

1 **Revised**

2 **Short communication to the Journal of Microbiological Methods**

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

5 **Authors**

6 David M. Hartnell^{1,2}, Ian J. Chapman¹, Genoveva F. Esteban¹ and Daniel J. Franklin¹

7 **Affiliations**

8 (1) Bournemouth University, Centre for Ecology, Environment and Sustainability,
9 Department of Life and Environmental Sciences, Faculty of Science and Technology, Dorset
10 BH12 5BB, U.K.

11 (2) Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Rd, The
12 Nothe, Weymouth, Dorset, DT4 8UB, U.K.

13 **Corresponding Author**

14 David M. Hartnell

15 *Tel.:* 01202 963795

16 *Email:* dhartnell@bournemouth.ac.uk

17 **Abstract**

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

23 **Keywords**

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

25 **Main text**

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

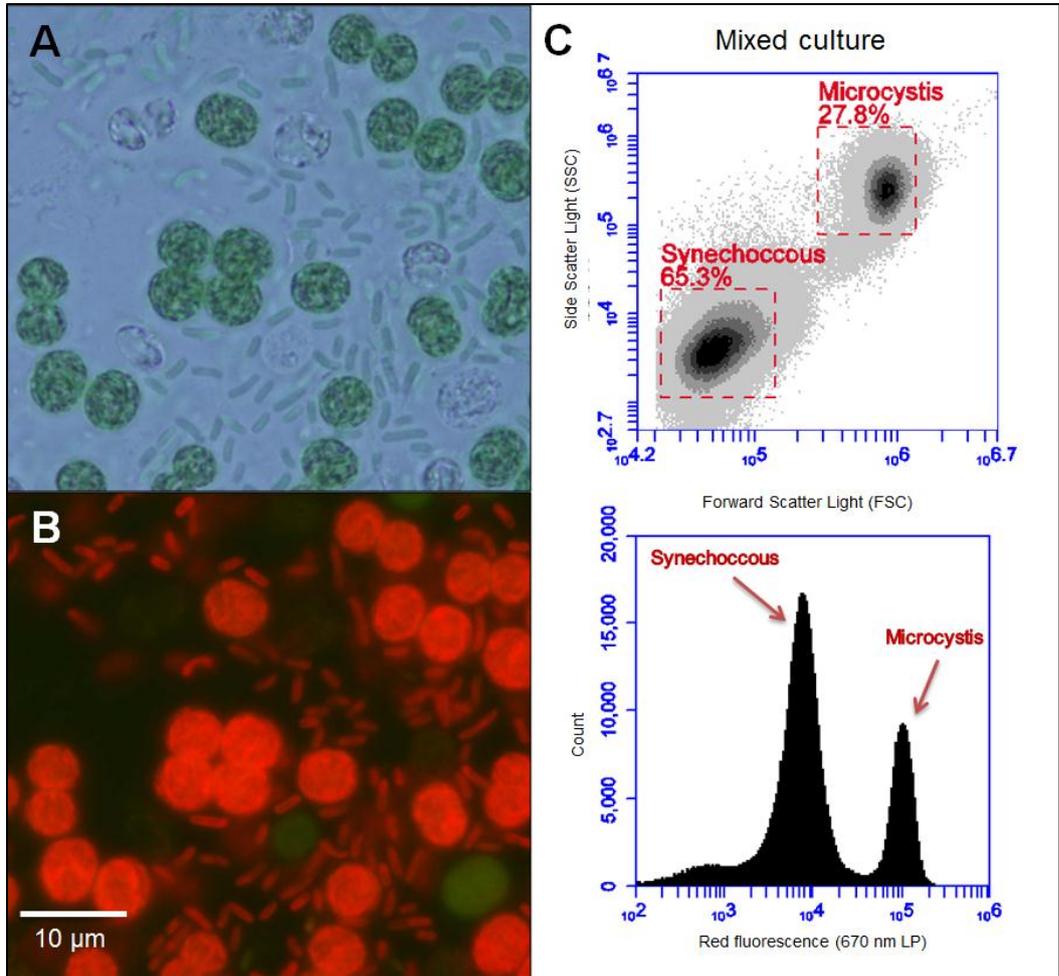
33 The physiological representativeness of these laboratory strains can be questioned
34 (Lakeman *et al.* 2009) providing impetus for the development of simple protocols to isolate
35 fresh cultures from natural populations.

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

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

62 After 10 days there was an increase in the biomass of *Microcystis* cells as cell density
63 increased from 270,000 to 4,000,000 per millilitre. However, closer examination under light
64 microscopy revealed cultures of *Microcystis* were contaminated with smaller bacillus cells
65 (Figure 1, A.). These were found to be photosynthetic by epifluorescence microscopy (Figure
66 1, B.) and the red auto-fluorescence signal from flow cytometry (Figure 2, C.). The non-
67 target cyanobacterial cells were identified as *Synechococcus* and tentatively assigned to S.

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



70

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

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

86 erythromycin (5µg), fusidic acid (10µg), oxacillin (5µg), novobiocin (5µg), penicillin G (1 unit),
87 streptomycin (10µg) and tetracycline (25µg). 3) A 5 mL subsample was centrifugally
88 separated in a sucrose gradient using 5, 10, 20, 30 and 40% in 1 mL volumes. Each sucrose
89 concentration was placed in a 50 mL conical flask with 20 mL 100% BG-11. The aim was to
90 separate the cyanobacteria by using differences in buoyancy through an increasingly
91 viscose media. 4) The next approach was to place 5 mL in a 50 mL conical flask with 20ml of
92 100% BG-11 and the addition of 7µg microcystin-LR (Sigma-Aldrich, 33893) to test
93 susceptibility of *Synechococcus* to the *Microcystis* toxin. 5) The cyanobacteria were
94 separated using an automated cell sorter (BD, FACSAria) into densities of 100, 1000, 10,000
95 and 100,000 cells per mL, respectively and placed into 20 mL of 100% BG-11 in 50 mL
96 conical flasks. Cells were discriminated by using a known unialgal *Microcystis* culture (PCC
97 7806 wild type) as a template. In all the techniques attempted culture flasks and plates were
98 incubated as per the parameters described in step one. The relative cell growth of the
99 cyanobacteria was monitored by light microscopy and flow cytometry for up to three weeks.

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

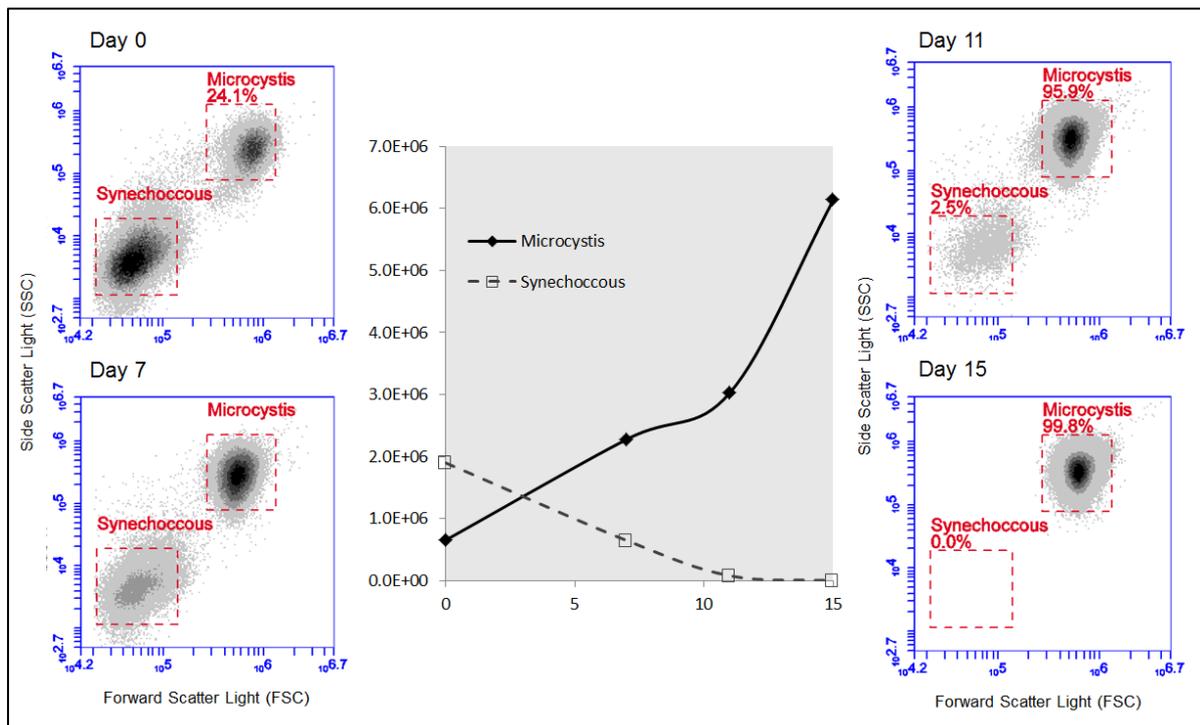
110 The main drivers of cyanobacterial blooms are increasing water temperature and increased
111 nutrient input (Paerl *et al.* 2001, Schindler *et al.* 2008). Rigosi and co-workers (2014)
112 conducted an analysis of data collected from over 1000 lakes in the United States and
113 reported evidence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

118 *Step two (physiological separation):*

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

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

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



127

128 **Figure 2.** The cell densities of *Microcystis* and *Synechococcus* in 20% BG-11 medium at 32°
129 C monitored over a 15 day period by flow cytometry, density plot cytograms of cell size
130 forward scatter light (FSC) and cell granularity, side scatter light (SSC). FSC signal threshold
131 20,000 arbitrary units (au), with gates on red fluorescence (670nm LP) between 2,000 and
132 300,000 au and far-red auto-fluorescence (675nm +/- 12nm) between 1,000 and 800,000 au
133 to remove noise from the light signal.

134 The effect was seen in all concentrations of BG-11, with the greatest *Microcystis* biomass in
135 20 and 30%. Inspection by microscopy and return to 100% BG-11 and 25°C culture
136 conditions confirmed a unialgal culture had been isolated. The *Microcystis* strain isolated
137 from 20% BG-11 is now deposited in the Culture Collection of Algae and Protozoa (CCAP,
138 UK) accession number 1450/17.

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

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

145 **Acknowledgments**

146 The authors would like to thank Sembcorp and Wessex Water for granting access to their
147 reservoirs for this project. Special thanks to Eddie McCarthy for his lab assistance and to the
148 Culture Collection of Algae and Protozoa (CCAP, UK) for helpful discussions. DMH was
149 funded by a Bournemouth University FUSION investment fund grant.

150 **References**

- 151 Bloch, C. J. S. and Blackburn, S. I., 1995. Isolation and purification of Australian isolates of
152 the toxic cyanobacterium *Microcystis aeruginosa* Kutz. *J. Appl. Phycol.*, 8, 5 – 13.
- 153 Carey, C. C., Ibelings, B. W., Hoffman, E. P., Hamilton, D. P. and Brookes, J. D., 2012. Eco-
154 physiological adaptations that favour freshwater cyanobacteria in a changing climate.
155 *Water Res.*, 46, 1394 – 1407.
- 156 Lakeman, M. B., von Dassow, P., and Cattolico, R. A., 2009. The strain concept in
157 phytoplankton ecology. *Harmful Algae*, 8, 746 – 758.
- 158 Paerl, H.W., Fulton, R.S., Moisander, P.H., Dyble, J., 2001. Harmful freshwater algal
159 blooms, with emphasis on cyanobacteria. *Sci. World* 1, 76–113.
- 160 Paerl, H. W. and Huisman, J., 2009. Climate change: a catalyst for global expansion of
161 harmful cyanobacterial blooms. *Environ. Microbiol. Rep.*, 1 (1), 27 – 37.
- 162 Rigosi, A., Carey, C. C., Ibelings, B. W. and Brookes, J. D., 2014. The interaction between
163 climate warming and eutrophication to promote cyanobacteria is dependent on
164 trophic state and varies among taxa. *Limnol. Oceanogr.*, 59 (1), 99 – 114.
- 165 Schindler, D. W., Hecky, R. E., Findlay, D. L., Staiton, M. P., Parker, B. R., Paterson, M. J.,
166 Beaty, K. G., Lyng, M. and Kasian, S. E. M., 2008. Eutrophication of lakes cannot be
167 controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem
168 experiment. *PNAS*, 105, 11254 – 11258.
- 169 Shirai, M., Matumaru, K., Ohotake, A., Takamura, Y., Aida, T. and Nakano, M., 1989.
170 Development of a solid medium for growth and isolation of axenic *Microcystis* strains
171 (Cyanobacteria). *Appl. Environ. Microbio.*, 55, 2569 – 2571.