Fine-scale cryogenic sampling of planktonic microbial communities: Application to toxic cyanobacterial blooms

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Abstract

A lack of fine-scale methods for sampling planktonic microbial populations hinders advancement in understanding the responses of these communities to environmental conditions. Current methods provide resolution at scales of centimeters to meters, but not at the millimeter-scale required to understand highly stratified communities. To address this we developed two cryogenic sampling tools to collect spatially-precise samples from aquatic environments while simultaneously preserving the microbial communities. The application of these samplers was examined over a 5.5 h period using a cyanobacterial scum (*Microcystis*) formed in experimental mesocosms. A cryogenic "surface snatcher" collected a discrete layer (ca. 1 mm) of surface water. Compared to conventional surface sampling methods, the surface snatcher samples contained up to 22-times more microcystin, indicating that less underlying water was incorporated into the sample. A cryogenic "cold finger" sampler was used to collect vertical profiles of the upper 40 mm of the water column. This profiler provided new insights into the fine-scale structure of *Microcystis* scums, demonstrating that more microcystin-producing *Microcystis* was contained in the surface 5 mm than the 35 mm below. The results also showed that upregulation of microcystin production was highly localized in the top 2.5 mm of the *Microcystis* scum. Our results demonstrate that extreme changes in cyanobacterial communities can occur over small distances, and indicate that sampling resolution is of great importance for improving knowledge on cyanobacterial blooms and toxin production. While this study focused on microcystin-producing *Microcystis*, the cryogenic sampling tools described here could be applied to any planktonic microbial community.

Introduction

Cyanobacterial blooms are an increasing problem globally. Significant research effort is being devoted to understanding their dynamics and the processes that lead to cyanobacterial bloom-formation and regulate toxin production. Some cyanobacteria, in particular *Microcystis*, can form dense accumulations (or scums) on the surface of lakes and reservoirs (Harke et al. 2016) and frequently produce the hepatotoxin microcystin (MC; Botes et al. 1984; Sivonen and Jones 1999; Tillet et al. 2000; Puddick et al. 2014). Microcys-

tins can accumulate in the liver of mammals, causing cell structure damage, potentially leading to liver tumors (Falconer and Yeung 1992; Fujiki and Suganuma 1993), and in high doses can result in death (Dawson 1998). The environmental triggers of MC production have not been fully elucidated, partly because of the coarse spatial and temporal scales adopted in most investigations (Oliver et al. 2012).

When *Microcystis* cells aggregate on the water surface and scums form, microcystin production can be markedly upregulated (18- to 28-fold; Wood et al. 2011, 2012). The low spatial precision of the conventional sampling techniques poses a severe limitation for accurately capturing these aggregations because biomass may vary on scales of millimeters. When surface water samples are collected using conventional methods (e.g., using grab samples or removing the surface layer with Pasteur pipettes), underlying water is introduced into the sample, potentially changing or diluting the microbial composition and toxin concentrations. Current methods for collecting vertical profiles of the water column have been designed for resolution at scales of meters (Van Dorn 1956) or

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Additional Supporting Information may be found in the online version of this article.

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centimeters (Baker 1970), and new sensor methods for phycocyanin measurements also have spatial precision of centimeters; not the millimeter-scale required to assess the variability of surface blooms. Furthermore, when operating in the field, there is often a delay before samples can be preserved, potentially leading to changes in the biological and chemical constituents of the samples.

Our aim was to address the need for a device capable of collecting meaningful samples where there are distinct spatial gradients in planktonic microbial communities. We developed two cryogenic sampling tools (cryo-samplers) to sample at higher spatial-precision (millimeter-scale) than has previously been obtainable, while simultaneously preserving the sample at low temperature ($-196°C$). In the present study we demonstrate the application of these tools to understand the processes stimulating microcystin production during cyanobacterial scum-formation.

Materials and procedures

Cryo-sampler design and construction

Two different cryo-sampling tools and a sectioning plate were designed in-house and manufactured by Topliss Bros Precision Engineering Ltd. (Nelson, New Zealand). The cryogenic samplers allow lake water to be frozen rapidly onto cooled metal surfaces with minimal disturbance, the ice sheets containing the samples can subsequently be removed for analysis. The efficacy of these tools was tested by sampling an experimentally formed *Microcystis* scum in on-land mesocosms.

A surface sampler (hereafter referred to as the "surface snatcher"; Fig. 1a) was designed to capture a discrete sample from the top 1 mm of surface water. It consisted of a 300 mm length of metal rod attached to a 50×50 mm stainless steel plate (thickness 4 mm). The steel plate was drilled with 16 holes (3 mm diameter), to prevent air bubbles forming between the steel plate and the water. The handle was insulated with polyvinyl chloride (PVC) tape. Further schematics for the surface snatcher are provided in Supporting Information S1 and a 3-dimensional rendering is provided in Supporting Information S2.

The vertical profiler (hereafter referred to as the "cold finger"; Fig. 1b) was designed to profile the top 4 cm of the water column. It consisted of a square aluminum tube of width 25.4 mm and length 200 mm which was sealed at one end with stainless steel plate (thickness 2 mm). The bottom 56 mm of the sampler (closest to the sealed end) was polished to aid in removal of the ice sheets formed against the metal surfaces. Several prototypes of the cold finger were tested, including designs where the end of the tube was sealed flush with the base. The design presented here has a 5 mm recess in the base which results in a gap in ice formation, allowing the vertical profile to be removed and processed in the field. An insulating handle was formed on the cold finger by wrapping the top portion of the sampler with

PVC tape. Further schematics for the cold finger are provided in Supporting Information S3 and a 3-dimensional rendering is provided in Supporting Information S4.

The "field sectioning plate" (Fig. 1c) was designed to allow vertical profiles removed from the cold finger to be sectioned in the field, by melting the ice sheets into the troughs of the device. It was constructed from a piece of aluminum plate of width 50 mm, length 115 mm and height 8 mm. The plate was drilled-out with 26 troughs, 2 mm wide and separated at 0.5 mm intervals. Further schematics for the sectioning plate are provided in Supporting Information S5 and a 3-dimensional rendering is provided in Supporting Information S6.

Scum-formation mesocosm experiment

To validate the effectiveness of the cryo-samplers, samples were collected from *Microcystis* scums formed in on-land mesocosms on 4 May 2015. Three high-density polypropylene containers were filled with lake water (40 L) and a concentrate of *Microcystis* cells (10 L) collected from Lake Horowhenua (Levin, New Zealand) using a plankton net (20-μm mesh size). The water and cells were mixed for 30 min using aquarium pumps. When mixing ceased at 11:45 h (hereafter referred to as 0 h), a *Microcystis* scum gradually formed on the surface. Surface samples were collected at 11:45, 13:45, 15:15, and 17:15 h (sample times 0, 2, 3.5, 5.5 h) from two of the mesocosms using a conventional pipette surface sampling method (described below) and the surface snatcher. Vertical profiles were collected from the third on-land mesocosm at the same sampling times (0, 2, 3.5, 5.5 h), using the cold finger cryo-sampler. Cold finger samples were sectioned immediately using the field sectioning plate.

Collection, preservation and extraction of surface water samples by pipette surface sampling method

Surface water samples were collected by carefully aspirating the surface layer of the water using a 3 mL Pasteur pipette (Interlab). A subsample for total microcystin analysis (1 mL) was placed in a cryo-vial (Nunc), frozen immediately in liquid nitrogen and stored at $-20°C$ until microcystin extraction. A subsample for extracellular microcystin analysis (1 mL) was prefiltered (20-μm netting) and syringe filtered (Whatman GF/C) into a microcentrifuge tube, before being frozen at $-20°C$.

The samples for total microcystin analysis were supplemented with formic acid (Merck) to a final concentration of 0.1% (v/v) and extracted using four freeze-thaw cycles which were interspersed with sonication (30 min; Kudos Ultrasonic Cleaner). The extract was diluted with 50% methanol (Fisher Scientific) + 0.1% formic acid (v/v; either 1/5 or 1/20 depending on expected sample concentration), sonicated for 30 min and clarified by centrifugation ($12,000 \times g$ for 5 min, 10°C; Eppendorf 5415R). The extracellular microcystin samples were thawed at ambient temperature and centrifuged ($12,000 \times g$ for 5 min, 10°C; Eppendorf 5415R). The supernatants from the total microcystin extracts and extracellular microcystin samples were transferred to septum-capped glass
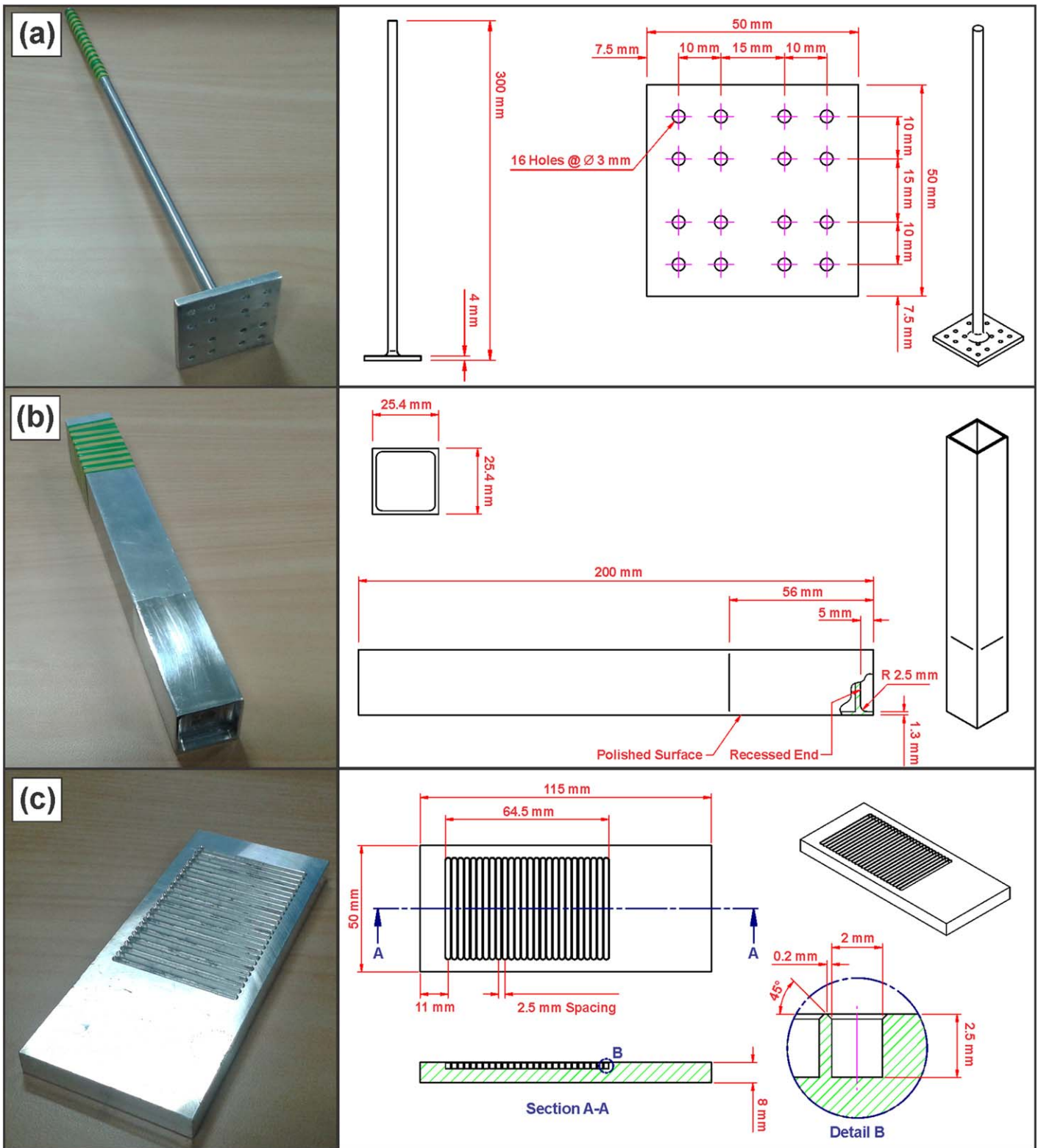
Fig. 1. Images and schematics of **(a)** the "surface snatcher" cryo-sampler, **(b)** the "cold finger" cryo-sampler, and **(c)** the "field sectioning plate".

Fig. 2. Images of vertical profiles collected using the "cold finger"; **(a)** immediately after being removed from the sampler, **(b)** after being separated into four ice sheets, and **(c)** melted into the troughs of the field sectioning plate.

vials (Phenomenex) and stored at −20°C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (within 1 week).

Collection of surface water samples using the surface snatcher

Surface water samples were collected using the "surface snatcher" by submerging the steel plate of the sampler in liquid nitrogen until the temperature equilibrated (when vigorous bubbling ceased; approximately 30 s; −196°C). The chilled surface snatcher was placed carefully on the surface of the water and held for 4 s before being removed. This duration allowed ca. 1 mm of surface water to freeze onto the plate, the equivalent of ca. 2.5 mL of sample; longer/shorter durations would increase/decrease the thickness of the ice sheet. The ice sample was transferred to a plastic sample container (500 mL) and crushed into shards to reduce the thawing time. The thawed samples were transferred to a cryo-vial (Nunc) and refrozen in liquid nitrogen. Samples were stored at −20°C until deoxynucleic acid (DNA) and microcystin extractions.

Collection of vertical profiles using the cold finger

Vertical profiles of the top 4 cm of the water column were collected by suspending the cold finger in the water column using a retort stand clamp. The sampler was positioned so that the bottom polished surface was 5 cm below the water and ca. 1 cm from the bottom of the profile was sacrificed to ensure consistent profiles between time-points. The apparatus was positioned while the mesocosms were still being mixed, to allow the *Microcystis* scum to form around the samplers. Each profile was acquired by dispensing ca. 40 mL of liquid nitrogen into the cavity of the sampler, waiting 20 s and withdrawing the sampler from the water. The 40 mL volume of liquid nitrogen was the maximum that could be used before drops of boiling liquid nitrogen would "spit" from the end of the sampler. The 20 s duration allowed a sheet of ice

of ca. 1 mm thickness to form on the outside of the sampler; longer/shorter durations would increase/decrease the thickness of the ice. The vertical profile was removed from the sampler in the field by dispensing warm water (ca. 25°C) into the cavity of the sampler and applying gentle pressure with tweezers to dislodge the ice. As the vertical profile was produced around the outside of the sampler, it resembled a box when removed (Fig. 2a). The four sheets of ice which formed this box were separated by applying uniform pressure on a flat surface along a single edge (Fig. 2b).

Vertical profiles were immediately sectioned in the field using the field sectioning plate. The ice sheets were laid over the sectioning plate with the top of the vertical profile in line with the top of a sectioning trough. The ice sheets quickly melted, partitioning the encapsulated cyanobacteria into the troughs corresponding to different depths below the surface (Fig. 2c). The samples were transferred into 0.2 mL tubes using wide-bore 200 μL pipettes tips and stored at −20°C until DNA and microcystin extractions. In the present study, ten samples were collected from each vertical profile; six at 2.5 mm increments from 0 mm to 15 mm, three at 5 mm increments from 15 mm to 30 mm and one from 30 mm to 40 mm.

Extraction of microcystin and DNA from the cryo-samples

In the laboratory, the samples collected using the surface snatcher and cold finger were defrosted at ambient temperature. The entire sample or 150 μL aliquots (when samples were > 150 μL) were transferred to new 0.2 mL tubes. The samples were heated at 99°C for 1 min in a polymerase chain reaction (PCR) thermal cycler (Eppendorf Mastercycler). An aliquot for microcystin analysis was placed in a 1.8 mL tube and an aliquot for molecular analysis in a 0.2 mL tube.

The aliquot for microcystin analysis was further extracted by sonication in 50% methanol + 0.1% formic acid (v/v) for

30 min (volumes were dependent on the expected dilution required, generally 1/10, 1/20 or 1/50). The extracts were clarified by centrifugation (12,000 $\times$ g for 5 min, 10°C; Eppendorf 5415R) and transferred to septum-capped glass vials. Samples were stored at -20°C until LC-MS/MS analysis (within one week).

The aliquot for molecular analysis was supplemented with 1% Tween-20 (v/v; Sigma) at a ratio of 9 : 1 (sample:Tween-20) to yield a final concentration of 0.1% Tween-20. The DNA samples were heated again at 99°C for 1 min in a PCR thermal cycler and were clarified by centrifugation (12,000 $\times$ g for 5 min, 10°C; Eppendorf 5415R). The supernatant was transferred to a new 0.2 mL tube and stored at -20°C until quantitative-PCR (qPCR; within six months). As DNA purification was not conducted on the samples for molecular analysis, spectrophotometric determination of the DNA content was not possible.

Quantitative PCR assay for microcystin-producing cyanobacteria

The concentration of microcystin-producing *Microcystis* was determined using a qPCR assay targeting the microcystin synthase E (*mcyE*) gene involved in microcystin production.

To generate qPCR standards, a culture of *Microcystis* CAWBG617 (RotoD) isolated from Lake Rotorua (Kaikoura, New Zealand; Rogers 2014) was grown in a plastic culturing container (100 mL) in MLA medium (Bolch and Blackburn 1996) under a light regime of 100 μmol·s^{-1}·m^{-2} with a 12 h : 12 h light/dark cycle and at a temperature of 25°C $\pm$ 1°C. When the culture was in the exponential growth phase, a subsample (10 mL) was preserved in Lugol's iodine for microscopic cell enumeration (as described below) and another subsample (50 mL) was frozen in a thin layer at -80°C. The frozen sample was thawed and DNA samples were prepared as described for cryo-samples; five aliquots (150 μL each) were boiled and combined into a 1.8 mL tube. This was supplemented 9:1 with 1% Tween-20 (v/v) and boiled again. The DNA extract was clarified by centrifugation and aliquots were stored at -20°C until required. The sample preserved for cell enumeration was diluted 10-fold in MQ-water and a known volume was pipetted into a 12-well plate (COSTAR). After the cells had settled (ca. 3 h), 10 random fields of view were counted at 800$\times$ magnification using an inverted microscope (CX41, Olympus). The average count from the 10 fields of view was used to determine the cell concentration in the original solution.

All samples were screened in duplicate for PCR inhibition using an internal control assay. Each 12.5 μL reaction contained 6.25 μL KAPA Probe Fast qPCR Master Mix (2$\times$), 1 μL of primers targeting the internal transcribed spacer (ITS) region 2 of the rRNA gene operon of *Oncorhynchus keta* salmon sperm (0.4 μM, Sketa-F2 and Sketa-R3; Integrated DNA Technologies; Haugland et al. 2005), 1 μL TaqMan probe synthesized with a FAM reporter dye at the 5′-end and

a Black Hole Quencher 2 at the 3′-end (0.2 μM; Sketa P2; Integrated DNA Technologies; Haugland et al. 2005), 1 μL extracted salmon sperm DNA (15 ng; Sigma) and 1 μL of template DNA. Reactions were analyzed on a Qiagen Rotor-Gene Q real-time PCR machine with the cycling profile; 95°C for 3 min, followed by 50 cycles of 95°C for 3 s and 58°C for 10 s. When PCR inhibition was observed, samples were diluted (1/10) and reanalyzed.

The *mcyE* qPCR assay was undertaken in triplicate for each sample in 12.5 μL of reaction mix containing 6.25 μL KAPA Probe Fast qPCR Master Mix (2$\times$), 1 μL of primers targeting a region within the *mcyE* open reading frame of the microcystin synthase gene (0.4 μM, mcyE-F2 and MicmcyE-R8; Integrated DNA Technologies; Vaitomaa et al. 2003), 0.2 μL of *mcyE* probe (Integrated DNA Technologies; Rueckert and Cary 2009) and 1 μL of template DNA per sample. The CAWBG617 qPCR standard was used to generate four-point calibration curves ranging from 9.4 $\times$ 10^3 to 4.7 $\times$ 10^6 cells mL^{-1}. Each point of the standard curve was analyzed in duplicate for each qPCR run. The standard curves were linear ($R^2 > 0.98$) and PCR efficiencies were > 0.8, consistent with the expectations of the original assay (Vaitomaa et al. 2003). When cell concentrations decreased below the standard detection limit (9400 toxic cells mL^{-1}), samples were reanalysed using 5 μL of template DNA to increase the sensitivity of the assay by fivefold.

Quantification of microcystins

Microcystin concentrations in all samples were determined by LC-MS/MS. Compounds were separated on an Acquity I-Class ultra-performance liquid chromatography system (Waters Co.) using a C$_{18}$ column (Waters Acquity BEH-C$_{18}$, 1.7-μm, 50 $\times$ 2.1 mm) maintained at 40°C in a column oven. Sample components were eluted using a flow rate of 0.4 mL min^{-1} and a gradient of 10% acetonitrile (mobile phase A) to 90% acetonitrile (mobile phase B), each containing 100 mM formic acid and 4 mM ammonia. The samples were injected at 5% B and held for 12 s before a linear gradient up to 35% B over 24 s, to 50% B over a further 72 s and to 65% B over a final 42 s, before flushing with 100% B and returning to the initial column conditions. Sample components were analysed on a Xevo-TQS mass spectrometer (Waters Co.) operated in positive-ion electrospray ionization mode (source temperature 150°C; capillary voltage 1.5 kV; nitrogen desolvation gas 1000 L h^{-1} at 500°C; cone gas 150 L h^{-1}). The microcystin/nodularin congeners assessed were nodularin-R, MC-RR, didesmethyl MC-RR, dimethyl MC-RR, MC-YR, MC-LR, didesmethyl MC-LR, desmethyl MC-LR, MC-AR, MC-FR, MC-WR, MC-RA, MC-RAba, MC-LA, MC-FA, MC-WA, MC-LAba, MC-FAba, MC-WAba, MC-LY, MC-LW and MC-LF. Multiple reaction monitoring channels assessing the *m/z* 135 fragment ion from the protonated molecular cations were used to quantify each toxin
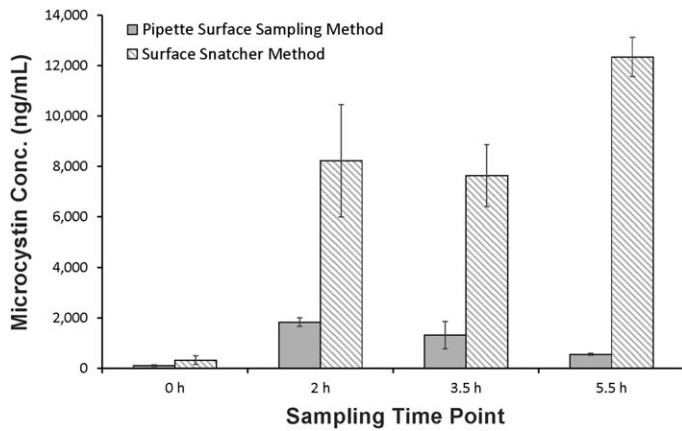
Fig. 3. Microcystin concentration for surface water samples collected from duplicate scum-formation mesocosm experiments using the pipette surface sampling method and the "surface snatcher" cryo-sampling method (Error bars indicate the minimum and maximum values observed; $n = 2$).

($[M + 2H]^{2+}$ for MC-RR and variants; $[M + H]^{+}$ for the others).

Primary standards of Nodularin-R, MC-RR, MC-YR and MC-LR (DHI Lab Products) were used to produce external calibration curves (2–100 ng mL^{-1}) and quantify the microcystin/nodularin congeners observed in the samples. Nodularin-R, MC-YR and the MC-RR congeners were calibrated from the Nodularin-R, MC-YR and MC-RR calibration curves respectively. The MC-LR calibration curve was used to quantify the other congeners. When sample concentrations were outside of the standard curve, the samples were diluted with 50% methanol and reanalysed. Microcystin quotas (the amount of microcystin per toxic cell) were calculated by summing the concentration of all congeners observed in the samples and dividing by the concentration of toxic cells (determined using *mcyE* qPCR).

Assessment

Collection of surface water samples using the surface snatcher

The mesocosm samples collected using the surface snatcher contained between 3- and 22-fold more microcystin than those collected using the Pasteur pipette (Fig. 3). The percentage deviation for the pipette surface sampling method ranged from 8% to 41% with an average of 21% over the four time points. A comparable deviation was observed with the surface snatcher samples, ranging from 6% to 53% with an average of 26%. When a more homogenous solution (i.e., an aqueous cyanobacterial extract) was sampled for microcystins using the two techniques the deviation observed was 4.4% for the pipette method and 6.1% using the surface snatcher (Supporting Information S7).

Microscopic enumeration was not possible for the surface snatcher samples as the cells were disrupted/lysed by ice

Table 1. Total and extracellular microcystin (MC) results for the pipette samples collected from the scum-formation mesocosm experiment.

Sample	Total MC (ng mL^{-1})	Extracellular MC (ng mL^{-1})	% Extracellular MC
0 h—A	187	0.6	0.32%
0 h—B	101	0.5	0.49%
2 h—A	8034	1.4	0.02%
2 h—B	6890	18.3	0.27%
3.5 h—A	2954	5.9	0.20%
3.5 h—B	6140	2.0	0.03%
5.5 h—A	2558	1.4	0.05%
5.5 h—B	2460	6.9	0.28%

crystals which form during the freezing/melting process. To overcome this, a qPCR assay for the *mcyE* gene was trialled with the surface snatcher samples. However, the qPCR assay results were not repeatable between days, some samples did not amplify and the cell concentrations obtained were not consistent with those obtained from the cold finger. For example, with the 5.5 h time point, similar microcystin concentrations were observed in the surface snatcher samples (12,350 ng mL^{-1}) and the cold finger sample from the top 2.5 mm (13,126 ng mL^{-1}). However, the *mcyE* results from the surface snatcher samples were ca. 70,000 *mcyE* copies mL^{-1} and for the cold finger ca. 4,000,000 *mcyE* copies mL^{-1}.

Despite the apparent underestimation of *mcyE* copies mL^{-1} in the surface snatcher samples, PCR inhibition was not observed when assessed using the internal control assay and by fortification of the samples with *mcyE* DNA standard. Attempts were made to improve the PCR amplification by supplementing the qPCR reaction mix with bovine serum albumin and using qPCR master mixes tailored to samples with high levels of PCR inhibitors. The samples were also "cleaned-up" using the MO BIO Power Soil DNA isolation kit, but none of these processes improved the repeatability of the qPCR results or amplification for the surface snatcher samples.

To determine whether cell lysis occurring during the mesocosm experiment (followed by degradation of the exposed DNA) was a factor in the poor qPCR amplification, the level of extracellular microcystin in the pipette samples was assessed. During the experiment, extracellular microcystin was consistently $< 0.5\%$ of the total microcystin measured (Table 1). As microcystins are relatively stable, this indicates that cell lysis levels remained low during the experiment.

Collection of vertical profiles using the cold finger

The concentrations of microcystin sampled in the top 1 cm of the water column with the cold finger increased 15- to 48-fold (114 ng mL^{-1} to 5527 ng mL^{-1}; Fig. 4a and
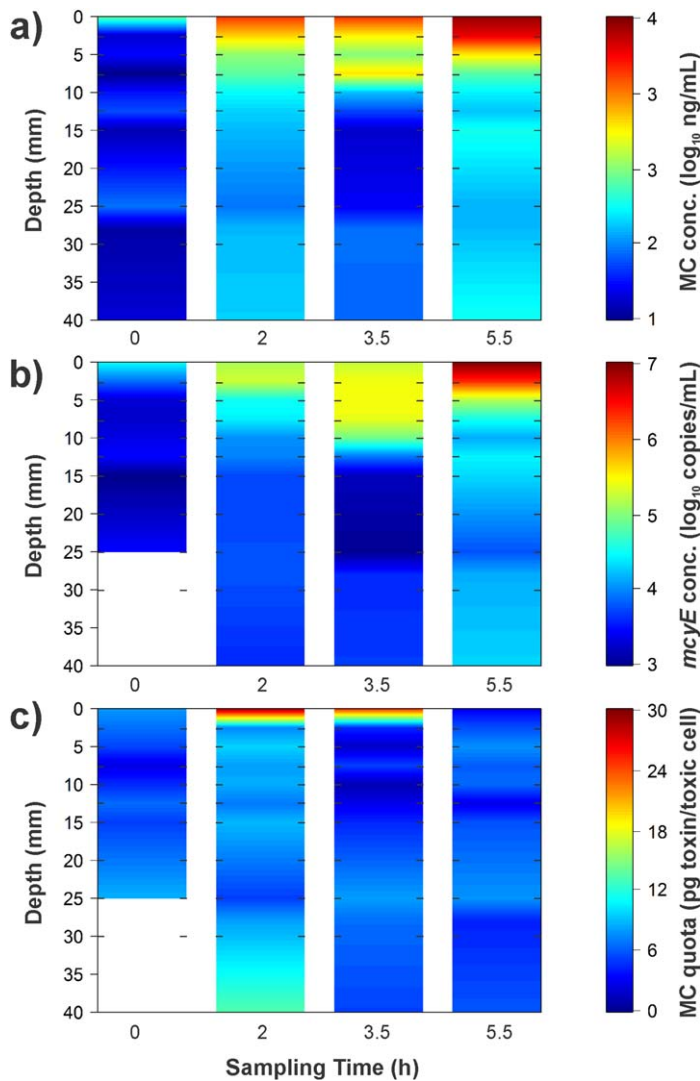
Fig. 4. Vertical profiles of the surface 4 cm of the water column collected using the "cold finger" cryo-sampler during a scum-formation mesocosm experiment; **(a)** microcystin concentration (note $\log_{10}$ scale), **(b)** *mcyE* concentration (note $\log_{10}$ scale), and **(c)** microcystin quota. Linear interpolation at 0.1 mm increments was used in each plot; indented lines indicate the sampling depths used; in b and c, data below 2.5 cm were not available as *mcyE* concentrations were below the method detection limit.

Supporting Information S8) during the 5.5 h time-course of the mesocosm experiment (from 11:45 to 17:15). In the 3 cm layer of water sampled immediately below this layer (i.e., 10–40 mm below the surface) microcystin concentrations were relatively consistent and only increased by fourfold over the duration of sampling. At each of the sampling points in the 5.5 h duration of the experiment, ca. 60% of the microcystin present within the 4 cm of the water column assessed was in the 0–2.5 mm depth (see Supporting Information S8 for microcystin measurements). Within

this layer the microcystin concentration increased from 390 ng mL^{-1} to 13,130 ng mL^{-1} during the experiment.

In some of the cold finger samples from the start of the experiment, the concentration of toxic *Microcystis* (expressed as *mcyE* mL^{-1}) was below the detection limit of the *mcyE* qPCR assay. Throughout the experiment there were marked changes in the concentration of toxic *Microcystis* in the top 1 cm of the water column (up to 72-fold; 20,000 cells mL^{-1} to 1,430,000 cells mL^{-1}; Fig. 4b), while the underlying 3 cm layer was relatively consistent, showing only a fivefold increase in toxic *Microcystis* concentrations. However, the toxic *Microcystis* was not as tightly constrained to the 0–2.5 mm depth as observed with the microcystin. In this instance, the majority of the toxic *Microcystis* was in the 0–5 mm layer and the concentration increased in this layer from 48,500 cells mL^{-1} to 4,210,000 cells mL^{-1} during the scum-formation experiment.

There was a very sharp decrease in microcystin quota between the 0–2.5 mm and 2.5–5 mm depths for the 2 h and 3.5 h time points (Fig. 4c). Below these layers microcystin quota was generally lower, varying between 1.2 pg/toxic cell and 13.4 pg/toxic cell (Supporting Information S8). In the 0–2.5 mm layer, the microcystin quota increased from 8 pg/toxic cell to 29.3 pg/toxic cell in the first 2 h of the experiment, remained relatively constant for the following 2 h, then decreased markedly to 3.1 pg/toxic cell.

Adoption of the described techniques

Construction of the cryo-samplers was relatively cost effective, with an individual surface snatcher costing ca. $30 US, a cold finger costing ca. $65 US and a sectioning plate costing ca. $30 US (in 2016). As the cold finger sampler needs to be placed in the environment of interest prior to stratification (e.g., prior to formation of a cyanobacterial scum), sufficient samplers must be manufactured to collect the desired number of time points or sample replicates. The surface snatchers and field sectioning plates are able to be cleaned and reused between sampling points.

The surface snatcher sampling method was very easy to use and mostly required an operator with a steady hand. Little training or prior practice was required in order for a novice to be able to collect and process samples. The cold finger required more care when samples were removed, to avoid damaging the thin ice sheets. However, with practice an operator with previous laboratory experience could quickly and confidently collect, remove and section a vertical profile.

Discussion

Experimental validation of the efficacy of the cryo-samplers

The techniques described in this article were designed to allow acquisition of samples from aquatic environments at spatially-precise scales. In this regard, both cryo-samplers

performed well, the surface snatcher collecting discrete samples from the top 1 mm of surface water and the cold finger collecting vertical profiles at mm-resolution. The vertical profiles collected with the cold finger allowed new insight into the structure of a *Microcystis* bloom; other studies have not been able to produce such a vivid image of the spatial distribution of toxic *Microcystis* as a scum forms (Fig. 4b). The 2 h and 3.5 h time points of the vertical profiles demonstrated that microcystin production was highly localized in the surface 2.5 mm of the water column (Fig. 4c) as microcystin quotas were ca. 75% lower in the remaining 37.5 mm of the 40 mm water column which was sampled. This observation fits with the theory that the ecological function of microcystins may be to alleviate oxidative stress in cyanobacteria (Zilliges et al. 2011). At the top of the water column where irradiance is most intense, photosynthesis is enhanced, causing increased levels of dissolved oxygen in the water.

Between 3.5 h and 5.5 h of the experiment, the microcystin quota in the 0–2.5 mm layer decreased substantially. This was associated with a large increase in the abundance of toxic *Microcystis* (23-fold increase) while the microcystin concentration increased by only threefold over the same time period. Therefore, the large decrease in microcystin quota observed at 5.5 h could be due to a large population of unaffected cells accumulating at the surface. The 5.5 h sample was collected at 17:15 h when irradiance was ca. 15% of the previous sample at 13.15 h (data not shown). Thus the new population of cyanobacteria may not have been affected by high light when it reached the surface and may not have upregulated microcystin production. The observed decrease in microcystin quota may have also been related to a different genotype of toxic *Microcystis*, which produced substantially less microcystin than the original population (Saker et al. 2005; Krüger et al. 2010). While the second hypothesis could be assessed through high-throughput sequencing of the DNA samples collected, this was not possible under the budgetary constraints of the present study.

Performance of the cryo-samplers

The surface snatcher enabled samples from the surface water to be obtained as a highly discrete layer (ca. 1 mm). While we were able to analyze these samples for microcystin concentrations, the use of qPCR with surface snatcher samples proved problematic and requires further assessment. The effects were not due to the "snap-freezing" process, as we have used this cryo-sampler to collect cyanobacterial colonies from the surface water and were able to perform qPCR assays on the samples (data not shown). The effects were also not due to cell lysis during the mesocosm experiment, as the levels of extracellular microcystin were $< 0.5\%$ of the total microcystin concentration throughout the scum-formation experiment in the pipette-collected samples.

The apparent underestimation of cell numbers and lack of consistent DNA amplification in the surface snatcher samples may have several explanations. Postsampling degradation of the DNA is possible, although this was not observed with the cold finger samples. However, the surface snatcher samples were larger and thawed more slowly, causing them to be exposed to near-ambient temperature for longer than the cold finger samples. As the surface snatcher samples were also very concentrated and likely to have contained deoxyribonucleases (DNases), rapid breakdown of DNA may have occurred during this transition period. There are a number of commercial products which stabilize nucleic acids during the thawing process, such as RNAlater-ICE (Ambion) and we suggest that future studies incorporate the use of such reagents when molecular analyses are to be conducted on the samples.

The cold finger sampler enabled vertical profiles of the top 4 cm of the water column to be collected, and using the field sectioning plate, the profiles were easily sectioned in the field. If finer increments in the sample depth were desired, sectioning plates with narrower troughs could be designed, however, this could lead to difficulties in retrieving the samples from the troughs. Alternatively, the cold finger samples can be kept frozen on dry ice until returning to the laboratory where the profile can be sectioned using laboratory equipment (e.g., a cryotome; Danovaro and Fraschetti 2002). The cold finger sampler has some similarities to a cryogenic sediment sampler described by Walkotten (1976, 1977), which uses compressed carbon dioxide to freeze sediment and interstitial water. However, the Walkotten sampler would not be practical for the collection of vertical profiles collected during the present study as removal of thin ice sheets from this sampler would result in their destruction.

The main limitation of the cold finger sampler was that it could not be used where there was a high level of turbulence (e.g., flowing current or when waves are present). However, in these mixed environments spatial resolution at the millimeter-level is unlikely to yield insightful results. Vertical profiles could still be collected from calm regions of lakes and marine environments or from sheltered pools in streams and rivers. As the profiles are collected quickly (20 s), the samples provide a snap-shot of the water column at that point in time. A second limitation of the sampler is that it should be placed in the environment of interest prior to stratification so that its placement does no disrupt the stratified environment. However, because of the relatively low manufacturing cost (ca. $65 US), the production of multiple devices would not burden most research teams, particularly as the sampler is reuseable. For the application presented in this study, profiling *Microcystis* scums, researchers only need to install the samplers on the day of sampling as surface scums generally form on a daily basis.

Future applications of the cryo-samplers

During the present study, the samples collected from the cryo-samplers were analysed for microcystin concentration by LC-MS/MS and for concentrations of microcystin-producing *Microcystis* by qPCR. Theoretically, other analytical techniques could be applied to these samples if there was sufficient sample size and the samples were assessed for total levels (as opposed to extracellular levels). Likewise, qPCR assays targeting other genes could be applied and, as the samples are snap frozen, RNA could be extracted and gene expression quantified. In this study we focused on toxic *Microcystis* and microcystins, however, the spatial distribution of other chemical compounds and microbes in aquatic environments could be similarly assessed using the cryogenic samplers. While the application of other analytical techniques is highly plausible, it is likely that different sample processing, extraction and clean-up procedures would be required, depending on the analyte of interest.

We envisage the analysis of fine-scale vertical profiles using high-throughput sequencing will provide new insights into the dynamics of microbial communities in aquatic ecosystems. For example, we have amplified the cyanobacterial intergenic spacer region of individual *Microcystis* colonies (data not shown). Another potential application is the use of fluorescent probes to understand the interactions between different microbial communities. This is likely to require additional development work, possibly using processes similar to those used when preparing histology samples or electron microscopy samples, but appears feasible. We also see a likely marriage between the techniques described here and methods for high-resolution *in situ* measurement of physio-chemical parameters (e.g., microelectrodes and optodes which measure pH and dissolved oxygen; Hawes et al. 2014; Wood et al. 2015). The combination of high-resolution profiling and fine-scale sampling could lead to much improved understanding of how microbial populations respond to environmental drivers.

Comments and recommendations

Aquatic environments can become stratified over small scales (mm to cm), especially in regards to their microbial community composition. To gain more insight into the chemical and community dynamics present in these environments we developed new cryogenic sampling tools which enabled samples to be collected from the water surface without dilution from the underlying water and high-resolution vertical profiles of the water column to be obtained. During this study we applied these techniques to the sampling of a cyanobacterial scum.

The surface snatcher enabled samples to be collected in a spatially-precise manner and the chemical analyses of these samples compared well with the vertical profile data from the cold finger. The cyanobacterial scum samples collected using the surface snatcher were not able to be accurately analysed using a *mcyE* qPCR assay, ruling-out the determination of cell concentrations in the samples. The vertical profiles collected using the cold finger sampler provided new insight into the distribution of toxic *Microcystis* during scum-formation and the localization of microcystin production in the top layer of the cyanobacterial scum. The cryo-samplers display great versatility and samples could be analyzed using multiple analytical platforms. This will likely enable many new research avenues in the field of microbial ecology.

Conflicts of Interest

The authors are named as inventors for New Zealand Patent Application No. 717237 which covers design elements of the "cold finger" and "field sectioning plate".

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