



Highlighting the promise of qPCR-based environmental monitoring: response of the ribosomal RNA:DNA ratio of calanoid copepods to toxic cyanobacteria

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Accepted: 6 February 2021 / Published online: 6 March 2021

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Abstract

Calanoid copepods are integral to aquatic food webs and may drive the bioaccumulation of toxins and heavy metals, spread of infectious diseases, and occurrence of toxic cyanobacterial harmful algal blooms (HABs) in freshwater aquatic systems. However, interrelationships between copepod and cyanobacterial population dynamics and ecophysiology remain unclear. Insights into these relationships are important to aquatic resource management, as they may help guide mitigation efforts. We developed a calanoid copepod qPCR assay to investigate how copepod abundance and physiological status relate to the abundance of cyanobacteria and the concentration of total microcystin in a HAB-prone freshwater multi-use eutrophic lake. Through *in silico* and *in vitro* validation of primers and analyses of time series, we demonstrate that our assay can be used as a reliable tool for environmental monitoring. Importantly, copepod RNA:DNA ratios on and shortly after the day when microcystin concentration was at its highest within the lake were not significantly lower (or higher) than before or after this period, suggesting that copepods may have been tolerant of microcystin levels observed and capable of perpetuating bloom events by consuming competitors of toxic cyanobacteria.

Keywords Cyanotoxin · Food web · Microcystin · Phytoplankton · Zooplankton

Introduction

Calanoid copepods are minute cosmopolitan crustaceans that are often the predominant zooplankton in freshwater lakes. They are integral to the food web, being consumers of mussel larvae, rotifers, phytoplankton, protozoa, and bacteria (Stemberger and Evans 1984; Liebig and Vanderploeg 1995; Burns and Schallenberg 2001) and themselves the prey or hosts of fish, adult mussels, and other aquatic organisms (Bayly 1963; Flinkman et al. 1994; Smith and Alexander 2008; Jonsson et al. 2009). As such, calanoid copepods have been found to be drivers of the

bioaccumulation of toxins (Magnusson and Tiselius 2010; Berry et al. 2012) and heavy metals (Kadiene et al. 2017; Kadiene et al. 2019) in aquatic systems and of the spread of infectious diseases among wildlife and commercial aquatic organisms (Piasecki et al. 2004). They are also thought to play a role in the occurrence of freshwater toxic cyanobacterial harmful algal blooms (HABs), a global environmental issue. However, in the case of HABs, the nature of the copepods' involvement is a source of dispute.

Evidence for calanoid copepod control of HABs has been mixed. Some studies suggest that HABs would likely be palliated or even eliminated by calanoid copepod feeding, if not for the copepods' populations being too low at the onset of HABs due to factors such as the nutritional inadequacy of diatoms (the most available phytoplankton at the start of the copepod growth season; Miralto et al. 1999) and the mortality caused by omnivorous fish (Gopalan et al. 1998). Gizzard shad (*Dorosoma cepedianum*), for example, purportedly create HAB-favoring conditions by feeding on copepods and other zooplankton and transporting nutrients from sediments to the water column (Schaus and Vanni

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2000); deliberate removal of gizzard shad has been found to increase the abundance of calanoid copepods and reduce the abundance of toxic cyanobacteria in some cases (Paerl et al. 2016). However, other studies indicate that calanoid copepods make for ineffective or even counter-effective top-down controls on HABs because they tend not to eat cyanobacteria (preferring to eat protozoa, algae, and the eggs of their competitors) and excrete more of the limiting nutrients that phytoplankton require for compensatory growth than do other zooplankton such as *Daphnia* spp. (Sommer and Sommer 2006). A review by Boon et al. (1994) of cases in Australian inland waters indicated that calanoid copepods can and do consume toxic cyanobacteria throughout their growth season, but at rates insufficient to control HABs. A study by Koski et al. (2002) similarly demonstrated that calanoid copepods can feed on toxic cyanobacteria and even produce eggs while doing so but noted that the copepods would switch to protozoa when the latter became more abundant. Hansson et al. (2007) found that calanoid copepods are sensitive to cyanotoxins, including congeners of microcystin, which, in combination with mortality from fish predation, allows the copepods to be supplanted by more tolerant, less efficient grazers such as rotifers.

Insights helping to resolve the relationship between calanoid copepods and HABs are worthwhile to pursue from the standpoint of natural resource management, as they may be used to guide mitigation efforts. If calanoid copepods are efficient grazers of toxic cyanobacteria but too low in abundance at the onset of HABs to present an effective control mechanism, then it may be cost-effective to grow the copepods to high population density in captivity and release them as part of an integrated pest management strategy. On the other hand, if copepods are inefficient grazers or facilitate HABs, regardless of their abundance and toxin tolerance, then the appropriate response may be to modify the environment to favor more effective controls on toxic cyanobacteria.

Part of the difficulty in determining whether calanoid copepods generally facilitate or inhibit HABs is the challenge of accurately monitoring copepod population dynamics and changes in their physiological status before, during, and after HAB events. Identifying and enumerating copepods repeatedly across multiple sites by itself can be time-consuming and labor-intensive and, furthermore, might be insufficient to address the issue. Even if one observes that the number of adult copepods remains consistent in parts of a lake where toxic cyanobacteria are the only available food source, this does not mean that these individuals are large and healthy enough to be efficient grazers of cyanobacteria or that they produce viable eggs; they may be sickly and slow-reproducing. Inferences and extrapolations regarding copepod physiological tolerance and vigor can be made from the results of laboratory

experiments, but this is not always ideal for monitoring purposes, as experimental conditions are intentionally less variable and complex than environmental conditions.

One way to simultaneously address the problems of sampling broadly, distinguishing calanoid copepods from other zooplankton, quantifying calanoid copepod abundance, and assessing calanoid copepod physiological status is to employ molecular methods. Such methods rely on the isolation of DNA or RNA from samples, which researchers can use to identify and enumerate target organisms without needing to perform morphological assessments or culturing. Species' chromosomal DNA content per cell tends to remain constant regardless of changes in the environment (each of the body's cell nuclei has the same DNA complement as all the others, determined by the number and size of the chromosomes that comprise the species' genome). In contrast, an individual's RNA varies based on its physiological status and metabolic activities. Previous studies have demonstrated that total RNA:DNA ratio in calanoid copepods can be a useful indicator of population health, with lower ratios generally being indicative of stress and morbidity (Wagner et al. 1998; Speekmann et al. 2007; Ning et al. 2013).

One of the most versatile and reliable molecular methods for monitoring aquatic species' population dynamics is that of real-time quantitative polymerase chain reaction (qPCR; Aw and Rose 2012; LeBlanc et al. 2020). For the present study, we developed a calanoid copepod qPCR assay to evaluate how copepod abundance and physiological status relate to the abundances of cyanobacteria and the concentrations of cyanotoxins.

Materials & methods

Sample collection

Our study site was Lake Harsha, a HAB-prone freshwater eutrophic lake located on the East Fork of the Little Miami River in southwest Ohio, USA. The lake is used for flood control, drinking water supply, and recreation, and serves as a wildlife habitat. Surface water samples (100 mL) were collected from Lake Harsha by boat at three different sites (CGB, EMB, BUOY) using autoclave-sterilized Nalgene bottles from May to September, 2015. At another location (EFL), water samples were siphoned into the same type of Nalgene bottles from intake pipes that pumped water from the surface and from 6 m below the surface, respectively (Fig. 1). Samples were collected at least once a week. In June, sampling frequency was increased to capture potential differences within shorter periods of time due to the occurrence of a cyanobacterial bloom, such that a total of 210 samples were ultimately fed into our analyses (40–45 samples per site).

Fig. 1 Map of Lake Harsha study sites. Samples were collected via boat from the surface water at all sites except for EFL, where they were collected from intake pipes that drew water from the surface and from ca. 6 m (20 feet) below

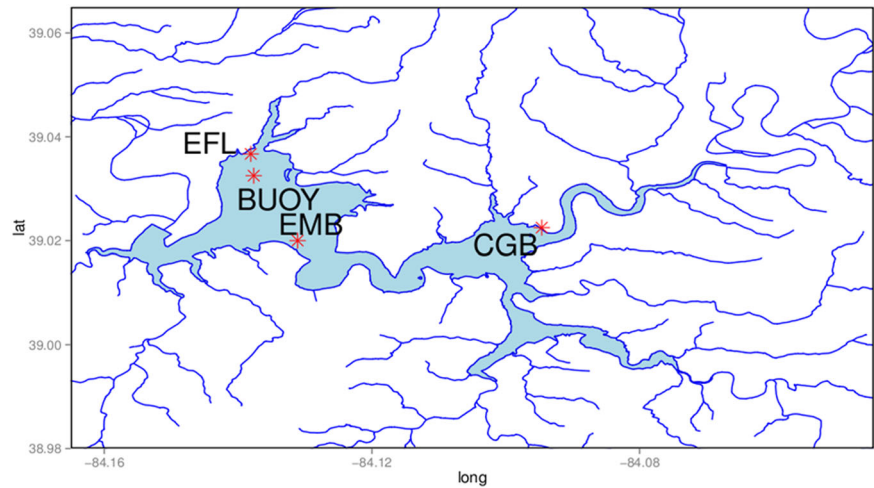


Table 1 Primer sequences evaluated for the assay

Primer set	Forward	Reverse
F1/R1	CTCGACTCACGGAGAGATGC	CGTCAAACCACTGTGAGCC
F3/R3	GCCGTCTCATTGGTGACTCT	AATCGAACCCCTGATTCCCCG
F10/R10	GGGCTGATCGTATGGCCTTG	CCGTCAAACCACTGTGAGC

Water samples were transported on ice to the laboratory (U.S. Environmental Protection Agency, AWBERB facility, Cincinnati, OH) within 2 h of collection. Phycocyanin and chlorophyll-a (along with temperature, pH, specific conductivity, dissolved oxygen, and turbidity) were measured at the time of sampling via a YSI EXO2 multiparameter water quality sonde (YSI Environmental, Yellow Springs, OH). The dominant class of cyanotoxin in the region was microcystin, with MC-LR being the most abundant congener in our system and the deadliest to humans. Concentrations ($\mu\text{g/L}$) of total microcystin and coarse estimates of the relative abundances of zooplankton within the lake ecological community were determined using mass spectrometry and metabarcoding of 18S rRNA genes, respectively, as described elsewhere (Banerji et al. 2018; Banerji et al. 2019).

Sample processing

Upon receipt in the laboratory, water samples were filtered onto 0.4- μm -pore-size, 47-mm-diameter polycarbonate membranes (GE Osmonics). The membranes were transferred to sterile microcentrifuge tubes and stored at -80°C for later manipulation. Nucleic acid extractions were performed as by Kapoor et al. (2016) with minor modifications. Briefly, nucleic acid was extracted directly from the filtered biomass on the membranes using AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of DNA and RNA was determined using Qubit dsDNA HS

assay kits and the Qubit 2.0 Fluorometer (Life Technologies). DNA and RNA extracts were stored for later use at -20 and -80°C , respectively. cDNA was synthesized from the purified total RNA extracts on the same day as RNA extraction using the Superscript III kit, following the manufacturer's instructions (Life Technologies, San Francisco, CA) and stored at -20°C .

qPCR Assay development & amplification

The consensus sequence for the most abundant calanoid operational taxonomic unit (OTU) identified in 18S rRNA gene sequencing libraries (Banerji et al. 2018) was used to develop different group-specific PCR assays. When the OTU sequence was challenged against the non-redundant nucleotide (nr) database in GenBank using BLAST (<https://blast.ncbi.nlm.nih.gov>), it was identified as *Leptodiatomus moorei* (Calanoida: Diaptomidae; GenBank accession number: AY339154.1). The *L. moorei* sequence was challenged against the Pick Blast software (Ye et al. 2012) to generate primer sets that amplify a PCR product ≤ 200 bp in length, with at least two mismatches at the 3' end of the primers of non-targeted copepods. Of ten different primer sets generated by the latter approach, three were selected for further evaluation based on the following criteria: T_m of 55 – 60°C , avoidance of self-priming, and absence of long homopolymers (Table 1).

Real-time qPCR was performed on a QuantStudio 6 Flex Real-Time PCR system (Life Technologies) with Power SYBR Green Master Mix (Applied Biosystems, Foster City,

CA). All qPCR assays were performed in 20 μ L volumes using the SYBR Green qPCR Master Mix, with 200 nM (each) of the forward and reverse primers and 2 μ L of the nucleic acid template. Cycling conditions involved an initial 10 min denaturing step at 95 °C, followed by 35 cycles of 10 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C.

Undiluted and 10-fold diluted nucleic acid extracted from water samples were used as qPCR templates, and results from qPCR runs were compared to determine the presence of potential PCR inhibitors within the filtered samples. Melting curves of each qPCR assay were examined to determine adequate amplification, and qPCR products were visualized on an agarose gel to confirm product sizes. The standards consisted of gBlock gene fragments (Integrated DNA Technologies, Inc., Iowa, USA) using the original *Leptodiatomus* OTU sequence. Five random bases were added to each end of the gBlock to provide better anchoring of primers.

qPCR Assay validation

The specificity of the primer sets was tested in silico against copepod sequences from the Silva128 database using Sequencher (Gene Codes). Primer sequences were also challenged against the nr database from GenBank to determine which of these sequences had a perfect match or minimal mismatches in the 3' end to the primers in question. Potential matches to other taxonomic groups were identified using PrimerTree (Cannon et al. 2016).

Additionally, DNA extracts of the copepods *Leptodiatomus tyrrelli*, *Cyclops scutifer*, and *Boeckella calcaris* and the cladocerans *Daphnia pulicaria*, *D. magna*, and *Ceriodaphnia dubia* were used to test the specificity of the qPCR assays experimentally. All specimens were obtained from culture collections (copepods were provided by Craig Williamson and Erin Overholt, Miami University, Ohio, and cladocerans by James Lazorchak, US-EPA, Cincinnati, Ohio). To further evaluate the primers, results of the qPCR assays on the Lake Harsha samples collected in 2015 were compared to the 18S rRNA gene metabarcoding data for OTU sequences assigned to the genus *Leptodiatomus*. DNA extracts of calanoids, daphnids, and cyclopoids collected from Lake Harsha were also used in specificity tests.

Individual copepod and cladoceran specimens noted above were used to assess the precision of the qPCR assays in enumerating calanoid-specific biomass. We determined the relationship between the number of copepods, total DNA extract concentration, and the number of 18S rDNA copies for Lake Harsha calanoid copepods. For these experiments, the specimens were identified using a dissection microscope before being transferred to sterile microcentrifuge tubes containing 30 μ L of sterile molecular grade

water. They were lifted using autoclaved tweezers that were flamed between uses to minimize cross-contamination. DNA from the specimens was released into the milieu by maceration with sterile pipette tips, heating of the suspension for 25 min at 100 °C, followed by a second round of maceration. Water samples from the tubes in which different plankton species had been stored together at 4 °C were used to determine potential background signal due to DNA released into the milieu.

We transferred adult calanoid copepods to tubes containing lysis buffer in sets of 1, 5, 10 and 20 individuals, each set replicated five times, and extracted the DNA using a Power Soil DNA Isolation kit following the manufacturer's instructions (MoBio Laboratories, Inc.). DNA concentration for each extracted copepod sample was determined using Qubit dsDNA HS kit (Invitrogen). Copepod DNA extracts were stored at -20 °C until used in molecular assays. The mean number of 18S rDNA copies per individual was determined by using all data points generated for 10-fold diluted DNA extracts. DNA was extracted for *D. magna* and *C. dubia* specimens in sets of 1, 5, and 10 individuals per replicate, following the same approach.

Calanoid qPCR signals were detected for all field samples tested in this study. However, it was necessary to dilute the extracts, as there were considerable differences between ten-fold diluted and the undiluted nucleic acid extracts, suggesting qPCR inhibition. In fact, several samples were negative for the undiluted extracts, while strong positive signals were obtained with the corresponding diluted extracts. We have successfully used this approach in several environmental studies using qPCR assays to deal with PCR inhibition (Ryu et al. 2013; Kapoor et al. 2015).

Statistical analysis

Analyses were performed in R (<https://www.r-project.org>). To support the validation of the qPCR assay, regression was used to determine the relationship between the number of 18S rDNA copies and the number of individual adult zooplankton (copepod and cladoceran). This relationship should be linear, yielding a species-specific mean number of 18S rDNA copies per adult individual. Multivariate analysis of variance (MANOVA) was used to test whether the DNA- and RNA-based qPCR measurements and the ratio between these differed among three phases of the sampling period (demarcated in terms of day-of-year, DOY): the pre-HAB period (DOY 126–156), when microcystin concentrations were below the detection limit of our method and the phytoplankton community was dominated by species other than toxic cyanobacteria; the HAB period (DOY 157–208), when microcystin concentrations were high enough to detect (peaking at 5.1 μ g/L) and species of the putatively

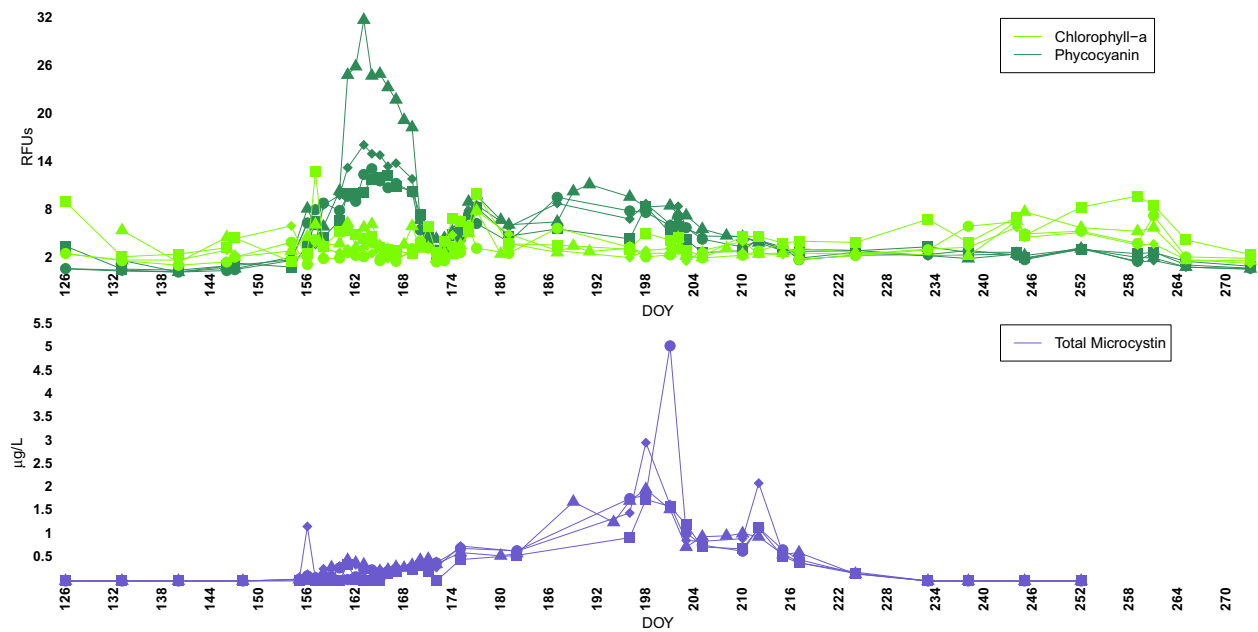


Fig. 2 Cyanobacterial Pigments & Toxins. Time series of photosynthetic pigments (relative fluorescent units of chlorophyll-a and phycocyanin) and cyanotoxin concentration (micrograms per liter of total microcystin) at each of the surface water sampling locations (DOY day-of-year). Shapes of the symbols represent the sampling locations (squares for CGB, circles for EMB, diamonds for BUOY,

and triangles for EFL). Phycocyanin measures exceeding those of chlorophyll-a are generally indicative of cyanobacterial dominance of the phytoplankton community (which we were able to verify via microscopy and comparison to the results from earlier 16S and 18S metabarcoding surveys of the system)

toxic cyanobacteria *Dolichospermum* and *Microcystis* dominated the phytoplankton community; and the post-HAB period (DOY 208–273), when the cyanobacteria began to senesce and microcystin concentrations fell again below our detection limit (Fig. 2). To determine whether differences among these phases were better interpreted as seasonal variations or as consequences of the unique features of certain sites, permutational analysis of variance (PERMANOVA) was used to evaluate differences in temporal trends among sampling sites (Anderson 2001; McArdle and Anderson 2001).

Results

In silico qPCR assay validation

Challenging each primer set against 18S rRNA gene sequences from the Silva 128 and GenBank nr databases revealed that the primers showed specificity to calanoid copepods. However, none of the primer sets were found to be specific to *Leptodiaptomus moorei*, as they could potentially anneal to 18S rRNA gene sequences of other closely related calanoid species/genera. The sets showed identical matches to species of the following Diaptomae genera: *Leptodiaptomus*, *Aglaodiaptomus*, *Skistodiaptomus*, *Diaptomus*, *Arctodiaptomus*, *Eudiaptomus*, *Hesperodiaptomus*, *Hemidiaptomus*,

Heliodiaptomus, *Onychodiaptomus*, *Mastigodiaptomus*, *Acanthodiaptomus*, *Sinodiaptomus*, *Neodiaptomus*, and *Copidodiaptomus*. Some of the primer pairs were identical to sequences from other calanoida families. For example, the F1R1 primers also matched sequences from Tortanidae (Tortanus), Sulcanidae (Sulcanus), Pontellidae (Anomalocera, Pontella, Pontellina), and Temoridae (Temora, Eurytemora).

Further in silico analyses using PrimerTree retrieved 65 sequences from Genbank showing high calanoid-specific amplification potential with the F1R1 assay. In contrast, F3R3 and F10R10 primers could potentially anneal to 500 and 184 sequences, respectively, including non-copepod species. Specifically, of the sequences possessing identical targets for the F1R1 assay, all were identified as calanoid species and, moreover, included a wide variety of genera. The other two primer sets showed great potential of generating false positive results with many microeukaryotes including non-arthropod groups. Based on this, only the F1R1 qPCR assay was appropriate for use in our subsequent analyses.

In vitro qPCR assay validation

Nucleic acid extracts from calanoids retrieved from Lake Harsha tested positive when the F1R1 qPCR assay was applied. The assay yielded weak signals when cyclopoid copepod and daphnid specimens collected from the lake were tested. Similarly, none of the cyclopoid copepod and

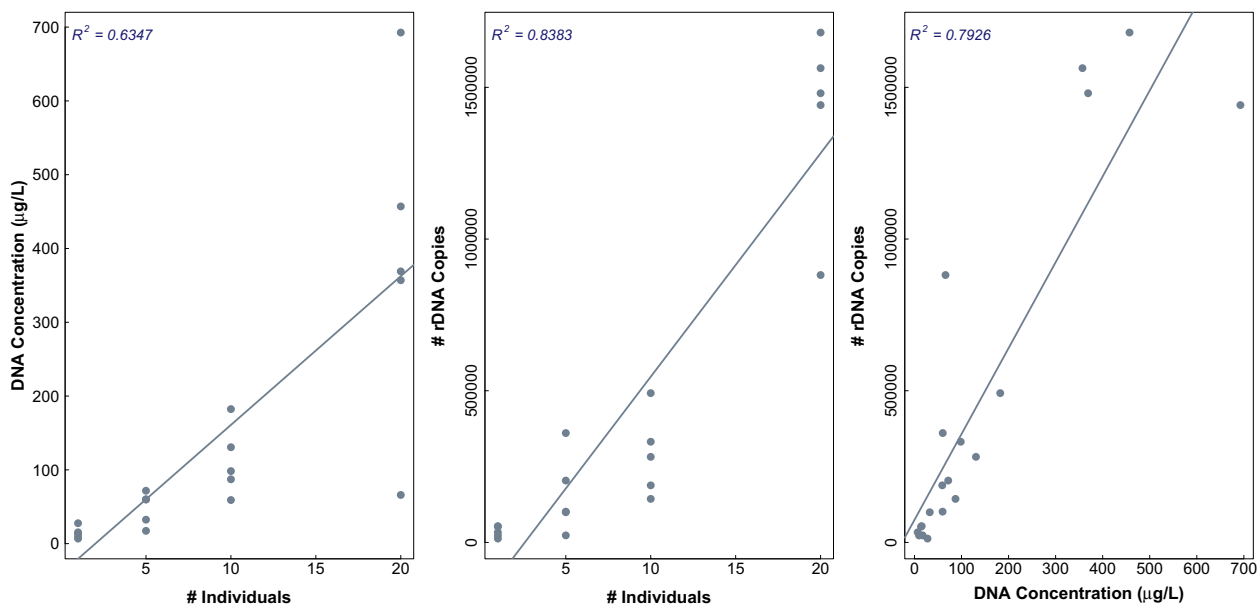


Fig. 3 Copepod Specimens vs. Nucleic Acid. Correlation between DNA concentration vs number of copepods (left panel), rDNA vs number of copepods (middle panel), and rDNA vs. DNA concentration (right panel)

daphnid species from culture collections tested positive, whereas the calanoid copepod culture specimens did test positive (data not shown). Regression analysis revealed a positive linear relationship between the number of individual adult copepods and rDNA copies ($R^2 = 0.84$, $p < 0.001$; Fig. 3). Likewise, trends in the DNA and RNA qPCR Ct's were correlated with trends in the reads of DNA ($\rho = 0.626$, $p < 0.001$) and RNA ($\rho = 0.832$, $p < 0.001$) of calanoid copepods reported in the earlier 18S metabarcoding study of the system (Banerji et al. 2018).

Calanoid copepod abundance and physiological dynamics in Lake Harsha

The analyses hereupon reported for the F1R1 qPCR assay were conducted with results obtained with 10-fold diluted DNA extracts (as per our assessments of primer specificity and PCR inhibition). The number of rRNA gene copies per sample fluctuated by approximately 3 to 4 orders of magnitude during the monitoring period at each site for both RNA (cDNA) and DNA. For DNA-based assays, the largest differences in abundance were observed at EMB, while the smallest were in the surface water of EFL. The detection of RNA-based qPCR signals indicated that the copepods were metabolically active at the time of sampling (as opposed to dormant or dead), which was consistent with microscopical observation of a subset of samples prior to fixation with ethanol (i.e., observation of swimming and feeding activity). For RNA-based assays, the deep water and surface water of EFL had the largest and smallest differences in copepod abundance/activity throughout the study, respectively.

Consistent with these variations in DNA and RNA, the RNA:DNA ratio varied over space and time, as well (Fig. 4). Analysis of the trends in DNA, RNA, and RNA:DNA ratio uncovered weak but significant differences among the separate temporal phases (pre-HAB, HAB, and post-HAB; MANOVA, $F_{2,207} = 2.910$, $p = 0.009$). However, the follow-up PERMANOVA revealed that these differences were attributable to site variation in DNA ($F_{4,204} = 14.738$, $R^2 = 0.224$, $p = 0.01$), RNA ($F_{4,204} = 13.667$, $R^2 = 0.211$, $p = 0.01$), and RNA:DNA ratio ($F_{4,204} = 5.519$, $R^2 = 0.098$, $p = 0.01$); there was no significant temporal variation once the site variation had been accounted for. Additionally, differences among the sites were no longer observed when the deep-water samples and a single anomalous spike in RNA at CGB were excluded from the analysis, and no significant correlations were found between any of the three qPCR measures and any of the standard water quality parameters that had been collected alongside the samples.

Discussion

Through the in silico and in vitro validations of the primers and the analyses of time series, we have demonstrated that our F1R1 qPCR assay can be used as a reliable tool for environmental monitoring. Copepod DNA and RNA signals were lowest in the deep-water samples, which is consistent with past findings regarding copepod ranges of tolerance and vertical distributions in lakes (Carter 1969; Benoît et al. 2002). Low abundances and activities in deep water could,

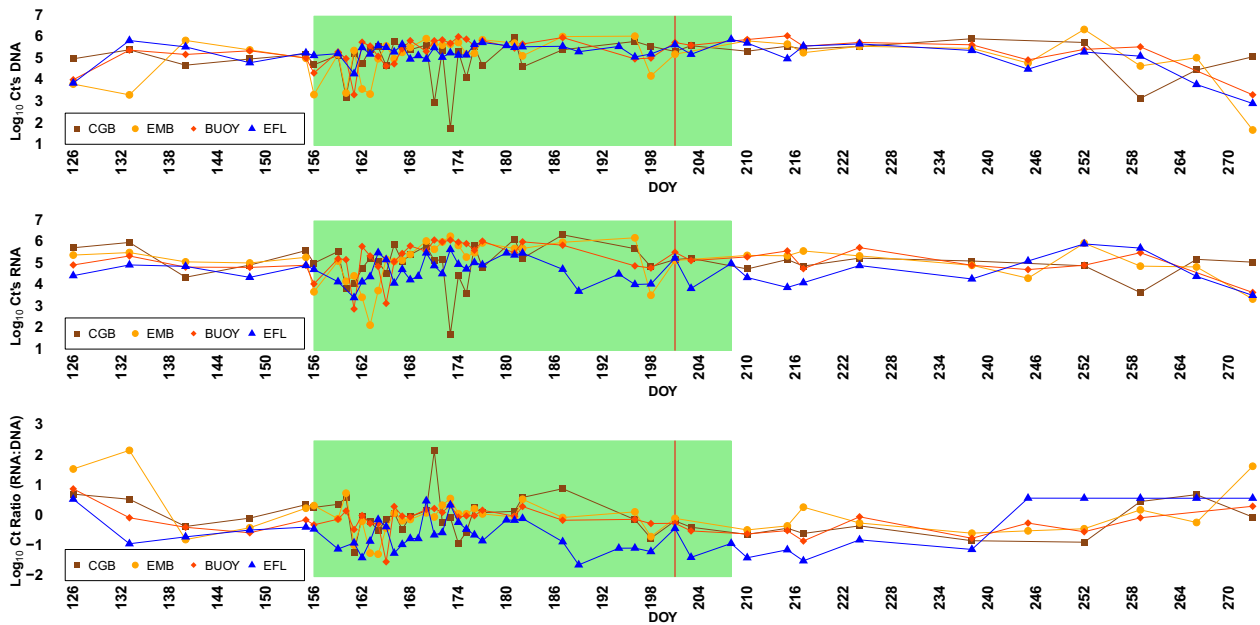


Fig. 4 Copepod Dynamics in the Field. Time series of copepod DNA, RNA, and RNA:DNA ratio signals in the surface water at each of the sampling locations (deep-water samples not shown; *DOY* day-of-year). The period during which cyanobacteria dominated the phytoplankton

community is shown within the green rectangles. The vertical red line indicates the day when total microcystin concentration within the lake was at its peak

for instance, have reflected lesser ability of copepods to tolerate deep-water conditions such as lower dissolved oxygen and lower temperature or the scarcity of food (Jamieson 2005; Meerhoff et al. 2007). Furthermore, our study has provided some insight regarding the relationship between copepods and freshwater HABs in our system. It is clear from the data we have collected that the copepods did not enter the HAB period with a significantly lower RNA:DNA ratio than they possessed during or after this period.

Importantly, the copepods' RNA:DNA ratios on and shortly after the day when microcystin concentration was at its highest within the lake were near the means for the entire study (i.e., neither on the low end nor the high end of the range of values that were observed). This reveals that the copepods were tolerant of microcystin (or, at least, tolerant of the levels that occurred). It is unlikely that the copepods simply avoided the cyanotoxin and other HAB-related environmental stressors, given that their DNA and RNA signals were detected in the same samples and that, being plankton, their ability to swim away was limited. It could be, however, that the copepods engaged in prey switching (Kiørboe et al. 1996), so that declines in particular prey had little effect on their population size and physiological status, and were able to capitalize on refuge sites within the patchy distributions of HAB-forming cyanobacteria to avoid constant exposure (Reichwaldt et al. 2013). Altogether, this is consistent with the hypothesis put forth by Mitra and Flynn (2006) and others (Kang et al. 2015) that dominant zooplankton such as copepods can coexist with toxic cyanobacteria perpetuate bloom

events by keeping competitors of the toxic cyanobacteria at low abundance.

The qPCR approach has some limitations when used for estimating copepod population densities and physiological status in environmental samples. For example, studies have shown that there could be large differences in rRNA gene copies per genome among different copepod groups, as observed for marine calanoid and freshwater cyclopoid species (Wyngaard et al. 1995). Differences in rRNA gene copies have been shown to be greater than two orders of magnitude between species of different copepod families (White and McLaren 2000). These differences may be the result of genome reduction/duplication and natural selection of genomes giving rise to adaptive developmental strategies (White and McLaren 2000) and adaptive morphologies (Rasch and Wyngaard 2006). On the other hand, for species of the same copepod family, these differences are generally less than two-fold (White and McLaren 2000). Given the limited number of different calanoid OTUs and morpho-species identified in Lake Harsha samples, and given that the F1/R1 qPCR assay is specific to Calanoida, differences in rRNA gene copies per genome cannot by themselves explain the fluctuations observed in rRNA gene copies in our study, particularly for the changes in relative abundance observed during consecutive sampling dates.

Another possibility is that, since the water samples were filtered using a small pore size membrane, it is likely that nauplii and copepodids were harvested along with the adults during filtration. As the number of cells per copepod will

change with each developmental stage, it is not feasible to measure the contribution of each stage to the total calanoid densities unless copepods are physically separated. In a study involving a COI-based qPCR assay, Jungbluth et al. (2013), using sieve fractionation, showed that the number of mtCOI gene copies significantly increases with each succeeding larval stage, with highest levels achieved for adult females. Assuming a similar trend for 18S rRNA gene copies, some of the changes observed in our study may be related to the ratio of immature to mature copepods, as well as changing sex ratios (gravid females having more rRNA gene copies than adult males, due to egg sac production and related somatic growth). While our approach may not accurately estimate total copepod densities, we have shown here that group-specific qPCR assays such as ours may be used for rapid calanoid detection and to study calanoid-specific spatial and temporal trends in aquatic systems (i.e., as a relative index of copepod biomass). That we were able to detect copepod signals in small sample volumes is evidence of the sensitivity and low detection limits of this approach.

Similar limitations may be true when using RNA (cDNA) instead of DNA as qPCR templates as an indication of physiological activity. Our rRNA transcript copies reflect numbers for multiple individuals filtered per sample. RNA content per individual within a species may vary considerably, depending on demographics (developmental stage, size, sex, etc.) as it does with DNA. However, RNA concentration in zooplankton has been shown to be sensitive to changes in food availability and other suboptimal environmental conditions (Holmborn and Gorokhova 2008). Thus, copepod rRNA data may be used as an indicator of relative physiological status of copepods in field studies (Saiz et al. 1998; Calliari et al. 2006). However, tracking the changes in rRNA:rDNA ratios may be a better indicator of copepod physiological status and how copepods react to changing environmental conditions (Wagner et al. 1998; Speckmann et al. 2007; Ning et al. 2013) than RNA (or DNA) alone, as our results also suggest.

An additional limitation of our study is that sampling itself might have triggered stress responses in the copepods. Quantifying such an effect in a field study like ours would require a method of determining the physiological status of copepods prior to sampling them, and eliminating such an effect would require one that ensures that the copepods' status remains the same upon being sampled (i.e., that euthanizes and preserves the copepods while they are still in the lake, without damaging their nuclear and cell membranes, before the copepods can produce more RNA). Unfortunately, we had no such methods at our disposal at the time of sampling. However, one of the advantages of whole-water grab sampling over depth-integrating and density-concentrating methods of zooplankton collection

(e.g., ring net vertical hauls) is that it does not expose the copepods to higher concentrations of cells and chemical cues (kairomones from adjacent copepods, etc.) than what was present in the equivalent volume of water surrounding them in the lake, nor to organisms that were deeper in the water column. Moreover, because we were consistent in our sampling method, we can infer that sampling-related stress alone could not have caused significant differences in observed copepod responses across sites and dates; these must rather have pertained to differing biotic and abiotic environmental conditions (which may or may not have been exacerbated by sampling).

Notwithstanding some of the limitations discussed above, group-specific genetic markers such as ours reduce the dependence of researchers on the use of pure cultures and artificial environments (e.g., laboratory microcosms) for evaluating physiological responses of zooplankton to changing physicochemical conditions. They might also represent a significant improvement from conventional approaches that depend on morphological taxonomy when studying the in situ occurrence, distribution, and physiological status of important zooplankton. The approach used in this study is adaptable to different environmental settings and compatible with robust monitoring schemes, including where it is necessary to track many planktonic groups simultaneously, regardless of composition and taxonomic diversity. As the technology advances and the use of sequencing-based monitoring becomes more available and popular, the opportunity to build upon molecular tools such as ours to study the ecology of zooplankton will expand, providing new ways to assess the responses of key biological networks to different types and levels of environmental perturbation.

Data availability

The data used in this study will be publicly accessible in the Sci-Hub repository upon publication of the study.

Acknowledgements We would like to thank Megan Berberich for technical assistance with laboratory procedures; Joel Allen, Mia Vijanderan, Dana Macke, Kit Daniels, and Armah de la Cruz for their role in collecting, transporting, and processing the lake water samples used in this study; Jade Young (Army Corps of Engineers) for sharing 2016 calanoid densities for Lake Harsha; Chris Nietch and Stefania Guglielmi for helping to validate discrete sonde water quality measurements; and John Olszewski and Samuel Hayes for critically reviewing an earlier draft of this manuscript.

Funding The U.S. Environmental Protection Agency, through its Office of Research and Development, partially funded and participated in the research described herein. *Any opinions expressed in this paper are those of the authors and do not necessarily reflect the views of the agency; therefore, no official endorsement should be inferred. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.*

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Consent to publish The authors hereby attest that this paper is their original unpublished work and is not currently under review elsewhere and hereby consent to its publication within Ecotoxicology.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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