IL-6 AS BIOMARKER FOR THE IMMUNOMODULATORY EFFECTS OF MICROCYSTINS

Report to the

Water Research Commission

on the project

"The effect of microcystins on the immune system using the pro-inflammatory hormone IL-6 as biomarker"

by

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EXECUTIVE SUMMARY

Background

Microcystins are hepatoxins produced by cyanobacteria. They are chemically very stable and pose a public health threat when they occur in water bodies used for human and animal consumption. Literature data indicate that animal fatalities due to microcystins have occurred in South Africa (Van Halderen, et al., 1995). Epidemiological studies done in China have also shown an increased risk of cancer associated with cyanobacteria contaminated drinking water resources (Zhou, et al., 2002).

Current routine monitoring systems for microcystin are mainly chemical and immunochemical assays. The chemical methods used for microcystin monitoring uses C18 chromatography followed by one of the following techniques: high performance liquid chromatography (HPLC), capillary electrophoresis (CE) or thin layer chromatography (TLC). The equipment used for HPLC and CE is very expensive. TLC is the only one of the chemical methods that can be readily performed in most laboratories. Most microcystin immunoassays are ELISA based. Several of these ELISA kits are available commercially. Due to the low cost per sample (R150 per sample assayed in duplicate) the ELISA has become one of the most popular methods for routine screening of water samples for microcystins.

Most of the current routine assays detects total mycrocystin peptides, but does give an indication of the toxicity or biological activity of the sample. This can result in false positives due to the fact that some microcystins are non-toxic. For this reason there is a great need to develop a routine test for microcystins that will give an indication of the toxicity and/or biological activity of the specific microcystin sample. There are indications in the literature suggesting that microcystin toxicity involve immune processes. Microcystin induces inflammation (the first phase of an immune response) of the liver and immunosuppressive drugs such as rafampicin completely abolish the toxic effects of microcystin (Theiss and Carmichal, 1986; Hermansky, 1991). Injection of microcystin into animals causes an increase in the plasma levels of the pro-inflammatory cytokines. The cytokines are hormones regulating the immune system and include the interleukins, interferons and also tumor necrosis factor. In vitro data have also shown that microcystins induce the synthesis and secretion of pro-inflammatory cytokines (IL-6 and TNF), similar to the in vivo situation (Lankoff, et al., 2004).

Objectives

The objectives of this study were to:

1. Determine whether microcystins modulate the immune system of animals *in vivo* and *in vitro* using the pro-inflammatory hormone interleukin 6 (IL-6) as biomarker;
2. Write standard operating procedures for microcystin immunomodulatory assays.

Methodology

In vivo immunomodulotory assays for microcystins.

BalbC mice were injected with one of the following: microcystin, endotoxin, saline or a mixture of microcystin and endotoxin. Blood was collected 6 hours after injection and analysed for IL-6 using a commercially available ELISA.

In vitro immunomodulatory assays for microcystins

Human whole blood was cultured overnight in the presence of one of the following solutions: microcystin, endotoxin, saline or a mixture of microcystin and endotoxin. The culture supernatants were collected and assayed for IL-6 using an in house ELISA. This assay was also used for monitoring the immunomodulatory effects of environmental water samples. In these assays environmental water samples were added to cultures in stead of isolated microcystin.

In vitro cytotoxicity assays for microcystins

Human whole blood was cultured overnight in the presence of one of the following solutions: microcystin, endotoxin, saline or a mixture of microcystin and endotoxin. The culture supernatants were collected and assayed for lactate dehydrogenase (LDH).

ELISA for microcystins

Environmental water samples were analysed for microcystins using the commercially available Envirologix ELISA Kit.

Summary of the major results

The levels of IL-6 synthesised by BalbC mice upon pathogen (endotoxin) exposure were very low and cannot be used for routine analysis.

Human whole blood cultures synthesise high levels of IL-6 upon endotoxin exposure. The amount of IL-6 synthesised is directly proportional to the endotoxin concentration used to stimulate the cells. Microcystin does not induce IL-6 synthesis in the absence of endotoxin. However, in the presence of microcystin, a specific endotoxin concentration induce more IL-

6 than in the absence of the microcystin, i.e. the microcystin acts as an upregulator of the endotoxin induced IL-6 response (an immunomodulator). The % upregulation by microcystin is microcystin concentration dependent. An environmental water sample that screened positive for microcystin using the HPLC analysis and also the Envirologix ELISA assay for microcystins, also upregulated the immune response due to endotoxin (increased IL-6 production in the presence of added endotoxin).

Conclusions

Microcystin acts as a modulator of the in vitro immune response of blood cultures to the bacterial endotoxin. Microcystin increase the level of the immune response that will normally be elicited by a given concentration of endotoxin (upregulates the immune response). Environmental samples containing microcystin have a similar effect on the endotoxin induced IL-6 synthesis and the immunomodulatory assay can thus be used as a potential bio-assay for microcystins. The methods used for determining the immunomodulatory effects of water samples were written as a standard operating procedures and these methods are attached to this report (Appendixes A, B and C). In conclusion, both objectives as set out in the initial proposal were met.

Recommendations and future research

Due to logistical reasons only one environmental sample containing microcystin was used in the current study. It is recommended that this study be extended to include more samples to evaluate the specificity of the assay system for microcystin.

The Envirologix ELISA kit (http://www.envirologix.com) for microcystin used in this study was found to be a user friendly, cost effective, rapid, quantitative and reproducible system for routine microcystin monitoring in environmental water samples. It is suggested that training of students/scientist to use these assays must be done to increase the human capacity to monitor microcystins. The cost of these kits has decreased substantially over the last four years and currently it cost approximately R 150 to analyse a sample in duplicate.

Proposals for the archiving of data generated during the project

The data generated are being stored on the Ecophysiology Laboratory, Zoology and Botany Department, University of Stellenbosch data storage system.

List of publications and other technology-transfer actions.

No papers have yet been submitted to journals. At present the data are being written up as a scientific publication under the working title: " The immunomodulatory activity of microcystins".

Standard operating procedure documents were prepared for the immune system activation and the IL-6 assay and these are attached (Appendix A and B).

A standard operating procedure document was prepared for the cytotoxic assays and this is attached (Appendix C).

The laboratory and human resources potential have been developed to conduct training programs for researchers and students in applied immunology in the Ecophysiology Laboratory, Stellenbosch University.

Capacity Building

Mr. Arries (black male) worked as a student-assistant on the project and was taught ELISA and tissue culture techniques. He obtained his B.Sc. in 2001.

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Cape Metropolitan Water for donating the water sample contaminated with microcystin-LR.

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THE EFFECT OF MICROCYSTIN ON THE IMMUNE SYSTEM

1. BACKGROUND

Members of the cyanobacteria such as *Microcystis, Oscillatoria* and *Anabaena* produce low molecular weight cyclopeptide hepatotoxins called microcystins (Krishnamurthy, et al., 1986; Botes, et al., 1984). Microcystin and also extracts and cells of these toxic species are cytotoxic and cause excessive haemorrhaging in the livers of animals (Falconer, et al., 1981; Theiss and Carmichael, 1986). Microcystin belongs to the okadaic acid type of tumor promotors (Fujiki and Saganuma, 1999). These molecules, although structurally very different from one another, act on plants and animals by inhibiting protein phosphatases-1 and –2A which dephosphorylate phosphoserine and phosphothreonine in proteins (Herscheler, et al., 1988; Suganuma, et al., 1992). They have been implicated in the death of wildlife and cattle consuming water containing dense blooms of cyanobacteria (Beasly, et al., 1991). Due to the toxic nature of some of the microcystins the WHO has set guidelines for maximum levels of these compounds in drinking water (1 µg/L for total microcystin-LR).

1.1 Bio-assays for the detection of microcystin

Several different bioassay methods have been developed to screen samples for microcystin. These assays include several *in vivo* whole animal toxicity assays, plant growth inhibition assays and *in vitro* assays such as cellular toxicity assays, enzyme inhibition assays and ELISA. Most of these systems only give an assessment of the general toxicity of the sample and cannot be used for quantitation of the individual microcystins in a sample. Table 1.1 is a summary of the various bio-assays used for microcystin detection.

The mouse assay for microcystin is the most commonly used *in vivo* assay for microcystin (Falconer, 1993). In this assay mice are injected with various quantities of water extract and then observed for 24 hours. After the observation period the mice are sacrificed and the liver is then examined to see if there is tissue injury. This assay can be used to screen for most of the cyanobacterial toxins. The observed symptoms are

indicative of the type of toxin that the animal was exposed to. Several invertebrate species have also been used to screen for microcystins. These include brine shrimp (Kiviranta, et al., 1991), Daphnia (Lawton, et al., 1994), mosquito (Kiviranta et al., 1993) and fruit fly (Swoboda, et al., 1994). Plant assays based on the inhibition of plant growth in the presence of microcystins have also been reported (Kos, et al., 1995, Gehringer, et al., 2003).

Primary cultures of hepatocytes from several species have been evaluated as potential replacements for the mouse toxicity assays. A good correlation between the mouse assay and cytotoxicity, measured by lactate dehydrogenase (LDH) leakage from cultured cells, was obtained (Aune and Berg, 1986). Data obtained by Heinze, 1996 showed that the sensitivity of the hepatocyte culture assay can be increased by longer culture periods and the same authors showed that the different microcystin variants vary in the respective LC_{50} value. The variations in LC_{50} were consistent with *in vivo* toxicity data. Fibroblasts have also been investigated as a potential alternative for the mouse toxicity assay (Lawton, et al., 1994). However due to the fact that some false negatives were observed when using this assay, this method was not pursued any further.

Microcystins induced inhibition of protein phosphatase 1 and 2A have been used extensively for very sensitive bioassays to screen for microcystin concentration. The level of protein phosphatase inhibition is proportional to the amount of microcystin in the assay mixture. Two general systems have been used in the literature. The first system uses radioactively labeled phosphate release from phosphoprotein to measure enzyme activity (Holmes, 1991). This method can be used to screen for enzyme activity in tissue homogenates from *in vivo* exposed animals and also from cultured cells. The problem with this assay method is that special equipment and facilities are required for radioactive work. The alternative assay system employs a colorimetric assay system to measure protein phosphatase activtiy (An and Carmichael, 1994). Commercial kits employing this assay method are available from several suppliers. Data obtained from ring tests however questions the repeatability and reliability of the colorimetric phosphatase inhibition assay (An and Carmichael, 1994).

TABLE 1.1: Bioassays for microcystin detection.

Method	Comments	Critical problem factors	Reference/s
Mouse *in vivo*	Determine total microcystins.	Ethical problems.	Falconer, 1993
Brine shrimp *in vivo*	Determine total microcystins. Commercial kits are available.	Expensive.	Kivitra et al., 1991
Daphnia *in vivo*	Determine total microcystins.	Labour intensive.	
Mosquito *in vivo*	Determine total microcystins.	Difficult to inject.	Kivitra et al., 1993
Fruitfly *in vivo*	Determine total microcystins.	Easy to culture.	Swoboda et al., 1994.
Plant growth assays	Determine total microcystins.	Requires long exposure periods.	Kos et al., 1995
Radio-active protein phosphatase inhibition	Requires special facilities and equipment. Very sensitive.	Radio-active waste generated.	Holmes, 1991
Chromogenic protein phosphatase inhibition	Requires purified enzyme (expensive). Very sensitive. Commercial kits available.	Expensive. Reproducibility a problem.	An and Carmichael, 1994
ELISA	Very sensitive. Reactivity for various microcystins must be checked. Commercial kits available. Does not require sample extractions. Rapid, reproducible and large numbers of samples can be assayed simultaneously. Samples are assayed directly thus eliminating time-consuming and costly extraction procedures.	Intermediate cost (For Envirologix kits: R 6000 per 96 well kit that can be used for assaying 40 samples in duplicate).	Chu et al., 1989 Ueno et al., 1996
Hepatocyte cultures	Requires sterile culture facilities. Toxicity of microcystins to hepatocytes similar to mouse *in vivo* toxicity.	Requires a highly trained scientist to do assays.	Aune and Berg, 1986 Heinze, 1996
Mammalian fibroblast cell culture assays	Requires sterile culture facilities.	Requires a highly trained scientist to do assays. False negatives a problem.	Lawton, et al., 1994

The ELISA technique is currently the most promising method for rapid sample screening for microcystins due to its sensitivity, specificity and ease of operation. Both monoclonal (Kfir, et al., 1986) and polyclonal antibodies (Chu et al., 1989) have been made against microcystins. The polyclonal antibodies have good cross-reactivity with microcystin LR, RR, YR and nodularin and to a lesser extent with microcystin LY and LA. An ELISA employing these polyclonal antibodies have successfully been employed for screening water samples containing microcystin levels as low as 0,2 μg/l (Chu et al., 1990). A major advantage of ELISA tests over other assay systems is the fact that samples can be assayed without prior extraction. All the commercial ELISAs have detection limits for microcystin-LR that is significantly lower than the WHO limit of 1 μg/l. A minor draw-back of the ELISA is that this assay tests for the presence of microcystins and does not give information regarding the toxicity or biological activity of the sample (e.g. a sample testing positive on the ELISA might be solely composed of non-toxic forms of microcystin and harmless to humans and animals i.e. false positive).

1.2 The biochemical changes induced by microcystin in vivo and in vitro

Addition of mycrocystins to cells results in the hyperphosphorylation of several proteins due to the inactivation of the protein phosphatases (Guy, et al., 1992). Injection of microcystin into mice causes an increase in the plasma levels of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), preceding the death of the animal due to hemorrhaging. Guzman, et al., 1999 reported that microcystin causes hepatic inflammation. The inflammation results in macrophage, neutrophil and lymphocyte infiltration of the liver. Several hormones, collectively called the cytokines, regulate the inflammatory response of mammals. Pretreatment of animals with antiserum against TNF-α (a member of the pro-inflammatory cytokines) neutralise the lethal effects of microcystin in a dose dependent manner (Nakano, et al., 1991). Further indirect evidence pointing to the involvement of the immune system in the toxicity of microcystin comes from studies that showed that the immunosuppressive drugs rifampicin and cyclosporin-A produced complete protection against the toxicity of microcystins (Hermansky, et al., 1991). Interleukin-6 is also pro-inflammatory cytokines central in several of the immune processes. When microbes or microbial contaminants are introduced into mammals, an

inflammatory response, which is characterized by fever, diarrhoea and in severe cases by anaphylactic shock (Bauman and Gauldie, 1994; Mimms, 1990), results. IL-6 is directly responsible for the synthesis of all the hepatic acute phase proteins, and this hormone is also responsible for fever and antibody synthesis in mammals (Gauldie, et al., 1987). Figure 1.1 is a graphic presentation of the effect of pathogens on IL-6 secretion by cells.

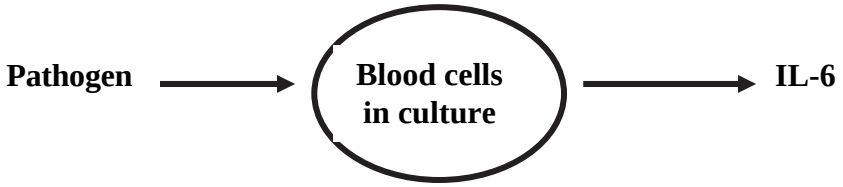

Figure 1.1: The effect of pathogens on blood cells. Pathogens induce IL-6 secretion by blood monocyte-macrophages.

Studies have shown that the isolated gram negative microbial toxin, lipopolysaccharide (LPS) or also called endotoxin, can be used in stead of microbes to induce inflammatory reactions (Pool, et al., 1998). Initial investigations on the effects of microcystin on IL-6 secretion by isolated macrophages and macrophage cell lines have shown that microcystin on its own have no effect on cytokine secretion. However, when microcystin is added to LPS, it results in an increase in the LPS induced IL-6 secretion (Lankoff, et al., 2004). Figure 2 is a graphic representation of the effect of microcystin on IL-6 secretion. When IL-6 recovery is calculated for a specific LPS concentration, it will be 100 % if there is no microcystin in the reaction mixture. If microcystin is present in the reaction mixture the IL-6 recovery will be greater than 100 %.

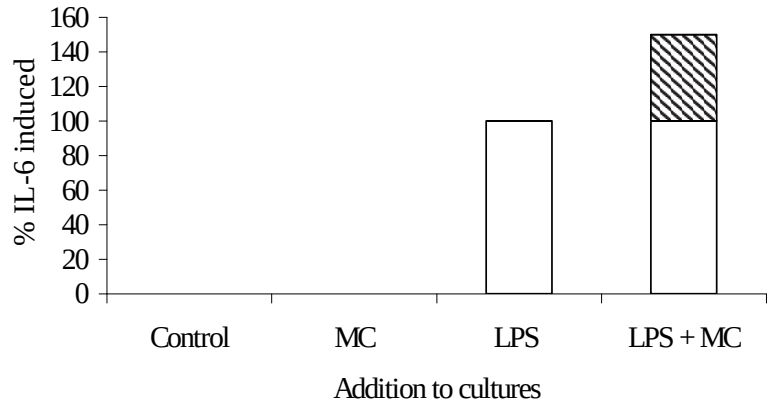

Figure 1.2: Graphic representation of the effect of microcystin on IL-6 secretion by blood cells. Key to additions: Control – water, MC – microcystin, LPS – endotoxin. Shaded area is the elevation of the IL-6 secretion due synergism of microcystin with endotoxin (Lankoff, et al., 2004).

2. OBJECTIVES

The objective of this study was to:

2.1 Determine if microcystin has an immunomodulatory effect using IL-6 as a biomarker.

2.2 Write standard operating procedures for the immunomodulatory assays so that the assays can also be done in other laboratories.

3. METHODS

3.1 In vivo studies using Balb/c mice.

Four groups of 2 mice each were injected intraperitoneally. Group 1 received 200 µl saline only, Group 2 received 200 µl saline containing 100 ng endotoxin, Group 3 received 200 µl saline containing 200 µg/ml microcystin while Group 4 received 200 µl saline containing 100 ng endotoxin and 200 ng microcystin. Blood was obtained by heart puncture 3 hours after the injection with chemicals and then assayed for IL-6 using the Amersham mouse IL-6 ELISA kit (AECI Amersham, SA).

3.2 Collection and culture of human whole blood

Blood from healthy male donors was collected in vacuum tubes containing ethylene-diamine tetra-acetic acid (EDTA) anticoagulant. The blood was diluted 1/10 with Rosswell Park Memorial Institute medium 1640 (RPMI 1640, Highveld Biologicals). Cultures were set up in 96 well plates (Nunc, Denmark)). Samples containing combinations of microcystin-LR (Sigma-Aldrich, US), okadaic acid (Sigma-Aldrich, US) and the E. coli endotoxin (Associates of Cape Cod, US) were diluted in water for injection (WFI, Vaccine Institute, SA). WFI was also included in all assays as a negative control. Samples or controls were added to the wells (20 µl/well). Diluted blood (200 µl/well) was added to the samples. The blood was then cultured overnight at 37 °C. At the end of the culture period the culture supernatants were assayed for IL-6 using an in-house ELISA previously described (Pool et al. 1998). A recombinant

IL-6 (Roche Molecular Biochemicals, South Africa) standard curve was included on each ELISA plate. This ELISA is linear for IL-6 concentrations between 12,5 and 400 pg/ml. A standard curve was generated for each ELISA plate using the Excell computer package and the IL-6 concentrations induced by the samples or controls were read off this curve. See annexure A and B for detailed standard operating procedures (SOPs) of the method used.

3.3 Environmental water samples

Four drinking water samples collected from areas in the Western Cape, South Africa was assayed using the whole blood culture assay described above. Water samples (10 μl/well) were added to the wells of a 96 well culture plate. WFI (10 μl/well) or 2 ng/ml endotoxin in WFI (10 μl/well) was added to the water samples. RPMI was added to the wells to a final concentration of 100 μl/well. The assay was started by the addition of 100 μl/well whole blood diluted 1:4 with RPMI. Cultures were incubated and assayed for IL-6 as described above.

4. RESULTS

4.1 In vivo studies using Balb/c mice

IL-6 was not detected in the mice injected with saline (group 1) or microcystin (group 3) only. Animals that were injected with saline and endotoxin (group 2) or microcystin and endotoxin (group 4) synthesised detectable microcystin concentrations (Table 4.1). These data indicate that higher LPS and/or microcystin levels should be injected in future experiments. Due to the very limited endotoxin and microcystin stocks obtained for this project it was thus decided to concentrate on the *in vitro* whole blood culture assay for subsequent experiments.

Table 4.1: The effect of microcystin on serum IL-6 levels of Balb/c mice.

Group number	Pg/ml IL-6 induced
1 (saline)	ND
2 (saline plus endotoxin)	60 pg/ml
3 (microcystin)	ND
4 (microcystin plus endotoxin)	78

4.2 The effect of microcystin on IL-6 secretion by human whole blood cultures.

Studies done have shown that IL-6 secretion is linear with respect to endotoxin concentration between 25 and 500 pg/ml E.coli endotoxin (Pool, et al., 1998). In this study all assays were done in the linear range for IL-6 secretion. It is postulated that endotoxin spike recovery from water samples not contaminated with microcystin will be approximately 100 % (expressed as a % of IL-6 secretion due to endotoxin only). Samples containing microcystin will have a greater than 100 % endotoxin spike recovery (expressed as a % of IL-6 secretion due to endotoxin only) due to the synergistic effects of microcystin and endotoxin.

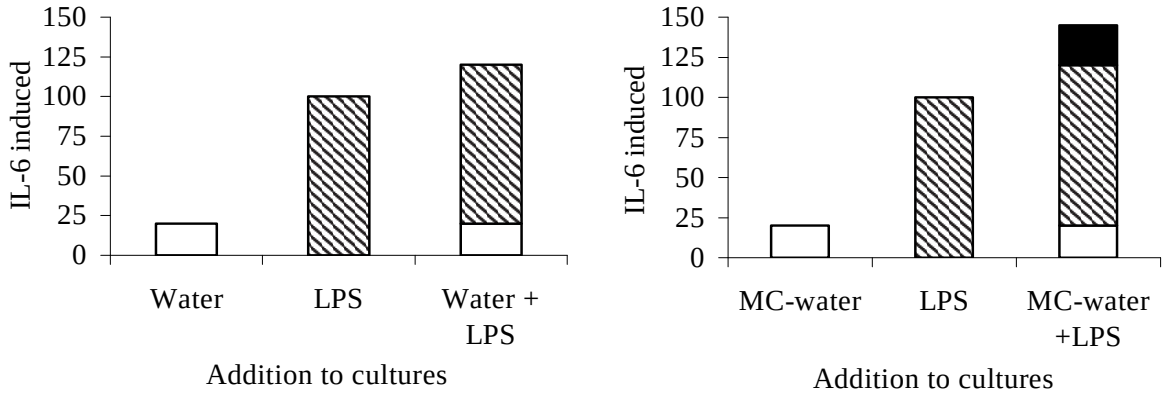

Figure 4.1: Graphic representation of postulate regarding IL-6 induction by samples containing microcystin. It is postulated that IL-6 recovery upon endotoxin addition will be 100 % if there is no microcystin in the sample (shaded area). If there is microcystin in the sample it will act synergistically with the endotoxin and this will cause an increase in IL-6 secretion which is more than 100 % (black area).

In the present study a concentration of 200 pg/ml endotoxin was used for stimulation as this falls within the linear range of the assay. In the absence of LPS very little IL-6 was secreted by whole blood cultures (Table 4.2). The addition of microcystin to cultures in the absence of LPS had no effect on LPS secretion by these cultures. Addition of LPS to the cultures caused a significant increase in the IL-6 secretion of the cells. The addition of microcystin to endotoxin result in Il-6 secretion and the amount of IL-6 secreted was higher then with endotoxin only. This increased IL-6 secretion was maximal at 0.5 ng/ml microcystin. At higher levels of microcystin the amount of IL-6 secreted drop. However, IL-6 synthesis remains above 100 % for microcystin levels between 0.5 to 50 ng/ml. LDH leaching into the medium was monitored as potential biomarker for cytotoxicity due to additives (See Appendix C for SOP of LDH assay). It was found that 5 and 50 ng/ml microcystin significantly increased the amount of LDL leaching. This indicated damage of the cell membranes of cells at these concentrations. The lower IL-6 secretion at 50 ng/ml microcystin compared to 0.5 ng/ml microcystin might be due to cytoxicity at higher microcystin levels.

Table 4.2: The effect of microcystin LR on human whole blood culture.

Additions to cultures		
ng/ml microcystin	**ng/ml LPS**	**pg/ml IL-6 secreted**
0	0.2	113 ± 7
0.5		249 ± 48
5		123 ± 66
50		176 ± 42
0	None	9 ± 0.5
0.5		8 ± 1
5		9 ± 2
50		13 ± 7

4.3 The effect microcystin in drinking water samples on IL-6 secretion by human whole blood cultures.

Drinking water samples were collected from areas in the Western Cape and were initially screened for microcystin using the Envirologix ELISA assay (http://www.envirologix.com). Only one sample (subsequently labeled as MC-LR positive) screened positive for microcystins (0.1 ng/ml). This sample was a kind donation by one of the major water suppliers in the country. Their laboratories also found that this sample contained microcystin LR using HPLC assays. Water samples were assayed using the whole blood culture assay as described above. Water samples were spiked with WFI or endotoxin (see Table 4.3 for details). The results obtained show that samples that are negative for microcystin does not affect the amount of IL-6 secretion due to endotoxin spiking (spike recoveries range from 98 – 101 %). However, the sample containing microcystin-LR caused a major activation of the IL-6 response (37 %). This study shows that the IL-6 response to endotoxin is upregulated in the presence of environmental microcystins.

Table 4.3: The effect of microcystin in drinking water on the IL-6 assay. Water samples were transferred to duplicate tubes. To the control tube only diluted human whole was added. Whole blood and endotoxin to a final concentration of 200 pg/ml was added to the other tube. The whole blood cultures were then incubated overnight at 37 °C. The IL-6 levels secreted into the blood culture supernatants were monitored using an in-house ELISA.

	IL-6 (pg/ml)			
Water sample	**No addition a**	**+ Endotoxin b**	**Spike recovery b – a**	**Spike recovery %**
RO water	9 ± 2	3400 ± 56	3394	100
Stellenbosch	49 ± 6	3372 ± 74	3323	98
Somerset West	9 ± 2	3451 ± 32	3442	101
Kuils River	103 ± 12	3432 ± 41	3329	98
MC-LR positive	234 ± 5	4897 ± 163	4663	137

5. CONCLUSIONS

Several studies on cultured cells have showed that there is a synergistic effect between purified microcystins and endotoxin on tumour necrosis factor secretion (Pahan et al., 1998). The present study shows that a similar synergistic effect for microcystin and endotoxin is seen when IL-6 secretion by whole blood cultures is monitored. A sample of environmental water contaminated with microcystin acted synergistically with added endotoxin, and this resulted in an IL-6 induction that was 37 % higher than the expected value. This study confirms the initial hypothesis and indicates that the IL-6 secretion method might be a valuable system in assessing the biological effects of microcystin containing samples on the immune system.

6. RECOMMENDATIONS.

It is recommended that the IL-6 induction experiments must be repeated with larger numbers of environmental samples (that have been screened for microcystins) to assess the repeatability and reliability of the IL-6 recovery assay as a bio-assay for microcystins.

7. REFERENCES

An, J., Carmichael, W.W. 1994. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immuno sorbent assay for the study of microcystins and nodularins. Toxicon. 32, 1495-1507.

Aune, T., Berg, K. 1986. Use of freshly prepared hepatocytes to study toxicity of blooms of blue-green algae *Microcystis aeroginosa* and *Oscillatoria agardii*. J. Toxic. Environ. Health. 19, 325-336.

Baumann, H, Gauldie, J. 1994. The acute phase response. Immunol. Today. 15, 74-80.

Beasley, V.R., Cook, W.O., Dahlem, A.M., Hooser, S.B., Lovell, R.A., Valentine, W.M. 1989 Algae intoxication in livestock and waterfowl. Vet. Clin. North. A. 5, 345-61.

Botes, D.P., Tuinman, A.A., Wessels, P.L., Viljoen, C.C., Kruger, H., Williams, D.H., Santikarn, S., Smith, R.J., Hammond, S.J. 1984. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. J. Chem. Soc. Perkin Trans. 1, 2311-2318.

Chu, F.S., Huang, X., Wei, R.D. 1990. Enzyme-linked immunosorbent assay for microcystins in blue-green algal blooms. J. Assoc. Analyt. Chem. 73(3), 451-456.

Chu, F.S., Huang, X., Wei, R.D., Carmichael, 1989. Production and characterisation of antibodies against microcystins. Appl. Environ. Microbiol. 55, 1928-1933.

Falconer, I.R. 1993. Measurement of toxins from blue-green algae in water and foodstuffs. In: I.R. Falconer (ed) Algal toxins in seafood and drinking water. Academic press, London. 165-175.

Falconer, I.R., Jackson, A.R.B., Langley, J., Runnegar, M.T. 1981. Liver pathology in mice in poisoning by the blue-green algae *Microcystis aeruginosa*. Aust. J. Biol. Sci. 43, 179-187.

Fujiki, H., Saganuma, M. 1999. Unique features of the okadaic acid activity class of tumor promotors. Cancer Res. Clin. Oncol. 125, 150-155.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., Baumann, H. 1987. Interferon beta2/ B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. U.S.A. 84(20), 7251-7255.

Gehringer MM, Kewada V, Coates N, Downing TG. 2003. The use of *Lepidium sativum* in a plant bioassay system for the detection of microcystin-LR. Toxicon. 41(7), 871-876.

Guy GR, Cao X, Chua SP, Tan YH. 1992. Okadaic acid mimics multiple changes in early protein phosphorylation and gene expression induced by tumor necrosis factor or interleukin-1. J Biol Chem. 267(3), 1846-1852.

Guzman, R.E., Solter P.F. 1999. Hepatic oxidative stress following prolonged sublethal microcystin LR exposure. Toxicol. Pathol. 27(5), 582-588.

Heinze, R. 1996. A biotest for hepatotoxins using primary hepatocytes. Phycologia. 35 (Supplement), 89-93.

Hermansky, S.J., Stohs, S.J., Eideen, Z.M., Roche, V.F., Mereish, K.A. 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. J. Appl. Toxicol. 11, 65-73.

Hescheler, J., Mieskes, G., Ruegg, J.C., Takai, A., Trautwein, W. 1988. Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. Pflugers Arch. 412, 248-252.

Holmes, C.F.B. 1991. Liquid chromatography linked protein phosphatase bio-assay; a highly sensitive marine bioscreen for okadaic acid and related diarrhetic shellfish toxins. Toxicon. 29, 469-477.

Kfir, R., Johannsen, E., Botes, D.P. 1986. Monoclonal antibodies specific for cyanoginosin-LA: Preparation and characterisation. Toxicon. 24, 543-552.

Kiviranta, J., Abdel-Hameed, A., Sivonen, K., Niemela, S.I., Carlberg, G. 1993. Toxicity of cyanobacteria to mosquito larvae: screening of active compounds. Environ. Toxicol. Water Qual. 8, 63-71.

Kiviranta, J., Sivonen, K., Niemela, S.I. 1991. Detection and toxicity of cyanobacteria by *Artemia salina* bioassay. Environ. Toxicol. Water Qual. 6, 423-426.

Kos, P., Gorzo, G., Suranyi, G., Borbely, G. 1995. Simple and efficient method for isolation and measurement of cyanobacterial hepatotoxins by plant tests (*Sinapis alba L.*). Anal Biochem 225(1):49-53.

Krishnamurthy, T., Carmichael, W.W., Sarver, E.W. 1986. Toxic peptides from freshwater cyanobacteria (blue-green algae). Isolation, purification and characterisation of peptides from *Microcystis aerugenosa and Anabaena flosaguae*. Toxicon. 24, 865-873.

Lankoff, A., Carmicheal, W.W., Grasman, K.A., Yuan, M. 2004. The uptake kinetics and immunotoxic effects of microcystin-LR in human and chicken peripheral lymphocytes in vitro. Toxicology 204, 23-40.

Lawton, L.A., Beattie, K.A., Hawser, S.P., Campbell, D.L., Codd, G.A. 1994. Evaluation of assay methods for the determination of cyanobacterial hepatotoxicity. In: G.A. Codd, T.M. Jefferies, C.W. Keevil and E. Potter (eds) Detection methods for cyanobacterial toxins, Special publication No. 149, The royal society of chemistry, Cambridge. 111-116.

Nakano, Y., Shirai, M., Mori, N., Nakano, M 1991. Neutralisation of microcystin shock in mice by tumor necrosis factor alpha antiserum. Appl. Environ. Microbiol. 57(1), 327-330.

Mimms, C.A. 1990. The pathogenesis of Infectious Disease. London: Academic Press; San Diego.

Pool, E.J., Johaar, G., James, S., Petersen, I., Bouic, P. J. 1998. The detection of pyrogens in blood products using an *ex vivo* whole blood culture assay. Immunoassay 19(2&3), 95-111.

Suganuma, M., Fujiki, H., Okabe, S., Nishiwaki, S., Brautigan, D., Ingebritsen, T.S., Rosner, M.R. 1992. Structurally different members of the okadaic class selectively inhibit protein serine/threonine but not tyrosine phosphatase activity. Toxicon. 30, 873-878.

Swoboda, U.K., Dow, C.S., Chaivimol, J., Smith, N., Pound, B.P. 1994. Alternatives to the mouse bioassay for cyanobacterial toxicity assessment in: G.A. Codd, T.M. Jefferies, C.W. Keevil and E. Potter (eds). Detection methods for cyanobacterial toxins. Special publication No. 149, The royal society of chemistry, Cambridge. 106-110.

Theis, W.W., Carmichael, W.W. 1986. Physiological effect of a peptide toxin produced by the freshwater cyanobacteriua (blue-green algae) *Microcystis aeruginosa* strain 7820. In P.S. Steyn and R. Vieggaar (ed), Mycotoxins and phycotoxins. Elsevier Science Publishers, Amsterdam. 353-364.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Yoshida, F., Suttajit, M., Mebs, D., Putsch, M., Vasconcelos, V. 1996. Survey of microcystins in environmental water by a highly sensitive immunoassay based on monoclonal antibody.

Survey of microcystins in environmental water by a highly sensitive immunoassay based on monoclonal antibody. Nat Toxins. 4(6), 271-276.

Van Halderen, A., Harding, W.R., Wessels, J.C., Schneider, D.J., Heine, E.W., Van der Merwe, J., Fourie, J.M. 1995. Cyanobacterial (blue-green algae) poisoning of livestock in the Western Cape Province of South Africa. J. S. Afr. Vet. Assoc. 66(4), 260-264.

Zhou, L., Yu, H., Chen, K. 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed. Environ. Sci. 15(2),166-171.

Appendix A

ZOOLOGY DEPARTMENT
NATUURWETENSKAPPE BUILDING
MERIMAN AVENUE

WHOLE BLOOD CULTURE (WBC) ASSAY FOR PYROGENS
1: ACTIVATION OF WHOLE BLOOD CULTURES (2PAGES)

SOP **PREPARED BY: Dr. EJ Pool**

TASK	Done by	Date
1. COLLECTION OF BLOOD		
1.1 Collect blood in 5ml heparin/EDTA vacuum tube	…….	
1.2 Donor: ……..…….……. Sex: …….	…….	
1.3 Age of Donor: …….years	…….	
Type of tube: …….. Batch number tubes: …….	…….	……...
2. STANDARDS and SAMPLES		
2.1 Endotoxin standard Batch number: ……. Endotoxin concentration: …….	…….	…….
2.2 Samples Description:……	…….	…….
3. ACTIVATION PROCEDURE		
Pipette 20 µl sample/standard per well of a tissue culture plate or individual 1.5 ml platic culture tubes (use plate format on next page to fill in sample numbers).	…….	…….
RPMI Medium Batch:……. Manufacturer:…….	…….	…….
Add 80 µl RPMI medium to each sample well or tube	…….	…….
Dilute heparinised blood (1ml Blood + 4 ml RPMI)	…….	…….
Add 100 µl diluted heparinised blood to each sample well or tube	…….	…….
Incubate overnight at 37 °C. Temperature of incubator: …….	…….	…….
Incubation starting time: …….	…….	…….
Incubation finished: …….	…….	…….
4. COLLECTION OF CULTURE SUPERNATANTS		
Pipette 75 µl culture supernatant of culture into microfuge tubes.	…….	…….
[N.B. take precautions not to disperse cells. If cells are taken of accidentally, centrifuge the culture for 1 min at 5000 rpm and then proceed to take off supernatant.]. Use for IL-6 ELISA.	…….	…….
Store supernatants at -20 °C.	…….	…….

Remarks	Date
……	
Signed: …….	…….

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Appendix B

ZOOLOGY DEPARTMENT
NATUURWETENSKAPPE GEBOU
MERIMAN AVENUE
STELLENBOSCH

ASSAY OF SAMPLES FOR IL-6

SOP NUMBER: #0002 **PREPARED BY: Dr. EJ Pool**

EFECTIVE DATE: 1 November 2000 **EXPIRY DATE: 1 November 2002**

TASK	Done by	Date
1. Remove appropriate number of coated strips from fridge.	…….	……..
2. **APPLICATION OF STANDARDS AND SAMPLES** Apply 25 µl sample or standard per well. Use the plate lay-out supplied on last page and fill in the identity of the samples. Standards Batch number: …….	…….	……..
3. **DETECTING ANTIBODY** Prepare detecting antibody by mixing 2 volumes of antibody concentrate with 1000 volumes of diluent. Antibody concentrate Batch number: ……. Volume: 10 µl Diluent Batch number: ……. Volume: 5 ml. Add 25 µl of antibody to each well Incubate for 2 hours at 37 °C on an orbital shaker. Incubation starting time: ……. Incubation finished: ……. Wash plate 4 x with wash buffer. Use 200 µl per well	…….	…….
4. **ENZYME CONJUGATE** Prepare conjugate by mixing 4 volume of avidin-enzyme conjugate with 10000 volumes of diluent. Volume of avidin-enzyme conjugate: 4µl Volume of diluent: 10 ml Add 50 µl of avidin-enzyme conjugate to each well Incubate for 20 minutes at room temperature on an orbital shaker. Incubation starting time: ……. Incubation finished: ……. Wash plate 4 x with wash buffer. Use 200 µl per well	…….	…….

Remarks	Date
…… …… Signed: …….	…….

TASK	Done by	Date
5. **ENZYME REACTION** Batch number of substrate: …… Add 50 µl substrate per well. Incubate 10 minutes at ambient temperature. Add 50 µl H_2SO_4 per well. Read the optical density of the wells at 450 nm on a plate reader. Attach print-out to SOP	 ……. …….	 …….. ……..
6. **CALCULATION OF IL-6 CONCENTRATIONS** Plot OD450 of standards against the IL-6 concentration of the standards Read the IL-6 concentration of the samples off this curve (A computer package such as Excell can be used for this purpose)	 …….	 ……..

Remarks	Date
…… …… Signed: …….	 …….

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
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H												

Appendix C

SOP: 03 **Cytotoxicity assay** **Prepared by**: EJ Pool	**University of Stellenbosch**	Page 1 of 3 **Effective date**: 4 March 2002

1. DESCRIPTION

This procedure describes the use of the Sigma colorimetric assay for lactate dehydrogenase (LDH).

2. PRINCIPLE

LDH catalyses the reaction

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactic acid} + \text{NAD}$$

The reaction strongly favours reduction of pyruvate to lactate at a rate proportional to the amount of LDH. Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely coloured hydrazone which has a peak absorbance between 400 and 550 nm. The amount of pyruvate remaining after the reaction is inversely proportional to the amount of LDH in the sample. Thus the intensity of the colour reaction is also inversely proportional to the amount of LDH in the sample.

3. REAGENTS

3.1 Sigma LDH kit (cat#500C-1KT) containing

Substrate

Colour reagent

NADH

3.2 LDH standard (100 %)

3.3 2M NaOH stop solution (4 g NaOH per 50 ml)

Checked by: ……..

Date: ……..

4. METHOD

4.1 Prepare working substrate by mixing 1 ml substrate to one container of NADH.

4.2 Prepare standards and samples in well on a 96 well plate using the table:

Well No	ID	µl saline	µl Sample	Substrate
A1;A2	No LDH	10	0	50
B1;B2	20 % LDH	20	0	40
C1;C2	40 % LDH	30	0	30
D1;D2	60 % LDH	40	0	20
E1;E2	80 % LDH	50	0	10
F1;F2	100 % LDH	60	0	0
G1;G2	Unknown 1	0	10	50
H1;H2	Unknown 2	0	10	50

4.3 Mix by tapping the plate.

4.4 Incubate plate at 37 °C for 30 minutes.

4.5 Add 50 µl colour reagent per well. Mix by tapping the plate.

4.6 Incubate plate at room temperature for 20 minutes.

4.7 Stop the reaction by adding 50 µl NaOH solution per well. Mix by tapping the plate.

4.8 Read absorbance on a plate reader at 450 nm. Attach to SOP.

Done by: ……..

Date: ……..

5. RESULTS

5.1 Create a standard curve by plotting the average absorbance at 450 nm against the % LDH standard. Use graph paper or a computer package such as Excell to prepare the standard curve.

Attach graph of standard curve to this SOP.

Done by: ……..

Date: ……..

5.3 Obtain the % LDH of the unknowns using the standard curve. Attach results to SOP.

Done by: ……..

Date: ……..

6. COMMENTS

Comment	**Signature**	**Date**