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

Progress Report for Period
February 1993 - April 1993

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ENVIRONMENT AGENCY
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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

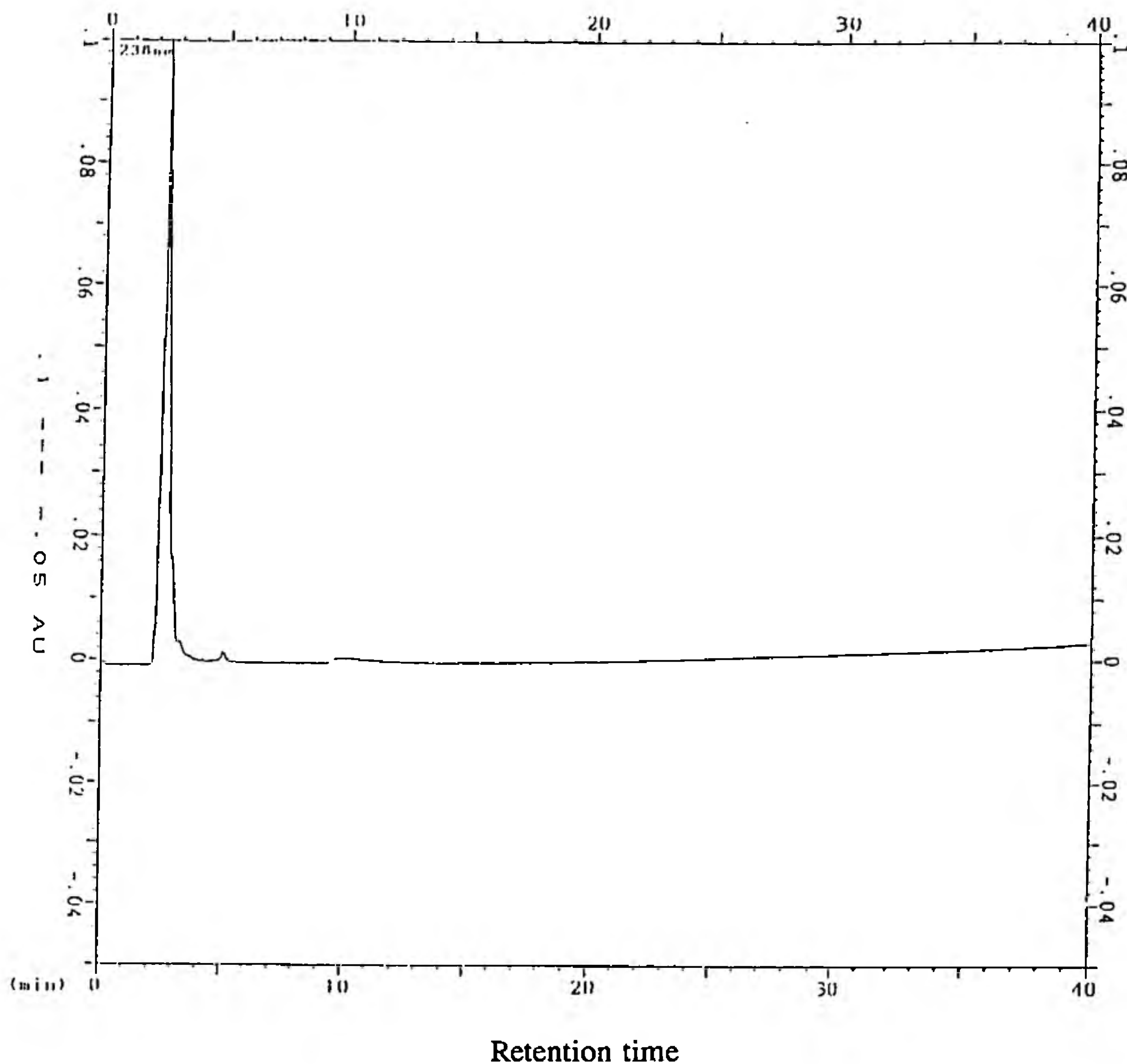

Figure 2.4.1 HPLC analysis[a] of a methanol extract of freeze-dried *M. elabens* NIES-177

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.