

Effect of Irrigation with Lake Water Containing Microcystins on Microcystin Content and Growth of Ryegrass, Clover, Rape, and Lettuce

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ABSTRACT: The effect of irrigation with lake water containing a variety of microcystins on accumulation of toxins, or toxin metabolites, and plant growth in ryegrass, clover, rape, and lettuce, was investigated in a glasshouse experiment. The plants were grown in sand culture and received either three or six applications of lake water, which was applied either directly to the sand surface or to the plant shoots. As determined by LC–MS, each plant received 170 μg of a mixture of 10 different microcystins per application. Microcystins in plant samples were extracted with 70% methanol and analyzed by Adda-specific ELISA. For the shoot application treatment, microcystins were not present at measurable levels in shoots of ryegrass or rape, but were present in lettuce [0.79 mg/kg dry weight (DW)] and clover (0.20 mg/kg DW). Total microcystin concentration in roots did not vary greatly depending on whether treatment water was applied directly to the sand, or reached the roots via run-off from the shoots. Microcystins in roots were highest in clover (1.45 mg/kg DW), intermediate in lettuce (0.68 mg/kg DW) and low in ryegrass (0.20 mg/kg DW), and rape (0.12 mg/kg DW). There was no evidence for root-to-shoot translocation of microcystins. Three applications of microcystins reduced shoot DW of ryegrass, rape and lettuce, and increased root DW of ryegrass and lettuce. Clover DW was not changed by treatment with microcystins. The results show that irrigation with water containing microcystins has the potential to move microcystins into farm animal and human food chains at concentrations that can exceed recommended tolerable limits. © 2008 Wiley Periodicals, Inc. *Environ Toxicol* 23: 246–252, 2008.

Keywords: cyanobacteria; microcystins; plant uptake; *Lactuca*; *Brassica*; *Lolium*; *Trifolium*; toxin accumulation

INTRODUCTION

Contamination of lake and reservoir waters by cyanobacteria such as *Microcystis* is a world-wide problem. Some *Microcystis* species produce microcystins—cyclic heptapeptides with over 70 structural variants that exhibit a range of hepatotoxicities (Zurawell et al., 2005). A β -amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-deca-dieonic acid (Adda) (Rinehart et al., 1988) is present in over 80% of the analogs which are derived from changes to

the L-amino acids or the presence or absence of methyl groups at other nonconserved sites (Carmichael, 1992). The microcystins have been shown to be potent and specific inhibitors of protein phosphatases 1 and 2A from both animals and higher plants, and this inhibition accounts for their extreme toxicity (MacKintosh et al., 1990; Hastie et al., 2005).

Both submerged and emergent aquatic plants have been shown to absorb microcystin-LR (MC-LR) from low external concentrations (0.5 $\mu\text{g}/\text{mL}$) (Pflugmacher et al., 1998; Pflugmacher et al., 2001; Yin et al., 2005), and accumulate it in the shoot tissue. MC-LR inhibited plant growth and photosynthetic oxygen production, and bleached chlorophyll pigments (Pflugmacher, 2002). Watercress (*Lepidium*

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sativum) exposed to 1 $\mu\text{g/L}$ MC-LR had significantly reduced root growth, and at 10 $\mu\text{g/L}$ of MC-LR, whole plant growth was reduced (Gehring et al., 2003). In *Ceratophyllum* and watercress, MC-LR stimulated glutathione S-transferase's activity (Pflugmacher et al., 1999; Gehring et al., 2003), indicating that a detoxification pathway for MC-LR exists in aquatic plants.

In terrestrial plants, microcystin-RR at 5 $\mu\text{g/L}$ caused malformations in white mustard (*Sinapis alba*) seedlings, reduced seedling weights and lateral root formation, and inhibited protein phosphatase 1 and 2A (Kurki-Helaso and Meriluoto, 1998). Watering broccoli (*Brassica oleraceae*) and white mustard seedlings with water containing 0, 1, or 10 $\mu\text{g/L}$ microcystins had no effect on chlorophyll concentrations or fluorescence, and only a slight (<10%) growth inhibition in broccoli (Järvenpää et al., 2007). Of the four microcystin variants present in the exposure mixture, only MC-LR was detectable in plant samples, and then only in the roots. Uptake of MC-LR and microcystin-LF through roots and translocation of the toxins to shoots was reported for seedlings of 11 agricultural plants by Puthert et al. (2007). Growth and chlorophyll content of potato (*Solanum tuberosum*) cultures were reduced at 0.005 mg/kg MC-LR in the culture medium, and inhibited completely at 0.5–5 mg/kg MC-LR, while growth of bean (*Phaseolus vulgaris*) plants in culture was inhibited by MC-LR at 1.12 mg/kg (McElhiney et al., 2001). The authors concluded that uptake of these toxins by edible plants may have significant implications for human health. Spray irrigation of commercial lettuce (*Lactuca sativa*) plants with water containing *Microcystis* resulted in colonies and single cells of the cyanobacterium being lodged on the leaves 10 days after the last irrigation (Codd et al., 1999). MC-LR was present at 2.5 mg/kg dry weight (DW) in the central leaves, 0.833 mg/kg (DW) in the distal zone of mature leaves, and 0.094 mg/kg (DW) in the basal zone of mature leaves. The study indicated that toxins were absorbed by the plants as the central leaves would have been protected from irrigation. The cyanobacterial cells were not removed by washing the leaves in water. The authors recommended investigation of the fate of cyanobacterial cells and toxins during and after spray irrigation with water containing cyanobacteria, to contribute to development of policies on the use of such water and the acceptability of plants for human consumption after irrigation with contaminated water. Similar conclusions were reached for rice (*Oryza sativa*) and rape (*Brassica napus*) by Chen et al. (2004). They suggest that consumption of edible plants exposed to microcystins via irrigation may have health risks. Greater quantities of microcystins were recovered from the shoots of rape than from rice, indicating that different plant species may accumulate microcystins at different rates. We report here on the effects of lake water containing microcystins, on growth and accumulation of toxins or metabolites in three forage plants and lettuce.

MATERIALS AND METHODS

Experimental

Water containing cyanobacterial cells and microcystin toxins was collected in autumn (April 2005) from the outlet of Lake Hakanoa, (37°33.172S, 175°10.082E), North Island, New Zealand. Hakanoa is a shallow, nutrient-enriched lake with very high levels of algal biomass. A regular monitoring program by Environment Waikato Regional Authority showed that in April 2005, the lake water contained 152 200 cells/mL of *Microcystis aeruginosa* and 28 200 cells/mL of *Anabaena* c.f. *smithii* (B. Vant, Environment Waikato, pers. comm.). The water samples were frozen immediately after collection and stored at -20°C . They were thawed, to release microcystins from lysed cells, mixed thoroughly and samples taken for analysis for total microcystins (see below) and plant nutrient concentrations. The water was refrozen in 2-L containers and re-thawed prior to application to the test plants.

Plants of lettuce (*L. sativa* cv Green Oak Leaf), rape (*Brassica napus* cv Winfred), perennial ryegrass (*Lolium perenne* cv Grasslands Samson), and white clover (*Trifolium repens* cv Grasslands Kopu II) were raised from seed in 15-cm pots of sand culture. The faster growing species were sown later than the slower species so that differences in plant sizes were minimized when treatments commenced. The pots were in a temperature-controlled glasshouse with average day/night temperatures of 20.1 and 14.1 $^{\circ}\text{C}$, and irrigated regularly with a complete nutrient solution (Hewitt, 1966). The experimental layout was a row column design. Treatments with lake water were started on all plants on the same day, when the plants covered most of the surface of the sand. At this stage, the lettuce was 42-days old, the rape 67 days, the ryegrass 73 days, and the clover 75 days.

The frozen lake water was thawed in a water bath and brought to ambient glasshouse air temperature, before being passed through a 104- μm mesh sieve to break up any large algal masses and ensure the homogeneity of the liquid. Plants were separated into two treatment groups with each plant receiving 100 mL of filtrate applied either directly to the sand surface, or over the foliage using a small watering can fitted with a sprinkler. Shoots of the plants receiving the surface treatment were raised slightly above the sand with acrylic sheeting to prevent accidental contamination of the shoot tissue. Control plants received 100 mL of deionized water amended with potassium (8.2 mg/L), ammonium nitrogen (11.4 mg/L), phosphate (4.8 mg/L) and sulfur (0.03 mg/L), so that total nutrient inputs were equivalent across all treatments. The control treatment was applied in the same way and at the same temperature as the lake water treatments. There were 10 replicates of each of the four plant species \times lake water or control \times surface or shoot application treatments i.e., 160 plants in total.

Subsamples (1 mL) of each treatment water were retained frozen until analysis by ELISA for microcystin toxins. The treatments were repeated on days 3, 5, 8, 10, and 12. On day 8, after three treatments and prior to the application of the fourth, five replicates of every treatment were harvested. Harvesting of the remaining five replicates for each treatment group was done on day 15 after six treatments.

The plant roots were washed out of the sand, taking care not to wet the shoots, the root and shoot systems were separated and chilled with dry ice, frozen at -80°C and then freeze dried. Root and shoot DW were recorded, and the plant material was milled using a UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, CO).

Analytical Methods

Water Samples

Stored aliquots of water retained from each of the six treatment applications were thawed and analyzed using an in-house competitive indirect ELISA for Adda-containing microcystins based on the reagents and method described by Fischer et al. (2001). The antibodies used in this ELISA were raised against the Adda moiety, which gives the assay broad specificity and theoretically cross-reactivity with over 80% of the known microcystin analogs. The horseradish peroxidase substrate used was BioFX substrate solution (BioFX Laboratories, MD, USA). Samples were diluted in assay buffer (PBS containing 0.05% Tween 20) to bring the toxin concentration into the working range of the assay (150–9000 ng/L, I_{20} – I_{80}). An additional pooled sample was also supplied to the Cawthron commercial analytical laboratory (Nelson, New Zealand) for a single analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Wood et al., 2006) to quantify 13 individual microcystin analogs and nodularin.

Plant Shoot and Root Samples

Each sample (~ 0.5 g) of freeze dried and milled plant material was weighed into 15-mL tubes and extracted with 10 mL of 70% methanol in water by end-over-end rotation for 30 min. Aliquots (1.5 mL) of the extracts were centrifuged (Eppendorf Microfuge 5415 C) at $8600 \times g$ for 5 min and the supernatant retained. Methanol concentration in the extract was reduced to 2% by dilution in assay buffer. All extracts were analyzed at a minimum dilution of 1 in 105 to overcome matrix effects, and at a second dilution of 1 in 210, and each dilution was analyzed in duplicate wells. ELISA standard curves were prepared for each assay using MC-LR (Alexis, Switzerland) as the reference standard. Results were reported in MC-LR immunoreactive equivalents.

Statistical Methods

The experiment was laid down over three adjacent tables in a glasshouse using a row–column design. The data were an-

alyzed using GenStat (Ninth Edition, version 9.1.0.148). Analysis of the data showed that there was no benefit in accounting for the row–column arrangement, so the data were analyzed as a randomized block design using analysis of variance. Differences among the four plant species were investigated using least significant difference where the ANOVA indicated overall significant treatment effects. Some data required log transformation to satisfy the assumptions of the analysis of variance, and in these cases we quote back-transformed means with least significant ratios. Microcystins were not detected in any of the control samples, so these treatments were omitted from the analysis of the total microcystins data. The minimum limit of quantification for total microcystins by ELISA (0.04 mg/kg DW), was used in the data analysis for seven plants where microcystins were detectable, but below measurable limits.

RESULTS

Chemical Analyses

Water Samples

Mean total microcystins concentration determined (MC-LR immunoreactive equivalents) for the aliquots retained from water applied in each of the six treatment applications, was 2.1 mg/L (SD = 0.08, CV = 3.7%), and this is within the range of toxin concentrations reported for New Zealand water bodies (Wood et al., 2006). The sum of the concentration of individual analogs determined by LC–MS/MS in a pooled sample of the treatment waters, was 1.6 mg/L and 10 different microcystin analogs were detected in the sample (Fig. 1). The major components were microcystin-RR (31%), -LR (25%), -FR (15%) and -WR (15%) while microcystin-desMeRR, -LY, -desMeLR, -AR, -LA, and -YR ranged from 0.3% to 5%.

Plant Shoot and Root Samples

No Adda-containing microcystins were measured in any of the control plants so the statistical analysis of plant microcystins was restricted to the lake-water treatments. The treatment water ran off the rape and ryegrass leaves with minimal visible wetting of the leaf surfaces, the lettuce leaves were thoroughly wetted, while the clover leaves were moderately wetted. For the shoot application treatment, microcystins were not present at measurable levels in shoots of ryegrass or rape, but were present at measurable levels in lettuce and clover shoot samples (Table I). Total microcystin concentrations in lettuce and clover shoots did not change significantly between harvests, and averaged 0.79 and 0.20 mg/kg DW for lettuce and clover, respectively. No microcystins were found in the shoots of any plants when lake water was applied to the roots (Table I). Total microcystins concentration in roots differed

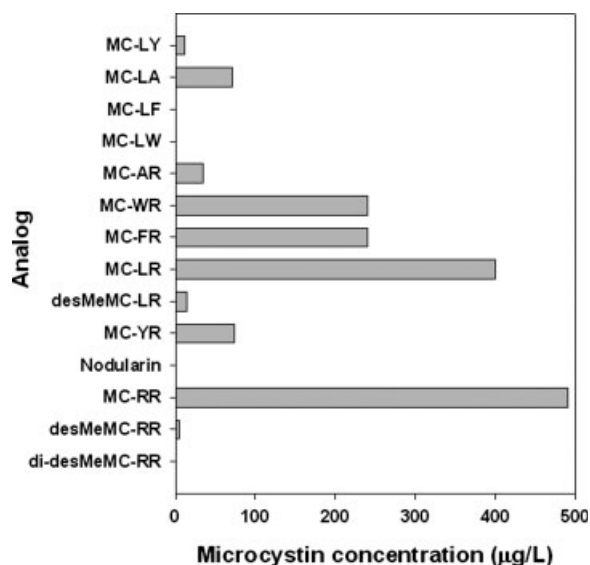


Fig. 1. Profile of microcystin analogs in a pooled sample of lake water applied to ryegrass, clover, rape, and lettuce plants. Toxin concentrations determined by LC–MS/MS, where limits of detection for MC-LF, MC-LW, nodularin, and di-desMeMC-RR, were 1, 0.5, 1, and 1 µg/L, respectively.

($P < 0.001$) among the plant species and was highest in clover (1.45 mg/kg DW), intermediate in lettuce (0.68 mg/kg), and low in ryegrass (0.20 mg/kg) and rape (0.12 mg/kg). Root total microcystins concentrations were lower ($P < 0.01$) at harvest 2, especially in the shoot application treatment (Table I), resulting from a significant ($P < 0.01$) time × lake water application-point interaction.

TABLE I. Adda-containing total microcystin concentration in shoots and roots of ryegrass, clover, rape, and lettuce plants at harvest 1 (H1) and harvest 2 (H2) after application of lake water containing microcystins to shoots or roots

	Microcystin Application	Shoot Samples		Root Samples	
		H1	H2	H1	H2
Ryegrass	Roots	nd ^a	nd	0.22	0.24
	Shoots	nd	nd	0.23	0.12
Clover	Roots	nd	nd	1.27	1.37
	Shoots	0.22	0.17	1.89	1.28
Rape	Roots	nd	nd	0.15	0.12
	Shoots	nd	nd	0.18	0.05
Lettuce	Roots	nd	nd	0.77	0.78
	Shoots	0.74	0.84	0.87	0.30
LSR _{0.05} ^b				2.19	2.19

All results are in milligram of microcystin-LR immunoreactive equivalents per kg DW and were determined by ELISA.

^aNot measurable.

^bLeast significant ratio ($P < 0.05$).

Shoot DW of ryegrass, rape, and lettuce were reduced by three applications of lake water directly to the roots (Table II). After six applications of lake water to the roots, all the shoot systems were heavier than at the first harvest and only lettuce showed any reduction in shoot DW compared with the control. After three applications of lake water to the roots, root DW of ryegrass and rape were heavier than the controls, but roots of clover and lettuce were not changed by the treatment (Table II). After six applications of lake water to the roots, ryegrass root DW was less than the controls, but rape root DW was still heavier than the controls. Clover and lettuce root DW did not differ from the controls. Application of lake water to the shoots had no effect on shoot DW after three applications (Table II), but after six applications, shoot DW of treated ryegrass and lettuce plants was less than for the control plants. After three applications of lake water to the shoots, root DW of treated rape plants was greater than for controls, but root DW of the other species did not differ from their controls. Six applications of lake water to the shoots resulted in the DW of rape roots being heavier than controls and lettuce root DW being lighter than controls (Table II).

The total amount of microcystins accumulated in the plants differed ($P < 0.001$) among plant species, with the highest levels in lettuce plants with lake water applied to the shoots (Table III). Microcystins accumulation did not differ between harvests. The location of lake water

TABLE II. Effect of three and six applications of contaminated lake water to either roots or shoots of ryegrass, clover, rape and lettuce on shoot and root DW

	Application No.	Root Application		Shoot Application	
		Control	Lake Water	Control	Lake Water
<i>Shoot DW</i>					
Ryegrass	3	3.83	3.23 ^a	3.04	3.26
Clover	3	2.06	2.42	2.94	2.76
Rape	3	6.32	5.76 ^a	5.84	6.02
Lettuce	3	6.66	6.02 ^a	5.93	5.90
Ryegrass	6	5.50	5.73	5.68	5.03 ^a
Clover	6	4.45	4.42	4.08	3.99
Rape	6	8.28	8.02	8.09	7.87
Lettuce	6	9.33	8.35 ^a	11.04	7.44 ^a
<i>Root DW</i>					
Ryegrass	3	3.02	3.38 ^a	2.51	2.37
Clover	3	0.84	0.99	1.23	1.13
Rape	3	3.00	3.61 ^a	3.15	3.71 ^a
Lettuce	3	2.03	1.82	1.79	1.93
Ryegrass	6	5.74	4.58 ^a	3.83	3.53
Clover	6	1.93	1.75	1.78	2.00
Rape	6	4.46	5.17 ^a	4.37	4.87 ^a
Lettuce	6	3.39	3.11	4.54	3.35 ^a

All values are in g DW.

^aSignificantly different ($P < 0.05$) compared with the control.

TABLE III. Accumulation of Adda-containing compounds in ryegrass, clover, rape, and lettuce plants after application of lake water containing microcystins to shoots or roots

	Microcystin Application	
	Roots	Shoots
Ryegrass	0.97	0.55
Clover	2.14	3.32
Rape	0.78	0.53
Lettuce	2.25	7.17

All results are in microgram of microcystin-LR immunoreactive equivalents per plant and were determined by ELISA. Least significant ratio ($P < 0.05$) = 0.72.

application had an effect ($P < 0.001$) on plant microcystins yields, and there was a plant species \times location of lake water application interaction ($P < 0.001$) on plant microcystin content. Shoot application resulted in higher plant microcystin yield in clover and lettuce, and root application gave higher yields in ryegrass and rape (Table III).

DISCUSSION

The total concentration of microcystin analogs determined in the lake water by LC-MS was less than that determined by ELISA. Over 70 microcystin analogs have been identified, 80% of which contain Adda. The ELISA used in this study will detect Adda-containing metabolites while the LC-MS method scans only for 13 microcystin analogs. The immunoassay will also measure Adda fragments resulting from microcystin degradation, and this may explain some of the difference in values obtained by the two methods. *In vitro* studies by Pflugmacher et al. (2001) on a reed plant (*Phragmites australis*) indicated that it is likely that glutathione and cysteine conjugates of microcystins would be produced during plant detoxification. The compounds recognized by the antibodies used in the ELISA are known to contain the Adda moiety which has a role in the toxicity of microcystins through interaction with protein phosphatases (Goldberg et al., 1995), and changes to the Adda structure can result in the formation of nontoxic compounds (Harada et al., 1990). It can also be postulated that Adda-containing plant metabolites could be toxic. In this study, 70% methanol in water was used as the extracting solvent which would be expected to extract free toxins and, if present, some toxin-peptide conjugates, while any higher molecular weight toxin-protein conjugates and/or complexes would be precipitated and excluded.

The average total microcystin concentration measured in whole lettuce shoot samples was 0.79 mg/kg (DW). This is in the concentration range (0.094–2.487 mg/kg DW), depending on the type of leaf sampled, measured by immu-

noassay in a commercial lettuce crop irrigated with water containing cyanobacteria (Codd et al., 1999). Taken together, these results show that microcystins applied to terrestrial plants at naturally occurring concentrations can be retained by the plants.

We cannot exclude the possibility that using lake water as a source of microcystins may have introduced other unknown bioactive compounds, including other cyanobacterial secondary metabolites into our experiment, and that these may have contributed to the observed effects on plant growth. However, the importance of *M. aeruginosa* in the cyanobacterial population in the lake, and the recovery of microcystins from plant samples, suggest that microcystins were the likely cause of the plant growth effects we observed, which were similar to those reported from previous studies using purified microcystins. The water applied to the control treatments was amended with plant nutrients to match those in the lake water so there were no plant mineral nutrition imbalances among the treatments. Because we are interested in the potential for microcystins to move from surface water into terrestrial food chains through activities such as irrigation, use of lake water seemed the most appropriate option for this initial experiment.

The results obtained for microcystin retention were plant-species specific, especially for microcystins applied to the shoot systems. Water applied to the shoots ran off the leaves of the rape and ryegrass, with very little wetting of the foliage, and no measurable absorption of microcystins by the shoots. In contrast, the lettuce and clover leaves were visibly wetted and retained microcystins. This suggests that leaf cuticle properties are very important in controlling absorption of microcystin applied to plant shoots, as is well established for organic agricultural compounds (Santier and Chanel, 1998).

The effects of microcystins on plant growth were more apparent at harvest one than at harvest two. This suggests that the plants were adapting to the presence of the toxins. At least part of this response in ryegrass and rape was an increase in root DW when the microcystins were applied directly to the roots, and to the shoots for rape. Järvenpää et al. (2007) reported no effect of microcystins on root growth of broccoli or mustard from microcystins applied at 10 μ g microcystins/L, but this was a much lower microcystin concentration than we used. Microcystins are strong inhibitors of protein phosphatases in higher plant cells (MacKintosh et al., 1990; Hastie et al., 2005), so they could readily disrupt absorption and transfer of nutrients or root phloem function. These effects could stimulate additional root growth to compensate for impaired root function at the expense of shoot growth. Clover plant growth was not affected by having the highest concentration of microcystins in the roots while lettuce plants showed the most consistent growth inhibitions in the presence of the microcystins. This shows that plants differ in their sensitivity to microcystins. The experiment yielded no evidence for root-

to-shoot translocation of microcystins in ryegrass, clover, rape, or lettuce. Translocation of microcystins from roots to shoots was reported for seedlings of other agricultural plants by Peuthert et al. (2007), but not by Järvenpää et al. (2007). A systematic investigation of plant species × microcystin concentration interactions is required to explain the contrasting results in the available data.

Quantitatively, less than 1% of the applied immunoreactivity was recovered in the plants. The highest levels of immunoreactivity were measured with shoot application of microcystins to lettuce and clover. These two species showed the most visible wetting of leaves during the treatment. This suggests that most of the microcystins applied in the lake water ran off the leaves before the toxins could be absorbed.

Should our results for the levels of microcystins in exposed plants be shown to be quantitative, then a 60-kg person, consuming a typical 65–75 g fresh weight salad (i.e., 6.8–7.9 g DW) made from the treated lettuce, would consume about 5.8 µg of microcystins per meal (0.10 µg/kg of body weight). This would exceed the tolerable daily intake of 0.04 µg/kg of body weight/day recommended by the World Health Organization (Chorus and Bartram, 1999) by a factor of two. This supports earlier suggestions that uptake of microcystins by edible plants may have significant implications for human health (Codd et al., 1999; McElhiney et al., 2001). This health concern has now increased since the assessment of the carcinogenicity of MC-LR by the International Agency for Research on Cancer that led to the conclusion that it is “possibly carcinogenic to humans” (group 2B) (Grosse et al., 2006). Our results indicate that the degree of risk will be plant-specific for leafy foods. All four species we tested contained microcystins in their roots, and the significance of microcystins for toxicity of root crops requires investigation.

There are no feed standards for microcystin intake by farm animals. A dairy cow consuming 15 kg DW per day of pasture containing 20% clover would consume 3 kg of clover (DW). In our study, measured clover herbage microcystin concentration was 0.21 mg/kg (DW) indicating that a cow would consume 630 µg of microcystins per day (1.26 µg/kg of body weight/day for a 500-kg live weight animal). This would exceed the recommended limit for microcystin intake in drinking water by farm animals (ANZECC, 2000), by a factor of four. The results support the hypothesis that irrigation with lake water has the potential to add significantly to the biotoxin load of farm animals.

Cyanotoxins released by cyanobacteria are widespread in New Zealand lakes with 60% of cyanobacterial blooms or benthic mats testing positive for microcystins, 10% for anatoxin-a and 77% for saxitoxins (Wood et al., 2006). There have been intermittent reports of stock and dog deaths in New Zealand resulting from animals consuming water with heavy blooms of cyanobacteria (Flint, 1966; Conner, 1977; Hamill, 2001). Wood et al. (2006) concluded

that impacts of cyanotoxins on farm animals may be under-reported in New Zealand, given the widespread occurrence of cyanobacteria in water that is accessible to stock. Our results indicate the potential for movement of microcystins into human and animal food chains via irrigation water. Microcystins may make animals grazing contaminated herbage more susceptible to other stresses (Babica et al., 2006), particularly those animals that are already ingesting a range of fungal toxins. The implications for human health are unknown until the nature and toxicity of the immunoreactive components detected in the plants are determined.

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