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Internal nutrient loading may increase microcystin concentrations in freshwater lakes by promoting growth of *Microcystis* populations

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Abstract – Nutrient release from lake sediments may increase concentrations of harmful algal toxins – such as microcystins – by stimulating blooms of toxigenic cyanobacteria. This hypothesis is supported by a series of experiments in which intact cores of sediment were incubated under different environmental conditions, after which the water overlying the sediments was harvested as a culture medium for growing a toxic strain of the common cyanobacterium *Microcystis*. Both littoral and profundal sediments from Lake Kinneret, the largest freshwater lake in Israel, released substantial amounts of dissolved phosphorus (1.0 and 4.0 $mg.m^{-2}.d^{-1}$, respectively) and nitrogen (44.2 and 24.3 $mg.m^{-2}.d^{-1}$, respectively) under simulated summer conditions in the laboratory. In comparison, nutrient fluxes from sediments under simulated winter conditions were considerably smaller or negative. The addition of nutrient-rich overlying water harvested from profundal sediments, and to a lesser extent from littoral sediments, increased both chlorophyll a and microcystin concentrations in *Microcystis* cultures. In contrast, when *Microcystis* cells were inoculated in natural surface waters only, the cultures did not grow or produce microcystins, and soon collapsed. This study provides experimental evidence of a link between internal nutrient loading from sediments and microcystin concentrations in freshwaters, and demonstrates how environmental factors may indirectly exert control over toxin concentrations in freshwater lakes.

Key words: Microcystins / cyanobacteria / lake sediments / internal loading / phosphorus

Introduction

Microcystins are a family of monocyclic heptapeptide toxins, which are commonly found in nutrient-polluted freshwaters (Carmichael, 1992). Microcystins are produced by certain strains of cyanobacteria, and are well known for their potent toxicity. These compounds strongly inhibit specific protein phosphatases in animal cells, triggering a cascade of events leading to cell necrosis or apoptosis (Campos and Vasconcelos, 2010). Microcystins can promote tumor development in mammals, and are classified as possible human carcinogens (Funari and Testai, 2008). Microcystin toxins have been implicated in poisonings of aquatic invertebrates and fish (Landsberg, 2002), wild and domestic terrestrial animals (Briand *et al.*, 2003) and humans (Chorus *et al.*, 2000). Because of the potential for microcystins to inflict illness or death, the increasing and widespread proliferation of toxin-producing cyanobacteria in freshwaters is serious cause for concern (Carmichael, 2008).

High concentrations of microcystins are frequently associated with blooms of the cyanobacterium *Microcystis* (order Chroococcales) in eutrophic water bodies (Sivonen and Jones, 1999). *Microcystis* is considered a global threat to human health because of its cosmopolitan distribution and ability to thrive in a wide range of climates (Pearson *et al.*, 2010). Microcystin production probably evolved in an ancient ancestor of modern cyanobacteria (Rantala *et al.*, 2004), and several extant species of *Microcystis* have retained the genes for microcystin synthesis (Via-Ordorika *et al.*, 2004). Although the biological function of microcystins is still under debate, these compounds may serve as infochemicals (Kaplan *et al.*, 2012), or perhaps as protectants against oxidative stress (Zilliges *et al.*, 2011).

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Dynamics of microcystins in freshwater lakes have been related to the biomass of *Microcystis* species (Kotak *et al.*, 2000; Ozawa *et al.*, 2005) and seasonal succession of toxic *Microcystis* genotypes (Kardinaal *et al.*, 2007; Davis *et al.*, 2009), confirming the role of *Microcystis* as an important source of these toxins in the natural environment.

Microcystin concentrations in freshwater lakes are often correlated with environmental parameters (Wicks and Thiel, 1990; Kotak *et al.*, 2000; Giani *et al.*, 2005; Rantala *et al.*, 2006; Rinta-Kanto *et al.*, 2009). This observation led to the hypothesis that toxin production by *Microcystis* is controlled, in part, by changes in environmental factors. Indeed, laboratory experiments have demonstrated that physicochemical variables can directly affect microcystin production by *Microcystis* cells. For example, rates of toxin production have been shown to be altered by temperature (Amé and Wunderlin, 2005), light (Deblois and Juneau, 2010), nitrogen (Dai *et al.*, 2008), phosphorus (Bickel *et al.*, 2000; Oh *et al.*, 2000), inorganic carbon (Jahnichen *et al.*, 2007), iron (Lukač and Aegerter, 1993; Sevilla *et al.*, 2008) and sulfur (Long, 2010). However, cellular microcystin quotas of cyanobacteria exposed to different environmental conditions usually do not vary by more than five-fold (Sivonen and Jones, 1999). Given that microcystin concentrations in surface waters of eutrophic lakes and ponds can vary by several orders-of-magnitude (Kotak and Zurawell, 2007), the direct effects of environmental factors on cellular toxin production cannot alone explain the observed variation in microcystin concentrations in aquatic ecosystems.

While the direct effects of environmental factors on microcystin production have been intensively studied at the cellular level, the complex pathways by which environmental factors indirectly influence microcystin concentrations in aquatic ecosystems are poorly understood. Microcystin concentrations in freshwater lakes are determined by the abundance of cyanobacterial cells, the relative dominance of toxic versus non-toxic strains and the microcystin production rate of toxic cells (Zurawell *et al.*, 2005). Environmental factors potentially exert control over concentrations of microcystins in freshwater lakes by triggering natural geochemical processes that stimulate the proliferation of toxigenic cyanobacteria. Specifically, changes in environmental conditions can activate the release of important algal nutrients, such as phosphorus and nitrogen, from bottom sediments. This phenomenon, known as "internal loading" (Søndergaard *et al.*, 2009) or "internal eutrophication" (Smolders *et al.*, 2006), can fuel blooms of cyanobacteria in eutrophic lakes (*e.g.*, Burger *et al.*, 2008; Grace *et al.*, 2010). However, the link between episodes of nutrient release from sediments and elevated concentrations of microcystin toxins is not well established.

In the present study, we provide experimental evidence supporting the "indirect effect" hypothesis to explain the relationship between environmental factors and microcystin concentrations in freshwater lakes. Specifically, we tested the hypothesis that environmental conditions that enhance the release of nutrients from sediments increase microcystin concentrations in lake water by stimulating the growth of *Microcystis* populations. To test this hypothesis, we first exposed natural lake sediments from an important freshwater lake – Israel's Lake Kinneret – to different environmental conditions to mimic seasonal nutrient fluxes from sediments. Next, we cultured a toxigenic strain of *Microcystis* in natural surface waters from Lake Kinneret amended with overlying water harvested from incubated sediments. This latter experiment examined how nutrients released from sediments affect the growth of *Microcystis* populations, and how this change in biomass alters concentrations of microcystins in lake water.

Materials and methods

Study lake

We conducted our experiments using sediments collected from Lake Kinneret ("Sea of Galilee"), the largest freshwater lake in Israel (32°42'-32°55' N; 35°31'–35°39' E). This subtropical lake covers an area of 170 km^2 and has a maximum depth of 43 m (Serruya, 1978). During the stratification period (April–December), the thermocline depth is typically between 15 and 20 m, and the epilimnion is warm (24–30 °C) and well-oxygenated, whereas the hypolimnion is relatively colder (14–16 °C) and anoxic. Lake Kinneret is mesotrophic with annual mean concentrations of total phosphorus and total nitrogen of approximately 20 μg $P.L^{-1}$ and 0.6 mg $N.L^{-1}$, respectively (Zohary, 2004). While cyanobacteria were once a minor component of the phytoplankton community of Lake Kinneret, a major regime shift occurred in the mid-1990s toward more frequent and more intense blooms of cyanobacteria (Zohary 2004). Since 1995, toxic populations of *Microcystis* commonly form surface scums in Lake Kinneret (Ostrovsky *et al.*, 2013).

Sediment core experiment

Collection of sediment cores

On 10 March 2010, sediment cores ($n = 12$) were retrieved from Lake Kinneret in polycarbonate tubes (inner diameter: 5.5 cm; height: 60 cm) using a Tessenow gravity sampler (Tessenow *et al.*, 1977). Six "profundal" cores were collected from a depth of 32 m, and six "littoral" cores were collected from a depth of 10 m. Care was taken to collect intact sediment columns with an undisturbed sediment–water interface. The height of "overlying water" (*i.e.*, water above the sediment–water interface) was adjusted to exactly 25 cm to ensure each tube contained the same volume of overlying water (0.6 L).

Incubation conditions

Littoral and profundal sediment cores were randomly assigned to either "summer" or "winter" incubation

Table 1. Phosphorus and nitrogen fluxes (mean ± SD, $n = 3$) from Lake Kinneret sediments.

Code	Sediment	Treatment	Conditions	SRP flux (mg $m^{-2}.d^{-1}$)	TDP flux (mg $m^{-2}.d^{-1}$)	DIN flux (mg $m^{-2}.d^{-1}$)	TDN flux (mg $m^{-2}.d^{-1}$)
LW	Littoral	Winter	16 °C/oxic	− 0.1 ± 0.1	− 0.2 ± 0.1	2.5 ± 3.3	2.1 ± 2.2
LS	Littoral	Summer	25 °C/oxic	1.0 ± 0.2*	1.5 ± 0.2*	44.0 ± 10.1*	44.2 ± 9.6*
PW	Profundal	Winter	16 °C/oxic	− 0.7 ± 0.2*	− 0.5 ± 0.1*	8.8 ± 1.8*	12.0 ± 11.5
PS	Profundal	Summer	16 °C/anoxic	4.4 ± 0.6*	5.1 ± 0.9*	17.6 ± 3.5*	24.3 ± 3.7*

SRP, soluble reactive phosphorus; TDP,total dissolved phosphorus; DIN, dissolved inorganic nitrogen; TDN, total dissolved nitrogen; * mean flux is significantly different from zero (*t*-test; $P < 0.05$).

Table 2. Initial composition and nutrient concentrations (mean ± SD, $n = 4$) of growth media for the *Microcystis* experiment.

Code	Composition	SRP (µg.L^{-1})	NH_4^+ (mg.L^{-1})	NO_3^- (mg.L^{-1})	NO_2^- (mg.L^{-1})	DIN (mg.L^{-1})
SW	100% surface water	n.d.	n.d.	0.14 ± 0.01	0.02 ± 0.00	0.16 ± 0.01
LS	80% littoral core water; 20% surface water	n.d.	0.42 ± 0.02	0.21 ± 0.01	0.12 ± 0.01	0.75 ± 0.03
PS	80% profundal core water; 20% surface water	50.7 ± 2.6	0.61 ± 0.02	n.d.	0.01 ± 0.00	0.61 ± 0.02

SRP, soluble reactive phosphorus; DIN, dissolved inorganic nitrogen; n.d.,non-detectable.

conditions ($n = 3$ cores/treatment). The incubation conditions were carefully chosen (based on long-term monitoring data of Lake Kinneret collected by the Yigal Allon Kinneret Limnological Laboratory) to reflect the ambient temperature and oxygen regimes experienced by sediments in Lake Kinneret (see Table 1). Littoral cores under "winter" conditions (*i.e.*, cold/oxic) were incubated at 16 °C and continuously aerated, whereas littoral cores under "summer" conditions (*i.e.*, warm/oxic) were incubated at 25 °C and continuously aerated. Aeration was achieved by gently and continuously bubbling overlying water with filtered (0.2 µm) atmospheric air using aquarium pumps. Profundal cores under "winter" conditions (*i.e.*, cold/oxic) were incubated at 16 °C and aerated, whereas profundal cores under "summer" conditions (*i.e.*, cold/anoxic) were purged with N_2 gas for 10 min, capped and sealed with parafilm, and then incubated at 16 °C. All cores were incubated in environmental chambers in darkness.

Sampling and chemical analyses

Overlying water in each sediment core was sampled for nutrients on days 0 and 7 of the incubation period, and filtered (0.45 µm) immediately after collection. Samples were analyzed for soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), ammonia (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) using a Flow Injection Auto-Analyzer (QuikChem 8000, Automated Ion Analyzer, Lachat Instruments) following the manufacturer protocol or spectrophotometrically according to the Standard Methods (APHA *et al.*, 2005). Total dissolved nitrogen (TDN) was measured using a spectrophotometer (Kontron) following a modified Kjeldahl method (APHA *et al.*, 2005). Dissolved inorganic nitrogen (DIN) is expressed as the sum of NH_4^+, NO_3^- and NO_2^-. Flux rates of nutrient release from sediments were calculated by dividing the change in the nutrient concentration over the incubation period by the length of the incubation period and the surface area of the sediments.

***Microcystis* experiment**

Incubation of sediment cores

We collected and incubated a second set of sediment cores to obtain the growth media for our *Microcystis* experiment, following an approach similar to Cymbola *et al.* (2008). Four littoral and four profundal sediment cores were collected from Lake Kinneret and incubated in the laboratory under "summer" conditions for 1 week. As was done in the previous experiment for the summer treatments, littoral cores were subject to "oxic/warm" conditions, whereas profundal cores were subject to "anoxic/cold" conditions. At the end of the 7-day incubation period, overlying water from cores of the same treatment was pooled and filtered through pre-rinsed GF/C filters.

Growth media

Three types of growth media were prepared in acid-washed glass flasks (see Table 2). The control medium, referred to as the "SW" medium, consisted of only filtered (GF/C) surface water from Lake Kinneret. The "LS" medium consisted of 20% filtered surface water from Lake Kinneret and 80% filtered overlying water from the littoral sediment cores incubated under "summer" conditions. The "PS" medium consisted of 20% filtered surface water from Lake Kinneret and 80% filtered overlying water from the profundal sediment cores incubated under "summer" conditions.

Inoculum and experimental cultures

For our study, we used a *Microcystis* strain (MG-K) previously isolated from Lake Kinneret. This strain is green due to a lack of phycoerythrin, does not form colonies in the laboratory, and is known to produce microcystins (Beresovsky *et al.*, 2006). Prior to the experiment, an inoculum culture of *Microcystis* MG-K was prepared in the BG (-P) medium, and then transferred to 1 L of filtered (GF/C) surface water from Lake Kinneret and acclimated in an environmental growth room for 2 days prior to the experiment.

Four replicate experimental cultures were prepared in acid-washed flasks for each of the three treatments (*i.e.*, SW, LS and PS media). Growth medium (425 mL) was added to each 1-L flask, and then inoculated with 10 mL of the *Microcystis* inoculum culture to achieve a target nominal chlorophyll a (chl a) concentration of 4 $\mu g.L^{-1}$. This target was about 50% lower than the observed chl a concentration in Lake Kinneret at the time of the experiment. Cultures were incubated for 7 days in an environmental growth room at a temperature of 20 °C and under a constant light level of 50 µmol photons $m^{-2}.s^{-1}$. Cultures were gently swirled by hand twice each day.

Sampling and analyses

We assessed nutrient availability in the growth media, as well as the nutrient status, growth, and toxin concentrations of the *Microcystis* cultures. All parameters were measured in each culture on days 0, 1, 4 and 7 (with the exception of toxin levels, as noted below). To examine nutrient availability in the growth media, we measured concentrations of SRP, NH_4^+, NO_3^- and NO_2^- (following the methods described above for the sediment core experiment). Phosphorus status of algal cells was determined by measuring particulate phosphorus (PP) and alkaline phosphatase activity (APA). For PP determination, algal cells were collected on GF/C filters, digested with persulfate, and analyzed using the Flow Injection Auto-Analyzer. For APA determination, algal cells were collected on 2 µm Millipore filters, incubated with 4-methyl-umbelliferyl phosphate at 37 °C for 1 h (Hadas *et al.*, 1999), and measured on a Fluoroskan plate reader (Thermo Scientific Fluoroskan Ascent, USA). To measure growth of phytoplankton, chl a was measured fluorometrically using the method of Holm-Hansen *et al.* (1965). To assess toxin production, the inoculum culture was sampled on day 0 (50 mL), and the experimental cultures were sampled on day 7 (100 mL) for microcystin determination. Whole water samples for toxin analyses were frozen, freeze-dried, extracted in methanol and quantitively analyzed by High-Performance Liquid Chromatography (Lawton *et al.*, 1994).

Statistical analyses

All data analyses were performed using SigmaPlot for Windows (Version 11.0). When necessary, data were transformed to satisfy the assumption of normality for parametric statistical methods (assessed by Shapiro–Wilk's Tests). For the sediment core experiment, we performed two-way repeated measures analyses of variance (ANOVA) to compare P and N concentrations at the beginning and end of the incubation period among the four seasonal treatments. We assessed whether fluxes of P and N from sediments were significantly different from zero using *t*-tests. For the *Microcystis* experiment, we performed two-way repeated measures ANOVA to compare changes in chl a and total microcystin concentrations over time among *Microcystis* cultures with different growth media. Each two-way repeated measures ANOVA was followed by a pairwise multiple comparison procedure (Holm–Sidak Method).

Results

Sediment core experiment

Phosphorus release

At the beginning of the experiment, concentrations of SRP and TDP in overlying water were, as expected, similar among the six replicate cores collected at the same depth in Lake Kinneret (SE < 8% of the mean; Fig. 1(A) and (B)). In comparison, average phosphorus concentrations in profundal cores from the deep station (21 and 24 $\mu g.L^{-1}$, for SRP and TDP, respectively) were three- to fivefold higher than those of littoral cores from the shallow station (4 and 8 $\mu g.L^{-1}$, for SRP and TDP, respectively). Over the 7-day incubation period under different environmental conditions, average concentrations of SRP decreased in both winter treatments, from 4 $\mu g.L^{-1}$ to below the limit of detection in the littoral cores, and from 22 to 4 $\mu g.L^{-1}$ in the profundal cores. In contrast, SRP concentrations increased in both summer treatments, from 5 to 31 $\mu g.L^{-1}$ in the littoral cores and from 22 to 134 $\mu g.L^{-1}$ in the profundal cores, respectively. The dynamics of TDP in overlying water were similar to those of SRP (cf. Figs. 1(A) and (B)). Concentrations of P in overlying water of the sediment cores differed significantly over time and among treatments during the experiment for both SRP (ANOVA; time: $F = 54$, $P < 0.001$; treatment: $F = 50$, $P < 0.001$; Fig. 1(A)) and TDP (ANOVA; time: $F = 213$, $P < 0.001$; treatment: $F = 209$, $P < 0.001$; Fig. 1(B)).

The net flux of SRP and TDP from sediments to overlying water over the incubation period was, on average, negative for the winter treatments, and positive for the summer treatments (Table 1). All SRP and TDP fluxes were significantly different from zero, with the exception of the winter treatment of littoral cores. Phosphorus fluxes were notably higher from profundal than littoral sediments under summer conditions.

Nitrogen release

Concentrations of DIN and TDN in overlying water were similar among all sediment cores at the start

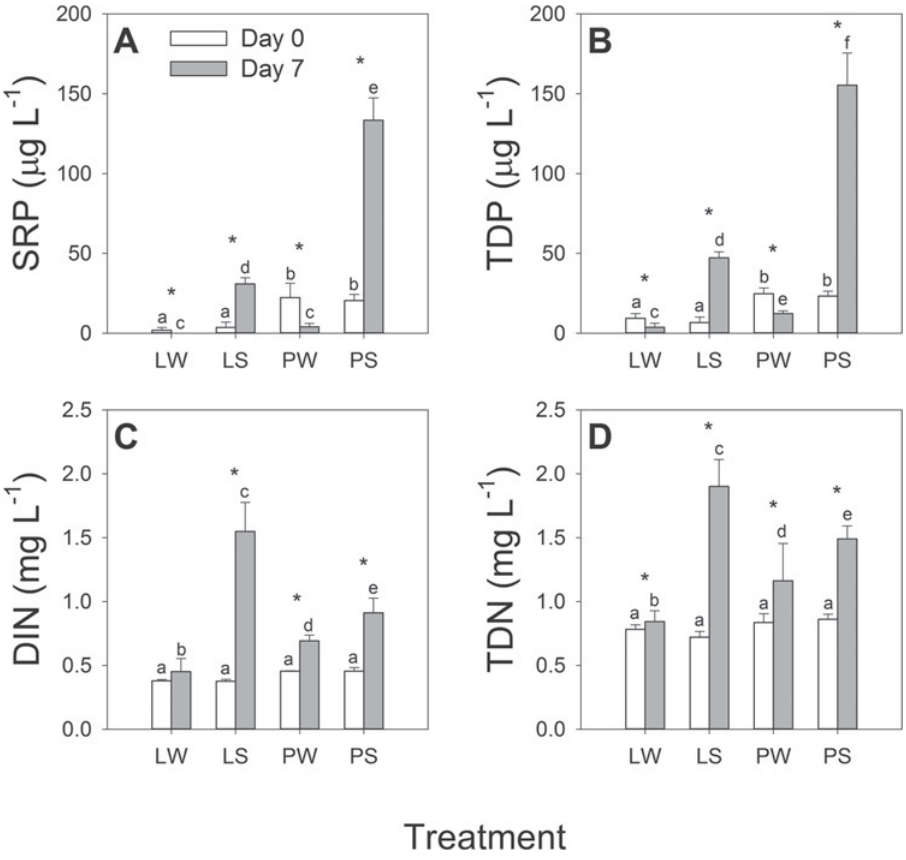

Fig. 1. Concentrations of SRP (A),TDP (B), DIN (C), and TDN (D) in overlying water of sediment cores on days 0 and 7 of the incubation experiment. Average (± SD) calculated from three cores per treatment. Treatments are defined in Table 1. Lower case letters denote significantly different values among treatments, and asterisks denote significantly different values between time periods ($P < 0.05$).

of the experiment collected at the same depth in Lake Kinneret (SE < 2% of the mean), with little difference in the average concentration between littoral (0.38 and 0.75 mg.L^{-1}, for DIN and TDN, respectively) and profundal cores (0.45 and 0.85 mg.L^{-1}, for DIN and TDN, respectively). At this time, the DIN pool was dominated by NO_3^- (see Supplementary Fig. S1 available online). Concentrations of DIN and TDN in the overlying water of the sediment cores increased over the 7-day incubation period in all treatments (Fig. 1(C) and (D)). The largest changes in DIN were observed for the summer treatments of both the littoral and profundal cores, which increased from 0.38 to 1.55 mg.L^{-1}, and from 0.45 to 0.91 mg.L^{-1}, respectively. Under summer conditions, the increase in dissolved nitrogen was largely due to an increase in NH_4^+ in the profundal cores, but due to an increase in both NH_4^+ and NO_2^- in the littoral cores (see Supplementary Fig. S1 available online). An increase in DIN between days 0 and 7 was also observed, but to a lesser extent, under winter conditions, where average concentrations increased modestly from 0.38 to 0.45 mg.L^{-1} in the littoral cores, and from 0.45 to 0.69 mg.L^{-1} in the profundal cores, respectively. Concentrations of N were significantly different over time and among treatments for both DIN (ANOVA; time: $F = 321$, $P < 0.001$; treatment: $F = 25$, $P < 0.001$; Fig. 1(C)) and TDN (ANOVA; time: $F = 91$, $P < 0.001$; treatment: $F = 14$, $P < 0.001$; Fig. 1(D)).

Net fluxes of DIN and TDN from sediments were positive and significantly different from zero under most conditions, with the exception of the littoral winter treatment (Table 1). Nitrogen fluxes were greater under summer than winter conditions, with the highest fluxes observed in the littoral sediment cores incubated under warm/oxic conditions (Table 1).

Microcystis experiment

Nutrient availability

Because sediments acted as sources of nutrients primarily in the summer treatments, we focused on the response of algae to nutrients released from littoral and profundal sediments incubated under summer conditions only. Initial phosphorus and nitrogen concentrations in the three growth media (SW, LS and PS), prior to inoculation with *Microcystis*, are provided in Table 2. Notably, the SRP concentration of the PS medium was an order of magnitude higher than in the LS and SW media,

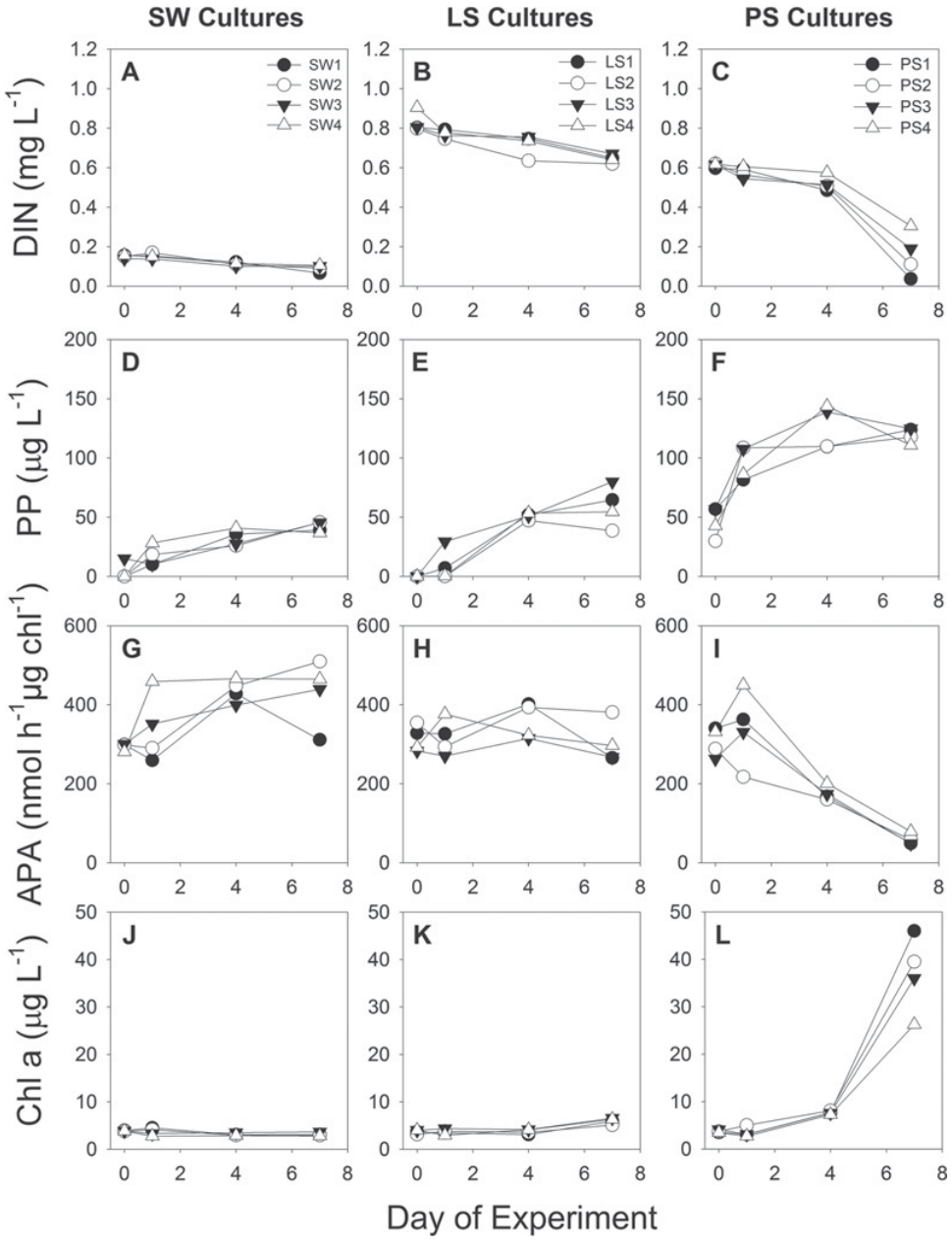

Fig. 2. Temporal changes in concentrations of dissolved inorganic nitrogen (A–C), PP (D–F), APA (G–I), and chlorophyll a (J–L) of *Microcystis* cultures over the 7-day experiment. Results for cultures in SW media are shown in panels A, D, G, J; for LS media in panels B, E, H, K; and for PS media in panels C, F, I and L. The four cultures of each treatment are indicated by different symbols. Growth media are defined in Table 2.

and the DIN concentrations of the LS and PS media were several-fold higher than in the SW media.

Although there was initially more SRP in the PS growth medium (51 $\mu g.L^{-1}$, on average), concentrations of SRP in all treatments were below detection limits immediately after the addition of *Microcystis*. In contrast to the rapid uptake of SRP, DIN concentrations exhibited little change before and after inoculation of *Microcystis* in all growth media (Figs. 2(A–C)). Over the course of the experiment, DIN slowly declined from an average concentration of 0.15–0.09 $mg.L^{-1}$ in the SW cultures, and from 0.83 to 0.64 $mg.L^{-1}$ in the LS cultures, but a substantial drop in DIN from 0.61 to 0.16 $mg.L^{-1}$ was observed in the PS cultures. Most of the decrease in DIN in growth media was due to losses in the NH_4^+ pool (see Supplementary Fig. S2 available online).

Steady uptake of phosphorus by *Microcystis* was observed in all cultures, based on the temporal trends in PP levels (Figs. 2(D–F)). The nominal PP concentration in the experimental cultures at time 0 (based on the concentration of the inoculum culture) was 4 $\mu g.L^{-1}$, but rapidly increased within the first 24 h of the experiment. *Microcystis* cultures in PS media achieved a maximum PP concentration over 100 $\mu g.L^{-1}$, which was greater

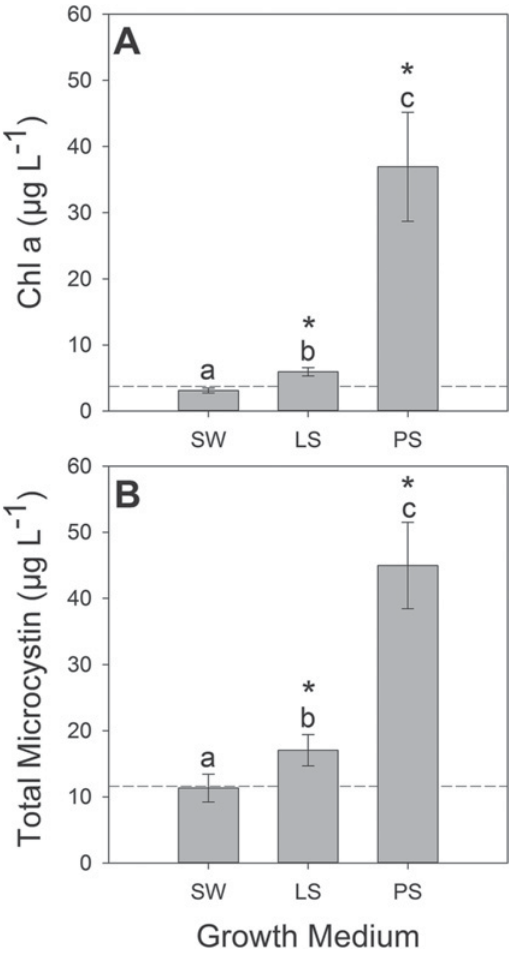

Fig. 3. Average concentrations of chlorophyll a (A) and total microcystin (B) in *Microcystis* cultures at the end of the experiment (day 7). Nominal concentrations of chlorophyll a and total microcystins on day 0 are indicated by horizontal dashed lines. Average ($\pm$ SD) calculated from four cultures per treatment. Growth media are defined in Table 2. Lower case letters denote significantly different values among treatments, and asterisks denote significantly different values between time periods ($P < 0.05$).

than that observed in the LS media (66 µg.L^{-1}) and SW media (58 µg.L^{-1}).

All cultures had similar APA activity when sampled on day 0 (range: 1065–1199 nmol.L^{-1}.h^{-1}), but differences among treatments in APA activity emerged over the course of the experiment. The chl-normalized APA activities of *Microcystis* grown in SW and LS media remained high during the experiment, whereas the APA activities of cultures in PS media decreased sharply (Figs. 2(G–I)).

Chlorophyll and microcystin *concentrations*

The observed chl a concentrations of *Microcystis* cultures at the start of the experiment (range: 3.1–4.1 µg.L^{-1}, $n = 12$) were similar and near the target nominal concentration of 4 µg.L^{-1}. *Microcystis* cultured only in surface water from Lake Kinneret (SW media) did not grow during the 7-day experiment (Fig. 2(J)). Contrary to these control cultures, *Microcystis* cultured in water previously exposed to sediments increased in biomass (Figs. 2(K) and (L)). Over 1 week of incubation, average chl a concentration of *Microcystis* cultures in LS media doubled (to 6 ± 1 µg.L^{-1}), whereas that of cultures in PS media increased by an order of magnitude (to 37 ± 8 µg.L^{-1}). Chl a concentrations among the three treatments were statistically different at the end of the experiment, and a significant increase in chl concentrations was observed over time in the LS and PS treatments (ANOVA; time: $F = 25$, $P < 0.001$; treatment: $F = 25$, $P < 0.001$; Fig. 3(A)).

The inoculum *Microcystis* culture contained six microcystin analogues: microcystin–LR, –RR and –YR, as well as three unidentified microcystin analogues (eluted at 16.47, 17.04 and 18.43 min). The total concentration of microcystins in the inoculum culture was 493 µg.L^{-1} on the day inocula were added to the experimental flasks. Based on this measured concentration in the inoculum, the nominal microcystin concentration in the experimental *Microcystis* cultures was 11.6 µg.L^{-1} at the beginning of the experiment (Fig. 3).

Microcystis cultures in SW and LS media produced microcystin–RR and three unidentified microcystin analogues (eluted at 16.47, 17.04, and 18.43 min). *Microcystis* cultures in PS media also produced these microcystin analogues, plus microcystin–LR and –YR. The four replicate cultures of each treatment showed close agreement in terms of both the amount and composition of microcystins (see Supplementary Fig. S3 available online). The total microcystin concentration of *Microcystis* cultures in SW media did not change during the experiment (11 µg.L^{-1}), whereas concentrations of total microcystins of *Microcystis* cultures in LS and PS media increased, on average, to 17 and 45 µg.L^{-1}, respectively. Total microcystin concentrations among the three treatments were statistically different at the end of the experiment, and a significant increase in microcystin levels was observed over time in the LS and PS treatments (ANOVA; time: $F = 155$, $P < 0.001$; treatment: $F = 82$, $P < 0.001$; Fig. 3(B)). Average ($\pm$ SD) microcystin levels of *Microcystis* cultures, normalized to the chl a content of each culture, were 3.62 ± 0.30, 2.89 ± 0.56 and 1.24 ± 0.12 µg toxin per µg chl for SW, LS and PS media, respectively.

Discussion

In this study, we postulated – and demonstrated experimentally – that nutrient release from lake sediments stimulates growth of the common cyanobacterium *Microcystis*, and this in turn, increases concentrations of microcystin toxins in lake water. Previous studies have been successful in demonstrating that physicochemical factors can directly affect cellular microcystin production by *Microcystis*, but the observed responses have been

modest. The effects of environmental factors on the population growth of *Microcystis* and other microcystin-producing cyanobacteria, rather than directly on toxin production, likely explain more of the observed variation in microcystin concentrations in freshwater lakes. Thus, in order to explain the dynamics in microcystin levels observed in natural ecosystems, it is critical to understand the contribution of biogeochemical processes to blooms of toxigenic cyanobacteria. One pathway by which environmental factors indirectly exert control on microcystin concentrations in freshwater ecosystems is through stimulating in-lake biogeochemical processes that supply the nutrients essential to the growth of toxin-producing cyanobacteria. In this study, we examined nutrient fluxes from intact cores of lake sediments incubated under different environmental conditions carefully chosen to mimic specific seasons. We then assessed the effects of adding nutrients harvested from incubated sediments to natural surface waters collected from Lake Kinneret and inoculated with an endemic, toxigenic strain of *Microcystis*.

Our first main finding was that *Microcystis* cultured in surface water from Lake Kinneret amended with sediment nutrients increased in biomass, whereas *Microcystis* cultured only in surface water experienced no growth. We observed that average chl a concentrations of *Microcystis* cultures increased nearly two- and tenfold when exposed to nutrients from littoral and profundal sediments, respectively. This result confirms that the pool of nutrients in natural surface waters is not sufficient to support algal growth even at low cell densities. In agreement with our study, the addition of hypolimnetic water to surface water (5–10%) in laboratory bioassays with a mixed phytoplankton sample from Lake Kinneret resulted in increased algal biomass and carbon fixation (Ostrovsky *et al.*, 1996). Notably, the biomass of *Microcystis* cultures doubled upon exposure to nutrients released from littoral sediments incubated under summer conditions (warm/oxic), but increased by an order of magnitude upon exposure to nutrients released from profundal sediments under summer conditions (cold/anoxic). This finding is consistent with the study by Cymbola *et al.* (2008), in which growth of a mixed phytoplankton community in laboratory cultures was stimulated to a greater extent by exposure to overlying water extracted from anaerobic than aerobic sediment cores.

Our observation that the growth of *Microcystis* from Lake Kinneret is enhanced upon exposure to sediment-derived nutrients adds to the existing body of literature that lake sediments provide bioavailable nutrients capable of supporting algal growth. Previous sediment-algal bioassays have demonstrated that a quantitatively small, but biologically important, fraction of nutrients in sediments is available for uptake by algae (Nalewajko and Murphy, 1998; Dzialowski *et al.*, 2008). These bioassays typically involve incubating an algal species in direct contact with sediment in a culture vessel (*e.g.*, Nalewajko and Murphy, 1998), or in indirect contact with sediment in a dual-chambered culture vessel separated by a membrane (*e.g.*, Ekholm and Krogerus, 2003). In contrast, we selected the method of Cymbola *et al.* (2008), in which algae are cultured in lake water harvested from sediment cores, because it allows for incubation of intact sediments under environmentally relevant conditions.

In our laboratory study, *Microcystis* was exposed to nutrients released from littoral or profundal sediments from Lake Kinneret. In aquatic ecosystems, cyanobacteria are exposed to nutrients released from lake sediments through several possible routes. For example, during periods of mixing, water currents entrain bottom waters rich in nutrients released from profundal sediments up to the euphotic zone where they are accessible to cyanobacteria and other algae. The same process brings nutrients from shallow sediments in the epilimnion when lakes are stratified. During periods of stratification, algae are also exposed to nutrients released from profundal sediments when wind-induced internal seiches cause mixing of epilimnetic and hypolimnetic waters at the lake's periphery – a mechanism that is pronounced in large lakes such as Lake Kinneret (Ostrovsky *et al.*, 1996). Certain species of cyanobacteria can also vertically migrate in the water column, which allows these organisms to translocate sediment-derived nutrients in the hypolimnion up into the epilimnion (Head *et al.*, 1999). Finally, colonies of cyanobacteria, such as *Microcystis*, that overwinter on the sediment surface (Preston *et al.*, 1980) likely accumulate sediment nutrients before migrating up to surface waters (Verspagen *et al.*, 2005).

A second main finding was that microcystin concentrations increased in surface water collected from Lake Kinneret and inoculated with *Microcystis* only when amended with nutrients from sediments. We observed that microcystin concentrations in *Microcystis* cultures exposed to nutrients released from littoral and profundal sediments increased to 17 and 45 $\mu g.L^{-1}$, respectively. These levels of microcystin are well above international drinking water guidelines (1.0 $\mu g.L^{-1}$) and would be considered a risk for health effects in the case of human recreational exposure (WHO, 2008). In contrast, microcystin concentrations in control cultures incubated in 100% surface water did not change over the course of the experiment.

Microcystin concentrations may have been higher in surface water amended with sediment nutrients simply because *Microcystis* populations achieved greater biomass under these conditions. Theoretically, an increase in the cellular rate of toxin production could have also contributed to the observed change in microcystin concentrations – because rates of toxin production and cell division are strongly positively correlated (Orr and Jones, 1998). However, we observed that microcystin-to-chlorophyll ratios were lower in *Microcystis* cultures amended with sediment nutrients in comparison to those in surface water only. In agreement with our study, Oh *et al.* (2000) reported that the microcystin content of *Microcystis aeruginosa* was lower at higher growth rates when cultured in media with different amounts of phosphorus.

In aquatic ecosystems, microcystin concentrations are determined in part by competition between toxic and non-toxic strains of cyanobacteria (Davis *et al.*, 2009). One limitation of our experimental design was that we isolated one toxigenic strain of *Microcystis* to examine its response to nutrients released from lake sediments. An interesting avenue of future research would be to compare the growth of toxic and nontoxic strains of *Microcystis* in response to sediment-derived nutrients, and to assess how competition for sediment nutrients among toxic and non-toxic strains affects microcystin concentrations in freshwaters.

In conclusion, our study provides experimental evidence of a linkage between nutrient release from sediments and concentrations of microcystins, known human hepatotoxins, in lake water. As high rates of nutrient regeneration from sediments are common in eutrophic lakes (Welch and Cooke, 1995; Jeppesen *et al.*, 2005), our hypothesized pathway of effects connecting internal nutrient loading and microcystin concentrations could potentially be operating in a broad range of freshwater systems. Furthermore, our study serves as an example of how environmental factors can be responsible for indirectly influencing microcystin concentrations in freshwater lakes by triggering the release of nutrients from sediments and stimulating the growth of *Microcystis*. We hope our study encourages future research on the impacts of anthropogenic activities on internal nutrient loading, and potentially microcystin concentrations, in freshwater lakes. Climate warming (Malmaeus and Rydin, 2006) and sulfur pollution (Caraco, 1993) are suspected of enhancing internal nutrient loading in freshwaters. If sediment nutrient release and microcystin production are indeed intimately linked, such global environmental problems may be having the unforeseen effect of increasing levels of harmful toxins in eutrophic freshwaters.

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