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Benchtop fluorometry of phycocyanin as a rapid approach for estimating cyanobacterial biovolume

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Cyanobacteria are the primary taxa responsible for freshwater harmful algal blooms (HABs), with several genera capable of producing potent intracellular toxins and off-flavor compounds. There is considerable growing interest in methods to rapidly quantify cyanobacteria in water samples. Past studies have demonstrated poor correlations between phycocyanin *in vivo* fluorescence and cyanobacterial cell densities. We conducted a series of laboratory experiments aimed at refining a protocol that uses benchtop fluorometry to measure the cyanobacterial pigment, phycocyanin, to accurately estimate cyanobacterial biovolume. In our study, we found strong correlations between phycocyanin concentration and cyanobacterial biovolume (but not for cell densities) both within and across ponds, which varied widely in productivity and algal diversity. Thus, benchtop fluorometry of phycocyanin is a viable method for water resource managers to quickly estimate cyanobacterial biovolume.

KEYWORDS: cyanobacteria; harmful algal bloom; HAB; *Microcystis*; monitoring

INTRODUCTION

Freshwater resources are under a variety of threats, including climate change, environmental degradation, increased demands and cultural eutrophication, that will further reduce water quality and quantity. Harmful algal blooms (HABs) are a common consequence of elevated temperatures and excess nutrient loading and can impair water quality in many freshwater and coastal marine ecosystems (Paerl and Huisman, 2009). Cyanobacteria are the primary phytoplankton taxa responsible for freshwater

HABs and have been implicated in the poisoning of pets and humans (Chorus and Bartram, 1999; Carmichael *et al.*, 2001) and the disruption of aquatic foodwebs (Zurawell *et al.*, 2005). In addition, several cyanobacterial genera produce off-flavor compounds, such as geosmin and methylisoborneol, which may contaminate municipal drinking water systems (Jüttner and Watson, 2007). Given the ecological, economic and human health concerns associated with cyanobacterial blooms, water resource managers need a rapid and accurate tool for quantifying the presence and abundance of cyanobacteria.

Microscopy has long been used to quantify phytoplankton biomass and species composition (Hasle, 1978; Komárek and Anagnostidis, 1986). Despite its advantages for fine-scale identification, microscopic enumeration of phytoplankton is extremely time consuming depending on the abundance and composition of plankton and other associated organic and inorganic material. Moreover, using microscopy to obtain accurate estimates of phytoplankton biovolume can be challenging, especially for cyanobacterial taxa with irregular morphologies (e.g. *Microcystis*), given that some preservatives can distort algal cells (Hawkins *et al.*, 2005). There has been a growing demand by regulatory agency and academic scientists for the development of new methods to rapidly assess cyanobacterial abundance in water samples (USEPA, 2009). For example molecular techniques, such as qPCR and gene detection, are often used to rapidly detect toxin-producing cyanobacteria (Tillett *et al.*, 2001; Rinta-Kanto *et al.*, 2009; Al-Tebrineh *et al.*, 2011). However, molecular-based approaches are more expensive than microscopy and also require extensive expertise. Furthermore, these molecular techniques often provide only crude estimates of cell density based on gene copies that are rarely calibrated with phytoplankton cell counts (Tillett *et al.*, 2001; Al-Tebrineh *et al.*, 2011). Phytoplankton contain a variety of pigments that are used for capturing sunlight for photosynthesis. Chlorophyll *a*, is widely used to estimate the biomass of freshwater and marine phytoplankton given its presence in all autotrophs and relative ease of analysis. Consequently, several approaches are available for measuring chlorophyll *a*, including high-performance liquid chromatography (HPLC), spectroscopy and fluorometry (Lorenzen, 1966; Leavitt *et al.*, 1989; Riemann *et al.*, 1989; Otsuki *et al.*, 1994; Randolph, 2007; Zimba, 2012). Cyanobacteria contain phycobilin pigments, including phycocyanin (freshwater taxa) and phycoerythrin (marine taxa), that have absorption maxima between 550 and 650 nm (phycocyanin: ~620 nm; phycoerythrin: ~580 nm, Turner Designs; Rowan, 1989; Lee *et al.*, 1995) and give many cyanobacterial taxa their distinctive blue-green color. Given its high market value in the food industry as a blue colorant (≈$10–50 million a year), the extraction and purification of phycocyanin is well-studied (Siegelman and Kycia, 1978; Santiagos-Santos *et al.*, 2004; Zhu *et al.*, 2007; Rinta-Kanto *et al.*, 2009; Chaiklahan *et al.*, 2012). There is a growing interest by water resource managers and scientists toward using cyanobacteria-specific pigments to quantify cyanobacterial abundance (Stewart and Farmer, 1984; Viskari and Colyer, 2003; Rinta-Kanto *et al.*, 2009; Lawrenz *et al.*, 2011; Zimba, 2012). For example most past studies have compared efficacies of multiple phycocyanin extraction protocols (Viskari and Colyer, 2003; Zimba, 2012) or used *in situ* fluorometers with attached phycocyanin probes to estimate cyanobacterial density (Lee *et al.*, 1995; Brient *et al.*, 2008; Rinta-Kanto *et al.*, 2009) or toxicity (Rinta-Kanto *et al.*, 2009; Bastien *et al.*, 2011; Marion *et al.*, 2012). While these studies have related phycocyanin or chlorophyll *a* measurements to cyanobacterial density, cyanobacteria can vary by several orders of magnitude in size within and across taxa (Reynolds, 1984). Thus, algal density estimates may be misleading and provide little information about the actual biomass of cyanobacteria present in a system. A growing body of studies has begun to explore the relationship between *in vivo* fluorescence and extracted phycocyanin measurements and cyanobacterial biovolume, with these studies often focusing on either monocultures of cyanobacteria in the laboratory (Bastien *et al.*, 2011; Chang *et al.*, 2012) or variation within a single body of water over time (McQuaid *et al.*, 2011; Horváth *et al.*, 2013). Given the risk that cyanobacteria pose to aquatic ecosystems, including drinking water reservoirs, it is necessary to develop tools to accurately estimate cyanobacterial biovolume both within and across waterbodies (Sobiechowska-Sasim *et al.*, 2014).

Previous studies focusing on a limited number of waterbodies (typically one) have demonstrated that *in vivo* phycocyanin fluorescence measurements can be strongly correlated with cyanobacterial biovolume. The primary objective of this study was to refine a rapid, robust protocol to estimate cyanobacterial biovolume across several lentic freshwater waterbodies from extracted phycocyanin concentrations measured with benchtop fluorometry (Randolph, 2007; Randolph *et al.*, 2008). Ultimately, our results show that this method can be used to quickly (i.e. same day) estimate cyanobacterial biovolume from freshwater ponds and reservoirs that contain diverse phytoplankton assemblages and vary widely in productivity.

METHOD

Study sites and sample collection

Seventeen ponds at the Auburn University E. W. Shell Fisheries Research Center that varied in productivity were used for this study (Table I). Four ponds (G13, G19, G20 and G52) were sampled on 22 May 2012 to refine

Table I: Characteristics of ponds at E. W. Shell Fisheries Research Center sampled for this study

Pond	Latitude	Longitude	Area (m^2)	Average depth (m)
E32	32°38′57.75″N	85°29′4.92″W	400	0.91
E33	32°38′57.13″N	85°29′4.97″W	400	0.91
E35	32°38′56.25″N	85°29′5.01″W	400	0.91
F4	32°39′11.62″N	85°29′17.12″W	400	0.91
F9	32°39′11.04″N	85°29′16.42″W	800	1.37
F11	32°39′10.16″N	85°29′15.35″W	800	1.37
G13	32°39′43.07″N	85°29′46.82″W	400	0.91
G19	32°39′41.30″N	85°29′46.95″W	400	0.91
G20	32°39′41.33″N	85°29′46.06″W	400	0.91
G48	32°39′42.05″N	85°29′42.92″W	400	0.91
G52	32°39′40.74″N	85°29′43.13″W	400	0.91
S3	32°40′49.55″N	85°30′54.95″W	38 400	1.71
S8	32°40′20.30″N	85°30′30.35″W	44 900	1.49
S10	32°40′10.37″N	85°30′30.22″W	13 200	1.74
S11	32°40′23.71″N	85°30′25.45″W	11 500	1.37
S22	32°40′45.28″N	85°30′56.16″W	8700	1.80
S28	32°40′12.30″N	85°30′19.99″W	19 200	1.65

Data source = Boyd and Shelton (Boyd and Shelton, 1984).

the extraction procedure. In addition to our pond samples, we included two unialgal cultures, including the non-phycocyanin producing chlorophyte, *Ankistrodesmus falcatus* (as a negative control since green algae do not produce phycocyanin), and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667) (as a positive control since cyanobacteria do produce phycocyanin). Thirteen additional ponds (E32, E33, E35, F4, F9, F11, G48, S3, S8, S10, S11, S22 and S28) and three previously sampled ponds (G19, G20 and G52) were sampled on 7 September 2012 to further develop the relationships between algal pigments and biovolume across systems that span a wider productivity gradient. During both sampling dates, integrated pond water samples (0−0.25 m) were collected with a clear polyvinyl chloride tube sampler (5 cm diameter) from multiple sides of each pond.

Water samples from each pond were stored in coolers with cold packs prior to being processed in the laboratory. Each sample was mixed well prior to collecting vacuum-filtered samples on 47-mm Pall A/E filters for chlorophyll *a* (two replicates per pond) and phycocyanin (four replicates per pond) analyses or poured into 20-mL glass vials and preserved with 1% Lugols solution for phytoplankton enumeration (two replicates per pond). Filters were stored in the dark at −9°C until processed.

Phycocyanin analysis and refinement

Filter grinding

To quantify phycocyanin, filters were first ground in 10 mL of 50 mM phosphate buffer (40 mL of concentrated buffer (Ricca Chemical Company, #5807-16) + 960 mL distilled water; pH 7.0 ± 0.1) under reduced light using a smooth Teflon grinder in a centrifuge tube for 1 min. Another 10 mL aliquot of phosphate buffer was used to rinse the grinder into the centrifuge tube, bringing the total extract volume to 20 mL. The centrifuge tube was capped, mixed well by hand, and immediately stored in darkness. The grinder was rinsed with clean buffer and dried between samples to prevent contamination.

Extraction refinement

To determine if there was an optimal extraction time at one of the two temperatures, 24 replicate samples were taken from a composite sample of water collected from each of three ponds (G13, G20 and G52) and extracted for 0, 4 or 24 h at 4°C (standard refrigerator) or 21°C (room temperature) (four replicates per pond per temperature and time combination). A second experiment was conducted to determine if a more specific extraction time at 4°C further maximized phycocyanin extraction. In this experiment, composite samples of water pooled from three ponds (G13, G19 and G20) were extracted for 0, 2, 4, 6, 8, 10 or 12 h (four replicates per time). Based on data from these two experiments, future samples were stored in darkness at 4°C for 2 h and then placed in a dark cabinet for another 2 h to warm to room temperature (≈21°C) prior to analysis. Thus, the total extraction time was 4 h.

Filtering samples

Extracts were filter-sterilized (<0.2 μm) to remove particulates prior to analysis. Following the 4 h extraction, a 4 mL aliquot of the extract was purified through a series of three separate 25-mm inline filters (A/E; 0.45 μm (VWR #28145-485); 0.20 μm (VWR #28145-483)). All syringes and filters were rinsed with fresh buffer between samples to prevent contamination.

Fluorometric analysis

Filtered extracts were analyzed for phycocyanin using a Turner Designs Trilogy fluorometer fitted with an orange module (Turner Designs #7200-044) that accommodated a four-sided clean, clear 10 mm, glass square cuvette (Nova Biotech, #G-550). Raw fluorescence units (RFUs) were converted to phycocyanin concentrations using a standard curve [concentrations (μg L^{-1}): 0, 10, 50, 100, 500, 1000 and 2000] created with phycocyanin (Sigma-Aldrich #P2172-10MG) dissolved in phosphate buffer. Prior to creating our standard curve, the stock phycocyanin standard concentration was confirmed by spectrophotometry using this equation: C-Phycocyanin (mg mL^{-1}) = (Abs 615 nm − (0.474 × Abs 652 nm))/5.34 (Siegelman and Kycia, 1978).

Chlorophyll *a* analysis

Chlorophyll *a* concentrations were measured by extracting phytoplankton from filters in 90% ethanol for 24 h in

the dark at 4°C (Sartory and Grobbelaar, 1984) followed by analysis on a Turner Designs Trilogy fluorometer complemented with a non-acidification chlorophyll module (Turner Designs #7200-046).

Phytoplankton enumeration

Samples collected from sixteen ponds on 7 September 2012, as well as the two algal cultures previously used, were analyzed to develop the relationship between algal pigment and biovolume across sites that varied widely in productivity and algal diversity. Phytoplankton species abundance and composition were determined using compound microscopy after settling phytoplankton samples in Palmer–Maloney chambers. On average, 25 fields were counted at each of three magnifications (×100, ×200 and ×400) for each replicate sample to include rare large and abundant small taxa (Chislock *et al.*, 2014). All samples were enumerated in duplicate. Average algal cell volumes were calculated for each taxon for each sample using standard measurements (Hillebrand *et al.*, 1999) of ≥10 individuals, when available. Biovolume for each species for each sample was calculated by multiplying cell density by cell volume. Dominant phytoplankton taxa (>10% biovolume) were determined for each pond sample (Table II).

Statistical analysis

Two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare phycocyanin concentration for three ponds (G13, G20 and G52) following varying extraction times (4 and 24 h) and temperatures (4 and 21°C). A paired *t*-test determined if phycocyanin extractions generally differed between temperatures (4 and 21°C) for two extraction times (4 and 24 h) across three ponds (G13, G20 and G52). ANOVA was used to compare phycocyanin concentrations for one composite sample pooled from three ponds (G13, G19 and G20) across several extraction times (0, 2, 4, 6, 8, 10 or 12 h) at 4°C. Pearson's correlation coefficients were calculated to examine the relationship between algal pigments and biovolume or density across 16 ponds. Given the wide range in algal biovolume and pigment concentrations across ponds, data were log-transformed, as needed, to conform to the assumptions of parametric statistics. For all tests, $\alpha = 0.05$.

Table II: Dominant phytoplankton taxa (>10% total biovolume) present in ponds that were used to develop a relationship between phycocyanin concentration and cyanobacterial biovolume

	Dominant phytoplankton taxa (% of total biovolume)	
Pond	Cyanobacteria	Other taxa
E32	*Planktothrix* (94.6%)	None
E33	*Planktothrix* (97.2%)	None
E35	*Planktothrix* (99.2%)	None
F4	*Cylindrospermopsis* (24.6%) *Phacus* (21.6%)	*Gymnodinium* (32.9%)
F9	*Cylindrospermopsis* (71.5%) *Microcystis* (24.2%)	None
F11	*Cylindrospermopsis* (84.3%) *Anabaena* (13.7%)	None
G19	*Cylindrospermopsis* (98%)	None
G20	*Cylindrospermopsis* (68.4%) *Planktolyngbya* (15%)	None
G48	*Anabaena* (84.4%) *Cylindrospermopsis* (15.2%)	None
G52	*Planktolyngbya* (99.9%)	None
S3	*Cylindrospermopsis* (93.4%)	None
S8	*Cylindrospermopsis* (70.8%) *Microcystis* (27.6%)	None
S10	*Cylindrospermopsis* (92.1%)	None
S11	*Cylindrospermopsis* (62.5%) *Microcystis* (32.3%)	None
S22	None	*Ceratium* (100%)
S28	*Pseudoanabaena* (47.2%) *Anabaena* (40.4%)	None

RESULTS

Optimization of extraction

Extraction time and temperature test

For all three ponds, we found significant interactions between extraction temperature and duration (temperature × time interaction $P < 0.05$, Fig. 1). In general, cold extractions (4 and 24 h across the three ponds) provided higher estimates of phycocyanin relative to warm extractions (paired *t*-test $P = 0.04$, Fig. 1). This difference was most pronounced at the 24 h extraction point ($P < 0.05$ for all three ponds, Fig. 1).

For the composite sample extracted at 4°C, longer extractions (10–12 h) showed relatively reduced phycocyanin concentration for a pooled water sample compared with shorter extraction times (2 vs. 10 h Tukey $P = 0.03$, 2 vs. 12 h Tukey $P = 0.08$), extraction times ranging from 2 to 8 h produced similar concentrations (all comparisons for these extraction times, Tukey $P > 0.59$, Fig. 2).

Relationships between algal pigments and phytoplankton biovolume

Across 16 ponds that varied widely in productivity and algal diversity and two strongly contrasting algal cultures (one chlorophyte and one cyanobacterium), we found strong relationships between chlorophyll *a* concentration ($\mu g\ L^{-1}$) and phytoplankton biovolume ($mm^3\ L^{-1}$)

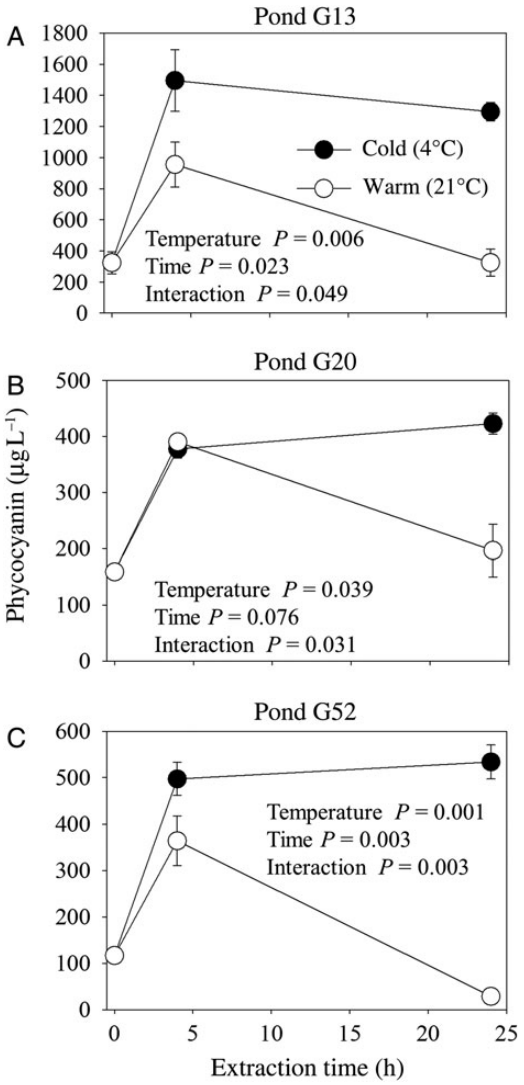

Fig. 1. Phycocyanin concentration ($\mu g\,L^{-1}$) from three ponds at the Auburn University E. W. Shell Fisheries Research Center [ponds (**A**) G13, (**B**) G20 and (**C**) G52] analyzed over a range of extraction times (0, 4 and 24 h) and under two temperatures (cold = 4°C and warm = 21°C). Replicate subsample mean ± standard error. All three pond samples showed significantly lower phycocyanin concentration after a 24 h extraction at 21°C relative to concentrations observed after a 24-h extraction at 4°C (paired *t*-test: G13 $P = 0.012$, G20 $P = 0.046$, G52 $P = 0.026$).

($P < 0.001$, $r^2 = 0.894$, Fig. 3A), and phycocyanin concentration ($\mu g\,L^{-1}$) and cyanobacterial biovolume ($mm^3\,L^{-1}$) ($P = 0.002$, $r^2 = 0.632$, Fig. 3B). Such robust patterns were surprising given that the pond algal communities were dominated by a wide range of phytoplankton, including *Anabaena*, *Ceratium*, *Cylindrospermopsis*, *Microcystis*, *Planktolyngbya*, *Planktothrix* and *Pseudoanabaena*. Regression equations associated with these relationships to estimate phytoplankton or cyanobacterial abundance

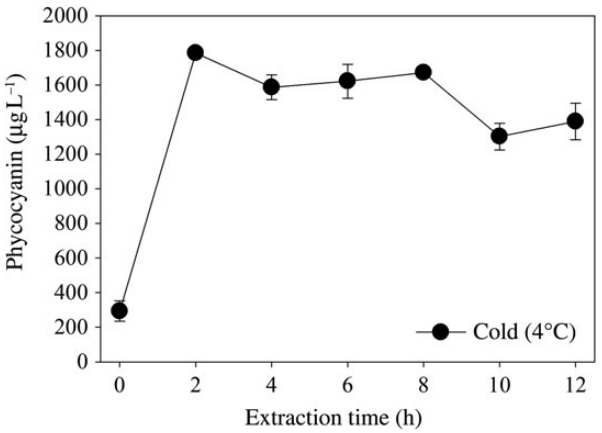

Fig. 2. Phycocyanin concentration ($\mu g\,L^{-1}$) of a mixture of seston collected from several ponds at the Auburn University E. W. Shell Fisheries Research Center analyzed over a range of extraction times (0–12 h) at 4°C. Replicate subsample mean ± standard error.

from algal pigments are below:

$$\log \text{phytoplankton biovolume}\,(mm^3\ L^{-1}) = (\log \text{chlorophyll}\,a\,(\mu g\,L^{-1}) \times 1.041) - 0.672$$

$$\log \text{cyanobacterial biovolume}\,(mm^3 L^{-1}) = (\log\ \text{phycocyanin}\,(\mu g\,L^{-1}) \times 0.573) + 0.296$$

Chlorophyll *a* and phycocyanin concentration also were highly correlated ($P < 0.001$, $r^2 = 0.837$, Fig. 3C). The relationship of the two algal pigments could also be used to help estimate abundance of cyanobacteria in water samples from only chlorophyll *a*. The regression equation used to predict phycocyanin concentration from chlorophyll *a* is below:

$$\log\ \text{phycocyanin}\,(\mu g\ L^{-1}) = (\log \text{chlorophyll}\,a\,(\mu g\ L^{-1}) \times 1.385) - 0.819$$

The correlations between chlorophyll or phycocyanin and algal or cyanobacterial cell densities were weak ($r^2 < 0.25$, Fig. 4). The only statistically significant pattern observed was the relationship between chlorophyll and phytoplankton cell density ($P = 0.043$, $r^2 = 0.231$, Fig. 4A). For our study, cyanobacterial cell density was poorly correlated to chlorophyll ($P = 0.142$, $r^2 = 0.148$, Fig. 4B) or phycocyanin ($P = 0.242$, $r^2 = 0.096$, Fig. 4C) concentrations.

DISCUSSION

While other methods exist for quantifying cyanobacterial abundance, such as qPCR or HPLC (Otsuki *et al.*, 1994;

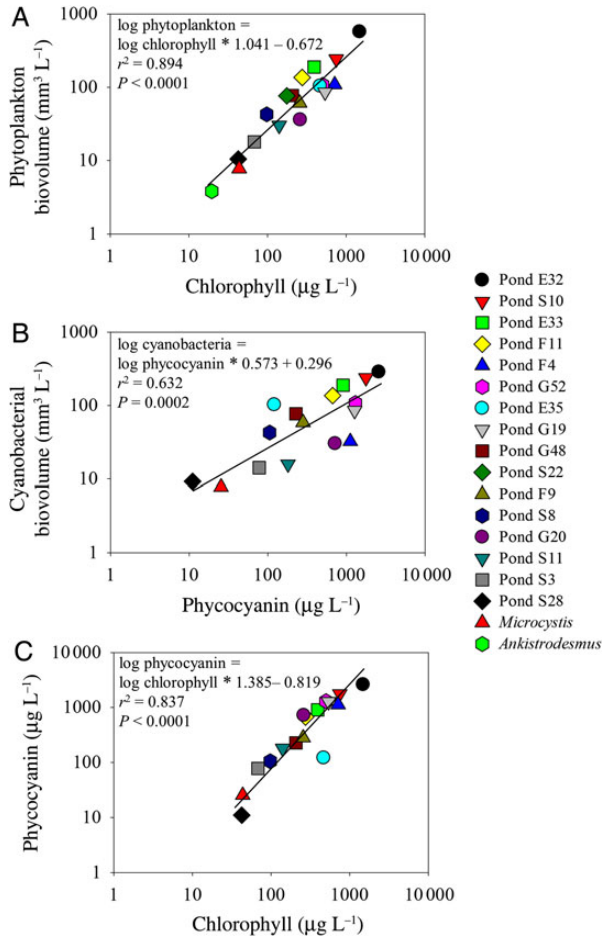

Fig. 3. General patterns between (**A**) chlorophyll *a* concentration (µg L^{-1}) and phytoplankton biovolume (µm^3 mL^{-1}), (**B**) phycocyanin concentration (µg L^{-1}) and cyanobacterial biovolume (µm^3 mL^{-1}) or (**C**) chlorophyll *a* concentration (µg L^{-1}) and phycocyanin concentration (µg L^{-1}) of two cultures [the chlorophyte, *Ankistrodesmus* and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)] and 16 ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, S22, S28, S3, S8, F9, F11, E33, F4, S10, E32 and E35).

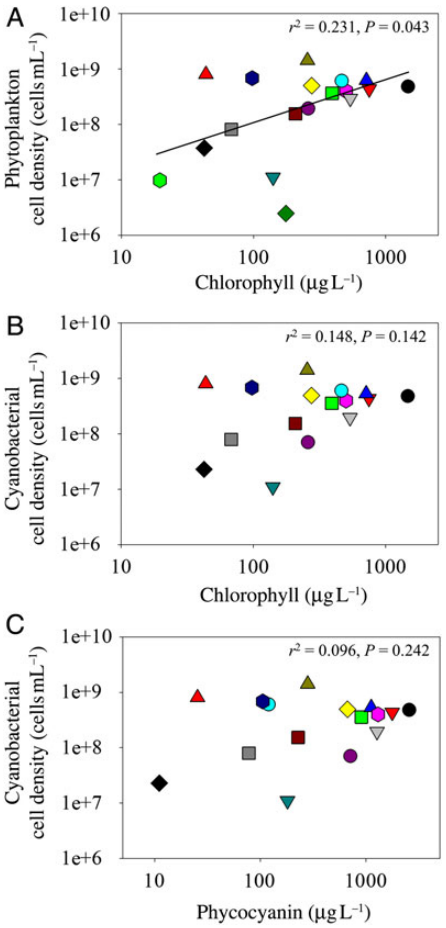

Fig. 4. General patterns between (**A**) chlorophyll a concentration (µg L^{-1}) and phytoplankton cell density (cells mL^{-1}), (**B**) chlorophyll a concentration (µg L^{-1}) and cyanobacterial cell density (cells mL^{-1}) or (**C**) phycocyanin a concentration (µg L^{-1}) and cyanobacterial cell density (cells mL^{-1}) of two cultures [the chlorophyte, Ankistrodesmus and the cyanobacterium, Microcystis aeruginosa (UTEX 2667)] and 16 ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, S22, S28, S3, S8, F9, F11, E33, F4, S10, E32 and E35; Fig. 3).

Rinta-Kanto *et al.*, 2009; Zimba, 2012), there is a need for a rapid, inexpensive and reliable method for estimating cyanobacterial biovolume from whole water samples. The aim of this study was to develop a protocol using benchtop fluorometry of the cyanobacterial pigment, phycocyanin, to accurately estimate cyanobacterial biovolume in freshwater habitats that varied in productivity and algal diversity.

Using two laboratory experiments that manipulated temperature and/or extraction time, we identified conditions that most effectively extracted phycocyanin from mixed phytoplankton communities using a standard phosphate buffer. The first experiment measured the effects of extraction time (4 vs. 24 h) and temperature (4 vs. 21°C) on phycocyanin concentration. Unlike standard chlorophyll *a* extraction protocols, we found that a 24-h extraction period significantly reduced phycocyanin concentrations, especially under the 21°C (Fig. 1). These results were not surprising given the sensitivity of phycocyanin pigment to elevated light and temperature. For example through our protocol development, we learned that phycocyanin degrades more rapidly under fluorescent laboratory light than chlorophyll *a*. Thus, all extractions and sample processing must be done under reduced laboratory light and absolutely no exposure to sunlight. These results are consistent with those of another study which examined the sensitivity of phycobilin pigments to pH, temperature and light (Moreth and Yentsch, 1970).

Our data also indicate that samples extracted at 4°C were less variable then the samples extracted at 21°C. Such

findings are consistent with other studies that have documented variation in phycocyanin extraction related to uncontrollable fluctuations in room temperature (Sarada *et al.*, 1999; Doke, 2005). Moreover, the extract temperature should be approximately the same as the temperature during standard curve development since colder extract temperatures cause higher fluorescence measurements (i.e. negative relationship between pigment temperature and fluorescence; Maxwell and Johnson, 2000). Such variation could negatively impact the utility of using algal pigment concentrations as a reliable analog for cyanobacterial concentrations. However, no fluorometric temperature correction factor currently exists for phycocyanin. Thus, a temperature correction factor was developed for phycocyanin by measuring RFUs of eight phycocyanin standards that varied in concentration across a large temperature gradient commonly found in laboratories (15–25°C; Fig. 5). Temperature (accuracy ± 0.1°C) was directly measured in the cuvette using a small probe attached to a digital thermometer. The temperature correction factor was calculated by calculating relative RFU differences compared with base measurements made near 21°C (i.e. RFU_t/RFU_{bt} where RFU_t and RFU_{bt} are the non-base and base temperature measurements, respectively). In general, similar small reductions in RFUs per degree increase in temperature were found regardless of pigment concentration (range = −2.2–0%; Fig. 5A). When pooling these temperature correction factors together (Fig. 5B), a small relative fluorescence reduction of −1.27%/°C (relative to 21°C) was observed which is consistent with corrections estimated for another algal pigment, chlorophyll (−1.4%/°C *in vivo* chlorophyll and −0.3%/°C in acetone-extracted chlorophyll; Lorenzen, 1966). Given that temperature effects were relatively minor for phycocyanin, corrected RFUs closely matched actual RFUs (Fig. 5C).

A second laboratory experiment highlighted that extractions lasting between 2 and 8 h at 4°C provide similar pigment concentrations. In addition, although 2- h extractions yielded the highest absolute phycocyanin concentration, it is necessary to allow an additional 2 h for the extracts to warm to room temperature prior to fluorometry. Thus, a total extraction period of 4 h was ultimately used for our final protocol. Our methods and results differ from a study which used HPLC to measure phycocyanin (Otsuki *et al.*, 1994), which showed the highest extractions at 12 h using 10 mM phosphate buffer at 3°C. One notable difference between Otsuki *et al.* (Otsuki *et al.*, 1994) and our study, besides the phosphate buffer, is that we ground our samples prior to extraction since we found that manually disrupting the cells helped shorten extractions times (unpublished data).

Our results show a strong relationship between phycocyanin concentration and cyanobacterial biovolume (Fig. 3B). Similar results have been reported for other studies (Brient *et al.*, 2008; McQuaid *et al.*, 2011) that have promoted the use of phycocyanin to estimate cyanobacterial cell density. These studies aimed to interpret their data within the World Health Organization (WHO) water quality guidelines (Ahn *et al.*, 2007; Brient *et al.*, 2008; McQuaid *et al.*, 2011). For example Brient *et al.* (Brient

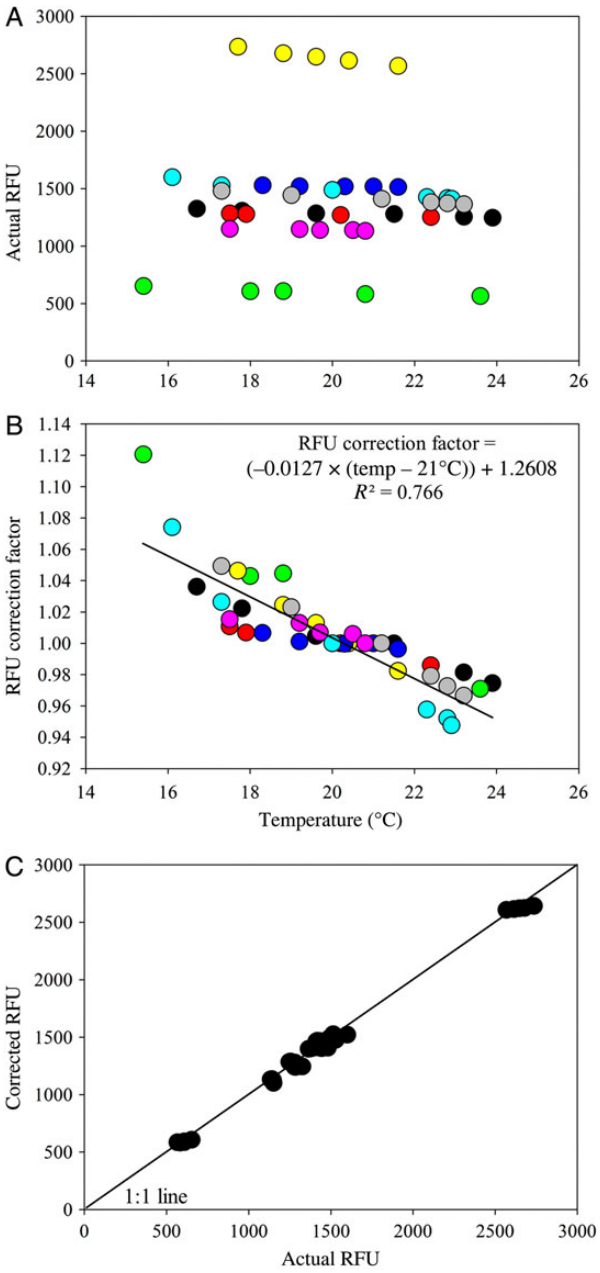

Fig. 5. General patterns between ambient temperature and (**A**) actual raw fluorescence units (RFU) or (**B**) RFU correction factors of eight different phycocyanin standards measured between 15 and 24°C. (**C**) Correlation between actual and corrected RFUs for phycocyanin standards measured between 15 and 24°C.

et al., 2008) reported that a phycocyanin concentration of 30 $\mu g\ L^{-1}$ is equivalent to the WHO alert level 1 of 20 000 cyanobacterial cells mL^{-1}. For our data, there was a highly variable, but statistically significant, relationship between chlorophyll *a* and phytoplankton cell density (Fig. 4A). However, no relationships were found for chlorophyll *a* and cyanobacterial cell density (Fig. 4B) or between phycocyanin concentration and cyanobacterial cell density (Fig. 4C). Therefore, we are unable to reliably assess the pigment concentrations relevant to WHO alert levels associated with cyanobacterial densities. Phytoplankton can vary several orders of magnitude in size both within and across species (Reynolds, 1984). Thus, it is not surprising that we were unable to find clear patterns between cell density and algal pigments. However, cyanobacterial biovolume was highly correlated with pigment concentration (Fig. 3B). Hence, we recommend that others consider using cell biovolume as opposed to cell density in water quality guidelines (e.g. Chang *et al.*, 2012; Horváth *et al.*, 2013; Lunetta *et al.*, 2014).

In situ fluorometers can measure phycocyanin across space and time, but there are many unresolved issues that may lead to inaccurate estimates of cyanobacterial biomass from these tools. For example several studies reported problems with estimating cyanobacterial abundance accurately because of technical problems related to the *in situ* fluorometer (e.g. clogging of the probe, light saturation interfering with the readings, calibration challenges), abiotic factors (e.g. turbidity, temperature) and intrinsic factors related to cyanobacteria (e.g. variation in phycocyanin production depending on cyanobacterial growth stage, morphology and size and relative abundance to other phytoplankton taxa) (Gregor *et al.*, 2007; Izydorczyk *et al.*, 2009; McQuaid *et al.*, 2011; Chang *et al.*, 2012; Richardson *et al.*, 2010). In addition, other technical issues associated with the calibration of *in situ* fluorometers may compromise the utility of these tools. For example in some studies, manufacturer settings are used without additional calibrations (Bowling *et al.*, 2012; Song *et al.*, 2013) or standards are made available in solvents that are not commonly used for pigment extraction. In other cases, phycocyanin probes are not calibrated but instead RFUs are converted into pigment concentrations (Song *et al.*, 2013). Since RFUs are specific to each instrument and protocol, it is impossible to accurately compare phycocyanin RFU measurements across studies. In other cases where *in situ* probes are calibrated, the probes are calibrated for one taxa, typically *M. aeruginosa*, regardless of the dominant cyanobacteria in the waterbody (Bastien *et al.*, 2011; McQuaid *et al.*, 2011). Despite the issues associated with *in situ* fluorometry, its use is spreading. However, some studies contend that *in situ* measurements should be confirmed with other techniques (Brient *et al.*, 2008; Bowling *et al.*, 2012). In our study, we showed strong relationships between algal pigments and biovolume.

Many studies have documented wide variation in extraction efficiencies of a variety of protocols (Siegelman and Kycia, 1978; Viskari and Colyer, 2003; Santiagos-Santos *et al.*, 2004; Zhu *et al.*, 2007; Rinta-Kanto *et al.*, 2009; Chaiklahan *et al.*, 2012; Zimba, 2012). Although phosphate buffers have been criticized for their relatively low extraction efficiencies (Zimba, 2012), this study aimed to develop a widely applicable relationship between phycocyanin and cyanobacterial biovolume using pragmatic protocols accessible to water resource scientists with diverse expertise and limited resources. Additionally, since the goal is to link algal pigments with biovolume, the absolute extraction efficiency does not have to be complete. Instead, relative extraction efficiencies can be used. We encourage scientists to develop their own relationships using their equipment following the protocols described in this study to predict cyanobacterial biovolume from phycocyanin concentration.

It is also important to note that cyanobacteria are not the only taxa to produce phycocyanin, but that cryptophytes have been shown to also produce this pigment (Hoef-Emden, 2008). So while phycocyanin is a more cyanobacteria-specific pigment compared with chlorophyll *a*, it is not exclusive to the taxa (Gantt, 1975; Zimba, 2012). Cryptophytes were not abundant in our samples, but it may be necessary to confirm phycocyanin measurements with microscopy to assure that cyanobacteria are the dominant taxa in samples. If not, then cryptophytes may cause overestimates of cyanobacterial abundance, and thus lead to inappropriate measures and associated management actions.

Overall, our study confirms that the use of benchtop fluorometry is an efficient way to estimate cyanobacterial biovolume from diverse water samples. The short (4 h) extraction time and relatively simple extraction method make it a reliable alternative for measuring cyanobacterial biovolume relative to complex molecular or chemical techniques that may take more time and be cost-prohibitive. This method may be useful for water resource managers interested in monitoring drinking water sources for the presence and abundance of cyanobacteria.

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