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# Salt tolerance of the harmful cyanobacterium *Microcystis aeruginosa*

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ABSTRACT: Increasing salinities in freshwater ecosystems caused by agricultural practices, droughts, or rise in sea level are likely to affect the species composition of phototrophic microorganisms. Cosmopolitan freshwater cyanobacteria of the *Microcystis* genus can produce the toxin microcystin, and present a potential health risk in many eutrophic lakes. In this study, *M. aeruginosa* Strain PCC 7806 was grown in semi-continuous turbidostats to investigate the effect of increasing salinity on growth rate, microcystin cell quota, microcystin production and extracellular microcystin concentration. Specific growth rate, microcystin cell quota and microcystin production remained more or less unaffected by salinity levels up to 10 g $l^{-1}$. Specific growth rate collapsed when salinity was increased beyond 10 g $l^{-1}$ for several weeks. Cell size and microcystin cell quota decreased while extracellular microcystin concentrations increased at salinities above 10 g $l^{-1}$, indicating leakage and/or cell lysis. Salt-shock experiments revealed that *M. aeruginosa* can temporarily endure salinities as high as 17.5 g $l^{-1}$. These results indicate that, for a freshwater species, *M. aeruginosa* has a high salt tolerance. Rising salinities in freshwater ecosystems are therefore unlikely to suppress *M. aeruginosa* blooms, and may in fact enhance the exposure of aquatic organisms to elevated concentrations of extracellular microcystins.

KEY WORDS: Harmful algal blooms · Harmful cyanobacteria · *Microcystis* · Microcystins · Salinity · HPLC



## INTRODUCTION

Freshwater ecosystems are confronted with increasing salinity on a world-wide scale, due to a variety of different processes, including long-term droughts, rising seawater levels, agricultural practices, or specific water management strategies (Williams 2001, Nielsen et al. 2003). Rising salt concentrations are known to affect numerous freshwater biota (Hart et al. 1991, 2003, James et al. 2003), and may shift the species composition of phytoplankton communities (Ahmed et al. 1985, Wilson et al. 1994, Mohapatra et al. 1998, Muylaert et al. 2000, Moisander et al. 2002, Bordalo & Vieira 2005).

Cyanobacteria are found across a wide range of different salinities, including hypersaline waters (Das-Sarma & Arora 2001, available at http://els.wiley.com). Most freshwater cyanobacteria, however, do not survive in brackish or seawater. *Microcystis aeruginosa* is a harmful cyanobacterium that occurs worldwide in freshwater lakes. The toxins produced by *M. aeruginosa* are commonly known as microcystins, and belong to an extensive group of cyclic heptapeptides comprising at least 71 described variants (Codd et al. 2005). Microcystins can cause liver damage through inhibition of protein phosphatases (MacKintosh et al. 1990). Usually, microcystins are concentrated inside *Microcystis* spp. cells, whereas extracellular microcystin concentrations remain low. The occurrence of *Microcystis* spp. is related to environmental factors such as excess nutrient availability, high temperature and a stable water column with little vertical mixing (Reynolds & Walsby 1975, Huisman et al. 2004, Visser et al. 2005). During calm summer weather, *Microcystis* spp. float towards the surface and form dense scums, thereby causing serious health risks (Chorus

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& Bartram 1999, Carmichael et al. 2001, Huisman et al. 2005).

In The Netherlands, characterized by large stretches of land reclaimed from sea (called 'polders'), *Microcystis* spp. blooms are a widespread nuisance. Because of increasing demands for freshwater irrigation and slowly intruding seawater, many of the eutrophic lakes in these polder areas face elevated salinities during summer. Furthermore, the possibility is being considered to convert freshwater lakes into brackish waters to reduce *Microcystis* spp. growth and to restore former estuarine ecosystems (Verspagen et al. 2006). As a result, the salinity in many *Microcystis* spp.-dominated lakes in The Netherlands is slowly increasing. However, little is known about the effect of elevated salinity on the growth rate and microcystin production of this genus.

Preliminary studies in batch cultures suggest that the growth rate of *Microcystis aeruginosa* might be stimulated by rising salinities, at least up to a salinity of ~2 g $l^{-1}$ (recalculated data of Prinsloo & Pieterse 1994). Furthermore, *M. aeruginosa* seems to have a relatively high salt tolerance, estimated at 7 g $l^{-1}$ by Otsuka et al. (1999), 10 g $l^{-1}$ by Orr et al. (2004) and 14 g $l^{-1}$ by Verspagen et al. (2006). If these observations are correct, *M. aeruginosa* blooms might actually be favored in slightly brackish waters.

In this study, the strain *Microcystis aeruginosa* PCC 7806 was grown in controlled semi-continuous turbidostats to investigate in detail how elevated salt concentrations affect its growth rate and intracellular and extracellular microcystin content. We used 2 approaches: in one set of experiments, cells were exposed to slowly rising levels of salinity, providing sufficient time for cells to adjust their physiology to the prevailing osmotic conditions; in the other set of experiments, *M. aeruginosa* cells were exposed to a sudden salt shock to examine whether cells would respond differently when confronted with a rapid rise in salinity.

## MATERIALS AND METHODS

**Organism and culture conditions.** *Microcystis aeruginosa* Strain PCC 7806 was provided by the Pasteur Institute, Paris. The strain was originally isolated from the Braakman Reservoir, The Netherlands, in 1972. We cultured this strain as single cells in semi-continuous cultures, using a turbidostat approach (Huisman et al. 2002). The optical density (750 nm) of the turbidostats was kept constant between 0.1 and 0.2 $cm^{-1}$ by diluting the culture with nutrient-rich mineral medium once every 2 d. Flat culture vessels with a working volume of 400 ml were used in combination with a constant aeration of filtered and moistened air to ensure homogeneous mixing and to provide sufficient amounts of inorganic carbon. A cooling vessel of the same dimensions as the culture vessel was placed between the culture vessel and the light source to maintain a constant temperature of 23 ± 1°C. Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P), directed towards the front surface of the culture vessel. Incident irradiance ($I_{in}$) and outgoing irradiance ($I_{out}$) were measured with a LI-COR LI-250 quantum photometer at 7 points on the front surface and 7 points on the back surface of the culture vessel, respectively. The average photon irradiance inside the culture vessel was calculated as $I_{avg} = (I_{in} - I_{out})/(\ln I_{in} - \ln I_{out})$ (Huisman et al. 2002). Cultures were run in triplicate on a 12:12 h light:dark cycle, with a depth-averaged photon irradiance of $I_{avg}$ = 110 ± 20 µmol photons $m^{-2}$ $s^{-1}$ during the light period.

**Experimental outline.** Our mineral medium was based on the O2 medium, which contains the macronutrients $MgSO_4 \cdot 7H_2O$ (50 mg $l^{-1}$), $NaNO_3$ (500 mg $l^{-1}$), $K_2HPO_4$ (25 mg $l^{-1}$), $CaCl_2 \cdot 2H_2O$ (13 mg $l^{-1}$), $NaHCO_3$ (20 mg $l^{-1}$) as well as a $FeCl_2 \cdot 4H_2O$ (2.2 mg $l^{-1}$) and a variety of trace metals (van Liere & Mur 1978). O2 medium has a total salinity of 0.6 g $l^{-1}$. In order to detect the effect of slowly rising salinity, NaCl was added to this mineral medium to increase the salinity in a stepwise fashion to the following levels: from 0.6 (O2 medium) to 0.7, 0.85, 1.1, 1.35, 1.6, 2.5, 3.6, 4.6, 5, 7.5, 10, 12.5, 15 and 17.5 g $l^{-1}$ (seawater has a salinity of ~35 g $l^{-1}$). Cultures were grown in triplicate at a constant salinity level for a period of 2 wk, 1 wk for acclimation and 1 wk for sampling. After each 2 wk period the salinity of the cultures was increased to the next level by adding the required amount of salt during a dilution event. Additionally, 3 salt-shock experiments were performed in which *Microcystis aeruginosa* cells, pregrown in O2 medium, were directly exposed to salinities of 10, 15 and 17.5 g $l^{-1}$, without the opportunity of gradually adjusting to intermediate salinities. The salt-shock experiments were again carried out in semi-continuous turbidostats, and the required amount of salt was added during a dilution event. The salt-shock experiments were run in triplicate using the same conditions as described above.

**Sampling and microcystin analysis.** The experiments with gradually increasing salinities were sampled 4 times during each 2 wk period, on Days 8, 10, 12 and 14 after the imposed salinity increase. The salt-shock experiments were monitored for a period of 10 d and sampled every other day. Samples were all taken on the same time of day to reduce possible variation in the data due to the light–dark cycle. Aliquots of all samples were analyzed in triplicate for cell concentration and intra- and extracellular microcystin concentration.

Intracellular microcystin concentrations were expressed per cell (cell quota). Cell concentrations and cell size were measured with a cell counter (Casy 1 TTC, Schärfe System). Since the semi-continuous turbidostats were diluted once every 2 d, the specific growth rate (μ) can be calculated as:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (1)$$

where $t_1$ is the time directly after a dilution event, $t_2$ the time directly before the next dilution event (2 d later), and $x_1$ and $x_2$ are the cell concentrations at Times $t_1$ and $t_2$, respectively. Note that Eq. (1) calculates net μ, because the cultures were exposed to diel fluctuations in the form of a light-dark cycle. Microcystin production was calculated according to Long et al. (2001) as the product of microcystin cell quota and μ.

For intracellular microcystin analysis, 10 ml of the culture suspension was filtered in triplicate using Whatman GF/C filters (pore size ~1.2 μm). Filters were freeze dried and stored at −20°C. Microcystin was extracted in 75% methanol (3 extraction rounds) as described by Fastner et al. (1998) with an extra step for grinding the filters in a Mini Beadbeater (Biospec Products) with 0.5 mm silica beads (Tonk et al. 2005). Dried extracts were stored at −20°C and dissolved in 50% MeOH for analysis of microcystin content using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron Instruments). The extracts were separated using a 30 to 70% acetonitrile gradient with 0.05% trifluoroacetic acid at a flow of 1 ml $min^{-1}$ and a LiChrospher 100 ODS 5 μm LiChorCART 250-4 cartridge system (Merck). The different microcystin variants were identified based on their characteristic UV-spectra and quantified by means of an MC-LR gravimetrical standard kindly provided by the Laboratory of Microbiology of the University of Dundee.

For extracellular microcystin analysis, the effluent from the filtered culture suspension used for intracellular microcystin analysis was freeze dried and resuspended in 1.5 ml Milli-Q. We expected that extracellular microcystin concentrations would be below the detection limit of the HPLC. Therefore, extracellular microcystin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA), according to the protocol of the 'Microcystin plate kit' (EnviroLogix).

**Data analysis.** We used ANOVA to test for significant differences between the 3 salt-shock experiments (10, 15 and 17.5 g $l^{-1}$) in combination with Tukey's (HSD) post-hoc test to compare the means. Homogeneity of the variances was checked by Levene's test. The statistical tests were carried out in SPSS Version 11.

## RESULTS

### Gradually increasing salinity

**Growth rates.** The specific growth rate (μ) of *Microcystis aeruginosa* Strain PCC 7806 remained constant at around 0.40 $d^{-1}$ (SD = 0.05; N = 12) up to a salinity of 10 g $l^{-1}$ (Fig. 1A). This was followed by an abrupt collapse to zero growth at 12.5 and 15 g $l^{-1}$.

**Cell size.** Mean cell size was 34 $\mu m^3$ (SD = 1.6; N = 5) in the salinity range of 0.6 to 10 g $l^{-1}$. At higher salinity, cell size decreased (Fig. 1B). The smallest cell size was approximately 20 $\mu m^3$ at a salinity of 15 g $l^{-1}$ (Fig. 1B).

**Intracellular microcystin.** *Microcystis aeruginosa* Strain PCC 7806 produced microcystin (MC) variants MC-LR and [$Asp^3$]MC-LR. The peaks of [$Asp^3$]MC-LR and MC-LR could not be separated completely by means of HPLC. Therefore both peaks were merged and are referred to as total microcystin. Total microcystin cell quota was on average 45 fg $cell^{-1}$ (SD = 6.7; N = 10) for salinity levels up to 8 g $l^{-1}$. Above 8 g $l^{-1}$ the total microcystin content decreased (Fig. 1C). Total microcystin production was on average 18 fg $cell^{-1}$ $d^{-1}$ (SD = 3.8; N = 11) in the salinity range from 0.6 to 10 g $l^{-1}$, and zero at higher salinities (Fig. 1D).

**Extracellular microcystin.** The extracellular microcystin concentration was not affected by salinity levels up to 10 g $l^{-1}$, where it amounted to approximately 16% of the total (intra- and extracellular) microcystin concentration in the culture vessels (SD = 1.6; N = 5). At 15 g $l^{-1}$, the extracellular microcystin concentration increased to almost 100% of the total microcystin concentration (Fig. 1E).

### Salt-shock experiments

**Growth rates.** *Microcystis aeruginosa* μ was reduced upon exposure to a sudden increase in salinity in the salt-shock experiments (Fig. 2A). μ recovered within a week in the salt-shock experiment with 10 g $l^{-1}$. The *M. aeruginosa* population was clearly negatively affected by the salt-shock experiments at higher salinities, but was nevertheless still able to grow for 9 d when suddenly exposed to salinities as high as 17.5 g $l^{-1}$. At Day 9, the growth rates at salinities 10, 15 and 17.5 g $l^{-1}$ differed statistically significantly from each other, decreasing at higher salinities (Fig. 2A; ANOVA: $F_{2,6} = 121$, $p < 0.001$).

**Cell size.** Mean cell size was 35 $\mu m^3$ (SD = 0.2; N = 54) throughout the salt-shock experiment, with a slight increase towards the end of the experiment (Fig. 2B). At Day 9, the cell size of cells grown at a salinity of 17.5 g $l^{-1}$ was statistically significantly larger than those cultured at 10 and 15 g $l^{-1}$; cell sizes at 10 and

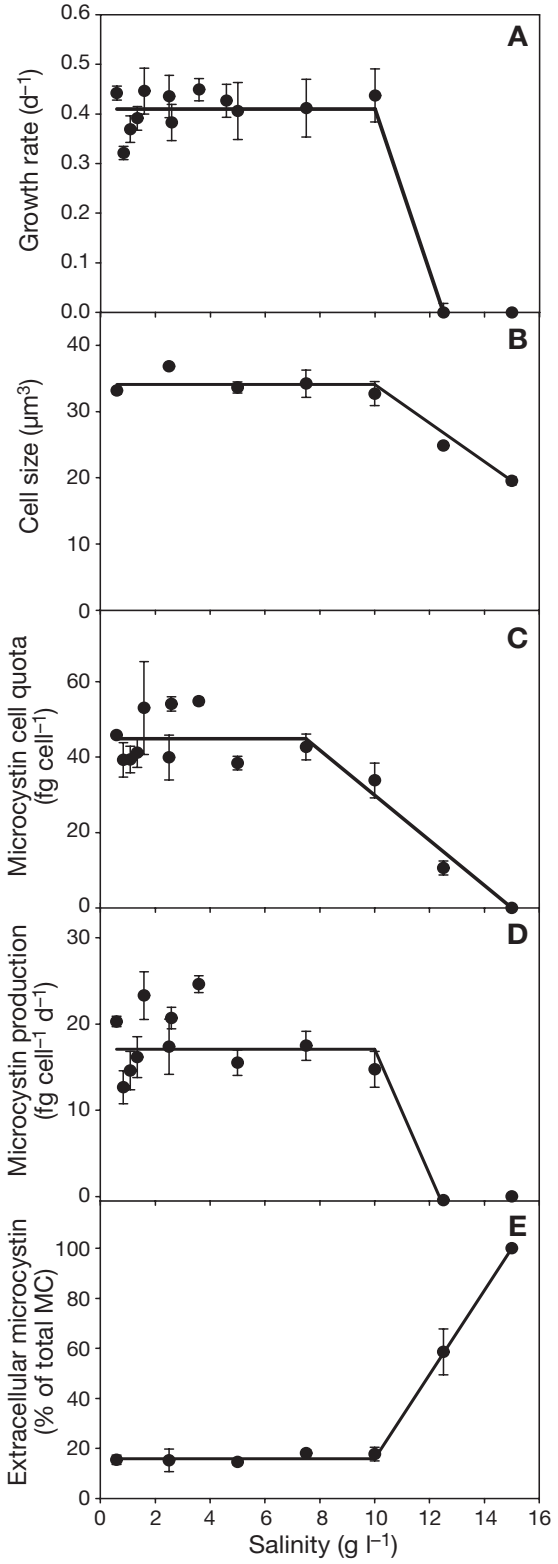


Fig. 1. *Microcystis aeruginosa*. Mean ± SD (N = 3) effects of salinity on (A) specific growth rate, (B) average cell size, (C) total microcystin cell quota, (D) microcystin production, and (E) extracellular microcystin concentration as a percentage of total (intra- and extracellular) microcystin concentration. Curves represent means at low salinities, and indicate steep changes at high salinities

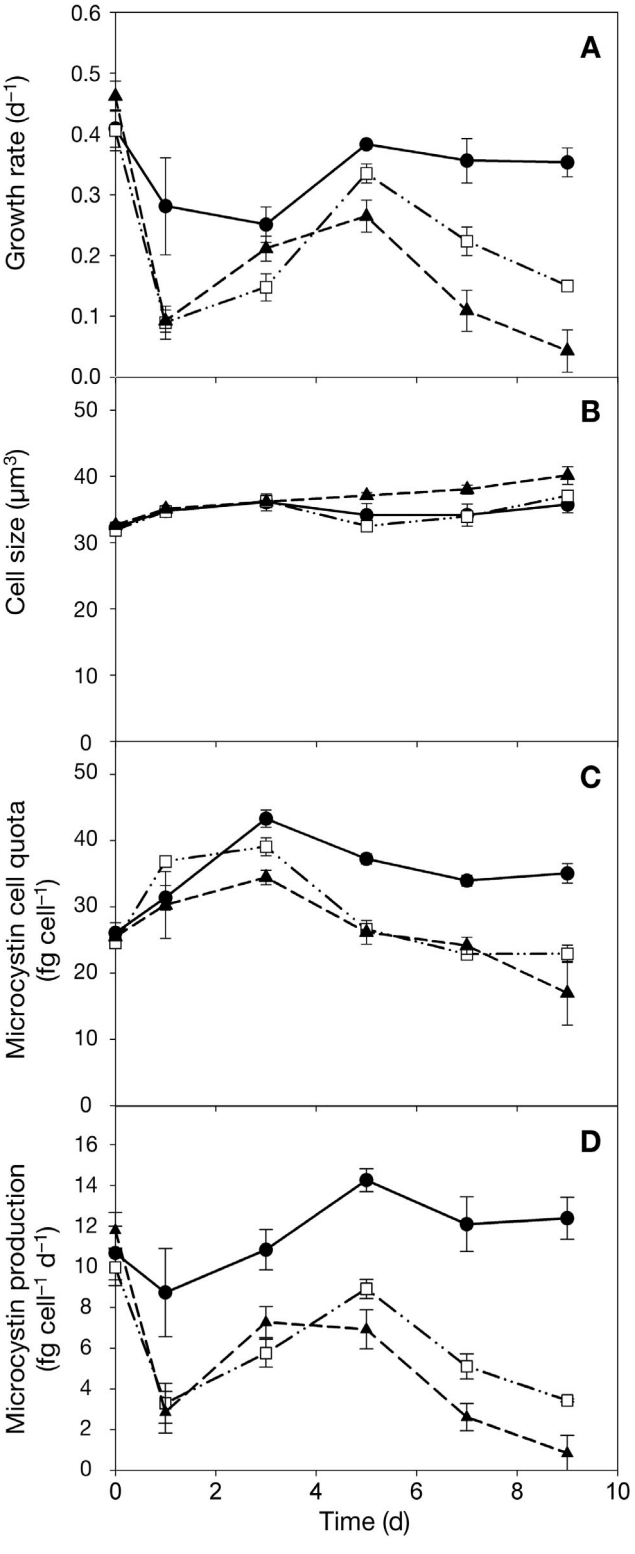


Fig. 2. *Microcystis aeruginosa*. Mean ± SD (N = 3) time course of (A) specific growth rate, (B) cell size, (C) total microcystin cell quota and (D) microcystin production during salt-shock experiments. (●) 10 g l$^{-1}$; (□) 15 g l$^{-1}$; (▲) 17.5 g l$^{-1}$. Salt was added at Day 0, directly after first sampling point

15 g $l^{-1}$ were not statistically significantly different (Fig. 2B; ANOVA: $F_{2,6} = 11.6$, $p < 0.01$).

**Intracellular microcystin.** The microcystin cell quota of cells exposed to salinities of 10, 15 and 17.5 g $l^{-1}$ initially increased (Fig. 2C). After 3 d, the microcystin contents of cells exposed to 10 g $l^{-1}$ remained high, while the microcystin contents at 15 and 17.5 g $l^{-1}$ decreased. At Day 9, microcystin cell quota was significantly higher at a salinity of 10 g $l^{-1}$ than at salinities of 15 and 17.5 g $l^{-1}$ (Fig. 2C; ANOVA: $F_{2,6} = 28$, $p < 0.01$).

Microcystin production of cells exposed to a salt shock of 10 g $l^{-1}$ decreased during the first day, but recovered fully during the following days (Fig. 2D). Total microcystin production of cells exposed to salt shocks of 15 and 17.5 g $l^{-1}$ also decreased during the first day, then increased during the next few days, after which the microcystin production collapsed. At Day 9, microcystin production at salinities of 10, 15 and 17.5 g $l^{-1}$ were significantly different from each other with a statistically significant decrease at higher salinities (Fig. 2D; ANOVA: $F_{2,6} = 121$, $p < 0.001$).

**Extracellular microcystin.** Extracellular microcystin concentrations measured at Day 7 were 17, 15 and 30% of the total microcystin concentration (intra- and extracellular) in the cultures exposed to salinities of 10, 15 and 17.5 g $l^{-1}$, respectively.

## DISCUSSION

The growth rate, microcystin cell quota, and microcystin production of the harmful cyanobacterium *Microcystis aeruginosa* Strain PCC 7806 remained unaffected up to a salinity of 10 g $l^{-1}$ (Fig. 1). These results from controlled semi-continuous turbidostats confirm earlier findings in batch cultures (Otsuka et al. 1999, Orr et al. 2004, Verspagen et al. 2006) that, for a freshwater species, *Microcystis* has a high salt tolerance. We investigated only a single *M. aeruginosa* strain. Therefore, it is not clear to what extent our findings can be extrapolated to other *M. aeruginosa* strains. However, Otsuka et al. (1999) reported a rather similar salt tolerance of 4 different *M. aeruginosa* strains. Moreover, the observed salt tolerance is consistent with observations of *Microcystis* spp. blooms in brackish waters, e.g. in the Patos Lagoon Estuary in Brazil (Matthiensen et al. 2000), the Oued Mellah reservoir, Morocco (Sabour et al. 2002), the Swan River Estuary, Australia (Robson & Hamilton 2003), San Francisco Bay, USA (Lehman et al. 2005), and the Kucukcekmece Lagoon, Turkey (Albay et al. 2005). The growth rate was not stimulated by salinity in our experiments. Hence, our results do not support preliminary observations of Prinsloo & Pieterse (1994) that the growth rate of *Microcystis* spp. might actually benefit from a slightly brackish environment. Cell size was reduced above 10 g $l^{-1}$ (Fig. 1B), indicating that osmoregulation capacity was exceeded, and cells were no longer able to uphold turgor, and began to leak and shrink when the salinity became too high. The reduction in cell size in response to osmotic stress is known as plasmolysis, and is also widely reported for other bacteria (Koch 1984, Csonka 1989, Shapiguzov et al. 2005). The combination of leakage and cell lysis most likely resulted in the high extracellular microcystin concentrations observed at salinities exceeding 10 g $l^{-1}$ (Fig. 2C). The growth rate and microcystin production rate collapsed when cells were exposed to salinities exceeding 10 g $l^{-1}$ for several weeks.

The salt-shock experiments show that *Microcystis aeruginosa* may temporarily withstand even higher salinities. The microcystin cell quota and microcystin production in the salt-shock experiment of 10 g $l^{-1}$ match the values found in the experiments with gradually rising salinity. When *M. aeruginosa* was exposed to stronger salt shocks of 15 and 17.5 g $l^{-1}$, growth rates and microcystin production declined, but *M. aeruginosa* was still able to grow and produce microcystins for at least 1 wk. During this period the average cell size did not decrease, indicating that the surviving cells were still able to withstand the high turgor pressure. However, extracellular microcystin concentration increased to 30% of the total microcystin concentration in the cultures exposed to a salinity of 17.5 g $l^{-1}$. These findings are consistent with recent salt-shock studies with a mixed *Microcystis* spp. population from the Swan River estuary in Western Australia, which revealed cell lysis and increased concentrations of extracellular microcystins when cells were exposed to a salinity of 21 g $l^{-1}$ (Orr et al. 2004).

What are the environmental implications? In the literature, surprisingly few data are available on the salt tolerance of freshwater phytoplankton species. A compilation of available data is shown in Fig. 3. In line with earlier suggestions (Hart et al. 1991, Nielsen et al. 2003), these data show that many of the freshwater diatoms and green algae thus far investigated have a relatively low salt tolerance. Accordingly, cyanobacteria with a higher salt tolerance, like *Microcystis aeruginosa* and *Anabaena* sp., may profit from rising salinities in freshwater ecosystems by gaining a competitive advantage over other freshwater phytoplankton. Conversely, when estuaries face a sudden input of freshwater, *M. aeruginosa* may gain an advantage over resident marine phytoplankton (Robson & Hamilton 2003). Current management evaluations consider whether several Dutch freshwater areas should be converted into brackish waters (e.g. Lake

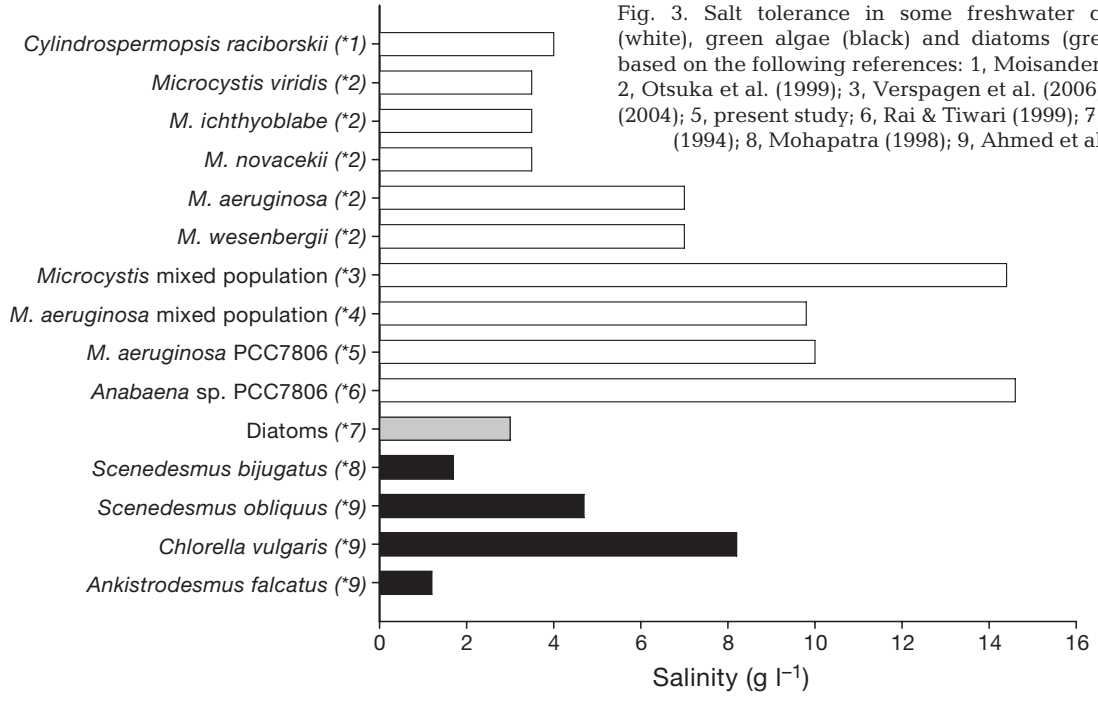


Fig. 3. Salt tolerance in some freshwater cyanobacteria (white), green algae (black) and diatoms (grey). Data are based on the following references: 1, Moisander et al. (2002); 2, Otsuka et al. (1999); 3, Verspagen et al. (2006); 4, Orr et al. (2004); 5, present study; 6, Rai & Tiwari (1999); 7, Wilson et al. (1994); 8, Mohapatra (1998); 9, Ahmed et al. (1985)

Volkerak; Verspagen et al. 2006). Our results indicate that such large-scale changes in water management might facilitate a competitive advantage of salt-tolerant cyanobacteria over salt-sensitive freshwater phytoplankton such as green algae and diatoms. Moreover, our results show that temporary exposure to salinities between 10 and 20 g $l^{-1}$ allows survival of *M. aeruginosa* populations, while extracellular microcystin concentrations will increase. This implies that salinity fluctuations in brackish waters may not only favor *M. aeruginosa* over other freshwater phytoplankton species, but may also increase the exposure of many aquatic organisms to elevated microcystin concentrations. In conclusion, a transition of freshwater ecosystems into brackish waters is unlikely to prevent *Microcystis* spp. blooms, unless salinities increase to sufficiently high levels at which *Microcystis* spp. no longer survives (i.e. above 17.5 g $l^{-1}$). Increases in salinity in freshwater ecosystems that remain below this threshold are more likely to enhance the risk of *Microcystis* spp. blooms and the exposure to elevated concentrations of microcystins.

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