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FATTY ACIDS FROM THE CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* WITH POTENT INHIBITORY EFFECTS ON FISH GILL Na^+/K^+-ATPase ACTIVITY

N. R. BURY[1,*], G. A. CODD[2], S. E. WENDELAAR BONGA[1] AND G. FLIK[1,†]

[1]*Department of Animal Physiology, University of Nijmegen, Nijmegen, The Netherlands* and [2]*Department of Biology, University of Dundee, Dundee DD1 4HN, Scotland, UK*

*Present address: Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1
†Author for correspondence (e-mail: gertflik@sci.kun.nl)

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Summary

Fatty acids from two strains of the cyanobacterium *Microcystis aeruginosa*, PCC 7820 (a strain that produces the hepatotoxin microcystin-LR, MC-LR) and CYA 43 (a strain that produces only small quantities of MC-LR), were extracted, partially characterised and tested for their inhibitory effect on the K^+-dependent *p*-nitrophenol phosphatase (*p*NPPase) activity of tilapia (*Oreochromis mossambicus*) gill basolateral membrane. Thin-layer chromatography of the lipids from dichloromethane:methanol extracts of *M. aeruginosa* PCC 7820 and CYA 43, using diethylether:isopropanol:formic acid (100:4.5:2.5) as solvent, yielded five inhibitory products from *M. aeruginosa* 7820 and six from *M. aeruginosa* CYA 43. None of these products could be related to MC-LR. The inhibitory behaviour of the products mimics that of a slow, tight-binding inhibitor. The inhibitory activity is removed by incubation of extracts with fatty-acid-free bovine serum albumin (FAF-BSA). However, FAF-BSA only partially reversed the inhibition of K^+-dependent *p*NPPase on fish gills pre-exposed to the extracted products. We conclude that *M. aeruginosa* strains PCC 7820 and CYA 43 produce fatty acids with potent inhibitory effects on K^+-dependent *p*NPPase. The release of these products following lysis of cyanobacterial blooms may help to explain fish kills through a disturbance of gill functioning.

Key words: *Microcystis aeruginosa*, Na^+/K^+-ATPases, lipids, fish, *Oreochromis mossambicus*, inhibition, tilapia.

Introduction

Eutrophication of fresh waters has led to an increased incidence of cyanobacterial blooms that may produce neuro- and hepatotoxins (Codd *et al.* 1989). Upon senescence of these blooms, the toxins that are released into the water may cause fish to die (e.g. Schwimmer and Schwimmer, 1968; Eriksson *et al.* 1986; Penaloza *et al.* 1990; Rodger *et al.* 1994).

The majority of such fish kills have been attributed to hypoxic water conditions resulting from the high oxygen demand caused by bloom respiration at night and/or by bloom senescence. However, dissolved oxygen levels were 90 % of normal values in Loch Leven, Scotland, when moribund brown trout (*Salmo trutta*) were found after lysis of an *Anabaena flos-aquae* bloom (Rodger *et al.* 1994). These authors found histopathological evidence both for gill damage and for severe liver damage in the brown trout, which was similar to that observed in fish treated with microcystins (Phillips *et al.* 1985; Råbergh *et al.* 1991; Tencalla *et al.* 1994). However, immersion trials using concentrations of aqueous extracts of the hepatotoxic cyanobacterial cells similar to those found in eutrophic environments did not cause deaths (Bury *et al.* 1995). Consequently, the exact cause of death, i.e. the biochemical mechanism underlying death following exposure to cyanobacterial blooms, has yet to be established.

Recent research has advanced a number of explanations for the fish kills. First, fish may ingest the toxins or toxic cyanobacteria, which may then result in liver malfunction (Tencalla *et al.* 1994). Second, fish exposed to extracts from cyanobacteria exhibit a stress response (Bury *et al.* 1995, 1996*a*), which may be detrimental to their health. Third, toxic compounds present in cyanobacteria affect fish gill ion transport by inhibiting ATPase activities in the plasma membranes of the branchial epithelium (Gaete *et al.* 1994; Bury *et al.* 1996*b*; Zambrano and Canelo, 1996).

The toxic compounds present in the cyanobacteria that inhibit fish gill ATPase activities are associated with hydrophobic fractions of methanol extracts of *M. aeruginosa*, but are unrelated to the hepatotoxin microcystin-LR (MC-LR; Bury *et al.* 1996*b*). These results contrast with those of Gaete *et al.* (1994) and Zambrano and Canelo (1996), who attributed an inhibitory action on carp gill microsomal ATPase activity to MC-LR. However, we were unable to reproduce these results using ultrapure MC-LR on carp or tilapia gill ATPases (Bury *et al.* 1996*b*). In view of these results, the aim of this

study was to characterise the compounds other than MC-LR found in *M. aeruginosa* that inhibit gill ATPase activity. K^+-dependent *p*NPPase activity in a basolateral plasma membrane (BLM) preparation of branchial epithelium of tilapia (*Oreochromis mossambicus*) was tested for its sensitivity to extracts from two strains of the cyanobacteria *Microcystis aeruginosa* PCC 7820 (which produces a range of microcystins), to *M. aeruginosa* CYA 43 (which produces toxins, but only very small quantities of microcystins) and to subfractions of these extracts.

Materials and methods

Fish-holding conditions

Tilapia (*Oreochromis mossambicus* Peters) with an appoximate mass of 250 g were obtained from laboratory freshwater stocks and were held in running Nijmegen tap water under a light régime 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

The method used for culturing *Microcystis aeruginosa* PCC 7820 and CYA 43 has been described previously (Bury *et al.* 1995). Cells were harvested in the early stationary phase of batch culture by continuous centrifugation in a Sharples centrifuge (Sharples Ltd, Surrey); the pelleted cells were collected and freeze-dried. The freeze-dried material was extracted twice in methanol (50 ml methanol per 0.15 g), centrifuged at 100 ***g*** for 10 min (Sorval RC-5B) and the supernatants combined. The methanol was then evaporated by heating in a waterbath at 35 °C. Dried extract was resuspended in 1 ml of methanol to a final concentration of extract equivalent to 150 mg dry mass ml^{-1} and stored at –20 °C. Samples of this stock were dried and resuspended in sucrose buffer (250 mmol l^{-1} sucrose and 10 mmol l^{-1} Hepes/Tris, pH 7.4) for the K^+-dependent *p*NPPase assay.

Gill membrane preparation

Methods for basolateral membrane isolation were described by Flik *et al.* (1985). Briefly, gills were excised from tilapia, washed in buffer containing 250 mmol l^{-1} sucrose, 5 mmol l^{-1} NaCl, 5 mmol l^{-1} Hepes/Tris, pH 7.4, aprotonin (0.01 trypsin inhibitor unit (TIU) ml^{-1}) 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 washing buffer for 2 min using a Polytron Ultra-Turrax homogeniser fitted with an Ultra-Turrax dispersing tool S25 and set at 20 % of its maximum speed. This procedure kept red blood cells intact while the branchial epithelium was disrupted, in a manner similar to previously published Douncer homogenisation techniques (Flik and Verbost, 1994; Bury *et al.* 1996*b*). The red blood cells and cellular debris were removed by centrifugation at 550 ***g*** for 10 min, and gill membranes were collected by centrifugation at 30 000 ***g*** for 30 min (Sorval RC-5B). The resulting pellet was resuspended in sucrose buffer containing 6 mmol l^{-1} dithiothreitol with a Douncer-type homogenisation device (100 strokes). The resulting suspension was differentially centrifuged: 1000 ***g*** for 10 min, and 10 000 ***g*** for 10 min. The supernatant was then removed and centrifuged (30 000 ***g*** for 30 min). The pelleted membranes were resuspended in buffer (150 mmol l^{-1} NaCl, 0.8 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Hepes/Tris, pH 7.4) by 10 passages through a 23 gauge needle fitted to a tuberculin syringe. This procedure produces a partly resealed, vesiculated membrane preparation (Flik *et al.* 1985; Verbost *et al.* 1994). To obtain maximum K^+-*p*NPPase activity, vesicles were permeabilised with saponin (0.2 mg ml^{-1} at a membrane concentration of 1 mg ml^{-1} bovine serum albumin equivalents) to ensure optimal substrate accessibility. The membrane protein content was estimated using a commercial kit (Bio-Rad); bovine serum albumin (BSA) was used as standard. Routinely, inhibition studies were performed using the material extracted from 23 mg of freeze-dried cyanobacterial material per milligram BSA equivalent of membrane protein, unless otherwise stated.

K^+-dependent p*NPPase activity*

K^+-dependent *p*-nitrophenol phosphatase (*p*NPPase) activity, which reflects the dephosphorylation step of the Na^+/K^+-ATPase reaction cycle, was determined as follows: toxin-treated or control membranes (10 µl) were mixed with 500 µl of either medium A or medium E and incubated for 20 min at 37 °C. Medium A contained 10 mmol l^{-1} KCl, 7.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} *trans*-1,2,-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (CDTA) and 5 mmol l^{-1} *p*-nitrophenolphosphate (*p*NPP), pH 7.4; medium E consisted of medium A to which 1 mmol l^{-1} ouabain had been added and from which KCl had been omitted. The reaction was stopped by the addition of 1 ml of ice-cold 1 mol l^{-1} NaOH. The K^+-dependent ouabain-sensitive *p*NPPase activity was defined as the difference in the amount of *p*-nitrophenol (*p*NP) released in media A and E, measured at 420 nm and calculated using the equation:

$$p\text{NPPase activity} = \frac{\Delta X}{\Delta Y} \times [p\text{NP standard}] \times \frac{1}{[\text{protein}]} \times \frac{1}{t},$$

where ΔX is the difference in absorbency at 420 nm between media A and E for experimental samples, ΔY is the difference in absorbency at 420 nm between a *p*NP standard and a blank sample, the concentration of *p*NP standard is in µmol l^{-1}; [protein] is the protein content of the experimental sample, and t is the duration of the assay (in h).

Enzyme inhibition assays

In the first series of experiments, membranes were either incubated on ice for 1 h with methanol extracts from *M. aeruginosa* PCC 7820 or CYA 43, or an equivalent quantity of cyanobacterial material was added to the membranes at the start of the assay. In this series of experiments, two concentrations of the methanol extracts from *M. aeruginosa*

PCC 7820 or CYA 43 were tested, 7.6 or 23 mg dry mass mg^{-1} membrane protein. In a second set of experiments, different quantities of membranes (between 1.875 and 30 μg of membrane protein) were incubated with various concentrations of cyanobacterial extracts; in the case of *M. aeruginosa* 7820, between 0 and 200 μg of freeze-dried material, and for *M. aeruginosa* CYA 43, between 0 and 150 μg of freeze-dried material.

Characterisation studies

Several methods were used to identify the compounds in *Microcystis aeruginosa* 7820 and CYA 43 that inhibit fish gill K^+-dependent *p*NPPase activity. The sequence in which these procedures were performed and details are described below.

Pharmacia 'Smart-System' fraction collection

The methanol extracts were fractionated with a Pharmacia 'Smart-System' using the eluents acetonitrile/0.05 % (v/v) trifluoroacetic acid (TFA) and milliQ water/0.05 % TFA with a flow rate of 150 μl min^{-1}. A 65 % acetonitrile (v/v) gradient was built up over 16 min, which was then increased to 90 % over the next 3 min; the flow then remained constant for 3 min before returning to 0 % acetonitrile by 25 min. Fractions (100 μl) were collected and pooled into five sequential groups, which were subsequently freeze-dried and resuspended in a volume of sucrose buffer equal to the volume injected onto the column. Following initial inhibition studies, individual fractions were collected from the region showing inhibitory activity.

Concanavalin A Sepharose and heat treatment

Concanavalin A Sepharose beads bind polysaccharides, primarily glycoproteins, and consequently this treatment was used to determine whether the compound possesses a sugar moiety. Concanavalin A Sepharose beads (0.5 ml) were washed with buffer (1 mol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $MnCl_2$, 1 mmol l^{-1} $CaCl_2$, 15 mmol l^{-1} Tris/HCl, pH 7.4), centrifuged (6500 ***g***) for 5 min and the supernatant removed. Samples (50 μl) of the methanol extracts from the cyanobacteria were dried down and resuspended in 250 μl of the same buffer. This was added to the beads, shaken for 1 h, then centrifuged at 6500 ***g*** for 5 min; the supernatant was removed and freeze-dried. Control medium was prepared using a similar procedure by incubating the beads with 250 μl of the buffer. The resulting residues were resuspended in sucrose buffer.

Additionally, the cyanobacterial material dissolved in sucrose buffer was heated to 90 °C for 30 min prior to testing for inhibitory activity.

Hexane separation

Methanol stock extracts of *M. aeruginosa* 7820 and CYA 43 (75 μl) were dried and resuspended in an equivalent volume of sucrose buffer. To this, 500 μl of hexane was added, and the resulting solution was shaken for 2 h and then left to stand for 1 h. The hexane layer was removed, and both fractions were left to dry in a fume hood. The residue from the hexane layer was resuspended in 75 μl of sucrose buffer, and the volume of the other layer was adjusted with sucrose buffer to an equivalent volume.

Dichloromethane:methanol separation

Methanol stock extracts from *M. aeruginosa* 7820 and CYA 43 (75 μl) were dried and resuspended in 75 μl of sucrose buffer. To this suspension, 0.73 ml of distilled water and 3 ml of dichloromethane:methanol (1:2) were added. This mixture was shaken for 15 min and, to obtain a two-phase system, a further 1 ml of dichloromethane and 1 ml of water were added and the solution was vigorously mixed. The layers were separated by centrifugation (550 ***g*** for 5 min), the dichloromethane layer was dried overnight in a fume hood, and the water/methanol layer was lyophilised. The residues were resuspended in 75 μl of sucrose buffer.

Thin-layer chromatography

Samples (20–60 μl) from the dichloromethane:methanol extracts from both strains of *M. aeruginosa* were dried and resuspended in an equivalent volume of methanol. In addition, the hydrophobic and inhibitory fractions obtained with the Pharmacia 'Smart-System' for either strain of *M. aeruginosa* were lyophilised and resuspended in a volume of methanol equivalent to that initially injected onto the column. Known volumes of these methanol suspensions were streaked along the origin of a Merck silica gel 60 (20 cm×20 cm×0.2 cm) plate (thin-layer chromatography, TLC, plates) and then exposed to a solvent system containing diethylether:isopropanol:formic acid (100:4.5:2.5).

TLC plates were divided into two batches, and one batch was sprayed with a sulphuric acid:methanol (1:1) solution. Regions of the silica gel plates were scraped off, including an area that had only been exposed to the solvent system to act as a control, and extracted three times with 2 ml of methanol. These extracts were centrifuged at 550 ***g*** to remove particulate matter, and the supernatant was dried in a waterbath at 40 °C. The resulting residues were resuspended in half the volume of methanol that was initially streaked onto the silica gel plate. This was then dried, and the residues were resuspended in sucrose buffer prior to inhibition assays. The final concentration of cyanobacterial material used for the assay was equivalent to 46 mg dry mass mg^{-1} membrane protein.

Fatty-acid-free bovine serum albumin (FAF-BSA) treatment

Samples (75 μl) of the dichloromethane:methanol extracts from *M. aeruginosa* PCC 7820 or CYA 43 were dried, resuspended in sucrose buffer to an equivalent volume and incubated for 1 h with 2 % (w/v) fatty-acid-free bovine serum albumin (FAF-BSA; Boehringer Mannheim, Germany). Samples from this solution were added to the membranes (equivalent to 30 mg dry mass mg^{-1} membrane protein) and incubated for 1 h. Additionally, membranes were initially incubated with 2 % (w/v) FAF-BSA for 1 h and then exposed

to an equivalent quantity of *M. aeruginosa* PCC 7820 or CYA 43. Appropriate controls using FAF-BSA were also run.

Statistics

Results are presented as mean ± S.E.M. Differences in the time course study and the concentration curves were assessed by analysis of variance (ANOVA) in combination with a Tukey's honestly significant differences (HSD) test. All other differences among groups were assessed using a paired *t*-test (SPSS-6 for Windows).

Results

Enzyme inhibition assays

Preincubation of membranes with extracts from the cyanobacteria *M. aeruginosa* 7820 or CYA 43 for 1 h prior to commencing the K^+-dependent *p*NPPase assay caused 70 % inhibition of activity compared with controls (Fig. 1B,D). Membranes that did not receive the extracts from the cyanobacteria at the beginning of the assay generally retained their enzyme activity at the level of controls (Fig. 1A,C). However, there was one exception to this: inhibition was seen in membranes that had received 23 mg dry mass of *M. aeruginosa* PCC 7820 per milligram membrane protein after 20 min of incubation (Fig. 1A).

An increase in the concentration of cyanobacterial extracts resulted in an increase in the level of inhibition of membrane K^+-dependent *p*NPPase activity. However, the degree of inhibition depended on the concentration of membrane (Fig. 2A,B). Extracts of 150 µg of freeze-dried material of *M. aeruginosa* CYA 43 gave 99 % inhibition at 1.875 µg of membrane protein (Fig. 2B), whilst *M. aeruginosa* PCC 7820 proved to be less potent (Fig. 2A); extracts from 200 µg of freeze-dried material of *M. aeruginosa* PCC 7820 gave 67 % inhibition at the same membrane concentration. In comparison, 30 µg of membrane protein exposed to the same concentration of *M. aeruginosa* 7820 or CYA 43 showed 19 % and 32 % inhibition, respectively.

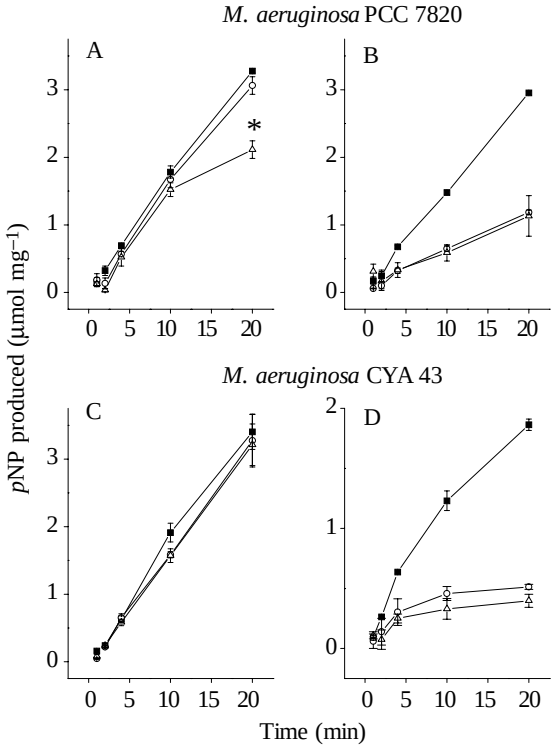

Fig. 1. Progress curves of *p*-nitrophenol (*p*NP) produced by gill basolateral plasma membranes after incubation with methanol extracts from either *Microcystis aeruginosa* PCC 7820 (A,B) or CYA 43 (C,D) at two concentrations of cyanobacterium (7.6 mg dry mass mg^{-1} membrane protein, open circles; 23 mg dry mass mg^{-1} membrane protein, open triangles) and in controls (filled squares). A and C show the profile when the extracts from either strain of *M. aeruginosa* are added at the beginning of the assay, whilst B and D give the profile after a preincubation of 1 h. Values are mean ± S.E.M. (N=6). The asterisk indicates a significant difference from the control in A and C (Tukey's HSD test, P<0.05). In B and D, all treated samples beyond 4 min of incubation are significantly different from controls, and asterisks have been omitted for clarity.

Characterisation of the compound(s)

Combining fractions from the Pharmacia 'Smart-System' confirmed a previous study (Bury *et al.* 1996*b*): the compounds that inhibited Na^+/K^+-ATPase were primarily found in the more hydrophobic fractions (data not shown). However, inhibition studies on the individual fractions show that there were four regions showing significant inhibitory activity (Table 1). These inhibitory fractions did not correspond to individual protein or peptide peaks on the chromatogram as

Table 1. *The K^+-dependent* p*NPPase activity of tilapia gill basolateral membranes treated with fractions from methanolic extracts of* M. aeruginosa *PCC 7820 separated by the Pharmacia 'Smart-System'*

Fraction	*p*NPPase activity (µmol mg^{-1} h^{-1})
Control	6.38±0.88
24	5.89±0.92
25	6.09±0.77
26	6.08±0.81
27	6.42±0.88
28	5.88±0.83
29	5.22±0.93
30	4.75±0.48*
31	4.31±0.61*
32	3.31±0.36*
33	4.19±0.72*
34	4.93±0.50
35	5.89±0.68

See Materials and methods for details of fractionation procedure.
Values are means ± S.E.M., N=4.
Asterisks indicate a significant difference from control values (paired *t*-test, P<0.05).
Activity is measured as µmol *p*NP mg^{-1} membrane protein h^{-1}.

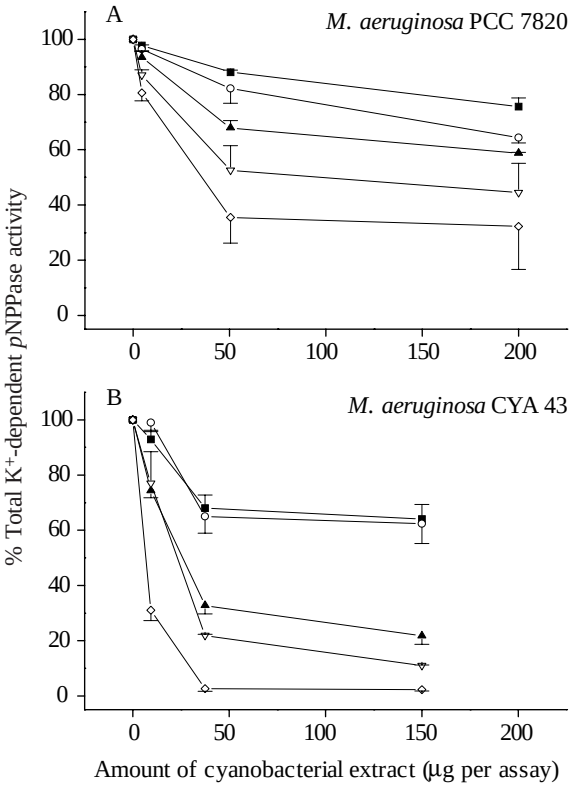

Fig. 2. Inhibition curves (activity expressed as a percentage of the activity of controls, designated 100 %) of gill plasma membrane K^+-dependent *p*NPPase activity, at various membrane protein concentrations (open diamonds, 1.875 µg membrane protein; open triangles, 3.75 µg; filled triangles, 7.5 µg; open circles, 15 µg; filled squares, 30 µg,) after incubation with differing levels of methanol extracts from *M. aeruginosa* PCC 7820 (A) or CYA 43 (B). Values are mean ± S.E.M. (*N*=3).

traced at wavelengths of 214 nm, 238 nm or 280 nm (chromatogram not shown).

Preheating extracts (resuspended in sucrose buffer) from either *M. aeruginosa* PCC 7820 or CYA 43 to 90 °C for 30 min increased the potency of the extract (Table 2), possibly because of a concentrating effect on the extract. Incubating the extracts with Concanavalin A Sepharose beads reduced the degree of inhibition of membrane K^+-dependent *p*NPPase activity, but this reduction was not complete (Table 2).

The hexane or dichloromethane:methanol separation procedure for either *M. aeruginosa* PCC 7820 (Fig. 3A) or CYA 43 (Fig. 3B) resulted in a partitioning of the inhibitory activity of membrane K^+-dependent *p*NPPase activity from the aqueous phase into dichloromethane:methanol, but not into hexane.

The separation of the dichloromethane:methanol extracts from *M. aeruginosa* PCC 7820 by thin-layer chromatography (TLC) revealed five regions that showed significant inhibition of membrane K^+-dependent *p*NPPase activity (Table 3); in the case of *M. aeruginosa* CYA 43, six regions were found to be inhibitory, the additional region having an R_F value (the distance from the origin that the compound migrated on the

Table 2. *The K^+-dependent* p*NPPase activity of tilapia gill basolateral membranes treated with methanolic extracts of* M. aeruginosa *PCC 7820 or CYA 43 resuspended in sucrose buffer and heated to 90 °C for 30 min or treated with Concanavalin A sepharose beads*

	*p*NPPase activity (µmol mg^{-1} h^{-1})	
	M. aeruginosa 7820	*M. aeruginosa* CYA 43
Control	7.66±0.44	
Untreated	5.95±0.53	4.2±0.46
Heated	5.03±0.60*	3.26±0.457*
Control	5.45±0.194	
Untreated	4.01±0.434	3.72±0.56
Concanavalin-A-treated	4.26±0.31*	4.67±0.28*

Values are means ± S.E.M.; *N*=9 for the heated samples and *N*=5 for those treated with Concanavalin A.

In all cases, untreated and treated values are significantly different from controls; asterisks indicate a significant difference between treated and untreated values (paired *t*-test, $P<0.05$).

Activity is measured as µmol *p*NP mg^{-1} membrane protein h^{-1}.

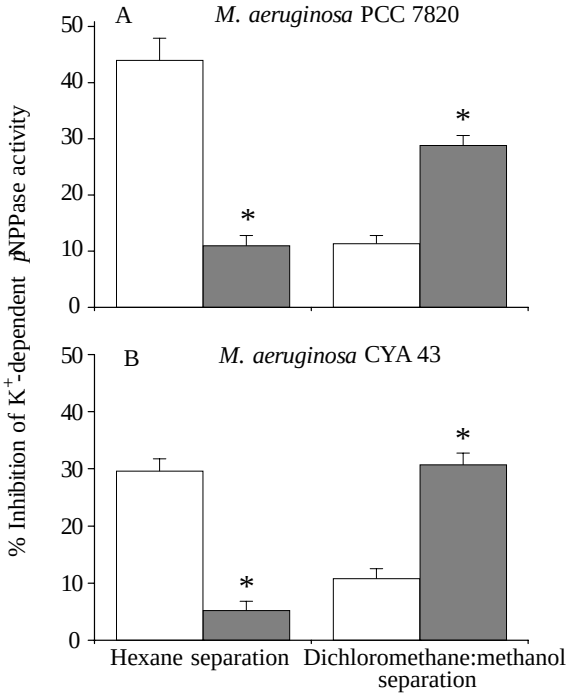

Fig. 3. Inhibition of gill plasma membrane K^+-dependent *p*NPPase activity (expressed as a percentage of the activity of controls, designated 100 %) after incubation with extracts from either *M. aeruginosa* PCC 7820 (A) or CYA 43 (B) after the hexane or dichloromethane:methanol separation procedure (see Materials and methods for further details). Open columns indicate the aqueous phase and tinted columns indicate the organic phase of the separation. Values are mean + S.E.M., *N*=9 for the hexane separation and *N*=5 for the dichloromethane:methanol separation. All statistics were performed on untransformed data; asterisks indicate a significant difference between the inhibition found in the aqueous phase (open column) and the organic phase (hatched column) of the two separation procedures (paired *t*-test, $P<0.05$).

Table 3. *The K^+-dependent* p*NPPase activity of tilapia gill basolateral membranes treated with different regions from a thin-layer chromatography plate following the separation of dichloromethane:methanol extracts from* M. aeruginosa *PCC 7820 or CYA 43 with a solvent phase of diethylether:isopropanol:formic acid (100:4.5:2.5)*

M. aeruginosa 7820		*M. aeruginosa* CYA 43	
R_F	*p*NPPase activity (μmol mg^{-1} h^{-1})	R_F	*p*NPPase activity (μmol mg^{-1} h^{-1})
Control	4.43±0.45	Control	4.01±1.04
1–0.96	4.67±0.50	1–0.97	5.03±0.96
0.96–0.91	4.94±0.64	0.97–0.89	4.36±0.90
0.91–0.76	3.78±0.49*	0.89–0.83	3.52±0.824
0.76–0.71	3.21±0.46*	0.83–0.73	2.80±0.50*
0.71–0.56	2.56±0.50*	0.73–0.65	2.02±0.43*
0.56–0.51	4.31±0.47	0.65–0.56	3.03±0.846*
0.51–0.34	4.64±0.48	0.56–0.43	2.64±0.81*
0.34–0.05	3.04±0.52*	0.43–0.35	3.72±0.83
0.05–0	1.74±0.53*	0.35–0.05	0.35±0.09*
		0.05–0	2.14±0.50*

Values are means ± S.E.M., N=5.
Asterisks indicate a significant difference from control values (paired t-test, P<0.05).
Activity is measured as μmol *p*NP mg^{-1} membrane protein h^{-1}.
R_F, distance migrated from the origin relative to the solvent front.

TLC plate relative to the solvent front) of 0.56–0.43 (Table 3). Separation of the hydrophobic region obtained from the Pharmacia 'Smart-System' by TLC revealed that *M. aeruginosa* PCC 7820 had two inhibitory regions and *M. aeruginosa* CYA 43 had three (Table 4). The additional region had an R_F value of 0.66–0.48 (Table 4). However, in the case of both separation procedures and for both cyanobacterium strains, the most potent region occurred in extracts taken from the origin of the plate (Tables 3, 4).

Incubation of the membranes with dichloromethane:methanol extracts from either *M. aeruginosa* PCC 7820 or CYA 43 followed by treatment with 2% (w/v) FAF-BSA significantly reduced the inhibitory activity of the extracts. The inhibition could be further and significantly reduced by pretreatment of the extracts with FAF-BSA (Fig. 4).

Discussion

The results from this study show that there are a number of cytotoxic compounds present in methanolic extracts of cyanobacteria that inhibit fish gill Na^+/K^+-ATPase, other than the hepatotoxin microcystin-LR (MC-LR). Characterisation shows that these compounds must be lipids. These findings are in line with and extend results from a previous study (Bury *et al.* 1996*b*).

TLC analysis of the lipids present in *M. aeruginosa* shows a profile that includes monoglycosyl-diglyceride (MGDG),

Table 4. *The K^+-dependent* p*NPPase activity of tilapia gill basolateral membranes treated with different regions of a thin-layer chromatography plate following separation of the most hydrophobic region collected from the Pharmacia 'Smart-System' for* M. aeruginosa *PCC 7820 or CYA 43 with a solvent phase of diethylether:isopropanol:formic acid (100:4.5:2.5)*

M. aeruginosa 7820		*M. aeruginosa* CYA 43	
R_F	*p*NPPase activity (μmol mg^{-1} h^{-1})	R_F	*p*NPPase activity (μmol mg^{-1} h^{-1})
1–0.94	6.20±0.865	1–0.9	4.53±0.55
0.94–0.81	6.18±0.95	0.9–0.77	4.36±0.55
0.81–0.61	5.94±0.89	0.77–0.66	4.35±0.57
0.61–0.45	6.02±0.91	0.66–0.48	3.24±0.44*
0.45–0.26	6.09±0.86	0.48–0.35	4.36±0.57
0.26–0.05	5.27±0.89*	0.35–0.04	4.07±0.50*
0.05–0	2.73±0.67*	0.04–0	2.61±0.27*

Values are means ± S.E.M., N=5.
Asterisks indicate a significant difference from control values (paired t-test, P<0.05).
Activity is measured as μmol *p*NP mg^{-1} membrane protein h^{-1}.
R_F, distance migrated from the origin relative to the solvent front.

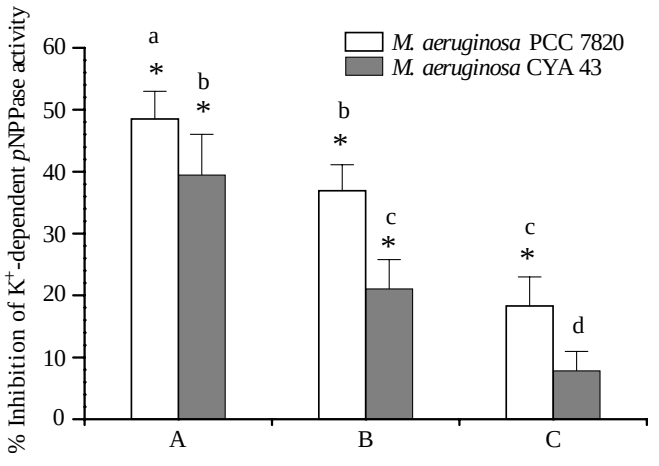

Fig. 4. Inhibition of basolateral membrane K^+-dependent *p*NPPase activity (expressed as a percentage of the control values, designated 100 %) after incubation with dichloromethane:methanol extracts from *M. aeruginosa* 7820 or CYA 43 (A), or following incubation with the extracts and a subsequent 1 h treatment with 2 % (w/v) fatty-acid-free bovine serum albumin (FAF-BSA) (B), or after the extracts have been pre-treated with FAF-BSA (C). Values are mean + S.E.M. (N=6). All statistics were performed on untransformed data; asterisks indicate a significant difference from the controls, whilst columns marked with the same letter were not significantly different from each other (paired *t*-tests, $P<0.05$).

diglycosyl-diglyceride (DGDG), phosphatidyl-glycerol and sulphoquinovosyl-diglyceride (sulpholipid), as well as orange, red and dark green pigments (Murata and Nishida, 1987; Ikawa *et al.* 1996; Walsh *et al.* 1997). It has previously been shown that lipids present in cyanobacteria may be bioactive (Murakami *et al.* 1991; Ikawa *et al.* 1994, 1996). MGDGs and DGDGs from *Phormidium tenue* have autolytic properties (Murakami *et al.* 1991), and unsaturated fatty acids (linoleic and oleic acids) extracted from *Aphanizomenon flos-aquae* and *M. aeruginosa* inhibit the growth of the green alga *Chlorella* (Ikawa *et al.* 1994, 1996). The present study indicates that the lipid compounds present in *M. aeruginosa* CYA 43 and *M. aeruginosa* PCC 7820 inhibit Na^+/K^+-ATPase activity in fish gills. In addition, the reduction in inhibiton observed when methanolic extracts of *M. aerugionosa* are treated with Concanavalin A suggests that at least one of the compounds may possess a sugar moiety. The inhibition profiles of the thin-layer chromatograms for each strain of cyanobacterium differ, with *M. aeruginosa* CYA 43 possessing an additional inhibitory region compared with *M. aeruginosa* PCC 7820.

Lipids have been shown to inhibit a number of ion-transporting enzymes in mammals, such as Na^+/K^+-ATPase (Kelly *et al.* 1986; Swarts *et al.* 1990), H^+/K^+-ATPase (Bin Im and Blakeman, 1982; Swarts *et al.* 1991; Beil *et al.* 1994), smooth endoplasmic reticulum Ca^{2+}-ATPases (SERCA ATPases) (Kim and LaBella, 1988) and Zn^{2+}-dependent ATPase (Ronquist and Frithz, 1992). In the brine shrimp *Artemia salina*, Morohashi *et al.* (1991) identified long-chain fatty acids as endogenous inhibitors of Na^+/K^+-ATPase. In most cases, inhibition is due to unsaturated long-chain fatty acids, rather than to the methylated or saturated forms (e.g. Davis *et al.* 1987; Swann, 1984; Swarts *et al.* 1990, 1991). Combining these findings with the known lipid profiles from *M. aeruginosa*, it is apparent that these cyanobacteria may produce unsaturated fatty acids inhibiting Na^+/K^+-ATPase. This hypothesis is corroborated by the observation that incubation of the dichloromethane:methanol extracts from *M. aeruginosa* PCC 7820 or CYA 43 with fatty-acid-free bovine serum albumin (FAF-BSA) reduced the extent of inhibition. However, the inhibition was not completely prevented, probably as a result of the concentrations of cyanobacterial extract used. Treatment of the branchial plasma membranes with FAF- BSA, following incubation with either strain of *M. aeruginosa*, only partially restored enzyme activity, indicating an avid binding of the inhibitor to the enzyme complex. Similar observations have been made for gastric H^+/K^+-ATPase activity after incubation with linoleic acid (Bin Im and Blakeman, 1982) and for Na^+/K^+-ATPase activity after incubation with oleic acid (Swarts *et al.* 1990).

Methanol extracts from *M. aeruginosa* have been shown to inhibit P-type and SERCA-type ATPases, as well as mitochondrial Ca^{2+}-sequestering mechanisms (either *via* the H^+-ATPase or the mitochondrial Ca^{2+} channel; Bury *et al.* 1996*b*). The use of assay conditions specific to the dephosphorylation step of the Na^+/K^+-ATPase reaction cycle indicates that lipids from the cyanobacterial extracts inhibit at the K^+-binding site of the enzyme. Studies have shown that unsaturated fatty acids may displace ouabain from the Na^+/K^+-ATPase and that this may be one of the mechanisms by which fatty acids inhibit Na^+/K^+-ATPase activity (Lamers and Hulsmann, 1977; Swann, 1984; Tamura *et al.* 1985; Swarts *et al.* 1990).

The degree of inhibition of the fish gill Na^+/K^+-ATPase by lipid compounds extracted from *M. aeruginosa* was dependent on the reaction conditions, i.e. the concentrations of enzyme and inhibitor (Fig. 2), as well as on incubation time (Fig. 1). Similar results have been observed by Swarts *et al.* (1990) for the inhibition of Na^+/K^+-ATPase activity by unsaturated fatty acids. The convex inhibition curves obtained when the enzyme was exposed to increasing concentrations of cyanobacterial extract, and at different concentrations of membrane protein, are characteristic of tight-binding inhibition (Morrison, 1969; Williams and Morrison, 1979). Furthermore, the time taken for the inhibition to occur (1 h) and the nature of this inhibition suggest 'slow, tight-binding' inhibition (Morrison and Walsh, 1988; Szedlacsek and Duggleby, 1995). However, we cannot yet further define the substances that cause inhibition and the kinetics of this inhibition because of the number of inhibitory compounds (see Tables 3, 4) extracted from both strains of *M. aeruginosa.*

What is the ecotoxicological importance of cyanobacterial lipids? They may reduce the growth of other phytoplankton (Ikawa *et al.* 1994, 1996), but it is not clear whether they adversely affect higher vertebrates when present in an aquatic

environment. Immersion trials, in which tilapia were exposed to methanol extracts of *M. aeruginosa*, showed inhibition of whole-body Ca^{2+} influx (Bury *et al.* 1996*b*). Our results suggest that lipids, rather than MC-LR, from cyanobacteria interfere with gill basolateral membrane ion-extrusion mechanisms and thus may contribute to the fish deaths seen after lysis of a cyanobacterial bloom.

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