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ASSESSING THE IMPORTANCE OF COBALT AS A MICRONUTRIENT FOR FRESHWATER CYANOBACTERIA[1]

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Micronutrients play key roles in numerous metabolic processes in cyanobacteria. However, our understanding of whether the micronutrient cobalt influences the productivity of freshwater systems or the occurrence of cyanobacterial blooms is limited. This study aimed to quantify the concentration of Co necessary for optimal cyanobacterial growth by exposing *Microcystis aeruginosa* to a range of Co concentrations under culture conditions. Extended exposure to concentrations below ~0.06 μg · L^{-1} resulted in notable inhibition of *M. aeruginosa* growth. A clear negative relationship was observed between Co concentration in solution and intracellular Fe quota of *M. aeruginosa*, possibly due to decreased transport of Fe at higher Co concentrations. Cyanocobalamin and any Co within the structure of cyanocobalamin appears to be non-bioavailable to *M. aeruginosa*, instead they likely rely on the synthesis of a structural variant – pseudocobalamin, which may have implications for the wider algal community as the variants of cobalamin are not necessarily functionally exchangeable. To evaluate the likelihood of Co limitation of cyanobacterial growth under field conditions, a survey of 10 freshwater reservoirs in South-Eastern Australia was conducted. Four of the ten sites had dissolved Co concentrations below the 0.06 μg · L^{-1} threshold value. All four of these sites rarely undergo cyanobacterial blooms, strengthening evidence of the potential for Co to limit growth, perhaps either alone or in combination with phosphorus.

Key index words: **blooms; growth limitation; trace metals; vitamin B_{12}**

***Abbreviations*: DOC, dissolved organic carbon; ICP-AES, inductively coupled plasma atomic emission spectroscopy; ICP-MS, inductively coupled plasma mass spectrometry; LOD, limits of detection; PVC, Polyvinyl chloride; RPM, revolutions per minute**

The importance of the macronutrients nitrogen (N) and phosphorus (P) in determining the structure and productivity of freshwater phytoplankton communities is well established (Dignum et al. 2005, Paerl and Fulton 2006). We know far less about the role of micronutrients, and in particular, their ability to regulate cyanobacterial blooms. The micronutrient cobalt (Co) is particularly understudied (Facey et al. 2019). Cobalt's biological significance is often associated with its ability to substitute for other micronutrients (Intwala et al. 2008); such as for zinc in the enzyme carbonic anhydrase (Quigg 2016). Although, some marine cyanobacteria (e.g., *Prochlorococcus*, *Trichodesmium*, and *Synechococcus*) appear to have an absolute Co requirement that cannot be replaced by substitution of other elements (Sunda and Huntsman 1995, Saito et al. 2002, Rodriguez and Ho 2015).

Further, there is some evidence that Co can influence marine cyanobacteria distribution and productivity (Saito et al. 2002, Panzeca et al. 2006, Koch et al. 2011, Huertas et al. 2014, Helliwell et al. 2016, Nef et al. 2019, Facey et al. 2021), as well as nitrogen fixation (Healey 1973, Rodriguez and Ho 2015) and phytoplankton community structure (Browning et al. 2017). Similarly, in freshwater systems the importance of Co has been observed by Downs et al. (2008) who noted a stimulation of primary productivity upon addition of Co during a bloom of the freshwater heterocystous cyanobacteria *Anabaena flos-aquae* in Lake Waihola, New Zealand.

Co is a component of vitamin B_{12}, a diverse group of corrinoids involved in the transfer of methyl

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groups and rearrangement reactions in cellular metabolism (Healey 1973, Huertas et al. 2014, Rodriguez and Ho 2015, Helliwell et al. 2016). There are a number of chemical variants of vitamin B_{12}, which share a characteristic planar corrin ring containing a central cobalt atom. Cobalamin, the form most bioavailable to many eukaryotic algae, is scarce in freshwater (Kurata 1986) and marine ecosystems (Sañudo-Wilhelmy et al. 2012), indicating competition for this resource is likely to be high. Rodriguez and Ho (2015) exposed *Trichodesmium* to varying concentrations of Co and cobalamin under culture conditions and observed that low Co concentrations appeared to decrease growth of *Trichodesmium*. Upon addition of cobalamin, growth was elevated. These results indicate the primary function of Co is its requirement for cobalamin synthesis and also that *Trichodesmium* can acquire its biological demand for cobalamin from the surrounding media. Interestingly, a recent study by Helliwell et al. (2016) found that a structural variant of cobalamin, called pseudocobalamin, is the form most widely produced by cyanobacteria. Pseudocobalamin was found to be several orders of magnitude less bioavailable to some vitamin B_{12}-dependent eukaryotic algae.

Currently little is known regarding typical Co concentrations required for optimal freshwater cyanobacterial growth and the availability of Co in Australian freshwaters. This study aims to: (1) determine what Co concentrations limit the growth of the toxic cyanobacteria *Microcystis aeruginosa*, (2) investigate the biochemical function of Co in *Microcystis aeruginosa*, particularly in relation to the production of vitamin B_{12}, and (3) perform a survey of Co concentrations in East-Australian freshwaters.

MATERIALS AND METHODS

Batch culture experiments were performed using the toxic *Microcystis aeruginosa* MASH01-AO5 (Australian National Algae Culture Collection, Hobart, Tasmania, Australia). Axenic cultures were maintained in MLA media (Bolch and Blackburn 1996) in an environmental chamber (Labec, HC-50). Incubation was at 22°C under 20–25 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} light with a 14:10 h light:dark cycle throughout the long-term maintenance of the cultures as well as the duration of the experiment.

Culture media. At the onset of the experiment, cells were subcultured into triplicate 700 mL sterile plastic culture flasks (Corning) at an initial cell density of 10^5 cells $\cdot$ mL^{-1}. Flasks had previously been soaked overnight in an acid bath (10% HNO_3) and repeatedly rinsed with Milli-Q water. An inoculum of 10^5 cells $\cdot$ mL^{-1} was added to each flask containing 600 mL of filter sterilized MLA growth media (Bolch and Blackburn 1996) with modified Co concentration. The composition of MLA growth media is outlined in Table 1. Standard MLA media contains Co in surplus and was used as the control. Four treatments were assessed, they are listed below with their nominal Co concentrations:

TABLE 1. The composition of unmodified MLA algal growth media

Nutrient/salt	Final concentration (mg $\cdot$ L^{-1})
K_2HPO_4	34.80
$NaNO_3$	170.00
$NaHCO_3$	16.80
$CaCl_2$	29.40
$Mg.SO_4.7H_2O$	49.10
H_3BO_3	2.40
$CoCl_2.6H_2O$	0.01
$CuSO_4.5H_2O$	0.01
$FeCl_3.6H_2O$	1.58
$Na_2EDTA.2H_2O$	4.56
$MnCl_2.4H_2O$	0.36
$Na_2MoO_4.2H_2O$	0.006
$ZnSO_4.7H_2O$	0.022
Thiamine HCl	0.10
Biotin	5×10^{-4}
Cyanocobalamin (B_{12})	5×10^{-4}

1 Control – filter sterilized MLA medium containing 2.48 μg $\cdot$ L^{-1} Co as $CoCl.6H_2O$ ("full Co").
2 MLA media without any addition of Co.
3 MLA media with 1% of standard MLA concentration of Co (~0.025 μg $\cdot$ L^{-1} Co as $CoCl.6H_2O$).
4 MLA media with 10% of standard MLA concentration of Co (~0.25 μg $\cdot$ L^{-1} Co as $CoCl.6H_2O$).

Transfers. On day 0, an inoculum of *Microcystis aeruginosa* was pelletized via centrifugation at 3500 rpm for 10 min. Preliminary tests showed that this process does not lyse *M. aeruginosa* cells. The supernatant was removed and the pellet washed with treatment media. The palletization and washing steps were repeated before the pellet was resuspended in treatment media. A cell count was performed to determine the volume required for transfer of 10^5 cells $\cdot$ mL^{-1} to the culture flasks.

Culture experiment sampling. Every 2-4 d cell counts were estimated via optical density (680 nm using Varian Cary 50 Bio UV Spectrophotometer). The relationship between *Microcystis aeruginosa* cell count and absorbance at 680 nm was previously determined ($R^2 = 0.98$). Manual cell counts were performed periodically using a hemocytometer to confirm manual cell counts were closely aligned with the optical density relationship.

The dissolved nutrient composition of the culture media was sampled on day 0, 10, 20, and 30 by filtering 25 mL of culture material through a 0.45 μm cellulose acetate syringe filter (Sartorius) pre-rinsed with 50 mL of 10% nitric acid followed by 100 mL milli-Q water. Samples were collected in acid washed 50 mL falcon tubes and refrigerated. Within 24 h of collection, samples were acidified with ultra-pure nitric acid to 0.2% (v/v). Intracellular iron was also sampled on day 0 from the inoculum and days 10, 20, 30 from the experimental flasks. Samples were prepared by transferring a volume of culture material corresponding to ~5×10^7 cells (or ~1.02 mg dry weight) into acid washed, pre-weighed 50 mL falcon tubes. Transfers were performed immediately following a cell count. The culture material was centrifuged at 3000 RPM for 10 min to form a pellet. The supernatant was removed after ensuring the absence of cells by pipetting 1 mL of solution into a Sedgewick rafter counting chamber for observation using a light microscope (Olympus BX41). Samples were frozen at −20°C.

Solution nutrient determination. The concentration of dissolved nutrients (P, Co, Cu, Fe, Mn, and Mo) in the filtered solution was analyzed with a combination of inductively coupled atomic emission spectrometry (ICP-AES; Varian 730 ES) and inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800). The spectrometer was operated according to the standard operating procedures outlined by the manufacturer. The instruments were calibrated using matrix-matched standards. At least 10% of samples were analyzed in duplicate to ensure the precision of the analyses. To check for potential matrix interferences at least 10% of samples had spike recoveries performed.

Intracellular iron sample preparation and analysis. Samples were freeze dried at 0.1 mbar and −80°C until all liquid was sublimated from the samples. The dried pellet was submerged in 500 µL distilled nitric acid and microwaved at 80°C with a 30 min holding time (CEM Mars 6). Samples were diluted with 4.5 mL Milli-Q (18 MΩ.cm) water and transferred to 5 mL acid washed vials for analysis via ICP-MS and ICP-AES. Analysis was performed using the instrument procedure outlined above.

Field evaluation of cobalt concentrations. Ten sites were sampled to assess the availability of dissolved Co in a variety of freshwater systems in South-Eastern Australia. A description of study sites is provided in Table 2. Cyanobacterial alerts were monitored on state government water authority websites (WaterNSW 2021). Land use characteristics was acquired from state government geospatial data (DPIE 2017).

Water samples were obtained from the shore using a PVC sampling pole with a 1 L acid washed Nalgene bottle fixed to the end. The bottle was rinsed once with site water, which was discarded away from the sampling location. Samples were taken at ~0.5 m depth. 100 mL of sample water was filtered through a 0.45 µm cellulose acetate syringe filter (Sartorius) pre-rinsed with 50 mL of 10% nitric acid followed by 100 mL milli-Q water. Samples were collected in 125 mL sample bottles pre-soaked in 10% nitric acid followed by repeated rinsing with milli-Q water. Field blanks were prepared at four sites by following the above procedure using milli-Q water. Immediately following sample collection, physicochemical measurements (pH, dissolved oxygen, conductivity and temperature) were taken using a multiparameter water quality sonde probe. All samples were refrigerated immediately following collection and were acidified with ultra-pure nitric acid to 0.2% v/v in a trace metal-clean room within 7 d of collection.

Co in the filtered solution was analyzed with a combination of inductively coupled atomic emission spectrometry (ICP-AES; Agilent 5110) and inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800). The spectrometers were operated according to the standard operating procedures outlined by the manufacturer. The instruments were calibrated using matrix-matched standards. At least 10% of samples were analyzed in duplicate to ensure the precision of the analyses. To check for potential matrix interferences at least 10% of samples had spike recoveries performed.

Dissolved organic carbon. Samples for dissolved organic carbon (DOC) were collected in the field using the procedure outlined above. Samples were stored in the 1 L Nalgene bottle used for filtered Co sample collection and refrigerated. DOC samples were filtered to 0.45 µm using a cellulose acetate syringe, acidified to 0.5% v/v HCl within a week of sampling, and purged with oxygen gas for 20 min to remove inorganic carbon. Analysis was performed by high-temperature combustion with a Shimadzu TOC-LCSH Total Organic Carbon Analyzer using the procedures recommended by the manufacturer.

PO_4-P determination. Samples for dissolved PO_4-P were collected in the field using the procedure outlined above. Samples were stored in the 1 L Nalgene bottle used for filtered Co sample collection and refrigerated. Prior to analysis, samples were filtered to 0.45 µm using a cellulose acetate syringe filter. PO_4-P was measured by the reduction of ascorbic acid using the molybdate blue colorimetric method (Murphy and Riley 1962, APHA 1995). Analysis was performed using a SEAL AQ400 Discrete Analyzer.

Data Analysis. Differences in Fe quotas between treatments and through time were analyzed with a Two-Way ANOVA with Tukey pairwise comparison. Tests were performed using SigmaPlot 12.5 with a significance level of $\alpha = 0.05$. The Levene statistic was used to test homogeneity of variance. Plots were created using the software R Version 1.2.1335 (R.C. 2018).

RESULTS

Cobalt limitation experiment. There were notable decreases in the growth of *Microcystis aeruginosa* when exposed to decreasing Co concentrations (Fig. 1). Cultures that had no added Co exhibited the most severe signs of growth limitation. The cell concentration was less than the full Co treatment after 13 d and maximum cell concentration occurred after 18 d, after which growth decreased. Similarly, the Co 1% treatment exhibited signs of limitation after 13 d of growth, but reached a higher maximum cell concentration, which occurred on day 24. The cell concentrations in the Co 10% treatment were similar to the full Co treatment until day 28, after which they showed minor signs of limitation.

The relationship between Co concentration and the percentage growth inhibition relative to the full Co treatment is illustrated in Figure 2. Growth was severely inhibited (>40% inhibition) after sustained (>10 d) exposure to concentrations below ~0.06 $\mu g \cdot L^{-1}$ cobalt in the no Co and Co 1% treatments. After 10 d, growth was similar across treatments. Growth was minimally impacted even after 30 d of exposure to ~0.25 $\mu g \cdot L^{-1}$.

There were significant differences in the intracellular Fe quota between treatments and through time (Two-Way ANOVA, $F_{2,24} = 109.652$, p-value <0.05; Fig. 3). Intracellular Fe quota was similar across treatments after 10 d, but after 20- and 30-d variations between treatments became evident. After 20- and 30-d the treatment without added Co had the largest intracellular Fe quota and was notably different to the full Co treatment. There was a trend of increasing Fe quota with decreasing Co concentration in solution at these time points. Tukey Multiple Comparison indicated significant differences between the no Co and full Co treatments after 20 and 30 d (p-value <0.05); however, there were no statistically significant differences between the Co 1% and Co 10% treatments compared to the full Co or any other treatments.

Field survey. Dissolved Co concentrations measured in various freshwater systems in Eastern Australia are presented in Table 3, along with a summary of the site and physicochemical

TABLE 2. Summary of study sites

Site	Coordinates	Geological setting	Altitude (m)	Capacity (ML)	Cyanobacterial alerts in summer immediately following sampling	Level of anthropogenic disturbance in catchment	Land use in catchment
Mannus Lake	-35.81809, 147.98329	Largely foliated granite, leucogranite, adamellite, granodiorite, tonalite.	487	2,350	>4 $mm^3 \cdot L^{-1}$ *Chrysosporum ovalisporum*, *Microcystis* sp.	Moderate	Grazing, plantation forests, native forestry
Carcoar Dam	-33.60478, 149.19042	Volcanics; granite and diorite	852	35,800	>4 $mm^3 \cdot L^{-1}$ *Microcystis* sp.	High	Grazing, cropping,
Lake Lyell	-33.52685, 150.07971	Granite and granodiorite	785	34,500	>4 $mm^3 \cdot L^{-1}$ of unknown cyanobacterial species	Low/moderate	Nature conservation, residential, grazing
Wentworth Falls Lake	-33.70505, 150.36908	Multicolored chert sandstone, quartzose sandstone, shale and claystone	880	300	No alert	Low/moderate	Marsh/wetland, residential, nature conservation
Glenbrook Lagoon	-33.75593, 150.61692	Hawkesbury Sandstone – quartz sandstone with some shale	209	168	No alert	Moderate	Nature conservation, residential
Lake Albert	-35.16636, 147.36932	Gravel, sand, silt, clay	189	4,000	>0.4 and <4 $mm^3 \cdot L^{-1}$ of unknown cyanobacterial species	High	Residential, cropping
Wyangala Dam	-33.96170, 148.96002	Granite and diorite; gray and black slate and quartz graywacke	386	1,217,000	<0.4 $mm^3 \cdot L^{-1}$ *Dolichospermum circinale*	Moderate	Grazing
Blowering Reservoir	-35.44750, 148.28265	Quartz feldspar porphyry with minor slate, graywacke, sandstone, quartzite, tuff, andesite	366	1,613,741	<0.4 $mm^3 \cdot L^{-1}$ *Radiocystis* sp.	Low	Nature conservation, native forest, grazing
Burrinjuck Dam	-34.99482, 148.60628	Mainly conglomerate, grit, shale, sandstone and minor limestone	345	1,026,000	>4 $mm^3 \cdot L^{-1}$ *Dolichospermum circinale*, *Microcystis* sp.	Low	Grazing, nature conservation
Lake Jindabyne	-36.38914, 148.64423	Largely massive intrusions	902	688,287	No alert	Low	Nature conservation, grazing

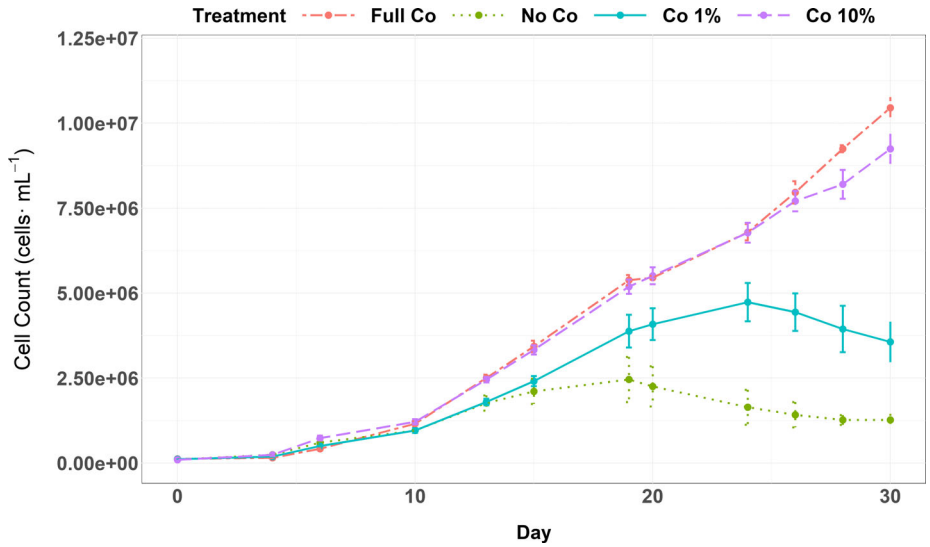

FIG. 1. *Microcystis aeruginosa* growth through time under variable trace metal conditions. Error bars are standard error of the mean (n = 3).

characteristics. Dissolved Co concentrations ranged from 0.016 ± 0.001 μg · L^{-1} at Lake Jindabyne to 0.562 ± 0.006 μg · L^{-1} at Carcoar Dam. Four sites had dissolved Co concentration below the threshold value for *Microcystis aeruginosa* growth of 0.06 μg · L^{-1} outlined in Figure 2; Wentworth Falls Lake, Glenbrook Lagoon, Blowering Reservoir and Lake Jindabyne. PO_4-P concentrations ranged from 18 μg · L^{-1} to below detection limit (LOD = 2 μg · L^{-1}). The highest concentrations were found at Lake Albert. Dissolved organic carbon concentration ranged from 13 mg · L^{-1} at Mannus Lake and Lake Albert to 2.0 mg · L^{-1} at Blowering Reservoir.

DISCUSSION

Microcystis aeruginosa was grown in batch cultures in media composed of varying Co concentrations to assess effects on growth and to provide insight into the biochemical role of Co in freshwater cyanobacteria. Co concentration had significant effects on the growth of the freshwater cyanobacterium *Microcystis aeruginosa* (Fig. 1). This demonstrates that Co is an essential nutrient, given that growth will cease in its absence and is optimal in its presence, and its role could not be replaced by any of the other major micronutrients present in the growth media. These results are consistent with similar studies that

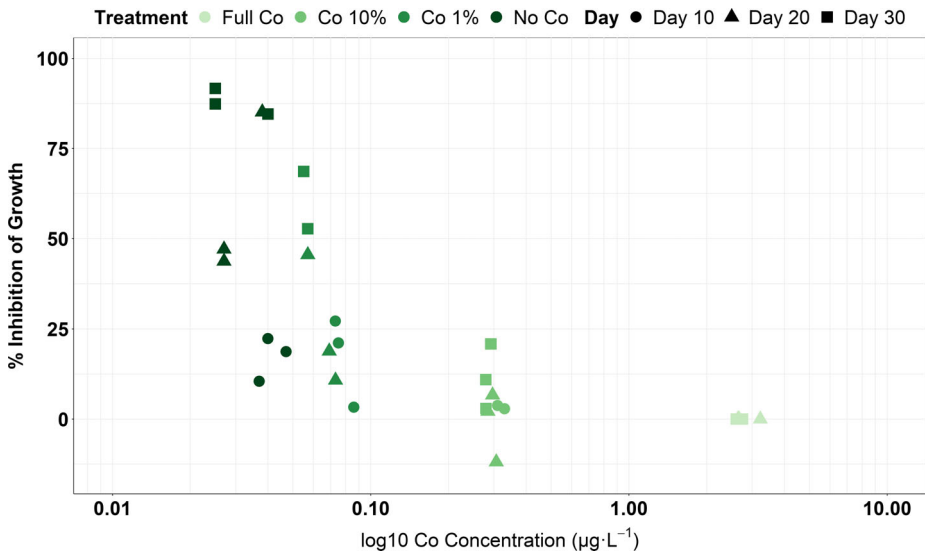

FIG. 2. Relationship between cobalt concentration in the culture media and the percentage growth inhibition compared to the full Co treatment.

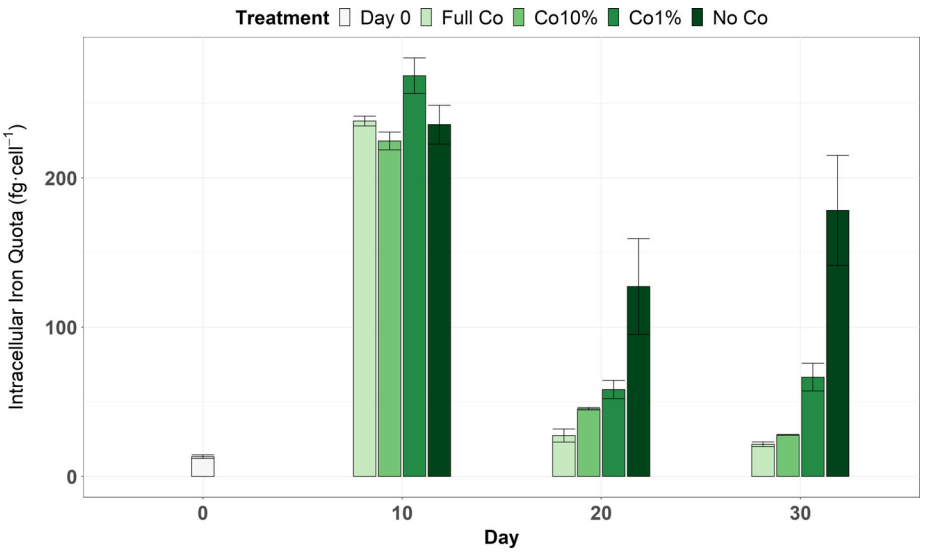

FIG. 3. Differences in the intracellular quota of iron in treatments exposed to varying cobalt concentrations. Error bars are standard error of the mean (n = 3).

TABLE 3. Dissolved cobalt concentrations and physicochemical parameters measured at 0.5 m depth at 10 freshwater sites in NSW, Australia

Site	pH	Dissolved oxygen (mg · L^{-1})	Conductivity (μS · cm^{-1})	Temperature (°C)	Dissolved organic carbon (mg · L^{-1})	PO_4-P (μg · L^{-1})	Dissolved cobalt concentration (μg · L^{-1})
Mannus Lake	7.7	6.5	103	20.7	13.0	8	0.228 ± 0.000
Carcoar Dam	8.6	9.6	381	21.9	12.0	5	0.562 ± 0.006
Lake Lyell	8.6	9.5	477	19.8	5.3	<2	0.072 ± 0.002
Wentworth Falls Lake	8.0	8.6	38	25.3	4.8	3	0.022 ± 0.002
Glenbrook Lagoon	7.7	6.2	139	22.61	8.0	<2	0.019 ± 0.001
Lake Albert	8.2	7.8	480	22.7	13.0	18	0.144 ± 0.004
Wyangala Dam	8.0	7.9	244	21.4	9.6	<2	0.091 ± 0.001
Blowering Reservoir	7.6	8.9	34	22.0	2.0	<2	0.026 ± 0.001
Burrinjuck Dam	8.4	9.1	169	22.5	6.4	3	0.071 ± 0.000
Lake Jindabyne	7.9	11.8	27	21.3	2.3	<2	0.016 ± 0.001

examined cobalt requirements of marine cyanobacteria (Saito et al. 2002, Rodriguez and Ho 2015).

Cobalamin. Co is a component of cobalamin derivatives required for transfer of methyl groups and rearrangement reactions in cellular metabolism (Huertas et al. 2014, Rodriguez and Ho 2015, Helliwell et al. 2016). A possible explanation for the growth limitation observed in treatments exposed to low Co concentrations is that there was insufficient Co to synthesize essential Co-containing molecules such as pseudocobalamin. Given that cyanocobalamin was present in the growth media, this suggests that the synthetic form of cobalamin added to MLA growth media is not bioavailable to *Microcystis aeruginosa* and, therefore, is not an essential component of growth media used for the culturing of *M. aeruginosa*. This is supported by recent findings by Helliwell et al. (2016) who found that two strains of *Microcystis*, along with the vast majority of cyanobacteria, lack the full suite of genes required for the synthesis of cobalamin. Instead, many genera synthesize pseudocobalamin, a structural variation of the form added to MLA media. This provides further evidence that *M. aeruginosa* is synthesizing pseudocobalamin in the presence of sufficient Co rather than bioaccumulating cyanocobalamin from solution. Cobalt can often metabolically substitute for zinc in many enzymes, such as carbonic anhydrase (Xu et al. 2008, Sunda 2012). Given that zinc is the preferred substrate in these enzymes (Intwala et al. 2008, Quigg 2016) and was available in surplus in the growth media, this relationship is unlikely to have caused the negative growth effects observed from low Co concentrations in this study.

Cobalamin and its structural variants can only be synthesized de novo by certain prokaryotes, including some cyanobacteria; however, the majority of microalgal species require it for growth (Watanabe and Bito 2018, Nef et al. 2019). This is achieved through direct uptake from the water column (Helliwell 2017). Pseudocobalamin produced by *Microcystis aeruginosa* and many other cyanobacteria is much

less bioavailable to eukaryotic algae than cobalamin – although some species are capable of converting pseudocobalamin to a bioavailable form (Helliwell et al. 2016). The implication of this is that the form of cobalamin produced, as well as the concentration of Co in surface waters, likely influence phytoplankton productivity and community composition.

Cobalt requirements – linking culture experiments and natural systems. There was a clear relationship between Co concentration in the media and the percentage inhibition of growth compared to the full Co treatment. The trend suggests a threshold value of ~0.06 $\mu g \cdot L^{-1}$, below which *Microcystis aeruginosa* growth was severely inhibited. Time is also a factor as *M. aeruginosa* cells were less affected by low Co availability in the solution after 10 d compared to 30 d, even at similar concentrations. This is likely due to luxury uptake of Co sustaining cell growth for ~10 d by maintenance of an adequate Co quota inside the cell causing a delay in the response (Droop 1973, Saito et al. 2008, Sunda 2012). Alternatively, this could be the result of the overall Co pool being shared between a larger number of cells later in the experiment. A similar study by Holm-Hansen et al. (1954) assessed the Co requirements of the cyanobacteria *Nostoc muscorum* and found that growth was optimal above 0.40 $\mu g \cdot L^{-1}$ although some growth was observed at concentrations as low as 0.002 $\mu g \cdot L^{-1}$.

Co concentrations in freshwaters can be quite variable and depend on the systems geology, hydrology, sediment composition, land use practices and other anthropogenic activity (Neal et al. 1996, Nagpal et al. 2004, Kim et al. 2006). For example Neal et al. (1996) compared trace metal concentrations in major rivers draining into the Humber estuary, England and found that dissolved Co concentrations were notably higher in urban locations compared to rural. In one of the rural sites the minimum dissolved Co concentration observed over a 12-month period was 0.07 $\mu g \cdot L^{-1}$. However, given that Co concentrations are more often reported within the context of potentially toxic levels, such as over application of fertilizers or wastewater from industries (Vetrimurugan et al. 2017), there is limited information on the concentrations of Co in freshwaters without contamination from industry and urban settlements.

In the present study, Co concentrations were surveyed in 10 freshwater lakes and reservoirs of varying inflow characteristics, algal bloom history, geology and anthropogenic disturbance and compared to the threshold value calculated in the culture experiment. To our knowledge this is the first survey of dissolved Co concentrations in Australian freshwaters. A wide range of Co concentrations were observed, ranging from 0.016 ± 0.001 $\mu g \cdot L^{-1}$ to 0.562 ± 0.006 $\mu g \cdot L^{-1}$. Interestingly, the four sites in the study that were below the 0.06 $\mu g \cdot L^{-1}$ threshold either had no cyanobacterial alerts in the following summer (Lake Jindabyne, Glenbrook Lagoon, Wentworth Falls Lake), or had very low cyanobacterial biovolume (Blowering Dam, <0.04 $mm^3 \cdot L^{-1}$), perhaps indicating the capacity for Co to limit the formation or severity of cyanobacterial blooms. On the other hand, systems that underwent blooms were all above the 0.06 $\mu g \cdot L^{-1}$ threshold value. For example, Carcoar Dam, a highly anthropogenically influenced reservoir, which frequently undergoes dense blooms had the highest Co concentration, likely reflecting runoff from a rural agricultural environment. However, as particulate Co was not measured, low dissolved Co concentrations may have also been due to high removal rates from dissolved to non-cyanobacterial particulate phases. PO_4-P concentrations followed a similar trend to those of Co, where sites that rarely bloom had the lowest concentrations. However, PO_4-P concentrations were all below those typically necessary for the proliferation of algae (Walker and Havens 1995, Zeng et al. 2016). This suggests temporal variation in P concentrations and possible colimitation of phytoplankton growth by P and Co.

Cobalt and intracellular iron. There was a clear negative relationship between Co concentration in media and intracellular Fe quotas after 20 and 30 d (Fig. 3). There were no notable differences between treatments after 10 d, likely due to luxury uptake of Co allowing cells to maintain sufficient Co quotas for optimal cellular functioning (Sunda 2012), thereby causing a delay in response in Fe quota. Alternatively, the large increase in Fe availability when cells were transferred on day 0 may have allowed the rapid accumulation of Fe in all treatments. However, the cause of this relationship is yet to be clarified.

High levels of Co induce Fe deficiency in plants by reducing absorption and inhibiting transport (Blaylock et al. 1986, Wallace and Abouzamzam 1989), potentially by displacing ions in binding sites (Gopal et al. 2003). A similar phenomenon may be occurring in *Microcystis aeruginosa* where at higher Co concentrations (in this case the control treatment), Co binds non-specifically with coordination sites normally occupied by Fe, displacing Fe and reducing intracellular concentrations. Cells appeared healthy and grew rapidly in the control treatment in the presence of the highest Co concentrations (2.48 $\mu g \cdot L^{-1}$), indicating that decreased Fe quotas were not a limiting factor. However, given this negative relationship, Fe deficiency will likely occur sooner in the presence of higher Co concentrations. We can also speculate that cyanobacterial siderophores required for the assimilation of chelated iron are also capable of binding other essential metals, including Co. Consequently, siderophore production could be triggered by low Co availability and as an unintended consequence lead to the increased uptake of iron from the culture media. Braud et al. (2009) observed that

pyochelin, a major siderophore produced by *Pseudomonas aeruginosa*, also binds Co^{2+}, which inhibited the uptake of Fe in vivo. Cells may have been able to accumulate greater amounts of intracellular iron when competition for the siderophore-mediated uptake pathway was decreased under low Co conditions.

Significance and future directions. Growth of *Microcystis aeruginosa* was decreased by exposure to low Co concentrations under culture conditions. Extended exposure (>10 d) to Co concentrations below 0.06 $\mu g \cdot L^{-1}$ resulted in significant inhibition of growth. Field concentrations of Co were assessed, and we observed concentrations well below this threshold value, and were exclusively at sites that are not known to undergo cyanobacterial blooms. These results support micronutrient limitation of cyanobacterial growth in freshwater reservoirs (Facey et al. 2021).

While we have provided evidence of growth-limiting concentrations of Co, PO_4-P concentrations were generally aligned to Co in this study, indicating phosphorus limitation may have also been occurring. Given that phosphorus and Co often have different sources, it is unlikely they will always be strongly correlated. However, future studies would benefit from analyzing the availability of macronutrients and physicochemical parameters over a larger temporal scale to fully understand the limiting factors in each system. Nutrient amendment mesocosm experiments conducted at lakes with low Co and P concentrations would also be useful to identify limiting nutrients and threshold concentrations for cyanobacterial growth. Further, our threshold value was based only on the growth response of *Microcystis aeruginosa*. Other cyanobacterial taxa, including nitrogen fixing species, will likely have different requirements for Co. Future studies should assess a broad range of cyanobacterial species to account for these differences.

As evidenced by the negative growth impacts of low Co even in the presence of cyanocobalamin, *Microcystis aeruginosa* appears to be unable to assimilate cyanocobalamin and unable to repurpose the Co held within cyanocobalamin. It appears that inorganic Co is required to produce the structural variant of cobalamin – pseudocobalamin. The variants of cobalamin are not necessarily functionally exchangeable and pseudocobalamin appears to be far less bioavailable (Helliwell et al. 2016). Given the important role of cyanobacteria in the production of cobalamin for utilization by eukaryotic algae, future studies should assess to what extent pseudocobalamin is bioavailable to these taxa, including further examination of the role of eukaryotic taxa capable of remodeling pseudocobalamin (Helliwell et al. 2016).

There was a clear negative relationship between the concentration of Co in solution and the intracellular Fe quota after more than 10 d of growth. To our knowledge this is the first time this relationship has been observed in cyanobacteria, although a similar relationship has been observed in higher plants in which Fe transport was inhibited by high Co concentrations. Future studies may seek to examine Co and Fe transport pathways and whether any competition or inhibition is occurring. Alternatively, reactive oxygen species could be measured to assess if Fe is being assimilated for their detoxification.

AUTHOR CONTRIBUTIONS

J.A. Facey: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal). **J.J. King:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal). **S.C. Apte:** Conceptualization (equal); Data curation (equal); Investigation (equal); Methodology (equal). **S.M. Mitrovic:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal).

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