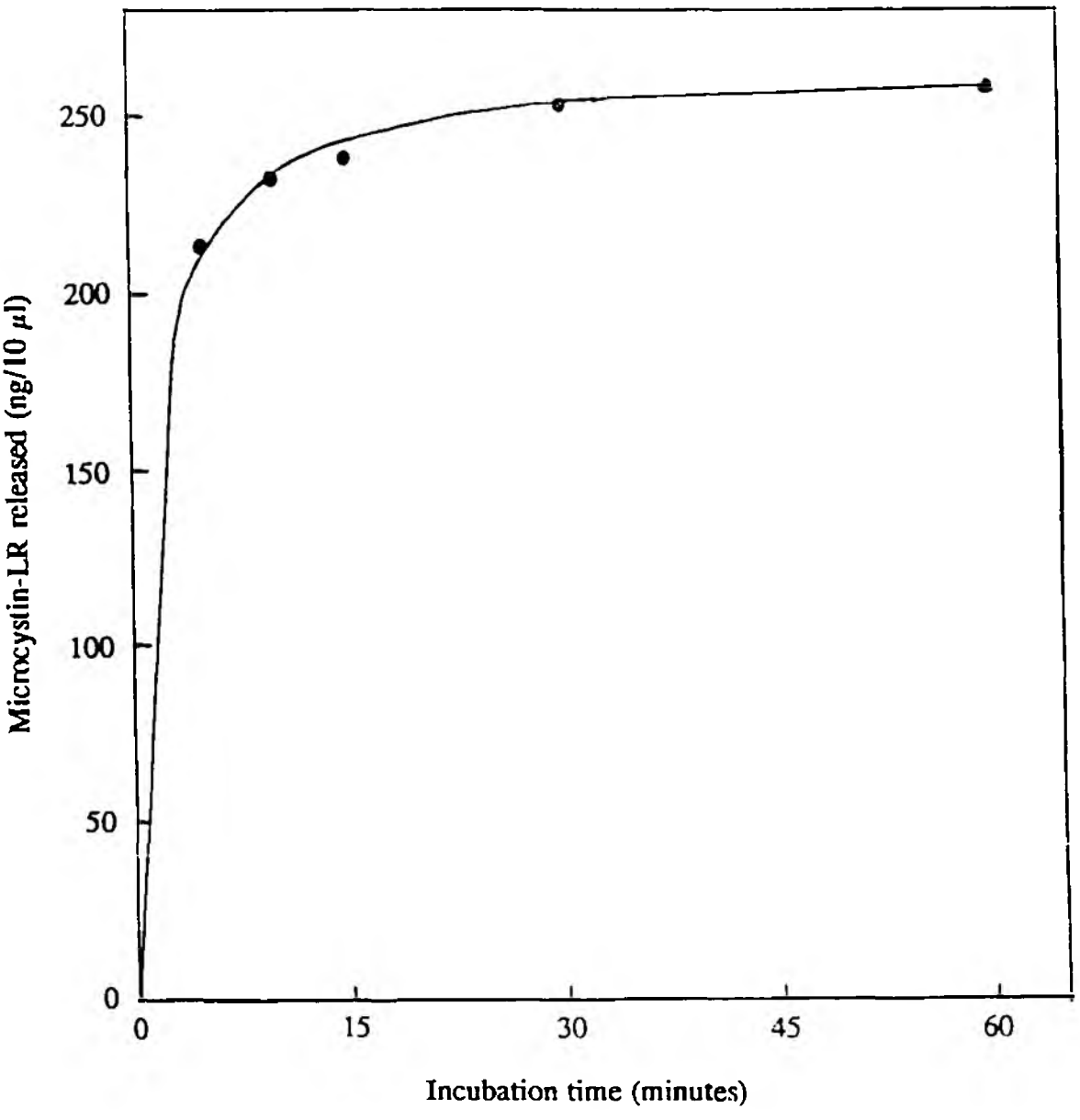


Figure 2.2.1 Release of microcystin-LR[a], from filter entrapped *Microcystis aeruginosa* PCC 7813 by 5 ml of 100% methanol[b], versus time.

a, analysed by HPLC

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

Table 2.2.3 Percentage release of microcystin-LR[a], from filter entrapped *Microcystis aeruginosa* PCC 7813 by 5 ml of 100% methanol[b], versus time.

Incubation time (minutes)	% MC-LR released
5	83 (±1.5)
10	90 (±1.5)
15	92 (±0.0)
30	99 (±4.2)
60	100 (±1.2)

a, analysed by HPLC

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

During the last Progress Meeting (Dundee University, 10.12.92) the lysis procedure was discussed and it was stated that the toluene method of lysis would be preferable. However, if methanol was to be included in the lysis reagent, it should be at as low a concentration as possible. In previous Progress Reports it was demonstrated that the toluene method of lysis was temperature-dependent and that MC-LR release was reduced in the presence of phosphate buffered saline. The latter problem was overcome by either reducing the concentration of sodium chloride in the PBS to 0.1% or by adding 10% methanol to the reagent. It was suggested that the temperature-dependency of the toluene reagent should be investigated in the presence of 10% methanol and /or in reduced sodium chloride (0.1%).

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% methanol in PBS (0.01M sodium phosphate, 0.85% w/v sodium chloride) 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 MC-LR content (Table 2.2.4).

The data presented infer that below 15°C the release of MC-LR from *M. aeruginosa* PCC 7813 by toluene in the presence of 10% methanol/PBS was temperature dependent. However, at temperatures above 15°C the release of MC-LR appeared to plateau out, being temperature-independent. This can be compared to previous findings when the release of MC-LR from *M. aeruginosa* PCC 7813 by toluene in the absence of methanol was temperature-dependent up to 20°C.

In Progress Report 3, 0349 (August 1992), the temperature-dependency of MC-LR release from *M. aeruginosa* PCC 7813 by 0.3% (v/v) toluene was observed in a procedure whereby the toluene was diluted in deionised water, i.e. in the absence of PBS. Therefore the investigation of the temperature-dependency of toluene-mediated MC-LR release in the presence of reduced sodium chloride was not necessary as this has already been shown to be temperature-dependent up to 20°C in a sodium chloride-free medium.

In an attempt to overcome the reduced release of MC-LR from *M. aeruginosa* PCC 7813 by toluene at 10°C, the concentration of methanol in the PBS was increased. The protocol involving 10% methanol in PBS and 0.3% (v/v) toluene, above, was repeated employing 10%, 20%, and 50% methanol (v/v). Again triplicate aliquots were incubated at 10°C, 15°C, 20°C, and 25°C. After centrifugation, resulting supernatants were analysed by HPLC for MC-LR content (Figure 2.2.2).

The results again infer that at 10% methanol the release of MC-LR is temperature dependent below 15°C, in the presence of 0.3% (v/v) toluene. This is also the case for the 20% methanol solution, but at 10°C more MC-LR is released with 20% methanol than with 10% methanol (88% cf 55%). At 50% methanol, MC-LR release is reduced at all the temperatures investigated, and appears to be temperature dependent.

During a recent visit to Biocode a standard operating procedure (SOP) for the preparation of phosphate buffered saline pH 7.4 was received. Upon examination it was observed that the recipe for this was different from that previously used by us, our buffer containing

Table 2.2.4 Release of microcystin-LR[a] from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene/10% methanol/phosphate buffered saline[b], at various incubation temperatures.

Incubation temperature (°C)	MC-LR content (ng/25μl)	% MC-LR released
10	192.7 ($\pm$21.4)	56.0
15	302.7 ($\pm$24.6)	87.9
20	324.2 ($\pm$14.6)	94.2
25	344.3 ($\pm$12.2)	100.0

a, analysed by HPLC

b, suspensions incubated for 15 minutes

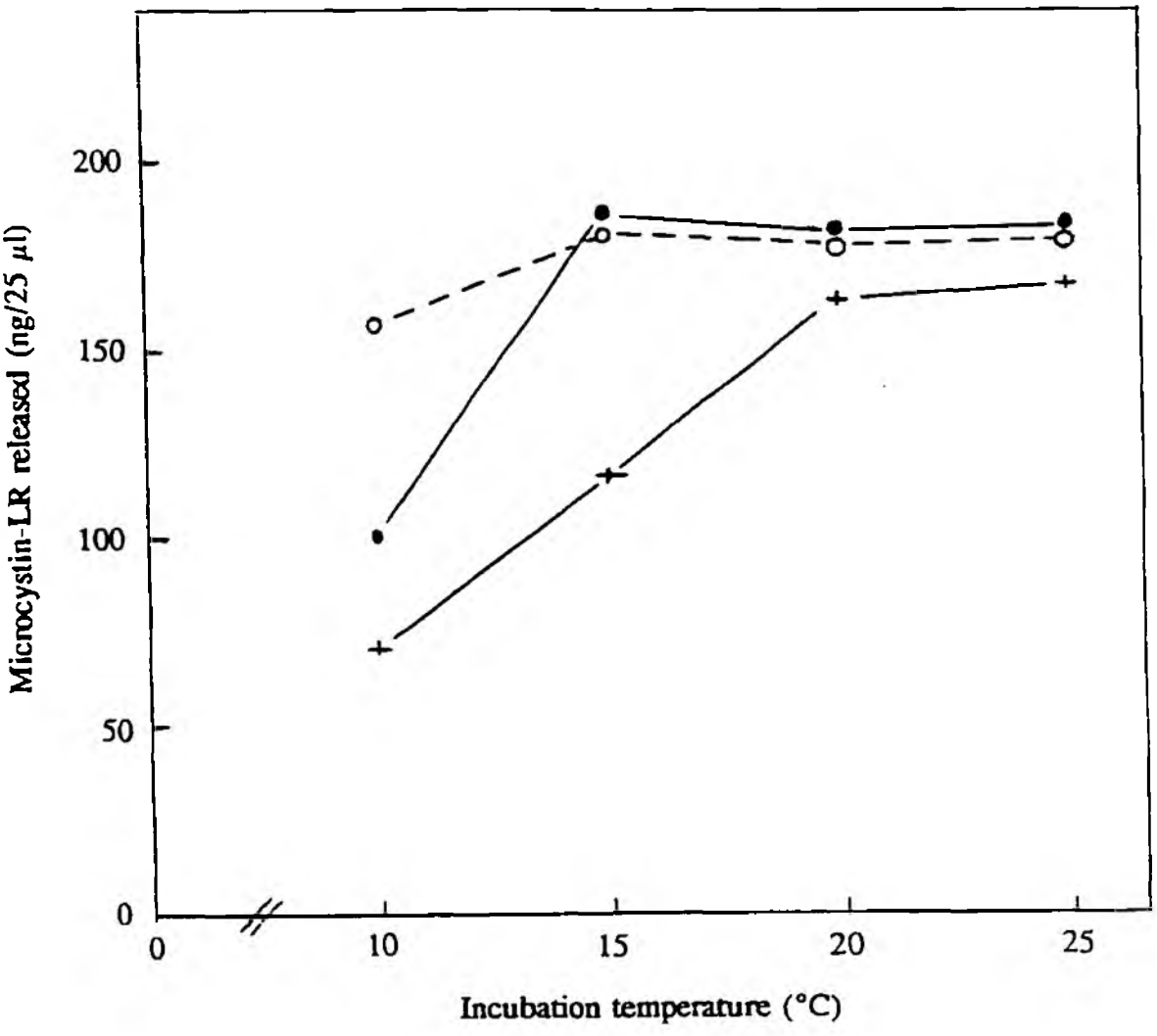


Figure 2.2.2 Release of microcystin-LR[a] from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene/methanol/phosphate buffered saline[b], at various concentrations of methanol and incubation temperatures.

a, analysed by HPLC

b, suspensions incubated for 15 minutes

● —— ● 10% (v/v) methanol/toluene/PBS

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

\+ —— + 50% (v/v) methanol/toluene/PBS

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. Once more 10%, 20%, and 50% methanol solutions were investigated at temperatures of 10°C, 15°C, 20°C, and 25°C, in the presence of 0.3% (v/v) toluene. After 15 minutes incubation at each temperature the suspensions were centrifuged and the supernatants analysed by HPLC for MC-LR content (Figure 2.2.3).

The data presented infer that the release of MC-LR from *M. aeruginosa* PCC 7813 by toluene/methanol/PBS (Biocode formulation) is different from that released by the formulation previously used. The release of MC-LR appears to be temperature dependent up to 20°C in the Biocode formulation of PBS, compared with 15°C in the previous formuation, at 15°C 81% being released, compared with 100% previously. The amount of MC-LR released appears to be generally lower in the Biocode formulation.

2.3 Field vacuum equipment

Equipment has been sourced to enable the filtering of water samples in the field, prior to analysis of the water in the test column, or lysis of the cells on the filter in the appropriate reagent. A side-arm vacuum flask would be fitted with a bung containing a filter funnel. Gelman Sciences supply a durable Polysulfone plastic filter funnel which will house the 2.5 cm diameter filters investigated at Dundee for use in cell lysis. This funnel can take up to 200 ml of solution. Other funnnels for housing larger filters are generally available. A hand operated vacuum pump is supplied by Nalgene, and this has been used to sucessfully draw samples of cyanobacterial solutions through the glass-fibre filters. A diagramatic representation of this system is presented in Figure 2.3.1.

2.4 Purification of microcystin variants

Purification of MC-LR and microcystin variants from *M. aeruginosa* PCC 7813 and other cyanobacterial cultures has continued. This has/will provide purified toxin for analysis of antibody cross-reactivity by ELISA procedures. The purified microcystins will also be employed in assessment of prototype affinity columns once these have been received from Biocode.

The feasibility of supplying tritiated-microcystin LR to Biocode has been examined, and it was concluded that up to 2 μCi of the radio-labelled toxin would be supplied once the requirement for this material was known to us. As this radio-labelled toxin will not now be required for affinity studies by Biocode, it was agreed that any future requirement will be given to us in writing, as for the untritiated toxin.

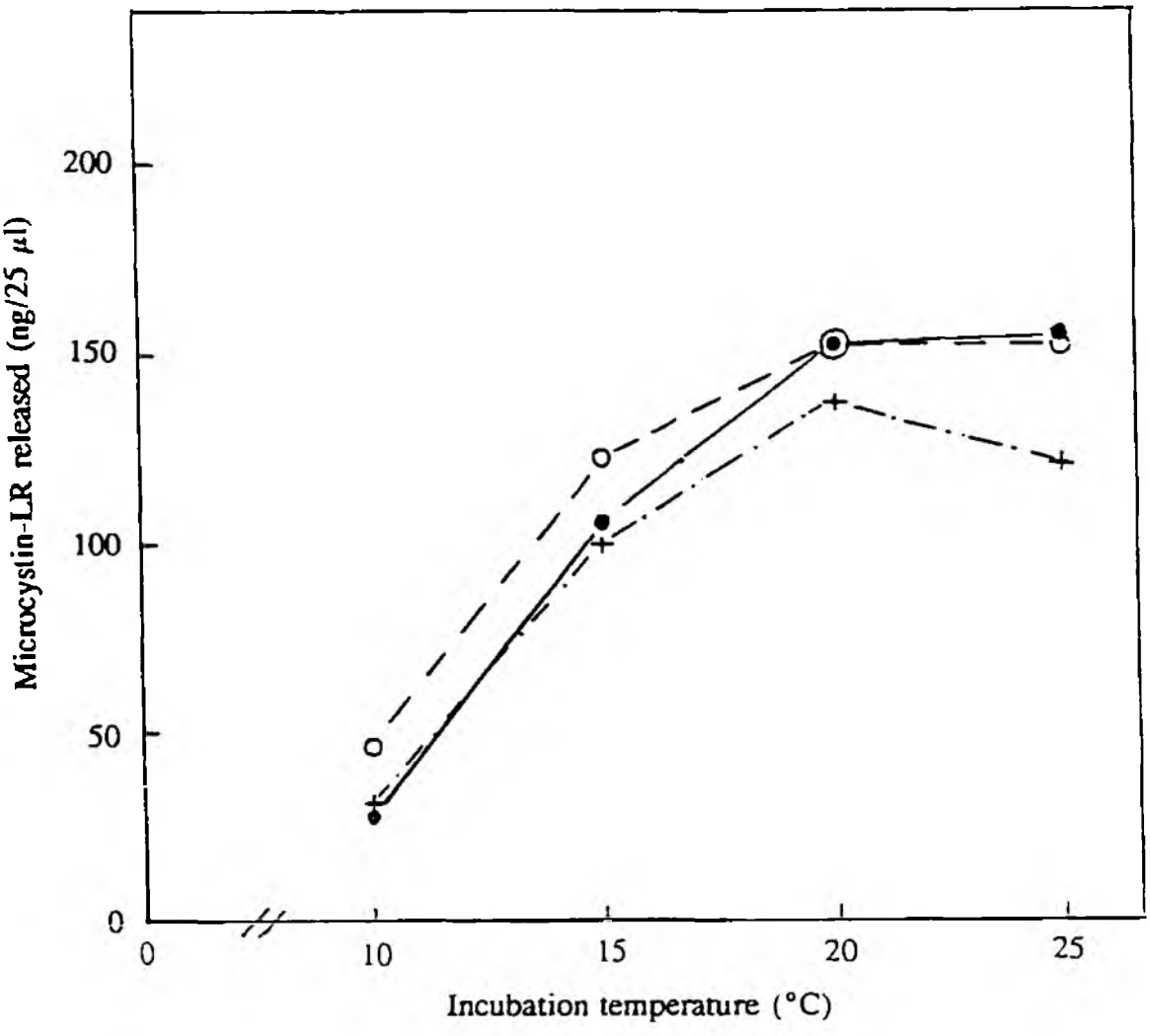


Figure 2.2.3 Release of microcystin-LR[a] from *Microcystis aeruginosa* PCC 7813 by 0.3% (v/v) toluene/methanol/phosphate buffered saline[b] (Biocode formulation), at various concentrations of methanol and incubation temperatures.

a, analysed by HPLC

b, suspensions incubated for 15 minutes

● ——— ● 10% (v/v) methanol/toluene/PBS

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

\+ —·—·— + 50% (v/v) methanol/toluene/PBS

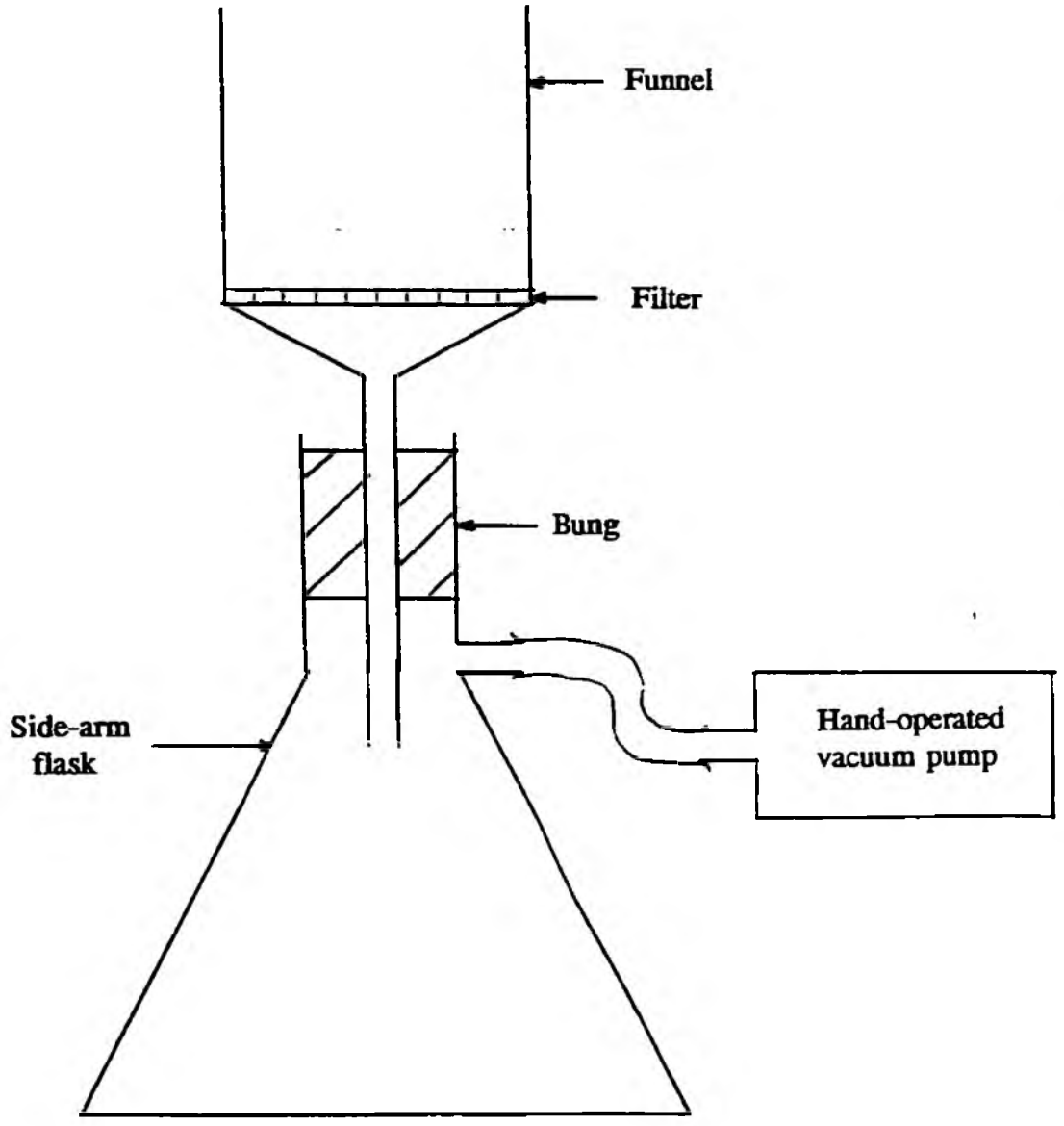


Figure 2.3.1 An example of a filtration system suitable for field use prior to analysis of water/cyanobacterial cells by the microcystin affinity column.

2.5 Visit to Biocode

On the 7th and 8th of January 1993, Dr Bell visited Biocode's facility at York, in order to assess and be trained in the ELISA techniques used. A futher purpose of the visit was to obtain relevant standard operating procedures (SOPs).

This visit was very successful, in that all relevant information was obtained, and this has allowed us to obtain all necessary reagents and materials to begin using the ELISA technique. This will begin in the very near future, and should enable us to perform cross-reaction studies on the monoclonal antibodies received from Biocode. These antibodies are believed to be the clone selected for large scale production by Biocode.

2.6 Future programme

Due to the 2-3 month delay envisaged for the project the future work for the next 3 months of the project will be similar to the last 3 months, i.e.:

- Continue optimisation of MC-LR release procedure
- Continue purification of microcystin variants
- Continue collection of natural bloom/water samples
- Evaluate MAbs (by ELISA) and validate preliminary columns
- Assess the linkage on MAbs to column support materials
- Compare Biocode test kits for specificity, recovery capacity, and detection limits for microcystins from aquatic environments with standard laboratory methods

3. DISCUSSION

Due to the unavailability of preliminary columns for testing, the work presented in this report has once more focused on the further refinement of methods of extracting microcystin-LR from cyanobacterial cells.

It appears that 100% methanol is required to ensure greater than 90% release of microcystin-LR (MC-LR) from *M. aeruginosa* PCC 7813, incubated for 10 minutes at room temperature. In 90% (v/v) methanol, in deionised water or 0.01 M phosphate buffer saline (PBS) pH 7.4, less than 90% MC-LR is released, and therefore these concentrations appear to be unacceptable. 100% methanol releases greater than 90% MC-LR from *M. aeruginosa* PCC 7813 cells entrapped on glass-fibre filter discs after 10 minutes incubation at room temperature, therefore reproducing the observation of MC-LR release with 100% methanol from free cyanobacterial cells.

It was decided however, that 100% methanol would be unacceptable as the MC-LR release reagent because of its high flammability. The toluene reagent has been shown in the past to

be temperature-dependent up to 20°C in releasing MC-LR from cyanobacterial cells. In an attempt to overcome this, the toluene method of release in the presence of methanol was investigated. At 10% (v/v) methanol concentrations, 55% MC-LR was released at 10°C, and at 20% (v/v) methanol, 88% MC-LR was released at the same temperature. 100% MC-LR release occurred at 15°C in both cases. It should be noted however, that this was only the case in PBS containing only sodium salts. When potassium chloride and potassium dihydrogen orthophosphate were present, the amount of MC-LR released was reduced, and temperature dependency was raised to 20°C once more. It would therefore be pertinent to use only sodium salts in PBS under the conditions described.

Equipment has been identified which would enable field filtration of water samples to be performed. This would involve the use of a filter-funnel connected to a vacuum flask which would be in turn connected to a hand operated vacuum pump. This would allow cyanobacterial cells to be filtered from the water so that intra- and extra- cellular toxins could be passed through the affinity column.

Purified microcystin variants that have been/will be obtained in the laboratory, will be analysed using ELISA techniques with recently obtained reagents and equipment. This will hopefully confirm data obtained by Biocode, and will involve the use of other non-microcystin compounds from cyanobacterial cells. SOPs have been obtained from Biocode and these will enable us to perform the ELISAs in exactly the same way as perfomed at York, thus allowing an accurate comparison of results.

4. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

As in the previous reporting period, the majority of work in the next period (February 1993 - April 1993) depends upon us receiving prototype antibody columns from Biocode colleagues.

5. COST OF WORK CARRIED OUT IN THE PERIOD NOVEMBER 1992 - JANUARY 1993

	This period (£)	Total to date (£)	Project total (£)
Staff salaries and overheads	8,982	44,910	57,938
Travel and subsistence	145	1,364	1,800
Laboratory consumables	925	5,653	6,000
Reports	160	640	1,000
Total	10,212	52,567	66,738

6. ESTIMATE OF COST OF WORK FOR THE PERIOD FEBRUARY 1993 - APRIL 1993

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	347
Reports	166
Total	10,813

R & D Project 0349
Field Validation of Algal Toxin Test Kit

Progress Report for Period
February 1993 - April 1993

University of Dundee
April 1993

CONTENTS

SUMMARY

1. Toluene/methanol/PBS lysis, using the filter-entrapment method, was performed on toxic *M. aeruginosa* PCC7813 spiked into fresh loch-water. Naturally occurring chlorophyll *a*-containing organisms in the water competed with the spiked cyanobacterial cells during filtration, resulting in lower than expected microcystin-LR recoveries. Lysis of spiked cells in prefiltered loch-water resulted in the expected recovery of microcystin-LR.

2. Assessment of anti-microcystin-LR monoclonal antibodies from Biocode Ltd, cell-line A005-1D 13 C6, batch 006, was performed in ELISAs. The antibodies cross-reacted with two microcystin variants and nodularin.

3. Microcystin variants have continued to be purified as an aid to validation of antibodies. Non-toxic *Microcystis* was supplied in a freeze-dried state to Biocode Ltd, as an aid to their assessment of antibodies.

4. Technical assessment of Biocodes' future Project 0348 options was made by this laboratory, by means of technical discussions with Biocode in York, followed by a subsequent report to the NRA.

1. OBJECTIVES

1.1 Overall project objectives

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

1.2 Specific objectives

The specific objectives to be carried out during the present reporting period were as follows:

- Continue optimisation of cell lysis procedure
- Continue purification of microcystin variants
- Continue collection of field samples of cyanobacteria/water
- Evaluate MAbs (by ELISA) and validate preliminary columns
- Assess the linkage on MAb(s) to column support materials
- Compare Biocode test kits for specificity, recovery capacity, and detection limits for microcystins from aquatic environments with standard laboratory methods

2. RESEARCH PROGRAMME

2.1 Technical progress

Due to technical problems encountered by Biocode Ltd, concerning the attachment of MAbs to affinity column support material, a substantial slippage of the project schedule has occurred. This has rendered the schedule in the Memorandum of Agreement of Research Contract NRD 040 inappropriate, and no work on validation of preliminary columns or test kits has been carried out.

Work has continued on cyanobacterial cell lysis, microcystin purification, and evaluation of antibodies produced by Biocode Ltd.

In addition to the technical development, and as a result of the problems encountered in attachment of antibodies to columns, an assessment of future project options was made by this laboratory at the request of the NRA. This involved a visit to Biocode in York, by Dr S.G.Bell, and a subsequent report (see section 2.5).

2.2 Lysis of cyanobacterial cells

In the last Progress Report (November 1992 - January 1993) data were presented demonstrating that addition of methanol to 0.3% (v/v) toluene in phosphate-buffered saline (PBS) reduced the inhibition of cyanobacterial cell lysis at temperatures below 20°C. The optimum conditions were 20% (v/v) methanol in potassium-free PBS, incubated at 15°C or above for 15 minutes.

The lysis conditions described above were investigated using a sample of freshwater from a natural waterbody, spiked with a laboratory culture of toxic *Microcystis aeruginosa* at different concentrations of chlorophyll *a*.

Water was collected from Loch Rescobie, a waterbody near to Dundee which has supported annual blooms of toxic *M. aeruginosa* for at least the past 10 years. This water was examined microscopically and was found to contain no obvious traces of any known toxic cyanobacterial species. The chlorophyll *a* concentration of the water was measured by filtering a known volume through a 7 cm diameter GF/C glass-fibre disc, which was subsequently placed in 5 ml of methanol and kept at 4°C in the dark for at least 16 hours. A 1 ml aliquot was then removed and measured at 663 nm in the spectrophotometer against a methanol blank. *M. aeruginosa* PCC7813 was then diluted 1/40 and 1/500 in the loch-water. Chlorophyll *a* concentrations were measured as described previously.

Known volumes of the diluted cyanobacteria and the loch-water were then passed through 2.5 cm diameter GF/C filters, in duplicate, until the flow had almost ceased, i.e. the filters became blocked. The filters were then placed in separate bottles containing 5 ml of 0.3% (v/v) toluene, 20% (v/v) methanol in PBS. The bottle was shaken vigorously to disrupt the filter, and then left for 15 minutes at room temperature. After this period, a 1 ml aliquot was removed from each bottle and centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge. The supernatants were removed and analyzed by HPLC for microcystin-LR content. The microcystin-LR concentration per μg of chlorophyll *a* was calculated, and is presented in Table 2.2.1.

The results presented in Table 2.2.1 demonstrate that, although no microplankton including cyanobacteria were observed in the loch water by light microscopy, chlorophyll *a* was measured in the water at a concentration of 0.016 μg/ml. This, plus the fact that the volume of material that was able to be passed through the 2.5 cm filter disks before blockage occurred was lower than expected, as were the resulting concentrations of microcystin-LR, indicated that the chlorophyll *a*-containing particles already present in the water was competing with the *M. aeruginosa* cells when filtered. It was therefore decided to pre-filter the loch-water through GF/C filters prior to performing the investigation.

Table 2.2.1 Chlorophyll *a* (Chl *a*) and microcystin-LR (MC-LR) content estimation of *M.aeruginosa* PCC7813 diluted in loch-water[a]

Dilution of cyanobacteria	Volume filtered[b] (Chl *a*)	[chl *a*][c] μg/ml	Volume filtered[d] (MC-LR) x	[MC-LR][e] ng/40μl y	[MC-LR][f] μg/μg chl *a*
1/40	200 ml	0.085 (±0.005)	80 ml	16.36	0.30
			90 ml	17.77	0.29
1/500	1000 ml	0.028 (±0.0007)	170 ml	0.00	0.00
			190 ml	0.00	0.00
loch-water	1000 ml	0.016 (±0.0003)	185 ml	0.00	0.00
			205 ml	0.00	0.00

a, water was collected from Loch Rescobie, a eutrophic freshwater loch near Dundee.

b, water was filtered through a 7 cm diameter glass-fibre filter disk, before incubating the disk in 5 ml methanol at 4°C for at least 16 hours.

c, [Chl *a*] $=(A_{663}$ [1ml MeOH extract] x 12.63 x 5 [volume of MeOH]) ÷ vol filtered.

d, water was filtered through a 2.5 cm diameter glass-fibre disk, before adding the disk to 5 ml of 0.3% (v/v) toluene, 20% (v/v) methanol in PBS for 15 minutes at room temperature, to lyse the cells.

e, microcystin-LR was analyzed by HPLC.

f, [MC-LR] μg/μg Chl *a* = (0.125y ÷ x) ÷ [Chl *a*] μg/ml

After filtering the water, it was used to dilute the *M. aeruginosa* PCC7813 1/10, 1/50 and 1/200. Known volumes of each dilution were filtered for chlorophyll *a* measurement and cell lysis as described previously. As a positive control, *M. aeruginosa* was diluted 1/5 in deionised water, then subjected to cell lysis and HPLC analysis as described previously. In order to estimate the efficiency of the lysis procedure, an equivalent volume of *M. aeruginosa* PCC7813 (10 ml) to that diluted in deionised water, filtered, and then lysed, was freeze-dried and then extracted with 5 ml of methanol to achieve maximum extraction of microcystin-LR. To achieve the chlorophyll *a* concentration of the undiluted cyanobacterial culture, duplicate 1 ml aliquots were centrifuged and the pellets were resuspended in an equal volume of methanol. After 2 hours at room temperature, the absorbance at 663 nm was measured in the spectrophotometer against a blank of methanol. The toluene/methanol/PBS extract was analyzed by HPLC for microcystin-LR content and results of this analysis, along with data obtained from the filtered loch-water diluted cells, are presented in Table 2.2.2.

The results demonstrate that, although the total microcystin-LR concentration recovered from filters was lower from the more diluted *M. aeruginosa* solutions, the absolute microcystin-LR concentrations per μg of chlorophyll *a* were approximately equal in all the diluted solutions recovered after filtration and cell lysis. The microcystin-LR recovery from the loch water-diluted cells was approximately equal to that recovered from cells diluted in deionised water, and the concentration of microcystin-LR per μg of chlorophyll *a* was also equivalent to the concentration recovered from freeze-dried/methanol extracted cells.

2.3 Evaluation of monoclonal antibodies

Monoclonal antibodies raised against microcystin-LR, cell-line number A005-1D 13C6, batch number 006, was received from Biocode Ltd on 7 January 1993, and kept stored at 4°C. Once all necessary reagents had been obtained, the antibodies 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 (50 μl) and left at 37°C for 3 hours, then 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 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 2.2.2 Chlorophyll *a* (Chl *a*) and microcystin-LR (MC-LR) content estimation of *M.aeruginosa* PCC7813 diluted in pre-filtered loch-water[a]

Dilution of cyanobacteria	Volume filtered[b] (Chl *a*)	[chl *a*][c] μg/ml	Volume filtered[d] (MC-LR) x	[MC-LR][e] ng/40μl y	[MC-LR][f] μg/μg chl *a*
1/10	100 ml	0.206 (±0.004)	100 ml	64.20	0.39
			100 ml	66.04	0.40
1/50	500 ml	0.042 (±0.001)	360 ml	51.14	0.42
			410 ml	62.21	0.45
1/200	500 ml	0.011 (±0.002)	950 ml	34.64	0.41
			840 ml	30.98	0.42
1/5[g]	-	-	50 ml	70.04	-
			50 ml	72.13	-
freeze-dried 7813	-[h]	2.179 (±0.146)	10 ml[i]	59.97	0.35
			10 ml	60.36	0.35

a, water was collected from Loch Rescobie, a eutrophic freshwater loch near Dundee, and filtered through 7 cm diameter glass-fibre disks.

b, water was filtered through a 7 cm diameter glass-fibre filter disk, before incubating the disk in 5 ml methanol at 4°C for at least 16 hours.

c, [Chl *a*] $=(A_{663}$ [1ml MeOH extract] x 12.63 x 5 [volume of MeOH]) ÷ vol filtered.

d, water was filtered through a 2.5 cm diameter glass-fibre disk, before adding the disk to 5 ml of 0.3% (v/v) toluene, 20% (v/v) methanol in PBS for 15 minutes at room temperature, to lyse the cells.

e, microcystin-LR was analyzed by HPLC.

f, [MC-LR] μg/μg Chl *a* = (0.125y ÷ x) ÷ [Chl *a*] μg/ml

g, the cyanobacterial cells were diluted in deionised water as a control, and no chlorophyll measurements were taken.

h, chlorophyll *a* measurements were taken from 1 ml aliquots of culture centrifuged and resuspended in an equal volume of methanol.

i, 10 ml of culture were centrifuged and the pellet freeze-dried before resuspension in 5 ml of methanol.

Table 2.3.1 presents data obtained from ELISA plates on which microcystin-LR, the microcystin variant "PK 3", 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-react with both microcystin variants and the nodularin. The mean absorbance values at 410 nm are approximately equal, although the value for nodularin is slightly higher.

2.4 Acquisition of assessment materials

The acquisition of material for assessment of lysis method, antibodies, and preliminary columns and kits has been, and will be, ongoing.

Loch-water has been collected and used to dilute *M. aeruginosa* cells in order to investigate the toluene/methanol lysis procedure, see section 2.2. Further loch water will be collected, along with cyanobacterial bloom samples, availability allowing, during the next few months. This should allow assessment of preliminary columns and kits, if available, along with further assessment of the lysis procedure and antibodies.

Microcystin variants and nodularin have been purified and used in ELISAs to assess the anti-microcystin-LR monoclonal antibodies, see section 2.3. Further purification is continuing in order to supply microcystin variants and nodularin for assessment of preliminary columns and kits.

A non-toxic *Microcystis, M.elabens* NIES-177 was cultured, centrifuged to harvest, washed in deionised water, and freeze-dried. A sample of this material was then resuspended in methanol and left at room temperature for 2 hours, to allow any microcystins present to be extracted. The methanol extract was then analyzed by HPLC to detect the presence of microcystins (Figure 2.4.1). The HPLC analysis of the methanol extract that no microcystins could be detected. An aliquot of freeze-dried material, equivalent to 500 ml of the original culture, was despatched to Biocode Ltd, for use in antibody assessment, as a microcystin-free control.

2.5 Assessment of Project 0348 options

In their last Progress Report, November 1992 - January 1993, Biocode Ltd reported the loss of toxin-binding capability after the microcystin-LR antibodies were immobilized in a column. At the last Progress meeting it was proposed that 3 options for obtaining a successful outcome to their Project, i.e a suitable prototype kit, could be attempted. These were as follows:-

A, continue attempts to attach the present IgM anti-microcystin-LR antibodies to column support material whilst retaining microcystin-binding activity.

Table 2.3.1 Cross-reactivity of A005-1D 13C6 batch 006 anti-microcystin-LR monoclonal antibodies with microcystin variants and nodularin in ELISA.

Variant investigated[a]	Absorbance 410 nm[b]
microcystin-LR	0.134 (±0.017)
microcystin-LR blank	0.016 (±0.008)
microcystin "PK 3"	0.127 (±0.018)
"PK 3" blank	0.014 (±0.007)
nodularin	0.151 (±0.019)
nodularin blank	0.013 (±0.007)

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

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

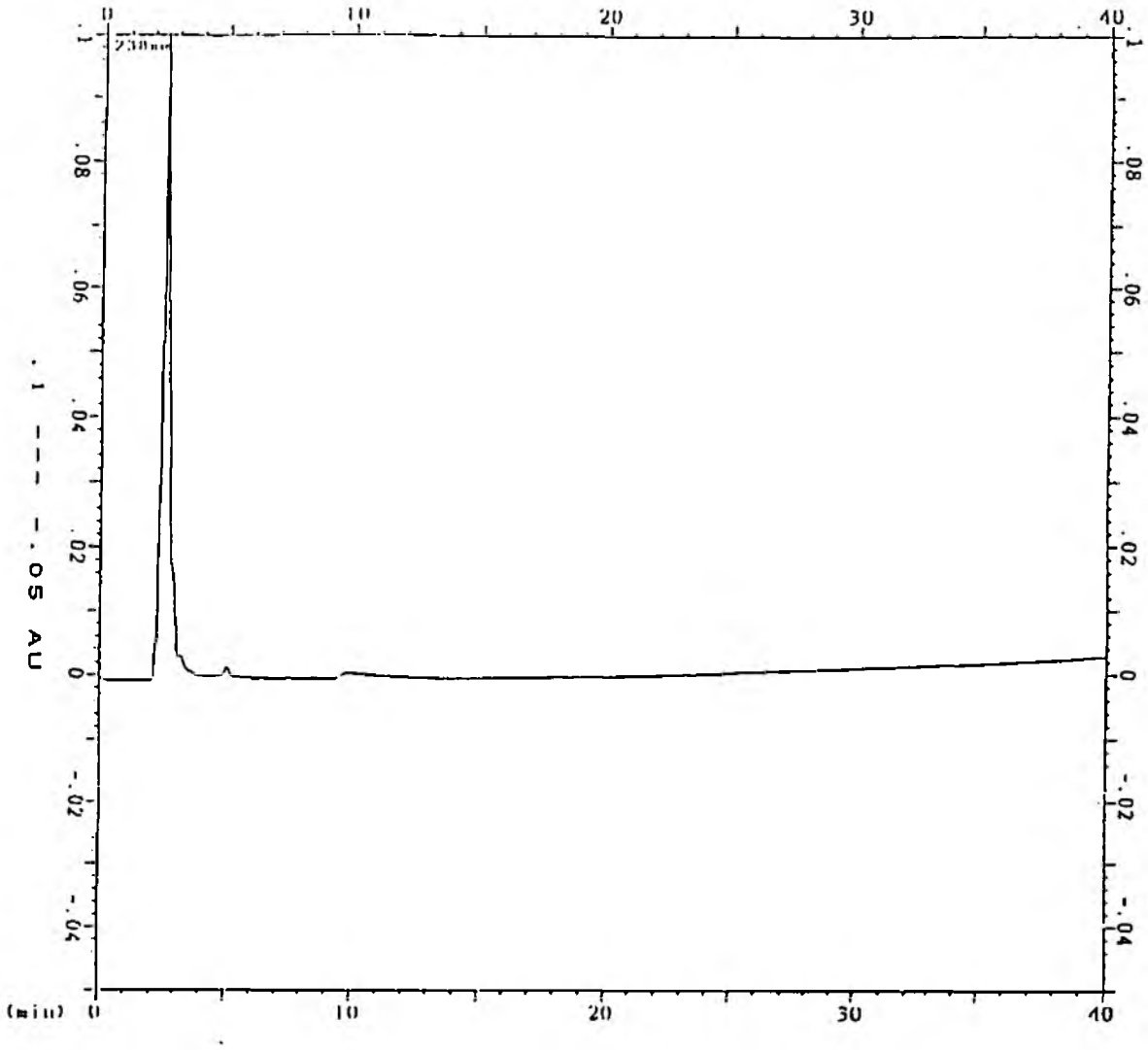


Figure 2.4.1 HPLC analysis[a] of a methanol extract of freeze-dried *M. elabens* NIES-177

a, 20 μl of methanol extract were loaded onto a Novapak 3.9 x 300 column and eluted with 30% - 70% acetonitrile in water containing 0.05% trifluoroacetic acid. Microcystins, if present, would have been detected as peaks with retention times of 15 - 40 minutes.

B, develop an alternative assay format utilising the present IgM anti-microcystin-LR antibodies, i.e a membrane format

C, utilise the ADDA fraction of the microcystin molecule to develop new monoclonal antibodies

This laboratory was asked to act as an independent assessor of these three options, and assign a level of the probability of technical success to each one. This involved a discussion of technical strategies with Biocode Ltd, followed by an assessment report.

Dr S Bell visited Biocode Ltd at York on 19 February 1993, where he had useful open technical discussions with Dr D Baron and Dr M Stow. The assessment of these technical proposals and their chance of technical success was then carried out in Dundee, and was documented in a report to the NRA.

2.6 Future work programme

- Investigate the toluene/methanol/phosphate-buffered saline lysis procedure with bloom samples of cyanobacteria.
- Asses monoclonal antibodies produced using modified purification procedures by Biocode Ltd.
- Continue to purify microcystin variants and nodularin, in order to asses antibodies, and preliminary columns.
- Validate preliminary columns, and asses the linkage on MAb(s) to column support materials.
- Compare Biocode test kits for specificity, recovery capacity. and detection limits for microcystins from aquatic environments with standard laboratory methods.

3 DISCUSSION

In order to investigate the cyanobacterial cell lysis procedure of a representative water sample, fresh loch-water was spiked with a culture of toxic *Microcystis aeruginosa*. This was performed as no naturally occurring toxic bloom water sample was available at the time of analysis. It was observed that, although no toxic cyanobacterial species were detected in the water sample by light microscopy, chlorophyll *a* was measurable, and this had a competitive effect with the spiked cyanobacteria when filtered through a glass-fibre disk. This reduced the expected volume of sample that could be filtered before the filter-disk became blocked, and also reduced the recovery of microcystin-LR after cell lysis, especially in the more dilute samples where added chlorophyll concentrations were low. When the toxic cyanobacterial cells were spiked into prefiltered loch-water, the recovery of microcystin-LR was at the expected level, independent of the chlorophyll concentration of the water. In naturally occurring blooms of cyanobacteria in freshwater the species present tend to be dominated by toxic forms. Thus competition during filtration by other chlorophyll *a*-containing non-toxic phytoplankton may not play such a part in natural blooms. It is also likely that, when analyzing a bloom sample, it would be preferable to obtain information of the overall toxicity of the total phytoplankton present, and so the competitive part played by non-toxic species in the lysis procedure may be essential. The concentrations of chlorophyll *a* investigated are representative of those previously found in the loch from which the water was sampled, the 0.01 μg/ml being representative of very low level bloom, and the 0.2 μg/ml being representative of one of the highest chlorophyll *a* concentrations recorded for a bloom. The species present in Loch Rescobie have been dominated by *M. aeruginosa* during the past 10 growth seasons. We are therefore confident that the lysis procedure employed is effective, but we hope to confirm this with naturally occurring toxic blooms later in the growth season.

The anti-microcystin-LR monoclonal antibodies from cell-line A005-1D 13C6, batch 006, have been assessed using an ELISA developed by Biocode Ltd. The results of this assessment indicate that this batch of antibodies cross-react with microcystin-LR, another microcystin, and nodularin. However, it should be noted that the batch of antibodies assessed was batch purified and stored before the column binding problems were encountered by Biocode. One of the approaches which Biocode considered employing to overcome this problem was to alter the antibody purification procedure, in an attempt to obtain more stable antibodies. If the purification procedure is changed, a subsequent batch of antibodies would also have to be assessed in this laboratory. We have now received such a batch.

As an aid to assessment of antibodies and preliminary kits, microcystin variants, nodularin, and non-toxic *Microcystis* have been purified/prepared. The non-toxic *Microcystis* was freeze-dried and extracted with methanol, prior to analysis by HPLC for the presence of microcystin(s). This was demonstrated to be microcystin-free. Further purification of microcystin variants/nodularin is planned in order to provide material for further antibody/ column evaluation.

4 FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

The problem of inhibited binding of microcystin to column-bound antibodies, encountered by Biocode Ltd, is a major one, and the ability to overcome it, and the decision by the NRA on the future options of Project 0348, will have an effect on the completion of Project 0349.

5. COST OF WORK CARRIED OUT IN THE PERIOD FEBRUARY 1993 - APRIL 1993

	This period (£)	Total to date (£)	Project total (£)
Staff salaries and overheads	8,982	53,892	57,938
Travel and subsistence	249	1,613	1,800
Laboratory consumables	347	6,000	6,000
Reports	220	860	1,000
Total	9,798	62,365	66,738

6. ESTIMATE OF COST OF FUTURE WORK

As the period of future work is unkown at the present time, and decisions pending the outcome of the next Progress Meeting on 5 may 1993 are necessary, an estimate of the cost of future work cannot be made.