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Toxic potential of five freshwater *Phormidium* species (Cyanoprokaryota)

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Abstract

Among the Cyanoprokaryota (blue-green algae), the genus *Phormidium* has thus far rarely been studied with respect to toxin production and potentially resulting human and environmental health effects. We here show that five previously unexplored freshwater species of this genus (*Ph. bijugatum*, *Ph. molle*, *Ph. papyraceum*, *Ph. uncinatum*, *Ph. autumnale*) are indeed capable of producing bioactive compounds. *Phormidium* extracts caused weight loss as well as neuro/hepatotoxic symptoms in mice, and in the case of *Ph. bijugatum* even death. Very low levels of saxitoxins and microcystins, as confirmed by ELISA, were insufficient to explain this toxicity and the differing toxic potencies of the *Phormidium* species. Qualitative HPLC analyses confirmed different substance patterns and in the future could aid in the separation of fractions for more detailed substance characterisation. The results in vivo were confirmed in vitro using cells of human, mouse and fish. The fish cells responded least sensitive but proved useful in studying the temperature dependence of the toxicity by the *Phormidium* samples. Further, the human cells were more sensitive than the mouse cells thus suggesting that the former may be a more appropriate choice for studying the impact of *Phormidium* to man. Among the human cells, two cancer cell lines were more responsive to one of the samples than a normal cell line, thereby indicating a potential anti-tumour activity. Thus, the five freshwater *Phormidium* species should be considered in environmental risk assessment but as well, as a source of therapeutic agents.

Keywords: Cyanoprokaryota; *Phormidium*; Toxins; Mouse bioassay; Fish and mammalian cell cultures; Cytotoxicity

1. Introduction

Cyanoprokaryota (blue-green algae) have been observed in aquatic environments around the world. Several strains of these microorganisms are known to produce a wide variety of toxins and/or biomedically interesting, bioactive compounds. The chemical structure of these compounds and their mechanisms of action in biological systems in vitro/in vivo have been intensively investigated during recent years. Most investigations in this field examined species belonging to the genera *Microcystis, Cylindrospermopsis, Anabaena, Oscillatoria (Planktothrix)* and *Aphanizomenon* (Lakshmana Rao et al., 2002; Haider et al., 2003).

Cyanotoxins cause direct intoxications of animals and humans through contact with bloom water or indirect

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poisoning due to consumption of contaminated food (Carmichael and Falconer, 1993; Carmichael, 1994, 1997; Jochimsen et al., 1998; Falconer, 1999; Ito et al., 2000). Well-known cyanotoxins can be divided, like algal toxins generally, into hepatotoxins (e.g. microcystins, nodularins, cylindrospermopsins), neurotoxins (e.g. anatoxin-a, homoanatoxin-a, saxitoxins) and dermatotoxins (e.g. lyngbyatoxins, aplysiatoxins). Hepatotoxins are inhibitors of serine/threonine specific protein phosphatases (PP1 and PP2A), neurotoxins block neurotransmission and dermatotoxins are the cause of skin irritations, allergic reactions and gastroenteritis. During the past years, the frequency and global distribution of toxic algal incidents appear to have increased, and human intoxications from novel algal sources have occurred. This has led to the revelation that numerous cyanoprokaryotic species not commonly investigated may be the source of potent toxins.

One genus of Cyanoprokaryota for which information on toxin production exists for only a few species is the genus *Phormidium*. In the marine environment, *Phormidium corallyticum* was identified as causing the black band disease of Atlantic reef corals (Mitsui et al., 1987). *Phormidium persicinum* was reported to produce unidentified compounds toxic to brine shrimps (Lincoln et al., 1991). The stereochemistry and structure of one marine *Phormidium* sp. metabolite highly toxic to brine shrimp was elucidated by Williamson et al. (2002) and termed phormidolide. According to Lilleheil et al. (1997), *Oscillatoria formosa*, or *Phormidium formosum* as classified by Anagnostidis and Komarek (1988), produces homoanatoxin-a. Likewise, Baker et al. (2001) observed lethal toxic effects to mice after intraperitoneal injection (400 mg kg^{-1}) of unfiltered extracts of *Ph. formosum* as well as of *Phormidium amoenum*.

Several studies have focussed on a more therapeutic viewpoint of biological activity of *Phormidium* species. Trichloracetic-acid-treated *Phormidium* extracts reduced croton oil-induced oedema in mice by about 60% in a dose-dependent manner (Garbacki et al., 2000). Glycolipids isolated from *Phormidium tenue* were found to inhibit enzymatic activity of HIV-1 reverse transcriptase to different extents (Reshef et al., 1997). Capsular polysaccharides isolated from different *Phormidium* strains showed anti-inflammatory properties. Several investigators presented data for anti-tumour or anti-plasmodial activity of compounds isolated from *Ph. tenue* and *Phormidium ectocarpi*. Two digalactosyl diacylglycerols isolated from the freshwater cyanobacterium *Ph. tenue* effectively inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin papilomas in ICR mice (Tokuda et al., 1986, 1996; Shirahashi et al., 1993). The methylene dichloride extract of *Ph. ectocarpi* showed anti-plasmodial activity towards *Plasmodium falciparum* (Papendorf et al., 1998). Williamson et al. (2002) reported a specific inhibitory activity of phormidolide to Ras-Raf protein–protein interaction, a critical component in the mitogen-activated signal transduction cascade in a number of cancer types.

The purpose of this study was to investigate whether five freshwater species of the genus *Phormidium* (*Ph. bijugatum, Ph. molle, Ph. papyraceum, Ph. uncinatum* and *Ph. autumnale*), unknown so far with regard to toxin production, are a source of intracellular and/or extracellular toxic compounds. Investigations were based both on analytical approaches as well as on in vivo/in vitro bioassays. These experiments revealed that the selected species indeed are toxigenic and produce identified as well as unidentified compounds with significant toxic activity.

2. Materials and methods

2.1. Phormidium cultures and preparation of extracts

Five different freshwater species of the genus *Phormidium* (Cyanoprokaryota) were studied:

- *Phormidium bijugatum* Kongiss. vel foleolarum (Mont.) Gomont—kept in PACC (Plovdiv Algal Culture Collection) as No 8602;
- *Phormidium molle* (Kutzing) Gomont—kept in PACC as No 8140;
- *Phormidium papyraceum* (Agardh) Gomont—kept in PACC as No 8600;
- *Phormidium uncinatum* (Agardh) Gomont—kept in PACC as No 8693;
- *Phormidium autumnale* (Agardh) Gomont—kept in PACC as No 5517.

Phormidium species were grown intensively under sterile conditions as described by Dilov et al. (1972) using a Z-nutrient medium (Staub, 1961). Cultures were synchronised by altering light/dark periods of 16/8 h. The temperature was 33 and 22 °C during the light and dark period, respectively. This culture regime was established in order to closely mimic the conditions for optimal growth of *Phormidium* in natural habitats in the summer months. The intensity of light during the light period was 224 μmol photon s^{-1} m^{-2} (Lux 12,000). The culture medium was aerated with 100 L of air per hour per 1 L of medium, adding 1% CO_2 during the light cycle. The period of cultivation was 14 days.

Extracts of the *Phormidium* species were obtained according to the method of Krishnamurthy et al. (1986) with slight modifications. Briefly, *Phormidium* species were removed from the Z-medium and weighed, then frozen and thawed, and extracted twice (3 h and overnight) with water–methanol–butanol solution (15:4:1, v:v:v, analytical grade) at 22 °C while stirring. The extracts were centrifuged at 10,000 rpm for 30 min. The supernatants of the two extracts were pooled and organic solvents removed via speed-vac centrifugation (SAVANT, Instruments, Inc. Farmingdate,

NY, USA) at 37 °C for 2 h. The resulting extract was filter-sterilized (0.22 μm Millipore filter) and prepared to give final equivalent concentrations of 150 mg/mL (wet weight/volume) suspended algal matter. Extracts were investigated for biological activity in both the in vivo as well as the in vitro assays.

To investigate whether *Phormidium* species release toxic products into their culture environment, the nutrient solution in which the Cyanoprokaryota were cultivated during the 14 days was filtered through a 0.22 μm Millipore filter. The final equivalent concentration of suspended algal matter per mL culture medium was 20 mg/mL (wet weight/volume). This medium was tested for its impact on cell viability in vitro.

2.2. Toxicity of Phormidium extracts in vivo

A total of 18 male DBA/1 mice (19–22 g) was used for the experiment (three mice per group). All mice were kept in a climate-controlled environment with 12 h light/dark cycles in polystyrene cages containing wood shavings. Mice were fed standard rodent chow and water ad libitum in a specific pathogen-free environment. Mice were injected i.p. with 0.5 mL test solution containing equivalent final concentrations per mouse of 15 mg suspended blue-green algae matter (682–790 mg/kg mouse). In order to obtain this test solution, the algal extracts were diluted 1:4 with phosphate buffered saline (PBS). Control mice were injected with 0.5 mL PBS. The animals were observed for 24 h after treatment. Behavioral symptoms, weight and survival times were recorded.

All animals were subjected to histological examination of the liver for pathology. After termination of the experiment, the liver slices were processed for light microscopy according to standard procedures. Briefly, the tissue samples were fixed in 4% buffered formalin for 24 h, dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Multiple sections from each block were prepared at 5 μm thickness and stained with hematoxylin and eosin (McManus and Mowry, 1965).

2.3. Toxicity of Phormidium extracts in vitro

2.3.1. Cell culture

Six different cell cultures were used. These consisted of one freshly established cell culture of mouse fibroblasts, four commercially available mammalian cell lines, FL (normal amniotic cells, human, ATCC CCL 62), A2058 (human metastatic melanoma, ECACC 91100402), RD (rhabdomyosarcoma, embryonic, human, ATCC CCL 136), 3T3 (fibroblasts, embryonic, mouse, ATCC CCL 92), and one cell line derived from a normal rainbow trout (*Oncorhynchus mykiss*) liver (RTL-W1, Lee et al., 1993).

2.3.1.1. Continuous mammalian cell lines. FL, A2058, RD and 3T3 cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Paisley, Scotland, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma, Steinheim, Germany), at 37 °C with 5% CO_2 in air and high humidity. Trypsin treatment and subculturing were done according to the Invittox protocols (1990, 1992). Cell viability was measured with the trypan blue exclusion test (Berg et al., 1972) prior to seeding.

2.3.1.2. Isolation and culture of primary mouse thymus fibroblasts. This cell culture was prepared from B10.Q mice. The thymus of each mouse was removed and stored immediately in sterile PBS containing 100 IU/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B (Serva, Heidelberg, Germany). All samples were finely minced with scissors and digested for 45 min at 37 °C in 5 mL PBS containing 0.1% trypsin (Gibco BRL, Paisley, Scotland, UK). Thereafter, samples were digested in 0.1% collagenase D (Roche Diagnostics GmbH, Mannheim, Germany) in DMEM, containing 10% fetal calf serum (FCS, heat inactivated, PAA Laboratories GmbH, Linz, Austria), for 90 min at 37 °C in an atmosphere with 5% CO_2. After passage of the cells through a 40 μm cell strainer (FALCON®, Becton Dickinson, Le Pont De Claix, France), cells were collected by centrifugation, washed twice with serum-free DMEM and cultured in complete DMEM as described above for the continuous mammalian cell lines. After three passages, the culture consisted of fibroblast-like cells only, as judged by cell morphology and by flow cytometry using CD106 (VCAM-1), CD68, and CD11b as markers (FACS, Becton Dickinson, San José, CA, USA) to exclude contamination with other cell types.

2.3.1.3. Continuous fish cell line. RTL-W1 cells were cultured as originally described by Lee et al. (1993) in an atmosphere of air in 75 cm^2 Nunc culture flasks at 19 °C in Leibovitz's L-15 medium without phenol red (Invitrogen, Karlsruhe, Germany). The medium was supplemented with 5% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and penicillin–streptomycin (20 IU/mL–20 μg/mL, Biochrom, Berlin, Germany). Subcultivation procedures were as previously described (Bols and Lee, 1994; Schirmer et al., 1994).

2.3.2. Exposure conditions. Prior to exposure, cells were plated in 96-well tissue culture plates at a density of 1.5×10^4 per 200 μL DMEM medium with 10% FCS for mammalian cells and 5×10^4 cells per 200 μL L-15 medium with 5% FBS for the piscine cell line. After 24 h of attachment, the medium was removed and replaced by the exposure medium as described below.

Exposure to the cyanoprokaryotic extracts. Mammalian cells were exposed to one concentration of the *Phormidium* extracts. This concentration was equivalent to 15 mg/mL (w/v) suspended algal matter (10% of the *Phormidium*

extract) and was obtained by adding 20 μl of the extract to 180 μl DMEM culture medium with 10% FCS. Control wells were prepared by adding 20 μl Millipore water to 180 μl culture medium. The cells were exposed to the extracts for 4 or 24 h prior to analysis of cytotoxicity by the MTT assay or of effects on proliferation by the [^{3}H]-thymidine incorporation assay as described below.

The piscine cell line was exposed to varying concentrations of the *Phormidium* extracts with the highest concentration being equivalent to an extract of 15 mg/mL suspended algal matter (10% of the *Phormidium* extract). Exposure was done in L-15 medium in the absence of serum. The exposure temperature was 19 °C as for routine maintenance. In some experiments, fish cells were exposed at 4 or 25 °C, in addition to 19 °C, in order to investigate if varying temperatures had an effect on the toxicity of the extracts. Cytotoxicity of the extracts was assessed after 24 h of exposure by a combined alamar Blue™/CFDA-AM cell viability assay as described below.

Exposure to the cyanoprokaryotic growth media. In addition to exposure to *Phormidium* extracts, the cells were also exposed to varying concentrations of media in which *Phormidium* species had been grown for 14 days. Mammalian cells were treated with *Phormidium* growth medium at a final concentration of 10% (20 μl of the algal medium to 180 μl DMEM) under the conditions mentioned above. A similar concentration of Z-medium was used as appropriate control. The highest concentration for the fish cell line was 50% of the growth medium, which was obtained by adding 100 μl of the algal Z-medium to 100 μl of L-15 medium in the absence of serum. Appropriate controls were prepared by mixing L-15 with up to 50% Z-medium. The exposure temperature for the fish cells was 19 °C as for routine maintenance. However, in some experiments, fish cells were exposed at 4 or 25 °C, in addition to 19 °C, in order to investigate if varying temperatures had an effect on the toxicity of the growth media. Cytotoxicity of algal growth media was assessed after 4 or 24 h of exposure by a combined alamar Blue™/CFDA-AM or the MTT cell viability assay as described below.

2.4. Cell viability assays

Viability assays were selected based on available, optimized procedures for the respective cell lines. Thus, the MTT assay was applied to the mammalian cell systems and the combined alamar Blue/CFDA-AM assay was applied to the fish cell line as described below.

2.4.1. MTT

The MTT (3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyl-tetrazolium bromide, Sigma, St Louis, MO, USA) assay was carried out in accordance with Edmondson et al. (1988). This assay is based on the capacity of mitochondrial succinyl dehydrogenase to convert the soluble yellow tetrazolium salt into an insoluble purple-blue formazan product. After the desired time of contact with *Phormidium* extracts or growth media (4 or 24 h), 20 μl of a 0.5% (w/v) solution of MTT in PBS were added directly to each well and incubated at 37 °C for 4 h in the dark. After incubation, the medium with the dye was aspirated and plates inverted to drain unreduced MTT, and 0.1 mL of 0.04 mol/l HCL in isopropanol were added to each well in order to facilitate solubilization of the formazan product. The plates were shaken, and absorbance was read at 570 nm.

2.4.2. Alamar Blue™ and CFDA-AM

Two fluorescent indicator dyes were used in combination as previously described using L-15/ex as a simplified culture medium (Schirmer et al., 1997). The two dyes were alamar Blue™ (BioSource, Solingen, Germany) which (similar to MTT) is a measure of the redox potential of a cell, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, Molecular Probes, Eugene, OR, USA), which measures cell membrane integrity. After exposure of the cells to *Phormidium* extracts or growth media, wells were emptied and filled with 100 μL of a mixuture of 5%, v/v alamar Blue™ and 4 μM CFDA-AM in L-15/ex and incubated in the dark for 30 min prior to fluorescence measurement. Fluorescence was analyzed using a SPECTRAmax Gemini spectrofluorometer (Molecular Devices, Munich, Germany) at optimized excitation/emission wavelengths for, respectively, alamar Blue™ and CFDA-AM of 530/595 and 493/541 nm.

2.5. Proliferation assay

Cells were plated and exposed to a final equivalent concentration of *Phormidium* extracts of 15 mg/mL as described above. During the last 18 h of exposure, cells were pulsed with 1 μCi [^{3}H]-thymidine per well (Amersham Labs, Buckinghamshire, England). After the complete 24 h of exposure, cultures were harvested in a Filtermate™ cell harvester (Packard Instrument, Meriden, CT, USA). Incorporation of [^{3}H]-thymidine was measured in a matrix 96 Direct Beta counter (Packard). The mean cpm values of triplicates were determined.

2.6. HPLC analysis

2.6.1. Chemicals and standards

HPLC Super gradient acetonitrile was purchased from Lab-Scan Analytical Sciences (Dublin, Ireland) and ammonium acetate from Scharlau Chemie S.A. (Barcelona, Spain). Water used for HPLC was purified with a Milli-Q plus PF system (Millipore, Molsheim, France). Anatoxin-a (AnTx-a) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), microcystin-LR (MC-LR) was from BIOMOL GmbH (Hamburg, Germany) and saxitoxin (STX) was from R-Biopharm GmbH (Darmstadt, Germany).

2.6.2. HPLC conditions

Chromatography was performed with an ÄKTA™ explorer 100 Air system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using the UNICORN V4.00 software. The analytical column was a Discovery® C_{18} (5×4 mm I.D., 5 μm) from Supelco (Bellefonte, PA, USA). The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate, pH 5.5) and solvent B (10 mM ammonium acetate–acetonitrile, 80:20, v/v) as follows: 0% of B at 0 min, 100% of B at 45–65 min using a linear gradient. Flow-rate was 0.8 mL/min and UV detection was performed at 238 nm. All runs were carried out at room temperature. The column was reequilibrated with 8 mL of solvent A between runs. Each standard was run separately (AnTx-a 5 μg/mL, MC-LR 5 μg/mL, STX 40.5 pg/mL, 200 μL injection volume) and thereafter a mixture of all standards with the same concentrations in 200 μL was run again. Two hundred microliters of each sample were injected for HPLC analysis. The patterns of substances in the samples were compared with the standards based on retention times.

2.7. ELISA

2.7.1. Saxitoxins

The samples were analyzed by the Ridascreen™ saxitoxin ELISA kit (R-Biopharm, Darmstadt, Germany). This is a competitive ELISA for the quantitative analysis of saxitoxin and related toxins based on the competition between the free toxins from samples or standards and an enzyme-conjugated saxitoxin for the same antibody. The mean lower detection limit of the Ridascreen™ saxitoxin assay is about 0.010 ppb.

2.7.2. Microcystins

Analysis of samples was performed using the Microcystin Plate kit (EnviroLogix, Inc., Portland, USA). As for the saxitoxin ELISA, this is a quantitative, competitive immunosorbent assay. The limit of detection of the EnviroLogix Microcystin Plate kit is 0.05 ppb.

2.8. Statistics

The results are reported as mean ± SD from individual determinations with at least three replicates. Statistical differences were analysed by Mann–Whitney *U* test using the StatView (SAS Institute, Inc.) programme. Values of $P < 0.05$ were regarded as significant.

3. Results

3.1. Toxicity of Phormidium extracts in vivo

Results from the in vivo mouse bioassay are provided in Table 1. Symptoms, typical for neurotoxin intoxications (reduced activity, convulsions, spasms and respiratory difficulties) were observed during the first 2 h after intraperitoneal injection of *Phormidium* extracts into DBA/1 mice. These symptoms were strongest in the group injected with extracts of *Ph. molle* and *Ph. uncinatum*. Lethality was observed only in the group injected with the *Ph. bijugatum* extract. Two of the three mice treated with this extract died 18 h after injection.

Mice treated with *Phormidium* extracts showed an average weight loss of between 0.6 ± 0.2 (*Ph. uncinatum*) and 3.35 ± 0.55 g (*Ph. papyraceum*) (Table 1), whereas control mice gained an average of 0.18 ± 0.16 g.

When the livers were removed for histopathological analysis it was found that those derived from mice treated with *Ph. molle* and *Ph. papyraceum* extracts were larger compared to the control. Histological examination of liver slices of treated mice revealed signs of hepatotoxic effects similar of those described in our previous study for *Lyngbya aerugineo-coerulea* extract (Teneva et al., 2003). Granulovacuolar degeneration with perinuclear clumping of cytoplasm and an increased number of cells undergoing mitosis were observed with highest frequency in liver specimens of the mice treated with extracts of *Ph. bijugatum* and *Ph. uncinatum*, while minor inflammations and sinosoid congestions were obtained mainly in the groups treated with extracts of *Ph. molle* and *Ph. papyraceum* (Fig. 1).

Table 1
Toxicity of *Phormidium* extracts determined in DBA/1 mice after i.p. injection of an equivalent of 682–790 mg algal matter/kg mouse

Phormidium extracts	Weight loss (g) 24 h after injection ($n=3$)	Symptoms of neurotoxity?	Symptoms of hepatotoxicity?	Death of mice?
Ph. bijugatum	2.85 ± 0.25	Yes	Yes	Yes (2 of 3)
Ph. molle	1.65 ± 0.35	Yes[a]	Yes[b]	No
Ph. papyraceum	3.35 ± 0.55	Yes	Yes[b]	No
Ph. uncinatum	0.6 ± 0.2	Yes[a]	Yes	No
Ph. autumnale	1.65 ± 0.25	Yes	Yes	No
Control	–[c]	No	No	No

[a] Strongest signs of neurotoxicity.
[b] Enlarged livers.
[c] Control mice gained an average weigth of 0.18 ± 0.16 g ($n=3$).

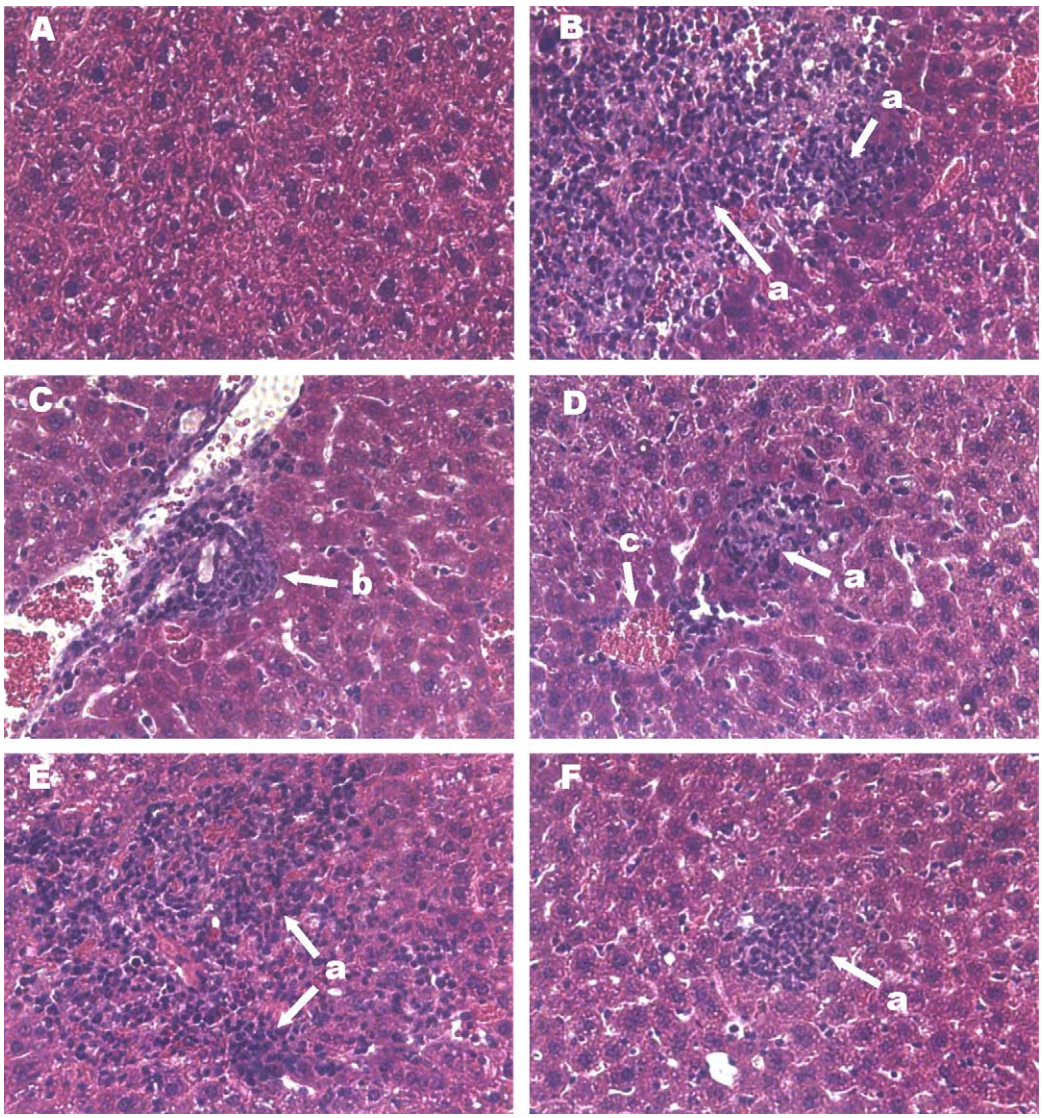

Fig. 1. Light microscopic histology of mice liver 24 h after i.p. injection into DBA/1 mice of 0.5 mL *Phormidium* extracts at an equivalent concentration of 15 mg suspended algal matter (682–790 mg algal matter/kg mouse). Liver tissue samples were prepared as described in Section 2. Shown are representative liver sections of mice treated with: (A) PBS (control mice), and extracts of (B) *Ph. bijugatum*, (C) *Ph. molle*, (D) *Ph. papyraceum*, (E) *Ph. uncinatum* and (F) *Ph. autumnale*. Alterations to normal histology are represented as a, granulovacuolar degeneration and mitosis; b, inflammation; and c, sinosoid congestion. The original magnification was 500×.

3.2. Toxicity of Phormidium extracts in vitro

3.2.1. Toxicity to mammalian cells

Distinct responses depending on the cell line and the exposure period were detected after exposure to *Phormidium* extracts. Both a stimulatory and a cytotoxic effect were observed after 4 h of exposure, whereas after 24 h all cells revealed different degrees of cytotoxicity (Fig. 2). Differentiation in responses was greatest after treatment of the cell lines with *Ph. autumnale* extract. A stimulatory effect on the human cell lines (FL, A2058 and RD) was indicated after 4 h of exposure with this extract compared to the controls (Fig. 2A, B and C), whereas the same concentration and time of exposure caused a weak cytotoxic effect in mouse fibroblasts (3T3) (Fig. 2D). These differences may be due to the distinct origin of the cells and indicate different degrees or mechanisms of the cellular stress elicited by the *Ph. autumnale* extract. After 24 h, however, exposure to all extracts invariably led to significant cytotoxicity of about 46–82%. All cell lines responded similarly to this cytotoxic insult with the exception of 3T3 cells which, in all cases, were least sensitive. Cytotoxicity after 24 h of exposure was

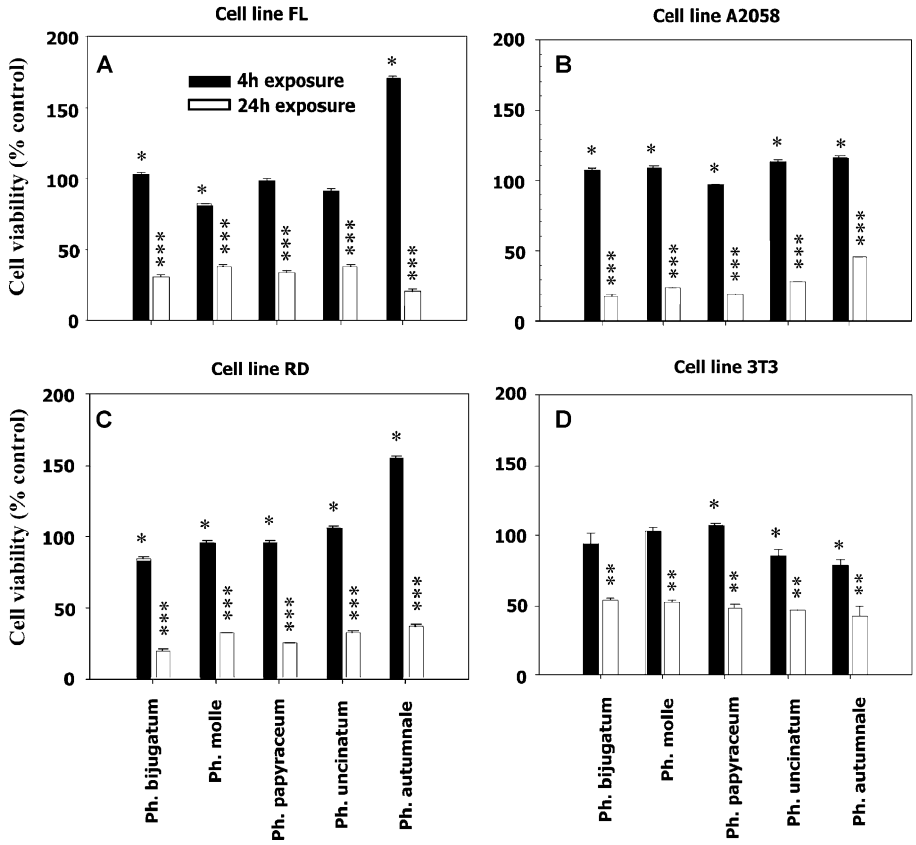

Fig. 2. Viability of mammalian cell lines treated with *Phormidium* extracts for 4 h (black bars) or 24 h (white bars) as determined with the MTT assay. Cells were exposed to an equivalent concentration of 15 mg/mL (w/v) suspended algal matter (10% of the *Phormidium* extracts). After exposure, MTT was applied as described in Section 2 and the absorbance of the formazan product assessed at 570 nM. Absorbance readings were expressed as % of the readings in the cultures receiving Millipore water as the control. One representative experiment is shown with each bar representing the average of three culture wells and vertical lines indicating the standard deviation. Asterisks indicate significant differences in cell viability compared to the control according to the Mann–Whitney *U* test (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

accompanied by the rounding up of many cells and detachment from the plates.

The influence of *Phormidium* extracts on cell proliferation was tested on a cell line, 3T3, and freshly established cell cultures of mouse fibroblasts. All *Phormidium* extracts significantly inhibited incorporation of [^{3}H]-thymidine in both 3T3 cells (Fig. 3A) and freshly established fibroblasts (Fig. 3B) to more than 85%, thus confirming the impact on cellular integrity observed by the MTT test.

3.2.2. Toxicity to the fish cell line, RTL-W1

To determine the effect of the *Phormidium* extracts on fish cells in vitro, RTL-W1 cells were treated with varying extract concentrations and incubated at 4, 19 and 25 °C for 24 h. This was done in the absence of serum as we have previously shown that RTL-W1 cells are more sensitive to toxic compounds derived from Cyanoprokaryota if serum is absent (Teneva et al., 2003). Despite these cytotoxicity-favouring exposure conditions, little to no impact on cell viability was observed for the fish cell line (Fig. 4). The most noticeable change in cell viability was a general stimulatory response of the cells upon exposure at 4 °C. Overall, the fish cells were much less sensitive to the exposure with *Phormidium* extracts compared to the mammalian cells.

3.3. Toxicity of the Phormidium growth media

In order to examine whether the *Phormidium* species release toxins or other biologically active compounds into their surrounding, the media in which the various species were grown for 14 days were tested as well.

3.3.1. Toxicity to mammalian cells

Mammalian cells were treated with 10% growth medium for 4 and 24 h. Data shown in Fig. 5 indicate that after 4 h of exposure to *Phormidium* growth media, cell viability was generally not significantly altered. Stimulatory effects were occasionally observed after the 4 h exposure. The most significant stimulatory effect was seen in A2058 cells upon

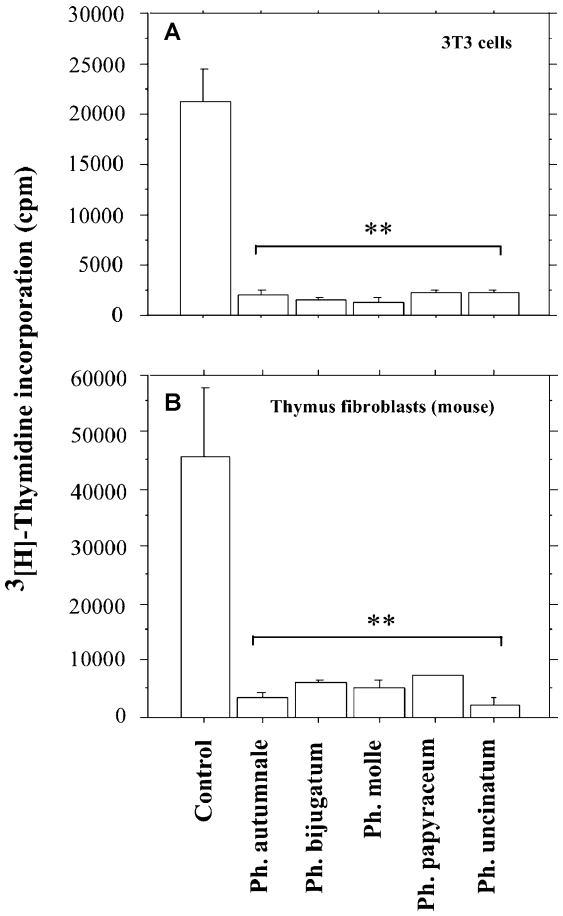

Fig. 3. [^{3}H]-thymidine incorporation into 3T3 cells (A) and mouse thymus fibroblasts (B) after 24 h treatment with *Phormidium* extracts. Cells were exposed to an equivalent concentration of 15 mg/mL (w/v) suspended algal matter (10% of the *Phormidium* extracts). During the last 18 h of exposure, cells were pulsed with 1 μCi [^{3}H]-thymidine per well and 6 h later processed as described in Section 2. One representative experiment is shown with each bar representing the average of three culture wells and vertical lines indicating the standard deviation. Asterisks denote significant differences in [^{3}H]-thymidine incorporation compared to the control according to the Mann–Whitney *U* test ($*P < 0.005$).

exposure to *Ph. autmnale* growth medium. After 24 h of exposure, impact on cell viability by the growth media was much more pronounced (Fig. 5). For example, the growth medium from *Ph. uncinatum* was cytotoxic to all cell lines although cell viability was never impaired by more than 50%. Taken together, cytotoxic effects of the *Phormidium* growth media to the mammalian cells were weaker in comparison to the *Phormidium* extracts.

3.3.2. Toxicity to the fish cell line, RTL-W1

RTL-W1 fish cells were exposed to 10, 30 and 50% *Phormidium* growth medium for 24 h at 4, 19 and 25 °C. In contrast to the *Phormidium* extracts, the viability of the cells was significantly affected after treatment with *Phormidium* media. For all *Phormidium* samples, the cytotoxic effects were temperature-dependent (Fig. 6). At the lower temperature (4 °C), a stimulatory effect was again observed and was highest for cells exposed to medium of *Ph. autumnale*. In contrast, cell viability dropped to far below 50% when cells were exposed at 25 °C to *Ph. papyraceum*, *Ph. uncinatum* and *Ph. autumnale* growth media (Fig. 6). These effects by the growth media were also dose-dependent. The cytotoxicity by the growth media was accompanied by irreversible morphological changes never observed upon exposure of cells to *Phormidium* extracts. Changes comprised alterations to the cytoskeleton, which were visible as the formation of long, thin extensions originating from the normally epithelial-like cells. These alterations were similar to the changes observed for RTL-W1 cells upon exposure to *Lyngbya aerugineo-coerulea* growth medium (Teneva et al., 2003).

3.4. HPLC analysis

Using a Discovery® C_{18} column and a linear gradient, the mobile phase was optimized to allow the simultaneous detection of cyanotoxins from different groups. The eluents were 10 mM ammonium acetate, pH 5.5 (A) and 10 mM ammonium acetate–acetonitrile, 80:20, v/v (B). A comparison between four different combinations of gradient mobile phases and five different reversed-phase columns (including Discovery® C_{18}) for HPLC determination of eight microcystins has been published by Spoof et al. (2001). With regard to the Discovery® C_{18} (4×3 mm I.D., 4.11 μm) column, these authors report asymmetry of 1.01 and a retention time of 18.72 min (for MC-LR) using mobile phases: A—10.5 mM ammonium acetate–acetonitrile (95:5), B—50 mM ammonium acetate–acetonitrile (20:80). Only MC-LR with retention time 7.09 min was detected when we used similar mobile phases (A—10 mM ammonium acetate–acetonitrile, 80:20; B—10 mM ammonium acetate–acetonitrile, 20:80) and a linear gradient (data not shown). We were not able to detect the other standards under these conditions. After modification of the mobile phases (see above), all standards were detected (Fig. 7A).

HPLC was applied in order to compare chromatograms between sample types and species of Cyanoprokaryota. As well, peak retention times were compared to those found in the mixture of standards. Apart from the HPLC chromatograms of the standard mixture of AnTx-a, STX and MC-LR, Fig. 7 shows two examples with different HPLC profiles: *Ph. bijugatum* (Fig. 7B and C) and *Ph. uncinatum* (Fig. 7D and E). The most remarkable differences in the chromatograms were the peaks between 4 and 7 min retention time, which occurred in the extracts (Fig. 7C and E) but not the media (Fig. 7B and D). For *Ph. bijugatum*, two peaks between 6 and 7 min retention time were observed and were also found for *Ph. molle* (data not shown). These peaks are close in retention time to the neurotoxin AnTx-a. For the extract of *Ph.*

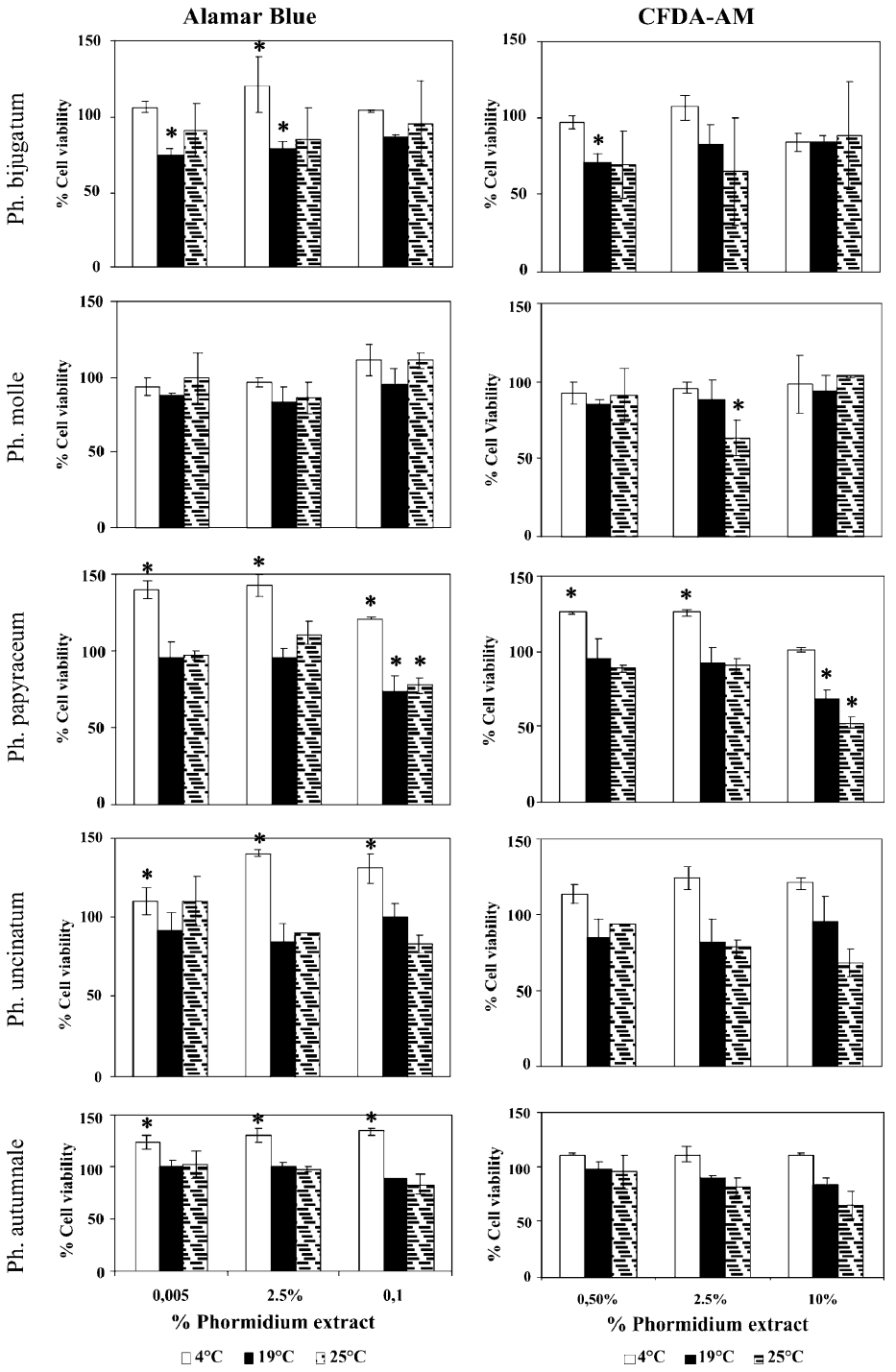

Fig. 4. Viability of RTL-W1 fish cells treated with *Phormidium* extracts for 24 h at 4 °C (white bars), 19 °C (black bars) and 25 °C (striped bars). Viability was assessed using a mixture of the alamar Blue (left panel) and CFDA-AM (right panel) fluorescent indicator dyes. Cells were exposed to equivalent concentration of suspended algal matter of 0.75 mg/mL (0.5% of extracts), 3.75 mg/mL (2.5% of extracts) and 15 mg/mL (10% of extracts). After exposure, alamar Blue and CFDA-AM were applied as described in Section 2 and fluorescence assessed at respective excitation/emission wavelengths of 530/595 and 493/541 nm. Fluorescence unit readings were expressed as % of the readings in the cultures receiving Millipore water as the control. One representative experiment is shown with each bar representing the average of three culture wells and vertical lines indicating the standard deviation. Asterisks indicate significant differences in cell viability compared to the control according to the Mann–Whitney *U* test (*$P < 0.05$).

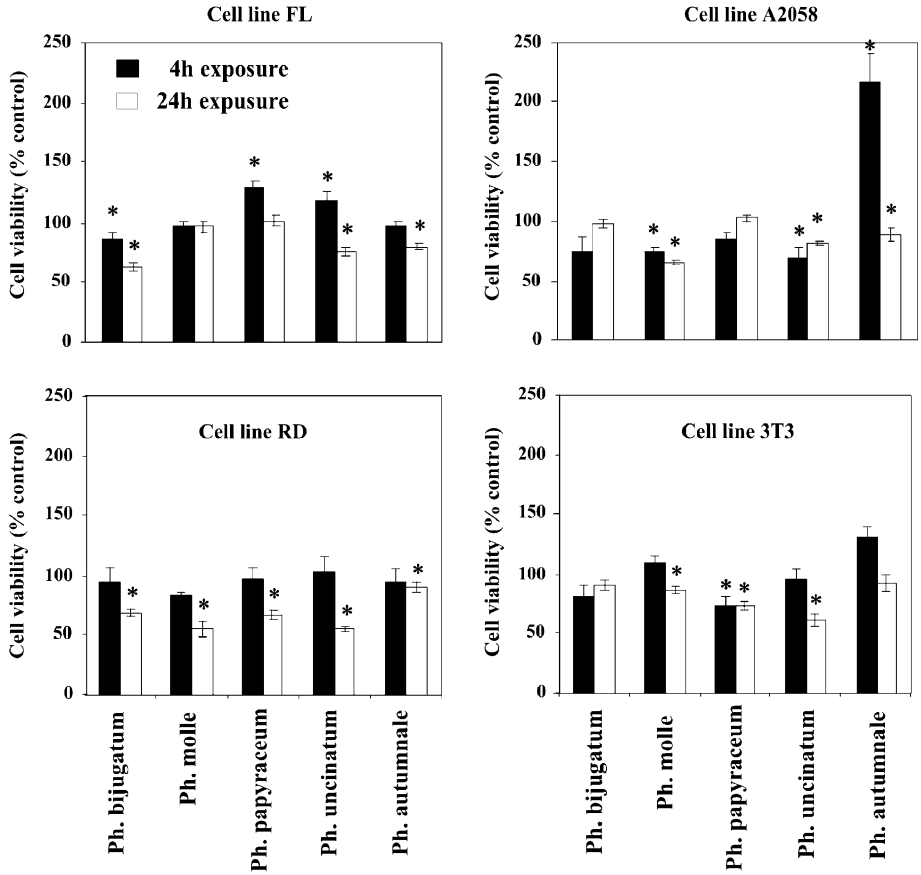

Fig. 5. Viability of mammalian cell lines treated with *Phormidium* growth media for 4 h (black bars) or 24 h (white bars) as determined with the MTT assay. Cells were exposed by diluting the cell culture medium with 10% of the *Phormidium* growth medium. An equivalent % of Z-medium (the medium in which the *Phormidium* species had been grown) was added to the control cultures. After exposure, MTT was applied as described in Section 2 and the absorbance of the formazan product assessed at 570 nM. Absorbance readings were expressed as % of the readings in the control cultures. One representative experiment is shown with each bar representing the average of three culture wells and vertical lines indicating the standard deviation. Asterisks indicate significant differences in cell viability compared to the control according to the Mann–Whitney *U* test ($*P < 0.05$).

uncinatum, as well as for *Ph. papyraceum* and *Ph. autumnale*, a peak at about 4.5 min was found. Microcystin-LR was eluted in the mixture of standards at 47.8 min, which was matched most closely by the peaks at, respectively, 47.3 and 47.2 min in the *Ph. uncinatum* medium and extract (Fig. 7D and E). Peaks with retention times between 37 and 47 min were also detected in the extracts and potentially represent microcystin and/or nodularin analogs. Within these limits, a peak at around 37.2–37.4 min retention time was most strikingly observed and would be one candidate for fractionation and detailed identification steps. All in all, peak patterns differed in the media compared to the extracts and peaks in the extracts were generally smaller.

3.5. ELISA analysis

To confirm the presence of cyanotoxins, the *Phormidium* extracts and media were also investigated by commercially available ELISAs for saxitoxins and microcystins. Neither group of toxins was detectable in the media. According to the microcystin ELISA, which cross-reacts with microcystin LR, LA, RR, YR and nodularin, all extracts contained minor levels of this group of toxins with concentrations ranging from 0.024 (*Ph. molle*) to 0.062 ppb (*Ph. autumnale*). As well in the saxitoxin ELISA, which has 10–30% cross reactivity to decarbamoyl saxitoxin, gonyautotoxins II, III, B1, C1 and C2, all *Phormidium* extracts responded close to the detection limit of the ELISA with concentrations ranging between 0.017 (*Ph. bijugatum*) and 0.033 ppb (*Ph. uncinatum*).

4. Discussion

Among the Cyanoprokaryota, the genus *Phormidium* has thus far rarely been studied with respect to toxin production

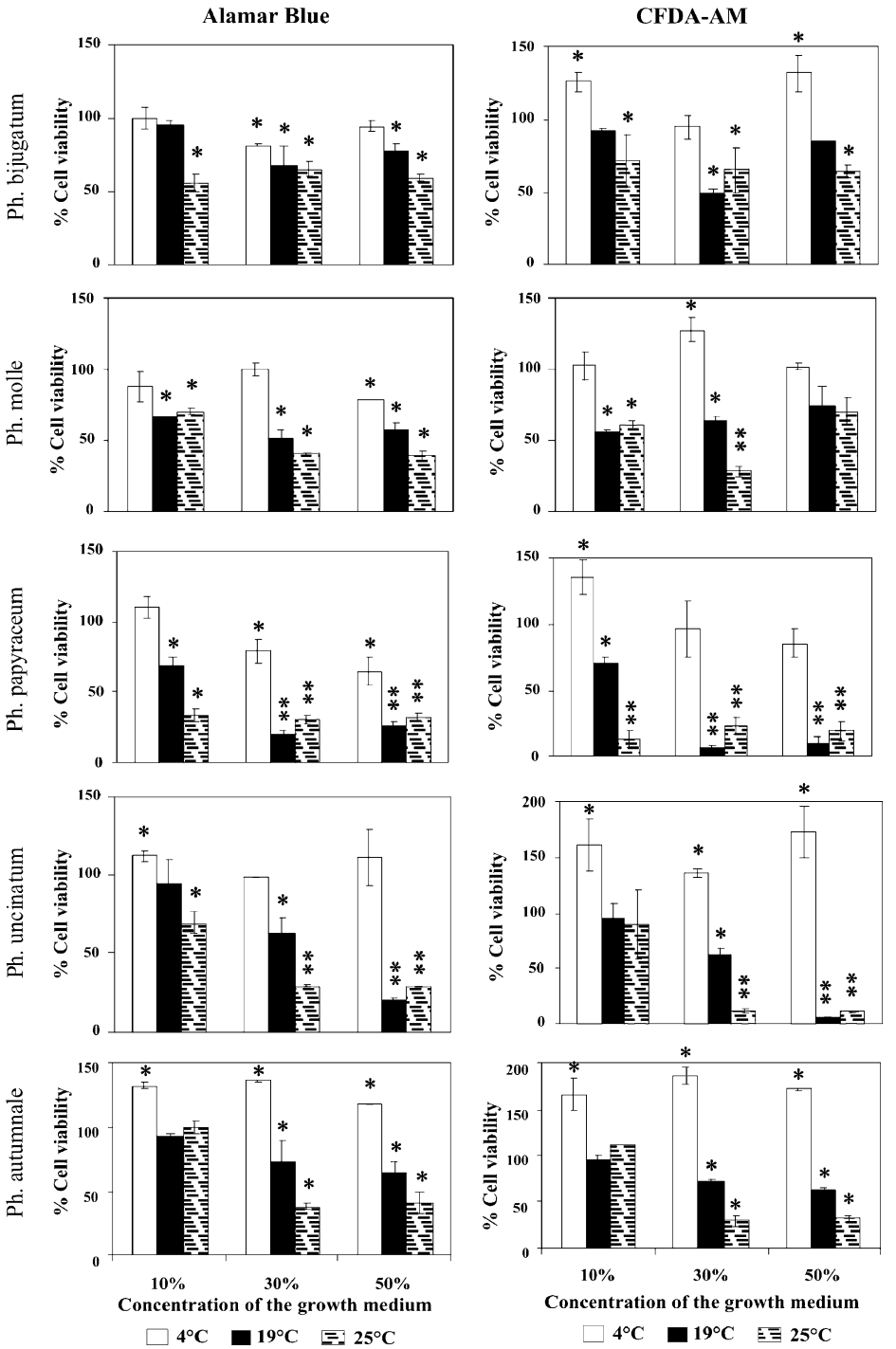

Fig. 6. Viability of RTL-W1 fish cells treated with *Phormidium* growth media for 24 h at 4 °C (white bars), 19 °C (black bars) and 25 °C (striped bars). Viability was assessed using a mixture of the alamar Blue (left panel) and CFDA-AM (right panel) fluorescent indicator dyes. Cells were exposed by diluting the culture medium with 10, 30 and 50% of the *Phormidium* growth medium. An equivalent % of Z-medium (the medium in which the *Phormidium* species had been grown) was added to the control cultures. After exposure, alamar Blue and CFDA-AM were applied as described in Section 2 and fluorescence assessed at respective excitation/emission wavelengths of 530/595 and 493/541 nm. Fluorescence unit readings were expressed as % of the readings in the control cultures. One representative experiment is shown with each bar representing the average of three culture wells and vertical lines indicating the standard deviation. Asterisks indicate significant differences in cell viability compared to the control according to the Mann–Whitney *U* test (*$P<0.05$; **$P<0.01$).

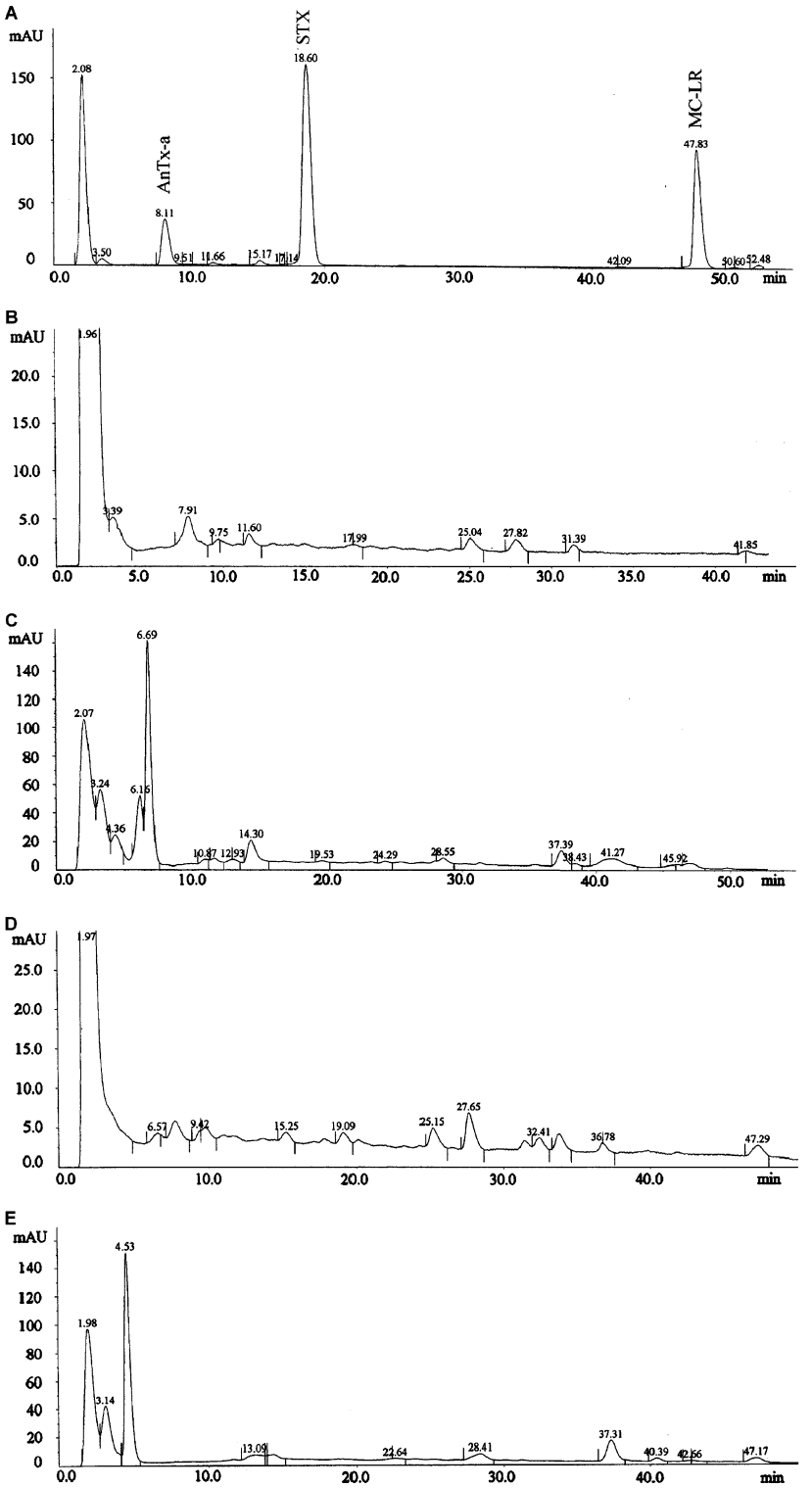

Fig. 7. HPLC chromatograms of selected extracts of *Phormidium* species and of corresponding media used to culture the Cyanoprokaryota. Shown are the chromatographic profiles of: (A) a mixture of standard toxins; (B) the growth medium of *Ph. bijugatum*; (C) the extract obtained from *Ph. bijugatum*; (D) the growth medium of *Ph. uncinatum* and (E) the extract obtained from *Ph. uncinatum*. A volume of 200 μL of each sample was used for HPLC analysis under the conditions described under Section 2.

and potentially resulting human health and ecological risks. By means of different assays, focusing on biological responses as well as on immunoassay-based or physico-chemical evidence for the presence of toxins, we here show that all of the five *Phormidium* species investigated produce bioactive compounds. These compounds, whose identity could only partly be assigned, occurred both intra- as well as extracellularily. Responses varied for the different extracts and bioassays but fish cells generally responded less sensitively to the impact of the cyanotoxins than the mammalian model systems.

The clinical symptoms in mice obtained after treatment with *Phormidium* extracts (convulsions, reduced activity, spasms and respiratory difficulties) strongly suggested the presence of neurotoxins. According to the ELISA, saxitoxins or neurotoxins cross-reacting with the ELISA were found in the lower ppb range in all extracts. Although the saxitoxin concentrations did not vary by more than a factor of two between the five *Phormidium* extracts, the highest measured saxitoxin concentration was found in extracts of *Ph. uncinatum*, which also yielded among the strongest signs of neurotoxicity within the first 2 h after injection. Lethality was observed within 18 h after treatment only with the *Ph. bijugatum* extract. This extract also yielded significant peaks in HPLC analysis that were close in retention time to anatoxin-a. Similar HPLC peaks were observed for the extract of *Ph. molle*, which elicited strong signs of neurotoxicity but no death. This comparison supports the notion that different profiles of toxic compounds must be produced by the different species upon growth under identical culture conditions. As well, the death of mice due to the extract of *Ph. bijugatum* cannot be assigned to one single toxin but appears to be caused by several toxins acting together.

Aside from signs of neurotoxicity, the observed histopathological changes in the liver of treated mice were indicative of hepatotoxic compounds. Signs of hepatotoxicity included granulovacuolar degeneration of cytoplasm and increased mitosis, observations similar to those reported after i.p. injection of the *Lyngbya aerugineo-coerulea* extract into mice (Teneva et al., 2003). In the latter case, microcystins were not observed using the microcystin ELISA, and rather low concentrations were measured in the ELISA in the current study. It has been reported recently that anatoxin-a induces apoptosis in rat thymocytes and Vero cells which is characterized by plasma membrane blebbing, condensed chromatin, nuclear fragmentation and formation of apoptotic bodies (Lakshmana Rao et al., 2002). Lyngbyatoxin-A, a dermatotoxin, also causes increased mitosis and small granulomas in livers of treated mice (Ito et al., 2002). Thus it is possible that histopathological changes in liver were caused by hepatotoxins as well as neurotoxins. Possible candidates are anatoxin-a, microcystin analoges not detectable in the ELISA but as well, previously unidentified compounds. Along this line, Baker et al. (2001) were unable to attribute the toxicity to mice of the freshwater species *Ph.* aff. *formosum* and *P.* aff. *amoneum* to either microcystins, cylindrospermopsin, saxitoxins, anatoxin-a or homoanatoxins.

Cyanobacterial toxins of different chemical groups were detectable in one analysis run by the HPLC method used therein. A number of HPLC methods are available for analysis of cyanotoxins using different high-resolution reversed-phase columns and UV detection (Harada et al., 1994; Lawton et al., 1994; James and Sherlock, 1996; Meriluoto, 1997; James et al., 1998; Yu et al., 1998; Spoof et al., 2001; Spoof and Meriluoto, 2002; Furey et al., 2002). The method developed here was specifically designed to provide a rapid overview of different groups of toxins potentially present in a sample and to allow for an overall comparison of patterns of peak height and retention times. This HPLC method could also be applied in the future to isolate fractions containing peaks of interest, which would then be subjected to more specific, quantitative analysis methods. For example, the peaks eluting around 4.5 min retention time in extracts of *Ph. uncinatum*, *Ph. papyraceum* and *Ph. autumnale* could be C-toxins, which have previously been described to occur at the beginning of the chromatographic run (Carmichael et al., 1997; Yu et al., 1998). These cross-react with the saxitoxin ELISA and thus cannot be deciphered as individual compounds by the ELISA.

The potency of *Phormidium* extracts to cause toxicity in mice was confirmed in vitro using mice and human cell lines. Cell proliferation, measured as [^{3}H]-thymidine incorporation, was severely inhibited by all *Phormidium* extracts in freshly isolated mouse thymus fibroblasts as well as in the mouse embryo cell line 3T3. Direct DNA damage could be one possible cause. For example, recent studies demonstrated that significant DNA strand breakages can be elicited in mammalian cells by cylindrospermopsin (Shen et al., 2002) and MC-LR (Žegura et al., 2003). In terms of cytotoxicity, the human cells appeared more sensitive than the mouse 3T3 cell line. This is in agreement with earlier investigations with an extract of another Cyanoprokaryota, *Lyngbya aerugineo-coerulea*, which was least toxic in 3T3 cells (Teneva et al., 2003). All mammalian cell lines were cultured identically, thus excluding different exposure conditions, such as varying serum concentrations, as a possible cause for different sensitivities. Thus, for studying toxins of Cyanoprokaryota to humans in vitro, human cells may indeed be a more appropriate choice.

The importance of considering species sensitivity was further reflected by the investigations into the cytotoxicity elicited by the *Phormidium* extracts in fish liver cells. Among all the cell cultures tested, the fish cell line was the least sensitive, and this was shown to be at least in part due to temperature. Inasmuch as fish cell exposures are done at temperatures close to those normally present for the fish in the wild, the current in vitro study indicates that fish may be less sensitive to toxins of *Phormidium* than humans. In fact, fish cells in vitro could be used in the future to study

the temperature-dependence of toxins of Cyanoprokaryota in general. Inasmuch as fish cells of one species can usually be grown within a wide range of temperatures (e.g. from 4 to 25 °C for *Salmonids*; Bols et al., 1992) and cells from different fish species cover an even wider temperature range, they could supplement studies on the relationship between the temperature for optimal toxin production by the Cyanoprokaryota and toxicity to vertebrates. For example, the greater number of calamities caused by Cyanoprokaryota in the tropical regions and regions with higher temperatures during the summer could be both due to higher toxin production and due to the temperature dependence of the toxic effects. On the other hand, Hitzfeld et al. (2000) reported production of cyanotoxins from Antarctic Cyanoprokaryota with cytotoxic effects on primary rainbow trout hepatocytes. Many of the toxin-producing prokaryota were identified as *Phormidium* species.

The sensitivity of the fish cells to the medium in which the *Phormidium* species had been grown for 14 days was much greater and more temperature-dependent than the sensitivity to the *Phormidium* extracts. As well, exposure to the medium led to morphological changes indicative of alterations to the cytoskeleton that were not observed upon exposure to the extracts. This is in line with our previous study on *Lyngbya aerugineo-coerulea*, which also revealed a different pattern of toxicity due to an intracellular extract vs. the growth medium (Teneva et al., 2003). Differences in the compounds present in the extracts and the medium were also supported by the HPLC profiles. For example, the medium of *Ph. uncinatum*, which caused the greatest response in the fish liver cell line, contained a relatively large peak at 27.65 min retention time. In the future, chemical fractionation combined with biological assays could be advanced to identify the substances that are most biologically active.

A bioassay-directed toxicity identification approach could also be applied to *Phormidium* growth media in order to identify compounds relevant to human health. For example, *Ph. molle* growth medium was toxic to the two cancer human cell lines (A2058 and RD cells), but not to the normal human FL cells. This finding can be interpreted as an indication that *Ph. molle* is a source of compounds with selective anti-tumour activity. Tokuda et al. (1996) reported inhibition of the papilloma formation in mouse skin after treatment with digalactosyl diacylglycerols isolated from *Ph. tenue*. Therefore, the *Phormidium* species could be useful sources of different bioactive compounds in general.

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