Effects of spatio-temporally varying lakeside human disturbance on nearshore periphyton communities in Flathead Lake (Montana, United States)

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**Abstract (250 of 250 words)**

Nutrients from lakeside developments can reshape aquatic ecosystems. Algal communities, especially in oligotrophic lakes, can rapidly remove nutrients from the water column, causing increased biomass and altered resources for grazers. However, associating times and locations of nutrient loading with biological responses can be complicated, as spatio-temporal heterogeneities in nutrient loading and biological succession can obfuscate patterns. To evaluate how variation in nutrient addition may be associated with algal succession and nutritional quality, we sampled 15 littoral sites in Flathead Lake (Montana U.S.) from June through September 2017, when tourism dynamics may create variation in nutrient influx. As indicators of anthropogenic nutrient additions, pharmaceutical and personal care product (PPCPs) concentrations were measured fortnightly. Periphyton community composition, stoichiometry, and fatty acids were assessed monthly. PPCPs, including caffeine, nicotine, and warfarin, were detected, and PPCP concentrations as well as periphyton branched- and odd-chain fatty acid abundance varied with seasonal tourist activity. Sewage indicators near centralized sewage treatment were more temporally consistent throughout the sampling regime in comparison to sites near decentralized treatment. These same locations near centralized treatment were more associated with increased chlorophyte abundance, lower periphyton C:N:P ratios, yet lower quality essential fatty acids. Conversely, locations near decentralized treatment were more associated with temporally transient periphyton community composition, higher periphyton C:N:P ratios, yet higher quality essential fatty acids. Overall, our results suggest that sewage treatment techniques and seasonal human activity can create hot spots and hot moments for nutrient addition, thereby reshaping algal community compositions and available nutrition to the food web.

**Introduction**

Pollutants in wastewater released from lakeside developments are a common human disturbance that can alter biological communities and reshape food webs. In particular, nutrients, such as nitrogen and phosphorus, in treated and untreated wastewater can increase biomass and eventually lead to system-wide changes in algal community composition and production (Edmondson 1970) with potential consequences for higher trophic levels (Edmondson 1994; Hampton et al. 2006; Galloway and Winder 2015). Although nutrients in sewage have been shown to drastically alter aquatic ecosystems, explicitly linking increased nutrient concentrations and biological responses to sewage can be challenging, as nutrients can originate from disparate anthropogenic and natural environmental sources. For example, agriculture (Powers et al. 2016), melting permafrost (Turetsky et al. 2000), wildfires (Gould et al. 2016), and even changing terrestrial plant communities (Goldman 1961; Moran et al. 2012) can all contribute nutrients to aquatic systems, potentially obfuscating sewage signals. In addition, biological processes can further confound sewage signals. Benthic primary producers, especially those in oligotrophic systems (Rosenberger et al. 2008; Hampton et al. 2011; Oleksy et al. 2020; Atkins et al. 2021), can remove nutrients quickly from the water column (e.g., hours; Hadwen and Bunn 2005), such that deviations in nutrient concentrations may not be detectable in water samples.

Because nutrients come from numerous non-sewage sources, indicators consistently associated with wastewater pollution, such as pharmaceuticals and personal care products (PPCPs) (Rosi-Marshall and Royer 2012; Meyer et al. 2019) as well as bacterial biomarkers (Seguel et al. 2001) have garnered attention for their usefulness as sewage indicators. PPCPs, such as caffeine, acetaminophen, and sulfamethoxazole, have been used to identify spatially and temporally heterogeneous sewage pollution in surface (Bendz et al. 2005) and subsurface (Yang et al. 2016) aquatic systems. Because biota seem not to actively uptake most PPCPs (Bai and Acharya 2017), their concentrations within the water column tend to be directly proportionate to increasing human population and inversely proportionate with distance from population centers (Meyer et al.; Bendz et al. 2005). Microbial and bacterial biomarkers, such as branched- and odd-chain fatty acids, also can be useful for defining areas and times of sewage loading (Seguel et al. 2001). In contrast to PPCPs, bacteria-associated fatty acids can be less specific sewage indicators, as bacteria can also originate from numerous non-sewage sources. Thus, tracking the changes in relative bacterial abundance with bacterial biomarkers can be a more reliable indicator of spatial and temporal patterns in sewage loading for both freshwater (Templar et al. 2016; Booncharoen et al. 2018) and marine systems (Abaya et al. 2018; Booncharoen et al. 2018). Together, the combined use of PPCP and bacterial biomarker measurements may provide more robust, multi-proxy information to infer the spatial extent and timing of sewage pollution into an ecosystem.

The effects of wastewater pollution are often first observed among littoral benthic communities, where increased nutrients can alter algal community succession by causing deviations in community composition and abundance (Meyer et al.; Rosenberger et al. 2008; Hampton et al. 2011; Timoshkin et al. 2016; Bondarenko et al. 2021; Atkins et al. 2021). Similar to patterns observed with phytoplankton succession (Sommer et al. 1986, 2012), benthic communities likewise tend to increase in chlorophyte abundance over the course of a growing season and then eventually decrease in abundance relative to diatoms and cyanobacteria (Hoagland et al. 1982; McCormick and Stevenson 1991; Stevenson et al. 1996; Azim et al. 2005). Mechanistically, this transition occurs when nitrogen and phosphorus become limiting for many taxa. In the case of sustained wastewater inputs, benthic chlorophyte taxa can establish and remain dominant within the community (Timoshkin et al. 2016, 2018; Volkova et al. 2018), potentially due to increased chlorophyte abundance being associated with increased nutrient uptake efficiency (Oleksy et al. 2021). In the case of intermittent wastewater inputs, chlorophyte taxa may increase in relative abundance in response to nutrient inputs, but then become displaced as nutrients become limiting and taxa compete for nutrients, space, and light (McCormick and Stevenson 1991; Marks and Lowe 1993; Stevenson et al. 1996). These two dichotomous disturbance regimes, therefore, present two similar yet diverging mechanisms where nutrient addition into aquatic environments can alter anticipated periphyton successional patterns.

By driving community compositional shifts, nutrients from wastewater can also influence nutritional quality of the periphyton, with consequences for higher trophic levels. Key elements of algal nutritional quality include fatty acid composition (Iverson et al. 2004; Kelly and Scheibling 2012) and C:N:P stoichiometric ratios (Elser et al. 2000), based on fundamental metabolic needs of aquatic grazers. Fatty acids reflect changing nutritional content as a function of altered community composition, where chlorophyte, cyanobacteria, and diatoms each contain taxon-specific multivariate fatty acid signatures (Kelly and Scheibling 2012; Taipale et al. 2013; Galloway and Winder 2015). In particular, the essential fatty acids (EFAs) are where these groups diverge the most, with diatoms being associated with 20:5ω3, chlorophytes being associated with 18:3ω3 and 18:2ω6, and cyanobacteria usually not containing EFAs (Taipale et al. 2013; Galloway and Winder 2015). Similarly, stoichiometric C:N:P ratios can also define nutritional content within periphyton for higher trophic levels (Kahlert et al. 2002; Fink et al. 2006), where lower C:N:P ratios indicate increased nutrition via increased nitrogen and phosphorus content. Unlike fatty acids, stoichiometric analysis does not allow inference of community taxonomic composition. Rather, algal stoichiometric ratios can be plastic (Thrane et al. 2017) and largely reflect environmental conditions such as light and nutrient availability (Frost and Elser 2002). Even so, these ratios may be modified by taxon-specific traits. Taxa that are associated with high nutrient uptake, such as filamentous chlorophytes, can decrease a community’s overall C:N:P ratios (Frost and Elser 2002) and potentially increase food quality for grazers. In the context of sewage-associated nutrient pollution, both periphyton stoichiometric ratios and fatty acid profiles can be useful for inferring taxonomic and environmental aspects of algal nutritional content. Sewage loading that drives successional shifts from diatom-dominated to chlorophyte-dominated communities may be expected to decrease 20-Carbon EFAs and increase less nutritious 18-Carbon EFAs; simultaneously, these communities may become more nutritious (i.e. decreased C:N and C:P ratios) from a stoichiometric perspective, as communities with more chlorophytes exhibit higher nutrient uptake rates (Nydick et al. 2004; Oleksy et al. 2021).

To investigate how sewage-associated nutrient addition can alter nearshore periphyton community composition, and thus standing nutritional resources for higher level consumers, we surveyed 15 littoral locations in Flathead Lake (Montana, United States) throughout the tourism season for indicators of sewage pollution and metrics of periphyton community abundance and nutritional content. Flathead Lake is a large, deep, oligotrophic lake in western Montana (Young 1935; Ellis et al. 2011). With Glacier National Park covering approximately one third of the lake’s watershed, Flathead Lake’s catchment is largely unpopulated, despite gradual increases in permanent and seasonal human population throughout the previous century (Stanford et al. 1994a). Within Flathead Lake’s watershed, areas with intensive development have shown distinct signs of sewage pollution, evidenced by pharmaceutical and personal care product (PPCPs) and volatile organic compounds detected in subsurface waters although not yet in the lake (Tappenbeck and Ellis 2010, 2011). As locations with dense human population are generally located away from the lake, (e.g., > 20 km), Flathead’s pelagic is broadly oligotrophic, likely due to the lake’s exceptionally low hydraulic residence time (~3 years; Stanford et al. 1983) relative to its size (Messager et al. 2016). While the human population in Flathead Lake’s watershed is low, the littoral areas with adjacent lakeside development have been suspected of localized sewage pollution (Stanford et al. 1994a), evidenced by sustained increases in periphyton biomass since the 1980s. In particular, littoral sites without adjacent centralized wastewater treatment infrastructure (e.g., sewage treatment plants) have demonstrated signs of nonpoint nutrient addition, which could be contributed from decentralized forms of wastewater management such as improperly functioning septic systems (Stanford et al. 1994a; Makepeace and Mladenich 1996; Moore et al. 2003; Withers et al. 2014). These same lakeside developments also experience large tourism activity throughout the summer, where a reliance on decentralized sewage treatment could create moments of increased nutrient loading during the tourism season followed by reduced sewage loading after the tourism season. Similarly, littoral areas near locations with centralized sewage treatment also experience increased tourism during the summer, but as these areas tend to have larger, less transient human populations, these adjacent nearshore locations also likely experience consistent but elevated wastewater-associated nutrient loading relative to nearshore communities with adjacent decentralized infrastructure.

Given prior evidence that Flathead’s periphyton communities may be responding to external nutrient loading, our goal was to understand how spatially and temporally heterogeneous nutrient addition from seasonal tourism may alter algal community composition and, thus, the available nutrition to the food web. This goal can be divided into three specific objectives:

1. identify areas and moments of wastewater-associated nutrient loading using sewage-specific indicators,
2. assess the relationship between sewage-specific indicators and periphyton community composition
3. evaluate how periphyton community compositional shifts may correspond with altered nutritional quality.

We hypothesized that (1) sewage-specific indicators, such as PPCP concentration as well as branched- and odd-chain fatty acids characteristic of bacteria, would increase near areas with increased population density and proximity to lakeside development as well as during moments of increased human activity; (2) an elevated sewage signal would correspond with increased abundance of filamentous benthic algae; (3) increased filamentous algal abundance should be associated with increased stoichiometric nutrition but decreased essential fatty acid nutrition of the periphyton community; and (4) sites with consistent sewage indicators (i.e., those near centralized wastewater treatment) would correspond with less temporal fluctuation in periphyton community composition and nutritional content relative to sites with temporally fluctuating sewage indicators (i.e., sites near decentralized wastewater treatment systems).

**Methods**

*Site Description*

Most of Flathead Lake’s 260-km shoreline contains some degree of lakeside development, but the vast majority of Flathead’s basin is unpopulated and roadless (Stanford et al. 1994a; Makepeace and Mladenich 1996). Located approximately 23 km from the lake, the city of Kalispell is the largest population hub within the basin with approximately 23,000 residents (U.S. Census, 2019). The Flathead River runs through Kalispell and is the main surface input to Flathead Lake. The Flathead River is Flathead Lake’s only surface outlet, where the river flows into Flathead Lake in the northeast region near the town of Bigfork with approximately 4,700 residents and exits in the southwest near the town of Polson with approximately 4,900 residents (U.S. Census, 2019). The Swan River is the second largest surface input into Flathead. Unlike the Flathead River, the Swan River originates within Glacier National Park and does not run through large population hubs.

While larger population hubs are concentrated away from Flathead’s shoreline, there are several small lakeside population hubs that host annual summertime tourists. Each of these main tourist hubs have different permanent population sizes and infrastructures for handling human population fluctuations, ranging from centralized wastewater treatment plants with tertiary treatment to septic systems. Previous studies have noted increased nutrient concentrations and periphyton abundance near these lakeside developments (Stanford et al. 1994a) and implicated sewage-associated nutrient loading as the source (Makepeace and Mladenich 1996). To expand on these previous works by specifically identifying sewage-associated nutrient loading and tracking co-located periphyton responses, our study focused on 15 littoral sampling locations throughout the lake from late May through late September 2017. All sampling sites were chosen to represent a range of lakeside development sizes - spanning from sites with centralized sewage treatment and housing for permanent residents to seasonally occupied developments with septic systems. Many sampling locations were located within Montana State Parks, which ensured consistent access to sampling locations throughout the course of a summer. To evaluate potential sewage contributions from Kalispell, one sampling site was located in the Flathead River, upstream of the town of Bigfork. Sampling occurred at a depth of approximately 0.75-1.25 m, depending on wave activity and periphyton accessibility, such that sampling locations ranged 0.5-8.0 m distance from shore. During each sampling event, we recorded air and surface water temperature with an alcohol thermometer, qualitative wave intensity (e.g., mild, moderate, strong waves), and whether or not tourists were present at time of sampling.

*Temporally-Scaled Inverse Distance Weighted (TSIDW) Population Calculation*

We recognized that sewage indicator presence was likely spatially related to the human population density of and distance from lakeside developments, as well as temporally related with the tourism season. Therefore, we created a metric that would represent both the human population density at a given sampling site as well as how that population might vary throughout a summer. Additionally, Makepeace and Mladenich (1996) suggested that anthropogenic nutrient loading into Flathead Lake was likely driven by subsurface flows, meaning that the length of development’s shoreline (or the size of a development's interface with the lake) was likely directly proportionate to the amount of wastewater that could enter the lake. Building on techniques described in Meyer et al (Under Revision) and concepts expressed in Bendz et al (2005), we created a temporally-scaled inverse distance weighted (TSIDW) population metric, which considers that sewage indicator levels are likely directly related to population density, a lakeside development’s shoreline length, and level of tourism as well as inversely related to a sampling location’s distance from lakeside developments.

The TSIDW population metric is meant to capture both spatial and temporal variation of human activity at each sampling location. The formulation of TSIDW population can be broken into two main components: (1) the static inverse distance weighted (IDW) population and (2) a temporal scalar of how much the lakeside human population changes through time. First, the static IDW population can be calculated in five main steps (Meyer et al, Under Revision). Municipality boundary and shoreline geometries were drawn using Google Earth and census delineations for five main lakeside municipalities. Shapefiles were then exported from Google Earth as a .kml file, and then imported into the R Statistical Environment (R Core Team 2019), in which shoreline length as well as municipality area and centroid were calculated using the sf package (Pebesma 2018). Static IDW population was then calculated by summing the distance-weighted, shoreline length-scaled population density to each sampling location relative to each of the five lakeside municipalities. Second, the static IDW population was scaled by the average monthly temporal change in human population. In this case, State Park visitation records were used to track changes in the human population relative to pre-tourism visitation levels. To track how the lakeside human population may change spatially and temporally, we collected data on visitor passes issued at each of the five State Parks around the lake (Figure 1). These data aggregated both day-visitor as well as overnight visitor passes purchased within a month, thereby representing the total number of visitors to each of the five lakeside Parks. To estimate a deviation in human population due to tourism, we normalized each Park’s monthly visitors by the number of visitors to the same Park in May, and as such this calculation assumes that the number of visitors in May is characteristic of the pre-tourism season. Because each State Park was not necessarily located within or adjacent to the five main lakeside population hubs, we weighted temporal scalars by the distance of a given sampling location from the centroid of each State Park. Lastly, we averaged monthly temporal scalars across Parks. Averaging the temporal scalars in a given month helped us account for populations that may not be visiting a local Park or correct for populations may have been dispersed when one of the Parks was closed near the end of the tourism season. TSIDW population (I) is expressed by equation 1:

where the left summation is the temporal scalar formulation and the right summation is the spatial IDW population formulation. The equation represents the TSIDW population *I* at site *i* in month *m*. The IDW formulation considers the population size *P*, area *A*, and shoreline length *L* at development site *j* and weights the shoreline population by the distance from site *i* to *j*. The temporal scalar formulation considers the number of visitor passes sold *n* in a month *m* at Park *q* relative to the number of passes sold in May at Park *q* and weights this ratio by the distance of sampling site *i* from Park *q*. When multiplied, the TSIDW population is meant to capture the temporal change and spatial position of human population around the lake’s perimeter, where sites with large populations but low tourism retain a high TSIDW population throughout the summer whereas sites with a smaller static population that experiences waves of tourism increase in TSIDW population at the beginning of the tourism season and then decrease in TSIDW population at the end of the tourism season.

*Pharmaceuticals and Personal Care Products (PPCPs)*

Water column Pharmaceutical and Personal Care Product (PPCP) samples were collected in duplicate fortnightly at a depth of approximately 0.5 m at each sampling location. Samples were collected in 1-L amber glass bottles that had been rinsed with lake water prior to collection. Samples were kept on ice and out of direct sunlight in a cooler while in the field and then placed in a refrigerator at ~4℃ upon return to the lab. While in the field, personnel collecting samples refrained from caffeine, nicotine, and other non-prescription PPCPs on the days of sample collection to avoid potential contamination.

Within 96 h of collection, samples underwent a solid phase extraction (SPE). Samples were filtered directly from the amber glass bottle using an in-line Teflon filter holder with glass microfiber GMF (1.0 µm pore size, WhatmanGrad 934-AH) in tandem with a solid phase extraction (SPE) cartridge (200 mg HLB, Waters Corporation, Milford, MA) connected to a 4-liter vacuum flask. Lab personnel wore gloves and face masks when handling samples in the lab to minimize potential for contamination. Prior to filtration, SPE cartridges were primed with at least 5 mL of either methanol or acetone and then washed with at least 5 mL of sample water. Rate of extraction was maintained at approximately 1 drop per second. Extraction proceeded until water could no longer pass through the SPE cartridge or until all collected water was filtered. Cartridges were stored in Whirlpacks at -20°C until analysis for 10 PPCP residues using high performance liquid chromatography tandem mass spectrometry (HPLC-MS) following methods of Furlong et al. (2008), Lee et al. (2016), and D’Alessio et al (2018). During the mass spectrometry sample processing, blank samples with methanol as well as a 20 µg/L standard were processed after every tenth sample. This step was added as a quality assurance protocol to flag instances of and correct for cross-sample contamination as well as peak drift when running samples over multiple hours. Regardless of detection thresholds, we manually integrated all PPCP peaks observed in the chromatogram and corrected for cross-sample contamination in post-processing. Samples were processed at the Water Environmental Research Laboratory at Washington State University.

*Nutrients*

Water column nutrient samples were collected at all 15 sampling locations and contemporaneously with the August and September PPCP sample collection that occurred at the end of the month (i.e., second fortnightly sampling campaign within a month). Samples were collected in 0.5-L plastic Nalgene bottles that had been rinsed with lake water prior to collection. Samples were kept on ice and out of direct sunlight in a cooler while in the field and then placed in a refrigerator at ~4°C upon return to the lab. Prior to freezing, samples intended for phosphorus analysis were transferred directly to a clear 60-mL HDPE Nalgene bottle. Samples intended for nitrate/nitrite analysis were first filtered through a 0.45 µm nitrocellulose filter to remove intracellular nitrogenous species. Samples were processed using a spectrophotometer. Detection limits were estimated to be 1.5 µg/L-N for nitrogen samples and 0.8 µg/L-P for phosphorus samples. When concentrations were estimated as below detection limits, we replaced values with a random number between 0 and half of the respective minimal detection limit.

*Periphyton Sample Collection*

Periphyton samples were collected at 13 of the 15 sampling locations and contemporaneously with PPCP sample collection that occurred at the end of the month (i.e., second fortnightly sampling campaign within a month). Two sampling locations (Holt and Ducharme) were largely characterized by clay and silt sediments, making standardized, systematic periphyton collection difficult. The remaining 13 locations were comparable with respect to the substrate, which could be characterized as a mix of large, oblate rocks and gravel. As such, we do not include data from HO and DU in our analysis.

At each of the periphyton sampling locations, nine rocks were haphazardly collected and placed into plastic, Ziplock bags within the field. Within 18 h of collection, a plastic stencil was used to define a surface area of each rock from which we scraped a standardized 1.61 cm2 patch of periphyton with a firm bristled toothbrush. Individual samples from three rocks were transferred to 50 mL plastic falcon tubes for abundance estimates. Glutaraldehyde (0.2% final volume) was added to each falcon tube as a preservative. Individual samples from four other rocks were transferred to GF/Fs for ash free dry mass estimates. Following standardized area scrapes, all remaining periphyton on each of the nine rocks was scraped from the rock and into an aluminum tray. The composite periphyton mixture for a given site and sampling time was then placed into 50 mL plastic falcon tubes, which were centrifuged at ~750 rpm for 20 minutes to concentrate periphyton at the bottom of the tube. Using a 5-mL transfer pipette, visibly compacted periphyton and sediment were removed from falcon tubes and transferred to 1.5-mL Eppendorf tubes until a maximum of 10 Eppendorf tubes were filled with periphyton and sediment. These composite periphyton samples were then placed in a -80℃ freezer until processing for stoichiometry and fatty acids.

*Benthic Algal Abundance*

Periphyton taxonomic identification and enumeration was performed by subsampling 30 μL aliquots from each preserved sample using a compound light microscope. For all 30 μL aliquots, cells and filaments were counted, for the entire subsample, until at least 300 cells were identified for a given sampling replicate. If the first aliquot contained less than 300 cells, we counted additional subsamples until we reached at least 300 cells in total. In instances when 300 cells were counted before finishing a subsample, we still counted the entire aliquot. Taxa were classified into broad categories consistent with periphyton algal taxonomy (Biggs and Kilroy 2000), using coarse groupings to capture general patterns in relative algal abundance. As a result, algal groups consisted of diatoms, chlorophytes, cryptophytes, chrysophytes, and cyanobacteria. For consistency, all samples were processed by one person (MFM) to reduce cross-observer error.

*Ash Free Dry Mass*

GF/Fs were removed from whirlpacks, placed on aluminum foil, and transferred to a drying oven at ~96°C overnight. Samples were then relocated to a dessicator for at least 24 h. After samples were dry, samples were weighed to the nearest 0.0001 g, and then placed in a muffle furnace at 550°C for ~1.5 h. After cooling for ~30 minutes, samples were massed again, and pre- and post-ignition were differenced. Samples were processed at the Water Environmental Research Laboratory at Washington State University.

*Periphyton Stoichiometry*

Two 1.5-mL Eppendorf tubes of concentrated periphyton for each sampling location and time were transferred to a foil pan and allowed to thaw and dry in a drying oven at 60°C overnight. Samples were then homogenized and placed in a desiccator for at least one hour prior to weighing.

Two subsamples of completely dried periphyton were transferred to two glass scintillation vials and covered with foil. Particulate carbon and nitrogen measurements could be processed together and required at least 50 mg of tissue, whereas particulate phosphorus required a separate instrument and no more than 5 mg of tissue for analysis. Samples were combusted at ~500°C for 4.5 h, and once cooled, 10 mL of 0.15 M hydrochloric acid was added. Samples were stored at 4°C until analyzed. Particulate carbon and nitrogen were estimated using a Leco CN628 analyzer. Particulate phosphorus was estimated using an Astoria Pacific, A2 segmented flow analyzer with an Astoria Pacific models 311 XYZ autosampler. The method detection limits were estimated to be 2 μg/L-P. Samples were processed at the Freshwater Research Laboratory at Flathead Lake Biological Station.

*Periphyton Fatty Acid Analysis*

All collected periphyton samples in 1.5-mL Eppendorf tubes that were not used for stoichiometric assessment were allocated for fatty acids. Samples were allowed to freeze-dry overnight. Following freeze-drying, samples were transferred to 10 mL glass centrifuge vials, and 2 mL of 100% chloroform was added to each under nitrogen gas. Samples remained in chloroform overnight at -80°C. Fatty acid extractions generally involved three phases: (1) 100% chloroform extraction, (2) chloroform-methanol extraction, and (3) fatty acid methylation. Fatty acid extraction methods were adapted from Schram et al. (2018).

After overnight chloroform extraction, samples underwent a chloroform-methanol extraction three times. To each sample, we added 1 mL cooled 100% methanol, 1 mL chloroform:methanol solution (2:1), and 0.8 mL 0.9% NaCl solution. Samples were inverted three times and sonicated on ice for 10 minutes. Next, samples were vortexed for 1 minute, and centrifuged for 5 minutes (3,000 rpm) at 4°C. Using a double pipette technique, the lower organic layer was removed and kept under nitrogen. After the third extraction, samples were evaporated under nitrogen flow, and resuspended in 1.5 mL chloroform and stored at -20°C overnight.

Once resuspended in chloroform, 1 mL of chloroform extract was transferred to a glass centrifuge tube with a glass syringe as well as an internal standard of 4 μL of 19-carbon fatty acid. Samples were then evaporated under nitrogen, and then 1 mL of toluene and 2 mL of 1% sulfuric acid-methanol was added. The vial was closed under nitrogen gas and then incubated in a 50°C water bath for 16 hours. After incubation, samples were removed from the bath, allowed to reach room temperature and stored on ice. Next, we performed a potassium carbonate-hexane extraction twice. To each sample, we added 2 mL of 2% potassium bicarbonate and 5 mL of 100% hexane, inverting the capped vial to mix the solution. Samples were centrifuged for 3 minutes (1,500 rpm) at 4°C. The upper hexane layer was then removed and placed in a vial to evaporate under nitrogen flow. Once almost evaporated, 1 mL of 100% hexane was added and stored in a glass amber autosampler vial for GC/MS quantification. GC/MS quantification was performed with a Shimadzu QP2020 GC/MS following Schram et al. (2018). Samples were processed at the Community Trophic Ecology Laboratory at the Oregon Institute for Marine Biology.

*Statistical Analysis*

To evaluate how lakeside development and tourism season may be associated with human activity and sewage indicator abundance, we tested whether total PPCP concentration, abundance of branched- and odd-chain fatty acids as indicators of bacteria, nitrogen and phosphorus concentrations, as well as ash free dry mass changed across sites and timepoints using ANOVA with sewage treatment technique (i.e., decentralized or centralized) and tourism season (i.e., in-season, out-of-season) and their interaction as predictors. To characterize patterns, we averaged a given site’s sewage indicator values observed in and out of the tourism season. Total PPCP and nutrient concentrations were log-transformed prior to analysis, as we assumed these variables were likely subject to non-linear physical processes, such as mixing and diffusion. Branched- and odd-chain fatty acid proportions were arcsine-square root-transformed prior to analysis to meet assumptions of normality. “In Season” samples were defined as samples collected in June, July, or August, and “Out of Season” samples were defined as samples collected in September. Months were classified as “In Season” or “Out of Season” based on the number of visitor passes sold in a particular month relative to the number sold in May, which we assume as being either before or at least at the beginning of the tourism season. Predictors were treated as fixed effects, and because the sampling design was unbalanced, we used a Type II SS formulation (Langsrud 2003). Raw data as well as model residuals were assessed for normality and homogeneity of variance.

To determine how spatial and temporal variation in lakeside development and human activity may influence periphyton community composition, we first performed a non-metric multidimensional scaling (NMDS) with Bray-Curtis similarity, and then visualized the NMDS in two-dimensional space. Because chrysophytes and cryptophytes were rare (i.e., < 2% of the intersite abundance), we omitted these taxa from analysis and visualization. To test differences among relative abundance of diatoms, chlorophytes, and cyanobacteria, we performed a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) with 4,999 permutations, where sewage treatment technique (i.e., decentralized or centralized) and tourism season (i.e., in-season, out-of-season) were predictors of relative periphyton community abundance. Unlike traditional multivariate analyses of variance (MANOVA), PERMANOVA does not require assumptions of multivariate normality (Anderson 2001). When significant differences were identified, post-hoc SIMPER analysis (Clarke 1993) was performed to identify which taxonomic groups most influenced site separation.

To assess variation in periphyton nutritional content, we performed ANOVAs with sewage treatment technique, tourism season and their interaction as predictors of periphyton stoichiometric ratios and relative fatty acid abundances. For stoichiometry, we assessed Carbon:Nitrogen, Carbon:Phosphorus, and Nitrogen:Phosphorus ratios. For fatty acids, we evaluated relative saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acid abundance, where proportions were arcsine-square root-transformed to improve normality.

To understand how sewage treatment technique and the tourism season may influence multivariate essential fatty acid signatures, we performed NMDS with Bray-Curtis similarity of all essential fatty acids constituting more than 1% of intersite proportions, and then visualized the NMDS in two-dimensional space. To test differences between relative essential fatty acid abundance in samples, we performed a PERMANOVA with 4,999 permutations, where sewage treatment technique and tourism season were predictors of the entire essential periphyton fatty acid profile. When significant differences were identified, post-hoc SIMPER analysis was performed to identify which fatty acids most influenced site separation.

All data aggregation routines and statistical analyses were performed using the R Statistical Environment (R Core Team 2019) with the tidyverse (Wickham et al. 2019), vegan (Oksanen et al. 2019), car (Fox and Weisberg 2019), lubridate (Grolemund and Wickham 2011), stringi (Gagolewski 2020), janitor (Firke 2020), ggpubr (Kassambara 2019), ggrepel (Slowikowski 2019), viridis (Garnier 2018), rnaturalearth (South 2017), ggspatial (Dunnington 2021), ggstar (Xu 2021), cowplot (Wilke 2019), and sf (Pebesma 2018) packages. All R code can be accessed from this project’s GitHub Repository (https://github.com/mishafredmeyer/flathead\_sewage), and all associated data can be found on this project’s Open Science Framework portal (Meyer et al. 2021). All R scripts were written by one person (MFM) and then independently reviewed by another co-author (MRB) to assess scripts for accuracy, reproducibility, and clarity of comments.

**Results**

*Temporally Scaled Inverse Distance Weighted (TSIDW) Population*

TSIDW tended to fluctuate through space and time in a way that reflected static and transient human populations, suggesting that our TSIDW metric effectively captured patterns in human population around Flathead Lake. Spatially, TSIDW population was higher in the northeastern part of the lake, which is likely due to the town of Bigfork being a larger population hub (Figure 2). Temporally, TSIDW population highlighted fluctuations in human population in the southernmost portion of Flathead Lake, near the town of Polson (Figure 2). Generally, TSIDW population values tended to decrease with the tourism season more than with differing sewage treatment technique (Figure 4A).

*Water Samples*

In general, water column nutrient concentrations did not differ across sites and timepoints. Nitrate/nitrite, total nitrogen, total phosphorous, and, to some extent, soluble reactive phosphorus (SRP) as well as ammonia concentrations were not different based on sewage treatment technique, tourism season, or interaction between site and tourism season (summarized in Table S1). SRP concentrations tended to be higher at sites with centralized sewage treatment relative to those near decentralized wastewater treatment, but neither sewage treatment technique (F = 3.313, p = 0.081; Table S1), tourism season (F = 0.009, p = 0.923; Table S1), nor the interaction between them (F = 0.114, p = 0.739; Table S1) were statistically significant. Ammonia (as nitrogen) concentrations likewise were higher at sites near centralized treatments relative to those near sites with decentralized treatment, although neither sewage treatment (F = 3.347, p = 0.080; Table S1), tourism season (F = 0.454, p = 0.507; Table S1), nor the interaction between them (F = 1.040, p = 0.318; Table S1) were statistically significant. Notably, a large majority of water column SRP concentrations (82% of samples) were below detection limits (<0.8 µg/L). In comparison to SRP, 27% of nitrate/nitrite, 20% of total phosphorus, and 3% of ammonia samples were below detection limits. No total nitrogen samples were below detection limits.

PPCPs detected within the water column included caffeine, paraxanthine/1,7-dimethylxanthine, acetaminophen/paracetamol, cotinine, cimetidine, and warfarin. PPCPs not detected included carbamazepine, codeine, diphenhydramine, sulfamethoxazole, thiabendazole, and trimethoprim. Total PPCP concentrations tended to be higher during the tourism season (F = 3.915, p = 0.059), although this result did not meet thresholds for statistical significance (i.e., p < 0.05; see Table S2). Neither sewage treatment technique (F = 0.649, p = 0.428) nor the interaction between sewage treatment and tourism season (F = 2.085, p = 0.161) were significant predictors of total PPCP concentration (summarized in Table S2).

*Periphyton Community Composition*

Periphyton community composition largely consisted of chlorophytes, cyanobacteria, and diatoms (Figure 5), with cyanobacteria abundance being higher at sites with decentralized wastewater treatment. NMDS suggested differentiation of chlorophyte and diatom abundance along the horizontal axis that potentially reflects temporal patterns, such as tourism season and community succession. In vertical space on the NMDS, relative cyanobacteria abundance helped differentiate sites as well, potentially reflecting sewage treatment technique, as sites near centralized sewage treatment tended to cluster (Figure 6). PERMANOVA results, however, suggested that groupings based on sewage treatment technique (F = 1.554, p = 0.160) and tourism season (F = 1.930, p = 0.098) were not statistically different (summarized in Table S3).

*Periphyton Stoichiometry*

Periphyton C:N, C:P, and N:P molar ratios varied widely across sites and timepoints. Most values were above stoichiometric ratios for sustained periphyton growth (119:17:1; Hillebrand and Sommer 1999). Neither C:P nor N:P ratios differed based on sewage treatment technique, tourism season, or interactions between tourism season and sewage treatment technique (Figure 7B, 7C; summarized in Table S4). C:N ratios were significantly lower at sites with centralized wastewater treatment in comparison to decentralized wastewater treatment (F = 5.830, p = 0.022) but did not differ with tourism season (F = 0.015, p = 0.904) nor interactions between tourism season and sewage treatment technique (F = 0.051, p = 0.823).

*Periphyton Fatty Acids*

To characterize benthic bacterial communities, our samples contained 15- and 17-Carbon saturated as well as 15-Carbon branched fatty acids. ANOVA results suggested that relative abundance of branched and odd-chain fatty acids was higher during the tourism season than outside of the tourism season (F = 4.565, p = 0.045). There was no difference in relative branched and odd-chain fatty acids based on sewage treatment technique (F = 0.307, p = 0.585) nor the interaction of sewage treatment technique and tourism season (F = 0.353, p = 0.559).

Other fatty acid groups, such as saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids, also varied across sites and timepoints but not between interactions of sewage treatment and time (Figure 8; Table S5). In particular, PUFAs increased in relative abundance following the tourism season (F = 14.470, p = 0.001) but did not change between types of sewage treatment (F = 0.160, p = 0.692) nor the interaction of sewage treatment and tourism season (F = 0.256, p = 0.617). Likewise, SAFAs decreased following the tourism season (F = 20.529, p < 0.001) and did not differ between types of sewage treatment (F = 0.070, p = 0.793) nor with interactions of sewage treatment and tourism season (F = 1.618, p = 0.214). MUFAs remained consistent across sewage treatment types (F = 0.046, p = 0.832), throughout the tourist season (F = 1.016, p = 0.322), and between interactions of sewage treatment and tourism season (F = 0.249, p = 0.622).

Deviations in PUFA abundance were largely driven by changes in essential fatty acid (EFA) relative abundance, which were likely reflective of differences in periphyton community composition. The EFAs with the highest coefficients of variation included 20:5ω3, 18:2ω6, and 18:3ω3. Periphyton EFAs at sites with centralized sewage treatment appeared to have more consistent proportions through time, whereas sites with decentralized treatment tended to have higher proportions of 20-Carbon EFAs, such as 20:5ω3. When analyzed in a multivariate framework (Figure 10A), visual inspection of the NMDS suggested that fatty acids more associated with chlorophytes (i.e., 18:2ω6, and 18:3ω3) and fatty acids more associated with diatoms (i.e., 20:5ω3) tended to separate in the horizontal dimension while changes with the tourism season were more apparent along the vertical axis. PERMANOVA results (summarized in Table S3) suggested tourism season as significant in discriminating groups (F = 2.775, p = 0.006), but sewage treatment technique was not significant (F = 0.726, p = 0.676). Post-hoc SIMPER analyses suggested 20:5ω3 as the most influential discriminating fatty acid (Cumulative Variance = 37.6%, p = 0.04). Together, 20:5ω3, 18:2ω6, and 18:3ω3 accounted for more than 85% of the total variance.

**Discussion**

Our combined results corroborate previous findings (e.g., Makepeace and Mladenich 1996) that sewage pollution is entering Flathead Lake’s nearshore area and likely is responsible for differences in nearshore periphyton community composition and nutritional content. Unlike previous studies in Flathead Lake (Stanford et al. 1994a; Makepeace and Mladenich 1996), we incorporated highly specific indicators of sewage pollution as well as periphyton nutritional content to describe how sewage pollution entering Flathead may influence periphyton stoichiometry and fatty acid composition. Thus, our results provide direct, quantitative evidence that sewage entering Flathead may alter nutrition available to the lake’s larger food web.

*Relating human population with sewage indicators*

In agreement with our expectations, sewage-specific indicators tended to increase during the tourism season, when lakeside human populations were larger. These patterns are noteworthy as Flathead’s watershed is largely unpopulated, but permanent and seasonal human populations have been growing since the 1950s (U.S. Census, 2020). In particular, many lakeside developments around Flathead Lake rely upon septic systems for wastewater treatment (Stanford et al. 1994a), and our data suggest that during the tourism season, sites with decentralized sewage treatment tended to have PPCP concentrations comparable to those near centralized treatment. In contrast, sites with centralized sewage treatment tended to have more temporally consistent sewage indicators throughout the summer. Furthermore, spatial and temporal patterns in sewage indicator concentrations mirrored patterns observed in TSIDW population, suggesting that our sampling successfully captured heterogeneous sewage loadings into Flathead’s nearshore.

Patterns in nutrient concentrations alone were not fully reflective of patterns observed with more specific sewage indicators such as PPCPs (Figure 3). These incongruencies are likely due to nutrients originating from non-sewage sources as well as nutrients being actively removed from and released into the water column through physicochemical and biochemical processes. For example, allochthonous nutrients can originate from fertilizers applied in directly adjacent lakeside orchards (Stanford et al. 1994a), pollen from conifers (Graham et al. 2006), and even forest fires (Gould et al. 2016) or atmospheric deposition more broadly (Elser et al. 2009). Throughout the course of our sampling campaign, wildfires, significant pollen deposition, and lakeside agriculture all occurred and likely contributed nutrients to Flathead’s nearshore communities, but these are currently not known to contribute significant nutrient loadings in Flathead Lake relative to sewage.

To our knowledge, this is the first study to detect PPCPs in Flathead Lake, a high-volume lake with a short hydraulic residence time relative to its size (Messager et al. 2016), and located in a largely unpopulated and undeveloped watershed (Stanford et al. 1994a). Previous investigations of sewage pollution into groundwater near Kalispell, Montana (the watershed’s most populous city with ~23,000 people; U.S. Census) have highlighted the potential for PPCPs to occur within Flathead’s watershed but from distal developments (Tappenbeck and Ellis 2010, 2011). Furthermore, unknown factors include: (1) how subsurface PPCP concentrations within Flathead’s basin may enter surface flows through groundwater-surface water connections or (2) how PPCPs contributed from Kalispell may affect subsurface, hyporheic, and potentially the nearshore biota such as stonefly larvae (Stanford et al. 1994b; Stewart 2002; Malison et al. 2020). Our sampling scheme included one location within the Flathead River (HO; Figure 1), which runs through Kalispell and is the main surface input into Flathead Lake. Although we detected PPCPs at this site, concentrations were lower than those observed near the lakeside development of Bigfork, implying that concentrations observed in Flathead Lake likely originated from adjacent lakeside settlements and were not contributed from distal sources. More broadly, our results suggest that wastewater infrastructure as well as the size and temporal variation of human populations at adjacent lakeside developments can contribute spatially and temporally varying sewage inputs, thereby creating hot spots as well as hot moments of sewage pulses into the nearshore.

Beyond Flathead Lake, these data and results are important for understanding PPCP prevalence in lakes and sewage contributions from decentralized wastewater treatment. Despite rapid worldwide growth in the PPCP literature over the past three decades, both lakes and forms of decentralized sewage treatment have remained far less represented in the literature relative to lotic environments and forms of centralized wastewater treatment (Meyer et al., 2019). These imbalances may be important for shaping our understanding of PPCPs in the environment. Lakes tend to have longer hydraulic residence times relative to lotic systems, meaning that pollutants within sewage may concentrate and elicit stronger biological responses. Likewise, decentralized wastewater treatment can contribute significant nutrient (Moore et al. 2003; Rosenberger et al. 2008; Hampton et al. 2011; Withers et al. 2014) and micropollutant (Meyer et al.; Bendz et al. 2005) loadings to proximal aquatic systems. Adding to this complexity, PPCPs contributed from septic systems have demonstrated complex transport dynamics that may influence their residence time, where some PPCPs, such as caffeine, can pulse through subsurface systems and enter into surface flows, yet others, such as acetaminophen, can sorb to proximal colloidal materials, thereby creating PPCP hotspots (Yang et al. 2016). Considering that more than half of the world’s population utilizes some form of decentralized wastewater treatment (Withers et al. 2014), the current uncertainties around PPCP prevalence, PPCPs’ potential to accumulate and create pollution hot spots, and PPCPs’ often uncertain biological effects, our data underscore the potential for PPCP concentrations to vary spatially and temporally in lakes and highlight the need for continued study of the biophysical processes influencing their abundance.

While our study focused on using PPCPs as indicators of sewage, PPCPs themselves can cause deleterious ecological consequences, even at concentrations we observed in Flathead (e.g., < 10 ng/L; Feijão et al. 2020). Biotic responses can include physiological (del Rey et al. 2011) and behavioral (Brodin et al. 2013) consequences as well as food web (Meador et al. 2016; Lagesson et al. 2016; Richmond et al. 2018) and ecosystem (Rosi-Marshall et al. 2013) alterations. Although our study was not designed to evaluate ecotoxicological effects of PPCPs on periphyton communities nor higher trophic levels, future studies could build on this study as well as the empirical results of others to address how wastewater treatment infrastructure and variation in human population can create diverse mixture of PPCPs that affect biological communities differently across various spatial and temporal scales.

*Relating human population with periphyton community composition*

Congruent with our hypotheses, areas and timepoints with increased human population and sewage indicator abundance tended to be associated with increased filamentous chlorophyte taxa. These same patterns have been noticed in the nearshore of other large, deep, oligotrophic lakes, such as Lake Baikal (Meyer et al.; Timoshkin et al. 2016, 2018; Volkova et al. 2018), Lake Crescent (Rosenberger et al. 2008; Hampton et al. 2011), Lake Pend Oreille and Priest Lake (Rosenberger et al., 2008), and Lake Tahoe (Naranjo et al. 2019; Atkins et al. 2021). In addition to sewage inputs providing increased nutrients, filamentous chlorophyte abundance in Flathead may also be responding to other nutrient co-limitation dynamics (Marks and Lowe 1993), seasonal shifts in light availability (Marks and Lowe 1993), and increasing surface temperatures (Thrane et al. 2017; Pilla et al. 2020). While much understanding of algal successional patterns is based on phytoplankton dynamics (Sommer et al. 1986, 2012), periphyton appear to undergo similar phenological shifts from spring through autumn (McCormick and Stevenson 1991; Stevenson et al. 1996; Azim et al. 2005). Some complications in understanding periphyton community succession patterns, relative to those of the pelagic algae, may emerge from complex algal life histories and adaptation such as diatom mucilage and stalk development (Roemer et al. 1984) or variation in mat thickness (Dodds et al. 1999). Recent syntheses have suggested that increased allochthonous nutrients into oligotrophic lakes’ littoral zones may be responsible for increased filamentous algal abundance worldwide and eventually contribute to nuisance filamentous algal blooms (Vadeboncoeur et al. 2021). In addition, other mechanisms may contribute to these patterns. For example, seasonal wave action can transport hypolimnetic nutrients to the littoral zone and stimulate increased filamentous algal production (reviewed in Vadeboncoeur et al. 2021). Likewise, shifting environmental conditions, such as temperature or dissolved oxygen, may limit the capacity for macroinvertebrates to graze, allowing for quickly growing, filamentous algae to outcompete diatom taxa (reviewed in Vadeboncoeur et al. 2021). Regardless of the exact mechanism occurring in Flathead Lake, our algal abundance data are consistent with other nearshore sewage-associated eutrophication patterns observed in other, large, deep, oligotrophic systems (e.g., Meyer et al.; Timoshkin et al. 2016, Rosenberger et al. 2008; Hampton et al. 2011). By mirroring patterns in sewage-specific indicator abundance, our data further suggest that relative abundance of filamentous taxa tends to be greatest near areas and during times of increased human activity at lakeside developments.

Beyond algal community composition, relative abundance of branched- and odd-chain fatty acids, which are highly specific indicators of bacterial communities, mirrored patterns observed in total PPCP concentrations and algal taxonomic abundances. While bacteria are core constituents of biofilm communities (Azim et al. 2005), their relative abundance can be useful for inferring sewage pollution (Seguel et al. 2001; Rocchetta et al. 2014). Similar studies at Lake Baikal measured *E. coli* and *Enterococcus* abundances near areas suspected of increased sewage pollution and noted that these areas tended to have higher fecal indicator bacteria abundances (Timoshkin et al. 2016). While branched- and odd-chain fatty acids can suggest bacterial abundance (Seguel et al. 2001; Rocchetta et al. 2014), the diversity of branched- and odd-chain fatty acids observed in our data could not identify specific taxonomic groups. These differences in heterotroph abundance may be consequential for autotrophic and heterotrophic productivity. Rosi-Marshall et al. (2013), for example, demonstrated how caffeine can act as generalist toxicant and reduce community respiration by reducing abundance of the entire community, whereas diphenhydramine can act as a specialist toxicant and reduce community respiration by reducing certain taxa’s relative abundance. In the context of our study, patterns in periphyton branched- and odd-chain fatty acids underscore the potential for heterogeneities in sewage loading to influence benthic bacterial communities. Our data, in addition to the uncertainties in how sewage may influence heterotroph composition and productivity in lotic and lentic systems (e.g., Rosi-Marshall et al. 2013; Shaw et al. 2015), suggest that non-autotrophic responses to sewage pollution warrant increased research emphasis.

*Relating human population with periphyton nutritional content*

Fatty acid analyses suggested that changes in periphyton community composition altered periphyton nutritional quality across sites and timepoints. Periphyton fatty acids at sites with centralized sewage treatment tended to have consistent fatty acid ratios through time, although one site following the tourism season did have increased PUFA proportions (YB; Figure 1). In contrast, periphyton fatty acid profiles at sites with decentralized sewage treatment tended to show a tradeoff of SAFAs and PUFAs throughout the course of the summer, where SAFAs decreased and PUFAs increased in relative abundance (Figure 8). These patterns are likely reflective of changes in the periphyton community composition, as algal taxa tend to have strongly differentiated multivariate fatty acid signatures (Taipale et al. 2013; Galloway and Winder 2015).

Among all PUFAs detected, increased PUFA abundance at decentralized sites following the tourism season was driven by increases in diatom-associated essential fatty acids (EFA). EFAs comprise a subgroup of PUFAs that accumulate in organisms, are mainly synthesized by primary producers, and can provide vital nutrition to higher trophic levels (Iverson et al. 2004; Kelly and Scheibling 2012). Among the eight common essential fatty acids, 18:3ω3, 18:2ω6, and 20:5ω3 had the highest coefficients of variation across sites and timepoints. Both 18:3ω3 and 18:2ω6 are associated with chlorophytes; 20:5ω3 is consistently associated with diatoms (Taipale et al. 2013; Galloway and Winder 2015). Comparing taxonomic compositions with fatty acid profiles, the increased diatom presence relative to chlorophytes at decentralized sites following the tourism season was likely responsible for increased 20:5ω3 abundance (Figure 9). Together, our analysis of sewage indicators, periphyton composition, and fatty acid results suggest that Flathead’s nearshore periphyton communities near centralized sewage treatment may experience more temporally consistent sewage loading that likely supports a consistent assemblage of chlorophytes and diatoms, and therefore these sites maintain a more temporally consistent fatty acid signature. In contrast, these same results suggest that Flathead’s nearshore periphyton communities near decentralized sewage treatment tend to experience more inconsistent sewage loading that likely allows for tradeoffs in chlorophyte, cyanobacteria, and diatom relative abundance. Therefore, these sites may maintain a temporally inconsistent fatty acid signature. In particular, sites with decentralized treatment tended to experience increased PUFA abundance, especially 20:5ω3, following the tourism season, which could provide necessary nutrition to higher trophic levels as they prepare to overwinter.

In contrast to fatty acids, periphyton stoichiometric ratios tended to be lower at sites with centralized treatment, implying that sites with centralized treatment have more nutritious periphyton. Unlike fatty acids, periphyton stoichiometric ratios do not to map to community composition, and furthermore, periphyton stoichiometric ratios may be influenced by ambient water column nutrient concentrations (Frost and Elser 2002), production of extracellular matrices or mucilage tubes (Frost et al. 2005), and variable nutrient uptake rates (Oleksy et al. 2021). Among each of the stoichiometric ratios considered, C:N ratios were the only ones that differed across locations and timepoints. The lack of differences between sites and timepoints for C:P and N:P ratios is not necessarily surprising, as phosphorus is usually a limiting element in oligotrophic Flathead (Axler and Reuter 1996). Conversely, C:N ratios were significantly lower at sites with centralized treatment than those at sites with decentralized treatment (Figure 7A). Mechanistically, the difference in C:N potentially stems from chlorophytes’ increased abundance at sites with centralized treatment, as periphyton assemblages containing chlorophytes have been associated with increased nitrogen uptake (Nydick et al. 2004; Hogan et al. 2014; Oleksy et al. 2021). Temporally, stoichiometric ratios did not differ. Thus, the combined results of our sewage indicator, periphyton community composition, and stoichiometric data suggest that algal communities near centralized wastewater treatment, can create temporally consistent, stoichiometrically nutritious periphyton assemblages. In contrast, the inconsistent sewage loading at sites with decentralized treatment may create stoichiometrically less nutritious periphyton assemblages.

Although differences in periphyton fatty acids and stoichiometries appear to tell diverging narratives about periphyton nutritional content in response to spatio-temporally heterogeneous sewage loading, each interpretation can be valid when considering the grazer guilds that might be consuming the periphyton. Through a stoichiometric lens, our results suggest that stoichiometric measures of nutrition vary more in space than in time. Sites near centralized wastewater treatment tended to have temporally consistent, low C:N periphyton that largely contained chlorophytes and diatoms. This periphyton assemblage may favor generalist grazers capable of consuming filamentous taxa as well as detritivores capable of consuming decomposing filamentous strands. Because these low C:N ratios and periphyton community composition appear to remain consistent throughout a growing season, these resources are likely to be reliable for consumer guilds throughout a summer. Through a fatty acid lens, our results suggest that fatty acid markers of nutrition vary more in time than in space. Following the tourism season, increased EFA abundance, particularly increased 20:5ω3 abundance at sites near decentralized wastewater treatment, was likely associated with increased diatom and decreased chlorophyte abundance. This periphyton assemblage may support grazers that require higher order PUFAs, which can be crucial for maintaining membrane fluidity (Nichols et al. 1993) as grazers prepare to overwinter. Because increased diatoms and 20:5ω3 tended to co-occur with decreased sewage indicator abundance, these resources may be temporally transient and potentially vulnerable to prolonged or enhanced sewage loading that could arise through an extended tourism season or increased lakeside development.

*Conclusions*

Over the past century, Flathead Lake’s watershed has experienced consistent growth in its permanent and seasonal human population (U.S. Census 2019). Although Flathead’s depth, volume, and short hydraulic residence time have likely aided in keeping the pelagic waters oligotrophic, there are distinct signs of eutrophication in the nearshore, particularly near areas with larger human populations. While Flathead receives nutrients from multiple sources, our repeated sampling of sewage specific indicators and co-located biological data throughout the lake implicate wastewater pollution as one of the nutrient sources. Our results corroborate previous work by Makepeace and Mladenich (1996), demonstrating how hot spots and hot moments of sewage pollution can occur within Flathead Lake’s nearshore. Unlike previous studies, our work pairs human population, highly specific sewage indicator, periphyton community composition, fatty acid, and stoichiometric data to understand how differences in human disturbance are associated with differences in algal community structure as well as nutrition available to the lake’s larger food web. While our data do not include higher trophic levels’ response to changing resources, our data do suggest that grazing macroinvertebrate communities would need to respond in some substantial way, either by migrating to where nutritious resources may be more abundant or altering community composition entirely to include more generalist or detritivorous grazers. In either event, our results suggest grazers requiring nutrition from diatom assemblages may be more vulnerable to increasing sewage pollution, especially in cases when tourism seasons may be extended or lakeside developments with decentralized sewage treatment increase in size.

*Beyond Flathead: A case for increased nearshore monitoring*

Our results stress the importance of nearshore monitoring for detecting sewage inputs in large lakes. Flathead Lake’s pelagic is considered oligotrophic (Ellis et al. 2011), but increased tourism and development along the shoreline and within the watershed may increase potential for eutrophication hot spots or hot moments within the nearshore. While pelagic samples are useful for describing the lake’s overall status, nearshore sampling can aid managers in identifying pollution sources before the entire system is affected. Flathead’s large volume, short hydraulic residence time, and general lack of intense development likely buffer the system from abrupt perturbations. However, once eutrophication of the pelagic has started, mitigation and restoration can be complex (Jeppesen et al. 2005) and require system-specific interventions (Jacoby et al. 1991) with long term strategies (Tong et al. 2020). Several other large, deep, oligotrophic lakes, such as Baikal (Timoshkin et al 2016; 2018; Bondarenko et al. 2021; Meyer et al, Under Revision), Tahoe (Njora et al., 2019; Atkins et al., 2021), and Superior (Camillieri & Ozerky, 2019), have likewise experienced localized eutrophication in the nearshore. Many of these cases implicated sewage as the source of increased nutrient concentrations and filamentous algal abundance. However, incorporating sewage specific indicators, such as PPCPs as well as branched- and odd-chain fatty acids, may be necessary to directly associate ecological consequences with sewage pollution. As observed in Flathead Lake, repeated PPCP sampling throughout the lake has the potential to define areas and times of greatest concern, especially in instances where wastewater treatment infrastructure and temporal swings in human population may vary. By pairing human population and sewage indicator data with co-located, contemporaneous biological data, managers can better contextualize and mitigate ecological consequences before they affect the entire lake. When compared across lakes, these same data can be useful tools to synthesize ramifications of sewage pollution, thereby enabling coordination between local, regional, and national management efforts.

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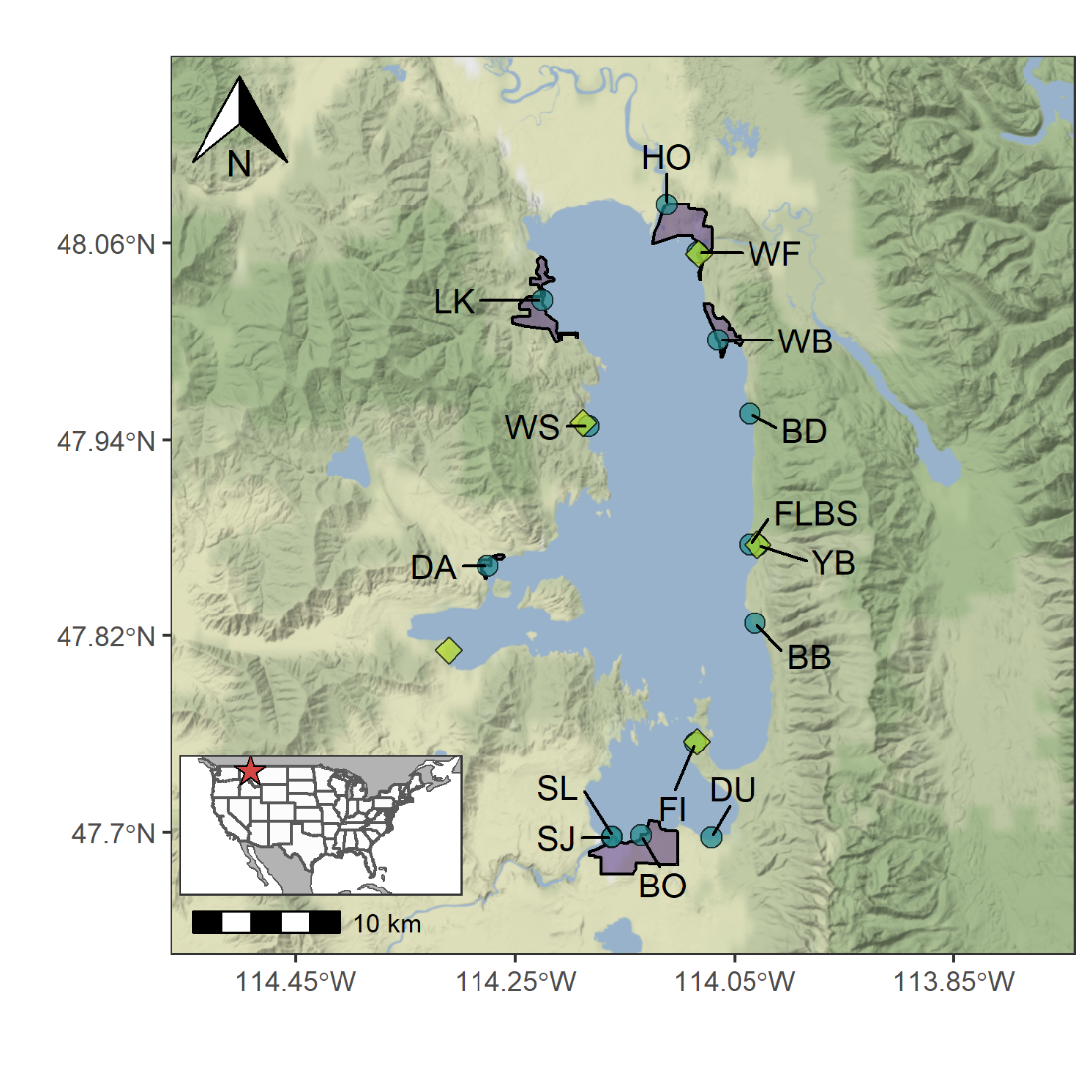


Figure 1: Map of sampling locations, State Parks, and major lakeside developments. Sampling locations are denoted by the blue circles and are labeled with the site name. HO and DU were considered reference sites, where HO was sampled within the Flathead River before it enters Flathead Lake and DU was sampled where there was no adjacent lakeside development. Green diamonds correspond to locations of five State Parks, from which we gather monthly data on visitor passes sold. Many sampling locations (blue circles) and State Parks (green diamonds) are co-located, and their respective symbols may overlap. Purple polygons refer to area delineated for five major lakeside developments (Bigfork, Lakeside, Wood’s Bay, Dayton, and Polson).

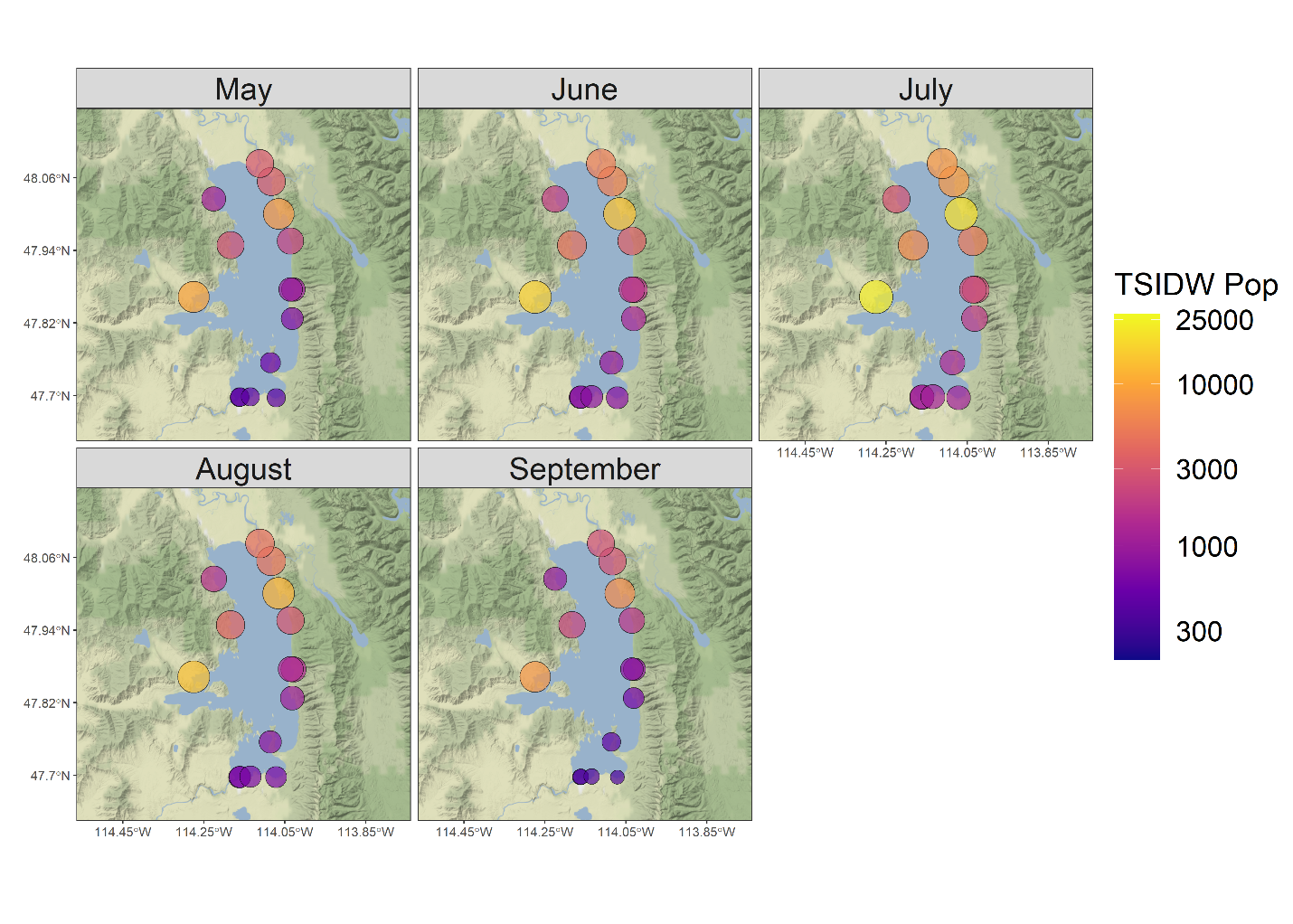


Figure 2: Temporally scaled inverse distance weighted (TSIDW) population for each sampling location and month. Larger, yellow points indicate sites with high TSIDW populations. Smaller, more purple points represent sites with lower TSIDW populations. Spatially, TSIDW population tended to be highest in the northeast portion of the lake, near the towns of Bigfork and Wood’s Bay. Temporally, TSIDW population tended to be highest in July. TSIDW population values have been log-transformed to make sites more comparable across multiple orders of magnitude.

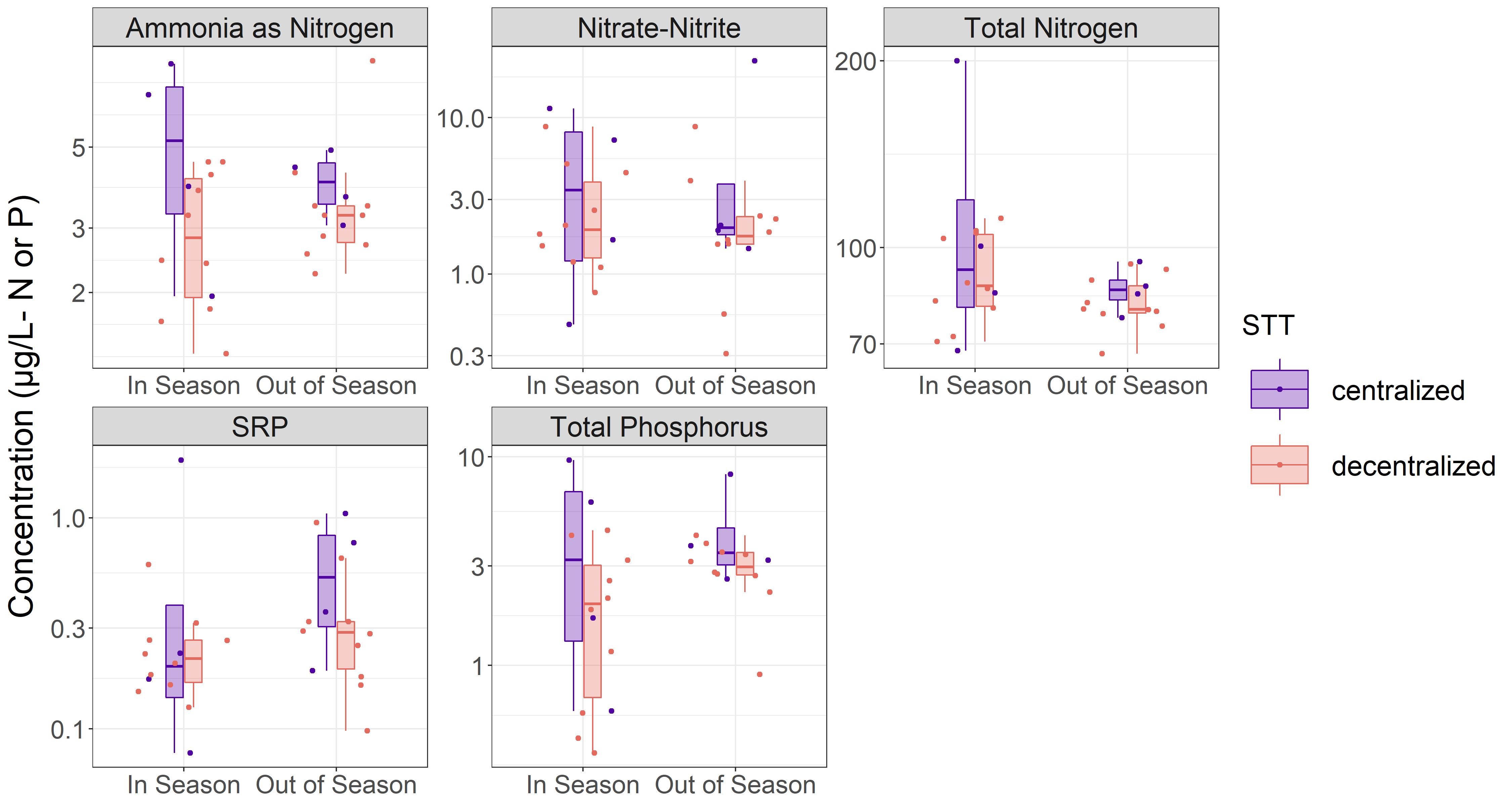


Figure 3: Littoral water column nutrient concentrations observed at each of the sampling locations. Concentrations are visualized on a log-scaled y-axis. While most nutrient concentrations were consistent between sewage treatment techniques and timepoints, SRP and Ammonia were often higher near sites with centralized sewage treatment in comparison to decentralized sewage treatment, although this may be a product of detection limits. When nutrient concentrations were below minimal detection limits (1.5 µg/L for ammonia, nitrate/nitrite, and total phosphorus; 25 µg/L for total nitrogen; and 0.8 µg/L for SRP), we assigned a random value between zero and half of the minimal detection limit.

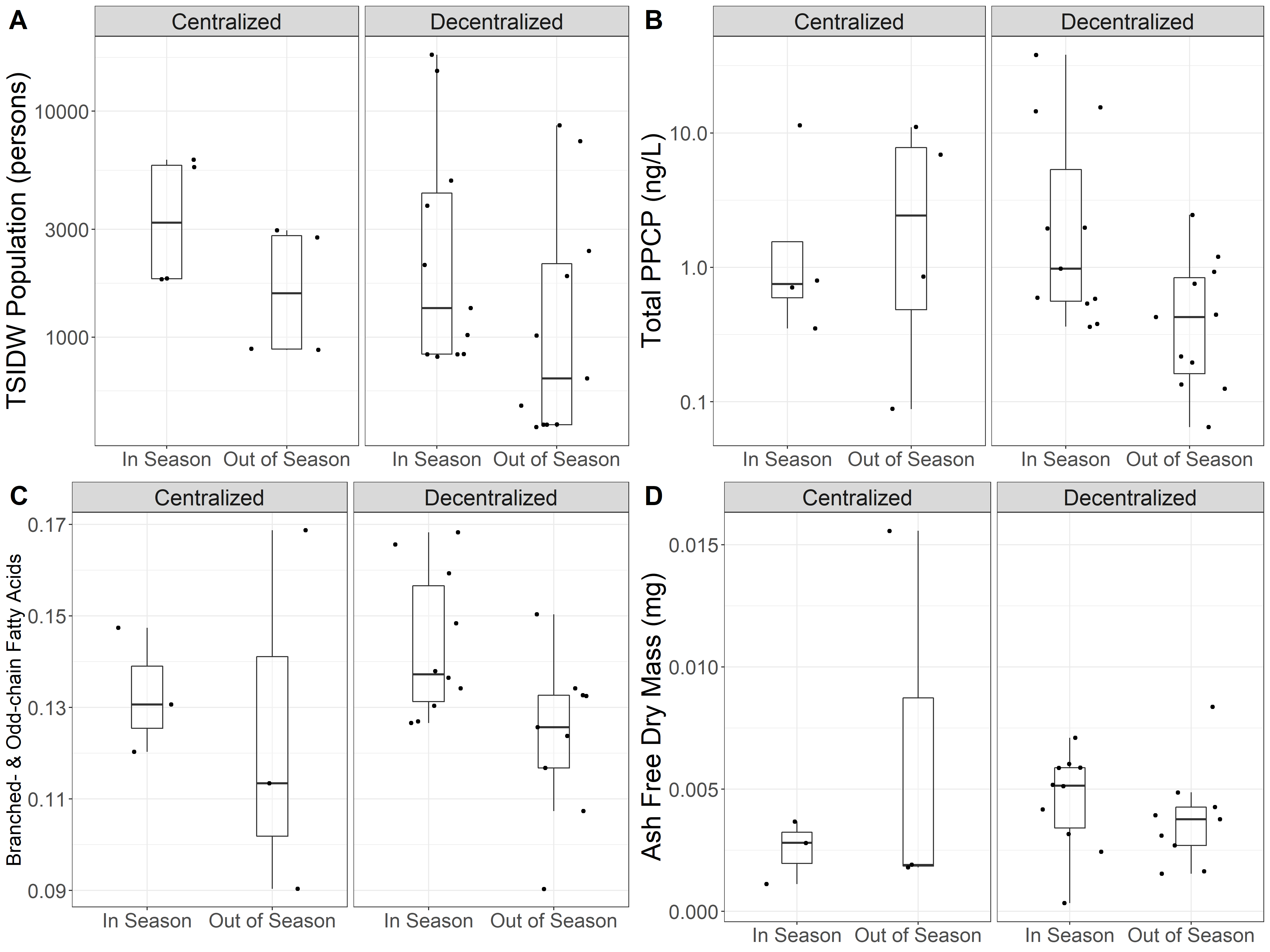


Figure 4: Boxplots of TSIDW population (A), total PPCP concentration (B), relative abundance of branched- and odd-chain fatty acids (C), and Ash Free Dry Mass (D) in response to tourism season (x-axis) and sewage treatment technique (faceted axis). To characterize overarching patterns, values are averaged within a sewage treatment technique and season for a given site. TSIDW Population (A) generally was higher at sites with centralized wastewater treatment and also decreased following the tourism season both for sites with centralized and decentralized wastewater treatment. Total PPCP concentrations (B) were often higher at sites with decentralized wastewater treatment during the tourism season than at those same sites following the tourism season. Periphyton branched- and odd-chain fatty acids (C) were higher during tourism season both for sites with centralized and decentralized wastewater treatment. These fatty acids are indicative of bacterial communities, which would be expected to be more abundant during times and at locations of increased wastewater loading. Ash Free Dry Mass (D), a proxy for periphyton productivity and detritus biomass, was consistent between sites in relation to sewage treatment technique and tourism season.

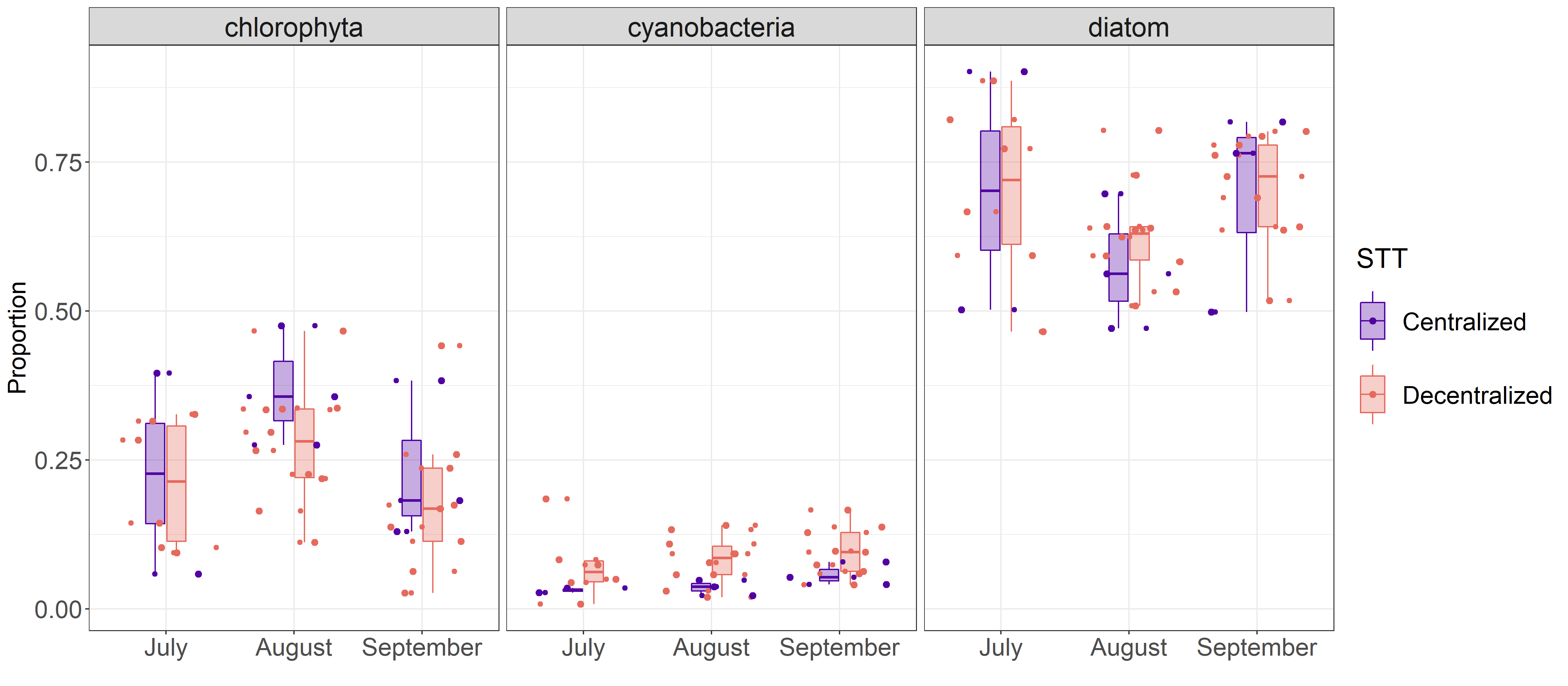


Figure 5: Boxplots of algal taxonomic group proportional abundance throughout the course of a summer. Sites with centralized wastewater treatment were generally dominated by chlorophyte and diatoms, whereas sites with decentralized treatment tended to have a more marked cyanobacteria abundance that gradually increased over the course of a summer.

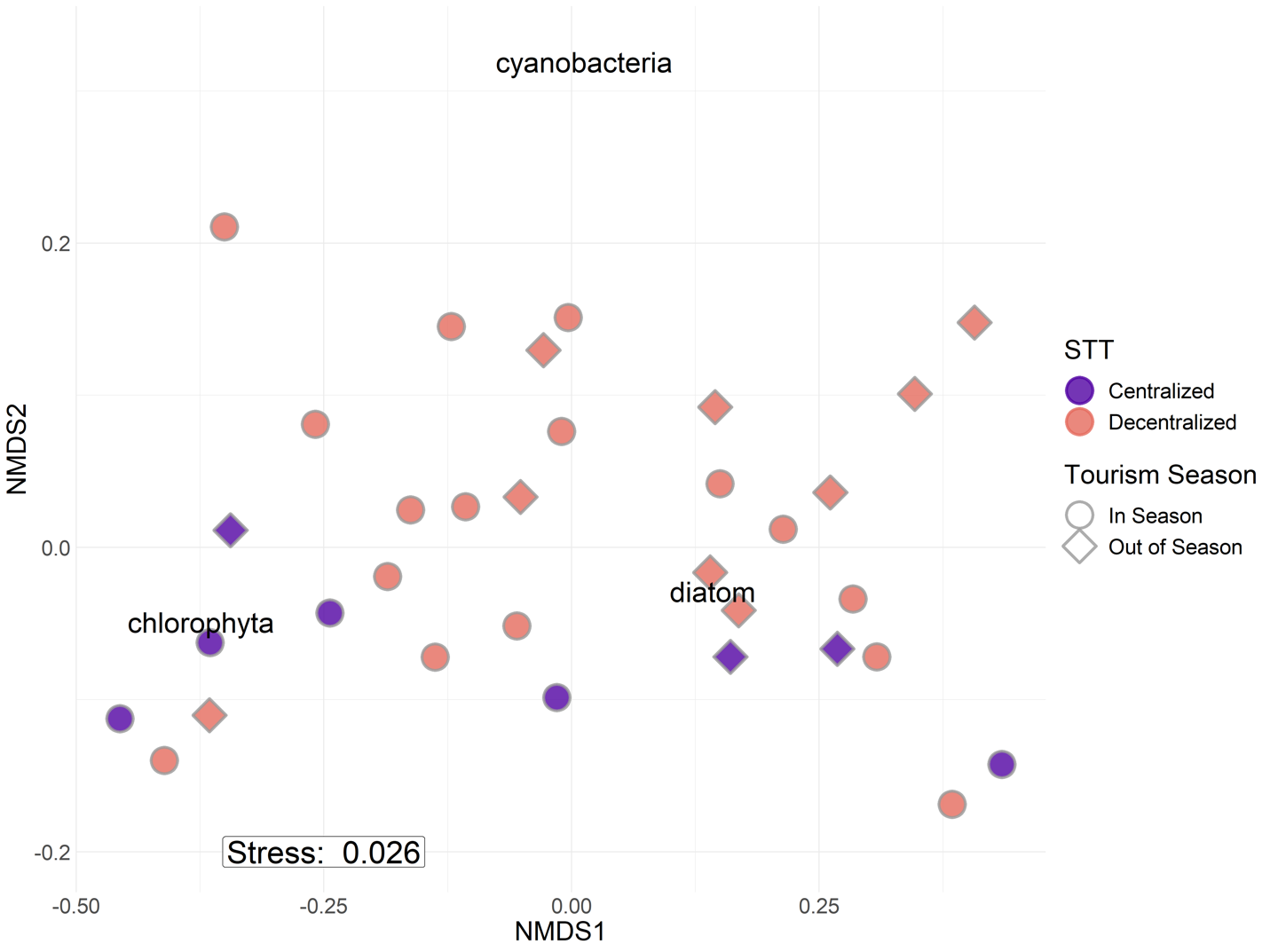


Figure 6: NMDS with Bray-Curtis similarity of relative periphyton abundance. The NMDS biplot suggested that sites did not separate based on multivariate patterns in algal community composition. Rather, sites with centralized wastewater treatment tended to cluster at the lower end of NMDS2, which likely was driven by these sites having lower relative cyanobacterial abundance. This same pattern was observed in the univariate analysis of each taxon’s relative abundance (Figure 5).

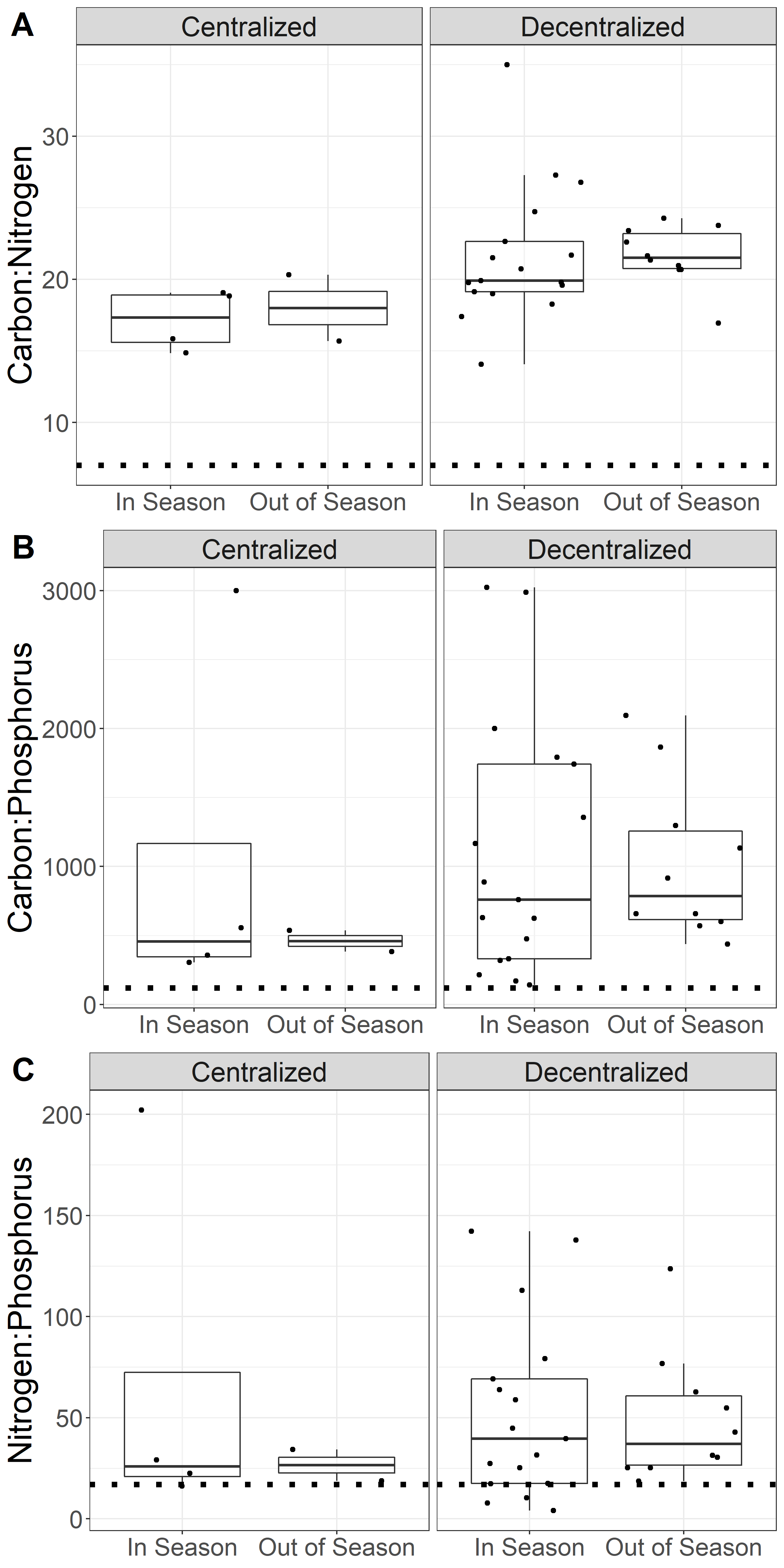


Figure 7: Molar Carbon:Nitrogen (A), Carbon:Phosphorus (B), and Nitrogen:Phosphorus (C) ratios for periphyton with samples groups by whether sites were located near centralized or decentralized wastewater treatment, or samples were collected during or following the tourism season. “In Season” denotes samples collected during June, July, or August, whereas “Out of Season” denotes samples collected in September. Dotted lines in each plot reflect the empirically derived stoichiometric ratios for non-nutrient limited growth of periphyton communities (i.e., 119:17:1; Hillebrand and Sommer 1999).

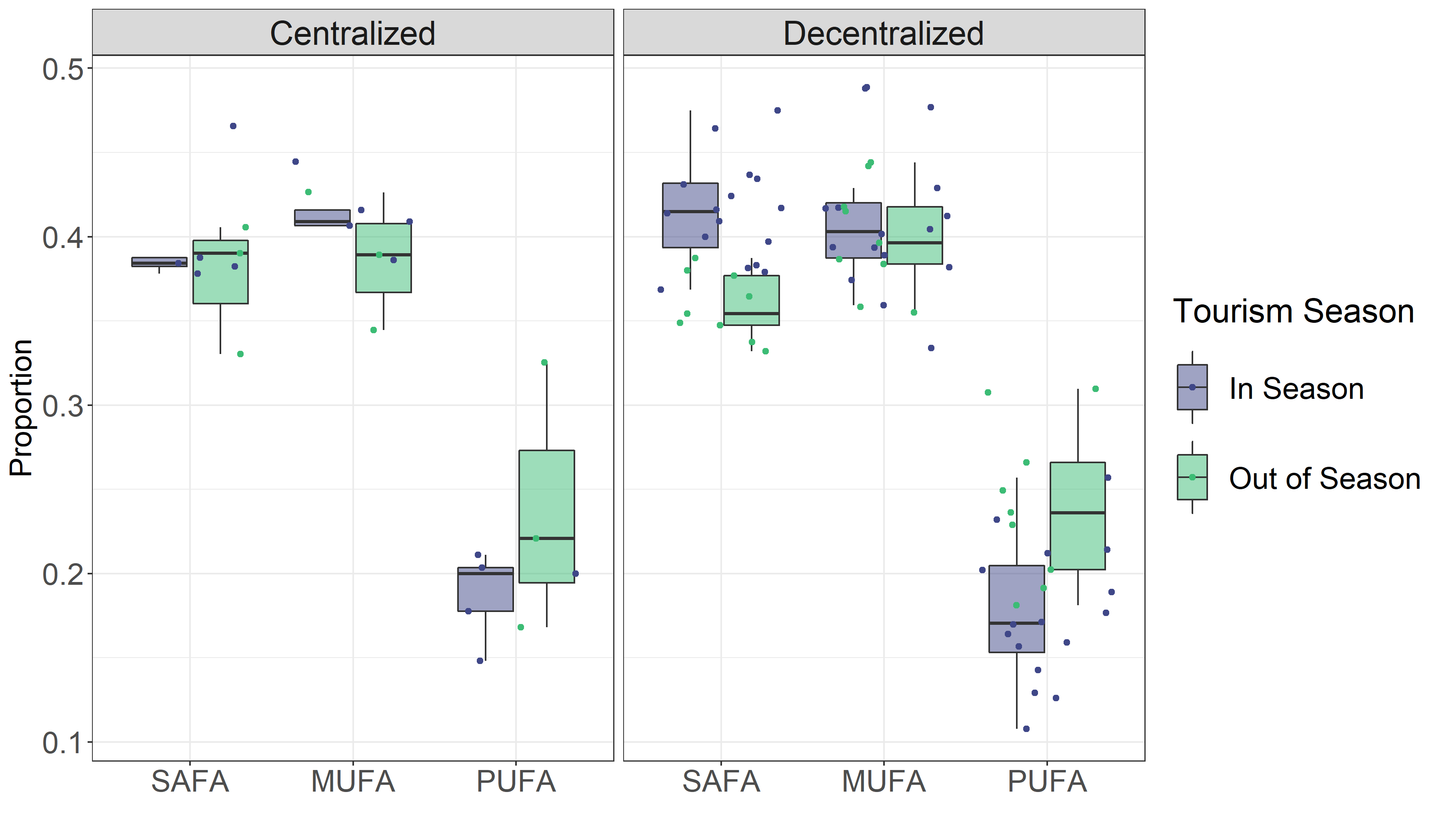


Figure 8: Relative concentrations of saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in periphyton with samples groups by whether sites were located near centralized or decentralized wastewater treatment or samples were collected during or following the tourism season. “In Season” denotes samples collected during June, July, or August, whereas “Out of Season” denotes samples collected in September.

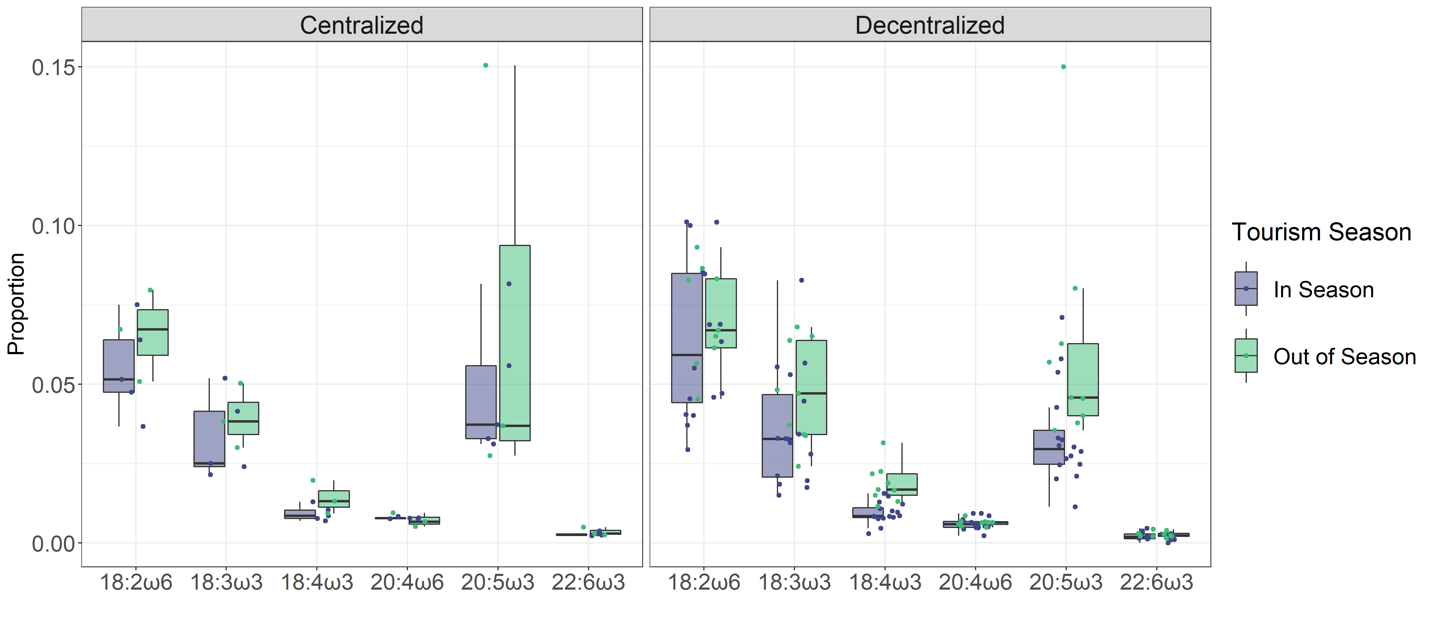


Figure 9: Relative concentrations of essential fatty acids (EFAs) in periphyton with samples groups by whether sites were located near centralized or decentralized wastewater treatment or samples were collected during or following the tourism season. “In Season” denotes samples collected during June, July, or August, whereas “Out of Season” denotes samples collected in September. EFAs are considered highly nutritious fatty acids for higher trophic levels and are primarily synthesized by primary producers. Greatest differences in EFAs between locations and timepoints occurred at sites with decentralized sewage treatment, which contained increased 20:5ω3 following the tourism season. Because fatty acids tend to map to algal community compositions, this increased 20:5ω3 signature was likely related with increased diatom abundance (Figure 5).

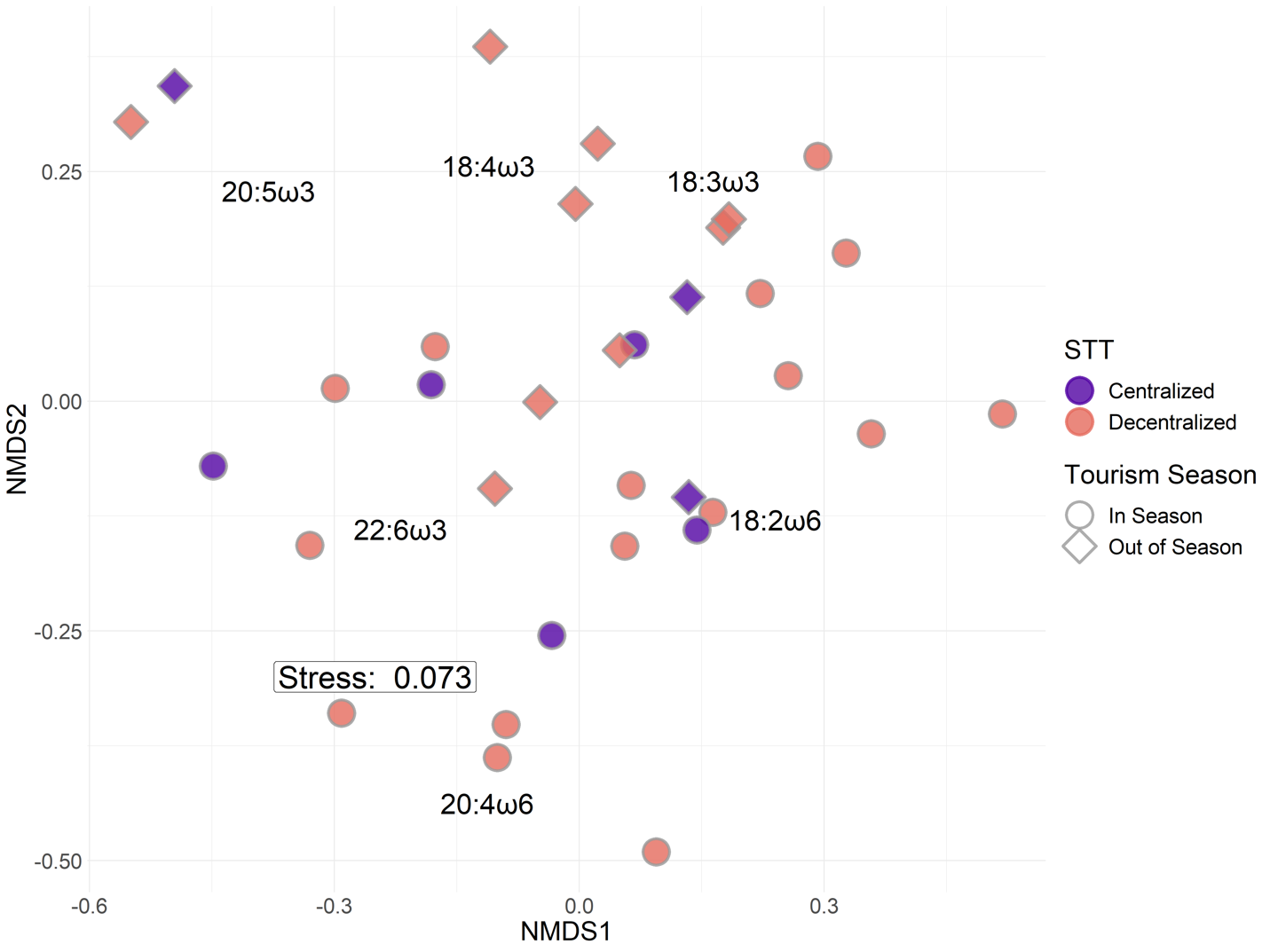


Figure 10: NMDS with Bray-Curtis similarity based on EFA relative abundance. The NMDS biplot suggested that sites separated by differences in tourism season (circles and diamonds). Post hoc SIMPER analyses suggested 20:5ω3 relative abundance as the most influential EFA in driving point separation, which was likewise supported by the univariate analysis of EFA abundance (Figure 9).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table S1: ANOVA tables for nutrient concentrations in response to sewage treatment technique (STT) and tourist season. | | | | |
| 1. **Ammonia** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.121 | 1 | 3.347 | 0.080 |
| Tourist Season | 0.016 | 1 | 0.454 | 0.507 |
| STT:Tourist Season | 0.038 | 1 | 1.040 | 0.318 |
| Residuals | 0.866 | 24 |  |  |
|  |  |  |  |  |
| 1. **Nitrate/Nitrite** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.255 | 1 | 1.058 | 0.314 |
| Tourist Season | 0.059 | 1 | 0.243 | 0.627 |
| STT:Tourist Season | 0.125 | 1 | 0.517 | 0.479 |
| Residuals | 5.793 | 24 |  |  |
|  |  |  |  |  |
| 1. **SRP** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.378 | 1 | 3.313 | 0.081 |
| Tourist Season | 0.001 | 1 | 0.009 | 0.923 |
| STT:Tourist Season | 0.013 | 1 | 0.114 | 0.739 |
| Residuals | 2.741 | 24 |  |  |
|  |  |  |  |  |
| 1. **Total Nitrogen** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.011 | 1 | 1.374 | 0.253 |
| Tourist Season | 0.020 | 1 | 2.554 | 0.123 |
| STT:Tourist Season | 0.003 | 1 | 0.316 | 0.579 |
| Residuals | 0.191 | 24 |  |  |
|  |  |  |  |  |
| 1. **Total Phosphorus** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.256 | 1 | 2.101 | 0.160 |
| Tourist Season | 0.400 | 1 | 3.282 | 0.083 |
| STT:Tourist Season | 0.018 | 1 | 0.148 | 0.704 |
| Residuals | 2.922 | 24 |  |  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table S2: ANOVA tables for sewage indicator values in response to sewage treatment technique (STT) and tourist season. | | | | |
| 1. **PPCPs** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.290 | 1 | 0.649 | 0.428 |
| Tourist Season | 1.750 | 1 | 3.915 | 0.059 |
| STT:Tourist Season | 0.932 | 1 | 2.085 | 0.161 |
| Residuals | 11.624 | 26 |  |  |
|  |  |  |  |  |
| 1. **Branched- and Odd-Chain Fatty Acids** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | < 0.001 | 1 | 0.307 | 0.585 |
| Tourist Season | 0.002 | 1 | 4.565 | 0.045 |
| STT:Tourist Season | < 0.01 | 1 | 0.353 | 0.559 |
| Residuals | 0.008 | 21 |  |  |
|  |  |  |  |  |
| 1. **Ash Free Dry Mass** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | < 0.001 | 1 | 0.039 | 0.845 |
| Tourist Season | < 0.001 | 1 | 0.096 | 0.760 |
| STT:Tourist Season | < 0.001 | 1 | 2.558 | 0.125 |
| Residuals | < 0.001 | 21 |  |  |

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| --- | --- | --- | --- | --- |
| Table S3: PERMANOVA tables for periphyton community and multivariate fatty profiles in response to sewage treatment technique (STT) and tourist season. | | | | |
| 1. **Periphyton Community Composition** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.127 | 1 | 0.127 | 0.127 |
| Tourist Season | 0.158 | 1 | 0.158 | 0.158 |
| Residuals | 2.459 | 30 |  |  |
|  |  |  |  |  |
| 1. **Fatty Acids** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 0.082 | 1 | 0.726 | 0.667 |
| Tourist Season | 0.311 | 1 | 2.775 | 0.008 |
| Residuals | 3.367 | 30 |  |  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table S4: ANOVA tables for periphyton stoichiometric ratios in response to sewage treatment technique (STT) and tourist season. | | | | |
| 1. **Carbon:Nitrogen** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 85.606 | 1 | 5.830 | 0.022 |
| Tourist Season | 0.215 | 1 | 0.015 | 0.904 |
| STT:Tourist Season | 0.752 | 1 | 0.051 | 0.823 |
| Residuals | 425.863 | 29 |  |  |
|  |  |  |  |  |
| 1. **Carbon:Phosphorus** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 233756.454 | 1 | 0.310 | 0.582 |
| Tourist Season | 204497.198 | 1 | 0.271 | 0.606 |
| STT:Tourist Season | 300369.615 | 1 | 0.399 | 0.533 |
| Residuals | 21848264.461 | 29 |  |  |
|  |  |  |  |  |
| 1. **Nitrogen:Phosphorus** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | 25.980 | 1 | 0.012 | 0.915 |
| Tourist Season | 728.571 | 1 | 0.328 | 0.571 |
| STT:Tourist Season | 1570.836 | 1 | 0.707 | 0.407 |
| Residuals | 64403.453 | 29 |  |  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table S5: ANOVA tables for periphyton relative fatty acid abundance in response to sewage treatment technique (STT) and tourist season. Prior to analysis, values were arcsine-square root transformed to normalize proportional data. | | | | |
| 1. **PUFA** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | < 0.001 | 1 | 0.160 | 0.692 |
| Tourist Season | 0.044 | 1 | 14.470 | 0.001 |
| STT:Tourist Season | 0.001 | 1 | 0.256 | 0.617 |
| Residuals | 0.089 | 29 |  |  |
|  |  |  |  |  |
| 1. **SAFA** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | < 0.001 | 1 | 0.070 | 0.793 |
| Tourist Season | 0.019 | 1 | 20.519 | < 0.001 |
| STT:Tourist Season | 0.001 | 1 | 1.618 | 0.213 |
| Residuals | 0.026 | 29 |  |  |
|  |  |  |  |  |
| 1. **MUFA** | | | | |
|  | Sum of Squares | Degrees of Freedom | F value | P value |
| STT | < 0.001 | 1 | 0.046 | 0.832 |
| Tourist Season | 0.002 | 1 | 1.016 | 0.322 |
| STT:Tourist Season | < 0.001 | 1 | 0.249 | 0.622 |
| Residuals | 0.044 | 29 |  |  |