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

Progress Report for Period
February 1992 - April 1992

University of Dundee
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NRA
National Rivers Authority

CONTENTS

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.

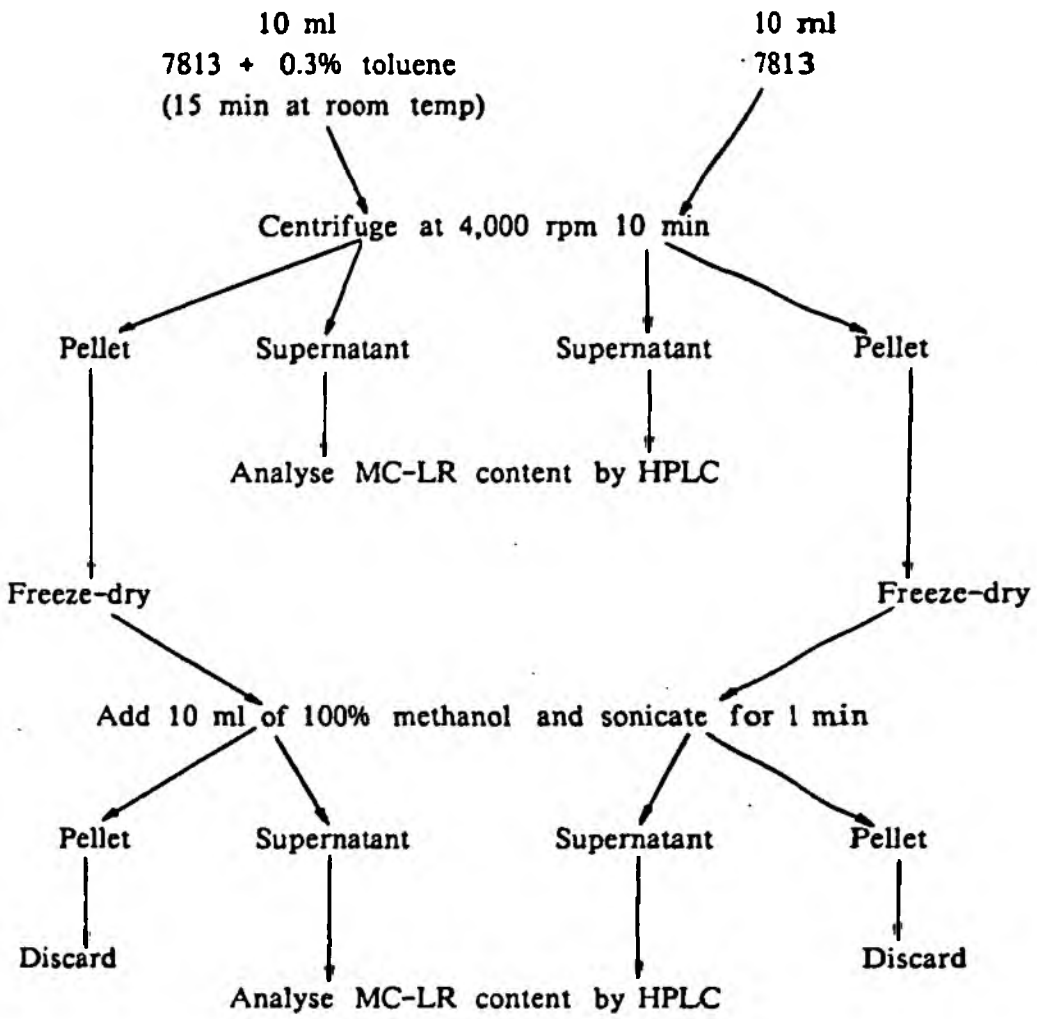
	Amount of MC-LR per 25 µl sample [a].	
Detergent added	0.01% (v/v) detergent	0.1% (v/v) detergent
Triton X-100	0.0 (± 0.0)	0.0 (± 0.0)
Sarkosyl	0.0 (± 0.0)	0.0 (± 0.0)
Tween 20	0.0 (± 0.0)	0.0 (± 0.0)
Nonidet P-40	0.0 (± 0.0)	0.0 (± 0.0)
Span 20	0.0 (± 0.0)	0.0 (± 0.0)
0.3% (v/v)[b] Toluene	92.2 (± 8.6)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

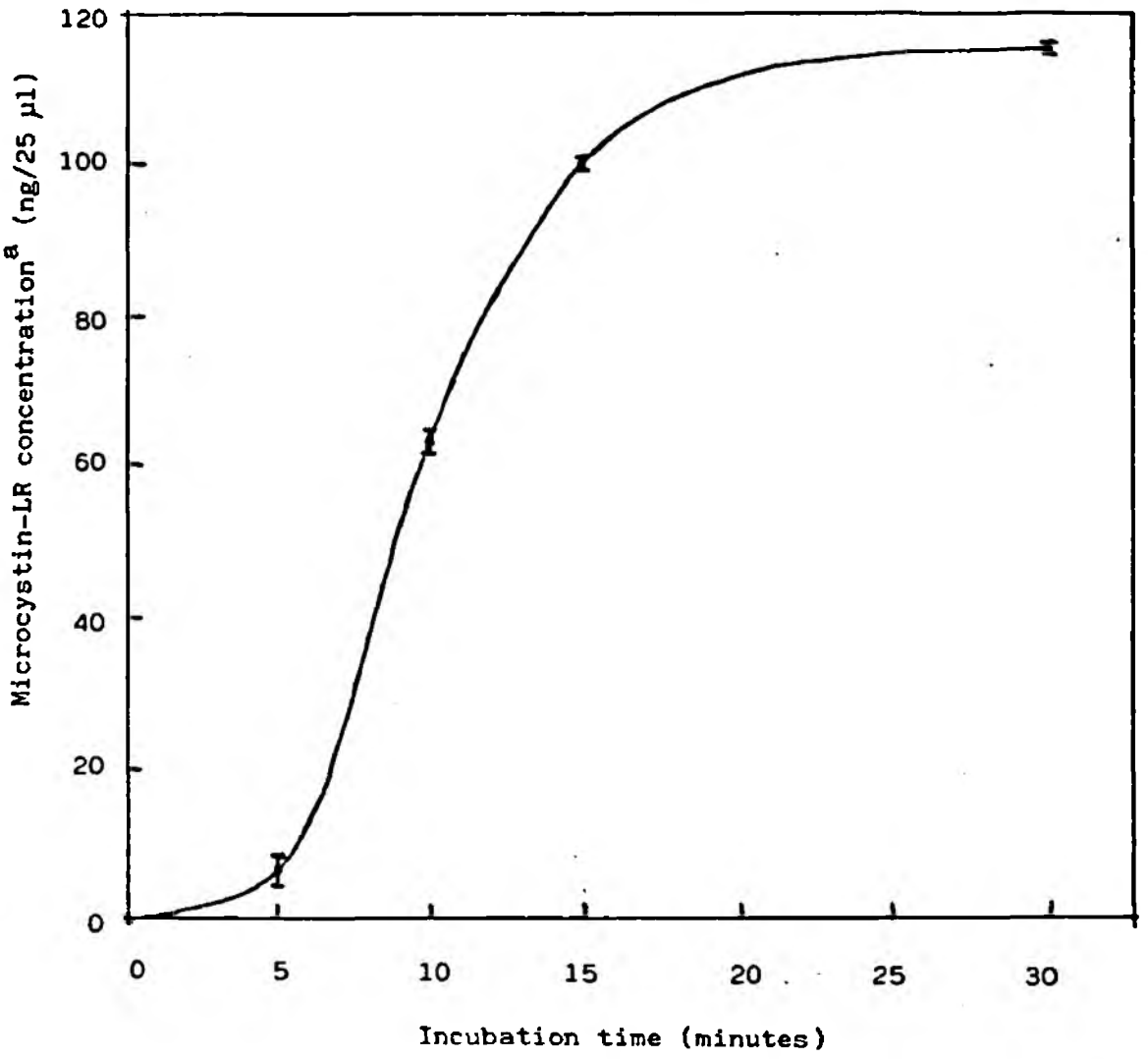


Figure 2.3.1 Release of microcystin-LR from *Microcystis aeruginosa* 7813 during incubation with 0.3% (v/v) toluene.

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.

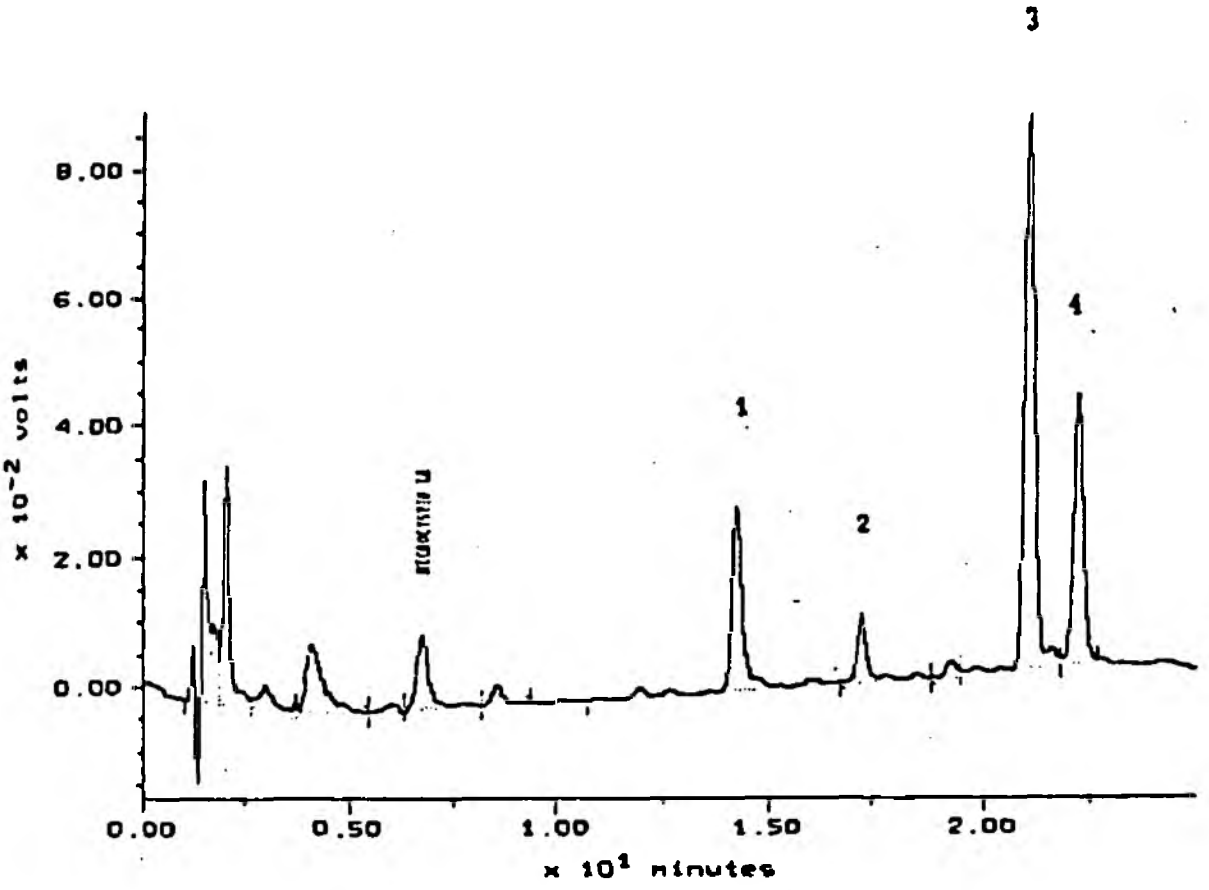
∴ chlorophyll a loading capacity of filter (GF/C) = 4.84 $\mu g/cm^2$.

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