

Impact of phosphorus enrichment patterns on stream periphyton microbial communities

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Delivery of excess nutrients, such as phosphorus (P), to nearby surface waters due to intensified agricultural and urban land use activities has resulted in the degradation of aquatic ecosystems such as lakes, coastal areas, and flowing waters. In stream ecosystems, excess P can influence the diversity and composition of periphyton communities. However, P enrichment can vary over time and in intensity according to the source of P and there is limited knowledge of the impacts of event-based loadings on the microbial community composition of stream periphyton. To address this knowledge gap, two related stream mesocosm experiments were conducted.

The first objective of my research was to evaluate whether P delivery patterns that are commonly associated with human land use practices influence the microbial community composition of stream periphyton. I achieved this objective by conducting a 25-day mesocosm experiment using nine artificial streams subjected to one of three P enrichment loading patterns in triplicate: unenriched, continuously enriched, or event-based enriched. Periphyton samples were collected at six time points and underwent high throughput sequencing of 16S and 18S rRNA genes to profile prokaryotic and eukaryotic microbial assemblages. Results showed that microbial community composition could not be differentiated among enriched delivery patterns, indicating the P load magnitude was more important than the P loading pattern. Thus, management of P enrichment in streams should prioritize targeting the most efficient way to reduce P loads, regardless of source.

The second research objective was to assess how stream periphyton microbial community composition responded along an increasing gradient of P event concentrations and test whether antecedent P availability influenced this response. Two artificial stream experiments were conducted under low or high antecedent P conditions, with each stream receiving one of nine different P event concentrations. Periphyton were collected one day before and 10 days after the event and high throughput sequencing of the 16S and 18S rRNA genes were used to characterize prokaryotic and eukaryotic members of stream periphyton communities. The results indicate that small levels of P enrichment from P events can lead to a common enriched stream periphyton community. Thus, bioassessments of periphytic communities in anthropogenic landscapes may underestimate the extent of enrichment in streams with high baseflow P concentrations.

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List of Abbreviations

AICc	corrected Akaike Information Criterion
AICw	Akaike's information criterion weight
ASV	Amplicon sequence variant
DADA2	Divisive Amplicon Denoising Algorithm 2
DNA	Deoxyribonucleic acid
GLM	General Linear Model
N	Nitrate
P	Phosphorus
PAR	Photosynthetic active radiation
PERMANOVA	Permutational Multivariate Analysis of Variance
PCoA	Principal Coordinate Analysis
QIIME2	Quantitative Insights Into Microbial Ecology 2
rRNA	Ribosomal ribonucleic acid
SRP	Soluble reactive phosphorus
SRS	Scaling with ranked subsampling
SIMPER	Similarity Percentage
TP	Total phosphorus
TRESS	Thames River Experimental Stream Sciences

Chapter 1: Introduction and literature review

1.1 Problem statement

Excess nutrient losses from anthropogenic activities, such as agriculture, industrial practices, and sewage treatment, have accelerated eutrophication of many aquatic ecosystems, leading to impaired water quality and ecosystem health at a global scale (Akinawo, 2023; Alexander et al., 2017; Zhang et al., 2023). In addition to an increase in primary production, eutrophication can cause a shift in the diversity and abundance of microbial species in aquatic ecosystems because fast growing autotrophic organisms (e.g., algae) that prefer high nutrient availability outcompete other microbial taxa (Alexander et al., 2017). In freshwater ecosystems, such as streams, eutrophication and associated shifts in the diversity and abundance of microbial communities is often controlled by the availability of phosphorus (P).

In stream ecosystems, anthropogenically sourced P can be delivered in two main patterns: continuously (i.e., point sources) or in short durations after storm events (i.e., runoff from non-point sources) (Wu and Chen, 2013). The effects of continuous P enrichment on stream ecosystems have been widely studied. However, there is limited understanding of how event-based P enrichment of stream ecosystems influences microbial community composition of stream periphyton.

Stream periphyton are comprised of diverse microbial communities found on rocks, wood, and other streambed debris. Periphyton play an important role in the cycling of P in streams and are known to be sensitive to P enrichment (Besemer, 2015). Most knowledge of P enrichment effects on the composition of periphyton is focused on a subset of the community (e.g., algae). However, periphyton also includes many other eukaryotic taxa as well as diverse prokaryotic assemblages. Although event-based P enrichment of streams is a common and increasing environmental problem, the relative importance of this enrichment pattern to stream periphyton communities is largely unknown. Thus, the goal of this thesis research was to assess how event-based loading can influence the whole microbial community composition of stream periphyton.

1.2 Nutrient enrichment in aquatic ecosystems

Efforts to restore and maintain the ecological structure and function of aquatic systems from environmental pollution has spanned multiple decades (Preisner et al., 2021; Schindler, 2006). In 1972, efforts to restore the integrity of the Great Lakes through implementation of P reduction programs were created by Great Lakes Water Quality Agreement (GLWQA) (Canada, 2015; Krantzberg, 2007). However, eutrophication driven by anthropogenic P enrichment remains a long-standing challenge affecting surface water quality. Excess P delivered to aquatic ecosystems increases primary productivity and can lead to murky waters from an overgrowth of macrophytes and algae, hypoxic zones that reduce fish populations, and increased abundance of algal toxins (e.g., microcystin) (Kangur et al., 2005; Mainstone and Parr, 2002; Mallin and Cahoon, 2020; Mishra et al., 2022; Song et al., 2023). As a result, there is potential for economic loss due to reduced recreational activities and waterfront real estate because of unpleasant sights and odours, biodiversity loss, and increased cost of water treatment (Dodds et al., 2009). Indeed, Dodds et al. (2009) estimated annual losses of \$2.4 billion due to eutrophication of U.S freshwaters. Although eutrophication has long been a concern in lakes and coastal areas, the implications for lotic ecosystems are often overlooked due to the dynamic hydrological nature of streams.

Although wadable streams (e.g., headwaters) are often neglected as impacted surface waters due to their small size, headwaters make up to 70-90% of total stream length (Evans-White et al., 2013; Leopold et al., 1964). Due to the high degree of connectedness with surrounding landscapes, headwater streams are vulnerable to excess nutrient inputs due to land-use changes (e.g., agriculture and urbanization) (Staponites et al., 2019; Withers and Jarvie, 2008). Anthropogenic influences on headwater streams can significantly influence the water quality, biodiversity, and ecological health of downstream ecosystems (e.g., larger rivers and lakes) (Evans-White et al., 2013; Lowe and Likens, 2005). However, nutrient availability in streams can be highly variable due to landscape (e.g., topography, physiography) and instream (e.g., flow and thermal regimes) conditions (Withers and Jarvie, 2008).

1.3 Phosphorus delivery patterns in streams

Anthropogenically sourced P can be delivered to streams continuously or through event-based loadings (Withers and Jarvie, 2008). Continuous P loading typically arises from point

sources that have a direct source of delivery (e.g., wastewater effluent) (Walsh, 2000; Withers and Jarvie, 2008; Wu and Chen, 2013). In contrast, event-based loadings are of short duration (i.e., hours to days) and arise from storm-driven runoff that transports nutrients to streams from non-point sources (e.g., agricultural and urban lands) (Walsh, 2000; Withers and Jarvie, 2008; Wu and Chen, 2013). However, depending on the activities occurring in the catchment, these runoff events can transport varying magnitudes of P along a range of pathways into nearby streams that are difficult to monitor and manage (Bennett et al., 2001; Fred Lee, 1973; Walsh, 2000; Wu and Chen, 2013). For example, along a waterway there can be multiple farms contributing to P loading, with P fertilizer loss to waters ranging from 25-75% of applications (Sutton, 2013). It is well established that varying sources of P can increase P concentration in streams and there are many in-stream processes that cycle various forms of P.

Newbold et al., (1981) identified water, particulates, and consumers (e.g., algae, zooplankton, fish) as the three major compartments that nutrients cycle through as they are transported downstream (Figure 1.1). For P measurements in streams, total P (TP) is a common metric that accounts for particulate, dissolved, organic, and inorganic forms of P (Allan et al., 2021). Particulate P is often less accessible biologically and can be either organic (e.g., part of cell walls or detrital particles) or inorganic (e.g., sediment bound) (Allan et al., 2021). Dissolved P can be organic (e.g., phospholipids) or inorganic (e.g., orthophosphate, PO_4^{3-} , also known as soluble reactive P, SRP) (Allan et al., 2021). Dissolved inorganic P (i.e., SRP) is readily available for uptake by autotrophic organisms (Figure 1.1), whereas particulate and organic forms require cleavage and transformation for biological use (Allan et al., 2021). Both TP and SRP are used commonly to measure the ecosystem productivity. However, due to the bioavailability of SRP, it is argued to be a better indicator of stream trophic status than TP (Allan et al., 2021; Ellison and Brett, 2006).

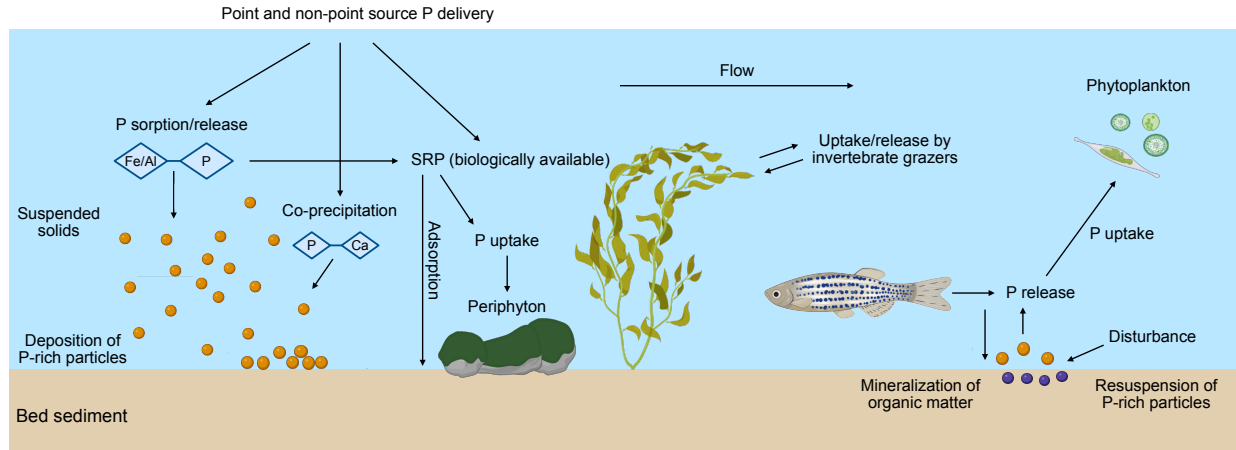


Figure 1.1 Biotic and abiotic in-stream processes influencing biogeochemical cycling of phosphorus (modified from Withers and Jarvie, 2008). Created in BioRender.

1.4 Stream periphyton

Stream periphyton communities play a key role in primary production, biogeochemical cycling, and bottom-up processes in stream food webs (Battin et al., 2016; Bunn et al., 2003; Guo et al., 2022). With an increase in P availability, studies have found an increase in P uptake (Jarvie et al., 2002; McCormick et al., 2006; Pearce et al., 2023, 2021) and biomass (i.e., ash-free dry mass and Chlorophyll *a*) (Biggs, 2000; Dodds et al., 2002; Rier and Stevenson, 2006; Stevenson et al., 2008). Thus, stream periphyton contribute significantly to ecosystem health, particularly by retaining and mitigating the transport of excess P to downstream ecosystems (Pearce et al., 2023).

It was previously assumed that P delivered during short duration events would have limited biological relevance due to the scouring effects of storm events and the short residence time of event P (Stamm et al., 2014; Withers and Jarvie, 2008). However, streams with larger bed sediments can experience flows from storm events that are “subscouring” (Power and Stewart, 1987; Stevenson, 1990). Additionally, microalgae can undergo a process known as “luxury uptake”, where P uptake occurs rapidly and above the necessary means required for growth and biomass accumulation (Pearce et al., 2023, 2020; Solovchenko et al., 2019). Thus, it is known that P events can be used by periphyton, but little is known about how different P loading patterns influence the community composition of stream periphyton.

1.5 Phosphorus enrichment and stream periphyton community composition

Eutrophication in stream ecosystems is often characterized by an overgrowth of periphyton, which are diverse communities of microorganisms composed of bacteria, archaea, fungi, and algae embedded in an extracellular polymer matrix on substrates such as rocks, woody debris, and sediments (Lock et al., 1984; McDowell et al., 2020). Under chronic P enrichment, stream periphyton are often dominated by autotrophic organisms, such as green algae (i.e., *Chlorophyta*), blue-green algae (i.e., *Cyanobacteria*), and diatoms (i.e., *Bacillariophyta*) (Bondar-Kunze et al., 2016; Davis et al., 1990). Many studies have found an overgrowth and dominance of filamentous green algae associated with nutrient enrichment (Biggs, 1990; Elwood et al., 1981; Perrin et al., 1987). Indeed, Chételat et al. (1999) found a dominance of *Cladophora*, a filamentous green alga, in streams with $TP \geq 20 \mu\text{g L}^{-1}$. Additionally, studies have also found compositional shifts in diatom abundances at high TP (Smucker et al., 2013; Winter and Duthie, 2000). For example, Winter & Duthie (2000) found species from the genera *Navicula* and *Nitzschia* to grow optimally with 62.5-118.9 $\mu\text{g L}^{-1}$ TP and be indicators of streams with high levels of P. In fact, watershed management have used P concentration guidelines to mitigate the overgrowth of algae in streams (Evans-White et al., 2013); however, it is not well known as to how the delivery pattern of P plays a role in shaping community composition.

Despite knowledge of the capability for periphyton to assimilate P from short duration events (Pearce et al., 2023; Solovchenko et al., 2019), few studies have assessed the influence on stream periphyton communities. A study by Pearce et al. (2020) found that the same P load resulted in similar algal assemblages (i.e., *Chlorophyta*, *Charophyta*, *Bacillariophyta*, and *Cyanobacteria*) in streams whether enriched continuously or in short duration events. Additionally, Stevenson et al. (2012) found an association with diatoms, filamentous green algae, red algae, and xanthophytes in streams experiencing P enrichment, most likely attributed to agricultural land use. Yet, the connection between event P enrichment and stream periphyton composition remains unclear, with a critical knowledge gap on whether periphyton composition changes with respect to varying magnitudes of P event sizes respective of land-use intensity in a catchment.

Although stream periphyton are diverse microbial communities, past studies have focused largely on algal assemblages, with little attention to other eukaryotic or prokaryotic

members (Biggs, 1990; Chételat et al., 1999; Mallin et al., 2004). This focus is attributable to limitations associated with organism identification using traditional microscopy techniques (Hagy III et al., 2020; MacKeigan et al., 2022). However, over the last decade, high-throughput sequencing of microbial communities has increasingly been promoted as an alternative means of establishing the composition of periphyton (Hagy III et al., 2020; Kermarrec et al., 2014). Molecular approaches, such as amplicon sequencing of target gene regions (e.g., 16S and 18S rRNA genes), offer rapid detection of community changes due to microbial sensitivity to environmental shifts and higher taxonomic resolution (Hagy III et al., 2020; Liu et al., 2021; Wang et al., 2023). Currently, many studies have adopted high-throughput sequencing of microbial communities for assessing their potential as indicators for anthropogenic disturbances in aquatic ecosystems (Gad et al., 2024; Lau et al., 2015; Zhu et al., 2019). Although amplicon sequencing serves as a valuable tool for detecting compositional changes, it comes with limitations because it provides data on relative abundances, rather than absolute abundances, and can only infer functional potential based on taxonomic affiliation (Liu et al., 2021). Nevertheless, high-throughput sequencing offers greater insight into periphyton community members (i.e., bacteria and archaea) that were unable to be detected or characterized using common alternative methods.

1.6 Artificial streams

Assessments of the impacts of changing environmental conditions on periphyton structure and function have commonly used artificial stream experiments (Menczelesz et al., 2020). Within real streams, there are multiple environmental factors that can influence the structure and function of stream periphyton. For example, light, temperature, flow, nutrients, and grazing activity are all dynamic spatially and temporally in real streams (Coelho et al., 2013; Menczelesz et al., 2020). As a result, identifying the effects of specific variables, such as P, is challenging in such complex environments.

Artificial streams offer an opportunity to minimize confounding factors while researching the influence of P loading patterns on microbial community composition of periphyton under conditions that mimic the natural environmental. However, mesocosm experiments can lack natural food web dynamics, spatial patchiness, and disturbances (e.g., precipitation and high flow events) that can control the succession of periphytic communities (Coelho et al., 2013; Wohl,

2016). Despite such drawbacks, artificial streams can provide precise experimental conditions that allow for true replication and low within-group variation, which can be difficult to achieve in field studies (Coelho et al., 2013).

1.7 Thesis outline

This thesis includes two chapters of research results describing changes in stream periphyton community composition when exposed to P enrichment in artificial streams with different delivery patterns relevant to anthropogenic activities in a catchment. Chapter 2 describes a mesocosm experiment that characterizes and compares periphyton community composition subjected to a continuous delivery or an event-based delivery of P. This experiment assessed if the pattern of P enrichment influences the community composition of various microbial assemblages in stream periphyton. Chapter 3 consists of two gradient design mesocosm experiments where stream periphyton communities were exposed to different sized P events under either low or high antecedent P conditions. This experiment assessed the relationship between composition and increasing P event sizes and if this relationship was modulated by the antecedent P availability.

Chapter 2: Periphyton microbial communities of artificial streams impacted similarly by distinct phosphorus enrichment patterns

2.1 Introduction

Anthropogenic activities have led to excess P in many streams, which impacts periphyton alpha and beta diversity (Peterson and Stevenson, 1992; Stevenson, 1990). For example, an increased abundance of periphytic organisms able to readily assimilate P reduces periphytic richness and evenness (Biggs and Smith, 2002; Dunck et al., 2015; Groendahl and Fink, 2017), likely due to a dominance of opportunistic taxa. Additionally, nutrient enrichment in streams commonly leads to shifts in algal community composition (Chételat et al., 1999; Munn et al., 2002; Smucker et al., 2013). However, current knowledge of how anthropogenically sourced P impacts stream periphyton diversity is predominantly from studies of continuously enriched ecosystems, but many streams may only be enriched in short duration P events.

Stream P enrichment can vary temporally, depending on the types and amount of human activity in the catchment. Delivery of P can be continuous through point sources (e.g., wastewater effluent) and groundwater fluxes, leading to chronically enriched P concentrations (Withers and Jarvie, 2008). In contrast, event-based enrichment can result from P being delivered during short duration events (i.e., hours to days) associated with storm-driven runoff that transports nutrients to streams from non-point sources (e.g., agricultural and urban lands) (Carpenter et al., 2018; Hanway and Laflen, 1974; Macrae et al., 2007). Others have hypothesized that P delivered during short duration events would have limited biological relevance due to the short residence time of event P and scouring effects of storm events (Stamm et al., 2014; Withers and Jarvie, 2008). However, many flows generated by storm events are subscouring (Power and Stewart, 1987; Stevenson, 1990), particularly in streams with larger bed particles (Power and Stewart, 1987). Additionally, microalgae can uptake excess P in greater quantities than necessary for growth when provided in short duration, a process known as luxury uptake (Pearce et al., 2023; Solovchenko et al., 2019), leading to periphytic growth and biomass accumulation similar to continuously enriched streams (Pearce et al., 2020). In addition, Pearce et al. (2020) found that periphytic assemblages receiving continuous or event-based P enrichment were dominated similarly by *Cyanobacteria* and *Chlorophyta*, although diatom assemblages exhibited differences in composition. Although past studies suggest that the variation in temporal

loadings of P may influence periphyton community composition, many of these studies focussed only on algae. However, in addition to algae, periphyton are represented by diverse communities of bacteria, archaea, and other eukaryotes; much less is known about the sensitivity of these groups to P enrichment patterns (Mallin and Cahoon, 2020).

Prokaryotes have high functional and taxonomic diversity, which makes them challenging to identify and enumerate using previous bioassessment methods (i.e., culturing and microscopy; Hagy III et al., 2020). With high-throughput sequencing, taxonomic characterization of prokaryotic members of periphyton can be done with higher taxonomic resolution and less time (Hagy III et al., 2020), providing an opportunity to assess ecological responses of various taxonomic groups (i.e., prokaryotes and eukaryotes) to varying temporal P enrichment patterns characteristic of anthropogenic land-use.

The goal of this study was to compare the effects of temporal patterns of P enrichment on stream periphyton. This was achieved by conducting an artificial stream experiment with three treatment levels (i.e., unenriched, continuously enriched, or event-based enriched) and analysis of 16S and 18S rRNA genes to characterize stream periphyton microbial communities. Our objectives were to determine if P enrichment patterns influence alpha and beta diversity of stream periphyton microbial communities and compare eukaryotic and prokaryotic assemblage responses to P enrichment.

2.2 Materials and methods

2.2.1 Experimental design

A one-factor experimental design was used to assess the effects of P loading patterns (i.e., continuous and event-based) on microbial community composition of stream periphyton (Figure 1A). A 25-day mesocosm experiment was conducted in summer of 2022 (July 14 to August 8), with artificial streams ($N = 9$) subjected to one of three SRP enrichment loading patterns in triplicate: 1) unenriched (consistent $10 \mu\text{g L}^{-1}$ P or $0.041 \text{ g P day}^{-1}$), 2) continuously enriched (consistent $40 \mu\text{g L}^{-1}$ P or $0.163 \text{ g P day}^{-1}$), and 3) event-based enriched (consistent $10 \mu\text{g L}^{-1}$ P or $0.041 \text{ g P day}^{-1}$ and one 48-hour event of $700 \mu\text{g L}^{-1}$ P or 3.14 g of P). By the end of the experiment (day 25), the cumulative SRP load delivered to the unenriched, event-based enriched, and continuously enriched streams were 1, 4, and 4 g, respectively (Figure 1A). Loads of SRP

were selected based on the background, chronically enriched (Chambers et al., 2012), and storm-event concentrations observed in streams in Southern Ontario, Canada (Williams et al., 2018).

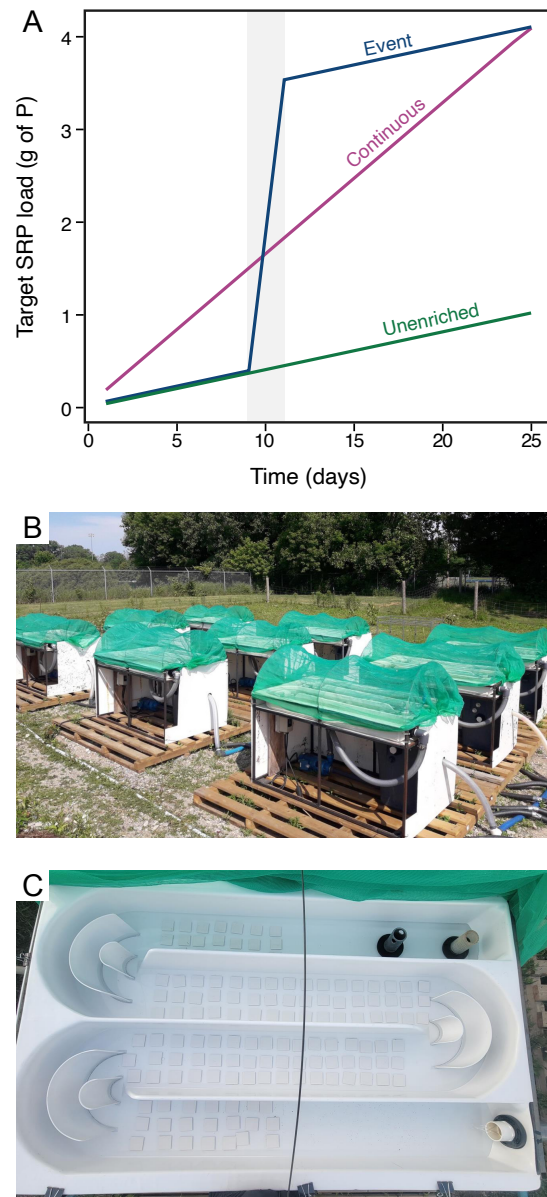


Figure 2.1 Target cumulative SRP loading for unenriched, event-based, and continuously enriched treated artificial streams over 25 days, with a grey bar indicating the timing and duration of the event-based P enrichment (A). Nine artificial streams at the Thames River Experimental Stream Sciences Centre (TRESS) in London, Ontario (B) and a view of the artificial stream and unglazed ceramic tiles used as the substrate (C). The four reaches shown are each 1.75 m long (7 m total), 0.2 m wide, and 0.15 m deep.

2.2.2 Experimental setup

The Thames River Experimental Stream Sciences (TRESS) Centre is an outdoor facility in London, Ontario, containing nine sinuous artificial streams, each with four reaches measuring 1.75 m long (7 m total), 0.2 m wide, and 0.15 m deep (Figure 2.1B, C). A total of 48 unglazed ceramic tiles (Olympia Quebec; 4.8 x 4.8 cm tile in Arctic White) were placed in the second and third reach (N = 96) to provide a consistent and collectable substratum for periphyton growth. Stream nutrient addition and calibrations were performed as described previously (Pearce et al., 2020). Water temperature was measured hourly using a pendant logger (Onset HOBO) at the bend between the second and third reach of each stream (average degree day of $504.3 \pm 1.4^{\circ}\text{C}$ across all streams). A 60% shade cloth was used to limit light availability. Two photosynthetic active radiation (PAR) loggers were placed beneath the shade cloths of two randomly selected streams to record light availability every five minutes and measured total incident PAR as $573.2 \pm 0.1 \text{ mol m}^{-2}$ and $617.4 \pm 0.1 \text{ mol m}^{-2}$, respectively. Stream velocity was maintained at 0.08 m s^{-1} for all streams through calibration of the recirculating pumps using a flow meter (OTT Hydromet).

To confirm target concentrations in streams, measurements of SRP and nitrate (N) concentrations were taken pre-event (i.e., day 1 for SRP and day 7 for N), during the event (i.e., day 10 for SRP and N), and after the event (i.e., day 24 for SRP and N). For each sample, 4 L of water was collected in a graduated cylinder from the inflow of the stream and homogenized. A 45 mL subsample was taken from the graduated cylinder to measure N concentration with a DR1900 Portable Spectrophotometer (Hach Canada). For SRP analysis, a 120 mL subsample was taken from the graduated cylinder and filtered through a sterile cellulose acetate membrane ($0.45 \mu\text{m}$). Subsamples of SRP were held at 4°C until shipment on ice for SRP analysis at the National Laboratory for Environmental Testing in Burlington, Ontario. Measurements of SRP concentration for all water samples were done with the standard molybdenum blue method and analyzed with a continuous flow analyzer. Analyses showed measured concentrations to be within acceptable range of the targeted concentrations for all samples (Figure 2.2). For unenriched streams during the event, samples were collected during refilling of the main dosing tank, underestimating all streams for less than two hours, with minimal impact on overall target.

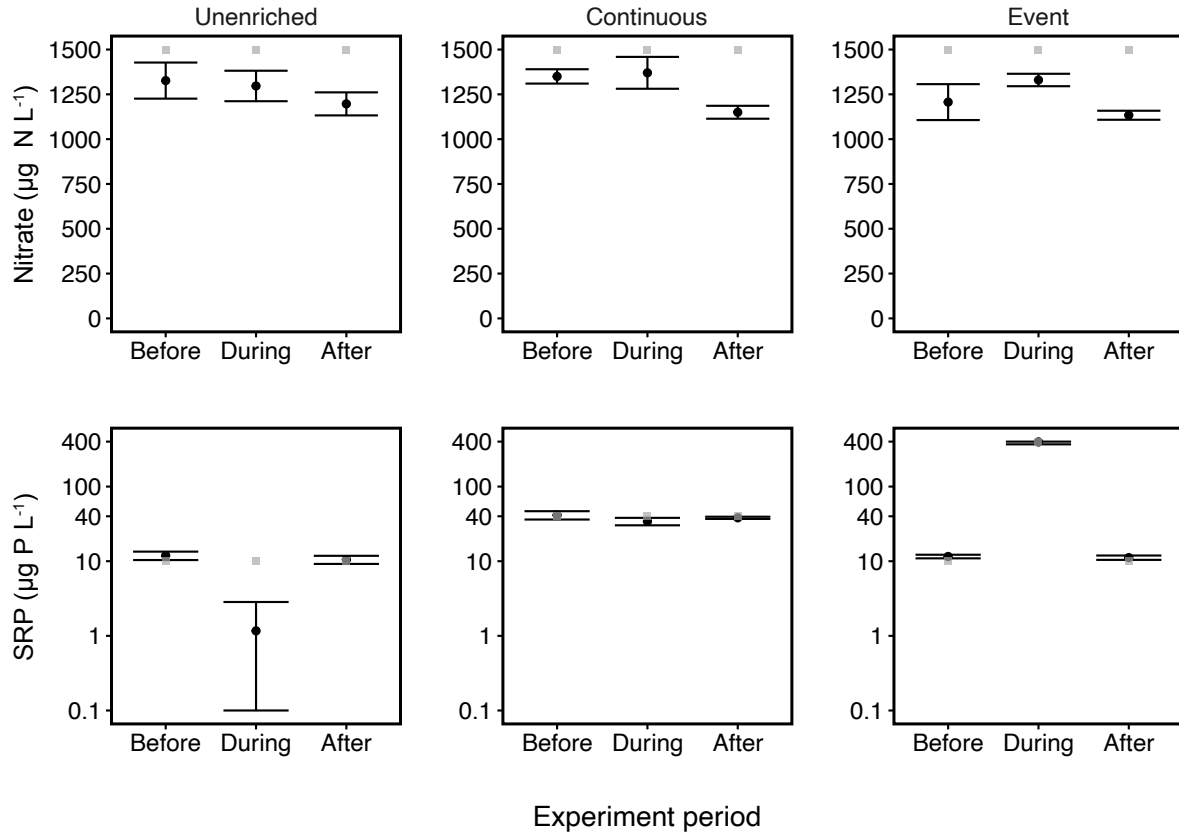


Figure 2.2 Average and standard deviations of nitrate (N) and soluble reactive phosphorus (SRP) concentrations (circles) for triplicate loading patterns (i.e., unenriched, continuous, and event) before, during, and after the event. Grey squares represent the target concentrations of N and SRP before, during, and after the event for each loading pattern.

Artificial streams were inoculated with periphyton collected from five Southern Ontario streams (Table 2.1). Streams had varying P concentrations to increase the diversity of collected inoculant. At each stream, periphyton was scraped from 20 randomly selected cobbles with a brush into 1 L Nalgene bottles. Large invertebrates were removed from collected periphyton. At TRESS, periphyton from each stream were combined in a bucket and homogenized. The resulting periphyton composite was divided equally into nine 300-mL aliquots that were poured into the upstream end of each stream to initiate the experiment. Triplicate samples of the periphyton composite (i.e., Comp1, Comp2, and Comp3) were collected to assess the composition of the inoculant microbial community. Periphyton subsamples from the five Southern Ontario streams ($N = 5$) and the composites used for artificial stream inoculation ($n = 3$) were collected and frozen on site in a portable freezer at -20°C and then transferred to a -80°C freezer within 24 hours.

Table 2.1 Five Southern Ontario streams and their corresponding Strahler order, location (coordinates), total phosphorus (TP), and total nitrogen (TN) concentration data obtained from Ontario Provincial Water Quality Monitoring Network.

Stream name	Strahler order	Location (coordinates)	TP ($\mu\text{g L}^{-1}$)	TN ($\mu\text{g L}^{-1}$)
Middle Thames	6	43.031385, -80.998540	46.0	4,170
Reynolds Creek	4	42.969076, -80.949771	191.0	3,100
South Thames A	5	43.018638, -80.927060	122.0	4,990
South Thames B	5	43.126534, -80.779388	158.0	5,380
Waubuno Creek	4	42.994523, -81.116698	25.0	4,390

Periphyton were sampled from the second and third reach of each artificial stream 6, 8, 11, 15, 20, and 25 days into the experiment (N = 54). To minimize within-sample variation associated with location in the streams, a sampling location was randomly selected at each time point, and the tile at that location sampled from each stream. Periphyton was scraped off tiles with a brush and mini glass scraper into cryo-tubes. After collection, samples were placed in a portable freezer at -20°C for less than 24 hours prior to being transferred to a -80°C freezer where samples were held until processing.

2.2.3 DNA extraction, sequencing, and analysis

All periphyton samples were sent to Integrative Microbiome Resource (IMR) at Dalhousie University for DNA extractions, library preparation, and paired-end sequencing as described previously (Comeau et al., 2017; Comeau and Kwawukume, 2023). Periphyton samples underwent total genomic DNA extractions using the DNeasy PowerSoil Pro QCHT kit (Qiagen). The V4-V5 region of the 16S rRNA gene was amplified using 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) primer sets for detection of prokaryotes (Parada et al., 2016; Walters et al., 2015). The V4 region of the 18S rRNA gene was amplified using E572F (5'-CYGCGGTAATTCCAGCTC) and E1009R (5'-AYGGTATCTRATCRTCCTTYG) primer sets for detection of eukaryotes (Comeau et al., 2011). Primers were customized to include unique adaptors and indexes for sequencing. The resulting library was sequenced on a MiSeq System (Illumina, CA, USA) with a 2 x 300 bp paired end V3

chemistry kit. Raw sequence data is available in the European Nucleotide Archive (PRJEB94294).

Sequence reads were demultiplexed with MiSeq Control Software and analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2024.5; Bolyen et al., 2019). Raw sequence data were processed for denoising and quality control with DADA2 (Callahan et al., 2016) to remove primer sequences, low quality reads, chimeric reads, and to join paired end reads. Taxonomic assignment to amplicon sequence variants (ASVs) was done using naïve Bayes classifier pre-trained with the SILVA 138 database (Quast et al., 2013) for prokaryotes and Protist Ribosomal Reference (PR²; version 5.0.0) database (Guillou et al., 2013) for eukaryotes. The prokaryote-specific sequence run had a cluster density of 845K and 87.5% Q30 and the eukaryote-specific run had a cluster density of 906K and 81.0% Q30. For the eukaryotic sequence run, of the five periphyton samples from Southern Ontario streams, Middle Thames failed to amplify. For the prokaryotic sequence run, there was an average of $56,382 \pm 19,020$ reads, with a minimum of 8,613 and a maximum of 93,136. Both chloroplast and mitochondria sequences were removed from ASV tables for the prokaryotic sequence run and resulted in an average of $34,577 \pm 16,451$ reads with a minimum of 5,262 and a maximum of 78,576 (Figure 2.3A). Although archaeal sequence reads were retained, due to low detection (< 0.003% of sequence reads), the term “bacteria” is used in place of “prokaryotes”. For the eukaryotic sequence run, the average number of reads was $23,982 \pm 9,278$, with a minimum of 4,816 and a maximum of 53,960. With the exclusion of metazoan sequences, the average reads were $23,593 \pm 9,723$, with a minimum of 2,399 and a maximum of 53,881 (Figure 2.3B).

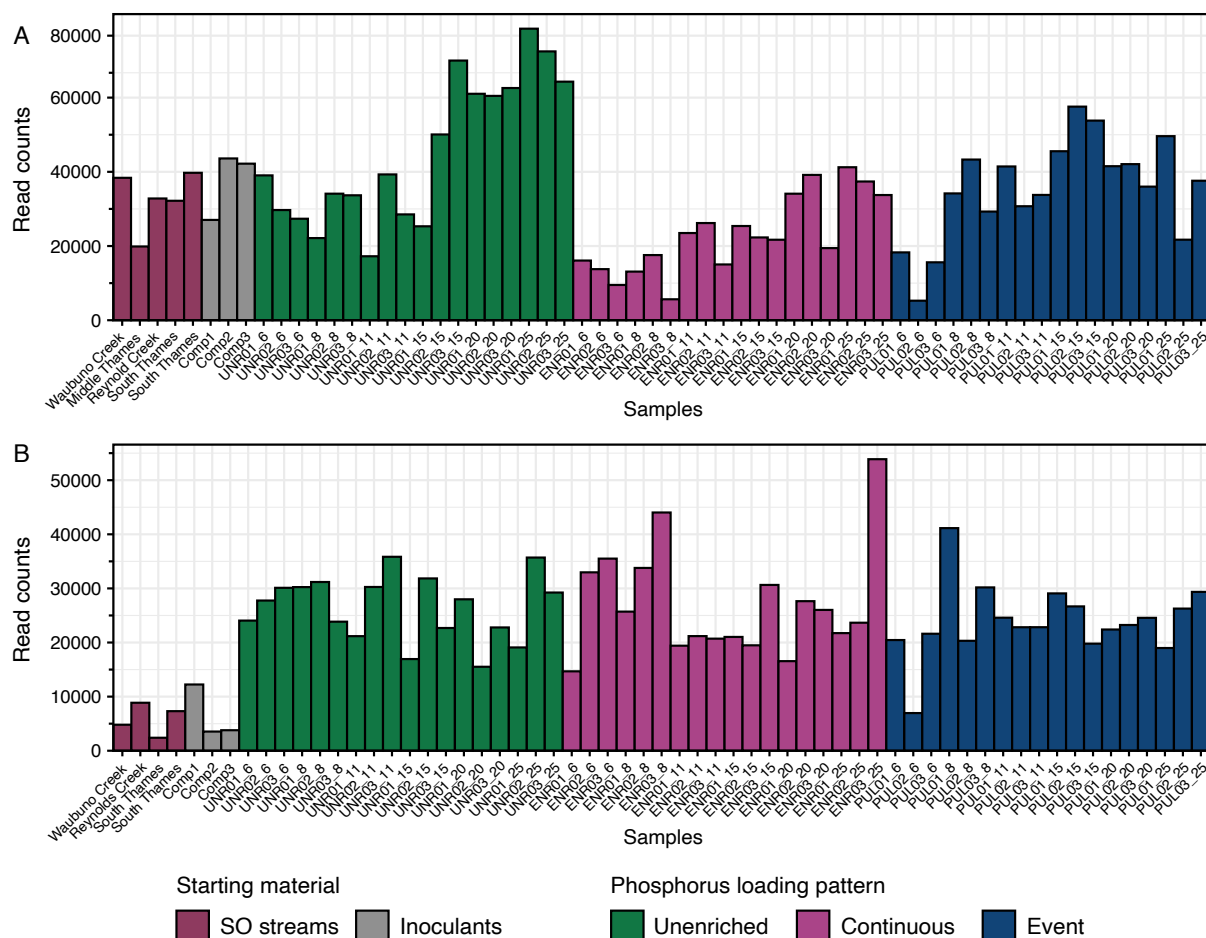


Figure 2.3 Sequence read counts for the bacterial sequence run (i.e., 16S rRNA gene) with mitochondria and chloroplast sequences removed (A) and eukaryotic sequence run (i.e., 18S rRNA gene) with metazoan sequences removed (B) across starting materials and artificial stream treatments.

2.2.4 Statistical analysis

The following analyses were all conducted in R (version 2024.04.01) and samples underwent normalization through rank subsampling (SRS) using the SRS package (version 0.2.3) (Beule and Karlovsky, 2020) prior to statistical analyses. A read depth of 5,262 and 6,960 for SRS was chosen based on the lowest read depth, with starting material (i.e., Southern Ontario streams and inoculants) excluded from statistical analysis from both bacterial and eukaryotic sequence runs.

Temporal changes in composition (i.e., beta diversity) of bacterial and eukaryotic assemblages in each P loading pattern were separately assessed using principal coordinate

analysis (PCoA) with Bray-Curtis dissimilarity in the vegan package (version 2.6.8). A one factor permutational analysis of variance (PERMANOVA) was performed to assess if assemblages differed among loading pattern for each sampling day. Homogeneity of variance was assessed across different groups within the same sampling day using vegan (Oksanen et al., 2025) and showed equal variance across all groups (Table 2.2).

Table 2.2 Summary of homogeneity of variance for bacterial (i.e., 16S rRNA gene) and eukaryotic (i.e., 18S rRNA gene) Bray-Curtis dissimilarity for each sampling day (N = 9).

Time (days)	Bacteria				Eukaryotes			
	df	SS	<i>F</i>	<i>p</i>	df	SS	<i>F</i>	<i>p</i>
6	2	<0.001	<0.1	0.98	2	0.028	1.8	0.26
8	2	0.006	0.6	0.60	2	0.034	0.9	0.51
11	2	<0.001	<0.1	0.97	2	0.028	2.7	0.15
15	2	0.009	0.4	0.74	2	0.039	1.6	0.28
20	2	0.005	2.1	0.18	2	0.010	1.3	0.35
25	2	0.002	1.2	0.36	2	0.010	2.0	0.18

If PERMANOVA indicated that loading pattern was a significant factor for a given sampling day, two additional analyses were conducted. First, a pairwise post-hoc among the three different levels (i.e., unenriched, continuous, and event) using pairwiseAdonis (version 0.4.1) to assess whether loading patterns contributed to dissimilarities among periphyton samples. Due to low sample size ($n = 3$ per group for each time point), statistical power was limited and permutation p values could not fall below 0.1. Second, a similarity percentage (SIMPER) analysis based on Bray-Curtis dissimilarity was used to identify taxa that contributed significantly to observed dissimilarities among loading patterns (Oksanen et al., 2025).

Alpha diversity (i.e., Shannon index, richness, and Pielou's evenness) of each sample was measured using the phyloseq package (version 1.46.0) (McMurdie and Holmes, 2013). A general linear model (GLM) was used to assess differences ($\alpha = 0.05$) of alpha diversity metrics between loading patterns on each day. For significant GLMs ($p \leq 0.05$), a Tukey's HSD post-hoc test was conducted to assess whether P loading patterns differed between one another using the emmeans package (version 1.10.2) (Lenth et al., 2025).

2.3 Results

2.3.1 Stream periphyton microbial community profiles and relative abundances

Stream periphyton samples that were collected from five Southern Ontario streams were used to prepare a composite for mesocosm inoculation to represent periphyton community diversity (Figure 2.4). Despite many shared taxa, profiles from these five streams varied in terms of the collections of ASVs present, and their relative abundances, for both bacteria and eukaryotes. The composite inoculant encompassed the ASV diversity of the bacterial and eukaryotic assemblages present in the periphyton of all five Southern Ontario streams (Figure 2.4). In addition, the three inoculant subsamples exhibited highly similar assemblages. Over the course of the experiment, many inoculum ASVs persisted in stream mesocosms during periphyton establishment for both bacterial (Figure 2.5) and eukaryotic taxa (Figure 2.6).

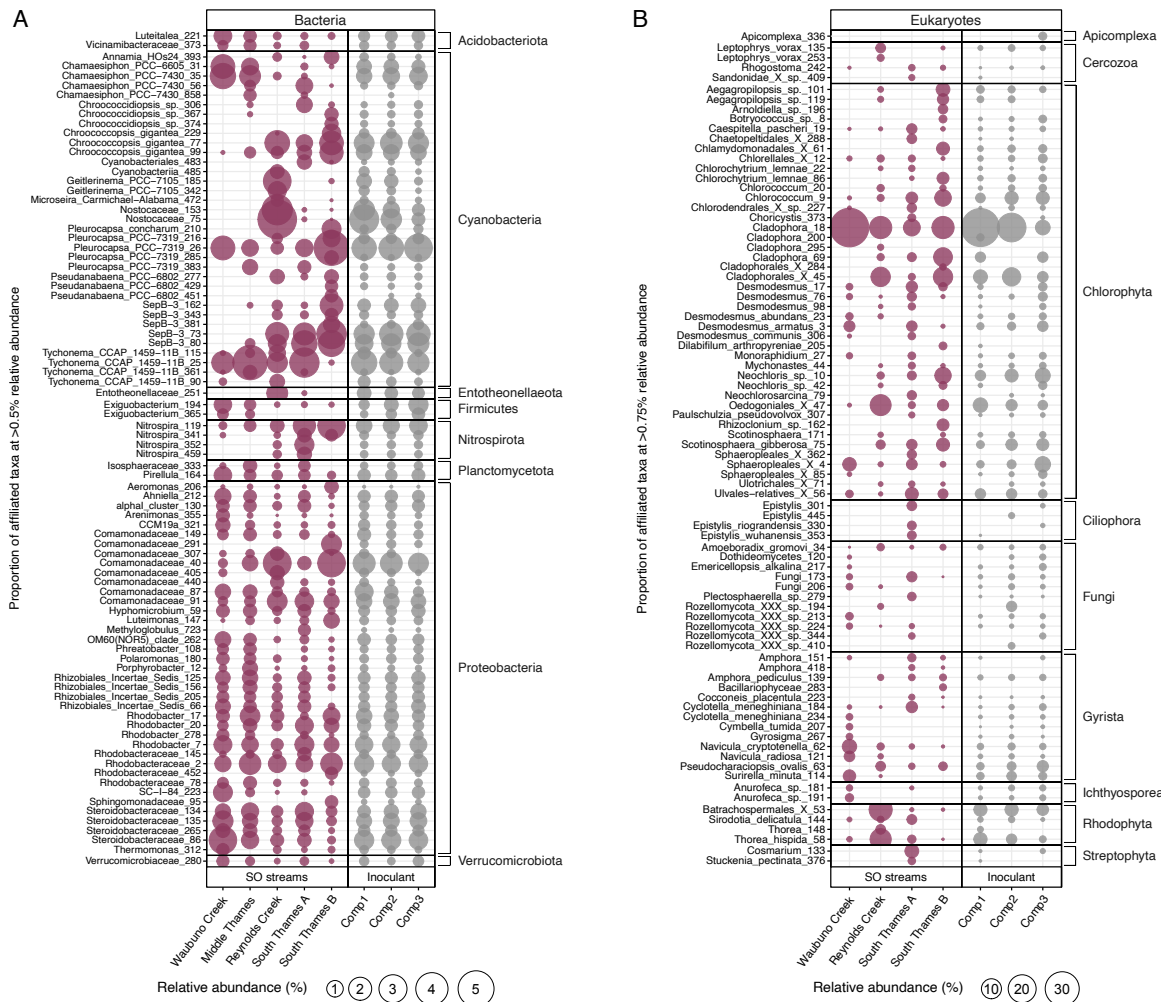


Figure 2.4 Periphyton microbial community profiles of 16S rRNA gene ASVs organized by phylum at $\geq 0.5\%$ relative abundance (A) and 18S rRNA gene ASVs organized by division and subdivision at $\geq 0.75\%$ relative abundance (B) for subsamples collected from inoculant material from Southern Ontario (SO) streams and from the composite inoculant sampled in triplicate.

Microbial community profiles demonstrated that microbial communities in artificial streams were dominated by two phyla: *Proteobacteria* (i.e., 70.4% of sequence reads) and *Cyanobacteria* (i.e., 13.1% of sequence reads) (Figure 2.5). Two ASVs (i.e., *Rhodobacteraceae_2* and *Rhodobacter_7*) were dominant in the Southern Ontario streams and inoculants and showed little to no change in relative abundance throughout all days and treatments. Additionally, ASVs affiliated with the family *Comamonadaceae* (i.e., *Comamonadaceae_4* and *Comamonadaceae_46*) were undetected in the five Southern Ontario streams and inoculants, but showed an increase in relative abundance on day 11 for both enrichment treatments, but not for the corresponding unenriched stream mesocosm samples.

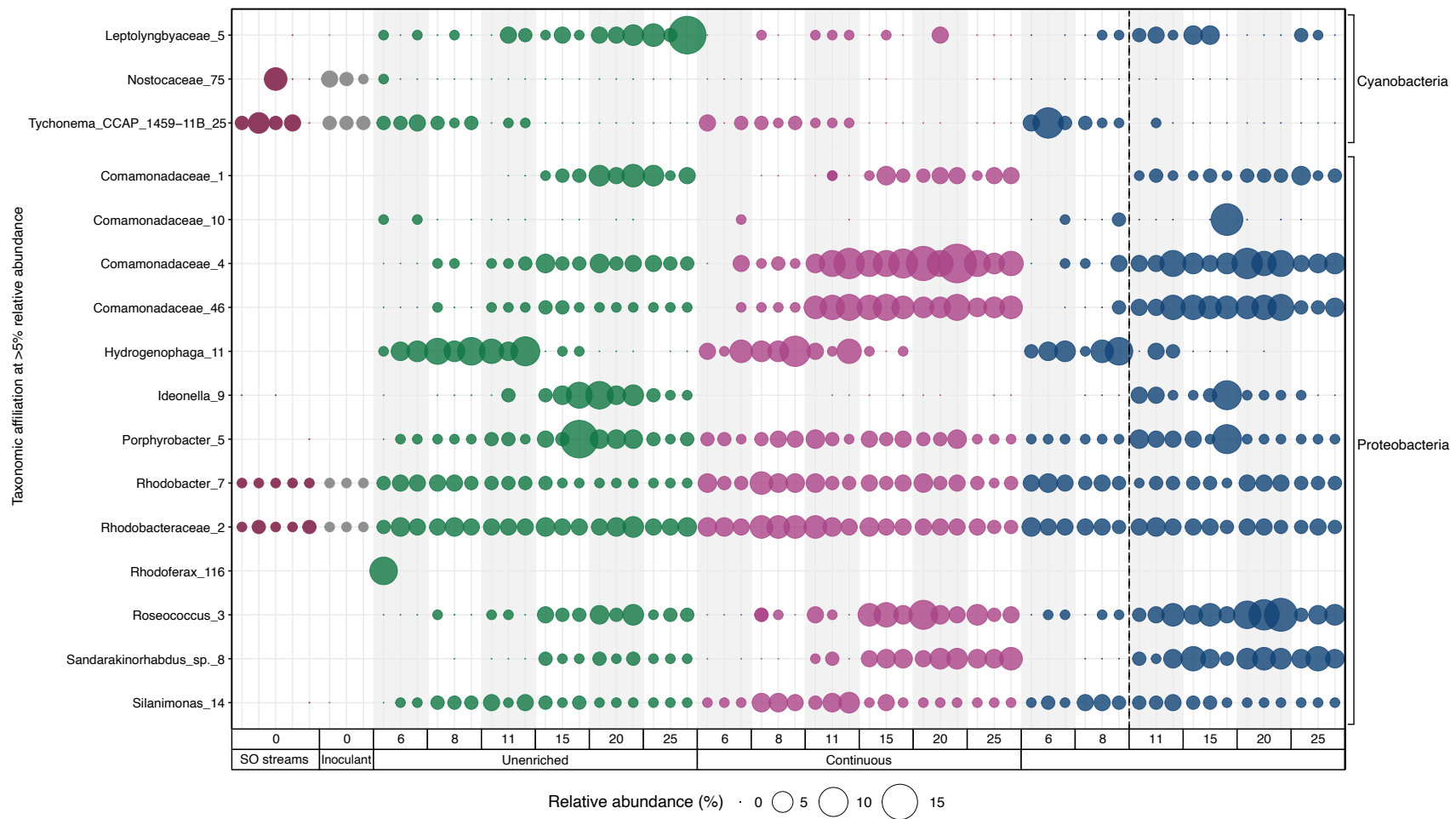


Figure 2.5 Distribution and relative abundance of 16S rRNA gene ASVs organized by phylum across Southern Ontario (SO) streams, inoculants, and artificial stream P loading treatments at $\geq 5\%$ relative abundance. Dashed line marks the short duration event that occurred between days 9 and 11.

Eukaryotic assemblages were dominated by green algae (i.e., *Chlorophyta*; 93.3% of sequence reads) in both starting materials and treatments (Figure 2.6). An ASV affiliated with the genus *Uronema* (i.e., Uronema_Chlorophyceae_2) was undetected in the Southern Ontario streams and inoculants but was dominant in the unenriched streams throughout the experiment. An ASV corresponding to the genus *Desmodesmus* (i.e., Desmodesmus_1) was detected in very low abundance in one of the Southern Ontario streams and undetected in the inoculum. However, Desmodesmus_1 had the highest relative abundance in the continuously enriched streams across all days of the experiment and after the P event (day 11) in the event-based enriched streams

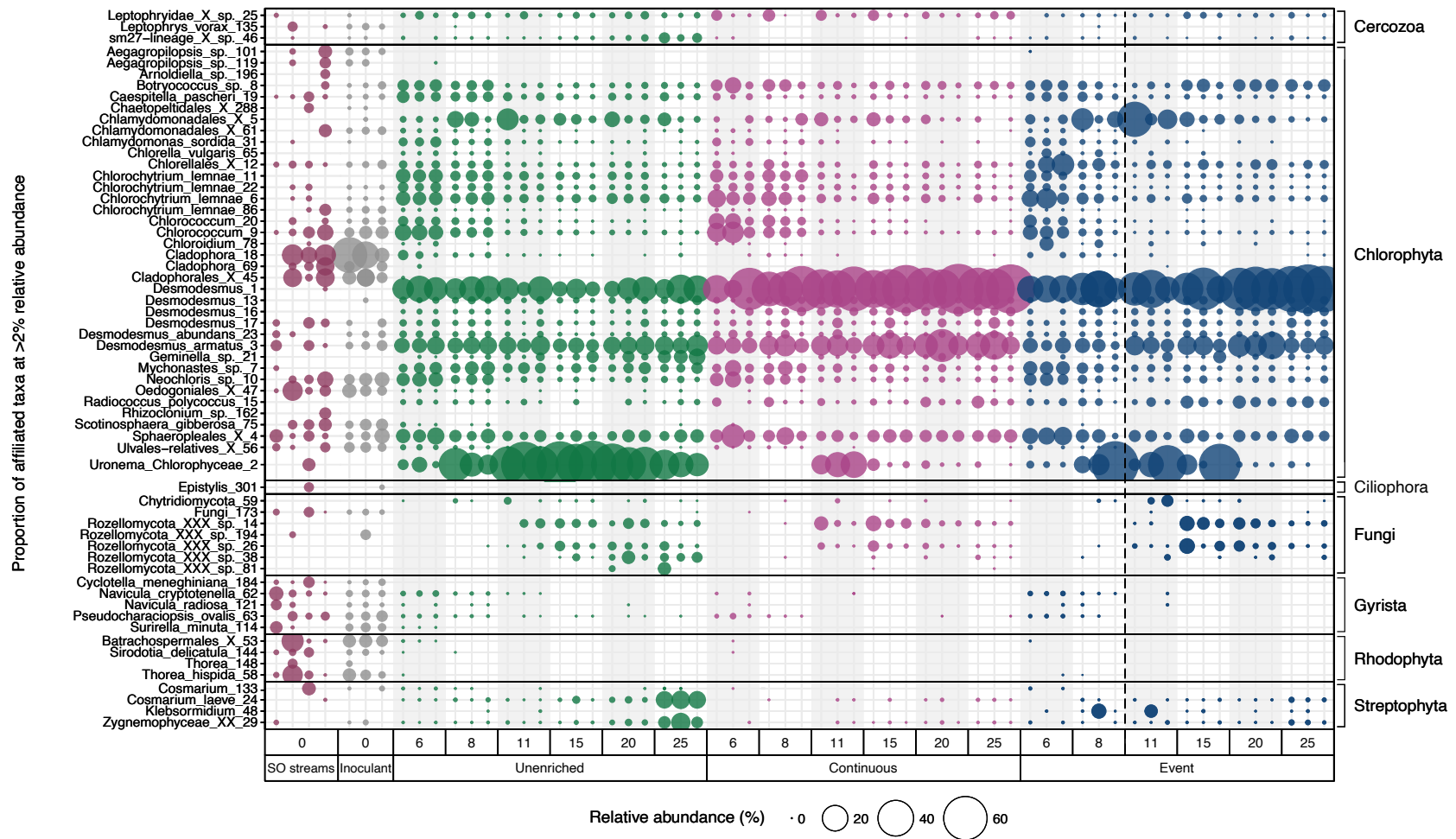


Figure 2.6 Distribution and relative abundance of 18S rRNA gene ASVs organized by division and subdivision across Southern Ontario (SO) streams, inoculants, and artificial stream P loading treatments at $\geq 2\%$ relative abundance. Dashed line marks the short duration event that occurred between days 9 and 11.

2.3.2 Beta diversity

A Bray-Curtis dissimilarity based PCoA revealed distinct periphyton communities between starting materials (i.e., Southern Ontario streams and inoculants) and artificial streams for both bacterial (Figure 2.7A) and eukaryotic (Figure 2.7B) assemblages. Indeed, the average dissimilarity between periphyton in the starting material and all treatments were 0.84 ± 0.08 for bacteria and 0.90 ± 0.06 for eukaryotes. Additionally, periphyton communities at the beginning of the experiment were most similar to the starting material compared periphyton by the end of the experiment for both bacteria and eukaryotes.

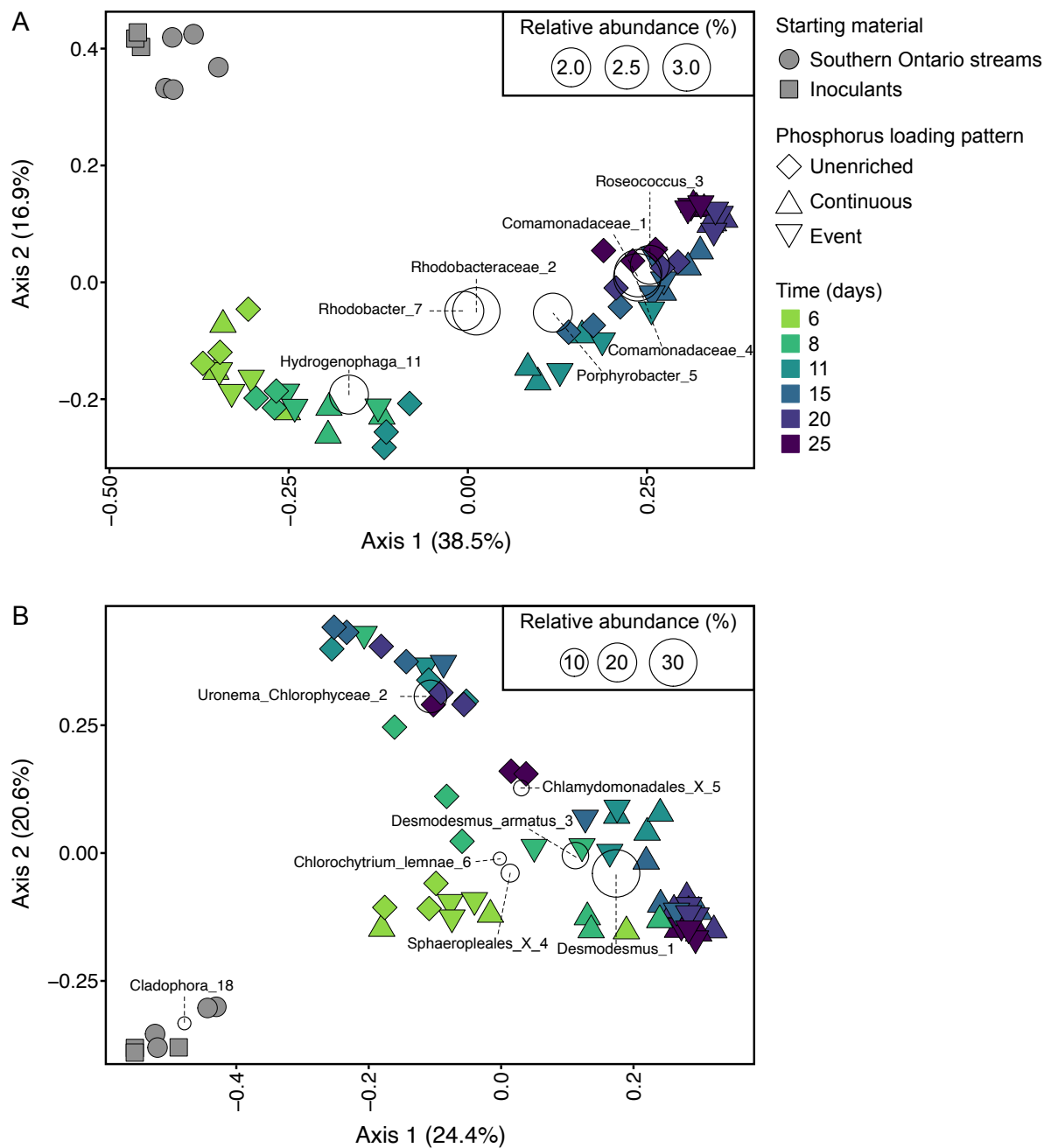


Figure 2.7 Bray-Curtis dissimilarity distance metric based principal coordinate analysis (PCoA) biplot of the 16S rRNA gene and ASVs (A) and 18S rRNA gene and ASVs (B) with $\geq 2\%$ relative abundance profiles of periphyton from Southern Ontario streams (grey circles), inoculants (grey squares), unenriched (diamonds), continuously enriched (triangles), and event-based enriched (upside down triangles) mesocosms.

An ordination based on Bray-Curtis dissimilarities among mesocosm bacterial assemblages indicated that the variation associated with axis 1 (45.0%) appeared to be with time (Figure 2.8A). Separation along the second axis (9.8%) reflected P loading patterns. In contrast, the first axis for the eukaryotic assemblages was most strongly associated with P loading pattern (31.8%) and the second axis with time (16.8%) (Figure 2.8B). Based on these ordinations, both bacterial and eukaryotic assemblages appeared to follow the same general patterns of change throughout the experiment (Figure 2.8). On the first sampling day (day 6), periphyton showed similar community profiles among all loading patterns (Table 2.3 & 2.4). Before the P event (day 8), continuously enriched periphyton diverged from unenriched and event-enriched communities. After the P event (day 11), a change was apparent for the event-based P treated communities away from the unenriched communities and towards the continuously enriched community composition. By day 25, communities were different between the unenriched streams and the enriched streams, but communities did not differ between the continuously and event-based enriched streams.

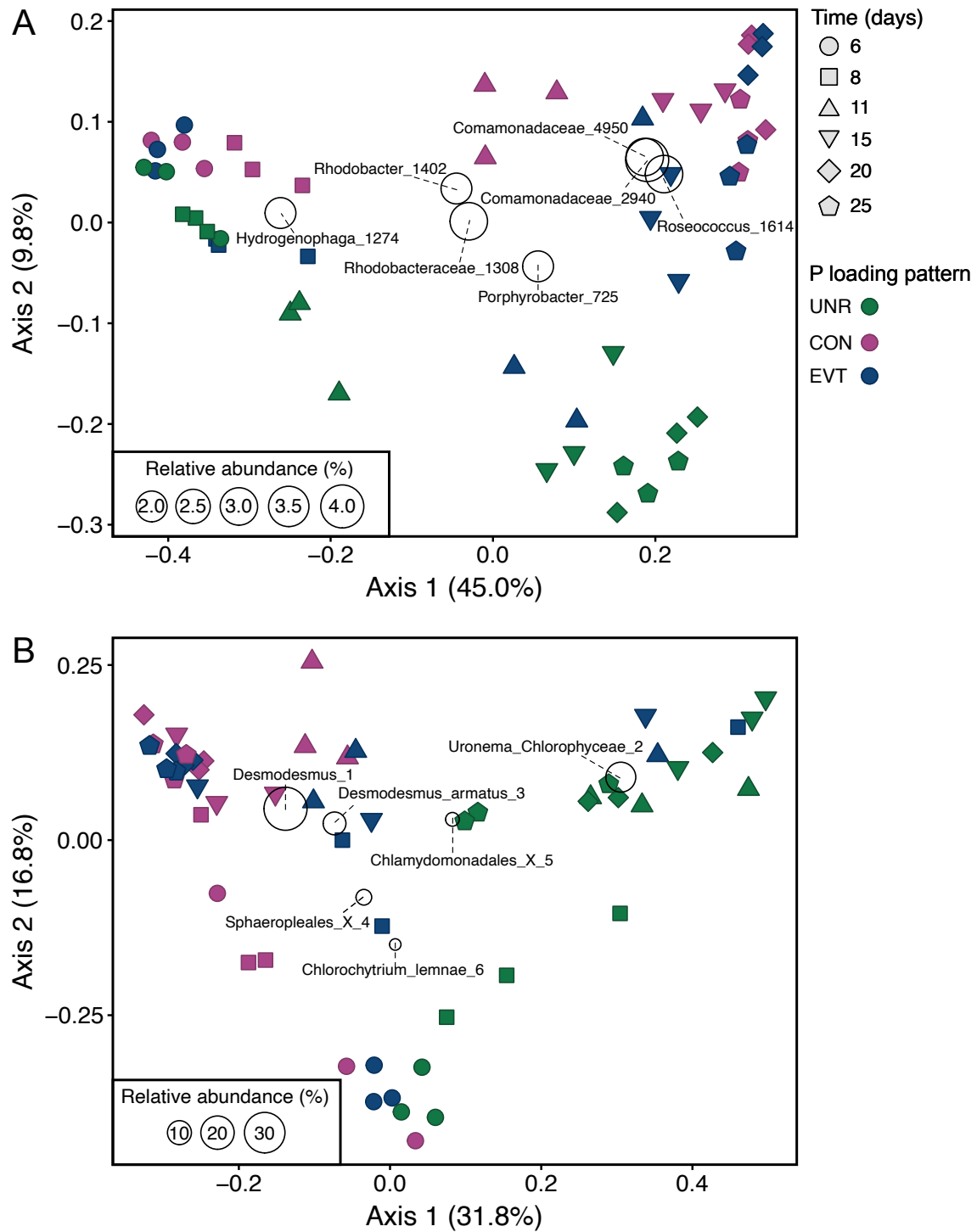


Figure 2.8 Bray-Curtis dissimilarity distance metric based principal coordinate analysis (PCoA) biplot of the 16S rRNA gene and ASVs (A) and 18S rRNA gene and ASVs (B) with $\geq 2\%$ relative abundance profiles of stream periphyton in unenriched (green), continuously enriched (pink), and event-based enriched (blue) artificial streams.

Table 2.3 Permutational Analysis of Variance (PERMANOVA) for bacterial (i.e., 16S rRNA gene) Bray Curtis dissimilarity matrix on each sampling day for three streams per loading pattern (N = 9) and pairwise post-hoc (N = 6) between loading patterns.

Time (days)	PERMANOVA					Pairwise post-hoc														
	df	SS	<i>F</i>	<i>p</i>	Unenriched vs Continuous					Unenriched vs Event					Continuous vs Event					
					df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	
6	2	0.19	1.3	0.093	1	-	-	-	-	1	-	-	-	-	1	-	-	-	-	
8	2	0.22	2.4	0.027	1	0.10	0.69	8.8	0.1	1	0.02	0.20	1.0	0.5	1	0.06	0.39	2.6	0.1	
11	2	0.38	3.2	0.007	1	0.08	0.61	6.2	0.1	1	0.07	0.56	5.1	0.1	1	0.03	0.30	1.7	0.2	
15	2	0.30	2.5	0.012	1	0.10	0.62	6.5	0.1	1	0.04	0.27	1.5	0.3	1	0.03	0.23	1.2	0.2	
20	2	0.42	6.1	0.004	1	0.19	0.81	17.0	0.1	1	0.24	0.88	28.8	0.1	1	0.02	0.36	2.3	0.1	
25	2	0.36	4.5	0.003	1	0.15	0.82	18.2	0.1	1	0.15	0.81	17.0	0.1	1	0.02	0.34	2.0	0.1	

Table 2.4 Permutational analysis of variance (PERMANOVA) for eukaryotic (i.e., 18S rRNA gene) Bray-Curtis dissimilarity matrix on each sampling day for three streams per loading pattern (N = 9) and pairwise post-hoc (N = 6) between loading patterns.

Time (days)	PERMANOVA					Pairwise post-hoc														
	df	SS	<i>F</i>	<i>p</i>	Unenriched vs Continuous					Unenriched vs Event					Continuous vs Event					
					df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	df	SS	<i>R</i> ²	<i>F</i>	<i>p</i>	
6	2	0.16	1.5	0.055	1	0.07	0.43	3.0	0.2	1	0.03	0.34	2.1	0.2	1	0.05	0.32	1.9	0.2	
8	2	0.38	2.6	0.057	1	0.11	0.64	7.0	0.1	1	0.05	0.23	1.2	0.5	1	0.06	0.29	1.6	0.2	
11	2	0.41	3.4	0.047	1	0.22	0.77	13.5	0.1	1	0.07	0.28	1.6	0.4	1	0.05	0.28	1.5	0.4	
15	2	0.78	6.7	0.012	1	0.48	0.93	57.5	0.1	1	0.21	0.51	4.1	0.1	1	0.07	0.25	1.3	0.4	
20	2	0.77	15.9	0.016	1	0.46	0.90	35.0	0.1	1	0.58	0.96	87.9	0.1	1	0.01	0.25	1.3	0.4	
25	2	0.66	14.9	0.010	1	0.43	0.89	32.8	0.1	1	0.52	0.94	61.7	0.1	1	0.02	0.34	2.0	0.3	

A SIMPER analysis indicated that the dissimilarity in bacterial assemblages across different P loading patterns was predominantly influenced by 15 ASVs (Figure 2.9). Dissimilarity among assemblages and their corresponding enrichment patterns before a P event (day 8) shared one dominant ASV. However, four days after the P event (day 15), both enrichment patterns shared five ASVs that comparably contributed to the dissimilarity of unenriched assemblages. Similarly, nine days post P event (day 20), the contribution pattern between enrichment pattern samples and unenriched mesocosm samples shifted to include three persistent ASVs from day 15 and four new ASVs. However, by the end of the experiment (day 25), two of the ASVs from day 20 remained and comparably contributed to the dissimilarity between enrichment patterns and unenriched.

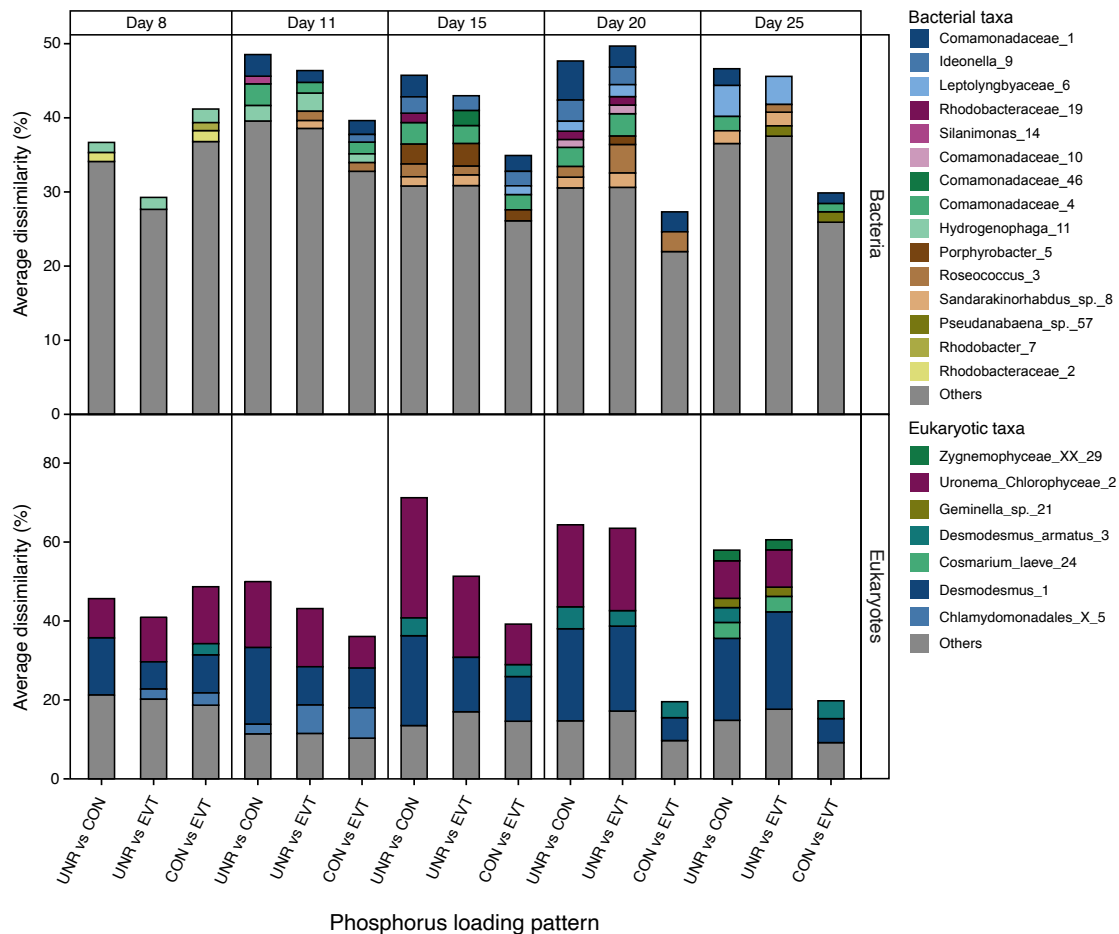


Figure 2.9 Similarity percentage (SIMPER) analysis of 16S and 18S rRNA gene profiles at $\geq 1\%$ and 2% contribution to the dissimilarity between pairwise loading patterns, respectively: unenriched (UNR) vs continuous (CON); unenriched (UNR) vs event (EVT); and continuous (CON) vs event (EVT).

The SIMPER analysis revealed dissimilarity amongst eukaryotic assemblages exposed to different P loading patterns was largely explained by changes in abundance of two green algal assigned ASVs, *Uronema_Chlorophyceae_2* and *Desmodesmus_1*. Prior to the P event (day 8), the dissimilarity of assemblages among all loading patterns varied but were driven primarily by *Uronema_Chlorophyceae_2* and *Desmodesmus_1* (Figure 2.9). However, nine days post P event (day 20), both enrichment patterns differed from unenriched assemblages due to the abundance of three green algal ASVs (i.e., *Uronema_Chlorophyceae_2*, *Desmodesmus_1*, and *Desmodesmus_armatus_3*). By the end of the experiment (day 25), with the addition of three new dominant ASVs, *Uronema_Chlorophyceae_2* and *Desmodesmus_1* remained major contributors to the differences between enriched and unenriched assemblages.

2.3.2 Alpha diversity

Using GLM analyses of Shannon index values, richness, and evenness, the results revealed significant differences with respect to P loading patterns over time (Table 2.5-2.7; Figure 2.10). For bacteria, Shannon index values differed prior to the P event (day 8), with unenriched and event-based enriched assemblages having higher Shannon indices than continuously enriched assemblages (Table 2.5). However, these values did not differ amongst any of the assemblages by the end of the experiment. In contrast, Shannon index values for eukaryotic assemblages were not significantly different between P loading patterns prior to the P event but did differ by the end of the experiment (day 25), such that unenriched assemblages had higher Shannon index values than both enriched assemblages.

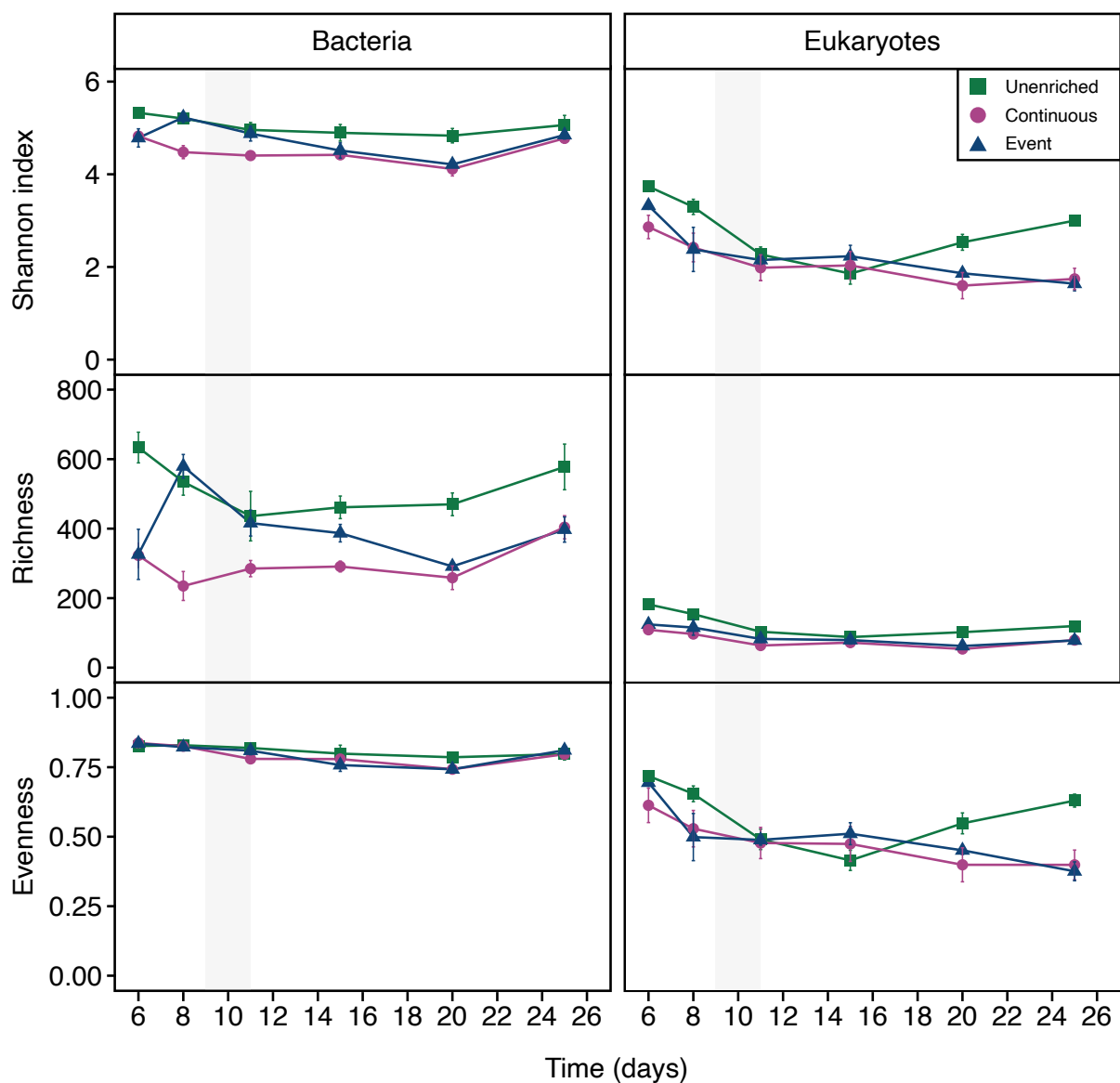


Figure 2.10 Mean Shannon index, richness, and Pielou's evenness metrics ($\pm$ standard error) for bacterial and eukaryotic members of periphyton in unenriched ($n = 3$), event-based enriched ($n = 3$), and continuously enriched ($n = 3$) P in streams ($N = 9$). Grey bars indicating the timing and duration of the event-based enrichment.

Table 2.5 Bacterial (i.e., 16S rRNA gene) and eukaryotic (i.e., 18S rRNA gene) Shannon index general linear model (GLM) containing sum of squares, *F* and *p* values (N = 9) and Tukey's post-hoc (N = 6) on each sampling day for three streams per loading pattern.

Assemblage	Time (days)	GLM			Tukey's post -hoc					
		SS	<i>F</i>	<i>p</i>	UNR vs CON		UNR vs EVT		CON vs EVT	
					<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>
Bacteria	6	0.547	5.6	0.042	2.8	0.071	3.0	0.054	0.2	0.976
	8	1.090	14.9	0.005	4.6	0.008	-0.2	0.985	-4.8	0.007
	11	0.538	4.6	0.062	-	-	-	-	-	-
	15	0.381	3.0	0.128	-	-	-	-	-	-
	20	0.911	9.3	0.014	4.0	0.017	3.4	0.032	-0.5	0.854
	25	0.134	1.1	0.389	-	-	-	-	-	-
Eukaryotes	6	1.149	7.0	0.027	3.7	0.023	1.8	0.257	-2.0	0.201
	8	1.611	2.3	0.181	-	-	-	-	-	-
	11	0.125	0.6	0.602	-	-	-	-	-	-
	15	0.211	0.5	0.611	-	-	-	-	-	-
	20	1.393	6.2	0.034	3.4	0.032	2.4	0.108	1.0	0.616
	25	3.446	22.2	0.002	5.5	0.004	6.0	0.002	0.5	0.893

Bacterial assemblages receiving unenriched and event-based enriched treatments had greater richness than assemblages under continuous enrichment before the P event (day 8; Table 2.6). In contrast, eukaryotic assemblage richness did not differ across treatments prior to the P event. By the end of the experiment (day 25), richness differed between the unenriched and both enrichment patterns by an average decrease of $30.6 \pm 0.8\%$ and $32.4 \pm 1.1\%$ for enrichment of bacteria and eukaryotes, respectively.

Table 2.6 Bacterial (i.e., 16S rRNA gene) and eukaryotic (i.e., 18S rRNA gene) richness general linear model (GLM) containing sum of squares, F and p values ($N = 9$) and Tukey's post-hoc ($N = 6$) on each sampling day for three streams per loading pattern.

Assemblage	Time (days)	GLM			Tukey's post -hoc					
		SS	F	p	UNR vs CON		UNR vs EVT		CON vs EVT	
					t	p	t	p	t	p
Bacteria	6	1.91E+05	11.2	0.009	4.1	0.015	4.1	0.015	<-0.1	0.999
	8	2.10E+05	24.0	0.001	5.5	0.004	-0.8	0.701	-6.4	0.002
	11	4.04E+04	2.9	0.133	-	-	-	-	-	-
	15	4.38E+04	11.4	0.009	4.8	0.008	2.1	0.175	-2.7	0.081
	20	7.77E+04	16.7	0.004	5.4	0.004	4.5	0.009	-0.8	0.700
	25	6.27E+04	4.7	0.060	2.6	0.091	2.7	0.080	0.1	0.995
Eukaryotes	6	8.890E+03	6.9	0.028	3.5	0.029	2.8	0.072	-0.7	0.751
	8	5.131E+03	4.6	0.061	-	-	-	-	-	-
	11	2.362E+03	4.6	0.062	-	-	-	-	-	-
	15	3.849E+02	0.5	0.638	-	-	-	-	-	-
	20	3.985E+03	21.7	0.002	6.2	0.002	5.1	0.006	-1.1	0.544
	25	3.336E+03	7.5	0.024	3.3	0.038	3.4	0.034	<0.1	0.996

Over the duration of the experiment, the evenness of bacterial assemblages was not significantly influenced by P loading pattern (Figure 2.10; Table 2.7). In contrast, eukaryotic evenness was impacted by P loading pattern toward the end of the experiment (day 25), with assemblages under both enrichment patterns having on average 38% lower evenness compared to unenriched.

Table 2.7 Bacterial (i.e., 16S rRNA gene) and eukaryotic (i.e., 18S rRNA gene) Pielou's evenness general linear model (GLM) containing sum of squares, *F* and *p* values (N = 9) and Tukey's post-hoc (N = 6) on each sampling day for three streams per loading pattern.

Assemblage	Time (days)	GLM			Tukey's post -hoc					
		SS	<i>F</i>	<i>p</i>	UNR vs CON		UNR vs EVT		CON vs EVT	
					<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>
Bacteria	6	<0.001	0.8	0.496	-	-	-	-	-	-
	8	<0.001	0.2	0.844	-	-	-	-	-	-
	11	0.003	4.1	0.076	-	-	-	-	-	-
	15	0.003	0.9	0.468	-	-	-	-	-	-
	20	0.004	4.9	0.056	2.7	0.080	2.7	0.079	<0.1	1.000
	25	<0.001	0.4	0.696	-	-	-	-	-	-
Eukaryotes	6	0.018	2.2	0.194	-	-	-	-	-	-
	8	0.041	1.7	0.266	-	-	-	-	-	-
	11	0.000	<0.1	0.970	-	-	-	-	-	-
	15	0.014	1.0	0.412	-	-	-	-	-	-
	20	0.034	3.2	0.113	-	-	-	-	-	-
	25	0.119	13.1	0.006	4.2	0.013	4.6	0.009	0.4	0.908

2.4 Discussion

Previous literature has argued that P load is a less relevant ecological metric for understanding instream eutrophication processes and associated shifts in periphyton composition, because event-driven P loads would not have sufficient residence time for biota to assimilate nutrients (Stamm et al., 2014). However, recent artificial stream studies have shown that, not only can stream periphyton take-up large quantities of P delivered in high concentration events of short duration (Pearce et al., 2023), but that periphyton can also assimilate this P to attain biomass levels comparable to chronically enriched streams (Pearce et al., 2020). Our findings further extend our understanding of the influence of event-based P on stream periphyton by providing evidence that continuous and event-based enrichments also generate similar periphyton communities, indicating that periphyton composition and diversity are regulated by the magnitude of P enrichment rather than the pattern.

2.4.1 Community composition

We hypothesized that two distinct periphyton communities would develop under different P enrichment patterns, with different taxa dominating in environments with a continuously enriched P supply versus those enriched by P events. This prediction was based on evidence that algal taxa vary in ability to perform luxury P uptake and in maximum growth rate under P enrichment (Grover, 1989; Stevenson and Stoermer, 1982). However, we found that similar periphyton communities developed under both P enrichment patterns. These results suggest that the same taxa were able to take advantage of P availability regardless of delivery patterns.

The lack of compositional differences amongst delivery patterns suggests an absence of taxa that specialize in a particular enrichment pattern. However, because our experiment only includes a single P event, it is possible that successive events could induce further shifts in composition that differentiate event and continuously enriched communities. Indeed, a previous artificial stream experiment observed compositional differences in diatom assemblages exposed to multiple P events compared to those continuously enriched in P (Pearce et al., 2020). Thus, future studies are needed to investigate the effect of successive event-based loads on the microbial community composition of stream periphyton to better elucidate the relative abilities of taxa to use event P.

Our results highlight that P enrichment can contribute significantly to diversity loss, with eukaryotic and bacterial assemblages having a comparable decline in richness under both enrichment patterns compared to unenriched assemblages. Declines in richness with enrichment was predicted because previous studies assessing changes in periphyton diversity reported decreases in richness for eukaryotes and bacteria in streams with chronic nutrient enrichment (Hagy III et al., 2020; Lear et al., 2009; Pilgrim et al., 2022). Although such studies of the impacts of continuous enrichment on periphyton richness are common, experiments isolating impacts on ecosystems receiving enrichment in events have been more limited. Thus, our finding that event-driven enrichment can reduce richness in periphyton communities extend our understanding of the impacts of nutrient enrichment on microbial diversity. In particular, the loss of diversity suggests that event-based enrichment may ultimately lead to reduced periphytic function and altered stream food web dynamics like that experienced in chronically enriched streams. However, because our study was conducted with a specific set of environmental conditions in artificial streams, future studies need to evaluate the event P to microbial richness relationship across a broader set of events (e.g., load sizes and durations) and ambient conditions (e.g., temperature, light, velocity) in real streams.

Shifts in evenness were more pronounced in eukaryotic than bacterial assemblages, with both P enrichment patterns showing comparable declines relative to unenriched assemblages. A decline in eukaryote evenness in response to enrichment was expected because past studies have reported increased dominance of a small number of algal taxa with increasing enrichment (e.g., Chételat et al., 1999; Smucker et al., 2013). In contrast, the relative abilities of bacterial taxa to use excess P loads is less well described, although others reported that abundances of autotrophic bacteria did not respond to P enrichment (Weigel et al., 2023). Our finding of no difference in evenness in unenriched and enriched streams thus provides further evidence that, unlike eukaryotic assemblages, bacterial assemblages may not have taxa that can disproportionately benefit from high P availability.

Dissimilarity among eukaryotic assemblages was mostly attributable to a shift in the most abundant green algae (i.e, *Chlorophyta*) from an ASV of *Uronema* in unenriched streams to an ASV of *Desmodesmus* in both types of enriched streams. The observed shifts in abundance align with known nutrient preferences for the broader taxonomic groups associated with these ASVs.

For example, *Desmodesmus* prefers nutrient-rich waters (Ferragut and de Campos Bicudo, 2012), exhibiting fast growth and high P uptake rates in wastewater treatment systems (Kim et al., 2015; Wang et al., 2022). Similarly, although there is limited reporting on *Uronema* as an obligate low P taxon, *Uronema* belongs to the order *Chaetophorales*, which a previous study found to have morphological adaptations that may facilitate P uptake under limiting conditions (Gibson and Whitton, 1987). The elucidation of a shift in dominant green algal taxa builds upon knowledge of the relative effects of continuous and event-based enrichment generated by a previous nutrient enrichment study at TRESS (Pearce et al., 2020).

The previous enrichment study at TRESS found that increased nutrient availability led to a succession of periphyton from early stage diatom dominance to green algal prevalence and that continuously and event-based enriched streams could be differentiated by the composition of diatoms present at the end of the experiment (Pearce et al., 2020). Diatoms are often abundant members of stream periphyton in nutrient enriched streams (Kelly and Whitton, 1995; Pringle, 1990) and diatom composition is often used to assess enrichment impacts (Lavoie et al., 2006; Potapova and Charles, 2007; Smucker et al., 2013). However, despite being common in the inoculant and unenriched treatment, evidence that the primers used were able to amplify diatom DNA, diatom-associated ASVs were either absent or at relatively low abundances within enriched treatment profiles. One possibility is that the lack of diatoms, and associated contrast with the microscopy-based results reported previously (Pearce et al., 2020), was a bias caused by our detection of multiple 18S rRNA genes from each multicellular organism present (i.e., *Desmodesmus*) relative to the contributions from unicellular organism (e.g., diatoms). We recommend incorporating diatom-specific primers (Kelly et al., 2020; Kermarrec et al., 2014; Rimet et al., 2019; Wang et al., 2024) alongside universal eukaryotic primers to better profile diatom populations in future studies using similar molecular methods.

Despite observable shifts in relative abundances of bacterial ASVs with P enrichment, the taxa most responsible for these shifts were less apparent than for eukaryotic assemblages. An ASV assigned to the genus *Roseococcus* and two ASVs affiliated with the family *Comamonadaceae* were associated with both continuously and event P enriched streams. *Roseococcus*, a phototrophic bacterium, was also observed in a previous study to have greater abundance at the latter stages of an experiment in treatments with high P and light availability

(Wagner et al., 2015). Despite an increase in relative abundance of two ASVs affiliated with *Comamonadaceae*, several *Comamonadaceae*-affiliated ASVs also showed no preference for P enrichment. Bacteria can exhibit considerable diversity amongst taxa of the same family (Hagy III et al., 2020) and a lack of ability to resolve to lower taxonomic levels can limit the ability to interpret the functional or ecological characteristics of ASVs. Currently, there is little information on the ecological preferences of most bacteria or knowledge of their interactions with eukaryotes in stream periphyton. Thus, although our findings contribute to addressing the knowledge gap regarding bacterial compositional responses to nutrient enrichment in stream ecosystems, further study is clearly needed to elucidate their mechanisms and role within enriched stream periphyton communities.

2.4.2 Implications and Applications

Our finding that P events can generate similar periphyton assemblages as observed previously for continuous enrichments suggest that current monitoring of P in stream water may not accurately reflect composition and diversity of periphyton assemblages. Instream P enrichment is often monitored through tracking baseflow concentrations on a weekly to monthly basis. For example, the Ontario Provincial Water Quality Monitoring Network collects stream water data on a monthly basis (Kaltenecker, 2023) and the National Water-Quality Assessment Program of the U.S. Geological Survey on a weekly to monthly basis (Gilliom et al., 1995). Such dispersed measurements of instream P concentration may fail to account for short duration event-based P loads due to infrequent sampling and ultimately underestimate the amount of P delivered to streams. Consequently, periodic water monitoring systems may be insufficient to target mitigation activities to limit stream eutrophication in agricultural and urban landscapes. Moreover, our findings emphasize the importance of using biomonitoring endpoints such as periphyton that integrate temporal variation in P loads.

Our research demonstrates that eukaryotic and bacterial assemblages have potential to be used for assessment of P enrichment of stream ecosystems. Indeed, both enrichment patterns resulted in bacterial and eukaryotic assemblages that were statistically distinct from unenriched assemblages. Strong deviation between assemblages at reference (i.e., unimpacted) ecosystems and impacted ecosystems is an important criterion for assemblages to be suitable for bioassessment because nearly all current programs use the reference condition approach where

biota at a potentially impacted site are compared to assemblages from reference sites (Reece and Richardson, 1999; Reynoldson et al., 1997). Although our findings come from artificial streams, the indicated potential of eukaryotic and bacterial assemblages to serve as measures of stream condition are consistent with a growing number of studies (Gad et al., 2024; Simonin et al., 2019; Wang et al., 2023). However, our study found a potential redundancy in studying both bacterial and eukaryotic assemblages given that both exhibited similar ecological responses to P enrichment when compared to reference conditions. It is thus recommended that field studies be undertaken to validate the relative sensitivity of these communities to different patterns and amounts of P enrichment in real streams.

2.5 Conclusion

This study expands on existing knowledge of P enrichment in stream ecosystems to build upon the argument that load, rather than load pattern, governs the composition of bacterial and eukaryotic assemblages in stream periphyton. Thus, management of P enrichment should focus on targeting the most efficient way to reduce P loads, regardless of source. In addition, our study offers evidence that microbial community composition can be a valuable metric for broad P enrichment assessments in stream ecosystems, but not the source of enrichment. Lastly, comparable compositional responses to sources of enrichment reveal a shortcoming in current monitoring practices, which may not effectively account for event-induced P enrichment. Due to the observed sensitivity of microbial communities to anthropogenic associated pressures, it is recommended that future research on the effect of P events in stream ecosystems incorporate genomic tools in real streams.

Chapter 3: Microbial community composition of stream periphyton under low or high antecedent P conditions exposed to event-based P enrichment

3.1 Introduction

Runoff resulting from storm events serves as a major transporter of excess P from anthropogenic landscapes (e.g., agriculture, residential areas, golf courses) to nearby aquatic ecosystems (Simonin et al., 2019). These P enrichment events are typically of short duration (i.e., hours to days) and vary in concentration depending on the amount of runoff and transportable P within the catchment (Carpenter et al., 1998; Macrae et al., 2007). The biological relevance of storm runoff events to stream periphyton lies in their ability to uptake and assimilate P into their biomass (Pearce et al., 2020; Weigelhofer et al., 2018). Previous research indicated that stream periphyton are able to uptake P logistically proportional to the magnitude of event P one day after the event, with an upper limit of $400 \mu\text{g L}^{-1}$ SRP (Pearce et al., 2023), yet biomass had a linear relationship 10 days after the event. Thus, there is evidence that dynamic P events can be leveraged by stream periphyton to support growth. However, research on the response of community composition with P events remains limited.

Periphyton communities exhibit compositional shifts with increasing amounts of P enrichment (Biggs, 1990; Smucker et al., 2013). In contrast, direct studies of the effects of enrichment from P events is limited to an artificial stream study by Pearce et al. (2020). They found that stream periphyton exhibited a shift from diatom dominated algal assemblages to assemblages dominated by green algae (i.e., *Chlorophyta*) when exposed to either event-based or continuous P enrichment. This previous study thus demonstrated that stream periphyton are not only able to use event P but are also influenced compositionally by events. However, they only assessed a single large P event concentration and thus it is unknown how the size of an event would influence periphyton composition.

In addition to the size of the event, the amount of antecedent P in a stream could modulate the impact of a P event on periphyton composition. Antecedent P refers to the background concentration of P available in a stream prior to a storm event. In streams, background levels of P can be highly variable spatially and temporally, depending on natural processes and anthropogenic sources within the catchment (Dillon and Kirchner, 1975; Withers and Jarvie, 2008). Naturally, P can enter streams through weathering and erosion of rocks and soils,

atmospheric deposition, riparian vegetation, decomposition of organic matter in which P is released, and groundwater inputs (Holtan et al., 1988; Withers and Jarvie, 2008). In most cases, P concentrations typically remain low in streams within undisturbed catchments due to little P release from natural processes (Binkley et al., 2004; Clark et al., 2000; Withers and Jarvie, 2008). However, groundwater, which often varies in P concentration due to anthropogenic land uses (e.g., residential septic systems), can deliver P in high concentration when contaminated (Mechtensimer and Toor, 2017). Point sources, such as wastewater treatment plants, can also lead to chronically enriched P conditions (Akinawo, 2023). Because chronically enriched streams present altered algal communities compared to unimpacted streams, it is predicted that higher antecedent P may inhibit periphyton communities from compositionally responding to additional P from events.

The first objective of this study was to assess how stream periphyton microbial community composition responds along an increasing gradient of P event concentrations. The second objective was to determine whether antecedent conditions regulate how stream periphyton microbial communities respond to increasing P event concentrations. To accomplish these objectives, two artificial stream experiments were conducted under low ($10 \mu\text{g L}^{-1}$ SRP) or high ($50 \mu\text{g L}^{-1}$ SRP) antecedent P with nine different P event concentrations. High-throughput sequencing of 16S and 18S rRNA genes were performed to characterize bacterial and eukaryotic assemblages, respectively.

3.2 Materials and methods

3.2.1 Experimental design

Two 26-day artificial stream experiments were conducted at TRESS during the summers of 2018 (July 26 – August 21) and 2020 (July 31 – August 25) to test periphytic responses to P event size under low ($10 \mu\text{g L}^{-1}$ SRP) and high ($50 \mu\text{g L}^{-1}$ SRP) antecedent P conditions, respectively. For both experiments, nine artificial streams were randomly assigned a P event concentration of 15, 40, 90, 140, 190, 240, 290, 390, or $690 \mu\text{g L}^{-1}$ SRP by which the antecedent concentration was increased over a 48-hour period (Figure 3.1). Both antecedent and event concentrations were based on observed baseflow and storm-event concentrations reported for Southern Ontario streams (DeBues et al., 2019; Raney and Eimers, 2014; Stevenson et al., 2008; Williams et al., 2018).

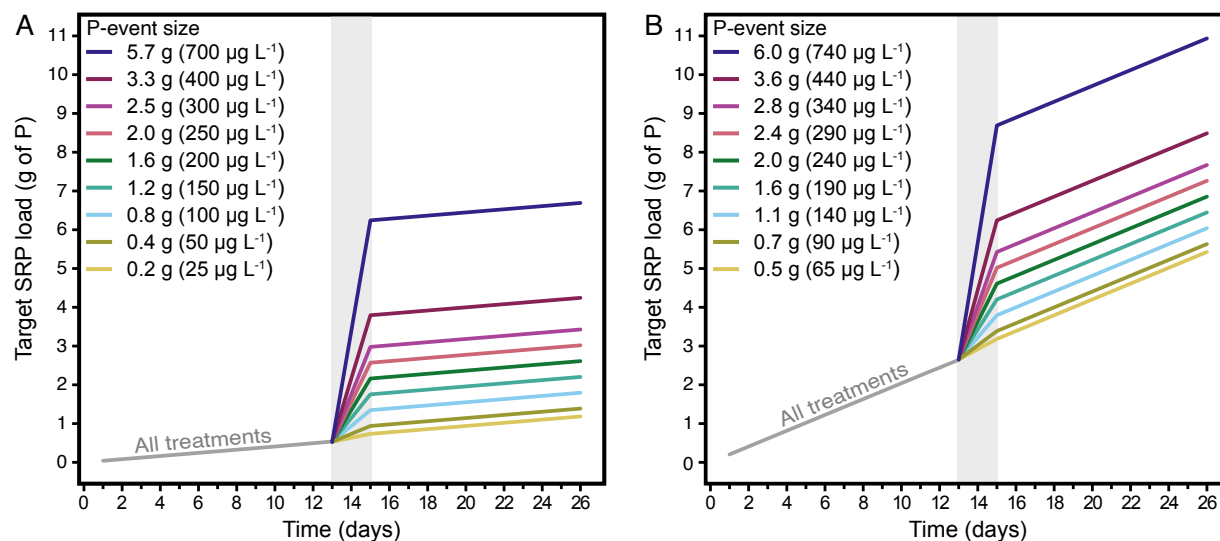


Figure 3.1 Target cumulative SRP load with an increasing gradient of P events under low antecedent P (A) and high antecedent P (B) conditions in artificial streams over 26 days. The grey bar indicates the timing and duration of the 48-hour P event.

3.2.2 Experimental setup

The loading of nutrients, dosing calibrations, SRP and N measurements, control for physiochemical factors (i.e., temperature, light availability, and velocity), and periphyton collection for inoculation followed methods described in Chapter 2, except for two differences. First, the addition of cobble substrates into artificial streams along with unglazed ceramic tiles. Both experiments placed quarried river rock in plastic mesh bags (2 x 2 cm) into Medway Creek (TP = $20 \mu\text{g L}^{-1}$ and TN = $3300 \mu\text{g L}^{-1}$) for four weeks prior to transferring them into artificial streams (Figure 3.2). Second, periphyton collection where inoculants were obtained from scraping 5-10 randomly selected cobbles from the five regional streams as described in Chapter 2 (Table 2.1). The average degree days associated with all artificial streams for low and high antecedent P experiments were $533.3 \pm 0.6 ^\circ\text{C}$ and $560.1 \pm 7.1 ^\circ\text{C}$, respectively. The average sum of PAR for low antecedent P was $546.5 \pm 7.4 \text{ mole m}^{-2}$; however, PAR for the high antecedent experiment was not collected due to broken PAR loggers. Additionally, due to a duplex pump failure, the high antecedent P experiment was missing an event size of $240 \mu\text{g L}^{-1}$ SRP (cumulatively $290 \mu\text{g L}^{-1}$ SRP).

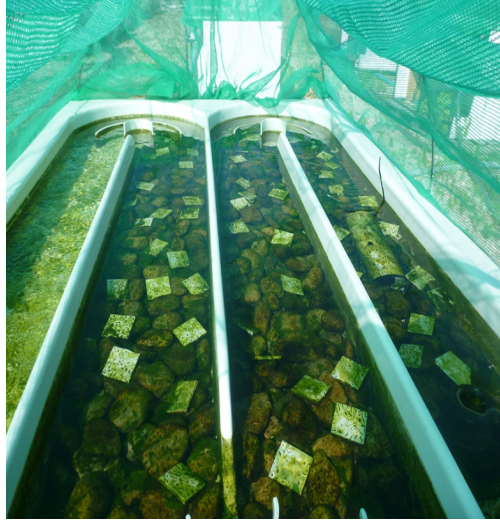


Figure 3.2 View of artificial stream halfway through the experiment with quarried river rock and tiles.

3.2.3 Sample collection

Periphyton were sampled from tiles on days 12 (one day before event) and 26 (end of experiment) for both low ($N = 18$) and high ($N = 16$) antecedent experiments. Sample collection and storage followed previously described methods in Chapter 2.

3.2.4 DNA extractions, sequencing, and analysis

DNA extractions, sequencing, and sequence analysis followed described methods in Chapter 2. Both low and high antecedent P experimental samples were processed in the same sequence run. The V4-V5 run had a CD of 737K and 86.3% Q30 and the V4 run had a CD of 682K and 86.9% Q30. Samples P25_D12 from the V4-V5 run and P140_D12, P300_D12, P340_D12, P400_D12, and P700_D12 from the V4 run failed during sequencing. Additionally, for the V4-V5 run, there was an average of $33,388 \pm 13,425$ reads, with a minimum of 3,366 and a maximum of 64,732. With the exclusion of chloroplast and mitochondrial sequences, the V4-V5 run had an average of $17,032 \pm 10,682$ reads, with a minimum of 1,959 and a maximum of 57,162 (Figure 3.3A). As archaeal sequence reads were undetected, the term “bacteria” is used in place of “prokaryotes”. For the V4 run, the average number of reads was $8,748 \pm 2,805$ with a minimum of 5,973 and a maximum of 17,891. With the exclusion of metazoan sequences, the V4 run had a minimum of 2,886 reads and a maximum of 17,140, and an average of $7,519 \pm 3,178$ (Figure 3.3B).

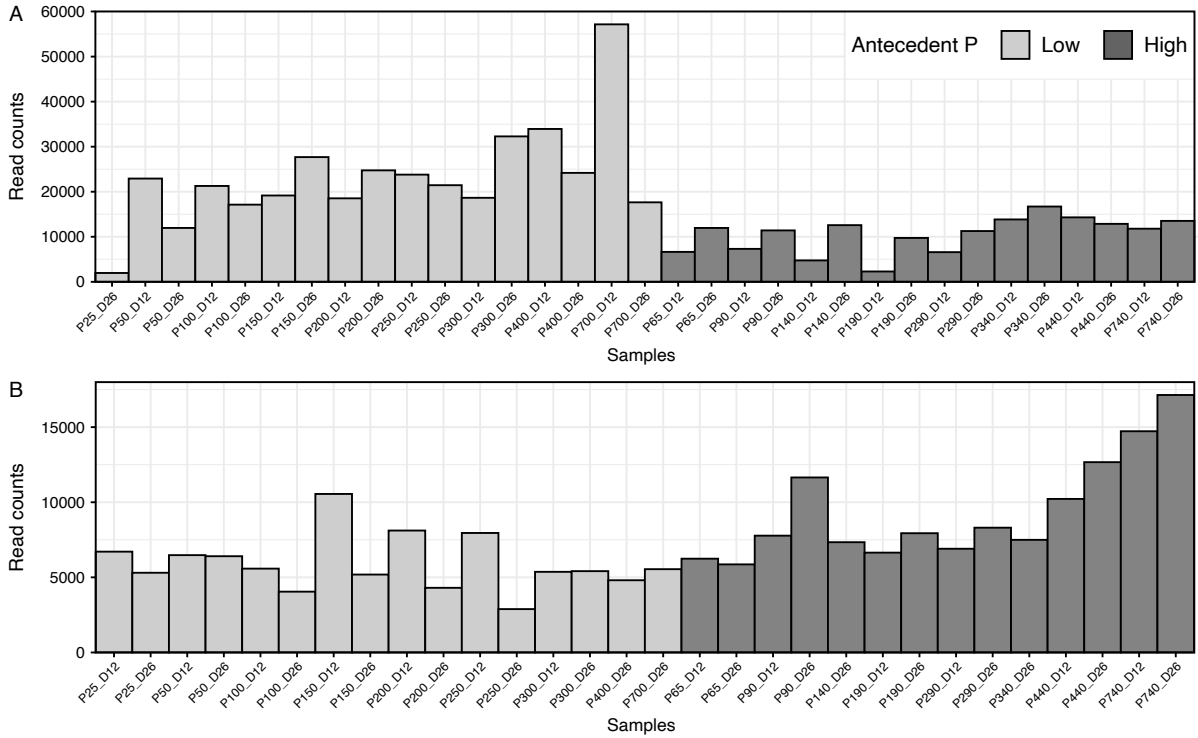


Figure 3.3 Sequence read counts for the bacterial sequence run (i.e., 16S rRNA gene) with mitochondria and chloroplast sequences removed (A) and eukaryotic sequence run (i.e., 18S rRNA gene) with metazoan sequences removed (B) under low and high antecedent P.

3.2.5 Statistical analysis

All analyses were conducted in R (version 2024.04.01). Prior to analyses, reads from all samples underwent normalization through rank subsampling (SRS) using the SRS package (version 0.2.3; Beule et al., 2022; Beule and Karlovsky, 2020). Low antecedent P conditions had a read depth of 1,959 and 2,886 for bacterial and eukaryotic sequence runs, respectively, whereas the high antecedent P conditions had a read depth of 2,290 for bacteria and 5,863 for eukaryotes.

Ordinations based on Bray-Curtis dissimilarity were used to separately assess changes in bacterial and eukaryotic assemblages with an increasing P event gradient for low and high antecedent conditions using vegan (version 2.6.8) (Oksanen et al., 2025). To assess if P event size was associated with community composition for each antecedent condition, the PCoA ordinations were generated to include only samples after a P event (i.e., day 26) and P event size was fitted onto ordination space using the envfit function in vegan (version 2.6.8).

Alpha diversity metrics (i.e., Shannon index, richness, and Pielou's evenness) were calculated as described in Chapter 2. Metrics were calculated using read counts normalized with SRS depths of 1,959 and 2,886 for bacteria and eukaryotes, respectively. Associations between P event concentration and alpha diversity metrics were assessed using general linear models to generate null and linear models. Models were compared using Akaike's information criterion corrected for small sample size (AICc) using the AICcmodavg package (version 2.3.3). Models were selected based on AICc differences, with those having $\Delta\text{AICc} > 2$ considered to have weak support. To evaluate the strength of evidence for each model, log-likelihoods and AIC weights (AICw) were calculated.

3.3 Results

3.3.1 Microbial community profiles and relative abundances

Microbial community profiles of bacteria under low P antecedent conditions demonstrated that the three most abundant phyla were *Proteobacteria* (48.4%), *Cyanobacteria* (21.5%), and *Bacteroidota* (13.0%) (Figure 3.4). On average there were between 4-6 and 2-4 ASVs comprising above 2% of the total relative abundance in each stream on days 12 and 26, respectively. Four ASVs (i.e., Comamonadaceae_2, Burkholderiales_3, Leptolyngbyaceae_4, and Leptolyngbyaceae_5) had a greater than 20% decrease in relative abundance on average across all periphytic assemblages after the P event. There was also on average a 69% increase in the relative abundance of *Pseudanabaena_PCC_7429_1* across all P event sizes at the end of the experiment, compared to before the event.

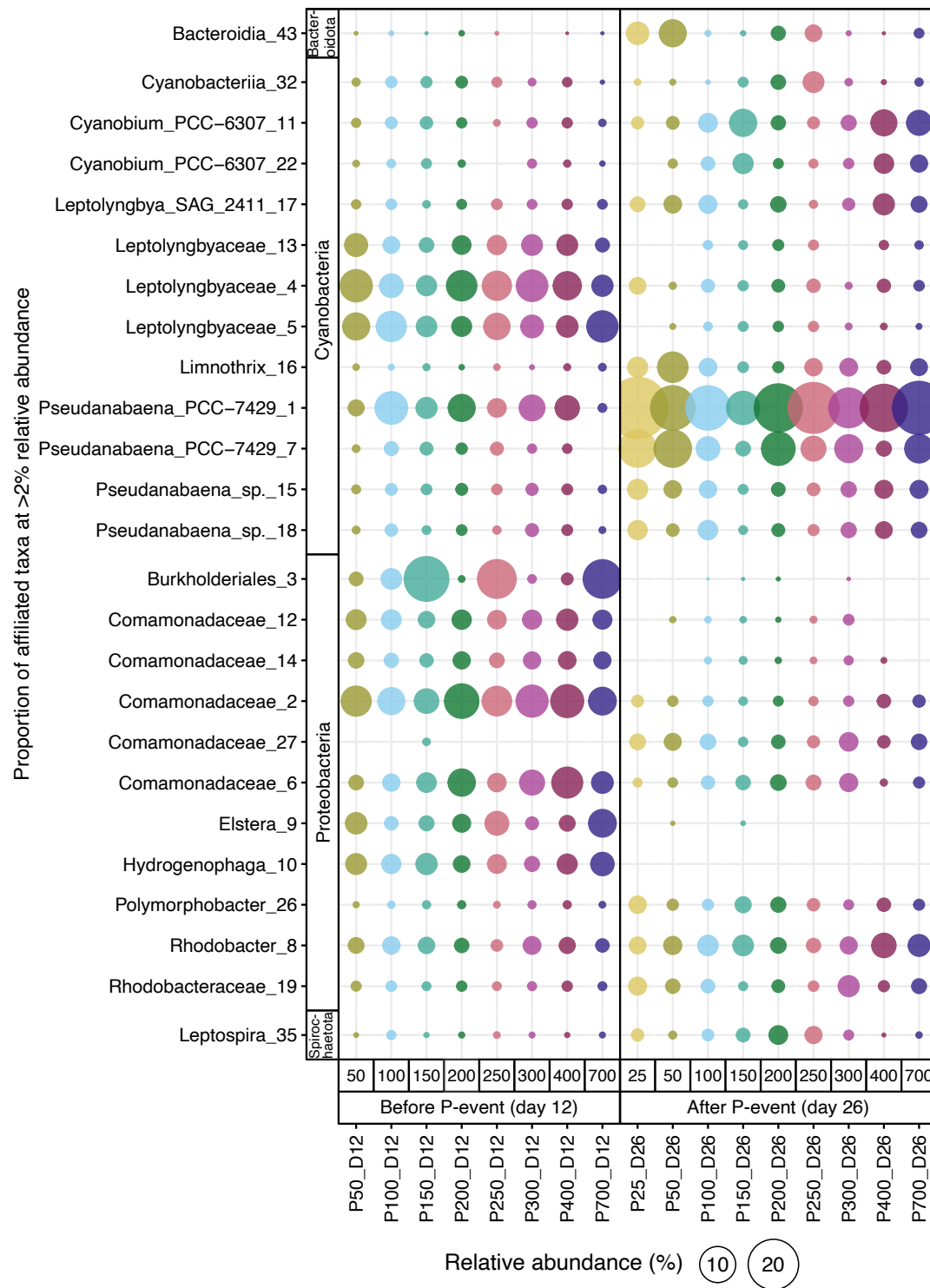


Figure 3.4 Distribution and relative abundance of 16S rRNA gene ASVs organized by phyla before (day 12) and after (day 26) P events at low antecedent P ($10 \mu\text{g L}^{-1}$) shown at $\geq 2\%$ relative abundance.

The most abundant taxa in the eukaryotic assemblages were *Chlorophyta*, *Streptophyta*, and *Gyrysta* contributing to 45.3%, 17.9%, and 16.9% of all sequence reads, respectively (Figure 3.5). Before events (day 12) and by the end of the experiment (day 26), there were between 9-11 and 11-15 ASVs at greater than 2% relative abundance in assemblages, respectively. Two ASVs (i.e., Uronema_Chlorophyceae_2 and Leptophrys_vorax_6) had an average decrease in relative abundance of greater than 10% after P events, whereas Geminella_sp_3 increased in relative abundance by 10%.

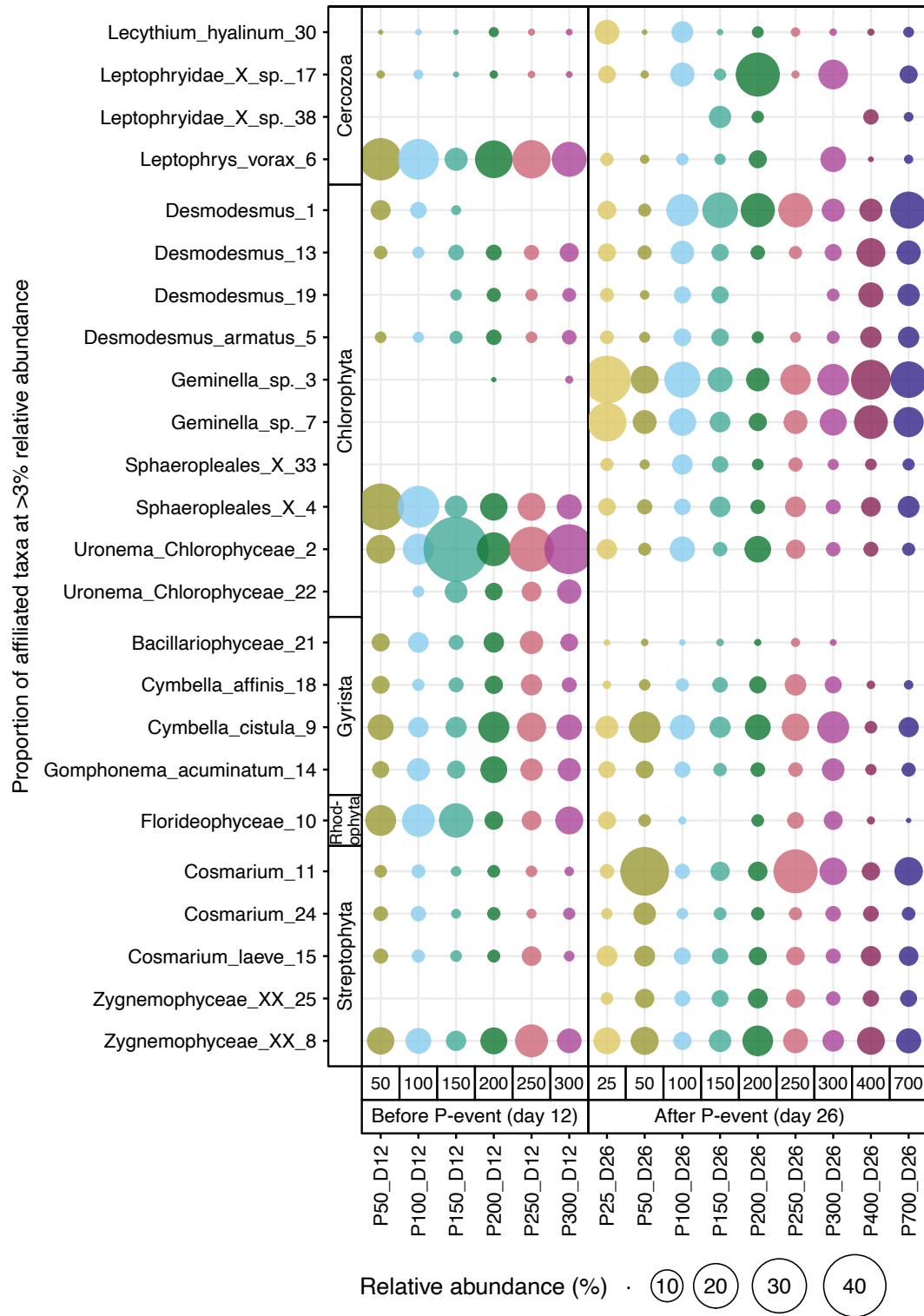


Figure 3.5 Distribution and relative abundance of 18S rRNA gene ASVs organized by phyla before (day 12) and after (day 26) P events at low antecedent P ($10 \mu\text{g L}^{-1}$) shown at $\geq 2\%$ relative abundance.

For high antecedent P conditions, *Proteobacteria*, *Bacteroidota*, and *Cyanobacteria* were the most abundant phyla before and after a P event and represented 48.1%, 16.2%, and 14.7% of all reads, respectively (Figure 3.6). An average of between 7-12 and 5-8 ASVs were above a 2% relative abundance threshold within assemblages on days 12 and 26, respectively. All ASVs maintained a relatively stable relative abundance before and after P events, with the average change in relative abundance no greater than 4% except for an average decrease of 7.8% for Comamonadaceae_3 across all events. Assemblages showed no clear shift in relative abundance across the increasing P event gradient, with all ASV relative abundances remaining relatively consistent regardless of event size.

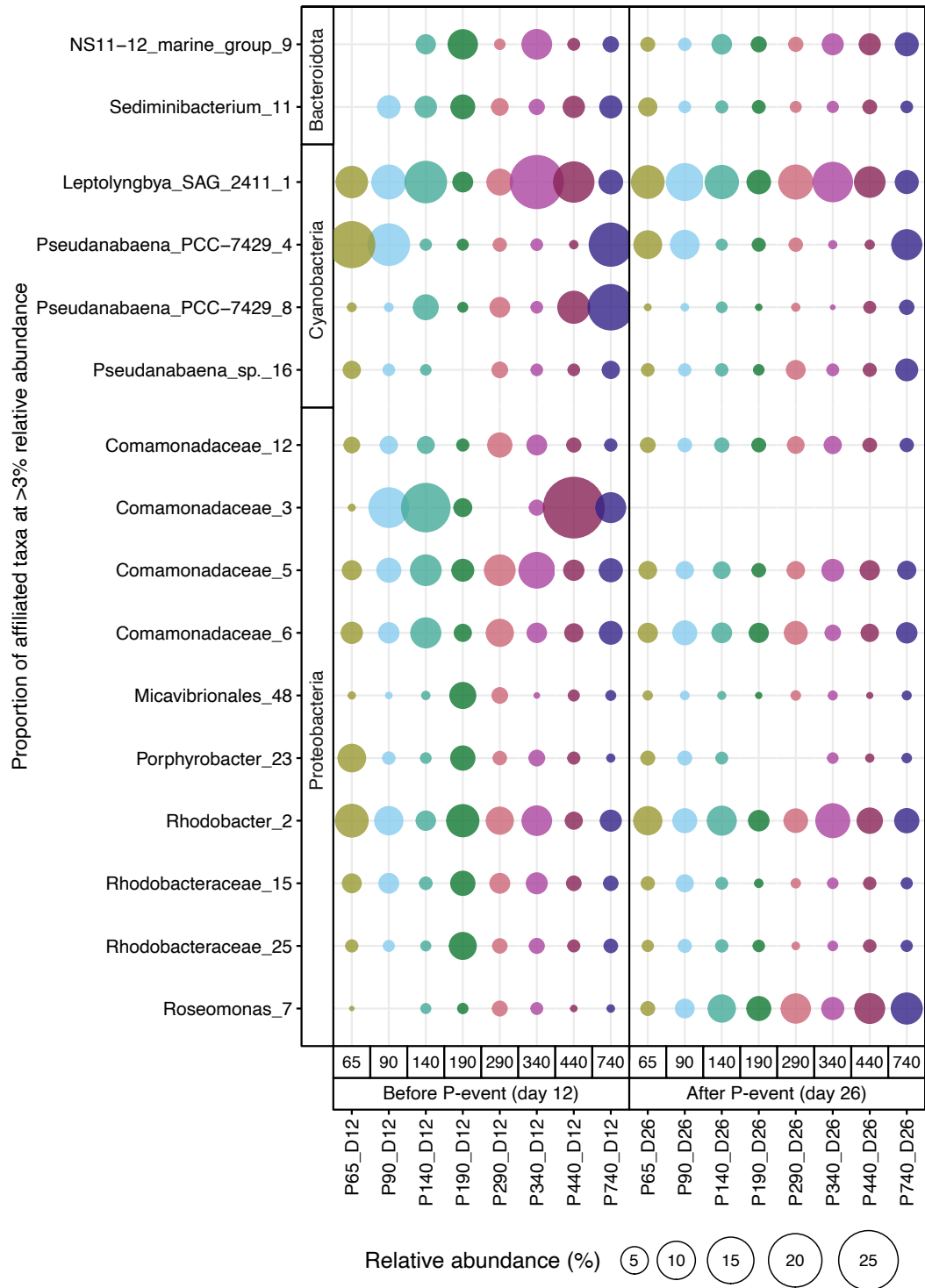


Figure 3.6 Distribution and relative abundance of 16S rRNA gene ASVs organized by phyla before (day 12) and after (day 26) P events at high antecedent P ($50 \mu\text{g L}^{-1}$) shown at $\geq 3\%$ relative abundance.

Eukaryotic assemblages under high antecedent P conditions were dominated (96.6% of sequence reads) by green algae (i.e., *Chlorophyta*) before and after the P event (Figure 3.7). Of the green algae, an ASV associated to the genus, *Desmodesmus* (i.e., *Desmodesmus_1*), comprised 73% of total sequence reads before and 10 days after the P event.

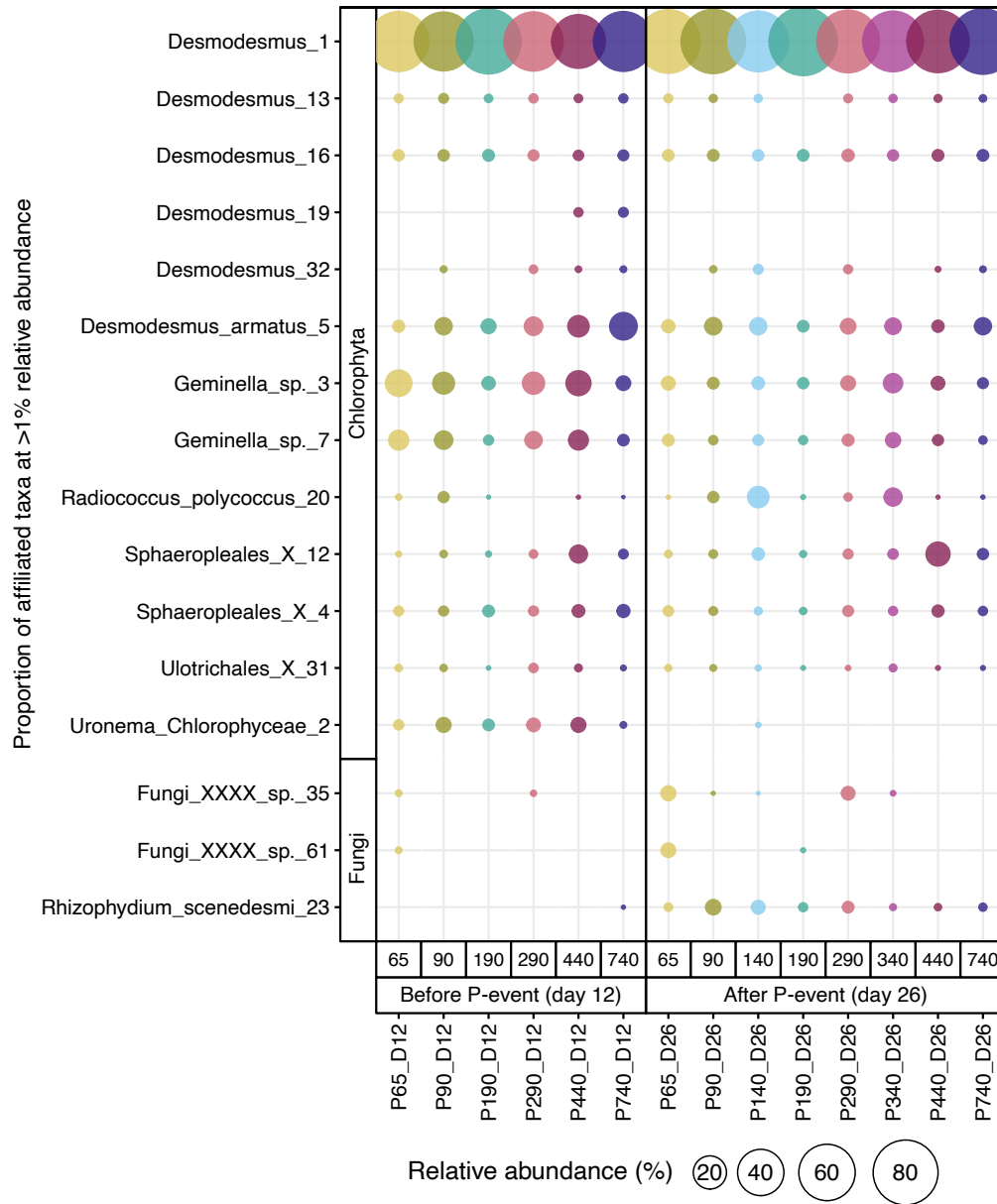


Figure 3.7 Distribution and relative abundance of 18S rRNA gene ASVs organized by phyla before (day 12) and after (day 26) P events at high antecedent P ($50 \mu\text{g L}^{-1}$) shown at $\geq 1\%$ relative abundance.

3.3.2 Beta diversity

A PCoA based on Bray-Curtis dissimilarity of the low antecedent P treated bacterial assemblage in axis 1 was associated with time (Figure 3.8A). Leptolyngbyaceae_4 and Comamonadaceae_2 were associated with before events (day 12); whereas Pseudanabaena_PCC-7429_1 and Pseudanabaena_PCC-7429_7 were affiliated with after events (day 26). Axis 2 separation showed no evidence that change in community composition was in relation to P event size ($R^2 = 0.06$, $p = 0.79$). Additionally, between communities of the same stream before and after an event, there was an average dissimilarity of 0.35 ± 0.03 . Similarly, axis 1 of the eukaryotic assemblage PCoA was linked to time (Figure 3.8B). Axis 2 of the eukaryotic assemblage likewise showed no correlation of P event size and community composition ($R^2 = 0.12$, $p = 0.70$). Average eukaryotic dissimilarity between the same streams before and after an event was 0.67 ± 0.05 .

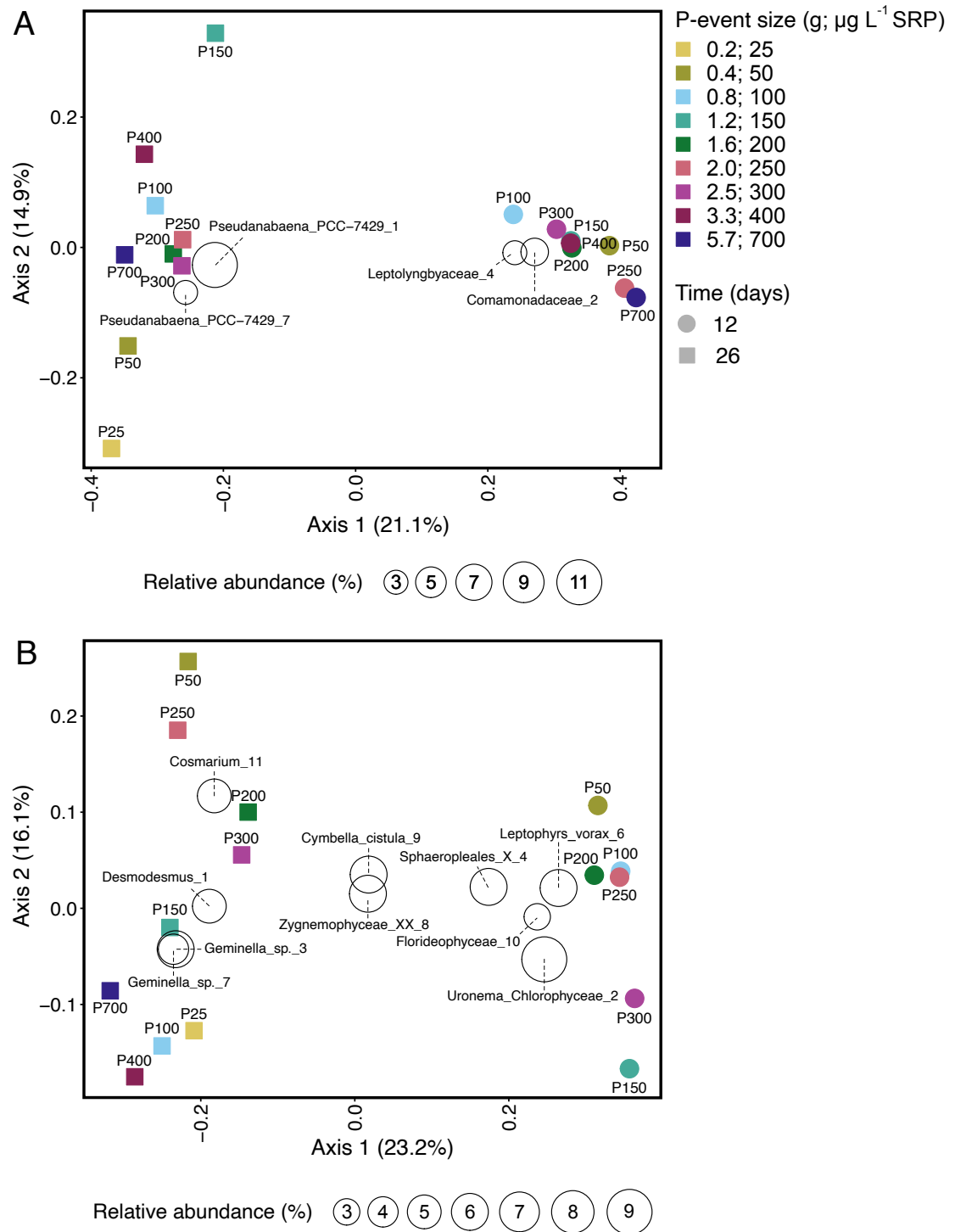


Figure 3.8 Bray-Curtis dissimilarity distance metric based principal coordinate analysis (PCoA) biplot of the 16S rRNA gene and ASVs with $\geq 3\%$ relative abundance (A) and 18S rRNA gene and ASVs with $\geq 3\%$ relative abundance (B) profiles before (day 12) and after (day 26) P events at low antecedent P ($10 \mu\text{g L}^{-1}$).

A PCoA of periphyton treated with high antecedent P showed that bacterial communities organized by time of sampling along axis 1 (Figure 3.9A). Similarly to low antecedent P, axis 2 was not correlated to P event size ($R^2 = 0.50$, $p = 0.19$). On average, bacterial communities had an average dissimilarity of 0.52 ± 0.08 between time points of the same stream. Unlike low antecedent P, abundant ASVs (i.e., *Leptolyngbya_SAG2411_1*, *Rhodobacter_2*, *Pseudanabaena_PCC-7429_4* Comamonadaceae_5) were not associated with either time points. For eukaryotes, axis 1 explained most of the variation but there was less of a separation with time because the dissimilarity between time points within the same stream on average was 0.18 ± 0.05 (Figure 3.9B). Indeed, abundant eukaryotic ASVs (i.e., *Desmodesmus_1*, *Geminella_sp._3*, and *Desmodesmus_armatus_5*) were associated with both time points. Similar to bacteria, axis 2 explained a small proportion of the variation and P event size was not significantly associated with eukaryotic community composition ($R^2 = 0.07$, $p = 0.85$).

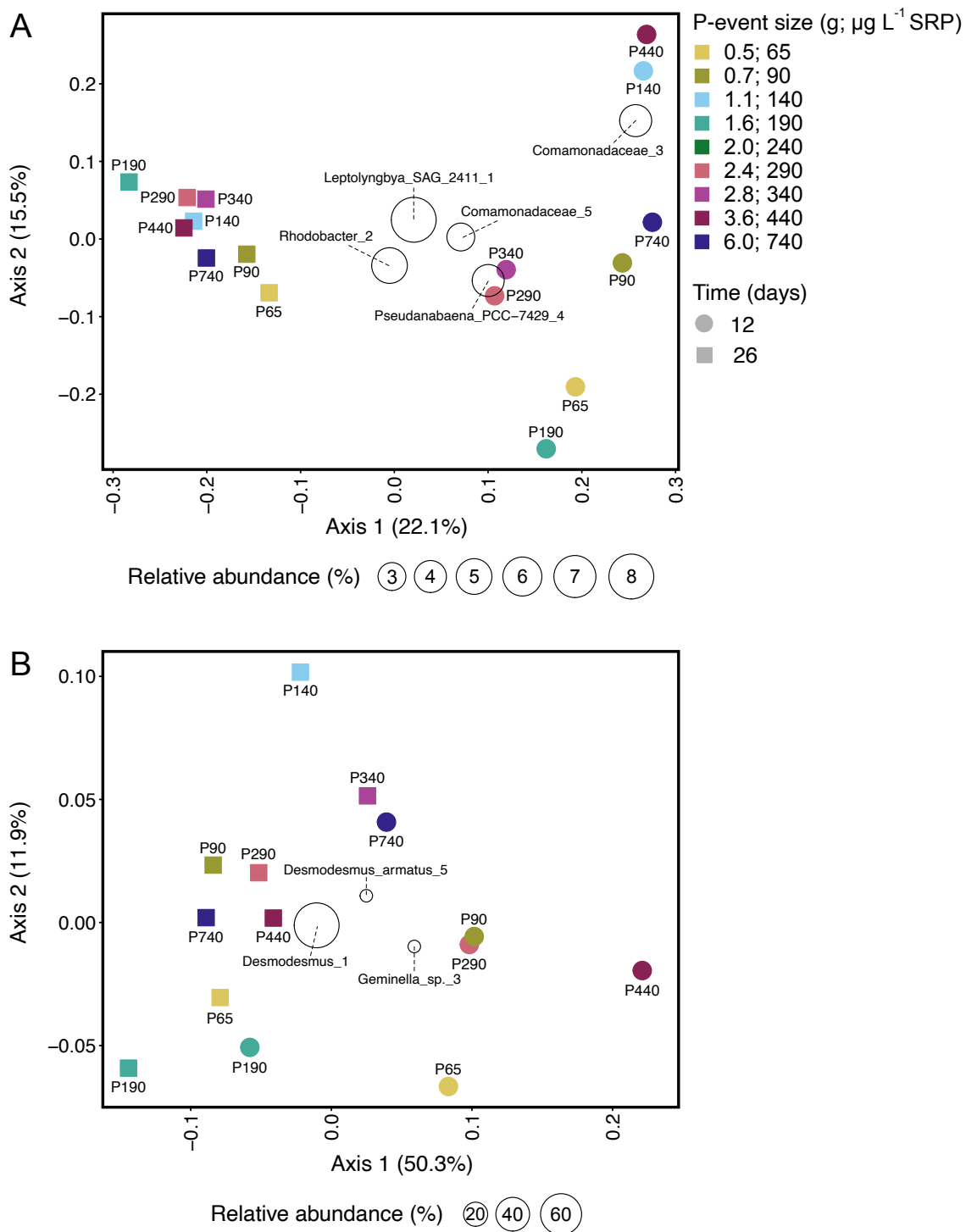


Figure 3.9 Bray-Curtis dissimilarity distance metric based principal coordinate analysis (PCoA) biplot of the 16S rRNA gene and ASVs with $\geq 3\%$ relative abundance (A) and 18S rRNA gene and ASVs with $\geq 3\%$ relative abundance (B) before (day 12) and after (day 26) P events at high antecedent P ($50 \mu\text{g L}^{-1}$).

3.3.3 Alpha diversity

The null model best fit the association between P event concentration and almost all alpha diversity measures for bacteria and eukaryotes under low and high antecedent P conditions (Figure 3.10-3.11; Table 3.1). Exceptions were the linear model being best for eukaryotic richness after a P event under high antecedent P and bacterial evenness before a P event under low antecedent P. A linear model for eukaryotic richness after a P event under high antecedent P may have been driven by a single unrepresentative stream (i.e., 190 $\mu\text{g L}^{-1}$ SRP event), as there was limited variation among streams. Additionally, the variation in evenness data (i.e., 0.52-0.54) was minimal and may not be ecologically relevant.

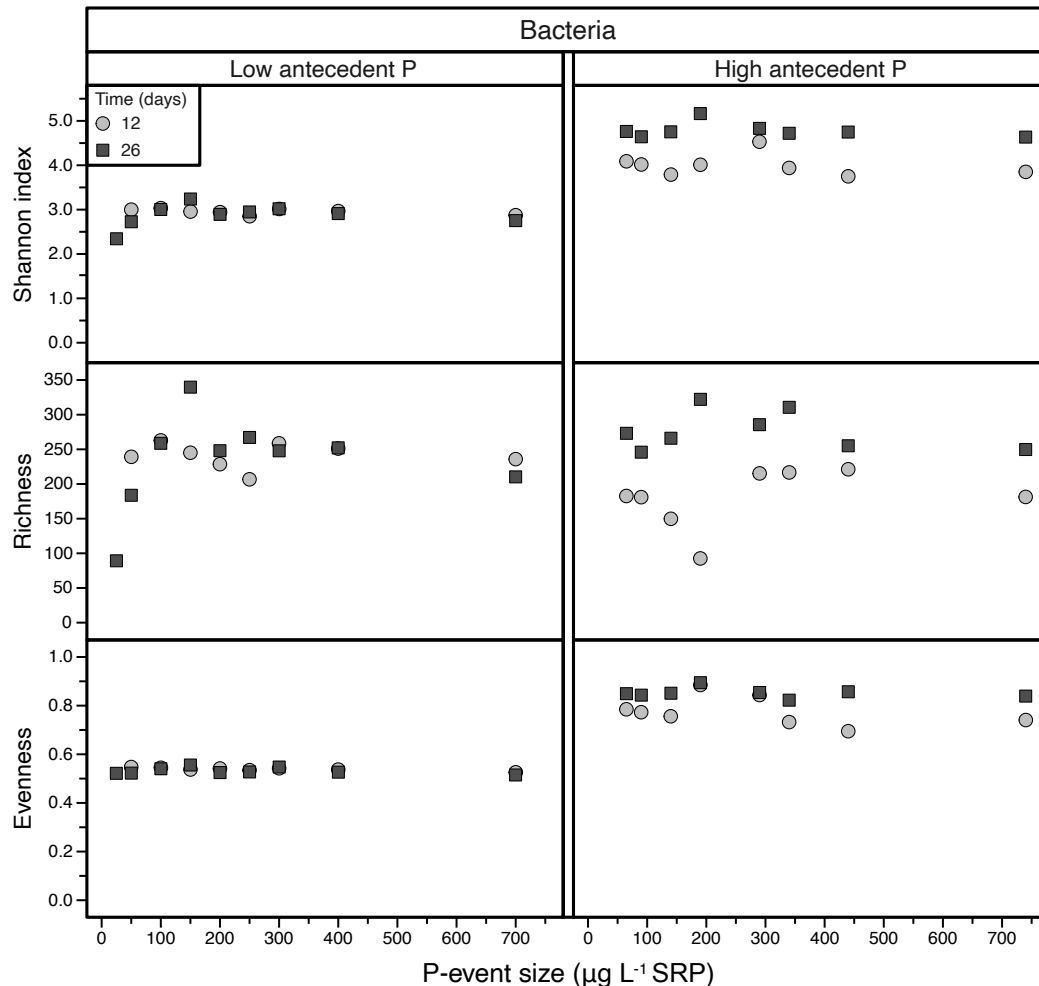


Figure 3.10 Shannon index, richness, and Pielou's evenness for bacterial members of stream periphyton before (day 12) and after P events (day 26) under low or high antecedent P conditions.

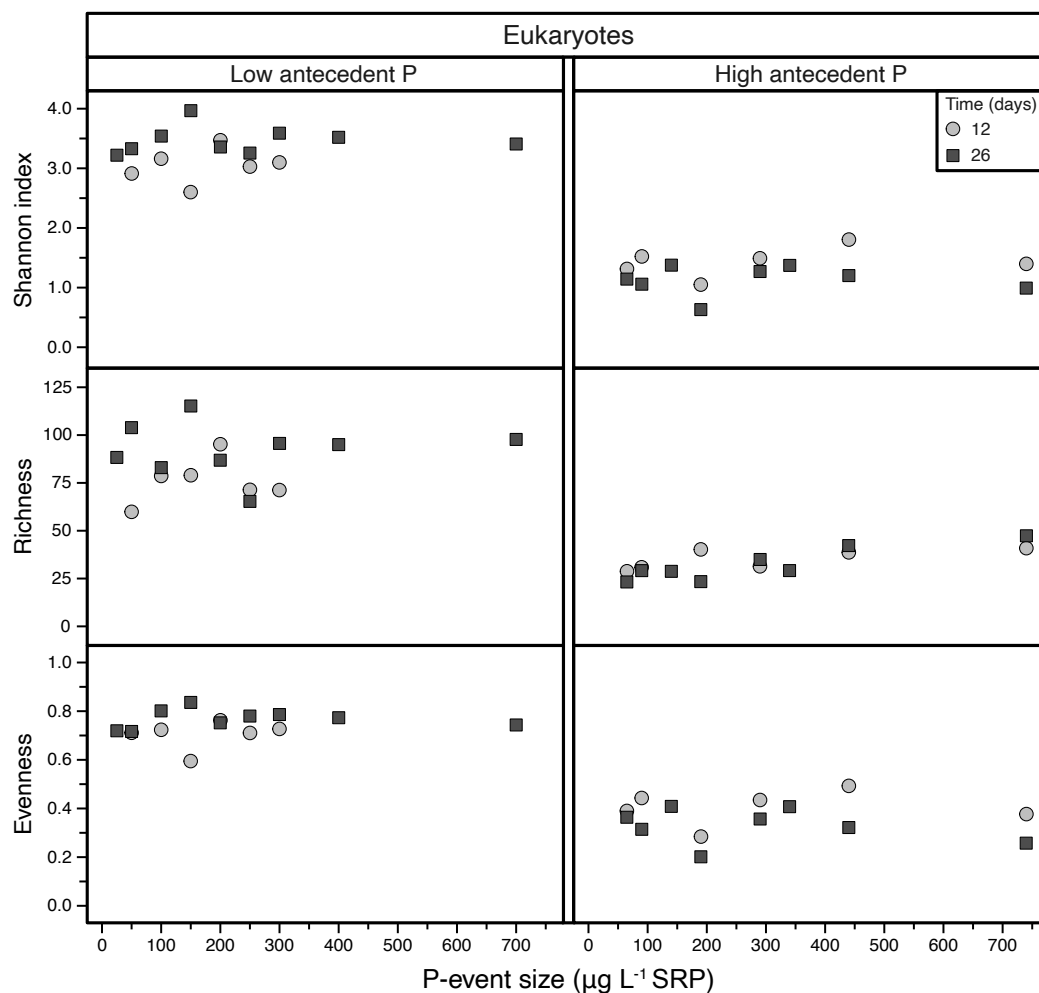


Figure 3.11 Shannon index, richness, and Pielou's evenness for eukaryotic members of stream periphyton before (day 12) and after P events (day 26) under low or high antecedent P conditions.

Table 3.1 Alpha diversity metric (i.e., Shannon index, richness, and Pielou’s evenness) models of bacterial and eukaryotic assemblages for stream periphyton under low antecedent P collected before a P event (day 12) and after a P event (day 26). Second order Akaike’s information criteria values (AICc), differences in AICc relative to the best model (Δ AICc), AICc weights (AICw), R^2 , number of estimated parameters (k), and log-likelihood values (LL) for each model. Bolded models indicate the most supported model.

Alpha diversity	Assemblage	Time (days)	Model	AICc	Δ AICc	AICw	R^2	k	LL
Shannon	Bacteria	Before event	Null	-15.7	0.0	0.79	0.00	1	11.1
		(day 12)	Linear	-13.0	2.7	0.21	0.30	2	12.5
		After event	Null	5.5	0.0	0.91	0.00	1	0.25
		(day 26)	Linear	10.2	4.7	0.09	0.01	2	0.32
	Eukaryotes	Before event	Null	9.0	0.0	0.99	0.00	1	-0.49
		(day 12)	Linear	18.6	9.6	<0.01	0.39	2	-0.28
		After event	Null	3.8	0.0	0.92	0.00	1	1.1
		(day 26)	Linear	8.6	4.8	0.08	0.4	2	1.1
Richness	Bacteria	Before event	Null	74.2	0.0	0.94	0.00	1	-34.0
		(day 12)	Linear	79.7	5.5	0.06	<0.01	2	-33.9
		After event	Null	106.6	0.0	0.91	0.00	1	-50.3
		(day 26)	Linear	111.1	4.5	0.09	0.03	2	-50.2
	Eukaryotes	Before event	Null	53.5	0.0	0.99	0.00	1	-22.7
		(day 12)	Linear	63.2	9.7	<0.01	0.05	2	-22.6
		After event	Null	78.1	0.0	0.92	0.00	1	-36.0
		(day 26)	Linear	82.9	4.8	0.08	<0.01	2	-36.0
Evenness	Bacteria	Before event	Linear	-57.7	0.0	0.95	0.76	2	34.8
		(day 12)	Null	-52.0	5.7	0.05	0.6	1	34.8
		After event	Null	-47.0	0.0	0.88	0.00	1	26.5
		(day 26)	Linear	-43.0	3.9	0.12	0.09	2	27.0
	Eukaryotes	Before event	Null	-10.4	0.0	0.99	0.00	1	9.2
		(day 12)	Linear	-0.7	9.8	<0.01	0.04	2	9.3
		After event	Null	-28.0	0.0	0.92	0.00	1	17.0
		(day 26)	Linear	-23.2	4.8	0.08	<0.01	2	17.0

Table 3.2 Alpha diversity metric (i.e., Shannon index, richness, and Pielou’s evenness) models of bacterial and eukaryotic assemblages for stream periphyton under high antecedent P collected before a P event (day 12) and after a P event (day 26). Second order Akaike’s information criteria values (AICc), differences in AICc relative to the best model (Δ AICc), AICc weights (AICw), R^2 , number of estimated parameters (k), and log-likelihood values (LL) for each model. Bolded models indicate the most supported model.

Alpha diversity	Assemblage	Time (days)	Model	AICc	Δ AICc	AICw	R^2	k	LL
Shannon	Bacteria	Before event	Null	5.6	0.0	0.93	0.00	1	0.42
		(day 12)	Linear	10.6	5.0	0.07	0.07	2	0.71
		After event	Null	-0.6	0.0	0.92	0.00	1	3.48
		(day 26)	Linear	4.3	4.9	0.08	0.08	2	3.84
	Eukaryotes	Before event	Null	7.3	0.0	0.99	0.00	1	0.35
		(day 12)	Linear	16.8	9.5	<0.01	0.08	2	0.59
		After event	Null	5.5	0.0	0.94	0.00	1	0.46
		(day 26)	Linear	11.1	5.6	0.06	<0.01	2	0.47
Richness	Bacteria	Before event	Null	88.1	0.0	0.92	0.00	1	-40.8
		(day 12)	Linear	92.9	4.8	0.08	0.10	2	-40.4
		After event	Null	81.5	0.0	0.93	0.00	1	-37.5
		(day 26)	Linear	86.7	5.2	0.07	0.05	2	-37.3
	Eukaryotes	Before event	Null	44.1	0.0	0.94	0.00	1	-18.1
		(day 12)	Linear	50.0	5.6	0.06	0.50	2	-15.9
		After event	Linear	55.4	0.0	0.97	0.80	2	-21.7
		(day 26)	Null	62.5	7.1	0.03	0.00	1	-28.1
Evenness	Bacteria	Before event	Null	-16.5	0.0	0.89	0.00	1	11.5
		(day 12)	Linear	-12.4	4.1	0.11	0.17	2	12.2
		After event	Null	-34.2	0.0	0.93	0.00	1	20.3
		(day 26)	Linear	-29.1	5.6	0.07	0.06	2	20.6
	Eukaryotes	Before event	Null	-7.7	0.0	0.99	0.00	1	7.9
		(day 12)	Linear	2.2	9.9	<0.01	<0.01	2	7.9
		After event	Null	-14.1	0.0	0.92	0.00	1	10.2
		(day 26)	Linear	-9.2	4.9	0.08	0.09	2	10.6

Under low antecedent P, all alpha diversity metrics for bacteria were lower by less than 4% following P events (Table 3.3). For eukaryotes, shifts in alpha diversity metrics before and after an event was opposite to the pattern observed in bacteria under low antecedent P. For example, richness in eukaryotic assemblages was 21% higher after a P event compared to before. In contrast, with high antecedent P, all bacterial alpha diversity metrics were higher by greater than 8% after an event, with richness being 35% higher than before an event. Whereas eukaryotic assemblages were higher by 9% across all metrics, with a maximum of 27% in Shannon values.

Table 3.3 Mean and standard deviation of Shannon index, richness, and Pielou's evenness for bacterial and eukaryotic assemblages before and after P events under low or high antecedent P conditions.

Condition (P)	Assemblage	Time (days)	Shannon	Richness	Evenness
Low antecedent	Bacteria	Before event (day 12)	3.0 ± 0.1	241 ± 18	0.54 ± 0.01
		After event (day 26)	2.9 ± 0.2	233 ± 69	0.53 ± 0.01
	Eukaryotes	Before event (day 12)	3.0 ± 0.3	76 ± 12	0.70 ± 0.06
		After event (day 26)	3.5 ± 0.2	92 ± 14	0.77 ± 0.04
High antecedent	Bacteria	Before event (day 12)	4.0 ± 0.2	180 ± 43	0.78 ± 0.06
		After event (day 26)	4.8 ± 0.2	275 ± 28	0.85 ± 0.02
	Eukaryotes	Before event (day 12)	1.4 ± 0.3	35 ± 5	0.40 ± 0.07
		After event (day 26)	1.1 ± 0.2	32 ± 9	0.33 ± 0.07

3.4 Discussion

Nutrient enrichment from short duration events is gaining recognition for its biological relevance to stream ecosystems, influencing community structure, productivity, and ecosystem function. In this study, antecedent P conditions typical of undisturbed and human impacted catchments were used to assess stream periphyton compositional responses to a gradient of P events sizes. The data demonstrate that (1) bacterial and eukaryotic assemblages undergo enrichment and ultimately reach a compositional plateau with a $25 \mu\text{g L}^{-1}$ SRP event, and (2) antecedent P constrains shifts in community structure with additional P delivered through events.

3.4.1 Response of microbial community composition to P event concentration

Event P concentration was not related to the composition of stream periphyton communities regardless of the antecedent condition. Although a lack event size effect was anticipated for the high antecedent streams, it was predicted that under low antecedent P, microbial community composition would respond proportionately to P event concentrations. The absence of a periphytic community response in proportion to P event size would be expected if periphyton had been unable to uptake and assimilate excess P during the events. However, additional data collected during this experiment focused on periphyton structure and function, demonstrating that periphytic microorganisms took up and assimilated P into biomass (i.e., AFDM and Chl *a*) in a positive linear proportion to a P event size (Schneider unpublished data, 2025). This suggests that periphyton were able to uptake and assimilate P proportionally to the magnitude of P event sizes and the reason for a lack of compositional change to P events was not due to an inability to uptake P during highly concentrated short duration P events.

Event size may not have driven shifts in periphyton community composition due to a low limit of response to nutrient enrichment. Evidence of a threshold comes from a stable community observed across all event sizes under low antecedent P, with a small event size ($25 \mu\text{g L}^{-1}$ SRP; 0.14 g SRP) sufficient to create an enriched community. Although the smaller event sizes did not greatly increase the total P load to the streams, there are precedents where relatively small P loads have been associated with limits in periphyton structure. Indeed, Hill et al. (2009) found biomass (i.e., Chl *a*) and % algal biovolume of periphyton dominated by diatoms reached a maximum threshold by $25 \mu\text{g L}^{-1}$ SRP in artificial streams. Likewise, studies in real streams have found compositional shifts of diatom communities from lower concentrations of TP (e.g., $22 \mu\text{g}$

L⁻¹), although others have observed much higher concentration limits (e.g., 280 µg L⁻¹) (Black et al., 2011; Chambers et al., 2012; Smucker et al., 2013). Although comparing our findings to studies using TP is challenging, because it is unknown what proportion of SRP makes up TP in these studies, the wide range of threshold values indicates that other variables common to real streams (e.g., temperature, flow, light) may influence the role of events. Thus, further work is needed to assess the relationship between microbial community composition of periphyton and SRP in real streams.

3.4.2 Microbial community composition constrained by antecedent P

Stream periphyton compositional responses to P events were constrained by antecedent P conditions given that periphyton under low antecedent P shifted in composition following the event, but those under high antecedent P did not. The lack of shift in the high antecedent streams appears to have been driven by the fact that the periphyton community exhibited a taxonomic composition characteristic of P enrichment prior to the events. The pre-event establishment of an “enriched” community under high antecedent conditions was consistent with the hypothesis that enriched background conditions would favour taxa associated with elevated nutrient levels, leading to periphytic community composition being largely unaffected by further P enrichment from events. This study thus provides evidence for the modulating role of antecedent P availability and indicates that chronically enriched streams will be unaffected by event loadings.

Although the influence of P events was modulated by antecedent P availability, the data showed that under low antecedent P, the P events ultimately resulted in communities comparable to those of the high antecedent P treatments. This similarity was primarily driven by the increased abundance of several green algae taxa (i.e., *Desmodesmus*, *Geminella*, and *Sphaeropleales*) following the P event in the low antecedent streams. The instigation of a common enriched community in both experiments indicates that the same taxa benefited from the events in the low antecedent streams as benefited from the high antecedent P. It thus appears that streams where baseflow waters are low in P but receive periodic P loading with runoff from anthropogenic activities may be indistinguishable from those experiencing high antecedent P concentrations due to nutrient contaminated baseflows or consistent inputs of treated wastewater. Assessments of periphytic composition to detect the relative effects of antecedent and event P may therefore be ineffective.

3.5 Conclusion

This study furthers understanding of event induced P enrichment in stream ecosystems by determining that small or large events reach a similar enriched microbial community. The underestimated potential for short duration P events to govern stream periphyton communities highlights a need for improved management of P loads from diffuse sources. In addition, this study found a constraining effect of high antecedent P on compositional responses to P events. These results highlight a potential difficulty in biomonitoring of event P, because the overlapping influence of high baseflow P and stormflow P on stream periphyton composition can make it difficult to disentangle their individual effects. Future research on the effect of P events in stream ecosystems should incorporate assessments of varying magnitudes of event sizes in real streams to gain additional insight into critical thresholds of nutrient loading that lead to shifts in periphyton composition associated with eutrophication.

Chapter 4: Discussion & conclusions

4.1 Summary of findings

Although stream research has lagged behind that of lakes, assessment of P eutrophication in stream ecosystems has received growing attention over the last few decades (Melles et al., 2012; Poole, 2010; Vannote et al., 1980). In addition, our understanding of P eutrophication effects on stream periphyton communities has been highly focused on algal assemblages (Biggs, 1990; Biggs and Smith, 2002; Black et al., 2011; Munn et al., 2002; Pearce et al., 2020; Smucker et al., 2013). However, more recent research on aquatic ecosystems over the last 20 years has shifted towards the use of high-throughput sequencing of whole microbial communities, with an increase in the use of amplicon sequencing for stream-related research over the last decade (Michán et al., 2021; Zeglin, 2015). Thus, this thesis provides a novel assessment of P eutrophication derived from common human land use activities at a broader periphyton community level (i.e., bacteria and eukaryotes) using high-throughput sequencing in a controlled mesocosm setting.

Results from Chapter 2 provide evidence that stream periphyton community composition is largely controlled by P load, and not the pattern of P delivery. Periphyton exposed to either continuous or event-based P enrichment resulted in similar eukaryotic and bacterial assemblages. Thus, this study found a potential limitation in current biweekly-monthly stream water monitoring practices that may not account for P loads from runoff events, leading to underestimation of the eutrophic status exhibited by stream periphyton communities. Additionally, differences in microbial communities were captured using high-throughput sequencing and demonstrated potential as an assemblage descriptor for bioassessment tools in P enriched streams.

Chapter 3 provided evidence that stream periphyton community composition does not respond proportionately to the P event load. This chapter built upon existing periphyton research that demonstrated benthic algae can uptake and maintain growth rates in proportion to the size of an event (Pearce et al., 2023). Previous studies have looked at specific taxa (e.g., diatoms; Chambers et al., 2012; Chételat et al., 1999; Lavoie et al., 2014) and algal metrics (e.g., richness; Azevedo et al., 2013; Biggs and Smith, 2002) to assess enrichment thresholds in field environments. Whereas the current study provides a novelty of whole microbial periphyton

communities reaching a compositional enrichment threshold at events of 25 $\mu\text{g L}^{-1}$ SRP. Additionally, this study found that antecedent conditions do limit periphyton compositional response as periphyton under high antecedent P were pre-established as enriched communities prior to an event.

4.2 Synthesis

This thesis indicates that P enrichment, whether small or large, and regardless of pattern, can result in a common enriched microbial community. Indeed, Chapter 2 and 3 found common enriched communities dominated by green algae (e.g., *Desmodesmus*), *Proteobacteria* (e.g., *Comamonadaceae*), and *Cyanobacteria* (e.g., *Pseudanabaena*), regardless of pattern or degree of P enrichment. Although these results need to be validated in real streams, the results have important implications. First, because periphyton communities represent the foundation of stream food webs, shifts in communities can affect the diet of higher trophic levels (Allan et al., 2021). For example, the consumption of periphyton by grazers (e.g., benthic invertebrates) can directly affect the growth of grazers, thereby indirectly affecting the diet of higher trophic organisms (e.g., fish, amphibians, and other macroinvertebrates). Thus, a common enriched community could lead to regional homogeneity and associated reductions in beta diversity at a regional level, despite substantial spatial variation in the pattern or amount of nutrient enrichment. Indeed there is some evidence (e.g., Krynak and Yates, 2018; Yates and Bailey, 2011) indicating our findings may reflect real streams as regional homogeneity in higher level trophic assemblages (e.g. benthic macroinvertebrates) has been observed in past studies of streams in mixed land use landscapes, such as those found in Southern Ontario.

Chapters 2 and 3 both revealed an inconsistent finding with common algal compositional patterns observed throughout literature. Throughout this thesis, high-throughput sequencing of the 18S rRNA gene potentially underestimated the abundance and diversity of diatom taxa found compared to previous TRESS studies done with microscopy (e.g., Pearce et al., 2020). Indeed, previous studies have reported inconsistencies in quantification of community members observed through microscopy and high-throughput sequencing (Hu et al., 2025; Turk Dermastia et al., 2023). Moreover, literature suggests that the 18S rRNA gene is a less suitable target compared to the *rbcL* gene for diatom identification (Turk Dermastia et al., 2023; Wang et al., 2024). Despite such drawbacks, sequencing provides greater taxonomic resolution than traditional microscopy

methods (Hu et al., 2025). Overall, diatom identification is a useful tool for assessments of ecosystem integrity due to their sensitivity and tolerance to pollution (Descy and Coste, 1991; Kelly, 1998; Lavoie et al., 2006). Because a previous TRESS study was able to distinguish between P events and continuous enrichment through diatoms assessed via microscopy, it is recommended that future P event studies use diatom specific and universal eukaryotic primers for high-throughput sequencing and quantitative PCR, as well as microscopy, for accurate identification and quantification of diatoms.

4.3 Future work

Findings from this thesis identified two areas for further research to deepen the understanding of P event induced eutrophication in streams and microbial communities of periphyton. First, this work highlights the sensitivity of microbial communities in stream periphyton to P enrichment, with promising potential to identify human-impacted streams from pristine streams. Thus, future studies should test integrating high-throughput sequencing of microbial communities with the reference condition approach for biomonitoring of P events in real streams. Second, this work sheds light on the comparable sensitivity of bacterial and eukaryotic assemblages and built upon fundamental characterization of bacterial members of stream periphyton exposed to P enrichment. Most stream studies have focused on algal members, and few have assessed bacterial assemblages and the potential ecological interactions and functions occurring within diverse communities of stream periphyton. Thus, this thesis draws attention to the limited understanding of bacterial structure and function in stream periphyton under broad P enrichment and generated opportunities for future research to elucidate the role of bacteria in stream periphyton under P events, and P enrichment more broadly.

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