Phase II Bioavailable Phosphorus Study
Study Outline
May 2012

Background:
In February 2011, Dr. Michael Brett and his laboratory at the University of Washington completed a study examining the bioavailability of phosphorus in multiple wastewater effluents discharging to the Spokane River as well as from the Spokane River itself. During and immediately following the study, there were questions raised about the procedures used to determine bioavailable phosphorus (BAP) and how the results can be incorporated in water quality management tools in the Spokane River – Lake Spokane watershed.

The work completed in February 2011 is now called the Phase I BAP study. This document outlines a proposed Phase II study to further the understanding of BAP for possible incorporation into water quality management.

This document includes the following sections:

- Experimental constraints – a summary of laboratory capacity to process samples.
- Phase II possible objectives – this outlines and describes questions raised during the Phase I study and provides a discussion of the merits of pursuing additional study on these points.
- Priorities for Phase II – this proposes the specific study tasks to be implemented in the new BAP study.
- QA/QC procedures – a summary of the analytical procedures for the study.

Following a review and agreement by the appropriate agencies on which tasks to pursue during Phase II, a full quality assurance project plan (QAPP) will be prepared for the study.

Experimental Constraints:
Bo Li is able to process a new BAP experiment every 3 weeks, with each experiment lasting 2 weeks. This means that she could likely complete 17 new experiments during a one-year lab study. In each BAP experiment, Bo can determine the phosphorus bioavailability of 6 separate samples.

Phase II Possible Objectives:
1. Determine if the low %BAP for some effluents was an artifact of effluent toxicity
During Phase I of the Spokane Basin BAP project, EPA and Ecology commented that some of the BAP results reported might have been biased towards low values because the pure effluents we tested for some dischargers may have been toxic for algae. This suggestion is most plausible for the BAP analyses conducted on water collected from the Inland Empire Paper and Spokane City facilities.

To address this concern, we will conduct two experiments with water collected from IEP and the new Spokane County facility to test whether pure effluents have growth inhibiting properties for the algae we used in our bioassay experiments. According to the EPA protocol

recommended by Brian Nickel (EPA 2002; Section 14.10.1.2.7), these experiments will include two treatments. The first treatment will be algae grown in the pure effluent matrix supplemented with 1 mL (of 1000X) synthetic algal growth media for each reagent including P. The second treatment will be algae grown in deoinized water supplemented with 1 mL (of 1000X) of synthetic algal growth media for each reagent including P. In most other regards, this experiment will employ the same methods as our normal BAP experiments (*i.e.*, growth chamber, temperature, light regime, cell counting). However, this EPA protocol only calls for 4-day experimental incubations, as opposed to the 14-day incubations we normally use for the BAP bioassays. If the pure effluents inhibit algal growth, the algal growth yield in the first treatment (100% effluent matrix) will be significantly less than that in the second (deionized water).

2. Determine if the low %BAP was an artifact of nutrient (*e.g.*, N) co-limitation

During Phase I of the Spokane Basin BAP project, EPA and Ecology commented that some of the BAP results reported might have been biased towards low values because alternative nutrients (*e.g.*, nitrogen) were limiting or co-limiting algal growth in these experiments. This suggestion is particularly plausible for the BAP analyses of water collected from the Spokane River, which could also have had low bioavailable N.

To address this concern, we will conduct a series of four BAP experiments with water collected from the Spokane River (as well as from Little Spokane River, Hangman Creek, Long Lake, and Inland Empire Paper) that would employ an experimental modification to address potential limitation by nutrients other than P. Specifically, instead of simply testing algal growth in raw water, the raw water will be amended with all of the components of synthetic algal growth media (*sans* P) with 1 mL (but 1000X) aliquots for each reagent. This protocol will be similar to the EPA method previously recommended by Brian Nickel. However, in this case the algal incubations will last 14 days, and we will not use a phosphorus addition treatment. To determine whether our earlier results for the Spokane River were biased downward, we will also include a treatment that is not amended with synthetic growth media in each experiment (however, this additional treatment will not be used for experiments using water collected from the Little Spokane River, Hangman Creek, and Long Lake).

3. For Spokane River samples determine if phosphorus is non-biovailable or has previously been sequestered by algae, periphyton, and/or macrophytes making it appear not present or "non-bioavailable."

Our hypothesis is that algae and bacteria rapidly strip-out the most bioavailable phosphorus forms from the water when their growth conditions are favorable, especially when light and other nutrients are readily available. According to this hypothesis, a large portion of the most labile P forms loaded to the Spokane River would be tied up within the periphyton biofilm on the bottom substrate of the Spokane River. Classically, stream ecologists have examined the exchange of bioavailable nutrients between the stream water and the bottom substrate biofilm using a "nutrient spiraling" experimental approach (Newbold et al. 1983). Because this approach entails addition of radioactive P isotopes, it is only practical in very small streams and it is not advisable in rivers such as Spokane that are open to public contact. Furthermore, because it is not possible to purchase or produce ^{32}P labeled effluents, even this approach can only be used to study a very small proportion of P containing compounds. We are unaware of

a more practical method for quantifying the flux of bioavailable P sequestered within the biofilm versus the flux of less bioavailable P fluxed with advected transport in the river. The later would be easy to determine with detailed river water characterization, but the former would at best be very difficult to determine and would require a very intensive sampling effort. We do not think it would be practical to pursue this objective in the Phase II project. However, it would make a fascinating research project in its own right.

4. Determine if autoclaving impacts the bioavailability of phosphorus in bioassays

In Phase I, it was necessary to quantify the %BAP for the bulk effluents, which included both dissolved and particulate phases of P. Further, our first BAP experiments (which were not autoclaved) were immediately and severely contaminated with indigenous algae. To address the question above, we have already completed a series of experiments that examined the impact of autoclaving versus filtration on our BAP estimates. These experiments showed a strong linear relationship between autoclaved and 0.45 µm filtered samples ($r^2 = 0.99$, n = 15) with the autoclaved samples giving on average 7% higher BAP estimates. Thus the BAP results reported in Phase I were most likely conservative.

5. Conduct phosphorus fraction testing on samples to better determine relationship between TP-TRP-SRP-BAP

All samples processed for BAP during Phase II, will also have a comprehensive operational P characterization, which would include total phosphorus (TP), total reactive phosphorus (TRP), soluble phosphorus (SP), and soluble reactive phosphorus (SRP). These direct determinations allow us to partition TP into the following operational categories: SRP, soluble non-reactive P (SnRP = SP - SRP), particulate reactive P (PRP = TRP - SRP), and particulate non-reactive P (PnRP = TP - SP - PRP). If needed, we could operationally characterize some P samples above and beyond those that will already be processed for our BAP determinations.

6. Test BAP in various samples using alternative algal types to see if BAP varies by algal culture

The EPA guidance manual on the use of algal bioassay experiments (Miller et al. 1978) discusses the merits of using *Selenastrum capricornutum* (currently *Pseudokirchneriella subcapitata*) as the algal bioassay "white rat". In fact, it should be noted that Miller et al. (1978) actually specifically mentioned *Selenastrum capricornutum* by name in the title of their guidance manual. This manual further stated *"this research was designed to determine . . . whether the growth response of* Selenastrum capricornutum *reflects the response of indigenous [phytoplankton] species."* Miller et al. (1978) also state *"when comparing algal growth potentials from a number of widely different water sources there are advantages in using the same species of algae for all waters . . . the recommended test alga [is]* Selenastrum capricornutum . . . *generally, indigenous phytoplankton bioassays are not necessary"*. In fact, these EPA authors reached this conclusion based on experiments they did in Long Lake itself, which compared the growth response of *Selenastrum capricornutum* to that of indigenous phytoplankton, the cyanobacterium *Ananbaena flos-aqua*, and a strain of the green alga *Sphaerocystis schroeteri* (that had been isolated from Long Lake). Greene et al. (1978) found that for a wide range nutrient and zinc enrichment treatments, the growth response for *Selenastrum capricornutum* correlated strongly to that of other phytoplankters.

Our take on this issue is that there are likely "first-order" affects on the bioavailability of the various P compounds that comprise the WWTP effluents and natural waters we will be testing. These first-order affects are related to the molecular properties of these compounds, and especially whether they are more or less susceptible to biological modification. These molecular properties will be the primary (= first-order) determinants of bioavailability. Based on the information provided in he EPA guidance manual on this topic (Miller et al. 1978) and our own professional judgment, we hypothesize that there are also likely secondary (= second-order) affects on phosphorus bioavailability that relate to the specific biological strategies of the various algae, cyanobacteria and bacteria that could be used for these types of experiments. Because much of the effort for each bioassay experiment goes to deriving a standard curve, adding additional phytoplankton species to our experimental protocol will double our effort and in affect halve the number of samples we can process during the Phase II project. Given the time and resource constraints that this project will have, we believe we will achieve the most useful data for the available resources by using a single algal species for the bioassay experiments.

7. Obtain and test additional effluent samples from the facilities that did not yield a consistent series of samples in Phase I

During Phase I of our Spokane BAP study, some treatment facilities provided us samples that yielded highly variable results for phosphorus concentrations and speciation. Where possible, we will operationally characterize the phosphorus on ca. four samples from each of these original facilities. These reassessments will only be possible in cases where the same facilities that we previously studied are currently in operation.

8. Test effluents from the new Spokane County Regional Water Reclamation Facility (SCRWRF) to determine their TP and %BAP

During the course of Phase II, we will process ten independent effluent samples from the new Spokane County Regional Water Reclamation Facility. For each of these samples, we will carry out a full operational categorization of the phosphorus (*i.e.*, quantify SRP, SnRP, PRP and PnRP), as well as determine the %BAP of the bulk effluent.

9. Test effluents from the new facilities in the basin to determine their TP and %BAP

If new treatment facilities or pilot plants become available prior to or during Phase II, we will process at least five independent effluent samples from each. For each sample, we will carry out a full operational categorization of the phosphorus and determine the %BAP of the bulk effluent. A list of the facilities that we will likely process samples from is provided below.

Existing Facilities available for Sampling	**Influent type**	**Treatment process**	**Tertiary (Y/N)**	**Pilot or full scale**
Coeur d'Alene Wastewater	Municipal wastewater	Membrane	yes	pilot
Spokane County Regional Water Reclam. Facility	Municipal wastewater	Membrane bioreactor	yes	full scale
Inland Empire Paper Co.	Paper manufacturing	Trident HS chemical precipitation	yes	full scale
Inland Empire Paper Co.	Paper manufacturing	Algal based system	yes	pilot
Existing Facilities but not yet requested samples				
Blue Water test Facility, Hayden, ID	Municipal wastewater	Blue Water technology	yes	pilot
Plummer, ID	Municipal wastewater	Blue Water technology	yes	full scale
Coeur d'Alene Casino	Resort/Hotel/Casino	Membrane bioreactor	yes	full scale
Mountainside Middle School	Middle school	Membrane bioreactor	yes	full scale

10. Test the natural surface waters collected from Hangman Creek, Little Spokane River and Long Lake to determine whether a substantial fraction of non-bioavailable P is present in these systems

As previously noted, we plan to test of the BAP of the P contained in samples from Hangman Creek, Little Spokane River and Long Lake. The protocol for these experiments will be modified to account for potential nutrient colimitation by nutrients other than P as previously described.

11. Test the hypothesis the recalcitrant dissolved P fraction is primarily Humic-metal complexed P.

Our hypothesis that much of the dissolved recalcitrant P is bound in humic-metal-complexes can be tested by size fractionating the "dissolved" phosphorus phase using ultra-filtration and a logarithmic series of filters. It is common practice to use ultra-filtration with nominal pore sizes of 1 kilo dalton[1] (kDa), 3 kDa, 10 kDa, 30 kDa and 100 kDa to characterize the dissolved organic carbon in water samples (Gerke 2010). Classic research indicates molecules up to 1 kDa can pass through cell membrane channels with permeation velocities inversely proportional to molecular size, whereas molecules $\geq$ 2 kDA can not cross cell membranes (Simpson et al. 1997). By comparison, humic-metal complexed phosphorus is associated with macromolecules that are in the 10-30 kDa size range and thus far too large to cross algal cell membranes (Battin et al. 2008, Gerke 2010). By using ultra-filtration, we should be able to isolate humic-complexed P and test whether this fraction comprises a large portion of the dissolved P in certain effluents. This is possible because few of the other dissolved P compounds that are likely to occur in effluents or natural surface waters are likely to be larger than 1 kDa.

For these analyses, we will subject each sample to a full ultra-filtration series to identify which colloidal size fractions contain the most dissolved P. The differences in the DOP and SRP content of each size fraction will identify where the most dissolved P is found. For example, if we ran water through a series of filters with the following nominal pore sizes (*e.g.,* 1, 3, 10, 30 and 100 kDa) and obtained the following total soluble P concentrations (*e.g.,* 15, 17, 25, 55 and 60 µg L^{-1}, respectively), we would infer that 25% of the SP is found in the smallest colloidal size range corresponding to most P containing compounds, 50% of the SP is found within the 10-30 kDa colloidal size range characteristic for humic many substances, and the remaining SP is found in all other size categories combined. For each sample processed we will conduct the classic 4-way characterization (*i.e.,* reactive *vs.* non-reactive and particulate *vs.* dissolved) on the bulk sample, as well as SRP and SP analyses on each of the five colloidal fractions (*i.e.,* < 1, < 3, < 10, < 30 and < 100 kDa). Thus each sample processed for these analyses will generate 14 separate phosphorus determinations. Once we have identified the colloidal size fraction where the most soluble P occurs, we will conduct BAP analyses on soluble P samples that have been fractionated above and below this cut off. This will result in two separate BAP analyses for each sample processed. This series of analyses and experiments will determine whether a significant portion of the recalcitrant soluble P pool of some effluents is found within the colloidal size fraction associated with humic complexes.

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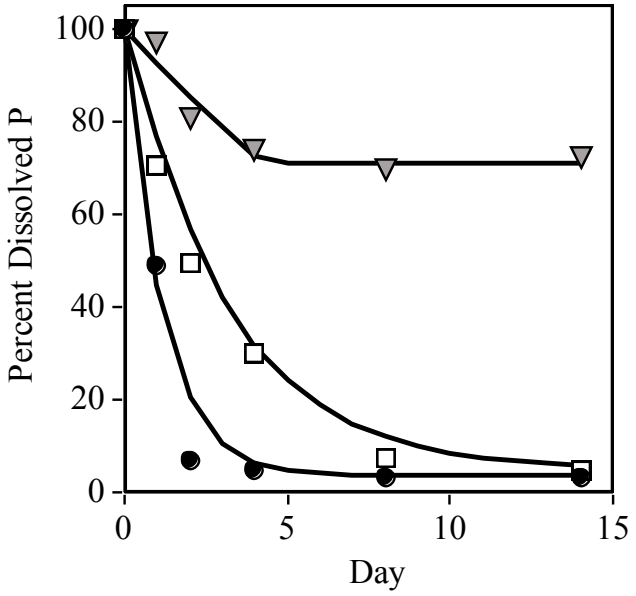
[1] Daltons are a molecular mass unit (corresponding to the molecular mass of a single proton or neutron) and approximately correspond to molecular size; for example PO_4^{3-} has a "size" of 95 Da.

12. During the recent workshop to discuss Phase II BAP research priorities, there was a strong consensus amongst the participants that in order to most easily implement differences in effluent phosphorus bioavailability into the eutrophication/biogeochemistry model being used to represent management scenarios in Long Lake, soluble phosphorus mineralization rates will need to be quantified for the main effluents to the Spokane River/Long Lake system.

We have recently completed a series of experiments that allowed us to quantify rates of soluble P uptake and utilization by algae. These experiments showed that some types of dissolved P were taken up by algae almost immediately (*i.e.*, within 2 days), whereas other soluble P compounds were mostly taken up after 4 days, and other phosphorus forms reached a rapid equilibrium and maintained a more or less constant concentration for a large part of the experiment. In these experiments, $\approx 100\ \mu g\ L^{-1}$ soluble P was added to Erlenmeyer flasks with a previously phosphorus starved *Selenastrum capricornutum* inoculum. We then determined the soluble P concentrations in these flasks at time 0, 1, 2, 4, 8 and 14 days. In this experimental design, the loss of soluble P, as opposed to the production of algae cells, is the experimental response metric. In most cases, it will be necessary to incubate simultaneous dark/algae free control treatments to account for the abiotic formation of P containing flocs or precipitates, as this methodology is unable to distinguish between soluble P lost to algal uptake and soluble P transferred to the particulate phase via abiotic processes. For example, previous experiments with Inland Empire Effluents indicated extensive humic floc formation during algal BAP bioassays.



In Figure 1, we present the recent results obtained using this experimental design and three different P containing compounds, *i.e.*, ATP (adenosine-5'-triphosphate; closed circles), DNA (deoxyribonucleic acid; open squares), and humics (shaded triangle). The loss rate of dissolved P contained in the ATP and DNA tested indicated the dissolved organic P is taken up extremely rapidly, *i.e.*, 51 and 30% in the first day, respectively. Conversely, the results for the humic bound P suggested these was a 30% loss of dissolved P in the first four days of the experiment, and no change in concentration in the final 10 days. We believe the initial 30% loss of dissolved P was due to abiotic humic floc formation and not biological uptake. Because any dissolved organic P mineralized to true phosphate will be immediately taken up by algae, as long as algae are present, it is not actually possible to measure dissolved P mineralization as the rate of PO_4 production. However, loss of soluble P due to algal uptake is a very good, and easily measured, proxy for P mineralization.

Priorities for Phase II:

During the Phase II BAP study we propose to do:

A. Determine if the %BAP of pure effluents (*i.e.*, IEP, Spokane County, Hayden Blue Water, etc.) is modified by effluent toxicity

We will conduct two experiments for at least three pure effluent types for a total of six experiments according to the recommended EPA protocol.

B. Determine if the low %BAP of the Spokane River samples processed during Phase I was an artifact of nutrient (*e.g.*, N) co-limitation

We will conduct five BAP experiments that will quantify the %BAP of the Spokane River, while accounting for possible nutrient co-limitation according to the recommended EPA protocol. These experiments will also include a without nutrient addition treatment to determine whether the prior %BAP results obtained for the Spokane River samples in Phase I were artificially low due to nutrient co-limitation.

C. Conduct phosphorus fraction testing on samples to better determine relationship between TP-TRP-SRP-BAP

All bulk water samples processed for %BAP determinations during the course of Phase II will be subjected to the classic phosphorus operational characterization, *i.e.*, reactive *vs.* non-reactive and particulate *vs.* dissolved in duplicate.

D. Obtain and test additional effluent samples from the facilities that did not yield a consistent series of samples in Phase I

We will analyze up to 10 samples for %BAP and phosphorus operational categories from treatment plants that provided highly variable results during the Phase I study.

E. Test effluents from the new Spokane County Regional Water Reclamation Facility (SCRWRF) to determine their TP and %BAP

We will complete %BAP and P characterization analyses on 10 independent samples collected from the new Spokane County WWTP.

F. Test effluents from the new facilities in the basin to determine their TP and %BAP

To be determined. This task will be based upon availability of effluent samples and participant's interest in testing.

G. Test the natural surface waters collected from Hangman Creek, Little Spokane River and Long Lake

We will conduct five %BAP determinations and P speciation characterizations on water samples collected from each of Hangman Creek, Little Spokane River and Long Lake. The BAP experiments will be modified to account for potential nutrient co-limitation according to the recommended EPA protocol. These samples will ideally be collected during the peak algal growth months.

H. Test the hypothesis the recalcitrant dissolved P fraction is primarily Humic-metal complexed P.

For the main effluent types discharged to the Spokane River/Long Lake system, we will use an ultra-filtration and BAP characterization procedure on two independent samples from each effluent (*i.e.*, IEP, Spokane County, Cd'A, etc.) for up to four effluent types (*i.e.*, a sum of eight humic-P characterizations).

I. Determine the rate of dissolved P conversion (or the loss rate to algal uptake) for the primary effluents discharges for the Spokane River/Long Lake system.

For the main effluent types discharged to the Spokane River/Long Lake system, we will conduct experiments designed to quantify the rates of dissolved P loss (a proxy for dissolved organic P mineralization and bioavailable P utilization) for two independent samples from each effluent (*i.e.*, IEP, Spokane County, Cd'A, etc.) for up to four effluent types (*i.e.*, a sum of eight utilization rate characterizations). These experiments will last 16 days and samples for dissolved P characterization will be collected on days 0, 1, 2, 4, 7, 11, and 16; all treatments will be carried out in triplicate.

See attached table for a summary of proposed experiments and samples.

QA/QC procedures:

Chemical analyses: Chemical analyses for each effluent sample will be used to determine whether the P in these samples is reactive and/or dissolved according to the acid-molybdate spectrophotometric method described in Standard Methods 4500-P (APHA 1998). This will yield the four classic operational categories, *i.e.*, total P (TP), soluble P (SP), total reactive P (TRP) and soluble reactive P (SRP). TP will be determined after 45 minutes of autoclave-mediated digestion (120 °C, 100 kPa, with $K_2S_2O_8$ and H_2SO_4) of unfiltered samples (APHA 1998). TRP will be determined using the same reaction on unfiltered samples without persulfate digestion. Samples for SP and SRP analyses (120 mL) will be first filtered through a 0.45 µm polycarbonate membrane filter (Millipore®). SP will be measured after persulfate digestion while SRP will be measured without persulfate digestion. All phosphorus analyses will be carried out in duplicate.

Solutions for the standard curve will be prepared from a 50 mg L^{-1} stock solution. Seven concentrations spanning the expected range of sample values plus a blank will be run in duplicate. The typical standard concentrations used are 0, 10, 20, 30, 40, 75 and 100 µg L^{-1}. A 200 µg L^{-1} standard can also be added if we have effluent samples in the 100-200 µg L^{-1} range. Intermediate spike solutions will be made from the same stock solution, and will be prepared fresh for each assay. A final spike concentration of 50 µg L^{-1} will be used for samples in the 0-100 µg L^{-1} range. Sample phosphate concentrations will be obtained from a regression equation after plotting the heteropoly blue absorbance versus the standard solution PO_4 concentrations. We will use separate standard curves for SRP and TP analyses because the reagents used to oxidize TP also slightly alter color development. If samples have been diluted, we will multiply the concentration from the regression equation by the appropriate dilution factor to calculate final concentrations.

Algal bioassays: The freshwater alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) will be used for these experiments, as indicated by Standard Method 8111 (APHA 1998). *P. subcapitata* will be maintained in synthetic nutrient growth media prior to and during the bioassay experiments. Seven to ten days prior to the bioassays, algae cultures will centrifuged and resuspended into P-free medium to induce P-stress. Fifty mL of each test sample will be placed into 125-mL Erlenmeyer flasks, which will be acid-washed (0.1 M HCl) and autoclaved prior to each experiment. Standard media with known concentrations of KH_2PO_4 (0, 5, 10, 15, 20, 25, 30, 40 and 50 µg $P \cdot L^{-1}$) will be incubated in triplicate to obtain a standard curve for the algal growth yield. Because the precision of this method is lower than for standard wet chemistry approaches, four replicates of each sample will be incubated and the results averaged for the final calculations. P-starved algae will be added to the samples at a starting concentration of 10^4 cell$\cdot mL^{-1}$ to initialize the experiments. Samples will be incubated at 24 ± 2 ºC under continuous cool white fluorescent lighting of 4300 ± 430 lm in a horizontal shaker at 110 rpm for 14 days. The 14-day incubation period is based upon the maximum growth potential for the test algae in laboratory conditions (APHA 1998). Following incubation, algal cell density in the test and standard curve samples will be determined using a Coulter Multisizer III particle size analyzer by passing the samples through a 100 µm aperture, with every sample read three times (Miller et al. 1978, APHA 1998). Prior to each reading, background particle concentrations will be estimated by testing parallel samples which have not been inoculated with algae. The regression equation between algal cell density and BAP will be derived from the standard solution concentrations and algal counts accordingly: BAP (µg L^{-1}) = (Cell Density)*A + B, where, A represents the slope and B the intercept of the standard curve.

References:

APHA (American Public Health Association), 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC.

Battin, T. J.; Kaplan, L. A.; Findlay, S.; Hopkinson, C. S.; Marti, E.; Packman, A. I.; Newbold, J. D.; Sabater, F., 2008. Biophysical controls on organic carbon fluxes in fluvial networks. Nat. Geosci. 1, 95-100.

EPA. 2002. Short-term Methods for Evaluating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Office of Water. U.S. Environmental Protection Agency. Washington, DC. Publication number EPA-821-R-02-013.

Gerke, J., 2010. Humic (Organic Matter)-Al(Fe)-phosphate complexes: an underestimated phosphate form in soils and source of plant-available phosphate. Soil Sci., 175, 417-425.

Greene, J. C., W. E. Miller, T. Shiroyama, R. A. Soltero, and K. Putnam. 1978. Use of laboratory cultures of *Selenastrum*, *Anabaena* and the indigenous isolate *Sphaerocystis* to predict effects of nutrient and zinc interactions upon phytoplankton growth in Long Lake, Washington. Mitt. Int. Ver. Limnol. 21: 372-384.

Miller, W. E.; Greene, J. C.; Shiroyama, T. 1978. Corvallis Environmental Research, L., The *Selenastrum capricornutum* Printz algal assay bottle test: experimental design, application, and data interpretation protocol. Environmental Protection Agency, Office of Research and Development, Corvallis Environmental Research Laboratory: Corvallis, Ore.

Newbold JD; Elwood JW; O'Neill RV; et al. 1983. Phosphorus dynamics in a woodland stream ecosystem - a study of nutrient spiraling. Ecology 64: 1249-1265.

Simpson I; Rose B; Loewenstein WR. 1977. Size limit of molecules permeating junctional membrane channels. Science 195: 294-296.

Phase II sample summary				**Total samples**	**52**
Task					
A -Effluent toxicity	Experiment 1	Effluent 1	Effluent 2	Effluent 3	3
	Experiment 2	Effluent 1	Effluent 2	Effluent 3	3
B -Spokane River, co-limitation	Experiment 1	Spokane River			1
	Experiment 2	Spokane River			1
	Experiment 3	Spokane River			1
	Experiment 4	Spokane River			1
	Experiment 5	Spokane River			1
C - Phosphorus fractions	Included for all sample sources				
D - Additonal previous effluents	Experiment 1	Effluent			1
	Experiment 2	Effluent			1
	Experiment 3	Effluent			1
	Experiment 4	Effluent			1
	Experiment 5	Effluent			1
	Experiment 6	Effluent			1
	Experiment 7	Effluent			1
	Experiment 8	Effluent			1
	Experiment 9	Effluent			1
	Experiment 10	Effluent			1
E - SCRWRF testing	Experiment 1	Effluent			1
	Experiment 2	Effluent			1
	Experiment 3	Effluent			1
	Experiment 4	Effluent			1
	Experiment 5	Effluent			1
	Experiment 6	Effluent			1
	Experiment 7	Effluent			1
	Experiment 8	Effluent			1
	Experiment 9	Effluent			1
	Experiment 10	Effluent			1
F - Additional facilities	To be deterimined				
G - Natural water samples	Experiment 1	Natrual water 1			1
Hangman, LSR, Spokane	Experiment 2	Natrual water 2			1
	Experiment 3	Natrual water 3			1
	Experiment 4	Natrual water 4			1
	Experiment 5	Natrual water 5			1
H - Humic-metal complex test	Experiment 1	Effluent 1	Effluent 1		2
	Experiment 2	Effluent 2	Effluent 2		2
	Experiment 3	Effluent 3	Effluent 3		2
	Experiment 4	Effluent 4	Effluent 4		2
I - Dissolved P conversion rate	Experiment 1	Effluent 1	Effluent 1		2
	Experiment 2	Effluent 2	Effluent 2		2
	Experiment 3	Effluent 3	Effluent 3		2
	Experiment 4	Effluent 4	Effluent 4		2