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

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## Abstract

Microbial communities form the base of 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, regardless of variation in lake characteristics. 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 both differential expression in day versus night and diel cycles in all three lakes. Specifically, genes related to photosynthesis were more expressed during daylight, and genes related to sugar transport were more expressed at night, suggesting that primary production and respiration are not only performed by different community members, but also performed at different times. These results indicate sophisticated organization within freshwater microbial communities that is generalizable across lake types.

**Scientific Significance Statement**

The importance 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 infer function and 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 both phototrophs and heterotrophs 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 potential 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 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 compositions 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 the community 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 release 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). Up to 80% of photosynthetic carbon is released extracellularly in marine systems, while up to 99% release has been reported in freshwaters (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 avoid this stress (Sommaruga et al. 1997).

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.

## 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, SP), eutrophic (Lake Mendota, ME), and humic (Trout Bog Lake, TB) (Table 1).ME is located in Madison, WI, USA, while TB and SP 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 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 phenol:chloroform and the same lysis method as in the RNA extraction protocol. Four additional DNA samples collected from SP 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 SP, 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 (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 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). Bins and unbinned contigs from the metagenome assemblies were classified by taking the consensus taxonomy of the best hit in the Integrated Microbial Genomes database (Markowitz et al. 2012) for each coding region on a contig/bin using in-house McMahon Lab scripts. Where contigs were too short to classify or had conflicting coding region classifications, the coding region classifications were used instead.

### Metatranscriptomics

Metatranscriptomic samples were sequenced by the JGI. 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. 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 SP, 30 from ME, and 21 from TB. Many samples from day two in the TB time series failed to meet quality control standards.

### Statistics

The statistical software R was used for expression analysis (R Core Team, 2018). 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. The summed expression of each pathway/process was input into DESeq2 to test for differential expression (Love et al. 2016). Using the internal standard to determine normalization size factors, we converted read counts to units of transcripts per liter. Therefore, these results are semi-quantitative. In addition to normalizing by the internal standard, samples were also normalized using a negative binomial distribution using DESeq2 to control for compositional bias before testing for differential expression (Anders and Huber 2010). RAIN was used to detect cyclic trends in gene expression (Thaben and Westermark 2014). Based on PAR, day time points were considered to be 9AM, 1PM, and 5PM, while night time points were considered to be 9PM, 1AM, and 5AM. 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 1). 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 ME and TB. These genes were most frequently attributed to *Cyanobacteria.*

Because genes related to phototrophy were overwhelmingly represented in the metatranscriptomes, we also ran this analysis excluding genes associated with phototrophy and unannotated genes (Figure 1). This showed that housekeeping genes such as RNA polymerase, chaperonin, and translation elongation factors were commonly expressed in all lakes. Many of the most highly expressed non-photosynthetic genes in ME belonged to *Actinobacteria* (acI-A and acI-B clades), including a sugar transporter. In TB, *Verrucomicrobia* and *Armatimonadetes* (formerly candidate phylum OP10) contributed some of the most expressed genes, while in SP, a chaperonin expressed by *Deltaproteobacteria* was among most highly expressed genes. Cytochrome subunits, presumably an indicator for respiration, were highly expressed in all lakes, ranking in the top 10 in TB and SP, and in the top 25 in ME.

*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 2). 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, *Cyanobacteria, Bacteroidetes,* and *Actinobacteria* were relatively highly expressed in all three lakes. *Betaproteobacteria* was highly expressed in TB and SP, while *Verrucomicrobia* was especially highly expressed in TB. 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 “clades” (Newton et al. 2011). Where taxonomy was resolved to the clade level, we noted which clades contributed to observed transcripts. Members of *Actinobacteria* acI-B were expressed and abundant in all lakes, but especially TB. This is consistent with previous research identifying acI-B2 as an acidic lake specialist (Newton et al. 2007). The betIV-A clade (LD28) contributed a surprising number of expressed genes in ME, as did the alfV-A clade (LD12) and the acI-A clade. The bacIII-B clade was highly expressed in SP, but had low abundance. Members of betIV-A were abundant in TB, but not proportionally highly expressed. The Pnec clade, known to be endemic to bog lakes and particularly TB, was 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.

### Assessing variability in freshwater metatranscriptomes

Because this study is among the largest metatranscriptomic sequencing efforts to date, we discuss the biological vs. 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 S1). 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 S2). Parameters that reflect the boundaries between layers within the water column, such as dissolved oxygen, temperature, pH, and conductivity, were strongly diel in ME, but less so in SP and TB (Figure S3). Chlorophyll concentrations, often used as an indicator of primary production, were diel in TB, 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 S4). No diel trends were observed in total and dissolved nitrogen or phosphorus concentrations.

### Gene expression in day vs. night

To test differential expression in day vs. night, we aggregated time points by day (9AM, 1PM, and 5PM) or night (9PM, 1AM, and 5AM). We identified many genes with significant differential expression in day vs. night and tested for significant differences in the number of reads assigned to genes in functional categories. We also used RAIN to reveal any cyclic trends with 12-hour periods among genes pre-screened for differential expression in day vs. night.

Genes related to photosynthesis were significantly more expressed in day vs. night and were likely to be cyclic in all lakes (Figure 3). In ME (Table 2), this expression was largely derived from *Cyanobacteria,* while photosynthesis-related gene expression in TB (Table 3) and SP (Table 4) was derived from a mix of *Cyanobacteria, Eukaryota,* and unclassified groups*.* Expression of genes encoding the key carbon fixation enzyme RuBisCO was only significantly different in day vs. night in TB, where it was 7-fold higher during the day; the associated gene clusters were largely taxonomically unclassified due to poor reference matching.

Genes related to sugar transport were often significantly more expressed at night in all three lakes. Specifically, genes annotated as general sugar transporters, ribose transporters, and raffinose/stachyose/melibiose (R/S/M) transporters were significantly more expressed at night in ME (Figure 4). General sugar transporters were expressed by *Actinobacteria* (acI-B1 and acTH1-A1)*, Cyanobacteria* (*Synechococcaceae* and *Microcystis*)*,* and *Bacteroidetes,* with a lower proportion of reads derived from *Cyanobacteria* at night compared to day. Ribose transporters and R/S/M transporters were mostly classified to *Actinobacteria* and *Bacteroidetes,* with little difference in profiles between day and night. In TB, genes annotated as transporters for general sugars, ribose, and xylose were significantly more expressed at night. *Actinobacteria* (acI-B)contributed the majority of expressed reads for all three types of sugar transporters, while *Alphaproteobacteria* were associated with xylose and general sugar transport, and *Cyanobacteria* and *Armatimonadetes* contributed to general sugar transport. The only significant differentially expressed sugar transport group in SP was R/S/M transport in *Actinobacteria*, although several other types were near the significance threshold.

Reactive oxygen species (ROS) defense is a critical function for microbes exposed to UV radiation. As expected, genes related to ROS defense were significantly more expressed in day vs night in all three lakes, with roughly 15% of genes identified as cyclic in ME and SP. Phyla expressing ROS defense-related genes in ME included *Cyanobacteria, Deltaproteobacteria, Planctomycetes, Verrucomicrobia, Betaproteobacteria, and Bacteroidetes.* In TB, ROS defense-related reads were assigned to *Actinobacteria, Alphaproteobacteria, Armatimonadetes, Bacteroidetes, Betaproteobacteria, Eukaryota, Gammaproteobacteria,* and *Verrucomicrobia,* with roughly a 3rd of reads mapping to unclassified gene clusters. ROS defense gene expression in SP was contributed by *Actinobacterica, Bacteroidetes, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, Eukaryota, Gammaproteobacteria,* and *Planctomycetes.* Interestingly, *Cyanobacteria* expression of ROS defense genes was comparatively low compared to other groups in both TB and SP.

Several functional gene categories differed in significance between lakes. Genes related to opsins, classified as *Actinobacteria* (acI-B1)and *Bacteroidetes,* were significantly more expressed in day in only ME, as were genes related to proteases (*Cyanobacteria, Betaproteobacteria*, *Gammaproteobacteria, Bacteroidetes,* and *Actinobacteria,* with a higher proportion of *Cyanobacteria* in daytime). Genes associated with xylose transport and RuBisCO were significantly differentially expressed only in TB. Additionally, amino acid transport genes were more expressed at night in TB, classified *Actinobacteria, Alphaproteobacteria, Armatimonadetes,* and *Betaproteobacteria*. Fewer functional groups were differentially expressed in SP compared to the other two lakes, with no groups found to be significant only in SP.

## Discussion

In this study, we sought to identify generalizable interactions in freshwater microbial communities through diel gene expression. Using metatranscriptomic time series, we were able to detect genes that were differentially expressed in day vs. night and identify those with cyclic trends. The functional annotations of those genes allowed us to hypothesize metabolic exchanges in freshwater microbial communities. We found similar trends in the timing and types of genes expressed across all lakes studied, regardless of trophic status.

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 in day and often cyclic. Photosynthesis and carbon fixation are often considered to be coupled in the process of primary production; however, we only saw differential expression of RuBisCO genes in TB. Still, expression does not necessarily correlate to direct protein abundance or function (Moran et al. 2013), indicating only that transcriptional regulation of RuBisCO is not diel in ME or SP.

Respiration is a broad 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 found significantly higher expression of genes related to sugar transport at night compared to day. Phototrophs are known to exude sugars (Maršálek and Rojíčková 1996), suggesting that sugars may be exchanged between phototrophs and heterotrophs.

We studied lakes representing eutrophic (ME), humic (TB), and oligotrophic (SP) trophic statuses. There were key differences in gene expression between these lakes, suggesting that microbial communities function differently under varying productivity levels. For example, genes related to amino acid transport were only differentially expressed in day vs. night in TB, while genes related to opsins were only differentially expressed in ME. Although sugar transporter expression was commonly observed, the types of transporters that were differentially expressed in day vs. night differed by lake. In ME, these genes encode ribose, general sugar, and raffinose/stachyose/melibiose transport, while for TB, they encoded ribose, general sugar, and xylose transport. Among the sugar transporters, only genes encoding raffinose/stachyose/melibiose transport were significantly differentially expressed in SP. Still, the similarities between these three lakes in sugar transport and photosynthesis diel expression indicate a potentially generalizable trend in freshwater.

There are two non-exclusive hypotheses as to why we observed diel trends in 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 at night. Although such diel release of sugars has not been documented, 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. The other hypothesis is that oxidative stress prevents heterotrophs from consuming sugar during the day, even if it is available. We observed significant differential expression in genes related to ROS defense, with higher expression during the day, in all three lakes. Although solar radiation is known to produce low molecular organic acids from high molecular weight organic matter, we did not observe differential expression of genes encoding transporters for typical photodegradation products, such as glycolate or carboxylic acids.

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 exudates 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 photoautotrophs 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” (Morris et al. 2012). Further concerted efforts to recover nearly complete reference genomes from freshwater systems should enable more rigorous testing of this hypothesis.

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). Here, we present a comparative metatranscriptomic analysis which demonstrates similar diel trends in photosynthesis, reactive oxygen species defense, and sugar transport in three different types of lakes, suggesting that these trends may be a general property of freshwater microbial communities. We outline 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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