Project 349

2027

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

Progress Report for Period
May 1992 - July 1992

University of Dundee
August 1992

NRA

National Rivers Authority

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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.

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

Table 2.2.2 Phycocyanin release from *Microcystis aeruginosa* 7813 incubated with n-dodecyl β-D maltoside or n-octyl β-D glucopyranoside in the presence of lysozyme.

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

a, see footnote a Table 2.2.1

b, lysozyme was added to two final concentrations, 0.1% (w/v) and 1.0% (w/v)

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

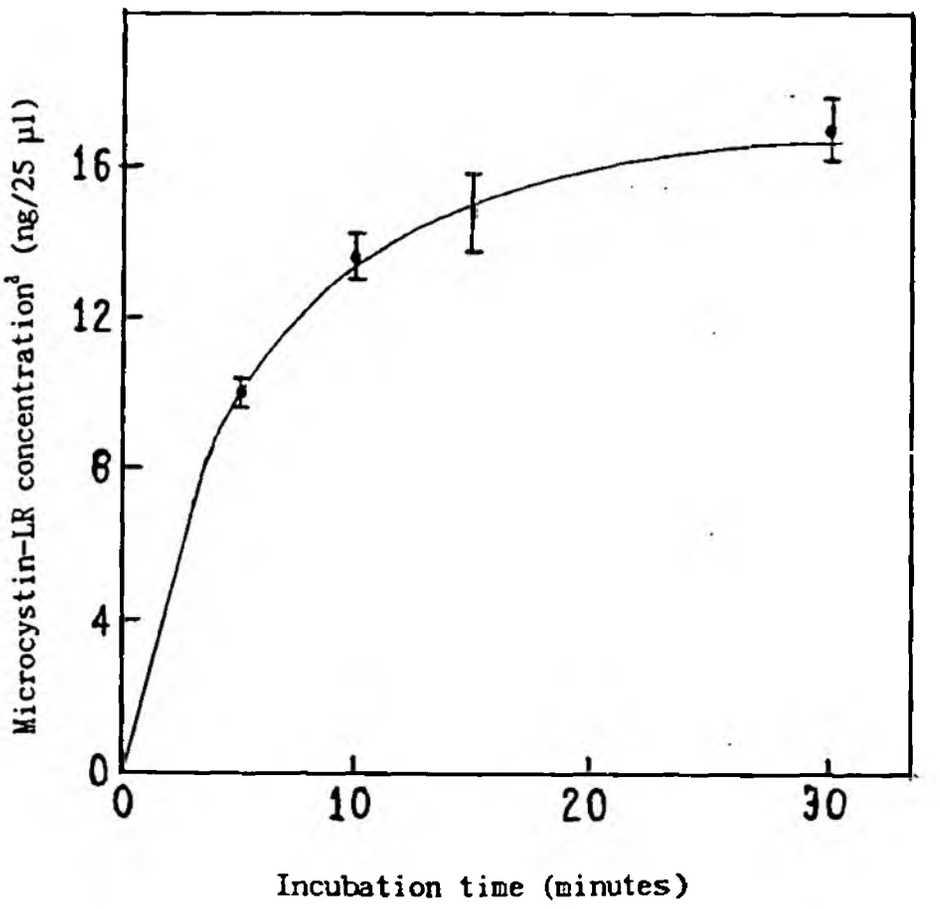

Figure 2.3.1 Release of microcystin-LR from *Microcystis aeruginosa* 7813 entrapped on a glass fibre filter and incubated in 0.3% (v/v) toluene compared with incubation time.

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 ($\pm$ 1.67)
10 ml	5.19 ($\pm$ 0.76)
20 ml	5.76 ($\pm$ 1.13)

a, measured by HPLC analysis

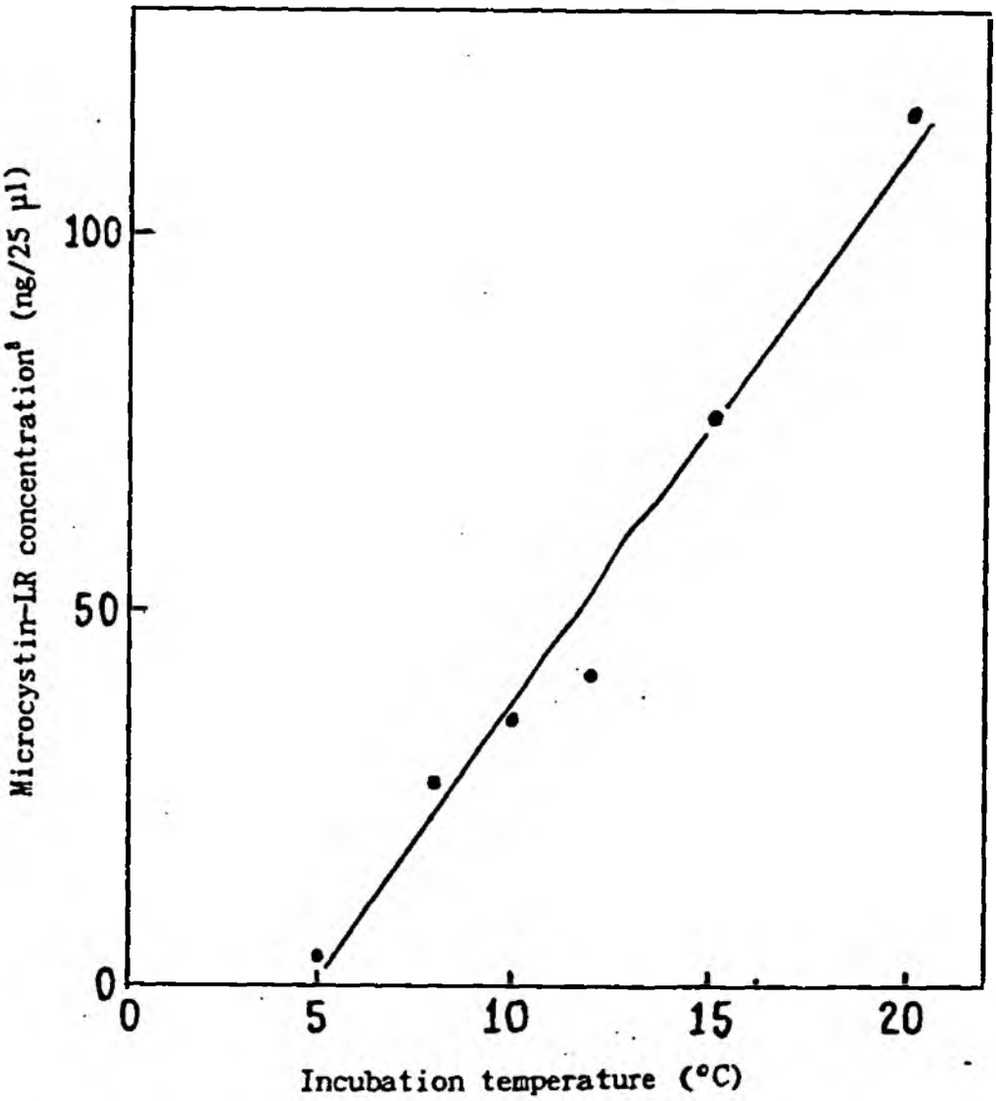

Figure 2.3.2 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with 0.3% (v/v) toluene compared with incubation temperatures to 20°C.

a, measured by HPLC analysis

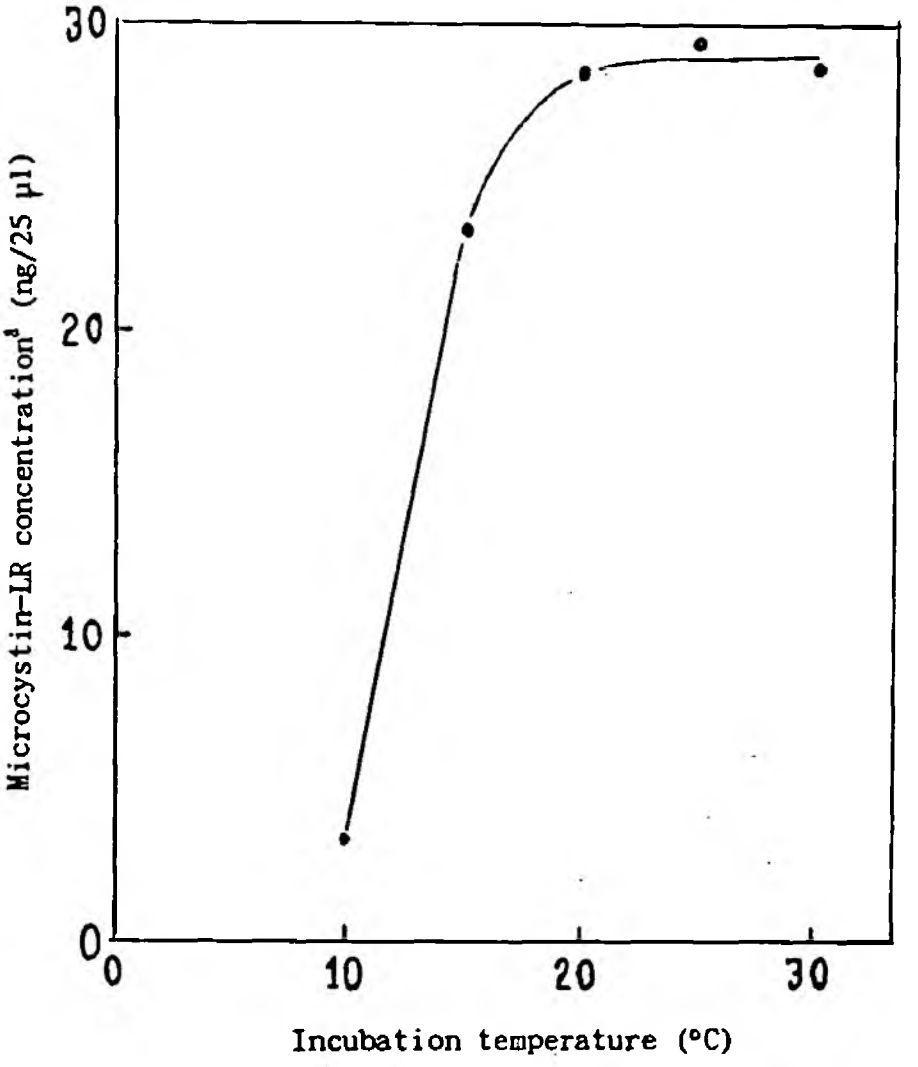

Figure 2.3.3 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with 0.3% (v/v) toluene compared with incubation temperatures to 30°C.

a, measured by HPLC analysis

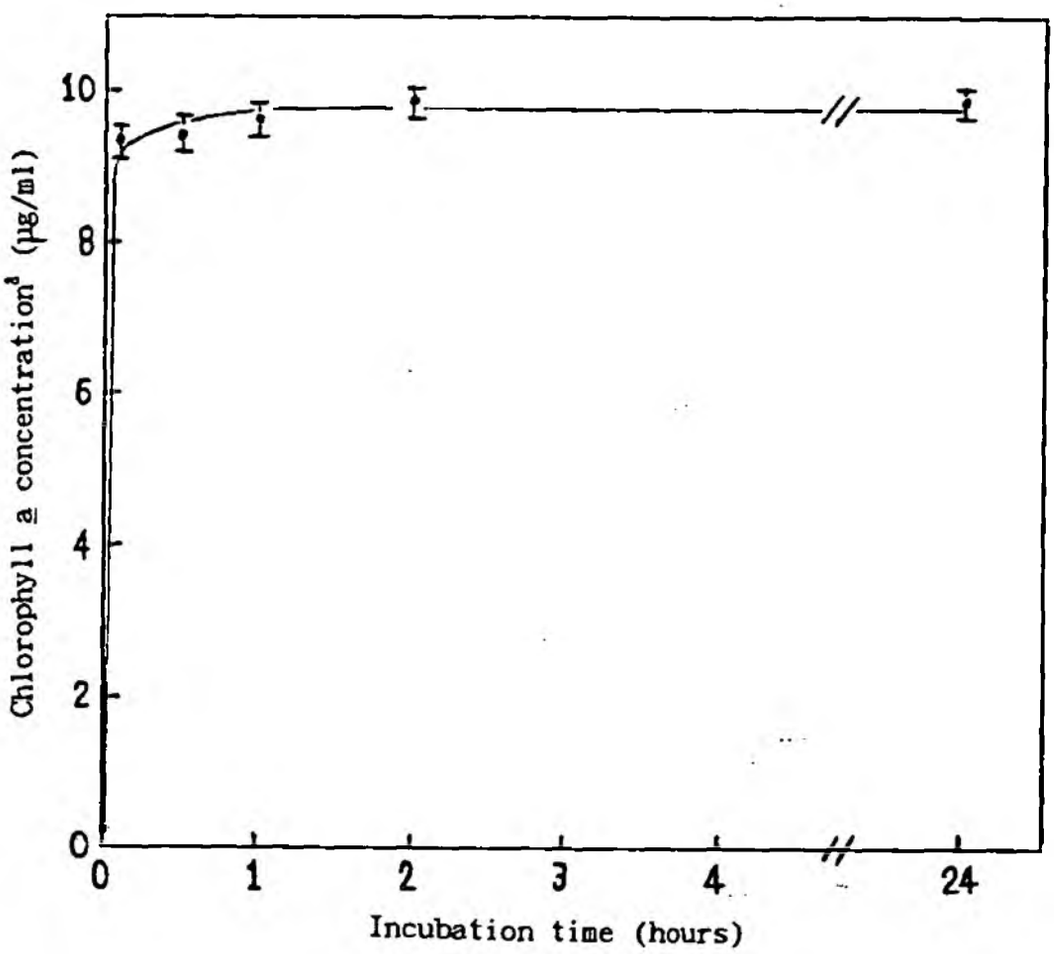

Figure 2.4.1 Chlorophyll a extraction from *Microcystis aeruginosa* 7813 with methanol compared with incubation time.

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

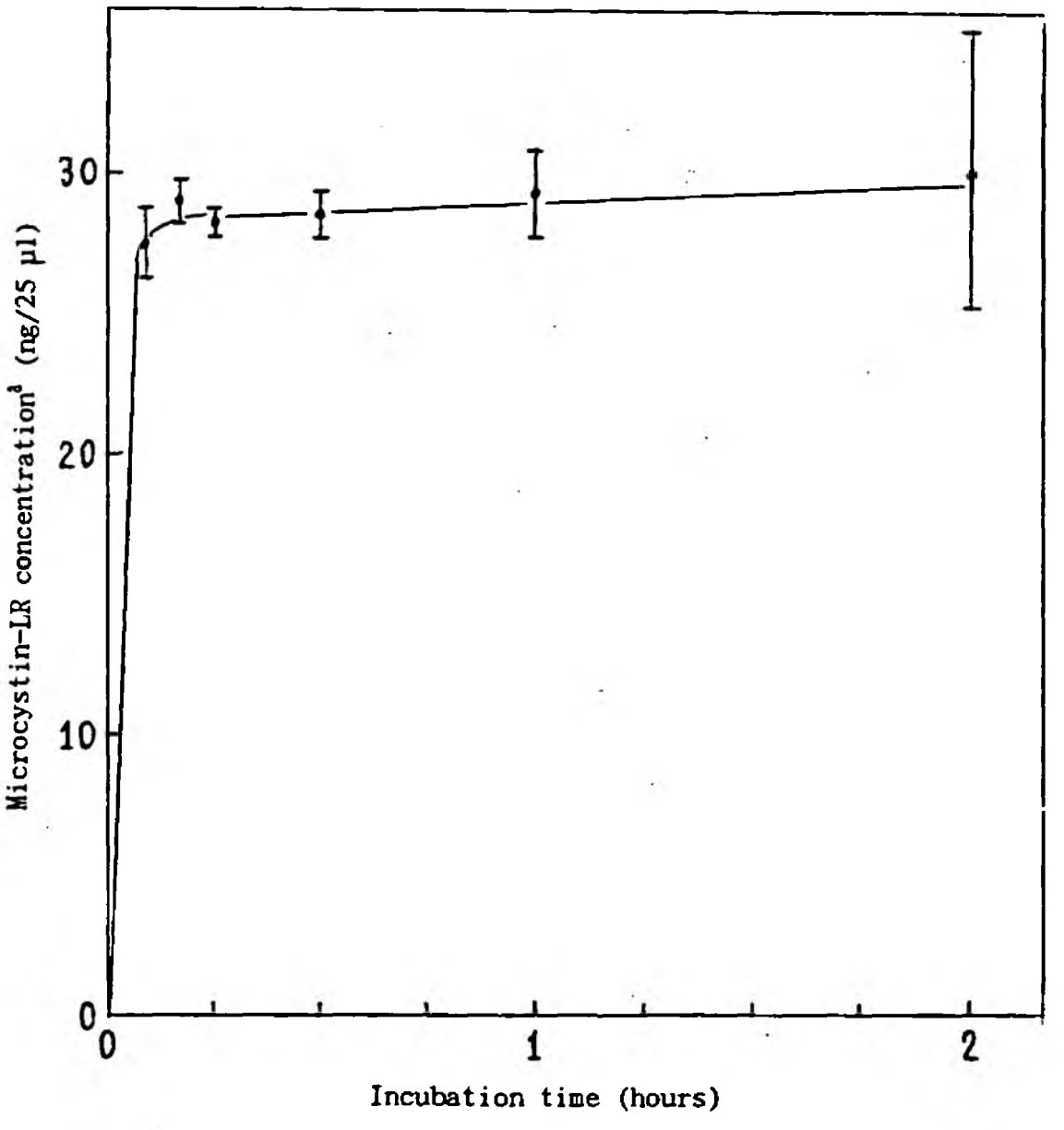

Figure 2.4.2 Release of microcystin-LR from *Microcystis aeruginosa* 7813 with methanol compared with incubation time.

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

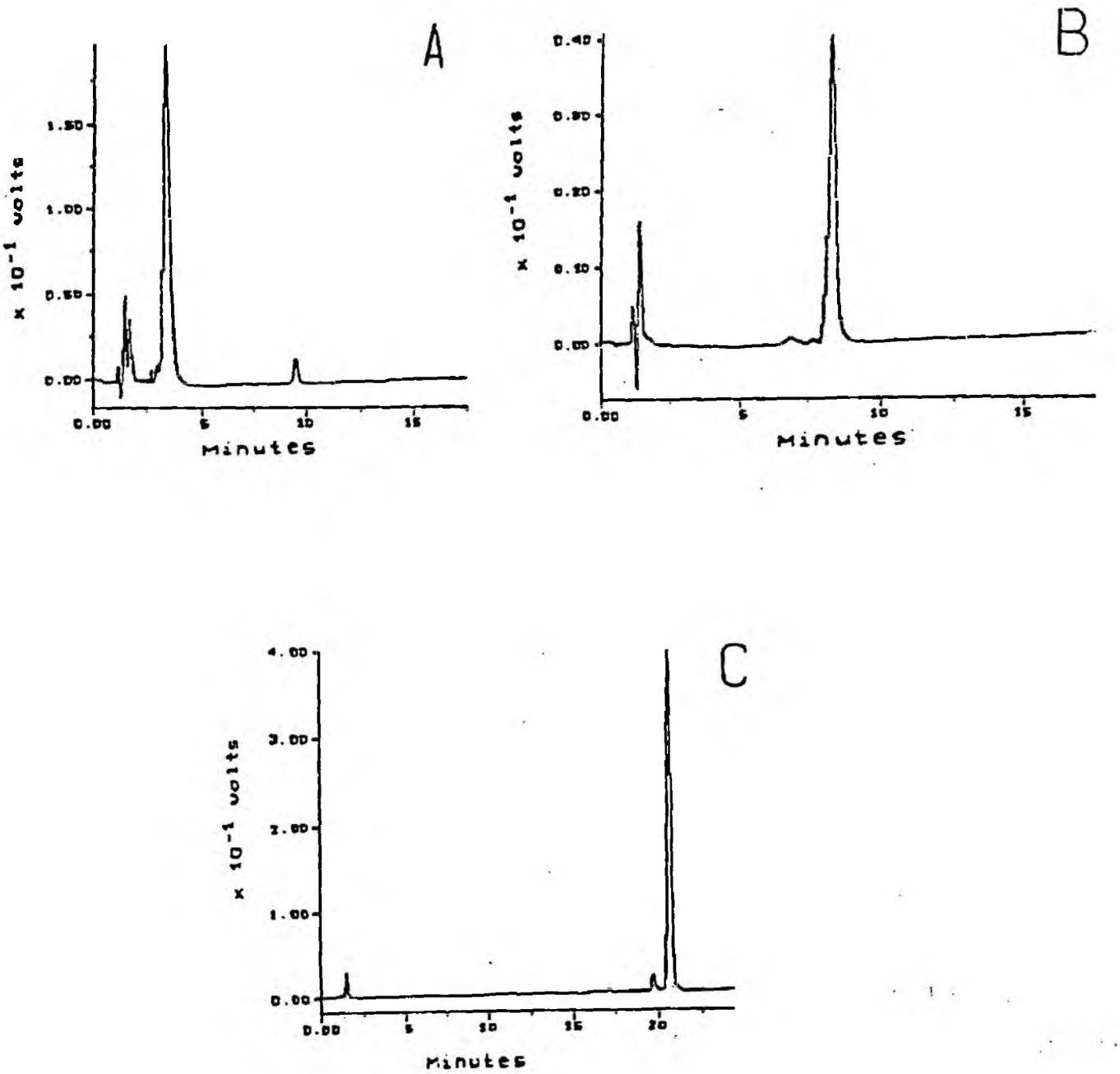

Figure 2.5.1 HPLC chromatograms of cyanobacterial toxin variants

A microcystin-3-desmethyl-RR

B nodularin

C PK-3

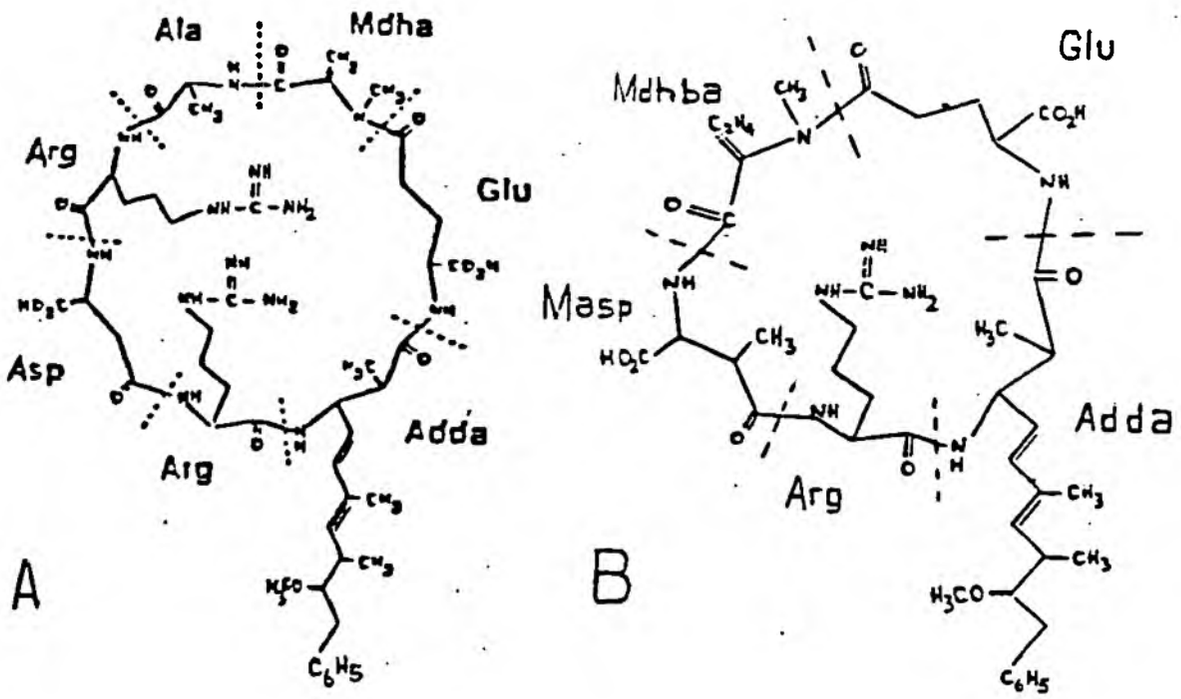

Figure 2.5.2 Structure of cyanobacterial toxin variants

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 (~ 29 ng MC-LR/25 μl released with methanol c.f. ~ 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