

Intracellular, environmental and biotic interactions influence recruitment of benthic *Microcystis* (Cyanophyceae) in a shallow eutrophic lake

HUGO BORGES^{1,2}, SUSANNA A. WOOD^{1,2*}, JONATHAN PUDDICK¹, EMILY BLANEY¹, IAN HAWES³, DANIEL R. DIETRICH⁴ AND DAVID P. HAMILTON²

¹CAWTHRON INSTITUTE, PRIVATE BAG 2, NELSON, 7042, NEW ZEALAND, ²ENVIRONMENTAL RESEARCH INSTITUTE, UNIVERSITY OF WAIKATO, PRIVATE BAG 3105, HAMILTON, 3240, NEW ZEALAND, ³WATERWAYS CENTRE FOR FRESHWATER MANAGEMENT, UNIVERSITY OF CANTERBURY, CHRISTCHURCH, 8140, NEW ZEALAND AND ⁴FACULTY OF BIOLOGY, UNIVERSITY OF KONSTANZ, KONSTANZ, D-78457, GERMANY

*CORRESPONDING AUTHOR: susie.wood@cawthron.org.nz

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Microcystis is known to overwinter on sediment surfaces and provide an inoculum to support water column blooms in lakes. There is uncertainty as to whether recruitment is an active process triggered by internal changes in buoyancy, or a passive process induced by resuspension. The effect on *Microcystis* recruitment of ammonium, light and temperature was assessed in laboratory experiments using sediments collected in a small eutrophic lake from two habitats: near-edge (containing *Microcystis* and *Aphanizomenon gracile*) and mid-lake (predominantly *Microcystis*). In all experiments single cells, rather than colonies, accounted for the majority (>55%) of recruited cells. *Microcystis* recruitment was significantly lower ($P < 0.001$) in near-shore samples suggesting that *A. gracile* elicits allelopathic effects on *Microcystis*. In mid-lake samples, *Microcystis* recruitment was significantly higher at moderate ammonium concentrations (0.1, 0.2 and 0.5 mg L⁻¹; $P < 0.001$), at two temperatures (16 and 25°C; $P < 0.001$) and high light intensities (50 and 100 μmol m⁻² s⁻¹; $P < 0.01$). *Microcystis* cells were isolated from sediment in spring, early and late summer and assessed using transmission electron microscopy. The percentage of cell area filled with gas vesicles increased significantly ($P < 0.001$). These data demonstrate that allelopathic interactions, ammonium, light and temperature can individually and synergistically regulate gas vesicle synthesis and *Microcystis* recruitment.

KEYWORDS: allelopathic interactions; cyanobacterial blooms; gas vesicles; light; ammonium; temperature; transmission electron microscopy

INTRODUCTION

Microcystis is a cosmopolitan cyanobacterial genus that forms surface blooms in eutrophic waterbodies (Harke *et al.*, 2016). It has gained notoriety because of its ability to produce cyclic heptapeptides known as microcystins. Microcystins can be hepato-, nephro- and neuro-toxic (Mackintosh *et al.*, 1990; Yu, 1995; Feurstein *et al.*, 2010) and ingestion of contaminated water has caused human and animal fatalities (Carmichael *et al.*, 2001; Codd *et al.*, 2005; Šejnohová and Maršálek, 2012).

In the environment *Microcystis* cells are usually organized in colonies that can consist of many thousands of cells aggregated together with a mucus secretion. One of the reasons for the global success of *Microcystis* is that cells within these colonies contain gas vesicles which enable them to regulate their buoyancy and gain access to vertically separated resources such as light and nutrients (Walsby, 1994). This provides them a competitive advantage over other phytoplankton species unable to migrate through the water column (Ibelings *et al.*, 1991). Buoyancy is primarily regulated through photosynthesis, where excess energy is stored as a carbohydrate ballast and the colonies sink, until respiration sufficiently uses up these stores and cells become buoyant again (Visser *et al.*, 1995).

Another related feature of *Microcystis* that may account for its global dominance is its ability to overwinter in the bottom sediment (Preston *et al.*, 1980; Fallon and Brock, 1981). In temperate environments summer *Microcystis* blooms are followed by autumnal settling of colonies to the bottom sediments due to an increase in cellular carbohydrate content at lower temperatures (Visser *et al.*, 1995). Colonies overwinter on the sediment surface until spring or summer when they are recruited back into the water column which can provide a substantial inoculum for summer blooms (Preston *et al.*, 1980; Verspagen *et al.*, 2005; Torres and Adámek, 2013). However, there is uncertainty as to whether recruitment is an active process triggered by intracellular changes in buoyancy, or a passive process induced by resuspension, or a combination of both mechanisms.

Amongst those advocating for intracellular changes, two scenarios are postulated: gas vesicle synthesis and a reduction in carbohydrate ballast. Gas vesicle synthesis is supported by the work of Šmarda and Maršálek (2008) and Šmarda (2009), who demonstrated that overwintering *Microcystis* cells contain no gas vesicles. They documented an increase in their abundance from spring to mid-summer when recruitment occurs. Whilst there is limited knowledge on the variables which stimulate gas vesicle synthesis, temperature, light and nutrient that have been postulated as triggers (Caceres and Reynolds,

1984; Walsby, 1994; Tsujimura *et al.*, 2000; Ståhl-Delbanco *et al.*, 2003; Tan *et al.*, 2008). Conversely, other researchers have found that benthic *Microcystis* cells always contain gas vesicles (Reynolds *et al.*, 1981) and that cells remain photochemically active on the sediment surface whilst overwintering (Verspagen *et al.*, 2004). Under this scenario rather than gas vesicle synthesis, cells become positively buoyant in spring or summer as environmental conditions such as temperature or light increase and enhance activity levels in combination with depletion of carbohydrate stores.

Others have suggested that benthic recruitment is passively induced by physical mixing by processes such as wind resuspension or bioturbation (Ståhl-Delbanco *et al.*, 2003; Verspagen *et al.*, 2004; Verspagen *et al.*, 2005; Misson and Latour, 2012; Torres and Adámek, 2013). However, unless cells are physiologically active and already contain gas vesicles it seems unlikely that these physical processes alone would explain observed levels of recruitment and allow cells to remain positively buoyant.

Variability in recruitment rates from different habitats within lakes has been documented (Livingstone and Reynolds, 1981; Reynolds *et al.*, 1981; Hansson *et al.*, 1994; Tsujimura *et al.*, 2000). Traditionally, deep environments have been thought to be the most important, as these sites receive the largest proportion of settling cells (Hansson *et al.*, 1994; Tsujimura *et al.*, 2000). However, Brunberg and Blomqvist (2003) showed much higher recruitment rates from shallow bays and suggested that this phenomenon could be due to early access to increased light in these habitats. Few studies have investigated whether there are variable recruitment rates amongst different habitats in shallow lakes. Whilst shallow lakes may vary little in depth, in those where cyanobacteria are dominant, scums are more likely to accumulate along shorelines or in bays, potentially providing a substantial inoculum.

To date research on benthic recruitment has focused mostly on Northern Hemisphere lakes where *Microcystis* persists throughout the summer. Lake Rotorua (South Island, New Zealand) is a shallow eutrophic lake which experiences summer blooms that are initially dominated by nitrogen fixers (*Dolichospermum* and *Aphanizomenon*) before a succession to *Microcystis* blooms later in summer (Wood *et al.*, 2010, 2012). Water temperature in Lake Rotorua is generally warmer (e.g. minimum surface temperature in 2014 was 5.8°C) than the lakes where benthic recruitment studies have previously been undertaken. Because of the observed cyanobacterial succession patterns and less extreme climatic conditions we hypothesized that triggers of benthic recruitment in Lake Rotorua may differ from those previously identified. We

investigated three key questions: (i) Does recruitment vary between near-shore and mid-lake sites in this shallow lake? (ii) Do variations in ammonium concentration, light or temperature trigger benthic recruitment of *Microcystis*? (iii) Do ultrastructural changes occur within *Microcystis* cells during the recruitment period? To answer these questions we undertook a series of laboratory experiments using sediment collected from near-shore (ca. 0.5 m depth) and mid-lake (ca. 2.6 m depth) sites. *Microcystis* recruitment was recorded under different ammonium concentrations, light intensities and temperatures. Transmission electron microscopy (TEM) was used to assess the cellular structure of *Microcystis* cells isolated from sediment samples collected from spring to late summer.

METHOD

Sampling site and sample collection

Lake Rotorua (42°24'05 S, 173°34'57 E) is a small (0.55 km), shallow (max. depth 3 m), eutrophic lake in the northeast of the South Island of New Zealand (Flint, 1975). It is surrounded by farmland, regenerating scrub and native bush, and has one outflow at its southern end (Fig. 1).

Sediment samples were collected for the laboratory experiments with a Ponar grab sampler (0.0225 m²) from six sites on 10 October 2014; three near-shore sites (ca. 0.5 m depth; sites 1–3; Fig. 1) and three mid-lake sites (ca. 2.6 m depth; sites 4–6; Fig. 1). Samples (ca. 40 mL) from the top layer (ca. 1 cm) of the sediment

were transferred to sterile tubes (50 mL) and stored in the dark at 4°C and transported to the laboratory within 24 h.

Enumeration of cyanobacteria in sediment

Subsamples (35 mL) were pooled for the three near-shore sites and the three mid-lake sites. An aliquot (2 mL) from each pooled sediment sample was added to Percoll[®] solution (5 mL; Sigma-Aldrich, USA) and nitrogen-free MLA medium (5 mL; Bolch and Blackburn, 1996) in 15 mL tubes. The tubes were centrifuged (600 × *g*, 15 min) to separate cyanobacterial cells from the sediment. The upper layer (ca. 2 mL) was removed and immediately preserved with Lugol's iodine. Preserved samples were pipetted into 12-well plates (COSTAR, USA) and allowed to settle (ca. 3 h). *Microcystis* single cells and *A. gracile* were enumerated from 1 to 2 transects, at 400× or 600× magnification using an inverted microscope (IX70, Olympus). *Microcystis* colonies were assessed by scanning the entire well at 200× magnification. The colonies were categorized according to their sizes (CS1 = 2–10, CS2 = 11–50, CS3 = 51–100, CS4 = 101–300 and CS5 > 300 cells). The approximate number of cells in each *Microcystis* size class was calculated by multiplying the number of colonies in each size class by the mid-point of each bin, e.g. for CS1 a mid-point of 6 cells was used.

Experimental setup

Ammonium experiment

Ammonium was chosen for the nutrient addition experiments as previous research suggests that nitrogen plays an important role in stimulating gas vesicle synthesis (Chu *et al.* (2007), and *Microcystis* is known to uptake and utilize ammonium rapidly (Blomqvist *et al.*, 1994). Subsamples (2 mL) from the pooled near-shore and mid-lake sediment samples were pipetted separately into gamma-sterilised polystyrene culturing containers (ThermoFisher Scientific, New Zealand) containing aliquots (40 mL) of Milli-Q water or Milli-Q supplemented with ammonium chloride at concentrations of 0.1, 0.2, 0.5, 1 and 5 mg N L⁻¹. Controls and treatments were tested in triplicate. The culture containers were incubated at 19°C (±1°C) at an irradiance of 5 μmol photons m⁻² s⁻¹ on a 12 h:12 h light/dark cycle.

To determine whether dissolved nutrients from the sediments influenced the concentrations in the water, an additional three culture containers were filled with Milli-Q water (40 mL) and set-up as described above. After 48 h, water samples (30 mL) were collected,

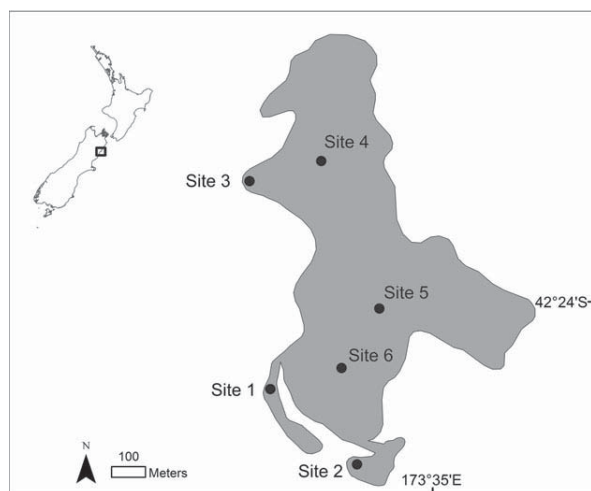


Fig. 1. Map of Lake Rotorua (Kaikoura, New Zealand) and the six sediment sampling sites. Inset: map of New Zealand showing location of the lake.

filtered (Whatman GF/C), and stored at -20°C . These were analysed with a LachatQuickChem[®] flow injection analyser (FIA + 8000 Series, Zellweger Analytics, Inc.) using APHA (2012) 4500 methods for ammoniacal nitrogen ($\text{NH}_4\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), nitrite ($\text{NO}_2\text{-N}$) and dissolved reactive phosphorus (DRP).

Temperature and light intensity experiments

Subsamples (2 mL) of the pooled near-shore and mid-lake sediment samples were pipetted into 40 mL aliquots of Milli-Q water in culture containers (two sets of 30). For the temperature experiments, the culture containers were incubated in triplicate at low irradiance ($5\ \mu\text{molm}^{-2}\text{s}^{-1}$) and at five different temperatures; 4, 13, 16, 19 and 25°C , using temperature-controlled cabinets (SKOPE, 1000 K). For the light experiments, the triplicate culture containers were incubated at 19°C in the dark or at four different light intensities: 1.5, 10, 50 and $100\ \mu\text{molm}^{-2}\text{s}^{-1}$ (12 h:12 h light/dark cycle). The different light intensities were achieved by varying the numbers of layers of high density polyethylene cloth covering the culture containers.

Sampling of benthic recruitment experiment

For all experiments, subsamples (10 mL) were collected from the surface of each culture container after 1, 3, 6, 9 and 12 days, and were immediately preserved with Lugol's iodine. Each container was carefully supplemented with Milli-Q water (10 mL) or the appropriate ammonium chloride solution (ammonium experiment only) to replace the sampled volume, taking care to avoid mixing of sediment. Aliquots of the preserved samples (5 mL) were pipetted into 12-well plates (COSTAR, USA), allowed to settle (ca. 3 h) and enumerated as described above.

Statistical analysis

Linear Mixed Effect Models (LMEMs) were used to test for interactions between habitat (near-shore and mid-lake) and the different environmental variables (ammonium, temperature and light intensity). The three environmental variables and habitat were tested as fixed effects; time was the continuous covariate and sample was a randomized effect to account for repeated measures. The LMEM analysis was undertaken separately on samples from the near-shore and mid-lake habitats using the total *Microcystis* concentration data. The *P*-values were obtained using ANOVA and adjusted post-hoc pair-wise tests (Chi-squared test) were undertaken.

Assessment of the ultrastructure of benthic *Microcystis*

To identify changes in the ultrastructure of benthic *Microcystis* cells (gas vesicles, polyphosphate bodies and structured granules), sediment samples were collected from site 1 (Fig. 1) on 10 October 2014, 26 January 2015 and 29 March 2015 as described above. Sediments were sub-sampled (10 mL) and preserved with 2% glutaraldehyde in 0.1 M sodium cacodylate (40 mL) for later TEM imaging. Samples were stored in the dark at 4°C and processed for 48 h. Surface water samples (250 mL) were collected on the same days, preserved with Lugol's and stored in the dark at 4°C . Cyanobacteria in these samples were identified and enumerated as described above.

In the laboratory, sediment samples were homogenized by gently inverting 10 times. To separate *Microcystis* cells from the sediment, subsamples (ca. 6 mL) were suspended in Percoll solution (1:1 Percoll/MLA medium, adapted from Van Liere and Mur, 1978) and centrifuged ($600 \times g$, 15 min). The supernatant containing the cells (ca. 0.5 mL) was transferred into 1.5 mL tubes and centrifuged ($600 \times g$, 10 min) with 2% glutaraldehyde in 0.1 M sodium cacodylate (1.5 mL) to remove residue sediment. The supernatant was transferred into a new 1.5 mL tube, centrifuged ($1000 \times g$, 5 min), the supernatant discarded and fresh 2% glutaraldehyde in 0.1 M sodium cacodylate (1.5 mL) was added. The samples were gently mixed on a rotating wheel (1 rpm, 1 h, 20°C), centrifuged ($1000 \times g$, 5 min) and the supernatant replaced with 0.1 M sodium cacodylate (1.5 mL). This process was repeated twice to give a total of three washes.

The supernatant was removed and samples were fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (1 h, gentle mixing, 20°C). Samples were centrifuged ($1000 \times g$, 5 min) and the supernatant replaced with 0.1 M sodium cacodylate (1.5 mL). The process was repeated twice more to give a total of three washes. The samples were then dehydrated through a series of ethanol concentrations (50%, 70%, 90%, $2 \times 100\%$) and infiltrated with Spurr's resin, initially diluted 1:1 with 100% ethanol. Infiltration involved three changes of resin over two days. To assist in concentrating the cells in the resin, the samples were centrifuged ($1500 \times g$, 15 min). For the embedding process in the final resin, the resulting pellet was carefully re-suspended in a small volume of the remaining resin supernatant and resin with cells was transferred by pipette to a polyethylene embedding capsule (BEEM capsule). To ensure minimum loss of specimen during the transfer, the processing vial (a 1.5 mL tube) was "rinsed"

repeatedly with small volumes of fresh resin that was then used to top up the BEEM capsule. The resin was cured at 60°C for 48 h.

The cured block was sectioned using a Leica UC6 Ultramicrotome. Sections (0.5 µm-thick) were initially screened for cells under a light microscope (CX31, Olympus). Sections showing cells were recut (80 nm-thick) and placed onto Formvar-covered slot grids. The sections were contrasted with uranyl acetate and lead citrate using an LKB 2168 Ultrastainer. The sections were viewed on a Philips CM100 TEM and images captured using an Olympus/SIS MegaView 3 camera.

Using Standard CellSens (Version 1.0, Olympus), 30 cells from each of the three samples were assessed to measure the biovolume and the percentage of cell area occupied by gas vesicles, polyphosphate bodies and structured granules. Differences in the mean biovolume and the cellular components among collection dates were assessed using one way ANOVA and Tukey's honestly significant difference (HSD) post-hoc tests in R Studio for Windows (Version 0.98.501, Intel Corp).

RESULTS

Recruitment experiment

Total *Microcystis* cell densities in the pooled near-shore and mid-lake sediment samples were 3750 and 3600 cells mL⁻¹, respectively. Fifty-seven percent of cells in the near-shore sample were single cells, compared to 18% for the mid-lake sample. The remainder of the cells were colonies (CS1-CS4 size classes; Supplementary Information Table 1). *Aphanizomenon gracile* filaments (24 800 cells mL⁻¹) were present in the near-shore samples, but none were observed in mid-lake samples.

Average nutrient concentrations from the three Milli-Q samples with sediment, which used to assess the effect of nutrient inputs from the sediment were: NH₄-N = 0.05 mg L⁻¹, NO₃-N = 0.2 mg L⁻¹, NO₂-N = 0.02 mg L⁻¹ and DRP = 0.01 mg L⁻¹. This increase in dissolved nutrient concentrations in the Milli-Q water would have been consistent across all experiments and treatments and therefore had an equal effect on recruitment

In all experiments and all treatments *Microcystis* recruitment was observed on day 1 (Fig. 2). *Microcystis* densities were always lower (ca. 2–5 fold) on day 1 using the near-shore sediment compared to the mid-lake sediment (Fig. 2). At the conclusion of the experiment *Microcystis* cell densities were similar in the near-shore samples among experiments (max. day 12 ca. 2000 cells mL⁻¹; Fig. 2). In contrast, total *Microcystis* cell density in the

mid-lake samples varied, for example, were highest in the 100 µmol m⁻² s⁻¹ light treatment (5380 cells mL⁻¹; 72% of cells in original sediment sample), followed by the ammonium treatment at 0.1 mg N L⁻¹ (4660 cells mL⁻¹; 65%), and lowest in the treatment at 13°C (1570 cells mL⁻¹; 21%; Fig. 2; Supplementary Information Table 2).

Single cells of *Microcystis* were dominant in the near-shore and mid-lake samples in all experiments at each time point (Fig. 3a–f; Supplementary Information Figs. 1–3). Single cells represented >80% of total *Microcystis* density for the ammonium and light intensity experiments, and >55% for the temperature experiment in the near-shore samples at day 12 (Fig. 3a–f). In all mid-lake samples single cells comprised over 90% of the total *Microcystis* density at day 12 (Fig. 3). The range of colony sizes was greatest in the temperature experiment, e.g. after 12 days in the 25°C treatment all six colony size categories were present (Supplementary Information Fig. 2).

Cumulative *Microcystis* recruitment increased significantly with time across all treatments in the ammonium, temperature and light experiments (Time: $P < 0.001$, Fig. 2). The effect of treatment significantly interacted with habitat type (ammonium, temperature or light × habitat: $P < 0.001$, Fig. 2).

Adjusted post-hoc pair-wise tests showed there was no significant difference within treatments in near-shore samples. However, in the mid-lake samples *Microcystis* recruitment was significantly higher in sample: (1) exposed to ammonium concentrations of 0.1, 0.2 and 0.5 mg L⁻¹ compared to the 0 mg L⁻¹, 1 and 5 mg L⁻¹ ($P < 0.01$), (2) kept at 16 and 25°C compared to 4, 13 and 19°C ($P < 0.001$) and (3) exposed to light intensities of 50 and 100 µmol m⁻² s⁻¹ compared to 0, 1.5 and 10 µmol m⁻² s⁻¹ ($P < 0.001$), whilst recruitment in the 100 µmol m⁻² s⁻¹ was also higher than at 50 µmol m⁻² s⁻¹ ($p < 0.01$; Supplementary Information Table 3).

Aphanizomenon gracile (>800 cells mL⁻¹ on day 12) recruitment was observed in all experiments and treatments using near-shore sediment samples, while in general there was very little recruitment from mid-lake samples by day 12 (Fig. 2). After 12 days the highest recruitment was observed in near-shore treatments at 13°C (8210 cells mL⁻¹), followed by ammonium at 0.2 mg N L⁻¹ (4420 cells mL⁻¹), and light at 10 µmol m⁻² s⁻¹ (4020 cells mL⁻¹).

In general the *Microcystis* recruitment rate for all experiments was highest on Day 1 (Fig. 4). In contrast, the recruitment rate of *A. gracile* in near-shore samples was highest on day 9 in the ammonium and temperature experiments for all treatments, except for at 25°C which had the highest recruitment rate on day 3 (Fig. 4). The highest *A. gracile* recruitment rate was recorded on

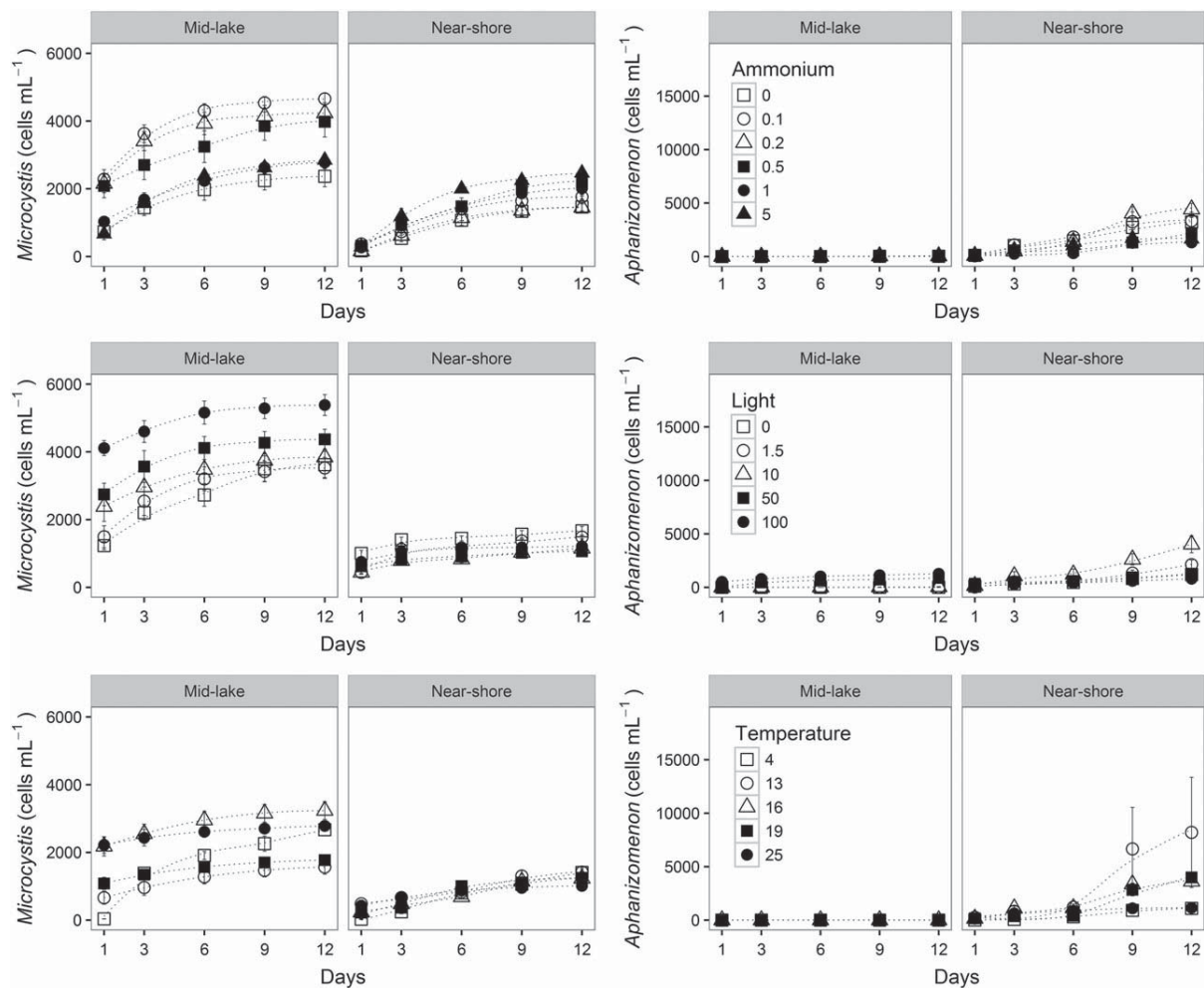


Fig. 2. Total cumulative cell density of *Microcystis* sp. (left) and *Aphanizomenon gracile* (right) in the (top panel) ammonium (mg N L^{-1}), (mid panel) light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$), and (bottom panel) temperature ($^{\circ}\text{C}$) experiments over the 12-day sampling period for the mid-lake and near-shore sediment samples. Note: Y -axis scales differ between the left and right panels.

Day 1 for light treatments of 50 and $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, and Day 12 for 0, 1.5, and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 4). Recruitment rates of *A. gracile* in the mid-lake samples were negligible (Fig. 4).

Changes in the ultrastructure of benthic *Microcystis*

When benthic *Microcystis* cells isolated from the sediment were analysed by TEM, the images showed changes in cellular biovolume and in the abundance of cellular ultrastructure components over the sampling period (Fig. 5). The highest biovolumes were measured in January 2015 and this was significantly reduced by March 2015 ($df = 2$, $F = 4.2$, $P < 0.02$; Table I). The percentage of transverse cell section occupied by gas

vesicles increased significantly from 0.02% to 11.27% over the three sampling time points ($df = 2$, $F = 22.51$, $P < 0.001$; Fig. 5; Table I). The percentage of area occupied by structured granules also varied significantly with cells collected in January 2015 having a lower percentage than those from October 2014 or March 2015 ($df = 2$, $F = 10.04$, $P < 0.001$; Fig. 5; Table I). The polyphosphate bodies percentage area varied markedly among cells regardless of sampling period and no significant differences were observed (Fig. 5; Table I).

Between October 2014 and March 2015, *Microcystis* concentrations increased in the surface water from 0 cells mL^{-1} to $36\,730 \text{ cells mL}^{-1}$ (Table II). This increase in the surface water corresponded with the increase in the abundance of gas vesicles in the sediment-bound *Microcystis* observed by TEM (Fig. 5; Table I).

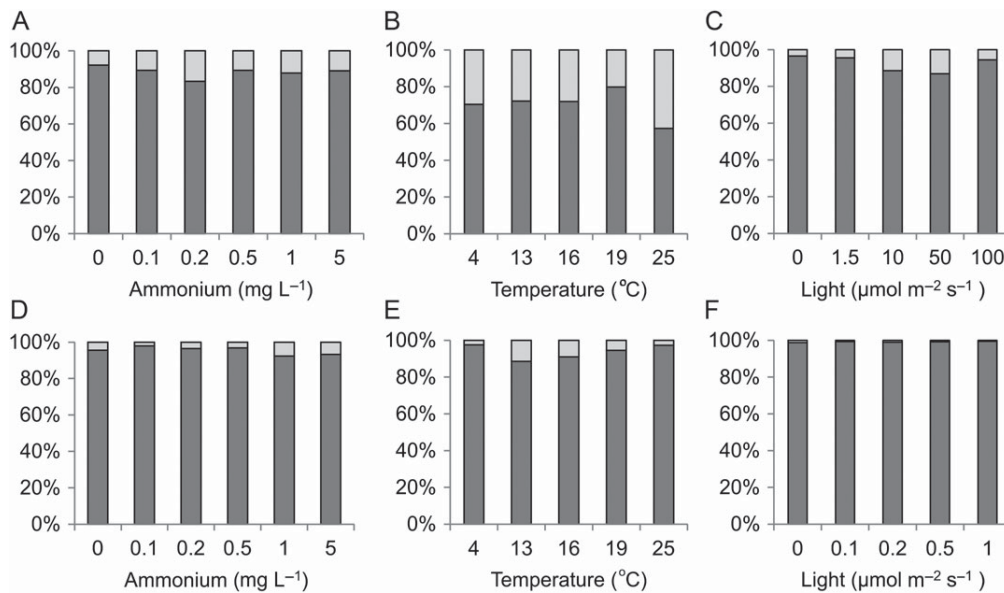


Fig. 3. Relative abundance of *Microcystis* sp. single cells (dark grey) and colonies (light grey: all colony sizes grouped together) at Day 12 of the ammonium, light intensity and temperature experiments, for each treatment. A, B, C are near-shore and D, E, F are mid-lake samples.

DISCUSSION

Previous studies using in-lake migration traps have shown differential recruitment associated with shallow versus deep regions of lakes (Rengefors *et al.*, 2004). Most studies suggest that deep regions are of greater importance as they receive a larger portion of cells that sediment out of the water column (Verspagen *et al.*, 2005). However, Brunberg and Blomqvist (2003) observed high recruitment rates in the shallow bays of Lake Limmaren (Sweden). They attributed this to high concentrations of sinking cells following wind-accumulated blooms, and suggested that these sites are generally exposed earlier than deep sites to environmental triggers, e.g. light and temperature, that promote recruitment. Only a limited number of studies have compared recruitment rates for samples collected from different lake habitats in laboratory-based experiments where environmental parameters can be controlled (Rengefors *et al.*, 2004). In the present study, *Microcystis* recruitment was significantly higher in samples collected from the mid-lake sites compared to near-shore sites. The initial *Microcystis* inoculum in the sediment samples was similar, eliminating inoculum size as a causative factor. Unlike in-lake studies where environmental conditions such as light intensity and temperature can vary between near-shore and mid-lake habitats, in our experiments the samples from both habitat types were exposed to identical conditions. Although there was no difference in the total number of *Microcystis* cells in the

starting inoculums, the near shore sample contained a higher portion of single cells. It is plausible that greater turbulence at this site prior to settlement or faster breakdown rates in the sediment cause this discrepancy. This difference is not the reason for the observed variability in recruitment rates as there was significantly higher recruitment of single cells in treatments inoculated with mid-lake samples.

The most striking difference between the two sample types was the presence of high concentrations of *A. gracile* in the near-shore sediment samples. *Aphanizomenon gracile* was not observed in the mid-lake sediment samples microscopically, although low recruitment rates were recorded which suggests that it was present at very low concentrations (Fig. 2). It is not apparent why the *A. gracile* cell density differed between the near-shore and the mid-lake sediment, but it could be the result of *A. gracile* cells settling after a wind-accumulated bloom along the lake edge.

To explain the decreased *Microcystis* recruitment in the presence of *A. gracile* we suggest two possible scenarios: (1) *A. gracile* out-competes *Microcystis* for resources, thereby constraining recruitment, or (2) *A. gracile* has an allelopathic effect on *Microcystis*, inhibiting processes involved in recruitment. Under scenario 1, while resources such as light and nutrients are adequate there is a decrease in the suppression of *Microcystis*. However, no significant differences in recruitment rates were observed within treatments (ammonium concentration, light intensity or temperature) in the near-shore samples.

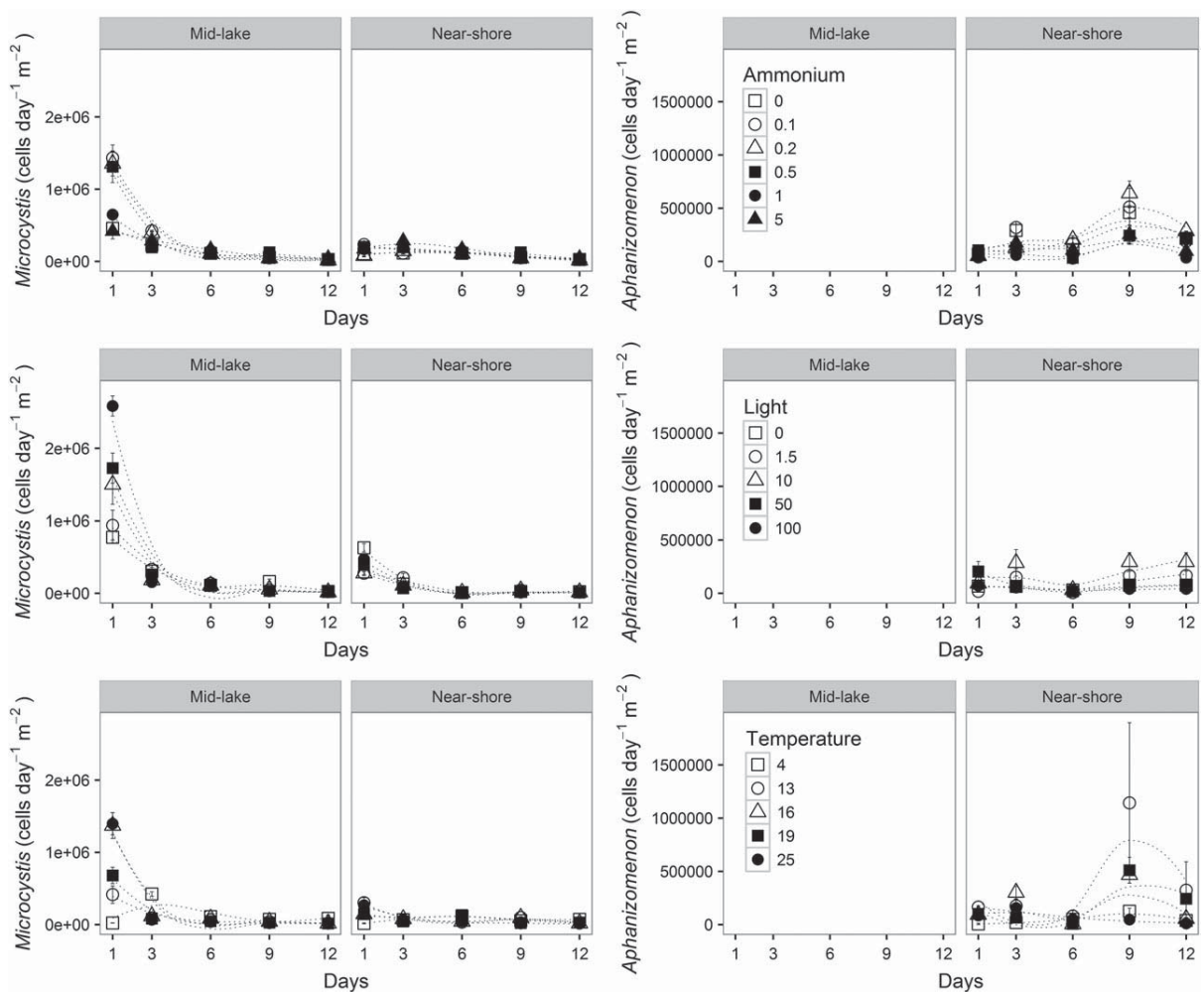


Fig. 4. Recruitment rate (cells day⁻¹ m⁻²) of *Microcystis* (left) *Aphanizomenon gracile* (right) in (top panel) ammonium (mg L⁻¹), (mid panel) light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and (bottom panel) temperature (°C), experiments over the 12-day sampling period.

Extracts of *Aphanizomenon flosaquae* have been shown to have allelopathic effects on *Rhodomonas* sp. by reducing its cellular chlorophyll-a content and CO₂ uptake (Suikkanen *et al.*, 2004, 2006). In the reverse situation to that proposed here, *Microcystis* extracts have been shown to inhibit *A. flosaquae* growth (Ma *et al.*, 2015). Further investigations are required to confirm the potential allelopathic effects of *A. gracile* on *Microcystis* recruitment.

Another possible explanation for the differences in *Microcystis* recruitment between the near-shore and mid-lake samples is that the physiological state of the cells could differ among habitats. Variations in the physiological state may occur, for example, if cells were exposed to differences associated with exposure to anoxia, temperature or decomposition processes. Whilst we did not assess the physiological state of cells prior to commencing the experiments, microscopic analysis of

cells from the sediment samples provided no evidence to support this theory, and unlike other studies the difference in depth (2 m) and distance (2–300 m) between the near-shore and mid-lake sampling sites would be less likely to support major environmental gradients between the two sites.

A substantial difference between the results of this study and those reported previously was the presence of high concentrations of single *Microcystis* cells. To our knowledge, all previous recruitment studies only report on the abundance of *Microcystis* colonies. It is unclear whether single cells were not present in these studies or overlooked due to methodological approaches. For example, some studies report the use of sonication to dissociate colonies and aid in microscopic enumeration (Misson *et al.*, 2011), others filter samples with a mesh size that would not retain single cells or small colonies

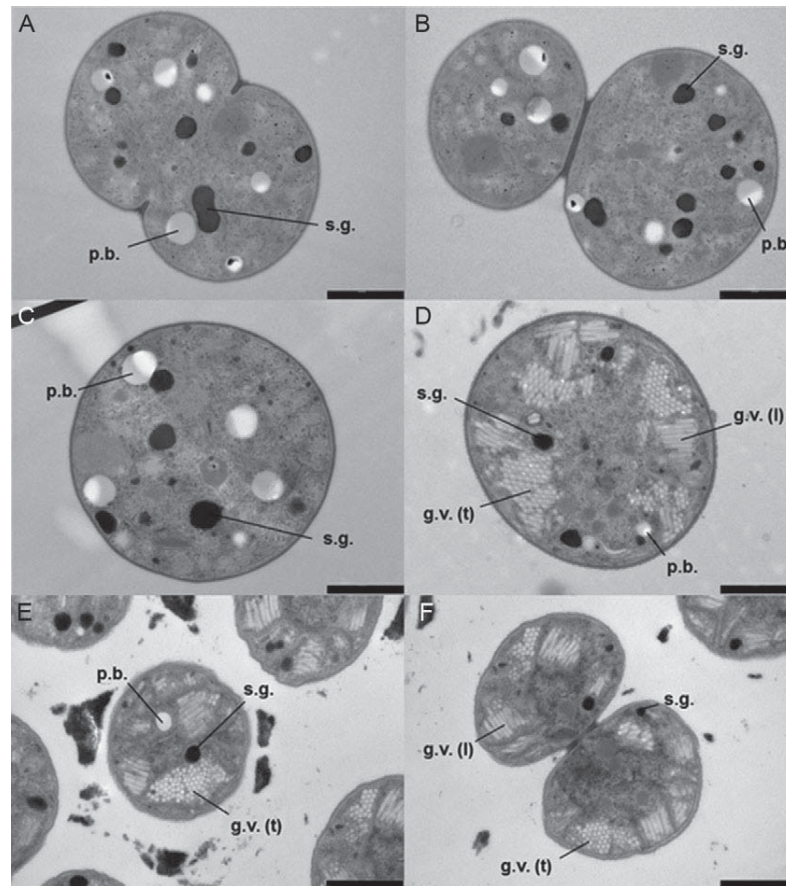


Fig. 5. A selection of the images analysed for the ultrastructure of benthic *Microcystis* cells sampled in October 2014 (**A** and **B**), January 2015 (**C** and **D**) and March 2015 (**E** and **F**) from Lake Rotorua, New Zealand. Scale bar (bottom right) = 1 μm ; s.g. = structured granules; p.b. = polyphosphate bodies; g.v. (t) = gas vesicles (transversal section); g.v. (l) = gas vesicles (longitudinal section). The complete set of images is provided in Supplementary Information Fig. 4–6.

Table I: Microcystis cells biovolumes and percentage of transverse sections of Microcystis cells occupied by gas vesicles (GVs), polyphosphate bodies (PBs), and structured granules (SGs) in each studied month. Data are means ($n = 30$) \pm one standard deviation. Super-scripts indicate Tukey's HSD pairwise test groupings ($P < 0.05$)

Date	Biovolume ($\mu\text{m}^3/\text{cell}$)	GV (%)	PB (%)	SG (%)
Oct-14	19.01 \pm 6.77 ^{AB}	0.02 \pm 0.12 ^A	4.22 \pm 2.24	4.78 \pm 2.06 ^A
Jan-15	22.34 \pm 6.42 ^B	1.31 \pm 6.62 ^A	2.89 \pm 3.14	2.54 \pm 1.69 ^B
Mar-15	17.74 \pm 5.81 ^A	11.27 \pm 10.37 ^B	4.08 \pm 3.56	4.98 \pm 3.05 ^A

(63 μm ; Tsujimura *et al.*, 2000), and many have used magnifications of less than 400 \times for enumerations, which would likely prevent identification of single cells (Misson and Latour, 2012). In our study, single

Microcystis cells accounted for greater than 55% of total cell concentrations in all experiments and treatments, and we recommend that future studies ensure methods are adequate to enable their detection. Previous studies have shown that single cells can aggregate relatively rapidly (Yang *et al.*, 2006), thus enabling colony formation and activation of some of the advantages it confers post recruitment (e.g. buoyancy regulation and predation defence; Oliver, 1994; Wu and Song, 2008; Yang and Kong, 2012). During our study, single cells were recruited from the sediment faster than colonies. Cells within colonies may be exposed to environmental gradients, whereas cells in the middle of a colony receive reduced light and access to nutrients compared to those on the outside (Paerl, 1983; Mulling *et al.*, 2014). Light, nutrients and other possible environmental triggers may play a role in initiating gas vesicle synthesis (Walsby, 1994). Theoretically, if gas vesicle synthesis is delayed in central cells of a colony these may collectively “weigh

Table II: Cyanobacterial concentrations (cells mL⁻¹) in surface water samples from Lake Rotorua collected on the same day as sediment samples (Table I)

Date	<i>Dolichospermum</i> sp.	<i>Aphanizomenon gracile</i>	<i>Aphanocapsa</i> sp.	<i>Microcystis</i> sp.
Oct-14	1210	102 150	0	0
Jan-15	2 260	25 090	4 782 000	16 460
Mar-15	19 720	11 690	544 200	36 730

down” the colony and delay buoyancy. Evidence to support our theory comes from several studies which have shown a higher abundance of small colony sizes (ca. 2–8 cells) in newly established planktonic populations (Kurmayer *et al.*, 2003; Tao *et al.*, 2005).

Recruitment of *Microcystis* in the laboratory experiments was rapid, with the highest rates occurring within 24 h (Fig. 4). Similar recruitment rates have been observed in laboratory studies even when colonies have been buried in sediment for almost three years (Misson and Latour, 2012). The TEM analysis (discussed below) suggested that when cells were collected for the benthic recruitment experiments, the percentage containing gas vesicles was low (ca. 0.02%). Lehmann and Jost (1971) demonstrated that gas vesicle synthesis can occur rapidly (within 12 h). Whilst we did not assess gas vesicle formation during our experiments, the high recruitment rates after 24 h support the suggestion of rapid gas vesicle synthesis.

The absence of *A. gracile* from the mid-lake samples enabled us to assess the relative importance and effects of varying ammonium concentrations, light intensity and temperature on *Microcystis* recruitment. Recruitment occurred in all experiments and treatments, even in conditions we had anticipated would not facilitate it, such as complete darkness. These data indicate that multiple factors may regulate gas vesicle synthesis in Lake Rotorua. Within each experiment significant differences among treatments were observed. In the ammonium experiment recruitment was highest in the mid-range of concentrations (0.1, 0.2 and 0.5 mg L⁻¹). Ståhl-Delbanco *et al.* (2003) observed a similar response in enclosure experiments, with the highest *Microcystis* recruitment recorded at intermediate concentrations of dissolved inorganic nitrogen concentrations (ca. 0.1 and 0.5 mg L⁻¹). Likewise, Chu *et al.* (2007) observed significant decreases in gas vesicle abundance when nitrogen was limited, which highlights a likely role for this nutrient in their synthesis.

Temperatures above 15–20°C have repeatedly been implicated in promoting *Microcystis* recruitment (Tsujimura *et al.*, 2000; Tao *et al.*, 2005; Yamamoto, 2009). Some studies have shown discrete thresholds below which no recruitment occurs; e.g. <9°C Tang *et al.* (2010), <14°C Tao *et al.* (2005), and <15°C Li *et al.* (2003). The results of our study, which showed significantly greater recruitment at 16°C and 25°C, are consistent with enhanced recruitment

at higher temperatures. Reasons for the lower recruitment at 19°C are unknown and further investigation is required to explore whether this pattern is consistently observed. In contrast to some previous studies, recruitment was still observed at low temperatures (4°C). In these samples recruitment rates were markedly lower and delayed. It appeared that the low light used in this experiment, regardless of temperature, was sufficient to stimulate recruitment, albeit with a delay and at reduced levels.

In-lake studies have demonstrated the importance of light penetration to bottom sediment as a factor for stimulating recruitment (Reynolds *et al.*, 1981; Brunberg and Blomqvist, 2003). In our study, as *Microcystis* recruitment was significantly increased at the two highest intensities tested (50 and 100 µmol m⁻² s⁻¹). Furthermore, of the three environmental parameters tested, high light resulted in the greatest total recruitment. Deacon and Walsby (1990) showed that the optimal photon irradiance for the production of new gas vesicles in *Microcystis* is 35 µmol m⁻² s⁻¹ with a marked decrease at higher intensities. This was in contrast to the observations of our study, although we did not test the specific intensity (the closest were 10 and 50 µmol m⁻² s⁻¹) and this optimum may therefore have been missed. Although lower than the other light intensities tested, recruitment was still observed in the samples maintained in darkness. Deacon and Walsby (1990) demonstrated that some gas vesicle production can occur in darkness, but only if the cells have accumulated energy reserves. If the cells used in our experiment had maintained some photochemical activity on the sediment surface during overwintering (Verspagen *et al.*, 2004) then this may explain the ability of these cells to recruit to the water column. Thomas and Walsby (1986) also showed that non-buoyant colonies regained buoyancy in the dark, and that this was enhanced when temperatures were 20°C.

Significant differences were observed in the cellular structure of the *Microcystis* cells isolated from the sediment during spring, early and late summer. Most notable was the low number of gas vesicles in samples collected in spring. Similar observations have been made by Šmarda and Maršálek (2008) and Šmarda (2009) who observed no gas vesicles in overwintering populations. In contrast Reynolds *et al.* (1981) collected

colonies from sediment over a three-year period and found that every cell examined contained gas vesicles. Such discrepancies between studies highlight that the processes involved in benthic *Microcystis* recruitment likely vary between lakes, with factors such as competition (e.g. with other cyanobacterial or algal species), the presence of organisms involved in bioturbation (e.g. fish), water temperature, depth and nutrient status synergistically influencing recruitment.

CONCLUSIONS

Many previous studies have investigated the effect of one or several physical or chemical variables on *Microcystis* recruitment. This study has highlighted the interactive nature of the variables which regulate *Microcystis* recruitment, and that the relative importance of these can differ among lakes. We have demonstrated, for the first time, that even in a small shallow lake there is variability in the rate of recruitment of benthic *Microcystis* collected from different habitats. Recruitment was significantly reduced in the near-shore samples compared to the mid-lake samples, and the main difference between habitat types was the presence of high densities of *A. gracile* in the initial sediment sample. Species from this genus are known to elicit allelopathic effects on other algae and the possibility that *A. gracile* inhibits *Microcystis* recruitment requires further investigation. To our knowledge, this is the first study to show a direct effect of another organism on recruitment of benthic *Microcystis*. A major difference in this study compared to those reported previously was the high percentage of recruited single cells, as opposed to colonies. Single cyanobacterial cells may have more immediate access to factors that stimulate gas vesicle synthesis than colonies and may therefore regain buoyancy more rapidly. This aspect of benthic *Microcystis* recruitment points to the ongoing need to better understand and differentiate inter-colony variations (Mulling *et al.*, 2014). In the mid-lake samples ammonium concentration, temperature and light intensity all had effects on recruitment, suggesting that all these parameters individually or synergistically regulate recruitment, with exposure to high light intensities promoting the greatest recruitment. The very low abundance of cells with gas vesicles in the sediment in spring (the inoculum used for laboratory experiments) suggested that synthesis of these cellular structures is necessary for benthic *Microcystis* recruitment in Lake Rotorua.

SUPPLEMENTARY DATA

Supplementary data can be found online at <http://plankt.oxfordjournals.org>

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