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Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp.

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Abstract

In a novel approach to separate the co-occurring freshwater cyanobacteria *Microcystis* and *Synechoccous,* published ecological characteristics are used to manipulate temperature and nutrient concentrations to successfully establish a unialgal *Microcystis* strain. The simple protocol has implications for future cyanobacterial culturing approaches and the establishment of new cyanobacteria strains.

Keywords

Cyanobacteria, Isolation, *Microcystis,* Purification, *Synechococcus*.

Main text

Dense blooms of cyanobacteria (blue-green algae) can adversely affect freshwater supplies and ecosystems by releasing toxins, fouling infrastructure and causing anoxic conditions (Paerl and Huisman 2009). Bloom forming genera of cyanobacteria have an array of eco-physiological adaptations which can allow their domination of freshwater ecosystems under favourable environmental conditions (Carey *et al.* 2012). Experimental work into the ecology and physiology of these important organisms often relies on long-established culture strains which may have been maintained under laboratory conditions for years or even decades.

The physiological representativeness of these laboratory strains can be questioned (Lakeman *et al.* 2009) providing impetus for the development of simple protocols to isolate fresh cultures from natural populations.

Cyanobacteria isolation and purification techniques fall into two broad categories; mechanical (e.g. micro-pipetting, centrifugal washing and cell sorting) and physiological separation (e.g. antibiotic resistance, or changes in environmental parameters; light, temperature, nutrients). The majority of these isolation techniques require only simple apparatus. In this work we tested several techniques, alone and in combination, in order to design a protocol which yielded a unialgal culture of *Microcystis* after separation from the co-occurring *Synechococcus*.

Step one (picking and crude selection): Water samples were collected using 1 L Duran bottles from Ivy Lake, a flooded gravel pit, now used for drinking water storage and as a nature reserve in Dorset, UK (50°52'7.86"N, 1°47'7.87"W). Using light microscopy (10 x light magnification) *Microcystis* sp. colonies were transferred by micro-pipette into a 25 mL conical flask containing 10 mL of 0.1 µm filtered reservoir water (collected along with the sample) and maintained at room temperature (18-22˚C) in a North facing window for 7 days. One mL of this mixed culture was transferred by pipette into a 15 mL centrifuge tube and diluted 1 in 6 with ultra-pure water (Millipore, USA) and then centrifuged at 1000 *x g* for 20 minutes. The supernatant biomass was removed by pipetting and transferred to a separate sterile centrifuge tube. This process was repeated three times to select for buoyant cyanobacteria following the methods of Bloch and Blackburn (1995). Processed cells were placed in 150 mL conical flasks with 50 mL of 50% BG-11 medium (Sigma-Aldrich, C3061) diluted by adding 0.5 mL to 50 mL ultra-pure water. All samples were then placed into an incubator (Conviron, CMP6010) at 25 ±1°C, on 12 hour light/dark. Light was provided by a single 58 watt fluorescent tube (Luminex, Cool white) at 25 µmol quanta m^{-2} s^{-1} (Biospherical Instrument Inc., PAR Scalar Irradiance sensor) for 18 days. Colonies were homogenised by vortex and cell division monitored by flow cytometry (Accuri, C6) for 2 minutes using a 10 µl core size, 14 µl/min flow rate and threshold set at 20,000 on forward scatter light (FSC) signal.

After 10 days there was an increase in the biomass of *Microcystis* cells as cell density increased from 270,000 to 4,000,000 per millilitre. However, closer examination under light microscopy revealed cultures of *Microcystis* were contaminated with smaller bacillus cells (Figure 1, A.). These were found to be photosynthetic by epifluorescence microscopy (Figure 1, B.) and the red auto-fluorescence signal from flow cytometry (Figure 2, C.). The non-target cyanobacterial cells were identified as *Synechococcus* and tentatively assigned to *S.*

elongatus (John Day, Culture Collection of Algae and Protozoa, pers. comm.). Therefore further techniques were required to separate the two cyanobacteria.

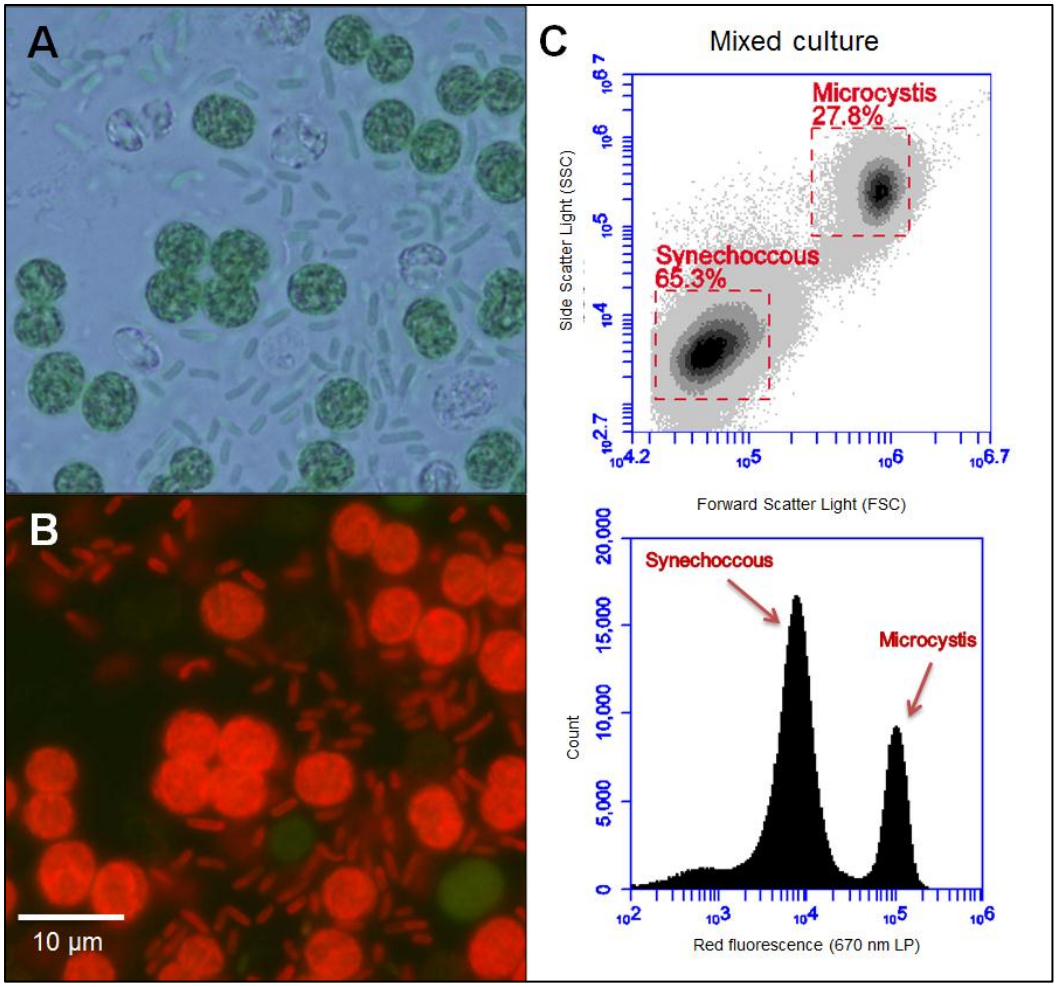

Figure 1. (A) True colour micrograph of isolated *Microcystis* sp., spherical cells with darker green pigment, and *Synechococcus* sp., smaller bacillus-shaped cells with lighter green pigment. **(B)** The same image through epifluorescence microscopy, red fluorescence indicates chlorophyll content in both cell types and green fluorescence indicates chlorotic (non-photosynthesising) cells. **(C)** Flow cytometry cytogram showing *Microcystis* cells (R1) and *Synechococcus* cells (R2) and red auto-fluorescence histogram, smaller peak *Microcystis* larger peak *Synechococcus.*

Other separation techniques: The contaminated cultures of *Microcystis* were subjected to additional techniques to attempt separation from the *Synechococcus.* 1) A 2 mL subsample of culture was homogenised and serially diluted to -3 log, the final dilution was spread on 10 solid agar petri dishes at 1 or 5% and 10 solid agarose petri dishes at 1 or 5%. All agar and agarose plates were prepared using 100% BG-11 adapting the work of Shirai and co-workers (1989). 2) Ten additional agarose petri dishes were prepared with the addition of antibiotic disks (Mastring-S, M13) to test for difference in resistance or susceptibility between *Microcystis* and *Synechococcus.* Antibiotics and dose applied; chloramphenicol (25µg),

erythromycin (5µg), fusidic acid (10µg), oxacillin (5µg), novobiocin (5µg), penicillin G (1 unit), streptomycin (10µg) and tetracycline (25µg). 3) A 5 mL subsample was centrifugally separated in a sucrose gradient using 5, 10, 20, 30 and 40% in 1 mL volumes. Each sucrose concentration was placed in a 50 mL conical flask with 20 mL 100% BG-11. The aim was to separate the cyanobacteria by using differences in buoyancy through an increasingly viscose media. 4) The next approach was to place 5 mL in a 50 mL conical flask with 20ml of 100% BG-11 and the addition of 7µg microcystin-LR (Sigma-Aldrich, 33893) to test susceptibility of *Synechococcus* to the *Microcystis* toxin. 5) The cyanobacteria were separated using an automated cell sorter (BD, FACSAria) into densities of 100, 1000, 10,000 and 100,000 cells per mL, respectively and placed into 20 mL of 100% BG-11 in 50 mL conical flasks. Cells were discriminated by using a known unialgal *Microcystis* culture (PCC 7806 wild type) as a template. In all the techniques attempted culture flasks and plates were incubated as per the parameters described in step one. The relative cell growth of the cyanobacteria was monitored by light microscopy and flow cytometry for up to three weeks.

None of the techniques attempted were successful in separating the *Microcystis* sp. cells from the *Synechococcus* sp. cells. The solid plating technique there was no observed difference in the resistance or susceptibility between the two cyanobacteria to all antibiotic types and doses. The sucrose gradient centrifuging also failed due to both cyanobacteria having the same fractionation position in the gradient. Not one of the cell densities obtained from automated cell sorting showed any growth when incubated, the cyanobacteria could have been adversely affected by fluid acceleration, electrical shock or photo-bleaching (light shock). In techniques where cyanobacterial growth was observed, it was apparent that a proportion of the smaller *Synechococcus* cells were remaining attached to the *Microcystis* mucus sheath and subsequently overgrowing the assumed unialgal cultures.

The main drivers of cyanobacterial blooms are increasing water temperature and increased nutrient input (Paerl *et al.* 2001, Schindler *et al.* 2008). Rigosi and co-workers (2014) conducted an analysis of data collected from over 1000 lakes in the United States and reported evidence of the growth of cyanobacteria genera responding differently to temperature and nutrients. For example, *Microcystis* dominance within a bloom is primarily driven by increased temperature, whereas *Synechococcus* dominance is primarily driven by increased nutrients. This posed the hypothesis, could the two cyanobacteria in culture be separated by increasing temperature and decreasing nutrients?

Step two (physiological separation):

Five decreased concentrations of BG-11 at 40, 30, 20, 10 and 0% were put into 5 test tubes in 5 mL volumes and 0.5 mL of mixed culture was transferred into each. The test tubes were

placed in an incubator (AlgaeTron, AG230) at 32 ±1°C, on a 12 hour light/dark sine wave regime provided by white and infra-red LED's at 20 µmol quanta m^{-2} s^{-1}. The relative cell growth of both cyanobacteria was monitored by flow cytometry over 15 days.

The increase in temperature and decrease in nutrients were conditions that favoured the target cyanobacteria. Flow cytometry data showed an increase of *Microcystis* cells and a reciprocal decrease in *Synechococcus* cells over 15 days (Figure 2.).

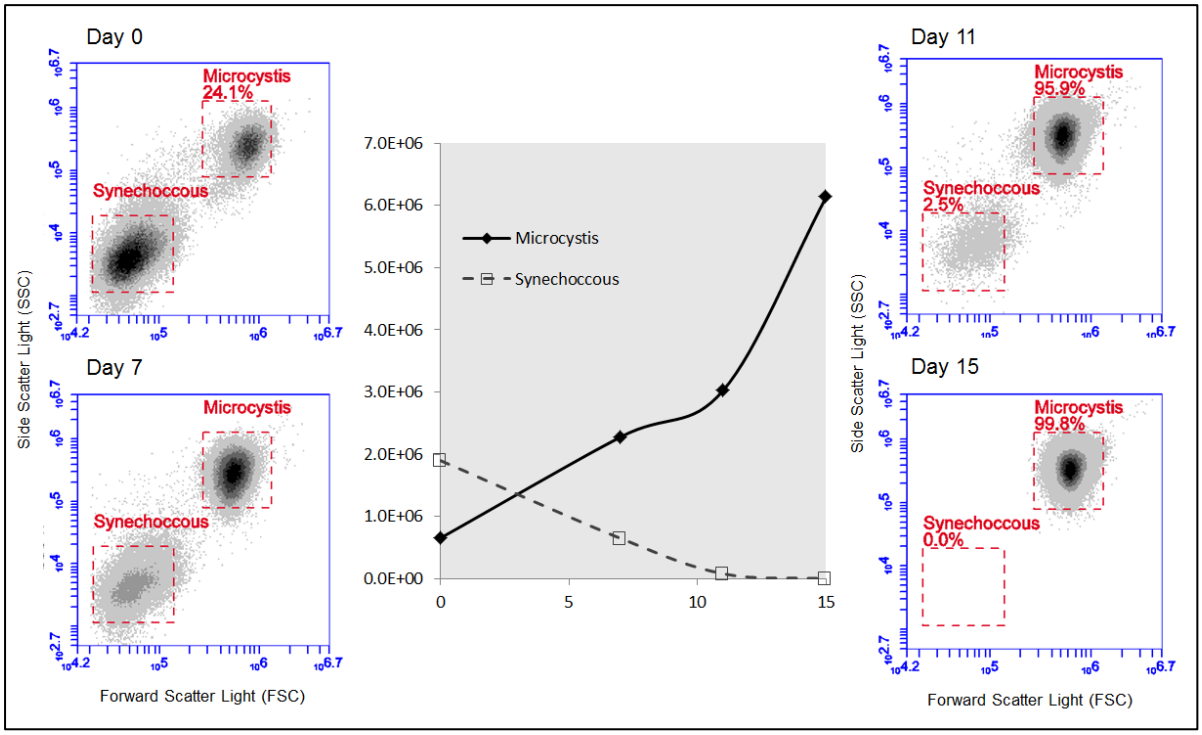

Figure 2. The cell densities of *Microcystis* and *Synechococcus* in 20% BG-11 medium at 32° C monitored over a 15 day period by flow cytometry, density plot cytograms of cell size forward scatter light (FSC) and cell granularity, side scatter light (SSC). FSC signal threshold 20,000 arbitrary units (au), with gates on red fluorescence (670nm LP) between 2,000 and 300,000 au and far-red auto-fluorescence (675nm +/- 12nm) between 1,000 and 800,000 au to remove noise from the light signal.

The effect was seen in all concentrations of BG-11, with the greatest *Microcystis* biomass in 20 and 30%. Inspection by microscopy and return to 100% BG-11 and 25°C culture conditions confirmed a unialgal culture had been isolated. The *Microcystis* strain isolated from 20% BG-11 is now deposited in the Culture Collection of Algae and Protozoa (CCAP, UK) accession number 1450/17.

The isolation and purification of cyanobacteria is a time consuming process, where techniques need to be refined for the target organism. Time invested is, however, rewarded by the establishment of new culture lines for ecological and physiological research into these important organisms. We conclude that modifying culture conditions in accordance with

published eco-physiological niche requirements was the best method for artificially selecting for the target organism, in this case *Microcystis,* and thereby creating a new culture lineage.

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