Chapter 2

Characterization of the Kootenai River Algae Community and Primary Productivity before and after Experimental Nutrient Addition, 2004–2007

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EXECUTIVE SUMMARY

The Kootenai River ecosystem (spelled Kootenay in Canada) has experienced numerous ecological changes since the early 1900s. Some of the largest impacts to habitat, biological communities, and ecological function resulted from levee construction along the 120 km of river upstream from Kootenay Lake, completed by the 1950s, and the construction and operation of Libby Dam on the river near Libby Montana, completed in 1972. Levee construction isolated tens of thousands of hectares of historic functioning floodplain habitat from the river channel downstream in Idaho and British Columbia (B.C.) severely reducing natural biological productivity and habitat diversity crucial to large river-floodplain ecosystem function. Libby Dam greatly reduces sediment and nutrient transport to downstream river reaches, and dam operations cause large changes in the timing, duration, and magnitude of river flows. These and other changes have contributed to the ecological collapse of the post-development Kootenai River ecosystem and its native biological communities.

In response to large scale loss of nutrients, experimental nutrient addition was initiated in the North Arm of Kootenay Lake in 1992, in the South Arm of Kootenay Lake in 2004, and in the Kootenai River at the Idaho-Montana border during 2005. This report characterizes baseline chlorophyll concentration and accrual (primary productivity) rates and diatom and algal community composition and ecological metrics in the Kootenai River for four years, one (2004) before, and three (2005 through 2007) after nutrient addition.

The study area encompassed a 325 km river reach from the upper Kootenay River at Wardner, B.C. (river kilometer (rkm) 445) downstream through Montana and Idaho to Kootenay Lake in B.C. (rkm 120). Sampling reaches included an unimpounded reach furthest upstream and four reaches downstream from Libby Dam affected by impoundment: two in the canyon reach (one with and one without nutrient addition), a braided reach, and a meandering reach. The study design included 14 sampling sites: an upstream, unimpounded reference site (KR-14), four control (non-fertilized) canyon sites downstream from Libby Dam, but upstream from nutrient addition (KR-10 through KR-13), two treatment sites referred to collectively as the nutrient addition zone (KR-9 and KR-9.1, located at and 5 km downstream from the nutrient addition site), two braided reach sites (KR-6 and KR-7), and four meander reach sites (KR-1 through KR-4).

A series of qualitative evaluations and quantitative analyses were used to assess baseline conditions and effects of experimental nutrient addition treatments on chlorophyll, primary productivity, and taxonomic composition and metric arrays for the diatom and green algae communities. Insufficient density in the samples precluded analyses of bluegreen algae taxa and metrics for pre- and post-nutrient addition periods. Chlorophyll *a* concentration (mg/m^2), chlorophyll accrual rate ($mg/m^2/30d$), total chlorophyll concentration (chlorophyll *a* and *b* (mg/m^2), and total chlorophyll accrual rate ($mg/m^2/30d$) were calculated. Algal taxa were identified and grouped by taxonomic order as Cyanophyta (blue-greens), Chlorophyta (greens), Bacillariophyta (diatoms), Chrysophyta (goldens), and dominant species from each sample site were identified. Algal densities (#/ml) in periphyton samples were calculated for each sample site and sampling date. Principal Component Analysis (PCA) was performed to

reduce the dimension of diatom and algae data and to determine which taxonomic groups and metrics were contributing significantly to the observed variation. PCA analyses were tabulated to indicate eigenvalues, proportion, and cumulative percent variation, as well as eigenvectors (loadings) for each of the components. Biplot graphic displays of PCA axes were also generated to characterize the pattern and structure of the underlying variation. Taxonomic data and a series of biological and ecological metrics were used with PCA for diatoms and algae. Algal metrics included a suite of abundance, diversity, richness, dominance, and other measures, whereas additional trophic status and chemical limnology metrics, Van Dam indices and morphological groupings were employed in diatom PCAs. Analysis of Variance (ANOVA) was carried out using chlorophyll metrics and taxa and metric arrays for the diatom and green algae community data for comparing site differences from 2004 through 2007.

Clear, statistically significant, biological responses from chlorophyll metrics, and taxa and metrics of the diatom and algal communities were revealed following experimental nutrient addition in the Kootenai River. Chlorophyll metric responses were more often significant and generally greater in magnitude than diatom and green algae taxa and metric responses.
ANOVA revealed significant nutrient addition treatment and site effects for all four chlorophyll metrics (chlorophyll *a* and total chlorophyll biomass and accrual rates for both), and a significant site*fertilization interaction for total chlorophyll accrual. The four chlorophyll metrics responded significantly and consistently to experimental nutrient addition in the Kootenai River. Values for all chlorophyll metrics were lowest during 2004, they more than doubled in 2005 and increased by as much as ten-fold or more during 2006, then consistently decreased by up to 75% during 2007. This multi-year annual pattern has been observed at lower trophic levels in other experimentally nutrified rivers and streams, and has been attributed to time-lagged cascading trophic interactions.

A significant shift in overall diatom taxa composition between pre- and post-nutrient addition (treatment) periods in the nutrient addition zone was revealed by PCA. During the pre-treatment period, four diatom metrics exhibited significant site effects, involving trophic status and richness measures (e.g. Trophic state, Richness-Eutrophic, Richness-hypereutrophic, and Overall Richness). During the post-treatment period, 7 diatom metrics exhibited significant site effects, again mainly associated with trophic status and richness measures. The number of significant ANOVA site and reach contrasts tripled from 5 to 15 of 104 from pre-and post-treatment periods (or from about 5 to 15% of all contrasts), but remained a minor shift in taxonomic composition.

The overall abundance of green algae taxa was variable and generally increased following nutrient addition. Regarding abundance of green algae by morphological type, one taxa group (prostrate) increased during every year, two taxa groups (stalked and unattached) decreased during 2005 and 2007, but increased markedly during 2006, and one (variable) declined slightly across all years. Although up to 92% of the variability associated with green algae taxa and metrics was accounted for using PCA, as with diatoms, no discernable, ecologically insightful trends or patterns emerged when comparing green algae taxa and metrics between pre-and post-treatment periods. Only two green algae taxa (*Stigeoclonium and Ulothrix*) exhibited a significant treatment effect; no other green algae taxa exhibited significant

treatment effects or site*fertilization interactions. Significant site effects for green algae and diatom metrics were also observed during the post-treatment period. These results were interpreted as ecologically beneficial responses, resulting from nutrient addition, within the context of increased food web structure, diversity, and diet item availability. Ongoing and future cascading interaction analyses and modeling of chlorophyll, diatom, and algal metrics and taxa, along with data from water quality, macroinvertebrate, and fish communities in the Kootenai River will better characterize effects of nutrient addition.

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INTRODUCTION

The Kootenai River ecosystem (spelled Kootenay in Canada) has experienced numerous ecological changes since the early 1900s. Some of the largest impacts to habitat, biological communities, and ecological function resulted from levee construction along the 120 km of river upstream from Kootenay Lake, completed by the 1950s, and the construction and operation of Libby Dam on the river near Libby Montana, completed in 1972. Levee construction isolated tens of thousands of hectares of historic functioning floodplain habitat from the river channel downstream in Idaho and British Columbia, severely reducing natural biological productivity and habitat diversity crucial to large river-floodplain ecosystem function (Anders et al. 2002). Libby Dam greatly reduces sediment and nutrient transport to downstream river reaches, and dam operations cause large changes in the timing, duration, and magnitude of river flows. These changes have contributed to the ecological collapse of the post-development Kootenai River ecosystem and its native biological communities.

In response to the previously documented loss of nutrients at the Kootenai Basin scale, experimental nutrient addition was initiated in the North Arm of Kootenay Lake in 1992, in the South Arm of Kootenay Lake in 2004, and in the Kootenai River at the Idaho-Montana border during 2005. This report characterizes baseline chlorophyll concentration and accrual (primary productivity) rates and diatom and algal community composition and ecological metrics in the Kootenai River for four years, one (2004) before, and three (2005 through 2007) after nutrient addition.

Evaluation of aquatic algal community composition, dynamics, and primary production provides a valuable approach for characterizing natural and altered large river ecosystems. Primary productivity in aquatic and terrestrial ecosystems represents the organic matter fixed by autotrophic organisms (using solar energy) through the process of photosynthesis (Odom 1997). In mid-order lotic ecosystems, primary productivity is the main source of biogenic development upon which all higher trophic levels (macroinvertebrates and fishes) depend (Vannote et al. 1980; Allan 1995; Allan and Castillo 2007). Although a substantial loss in energy occurs when ascending from one trophic level to the next, in general, the greater the level of primary productivity, the greater the potential for increased production at higher trophic levels (i.e. bottom-up enhancement).

Besides its importance in photosynthesis, chlorophyll a is a central indicator of algal biomass in lakes and streams in North America (USGS 2007). Chlorophyll provides an estimate for measuring algal weight and volume, and provides a measurable, empirical link between nutrient availability and biological phenomena in aquatic ecosystems. Collectively, nutrients water chemistry, temperature, and light affect the biomass production of algal production, which, in turn, regulates the entire biological structure of an ecosystem.

Benthic algae represent a critical food web component in rivers (Allan 1995; Stevenson 1996; Blinn and Herbst 2003; Wehr and Sheath 2003). Many authors have reported diatoms as good indicators of the environmental integrity in lotic ecosystems because diatoms are common, abundant, are sensitive to nutrient availability, and provide a primary food source for many riverine invertebrate and some juvenile fish taxa (Dixit et al. 1992; Lowe and Pan

1996; Stevenson and Pan 1999; Blinn and Herbst 2003; Lavoie et al. 2006). Other authors also reported diatoms as useful indicators of environmental change because: 1) they are readily dispersed and can invade a variety of habitats; 2) they are relatively easy to sample and create minimal impact to resident biota during collections; 3) their response time (generation time) lies between bacteria (hourly) and macroinvertebrates (triannual); and 4) they are sensitive to subtle changes in environmental conditions and/or disturbances that may not visibly affect other communities, or may only affect other communities at elevated levels of disturbance (Bahls 1993; Stevenson and Pan 1999; Stevenson and Bahls 1999).

As summarized by Blinn and Herbst (2003), additional investigators have used various diatom metrics to assess and monitor environmental conditions in rivers and streams, including: discharge and hydraulic fluctuations (Duncan and Blinn 1989; Biggs and Hicky 1994; Benenati et al. 1998), light (Duncan and Blinn 1989; Hardwick et al. 1992), temperature (Squires et al. 1979; Blinn et al. 1989), salinity (Blinn and Bailey 2001), nutrients (Patrick 1977; Bahls et al. 1992; Van Dam et al. 1994; Hill et al. 2000; Blinn and Bailey 2001), and herbivory (Colletti et al. 1987; Steinman et al. 1987).

Soft algae (non-diatoms) have also been widely reported as useful indicators of biological integrity (Palmer 1969; Fjerdingstadt 1965; Palmer 1979; Hill et al. 2000; Blinn and Herbst 2003), but may be less useful indicators than diatoms due to highly variable algal morphologies (Stevenson et al. 1996; Blinn and Herbst 2003). Furthermore, successful taxonomic identification of soft algae to species may be needed, which may require reproductive structures and material only attainable with cultures in the laboratory (Stevenson and Pan 1999).

Study area

The Kootenai River Subbasin is situated between 48° and 51° north latitude and 115° and 118° west longitude and includes parts of southeastern British Columbia (B.C.), northern Idaho, and northwestern Montana. It measures approximately 238 miles by 153 miles and has an area 16,180 sq miles (Figure 1). Nearly two-thirds of the Kootenai River's 485-mile-long channel and almost 70 percent of its watershed area are located in B.C. An additional 23% percent of the watershed is in Montana, with the remaining 6.5% in Idaho (Knudson 1994).

Sampling reaches and sites

The study area encompassed a 325 km reach from the upper Kootenay River at Wardner B.C. (rkm 445) downstream through Montana and Idaho to Kootenay Lake in B.C. (< rkm 120; Figure 1). Sampling reaches included an unimpounded reach furthest upstream, and four reaches downstream from Libby Dam affected by impoundment: two in the canyon reach (one with and one without nutrient addition), a braided reach, and a meandering reach (Table 1).

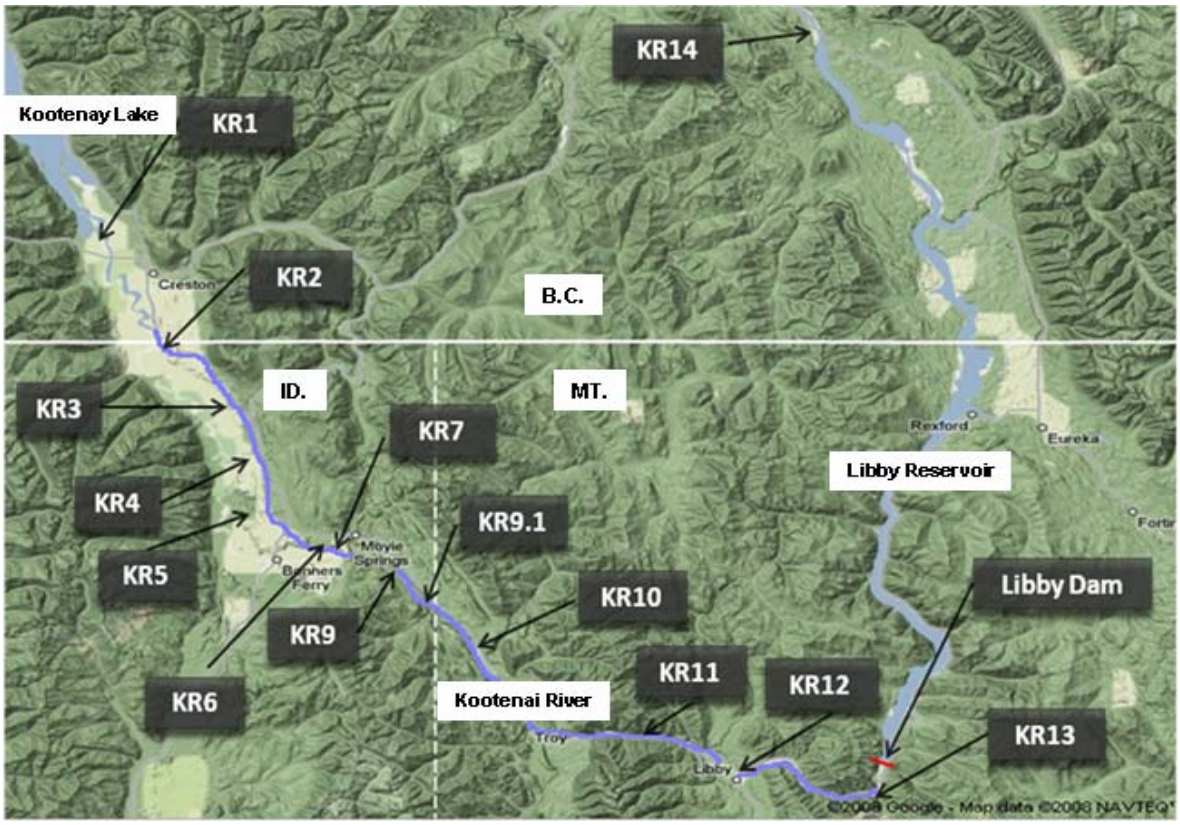


Figure 1. Study area and algae sampling sites (2004–2007) (Figure provided by SCS).

Table 1. Algae and chlorophyll sampling reach and site attributes.

Orientation	Upstream ..... Downstream				
Reach	Unimpounded Reach	Montana Canyon (Control)	Idaho Canyon (Treatment)	Braided Reach (Treatment)	Meander Reach (Treatment)
Locations	BC upstream of Libby Res.	Libby Dam downstream to ID/MT state line	ID/MT state line to Moyie River	Moyie River to Bonners Ferry	Bonners Ferry to Kootenay Lake
Reach (rkm) boundaries		357-276	276-258	258-246	246-120
Sampling Sites (rkm)	KR-14 (445.0)	KR-10 (285.6) KR-11 (310.7) KR-12 (325.0) KR-13 (347.4)	KR-9 (262.2) KR-9.1 (267.1)	KR-6 (250.0) KR-7 (255.4)	KR-1 (123.5) KR-2 (170.0) KR-3 (203.6) KR-4 (231.4)
Features	Natural river conditions; upstream reference site	Canyon habitat with hydropower effects	Canyon habitat with hydropower and fertilization effects	Braided channel reach with hydropower and fertilization effects	Leveed meander habitat with hydropower and fertilization effects
Trophic status[a]	Autotrophic	Autotrophic	Autotrophic	Autotrophic	Heterotrophic

a: Autotrophic: Photosynthesis > respiration Heterotrophic: Photosynthesis < respiration (Snyder and Minshall 1996, 2005)

At a larger spatial scale than the experimental reaches presented in Table 2 (treatment, control, and reference reaches), the Kootenai River has three major geomorphically distinct reaches in downstream orientation: the canyon, braided, and meandering reaches. The canyon reach (rkm 352–258) extends from Libby Dam downstream to the mouth of the Moyie River in Idaho. This reach is characterized by relatively high gradient and hydraulic energy. Substrates in the canyon reach range from exposed bedrock to boulders, cobble, and gravels.

Immediately downstream from the canyon reach, the short braided reach (rkm 258–246) extends from the mouth of the Moyie River to the Highway 95 Bridge at Bonners Ferry, Idaho. The braided reach is characterized by a series of braided channels with reduced slope and hydraulic energy compared to the upstream canyon reach. Braided reach substrates are typically gravels and cobbles in the main channels to sand and fine sediments in the off-channel habitats.

Further downstream, the meander reach extends from Bonners Ferry downstream to the river delta at Kootenay Lake (rkm 246-120). The meander reach lies entirely within the historic floodplain within the Purcell Trench, a glacial valley that runs north from Bonners Ferry into British Columbia, forming the basin for Kootenay Lake (Figure 1). The meander reach is characterized by very low gradient, low hydraulic energy, and fine substrates, mainly silt and sand.

METHODS

Experimental nutrient addition treatment

Experimental nutrient addition treatment began in the Kootenai River at the Idaho-Montana border (KR-9.1, rkm 276.1; Figure 1, Table 2) in July of 2005 in response to a series of previous studies that documented the nutrient poor (ultraoligotrophic) conditions in the Kootenai River following loss of the historic floodplain and impoundment (Daley et al. 1981; Woods 1982; Snyder 2001; Snyder and Minshall 1996, 2005). Target in-river phosphorus concentrations were 1.5 µg/L during 2005 and 3.0 µg/L during 2006 and 2007 (KTOI 2005, 2006, 2007). Nitrate-nitrogen was not added to the river during these years because nitrogen limitation (defined as < 60 µg/L nitrate when adding 3 ug/L P) was never approached, based on weekly water quality analysis of the Kootenai River upstream and downstream from the nutrient addition site from June through September, 2005 through 2007 (KTOI 2005, 2006, 2007). (It should be noted that a small amount of ammonia-nitrogen, 1-2 µg/L, was added passively, as a stabilizing element within the phosphorus fertilizer).

Chlorophyll - Data availability

All chlorophyll analyses in this report involved data from 2004 through 2007, representing two pre-treatment years and two post-treatment years. Chlorophyll data were collected from all sites from 2004 through 2007 (Table 2).

Table 2. Chlorophyll data availability by year and by site.

Year \ Site	KR1	KR2	KR3	KR4	KR6	KR7	KR9	KR9.1	KR10	KR11	KR12	KR13	KR14
2004													
2005													
2006													
2007													

- Data Available
- No Data

Algae taxa and metrics – Data availability

Algal taxa and metric data were collected during all years at all sites from 2004 through 2007 with the exception of the four meander reach sites (KR-1 through KR-4) during 2005 (Table 3

Table 3. Algae and diatom metric and taxa data availability by year and by site.

Year \ Site	KR1	KR2	KR3	KR4	KR6	KR7	KR9	KR9.1	KR10	KR11	KR12	KR13	KR14
2004													
2005													
2006													
2007													

- Data Available
- No Data

Field sampling protocols

Field sampling was designed to represent and characterize aquatic algae and periphyton community attributes and primary productivity rates along a longitudinal gradient of environmental conditions in the Kootenai River: (1) upstream and downstream from Libby Dam; (2) before and after experimental nutrient addition; and (3) upstream and downstream from nutrient addition.

Chlorophyll - Chlorophyll samples were collected monthly from late March through September in the years 2004 through 2007. Due to differences in current velocity and substrate conditions at upriver and downriver sites, different but comparable chlorophyll sampling methods were implemented in these reaches. At each of the upriver sites (KR-6 through KR-14), two 8"x 8"x 2" concrete blocks fitted with a 1 inch Styrofoam surface were placed on the substrate to sample chlorophyll. The Styrofoam surface was etched with a one by one inch sampling grid. Samplers were placed on the bottom of the river in a shallow excavation to prevent water flow underneath the sampler, and to closely approximate the physical conditions at the substrate level. The tiles were placed in a sunny location in riffle-run habitats with similar water velocity and depth characteristics, both within and across sites (mean depth 0.5 to 0.75 m; mean water velocity 0.20-0.50 m/s).

Six Styrofoam core samples (n= 3 from each sampler; n=6 total) were taken each month of the growing season at each site. (One exception occurred at KR-9.1 and KR-9, where bimonthly sampling occurred in mid-summer 2006 to account for rapid algae growth). Sample cores were taken using a modified spark-plug socket, with an approximately 1 inch diameter. Individual core locations were randomly chosen from the Styrofoam grid using a random numbers table. Samples were then placed in a Whirl Pac® plastic bag, labeled, sealed, and put into a 75 ml dark brown plastic bottle to avoid further photosynthetic activity. The bottles were placed in a cooler on ice until placed in a freezer at the KTOI lab. Frozen samples were shipped (within 3 months) to the University of Idaho Holm Research Center for chlorophyll *a* and *b* analysis using a Styrofoam method and Biomate 3 Spectrophotometer® (See Appendix B: Diatom and algae lab protocols for detailed methods of chlorophyll lab protocols). Sample data were then used to calculate chlorophyll concentration, and algal biomass as a function of area (mg/m^2).

At sites KR-1 through KR-4, three 1.5 m tile ropes were hung from large, floating dead wood. Five 10.2 cm x 10.2 cm polyvinyl tiles were attached to a rope and suspended in the water column up to 1.5-m (0.3, 0.61, 0.92, 1.22, and 1.5 m) in depth to collect chlorophyll biomass samples (n=8). The ropes were weighted at the bottom of the rope to ensure a relatively vertical water column position (n=3 ropes/site). Each polyvinyl tile was covered with Styrofoam 1.27 cm deep.

Individual tile ropes were pulled from the water column, and sample cores were taken using the same modified spark-plug socket, with an approximate 1 inch diameter. Three cores were taken from each tile from two sets and two cores out of each tile from the remaining set (n=8). Styrofoam core samples were placed in Whirl-paks, stored in brown plastic bottles, frozen at -20°C, and shipped for processing at the University of Idaho Analytical Lab at the Holm Center in Moscow, Idaho.

Algae Taxonomy **-** Algae samples, with an area of 51.6 cm^{-2}, were collected at KR-1 through KR-4 from the underside of the uppermost polyvinyl tile, scraped into a glass jar, and rinsed to a 50 ml brown bottle. Samples were preserved with Lugol's solution (1 $drop \cdot ml^{-1}$). The identification of representative algal taxa for each treatment was provided by Aquatic Taxonomy Specialists in Malinta, Ohio. Soft-bodied periphyton cells were identified by viewing 300 cell-count wet mounts at 400X magnification. Diatom species were identified using sub-sample burn mounts magnified up to 1000X. All algal taxa present were accounted for and identified to genus, and when possible to species.

Algae samples were collected on a monthly basis (April through October) at 9 sites, 5 between the Kootenai Tribal Hatchery and the Montana border (upriver), and 4 sites between Bonners Ferry and Kootenay Lake (downriver) from 2004 through 2007 (Figure 1). Samples were collected to estimate composition and primary production rates during the biological production period (when flow conditions allowed). The Kootenai Tribe of Idaho and the Idaho Department of Fish and Game performed upriver (KR-6 through KR-14) data collection and reporting and Free Run Aquatic Research sampled the downriver sites (KR-1 through KR-4). At sites KR-6 through KR-14, three random cobbles were selected from the substrate and all periphyton was removed from the surface using a rubber spatula. The removed periphyton was placed into a 125 ml brown plastic bottle and filled with 10% Lugol's solution for preservation. Preserved samples were taken to the KTOI lab and placed under refrigeration for 1-3 months prior to sending to the lab for identification.

Diatom and Algae Lab protocols

All diatom and algae samples were logged into the lab using a sample data sheet that provided the following information for each sample: sample identification number, pertinent location and replicate information, sample date, habitat type, area sampled, and volume if available. All samples were subsampled and carefully but thoroughly shaken to evenly disperse the diatoms or algae. Sub-samples were taken from the middle of the sample and placed into labeled vials with a unique sample ID # with the suffixes for diatom and algal samples. The volume of each sub-sample was then recorded both on the vials and on the lab sheet for all subsamples.

Diatom slides were prepared to count and identify diatoms to the lowest practical taxon, usually to the genus or species level. All slide preparations were performed using three standard digestion methods (acid, hydrogen peroxide, and burn mount). More detailed methods are provided in Appendix A.

Diatoms were counted and identified to the lowest practical taxon using the strip method until at least 300 cells (600 valves) are encountered. Counting and identification occurred using 1000X magnification with a Nikon light microscope and the latest taxonomic keys and references.

After homogenizing subsamples by thorough agitation, exactly 0.1ml was placed on a slide using a micropipette. Algae samples were examined at 400X magnification using a light microscope to assess if periphyton was too dense or dilute for identification and enumeration.

(The original sample was diluted or concentrated if necessary to achieve desirable cell density approximately 15-20 counting units per field of view). If dilution or concentration were needed, the new volume and concentration ratio was recorded on the data sheet and incorporated into relevant calculations.

Soft algae were counted and identified to the lowest practical taxon using the strip method until at least 300 counting units (or another target number specified by DRBC) were encountered. For colonial algae, each colony was counted as one algal unit per 10 by 10 micron area. In the case of filamentous algae, each 10 micron length represented one algal unit, for purposes of tallying 300 counting units in a count. Counting and identification was performed at 400X magnification using a Nikon light microscope and the latest taxonomic keys and references. Diatoms and soft algae in subsamples were counted and identified to the lowest practical taxon.

Total algal density (cells/substrate area) was calculated as {[N / (Vsc * CR)] * VT} / AS, where:

N = the total number of cells counted

Vsc = the volume of sample counted

CR = the concentration or dilution factor (if applicable, or CR = 1)

VT = total sample volume

AS = substrate area sampled

A series of steps were taken to ensure quality assurance of diatom and algae lab work:

(1) High quality digital images were taken of each taxon encountered in the project, including names, photographer/taxonomist name, date, and project ID number;
(2) Diatom slides were archived in slide boxes with the project name;
(3) A minimum of 10% of all samples were analyzed by an independent phycologist (at the Philadelphia Academy of Natural Sciences) to ensure taxonomic accuracy and reproducibility of the processing and analysis methods;
(4) Consensus levels of at least 90% for the common taxa in each sample were implemented, with multiple professional taxonomists consulting to resolve any taxonomic identification discrepancies. Additional quality assurance criteria and actions are provided in Appendix A.

All diatom and algae data were entered into a custom-built taxonomy counting program that created an electronic file for each project, including a counting function that automatically tallied the number of cells for each taxon.

Summary statistics

Chlorophyll **–** Chlorophyll a (mg/m2), chlorophyll a accrual rate (mg/m2/30d), total chlorophyll (chlorophyll a and b (mg/m2), and total chlorophyll accrual rate (mg/m2/30d) were calculated for each sample site and sampling date.

Algae **-** Algal taxa were identified and grouped by taxonomic order as Cyanophyta (blue-greens), Chlorophyta (greens), Bacillariophyta (diatoms), or Chrysophyta (goldens).

Dominant algal species were also identified for each sample site. Algal densities (#/ml) in periphyton samples were calculated for each sample site and date.

Quantitative methods

***Principal Components Analysis (PCA).* -** Principal components analysis was performed to reduce the dimension of diatom and algae data and to determine which taxonomic groups or metrics were contributing significantly to the observed variation. Data for all PCA runs were selected to represent taxonomic orders and metrics that were in common across samples from all dates at those sites or site combinations. Numerical results of the PCA analyses reported were eigenvalues, proportion, and cumulative percent variation, as well as eigenvector loadings for each of the components. Biplot graphic displays (Shafii 1993; Shafii and Price 1998) were also generated to investigate the pattern and structure of the underlying variation. Additional PCA analyses were presented for pre- and post-nutrient addition periods at the KR-9 and KR-9.1 combination of sites and at KR-10. Principal Components Analysis of response variables was conducted for sites KR-9.1, KR-10 and KR-14 separately and combined sites KR-9 and KR-9.1 (subsequently referred to as the "nutrient addition zone"), and for all sites and years for which data were available. All principal components analyses and graphics were performed and created using SAS (2004).

Taxonomic data and a series biological and ecological metrics were used with PCA for diatoms and algae. Algal metrics included a suite of abundance, diversity, richness, dominance, and other measures. Additional trophic status and chemical limnology metrics, Van Dam indices (Van Dam et al. 1994), and morphological groupings (Stevenson et al. 1996) were employed in diatom PCAs (Aappendix d: Algae and diatom metrics). The van Dam indices were applied to each diatom taxon that consisted of an 8-letter code eight ecological indicator values for pH, salinity, nitrogen uptake metabolism, oxygen, saprobity, trophic state, and moisture)(Van Dam et al. 1994). Algae were also described and analyzed according to morphological types reported by Stevenson et al. (1996) as: E (erect), F (filamentous), S (stalked), U (Unattached), and V (variable).

***Analysis of variance (ANOVA).* -** ANOVA was carried out on chlorophyll a (Chl_a), chlorophyll accrual (Chl_acc), total chlorophyll (Total), and total chlorophyll accrual (Total_acc) data to assess site differences and mean contrasts from 2004 through 2007. Preplanned site or grouped site contrasts included:

1. Meander (KR1-KR4) vs. Treated Zone (KR9 and KR9.1)
2. Fertilization Control (KR10) vs. Treated zone (KR9 and KR9.1)
3. Natural (KR14) vs. Hydro (KR13)
4. Braided (KR6 and KR7) vs. Treated Zone (KR9 and KR9.1)
5. Braided (KR6 and KR7) vs. Meander (KR1-KR4)
6. Braided (KR6 and KR7) vs. Fertilization Control (KR10)
7. Braided (KR6 and KR7) vs. Natural (KR14)
8. Lower meander (KR-1 and KR-2) vs. Upper meander (KR-3 and KR-4)

ANOVA was also used to test for nutrient addition (treatment) and site effects on chlorophyll a, chlorophyll a accrual, total chlorophyll, and total chlorophyll accrual. These contrasts involved pre- and post-treatment data (before and after 7/2005) exclusively from sites KR-9, KR-9.1 (treatment) and KR-10 (control). ANOVA was also performed using data from 2004 through 2007 to investigate the average algal abundance, biomass, and richness responses to assess the nutrient addition treatment and to test for site effects on these metrics. Abundance and biomass responses were logarithmically transformed to meet statistical requirements of the analyses. Analysis of variance tables, least squares means tables, and a table of preplanned contrasts for reach effects was provided as output. All ANOVA procedures were performed using SAS (SAS 2004).

Specific Hypotheses

A series of hypotheses were tested to assess effects of experimental nutrient addition on algal abundance, biomass, richness, order composition, chlorophyll *a*, and total chlorophyll concentration and accrual rate before and after addition, as well as upstream and downstream from the nutrient addition site (Table 4).

Table 4. Hypotheses tested with ANOVA to assess effects of experimental nutrient addition on algal abundance, biomass, richness, and taxonomic order composition in the Kootenai River at sites KR-9 and KR-9.1 for all years sampled.

Response Metric-Algae	Hypotheses
Abundance	Experimental nutrient addition had no significant effect on average aggregated algal abundance at sites KR-9 and KR-9.1 combined.
Biomass	Experimental nutrient addition had no significant effect on average aggregated algal biomass at sites KR-9 and KR-9.1 combined.
Richness	Experimental nutrient addition had no significant effect on average aggregated algal richness at sites KR-9 and KR-9.1 combined.
Order composition	Experimental nutrient addition had no significant effect on average aggregated order composition abundance at sites KR-9 and KR-9.1 combined.
Response Metric-Chlorophyll *a*	
Concentration	Experimental nutrient addition had no significant effect on average chlorophyll *a* biomass at sites KR-9 and KR-9.1 combined.
Accrual rate	Experimental nutrient addition had no significant effect on average chlorophyll *a* accrual rate at sites KR-9 and KR-9.1 combined.
Response Metric-Total Chlorophyll (*a* + *b*)	
Concentration	Experimental nutrient addition had no significant effect on average total chlorophyll biomass at sites KR-9 and KR-9.1 combined.
Accrual rate	Experimental nutrient addition had no significant effect on average total chlorophyll (algal) accrual rate at sites KR-9 and KR-9.1 combined.

RESULTS

Chlorophyll

Longitudinal, in-river gradients of chlorophyll *a* and total chlorophyll (*a* + *b*) biomass and accrual rates were observed during all years, along with increased responses at nearly all sites during all treatment years (2005 through 2007) relative to the pre-treatment year (2004). For all within-year comparisons (2004-2007), chlorophyll *a* and total chlorophyll values were lower in the canyon reach upstream from nutrient addition (KR-10 through KR-14) than at sites downstream from nutrient addition (KR-1 through KR-9.1; Figure 2 and Figure 3). Peak chlorophyll *a* and chlorophyll *a* accrual rate values occurred at sites KR-4 and KR-9.1 during all treatment years (Figure 2 and Figure 3). Elevated chlorophyll *a* values were consistently observed at KR-4 during all pre- and post-fertilization years (Figure 2). With the exception of site KR-7 through KR-10, chlorophyll *a* and total chlorophyll *a* accrual rates increased during the three consecutive treatment years (2005 through 2007; Figure 2 and Figure 3).

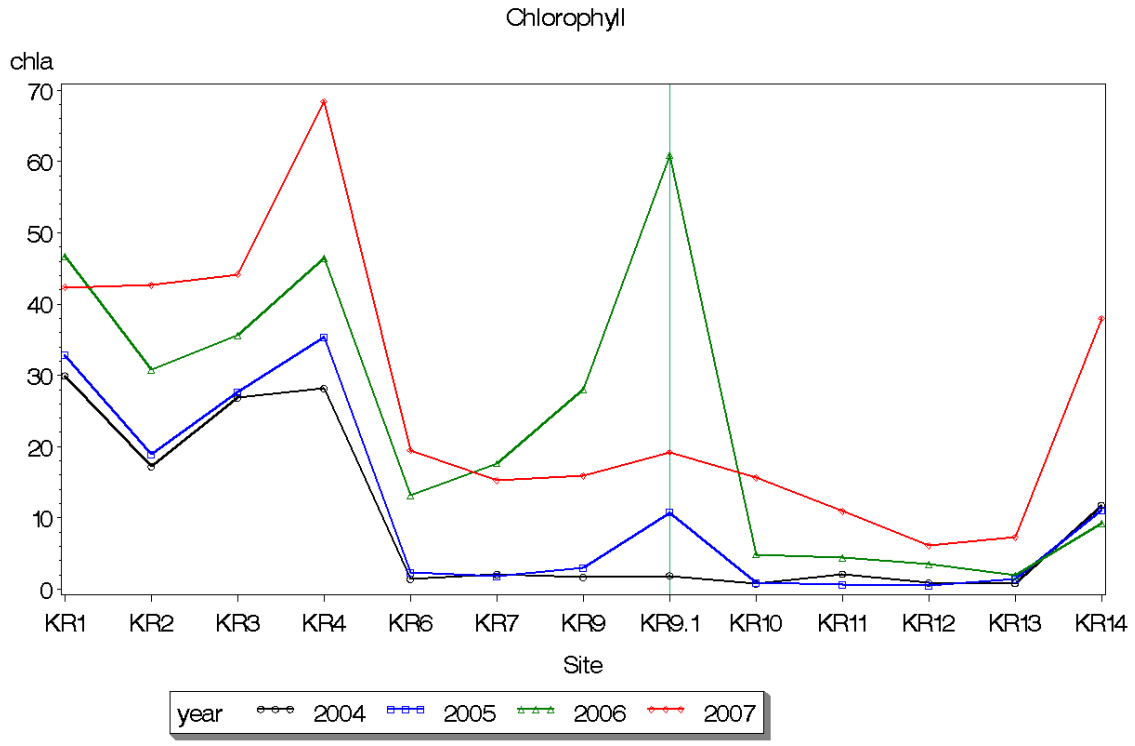


Figure 2. Mean chlorophyll *a* biomass values (mg/m^2) by site (KR-1 through KR-14) among years before (2004) and after (2005-2007) experimental nutrient addition in the Kootenai River.

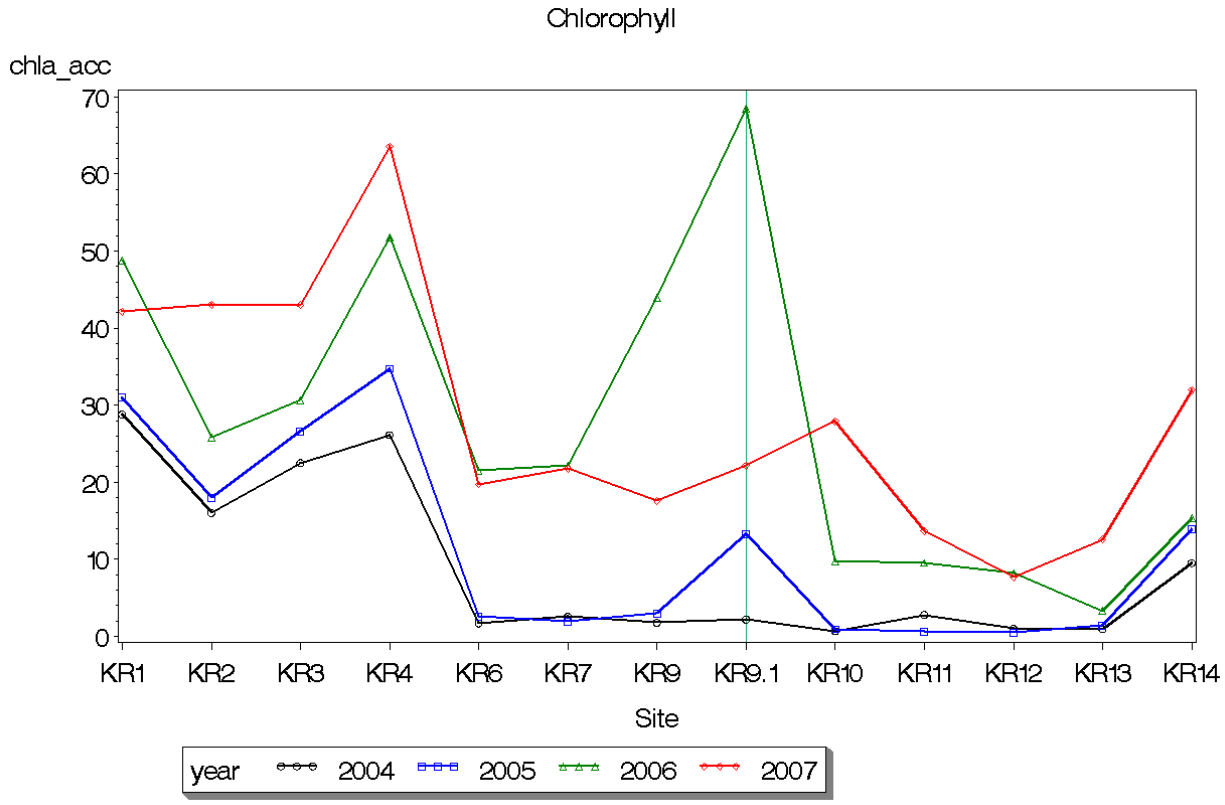


Figure 3. Mean chlorophyll *a* accrual rates (mg/m^2/30d) by site (KR-1 through KR-14) among years before (2004) and after (2005-2007) experimental nutrient addition in the Kootenai River.

As with chlorophyll *a* concentration, similar longitudinal, in-river patterns of total chlorophyll and total chlorophyll accrual rates were observed during all years, with values elevated at nearly all sites during all treatment years (2005 through 2007) compared to the pre-treatment year (2004; Figure 4 and 5). However, mean total chlorophyll biomass and accrual rates were almost twice as high as chlorophyll *a* and chlorophyll *a* biomass and accrual (Figure 2-Figure 4). Similarly, total chlorophyll biomass and accrual rate values typically increased with each subsequent year of nutrient addition, with the exception of KR-9.1 during 2006 and 2007, where this trend was consistently reversed (Figure 4 and 5).

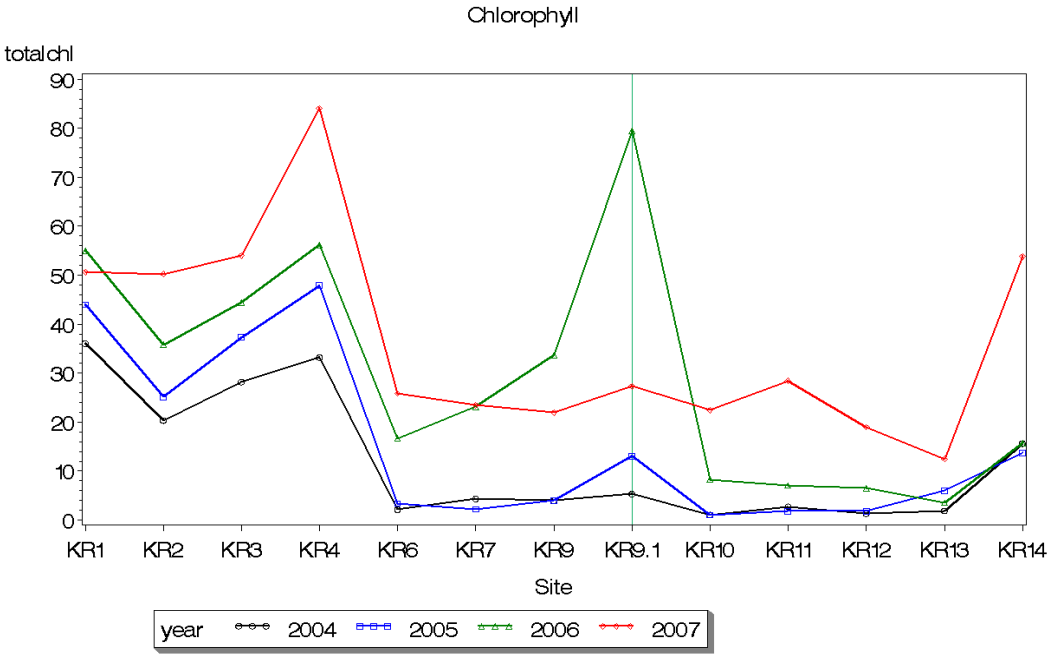


Figure 4. Total chlorophyll (*a*+*b*) values (mg/m^2) by site (KR-1 through KR-14) among years before (2004) and after (2005-2007) experimental nutrient addition in the Kootenai River.

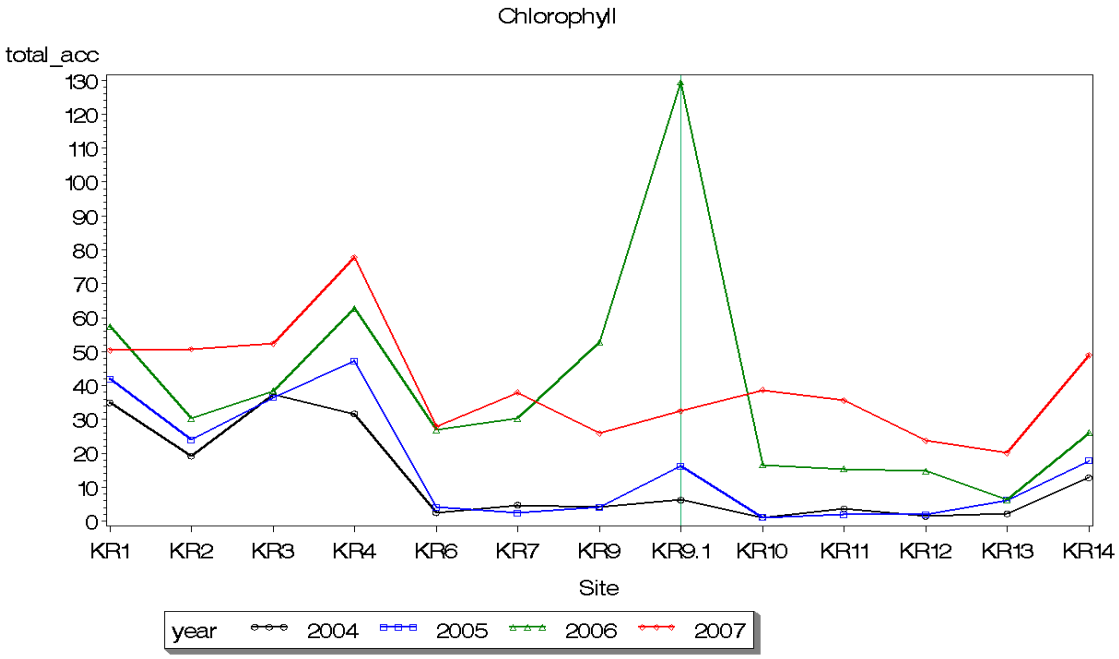


Figure 5. Total chlorophyll (*a* + *b*) accrual rates (mg/m^2/30d) by site (KR-1 through KR-14) among years before (2004) and after (2005-2007) experimental nutrient addition in the Kootenai River.

Mean annual chlorophyll *a*, chlorophyll *a* accrual, total chlorophyll, and total chlorophyll accrual exhibited the same temporal pattern among pre- and post-fertilization years in the nutrient addition zone (KR-9 and KR-9.1; Figure 6).

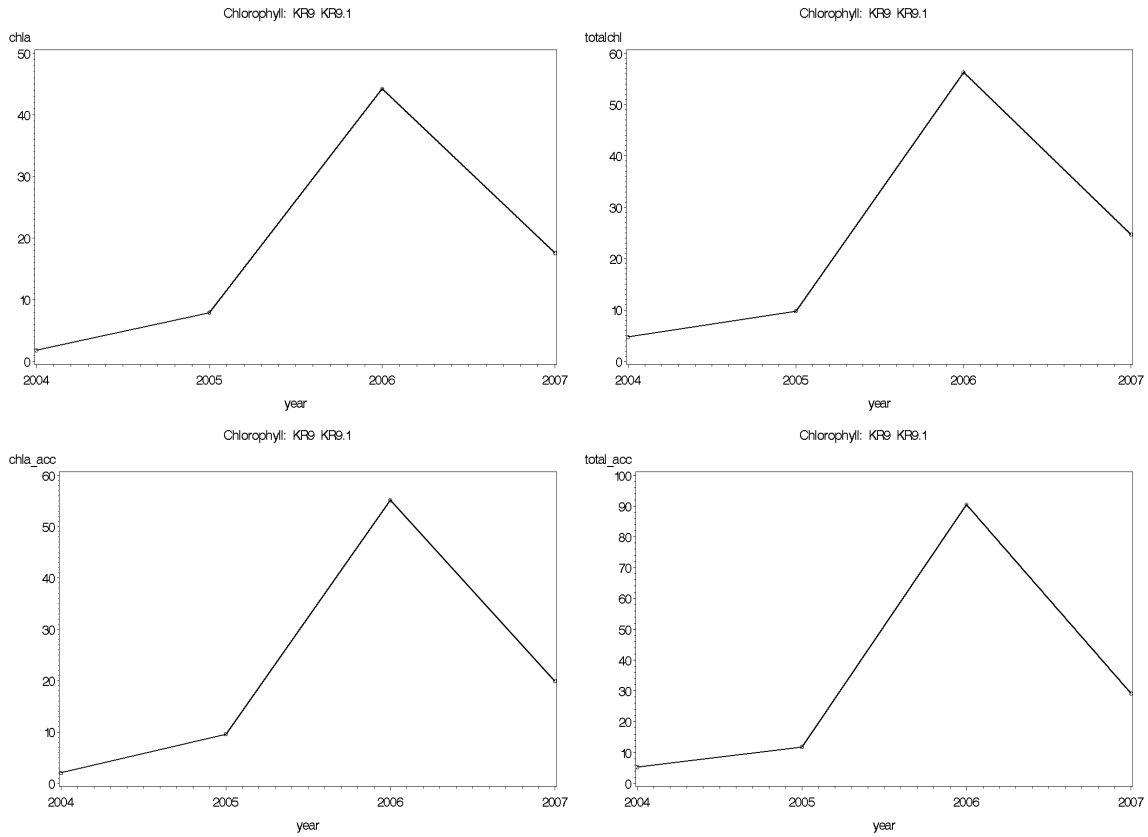


Figure 6. Mean annual chlorophyll *a* biomnass (mg/m^2; upper left), chlorophyll a accrual rates (mg/m^2/30d) lower left), total chlorophyll (mg/m^2; upper right), and total chlorophyll accrual rates (mg/m^2/30d) in the nutrient addition zone (KR-9 and KR-9.1) from 2004 through 2007. (Note difference in vertical axis scale on bottom right plot).

Pre- and post-fertilization average values for all chlorophyll metrics at treatment (KR-9 and KR-9.1) and control sites (KR-10) also increased following nutrient addition, with the largest increases consistently occurring at the nutrient addition site, KR-9.1 (Figure 7). Responses at KR-9 exceeded those at KR-10 with the exception of total chlorophyll accrual (Figure 7).

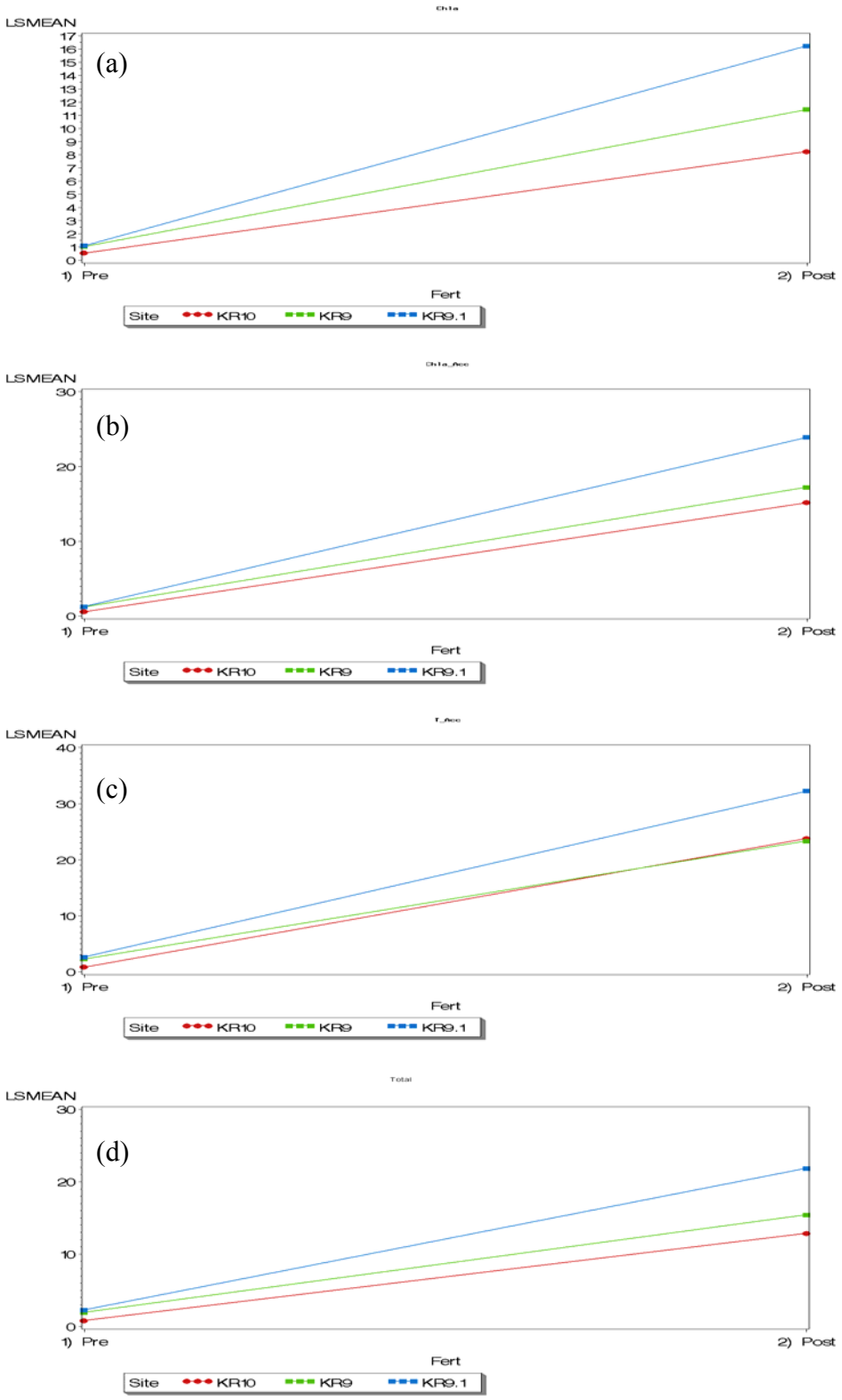


Figure 7. Pre- and post-fertilization least square mean values for (a) chlorophyll *a*, (b) chlorophyll *a* accrual, (c) total chlorophyll, and (d) total chlorophyll accrual.

Analysis of Variance – Chlorophyll – site effects

ANOVA revealed consistent significant site effects for each year for chlorophyll *a* biomass, chlorophyll *a* accrual, total chlorophyll biomass, and total chlorophyll accrual (Table 4, Table 5, Table 6, and Table 7). Exceptions included: 1) non-significant differences for chlorophyll *a* biomass and accrual, and total chlorophyll concentration between the braided and treated zones; 2) consistent non significant differences between the braided vs. natural reach and lower and upper meanders reach comparisons in 2006; and 3) several site and site group comparisons in 2007 (Table 5, Table 6, Table 7, and Table 8).

Table 5. Individual and grouped site contrasts for chlorophyll *a* biomass (mg/m^2) by year. Shaded cells indicate significance at α=0.05.

Site Contrast	Pr > F			
Chlorophyll *a* Biomass	**2004**	**2005**	**2006**	**2007**
Meander vs. Treated Zone	<.0001	<.0001	<.0001	<.0001
Control vs. Treated Zone	0.0041	<.0001	<.0001	0.4679
Natural vs. Hydro	<.0001	<.0001	<.0001	0.0011
Braided vs. Treated Zone	0.1743	<.0001	<.0001	0.5456
Braided vs. Meander	<.0001	<.0001	<.0001	<.0001
Braided vs. Control	0.0001	0.0101	<.0001	0.2632
Braided vs. Natural	<.0001	<.0001	0.0979	0.0049
Lower Meander vs. Upper Meander	0.0151	0.0071	0.4585	0.0842

Table 6. Individual and grouped site contrasts for chlorophyll *a* accrual (mg/m^2/30 d) by year. Shaded cells indicate significance at α=0.05.

Site Contrast	Pr > F			
Chlorophyll *a* Accrual	**2004**	**2005**	**2006**	**2007**
Meander vs. Treated Zone	<.0001	<.0001	0.5831	<.0001
Control vs. Treated Zone	0.0014	<.0001	<.0001	0.0614
Natural vs. Hydro	<.0001	<.0001	<.0001	0.0002
Braided vs. Treated Zone	0.2076	<.0001	<.0001	0.7144
Braided vs. Meander	<.0001	<.0001	<.0001	<.0001
Braided vs. Control	<.0001	0.0027	0.0030	0.0360
Braided vs. Natural	<.0001	<.0001	0.3032	0.0005
Lower Meander vs. Upper Meander	0.0561	0.0017	0.3946	0.0330

Table 7. Individual and grouped site contrasts for total chlorophyll biomass (mg/m^2) by year. Shaded cells indicate significance at α=0.05.

Site Contrast	Pr > F			
Total Chlorophyll Biomass	2004	2005	2006	2007
Meander vs. Treated Zone	<.0001	<.0001	0.0004	<.0001
Control vs. Treated Zone	0.0001	<.0001	<.0001	0.3823
Natural vs. Hydro	<.0001	0.0020	<.0001	0.0002
Braided vs. Treated Zone	0.7331	<.0001	<.0001	0.8562
Braided vs. Meander	<.0001	<.0001	<.0001	<.0001
Braided vs. Control	0.0004	0.0027	0.0017	0.3252
Braided vs. Natural	<.0001	<.0001	0.4150	0.0036
Lower Meander vs. Upper Meander	0.0193	0.0045	0.3090	0.0484

Table 8. Individual and grouped site contrasts for total chlorophyll accrual (mg/m^2/ 30 d) by year. Shaded cells indicate significance at α=0.05.

Site Contrast	Pr > F			
Total Chlorophyll Accrual	2004	2005	2006	2007
Meander vs. Treated Zone	<.0001	<.0001	0.2648	<.0001
Control vs. Treated Zone	<.0001	<.0001	<.0001	0.0325
Natural vs. Hydro	<.0001	<.0001	<.0001	<.0001
Braided vs. Treated Zone	<.0001	<.0001	<.0001	0.6529
Braided vs. Meander	<.0001	<.0001	<.0001	<.0001
Braided vs. Control	0.0101	0.0006	0.0363	0.0689
Braided vs. Natural	<.0001	<.0001	0.8922	0.0005
Lower Meander vs. Upper Meander	0.0071	0.0013	0.2546	0.0124

Analysis of Variance – Chlorophyll – Fertilizer and site effects

ANOVA was also used to directly test site, fertilization, and site*fertilization interaction effects, using data exclusively from KR-9 and KR-9.1 combined as the nutrient addition zone, and KR-10 as the upstream control site (no nutrient addition). All responses were log transformed to meet the assumptions of the analyses. ANOVA revealed significant treatment and site effects for all chlorophyll metrics (chlorophyll *a* biomass, chlorophyll *a* accrual rate, total chlorophyll biomass, and total chlorophyll accrual rate; Table 9). However, a significant Fert*Site interaction occurred only for total chlorophyll accrual rate (Table 9). Least squares mean values for all chlorophyll metrics consistently increased by an order of magnitude following nutrient addition, with the exception of KR-9.1, which increased three-fold (Table 10).

Table 9. ANOVA results for site, fertilization, and fertilization*site interaction for log chlorophyll *a* and total chlorophyll biomass and accrual rates. Shaded cells indicate significance at α=0.05.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Log Chlorophyll a Biomass					
Fert	1	320.8296390	320.8296390	257.34	<.0001
Site	2	13.2961532	6.6480766	5.33	0.0054
Fert*Site	2	1.0625004	0.5312502	0.43	0.6535
Log Chlorophyll a Accrual					
Fert	1	419.3222123	419.3222123	422.27	<.0001
Site	2	10.8769879	5.4384939	5.48	0.0047
Fert*Site	2	2.4829870	1.2414935	1.25	0.2882
Log Total Chlorophyll Biomass					
Fert	1	274.3772928	274.3772928	206.29	<.0001
Site	2	17.9662099	8.9831050	6.75	0.0014
Fert*Site	2	3.2971064	1.6485532	1.24	0.2913
Log Total Chlorophyll Accrual					
Fert	1	363.8262686	363.8262686	332.22	<.0001
Site	2	14.8886425	7.4443212	6.80	0.0013
Fert*Site	2	7.4336316	3.7168158	3.39	0.0351

Table 10. Least square means for pre- and post-nutrient addition periods for log chlorophyll *a* and total chlorophyll biomass and accrual rates at KR-9, KR-9.1 and KR-10. Shaded cells indicate significance at α=0.05.

Fert	Site	LSMEAN	Standard Error	Pr> |t|
Log Chlorophyll a Biomass				
1) Pre	**KR10**	-0.42557261	0.23282088	0.0687
1) Pre	**KR9**	0.14243741	0.20385681	0.4854
1) Pre	**KR9.1**	0.18510547	0.20385681	0.3647
2) Post	**KR10**	2.12004503	0.21101185	<.0001
2) Post	**KR9**	2.44567066	0.13744023	<.0001
2) Post	**KR9.1**	2.79399376	0.12406330	<.0001
Log Chlorophyll a Accrual				
1) Pre	**KR10**	-0.38571694	0.20778551	0.0646
1) Pre	**KR9**	0.29315763	0.18193596	0.1084
1) Pre	**KR9.1**	0.32483950	0.18193596	0.0754
2) Post	**KR10**	2.72672593	0.18832161	<.0001
2) Post	**KR9**	2.85315209	0.12266120	<.0001
2) Post	**KR9.1**	3.17837382	0.11072270	<.0001
Log Total Chlorophyll Biomass				
1) Pre	**KR10**	-0.09851771	0.24047284	0.6824
1) Pre	**KR9**	0.72661980	0.21055683	0.0007
1) Pre	**KR9.1**	0.87137811	0.21055683	<.0001
2) Post	**KR10**	2.56374662	0.21794702	<.0001
2) Post	**KR9**	2.74242996	0.14195739	<.0001
2) Post	**KR9.1**	3.09004645	0.12814081	<.0001
Log Total Chlorophyll a Accrual				
1) Pre	**KR10**	-0.04254386	0.21820926	0.8456
1) Pre	**KR9**	0.88907931	0.19106295	<.0001
1) Pre	**KR9.1**	1.01542300	0.19106295	<.0001
2) Post	**KR10**	3.17285714	0.19776894	<.0001
2) Post	**KR9**	3.15387054	0.12881461	<.0001
2) Post	**KR9.1**	3.47699473	0.11627721	<.0001

Diatom Taxa Abundance

Fifty-three distinct diatom taxa were represented in the diatom samples, with the four most abundant taxa (*Achnanthes*, *Acnanthidium*, *Diatoma*, and *Cymbella*) representing 56% of the total specimen count across samples (Table 11).

Table 11. Taxa, count, and percent contribution by taxa for diatom samples from all sites in the Kootenai River, 2004 through 2007.

Taxa	Count	Percent
Achnanthes	73224	34.5642
Achnanthidium	16765	7.9137
Diatoma	16585	7.8287
Cymbella	11987	5.6583
Fragilaria	7806	3.6847
Synedra	6766	3.1938
Gomphonema	5389	2.5438
Cyclotella	4286	2.0231
Navicula	3106	1.4661
Nitzschia	2954	1.3944
Cocconeis	2581	1.2183
Encyonema	1534	0.7241
Encyonopsis	1510	0.7128
Rhoicosphenia	1496	0.7062
Denticula	650	0.3068
Distrionella	587	0.2771
Didymosphenia	523	0.2469
Hannaea	495	0.2337
Stephanodiscus	316	0.1492
Melosira	314	0.1482
Amphora	289	0.1364
Reimeria	286	0.1350
Anomoeoneis	262	0.1237
Staurosira	204	0.0963
Planothidium	195	0.0920
Gomphoneis	179	0.0845
Meridion	155	0.0732

Taxa	Count	Percent
Asterionella	133	0.0628
Amphipleura	118	0.0557
Brachysira	91	0.0430
Epithemia	85	0.0401
Eunotia	70	0.0330
Stauroneis	62	0.0293
Staurosirella	51	0.0241
Rhopalodia	47	0.0222
Cymatopleura	42	0.0198
Gyrosigma	32	0.0151
Pinnularia	27	0.0127
Surirella	25	0.0118
Caloneis	23	0.0109
Aulacoseira	21	0.0099
Frustulia	21	0.0099
Diploneis	15	0.0071
Mastogloia	9	0.0042
Coscinodiscus	8	0.0038
Desmidium	6	0.0028
Dickieia	6	0.0028
Neidium	6	0.0028
Chrysidiatrum	5	0.0024
Hantzschia	3	0.0014
Geissleria	2	0.0009
Scoliopleura	2	0.0009
Gomphosphenia	1	0.0005

Principal Component Analysis

Diatom Taxa: Overall

Principal Component Analysis for diatom taxa abundance was performed using data in common across sampling dates and sites. Approximately 60% of the overall variability was accounted for in the first 3 PCA axes (Table 12). Loading in the first axis was dominated by *Denticula*, *Fragilaria*, *Navicula*, *Nitschia*, and *Synedra*, compared to *Cocconeis* and *Gomphonema*, which dominated the second axis loadings (Table 13).

Table 12. Eigenvalue information for the Principal Component Analysis of overall diatom taxa.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	Difference	Proportion	Cumulative
1	6.10868903	2.55736642	0.3215	0.3215
2	3.55132260	1.66350261	0.1869	0.5084
3	1.88781999		0.0994	0.6078

Table 13. Principal component loadings by taxonomic order for KR-9 and KR-9.1 combined during all years.

Eigenvectors			
	Prin1	Prin2	Prin3
Achnanthes	-.208533	0.143374	0.056180
Amphora	0.277558	0.240753	0.092054
Cocconeis	-.107799	0.370603	0.267790
Cyclotella	0.249442	0.055029	0.389820
Cymbella	0.240578	-.004464	-.159106
Denticula	0.339021	0.059815	0.072773
Diatoma	0.250667	0.133576	-.375856
Fragilaria	0.320924	0.023150	-.143999
Gomphonema	0.150651	-.327760	0.030541
Hannaea	-.013598	-.179490	0.125773
Navicula	0.317105	0.133680	0.270595
Nitzschia	0.330014	0.051229	0.351867
Synedra	0.311908	0.210260	-.215182
Tabellaria	-.150658	0.281863	-.220589
Amphipleura	0.042405	-.163951	0.191802
Anomoeoneis	0.212466	0.265845	-.331967
Didymosphenia	-.177968	0.359597	-.129588
Reimeria	-.118163	0.395480	0.179185
Rhoico sphenia	-.148625	0.303428	0.259708

Hence, these taxa contributed significantly to the overall variability of diatom abundance. A significant shift in diatom taxa composition following nutrient addition was also revealed by PCA, biplots, where the two classes (pre- and post-nutrient addition) formed non-overlapping groupings (Figure 8).

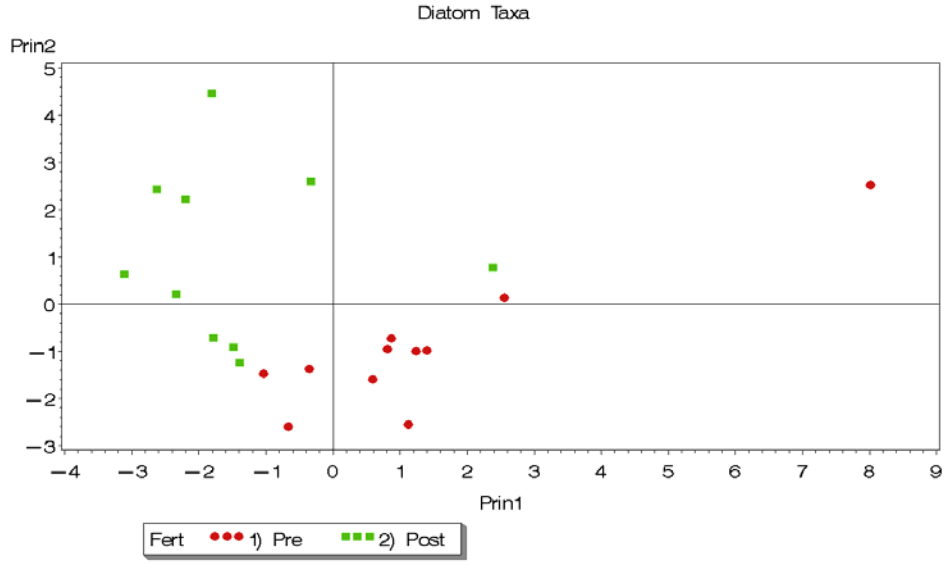


Figure 8. Principal Component Analysis Bi-plot for overall diatom taxa decomposition.

Diatom Taxa by Nutrient Timing

PCA of diatom taxa accounted for slightly over 60% of variability in the first 3 axes during the pre- and post-fertilization time periods (Table 14).

Table 14. Eigenvalue information for the Principal Component Analysis of diatom taxa during the pre-nutrient addition period.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
Pre-fertilization				
1	6.40523553	3.43669633	0.3371	0.3371
2	2.96853921	0.48462090	0.1562	0.4934
3	2.48391830		0.1307	0.6241
Post-fertilization				
1	5.09262869	1.51694711	0.2680	0.2680
2	3.57568157	0.62930489	0.1882	0.4562
3	2.94637668		0.1551	0.6113

In the pre-treatment diatom taxa PCA, the first axis was represented by the genera *Amphora, Cyclotella, Denticula, Navicula, Nitzschia, Anomoeoneis, and Reimeri*, compared to dominance in the second axis by the genera *Achnanthes*, *Cymbella*, *Diatoma*, and *Tablellaria* (Table 15. Pre-nutrient addition Principal Component Analysis eigenvector loadings for diatom taxa. Shaded cells indicate contributing taxa.).

Table 15. Pre-nutrient addition Principal Component Analysis eigenvector loadings for diatom taxa. Shaded cells indicate contributing taxa.

Eigenvectors			
	Prin1	**Prin2**	**Prin3**
Achnanthes	0.114178	0.329209	0.138059
Amphora	0.307112	0.152087	0.234943
Cocconeis	0.054016	0.177755	0.060531
Cyclotella	0.306165	0.067406	-.260205
Cymbella	0.117014	0.358777	0.197083
Denticula	0.352353	0.088272	-.133750
Diatoma	0.199189	-.401615	0.034532
Fragilaria	0.258095	-.231449	0.227229
Gomphonema	-.077810	0.132515	-.395897
Hannaea	-.094075	0.273563	0.414395
Navicula	0.363204	0.163868	-.020366
Nitzschia	0.316414	0.245136	-.058796
Synedra	0.291972	-.144118	-.079277
Tabellaria	-.014950	0.401422	-.073982
Amphipleura	-.054626	0.208128	-.227896
Anomoeoneis	0.302792	-.254754	0.115323
Didymosphenia	-.088645	0.014265	0.535892
Reimeria	0.341469	-.037637	0.021359
Rhoicosphenia	0.017903	0.099435	-.218757

In the post-treatment diatom taxa PCA, the first axis was dominated by *Achnanthes*, *Amphora*, *Diatoma*, *Nitschia*, *Didymosphenia*, compared to *Denticula*, *Gomphonema*, *Reimeria*, *Rhoicosphenia* for the second axis (Table 16. Post-nutrient addition Principal Component Analysis eigenvector loadings for diatom taxa. Shaded cells indicate contributing taxa.).

Table 16. Post-nutrient addition Principal Component Analysis eigenvector loadings for diatom taxa. Shaded cells indicate contributing taxa.

Eigenvectors			
	Prin1	**Prin2**	**Prin3**
Achnanthes	-.403427	0.032058	-.161322
Amphora	0.333011	-.016219	0.007207
Cocconeis	0.122141	0.224520	0.399557
Cyclotella	0.178737	-.287192	0.133424
Cymbella	0.254142	0.168785	0.099398
Denticula	0.150868	-.376378	-.029012
Diatoma	0.331681	-.012396	-.156743
Fragilaria	0.183595	0.180450	-.185326
Gomphonema	0.032512	-.381335	0.024941
Hannaea	-.189390	0.010836	0.088494
Navicula	0.268370	-.234063	0.158861
Nitzschia	0.342495	-.092954	0.153800
Synedra	0.269395	0.128750	-.208745
Tabellaria	0.053625	0.212789	-.427618
Amphipleura	0.069572	0.187967	-.305315
Anomoeoneis	0.223391	0.297873	-.267818
Didymosphenia	0.302586	0.009531	-.005331
Reimeria	0.020362	0.373685	0.367258
Rhoicosphenia	0.000019	0.358260	0.378436

Hence, notable change in diatom taxonomic structure between pre- and post-fertilization time periods was revealed by the PCA and is graphically represented in the biplots shown in Figure 9. The KR-9 and KR-9.1 observations on the right side of the post-fertilization plot were related to a single sampling date (Oct 22, 2007; Figure 9).

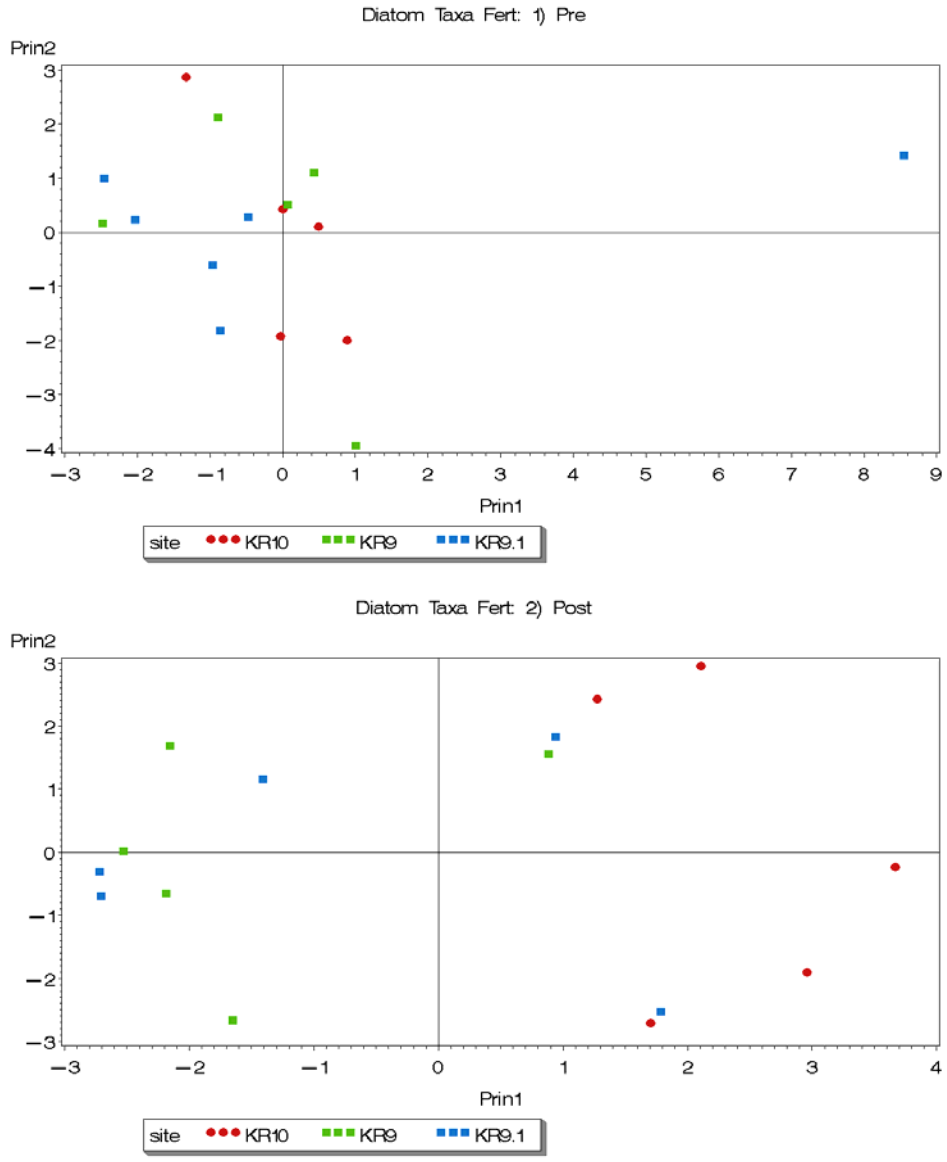


Figure 9. Principal Component Analysis Bi-plots for overall diatom taxa decomposition for 1) pre- and 2) post-nutrient addition periods.

ANOVA - Diatom Taxa

Pre-fertilization - During the pre-treatment period, 8 of 104 ANOVA site contrasts (~5%) for diatom taxa were significant. Two diatom taxa exhibited significant site effects *(Diatoma* and *Gomphonema).* A total of 8 individual site or reach contrasts were significantly different for 5 diatom taxa, however, the additional three taxa (*Tabellaria*, *Anomoeoneis*, and *Didymosphenia*) were significant in unprotected tests (i.e. significant without a significant main site effect; Table 17).

Post-fertilization – During the post-treatment period, 15 of the 104 ANOVA site contrasts (~14%) for diatom taxa were significant. Two diatom taxa, *Anomoeoneis* and *Didymosphenia* exhibited significant site effects, whereas an additional 5 diatom taxa (*Cocconeis*, *Diatoma*, *Fragellaria*, *Navicula*, and *Nitzchia*) collectively revealed 10 significant site differences (Table 17).

ANOVA for diatom taxa by nutrient addition timing (pre vs. post) revealed significant treatment effects for *Achnanthes*, *Gomphonema*, and *Didymosphenia*, and a significant site effect for *Achnanthes.*

Pre Treatment (prior to 7/2005)

Contrast	Taxa												
	Achnanthes	Cocconeis	Cymbella	Diatoma	Fragilaria	Gomphonema	Navicula	Nitzschia	Synedra	Tabellaria	Anomoeoneis	Didymosphenia	Reimeria
Site													
Meander vs Treated Zone													
Control (KR10) vs Treated Zone													
Natural (KR14) vs Hydro (KR13)													
Braided vs Treated Zone													
Braided vs Meander													
Braided vs Control (KR10)													
Braided vs Natural (KR14)													
Lower Meander vs Upper Meander													

Post Treatment (after 7/2005)

Contrast	Taxa												
	Achnanthes	Cocconeis	Cymbella	Diatoma	Fragilaria	Gomphonema	Navicula	Nitzschia	Synedra	Tabellaria	Anomoeoneis	Didymosphenia	Reimeria
Site													
Meander vs Treated Zone													
Control (KR10) vs Treated Zone													
Natural (KR14) vs Hydro (KR13)													
Braided vs Treated Zone													
Braided vs Meander													
Braided vs Control (KR10)													
Braided vs Natural (KR14)													
Lower Meander vs Upper Meander													

Non-significant
Significant*
Protected Test (overall Site effect significant)

Meander: KR1, KR2, KR3, KR4
Braided: KR6, KR7
Treated Zone: KR9, KR9.1

Table 17. Pre-and post-nutrient addition ANOVA site contrast results for diatom taxa in the Kootenai River.

Diatom Metrics: Overall

Principal Component Analysis for diatom metrics was performed using data consistent across sampling dates and times. Approximately 68% of the overall variability was accounted for in the first 3 axes (Table 18). Loading in the first axis was dominated by Richness, Richness-Mesotrophic, Richness-Prostrate, compared to Abundance, Oxygen Tolerance, Richness-Oligo-eutrophic, Richness-Erect, which dominated the second axis (Table 19).

Table 18. Eigenvalue information for Principal Component Analysis of diatom metrics.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	Difference	Proportion	Cumulative
1	7.65339028	5.12626347	0.4252	0.4252
2	2.52712681	0.52567659	0.1404	0.5656
3	2.00145022		0.1112	0.6768

Table 19. Eigenvector loadings for Principal Component Analysis of diatom metrics. Shaded cells indicate contributing metrics.

Eigenvectors				
		Prin1	Prin2	Prin3
Richness	Richness	0.345562	-.091062	0.085339
Abundance	Abundance	-.091761	0.445770	-.244682
Shannon_Weaver_H___log_e_	Shannon_Weaver_H___log_e_	0.284075	-.152042	-.266415
Nitrogen_Uptake	Nitrogen_Uptake	-.260570	-.169209	0.136701
Oxygen_Tolerance	Oxygen_Tolerance	0.004186	-.362535	-.496553
Trophic_State	Trophic_State	-.199241	0.245795	0.446453
Richness_Oligotrophic	Richness_Oligotrophic	0.264547	0.010689	0.059874
Richness_Oligo_Mesotrophic	Richness_Oligo_Mesotrophic	0.225169	0.049507	0.125897
Richness_Mesotrophic	Richness_Mesotrophic	0.318649	0.055158	0.057883
Richness_Meso_Eutrophic	Richness_Meso_Eutrophic	0.261166	0.126923	0.240795
Richness_Eutrophic	Richness_Eutrophic	0.201990	-.326809	-.004350
Richness_Hypereutrophic	Richness_Hypereutrophic	-.105332	-.131396	0.397786
Richness_Oligo_to_Eutrophic	Richness_Oligo_to_Eutrophic	0.207881	0.324310	-.012458
Richness_Erect	Richness_Erect	-.068696	0.384054	-.341871
Richness_Stalked	Richness_Stalked	0.209623	0.195834	-.113494
Richness_Unattached	Richness_Unattached	0.260518	-.182657	0.137335
Richness_Prostrate_Adnate	Richness_Prostrate_Adnate	0.332414	-.000653	0.097040
Richness_Variable	Richness_Variable	0.266997	0.281471	0.009396

A moderate split in axes relative to pre- and post-treatment periods was revealed by the PCA biplots for overall diatom metrics (Figure 10). However, the extent of the shift for diatom metrics (Figure 10) was not as consistent or definitive as that observed previously for diatom taxa between pre- and post-treatment periods (Figure 9).

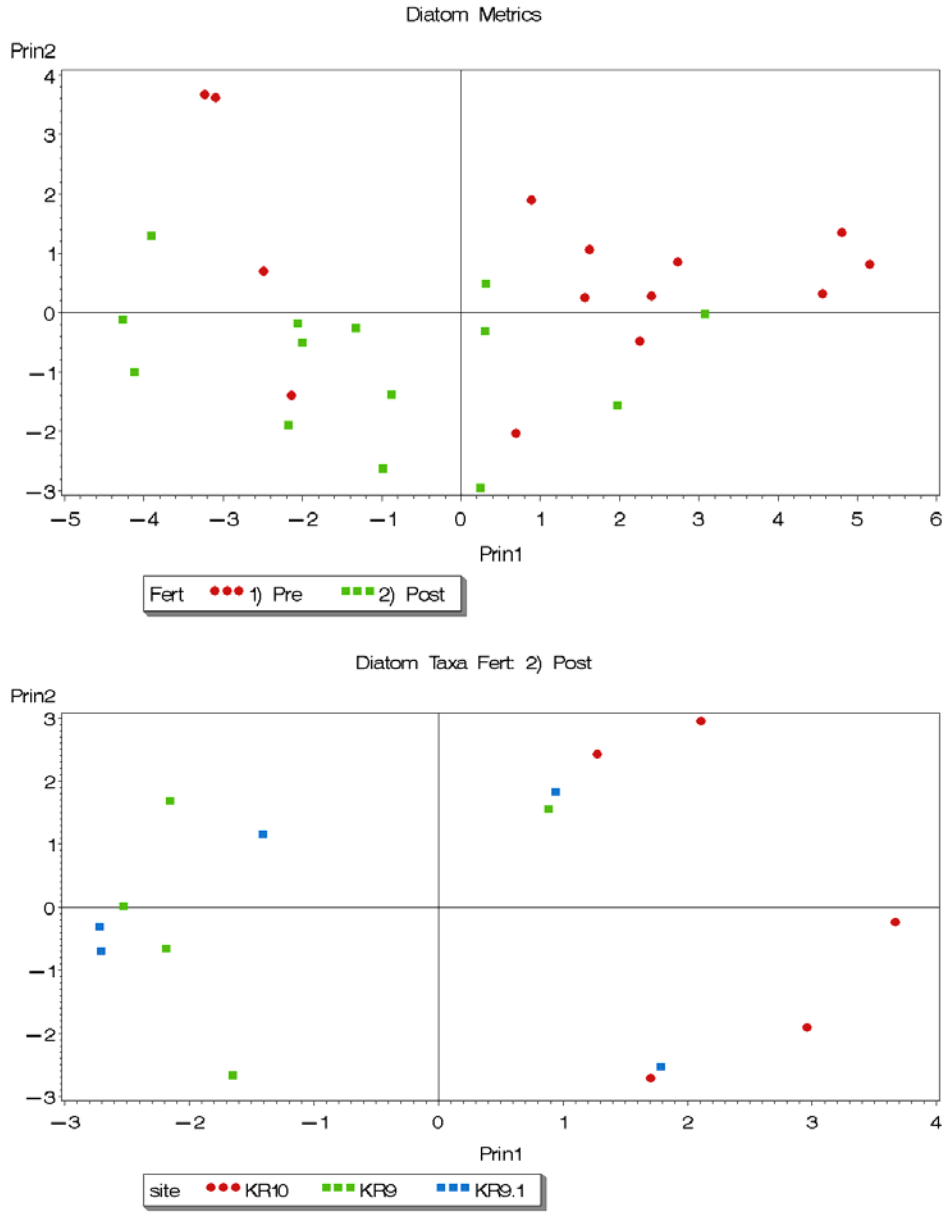


Figure 10. **Principal Component Analysis Bi-plots for overall diatom metric decomposition for 1) pre- and 2) post-nutrient addition periods.**

PCA Diatom Metrics: Richness-evenness Measures

Approximately 89% of the empirical variability associated with diatom richness-evenness metrics was accounted for in the first three PCA axes. PCA eigenvector loadings indicated that the first axis was represented by Richness, Shannon-Weaver, Trophic State metrics, whereas the second axis was dominated by Nitrogen Uptake and Oxygen Tolerance. Although PCA accounted for high levels of variability in the richness-evenness measures for diatoms, it revealed no definitive patterns or shifts for these metrics between pre- and post-treatment periods.

PCA Diatom Metrics: Trophic State

Approximately 75% of the variability accompanying diatom trophic status was accounted for in the first three PCA axes. The first axis was dominated by the Richness-oligotrophic, Richness-mesotrophic, Richness-meso-Eutrophic metrics, whereas the second axis was dominated by Richness-eutrophic, Richness-hypertrophic, Richness-oligo-eutrophic metrics. This PCA also revealed a more distinctive pattern relative to pre- and post treatment changes than the overall analysis, with dominant measures consistent with those from previous PCAs.

PCA Diatom Metrics: Morphology

Approximately 88% of the variability associated with diatom morphology metrics was accounted for in the first three PCA axes. PCA eigenvector loadings for the first axis were dominated by Richness-unattached, Richness-prostrate, Richness-variable metrics, whereas the second axis had high loadings for the Richness-erect, richness-stalked metrics. A weak pattern in PCA structure of diatom morphology response was revealed by the accompanying PCA biplots. However, this pattern for diatom morphology was not as clear as it was for the diatom taxa PCA.

PCA Diatom Metrics: Overall, by Nutrient Timing

PCA accounted for approximately 73 and 66% of the variability associated with the PCA of overall diatom metrics pre-and post-nutrient periods respectively (Table 20). In terms of eigenvalue loadings, for the pre-treatment period, the first axis was represented by the metrics of Richness, Richness-mesotrophic, Richness-prostrate, compared to Shannon-Weaver, Oxygen Tolerance, trophic State, Richness Eutrophic as contributing metrics for the second axis (Table 21). The post-treatment PCA of overall diatom metrics was dominated by Richness, Shannon-Weaver, Richness-prostrate, whereas the second axis was represented by Abundance, Trophic State, Richness-meso-eutrophic, Richness hypertrophic (Table 22).

Table 20. **Eigenvalue information for the Principal Component Analysis of diatom metrics overall during pre-and post-nutrient addition periods.**

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
Pre-fertilization				
1	8.15320808	5.16226892	0.4530	0.4530
2	2.99093915	1.07460498	0.1662	0.6191
3	1.91633417		0.1065	0.7256
Post-fertilization				
1	7.75529747	5.52346208	0.4308	0.4308
2	2.23183539	0.42440656	0.1240	0.5548
3	1.80742883		0.1004	0.6553

Table 21. **Eigenvector loadings for the Principal Component Analysis of overall diatom metrics for the pre-nutrient addition period. Shaded cells indicate contributing metrics.**

Eigenvectors				
		Prin1	**Prin2**	**Prin3**
Richness	Richness	0.337884	-.051857	0.122220
Abundance	Abundance	-.246388	0.276562	-.115857
Shannon_Weaver_H___log_e_	Shannon_Weaver_H___log_e_	0.262836	-.314168	-.184359
Nitrogen_Uptake	Nitrogen_Uptake	-.245929	-.133211	0.282247
Oxygen_Tolerance	Oxygen_Tolerance	-.058007	-.374299	-.422159
Trophic_State	Trophic_State	-.163125	0.348272	0.412894
Richness_Oligotrophic	Richness_Oligotrophic	0.295899	0.251400	0.080937
Richness_Oligo_Mesotrophic	Richness_Oligo_Mesotrophic	0.269329	0.038221	-.005465
Richness_Mesotrophic	Richness_Mesotrophic	0.316058	0.096787	0.054629
Richness_Meso_Eutrophic	Richness_Meso_Eutrophic	0.254148	0.211091	-.096108
Richness_Eutrophic	Richness_Eutrophic	0.137156	-.363798	0.237368
Richness_Hypereutrophic	Richness_Hypereutrophic	-.069557	-.241659	0.365249
Richness_Oligo_to_Eutrophic	Richness_Oligo_to_Eutrophic	0.185874	0.255539	0.024754
Richness_Erect	Richness_Erect	-.199716	0.278893	-.215449
Richness_Stalked	Richness_Stalked	0.057897	0.069891	0.395518
Richness_Unattached	Richness_Unattached	0.289621	-.063561	0.126531
Richness_Prostrate_Adnate	Richness_Prostrate_Adnate	0.325806	-.018885	0.069871
Richness_Variable	Richness_Variable	0.222585	0.285101	-.276368

Table 22. Eigenvector loadings for the Principal Component Analysis of overall diatom metrics for the post-nutrient addition period. Shaded cells indicate contributing metrics.

Eigenvectors				
		Prin1	**Prin2**	**Prin3**
Richness	Richness	0.346600	0.061060	-.034224
Abundance	Abundance	0.040821	-.420101	0.369847
Shannon_Weaver_H___log_e_	Shannon_Weaver_H___log_e_	0.306919	-.132357	-.178243
Nitrogen_Uptake	Nitrogen_Uptake	-.254847	0.215455	-.084960
Oxygen_Tolerance	Oxygen_Tolerance	0.108206	-.304824	-.507831
Trophic_State	Trophic_State	-.267438	0.375557	0.158996
Richness_Oligotrophic	Richness_Oligotrophic	0.162469	-.192100	0.069627
Richness_Oligo_Mesotrophic	Richness_Oligo_Mesotrophic	0.246357	-.009068	0.206596
Richness_Mesotrophic	Richness_Mesotrophic	0.267897	-.210954	0.064217
Richness_Meso_Eutrophic	Richness_Meso_Eutrophic	0.261052	0.335061	0.120421
Richness_Eutrophic	Richness_Eutrophic	0.291450	0.151090	-.178417
Richness_Hypereutrophic	Richness_Hypereutrophic	0.074828	0.351897	0.234630
Richness_Oligo_to_Eutrophic	Richness_Oligo_to_Eutrophic	0.227728	0.259205	0.029845
Richness_Erect	Richness_Erect	0.060509	-.246368	0.505550
Richness_Stalked	Richness_Stalked	0.242160	0.045062	0.155990
Richness_Unattached	Richness_Unattached	0.228957	0.130807	-.263290
Richness_Prostrate_Adnate	Richness_Prostrate_Adnate	0.313013	0.157163	-.025582
Richness_Variable	Richness_Variable	0.231768	0.098740	0.189902

Although a discernable pattern in overall diatom metrics was revealed between treated sites (KR-9, KR-9.1) and the control site (KR-10), little structural change was revealed by the PCA biplots for the pre- and post-fertilization time periods, with a minor exception for KR-9.1 (Figure 11).

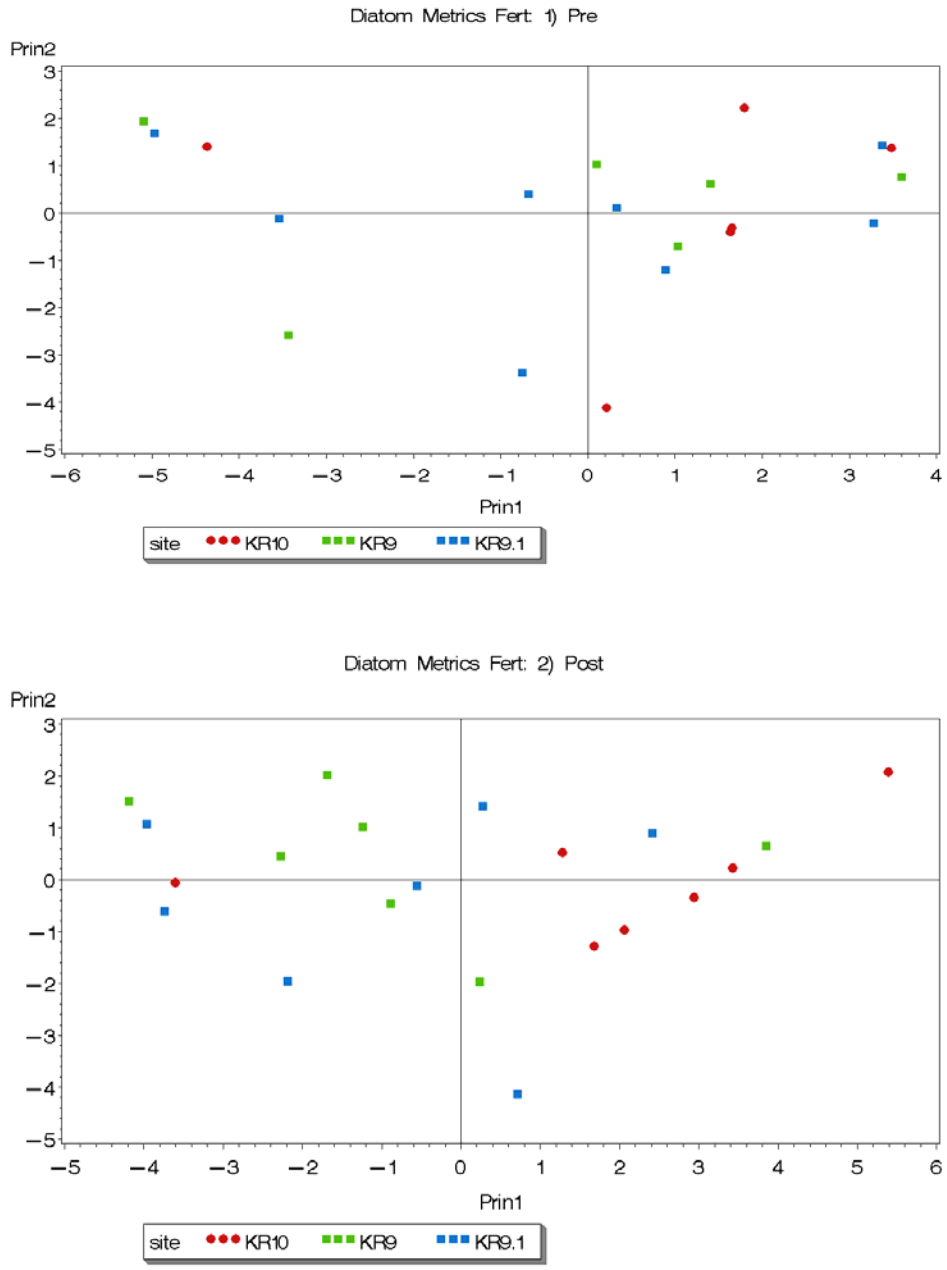


Figure 11. Principal Component Analysis Bi-plots for overall diatom metric decomposition for 1) pre- and 2) post-nutrient addition periods.

PCA Diatom Metrics: Richness Evenness measures, by Nutrient Timing

Very high levels of variability (>90%) associated with diatom richness-evenness measures were accounted for in PCAs for both the pre- and post-treatment periods. During the pre-treatment period, Shannon-Weaver and Trophic State measures explained most of the variability in the first principal component, compared to measures of Richness and Oxygen Tolerance for the second. The same two metrics (Shannon-Weaver and Trophic State) were highly represented in the first principal component in the pre-nutrient addition period, whereas the second axis was dominated by the metrics of Abundance and Oxygen Tolerance. A moderate or small shift was noted in richness and evenness measures by nutrient timing at KR-10 and KR-9.1. Based on the PCA biplots for this analysis (not shown), the first axes of the PCAs for pre- and post-treatment periods largely carried the same dominant elements. However, the signs (+/-) changed, suggesting a reversal of these responses in these metrics between pre-and post-treatment periods.

PCA Diatom Metrics: Trophic State, by Nutrient Timing

High levels of variability of diatom Trophic State by nutrient timing were accounted for in the PCA of the pre- and post-treatment periods (76 and 84% respectively). During the pre-treatment period, Richness-oligotrophic, Richness-mesotrophic, Richness-meso-eutrophic metrics dominated the first component, compared to Richness-eutrophic, Richness-hypertrophic metrics for the second. During the post-fertilization period, variability was accounted for by Richness-meso-eutrophic, Richness-eutrophic in the first principal component, compared to Richness-mesotrophic and Richness-hypertrophic metrics in the second axis. Although some shift was noted for trophic state by nutrient timing at KR-10, no strong discernable patterns of change were observed for Trophic State between pre- and post-fertilization periods.

PCA Diatom Metrics: Morphology, by Nutrient Timing

A large proportion of variability for diatom morphology in pre- and post-treatment periods were also revealed (85 and 86% respectively). During the pre-treatment period, the first axis was dominated by the morphological metrics of Richness-unattached, Richness, Prostrate, compared to Richness-stalked, Richness-variable for the second. In the analysis of the post-treatment period, the first axis was dominated by the morphological metrics of Richness-stalked, Richness-prostrate, Richness-variable, compared to Richness-erect and Richness-unattached for the second axis. Although the control site (KR-10) was somewhat differentiated from the treated sites (KR-9, KR-9.1), no changes in structure were observed between pre- and post-treatment periods.

ANOVA – Diatom Metrics

Pre-treatment - During the pre-treatment period, four diatom metrics exhibited significant site effects, involving trophic status and richness measures (Trophic state, Richness-Eutrophic, Richness-hypereutrophic, and Richness-variable). Within these metrics, a total of 21 site or reach comparisons were significant, dominated by richness measures (Table 23). However, of these 21 significant responses, 8 were unprotected tests, meaning that the metric they represented did not exhibit a significant overall site effect (Table 23).

Post-treatment - During the post-treatment period, 7 diatom metrics exhibited significant site effects, as similarly observed during the pre-fertilization, mainly associated with trophic status and richness measures (Abundance, Nitrogen uptake, Trophic state, Richness-Oligotrophic, Richness-oligo-mesotrophic, Richness-oligo-eutrophic, and richness-unattached morphology). Within these metrics, a total of 30 site or reach comparisons were significant. Of these significant site effects for diatom metrics, two-thirds (20 of 30) involved richness or diversity measures (Table 23). However, of these 30 significant responses, 11 involved unprotected tests.

Pre Treatment (before 7/2005)

Contrast	Metric																	
	Richness	Abundance	Shannon Weaver	Nitrogen Uptake	Oxygen Tolerance	Trophic State	Richness Oligotroph	Richness Oligo-Meso	Richness Mesotrophic	Richness Meso-Eu	Richness Eutrophic	Richness Hypereu	Richness Oligo-	Richness Erect	Richness Stalked	Richness Unattached	Richness Prostrate	Richness Variable
Site																		
Meander vs Treated Zone																		
Control (KR10) vs Treated Zone																		
Natural (KR14) vs Hydro (KR13)																		
Braided vs Treated Zone																		
Braided vs Meander																		
Braided vs Control (KR10)																		
Braided vs Natural (KR14)																		
Lower Meander vs Upper Meander																		

Post Treatment (after 7/2005)

Contrast	Metric																	
	Richness	Abundance	Shannon Weaver	Nitrogen Uptake	Oxygen Tolerance	Trophic State	Richness Oligotroph	Richness Oligo-Meso	Richness Mesotroph	Richness Meso-Eu	Richness Eutrophic	Richness Hypereu	Richness Oligo-Eutrophic	Richness Erect	Richness Stalked	Richness Unattached	Richness Prostrate	Richness Variable
Site																		
Meander vs Treated Zone																		
Control (KR10) vs Treated Zone																		
Natural (KR14) vs Hydro (KR13)																		
Braided vs Treated Zone																		
Braided vs Meander																		
Braided vs Control (KR10)																		
Braided vs Natural (KR14)																		
Lower Meander vs Upper Meander																		

Non-significant
Significant*
Protected Test (overall Site effect significant)

Meander: KR1, KR2, KR3, KR4
Braided: KR6, KR7
Treated Zone: KR9, KR9.1

Table 23. [Pre-and post-nutrient addition ANOVA site contrast results for diatom metrics in the Kootenai River](#).

Green Algae

Algal Abundance by Morphological Type

Green algae morphological types included: E (erect), P (prostrate), S (stalked), U (Unattached), and V (variable) (Stevenson et al. 1996). Plotting total abundance of green algae by year and by morphological type in the nutrient addition zone (KR-9 and KR-9.1) revealed that prostrate taxa abundance increased every year from 2004 through 2007, abundance of stalked and unattached taxa both decreased from 2004 to 2005, peaked during 2006 and decreased in 2007, while abundance of variable morphology taxa exhibited a slight decrease in abundance across all four years (Figure 12).

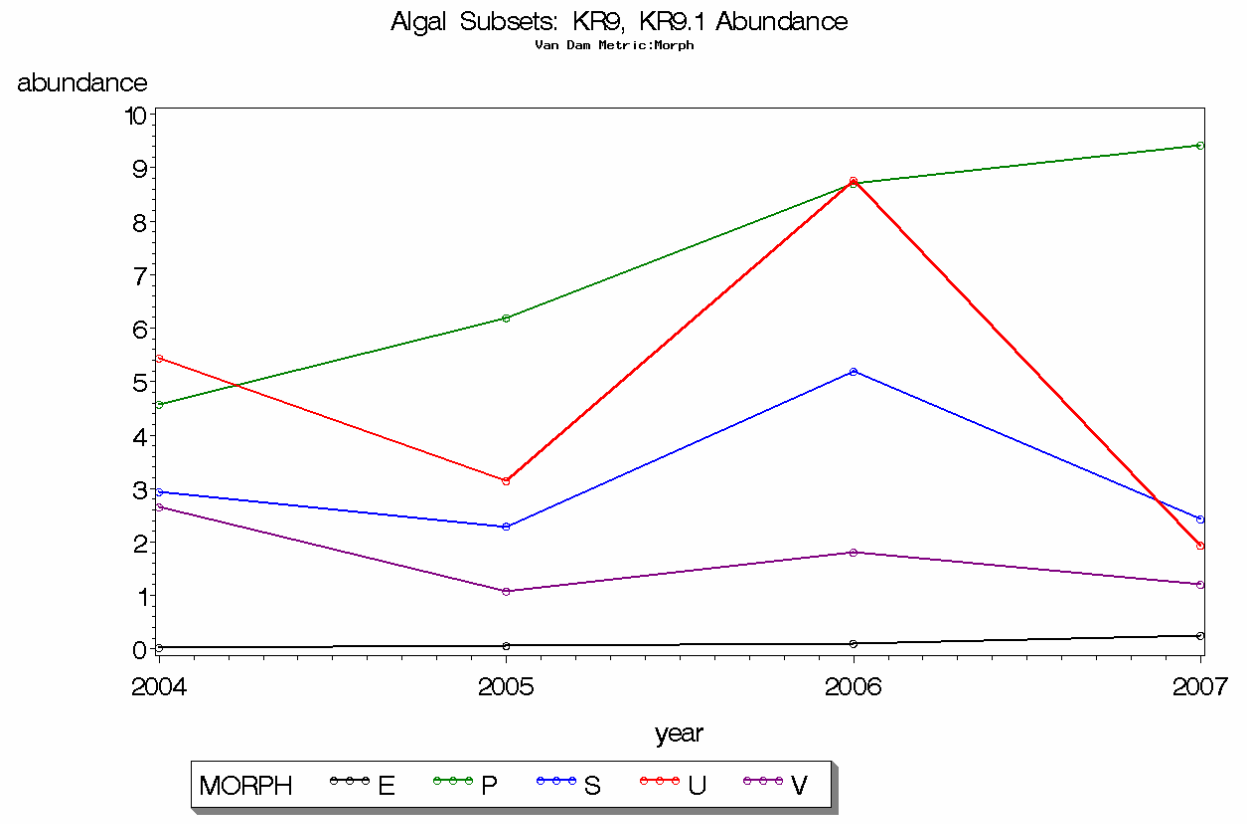


Figure 12. Algal abundance by morphological type in the nutrient addition zone (KR-9 and KR-9.1 combined) from 2004 through 2007, before and after experimental nutrient addition. Algal morphological types included E (erect), F (filamentous), P (Prostrate), S (stalked), U (Unattached), and V (variable) after Stevenson et al. (1996).

However, when abundance of green algae by morphological type by year and by site was plotted, these taxa exhibited various weak and inconsistent spatial (longitudinal) and temporal (among years and between pre- and post-treatment periods) trends or patterns. Patterns were more distinguished by morphological type within and among years than by among-year shifts or by pre- and post-treatment shifts within types. In summary, no prevalent responses to nutrient addition were seen among green algae taxa when portrayed by morphological type (Figure 13(a-

e). Algal abundance by year, site, and morphological type for all sites from 2004 through 2007. Algal morphological types included E (erect), F (filamentous), P (Prostrate), S (stalked), U (Unattached), and V (variable) after Stevenson et al. (1996).

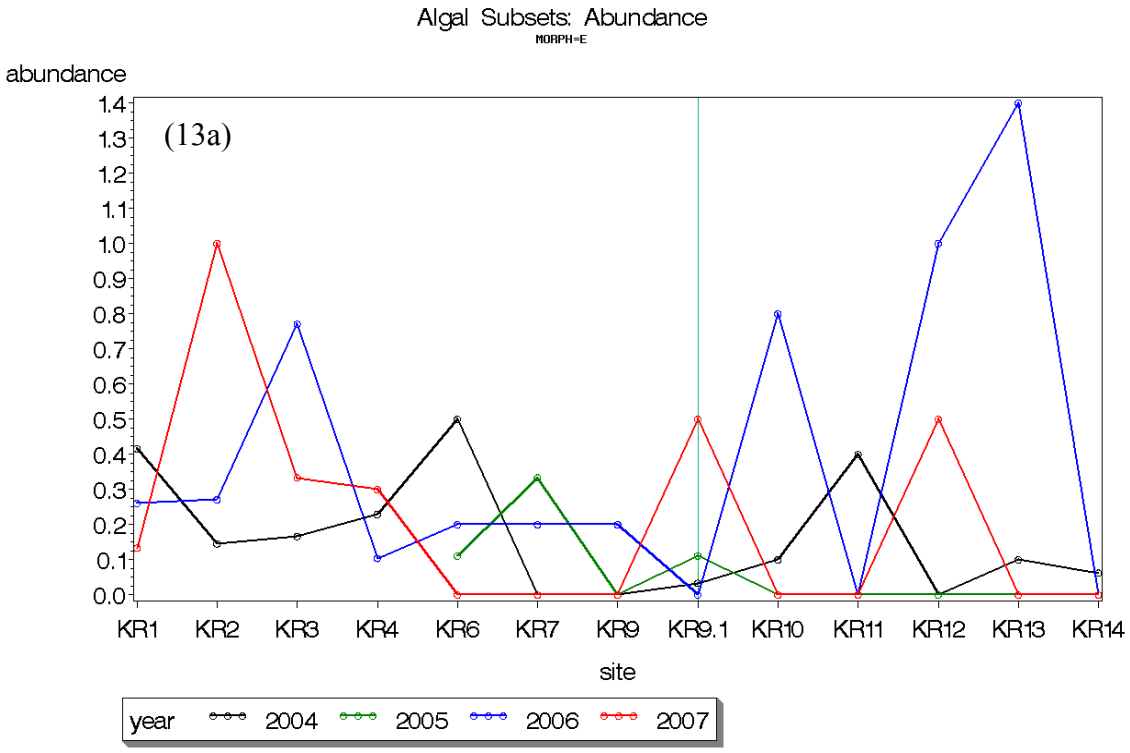
Erect morphology taxa exhibited considerable variability across sites during most years. Of particular note were the extreme and variable abundance values for erect taxa during 2006 upstream from the nutrient addition site (Figure 13a).

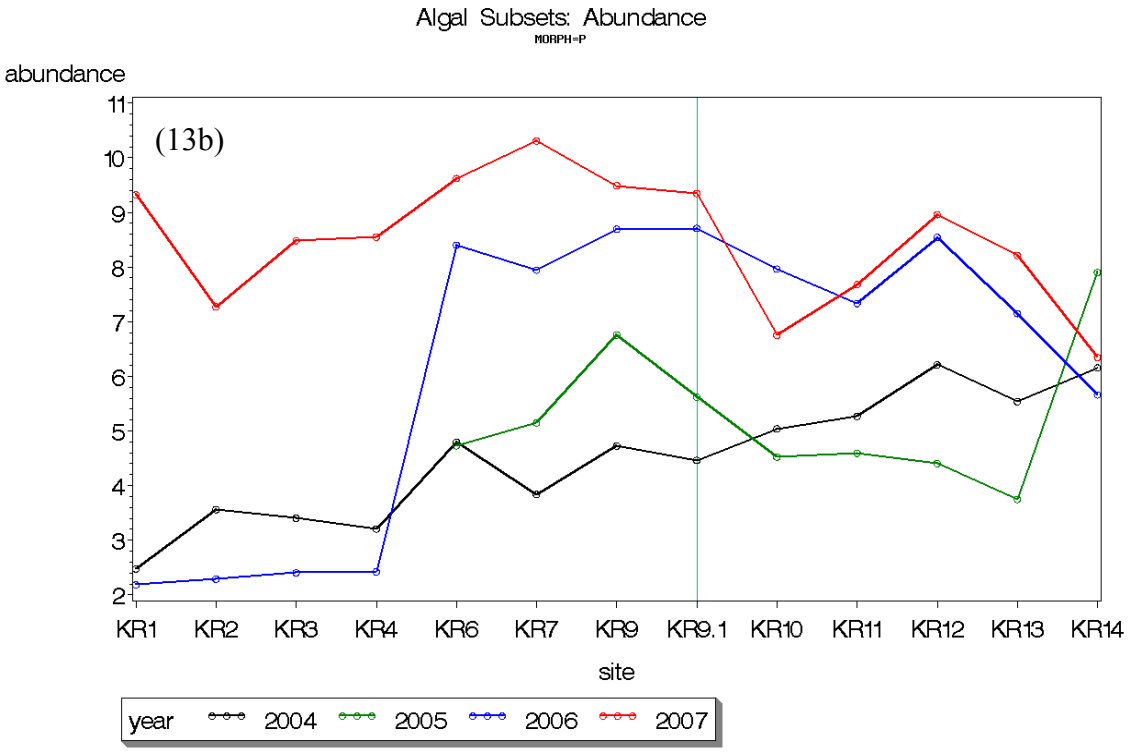
Prostrate taxa also exhibited considerable longitudinal variation among years, initially (2004) decreasing in a downstream orientation, but increasing considerably during the three nutrient addition years by up to a three-fold increase at KR-1 through KR-4 during 2007 (Figure 13b).

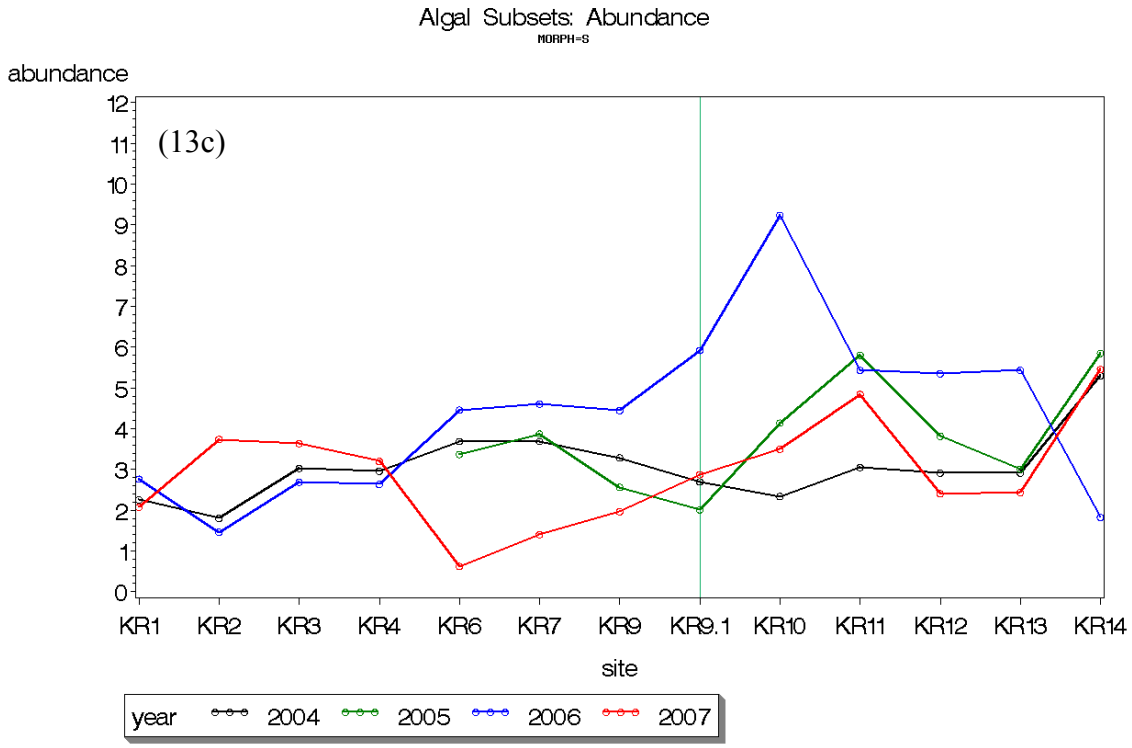
Stalked taxa exhibited relatively consistent longitudinal and temporal trends across all years and sites. These taxa also exhibited elevated abundance at several sites upstream from the nutrient addition site during 2005 and 2006, and the lowest abundance value at KR-6 during 2007 (Figure 13c).

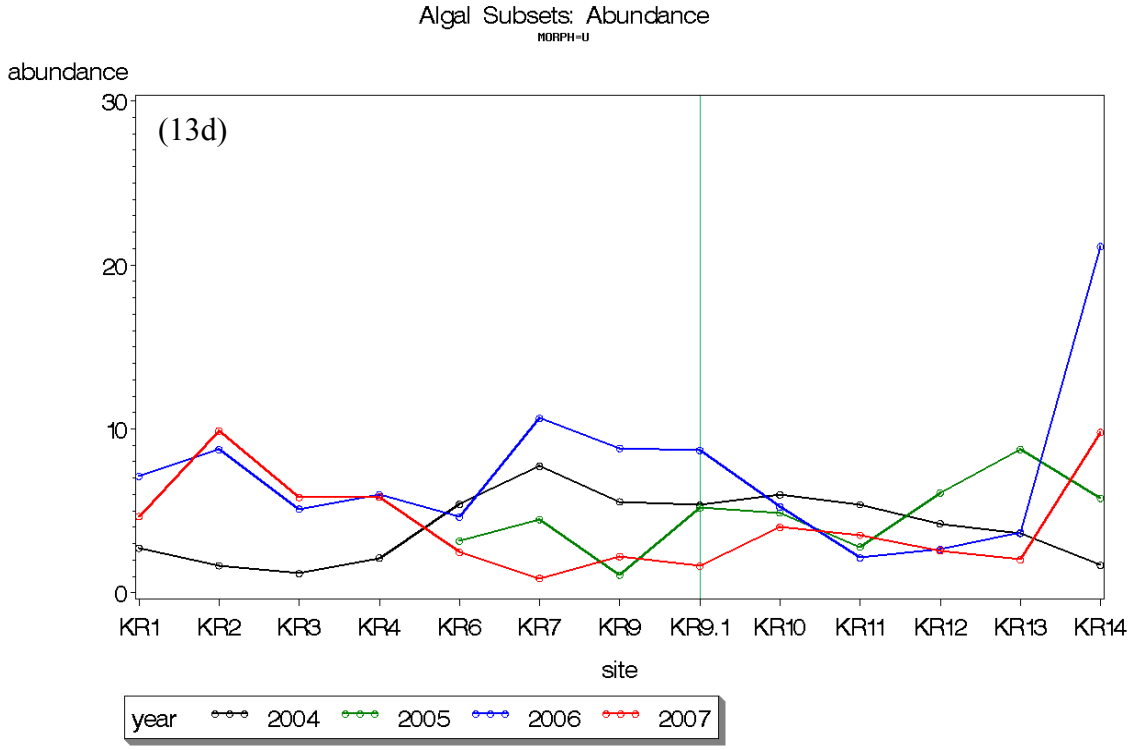
Unattached taxa exhibited relatively stable abundance distribution at all sites among all years with the exceptions of two extremely elevated abundance values at the unimpounded upriver reference site KR-14 during 2006 and 2007, and increases in abundance in the meander reach (sites KR-1 through KR-4 during 2006 and 2007 (Figure 13d).

Variable morphology taxa also exhibited a relatively flat abundance distribution across sites and years, with the exceptions of dramatic peaks and drops in abundance from KR-7 upstream to and including KR-14 during 2007, and a series of elevated values from KR-4 through KR-9.1 during 2004 (Figure 13e).


Algal Subsets: Abundance
MORPH=E
abundance
(13a)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
KR1 KR2 KR3 KR4 KR6 KR7 KR9 KR9.1 KR10 KR11 KR12 KR13 KR14
site
year 2004 2005 2006 2007


Algal Subsets: Abundance
MORPH=P
abundance
(13b)
11
10
9
8
7
6
5
4
3
2
KR1 KR2 KR3 KR4 KR6 KR7 KR9 KR9.1 KR10 KR11 KR12 KR13 KR14
site
year 2004 2005 2006 2007


Algal Subsets: Abundance
MORPH=S
abundance
(13c)
0
1
2
3
4
5
6
7
8
9
10
11
12
KR1
KR2
KR3
KR4
KR6
KR7
KR9
KR9.1
KR10
KR11
KR12
KR13
KR14
site
year
2004
2005
2006
2007


Algal Subsets: Abundance
MORPH=U
abundance
(13d)
0
10
20
30
KR1
KR2
KR3
KR4
KR6
KR7
KR9
KR9.1
KR10
KR11
KR12
KR13
KR14
site
year
2004
2005
2006
2007

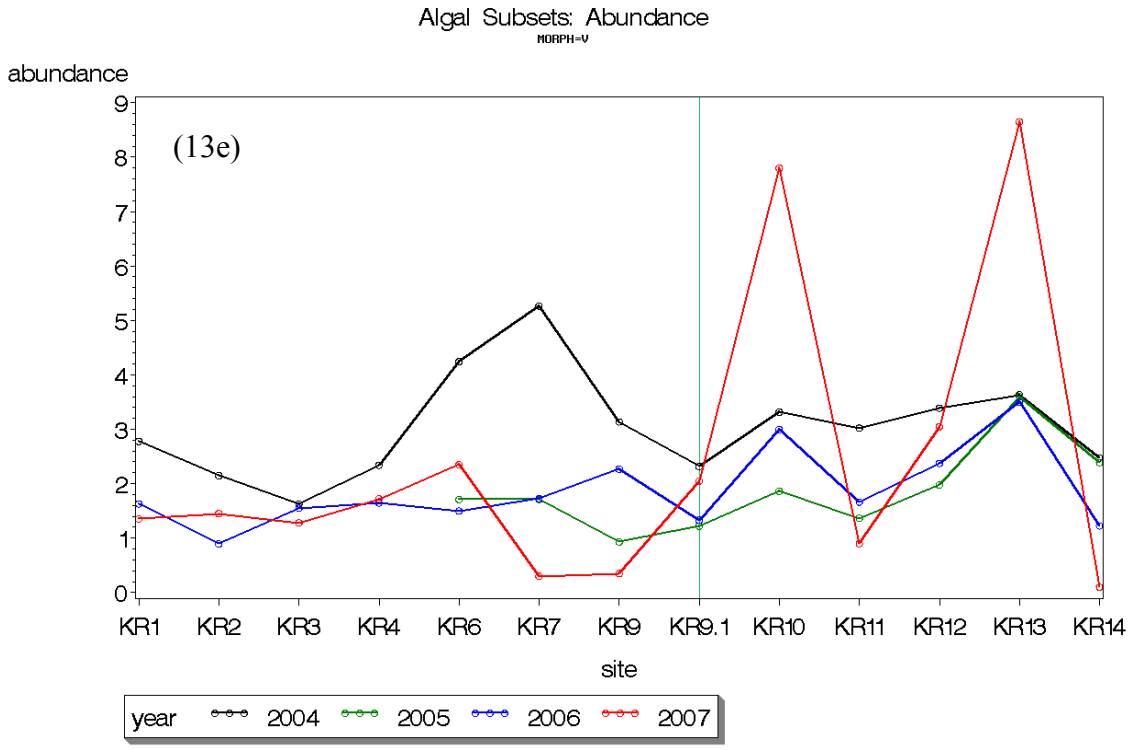


Figure 13(a-e). Algal abundance by year, site, and morphological type for all sites from 2004 through 2007. Algal morphological types included E (erect), F (filamentous), P (Prostrate), S (stalked), U (Unattached), and V (variable) after Stevenson et al. (1996).

PCA Soft-body Algae (green) Taxa

As with all PCA results presented in this report, green soft-bodied algal taxa with consistent information were used in this analysis. A very high level of variability in green algae taxa (92%) was accounted for by the first two principal components. The first axis was dominated by the genera *Closterium*, *Stigeoclonium*, *Ulothrix*, compared to *Ankistrodesmus* and *Scenedesmus* for the second axis. A notable change in structure in green algal taxa was observed between pre- and post-treatment periods. Insufficient taxa were represented in the data to perform PCAs on bluegreen algae taxa.

PCA Soft-body (green) Taxa, by Nutrient Timing

Green algae taxa PCA by nutrient timing also had a very high level of variability accounted for (88%), although observations were limited, prohibiting any interpretation of analytical results of this post-treatment PCA (pre-treatment, 16 observations and 7 variables; post-treatment, 6 observations, 7 variables). A similar suite of green algae genera accounted for PCA eigenvector loadings. During the pre-treatment period, *Closterium* and *Ulothrix* dominated the first axis compared to *Ankistrodesmus*, *Scenedesmus*, and *Stigeoclonium* for the second axis. During the post-fertilization period, the first axis was represented by *Closterium*, *Stigeoclonium*, and *Ulothrix,* and by *Cosmerium* and *Zygnema* for the second. No changes in structure were evident between pre- and post-treatment periods.

PCA Soft-body (green) Metrics

Seventy-six percent of variability associated with green algae metrics was accounted for by soft-body metrics PCA. Richness, Shannon-Weaver, Margalef diversity metrics dominated the first axis, with Percent Siltation and Percent Stability Index accounting for a majority of the loading of the seconds axis. No discernable patterns or directional changes were revealed between pre- and post-treatment periods for green algae metrics. Insufficient representation of bluegreen algae in the database prohibited PCA of bluegreen algae metrics.

PCA Soft-body (green) Metrics, by Nutrient Timing

Seventy-seven and 86% of variability associated with green algae metrics by nutrient addition timing was accounted for by PCA. For both the pre- and post-treatment periods the first PCA axis was dominated by Richness and the Margalef diversity index, and the second axis loading was dominated by the Percent Siltation Index and Siltation Richness. No changes in structure between the pre- and post-treatment periods were revealed by this PCA.

ANOVA - Green algae

ANOVA for green algae taxa included only abundance data because insufficient data existed to perform taxa analyses by year and genus. Four green algal taxa exhibited significant site or reach contrasts for abundance during the pre-treatment period (*Ankistrodesmus, Scenedesmus, Stigoclonium,* and *Ulothrix*), compared to a single genus (*Ulothrix*), which exhibited a significant site effect on abundance during the post-fertilization period. During the post-treatment period, ANOVA revealed significant differences in *Ulothrix* abundance in the Meander vs. Treated zone and the Braided vs. Meander reach comparisons.

ANOVA for site, fertilization, and site*fertilization interaction revealed that two green algae genera (*Stigoclonium,* and *Ulothrix*) exhibited significant treatment effects. No other site, fertilization, or site*fertilization interactions were significant for green algae taxa.
ANOVA for fertilization, site, and site*fertilization interaction revealed that two response metrics (% Centrics and % Stability Index) exhibited a significant site effect. No other fertilization, site, or site* fertilization interactions were significant for green algae metrics.

DISCUSSION

Clear, statistically significant, biological responses from diatom and algal communities were evident in the analysis of experimental nutrient addition to the Kootenai River. These responses are discussed sequentially in the following sections.

Chlorophyll

The four chlorophyll metrics (chlorophyll a and total chlorophyll biomass and accrual rates for both) consistently responded significantly to the experimental nutrient addition treatments in the Kootenai River. Chlorophyll metric values were consistently lowest during 2004, more than doubled in 2005, increased ten-fold or more during 2006, then consistently decreased to roughly 25% of their 2006 values during 2007. This pattern indicated possible annual dose-dependent responses in 2004 through 2006, given that 2004 was a pre-treatment year (low P availability), and that in-river target concentrations for phosphorus (P) were 1.5 µg/L for 2005 and 3.0 µg/L for 2006 and 2007. The 1.5 µg/L in-river P target for 2005 was part of the intended precautionary ramping-up approach of experimental nutrient addition in the Kootenai River, for biological and political reasons, and was not expected to produce or contribute to order of magnitude response changes in primary productivity. As with all post-treatment metric responses in this study, the magnitude of response was typically largest in the nutrient addition zone, and during 2006, the first year of the 3 µg/L P in-river target.

The order of magnitude increases in all four chlorophyll metric values in 2006 compared to 2004, and in some cases during 2005, were likely biological responses to the doubling of the in-river P target to 3µg/L for 2006. These disproportionately large increases in all the chlorophyll metric values likely resulted from initial increases in nutrient availability during 2006, and subsequent, lagged, commensurate top-down regulation by grazing pressure from the overlying macroinvertebrate trophic level. This multi-year annual pattern has been seen in lower trophic level responses in other experimentally nutrified rivers and streams (Peterson et al. 1985; Wilson et al. 2003).

ANOVA revealed significant nutrient addition treatment and site effects for all four chlorophyll metrics and a significant site*fertilizer interaction for total chlorophyll accrual. When ANOVA and qualitative metric values differed between chlorophyll *a* and total chlorophyll, total chlorophyll biomass and accrual rates were typically larger. This consistent pattern was likely due to the additional production from periphyton taxa that include chlorophyll *b* in their metabolism, (primarily green algae, Wetzel 2001).

ANOVA results also demonstrated consistent significant differences in all chlorophyll metrics for nearly all site and reach comparisons during pre- and post-treatment years. This finding may have been due in part to the pre-existing natural (habitat-based) longitudinal variation or differences in biological productivity in the Kootenai River (e.g. canyon vs. braided vs. meander reaches, and autotrophic vs. heterotrophic reaches). For example, ecosystem metabolism reported by Snyder (2001) and Snyder and Minshall (1996, 2005) found that the canyon and braided reaches of the Kootenai River (> KR-4) were autotrophic, a condition in which net photosynthesis exceeds net respiration (i.e. an oxygen producing reach), but the downstream meander reach (KR-1 through KR-4)was heterotrophic (net photosynthesis < net respiration), making it an oxygen sink. The degree of difference in oxygen availability between and among

these reaches would be expected to play a potentially major role in tests for site effect on primary productivity, as reported in this study.

Diatoms

PCA - A significant shift in overall diatom taxa composition between pre- and post-treatment periods in the zone of maximum biological response (KR-9 and KR-9.1) was revealed by PCA. Although approximately 60 to 73% of the variability associated with diatom taxa and metrics were consistently accounted for by the PCA analyses, patterns among taxa in the first two or three principal components were not evident. This suggests that PCAs of diatom taxa did not reveal consistent trends or dominant loading patterns. However, richness and diversity metrics were typically most responsive in the diatom metric PCAs. This was viewed positively in terms of benefits to productivity, based on the inherent value of increased food web complexity and diet item diversification at this trophic level (primary productivity). Just as increased physical habitat diversity serves as the basis for increased biological diversity, increased food item diversity (and abundance) can promote and support increased biological diversity.

ANOVA - Diatom taxa – Minimal responses in diatom taxa composition were observed as a function of experimental nutrient addition at the river scale. Although the number of significant ANOVA site and reach contrasts tripled from 5 to 15 of 104 from pre-and post-treatment periods (or from about 5 to 15% of all contrasts), this constituted a relatively minor taxa composition shift.

ANOVA Diatom metrics – As with diatom taxa results, minimal diatom metric responses were observed in response to nutrient addition at the river scale. During the pre-fertilization period, 20 of the 144 total ANOVA site and reach contrasts for diatom metrics (~14%) were significant (including 8 unprotected tests), compared to 30 significant tests, or about 21% in the post-treatment period, of which 15 were unprotected tests.

Green Algae

Abundance – The overall abundance of green algae taxa by morphological type was variable, but generally increased following nutrient addition. However, when analyzed by morphological type, results did not follow any consistent trends or patterns.

PCA – Although up to 92% of the variability associated with algae taxa and metrics were accounted for using PCA, no discernable, ecologically insightful trends or patterns emerged when comparing green algae taxa or metrics between pre-and post-treatment periods. Likewise, no consistent eigenvector loading patterns for taxa or metrics were observed in the PCAs for green algae.

ANOVA – Green algae taxa – Surprisingly, little algal taxa composition shifting was revealed by ANOVA comparing pre- and post-fertilization periods. Only two taxa (*Stigeoclonium and Ulothrix*) exhibited a significant treatment effect. During the pre-treatment period, four taxa exhibited significant site or reach contrasts, although all four were unprotected tests (i.e. no associated main site effect). Furthermore, during the post-treatment period, a single taxon,

Ulothrix, exhibited a significant site effect and three significant site or reach contrasts. This lack of significant algal composition responses may have been attributed to a lack of resolution in the green algae data.

ANOVA – Green algae metrics – As with the minimal responses noted of green algae taxonomic composition above, ANOVA results for green algae metrics also showed only infrequent, low magnitude responses following nutrient addition. Only two metrics (% Centrics and % Stability Index) exhibited a significant treatment effect, and no other metrics exhibited a significant treatment effect or a significant site*fertilization interaction.

However, unlike the ANOVA results for green algae taxa, green algae metric ANOVA revealed more significant site effects for metrics between pre- and post-treatment periods. During the pre-treatment period, two metrics exhibited a significant site effect (% Siltation Index and % Stability Index), compared to five metrics with significant site effects during the post-treatment period (Richness, Margalef, Siltation Richness, % Siltation Index and % Stability Index). It was noted that two of these latter metrics were important in the pre-treatment analysis. The addition of significant site effects for the Richness and Margalef's diversity metrics was indicative of increased diversity or richness in the green algae community, which as mentioned in the previous discussion of diatom responses, is inherently beneficial, based on the value of increasingly diverse food web structure and diet item availability.

Green Algae composition by morphological type- Plotting total abundance of green algae by year and by morphological type in the nutrient addition zone (KR-9 and KR-9.1) revealed that prostrate taxa abundance increased every year from 2004 through 2007, abundance of stalked and unattached taxa both decreased from 2004 to 2005, peaked during 2006 and decreased in 2007, while abundance of variable morphology exhibited a relatively flat abundance distribution across sites and years.

However, abundance of green algae by morphological type by year and by site exhibited various weak and inconsistent spatial (longitudinal) and temporal (among years and between pre- and post-treatment periods) trends or patterns. Patterns were more distinguished by morphological type within and among years than by among-year shifts or by pre- and post-treatment shifts within types. In summary, no prevalent responses to nutrient addition were seen among green algae taxa when portrayed by morphological type.

CONCLUDING REMARKS

Algal communities in rivers and streams are important in terms of potential top-down and bottom-up regulation of ecological function, trophic structure, and taxonomic composition. Reported algal community metrics and chlorophyll concentrations and accrual rates provided a good characterization of the ecological responses to experimental nutrient addition and for the assessment of inter-trophic relationships in a large altered river-floodplain ecosystem. Nutrient addition in the nutrient addition zone produced relatively strong macroinvertebrate responses that highlighted potential cascading interactions across trophic levels. The upstream control sites also provided meaningfully partitioned background variability to assess treatment effects. This was particularly true for algae and diatoms, which did not have any ability to move evidence of treatment effect upstream beyond the nutrient addition site as may happen with migratory fishes or subsequent generations of emergent insects.

Future algal sampling should be coordinated with ongoing annual fish condition and community sampling to better assess trophic linkages and interactions between the two communities. Future fish sampling should also be expanded to at least provide comparable fish diet information to complement invertebrate community data collected at KR-9.1. Implementing these actions will provide a more accurate quantitative assessment of the potential role of nutrient addition in restorative large river ecology.

Finally, this project represents the first large-scale experimental approach on the Kootenai River, and the largest known experimental nutrient treatment in any river in the world to the best of the authors' knowledge. The project is aimed at one of the critical sources of biological limitation among the entire array of communities and populations in the Kootenai River, hydrologic, energetic, and nutrient delivery alterations caused by the construction and operation of Libby Dam, in addition to floodplain loss from levee construction.

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APPENDIX A: ANALYTIC METHODS FOR CLOROPHYLL METRICS

Determination of Chlorophylls a and b in Filters Using an Acetone Extraction and the Biomate 3 Spectrophotometer

Status: *This document is considered current standard procedure of the Analytical Sciences Laboratory when management approval is documented by signature above. This Standard Method is effective on the date of approval signature and supersedes all other versions until historically archived by the QAU as indicated below.*

Abstract:

This method is used to determine the absorbance of chlorophyll a and b in 1:1 acetone/95% ethanol sample extracts. Samples in the form of glass fiber filters are macerated in acetone/water (90:10) extraction solvent, steeped in the dark at 4 ºC for a minimum of 2 hours, centrifuged for 10 min at 2000 rpm, and filtered into amber vials. Sample absorbance at 665 nm and 649 nm is measured by spectrophotometry. Using known absorptivity coefficients, the sample concentrations of chlorophylls a and b are calculated by Beer's Law (Wintermans & De Motts, 1965). An alternative approach for sample absorbance and chlorophyll a, b, and c calculations can be performed using the spectrophotometric procedures as outlined in EPA 446 and described in this method.

Samples should be frozen upon receipt and protected from light. All chlorophyll analyses must be performed in reduced light conditions. This method has been validated by analyzing multiple spiked samples to verify that the recovery of spiked chlorophyll was equal to or better than a previously employed method (i.e., ethyl ether/water partitioning with a separatory funnel).

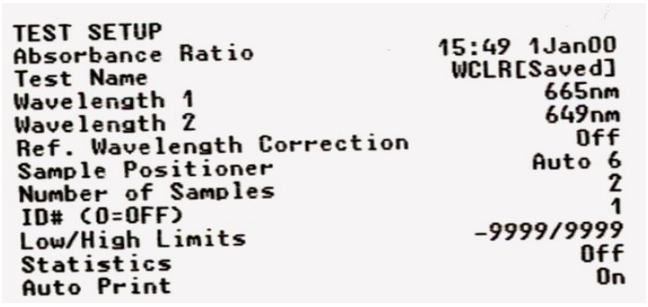
I. EQUIPMENT AND APPARATUS

- **A.** 50 mL Pyrex centrifuge tubes with caps
- **B.** Centrifuge
- **C.** 100 µL and 50 µL Drummond pipettes
- **D.** Thermo Spectronic Biomate 3 Spectrophotometer
  1. Aluminum foil
  2. 1-cm path length cuvettes (Fisher catalog #13-688-74 chemical resistant, quartz/glass or equivalent)
  3. Syringe filters: Nylon 25 mm, 0.45 µm pore size
  4. Homogenizer (e.g. VirTis '23')
  5. 8-mL amber vials with Teflon-lined caps

Important: If amber vials are being reused, DO NOT REUSE CAPS.

II. INSTRUMENT OPERATION PARAMETERS (EXAMPLE)

A. Biomate 3 Test Setup: Method Name WCLR



B. Biomate 3 Test Setup: Method Name EPA 446 (not shown)
(Wavelengths 1, 2, & 3 are 664, 647, & 630 nm respectively)

C. Biomate 3 Test Set up: Method Name EPA 446A (not shown)
(Wavelength 1 & 2 are 665 & 750 nm)

III. REAGENTS

A. Acetone (Pesticide or Optima grade)

B. HCL, 0.1 N

IV. QUALITY CONTROL STANDARDS

Chlorophyll (Absolute Standards catalog # 71592 or equivalent) standard is diluted in acetone to a concentration of 100 µg/mL. Two QC samples are prepared by spiking an acetone-soaked piece of blank glass fiber filter paper with chlorophyll at an appropriate concentration. Usually, 150 µL of chlorophyll standard is spiked into each of the two QC samples so that the final concentration of total chlorophyll is around 1 ppm in the 15 mL final volume of 90:10 acetone/water. (Higher spike levels should be used in conjunction with samples containing large amounts of chlorophyll.)

V. SAMPLE PREPARATION

NOTE: Chlorophyll samples should be extracted under limited light conditions (i.e. lights off).

A. Cut up and transfer filters to labeled 50 mL centrifuge tubes and add 15 mL 90:10 acetone/water. Samples may require 1 hour to thaw before they can be transferred into centrifuge tubes. Prepare QC samples by spiking a blank piece of filter paper with 150 µL of the chlorophyll reference standard and add 15 mL 90:10 acetone/water. For the sample blank (EBLK), place a piece of blank filter paper in a centrifuge tube and add 15 mL 90:10 acetone/water.

B. Prepare a QC check sample at this time by spiking an equivalent amount of standard into 15 mL of 90:10 acetone/water (the QC check sample does not go through further sample preparation steps).

C. Macerate about 30 seconds with homogenizer. Rinse probe with 90:10 acetone/water in between each sample. Place all samples in refrigerator to steep for at least 2 hours at approximately 4 °C. Samples must be placed in a closed box to prevent light exposure.

D. Centrifuge samples at 2000 rpm for 10 minutes on the TJ-6 centrifuge. Use a refrigerated centrifuge if possible. Syringe filter samples into labeled amber vials and close securely with lids.

VI. SAMPLE ANALYSIS (PERFORM WITH THE LABORATORY LIGHTS OFF)

A. Turn on the main power control for the Biomate 3 Spectrophotometer and allow the instrument to warm up for 1 hour.

B. Check to determine if the instrument operation parameters are as stated in Section II. Change the "Number of Samples" to match the number of samples in the analytical batch. Be sure to include the QC, blank, and spikes when calculating the "Number of Samples." It is also recommended that a few samples be read in duplicate.

C. Place 3 mL of 90:10 acetone/water into the cuvette holder that is marked 'B'. **Be sure to use acetone-resistant or quartz/glass cuvettes.**

D. Place 3 mL of each filtered sample into the other five cuvette holders that are marked 1-5 within the six-place cell rotor.

E. Close the lid of the cell compartment and press "Run Samples." After the instrument has read and printed the results for the first five samples, replace the cuvettes with the next set of samples and press "Enter". Continue until you have read all of the samples.

All EcoAnalytst samples by EPA 446.0: When performing EPA 446.0, all samples must be read at 664 nm, 647 nm, and 630 nm (**Biomate 3 Method**: **EPA 446**). After this initial reading, add 90 µL of 0.1 N HCl, and then use a Pasteur pipette to mix each sample, making sure to keep the tip below the surface of liquid to avoid aeration. Wait 90 seconds and measure the samples' absorbance values at 665 nm and 750 nm (**Biomate 3 Method**: **EPA 446A**).

F. Dilute sample if necessary (if absorbance >1.00 at any wavelength). Note amount of dilution.

G. Keep the extracts in amber vials below 0 ºC until no longer needed. Store vials so they are upright and protected from light.

VII. CALCULATIONS

A. Wintermans and De Mots Equations (**Kootenai Tribe of Idaho samples**)

$C_a = 13.70\ A_{665\ nm} - 5.76\ A_{649\ nm}$ (in µg/mL), where C_a is concentration of chlorophyll a in the extract.

$C_b = 25.80\ A_{649\ nm} - 7.60\ A_{665\ nm}$ (in µg/mL), where C_b is concentration of chlorophyll b in the extract.

Volume Ratio Adjustment = initial volume/final volume

C_a/ volume ratio adjustment = A (µg/mL)
C_b/ volume ratio adjustment = B (µg/mL)
Total chlorophyll = A + B

B. Jeffrey and Humphrey's Trichromatic Equations (EPA 446.0)

$C_a = 11.85\ A_{664\ nm} - 1.54\ A_{647\ nm} - .08\ A_{630}$ (in µg/mL), where C_a is concentration of chlorophyll a in the extract.
$C_b = 21.03\ A_{667\ nm} - 5.43\ A_{644\ nm} - 2.66\ A_{630}$ (in µg/mL), where C_b is concentration of chlorophyll b in the extract.
$C_c = 24.52\ A_{630\ nm} - 7.60\ A_{647\ nm} - 1.67\ A_{664}$ (in µg/mL), where C_c is concentration of chlorophyll $c_1 + c_2$ in the extract.
Volume Ratio Adjustment = initial volume/final volume
C_a/ volume ratio adjustment = A (µg/mL)
C_b/ volume ratio adjustment = B (µg/mL)
C_c/ volume ratio adjustment = C (µg/mL)
Total chlorophyll = A + B + C

C. Lorenzen's Pheopigment-corrected Chl *a* and Pheo *a* Equations (EPA 446.0)

$C_a = 26.7\ (A_{664b} - A_{665a})$
concentration (mg/L) of chlorophyll a in the extract solution measured
$P_a = 26.7(1.7\ A_{665a} - A_{664b})$
Concentration (mg/L) of pheophytin *a* in the extract solution measured
A_{664b} = sample absorbance at 664 nm (minus absorbance at 750 nm) measured before acidification
A_{665a} = sample absorbance at 665 nm (minus absorbance at 750 nm) measured after acidification

VIII. QUALITY CONTROL AND REFERENCE MATERIAL

- **A.** A 90:10 acetone/water blank is utilized as a reference for the spectrophotometer.
- **B.** QC check is prepared in 90:10 acetone/water to verify the amounts of chlorophyll a and b that should correlate with the two QC samples that were spiked and extracted with the sample batch. Acceptable spike recoveries are between 50-120%.

IX. CORRECTIVE ACTION

It is difficult to determine the best course of corrective action for this method due to the fact that the quantity of sample provided is enough for only one extraction. If a spike recovery is not acceptable, it should be noted on the QC sheet. However, a single failure of a QC sample spike does not constitute a batch failure. The two QC sample spikes should agree within 10% of each other, and any larger discrepancy should be noted on the QC sheet. If the absorbance of the QC check shows more than the normal variation from batch to batch (> ±0.001), a new chlorophyll standard should be prepared and checked against the current one for degradation.

If a chemist makes an error during the extraction, every effort should be made to bring the sample to completion due to the impossibility of repeating the extraction. The best course of corrective action should be discussed with the Group Leader in this case, and whether or not the sample should be reported.

X. DOCUMENTATION REQUIREMENTS

A completed bench sheet and a sample analysis worksheet are required for all analyses. The bench sheet will verify that all steps in the analytical method were performed and by whom, and records the reagent lot numbers. Bench sheets and sample analysis worksheets can be found at P:\bench\organic\water\epaother\chloroph\2006.

XI. SAFETY AND HEALTH

The toxicity and carcinogenicity of chemicals used in this method have not been precisely defined; each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining awareness of OSHA regulations regarding handling of chemicals used in this method.

XII. REFERENCES

Wintermans, J.F.G.M., and A. De Mots, "Spectrophotometric Characteristics of Chlorophylls a and b and Their Pheophytins in Ethanol." Biochim. Biophys. Acta, 109 (1965) 448 - 453.

EPA Method 446.0: In Vitro Determination of Chlorophyll a, b, c1 + c2, and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry. Revision 1.2, September 1997.

10200 H. Chlorophyll: Standard Methods for the Examination of Water and Wastewater, 19th Edition 1995.

XIII. VALIDATION

This method has been validated by analyzing multiple spiked samples to verify that the recovery of spiked chlorophyll was equal to or better than a previously employed method (i.e., ethyl ether/water partitioning with a separatory funnel). During the 2005 sampling season, spiked samples were analyzed, giving an average recovery ± standard deviation of 90 ± 11% (n=10).

APPENDIX B: DIATOM AND ALGAE LAB PROTOCOLS

SAMPLE PREPARATION

The purpose of this process is two-fold: (1) to create a laboratory data sheet for each unique sample, and (2) to sub-sample the original sample for soft-bodied algae and diatom splits.

CREATING THE SAMPLE DATA SHEET

A sample is removed from storage and the lab technician fills out a sample data sheet with the following information: sample identification number, pertinent location and replicate information, sample date, habitat type, area sampled and volume if available. If sample volume is not available, the following procedure is followed:

MEASURING INITIAL SAMPLE VOLUME

- Sample volume is carefully measured using graduated cylinders of appropriate size and the volume is recorded on the data sheet.
- The sample is returned to its original container, resealed, and properly stored until further analyses are made.
- Glassware is acid-washed and rinsed with de-ionized water before volume measurements are made for each sample.

SUBSAMPLING FOR SOFT-BODIED ALGAE AND DIATOM SPLITS

- The sample is vigorously shaken to evenly disperse the periphyton within the sample.
- Sub-samples are taken from the middle of the sample and placed into labeled vials which have the unique sample ID # with the suffixes –soft and –diatom. Note: It takes substantial experience to make a sound judgment call regarding how much to sub-sample for soft algae and diatoms. If the sample is very silty, it will be better to sub-sample a minimum of 10 ml, if the sample looks clear, sample at least 20 ml. In general, EcoAnalysts samples at least 10 ml for soft algae and at least 20 ml for diatoms.
- The volume of the sub-sample is recorded both on the vials and on the lab sheet for both the soft body algae and the diatom sub-samples.
- The original sample is returned to the shipping container it was sent in and set aside for shipping after the project is finished.

IDENTIFICATION AND ENUMERATION OF SOFT ALGAE

In this process EcoAnalysts counts and identifies soft algae to the lowest practical taxon, with a count of live/dead diatoms (i.e., soft algae counts).

ACHIEVING DESIRED CELL DENSITY

- The sample is vigorously shaken to evenly disperse the periphyton within the sample.
- Exactly 0.1ml of the homogenized sample is placed on a slide (or a Palmer Maloney counting chamber if requested by the client) using a micropipette.
- Algae will be examined at 400X magnification using a light microscope to assess if periphyton is too dense or dilute for identification and enumeration. The original sample will be diluted or concentrated if necessary to achieve desirable cell density approximately 15-20 counting units per field of view). If dilution or concentration is needed, the new volume and concentration ratio will be recorded on the data sheet.

ENUMERATING SOFT ALGAE

- Soft algae are counted and identified to the lowest practical taxon using the strip method until at least 300 counting units (or another target number specified by DRBC) are encountered. For colonial algae, each colony is counted as one algal unit per 10 by 10 micron area, or in the case of filaments, each 10 micron length is one algal unit, for purposes of tallying 300 counting units in a count. Diatoms may be identified to the genus level, or enumerated as either 'live' or 'dead,' per DRBC.
- Counting and identification is made at 400X magnification using a Nikon light microscope and the latest taxonomical references. All identifications and counts are recorded on approved data sheets, as described previously.

CALCULATING CELL DENSITY

To estimate algal cell density, we keep track of the volume of the sample counted in the following way:

- Record the strip length and width and then multiply the area by the depth.
- If applicable, the counted volume is then multiplied by the dilution or concentration factor.
- The total algal cell density is calculated as follows:

The total algal density (cells / substrate area)= {[N / (Vsc * CR)] * VT} / AS, where:

N = the total number of cells counted
Vsc = the volume of sample counted
CR = the concentration or dilution factor (if applicable, or CR = 1)
VT = total sample volume
AS = substrate area sampled

- Each taxon's density can also be calculated using the same formula except N will represent the number of counts for the individual taxa.

DIATOM SLIDE PREPARATION

The purpose of this process is to count and identify diatoms to the lowest practical taxon, usually to the species or variety level.

DIGESTION

Method A – Acid

- The diatom sub-sample split is digested under the hood on a hot plate set to 100 degrees Celsius.
- Nitric acid (or Sulfuric acid) is carefully added to the sample in a 1:2 sample-to- acid ratio.
- A dash of Potassium dichromate is added to catalyze the reaction and the acid and sample mixture is heated for 1 to 2 hours.
- The mixture is removed from the heat and left to cool for a few hours.
- De-ionized water is added to the digested sample to fill the 300 ml beaker. The sample is left overnight (or for at least 8 hours).
- Water is siphoned from the center of the water column (under the surface) to the 50 ml mark on the beaker.
- De-ionized water is added to top off the beaker and left for at least 8 hours to let the diatom cells settle. Note: the rule is one hour per centimeter so the smallest diatoms settle.
- After a neutral pH is achieved, usually after 6-8 rinses, the digested diatom material is transferred to a clean vial and labeled with the unique sample ID # with the suffix –clean diatom.

Method B – Hydrogen Peroxide

- The diatom sub-sample split is digested under the hood.
- Hydrogen Peroxide is carefully added to the sample in a 1:2 sample-to-peroxide ratio and allowed to sit for 2 hours.
- A dash of Potassium dichromate is added to catalyze the reaction and the peroxide and sample mixture.
- De-ionized water is added to the digested sample to fill the 300 ml beaker. The sample is left overnight (or for at least 8 hours).
- Water is siphoned from the center of the water column (under the surface) to the 50 ml mark on the beaker.
- De-ionized water is added to top off the beaker and left for at least 8 hours so that diatom cells settle. Note: the rule is one hour per centimeter so the smallest diatoms can settle.
- After a loss of orange color is achieved, usually after 6-8 rinses, the digested diatom material is transferred to a clean vial and labeled with the unique sample ID # with the suffix – clean diatom.

Method C – Burn Mount

- Cover slips are arranged on a slide warmer with scribe numbers identifying the unique sample ID of each sample.
- An unclean diatom sample is vigorously shaken and then dripped onto the cover slip.
- After drying, the cover slips are examined at 400X magnification. Note: A density of 15-30 diatom valves per field of view is best for counting purposes. If the desired density is not achieved, the cover slip is remade after the proper dilution or concentration of the sample has been made and recorded on the data sheet.

- The cover slips are heated on a hot plate or in an oven for 4 to 6 hours until the organic material is incinerated.
- Cover slips with the desired cell density are permanently mounted on labeled slides using a high resolution mounting medium (NAPHRAX®).
- The slides are heated on a hot plate until the resin stops bubbling and are then left to cool and harden overnight.
- The finished slides are placed in a slide box labeled with the project name and dentifying the method as a burn mount.

DIATOM IDENTIFICATION AND ENUMERATION

- Diatoms are counted and identified to the lowest practical taxon using the strip method until at least 300 cells (600 valves) are encountered. Counting and identification is made at 1000X magnification using a Nikon light microscope and the latest taxonomical references
- All identifications and counts are recorded on approved data sheets.

SPECIAL HANDLING FOR LOW DENSITY OF DIATOMS

- In some cases, the number of diatoms in a sample is very sparse. This is usually because diatoms were rare in the habitats sampled, or the sample bottles contain a small amount of material. In these cases, additional procedures are required to either make a satisfactory slide for analysis or to determine that analysis of a sample is not practical.
- If a satisfactory slide could be made by increasing the concentration of cleaned diatom material by two to five times, then we do this by using a micropipettor to remove the required amount of water from the vial of material after it has been allowed to settle for at least eight hours. The concentration factor is then recorded.
- If a concentration of cleaned material greater than two to five times is required, then the original sample is resub-sampled. We will take a sub-sample of a size sufficient to prepare satisfactory slides, and use all the remaining sample only if absolutely necessary. Then the sub-sample is digested and a new vial of cleaned material is prepared. If it is still too dilute, the two vials of cleaned sub-sample materials are combined. EcoAnalysts records all steps and volumes along the way, including the final concentration factor.
- If, after following the steps above to concentrate the cleaned material, the density of diatoms on a cover slip still does not meet the criteria of 30 to 40 cells per field at 400 - 450x magnification, then EcoAnalysts will proceed to make the densest slide possible. First, the taxonomist will make a determination of whether it is practical to analyze the sample. To do this, he/she will quickly scan the slide in its entirety under 100X magnification, and estimate the total number of individuals on the slide. To make their determination of whether the slide is countable, the taxonomist will take into account the density of diatoms, evidence of dissolution, and amount of debris (silt, clay, broken remains of diatoms and other siliceous organisms) that would make it difficult to identify specimens accurately. As a general guideline, if accurate identifications are possible, and at least 100 specimens could be counted within four hours, a determination will be made to analyze the slide. If the diatom taxonomist determines that the slide should not be counted, the Project Coordinator will be informed. Only under very special

circumstances will the taxonomist be asked to take the extraordinary measure of counting a slide for more than four hours.

- When evaluating a slide with few diatoms, a taxonomist may occasionally see evidence suggesting that a sample contains lightly silicified diatoms that may not have survived the digestion process. In these rare instances, they may suggest that a "burn mount" be made to determine whether diatoms did exist in the original sample (This is one reason why a small portion of the initial sample should always be saved, even for phytoplankton.) The burn mount procedure was used extensively to create slides for diatom analysis before the introduction of methods incorporating acids for the digestion of organic material. Even though this method does not rid sample material entirely of organic debris, diatoms on the slide can at least be identified as diatoms. After it is prepared, the burn mount slide will be examined by a diatom taxonomist. It will be determined if diatoms are present and whether analysis of the slide is warranted. Slides prepared using the burn mount method cannot be counted if too much organic material remains on the slide due to it not being possible to make accurate taxonomic identifications. Generally, burn mounts are used only as a last resort, and to confirm that weakly silicified diatoms are not present in the sample.

QUALITY ASSURANCE OF TAXONOMIC IDENTIFICATIONS

This series of steps represents a proven method to help ensure the accuracy of our taxonomy.

- High quality digital images are taken of each taxon encountered in the project. This is one of the best voucher systems for permanently archiving soft algae specimens. These images include taxa names, photographer/taxonomist name, date, and project ID number.
- Diatom slides are archived in slide boxes with the project name.
- A minimum of 10% of all samples will be analyzed by an independent phycologist to ensure taxonomic accuracy and reproducibility of the processing and analysis methods. The independent taxonomist will be the Philadelphia Academy of Natural Sciences. We regularly use the Academy for our QA of diatom taxonomy.
- There will be a consensus of at least 90% of the common taxa in each sample.
- Both taxonomists meet via phone conference to discuss any discrepancies. In some cases it is necessary to re-examine the digital images and/or specimens to resolve discrepancies.
- The final data are adjusted according to the recommendations of both taxonomists.

QUALITY ASSURANCE METHODS

Soft-bodied algae and diatom proportional counts will be subject to the following criteria:

- The common algae identified by both taxonomists should match.
- Diatom taxa accounting for more than 10% relative abundance should be identified similarly by both taxonomists (synonyms are acceptable).
- The percent community similarity index calculated from the two diatom counts should exceed 90%.
- If any of these criteria are not met, the sample will be re-analyzed and any discrepancies will be resolved.

- A quality control report describing results and corrective steps taken, if necessary, will be provided concurrently with data delivery.

VOUCHERING

- Each microscope slide will be labeled with all information necessary for the identification of the sample, including water body name, site identification number, and sampling date.
- Samples will be placed into slide boxes in an orderly fashion and shipped to you upon completion of the project.
- A second set of slides will be vouchered in a diatom herbarium at the Philadelphia Academy of Natural Sciences, pending permission from the client.

DATA MANAGEMENT AND REPORTING

- Data are entered into a custom-built taxonomy counting program which creates an electronic file for each project.
- Since the counting program automatically tallies the number of cells for each taxon, no handwritten bench sheets are required – this entirely eliminates the potential for transcription error. We can print an electronic benchsheet on demand if the Commission requests such during any point in the contract period.
- Sample identifier information is entered, followed by the taxa and counts and notes.
- After all samples in the project are identified, the data are formatted in the output specified by the client, in this case a database format outlined in the Request for Proposals.
- A CD of taxa photographs will be compiled.
- Any remaining sample materials will be returned to the client if requested.

LIST OF PERIPHYTON EQUIPMENT

A brief list of equipment available for this project:

- A digital image reference collection
- A library of taxonomic literature for identifying algae and the ability to add to it if more references for a specific project are needed
- Aluminum foil
- Collection bottles
- Digital imaging cameras and software for high quality microscope and field images
- Fume hood
- Glassware (beakers, graduated cylinders, centrifuge tubes) and micropipettes for accurately measuring sample volume
- GPS
- High speed internet connection
- Hot plates

- Knife
- Labels
- Laser printers
- Locked store rooms for sample storage security
- Lugol's solution
- NAPHRAX® high refractive index mounting media for archiving diatom slides
- Nikon Compound Microscope has differential interference contrast (DIC) capabilities, capable of 40, 100, 200, 400 and 1000X magnification
- Palmer-Maloney counting chambers (if requested by the client)
- Periphytometers
- Rock brushes
- Slide warmers
- Tally program for counting algae
- Wash bottles
- Windows based computers and network

APPENDIX C: DIATOM AND ALGAE TAXA SAMPLED

Diatom Taxa

1 Achnanthes
2 Achnanthidium
3 Amphipleura
4 Amphora
5 Anomoeoneis
6 Asterionella
7 Aulacoseira
8 Brachysira
9 Caloneis
10 Chrysidiatrum
11 Cocconeis
12 Coscinodiscus
13 Cyclotella
14 Cymatopleura
15 Cymbella
16 Denticula
17 Desmidium
18 Diatoma
19 Dickieia
20 Didymosphenia
21 Diploneis
22 Distrionella
23 Encyonema
24 Encyonopsis
25 Epithemia
26 Eunotia
27 Fragilaria
28 Frustulia
29 Geissleria
30 Gomphoneis
31 Gomphonema
32 Gomphosphenia
33 Gyrosigma
34 Hannaea
35 Hantzschia
36 Mastogloia
37 Melosira
38 Meridion
39 Navicula
40 Neidium
41 Nitzschia
42 Pinnularia
43 Planothidium
44 Reimeria
45 Rhoicosphenia
46 Rhopalodia
47 Scoliopleura
48 Stauroneis
49 Staurosira
50 Staurosirella
51 Stephanodiscus
52 Surirella
53 Synedra
54 Tabellaria

Soft-Body Taxa (Blue-Green Algae)

1 Anabaena
2 Chroococcus
3 Gloeocapsa
4 Gloeocystis
5 Microcystis
6 Oscillatoria
7 Sphaerocystis
8 Stichosiphon

Soft-Body Taxa (Green Algae)

1 Ankistrodesmus
2 Botryococcus
3 Chlamydononas
4 Chlorella
5 Closterium
6 Coelastrum
7 Cosmarium
8 Microspora
9 Oedogonium
10 Oocystis
11 Protoderma
12 Rhizoclonium
13 Roya
14 Scenedesmus
15 Selenastrum
16 Stigeoclonium
17 Ulothrix
18 Volvox
19 Zygnema

Soft-BodyTaxa (Others)

1 Cryptomonas
2 Dinobryon
3 Euglena

APPENDIX D: ALGAE AND DIATOM METRICS

SOFT BODY (GREEN ALGAE) METRICS

Abundance Measures
Species Richness
Number of Valves Counted
Total Abundance for Sample

Dominance Measures
1st Dominant Taxon
1st Dominant Taxon Abundance
2nd Dominant Taxon
2nd Dominant Taxon Abundance
3rd Dominant Taxon
3rd Dominant Taxon Abundance
4th Dominant Taxon
4th Dominant Taxon Abundance
5th Dominant Taxon
5th Dominant Taxon Abundance
% 1st Dominant Taxon
% 2nd Dominant Taxon
% 3rd Dominant Taxon
% 4th Dominant Taxon
% 5th Dominant Taxon

Diversity/Evenness Measures
Shannon-Weaver H' (log e)
Shannon-Weaver H' (log 10)
Shannon-Weaver H' (log 2)
Margalef's Richness

Other Measures
% Rhopalodiales
% Achnanthes minutissima (Disturbance Index)
% Siltation Index
Siltation Richness
% Aerophiles
% Centrics
% Stability Index
% Heavy Metals Index

Diatom Metrics

Abundance Measures
Species Richness
Number of Valves Counted
Total Abundance for Sample

Dominance Measures
1st Dominant Taxon
1st Dominant Taxon Abundance
2nd Dominant Taxon
2nd Dominant Taxon Abundance
3rd Dominant Taxon
3rd Dominant Taxon Abundance
4th Dominant Taxon
4th Dominant Taxon Abundance
5th Dominant Taxon
5th Dominant Taxon Abundance
% 1st Dominant Taxon
% 2nd Dominant Taxon
% 3rd Dominant Taxon
% 4th Dominant Taxon
% 5th Dominant Taxon

Diversity/Evenness Measures
Shannon-Weaver H' (log e)
Shannon-Weaver H' (log 10)
Shannon-Weaver H' (log 2)
Margalef's Richness

Van Dam Indices
pH
Salinity
Nitrogen Uptake
Oxygen Tolerance
Saprobility
Trophic State
Moisture

pH Preference
% at Optimal Occurance pH 5.5
% Mainly Occuring at pH LT 7
% Mainly Occuring at pH~7
% Mainly Occuring at pH GT 7
% Exclusively Occuring at pH GT 7
% at no Apparent Optimum
% Unclassified pH
Richness at pH LT 5.5

Richness at pH LT 7
Richness at pH ~7
Richness at pH GT 7
Richness Exclusively at pH GT 7
Richness at no Optimum

Salinity Preference
% Fresh
% Fresh-Brackish
% Brackish-Fresh
% Brackish
% Marine
% Unclassified Salinity
Richness Fresh
Richness Fresh-Brackish
Richness Brackish-Fresh
Richness Brackish
Richness Marine

Nitrogen Uptake Metabolism
% Very Small Tolerance N
% Elevated Tolerance N
% Needing Periodically Elevated N
% Needing Continuously Elevated N
% Unclassified Nitrogen
Richness at Small Tolerance N
Richness at Elevated Tolerance N
Richness at Periodic Elevated N
Richness at Continuously Elevated N

Oxygen Requirements
% at Continuously High Oxygen
% at Fairly High Oxygen
% at Moderate Oxygen
% at Low Oxygen
% at Very Low Oxygen
% Unclassified Oxygen
Richness at High Oxygen
Richness at Fairly High Oxygen
Richness at Moderate Oxygen
Richness at Low Oxygen
Richness at Very Low Oxygen

Saprobity
% Very Slightly Polluted
% Moderately Polluted

% Moderate-Heavily Polluted
% Heavily Polluted
% Very Heavily Polluted
% Unclassified Saprobity
Richness Very Slightly Polluted
Richness Moderately Polluted
Richness Moderate-Heavily Polluted
Richness Heavily Polluted
Richness Very Heavily Polluted

Trophic State
% Oligotrophic
% Oligo-Mesotrophic
% Mesotrophic
% Meso-Eutrophic
% Eutrophic
% Hypereutrophic
% Oligo-to-Eutrophic
% Dystrophic
% Unclassified Trophic
Richness Oligotrophic
Richness Oligo-Mesotrophic
Richness Mesotrophic
Richness Meso-Eutrophic
Richness Eutrophic
Richness Hypereutrophic
Richness Oligo-to-Eutrophic
Richness Dystrophic

Moisture
% Never Outside Water
% Mainly in Water, Sometimes Wet
% Mainly in Water, Regularly Wet
% Mainly Wet, Moist or Temporarily Dry
% Nearly Exclusively Outside Water
% Unclassified Moisture
Richness Never Outside Water
Richness Mainly in Water, Sometimes Wet
Richness Mainly in Water, Regularly Wet
Richness Mainly Wet, Moist or Temporarily Dry
Richness Nearly Exclusively Outside Water

Morphological Guild (JS)
% Erect
% Stalked
% Unattached
% Prostrate/Adnate
% Variable
% Unclassified Morphological Guild
Richness Erect
Richness Stalked

Richness Unattached
Richness Prostrate/Adnate
Richness Variable

Motility (JS)
% Highly Motile
% Moderately Motile
% Not Motile
% Variable Motility
% Unclassified Motility
Richness Highly Motile
Richness Moderately Motile
Richness Not Motile
Richness Variable Motility

Pollution Tolerance (LB)
% Very Tolerant
% Moderately Tolerant
% Sensitive/Intolerant
Richness Very Tolerant
Richness Moderately Tolerant
Richness Sensitive/Intolerant
L-B Pollution Tolerance Index

Other Measures
% Rhopalodiales
% Achnanthes minutissima (Disturbance Index)
% Siltation Index
Siltation Richness
% Aerophiles
% Centrics
% Stability Index
% Heavy Metals Index