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THE EFFECTS OF CYANOBACTERIA AND THE CYANOBACTERIAL TOXIN MICROCYSTIN-LR ON Ca^{2+} TRANSPORT AND Na^+/K^+-ATPase IN TILAPIA GILLS

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Summary

The effects of cytotoxic substances from cyanobacteria on ionic transport processes in tilapia (*Oreochromis mossambicus*) were examined. Inhibitory effects on ionic transport including whole-body Ca^{2+} fluxes and P-type ATPases of the gill were found. The compounds tested were (1) purified microcystin-LR (MC-LR), a heptapeptide hepatotoxin produced by the cyanobacterium *Microcystis aeruginosa*, (2) extracts from *M. aeruginosa* strain PCC 7820, a strain producing MC-LR and other microcystin variants, and (3) extracts of *M. aeruginosa* CYA 43, a strain producing toxins including small quantities of MC-LR. Whole-body Ca^{2+} influx was inhibited by a 24 h exposure to extracts of *M. aeruginosa* CYA 43 and 7820, but not by exposure to an equivalent amount ($90\,mg\,l^{-1}$) of purified MC-LR. Shorter exposure times (4 h) were ineffective. Fish exposed to extracts from *M. aeruginosa* CYA 43 showed significant plasma hypocalcaemia. Both strains of *M. aeruginosa* inhibited Ca^{2+} uptake by basolateral plasma membrane vesicles (BLMVs), endoplasmic reticulum (ER) and mitochondria, as well as BLMV K^+-dependent *p*-nitrophenol phosphatase (pNPPase) activity. The hydrophobic fractions of the cyanobacterial extracts were the most potent, inhibiting BLMV, ER and mitochondrial Ca^{2+} uptake by up to 99 %, but they were less inhibitory of BLMV K^+-dependent pNPPase activity. Purified MC-LR was without effect on these preparations. In conclusion, cytotoxic substances from cyanobacteria have the potential to disrupt normal physiological processes dependent upon Ca^{2+} transport processes in tilapia gills.

Key words: Ca^{2+}-ATPase, plasma membranes, fish gills, *Oreochromis mossambicus*, microcystin, cyanobacterial toxins.

Introduction

In the gills of freshwater fish, Ca^{2+} influx is facilitated by a Ca^{2+} channel in the apical membrane of the chloride cells (Verbost *et al.* 1989); extrusion of Ca^{2+} from the cell across the basolateral membrane is mediated by a high-affinity Ca^{2+}-ATPase and possibly by a Na^+/Ca^{2+} exchanger (Flik *et al.* 1993; Verbost *et al.* 1994). The intracellular Ca^{2+} concentration could be regulated by this extrusion process, by Ca^{2+}-binding proteins, by active Ca^{2+} uptake into endoplasmic reticulum (ER) (Somlyo, 1985) or by sequestration by mitochondria *via* a uniporter (Carafoli, 1982; Gunter and Pfieffer, 1990; Gunter *et al.* 1994). Inhibition of the Ca^{2+}-ATPase of the basolateral membrane of chloride cells by Cd^{2+} (Verbost *et al.* 1987*a*, 1988, 1989) causes hypocalcaemia (Giles, 1984), indicating the importance of this branchial Ca^{2+} pump in calcium homeostasis.

Eutrophication of water bodies has resulted in an increased frequency of cyanobacterial (blue-green algal) blooms that may produce neuro- and/or hepatotoxins (Codd *et al.* 1989; Carmichael, 1992). On senescence of a bloom, these toxins are released into the water and they may cause fish kills (e.g. Schwimmer and Schwimmer, 1968). The majority of kills have been linked with hypoxia caused by the high biological oxygen demand on senescence of cyanobacterial blooms and scums. However, oxygen levels were above 90 % saturation when moribund brown trout were retrieved after the lysis of a bloom of the toxic blue-green alga *Anabaena flos-aqua* at Loch Leven, Scotland (Rodger *et al.* 1994). Histopathology revealed that these fish had severe liver damage similar to that of fish administered extracts of *Microcystis* spp. containing the hepatotoxin microcystin-LR (MC-LR) or purified MC-LR injected intraperitoneally (Phillips *et al.* 1985; Sugaya *et al.* 1990; Råbergh *et al.* 1991) or after gavage experiments with microcystin-producing cyanobacteria (Tencalla *et al.* 1994). However, immersion trials using concentrations of extracts of the hepatotoxic cyanobacterial cells similar to those found in eutrophic environments have produced sublethal effects in fish, e.g. increased plasma cortisol and glucose levels, while plasma Na^+ and Cl^- concentrations decreased over a 4 h exposure

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period (Bury *et al.* 1996). Long-term exposure, for 63 days, was detrimental to fish growth and resulted in disturbances to ionic regulation (Bury *et al.* 1995).

In vitro, MC-LR specifically inhibits protein phosphatases 1 and 2A (Eriksson *et al.* 1990; Honkanen *et al.* 1990; MacKintosh *et al.* 1990; Matsushima *et al.* 1990). There is also one report that MC-LR inhibits Na^+/K^+-ATPase activity in carp gill microsomes (Gaete *et al.* 1994), and these observations imply that aspartic acid–phosphatase hydrolase activity of P-type ATPases is also a target for MC-LR. In view of these results, the present study examines the effects of purified MC-LR and of extracts of cells from the cyanobacteria *Microcystis aeruginosa* 7820 (which produces a range of microcystins including MC-LR) or *M. aeruginosa* CYA 43 (which produces toxins containing very small quantities of MC-LR) on Ca^{2+} transport in tilapia, *Oreochromis mossambicus.* This investigation focuses on whole-body Ca^{2+} influxes, on Ca^{2+} transport in a gill basolateral membrane vesicle (BLMV) preparation and in endoplasmic reticulum (ER) and mitochondria and on Na^+/K^+-ATPase activity of the gill BLMVs.

Materials and methods

Fish holding conditions

Freshwater tilapia (*Oreochromis mossambicus* Peters) were obtained from laboratory stocks and were held in running Nijmegen tap water (in $mmol\,l^{-1}$, 0.7 Ca^{2+}, 0.5 Na^+, 0.06 K^+, pH 7.8, 27 °C) under a light regime of 12 h:12 h light:dark. Fish were fed Trouvit fish pellets (Trouw and Co., Putten, The Netherlands) at a ration of 1.5 % body mass per day.

Cyanobacterial culture, toxin purification and fraction preparation

A method for culturing *Microcystis aeruginosa* PCC 7820 has been described previously (Bury *et al.* 1995). Chlorophyll *a* levels were measured according to MacKinney (1941). Cells were harvested in the early stationary phase of batch culture by continuous centrifugation in a Sharples centrifuge (Sharples Ltd, Surrey), the cell pellet was collected, freeze-dried and microcystin-LR (MC-LR; molecular mass 994 kDa) extracted by high-performance liquid chromatography (HPLC) (Lawton *et al.* 1994). *M. aeruginosa* CYA 43 was similarly cultured, but does not yield MC-LR at levels detectable by HPLC (detection limit, 5 ng of microcystin on the HPLC column).

For *in vitro* Ca^{2+} transport and Na^+/K^+-ATPase assays, freeze-dried cyanobacterial cells were twice extracted in methanol (0.314 g per 50 ml methanol), centrifuged at 100 ***g*** for 15 min (Sorval RC-5B), the supernatants were combined, dried under vacuum, and the extract was resuspended in 2 ml of methanol and stored at −20 °C. Samples were dried and resuspended in sucrose buffer containing 250 $mmol\,l^{-1}$ sucrose and 10 $mmol\,l^{-1}$ Hepes/Tris, pH 7.4, to give a final concentration of 150 mg dry mass ml^{-1}, equivalent to 0.5 mg ml^{-1} MC-LR for *M. aeruginosa* PCC 7820 extracts. Additions of the cyanobacterial extracts will be referred to as milligrams of freeze-dried cells per milligram of membrane protein. Purified microcystin-LR was resuspended in sucrose buffer to a concentration of 2 $\mu g\,\mu l^{-1}$ MC-LR.

The methanol extracts from *M. aeruginosa* 7820 and CYA 43 were fractionated by a Pharmacia 'Smart' system using the eluents acetonitrile/0.5 % trifluoroacetic acid (TFA) and milliQ water/0.05 % TFA. A 65 % acetonitrile (v/v) gradient was built up over a 25 min period with a flow of 150 $\mu l\,min^{-1}$, during which fractions of 100 μl were collected and pooled into six sequential groups, fraction 1 being the most hydrophilic and fraction 6 being the most hydrophobic. Fractions were resuspended in sucrose buffer in a volume equivalent to the volume of methanol injected onto the column to allow comparisons of toxin levels in fractions and cell extracts (see also toxin application).

In vivo *exposure*

Purified MC-LR (1 mg) was dissolved in 2 ml of ethanol and diluted in 100 ml of distilled water. Freeze-dried *M. aeruginosa* 7820 (0.314 g yielded 1 mg of MC-LR) was extracted twice with 50 ml of 4 % (v/v) ethanol in water and centrifuged at 1000 ***g*** (Sorval RC-5B) and the supernatants combined. A similar quantity of *M. aeruginosa* CYA 43 was similarly extracted.

Tilapia (13–35.9 g) were starved for 48 h and then six fish were held for 24 h in 4 l vessels containing Nijmegen tap water at 27 °C, to which 90 $\mu g\,l^{-1}$ MC-LR, freeze-dried extract of *M. aeruginosa* 7820 (86.4 $\mu g\,l^{-1}$ MC-LR, 27 mg dry mass l^{-1}) or *M. aeruginosa* CYA 43 (27 mg dry mass l^{-1}) was added. MC-LR levels were determined by HPLC. Fish exposed to 0.025 % (v/v) ethanol in water acted as controls.

Exposure was for 4 and 24 h. Whole-body Ca^{2+} influx was measured by the uptake of $^{45}Ca^{2+}$ (0.11 $MBq\,l^{-1}$, Dupont) over 4 h. Fish were killed after this time by an overdose of the anaesthetic 2-phenoxyethanol, blood was removed *via* the caudal vessel and the plasma was collected by centrifugation (1 min at 13 000 ***g***).

Carcasses were digested for 48 h in 50 ml of H_2O_2 at 60 °C, 1 ml of digest was removed and 4 ml of Aqualumar added. $^{45}Ca^{2+}$ was measured in a Pharmacia Wallac 1410 liquid scintillation counter (all subsequent measurements of $^{45}Ca^{2+}$ levels are made with the same counter) and calculated on the basis of carcass total radioactivity (disints min^{-1}) and water specific activity (disints $min^{-1}\,ml^{-1}$). Plasma osmolality was measured with a Vogel osmometer using distilled water and a 300 mosmol kg^{-1} standard as references. Plasma total Ca^{2+} concentrations were measured using a calcium kit (Sigma Diagnostics), Na^+ concentrations were measured by atomic absorption (Pye-Unicam SP9 atomic absorption spectrophotometer) and Cl^- concentrations were determined with a chloride meter (Jenway PCLM3 chloride meter).

Gill membranes and permeabilised cell preparations

Methods for basolateral membrane vesicle preparations are described by Flik *et al.* (1985). Briefly, gills were excised from tilapia (250 g), washed in buffer containing 250 $mmol\,l^{-1}$

sucrose, 5 mmol l^{-1} NaCl and 5 mmol l^{-1} Hepes/Tris, pH 7.4, and kept on ice. All subsequent procedures were performed at 1–4 °C. The epithelium was scraped off with a glass microscope slide and homogenised in the above buffer for 2 min using a Polytron ultra-turrax homogeniser set at 20 % of its maximum speed. This procedure kept red blood cells intact while branchial epithelium was disrupted. In this respect, it is similar to a previously described technique using a Douncer homogenisation device instead of a Polytron ultra-turrax homogeniser (Flik and Verbost, 1994).

The red blood cells and cellular debris were removed from the homogenate by centrifugation at 550 ***g*** for 10 min. Membranes were collected by centrifugation at 30 000 ***g*** (Sorval RC-5B) and were resuspended in buffer using a Douncer-type homogenisation device (100 strokes). The resulting suspension was differentially centrifuged; 1000 ***g*** for 10 min, 10 000 ***g*** for 10 min and 30 000 ***g*** for 30 min, and the final pellet was resuspended in buffer containing 150 mmol l^{-1} NaCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4, for the Na^+/K^+-ATPase assay or 150 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4, for Ca^{2+} transport measurements. The vesicles were resealed by 10 passages through a 23 gauge needle, giving 19.2–30 % inside-out (IOV) and 29–44 % rightside-out vesicles (ROV) (Flik *et al.* 1985; Verbost *et al.* 1994). The amount of membrane protein present was determined using a commercial kit (Bio-Rad) with bovine serum albumin (BSA) as a standard and was adjusted to 1.5 mg ml^{-1}. To obtain maximum Na^+/K^+-ATPase activity, vesicles were permeabilised with saponin (0.2 mg mg^{-1} protein) to ensure optimal substrate accessibility.

The preparation of permeabilised gill cells was based on the methods of Verbost *et al.* (1994). Gills were excised and the epithelial scrapings incubated in lysis medium containing 9 parts of 0.17 mmol l^{-1} NH_4Cl and 1 part of 0.17 mmol l^{-1} Tris/HCl, pH 7.4, for 20 min. The cells and red cell material were resuspended at the beginning and end of lysis by passage through a 10 ml pipette, large cell clusters were removed by passage through a 100 μm nylon mesh. Cells were collected, and the lysed red cells removed by centrifugation at 150 ***g*** for 5 min. The resulting pellet was resuspended in buffer containing 150 mmol l^{-1} NaCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris, pH 7.4, and incubated with 0.3 mg ml^{-1} saponin at 37 °C for 5 min. The cells were centrifuged for 5 min at 150 ***g*** and the pellet washed twice and finally resuspended in assay buffer containing 120 mmol l^{-1} KCl, 1.2 mmol l^{-1} KH_2PO_4, 5 mmol l^{-1} succinate, 5 mmol l^{-1} pyruvate, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} nitrilotriacetic acid (NTA) and 24 mmol l^{-1} Hepes/KOH, pH 7.1. The protein content was adjusted to 2 mg ml^{-1}.

Toxin application

The BLMVs and permeabilised cell preparations were exposed to purified MC-LR at concentrations in the range 1.56–333 μg mg^{-1} membrane protein (equivalent to 1.57–335 nmol mg^{-1} membrane protein) and for experiments with methanol extracts of cyanobacteria in the range 0.12–25 mg of freeze-dried material per milligram membrane protein. For experiments with the six fractions obtained from the methanol extracts, a volume of 83.3 μl of cyanobacterial extract fraction per milligram membrane protein was applied, which was equivalent to 12.5 mg of freeze-dried material per milligram membrane protein. For permeabilised cell preparations, thapsigargin (10 μmol l^{-1}) was used to inhibit endoplasmic reticulum Ca^{2+} transport and Ruthenium Red (1 μmol l^{-1}) to inhibit mitochondrial Ca^{2+} transport. All preparations were incubated for 2 h on ice prior to assays.

K^+-dependent pNPPase activity

K^+-dependent *p*-nitrophenol phosphatase (pNPPase) activity, which reflects the dephosphorylation step (i.e. the phosphatase activity) of the Na^+/K^+-ATPase, was determined. Medium A contained 100 mmol l^{-1} KCl, 75 mmol l^{-1} $MgCl_2$, 300 mmol l^{-1} imidazole, 10 mmol l^{-1} *trans*-1,2,-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) and 5 mmol l^{-1} *p*-nitrophenolphosphate (pNPP), pH 7.4, whilst medium E consisted of medium A to which 1 mmol l^{-1} ouabain was added and from which KCl was omitted. Toxin-treated vesicles (10 μl) were mixed with 500 μl of either medium A or medium E and incubated for 15 min at 37 °C. The K^+-dependent ouabain-sensitive pNPPase activity was defined as the difference in activity measured between medium A and medium E at 420 nm.

Ca^{2+} transport

Basolateral membrane vesicles

Toxin-treated vesicles (12.5 μl) were added to 50 μl of assay medium; 150 mmol l^{-1} KCl, 1 μmol l^{-1} 'free' Ca^{2+}, 0.8 mmol l^{-1} 'free' Mg^{2+}, buffered with 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} *N*-(2-hydroxyethyl)-ethylenediamine-*N,N′,N′*-triacetic acid (HEEDTA), 0.5 mmol l^{-1} NTA, with or without 3 mmol l^{-1} ATP and containing $^{45}Ca^{2+}$. Both solutions were

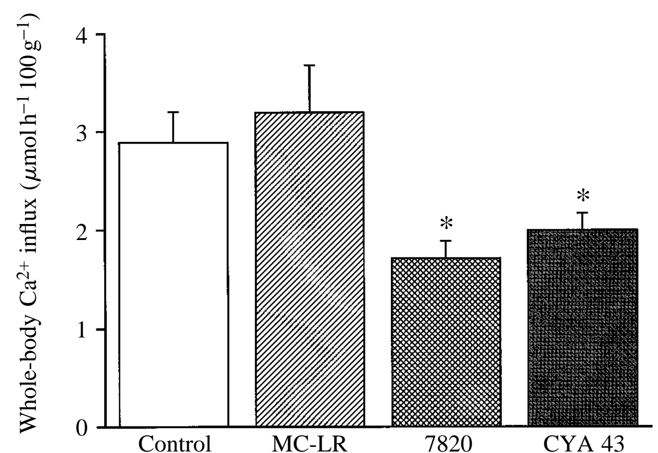

Fig. 1. Whole-body Ca^{2+} influx (μmol h^{-1} 100 g^{-1}) in tilapia after 24 h of exposure to purified microcystin-LR (MC-LR, 90 μg l^{-1}) or extracts of *Microcystis aeruginosa* 7820 (7820, 86 μg l^{-1}, 27 mg dry mass l^{-1}) or *M. aeruginosa* CYA 43 (CYA 43, 27 mg dry mass l^{-1}). Values are means + S.E.M., *N*=6, apart from controls where *N*=5. Asterisks indicate a significant difference from control values (P<0.05).

Table 1. *Plasma osmolality and Ca^{2+}, Na^{+} and Cl^{-} concentrations in tilapia exposed for 24 h to purified microcystin (MC-LR, 90.8 μg l^{-1} MC-LR) or extracts of* Microcystis aeruginosa *7820 (7820, 86 μg l^{-1} MC-LR, 27 mg l^{-1} of freeze-dried material) or* M. aeruginosa *CYA 43 (CYA 43, 27 mg l^{-1} of freeze-dried material)*

	Control	MC-LR	7820	CYA 43
Osmolality (mosmol kg^{-1})	301±1.9	294±5	306±1.9	299±1.5
[Ca^{2+}] (mmol l^{-1})	2.71±0.15	2.68±0.05	2.39±0.26	2.21±0.08*
[Na^{+}] (mmol l^{-1})	153±4.6	161±4	169±26*	158±2.2
[Cl^{-}] (mmol l^{-1})	129.5±2.7	125±2.8	139±1.4*	131±1.3

Values are mean ± S.E.M., N=6, apart from controls where N=5.
Asterisks indicate significant differences (P<0.05) compared with control values at 24 h.

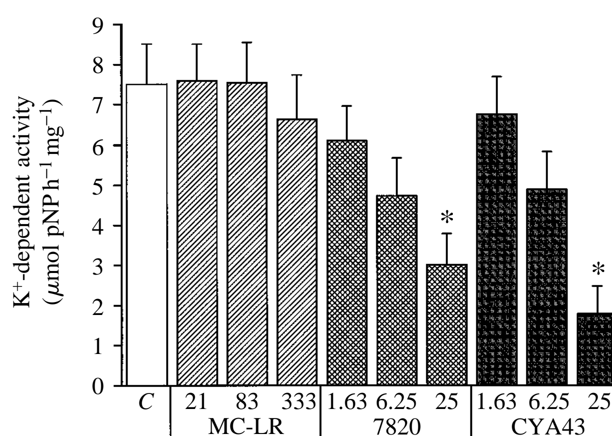

Fig. 2. K^{+}-dependent pNPPase activity (μmol h^{-1} mg^{-1}) of gill basolateral membrane vesicles (BLMVs) of controls (*C*) and following exposure to varying concentrations of MC-LR (in μg mg^{-1} membrane protein) or methanol extracts of *Microcystis aeruginosa* 7820 or CYA 43 (in mg freeze-dried material mg^{-1} membrane protein). Values are means + S.E.M., N=5. Asterisks indicate a significant difference from control values (P<0.05).

pre-warmed to 37 °C and subsequent incubations performed at this temperature for 1 min, to determine initial velocities. The transport was stopped by the addition of 1 ml of ice-cold stop buffer containing 150 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA and 10 mmol l^{-1} Hepes/Tris, pH 7.4. Vesicles were collected by rapid filtration using Schleicher and Schuell ME25 mixed cellulose filters, pore size 0.45 μm, and were rinsed twice with ice-cold stop buffer. Filters were placed in scintillation vials and dissolved in 4 ml of Aqualuma for 30 min before activity was counted.

Permeabilised gill cells

Permeabilised gill cell preparations were mixed thoroughly and 10 μl of the suspension was added to 50 μl of assay medium, both having been pre-warmed to 28 °C. Assay medium contained the same concentration of ligands and KCl as above, but with 5 mmol l^{-1} ATP. 'Free' [Mg^{2+}] was set at 0.8 mmol l^{-1}; for endoplasmic reticulum Ca^{2+} transport measurements, 'free' [Ca^{2+}] was set at 0.1 μmol l^{-1}, and for mitochondrial Ca^{2+} transport measurements, 'free' [Ca^{2+}] was 1 μmol l^{-1} (Verbost *et al.* 1987*b*). Cells were incubated at 28 °C for 2 min and transport was stopped by the addition of 1 ml of stop buffer, as above. Cells were collected on Schleicher and Schuell (GF92 diameter 25 mm) glass filters, and rinsed and counted as above.

Statistics and calculations

All results were compared with control values using a Student's *t*-test on the Minitab computer package. 'Free' Ca^{2+} and Mg^{2+} concentrations were calculated using a matrix computer program developed by Schoenmakers *et al.* (1992).

Results

In vivo *experiments*

Whole-body Ca^{2+} influx rates were inhibited by 40 % after 24 h of exposure to extracts of *M. aeruginosa* 7820 and by 31 % with *M. aeruginosa* CYA 43 extracts (Fig. 1). Table 1 shows that plasma Ca^{2+} concentrations were significantly lower in fish exposed for 24 h to *M. aeruginosa* CYA 43, while plasma Na^{+} and Cl^{-} levels showed a small but significant increase in fish exposed to *M. aeruginosa* 7820. Purified microcystin-LR (MC-LR) had no effect on plasma electrolyte levels or whole-body Ca^{2+} influx when compared with controls at 24 h (Table 1; Fig. 1). None of the treatments had a significant effect on whole-body Ca^{2+} influx or body ion concentrations after 4 h of exposure.

Table 2. *Percentage inhibition of K^{+}-dependent pNPPase activity in gill basolateral membrane vesicles (BLMVs) exposed to fractions 1–6 from methanol extracts of* Microcystis aeruginosa *7820 or CYA 43*

	Fraction number					
	1	2	3	4	5	6
Inhibition by 7820 (%)	9±17	8±14	5±10	7±17	35±14	12±13
Inhibition by CYA 43 (%)	12±15	5±14	0±17	7±13	37±14	27±13

See Materials and methods for details of fraction preparation.
Values are percentage of control values ± S.E.M., N=5.

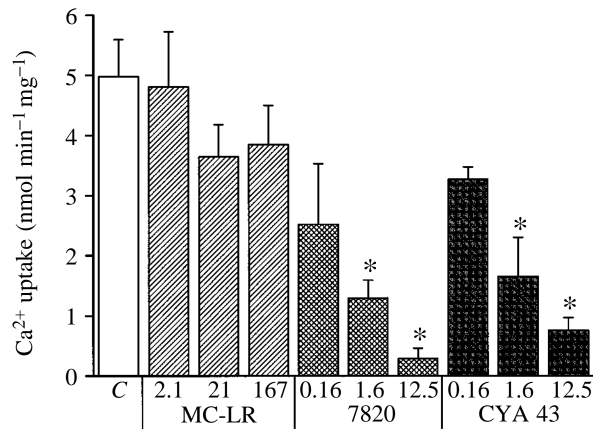

Fig. 3. Initial rates of ATP-driven Ca^{2+} uptake (nmol min^{-1} mg^{-1}) measured at 1 μmol l^{-1} Ca^{2+} in BLMV preparations of controls (*C*) and following exposure to varying concentrations of MC-LR or to methanol extracts of *Microcystis aeruginosa* 7820 or *M. aeruginosa* CYA 43 (see legend to Fig. 2 for details). Values are means + S.E.M., *N*=5. Asterisks indicate a significant difference from control values ($P<0.05$).

K^+-dependent pNPPase activity

BLMV K^+-dependent pNPPase activity was not affected by purified MC-LR, but was inhibited by increasing doses of methanol extracts of both *M. aeruginosa* 7820 and CYA 43 (Fig. 2). Fraction 5 of the cyanobacterial extracts appeared to produce the greatest reduction in K^+-dependent pNPPase activity (Table 2), but this was not significant when compared with the effects of other fractions.

Ca^{2+} transport

Basolateral membrane vesicles

Purified MC-LR had no effect on Ca^{2+} uptake in BLMVs, although methanol extracts of both *M. aeruginosa* 7820 and CYA 43 progressively inhibited Ca^{2+} uptake with increasing doses (Fig. 3). *M. aeruginosa* 7820 and CYA 43 extract fractions 4, 5 and 6 significantly inhibited Ca^{2+} uptake in BLMVs compared with controls (Table 3).

Permeabilised gill cells

Ca^{2+} uptake specific to the endoplasmic reticulum (ER) of the permeabilised cells was verified using the inhibitor thapsigargin at 0.1 μmol l^{-1} Ca^{2+}, which produced an 82 % inhibition. Ca^{2+} uptake specific to mitochondria of the permeabilised cells was verified using Ruthenium Red at 1 μmol l^{-1} Ca^{2+} as an inhibitor, which resulted in 80 % inhibition. Purified MC-LR had no effect on ER or mitochondrial uptake of Ca^{2+} (Figs 4, 5). The methanol extracts from both strains of *M. aeruginosa* inhibited Ca^{2+} uptake by ER (Fig. 4) and mitochondria (Fig. 5), most noticeably at higher concentrations. *M. aeruginosa* 7820 and CYA 43 extract fractions 4, 5 and 6 all inhibited Ca^{2+} transport in both systems compared with controls, with fraction 5 being the most potent (Table 4).

Discussion

The toxins from cyanobacteria have been reported to impede Na^+/K^+-ATPase activity of carp gill microsomes (Gaete *et al.* 1994), and so we focused our studies on the ATPases that mediate Ca^{2+} transport in the gill of tilapia. Our results show that, *in vitro*, MC-LR did not inhibit Na^+/K^+-ATPase activity, measured as K^+-dependent pNPPase (Fig. 2), or the ATP-driven Ca^{2+} transport of the gill basolateral plasma membrane (Fig. 3); in addition, there was no inhibition of SERCA-type ATPases of the endoplasmic reticulum (ER, Fig. 4) or of the Ca^{2+} uniporter of the mitochondria (Fig. 5). In contrast, extracts, particularly in the hydrophobic fractions, from the 7820 and CYA 43 strains of *M. aeruginosa* inhibited all the systems investigated. Na^+/K^+-ATPase activity appeared to be less sensitive than the Ca^{2+}-dependent transporters to the toxic substances within the cyanobacteria.

The cyanobacterial toxin microcystin-LR (MC-LR) inhibits protein phosphatases 1 and 2A (PP1 and PP2A) (Eriksson *et al.* 1990; Honkanen *et al.* 1990; MacKintosh *et al.* 1990; Matsushima *et al.* 1990). If MC-LR also inhibits Na^+/K^+-ATPase activity of carp gill microsomes (Gaete *et al.* 1994), this would imply either that the inhibitory action of MC-LR is wider, i.e. it would also inhibit P-type ATPase activity (where the phosphatases show an aspartic acid–phosphate hydrolase activity) and/or that the fish ATPase activity is aberrant in having a PP1/PP2A-type phosphatase activity as part of its Na^+/K^+-ATPase cycle. However, in the present study, MC-LR did not inhibit any Na^+/K^+-ATPase activity in tilapia gill (Fig. 3), nor has MC-LR inhibition been found in carp gill,

Table 3. *Percentage inhibition of Ca^{2+} transport in gill BLMVs exposed to fractions 1–6 from methanol extracts of* Microcystis aeruginosa *7820 or CYA 43*

	Fraction number					
	1	2	3	4	5	6
Inhibition by 7820 (%)	12±11	5±10	19±16	32±5*	88±5*	77±9*
Inhibition by CYA 43 (%)	26±12	31±12	12±9	40±12*	88±5*	74±9*

See Materials and methods for details of fraction preparation.
Values are percentage of control values ± S.E.M.
Asterisks indicate significant differences ($P<0.05$) compared with control values before percentage transformation, *N*=5.

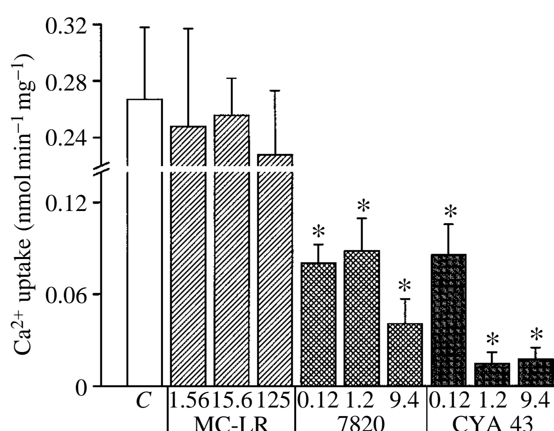

Fig. 4. Initial rates of ATP-driven Ca^{2+} uptake (nmol min^{-1} mg^{-1}), measured at 0.1 μmol l^{-1} Ca^{2+}, by endoplasmic reticulum in controls (*C*) and after exposure to varying concentrations of MC-LR or to methanol extracts of *Microcystis aeruginosa* 7820 or CYA 43 (see legend to Fig. 2 for details). Values are means + S.E.M., *N*=5. Asterisks indicate a significant difference from control values (P<0.05).

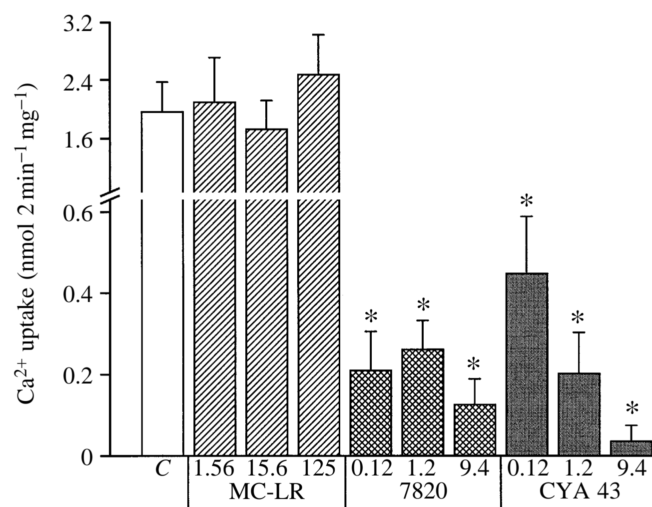

Fig. 5. Initial rates of Ca^{2+} uptake (nmol 2 min^{-1} mg^{-1}), measured at 1 μmol l^{-1} Ca^{2+}, by mitochondria in controls (*C*) and after exposure to varying concentrations of MC-LR or methanol extracts of *Microcystis aeruginosa* 7820 or CYA 43 (see Fig. 2 for details). Values are means + S.E.M., *N*=5. Asterisks indicate a significant difference from control values (P<0.05).

dog kidney or human erythrocytes (N. R. Bury and G. Flik, personal observations). Moreover, other Ca^{2+}-ATPases in the plasma membranes and ER proved to be unaffected by the toxin, in line with the specificity of the toxin to PP1 and PP2A. We have no doubt of the potency of our MC-LR preparations, as acute hepatotoxicity had been ascertained by intraperitoneal injection in mice (Falconer *et al.* 1981). Moreover, the preparation appeared as a single homogeneous peak on HPLC with the same retention time as that previously published for MC-LR (Lawton *et al.* 1994).

Tilapia exposed for 24 h to extracts from either strain of *M. aeruginosa,* 7820 or CYA 43, showed inhibited Ca^{2+} influx (Fig. 1), which resulted in hypocalcaemia in fish exposed to *M. aeruginosa* CYA 43 (Table 1). The small but significant increase in plasma Na^{+} and Cl^{-} concentrations in fish exposed to *M. aeruginosa* 7820 (Table 1) was unexpected, and there is no immediate explanation. A possible explanation for the lack of *in vivo* effects at 4 h is that the toxin has yet to exert an inhibitory effect at the basolateral membrane on Ca^{2+} transporting mechanisms (see Flik *et al.* 1985, 1993).

Toxicity tests with invertebrates and bacteria have verified the toxicity of the microcystins (Penaloza *et al.* 1990; Demott *et al.* 1991; Kiviranta *et al.* 1991), but have also illustrated the presence of additional cytotoxic substances, as yet unidentified, within cyanobacteria (Nizan *et al.* 1986; Jungmann *et al.* 1991; Jungmann, 1992; Jungmann and Benndorf, 1994; Campbell *et al.* 1994). Penaloza *et al.* (1990) found that cyanobacterial fractions toxic to zooplankton contained factors with a molecular mass similar to that of MC-LR, but toxicity was lost upon boiling, while microcystins are heat-stable to 160 °C (e.g. Jungmann and Benndorf, 1994). Cyanobacteria have been screened for compounds with therapeutic potential and are a rich source of novel bioactive substances; for example, antineoplastic compounds (Patterson

Table 4. *Percentage inhibition of Ca^{2+} uptake by gill endoplasmic reticulum (ER) and mitochondria in permeabilised cell preparations exposed to fractions 1–6 from methanol extracts of* Microcystis aeruginosa *7820 or CYA 43*

	Fraction number					
	1	2	3	4	5	6
Inhibition of ER Ca^{2+} transport (%)						
7820	0±23	0±18	10±12	33±12	88±7*	75±9*
CYA 43	8±21	10±21	1±11	35±12*	90±6*	50±19
Inhibition of mitochondrial Ca^{2+} transport (%)						
7820	20±13	3±16	12±18	57±6	99±1*	96±2*
CYA43	11±21	0±22	8±18	59±7	99±1*	97±2*

See Materials and methods for details of fraction preparation.
Values are percentage of control values ± S.E.M.
Asterisks indicate significant differences (P<0.05) compared with control values before percentage transformation, *N*=5.

et al. 1991), and lipophilic cyclic peptides (laxaphycins) that are antifungal and cytotoxic (Frankmolle *et al.* 1992). There is no account of these substances inhibiting ATPases, but fungi have been shown to possess citreoviridin, a polyene neurotoxin which inhibits mitochondrial ATPase (Sayood *et al.* 1989), and tentotoxin, a cyclic tetrapeptide which inhibits ATPase activity in cyanobacteria (Ohta *et al.* 1993).

In conclusion, we have shown that strains of the cyanobacterium *Microcystis aeruginosa* produce compound(s) that inhibit Ca^{2+} uptake and gill K^+-dependent pNPPase activity in tilapia. We also present evidence that these inhibitory effects are not due to the protein phosphatase inhibitor MC-LR. However, further work is required to determine the exact structure and inhibitory mode of action of the compound(s) which affect gill Ca^{2+} transport and may inhibit physiological processes dependent upon Ca^{2+}, thus contributing to the fish death that often accompanies senescence of a cyanobacterial bloom.

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References

Bury, N. R., Eddy, F. B. and Codd, G. A. (1995). The effects of the cyanobacterium *Microcystis aeruginosa*, the cyanobacterial toxin microcystin-LR and ammonia on growth rate and ionic regulation of brown trout (*Salmo trutta* L.). *J. Fish Biol.* **46**, 1042–1054.

Bury, N. R., Eddy, F. B. and Codd, G. A. (1996). The stress responses of the brown trout, *Salmo trutta*, to the cyanobacterium, *Microcystis aeruginosa*. *Environ. Tox. Water Qual.* (in press).

Campbell, D. L., Lawton, L. A., Beattie, K. A. and Codd, G. A. (1994). Comparative assessment of the specificity of the brine shrimp and Microtox assays to hepatotoxic (microcystin-LR-containing) cyanobacteria. *Environ. Tox. Water Qual.* **9**, 71–77.

Carafoli, E. (1982). The transport of calcium across the inner membrane of mitochondria. In *Membrane Transport of Calcium* (ed. E. Carafoli), pp. 109–140. London: Academic Press.

Carmichael, W. W. (1992). Cyanobacteria secondary metabolites. *J. appl. Bacteriol.* **72**, 445–459.

Codd, G. A., Bell, S. G. and Brooks, W. P. (1989). Cyanobacterial toxins in water. *Water Sci. Technol.* **23**, 1–13.

Demott, W. R., Zhang, Q.-X. and Carmichael, W. W. (1991). Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnol. Oceanogr.* **36**, 1346–1357.

Eriksson, J. E., Toivola, D., Meriluoto, J. A. O., Karaki, H., Han, Y.-G. and Hartshorne, D. (1990). Hepatocyte deformation induced by cyanobacterial toxins reflect inhibition of protein phosphatase. *Biochem. biophys. Res. Commun.* **173**, 1347–1353.

Falconer, I. R., Jackson, A. R. B., Langley, J. V. and Runnegar, M. T. C. (1981). Liver pathology in mice in poisoning by the blue-green alga *Microcystis aeruginosa*. *Aust. J. biol. Sci.* **34**, 179–187.

Flik, G., van der Velden, J. A., Dechering, K. J., Verbost, P. M., Schoenmakers, T. J. M., Kolar, Z. I. and Wendelaar Bonga, S. E. (1993). Ca^{2+} and Mg^{2+} transport in gills and gut of tilapia *Oreochromis mossambicus*: a review. *J. exp. Zool.* **265**, 356–365.

Flik, G., van Rijs, J. H. and Wendelaar Bonga, S. E. (1985). Evidence for high-affinity Ca^{2+}-ATPase activity and ATP-driven Ca^{2+}-transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus*. *J. exp. Biol.* **119**, 335–347.

Flik, G. and Verbost, P. M. (1994). Ca^{2+} transport across plasma membranes. In *Biochemistry and Molecular Biology of Fishes*, vol. 3 (ed. P. W. Hochachka and T. P. Mommsen), pp. 625–637. Amsterdam, The Netherlands: Elsevier Science Press.

Frankmolle, W. P., Larsen, L. K., Caplan, F. R., Patterson, G. M. L., Knubel, G., Levine, I. A. and Moore, R. E. (1992). Antifungal cyclic peptides from the terrestrial blue-green alga *Anabaena laxa*. I. Isolation and biological properties. *J. Antibiotics* **45**, 1451–1457.

Gaete, V., Canelo, E., Lagos, N. and Zambrano, F. (1994). Inhibitory effects of *Microcystis aeruginosa* toxin on ion pumps of the gill of freshwater fish. *Toxicon* **32**, 121–127.

Giles, M. A. (1984). Electrolyte and water balance in plasma and urine of rainbow trout (*Salmo gairdneri*) during chronic exposure to cadmium. *Can. J. Fish. aquat. Sci.* **41**, 1678–1685.

Gunter, T. E., Gunter, K. K., Sheu, S.-S. and Gavin, C. E. (1994). Mitochondrial calcium transport: physiological and pathological relevance. *Am. J. Physiol.* **267**, C313–C339.

Gunter, T. E. and Pfieffer, D. R. (1990). Mechanisms by which mitochondria transport calcium. *Am. J. Physiol* **258**, C755–C786.

Honkanen, R. E., Zwillers, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M. and Boynton, A. L. (1990). Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatase. *J. biol. Chem.* **265**, 19401–19404.

Jungmann, D. (1992). Toxic compounds isolated from *Microcystis* PCC 7806 that are more active against *Daphnia* than two microcystins. *Limnol. Oceanogr.* **37**, 1777–1783.

Jungmann, D. and Benndorf, J. (1994). Toxicity to *Daphnia* of a compound extracted from laboratory and natural *Microcystis* spp. and the role of microcystins. *Freshwater Biol.* **32**, 13–20.

Jungmann, D., Henning, M. and Juttner, F. (1991). Are the same compounds in *Microcystis* responsible for toxicity to *Daphnia* and inhibition of its filtering rate. *Int. Rev. Ges. Hydrobiol.* **76**, 47–56.

Kiviranta J., Sivonen, K. and Niemelä, S. I. (1991). Detection of toxicity of cyanobacteria by *Artemia salina* bioassay. *Environ. Tox. Water Qual.* **6**, 423–436.

Lawton, L. A., Edwards, C. and Codd, G. A. (1994). Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated water. *Analyst* **119**, 1525–1530.

MacKinney, G. (1941). Absorption of light by chlorophyll solutions. *J. biol. Chem.* **140**, 315–322.

MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatase 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**, 187–192.

Matsushima, R., Yoshizawa, S., Watanabe, M. F., Harada, K. L., Furusawa, M., Carmichael, W. W. and Fujiki, H. (1990). *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin and fibroblasts. *Biochem. biophys. Res. Commun.* **171**, 867–874.

Nizan, S., Dimentman, C. and Shilo, M. (1986). Acute toxic effects

of the cyanobacterium *Microcystis aeruginosa* on *Daphnia magna. Limnol. Oceanogr*. **31**, 497–502.

OHTA, Y., YOSHIOKA, T., MOCHIMARU, M., HISABORI, T. AND SAKURAI, H. (1993). Tentotoxin inhibits both phosphophorylation in thylakoids and the ATPase activity of isolated coupling factor F_1 from the cyanobacterium *Anacystis nidulans*. *Plant Cell Physiol*. **34**, 523–529.

PATTERSON, G. M. L., BALDWIN, C. L., BOLIS, C. M., CAPLAN, F. R., KARUSO, H., LARSEN, L. K., LEVINE, I. A., MOORE, R. E., NELSON, C. S., TSCHAPPAT, K. D., TUANG, G. D., FURUSAWA, E., FURUSAWA, S., NEWTON, T. R. AND RAYBOURNE, R. B. (1991). Antineoplast activity of cultured blue-green algae (Cyanophyta). *J. Phycol*. **27**, 520–536.

PENALOZA, R., ROJAS, M., VILA, I. AND ZAMBRANO, F. (1990). Toxicity of a soluble peptide from *Microcystis* sp. to zooplankton and fish. *Freshwater Biol*. **24**, 233–240.

PHILLIPS, M. J., ROBERTS, R. J., STEWART, J. A. AND CODD, G. A. (1985). The toxicity of the cyanobacterium *Microcystis aeruginosa* to rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis*. **8**, 339–344.

RÅBERGH, C. M. I., BYLUND, G. AND ERIKSSON, J. E. (1991). Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium (blue-green alga) *Microcystis aeruginosa* on common carp (*Cyprinius carpio* L.). *Aquat. Toxicol*. **20**, 131–146.

RODGER, H. D., TURNBULL, T., EDWARDS, C. AND CODD, G. A. (1994). Cyanobacterial (blue-green algal) bloom associated pathology in brown trout (*Salmo trutta* L.) in Loch Leven, Scotland. *J. Fish Dis.* **17**, 177–181.

SAYOOD, S. F., SUH, H., WILCOX, C. S. AND SCHUSTER, S. M. (1989). Effect of citreoviridin and isocitreoviridin on beef heart mitochondrial ATPase. *Archs Biochem. Biophys*. **270**, 714–721.

SCHOENMAKERS, T. J. M., VISSER, G. J., FLIK, G. AND THEUVENET, A. P. R. (1992). Chelator: an improved method for computing metal ion concentrations in physiological solutions. *Biotechniques* **12**, 870–879.

SCHWIMMER, M. AND SCHWIMMER, D. (1968). Medical aspects of phycology. In *Algae, Man and the Environment* (ed. D. F. Jackson), pp. 279–358. New York: Syracuse University Press.

SOMLYO, A. P. (1985). Cellular site of calcium regulation. *Nature* **309**, 516–517.

SUGAYA, Y., YASUNO, M. AND YANOI, T. (1990). Effects of toxic *Microcystis viridis* and isolated toxins on goldfish. *Jap. J. Limnol*. **51**, 149–153.

TENCALLA, F. G., DIETRICH, D. R. AND SCHLATTER, C. (1994). Toxicity of *Microcystis aeruginosa* peptide toxin to yearling rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol*. **30**, 215–224.

VERBOST, P. M., FLIK, G. AND COOK, H. (1994). Isolation of gill cells. In *Biochemistry and Molecular Biology of Fishes*, vol. 3 (ed. P. W. Hochachka and T. P. Mommsen), pp. 239–247. Amsterdam, The Netherlands: Elsevier Science Press.

VERBOST, P. M., FLIK, G., LOCK, R. A. C. AND WENDELAAR BONGA, S. E. (1987*a*). Cadmium inhibition of Ca^{2+}-uptake in rainbow trout gills. *Am. J. Physiol*. **253**, R216–R221.

VERBOST, P. M., FLIK, G., LOCK, R. A. C. AND WENDELAAR BONGA, S. E. (1988). Cadmium inhibits plasma membrane calcium transport. *J. Membr. Biol*. **102**, 97–104.

VERBOST, P. M., SENDEN, M. H. M. N. AND VAN OS, C. H. (1987*b*). Nanomolar concentrations of Cd^{2+} inhibit Ca^{2+} transport systems in plasma membranes and intracellular calcium stores in rat intestinal epithelium. *Biochim. biophys. Acta* **902**, 247–252.

VERBOST, P. M., VAN ROOIJ, J., FLIK, G., LOCK, R. A. C. AND WENDELAAR BONGA, S. E. (1989). The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J. exp. Biol*. **145**, 185–197.