

The Cooperative Research Centre for

**Water Quality and Treatment**



# Acute Skin Irritant Effects of Blue-Green Algae in Healthy Volunteers



Research Report

25

# **Acute Skin Irritant Effects of Blue-Green Algae in Healthy Volunteers**

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Research Report: Acute Skin Irritant Effects of Blue-Green Algae in Healthy Volunteers

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## **FOREWORD**

Title: Acute Skin Irritant Effects of Blue-Green Algae in Healthy Volunteers

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CRC for Water Quality and Treatment Project No. 1.3.1.5 – Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers

## EXECUTIVE SUMMARY

This project was initiated by the CRC for Water Quality and Treatment in 2000 with guidance and support from the New South Wales Health Department, Eraring Energy Pty Ltd and the Sydney Catchment Authority. These partners recognised at that time that the local and international recreational guidelines for cyanobacteria were not soundly evidence-based. It was felt that the revision and development of new guidelines for the cyanobacterial risks in recreational water, being undertaken by the National Health and Medical Research Council (NHMRC) in Australia, would benefit from some specific research on skin irritation effects of cyanobacteria on humans. This was perceived as a knowledge gap for determining the importance of skin contact with cyanobacteria during water recreational activity.

The project was developed in the context of a debate within Australia that the management practice of restricting access and bathing, when the currently accepted cyanobacterial guideline value for cell density of 15,000 or 20,000 cells/mL (at that time) was exceeded, was unduly restrictive to water users. This was seen as particularly the case where there were few documented or even anecdotal reports of adverse health effects at these cell densities. Furthermore, these closures of lakes or water bodies caused both economic and social hardship in areas where affected water bodies are an important social amenity, without any demonstrated benefit of improved public health outcomes.

The aim of this study was to assess the skin irritant potential of a range of cyanobacterial types or species with human volunteers to determine thresholds for these effects. The irritant effects were assessed both qualitatively and quantitatively, and the cyanobacteria were characterised in terms of species type and cell density and analysed for a range of known cyanotoxins (ie. microcystin, nodularin, cylindrospermopsin and saxitoxins).

In addition, the study also set out to determine the quantitative nature of dose-response for skin irritation by cyanobacteria in terms of both cell numbers and cell mass, represented by biovolume. The guidelines in use at the time for cyanobacteria in recreational waters were based upon cyanobacterial cell counts (WHO, 2003). However, there has been discussion that it may not be appropriate to use cell counts as the single quantitative indicator of cyanobacterial material. The reaction to cyanobacterial contamination may be expected in some cases to be proportional to contact with the mass of cell material (estimated by biovolume), which can be quite different for equivalent cell densities (cells/mL) of different species. This is because the biovolume, derived by calculation from the cell count and the average cell volume, varies over a wide range for the equivalent cell density of different species, due to their properties of widely different cell shapes and sizes.

### Research Design

This study was designed to determine both qualitative and quantitative irritant effects of cyanobacteria to the skin of healthy individuals. An additional component investigated the inherent variability associated with laboratory assessment of cyanobacteria in water, by assessing the variability between experienced laboratories that routinely carry out cell count and cell volume measurements.

Cell suspensions and extracts of cyanobacterial cultures of *Microcystis aeruginosa* (non-toxic strain), *Anabaena circinalis* and *Nodularia spumigena* were applied to 64 volunteers in one trial, and *Microcystis aeruginosa* (toxic strain), *Aphanocapsa incerta* and *Cylindrospermopsis raciborskii* were applied to 50 volunteers in a second trial. The cell suspensions were applied across a concentration range for each organism ranging from less than 5,000 to greater than 200,000 cells/mL. The suspensions were applied in random order to the upper torso (back) of healthy human volunteers using standard adhesive skin patches. In addition, the applications included two different treatments of each cyanobacterial culture: whole and lysed cells. Positive (sodium lauryl sulphate) and negative (culture media) controls were also applied in the patches. Patches were removed after 24 hours and assessment of erythema was undertaken by a specialist dermatologist physician. The trial was a double-blind design, ie. both the researchers applying the patches and the physician reading the reaction were not made aware of the particular cyanobacterial species, toxicity status and cell density in the patch distributions across each trial.

The study also investigated the variability of cell count and biovolume measurements both within and between three different laboratories for the cyanobacterial suspensions applied to volunteers in this trial. The laboratories used equivalent methods for determining cell numbers and for the measurement of cell volume. Two of the laboratories were NATA-registered and all were staffed by experienced and trained algal technicians. The study was designed to identify variability associated with conventional estimates of cell numbers in water quality laboratories, and to determine whether these analytical estimates would need to be considered in comparison of algal estimates against guideline values.

### Results

On average, between 20% and 24% of individuals  $\pm 16\%$  (95% confidence interval, CI) reacted across the concentration range tested for the six cyanobacteria. The reaction rates were lower, between 11% and 15%, with a CI of  $\pm 8\%$ , when those individuals who also reacted to negative controls were removed. The reactions were mostly mild, and in all cases were resolved without

treatment within a short time period (24-72 hours). This was the case for both whole and lysed cells with little difference in reaction rates between these two treatments. There was also no dose-response across the concentration range for any of the cyanobacteria tested. Similar patterns of reaction were observed for atopic and non-atopic individuals. Atopic individuals were defined as those who had reported a previous diagnosis of hay fever, asthma, eczema or other severe skin irritation.

In the component of the study involving the evaluation of cell count analyses between different laboratories, the comparison revealed a very large coefficient of variation (CV) for cell counts between laboratories. However, no trend could be identified either within the concentration range analysed or between the participants. For example, for *Nodularia spumigena* the CV was 18 - 43% for the lowest cell density sample, and decreased to 4 - 12% at the highest cell density. However, the reverse was observed for samples of *Cylindrospermopsis raciborskii* with CV increasing from 21% at lowest cell densities to 58% for the highest cell density samples. For the remainder of the cyanobacteria, including both *Microcystis aeruginosa* strains, *Aphanocapsa incerta* and *Anabaena circinalis*, the CV did not change significantly across the cell concentration range. The calculated cell volume estimates for each cyanobacterial species also varied between laboratories. The greatest variation occurred for *Nodularia spumigena*, which showed a CV of 66%, and the lowest variation of 4%, was recorded for *Cylindrospermopsis raciborskii*.

## Conclusions

This study has shown that only a small percentage of individuals exhibited skin irritation when exposed to cyanobacteria at a range of environmentally relevant concentrations that are normally encountered in freshwaters used for contact recreation. This suggests that a small proportion of the healthy people in the population (around 20%) may develop a skin reaction to cyanobacteria in the course of normal water recreation. Further, the reactions were mild and were resolved without treatment. The response was not dose-related making it impossible to determine a minimum exposure level to fully prevent the occurrence of skin irritation during bathing and recreational activity. The results are also not conclusive in identifying a link between atopic individuals and skin irritation making it difficult to target any particular groups with warnings about their susceptibility if exposed to waters containing cyanobacteria. The study suggests that the potential for skin irritation cannot be readily translated into a quantitative bathing or water recreation guideline for cyanobacterial cell levels. This could only be done with further research to better characterise those individuals that do experience skin irritation reactions or possibly allergic reactions associated with cyanobacteria. In any case, the minor nature of the potential for irritant

dermatitis from this type of exposure can be regarded as less important than the need to protect against potential adverse outcomes from oral ingestion of cyanobacteria (hepato- and neurotoxins) in recreational waters.

The study indicated that large variations can occur between experienced laboratories in analyses for both cell count and cell volume measurements. This is in spite of the use of standard methods that aim to minimise analytical error. This inherent variability needs to be considered in assessment of algal analytical data, and be part of the judgement for comparison of results against guidelines.

## Recommendations

It is not possible or appropriate to derive a quantitative guideline for protection against skin irritation reactions caused by cyanobacteria in normal water recreation situations. This is because even though a small percentage of individuals may develop a skin irritation reaction, the reaction is mild and idiosyncratic, and there is no threshold or dose-response. However, it may be appropriate to issue a general precautionary warning in circumstances where cell numbers are elevated and exposure is likely.

Further, it is recommended that recreational guidelines should be derived based primarily upon the better known and characterised health risks associated with ingestion of cyanobacteria and their known toxins. The guideline in this case could be developed using animal toxicity data and conventional toxicological calculations to derive a protective level for sub-chronic exposure to cyanobacteria and cyanotoxins via ingestion in a typical recreational situation.

The high degree of variability associated with estimates of cell numbers of cyanobacteria in water by conventional counting techniques needs to be considered in both setting and interpreting cell-based and biovolume-based water guidelines. This may require setting conservative levels that take into account large variations in estimates of cell density and cell volume in the sampling and analytical process.

## Guideline Development

The findings of this study have been considered as part of the revision of NHMRC guidelines for recreational exposure to cyanobacteria and algae in freshwater (NHMRC, 2005).

The review assessed the results of this study (also published as Pilotto *et al* (2004)) and noted that "human skin contact with cyanobacteria across a wide cell density range results in a somewhat idiosyncratic response." (NHMRC, 2005). In addition, "these mild skin irritative

effects, which are readily resolved without medical treatment, do not warrant consideration in the setting of a quantitative guideline for recreational exposure” (NHMRC, 2005).

As a result, the revised Level I of the Australian guideline for cyanobacteria in freshwater recreational situations is therefore based upon risk of exposure to microcystin toxins via ingestion. The derivation used animal toxicity

data for microcystin toxins and conventional toxicological calculations recommend a guideline for short-term (14-day) exposure to microcystins via ingestion for both children and adults (NHMRC, 2005). This research project has therefore provided useful information for the development of public health guidelines in the Australian context.

## TABLE OF CONTENTS

<b>Foreword .....</b>	<b>3</b>
<b>Executive Summary.....</b>	<b>4</b>
<b>Contents.....</b>	<b>7</b>
<b>List of Figures .....</b>	<b>8</b>
<b>List of Tables.....</b>	<b>9</b>
<b>List of Plates .....</b>	<b>10</b>
<b>Abbreviations.....</b>	<b>10</b>
<b>1. Introduction .....</b>	<b>11</b>
1.1 Aim.....	11
1.2 Background .....	11
<b>2. Material and Methods .....</b>	<b>15</b>
2.1 Recruitment of Volunteers and Ethical Approval .....	15
2.2 Preparation of Cyanobacterial Cultures.....	15
2.3 Skin Irritation Tests .....	15
2.4 Statistical Methods .....	18
2.5 Cell Counting and Biovolume Measurement .....	20
<b>3. Results.....</b>	<b>21</b>
3.1 Skin Irritation.....	21
3.1.1 Clinical Gradings (Subjective Readings).....	21
3.1.2 Mexameter Data (Objective Readings).....	21
3.1.3 Atopy Status .....	22
3.2 Cell Counting and Biovolume Measurement .....	22
<b>4. Discussion.....</b>	<b>39</b>
4.1 Skin Irritation.....	39
4.2 Cell Counting and Biovolume Measurement .....	41
<b>5. Summary and Conclusions.....</b>	<b>43</b>
5.1 Skin Irritation.....	43
5.2 Cell Counting and Biovolume Measurement .....	43
<b>6. Recommendations .....</b>	<b>44</b>
6.1 Skin Irritation.....	44
6.2 Cell Counting and Biovolume Measurement .....	44
<b>7. Acknowledgements .....</b>	<b>45</b>
<b>8. References .....</b>	<b>46</b>
<b>Appendix I: Ethics Approval .....</b>	<b>48</b>
<b>Appendix II: Guideline Development, Publications and Conferences.....</b>	<b>51</b>

**LIST OF FIGURES**

Figure 1:	Percentage of subjects with clinical gradings (bars) and mean mexameter measurements (dots) for negative and positive control patches in each round.....	23
Figure 2a:	Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for all 64 subjects in Rounds 1 and 2.....	24
Figure 2b:	Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for 49 subjects in Rounds 1 and 2 who did not react to negative control patches.....	25
Figure 3a:	Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for all 50 subjects in Round 3.....	26
Figure 3b:	Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for 39 subjects in Round 3 who did not react to negative control patches .....	27
Figure 4a:	Distribution of clinical gradings for whole and lysed cyanobacterial patches (MA338, AC and NS) for 20 non-atopic and 29 atopic subjects with no response to negative controls in Rounds 1 and 2.....	36
Figure 4b:	Distribution of clinical gradings for whole and lysed cyanobacterial patches (MA309, CR and AI) for 24 non-atopic and 15 atopic subjects with no response to negative controls in Round 3 .....	36

## LIST OF TABLES

Table 1:	General features of the cyanotoxins (after Sivonen and Jones, 1999; +Li <i>et al</i> , 2001; *Moore 1977; #Fujiki <i>et al</i> , 1981; †Cox <i>et al</i> , 2003).....	12
Table 2:	Intracellular and total toxin concentration for cultures of cyanobacteria with highest cell densities used in Rounds 1, 2 and 3 and corresponding total toxin and intracellular toxin concentration per cell.....	16
Table 3:	Cyanobacteria and cell densities used in Rounds 1, 2 and 3 of the skin irritation study as measured in-house by the Biology Research Laboratory at the Australian Water Quality Centre.....	18
Table 4:	Equations used to calculate cell volumes and methods for measuring length and breadth of cells.....	20
Table 5:	Odds ratios (OR) and 95% confidence intervals (CI) for clinical response (grades 1 to 4) estimated in logistic regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) .....	28
Table 6:	Number (n) and percentage (%) of subjects with positive gradings to at least one active (non-control) patch overall and subdivided by whether or not subjects were graded with positive responses to negative controls.....	29
Table 7:	Mean percentages of subjects and 95% confidence intervals (CI) reacting to an active (non-control) patch by species, overall (Total) and for the subset of subjects not reacting to negative controls.....	29
Table 8a.	Odds ratios (OR) and 95% confidence intervals (CI) for clinical response estimated in logistic regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3).....	30
Table 8b.	Odds ratios (OR) and 95% confidence intervals (CI) for clinical response estimated in logistic regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls .....	31
Table 9a.	Mean difference and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) .....	32
Table 9b.	Mean difference and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls.....	33
Table 10a.	Means and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) .....	34
Table 10b.	Means and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls .....	35
Table 11:	Comparison of cell counts from three laboratories for six different cyanobacteria at six different concentrations.....	37
Table 12:	Cell measurements and calculated cell volumes for each of the six cyanobacteria at all three laboratories. ....	38

## LIST OF PLATES

Plate 1:	Cyanobacterial cell suspensions added to wells in adhesive patches ready for application to subjects' backs.....	17
Plate 2:	Adhesive patches applied to subject's back.....	17
Plate 3:	Close up view of subject's back after removal of patches. Area of positive reaction is shown.....	19
Plate 4:	Measurement of skin reaction to cyanobacterial cultures using Mexameter.....	19

## ABBREVIATIONS

AC	<i>Anabaena circinalis</i> strain 118AR
Active Patches	Patches containing cyanobacteria
AI	<i>Aphanocapsa incerta</i> strain 001
CI	95% confidence interval
CR	<i>Cylindrospermopsis raciborskii</i> strain 031C
CV	Coefficient of variation (CV) ie. standard deviation as a percentage of mean
GEE	Generalised Estimating Equation
LPS	Lipopolysaccharides
MA338	<i>Microcystis aeruginosa</i> strain 338
MA309	<i>Microcystis aeruginosa</i> strain 309 (1)CA
n	Number of individuals
NS	<i>Nodularia spumigena</i> strain 001E
OR	Odds ratio

## I INTRODUCTION

### I.1 AIM

The aim of this study was to assess the skin irritant potential of a range of cyanobacterial types or species with human volunteers to determine thresholds for these effects. The irritant effects were assessed both qualitatively and quantitatively, and the cyanobacteria were characterised in terms of species type, cell density and analysed for a range of known cyanotoxins (ie. microcystin, nodularin, cylindrospermopsin and saxitoxins).

### I.2 BACKGROUND

A significant proportion of cyanobacteria produce one or more of a range of compounds that are potent toxins to vertebrates including mammals (Table I). If high concentrations of toxic cyanobacteria are ingested in drinking water, or accidentally during recreation, they can represent a hazard to human health. Cyanotoxins belong to a chemically diverse group of substances, each of which shows specific toxic mechanisms in animals. Some cyanotoxins are strong neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins). Others are primarily hepatotoxins ie. toxic to the liver (microcystins, nodularin and cylindrospermopsin), and yet others (eg lipopolysaccharides) may cause health impairments such as gastroenteritis or dermal effects, and the importance and health significance of these are poorly understood (Sivonen and Jones, 1999). There is a long history of animal poisonings and adverse health outcomes associated with exposure to cyanobacteria in freshwaters. The best documented early report in Australia confirming poisoning of animals is that of Francis (1878).

The evidence surrounding adverse health effects from recreational activity in water contaminated by cyanobacteria is more recent, varies in quality and is often anecdotal (Box I).

There are three possible routes of exposure to water contaminated with cyanobacteria or cyanotoxins during recreational activity: direct oral ingestion, inhalation via aerosols, and dermal contact. The relative importance of these routes has not been studied in a systematic way. Furthermore, the nature of reports for both dermal and allergic reactions is sporadic and largely anecdotal. Dermal effects from cyanobacterial exposure have been reported at Lakes Alexandrina and Albert in South Australia involving *Nodularia spumigena* blooms (Soong, 1992) and in the Murray River, South Australia involving the genera *Anabaena*, *Aphanizomenon* and *Oscillatoria* (El Saadi et al, 1995). Pilotto et al (1997) carried out a comprehensive survey of health effects in people undertaking normal water recreation in waters contaminated by cyanobacteria at several sites across south-eastern Australia. The participants recorded skin irritations as one of a class of

symptoms after exposure to relatively low cell densities of cyanobacteria.

Dermal toxicity of cyanobacteria has been ascribed to lipopolysaccharides (LPS), which are produced by all cyanobacteria (Ressom et al, 1994). LPS are an integral component of the cell wall of all Gram-negative bacteria, including cyanobacteria. Some LPS can elicit irritant and allergenic responses in human and animal tissues that come into contact with the compounds (Sivonen and Jones, 1999). However cyanobacterial LPS has been shown to be less potent than LPS from pathogenic gram-negative bacteria such as *Salmonella* (Keleti and Sykora, 1982; Raziuddin et al, 1983). Torokne et al (2001) even argues that the lipopolysaccharides of bacteria living in association with the cyanobacteria may be the actual cause of skin irritation.

The few studies linking cyanobacteria to skin contact irritant effects are observational, with limited or no measurement of personal levels of exposure (eg. Pilotto et al, 1997), and therefore do not provide a basis for the establishment of guidelines for recreational activities involving water contact in water contaminated with cyanobacteria.

The issue of guidelines to protect recreational users in Australia from cyanobacteria was first discussed by the NSW Blue-Green Algal Task Force in 1992 (NSWBGATF, 1992). They suggested 15,000 cells/mL of total cyanobacterial cells for a "High Alert Level", regardless of species. This was based upon drinking water alert levels for protection against human exposure to toxins at that time. This was followed by a "guideline" or advisory level proposed by ARMCANZ (Agriculture and Resource Management Council of Australia and New Zealand) in a paper by Johnstone (1993), which stated the following in relation to guidelines for recreational use and cyanobacteria:

"On the basis of reports that have established links between skin contact with cyanobacteria and adverse health effects and the apparent variability in sensitivity it is reasonable to conclude that contact with visible levels of cyanobacteria may constitute a health risk for sensitive individuals....20,000 cells/mL would correspond to a slight discolouration of water and would satisfy the criteria for accepting that discoloured water poses a potential health risk."

This value, while also being non-specific for different types of cyanobacteria, has been widely applied in Australia for advice for guidance, and in many circumstances for restriction of recreational activity in water contaminated by cyanobacteria.

Table 1: General features of the cyanotoxins (after Sivonen and Jones, 1999; +Li et al, 2001; \*Moore 1977; #Fujiki et al, 1981; †Cox et al, 2003).

Toxin Group	Primary target organ in mammals	Cyanobacterial genera
<b>Cyclic Peptides</b>		
Microcystins	Liver. Possible carcinogen in this and other tissues	<i>Microcystis</i> , <i>Anabaena</i> , <i>Planktothrix</i> ( <i>Oscillatoria</i> ), <i>Nostoc</i> , <i>Hapalosiphon</i> , <i>Anabaenopsis</i> , <i>Aphanizomenon ovalisporum</i>
Nodularin	Liver. Possible carcinogen	<i>Anabaena</i> , <i>Planktothrix</i> ( <i>Oscillatoria</i> ), <i>Aphanizomenon</i>
<b>Alkaloids</b>		
Anatoxin-a	Nerve synapse	<i>Anabaena</i> , <i>Planktothrix</i> ( <i>Oscillatoria</i> ), <i>Aphanizomenon</i> , <i>Cylindrospermopsis</i>
Anatoxin-a(S)	Nerve synapse	<i>Anabaena</i>
Aplysiatoxins	Skin. *Possible tumour promoter	<i>Lyngbya</i> , <i>Schizothrix</i> , <i>Planktothrix</i> ( <i>Oscillatoria</i> )
Cylindrospermopsins	Liver and possibly kidney. Possibly genotoxic and carcinogenic	<i>Cylindrospermopsis</i> , <i>Aphanizomenon</i> , <i>Umezakia</i> , * <i>Raphidiopsis</i>
Lyngbyatoxin-a	Skin, gastrointestinal tract. #Possible tumour promoter	<i>Lyngbya</i>
Saxitoxins	Nerve axons	<i>Anabaena</i> , <i>Aphanizomenon</i> , <i>Lyngbya</i> , <i>Cylindrospermopsis</i>
Lipopolysaccharides (LPS)	Potential irritant; affects any exposed tissue	All
†β-methylamino-L-alanine (BMAA)	†Possible cause of Amyotrophic lateral sclerosis/Parkinson-dementia in Chamorro people of Guam	†Nostoc - Cycad produces the toxin which is consumed by flying foxes which are in turn consumed by humans

**Box 1: Cases of adverse health effects associated with exposure to cyanobacteria and cyanotoxins in recreational water.**

- 1959: **Canada:** In spite of a kill of livestock and warnings against recreational use, people still swam in a lake infested with cyanobacteria. Thirteen persons became ill (headaches, nausea, muscular pains, painful diarrhoea). In the excreta of one patient numerous cells of *Microcystis* spp. and some trichomes of *Anabaena circinalis* could be identified (Dillenberg & Dehnel, 1960).
- 1981: **USA:** Various locations in Pennsylvania and Nevada, USA, *Anabaena* and *Aphanizomenon* in lakes where recreational activities of swimming and water skiing occurred. Over 100 persons affected by skin and eye irritation, earache, hayfever, and gastroenteritis (Carmichael *et al*, 1985).
- 1989: **England:** Ten out of 20 soldiers became ill after swimming and canoe training in water with a heavy bloom of *Microcystis* spp.; two of them developed severe pneumonia attributed to the inhalation of a *Microcystis* toxin and needed hospitalisation and intensive care (Turner *et al*, 1990). Swimming skills and the amount of water ingested appear to have been related to the degree of illness.
- 1992: **Australia:** Dermal irritation from cyanobacteria at Lakes Alexandrina and Albert in South Australia involving *Nodularia* blooms (Soong *et al*, 1992).
- 1995: **Australia:** Reports of allergic and dermal reactions in the Murray River, South Australia involving *Anabaena*, *Aphanizomenon*, and *Oscillatoria*. (El Saadi *et al*, 1995).
- 1997: **Australia:** Epidemiological evidence of adverse health effects after recreational water contact from a prospective study involving 852 participants showed elevated incidence of diarrhoea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, and eye or ear irritations within 2–7 days after exposure (Pilotto *et al*, 1997). Symptoms increased significantly with duration of water contact and density of cyanobacterial cells, but were not related to the content of known cyanotoxins.
- 2001: **Australia:** Prospective cohort study which included 1,331 individuals recruited prior to engaging in various water recreation activities at freshwater and brackish lakes and reservoirs in southern Queensland, the Myall Lakes area in New South Wales, and northeast and central Florida, USA (Stewart, 2004). Individuals exposed to recreational waters from which total cyanobacterial cell surface areas exceeded 12mm<sup>2</sup>/mL (100,000 cells/mL) were more likely to report symptoms after exposure than those exposed to waters where cyanobacterial surface areas were less than 2.4mm<sup>2</sup>/mL (20,000 cells/mL). Mild respiratory symptoms appeared to be the most influential contribution to overall symptom reporting.

In 2000, a range of surface waters in New South Wales experienced a number of occurrences of relatively small-celled cyanobacteria that led to restrictions on the use of the water bodies when the “High Alert” guideline of 15,000 cells/mL was exceeded. However, this was perceived to be too restrictive due to the low biovolume (biomass) for these types of cyanobacteria at this cell density compared to that which would be encountered for other known toxigenic species of *Microcystis* or *Anabaena*. This prompted the development of an amended guideline by the NSW State Algal Coordinating Committee in January, 2001. This guideline still applied a High Alert Level when the abundance of known toxigenic (potentially toxic) species of cyanobacteria (eg. *Microcystis aeruginosa*, *Anabaena circinalis*) exceeded 15,000 cells/mL. However, where total cyanobacteria cell numbers exceed 15,000 cell/mL, but non-toxigenic species were predominant, total cyanobacterial biovolume was used to determine whether to declare a High Alert. In addition where the total biovolume of all

cyanobacterial species exceed 2mm<sup>3</sup>/L a High Alert was applied. The rationale for this amended guideline was the perception that the hazard posed by cyanobacteria in recreational situations, particularly for skin contact, was principally related to the mass of cell material, which could in this case be quantified by the concentration of total cyanobacterial cell biovolume.

In the international arena the World Health Organization released guidelines for cyanobacteria in recreational water in the “Guidelines for safe recreational water environments, Volume 1, Coastal and Fresh Waters” (WHO, 2003). These guidelines differentiate the health impairments from cyanobacteria in recreational waters between irritative symptoms and the potentially more severe hazard of ingestion of cyanotoxins. The recommendation is for a series of guideline values associated with incremental severity and probability of health effects defined as follows:

1. *Relatively mild and/or low probabilities of adverse health effects:* For protection from health outcomes not due to microcystin toxicity, but rather to the irritative effects of other cyanobacterial compounds, a guideline level of 20,000 cyanobacterial cells/mL was derived from the epidemiological study by Pilotto et al (1997).
2. *Moderate probability of adverse health effects:* A level of 100,000 cyanobacterial cells/mL, represents a guideline value for a moderate health alert in recreational waters. At these concentrations of cyanobacterial cells the probability of irritative symptoms is elevated. Additionally, cyanotoxins (usually cell-bound) may reach concentrations with potential health impacts in terms of ingestion.
3. *High risk of adverse health effects:* This level is defined as the presence of a cyanobacterial scum which can represent a thousandfold to a millionfold concentration of cyanobacterial cells. Calculations suggest that a child playing in a *Microcystis* scum for a protracted period and ingesting a significant volume could receive a lethal dose.

In 2001, which was around the time of the development and release of the above WHO guidelines, the National Health and Medical Research Council (NHMRC) established the Working Party on Healthy Recreational Water Use to oversee the development of guidelines on the use of recreational water. The guidelines would address natural fresh, estuarine and marine recreational water bodies and were required to address recent developments in risk assessment approaches to the management of microbiological water quality, and to include guidance on cyanotoxins along with other physical and chemical hazards.

The current project was initiated around the time of the NHMRC review and was developed by the CRC for Water Quality and Treatment in 2000 with guidance and support from the New South Wales Health Department, Eraring Energy Pty Ltd and the Sydney Catchment Authority. These partners recognised at that time the local and international recreational guidelines for cyanobacteria were not soundly evidence-based. It was felt that the revision and development of new guidelines for the cyanobacterial risks in recreational water being undertaken by the NHMRC would benefit from some specific research on skin irritation effects of cyanobacteria on humans. This was perceived as a knowledge gap for determining the importance of skin contact with cyanobacteria during water recreational activity.

The project was developed in the context of a debate within Australia that the management practice of restricting access and bathing, when the currently accepted cyanobacterial guideline value for cell density of 15,000 or 20,000 cells/mL (at that time) was exceeded, was unduly restrictive to water users. This was seen as particularly the case where there were few documented or even anecdotal reports of adverse health effects at these cell densities. Furthermore, these closures of lakes or water bodies caused both economic and social hardship in areas where affected water bodies are an important social amenity, without any demonstrated benefit of improved public health outcomes.

The study set out to investigate the class of relatively minor dermal irritant effects, which have been frequently reported but are not well characterised. The experimental studies with human volunteers were designed to confirm the nature of the hazards associated with dermal contact with cyanobacteria, and to establish quantitative dose-response data for human health risk assessment and guideline setting.

In addition, the study also set out to determine the quantitative nature of dose-response for skin irritation by cyanobacteria in terms of both cell numbers and cell mass, represented by biovolume. The guidelines in use were based upon cyanobacterial cell counts (WHO, 2003). However, there has been discussion that it may not be appropriate to use cell counts as the single quantitative indicator of cyanobacterial material. The reaction to cyanobacterial contamination may be expected in some cases to be proportional to contact with the mass of cell material (estimated by biovolume), which can be quite different for equivalent cell densities (cells/mL) of different species. This is because the biovolume, derived by calculation from the cell count and the average cell volume, varies over a wide range for the equivalent cell density of different species, due to their properties of widely different cell shapes and sizes.

The study also investigated the variability of cell count and biovolume measurements both within and between three different laboratories for the cyanobacterial suspensions applied to volunteers in this trial. The laboratories used equivalent methods for determining cell numbers and for the measurement of cell volume. The aim was to identify variability associated with conventional estimates of cell numbers in water quality laboratories, and to determine whether these analytical estimates would need to be considered in comparison of algal estimates against guideline values.

## 2 MATERIAL AND METHODS

### 2.1 RECRUITMENT OF VOLUNTEERS AND ETHICAL APPROVAL

A total of 114 volunteers were recruited from the School of Medicine at Flinders University in South Australia. Males and females aged 18 years or older were eligible to take part. Participants completed a questionnaire regarding their medical history and family history of atopy prior to patch application. People who had been diagnosed as having eczema, hay fever, asthma or an atopic condition were included in the study. However, individuals with severe recurrent skin conditions were excluded from participating, as were pregnant women. Each participant was paid \$50 to cover the cost of travel expenses. Ethics approval was obtained from the Ethics Committee of the Flinders University of South Australia (Research Application 103/00). A copy of the correspondence indicating approval is included as Appendix I.

### 2.2 PREPARATION OF CYANOBACTERIAL CULTURES

Cyanobacterial cultures were provided by Peter Baker, Australian Water Quality Centre, Bolivar, South Australia. Species and strains tested were:

*Microcystis aeruginosa* strain 338 (non-toxic strain)  
*Anabaena circinalis* strain 118AR  
*Nodularia spumigena* strain 001E  
*Microcystis aeruginosa* strain 309(1)CA (hepato-toxic strain)  
*Aphanocapsa incerta* strain 001  
*Cylindrospermopsis raciborskii* strain 031C.

Cultures were grown under laboratory conditions, (25°C and 15  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and harvested in log (active growth) phase on the same day of application to the volunteers. This avoided cell lysis and loss in cell integrity. The cultures were mono-cyanobacterial but not axenic (ie. contains contaminant bacteria). Experience has shown that the relative numbers of contaminant bacteria are not high for cultures harvested in early log phase.

The cyanobacterial culture samples used and applied in each separate round in this study were individually analysed for the full range of cyanobacterial toxins for which analytical tests were available in our laboratory. These included microcystins, nodularin, saxitoxins and cylindrospermopsin (Table 2). The results for the analyses shown in Table 2 give the toxin concentrations for the sample with highest cell density applied to the skin. The results are presented both in terms of intracellular and total toxin levels per litre. Intracellular and total toxin (intracellular and extracellular) levels of cultures are also presented on a per cell basis. Toxin levels were measured to identify if there was any link between toxin content and skin irritation.

### 2.3 SKIN IRRITATION TESTS

The study was made up of two trials. The first trial was composed of two separate rounds. Round 1 was conducted in May 2001 and Round 2 was conducted in October 2001. This first trial was used to investigate *Microcystis aeruginosa* strain 338 (non-toxic strain), *Anabaena circinalis* strain 118AR and *Nodularia spumigena* strain 001E. The second trial (50 volunteers) was composed of only one Round which was conducted in May 2002 and was used to investigate *Microcystis aeruginosa* strain 309(1)CA (hepato-toxic strain), *Aphanocapsa incerta* strain 001 and *Cylindrospermopsis raciborskii* strain 031C. Volunteers from the three rounds were treated in the same way with cyanobacterial cultures having similar cell densities (Table 3). Cell densities differed within and between the three rounds due to the inherent variability of cyanobacterial counts. Some volunteers were included in both trials.

Part of the experiment design was to apply cyanobacterial cultures in two ways: both as whole cell suspensions and also as solutions of fully lysed (disrupted) cells. This was done to determine the effect of any intracellular components or metabolites on the skin over the exposure period. In the normal circumstances of exposure to cyanobacteria in a recreational water body, individuals would be exposed to a varying solution of whole and lysed cyanobacterial cells and associated cell components. It is possible that the components could have different irritant potential, and this treatment allowed for the potential for full contact for intracellular contents to the skin.

Solutions of known concentrations of cyanobacterial cultures were applied to the skin using adhesive patches containing 10 x 8 mm chambers each containing a small filter pad (Finn Chambers®). Each chamber held a 20  $\mu\text{L}$  volume of culture, which was placed onto the patch using a micro-pipette (Plate 1). Four patches were applied in a random sequence on each of the subjects' upper backs (Plate 2). Participants were advised not to get the patch wet ie. not to shower or work up a sweat until the patches were removed.

Each volunteer was exposed to three different cyanobacterial strains at six different cell concentrations. Table 3 gives the dose-concentrations for each species tested. Both whole and lysed preparations of each cyanobacterial species at each dose concentration were applied to each participant's skin. Cultures were lysed by placing 10 mL of each culture into a 25 mL plastic centrifuge tube which was placed into a beaker of ice and then sonicated for 15 minutes (30 minutes for the two *Microcystis aeruginosa* cultures) using a probe sonicator (Branson 250 Sonifier®) fitted with a 1/8" tapered micro tip.

Table 2: Intracellular and total toxin concentration for cultures of cyanobacteria with highest cell densities used in Rounds 1, 2 and 3 and corresponding total toxin and intracellular toxin concentration per cell.

### Round 1

Cyanobacteria	Cell Density (cells/mL)	Toxin	Conc. Intracellular ug/L	Conc. Total ug/L	Intracellular Conc. per cell ug/cell	Total (intra & extracellular) Conc. per cell ug/cell
<i>Anabaena circinalis</i> strain 118AR (neurotoxic)	287,000	GTX2	4.9	6.4	$1.7 \times 10^{-8}$	$2.23 \times 10^{-8}$
		GTX3	2.0	3.1	$6.94 \times 10^{-9}$	$1.07 \times 10^{-8}$
		C-1	33.7	35.7	$1.18 \times 10^{-7}$	$1.25 \times 10^{-7}$
		C-2	11.0	11.8	$3.83 \times 10^{-8}$	$4.12 \times 10^{-8}$
		STX	0.16	0.62	$5.75 \times 10^{-10}$	$2.16 \times 10^{-9}$
<i>Nodularia spumigena</i> strain 001E (hepatotoxic)	214,000	Nodularin	16.8	19.8	$7.84 \times 10^{-8}$	$9.04 \times 10^{-8}$
<i>Microcystis aeruginosa</i> strain 338 (non-toxic)	289,000	Non-detected				

### Round 2

Cyanobacteria	Cell Density (cells/mL)	Toxin	Conc. Intracellular ug/L	Conc. Total ug/L	Intracellular Conc. per cell ug/cell	Total (intra & extracellular) Conc. per cell ug/cell
<i>Anabaena circinalis</i> strain 118AR (neurotoxic)	324,360	GTX2	2.0	7.5	$6.20 \times 10^{-9}$	$2.30 \times 10^{-8}$
		GTX3	0.80	6.2	$2.45 \times 10^{-9}$	$1.92 \times 10^{-8}$
		C-1	7.1	25.6	$2.19 \times 10^{-8}$	$7.87 \times 10^{-8}$
		C-2	3.3	19.0	$1.01 \times 10^{-8}$	$3.07 \times 10^{-8}$
		STX	0.34	2.2	$1.03 \times 10^{-9}$	$6.84 \times 10^{-9}$
<i>Nodularia spumigena</i> strain 001E (hepatotoxic)	304,560	Nodularin	35.8	49.3	$1.18 \times 10^{-7}$	$1.62 \times 10^{-7}$
<i>Microcystis aeruginosa</i> strain 338 (non-toxic)	364,800	Non-detected				

### Round 3

Cyanobacteria	Cell Density (cells/mL)	Toxin	Conc. Intracellular ug/L	Conc. Total ug/L	Intracellular Conc. per cell ug/cell	Total (intra & extracellular) Conc. per cell ug/cell
<i>Microcystis aeruginosa</i> strain 309 (hepatotoxic)	300,500	MIC-LR	2.3	8.6	$7.65 \times 10^{-9}$	$2.86 \times 10^{-8}$
<i>Cylindrospermopsis raciborskii</i> strain 031C (toxic)	150,792	CYP	12.1	23.82	$8.02 \times 10^{-8}$	$1.57 \times 10^{-7}$
<i>Aphanocapsa incerta</i> strain 001 (non-toxic)	351,900	Non-detected				

### Abbreviations:

C1: C-toxin

C2: C-toxin

GTX2: gonyautoxin

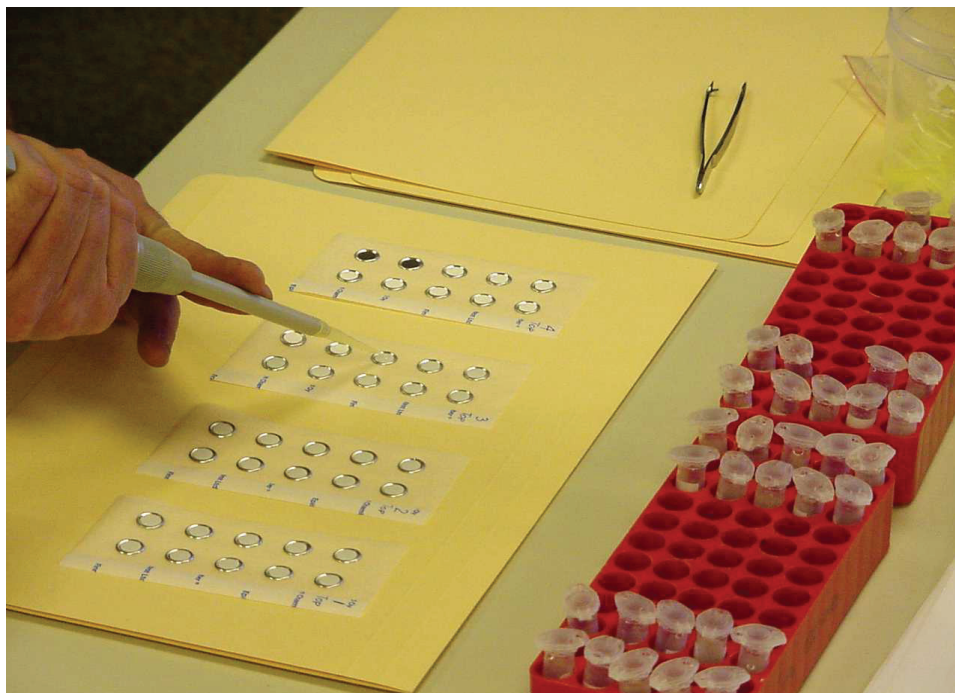
GTX3: gonyautoxin

STX: saxitoxin

NOD: nodularin

CYP: cylindrospermopsin

MIC-LR: microcystin-LR equivalents



*Plate 1: Cyanobacterial cell suspensions added to wells in adhesive patches ready for application to subjects' backs.*



*Plate 2: Adhesive patches applied to subject's back.*

Two positive controls (1 and 5% solutions of sodium lauryl sulphate) and two negative controls (culture media and an empty patch) were also included. The 1% solution was introduced in Round 2 due to the strong reaction to the 5% solution in Round 1.

Patches were removed from the subjects' skin after 24 hours and a subjective assessment of the erythematous reaction was made by a specialist dermatologist physician using the following clinical grades: 0 (no reaction), 1+, 2+, 3+ and 4+ (highest level of erythema) (Plate 3). Assessments were made for each of the 40 patches, as well as for normal skin. Erythema readings were repeated 48 hours later in Round 1 (a total of 72 hours after the patches were applied). The second assessment was not included in Rounds 2 and 3 due to the marked reduction in response after 72 hours, as observed in Round 1. Due to the reduced response after 72 hours only the 24 hour results from Round 1 are included in the analysis.

An objective assessment of the skin reactions was also made for each patch and normal skin using the MX-18 Mexameter (Courage & Khazaka Electronic®) (Plate 4). A mexameter provides a measure of melanin (pigment) and erythema (haemoglobin) content of the skin. A special probe emits light at known wavelengths into the skin. The same probe then measures the light reflected back by the skin. Different amounts of light are absorbed depending upon the amount of melanin and erythema. The quantity of light absorbed is then used to calculate melanin or erythema content. Erythema levels only are presented here and are reported as the deviation from the erythema measurement achieved for the negative control. It should also be noted that the mexameter measurement is the same as an erythema measurement in this report.

The rounds were carried out as double-blind experiments. Both the project officer applying the patches and the

dermatologist reading the skin reactions were unaware of the type and cell concentration of the cyanobacterial preparations applied to each individual.

## 2.4 STATISTICAL METHODS

The distribution of clinical gradings by patch type (control or active), species, cell type and cell concentration was assessed by both tabulations and graphical methods.

Sets of stacked bar charts display the percentage of subjects assigned each clinical grade for each patch type ie. control type (Figure 1 – see Results section) or cell density (Figures 2-4 – see Results section) for each species and cell type (whole or lysed).

Due to the relatively small number of high level clinical gradings, each observation was dichotomised into no reaction (grade 0) and a positive reaction (1+, 2+, 3+ and 4+) prior to modelling. Two sets of logistic regression models were then fitted. Firstly, reactions to active patches were compared to reactions to negative control patches producing odds ratios (and 95% confidence intervals) for reactions for exposure to an active versus a negative control patch. The average percentage of reactions to an active patch (and 95% confidence interval) was also calculated based on estimates from this model for each species. Secondly, dose-response models were fitted based on the cell counts of the active patches estimating the odds ratio for reactions per 10,000 cells/mL. Separate regression models were fitted for each species (whole and lysed cells combined and whole and lysed cells separately) for data from each round and combined. Dose-response models were also fitted excluding subjects who reacted to the negative control patches.

Table 3: Cyanobacteria and cell densities used in Rounds 1, 2 and 3 of the skin irritation study as measured in-house by the Biology Research Laboratory at the Australian Water Quality Centre.

Species and Strains Tested	<i>Microcystis aeruginosa</i> strain 338 (non-toxic)		<i>Anabaena circinalis</i> strain 118AR (neurotoxic)		<i>Nodularia spumigena</i> strain 001E (hepatotoxic)		<i>Microcystis aeruginosa</i> strain 309(1) CA (hepatotoxic)	<i>Cylindrospermopsis raciborskii</i> strain 031C (toxic)	<i>Aphanocapsa incerta</i> strain 001 (non-toxic)
Round	1	2	1	2	1	2	3	3	3
Cell density (cells/mL)	5,260	8,708	3,792	3,854	4,884	5,582	5,450	4,670	82,578
	20,800	35,571	13,490	20,416	15,800	24,385	22,750	14,458	107,583
	55,200	100,600	56,700	38,976	59,700	61,104	70,667	51,015	225,944
	103,000	155,273	82,100	86,700	79,500	86,156	109,500	81,795	200,667
	204,000	295,333	163,000	232,202	121,000	186,750	203,000	123,675	311,567
	289,000	364,800	287,000	324,360	214,000	304,560	300,500	150,792	351,900



*Plate 3: Close up view of subject's back after removal of patches. Area of positive reaction is shown.*



*Plate 4: Measurement of skin reaction to cyanobacterial cultures using Mexameter.*

The repeated nature of the data (multiple observations per subject) was taken into account using the Generalised Estimating Equation (GEE) approach as implemented in the STATA Version 7 (Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA) procedure 'xtlogit' using robust standard error estimation.

The results were also disaggregated by atopy status (defined as self report of ever being diagnosed with asthma, eczema, hayfever or atopy).

This analysis was repeated for the mean erythema mexameter readings using a normal model rather than the binomial model. The relevant estimates from the two sets of regressions were the mean difference between the readings for the active and control patches and the slope of the regression on cell counts (ie. mean increase in the readings per 1000 cells). These models were fitted using the 'xtgee' procedure in STATA.

Tests with significance levels below 0.05 are interpreted as statistically significant.

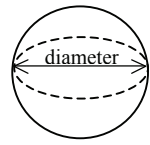
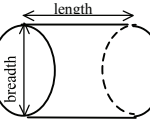
## 2.5 CELL COUNTING AND BIOVOLUME MEASUREMENT

Culture samples for cell counts and cell volume measurements were preserved in Lugols Iodine. Samples of each cyanobacteria tested were sent to three separate laboratories for cell counting and for biovolume determination. The cultures included samples from the full concentration range tested in Rounds 2 and 3. One laboratory was the Biology Research Laboratory at the Australian Water Quality Centre and the other two were NATA-registered water authority analytical laboratories that carry out algal cell counts as a standard commercial test for water quality monitoring. Two of the laboratories used Sedgewick-Rafter counting chambers and the third used Lund Cells to carry out cell counts using the following methods:

1. *Microcystis aeruginosa* 338, *Microcystis aeruginosa* 309(1)CA and *Aphanocapsa incerta* 001: At least 400 cells were counted and cell counts calculated using the appropriate dilution factor. This achieved a counting error of  $\pm 10\%$  (Hötzels and Croome, 1999).
2. *Nodularia spumigena* 001E, *Cylindrospermopsis raciborskii* 031C: The cell length of 30 *Nodularia* cells from 6 different trichomes (ie. 5 cells from each trichome) was measured under oil emersion using 1000 $\times$  magnification and mean cell length calculated. At least 100 trichomes were counted measuring the length of the first 50 with an eyepiece micrometer. The mean trichome length of the first 50 trichomes was calculated and then divided by mean cell length to calculate number of cells per trichome. Number of cells per trichome was multiplied by number of trichomes counted and then converted to cell count using an appropriate dilution factor. This achieved a counting error of  $\pm 20\%$  (Hötzels and Croome, 1999).
3. *Anabaena circinalis* 118AR: At least 100 trichomes were counted enumerating the number of cells in the first 50 trichomes. The mean number of cells per trichome was then calculated for the first 50 trichomes. The number of cells per trichome was multiplied by number of trichomes counted and then converted to cell count using appropriate dilution factor. This achieved a counting error of  $\pm 20\%$  (Hötzels and Croome, 1999).

Table 4 shows the equations used to calculate the cell volume for each of the cyanobacterial species and how cell dimensions were measured. Cell dimensions were measured at 1000 $\times$  magnification using oil emersion. Measurement of the dimensions of 30 cells was recommended by Hötzels and Croome (1999). To improve the accuracy for filamentous cyanobacteria, 5 cells were counted from 6 different trichomes.

Table 4: Equations used to calculate cell volumes and methods for measuring length and breadth of cells.

Cyanobacteria	Equation for Volume	Procedure for measurement of cells	Cell dimensions
<i>Microcystis aeruginosa</i> 338 <i>Microcystis aeruginosa</i> 309(1)CA <i>Aphanocapsa incerta</i> 001 <i>Anabaena circinalis</i> 118AR	$\frac{4}{3}\pi r^3$ ( $r = \text{diameter} \div 2$ )	Measure length of 30 cells and calculate mean diameter. The diameter of <i>Anabaena circinalis</i> 118AR cells was measured both vertically and horizontally for 30 cells and a mean diameter was calculated.	
<i>Nodularia spumigena</i> 001E <i>Cylindrospermopsis raciborskii</i> 031C	$\pi r^2 \times \text{length}$ ( $r = \text{breadth} \div 2$ )	Measure length and breadth of 30 cells and calculate mean cell diameter. <b>Note:</b> measure cell length and breadth at widest point of cell.	

### 3 RESULTS

#### 3.1 SKIN IRRITATION

The 114 subjects included in the study ranged in age from 17 to 54 years (mean 28, standard deviation 7, median 26 years). There were 44 men and 70 women. Half were atopic (50%).

##### 3.1.1 CLINICAL GRADINGS (SUBJECTIVE READINGS)

Most subjects (88%, 100 out of 114) had an erythematous reaction to the positive control patches. However, reactions were also recorded for the negative control or blank patches in almost one quarter (23%, 26 out of 114) of the subjects (Figure 1).

The stacked bars in Figures 2a and 2b show the percentage of individuals assigned different clinical gradings for the three cyanobacterial species tested in Rounds 1 and 2. Figures 3a and 3b show results for the three cyanobacterial species tested in Round 3. Figures 2a and 3a present results for all subjects tested, including those individuals that reacted to negative controls, and show that for any single concentration, between 10% and 40% of subjects are graded with a clinical response. Figures 2b and 3b present results when subjects reacting to negative control patches are excluded and show that reaction rates dropped to between 10% and 30%.

Subjects were more likely to react to the active patches than to the negative control patches, for all species, and for both whole and lysed cells (Table 5). Almost all results are statistically significant ( $p < 0.05$ ) and the odds ratios (OR) for active versus control patches range between 1.5 and 3.5.

Table 6 shows that approximately 70% (identified as Any) of all subjects were graded with positive reactions to at least one of the active patches ie. reacted with cyanobacteria irrespective of concentration. The percentages are in the general range of 40% to 50% for individual species for both whole and lysed preparations (Table 6). The response percentages are lower (between 20% and 40%) among the subjects who were not graded as reacting to the negative control patches. Interestingly, almost all the subjects (>80%) who were graded with positive reactions to the negative control patches were also graded with positive reactions to the active patches (Table 6).

When the results for both whole and lysed are combined and a mean estimate based on a logistic regression model of the probability of reacting to a patch is calculated, the reaction rates estimated for the six cyanobacteria lie in the range 20% to 24% with 95% confidence intervals of approximately  $\pm 16\%$  (Table 7). If the volunteers who exhibited reactions to the negative control patches are excluded from this analysis the reaction rates decrease to

between 11% and 15% with 95% confidence intervals of approximately  $\pm 8\%$  (Table 7).

There was no evidence of a consistent increasing dose-response relationship between positive gradings and increasing cell concentrations for the majority of the cyanobacterial species tested, whether they were whole or lysed. Tables 8a and 8b present statistical analysis of results to determine a dose-response relationship for all subjects and with subjects who reacted to negative controls removed respectively. Statistical significance ( $p < 0.05$ ) was reached for three species but no trend could be identified. MA309 and CR showed a significant dose relationship as whole cell applications for results that included individuals who reacted to negative controls. MA338 as a whole cell application and CR for combined results for whole and lysed cell applications also showed a significant dose-response relationship. However, in one of the cases, CR as a whole cell application with individuals who reacted to negative controls included, the relationship was decreasing (ie. reduction in clinical response with increasing cell concentration), not increasing. Furthermore, there is no evidence for a threshold effect for any of the species studied ie. a particular concentration above which there were frequent or strong reactions for any of the species tested.

The cyanobacteria used in this study were analysed for a range of known toxins (Table 2). However, it was found that there was no link between toxicity and an increased irritative effect. In particular, both a toxic (strain 309(I)CA) and non-toxic (strain 338) *Microcystis* species was tested and no significant difference in irritative effect was observed between the two strains.

##### 3.1.2 MEXAMETER DATA (OBJECTIVE READINGS)

The patterns of mean mexameter readings for erythema for the various control patches mirror the pattern of overall clinical responses in all rounds (Figure 1).

The mexameter readings for erythema are consistently and statistically significantly higher for reactions to active patches compared with control patches in each round, for both whole and lysed cells from each species, regardless of whether subjects reacting to negative controls are included (Table 9a) or excluded (Table 9b).

As was observed for clinical gradings, there is no evidence of consistent increasing dose-response relationships or threshold effects between mexameter levels and cell concentrations. Table 10a presents statistical analysis of results to determine a dose-response relationship for all subjects and Table 10b presents results when subjects who reacted to negative controls are removed. *Aphanocapsa incerta* in Round 3 is the only species for which statistical

significance is reached (Tables 10a and 10b), but the direction of the relationship is inverse to what would be expected i.e. lower mexameter levels at higher cell densities.

Importantly, there is little consistency between clinical grading levels and mean mexameter deviations across the different cell densities for the various species (Figures 2a, 2b, 3a, 3b). Results have shown that the Mexameter readings mirrored results for clinical gradings for controls but not for cyanobacterial extracts. Therefore, it is possible that there is an unknown agent in the extracts that is interfering with the mexameter. A possible candidate for this is cyanobacterial pigments.

### 3.1.3 ATOPY STATUS

Clinical gradings show that the percentage of subjects who reacted to the active patches was not consistently higher or lower for atopic versus non-atopic subjects for any species or concentration. The extent of reaction appears higher for atopic subjects in Rounds 1 and 2 (Figure 4a), but is not statistically significant ( $p > 0.05$ ) for any species and was not replicated in Round 3 for the other species (Figure 4b). In fact, results for Round 3 seem to show an increased reaction rate for non-atopic subjects.

The difference in mexameter readings between active and negative control patches also appears higher for atopic subjects for Round 1 and 2 species and lower for Round 3 species compared to results for non-atopic people, however none of the differences were statistically significant ( $p > 0.05$ ).

## 3.2 CELL COUNTING AND BIOVOLUME MEASUREMENT

The evaluation of variability of cell count analyses between different laboratories revealed a very large coefficient of variation (CV) for the measurements upon identical samples between the three laboratories (Table 11). However, no trend could be identified either within the concentration range analysed or between the participants. For example, for *Nodularia spumigena* the CV was 18 - 43% for the lowest cell density samples, and decreased to 4 - 12% at the highest cell density samples. However, the reverse was observed for samples of *Cylindrospermopsis raciborskii* with CV increasing from 21% at lowest cell density to 58% for the highest cell density. For the remainder of the species, including both *Microcystis aeruginosa* strains, *Aphanocapsa incerta* and *Anabaena circinalis*, the CV did not change significantly across the cell density range.

The calculated cell volume estimates for each cyanobacterial species also varied between laboratories (Table 12). The greatest variation occurred for *Nodularia spumigena*, which showed a CV of 66%. The two *Microcystis aeruginosa* strains recorded the next highest CV with values of 46% and 21% for strains 338 and 309(1) CA respectively. Analysis of *Anabaena circinalis* gave a CV of 16% and *Aphanocapsa incerta* and *Cylindrospermopsis raciborskii* had the lowest CV's of 5% and 4% respectively.

Figure 1: Percentage of subjects with clinical gradings (bars) and mean mexameter measurements (dots) for negative and positive control patches in each round.

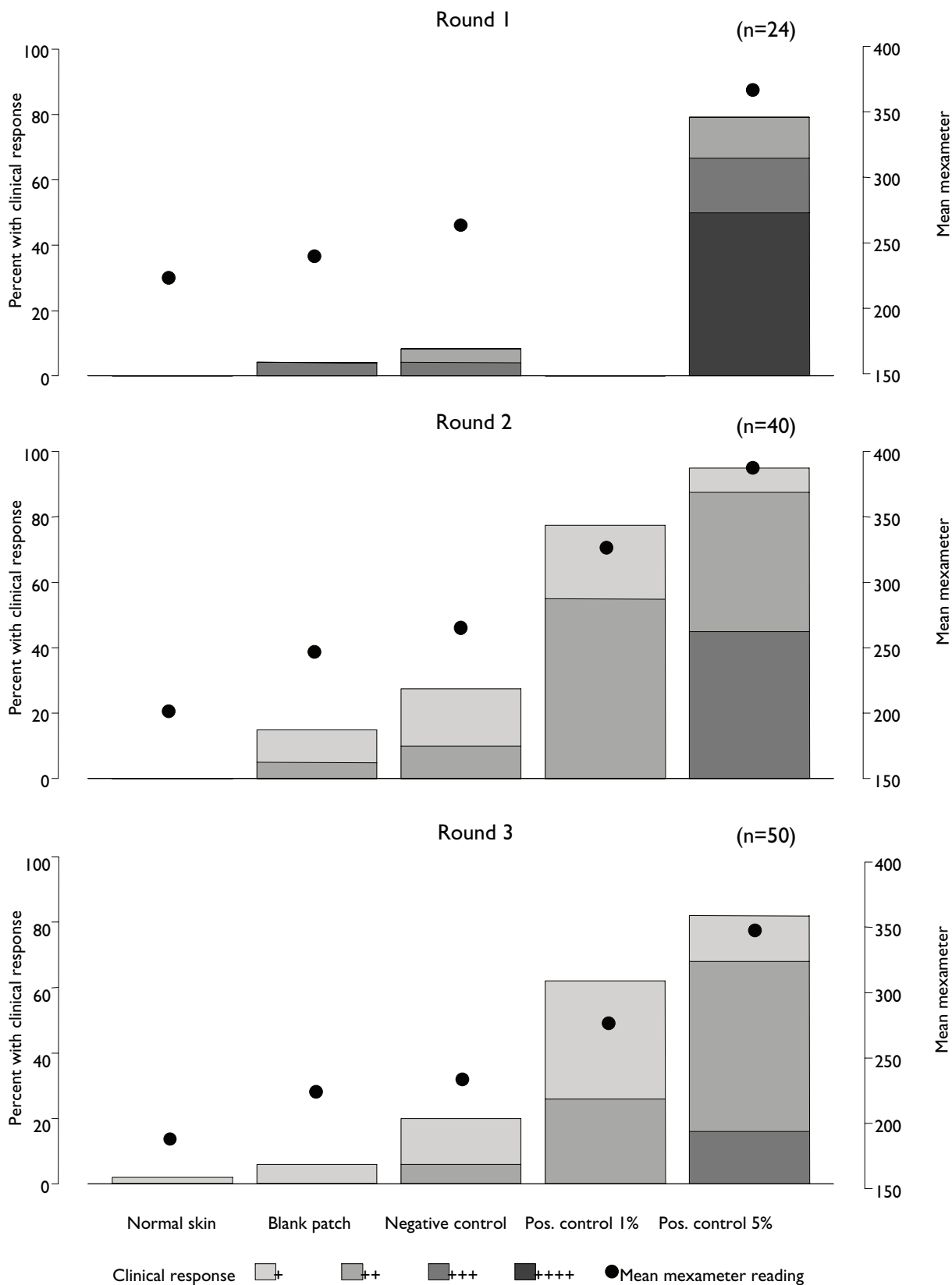


Figure 2a: Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for all 64 subjects in Rounds 1 and 2.

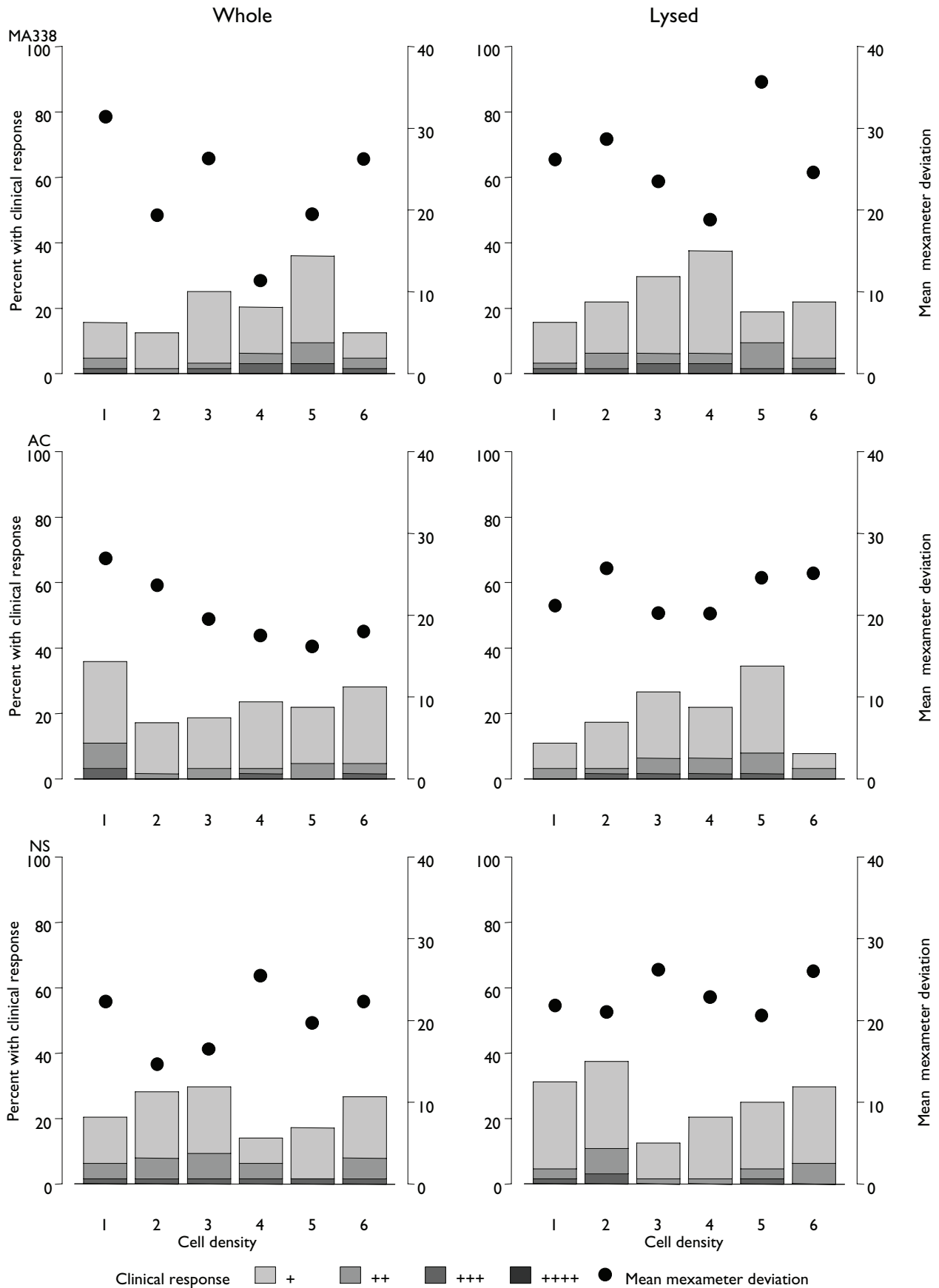


Figure 2b: Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for 49 subjects in Rounds 1 and 2 who did not react to negative control patches.

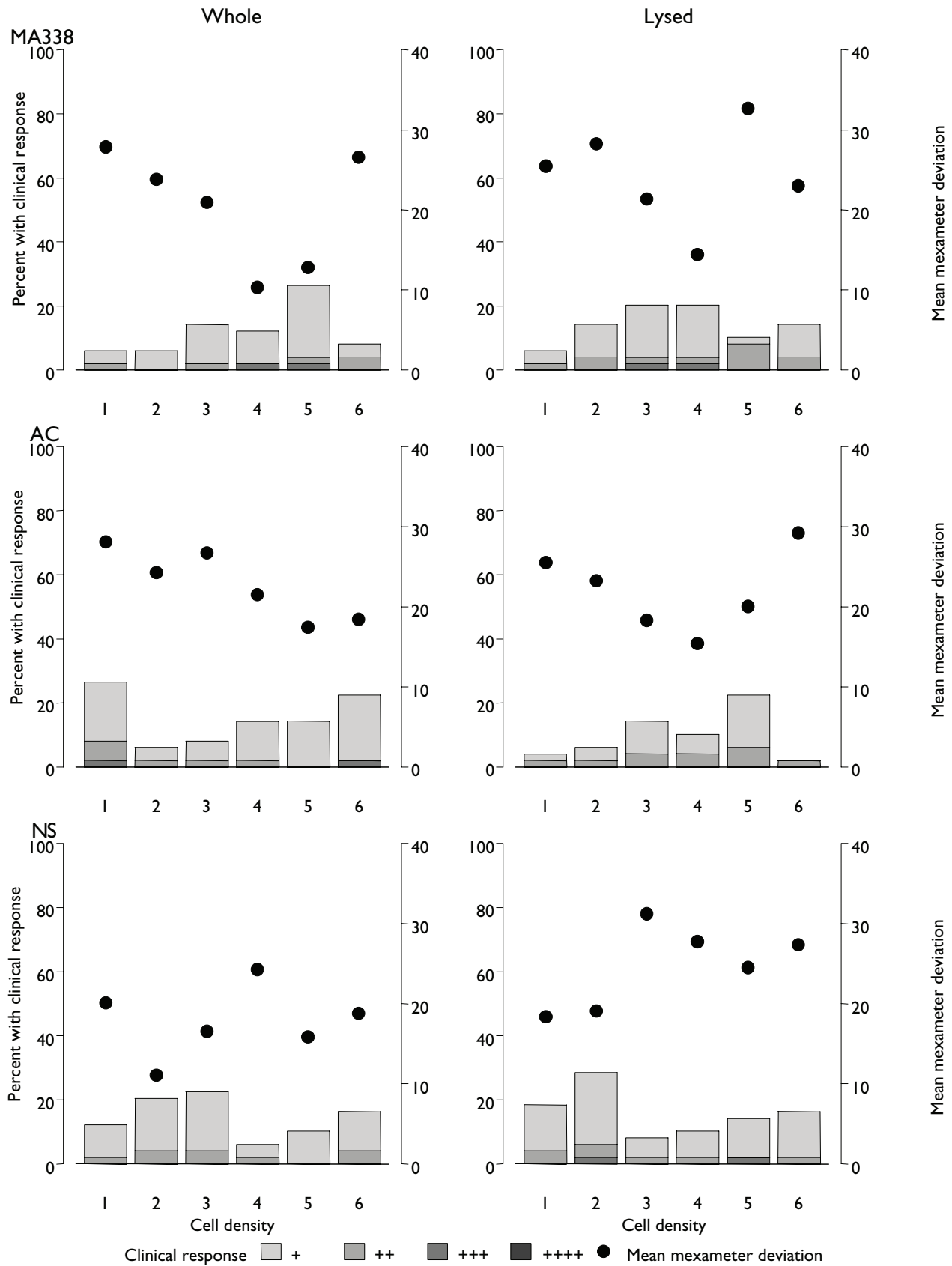


Figure 3a: Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for all 50 subjects in Round 3.

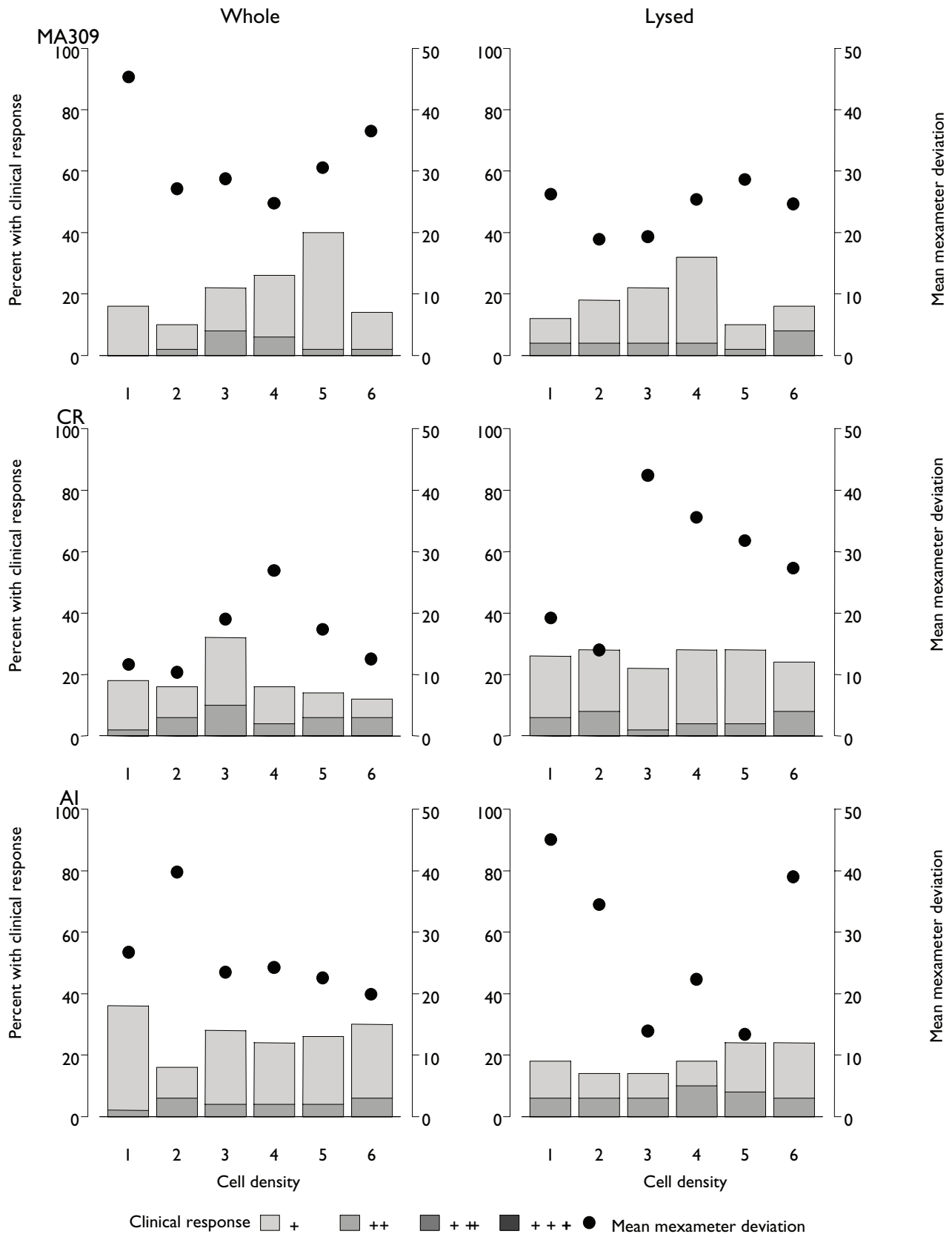


Figure 3b: Percentage of subjects with clinical gradings (bars) and mean mexameter deviations from negative control measurements (dots) for active patches of increasing cell densities for 39 subjects in Round 3 who did not react to negative control patches.

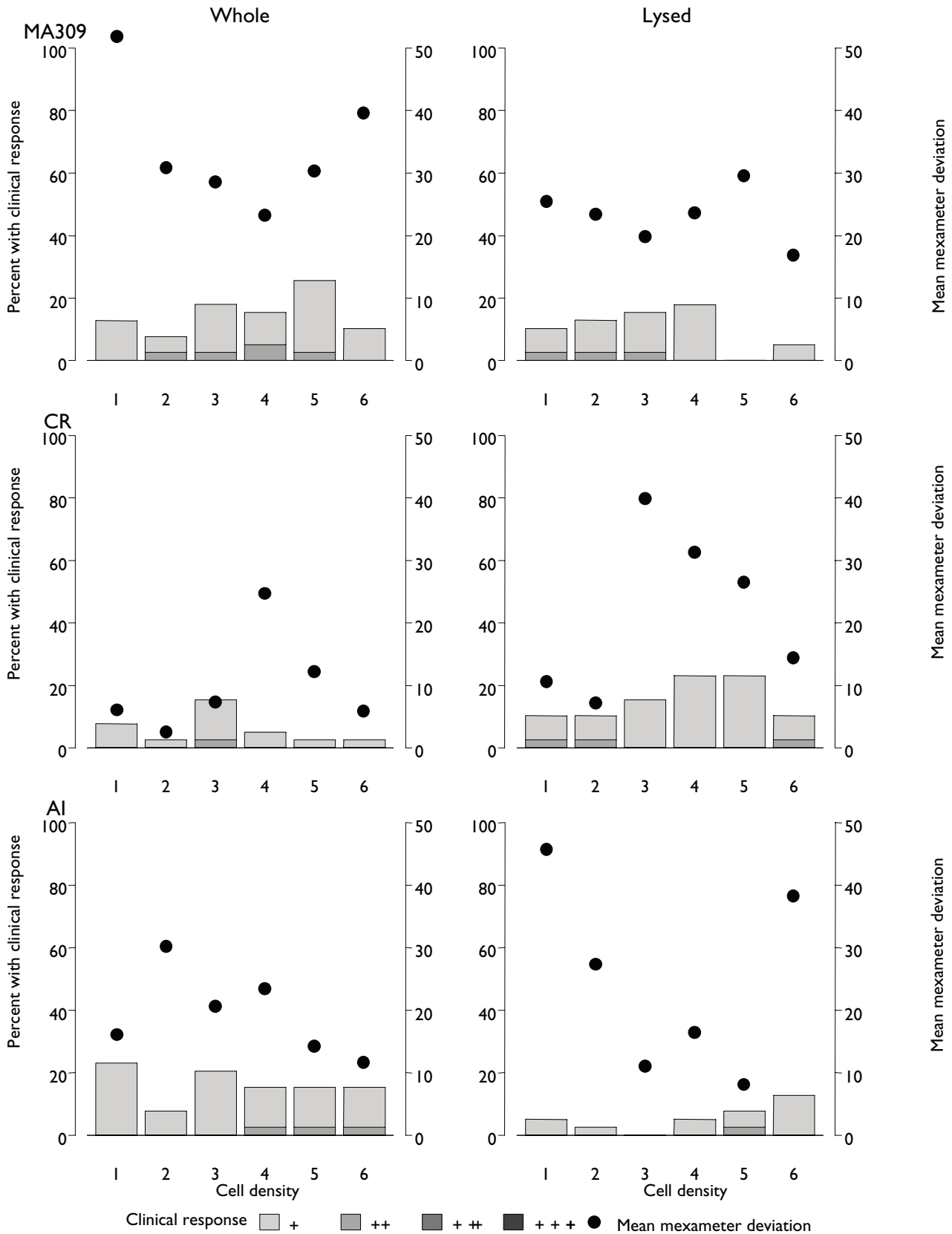


Table 5: Odds ratios (OR) and 95% confidence intervals (CI) for clinical response (grades 1 to 4) estimated in logistic regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3).

Rounds 1 & 2 (n=64)				
Term	OR*	CI		p
MA338	1.76	1.08	2.86	<b>0.02</b>
Whole	1.53	0.90	2.61	0.1
Lysed	1.98	1.21	3.23	<b>0.007</b>
AC	1.79	1.13	2.84	<b>0.01</b>
Whole	2.04	1.17	3.56	<b>0.01</b>
Lysed	1.51	0.95	2.41	0.09
NS	1.98	1.21	3.23	<b>0.006</b>
Whole	1.77	1.04	3.03	<b>0.04</b>
Lysed	2.18	1.33	3.59	<b>0.002</b>
Round 3 (n=50)				
Term	OR*	CI		p
MA309	2.40	1.33	4.35	<b>0.004</b>
Whole	2.63	1.35	5.14	<b>0.005</b>
Lysed	2.18	1.27	3.74	<b>0.005</b>
CR	2.74	1.79	4.21	<b>&lt;.001</b>
Whole	2.13	1.42	3.21	<b>&lt;.001</b>
Lysed	3.41	2.00	5.84	<b>&lt;.001</b>
AI	2.85	1.93	4.20	<b>&lt;.001</b>
Whole	3.53	2.13	5.87	<b>&lt;.001</b>
Lysed	2.23	1.55	3.21	<b>&lt;.001</b>

\* OR is Odds ratio for clinical response at 24 h for active patches vs control patches

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 6: Number (n) and percentage (%) of subjects with positive gradings to at least one active (non-control) patch overall and subdivided by whether or not subjects were graded with positive responses to negative controls.

Rounds 1 and 2		Total		Positive response to negative controls			
Species	Cell type	(n=64)		No (n=49)		Yes (n=15)	
		Number & % with positive gradings to active patches					
		n	%	n	%	n	%
MA338	Whole	27	42%	14	29%	13	87%
MA338	Lysed	32	50%	18	37%	14	93%
AC	Whole	29	45%	17	35%	12	80%
AC	Lysed	27	42%	14	29%	13	87%
NS	Whole	30	47%	18	37%	12	80%
NS	Lysed	34	53%	20	41%	14	93%
Any	Any	44	69%	30	61%	14	93%
Round 3		(n=50)		(n=39)		(n=11)	
MA309	Whole	24	48%	13	33%	11	100%
MA309	Lysed	22	44%	12	31%	10	91%
CR	Whole	19	38%	9	23%	10	91%
CR	Lysed	22	44%	11	28%	11	100%
AI	Whole	26	52%	16	41%	10	91%
AI	Lysed	22	44%	11	28%	11	100%
Any	Any	35	70%	24	62%	11	100%

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*  
 CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 7: Mean percentages of subjects and 95% confidence intervals (CI) reacting to an active (non-control) patch by species, overall (Total) and for the subset of subjects not reacting to negative controls.

Rounds 1 and 2		Total subjects			Subjects not reacting to negative controls		
Species	Mean	(n=64)		Mean	(n=49)		CI
		Mean	CI		Mean	CI	
MA338	22%	16%	31%	13%	8%	21%	
AC	22%	16%	29%	13%	8%	19%	
NS	24%	18%	33%	15%	10%	23%	
Round 3		(n=50)			(n=39)		
MA309	20%	13%	29%	13%	7%	22%	
CR	22%	15%	31%	11%	6%	18%	
AI	23%	16%	32%	11%	7%	17%	

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*  
 CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 8a. Odds ratios (OR) and 95% confidence intervals (CI) for clinical response estimated in logistic regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3).

Rounds 1 & 2 (n=64)				
Term	OR*	CI		p
MA338	1.001	1.000	1.001	0.2
Whole	1.001	1.000	1.003	0.1
Lysed	1.000	0.998	1.002	1.0
AC	1.000	0.999	1.001	1.0
Whole	1.000	0.998	1.002	0.8
Lysed	1.000	0.999	1.001	0.6
NS	1.000	0.998	1.001	0.8
Whole	1.000	0.998	1.002	1.0
Lysed	1.000	0.997	1.002	0.8
Round 3 (n=50)				
Term	OR*	CI		p
MA309	1.001	0.999	1.002	0.6
Whole	1.002	1.000	1.003	0.02
Lysed	0.999	0.996	1.003	0.7
CR	0.998	0.996	1.001	0.3
Whole	0.997	0.993	1.000	0.03
Lysed	1.000	0.996	1.004	0.9
AI	1.001	1.000	1.003	0.2
Whole	1.000	0.998	1.002	0.8
Lysed	1.002	0.999	1.005	0.1

\* OR is Odds ratio for clinical response per 1000 cell counts

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 8b. Odds ratios (OR) and 95% confidence intervals (CI) for clinical response estimated in logistic regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls.

Rounds 1 & 2 excluding subjects reacting to neg. controls(n=49)				
Term	OR*	CI		P
MA338	1.001	1.000	1.003	0.09
Whole	1.002	1.000	1.004	<b>0.02</b>
Lysed	1.000	0.998	1.003	0.9
AC	1.001	0.999	1.003	0.2
Whole	1.002	0.999	1.004	0.2
Lysed	1.000	0.998	1.002	0.7
NS	0.999	0.997	1.001	0.3
Whole	0.999	0.997	1.002	0.6
Lysed	0.998	0.995	1.002	0.4
Round 3 excluding subjects reacting to neg. controls(n=39)				
Term	OR*	CI		P
MA309	1.001	1.000	1.002	0.2
Whole	0.995	0.990	1.000	0.07
Lysed	1.000	0.996	1.005	0.8
CR	0.994	0.989	0.999	<b>0.02</b>
Whole	1.003	0.998	1.008	0.2
Lysed	1.001	0.999	1.004	0.4
AI	1.000	0.997	1.002	0.8
Whole	1.006	0.998	1.014	0.2
Lysed	1.001	1.000	1.002	0.2

\* OR is Odds ratio for clinical response per 1000 cell counts

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 9a. Mean difference and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3).

Rounds 1 & 2 (n=64)			
Term	Mean*	CI	p
MA338	24.34	16.00 32.68	<.001
Whole	22.35	13.17 31.53	<.001
Lysed	26.28	18.21 34.36	<.001
AC	21.37	14.79 27.95	<.001
Whole	20.29	10.88 29.69	<.001
Lysed	22.51	16.06 28.97	<.001
NS	21.33	13.22 29.44	<.001
Whole	19.98	10.01 29.94	<.001
Lysed	22.79	15.06 30.53	<.001
Round 3 (n=50)			
Term	Mean*	CI	p
MA309	27.98	17.59 38.38	<.001
Whole	32.14	20.47 43.81	<.001
Lysed	23.82	13.78 33.87	<.001
CR	22.30	13.72 30.89	<.001
Whole	16.26	5.93 26.60	0.002
Lysed	28.34	18.82 37.86	<.001
AI	27.05	19.42 34.67	<.001
Whole	26.09	14.93 37.25	<.001
Lysed	28.00	19.72 36.29	<.001

\* Mean is mean difference in mexameter readings for active patches vs control patches

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 9b. Mean difference and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression models for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls.

Rounds 1 & 2 excluding subjects reacting to neg. controls (n=49)				
Term	Mean*	CI		P
MA338	22.01	12.91	31.10	<.001
Whole	20.08	9.91	30.24	<.001
Lysed	23.99	15.15	32.83	<.001
AC	22.10	14.50	29.70	<.001
Whole	22.63	11.54	33.73	<.001
Lysed	21.62	14.70	28.54	<.001
NS	20.59	11.30	29.89	<.001
Whole	17.22	6.17	28.27	0.002
Lysed	24.22	15.36	33.08	<.001
Round 3 excluding subjects reacting to neg. controls (n=39)				
Term	Mean*	CI		P
MA309	28.57	17.08	40.06	<.001
Whole	34.06	20.98	47.13	<.001
Lysed	23.09	12.13	34.04	<.001
CR	15.70	6.28	25.12	0.001
Whole	9.79	-1.85	21.42	0.1
Lysed	21.62	11.22	32.02	<.001
AI	21.91	13.96	29.86	<.001
Whole	19.36	6.67	32.04	0.003
Lysed	24.47	15.64	33.29	<.001

\* Mean is mean difference in mexameter readings for active patches vs control patches

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Table 10a. Means and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3).

Rounds 1 & 2 (n=64)				
Term	Mean*	CI		p
MA338	0.004	-0.019	0.027	0.7
Whole	-0.006	-0.035	0.023	0.7
Lysed	0.014	-0.026	0.054	0.5
AC	-0.007	-0.030	0.016	0.6
Whole	-0.022	-0.061	0.016	0.3
Lysed	0.009	-0.024	0.041	0.6
NS	0.013	-0.018	0.044	0.4
Whole	0.012	-0.022	0.045	0.5
Lysed	0.015	-0.034	0.064	0.6
Round 3 (n=50)				
Term	Mean*	CI		p
MA309	0.006	-0.033	0.045	0.7
Whole	-0.002	-0.030	0.027	0.9
Lysed	0.015	-0.053	0.083	0.7
CR	0.051	-0.006	0.109	0.08
Whole	0.027	-0.037	0.09	0.4
Lysed	0.076	-0.036	0.188	0.2
AI	-0.048	-0.076	-0.019	<b>0.001</b>
Whole	-0.048	-0.083	-0.012	<b>0.009</b>
Lysed	-0.048	-0.086	-0.009	<b>0.02</b>

\* Mean is increase in mexameter reading per 1000 cell counts

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa increta*

Table 10b. Means and 95% confidence intervals (CI) for erythema mexameter readings estimated in regression dose-response models with cell counts for whole and lysed cells from different species (MA338, AC, NS in Rounds 1 and 2; MA309, CR, AI in Round 3) excluding subjects reacting to negative controls.

Rounds 1 & 2 excluding subjects reacting to neg. controls (n=49)				
Term	Mean*	CI		p
MA338	0.000	-0.025	0.026	1.0
Whole	-0.011	-0.042	0.021	0.5
Lysed	0.011	-0.035	0.057	0.6
AC	-0.008	-0.031	0.014	0.5
Whole	-0.031	-0.071	0.009	0.1
Lysed	0.015	-0.022	0.052	0.4
NS	0.016	-0.019	0.051	0.4
Whole	0.004	-0.032	0.039	0.8
Lysed	0.027	-0.028	0.083	0.3
Round 3 excluding subjects reacting to neg. controls (n=39)				
Term	Mean*	CI		p
MA309	-0.011	-0.050	0.029	0.6
Whole	-0.010	-0.040	0.020	0.5
Lysed	-0.011	-0.083	0.060	0.8
CR	0.048	-0.013	0.109	0.1
Whole	0.040	-0.016	0.096	0.2
Lysed	0.056	-0.069	0.181	0.4
AI	-0.043	-0.072	-0.014	<b>0.004</b>
Whole	-0.039	-0.077	-0.002	<b>0.04</b>
Lysed	-0.047	-0.085	-0.009	<b>0.01</b>

\* Mean is increase in mexameter reading per 1000 cell counts

MA = *Microcystis aeruginosa* AC = *Anabaena circinalis* NS = *Nodularia spumigena*

CR = *Cylindrospermopsis raciborskii* AI = *Aphanocapsa incerta*

Figure 4a: Distribution of clinical gradings for whole and lysed cyanobacterial patches (MA338, AC and NS) for 20 non-atopic and 29 atopic subjects with no response to negative controls in Rounds 1 and 2.

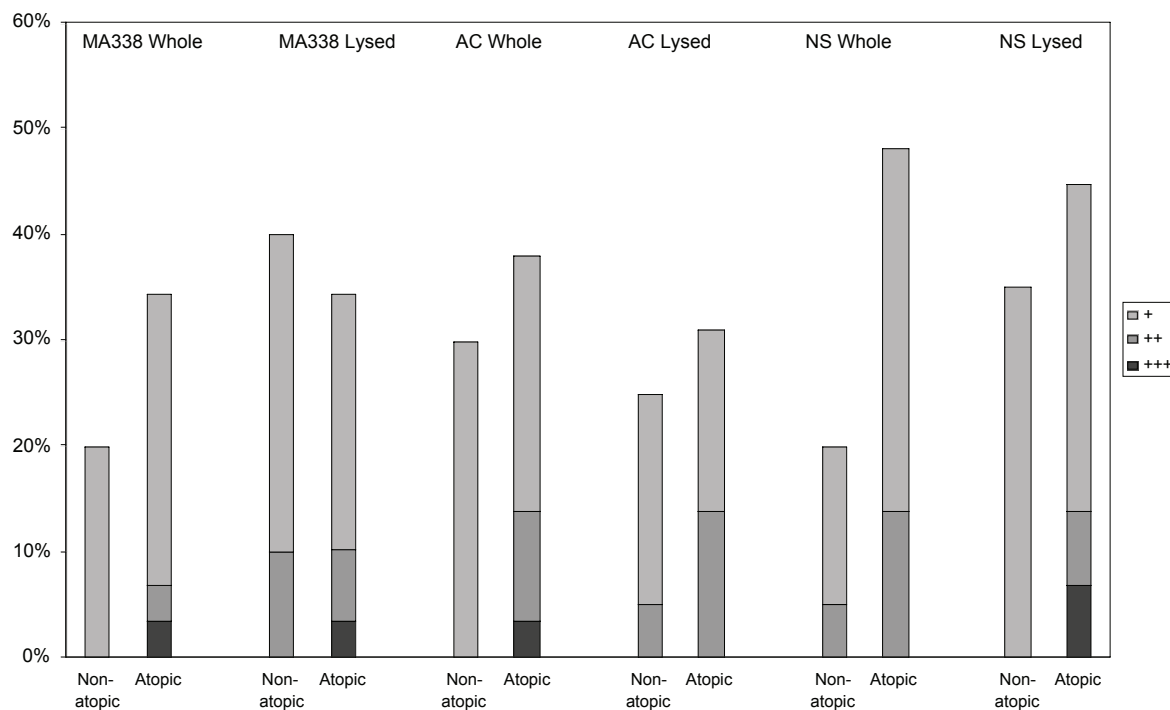


Figure 4b: Distribution of clinical gradings for whole and lysed cyanobacterial patches (MA309, CR and AI) for 24 non-atopic and 15 atopic subjects with no response to negative controls in Round 3.

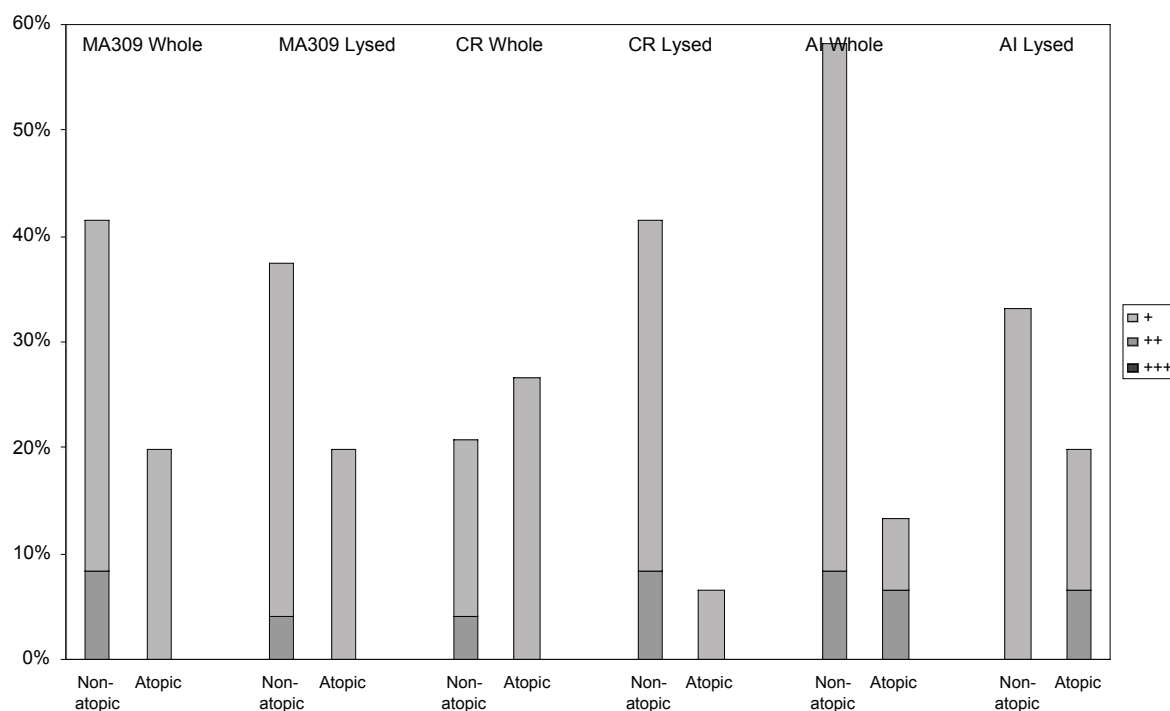


Table 11: Comparison of Cell counts from three laboratories for six different cyanobacteria at six different concentrations.

Organism	Mean cell count for three laboratories (n=3)	Standard deviation (S)	Coefficient of variation (CV) ie. standard deviation as a percentage of mean
<b><i>Microcystis aeruginosa</i> 338</b>	7,325	1,200	16
	28,272	6,668	24
	81,284	20,872	26
	119,360	37,755	32
	283,753	26,902	9
	366,238	62,969	17
<b><i>Nodularia spumigena</i> 001E</b>	7,028	1,257	18
	22,851	9,808	43
	61,057	8,775	14
	107,288	21,444	20
	209,449	25,451	12
	292,124	11,522	4
<b><i>Anabaena circinalis</i> 118AR</b>	4,857	2,266	47
	23,905	10,592	44
	52,044	24,542	47
	79,852	18,782	24
	193,119	67,729	35
	244,679	96,005	39
<b><i>Cylindrospermopsis raciborskii</i> 031C</b>	5,538	1,144	21
	18,876	3,865	20
	76,148	32,023	42
	137,568	50,424	37
	274,035	149,294	54
	375,149	217,771	58
<b><i>Microcystis aeruginosa</i> 309(1)CA</b>	5,832	1,027	18
	20,991	2,298	11
	82,008	11,765	14
	127,329	27,313	21
	263,852	73,630	28
	396,325	83,262	21
<b><i>Aphanocapsa incerta</i> 001</b>	108,714	50,388	46
	130,976	68,396	52
	192,946	60,210	31
	228,322	130,601	57
	309,775	152,419	49
	347,928	224,455	65

Table 12: Cell measurements and calculated cell volumes for each of the six cyanobacteria at all three laboratories.

Organism	Laboratory	Mean length (m)	Mean breadth (m)	Volume of single cell calculated from mean length/breadth (m <sup>3</sup> )	Mean	Standard deviation (s)	Coefficient of variation (CV) ie. standard deviation as a percentage of mean
<i>Microcystis aeruginosa</i> 338	1	4.2		38.79	26.00	12.07	46
	2	3.6		24.42			
	3	3.05		14.8			
<i>Nodularia spumigena</i> 001E	1	4.02	9.45	281.96	161.31	106.06	66
	2	3	5.93	82.8			
	3	3.81	6.31	119.16			
<i>Anabaena circinalis</i> 118AR	1	5.49		86.64	75.46	11.82	16
	2	4.94		63.09			
	3	5.27		76.64			
<i>Cylindrospermopsis raciborskii</i> 031C	1	6.16	2.45	29.04	29.53	1.21	4
	2	5.82	2.6	30.9			
	3	5.65	2.54	28.64			
<i>Microcystis aeruginosa</i> 309(1)CA	1	4.43		45.52	42.03	8.78	21
	2	4.52		48.53			
	3	3.94		32.04			
<i>Aphanocapsa incerta</i> 001	1	1.03		0.57	0.56	0.031	5
	2	1		0.53			
	3	1.04		0.59			

## 4 DISCUSSION

### 4.1 SKIN IRRITATION

The results of these skin irritation trials have shown that between 20% and 24% of both atopic and non-atopic individuals reacted to at least one of the active patches of the six cyanobacteria studied across the concentration range tested. These reaction rates may be overestimated by approximately 10% since a significant number of individuals reacted to the negative control patches. After removal of individuals who reacted to negative controls, the reaction rate dropped to between 11% and 15%. This was the case for both whole and lysed cells, with little difference in reaction rates between these two skin treatments.

No difference in the irritant effect was observed between the six cyanobacteria evaluated. There was considerable variation in the pattern of reactions across the range of cell densities and no consistently sustained increasing dose-response relationship was identified for any of the cyanobacterial species tested.

There appeared to be a more frequent and higher reaction intensity to both whole and lysed cyanobacterial cultures by atopic people compared to non-atopic people in Rounds 1 and 2. However, the study was not powered adequately to address this and the higher reaction intensity was not replicated in the third Round with different species.

A number of studies have investigated the skin irritant effects of cyanobacteria but have used intradermal application of test solutions rather than surface patch testing. Heise (1951) observed allergic reactions in persons given intradermal injections of glycerosaline extracts of dried *Microcystis* and *Oscillatoriaceae* species. Ten people were found to react to both groups of cyanobacteria. However, another 50 people showed no reaction to any of the test organisms. They concluded that both *Microcystis* and *Oscillatoriaceae* species contained similar antigens and that only certain individuals would show an allergic response.

McElhenny *et al* (1962) performed intradermal skin tests using four different green algae species on 140 children. This included 20 non-allergic children who did not have pollen and/or other inhalant sensitivities. None of the non-allergic group showed a reaction. Of the 120 allergic children, 98 showed positive reactions to one or more of the algal species with 22 showing no reaction. These results suggest a strong link between allergic individuals and an allergic response to algal species.

Mittal *et al* (1979) carried out a study investigating the clinical aspects of respiratory allergy to algae. Results were presented for 4,000 skin tests (intradermal) performed on 400 subjects suffering from nasal-bronchial allergy and 300 skin tests on 30 healthy persons. Ten common algae and cyanobacteria isolated from the atmosphere of Delhi,

India were used as test organisms and included members of the genera *Lyngbya*, *Phormidium*, *Anabaena*, *Scytonema*, *Chlorella*, *Westiellopsis*, *Anabaenopsis*, *Oscillatoria*, *Nostoc* and *Chlorococcum*. Positive skin reactions ranged from 25.7% for *Lyngbya* to 1.7% for *Oscillatoria* in allergic volunteers. For individuals who did not suffer from nasal-bronchial allergies there were no positive reactions. Prausnitz-Küstner tests (a test for the presence of an immediate hypersensitivity), bronchial provocation and conjunctival tests were all negative in subjects with negative skin reactions. However, in subjects with positive skin reactions Prausnitz-Küstner was positive for 70.9%, bronchial provocation in 50% and conjunctival in 48%. Levels of total human serum immunoglobulin E (IgE) in subjects with naso-bronchial allergy were higher, ranging from 1225 to 1550 international units per mL, while healthy volunteers had values less than 885 international units per mL. An international unit is a quantity of a biologic material, in this case IgE, that produces a particular biological effect agreed upon as an international standard. The 1<sup>st</sup> WHO reference preparation of IgE, as referred to in this paper, was established by WHO in 1973 (WHO, 1973).

While Heise (1951) suggested that certain individuals are specifically allergic to cyanobacteria, Mittal *et al* (1979) and McElhenny *et al* (1962) showed a direct link between those people who suffered from nasal-bronchial allergies and skin irritation due to exposure to cyanobacteria. In contrast, the present study showed that individuals who were atopic, ie. people who suffered from eczema, hay fever, asthma or who had been diagnosed as atopic, did not show a statistically significant increase in response rate compared to those that were not atopic.

Heise, (1951), McElhenny *et al* (1962) and Mittal *et al* (1979) all used intradermal applications of cyanobacteria. In breaking the skin barrier it could be expected that a more severe reaction would be observed compared to exposure of unbroken skin. In general, bathing or recreational activities would not result in exposure of people to cyanobacteria via broken skin. Therefore, the use of skin patches in the current study is more representative of normal exposure to cyanobacteria in the environment. However, irritation can occur around mucous membranes, eyes or broken skin and in situations where the cyanobacterial cells are rubbed into the skin (eg. underneath swimsuits and wetsuits). Exposure in these situations may have more in common with the experimental methods of Heise, (1951), McElhenny *et al* (1962) and Mittal *et al* (1979).

Stewart (2004) conducted a clinical dermatological study to examine delayed-contact hypersensitivity reactions to cyanobacterial extracts. The study groups were 20 patients presenting for diagnostic skin patch testing at the Royal Brisbane Hospital's dermatology outpatient clinic and a convenience sample of 20 individuals were recruited from outside the hospital as a control group. One patient

developed unequivocal reactions to several cyanobacteria extracts, with no dose-response pattern seen which they argue showed that the reactions were allergic in nature.

The significance of the characterised toxins produced by the cyanobacteria (ie. hepato- and neurotoxins) in this study requires comment. There was no difference in skin irritation reaction for the cyanobacteria that produced known toxins (*Microcystis aeruginosa*, *Cylindrospermopsis raciborskii*, *Nodularia spumigena* and *Anabaena circinalis*) compared to the non-toxic cyanobacteria (*Microcystis aeruginosa* and *Aphanocapsa incerta*). Toronke et al (2001) investigated the irritant effects of cyanobacterial toxins using sensitisation tests on albino guinea pigs and intradermal reactivity and ocular irritation tests on albino rabbits. Cultures used included *Microcystis*, *Anabaena*, *Cylindrospermopsis* and *Aphanizomenon* bloom samples and axenic cultures of *Anabaena*, *Oscillatoria* and *Microcystis*. Freeze dried algal suspensions in physiological salt solution were used for the tests. Slight skin irritation was recorded for *Anabaena*, *Microcystis* and *Aphanizomenon*, however, no correlation was found between the toxin content and the allergenic character. Toronke et al 2001 also made both water and lipid soluble fractions of a lyophilised *Aphanizomenon* bloom sample using water and chloroform extraction. A slight irritative effect was shown by the water soluble fraction with a negligible reaction to the lipid soluble fraction. Toronke et al (2001) argue that the irritation effect is caused by lipopolysaccharides and suggest that their source is from the bacteria present in the bloom samples and not from the cyanobacterial cell wall. They argue that this was shown by no irritation effects recorded for axenic cyanobacterial cultures.

Stewart (2004) used the mouse model of delayed-contact hypersensitivity, the mouse ear swelling test, and demonstrated that the purified toxin cylindrospermopsin is capable of producing cutaneous injury. The procedure involved four applications of test material to the abdomen (100 µg cylindrospermopsin/mL) – the induction phase – followed by challenge application to the ear. A micrometer gauge is used to measure ear thickness; an increase in ear swelling of 20 per cent or greater in one or more test mice is regarded as a positive result. Stewart (2004) recorded pronounced exfoliative lesions on abdominal skin during the induction phase of the experiments and delayed-contact hypersensitivity reactions were also demonstrated.

Epidemiological studies have also been used to try and identify an association between cyanobacteria and skin irritation. Skin irritations were a frequently reported symptom found in an epidemiological study by Pilotto et al (1997) on health effects after recreational exposure to cyanobacteria of 852 participants, 777 of whom had water contact and were considered exposed, and 75 who did not have water contact and were considered not exposed. Less than a quarter of the participants experienced one

or more symptoms which included vomiting or diarrhoea, cold and flu symptoms, mouth ulcers, eye irritation, ear irritation, skin rash and fever. Cold and flu like symptoms were the most common. For each symptom apart from eye irritation, there tended to be a higher rate of occurrence in the exposed participants. However, since the occurrence for each individual symptom was low, the presence of one or more symptoms was chosen as the outcome variable for comparative analysis. Results showed that participants who were exposed to more than 5,000 cells/mL had a significantly higher symptom occurrence rate than the unexposed. They argued that the results showed that symptom occurrence was associated with duration of contact with water containing cyanobacteria and with cyanobacterial cell density.

Stewart (2004) undertook a study of recreational exposure to freshwater cyanobacteria in southern Queensland, the Myall Lakes area in New South Wales, and northeast and central Florida, USA. The study design was a prospective cohort study which included 1,331 individuals recruited prior to engaging in various water recreation activities at freshwater and brackish lakes and reservoirs. Participants were given a self-administered questionnaire on the day, which provided basic demographic information. This was followed-up by a telephone interview after the third post-exposure day to determine symptom occurrence rate and severity. Water samples were collected on the recruitment day for cyanobacterial counts. Reported symptoms of people exposed included ear irritation, eye irritation, gastrointestinal problems (eg. vomiting, diarrhoea), respiratory problems (eg. sore throat, breathing difficulties), skin irritation (eg. rash, itchiness) and fever. The study divided exposure into three classes, characterised in units of cell density, total cell surface area, and total cell biovolume: low (<20,000 cells/mL, <2.4mm<sup>2</sup>/mL, 2.5mm<sup>3</sup>/L), intermediate (20,000 – 100,000 cells/mL, 2.4 – 12 mm<sup>2</sup>/L, 2.5mm<sup>3</sup>/L – 12.5mm<sup>3</sup>/L) and high (>100,000 cells/mL, >12 mm<sup>2</sup>/L, >12.5mm<sup>3</sup>/L). Surface areas and biovolumes were calculated using cell counts and a spherical cell of 6.2µm diameter, which is typical for *Microcystis aeruginosa* or *Anabaena circinalis*. The study showed that individuals exposed to recreational waters from which total cyanobacterial cell surface areas exceeded 12mm<sup>2</sup>/mL (100,000 cells/mL) were more likely to report symptoms after exposure than those exposed to waters where cyanobacterial surface areas were less than 2.4mm<sup>2</sup>/mL (20,000 cells/mL). Mild respiratory symptoms appeared to be the most influential contribution to overall symptom reporting. The relative odds ratio reported were that respiratory symptoms were 2.08 times more likely to be reported by subjects exposed to high levels of cyanobacteria than by those exposed to low levels.

It is possible that the lack of dose-response in the current study may be due to the limitations of the skin patch test method and requires discussion. A number of studies have

investigated the reproducibility of patch tests. Bourke *et al* (1999) explains that there is conflicting evidence regarding the reproducibility of patch testing. They carried out duplicate patch testing on opposite sides of the upper back of 383 individuals with 10 allergens. Discordant patch tests were recorded in 30 (8%) of the subjects. Patch tests were performed by Gollhausen *et al* (1989a) with a series of 39 substances on 401 subjects. Of all positive reactions, 40% were non-reproducible at sequential testing (1-week later) and 43.8% were non-reproducible at concomitant testing (applied on both sides of the back). It was also noted that weakly positive reactions were more often non-reproducible than stronger reactions. Gollhausen *et al* (1989b) also carried out a duplicate patch test series comprising 12 allergens simultaneously applied to either side of the backs of 63 subjects using both Finn Chambers (as used in the current study) and TRUE Test chambers. Non-reproducible positive results were recorded for 37.9% of Finn Chambers and 17.9% of the TRUE Test chambers. In the study by Gollhausen *et al* (1989a) the lack of reproducibility in patch tests was approaching 50%.

Finally, there was little consistency between clinical grading levels and mean mexameter measurements across the different cell densities for the six cyanobacteria used in the current study. The consultant dermatologist involved in this study considered that the clinical gradings were a more accurate method for the measurement of erythema. An experienced dermatologist is better able to discern between areas suffering from erythema compared to the surrounding unaffected skin.

#### 4.2 CELL COUNTING AND BIOVOLUME MEASUREMENT

Increasing counting errors with decreasing cell concentration was observed in the current study for *Nodularia spumigena* where CV decreased from between 18% - 43% at the lowest cell densities to 4% - 12% at the highest cell densities. However, the reverse was observed for *Cylindrospermopsis raciborskii*. The remainder of the cyanobacteria showed no trend.

Hamilton *et al* (2001) observed increasing counting errors with decreasing cell counts for *Tetrahedron minimum*, *Cryptomonas erosa* and *Anabaena spp.* when using a modified settling chamber and lake water samples. Hamilton *et al* (2001) found that the co-efficient of variation decreased from 53.2% for a mean cell count of 33 cells/mL to 12.9% for a mean cell count of 1017 cells/mL.

In this study the determination of cell volume measurements for each of the cyanobacteria also varied significantly between the three laboratories. The highest variation occurred for *Nodularia spumigena*, with a CV of 66%. The lowest CV, 4%, was recorded for *Cylindrospermopsis raciborskii*.

Rott (1981) investigated variation in total algal cell volume ( $\text{mm}^3/\text{L}$ ) of water samples from seven different European lakes using fourteen different participants. They also found large variation between participants and laboratories with confidence limits (95%) ranging from 30% – 50% for two oligotrophic lakes and from 10% – 30% for two mesotrophic and five eutrophic lakes. Hobro and Willén (1977) compared total cell volume ( $\text{mm}^3/\text{L}$ ) measurement of a water sample collected from the Baltic Sea between 3 laboratories and recorded a CV of 77%. They explained that the difference was due to low numbers of individual species and different preservation fluids.

Baker (1986) explained that errors in enumeration of phytoplankton can be divided into two basic types, systematic and random. He identified systematic errors as those that arise from faulty methods and include:

- Errors in filling the counting chamber – less than full volume or biased distribution of organisms due to pipette delivery
- Inadequate homogenisation of samples prior to the filling of counting chambers
- Inadequate settling time prior to counting of small phytoplankton
- Errors in sedimentation technique, ie. cyanobacteria may take longer to settle
- Error associated with enumeration at the edges of the microscope field of view.

Random errors affect the precision and accuracy of results and arise from:

- The spatial and temporal variation of phytoplankton distribution
- The sampling procedure ie. the number, size and frequency of samples and subsamples
- The enumeration procedure ie. the number of organisms counted, estimation of the number of cells per colony or filament and observer error.

Hillebrand *et al* (1999) notes that variation in cell volumes can be caused by errors in measurement of cell dimensions caused by light halos around cells which mask actual dimensions.

It should be noted that slight variations in mean cell length and mean cell breadth can result in very large changes in the final cell volume. The magnitude of this effect appears to be influenced by the equation used. This is most pronounced when the equation for a sphere is used. An example of this can be seen for *Microcystis aeruginosa* where a 1  $\mu\text{m}$  difference in cell length between laboratories resulted in up to a 50% difference in cell volume (Table 12). If these variations in cell dimensions are coupled with large differences in cell count then the errors in total cell volume become magnified.

Methods to reduce errors in cell counts and cell volume measurements as described above have been presented by many authors (Rott, 1981; Hötzel and Croome, 1999; Hillebrand *et al*, 1999; Eaton *et al*, 1995). The methods used for enumerating cell numbers and measurement of cell volume used in this study are accepted for use

by algal laboratories in Australia (Hötzel and Croome, 1999). However, results have shown that large variations in cell counts and cell volumes for cyanobacteria were still recorded between laboratories even when these methods were used.

## 5 SUMMARY AND CONCLUSIONS

### 5.1 SKIN IRRITATION

This study has shown that only a small percentage of individuals exhibited skin irritation when exposed to cyanobacteria at a range of environmentally relevant concentrations that are normally encountered in freshwaters used for contact recreation. This suggests that a small proportion of the healthy people in the population (around 20%) may develop a skin reaction to cyanobacteria in the course of normal water recreation. Further, the reactions were mild and were resolved without treatment. The response was not dose-related making it impossible to determine a minimum exposure level to fully prevent the occurrence of skin irritation during bathing and recreational activity. The results are also not conclusive in identifying a link between atopic individuals and skin irritation making it difficult to target any particular groups with warnings about their susceptibility if exposed to waters containing cyanobacteria. The study suggests that the potential for skin irritation cannot be readily translated into a quantitative bathing or water recreation

guideline for cyanobacterial cell levels. This could only be done with further research to better characterise those individuals that do experience skin irritation reactions or possibly allergic reactions associated with cyanobacteria. In any case, the minor nature of the potential for irritant dermatitis from this type of exposure can be regarded as less important than the need to protect against potential adverse outcomes from oral ingestion of cyanobacteria (hepato- and neurotoxins) in recreational waters.

### 5.2 CELL COUNTING AND BIOVOLUME MEASUREMENT

The study indicated that large variations can occur between experienced laboratories in analyses for both cell count and cell volume measurements. This is in spite of the use of standard methods that aim to minimise analytical error. This inherent variability needs to be considered in assessment of algal analytical data, and be part of the judgement for comparison of results against guidelines.

## **6 RECOMMENDATIONS**

### **6.1 SKIN IRRITATION**

It is not possible or appropriate to derive a quantitative guideline for protection against skin irritation reactions caused by cyanobacteria in normal water recreation situations. This is because even though a small percentage of individuals may develop a skin irritation reaction, the reaction is mild and idiosyncratic, and there is no threshold or dose-response. However, it may be appropriate to issue a general precautionary warning in circumstances where cell numbers are elevated and exposure is likely.

Further, it is recommended that recreational guidelines should be derived based primarily upon the better known and characterised health risks associated with ingestion of cyanobacteria and their known toxins. The guideline in this case could be developed using animal toxicity data and conventional toxicological calculations to derive a protective level for sub-chronic exposure to cyanobacteria and cyanotoxins via ingestion in a typical recreational situation.

### **6.2 CELL COUNTING AND BIOVOLUME MEASUREMENT**

The high degree of variability associated with estimates of cell numbers of cyanobacteria in water by conventional counting techniques needs to be considered in both setting and interpreting cell-based and biovolume-based water guidelines. This may require setting conservative levels that take into account large variations in estimates of cell density and cell volume in the sampling and analytical process.

## **7 ACKNOWLEDGEMENTS**

Financial support for this study was provided by the Cooperative Research Centre for Water Quality and Treatment, Eraring Energy Pty Ltd., the New South Wales Health Department and the Sydney Catchment Authority. Particular thanks are due to Bruce Hodgson, Frank Mieszala, Christine Cowie and Daniel Deere from these organisations for their support to facilitate this project. Many thanks to

the students and staff of the School of General Practice, Flinders University for participating as volunteers in this study. Special thanks go to Raelene Burnley, Caroline Fazekas, Cecilia Freeman and Leon Linden for assistance with volunteer trials and to Peter Baker for supplying the cyanobacterial cultures used for this work.

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## APPENDIX I: ETHICS APPROVAL

**Flinders Medical Centre**  
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**Flinders Clinical Research Ethics Committee**

6 September 2000

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email: Carol.Hakof@fmc.sa.gov.au

### MEMORANDUM

TO: Prof. L. Pilotto  
FROM: Ms. C. Hakof, Executive Officer, Flinders Clinical Research Ethics Committee  
TOPIC: **Research Application – 103/00**

I am pleased to advise that the Flinders Clinical Research Ethics Committee has approved your research application in accordance with the following extract from the Minutes of its meeting held on 28 August 2000.

4867 RESEARCH APPLICATION 103/00 – PROF. L. PILOTTO

Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers.

Reviewer: Dr. C. Karapetis

This application was approved subject to the following:

1. Advertisement to be sighted and approved by this Committee.
2. Payment to volunteers – clarify what will happen if a volunteer is to withdraw from the study.
3. Clarify who the study co-ordinator is, and by whom consent will be obtained.
4. Patient information sheet and consent form require amendments which have been conveyed to the investigator.
5. Clarify who will be providing the funding for this study.

**If conditional** ('subject to' or 'in principle') approval is granted, research involving human subjects **may proceed only after written acceptance of the conditions of approval** (including a copy of the modified research protocol) has been received by the Committee.

**This approval is for a period of one year. Application for re-approval must be made annually.** Please note that if this trial involves normal volunteers it will be necessary for you to keep a record of their names and you may be required to supply this list with your annual report.

You are reminded that the Flinders Clinical Research Ethics Committee must approve the content and placement of advertisements for the recruitment of volunteers.

**The Committee must be notified and approve any changes** (e.g. additional procedures, modification of drug dosage, changes to inclusion or withdrawal criteria, changes in mode and content of advertising) in the investigational plan particularly if these changes involve human subjects.

The safe and ethical conduct of a trial is entirely the responsibility of the investigators. While the Flinders Clinical Research Ethics Committee takes care to review and give advice on the conduct of trials, approval by the Committee is not an absolute confirmation of safety, nor does approval alter in any way the obligations and responsibilities of investigators.

It is the duty of the chief investigator to give prompt notification to the Flinders Clinical Research Ethics Committee of matters which might affect continued ethical acceptability of the project, including:

1. Adverse effects of the project on subjects, including the total number of subjects recruited, and of steps taken to deal with these adverse effects.
2. Other unforeseen events.
3. A change in the base for a decision made by the Committee, e.g. new scientific information that may invalidate the ethical integrity of the study.

If patients are involved the chief investigator is also responsible for the process of notification, seeking approval or permission of Departments, Divisions or individual consultants.

#### **C. Hakof**

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4 October 2000

## MEMORANDUM

TO: Prof. L. Pilotto, Department of General Practice


FROM: Ms. C. Hakof, Executive Officer, Flinders Clinical Research Ethics Committee

TOPIC: **Research Application 103/00**

Your attention is drawn to the following extract from the Minutes of the Committee's meeting held 25 September 2000.

4884.11 Research Application 103/00 – Prof. L. Pilotto  
Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers.  
Reviewer: Dr. C. Karapetis

Memorandum dated 20 September 2000 satisfactorily responding to this Committee's provisos was received and noted. Revised information sheet and consent form were approved.



**C. Hakof**

## APPENDIX II: GUIDELINE DEVELOPMENT, PUBLICATIONS AND CONFERENCES

### ***Development of Recreational Guidelines***

The findings from this study have been reviewed and contributed to the development of guidelines for cyanobacteria and cyanotoxins as part of the National Health and Medical Research Council “Guidelines for Managing Risks in Recreational Water” (NHMRC, 2005).

### ***Publications***

- Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers

Louis Pilotto, Peter Hobson, Michael Burch, Geetha Ranmuthugala, Robin Attewell, Warren Weightman

*Australian and New Zealand Journal of Public Health*, 2004; 28: 220-224

### ***Conference Presentations***

- Skin Contact with Cyanobacteria (blue-green algae) - Does it Matter?

Peter Hobson, Michael Burch, Louis Pilotto, Goethe Ranmuthugala, Robin Attewell, Warren Weightman

Conference Proceedings of the AWA and IWA Chemicals of Concern in Water Specialty Conference

Sydney, NSW, 4-5 June, 2003

- Skin Contact with Cyanobacteria and Development of a Recreational Guideline

Peter Hobson, Michael Burch, Louis Pilotto, Goethe Ranmuthugala, Robin Attewell, Warren Weightman

Sixth International Conference on Toxic Cyanobacteria (ICTC)

Bergen, Norway, 21-27 June, 2004

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CRC for Water Quality  
and Treatment



The Cooperative Research Centre (CRC) for Water Quality and Treatment is Australia's national drinking water research centre. An unincorporated joint venture between 29 different organisations from the Australian water industry, major universities, CSIRO, and local and state governments, the CRC combines expertise in water quality and public health.

The CRC for Water Quality and Treatment is established and supported under the Federal Government's Cooperative Research Centres Program.

The Cooperative Research Centre for Water Quality and Treatment is an unincorporated joint venture between:

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- Power and Water Corporation
- Queensland Health Pathology & Scientific Services
- RMIT University
- South Australian Water Corporation
- South East Water Ltd
- Sydney Catchment Authority
- Sydney Water Corporation
- The University of Adelaide
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- The University of Queensland
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- University of Technology, Sydney
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- Yarra Valley Water Ltd