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 (± 0.25)
Phosphate buffer	+	40.35 (± 2.05)
Phosphate buffer + 0.1% (w/v) NaCl	+	40.45 (± 3.75)
Phosphate buffer + 0.25% (w/v) NaCl	+	35.95 (± 5.05)
Phosphate buffer + 0.5% (w/v) NaCl	+	36.00 (± 2.00)
Phosphate buffer + 0.85% (w/v) NaCl	+	34.80 (± 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 (± 2.05)
PBS	+	34.80 (± 3.10)
PBS + 10% (v/v) MeOH	-	1.20 (± 1.20)
PBS + 10% (v/v) MeOH	+	39.65 (± 5.25)
PBS + 25% (v/v) MeOH	-	8.30 (± 1.30)
PBS + 25% (v/v) MeOH	+	37.4 (± 2.50)
PBS + 50% (v/v) MeOH	-	25.25 (± 1.95)
PBS + 50% (v/v) MeOH	+	38.75 (± 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

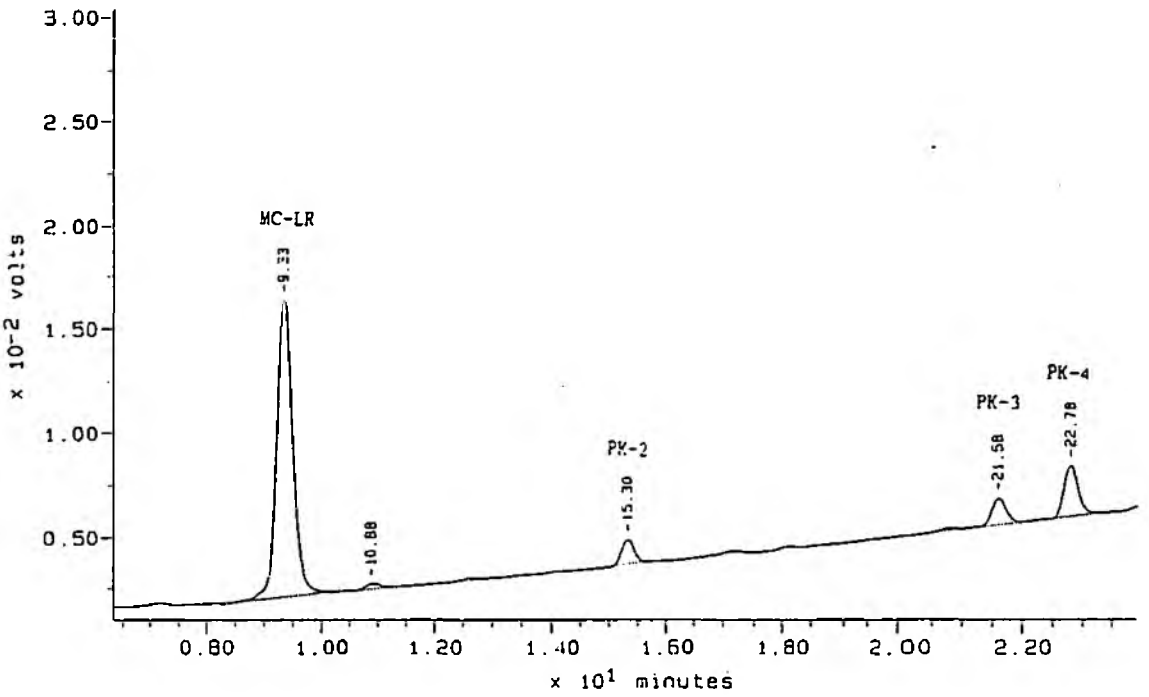

Figure 2.3.1 HPLC chromatogram of methanol extract of *M. aeruginosa* PCC 7813

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.

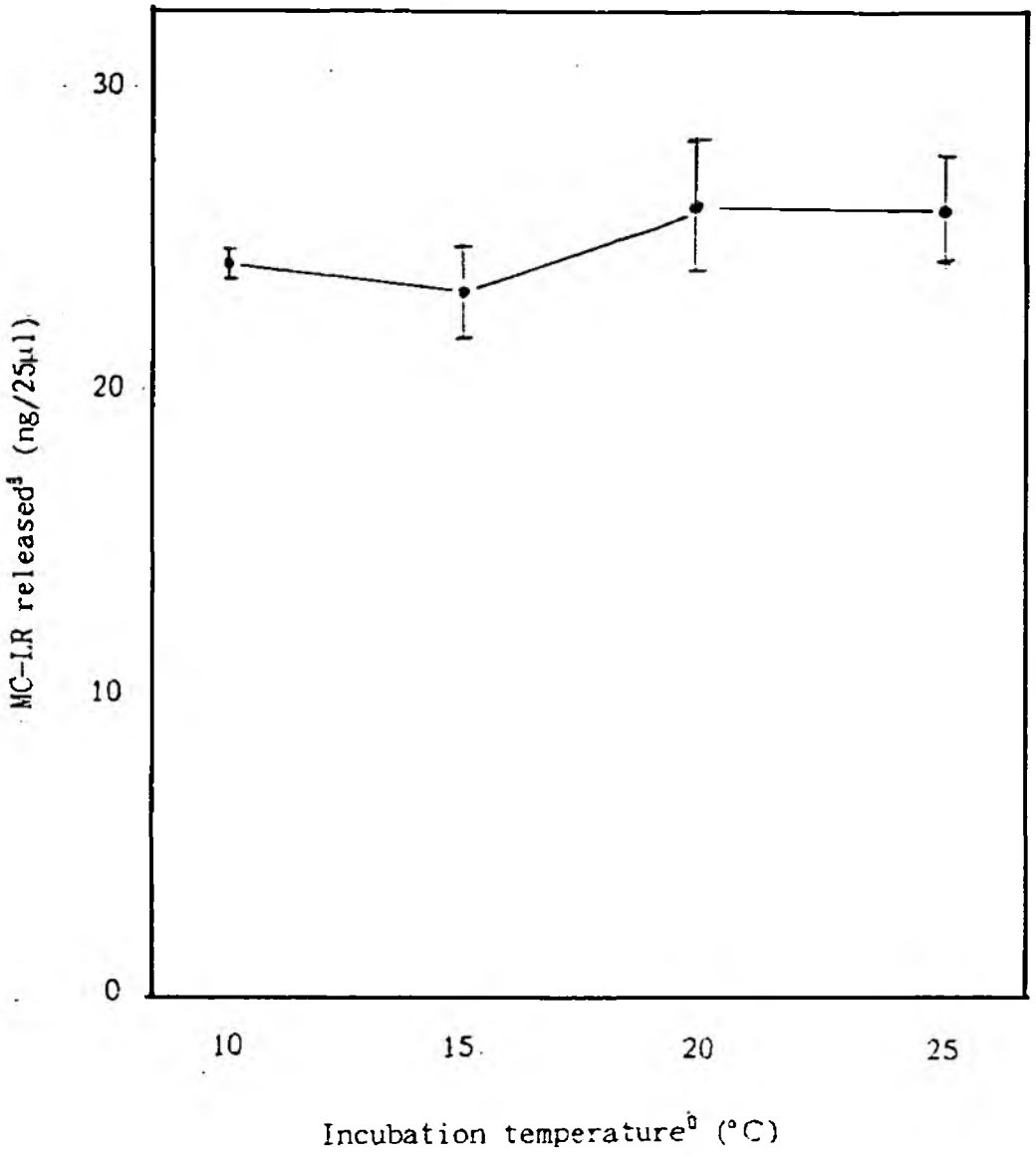

Figure 2.3.2 Extraction of microcystin-LR from *M. aeruginosa* PCC 7813 with methanol versus incubation temperature.

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