# Time-series metatranscriptomes reveal conserved patterns between phototrophic and heterotrophic microbes in diverse freshwater systems

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## Abstract

Microbial communities form the base of food webs in freshwater ecosystems, yet the interactions within these diverse assemblages are poorly understood. Based on evidence showing that primary production and respiration follow diurnal trends in lakes, we hypothesized that gene expression in freshwater microbes would have similar diel cycles. We used three two-day time series of metatranscriptomes to test this hypothesis in a eutrophic lake, an oligotrophic lake, and a humic lake. We identified prominent diel cycles in all three lakes, particularly in genes related to photosynthesis, sugar transport, and carbon fixation. The maximal time of expression for sugar genes tended to trail that of photosynthesis genes by several hours, indicating possible metabolic exchange between co-occurring microbial lineages. These results provide an important step in documenting the sophisticated multispecies transcriptional organization that exists within freshwater microbial communities.

**Scientific Significance Statement**

The functioning and diversity of microbial communities in aquatic ecosystems has become increasingly apparent as next-generation sequencing techniques provide extensive data on unculturable microbes. Still, one of the grand challenges in aquatic microbiology is linking taxonomic groups to their functions and understanding how community members contribute to emergent ecosystem-level processes. In this study, we generated one of the largest metatranscriptomic datasets to date and used it to propose interactions between microbes based on trends in gene expression. The results of our work shed light on how organization within microbial communities leads to patterns that are detectable at the ecosystem level.

**Data Availability**

Datasets used in this study are available on the Open Science Framework (DOI 10.17605/OSF.IO/9GR62). All code is publicly available at <https://github.com/McMahonLab/geodes>. Raw sequence files are available through the JGI Genome Portal.

**Keywords**

Microbial communities, metatranscriptomics, diel cycles

## Introduction

Many of the core ecosystem functions in freshwater lakes are driven by microbial communities. While the impact of each cell is miniscule, their collective actions form a dynamic, interconnected community whose emergent functions are visible on the ecosystem-level (Sunagawa et al. 2015; Goldford et al. 2018). Previous research in a wide range of freshwater ecosystems indicates that diurnal cycles drive photosynthesis, respiration, and dissolved organic matter (DOM) concentrations (Kaplan and Bott 1989; Bertilsson and Jones 2003; Solomon et al. 2013). This implies metabolic interactions between the phototrophic (photosynthetic, also known as phytoplankton) and heterotrophic (non-photosynthetic, also known as bacterioplankton) microbial communities. We hypothesized that these diel trends would be reflected in gene expression. Therefore, we investigated the mechanisms of specific community interactions by studying the timing of gene expression across the community. To this end, we produced three two-day time series of metatranscriptomes from three lakes with contrasting biogeochemistry. We hypothesized that diel trends in gene expression occur in heterotrophs as well as phototrophs due to the direct impacts of sunlight (such as photosynthesis and reactive oxygen species) and its indirect effects (such as metabolite exchange), regardless of the features that distinguish individual lakes.

Previous metatranscriptomic work in marine and freshwater systems has highlighted multispecies diel trends that may underpin metabolic links between phototrophic and heterotrophic microbes. One metatranscriptomic study in a phosphorus-limited mountain lake found differential gene expression between day and night in both phototrophs and heterotrophs, particularly in energy acquisition pathways and pyrophosphatase (Vila-Costa et al. 2013). Another study in marine systems also observed enhanced expression of energy acquisition pathways during the day and higher expression of biosynthesis and housekeeping pathways at night (Poretsky et al. 2009). Strong diel patterns in phototrophic gene expression followed by a cascade of heterotrophic gene expression have also been observed in marine systems (Ottesen et al. 2014). Furthermore, patterns in transcriptional networks of gene expression were consistent in two different regions of the Pacific Ocean, potentially indicating that linkages between phototrophs and heterotrophs are a generalizable feature of marine microbial communities (Aylward et al. 2015). These studies suggest that diel transcriptional trends may be a universal characteristic of both phototrophs and heterotrophs in aquatic microbial communities.

Other methods also suggest strong connections between phototrophs and heterotrophs in aquatic ecosystems. Co-cultures of phototrophic algae and heterotrophic bacteria are often stable over time, indicating mutualistic interactions, although competition or predation have also been described in the laboratory (Cole 1982; Posch et al. 1999; Pernthaler et al. 2001). In both marine and freshwater systems, the composition of phototrophic and heterotrophic communities are inextricably linked (Verity et al. 1999; Teeling et al. 2012; Paver et al. 2013, 2015). Perturbations in one portion of these communities have been shown to quickly ripple through the rest (Šimek et al. 2002; Kent et al. 2006; Sjöstedt et al. 2012). One potential mechanism that can explain these trends is DOM exuded by phototrophs (Moran et al. 2016). This DOM is to a large extent composed of low molecular weight compounds, such as sugars, amino acids, organosulfur compounds, carboxylic acids, and alditols (Hellebust 1965; Maršálek and Rojíčková 1996; Fiore et al. 2015). Approximately 20% of photosynthetic carbon is released extracellularly in marine and freshwater systems (Bertilsson and Jones 2003). Although factors causing DOM release are not fully understood, this DOM likely supports a substantial portion of the heterotrophic community. In fact, some ubiquitous freshwater bacteria, such as *Limnohabitans,* appear to specialize in algal-derived DOM (Simek et al. 2011).

Exposure to solar radiation may be a major factor driving gene expression in aquatic ecosystems. Beyond the canonical oxygenic photosynthesis, the presence of opsins, extensively documented in both freshwater and marine heterotrophs, may also lead to cycles of diel gene expression in microbes (Atamna-Ismaeel et al. 2008; Pinhassi et al. 2016). Even without opsins, some freshwater microbes such as *Actinobacteria* may sense light in order to optimally time uptake and catabolism of organic substrates (Maresca et al. 2019). Photodegradation of complex DOM into more labile forms is another potential mechanism that could drive diel trends in heterotrophs (Jorgenson et al. 1998; Bertilsson and Tranvik 2000). Sunlight also causes oxidative stress, and heterotrophs may time their metabolisms to minimize the impact of such harmful conditions (Sommaruga et al. 1997). Additionally, photoheterotrophic microbes can also exist outside the categories of phototroph and heterotroph, such as those that perform aerobic anoxygenic photosynthesis (AAP). These microbes use sunlight for energy, but do not fix carbon (Eiler 2006; Martinez-Garcia et al. 2012).

To identify generalizable diel interactions in freshwater microbial communities, we sequenced metatranscriptomes from the epilimnia of three freshwater lakes representing oligotrophic, eutrophic, and dystrophic (humic) lake types. These metatranscriptomes form a two-day time series for each lake, with samples collected every four hours. We additionally sequenced metagenomes and single-cell amplifed genomes (SAGs) from each lake to generate highly specific references, allowing us to obtain higher quality annotations and classifications than possible through read-based annotations. We observed diel trends in both phototrophs and heterotrophs and were able to propose biotic and abiotic mechanisms for these trends based on annotations of expressed genes. Although different taxa and genes were expressed in the three lakes studied, we identified diel trends in all sites, particularly in genes related to photosynthesis and sugar transport, suggesting that common transcriptional patterns exist in these disparate systems.

## Methods

### Study design and in situ measurements

Three lakes in Wisconsin, USA, were chosen for this study based on their different trophic status: oligotrophic (Sparkling Lake), eutrophic (Lake Mendota), and humic (Trout Bog Lake) (Table 1).Lake Mendota is located in Madison, WI, USA, while Trout Bog and Sparkling Lake are located in Boulder Junction, WI, USA, approximately 350 km north of Madison. These lakes were chosen because they are core sites of the North Temperate Lakes - Long Term Ecological Research (NTL-LTER) program. Therefore, they have a rich context of historical environmental data and automated sensor platforms were deployed at all three sites at the time of sampling. Previous microbial studies have been performed in all three lakes, providing reference genomes specific to each site (Ghylin et al. 2014; Bendall et al. 2016; Linz et al. 2018).

Hereafter, we provide brief summaries of our methods; full protocols are available in Supplemental Document S1. The epilimnion (top thermal layer) of each lake was sampled twelve times at four-hour intervals in July 2016. We used an instrumented sonde (Hydrolab DS5X, OTT Hydromet, Kempten, Germany) equipped with sensors for temperature, dissolved oxygen concentrations, pH, conductivity, and turbidity to collect measurements of the epilimnion. Photosynthetically active radiation (PAR) was also measured at this time using a PAR meter (Li-Cor, Lincoln, NE, USA). Secchi depth was measured once per lake during the time series.

At each time point, we collected an integrated water sample of the epilimnion. The sampling depth was chosen based on the location of the thermocline on the day prior to initiation of the two-day time series in each lake. To collect RNA, water from the integrated epilimnion sample was pumped through four 0.22-m polyethylene filters (Pall, Port Washington, NY, USA). Filters were flash frozen in liquid nitrogen in the field and stored at -80oC until extraction and sequencing. Additional samples were collected for metagenomic sequencing, single cell sequencing, total and dissolved nitrogen and phosphorus concentrations, chlorophyll concentrations, and bacterial production assays using 14C-leucine (Chin-Leo and Kirchman 1988).

### RNA extraction

Samples were lysed with EDTA and SDS and incubated at 65oC, then subjected to bead-beating (FastDNA Spin Kit for Soil, MP Biomedicals, Santa Ana, CA, USA) with TRIzol (Thermo-Fisher, Waltham, MA, USA). An internal standard - an in vitro transcription of the cloning plasmid pFN18A was added to samples after beadbeating (Satinsky et al. 2013). Phenol:chloroform was used to isolate RNA from the lysate. Purified RNA was precipitated in ethanol, pelleted, and resuspended in nuclease-free water. The RNA was further purified using an RNeasy kit (QIAGEN, Hilden, Germany) with an on-column DNAse digestion.

### Additional lab-based measurements

Chlorophyll was extracted with methanol from frozen filters and subsequently acidified to measure phaeophytin. Total and dissolved nitrogen and phosphorus were measured with a colorimetric autoanalyzer. DNA was extracted using a phenol:chloroform protocol. Four additional DNA samples collected from Sparkling Lake in a similar manner in 2009 used as additional references for this lake.

*Reference genomes*

Single-cell amplified genomes were generated following the Department of Energy Joint Genome Institute’s (JGI) standard protocol (Rinke et al. 2014). Briefly, individual cells were sorted using an Influx flow cytometer (BD Biosciences) and treated with Ready-Lyse lysozyme (Epicentre; 5U/μl final concentration) for 15min at room temperature.  Next, cell lysis and whole-genome amplification was performed with the REPLI-g Single Cell Kit (Qiagen) in 2μl reactions.  Lysis and stop reagents from the REPLI-g kit received UV treatment to remove potential DNA contamination (Woyke et al. 2011). Cells for SAG sequencing were chosen with a preference for Sparkling Lake, the least well-represented lake in our pre-existing reference genome collection. An Illumina shotgun library was constructed from each single cell and sequenced on the Illumina NextSeq platform (Illumina, San Diego, CA, USA). Sequencing reads were filtered using BBTools (Bushnell et al. 2014) and assembled into SAGs using SPAdes (Bankevich et al. 2012).

Metagenomes were prepared for sequencing using the KAPA-Illumina library creation kit (KAPA Biosystems). Metagenomes were sequenced on the Illumina HiSeq platform utilizing a TruSeq paired-end cluster kit (Illumina, San Diego, CA, USA), producing paired ends of 150bp (2x150). Quality filtering was performed on the resulting reads before assembly. BBDuk adapter trimming was used to remove known Illumina adapters (Bushnell et al. 2014). Reads ends were trimmed where quality values were less than 12. Read pairs containing more than three 'N', or with quality scores (before trimming) averaging less than 3 over the read, or length under 51bp after trimming were discarded. Filtered reads were assembled using MegaHit (Li et al. 2016) with a range of kmers (--k-list 23, 43, 63, 83, 103, 123) and otherwise default settings.

Assembled metagenomic contigs, newly sequenced SAGs, genomes from previous McMahon Lab time series sequencing on these lakes (Martinez-Garcia et al. 2012; Ghylin et al. 2014; Garcia et al. 2018; Linz et al. 2018), and freshwater algal genomes from NCBI RefSeq (Pruitt and Maglott 2001) were used to build a nonredundant, highly specific database for subsequent mapping of metatranscriptomic reads (Table S1). After formatting each type of genome or contig’s fastq and gff files, coding regions were extracted and clustered at 97% ID using CD-HIT (Huang et al. 2010). The longest gene in each cluster (used as the seed sequence to generate each cluster) was chosen as the representative sequence and used as the mapping reference.

Individual metagenome assemblies were binned using MaxBin version 2.2.4 (Wu et al. 2014) and checked for completeness and contamination using CheckM version 1.0.10 (Parks et al. 2015). Many bins were incomplete, and many contigs were too short to classify using the phylogeny of single-copy genes; therefore, we aggregated the taxonomic assignments of coding regions in each contig/bin to classify these sequences. Coding regions were first classified based on their best hit in the Integrated Microbial Genomes database (IMG, accessed Jan. 2017) (Markowitz et al. 2012). Bins and unbinned contigs from the metagenome assemblies were classified by taking the consensus taxonomy of the best hit for each coding region on a contig/bin using in-house McMahon Lab scripts. If contigs or bins were too short to classify or had conflicting coding region classifications, we assigned no classification. In these cases, each coding region retained the IMG-derived classification of its best hit.

### Metatranscriptomics

Three samples from each timepoint were submitted for metatranscriptomic sequencing at the JGI. Some samples failed to pass quality control standards and were replaced by the fourth filter from that timepoint. When we were unable to sequence three filters for a timepoint, we instead sequenced a fourth replicate for another timepoint, resulting in between one and four replicate metatranscriptomes for each timepoint (Table S2). Ribosomal RNA was depleted using the Illumina Ribo-Zero rRNA Removal Kit, and samples were prepared for sequencing with the Illumina TruSeq Stranded Total RNA HT kit. Samples were sequenced using the Illumina HiSeq platform and a TruSeq paired-end clustering kit for paired-end, 150bp sequencing (2x150). BBDuk adapter trimming was used to remove known Illumina adapters. Reads ends were trimmed where quality values were less than 12. Read pairs containing more than three 'N', or with quality scores (before trimming) averaging less than 3 over the read, or length under 51bp after trimming were discarded. Ribosomal RNA reads were removed using SortMeRNA (Kopylova et al. 2012). Metatranscriptomic reads were competitively mapped to this database with a 90% ID cutoff using BBMap and requiring at least 75% overlap with a gene feature. Mapped reads were tabulated using FeatureCounts (Liao et al. 2014).

Addition of an internal RNA standard allowed for both normalization of expressed reads to transcripts per liter and assessment of extraction success (Satinsky et al. 2013). Samples with either too few counts of the internal standard (less than 50) or orders of magnitude higher expression of all genes after normalization when compared to replicates were discarded. After these quality control measures, 32 samples remained from Sparkling Lake, 30 from Lake Mendota, and 21 from Trout Bog. Nine samples from day two in the Trout Bog time series were not analyzed due to insufficient yield, resulting in two lost timepoints.

### Statistics

The statistical software R was used for expression analysis (R Core Team, 2018). Using the internal standard to determine normalization size factors, we converted read counts to units of transcripts per liter: all following read-based metrics are in transcripts per liter. To reduce noise in the dataset, the top 20,000 expressed genes in each lake were retained for differential expression analysis. From this subset, marker genes for metabolic processes were selected and aggregated by pathway. RAIN was used to detect cyclic trends in gene expression (Thaben and Westermark 2014). Genes with p-values less than 0.05 were considered cyclic. In addition to the abundance threshold imposed by taking the top 20,000 genes in each lake, we also required that genes included in the cyclic trend analysis have a coefficient of variance of at least 0.2. The maximal time of expression for each gene was determined by averaging metatranscriptomes taken at the same time of day over the two-day time series. Results were plotted using the R packages ggplot2 (Wickham, 2009) and cowplot (Wilke, 2017).

## Results

### What genes were expressed?

We first asked which genes were most expressed in each lake across all time points (Figure S1). Photosynthesis related genes, particularly those relating to photosystem II P680, were highly expressed in all three lakes. Genes encoding ribulose-1,5-bisphosphate carboxylase (RuBisCO), the key enzyme in carbon fixation via the Calvin-Benson-Bassham (CBB) pathway, were among the most highly expressed genes in Lake Mendota and Trout Bog. We also ran this analysis excluding genes associated with phototrophy and unannotated genes (Figure S1). Many of the most highly expressed non-photosynthetic genes in Lake Mendota belonged to *Actinobacteria* (acI-A and acI-B clades, both members of *Actinomycetales*), including translation elongation factors, a sodium:solute transporter, and a sugar transporter.

*Which taxa were expressing genes?*

We next aggregated expressed genes by taxonomic classifications to compare the most expressed taxa to the most abundant taxa based on metagenomic data (Figure 1). We used the same reference database to map metatranscriptomes and metagenomes, making such comparisons possible. No positive trend between gene expression and taxonomic abundance was observed. At the phylum level, genes from *Cyanobacteria, Bacteroidetes,* and *Actinobacteria* were relatively highly expressed in all three lakes. Genes from *Betaproteobacteria* were highly expressed in Trout Bog and Sparkling Lake, while *Verrucomicrobia* genes were especially highly expressed in Trout Bog. Widely recognized abundant and/or cosmopolitan taxa were also present, and a significant portion of transcripts could be associated with groups recognized as being freshwater-specific and largely cosmopolitan “clades” (Newton et al. 2011). Where taxonomy was resolved to the clade level, we noted which clades contributed to observed transcripts. Genes from members of *Actinobacteria* acI-B (*Actinomycetales)* were expressed and abundant in all lakes, but especially Trout Bog. This is consistent with previous research identifying acI-B2 as an acidic lake specialist (Newton et al. 2007). The betIV-A clade (LD28, *Methylophilales*) contributed a surprising number of expressed genes in Lake Mendota, as did the alfV-A clade (*Candidatus* Fonsibacter, also known as LD12 (Henson et al. 2018)) and the acI-A clade (*Actinomycetales)*. Genes from the bacIII-B clade (*Sphingobacteriales)* was highly expressed in Sparkling Lake, but had low abundance. Genes from members of betIV-A were abundant in Trout Bog, but not proportionally highly expressed. Pnec clade (*Polynucleobacter)* genes, known to be endemic to bog lakes and particularly Trout Bog, were not as expressed or abundant as we had expected it to be (Jezberová et al. 2010; Linz et al. 2017). However, we noted many additional transcripts classified as *Burkholderiales* that may be Pnec, but could not be classified to the clade level, likely due to the high diversity of this group (Jezberová et al. 2010; Hahn et al. 2016).

### Assessing variability in freshwater metatranscriptomes

Because this study is among the largest metatranscriptomic sequencing efforts to date, we discuss the biological versus technical variability observed in this dataset to add to our knowledge of variability in environmental metatranscriptomics and to inform future study designs (Tsementzi et al. 2014). We used the coefficient of variation (CoV), i.e. the ratio of standard deviation to average expression (%), to compare the amount of variability within replicate samples to the variation observed across different time points (Figure S2). Higher CoVs were observed across samples than within the replicates. Still, the upper limit for CoV within replicates approached 200%. This result highlights the importance of replication in metatranscriptomic studies.

### Trends in environmental variables

We examined a suite of potentially relevant environmental variables to compare trends in these to the dynamic shifts observed in gene expression, expecting that several of these trends would be diel. PAR data was used to classify time points as night or day (Figure S3). Parameters that reflect the boundaries between layers within the water column, such as dissolved oxygen, temperature, pH, and conductivity, were strongly diel in Lake Mendota, but less so in Sparkling Lake and Trout Bog (Figure S4). Chlorophyll concentrations, often used as an indicator of primary production, were diel in Trout Bog, but not in the other two sites. Bacterial production, measured via 14C-leucine incorporation, showed dynamics over the two-day time series in all three lakes, although the trends were not diel (Figure S5). No diel trends were observed in total and dissolved nitrogen or phosphorus concentrations.

### Diel trends in gene expression

We used the RAIN software (Thaben and Westermark 2014) to reveal any cyclic trends with 24-hour periods among our top 20,000 most abundant genes in each lake, with an additional requirement that genes must be sufficiently variable (coefficient of variation greater than 0.2). Many genes related to photosynthesis were cyclic in all lakes (Figure 3). However, the time of maximum expression differed in each lake. In Lake Mendota, most photosynthesis genes were maximally expressed at 13:00 or 17:00, while in Sparkling Lake, the majority were most highly expressed at 13:00. In Trout Bog, 9:00 was the most common time of maximum expression for photosynthesis genes. Similarly, many genes related to sugar transport were cyclic across our study sites, but had different times of maximum expression. These genes were most expressed at 17:00 in Lake Mendota, 1:00 in Sparkling Lake, and 9:00 in Trout Bog. We also investigated cyclic trends in genes annotated as RNA polymerase subunits as a measure of general community activity. While less predominantly cyclic than the other categories discussed here, the time of maximum expression for RNA polymerase genes was typically mid-morning (9:00 in Lake Mendota, 9:00 and 13:00 in Sparkling Lake, and 9:00 in Trout Bog). Many genes encoding subunits of the key carbon fixation enzyme ribulose-1,6-bisphosphate carboxylase (RuBisCo), were cyclic in all three lakes and typically most expressed in the morning (9:00 in Lake Mendota, 5:00 in Sparkling Lake, and 9:00 in Trout Bog). Genes related to opsin synthesis were primarily expressed in Lake Mendota and Sparkling Lake, contained both cyclic and non-cyclic trends, and peaked at 13:00 in Lake Mendota and 1:00 in Sparkling Lake. We used genes encoding photosynthetic reaction center M as a marker for AAP and found that these genes were predominantly cyclic and peaked at 1:00 in Lake Mendota and Sparkling Lake and 5:00 in Trout Bog. Notably, genes in many categories in Sparkling Lake showed peak expression at 1:00, while most maximal expression in Trout Bog occurred in the hours of 5:00-13:00.

We next investigated the taxonomic assignments of genes annotated as related to RNA polymerase, general sugar transport, and photosynthesis over time in each lake (Figure 4). As expected, a diversity of freshwater microbes were expressing genes encoding RNA polymerase. Fewer unique taxa expressing RNA polymerase were observed in Sparkling Lake, likely due to the reduced number of available reference genomes for this lake. *Cyanobacteria* dominated expression of genes related to photosynthesis in Lake Mendota, while Trout Bog photosynthesis expression was derived from a mix of *Cyanobacteria* and *Eukaryota* (most likely algae)*.* Genes related to photosynthesis expression in Sparkling Lake were largely unclassified, although some were assigned to *Cyanobacteria.* Expression of genes related to general sugar transport varied between lakes, both in timing and taxonomy. In Lake Mendota, this category was derived mainly from *Cyanobacteria* and *Actinobacteria. Actinobacteria* were also well represented in Trout Bog general sugar transport expression, as were *Betaproteobacteria.* In Sparkling Lake, genes related to general sugar transport were classified as *Actinobacteria, Armatimonadetes,* and *Betaproteobacteria.*

## Discussion

In this study, we sought to identify diel trends in freshwater microbial community gene expression to hypothesize metabolic interactions between community members. Using metatranscriptomic time series, we were able to detect genes with cyclic trends. We found diel trends in all lakes studied, regardless of trophic status, although the timing of these cycles varied by lake.

The balance of primary production and respiration is of interest to those seeking to create carbon budgets for freshwater lakes. Previous research has linked photosynthesis and respiration to diel cycles (Solomon et al. 2013), leading us to hypothesize that genes related to these processes would also show diel trends. In all three lakes, genes related to photosynthesis were highly expressed and frequently cyclic. Photosynthesis and carbon fixation are often considered to be coupled in the process of primary production; however, we only saw that expression of genes related to primary production occurred at different times of day in Lake Mendota and Sparkling Lake. We also investigated expression of other types of potential phototrophy, including AAP and opsins. Genes encoding proteins related to these processes were often cyclic, especially those involved in AAP. AAP genes typically had different times of maximal expression than the canonical oxygenic photosynthesis, possibly indicating adaptation to different times of day to avoid competition for light and nutrients.

Respiration is a broad functional category that encompasses the degradation of many carbon substrates. To identify the compounds being respired, we focused on genes related to carbon transport, as transporter expression has previously been used in marine systems to predict substrate use (Ottesen et al. 2013; Vorobev et al. 2018). In all three lakes studied, we identified cyclic trends in many genes related to sugar transport. Phototrophs are known to exude sugars (Maršálek and Rojíčková 1996), suggesting that sugars may be exchanged between phototrophs and heterotrophs. Therefore, it is of particular interest that expressed genes encoding sugar transporters in Lake Mendota are primarily classified as *Cyanobacteria* and *Actinobacteria.*

There are multiple non-exclusive hypotheses as to why we observed diel trends in some genes encoding sugar transport. One is biotic in origin – if these sugars are indeed algal exudates, they may be produced during the day and released in the evening and at night. Although such diel release of sugars has not been documented here, day/night partitioning of photosynthesis and sugar metabolism are known to occur in phototrophs (Masuda et al. 2018; Welkie et al. 2018). Such diel trends in individual populations may extend to community-level interactions. Another possible hypothesis is that oxidative stress prevents heterotrophs from consuming sugar during the day, even if such substrates are available. We also observed cyclic trends in expression in genes related to ROS defense or carboxylic acid transport, a common photodegradation product, in all three lakes.

There is ample evidence in marine microbial communities suggesting that carbon released by phototrophs influences heterotrophic community composition to improve phototroph fitness. In coral reefs, algal exudates can dramatically shift bacterial community composition, potentially providing algae with a competitive advantage over coral by selecting for coral pathogens in the heterotrophic community (Nelson et al. 2013). In marine microbial communities, heterotrophic bacterioplankton are highly dependent upon *Prochlorococcus* exudates and likely perform a critical community function in return, such as the detoxification of hydrogen peroxide or free radicals (Morris et al. 2011). *Prochlorococcus* has lost its genes for reactive oxygen species defense and depends on the associated heterotrophic bacteria to supply this function (Morris et al. 2016; Ma et al. 2018). *Prochlorococcus* likely exudes carbon to maintain redox balance, as it generates more reducing power via photosynthesis than it can allocate to anabolic processes (Bertilsson et al. 2005). However, a frequently observed adaptation to excess reducing power is to downregulate photosynthesis electron flux; this is not observed in *Prochlorococcus* and suggests alternative reasons for its release of carbon (Braakman et al. 2017)*.*

It is therefore reasonable to hypothesize that freshwater phototrophs may be releasing carbohydrates to shape the heterotrophic community, which in turn may benefit phototrophs. Most likely, heterotrophs perform functions that benefit the community, such as ROS defense and vitamin production. The origin of metabolic exchanges that lead to co-dependencies has been postulated to be an important driver of evolution in aquatic communities, as in the “Black Queen Hypothesis,” where interdependencies among microbial community members leads to genome reduction (Morris et al. 2012). It is intriguing to note that the dependency between phototrophs and heterotrophs and the diel partitioning of carbon fixation and respiration would be analogous to the organization and functioning of chloroplasts and mitochondria in plant cells (Braakman et al. 2017). Further concerted efforts to recover nearly complete reference genomes from freshwater systems should enable more rigorous testing of this hypothesis.

Here, we present a comparative metatranscriptomic analysis which demonstrates similar diel trends in photosynthesis and sugar transport in three different types of lakes. We hypothesize both biotic (algal exudates) and abiotic (oxidative stress) as drivers of community-level diel trends in freshwater microbiomes. Whether all of these microbes are responding to the same day-night stimulus or whether community interactions confer these diel trends remains to be determined. Given the consistent patterns across metatranscriptomes from biogeochemically disparate lakes, our results underscore the prevalence of conserved microbial interactions that underpin a broad diversity of freshwater environments.

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## References

Atamna-Ismaeel, N., G. Sabehi, I. Sharon, and others. 2008. Widespread distribution of proteorhodopsins in freshwater and brackish ecosystems. ISME J. **2**: 656–662. doi:10.1038/ismej.2008.27

Aylward, F. O., J. M. Eppley, J. M. Smith, F. P. Chavez, C. A. Scholin, and E. F. DeLong. 2015. Microbial community transcriptional networks are conserved in three domains at ocean basin scales. Proc. Natl. Acad. Sci. **112**: 5443–5448. doi:10.1073/PNAS.1502883112

Bankevich, A., S. Nurk, D. Antipov, and others. 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J. Comput. Biol. **19**: 455–477. doi:10.1089/cmb.2012.0021

Bendall, M. L., S. L. Stevens, L.-K. Chan, and others. 2016. Genome-wide selective sweeps and gene-specific sweeps in natural bacterial populations. ISME J. **10**: 1589–1601. doi:10.1038/ismej.2015.241

Bertilsson, S., O. Berglund, M. J. Pullin, and S. W. Chisholm. 2005. Release of dissolved organic matter by Prochlorococcus. Vie Milieu **55**: 225–232.

Bertilsson, S., and J. B. Jones. 2003. Supply of Dissolved Organic Matter to Aquatic Ecosystems: Autochthonous Sources. Aquat. Ecosyst. 3–24. doi:10.1016/B978-012256371-3/50002-0

Bertilsson, S., and L. J. Tranvik. 2000. Photochemical transformation of dissolved organic matter in lakes. Limnol. Oceanogr. **45**: 753–762. doi:10.4319/lo.2000.45.4.0753

Braakman, R., M. J. Follows, and S. W. Chisholm. 2017. Metabolic evolution and the self-organization of ecosystems. Proc. Natl. Acad. Sci. U. S. A. **114**: E3091–E3100. doi:10.1073/pnas.1619573114

Bushnell, B., R. Egan, A. Copeland, and others. 2014. BBMap: A Fast, Accurate, Splice-Aware Aligner.doi:10.1186/1471-2105-13-238

Chin-Leo, G., and D. L. Kirchman. 1988. Estimating bacterial production in marine waters from the simultaneous incorporation of thymidine and leucine. Appl. Environ. Microbiol. **54**: 1934–9.

Cole, J. J. 1982. INTERACTIONS BETWEEN BACTERIA AND ALGAE IN AQUATIC ECOSYSTEMS.

Eiler, A. 2006. Evidence for the Ubiquity of Mixotrophic Bacteria in the Upper Ocean: Implications and Consequences. Appl. Environ. Microbiol. **72**: 7431–7437. doi:10.1128/AEM.01559-06

Fiore, C. L., K. Longnecker, M. C. Kido Soule, and E. B. Kujawinski. 2015. Release of ecologically relevant metabolites by the cyanobacterium Synechococcus elongatus CCMP 1631. Environ. Microbiol. **17**: 3949–3963. doi:10.1111/1462-2920.12899

Garcia, S. L., S. L. R. Stevens, B. Crary, and others. 2018. Contrasting patterns of genome-level diversity across distinct co-occurring bacterial populations. ISME J. **12**: 742–755. doi:10.1038/s41396-017-0001-0

Ghylin, T. W., S. L. Garcia, F. Moya, and others. 2014. Comparative single-cell genomics reveals potential ecological niches for the freshwater acI Actinobacteria lineage. ISME J. **8**: 2503–16. doi:10.1038/ismej.2014.135

Goldford, J. E., N. Lu, D. Bajić, and others. 2018. Emergent simplicity in microbial community assembly. Science **361**: 469–474. doi:10.1126/science.aat1168

Hahn, M. W., J. Jezberová, U. Koll, T. Saueressig-Beck, and J. Schmidt. 2016. Complete ecological isolation and cryptic diversity in Polynucleobacter bacteria not resolved by 16S rRNA gene sequences. ISME J. **10**: 1642–1655. doi:10.1038/ismej.2015.237

Hellebust, J. A. 1965. EXCRETION OF SOME ORGANIC COMPOUNDS BY MARINE PHYTOPLANKTON1. Limnol. Oceanogr. **10**: 192–206. doi:10.4319/lo.1965.10.2.0192

Henson, M. W., V. C. Lanclos, B. C. Faircloth, and J. C. Thrash. 2018. Cultivation and genomics of the first freshwater SAR11 (LD12) isolate. ISME J. **12**: 1846–1860. doi:10.1038/s41396-018-0092-2

Huang, Y., B. Niu, Y. Gao, L. Fu, and W. Li. 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics **26**: 680–682. doi:10.1093/bioinformatics/btq003

Jezberová, J., J. Jezbera, U. Brandt, E. S. Lindström, S. Langenheder, and M. W. Hahn. 2010. Ubiquity of Polynucleobacter necessarius ssp. asymbioticus in lentic freshwater habitats of a heterogeneous 2000 km area. Environ. Microbiol. **12**: 658–69. doi:10.1111/j.1462-2920.2009.02106.x

Jorgenson, N. O., L. J. Tranvik, H. Edling, W. Graneli, and M. Lindell. 1998. Effects of sunlight on occurrence and bacterial turnover of specific carbon and nitrogen compounds in lake water. FEMS Microbiol. Ecol. **25**: 217–227.

Kaplan, L. A., and T. L. Bott. 1989. Diel fluctuations in bacterial activity on streambed substrata during vernal algal blooms: Effects of temperature, water chemistry, and habitat. Limnol. Oceanogr. **34**: 718–733. doi:10.4319/lo.1989.34.4.0718

Kent, A. D., S. E. Jones, G. H. Lauster, J. M. Graham, R. J. Newton, and K. D. McMahon. 2006. Experimental manipulations of microbial food web interactions in a humic lake: shifting biological drivers of bacterial community structure. Environ. Microbiol. **8**: 1448–1459. doi:10.1111/j.1462-2920.2006.01039.x

Kopylova, E., L. Noé, and H. Touzet. 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics **28**: 3211–3217. doi:10.1093/bioinformatics/bts611

Li, D., R. Luo, C.-M. Liu, C.-M. Leung, H.-F. Ting, K. Sadakane, H. Yamashita, and T.-W. Lam. 2016. MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods **102**: 3–11. doi:10.1016/J.YMETH.2016.02.020

Liao, Y., G. K. Smyth, and W. Shi. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **30**: 923–930. doi:10.1093/bioinformatics/btt656

Linz, A. M., B. C. Crary, A. Shade, S. Owens, J. A. Gilbert, R. Knight, and K. D. McMahon. 2017. Bacterial Community Composition and Dynamics Spanning Five Years in Freshwater Bog Lakes. mSphere **2**: 1–15. doi:e00169-17

Linz, A. M., S. He, S. L. R. Stevens, K. Anantharaman, R. R. Rohwer, R. R. Malmstrom, S. Bertilsson, and K. D. McMahon. 2018. Freshwater carbon and nutrient cycles revealed through reconstructed population genomes. PeerJ **6**: e6075. doi:10.7717/peerj.6075

Ma, L., B. C. Calfee, J. J. Morris, Z. I. Johnson, and E. R. Zinser. 2018. Degradation of hydrogen peroxide at the ocean’s surface: the influence of the microbial community on the realized thermal niche of Prochlorococcus. ISME J. **12**: 473–484. doi:10.1038/ismej.2017.182

Maresca, J. A., J. L. Keffer, P. Hempel, and others. 2019. Light modulates the physiology of non-phototrophic Actinobacteria. J. Bacteriol. JB.00740-18. doi:10.1128/JB.00740-18

Markowitz, V. M., I. M. A. Chen, K. Palaniappan, and others. 2012. IMG: The integrated microbial genomes database and comparative analysis system. Nucleic Acids Res. **40**: 115–122. doi:10.1093/nar/gkr1044

Maršálek, B., and R. Rojíčková. 1996. Stress Factors Enhancing Production of Algal Exudates: a Potential Self-Protective Mechanism? Zeitschrift für Naturforsch. C **51**: 646–650. doi:10.1515/znc-1996-9-1008

Martinez-Garcia, M., B. K. Swan, N. J. Poulton, M. L. Gomez, D. Masland, M. E. Sieracki, and R. Stepanauskas. 2012. High-throughput single-cell sequencing identifies photoheterotrophs and chemoautotrophs in freshwater bacterioplankton. ISME J. **6**: 113–123. doi:10.1038/ismej.2011.84

Masuda, T., G. Bernát, M. Bečková, E. Kotabová, E. Lawrenz, M. Lukeš, J. Komenda, and O. Prášil. 2018. Diel regulation of photosynthetic activity in the oceanic unicellular diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501. Environ. Microbiol. **20**: 546–560. doi:10.1111/1462-2920.13963

Moran, M. A., E. B. Kujawinski, A. Stubbins, and others. 2016. Deciphering ocean carbon in a changing world. Proc. Natl. Acad. Sci. U. S. A. **113**: 3143–51. doi:10.1073/pnas.1514645113

Morris, J. J., Z. I. Johnson, M. J. Szul, M. Keller, and E. R. Zinser. 2011. Dependence of the Cyanobacterium Prochlorococcus on Hydrogen Peroxide Scavenging Microbes for Growth at the Ocean’s Surface F. Rodriguez-Valera [ed.]. PLoS One **6**: e16805. doi:10.1371/journal.pone.0016805

Morris, J. J., Z. I. Johnson, S. W. Wilhelm, and E. R. Zinser. 2016. Diel regulation of hydrogen peroxide defenses by open ocean microbial communities. J. Plankton Res. **38**: 1103–1114. doi:10.1093/plankt/fbw016

Morris, J. J., R. E. Lenski, and E. R. Zinser. 2012. The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. MBio **3**: e00036-12. doi:10.1128/mBio.00036-12

Nelson, C. E., S. J. Goldberg, L. Wegley Kelly, A. F. Haas, J. E. Smith, F. Rohwer, and C. A. Carlson. 2013. Coral and macroalgal exudates vary in neutral sugar composition and differentially enrich reef bacterioplankton lineages. ISME J. **7**: 962–979. doi:10.1038/ismej.2012.161

Newton, R. J., S. E. Jones, A. Eiler, K. D. McMahon, and S. Bertilsson. 2011. A guide to the natural history of freshwater lake bacteria. Microbiol. Mol. Biol. Rev. **75**: 14–49. doi:10.1128/MMBR.00028-10

Newton, R. J., S. E. Jones, M. R. Helmus, and K. D. McMahon. 2007. Phylogenetic ecology of the freshwater Actinobacteria acI lineage. Appl. Environ. Microbiol. **73**: 7169–76. doi:10.1128/AEM.00794-07

Ottesen, E. A., C. R. Young, J. M. Eppley, J. P. Ryan, F. P. Chavez, C. A. Scholin, and E. F. DeLong. 2013. Pattern and synchrony of gene expression among sympatric marine microbial populations. Proc. Natl. Acad. Sci. U. S. A. **110**: E488-97. doi:10.1073/pnas.1222099110

Ottesen, E. A., C. R. Young, S. M. Gifford, J. M. Eppley, R. Marin, S. C. Schuster, C. A. Scholin, and E. F. DeLong. 2014. Ocean microbes. Multispecies diel transcriptional oscillations in open ocean heterotrophic bacterial assemblages. Science **345**: 207–12. doi:10.1126/science.1252476

Parks, D. H., M. Imelfort, C. T. Skennerton, P. Hugenholtz, and G. W. Tyson. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. **25**: 1043–55. doi:10.1101/gr.186072.114

Paver, S. F., K. R. Hayek, K. A. Gano, and others. 2013. Interactions between specific phytoplankton and bacteria affect lake bacterial community succession. Environ. Microbiol. **15**: 2489–2504. doi:10.1111/1462-2920.12131

Paver, S. F., N. D. Youngblut, R. J. Whitaker, and A. D. Kent. 2015. Phytoplankton succession affects the composition of *P* *olynucleobacter* subtypes in humic lakes. Environ. Microbiol. **17**: 816–828. doi:10.1111/1462-2920.12529

Pernthaler, J., T. Posch, K. Simek, and others. 2001. Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. Appl. Environ. Microbiol. **67**: 2145–55. doi:10.1128/AEM.67.5.2145-2155.2001

Pinhassi, J., E. F. DeLong, O. Béjà, J. M. González, and C. Pedrós-Alió. 2016. Marine Bacterial and Archaeal Ion-Pumping Rhodopsins: Genetic Diversity, Physiology, and Ecology. Microbiol. Mol. Biol. Rev. **80**: 929–954. doi:10.1128/MMBR.00003-16

Poretsky, R. S., I. Hewson, S. Sun, A. E. Allen, J. P. Zehr, and M. A. Moran. 2009. Comparative day/night metatranscriptomic analysis of microbial communities in the North Pacific subtropical gyre. Environ. Microbiol. **11**: 1358–1375. doi:10.1111/j.1462-2920.2008.01863.x

Posch, T., K. Simek, J. Vrba, J. Pernthaler, J. Nedoma, B. Sattler, B. Sonntag, and R. Psenner. 1999. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. Aquat. Microb. Ecol. **18**: 235–246. doi:10.3354/ame018235

Pruitt, K. D., and D. R. Maglott. 2001. RefSeq and LocusLink: NCBI gene-centered resources. Nucleic Acids Res. **29**: 137–140. doi:10.1093/nar/29.1.137

Rinke, C., J. Lee, N. Nath, and others. 2014. Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics. Nat. Protoc. **9**: 1038–1048. doi:10.1038/nprot.2014.067

Satinsky, B. M., S. M. Gifford, B. C. Crump, and M. A. Moran. 2013. Use of Internal Standards for Quantitative Metatranscriptome and Metagenome Analysis. Methods Enzymol. **531**: 237–250. doi:10.1016/B978-0-12-407863-5.00012-5

Simek, K., V. Kasalický, E. Zapomĕlová, and K. Hornák. 2011. Alga-derived substrates select for distinct Betaproteobacterial lineages and contribute to niche separation in Limnohabitans strains. Appl. Environ. Microbiol. **77**: 7307–15. doi:10.1128/AEM.05107-11

Šimek, K., J. Nedoma, J. Pernthaler, T. Posch, and J. R. Dolan. 2002. Altering the balance between bacterial production and protistan bacterivory triggers shifts in freshwater bacterial community composition. Antonie Van Leeuwenhoek **81**: 453–463. doi:10.1023/A:1020557221798

Sjöstedt, J., P. Koch-Schmidt, M. Pontarp, B. Canbäck, A. Tunlid, P. Lundberg, A. Hagström, and L. Riemann. 2012. Recruitment of members from the rare biosphere of marine bacterioplankton communities after an environmental disturbance. Appl. Environ. Microbiol. **78**: 1361–9. doi:10.1128/AEM.05542-11

Solomon, C. T., D. A. Bruesewitz, D. C. Richardson, and others. 2013. Ecosystem respiration: Drivers of daily variability and background respiration in lakes around the globe. Limnol. Oceanogr. **58**: 849–866. doi:10.4319/lo.2013.58.3.0849

Sommaruga, R., I. Obernosterer, G. J. Herndl, and R. Psenner. 1997. Inhibitory effect of solar radiation on thymidine and leucine incorporation by freshwater and marine bacterioplankton. Appl. Environ. Microbiol. **63**: 4178–84.

Sunagawa, S., L. P. Coelho, S. Chaffron, and others. 2015. Structure and function of the global ocean microbiome. Science **348**: 1261359. doi:10.1126/science.1261359

Teeling, H., B. M. Fuchs, D. Becher, and others. 2012. Substrate-Controlled Succession of Marine Bacterioplankton Populations Induced by a Phytoplankton Bloom. Science (80-. ). **336**: 608–611. doi:10.1126/science.1218344

Thaben, P. F., and P. O. Westermark. 2014. Detecting rhythms in time series with RAIN. J. Biol. Rhythms **29**: 391–400. doi:10.1177/0748730414553029

Tsementzi, D., R. Poretsky, L. M. Rodriguez-R, C. Luo, and K. T. Konstantinidis. 2014. Evaluation of metatranscriptomic protocols and application to the study of freshwater microbial communities. Environ. Microbiol. Rep. **6**: 640–655. doi:10.1111/1758-2229.12180

Verity, P. G., P. Wassmann, T. N. Ratkova, I. J. Andreassen, E. Nordby, and T. Høisæter. 1999. Seasonal patterns in composition and biomass of autotrophic and heterotrophic nano- and microplankton communities on the north Norwegian shelf. Sarsia **84**: 265–277. doi:10.1080/00364827.1999.10420431

Vila-Costa, M., S. Sharma, M. A. Moran, and E. O. Casamayor. 2013. Diel gene expression profiles of a phosphorus limited mountain lake using metatranscriptomics. Environ. Microbiol. **15**: 1190–1203. doi:10.1111/1462-2920.12033

Vorobev, A., S. Sharma, M. Yu, and others. 2018. Identifying labile DOM components in a coastal ocean through depleted bacterial transcripts and chemical signals. Environ. Microbiol. **20**: 3012–3030. doi:10.1111/1462-2920.14344

Welkie, D. G., B. E. Rubin, S. Diamond, R. D. Hood, D. F. Savage, and S. S. Golden. 2018. A Hard Day’s Night: Cyanobacteria in Diel Cycles. Trends Microbiol. doi:10.1016/J.TIM.2018.11.002

Woyke, T., A. Sczyrba, J. Lee, and others. 2011. Decontamination of MDA Reagents for Single Cell Whole Genome Amplification O. Lespinet [ed.]. PLoS One **6**: e26161. doi:10.1371/journal.pone.0026161

Wu, Y.-W., Y.-H. Tang, S. G. Tringe, B. A. Simmons, and S. W. Singer. 2014. MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome **2**: 26. doi:10.1186/2049-2618-2-26