# PHOSPHORUS REMOVAL CHARACTERISTICS ON BIOGENIC FERROUS IRON OXIDES

By

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A thesis submitted in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE IN ENVIRONMENTAL ENGINEERING

WASHINGTON STATE UNIVERSITY
Department of Civil and Environmental Engineering

DECEMBER 2008

To the faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ANTOINE CORDRAY find it satisfactory and recommend that it be accepted.

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Chair

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# ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Jeremy Rentz, whose expertise, availability, and advice in many different fields made my graduate program a rich experience. I would also like to thank the members of my committee, Dr. Jeffrey Ullman and Dr. David Yonge. They took time to share their knowledge and contribute to my work. Finally, I would like thank Dr. David McLean and Gisela Bouzon who both made this international experience possible by working on a partnership between Washington State University and EPF "Ecole d'ingénieurs".

I acknowledge Haley Falconer whose help was invaluable throughout the summer. She has been largely involved in the sampling and experimentation efforts. She, and my other office mate Katherine Schaffnit also helped me improve my English skills for the thesis writing. I am grateful to two undergraduate students, Cameron Turtle and Andrew McDonald, who worked during the summer in our laboratory and participated actively in my research experiments.

Finally, I would like to thank my family members, Michèle Sembeille, Jean-Pierre Cordray, Agathe Cordray, Jean-Baptiste Cordray, Maude Decombat, Philippe Corre, Axel and Romane Corre, and Corinne Bonnet for their long distance support during this year and a half and for the love they have given me. I would like to thank my two friends and roommates Guillaume Paternostre and Michael Miraglio, both graduate students, for the mutual help, motivation, and good times we spent together. I am grateful to Marine Fagot, who since high school has been a close friend and has always supported me.

# PHOSPHORUS REMOVAL CHARACTERISTICS ON BIOGENIC FERROUS IRON OXIDES

## Abstract

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December 2008

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Phosphorus is a main aquatic pollutant, which has led to an evolution in treatment technologies. However, it remains a cause of water body degradation. Filters using iron rich or iron oxide containing substrates appear to be an effective method to remove phosphorus from water. A recent study revealed that biogenic iron oxides used within a filter are an improved and more cost effective method (Rentz et al., 2008). Phosphorus resources are depleted, and recovery methods are studied for phosphorus reuse in both agriculture and industry. In order to investigate the characteristics of biogenic iron oxides generated by *Leptothrix ochracea* and their behavior on phosphorus adsorption and release, we used batch equilibrium experiment methods. Biogenic iron oxides adsorbed 19.02 mg P/g solids on average against 6.27 mg P/g solids for synthetic or chemical substrates. Variations in pH dramatically enhanced phosphorus adsorption. Fresh samples showed an increase in phosphorus by 315% and 307% between low and high pH. Older samples increased by only 50% and 78%. Results suggest that adsorption of phosphorus to biogenic iron oxides is made by weak electronic linkage. Hydroxide sodium was the best chemical to break down these linkages and release phosphorus. Sample released from 159.3% and 295.3% of the initially adsorbed phosphorus. DI water showed the second best phosphorus

release potential of 40.60% averaged over the four samples. The KCl solution had an average potential of 30.37% and the saline solution 20.23%. The origin of the released phosphorus is in-situ phosphorus adsorption or cell disruption. Release potential for fresh samples was greater than old samples only using sodium hydroxide. High sorption capacity, adsorption enhancement by lowering the pH, and great phosphorus release capacity should be incorporated to the next step of this project of phosphorus filtration.

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# CHAPTER ONE
# INTRODUCTION

Phosphorus pollution contributes to water body degradation (Moore and Hicks, 2004). It often results in loss of aquatic life due to oxygen depletion by eutrophication, diminished water quality, and depleted recreational activities. Phosphorus regulations have become increasingly strict and several methods have been developed to remove this contaminant (de-Bashan and Bashan, 2004). Chemical precipitation and enhanced phosphorus biological removal (EPBR) are currently the technologies used most frequently by wastewater treatment plants (Morse et al., 1998). Used at different steps during the wastewater treatment process, chemical precipitation offers high water quality. EPBR represents an effective method to treat larger amounts of wastewater than chemical precipitation (GLS, 2006). However, both of these technologies, even coupled, can have difficulties meeting limitation requirements. Often, meeting limits is cost prohibitive. New methods must be investigated to provide sustainable solutions to this excessive problem. Filtration using highly selective substrates represents one effective, simple, and cost effective way to improve phosphorus removal and water quality (Riahi et al., 2008). Added as tertiary treatment systems, filters could improve phosphorus removal during municipal and industrial wastewater treatment (Jeon et al., 2002).

Previous studies examined several substrates for phosphorus removal, particularly iron oxides that strongly bind phosphorus (Sparks, 1995; Kreller et al., 2002). This element is widespread in both soils and water bodies. As a result, it reacts with various elements, resulting in a wide variety of iron forms that are available at low prices to provide a sustainable substrate. Pure iron oxides such as goethite and akaganeite revealed phosphorus adsorption potential

(Chitrakar et al., 2006). Sand coated with iron oxides also showed phosphate removal capabilities (Boujelben et al., 2008; Arias et al., 2006; Del Bubba et al., 2003). Filters using iron rich recycled and engineered material as substrates showed their capacity for phosphorus removal (Boujelben et al., 2008; Bastin et al., 1999; Zeng et al., 2004; Han et al., 2005). A first attempt to use biogenic iron oxides resulted in high phosphorus loading (Rentz et al., 2008).

Biogenic iron oxides are naturally synthesized in water environments under specific conditions. Chemical iron oxidation occurs spontaneously in aerobic environments. However, iron oxidizing bacteria are able to increase this oxidation rate up to 18% and thus compete with chemical oxidation (Rentz et al., 2007; Neubauer, 2007). This reaction occurs in both marine and freshwater environments. Most of the steps in biogenic iron oxide formation are known, but the complete process remains unclear (Fortin and Langley, 2005). Reactions take place at the interface between the oxic and anoxic zone where the oxygen concentration is low.

Different bacteria have the potential for biogenic iron oxide synthesis as a metabolic byproduct. *Sideroxydans paludicola* oxidizes iron in the rhizosphere under microaerobic conditions to produce insoluble ferric hydroxide $Fe(OH)_3$ (Neubauer et al., 2007; Rentz et al., 2007; Weiss et al., 2007). Bacteria from the genus *Gallionella* produce an insoluble ferric hydroxide that develops a helical, stalk-like filament formation from excreted polymers (Emerson and Moyer, 2002). *Gallionella ferruginea* and *Leptothrix ochracea* share metabolism similarities.

*Leptothrix ochracea* is one freshwater iron oxidizing bacteria that produces tubular iron oxides with high surface area. *Leptothrix ochracea* belongs to the group *Sphaerotilus-Leptothrix*. This bacteria is a gram-negative and autotrophic betaproteobacterium. It grows in slow running iron-containing water poor in readily decomposable organic material (Emerson and Revsbech,

1994). Their respiratory pathway oxidizes Fe (II)-oxides to insoluble Fe (III)-oxides. *Leptothrix ochracea* cells live in close association, orienting themselves linearly forming a chain of organisms. As the bacteria grow, iron oxides accumulate on the outside of the cell that are associated with the exopolymers, forming an iron oxide sheath (Emerson et al., 1992; Emerson et al., 1993). Scanning electron microscopy, energy dispersive X-ray spectrometry, and X-ray dispersion descriptions of these sheaths have indicated large surface areas both inside and outside a tubular shape that suggest a potential for high adsorption (Hashimoto et al., 2007).

Previously, our laboratory showed that biogenic iron oxides removed phosphorus well from solution, but the studied biogenic iron oxides were collected from only one location (Rentz et al., 2008). For this study, we expended our experiments to showcase sorption and desorption characteristics for biogenic iron oxides from four geographically distinct sites. The objectives of this study were: (1) to confirm the previous studies that showed iron oxides were strong phosphorus adsorbents, (2) to evaluate how variations in pH affect phosphorus sorption, (3) to investigate the potential for phosphorus desorption, and (4) to characterize differences between iron oxides from different sites in order to predict sorption and desorption capacities.

# CHAPTER TWO
# MATERIALS AND METHODS

Sixteen samples of biogenic iron oxides were collected from four different freshwater locations. Three of them at Moose Creek Reservoir, ID (M10A and M10B collected the same day at two different site locations, and M12). The first attempt to study biogenic iron oxides used only samples from Moose Creek Reservoir (Rentz et al., 2008). In order to investigate biogenic iron oxides from different sites, four samples were collected at Myron Lake in Yakima, WA (YK1, YK2, YK3, and YK4), four at Spring Lake, WA (SL1, SL2, SL3, and SL4), and one at Rainbow Lake, WA (RL1). Samples Moose Creek 14 (M14), Yakima 5 (YK5), Spring Lake 5 (SL5), and Rainbow Lake 3 (RL3) were dedicated to the phosphorus recovery experiments. Each of these sites was characterized visually by significant amounts of orange iron oxides.

Samples were collected between April 2008 and August 2008, using sterile plastic pipettes. Samples SL3 and SL4 were collected using a clean plastic bucket. Sample RL1 was collected directly using a 1L bottle in which the sample was conserved. Samples were concentrated by letting the biogenic iron oxides settle during collection, decanting overlying water, and filling bottles with additional iron oxides. For each sample collected, in situ parameters were measured for the water directly overlying collected iron oxides, including pH, temperature, and ferrous iron (Hach FerroVer). Samples were transported to the lab on ice and stored at 4ºC prior to analysis and experimentation.

Biogenic iron oxides were chemically, physically, and morphologically characterized to compare samples with each other and with other iron-based substrates. Total iron was determined using the Hach FerroVer method following a 24 hour oxalic acid (0.25M) digestion (Rentz et al., 2007); three replicates were conducted for each sample. Total solids content and

organic content were determined by gravimetric methods using four replicates per sample. Iron oxide morphology was captured for each sample using a Leica DMLB light microscope with SPOT software (Diagnostic Instruments Inc.); acridine orange (10 mg/L) was used to visualize microbial cells. Aqueous total organic carbon and phosphorus were measured using the Hach Method 10129 and Hach Method 8048, respectively. pH of the settled sample supernatant was also recorded.

Prior to use in batch equilibrium or desorption experiments, the biogenic iron oxides were washed with a 100 mM sodium chloride solution to remove background phosphorus. A dilute saline solution was used as an isotopic solution that would not interfere with subsequent experimentation. This procedure replaced between 40% and 60% of initial water. All samples were once again chemically and physically characterized following this wash step.

Batch equilibrium experiments were performed using standard methods (Rentz et al., 2008). Saline washed biogenic iron oxide samples were evenly distributed to sterile plastic tubes. The nature of the sample determined the total iron concentration in the reaction tubes. Fresh and gelatinous iron mats were supplied at 150 mg Fe/L. Experiments on older and settled biofilms required 350 mg Fe/L. The volume of washed sample added to the tubes was adjusted accordingly. Initial phosphorus concentrations from 0.163 to 20.9 mg P/L were created using a 326 mg P/L stock solution and were examined with five replicates each. DI water was added to achieve a final 50 mL tube volume. All tubes were mixed by rotation (20 r.p.m.) for 24 hours, a time kinetic studies previously suggested was adequate to reach equilibrium (Rentz et al., 2008). Tubes were then centrifuged (4100 r.p.m. for 10 min) and supernatant phosphate concentrations were measured for each tube using Hach Method 8048.

In order to investigate pH impact on phosphorus sorption to biogenic iron oxides, pH adjusted batch equilibrium experiments were conducted similarly, as above. Prior to the batch equilibrium, samples were washed with a buffered saline wash solution (0.1M NaCl and 10mM sodium bicarbonate). pH was adjusted using either a 100 mM NaOH solution or 100mM $HNO_3$ solution to yield a range of 2.5 to 9. Once the pH was adjusted, the samples were added to reaction tubes (four replicates per initial pH) and phosphorus solution was added to reach a concentration of 20.9 mg P/L. DI water completed the volume of the 50mL tube. Subsequent steps were similar to the batch equilibrium experiment: rotation, centrifugation, and phosphate concentration readings. Finally, supernatant pH was recorded in every reaction tube following centrifugation.

The desorption experiments also used a modified batch equilibrium process. A 24 hour rotation cycle was run with 16 reaction tubes containing 20.9 mg P/L and saline washed samples as described previously. The tubes were then centrifuged and the supernatant phosphate was measured. Supernatant (30 mL) was removed and replaced with one of four stock solutions: deionized water, a 0.1 M saline wash solution, a 0.01 M KCl solution and a 0.01 M NaOH solution. Each solution was conducted in quadruplicate. These tubes were rotated again for 24 hours, centrifuged, and the supernatant phosphate concentration measured. Again 30 mL of supernatant was replaced by an equal volume of desorption solution. This 24 hour cycle was repeated four times. Prior to phosphorus analyses, pH was adjusted to 6-8 with 0.3 M MES buffer solution.

Batch equilibrium data were fit using non-linear curve fitting (Igor Pro, Wavemetrics, Portland, OR U.S.A.), producing 95% confidence interval statistics. The strength of various fit equations was estimated using Pearson analysis to generate $R^2$ values (Microsoft Excel). Five

isotherm fitting equations were used to describe batch equilibrium data: Langmuir (Eq. 1), Freundlich (Eq. 2), Temkin (Eq. 3), Redlich-Peterson (Eq.4), and Langmuir-Freundlich (Eq.5). Γ was the sorbed phosphorus concentration (mg P/g), C was the aqueous equilibrium concentration (mg P/L), $\Gamma_{Max}$ was the maximum sorbed concentration (mg P/g), b was the Langmuir equilibrium constant, $K_f$ was the Freundlich sorption capacity, n was the Freundlich sorption intensity constant. $A_1$ is an adjustable parameter and $B_1$ is the Temkin adsorption coefficient. $A_2$ and $B_2$ were Redlich-Peterson isotherm constants determined by regression of the experimental data.

$$\Gamma=(\Gamma_{Max}bC)/(1+bC) \quad (1)$$

$$\Gamma=K_f\, C\text{^}(1/n) \quad (2)$$

$$\Gamma=A_1+B_1 \ln C \quad (3)$$

$$\Gamma=A_2C/(1+B_2C^n) \quad (4)$$

$$\Gamma= (\Gamma_{Max}bC^{1/n})/(1+bC^{1/n}) \quad (5)$$

All equations were plotted and the coefficients were calculated. For each sample the Langmuir and Freundlich fitting curves were represented. The fitting equation ((3), (4), or (5)) that led to the greatest $R^2$ value was also plotted.

# CHAPTER THREE
# RESULTS AND DISCUSSION

Chemical characteristics of the four sampling sites were favorable for the growth of circum neutral iron-oxidizing bacteria (Table 1). pH ranged from 7 to 7.9 for all samples and the temperature ranged from 8 to 18°C. Moose Creek showed the lowest temperatures (8.33°C in average), whereas other site temperatures were higher (14.11°C in average). Differences were observed between different sites. Average aqueous iron concentration was 12.40 ± 1.24 mg/L at Moose Creek, 3.25 ± 0.25 mg/L at Myron Lake, 5.09 ± 0.56 mg/L at Spring Lake, and 0.38 ± 0.01 mg/L at Rainbow Lake. Low aqueous iron concentrations were favorable for iron oxidizing bacteria growth. In situ aqueous phosphorus concentrations were higher in Rainbow Lake (1.22 mg $PO_4^{3-}$/L) and in Spring Lake (1.21 ± 0.49 mg $PO_4^{3-}$/L in average), two lakes located in agricultural areas. In comparison, Moose Creek showed an average of 0.40 ± 0.23 mg $PO_4^{3-}$/L and 0.48 ± 0.25 mg $PO_4^{3-}$/L for Yakima.

Moose Creek and Rainbow Lake microbial iron mats were physically different from Myron Lake and Spring Lake. Two different structures of biogenic iron oxide biofilms were encountered. One was qualified as fresh due to macroscopic characteristics (Myron Lake and Spring Lake). It had a light orange color and covered a large volume below the water surface. This biofilm was voluminous and often had a gelatinous consistency. The other structure was considered an old sample. The color was brown-orange and had settled considerably more at the bottom sediments, therefore more concentrated. As a result, it appeared flat and spread on a thin layer within the water. The biofilm appeared liquified.

These differences were also observed microscopically (Figure 1 and 2). Fresh samples consisted of biogenic sheaths of iron oxides in great quantity. These tubes in contact to each

other gave to the microbial mat a greater volume. The microbial activity was also superior. Chemical iron oxides were also present in older samples. It appeared as a crusty material on the microscope pictures. This material in contact with the biogenic sheaths suggested that the microbial activity was lower at these sites. Indeed, the aqueous iron oxide, released from the sediments, was not directly used by the bacteria at the oxic-anoxic limit (due to low bacterial activity) and oxidized in the presence of oxygen.

The characteristics of the material collected were different between fresh and old samples. Fresh samples were characterized by a low total iron concentration: 450.63 ± 17.73 mg total Fe/L average at Myron Lake and 431.67 ± 109.58 mg total Fe/L averaged between Spring Lake samples 1, 2, and 3. Due to the direct relationship, dry density values were also low for these samples: 1.42 ± 0.06 mg/L at Myron Lake and 1.23 ± 0.06 mg/L for SL1, SL2, and SL3. SL4 had different macroscopic and microscopic properties within the Spring Lake samples. It was characterized by 2412.08 ± 36.43 mg total Fe/L and a dry density of 6.91 ± 0.23 mg/L. These high values were specific to an old sample. Moose Creek samples averaged a total iron concentration of 3027.50 ± 107.35 mg/L and a dry density of 7.39 ± 0.12 mg/L. The Rainbow Lake sample had 2630 ± 130.41 mg total Fe/L and a dry density of 8.85 ± 0.22 mg/L.

**Table 1.** Chemical and physical characteristics of evaluated biogenic iron oxides.

| Sample | pH | Temp. (°C) | $PO_4^{3-}$ (mg/L) | $Fe^{2+}$ (mg/L) | Tot. Fe (mg/L) | Dry wt. (g/L) |
|---|---|---|---|---|---|---|
| **M10A** | 7.8 | 8 | 0.29 | 21.9 ± 2.23 | 2678 ± 92.2 | 7.98 ± 0.11 |
| **M10B** | 7.1 | 8 | 0.66 | 14.7 ± 1.01 | 3378 ± 123 | 6.08 ± 0.13 |
| **M12** | 7.9 | 9 | 0.24 | 0.61 ± 0.47 | ND | ND |
| **RL1** | 7.8 | 13 | 1.22 | 0.38 ± 0.01 | 2630 ± 130 | 8.85 ± 0.22 |
| **YK1** | 7.6 | 12 | 0.38 | 3.29 ±0.06 | 455 ± 35 | 0.845 ± 0.12 |
| **YK2** | 7.1 | 14 | 0.45 | 4.27 ± 0.27 | 222 ± 5.05 | 0.317 ± 0.026 |
| **YK3** | 7.5 | 12 | 0.27 | 3.66 ± 0.03 | 551 ± 17.5 | 1.37 ± 0.036 |
| **YK4** | 7.2 | 18 | 0.84 | 1.77 ± 0.65 | 575 ± 13.4 | 3.15 ± 0.064 |
| **SL1** | 7.5 | 13 | 1.25 | 11.4 ± 0.87 | 391 ± 111 | 0.843 ± 0.025 |
| **SL2** | 7.1 | 14 | 1.27 | 6.30 ± 1.21 | 449 ± 28.1 | 1.19 ± 0.074 |
| **SL3** | 7.2 | 15 | 1.76 | 1.53 ± 0.15 | 455 ± 177 | 1.67 ± 0.093 |
| **SL4** | 7.0 | 16 | 0.57 | 1.16 ± 0.01 | 2412 ± 36.4 | 6.91 ± 0.226 |

ND – Not determined because no pre-wash sample was reserved for experimentation.

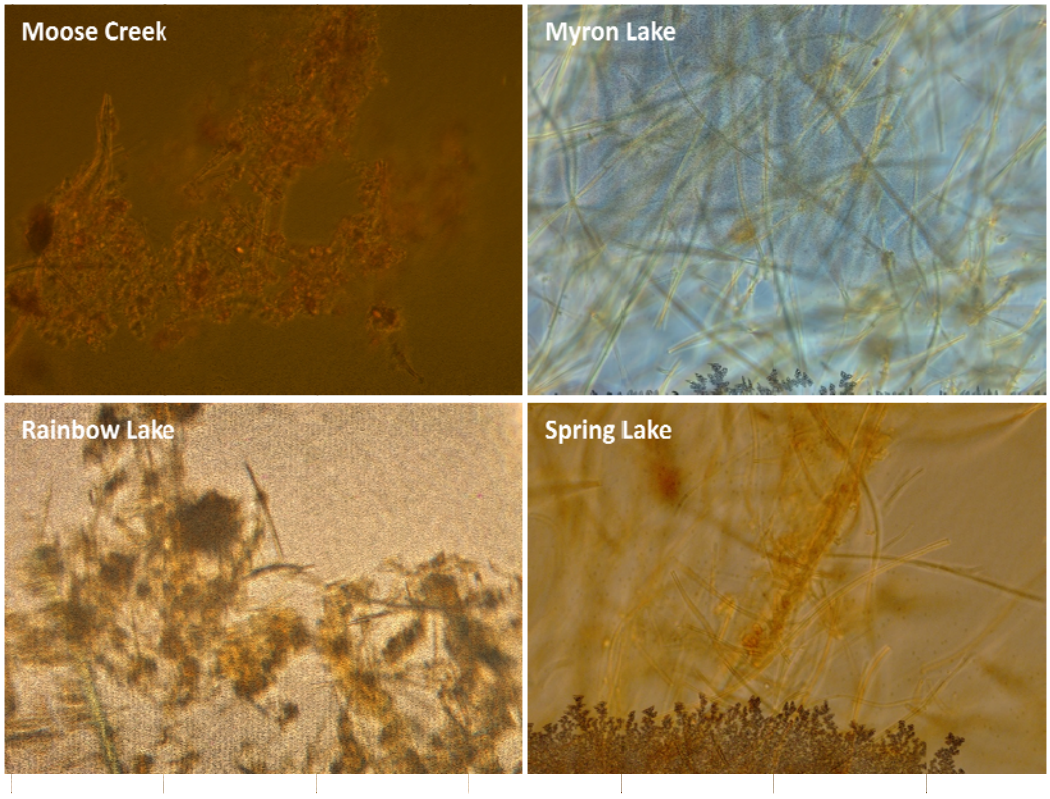


**Figure 1.** Iron oxide sheaths characteristic of *Leptothrix ochracea* that dominated the four sampling sites. Shown here are M10A, YK2, RL1, and SL2. The left hand samples (M10A and RL1) were old as illustrated by the presence of both smooth regular sheaths and crusty irregular shaped iron oxides. Samples YK2 and SL2 were fresh highlighted by the high concentration of biogenic iron oxide sheaths.

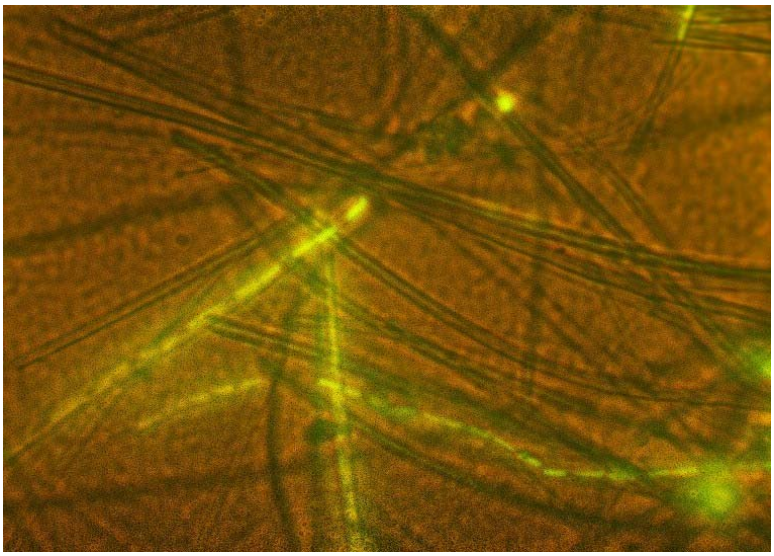

**Figure 2**. Iron oxide sheaths of *Leptothrix ochracea* from Moose Creek. This fluorescent picture showed the present of iron oxidizing bacteria within iron sheaths (yellow rods). These bacteria were present at the four sampling sites proof that the material is biogenic.

All samples showed potential for phosphorus adsorption and produced expected isotherms (Figure 3), which were consistent with previous studies (Zeng et al., 2004). The fitting equations used gave Pearson $R^2$ values between 0.939 and 0.972 for Langmuir, 0.814 and 0.895 for Freundlich, 0.981 for Redlich-Peterson, 0.946 for Langmuir-Freundlich, and 0.955 and 0.977 for Temkin. The Langmuir equation appeared to provide a better fit than the Freundlich equation, suggesting that a maximum sorption occurred and biogenic iron oxides were saturated with phosphorus. Redlich-Peterson, Langmuir-Freundlich, or Temkin equations always had the best $R^2$ values, because the calculated theoretical values were more similar to experimental data. The fitting coefficients were calculated to trace these isotherms (Table 2). The two old samples were coherent and lower than the fresh samples. Sample RL1 had less sorption, with a $\Gamma_{max}$ value of 24.21 ± 0.69 mg P/ mg Fe. Sample M12 showed a maximum sorption capacity of 29.14 ± 0.63 mg P/ mg Fe. Sample SL1 showed a $\Gamma_{max}$ value of 36.04 ± 1.42 mg P/ mg Fe and 40.37 ± 1.42 mg P/ mg Fe for YK2. The Langmuir parameter b value was between 0.91 ± 0.12 and 1.08 ± 0.11 for every sample except for M12 parameter that was five times higher. The Freundlich fitting equation gave a n value almost identical for each sample; between 2.61 ± 0.20 and 3.69 ± 0.28. Sample M10A had the greatest $\Gamma_{Max}$ value (106.82 mg P/ mg Fe) within the Moose Creek group. However, this sample was not represented because it was an outlier in this group. The initial concentration of biogenic iron oxides was too high, and the isotherm produced was not accurate enough for data analysis.

These biogenic iron oxides were efficient phosphorus sorbents and the phosphate sorption compared favorably to previous studies that evaluated iron rich or iron-oxide containing substrates (Figure 4). Here, phosphorus sorption was normalized to total solids composition (Table 3) and all samples were represented. Previously studied iron substrates all showed

significant phosphorus removal; biogenic iron oxides from Moose Creek Reservoir (Rentz et al., 2008), iron oxide coated sand, engineered materials (gypsum and brick), pure iron oxides (goethite and akaganeite), and recycled materials (tailings and two juniper fibers). The average adsorption of all of these iron-based substrates was 59.29 ± 7.28 mg P/ mg Fe and 14.80 ± 4.11 mg P/ mg solids. Thus, iron oxides are competitive substrates for phosphorus removal when compared to other substrates (Johansson Westholm, 2006).

Iron oxides possess different capabilities for phosphorus sorption. Biogenic substrates adsorbed more than three times the phosphorus than chemical iron oxides, thus representing a substrate of choice for phosphorus removal. The only Moose Creek sample studied for total solids normalization was consistent with the previous samples. The maximum sorption was 35.84 ± 17.48 mg P/ mg solids for Moose Creek sample. This value was the second highest. Samples M10A, YK1 (24.51 ± 1.22 mg P/ mg solids), YK2 (27.97 ± 0.98 mg P/ mg solids), YK3 (22.07 ± 1.67 mg P/ mg solids) were all above the highest value reported for engineered materials and recycled materials, which contained primarily iron oxides. Iron containing substrates (sand) resulted in a low sorption compared to other substrates. When both old and fresh samples were compared to synthetic iron oxides, a greater adsorption capacity was highlighted. On average, biogenic iron oxides adsorbed 19.02 mg P/g solids against 6.27 mg P/g solids for synthetic or chemical substrates referenced here.

Iron oxide characteristics determined the potential of phosphorus sorption. When normalized to total iron content, fresh samples had a higher sorbed phosphorus equilibrium concentration. The capacity of straight and active iron oxide sheaths to capture phosphate molecules was greater. Further studies should investigate biogenic iron oxide surface area. A relationship could be found between the sorbing capacity and the actual surface area of the fresh

and older sample. A number of environmental factors determine the surface area characteristics. The surface area interacted with gaseous and soluble species as well as bacterial cells (Cornell, 2006). Crusty iron oxides in contact with biogenic sheath might reduce the overall iron oxide loading capacity. It was likely that the surface area sorption potential varies dramatically from site to site. Interestingly, the relation was inversed when the phosphate loading was normalized to total solid content. Here, older samples had a greater capacity of sorption. This resulted in the fact that old sample iron oxides were much more concentrated. These samples had both biogenic and chemical iron oxides involved in the sorption processes.

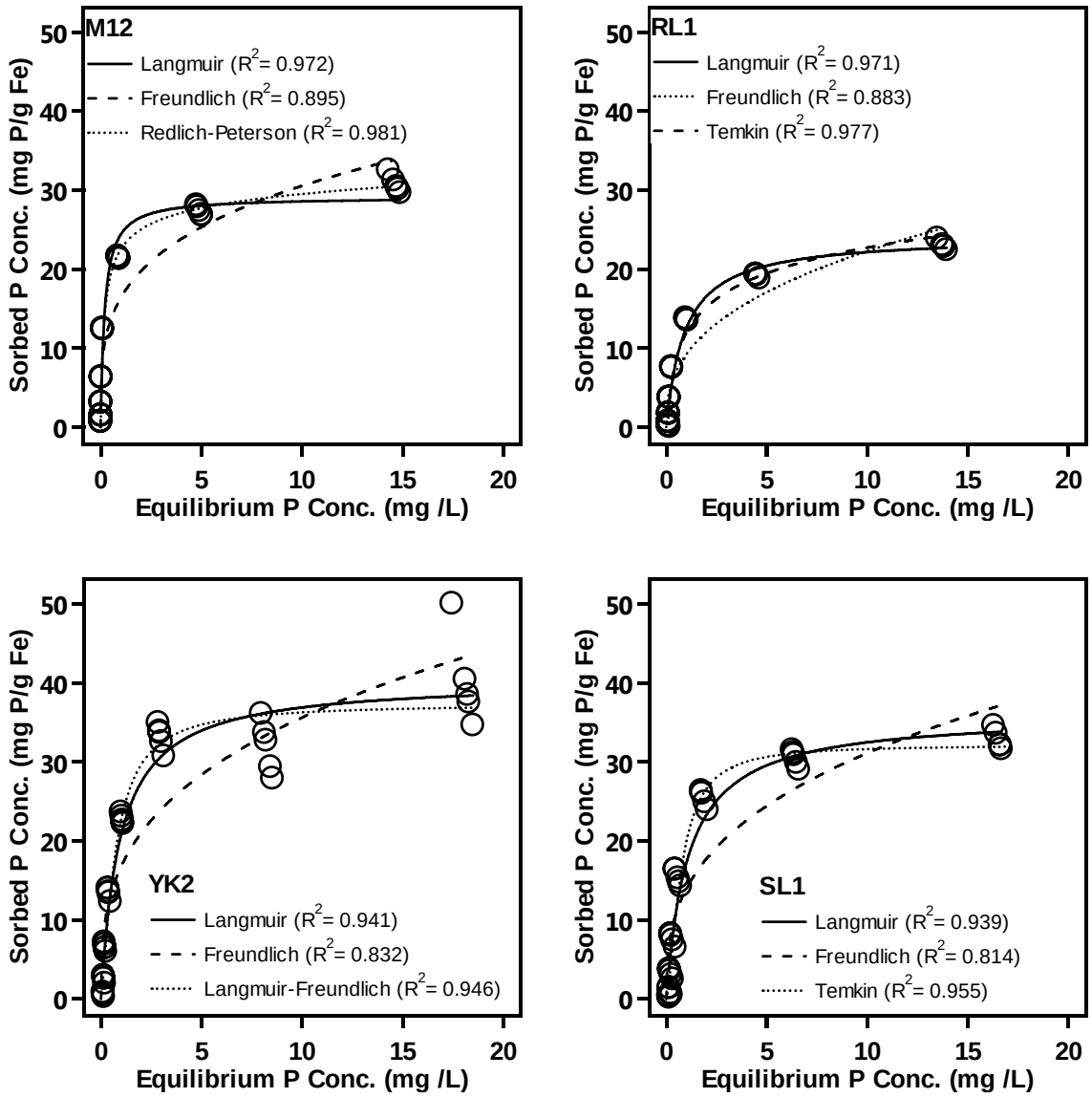


**Figure 3.** Representative isotherms showing phosphorus sorption to biogenic iron oxides. Langmuir and Freundlich equations were fit using non-linear regression (Igor Pro). The equation that gave the greatest Pearson value was also represented. Coefficients are summarized in Table 2. All five replicates are shown for each initial phosphate concentration. All samples shown in appendix A.

**Table 2.** Isotherm coefficients for biogenic iron oxide/phosphorus systems. Values reported with 95% confidence interval. M (Moose Creek), YK (Yakima), RL (Rainbow Lake), and SL (Spring Lake). All data represented in appendix A.

| | *Langmuir* | | | |
|---|---|---|---|---|
| | **$r_{Max\ Fe}$ (mg/g)** | **$r_{Max\ Solids}$ (mg/g)** | **b** | **Pearson $R^2$** |
| M10A | 106.82 ± 52.10 | 35.84 ± 17.48 | 0.12 ± 0.07 | 0.971 |
| M12 | 29.14 ± 0.63 | ND | 5.17 ± 0.55 | 0.972 |
| YK1 | 45.79 ± 2.28 | 24.51 ± 1.22 | 0.92 ± 0.15 | 0.914 |
| YK2 | 40.37 ± 1.42 | 27.97 ± 0.98 | 1.06 ± 0.14 | 0.941 |
| YK3 | 54.86 ± 4.15 | 22.07 ± 1.67 | 0.52 ± 0.10 | 0.886 |
| YK4 | 43.19 ± 3.14 | 7.88 ± 0.57 | 0.73 ± 0.14 | 0.88 |
| RL1 | 24.21 ± 0.69 | 7.19 ± 0.21 | 1.08 ± 0.11 | 0.971 |
| SL1 | 36.04 ± 1.42 | 16.77 ± 0.66 | 0.91 ± 0.12 | 0.939 |
| SL2 | 27.05 ± 0.96 | 10.21 ± 0.36 | 2.13 ± 0.31 | 0.916 |
| SL3 | 33.96 ± 1.77 | 9.25 ± 0.48 | 1.02 ± 0.18 | 0.884 |
| SL4 | 29.83 ± 1.65 | 10.41 ± 0.58 | 1.05 ± 0.18 | 0.903 |
| | *Freundlich* | | | |
| | **$K_{f\ Fe}$ (mg/g)** | **$K_{f\ Solids}$ (mg/g)** | **n** | **Pearson $R^2$** |
| M10A | 10.77 ± 0.33 | 3.61 ± 0.11 | 1.02 ± 0.05 | 0.959 |
| M12 | 16.38 ± 0.79 | ND | 3.69 ± 0.28 | 0.895 |
| YK1 | 16.83 ± 1.42 | 9.01 ± 0.76 | 2.62 ± 0.27 | 0.806 |
| YK2 | 16.76 ± 1.20 | 11.61 ± 0.83 | 3.05 ± 0.29 | 0.832 |
| YK3 | 16.12 ± 1.55 | 6.49 ± 0.62 | 2.15 ± 0.22 | 0.903 |
| YK4 | 14.06 ± 1.21 | 2.57 ± 0.22 | 2.12 ± 0.21 | 0.908 |
| RL1 | 9.24 ± 0.60 | 2.75 ± 0.18 | 2.61 ± 0.20 | 0.883 |
| SL1 | 13.95 ± 1.11 | 6.49 ± 0.52 | 2.87 ± 0.29 | 0.814 |
| SL2 | 14.15 ± 1.02 | 5.34 ± 0.39 | 4.04 ± 0.53 | 0.714 |
| SL3 | 13.98 ± 1.28 | 3.81 ± 0.35 | 3.11 ± 0.40 | 0.725 |
| SL4 | 1.22 ± 0.92 | 0.43 ± 0.32 | 2.52 ± 0.25 | 0.803 |

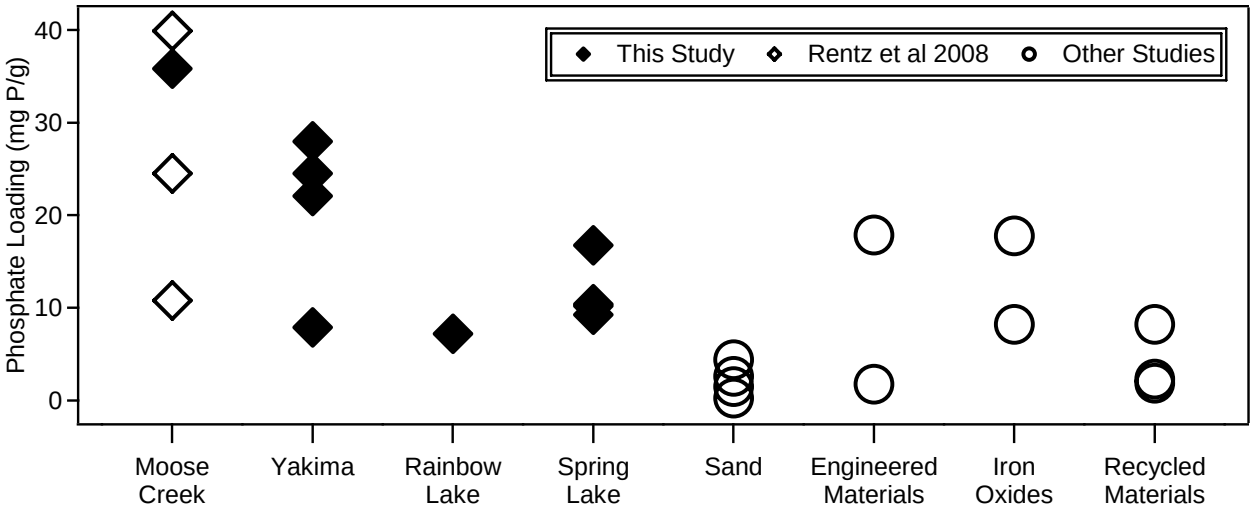


**Figure 4.** Maximum phosphate loadings (Langmuir ΓMax Solids) for various iron rich substrates. Within the Moose Creek group, only M10A was shown because the total solids were not evaluated for M12 and M10B. The values represented by the circles were either synthetic or natural sorbents investigated in previous studies. Sand substrates were determined from Boujelben et al. (2008), Arias et al. (2006), and Del Bubba et al. (2003). Engineered Materials were gypsum and brick, respectively, issued from Bastin et al. (1999) and Boujelben et al. (2008). Iron oxides were pure materials; goethite and akaganeite studied in Chitraker et al. (2006). Recycled materials were tailings from Zeng et al. (2004) and juniper fiber (precipitated acid mine drainage onto plant fibers) from Han et al. (2005).

All samples showed that iron oxide sorption potential was dramatically increased when pH was reduced (Figure 5). The general pattern of the pH adjusted batch equilibrium was that the lower the pH, the greater the sorption. Phosphorus sorptions were also greater when investigating fresh samples. Maximum sorption values were 69.68 mg P/ mg Fe and 95.17 mg P/ mg Fe for SL1 and YK2, respectively, at the lowest pH (5.71 and 5.48, respectively). Older sample maximum sorption was 31.41 mg P/ mg Fe RL1 and 39.37 mg P/ mg Fe for M12. The amplitude between the lowest and the highest sorption was then greater for fresh samples.

The range of pH values was reduced during the batch equilibrium experiment. For sample SL1, the pH ranged from 3.27 to 9.22. After the batch equilibrium, the pH ranged from 5.70 to 9.08. Sample YK2 ranged from 2.92 to 8.96 before batch equilibrium and ended at 5.48 to 9.17. These fresh samples started and ended with a wider interval of pH in comparison with the older samples. Sample M12 ranged from 4.26 to 7.37 and ended from 6.4 to 7.62. Sample RL1 ranged from 4.91 to 7.32 and ended from 6.71 to 7.98.

The liaison between iron oxides and phosphorus was impacted in pH-enhanced adsorption. When acid was added to reaction vials, quantities of $H^+$ ions were increased in solution. These positive ions were attracted to the surface of the iron oxides and bind to the negatively charged hydroxyl group linked to the iron atom. The net surface charge of the iron oxide was then positive (Figure 6). As a result, it was likely that phosphate anions bind to the iron oxide. This linkage would be electrostatic and result from Van der Waals forces. In contrast, when base was added to the reaction tubes to increase the pH, hydroxyl ions are increased in solution. These anions would catch the protons at the iron oxide surface. The net surface charge was then negative and cations are likely adsorbed. It explains the fact that phosphorus removal was greater at a lower pH.

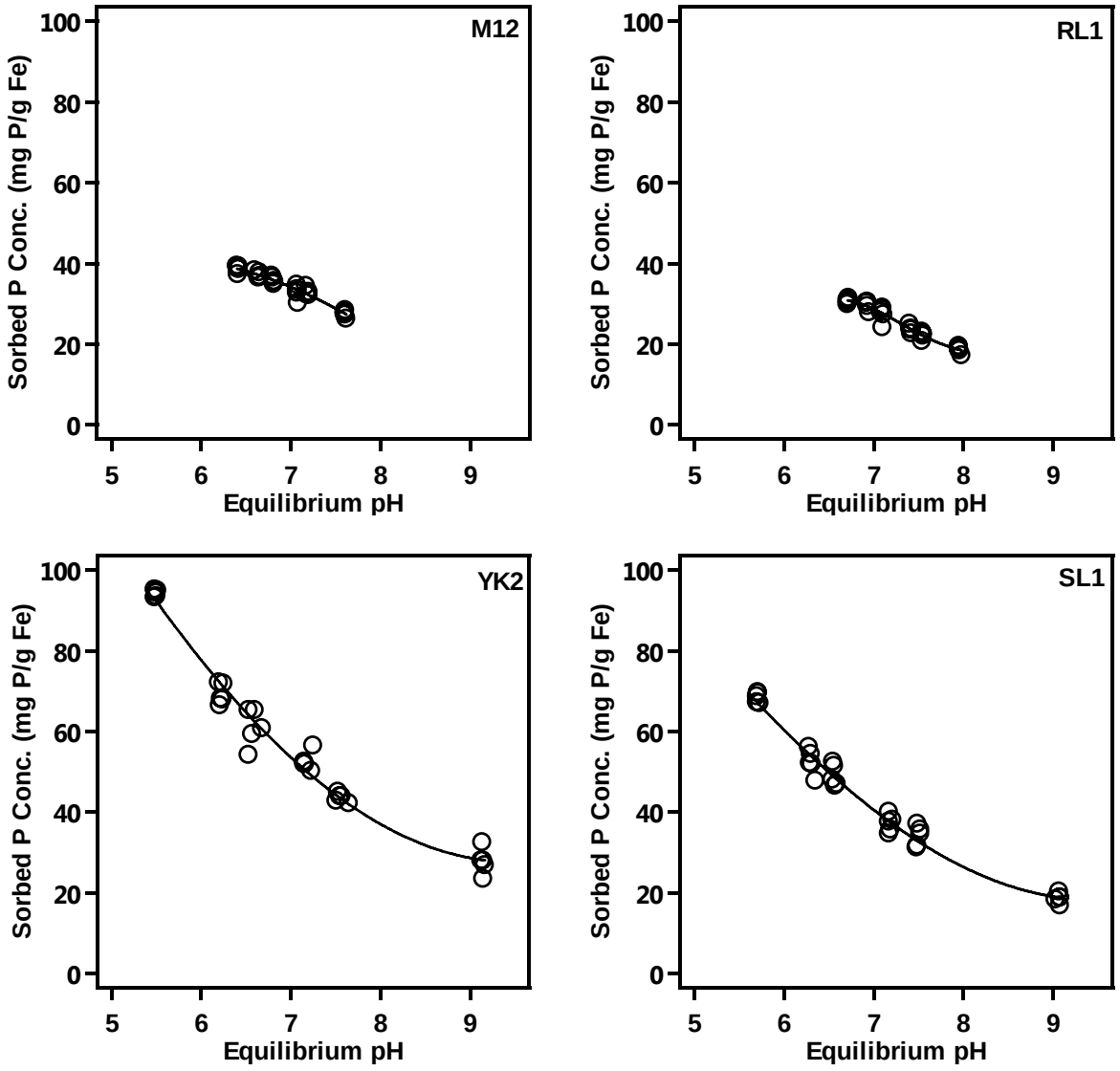


**Figure 5.** Impact of pH on phosphorus sorption to biogenic iron oxides. All five replicates shown for each initial pH. One sample of each site was represented. Selection of samples was made to be pertinent with Figure 3. M12 (Moose Creek), RL1 (Rainbow Lake), YK2 (Myron Lake in Yakima,WA), and SL (Spring Lake) were represented. All samples shown in appendix A.

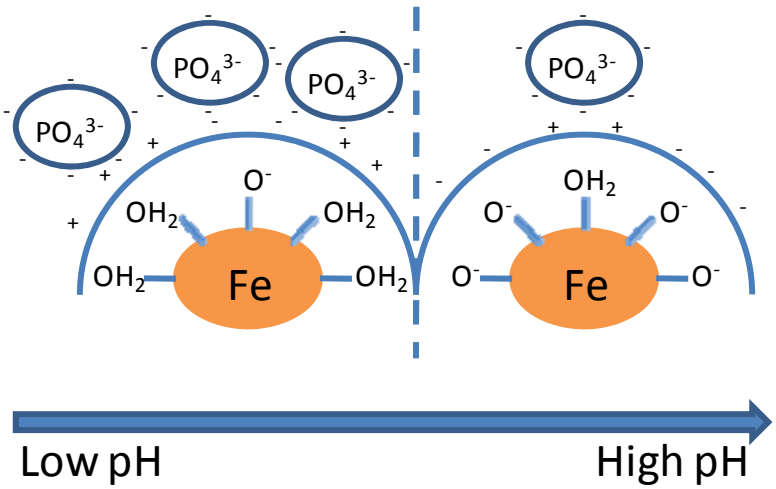


**Figure 6.** Chemical impact of pH variation on phosphate adsorption potential. Phosphate net surface charge was negatively charged. When the pH was lower, the $H^+$ cation was in excess in solution. It bond to the hydroxyl group of the iron oxide, which results in a more positive net surface charge. Phosphate anions electrostatically (Van der Waals forces) bind the iron oxide. At higher pH, hydroxide ions are in excess and bind protons from the hydroxyl group of the iron oxide to form water molecule. This results in a negative net surface charge to which a few phosphate anions are bound. Hydroxyl groups linked to the iron oxides are not representative of the iron oxides from *Leptothrix ochacea*.

Previous studies have also showed that pH control was required for optimal phosphorus adsorption. Phosphate adsorption on iron oxide tailings varied from 8.6 mg P/g solids at low pH and 4.6 mg P/g solids at high pH (Zeng et al., 2004). Adsorption was thus increased by 87% upon pH variation (from 9.5 to 3.2). For biogenic iron oxides, the pH intervals were smaller for all samples. However, a dramatic increase was seen for fresh materials (315% for SL1 and 307% for YK2). Phosphorus adsorption was 50% and 78% greater at lower pH for M12 and RL1, respectively.

Phosphorus recycling was limited for our biogenic iron oxides. Phosphorus release potential appeared low for all chemicals used except the basic solution (Figure 7). DI water showed the second best phosphorus release potential with a 40.60% average for the four samples. The KCL solution had an average potential of 30.37% and the saline solution 20.23%.

Differences in release potential were observed between the different chemicals used. Different reactions to the chemicals were also noticed from site to site. Sample M14 had the lowest release potential with an average of 6.08% between the three chemicals used (DI water, KCl, and NaCl). Samples RL3 and SL5 reacted similarly with averages of 34.64% and 32.13%, respectively. Finally, the greatest reaction was observed with sample YK5, with an averaged potential of 48.76%, yet phosphorus was not released from YK5 well with sodium hydroxide. The literature demonstrated that the adsorption process of phosphorus on iron oxides is not totally reversible, thus limiting the potential for phosphorus recycling (Zeng et al., 2004; Arias et al., 2006). Zeng et al. showed a desorbability of 13 to 14% using a solution of KCL regarding the initial phosphorus concentration. Our study revealed that the desorbability of biogenic iron oxides was two times higher than the potential of iron oxides tailings, although it may be too low in order to use in an engineered process.

A dramatic gap was observed between the release potential of the sodium hydroxide solution and the other solutions. This confirmed the fact that hydroxyl groups ($HO^-$) were involved in phosphorus detaching from the iron oxides. The sodium hydroxide solution provided large amounts of hydroxyl groups in solution. These likely bound protons from the $OH_2$ group at the iron oxide surface to form a water molecule (Figure 6). This capture resulted in a negative net surface charge on the iron oxide not favorable to keeping the phosphate molecule bound. As the phosphate linkage was a weak electrostatic bond, the sorption of phosphorus was reversible and phosphorus could be released. This phenomenon follows the same trends as the phosphoric acid dissociation into phosphate and a proton (Equation 1).

$$HPO_4^{2-} \Leftrightarrow PO_4^{3-} + H^+ \quad (6)$$

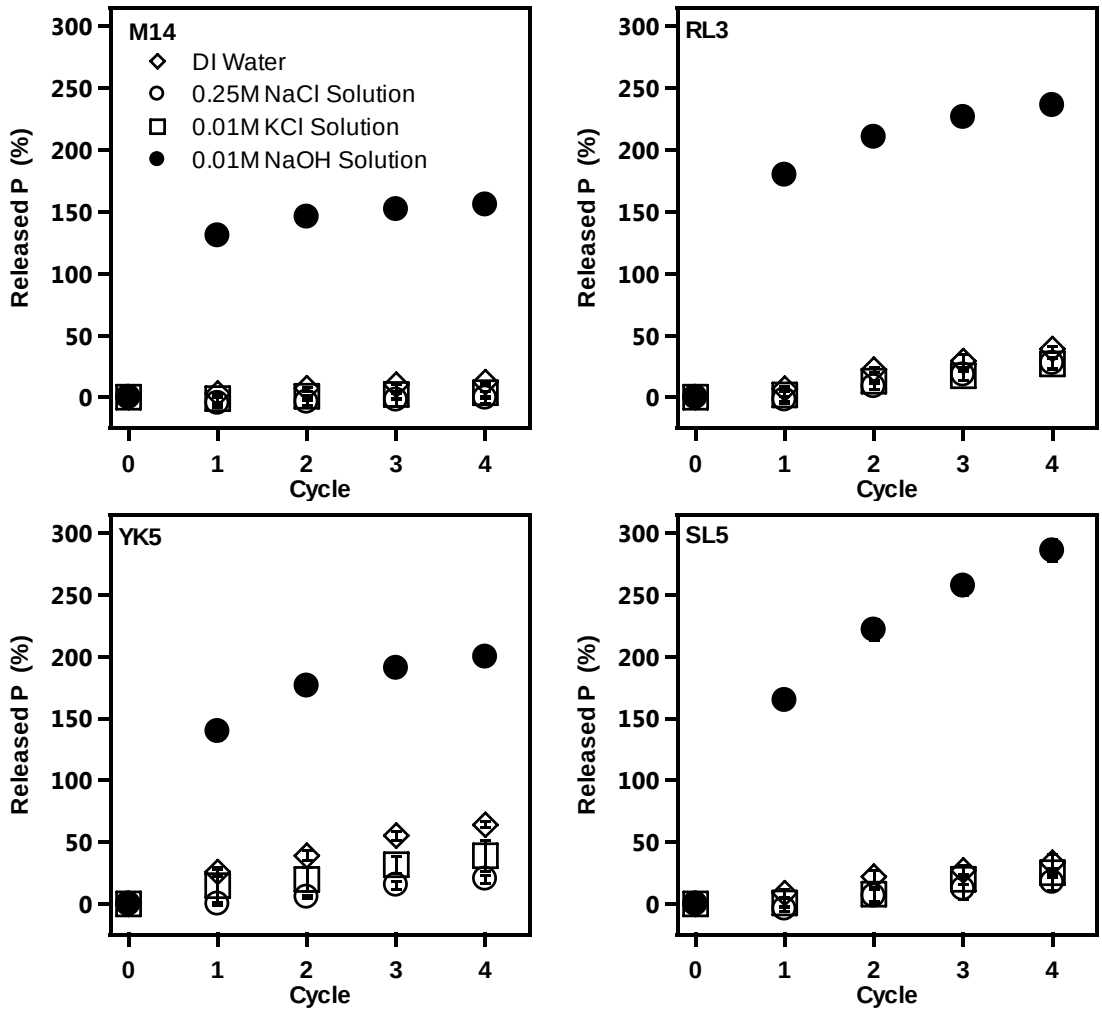


**Figure 7.** Phosphorus desorption from biogenic iron oxides facilitated by various solutions. Cycle 0 represented the first batch equilibrium run. Cycle 0 was to adsorb phosphorus prior to desorption. Cycle 1 to cycle 4 were the desorption cycles. A cycle was 24 hours of batch equilibrium. Between each cycle 30mL of supernatant was removed in each tube and replaced by different chemicals. The amount of phosphorus adsorbed at cycle 0 represented 100%. Negative percentages reflected the fact that adsorption was still occurring during the first cycles for some chemicals. Error bars represent the standard deviations from the mean of four replicates. Samples SL5 and RL3 had only three replicates for cycle 3 and cycle 4.

Basic conditions dramatically enhanced the phosphorus recycling potential. Samples from every site using all the different chemicals showed an increase in phosphorus concentration. In presence of sodium hydroxide, biogenic iron oxides yielded the highest amount of phosphorus for every sample. Sample M14 released a maximum of 159% of the first batch equilibrium adsorbed phosphorus. Sample RL3 released 239%, sample YK5 207%, and sample SL5 had the greatest recovery potential with a desorbability of 295%. A previous study showed that the increase of pH using a basic solution released most of the adsorbed phosphorus on goethite (Strauss et al., 1997). Our data suggested that most of the phosphorus was also released but other phenomena increased the phosphorus concentration within the reaction tubes. Biogenic iron oxides showed a better potential for recovery, suggesting again than the nature of the liaison between phosphate and substrate was favorable for recycling. In addition, our results suggested that a single desorption cycle was enough to release more phosphorus than what was previously adsorbed. After one cycle, sample M14 had a desorbability of 133%, 188% for RL3, 147% for YK5, and 172% for SL5. This represented 84%, 78% 70%, and 58% of the total desorbed phosphorus after four cycles for M14, RL3, YK5, and SL5, respectively.

pH variation can not be the only explanation for such great phosphorus release. The averaged maximal release of all replicates from all sites at cycle 4 was 213% using NaOH. On average, about two times more phosphorus was released than what was adsorbed during cycle 0. This supplementary phosphorus can either come from in-situ already sorbed phosphorus on the iron oxides or intra-cellular phosphorus released due to cell disruption. NaOH had the capacity to release most of the phosphorus from both sources. Concerning the other chemicals (Di, NaCL, and KCl), it was not possible to conclude the origin of the released phosphorus. Further studies need to demonstrate whether the recovered phosphorus was cellular or previously sorbed.

The oldest sample from Moose Creek showed the lowest capacity to recover phosphorus. Fresher samples had a higher releasing potential. Sample RL3 was actually fresher than the other samples from the same site and was similar to sample SL5. Those two had similar releasing behavior with chemicals other than sodium hydroxide (DI water, NaCl solution, and KCl solution average of 34.64% for RL3 and 32.13% for SL5). The freshest sample YK5 had the highest release activity on chemicals other than sodium hydroxide. This high capacity to release phosphorus was due to: (1) high cellular activity leading to large amounts of phosphorus released by cell disruption, and (2) the greater surface area of fresh iron oxides and the potential to release previously sorbed phosphorus.

# CHAPTER FOUR
# ENGINEERED APPLICATIONS

The *Leptothrix* group of bacteria is well known and growth conditions are already fully described (Van Veen et al., 1978). The growth cycle of bacteria from the group *Leptothrix* has already been outlined. Slow growth under minimal aeration at 20 to 25ºC on a salts-vitamin-pyruvate medium is feasible for *Leptothrix discophora* (Emerson and Ghiorse, 1992). The same experiments should be conducted with these laboratory-growing bacteria, which are iron oxide sheath-forming as well as *Leptothrix ochracea*. Phosphorus adsorption potential is likely to be the same between these two bacterial sheaths. As these two bacteria are similar phylogenetically and morphologically, *Leptothrix ochracea* growth should also be investigated under the previous growth conditions.

A limitation to chemical synthesized iron oxide is that they can not be regenerated within a filter. This leads to substrate replenishment, which is often expensive. Biogenic iron oxides present the potential of self-regeneration. During a standard filtration process, the phosphorus removal potential is self-regenerated. When used iron oxide is removed from the filter for phosphorus recycling, the microbial growth will produce iron oxides that replenish capacity. Use in a microbial filter requires the biogenic iron oxide sheath of *Leptothrix ochracea* to be kept at a certain pH range. As neutrophilic bacteria, they can not grow at pH below 6 or above 8. Ideal results would be found with a managed growth at pH 6, which results in already great increases in adsorption for samples SL1 and YK2 (Figure 5). Data show that even a small decrease in pH to reach 6 leads to significant sorption improvement.

In a hypothetical wastewater treatment process using biogenic iron oxide filters, a high pH tank would follow a low pH tank. In the low pH tank (pH=6), bacteria a grown and the

biogenic iron oxides formed are used to adsorb the phosphate in the wastewater. After 24 hours, the sludge constituted of the sheaths that bounded the phosphorus are extracted from the wastewater and introduced to a second high pH tank (pH=12). Here, phosphorus is recovered in large amounts after a 24 hours cycle. Even though a treatment using sodium hydroxide might be expensive, the use of a cheaper base to increase the pH should theoretically give similar results.

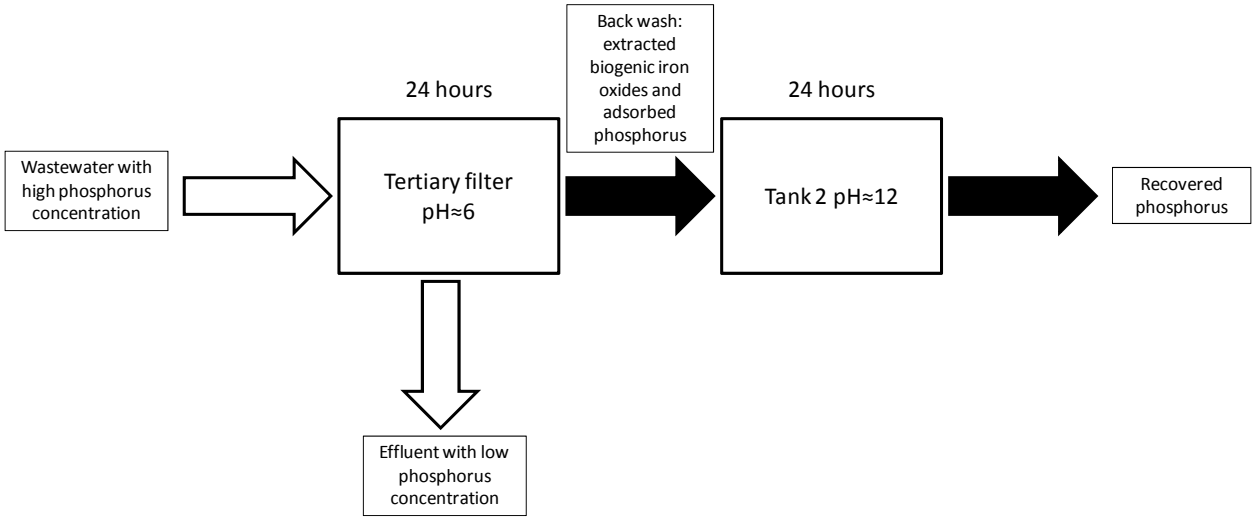


**Figure 8.** Simplified engineered wastewater treatment for phosphorus removal and recovery.

# CHAPTER FIVE
## CONCLUSIONS

Phosphate sorption characteristics to biogenic iron oxides and release were evaluated using batch equilibrium experiments. Our results support the following conclusions:

- Adsorption isotherms followed the Langmuir model.
- Maximum adsorption using biogenic iron oxides normalized to total solids was consistent with our previous study (Rentz et al, 2008) and was greater than reported for iron rich or iron oxide containing substrates.
- Phosphorus adsorption was dramatically enhanced at lower pH. A reduction to pH 6 revealed a significant phosphorus adsorption increase (1.5 to 2 fold increase).
- Phosphorus was recovered using sodium hydroxide, sodium chloride, potassium chloride, and DI water. The use of a base that increased the pH released both experimental and in-situ adsorbed phosphorus. It also releases intracellular phosphorus by cell disruption. Other chemicals released less phosphate and it is not possible to tell the origin of this phosphate.
- Fresh samples gave the best result in pH enhanced phosphorus adsorption and phosphorus release with every chemical used.
- Further studies should thus investigate fresh versus old samples differences such as composition, organic matter, or iron oxides surface area.
- pH 6 had the best potential for allowing both bacterial life and enhanced phosphorus removal in an engineered process.

# APPENDIX A
# SUPPLEMENTARY DATA

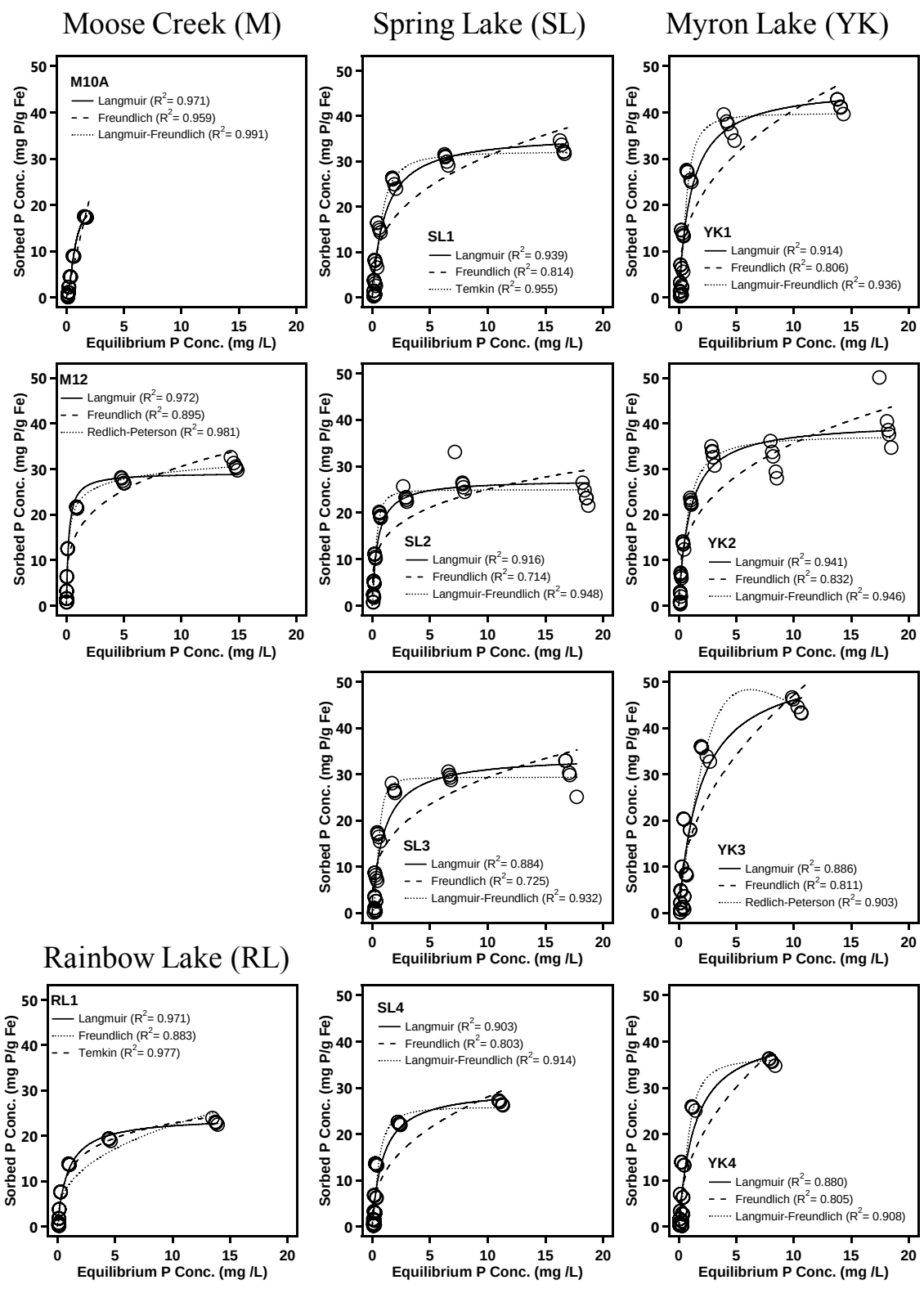


**Figure 9.** Isotherms showing phosphorus sorption to biogenic iron oxides. All samples are represented.

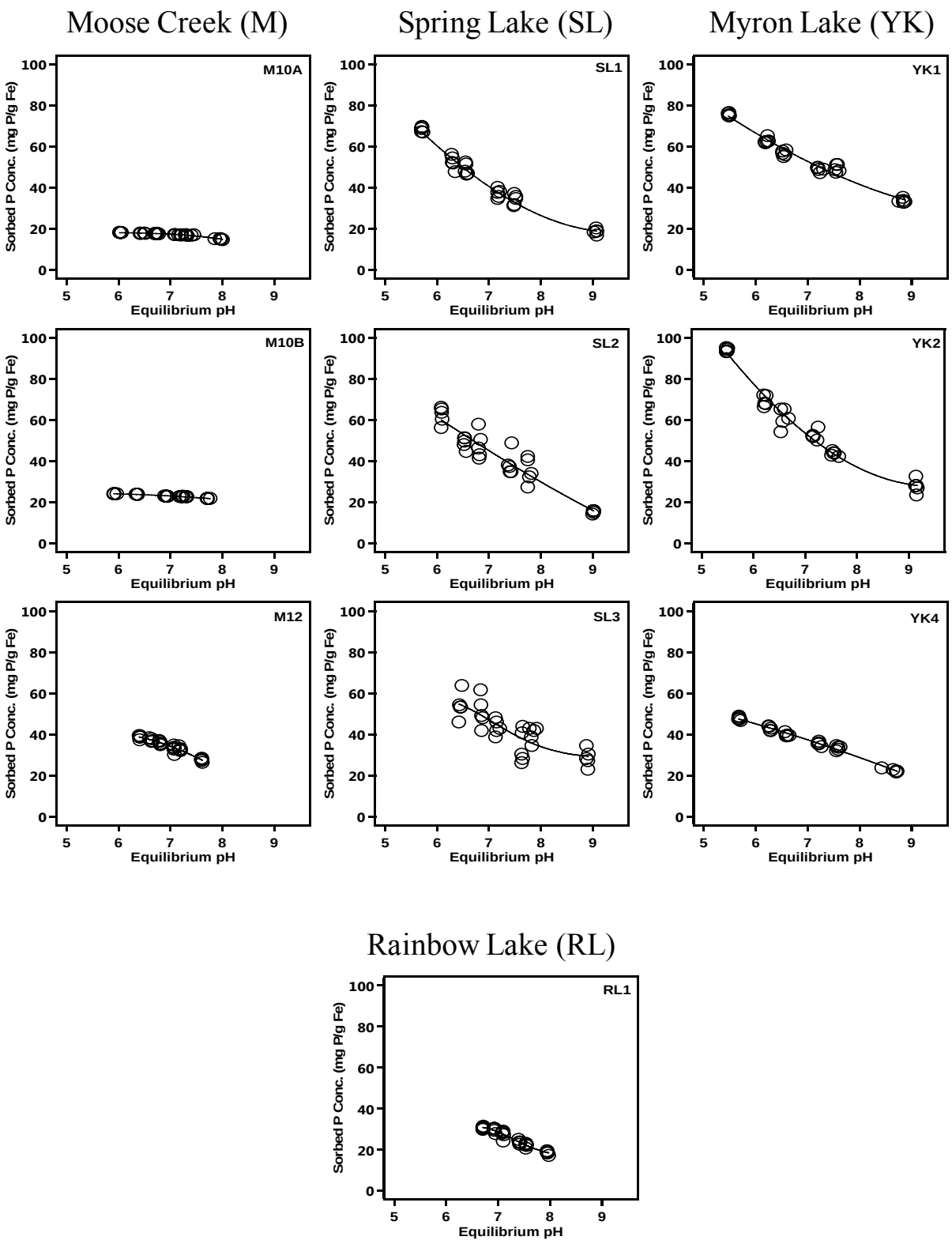


**Figure 10.** Impact of pH on phosphorus sorption to biogenic iron oxides. All samples are represented.

| Sample | | M10A | M12 | SL1 | SL2 | SL3 | SL4 | RL1 | YK1 | YK2 | YK3 | YK4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Langmuir | $\Gamma_{Max}$ | 107 ± 52.1 | 29.1 ± 0.628 | 36.0 ± 1.42 | 27.0 ± 0.963 | 33.9 ± 1.77 | 29.8 ± 1.65 | 24.2 ± 0.690 | 45.8 ± 2.28 | 40.4 ± 1.42 | 54.9 ± 4.15 | 43.2 ± 3.14 |
| | b | 0.117 ± 0.066 | 5.17 ± 0.548 | 0.914 ± 0.123 | 2.13 ± 0.305 | 1.02 ± 0.180 | 1.05 ± 0.179 | 1.08 ± 0.107 | 0.917 ± 0.146 | 1.06 ± 0.139 | 0.524 ± 0.103 | 0.728 ± 0.136 |
| | Pearson $R^2$ | 0.971 | 0.972 | 0.939 | 0.916 | 0.884 | 0.903 | 0.971 | 0.914 | 0.941 | 0.886 | 0.88 |
| Freundlich | $K_f$ | 10.8 ±0.329 | 16.4 ± 0.788 | 13.9 ± 1.11 | 14.1 ± 1.02 | 13.9 ± 1.28 | 1.22 ± 0.919 | 9.24 ± 0.602 | 16.8 ± 1.42 | 16.8 ± 1.20 | 16.1 ± 1.55 | 14.1 ± 1.21 |
| | n | 1.02 ± 0.049 | 3.69 ± 0.282 | 2.87 ± 0.292 | 4.04 ± 0.53 | 3.11 ± 0.398 | 2.52 ± 0.250 | 2.61 ± 0.201 | 2.62 ± 0.265 | 3.05 ± 0.290 | 2.15 ± 0.222 | 2.12 ± 0.207 |
| | Pearson $R^2$ | 0.959 | 0.895 | 0.814 | 0.714 | 0.725 | 0.803 | 0.883 | 0.806 | 0.832 | 0.811 | 0.805 |
| Temkin | $A_1$ | 12.7 ± 0.308 | 20.2 ± 0.308 | 16.2 ± 0.602 | 15.5 ± 0.676 | 15.5 ± 0.825 | 14.6 ± 0.585 | 11.9 ± 0.218 | 20.9 ± 0.840 | 19.3 ± 0.631 | 20.4 ± 1.07 | 18.7 ± 0.982 |
| | $B_1$ | 6.41 ± 0.196 | 4.50 ± 0.196 | 7.15 ± 0.361 | 4.70 ± 0.359 | 6.81 ± 0.513 | 5.89 ± 0.330 | 4.68 ± 0.115 | 9.12 ± 0.500 | 7.75 ± 0.355 | 10.8 ± 0.741 | 7.97 ± 0.572 |
| | Pearson $R^2$ | 0.966 | 0.976 | 0.918 | 0.83 | 0.838 | 0.899 | 0.977 | 0.902 | 0.928 | 0.869 | 0.843 |
| Redlich-Peterson | $A_2$ | 12.4 ± 0.654 | 187 ± 21.9 | 27.2 ± 3.55 | 45.1 ± 5.41 | 26.5 ± 3.82 | 27.1 ± 4.60 | 25.5 ± 3.05 | 36.3 ± 5.49 | 39.9 ± 6.40 | 19.7 ± 3.13 | 21.6 ± 3.00 |
| | $B_2$ | 0.015 ± 0.037 | 7.31 ± 1.08 | 0.533 ± 0.160 | 1.23 ± 0.264 | 0.450 ± 0.159 | 0.696 ± 0.263 | 1.01 ± 0.232 | 0.579 ± 0.207 | 0.901 ± 0.271 | 0.075 ± 0.085 | 0.052 ± 0.116 |
| | n | 4.99 ± 4.49 | 0.931 ± 0.019 | 1.14 ± 0.071 | 1.14 ± 0.047 | 1.23 ± 0.090 | 1.12 ± 0.0981 | 1.02 ± 0.050 | 1.13 ± 0.093 | 1.038 ± 0.059 | 1.66 ± 0.415 | 2.06 ± 0.991 |
| | Pearson $R^2$ | 0.98 | 0.981 | 0.946 | 0.935 | 0.907 | 0.906 | 0.971 | 0.919 | 0.941 | 0.903 | 0.908 |
| Langmuir-Freundlich | $\Gamma_{Max}$ | 20.3 ± 0.746 | 30.5 ± 0.993 | 32.2 ± 1.05 | 24.9 ± 0.638 | 29.3 ± 0.937 | 25.9 ± 1.36 | 22.4 ± 0.673 | 39.8 ± 1.46 | 37.4 ± 1.36 | 46.8 ± 3.20 | 36.5 ± 2.02 |
| | b | 2.08 ± 0.310 | 3.19 ± 0.711 | 1.62 ± 0.317 | 7.52 ± 2.53 | 4.52 ± 1.92 | 2.75 ± 1.29 | 1.62 ± 0.256 | 2.36 ± 0.629 | 1.53 ± 0.296 | 0.741 ± 0.165 | 1.52 ± 0.400 |
| | n | 0.499 ± 0.028 | 1.18 ± 0.098 | 0.639 ± 0.070 | 0.544 ± 0.063 | 0.404 ± 0.069 | 0.626 ± 0.120 | 0.797 ± 0.062 | 0.542 ± 0.072 | 0.750 ± 0.086 | 0.654 ± 0.104 | 0.562 ± 0.078 |
| | Pearson $R^2$ | 0.991 | 0.974 | 0.955 | 0.948 | 0.932 | 0.914 | 0.974 | 0.936 | 0.946 | 0.901 | 0.908 |

**Figure 11.** Calculated isotherm coefficients. All results are represented.

# APPENDIX B
# LITERATURE REVIEW

### 1. Phosphorus uses

Phosphorus is a highly reactive atom that is never found as a free element on Earth. It binds to different atoms to form phosphorus compounds as hydrides, halides, oxides, sulfides, acids, phosphates (most widespread form of phosphorus in the environment), phosphides, organophosphorus, and organophosphates.

As a result, this chemical is omnipresent in both natural and synthetic chemistry. First of all, phosphorus is a nutrient, essential element for any form of life. It is a framework constituent of bones, teeth, nucleic acids, energy carriers (ATP, NADH), proteins, and lipids (Madigan and Martinko, 2006). This nutrient is also encountered in pharmaceuticals and cosmetics. Phosphorus was largely used in all kinds of detergents and cleaners but due to their highly negative impact on the environment, their use is being reduced (Hoffman and Bishop, 1994; Wind, 2007). The largest use of phosphorus is for fertilizers (87% in Western Europe). Fertilizers are non-point (diffuse) source that reach the surface water by run-off, erosion or drainage (Figure 12). Industry, detergents and human wastes are the main phosphorus point-sources (direct).

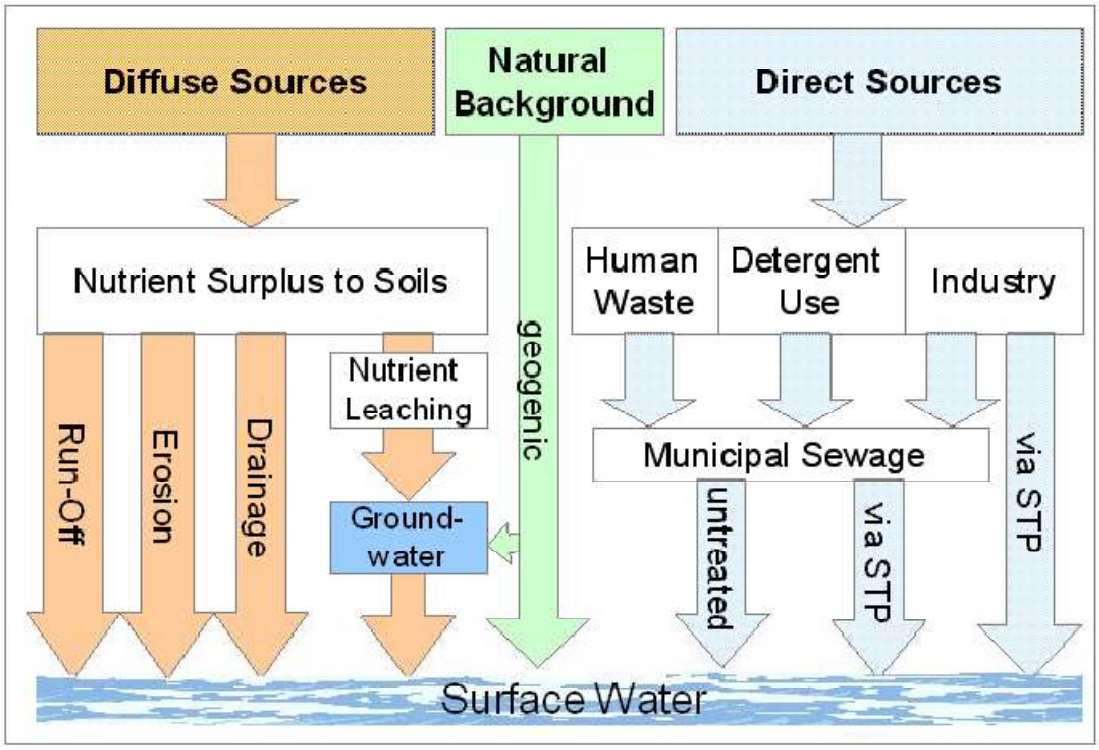


**Figure 12.** Repartition of phosphorus water into the surface water in Western Europe. STP stands for Sewage Treatment Plant (Wind, EWA, 2007).

The surface water receives both treated and untreated wastewater from point-sources and straight nutrients form non point-source. This leads to excess phosphorus, as well as nitrogen, available in lakes, rivers, and groundwater. In a water body, nutrients have the same functions as for agricultural uses, they act like fertilizers and enhance algal growth. This excessive algal development is a dramatic environment threat. In 1992, 19,155 acres of Washington State lake areas were affected by algal bloom (Department of ecology, 2004). This same study listed three environmental impacts of algal blooms:

- Loss of swimming, fishing, and aesthetic due to nuisance algal blooms, periphyton, and macrophyte growth.
- Loss of aquatic life due to dissolved oxygen depletion caused by excess algal and aquatic macrophyte respiration and decay.
- Loss of drinking water due to odor, clogging of filters, toxins.

**2. Phosphorus removal**

Efforts made to limit the inputs of phosphorus to water bodies are often unsuccessful due to the amount of input sources (Wind, 2007). As a result, different technologies have been developed to undergo the effect of high phosphorus concentration in water. These technologies are continually being refined. However, they present advantages and disadvantages that must be taken under account when designing a phosphorus removal process (Table3). Chemical precipitation and Enhanced Phosphorus Biological Removal (EPBR) are the most documented and used technologies (Morse et al., 1998).

- Chemical precipitation

Chemical precipitation was the first technology developed as an answer to the eutrophication problems. This physico-chemical reaction is accomplished by adding metal salts into the wastewater treatment process, primarily in the form of calcium, aluminum and iron. Phosphorus bonds the salt and forms an insoluble molecule. After a decanting process, the metallic phosphate is present in a sludge that is used for phosphate recovery. Calcium is a chemical difficult to use, while aluminum shows a great removal potential but is an expensive chemical. Thus, chemical precipitation using iron salts appears as the most attractive solution to phosphorus removal, although it often remains insufficient (Clark et al., 1997). This widespread process can be supplemented to other removal technologies and applied at different steps of the wastewater treatment process (Morse et al., 1998). Used during a primary, secondary, or tertiary treatment, this method offers high water quality.

**Table 3.** Phosphorus removal and recovery technologies. Adapted from Morse et al., 1998.

| Phosphorus removal technology | Recovery value | Technology advantages | Technology disadvantages |
|---|---|---|---|
| **Chemical precipitation** | Low: bound is too strong | Established technology, high potential, easy to implement | Low recyclability, sludge production, price extensive |
| **Enhanced Phosphorus Biological Removal (EPBR)** | Moderate: bound more recyclable | Established technology, no chemical used | Complex technology, sludge handling difficult |
| **Crystallisation** | High | Retrofitting, recyclability | Requires chemicals, process difficult |
| **Advanced chemical precipitation** | Low: possible but difficult to implement | Great potential | Requires chemicals, process difficult |
| **Ion exchange** | high (struvite) | High potential, use of struvite for agriculture | Requires chemicals, process difficult |
| **Magnetic** | Low | High potential | Requires chemicals, process difficult |
| **Phosphorus adsorbents** | Low | | Unproven technology |
| **Tertiary filtration** | None | Easy to use, proven technology | No useful product |
| **Sludge treatments** | High | Sludge value increased | Requires chemicals, process difficult |
| **Recovery from sludge ash** | High | High recovery | Undeveloped technology |

- Enhanced Phosphorus Biological Removal (EPBR)

EPBR is based on the selective growth of Polyphosphate Accumulating Organisms (PAO). This method consists of alternating anaerobic and aerobic incubation conditions for PAO growth in the wastewater. Under anaerobic environments, these organisms use organic matter and other carbon sources from the wastewater. The energy source comes from previously stocked intracellular polyphosphates by molecule cleavage. Released phosphates are expulsed from the cell into the wastewater. The energy generated is used to convert volatile fatty acids (VFAs) into stored poly-β-hydroxyalkanoates (PHAs) (Oehmen et al., 2007). In the following tank, under aerobic conditions, the previously stored PHAs are used as a carbon and energy source for uptake of large quantities of phosphates. Therefore, polyphosphates are synthesized and stored within the organism. The final step is to remove the biomass from the wastewater.

- Limitations

Although chemical precipitation is a simple process to implement, it remains an expensive and highly sludge productive process. The cost of the chemical precipitation is 15% of the total costs of exploitation against 1-2% for EPBR (GLS, 2006). As a result, chemical precipitation is not adapted to high capacity wastewater treatment plants whereas EPBR seems to be an adapted method. However, due to insufficient knowledge on microbial metabolism, environmental or wastewater factors are likely to disturb growth and therefore reduce efficiency (Morse et al, 1998). The process must be monitored, which increases costs. The plant configuration is complex and expensive. EPBR efficiency depends also on phosphorus load in wastewater and presents an overall efficiency between 50% and 60% against 80% for chemical precipitation. Using a coupled chemical and biological method enhances performances and

shows 7.5% of the total exploitation costs (GLS, 2006). Both methods present a low to moderate phosphorus recovery potential (Morse et al, 1998). Even though EPBR recovery potential is in theory higher than chemical precipitation, a lack of study in this field makes it difficult to assess for both agricultural and industrial use.

### 3. Phosphorus Recovery

Current focus is on phosphorus recovery for reuse in both agriculture and industry. Phosphorus originates from phosphate rock, a source that is currently depleting at high rate. Several technologies have been studied and demonstrate potential for phosphorus recycling. These technologies however are not well developed, resulting in difficult implementation and leading to expensive processes and poor recovery potential (Morse et al., 1998; de-Bashan et al., 2004). Literature indicated struvite as having the greatest potential for phosphorus recovery, since it forms spontaneously in wastewater treatment environments under specific conditions (Stratful et al., 2001). Sturvite is often higher quality than phosphate rock and sludge containing struvite from wastewater treatment can be directly applied to fields. Recovery of phosphorus from iron or aluminum oxides appears difficult because phosphorus binds tightly to these substrates.

### 4. Iron Cycle (Oxidation/Reduction)

Iron is the fourth most abundant element in the Earth crust. It is naturally present under two states of oxidation, Fe(II) (ferrous iron) and Fe(III) (ferric iron). Both chemical and biologic

reactions can transfer these cations from one to another in the process called iron cycle. Ferrous iron is oxidized into ferric iron, whereas ferric iron is reduced into ferrous iron. These reactions take place in marine and freshwater environments, such as lake sediments, aquifers, soils, wetlands, deep-sea vent environments and in man-made settings, like mine tailings impoundments (Fortin and Langley, 2005).

- Iron oxidation

The iron oxidation is known to occur naturally and spontaneously with oxygen. Otherwise, Ferrous-Oxidizing Bacteria (FeOB) are involved in the increase of iron oxidation rates. FeOB do not simply compete with the natural process, they also accelerate the oxidation rates up to 18% (Neubauer et al., 2007). *Leptothrix* or *Gallionella* are iron oxidizers that thrive under neutral pH conditions. This process occurs at the interface between the oxic and anoxic zone. Iron oxidizing bacteria influence Fe(II) oxidation kinetics at circumneutral pH and very low oxygen concentrations in the interface (Weiss et al., 2007). As ferrous iron oxides almost spontaneously with oxygen, bacteria use it as soon as it is released from the anoxic zone. *Sideroxydans paludicola* is shown to thrive under microaerobic conditions in the Fe(II) rich rhizosphere (Neubauer et al., 2007). This organism is contributing to the formation of iron oxides on the plant roots. The iron oxide produced forms insoluble ferric hydroxide $Fe(OH)_3$. The overall reaction of oxidation is:

$$Fe^{2+} + H^{+} + 0.25\ O_2 \rightarrow Fe^{3+} + 0.5\ H_2O \rightarrow\rightarrow Fe(OH)_3$$

At neutral pH, the reaction releases energy (E0'=0.77V). At lower pH, the reaction is less energetic but still releases energy (E0'=0.2V at pH=2).

Under acidic conditions, the $Fe^{2+}$ ion is stable and thus does not oxidize spontaneously. Acidophilic chemolithotrophs as *Acidithiobacillus ferrooxidans* are known to precede this reaction. *Leptosprillum ferroxidans* is known to oxidize ferrous iron as well. As this reaction is less energetic, large amounts of ferric irons have to be reduced to produce enough energy for microbial life.

Besides, a phylogenetically diverse group of bacteria is shown to gain energy from anaerobic iron oxidation assessing the following reaction (Weber et al., 2006).

$$HCO_3^- + 4\ Fe(II) + 10\ H_2O + h\nu \rightarrow CH_20 + 4\ Fe(OH)_3 + 7\ H^+$$

This oxidation utilizes light energy to fix $CO_2$ into biomass. No Achaea has been so far shown to assess that phototrophic reaction. However, various archaeal and bacterial genera are able to oxidize Fe(II) anaerobically in presence of nitrate. Thus, these organisms are suspected to be involved in both iron and nitrogen cycles. Even if that process has a minor impact on the terrestrial environment global iron cycles, it has been shown to have a significant local impact on iron redox cycling and mineral weathering.

- Iron reduction

The very ancient form of life rose 3.8 billon years ago from the hot, photochemical generation of Fe(III) and $H_2$, that were respectively electron acceptor and energy source (Vargas et al., 1998). Thus, iron respiration may have been the first form of microbial metabolism before

development of oxygen, nitrate and sulphate respiration (Weber et al., 2006). Under anoxic conditions, some iron oxides undergo iron reduction as a result of abiotic reactions or microbial anaerobic respiration. The reduction potential of the $Fe^{3+}/Fe^{2+}$ couple is lightly electropositive ($E_0$'=0.2V at pH=7). Ferric iron is an electron acceptor for energy metabolism of both chemoorganotrophic and chemolithotrophic bacteria in anaerobic respiration. Electrons travel through the electron transport chain that terminates in a ferric iron reductase system. This electron flow produces a proton motive force that generates ATP. *Shewanella putrefaciens*, *Geobacter*, *Geospirrillum*, and *Geovibrio* are known to be iron reducers (Madigan and Martinko, 2006). *S. putrefaciens* is a facultative anaerobe and substitutes Fe(III) for oxygen as a terminal electron acceptor to gain energy for growth and metabolism (Nealson and Saffarini, 1994). Then the same bacteria is able to use both natural organic matter (NOM) and Fe(III) as a terminal electron acceptor.

Dissimilatory ferric iron-reducing bacteria (DIRB) gain energy by coupling the oxidation of organic compounds or hydrogen to the reduction of ferric iron oxides (Straub et al., 2001). For instance, *Geobacter metallireducens* oxidize acetate with $Fe^{3+}$ as an electron acceptor:

$$CH_3COO^- + 8Fe^{3+} + 4H_20 \rightarrow 2HCO_3^- + 8Fe^{2+} + 9H^+$$

Humic coumpounds represent the majority of all organic matter, not only in soils but also in aquatic environments. Thus, it is the most probable energy source during all iron reduction processes (Straub et al., 2005) (Figure 13). Under anaerobic conditions, microorganisms can reduce NOM (as a terminal electron acceptor), that gives electrons available for iron reduction to

reduce $Fe^{2+}$ ions. Biogically mediated reduction of Fe(III) ions can be orders of magnitude faster than the abiotic reduction at circum neutral pH (Chen et al., 2003).

**Figure 13.** Biologically induced abiotic iron reduction. Dissimilatory iron-reducing bacteria use in their respiratory pathway organic matter, such as carbonhydrate, as the energy source. This lead to the fromation of carbon dioxide and the electron is used to the reduction of humic substances. The reduced humic substances are able to abiotically reduce Fe(III) to Fe(II) when they come into contact.

- Relation between iron oxidizing and iron reducing bacteria

As we have seen previously, in freshwater habitats, both parts of the iron cycle can occurs biotically or abiotically. FeOBs that oxidized Fe(II) into insoluble Fe(III) are likely to thrive at the interface between oxic and anoxic environments. Under these microaerobic conditions, the iron respiration leads to the formation of ferric iron. It can stay in suspension within water if it sticks to bacterial sheaths (*Leptothrix* or *gallionella*) or released as a free ions in water. It would likely sinks in the sediment anoxic zone where it would be reduced by DIRB either by direct respiration or by abiotic reduction. Then, the free soluble iron oxide (Fe(II)) is released and ready to approach the oxic zone to be once again oxidized (Figure 14).

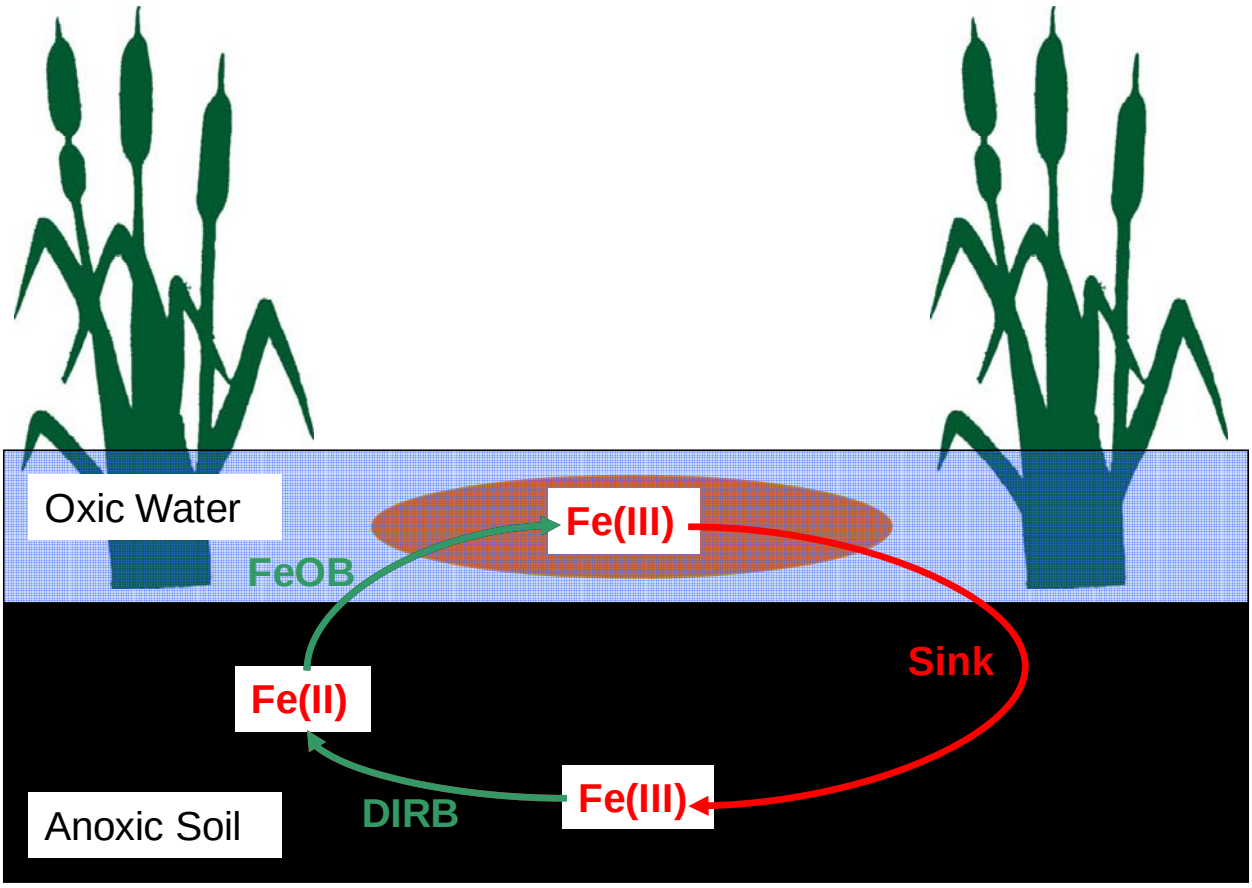


**Figure 14.** Circum neutral iron cycle within freshwater wetlands. The oxic freshwater is the receptor of the Fe(III) mat produced by FeOB at the interface between Oxic water and anoxic sediments. When the old iron oxide sinks in the sediments, it is reduced by DIRB to released in the anoxic-oxic interface Fe(II) available for a new cycle.

### 5. FeOB habitats and diversity

Table 4 references five different iron oxidizing bacteria studied by five scientific journal articles. For each bacteria, sampling site characteristics and laboratory growth requirements are compared. All referenced bacteria are freshwater growing bacteria growing at either circum-neutral pH (7.1 – 7.6) or acidic pH (4.0 – 4.5). However, under laboratory conditions, life can be maintained with a larger pH range (4.5 – 7.2) for most bacteria. Site water temperature is less than 14ºC. These organisms are thus psychrophiles but are grown in laboratory under warmer temperatures (13 - 37ºC). They all require a low oxygen concentration, close to anoxia, and use an opposite gradient between iron and oxygen for their respiratory pathway. Light appears to be an important for bacterial growth as well. Some of them need total obscurity whereas others grow under light alternation.

**Table 4.** Review of habitat characteristics requirements for iron oxidizing bacteria growth.

| | Reference | Site | | Laboratory | |
|---|---|---|---|---|---|
| ***Leptothrix ochracea*** | Emerson and Revsbech,1994 | **Location** | Iron Seep in Marselis Borg, Denmark. | **Isolation** | Gel-stabilized gradient method |
| | | | | **Oxygen** | Opposing gradient of $Fe^{2+}$ and $O_2$ |
| | | **Oxygen** | Anoxic Groundwater | **[Fe]** | 1200 nmol/h.$cm^3$ |
| | | **[Fe(II)]** | 250µM | **pH** | 6.9 - 7.2 |
| | | **Temperature** | 8 - 14°C | **Temperature** | 8 - 14 °C |
| | | **pH** | 7.1 - 7.6 | **Organic matter** | Natural organic matter used |
| | | **Flow** | < 2mL/s | **Note** | FeCl2 enhanced microbial activity |
| | Emerson and Moyer, 1997 | **Location** | Groundwater from a basement tile drain in East Lansing, rust colored floculant | **Isolation** | Agarose Medium |
| | | **[Fe(II)]** | 3 - 12 µM | **pH** | 6.2 - 6.4 |
| | | **Temperature** | 10°C | **Temperature** | 21 ± 1°C |
| | | **pH** | 7.1 | **Note** | Growth in the dark |
| ***Sideroxydans lithotrophicus (ES-1)*** | Weiss et al., 2007 | **Location** | Middle Atlantic, Roots of *Magnolia Virginia,* Fredericksburg, VA | **Isolation** | Gradient tubes and gradient plates, thin agarose-stabilized layer FeS in a Petri plate overlaid with liquid MWMM medium |
| | | **[Fe(II)]** | 453 µmol.$gdw^{-1}$ | | |
| | | **Temperature** | 10°C | | |
| | | **pH** | 4.5 | | |
| | | **Organic Matter** | High (13%) | **pH** | 4.5 - 7 |
| | | **Note** | soil [Fe(II)] = 72 µmol.gdw-1 | **Temperature** | 12 - 26°C |
| | Emerson et al., 2008 & Emerson and Moyer, 1997 | **Location** | Groundwater from a basement tile drain in East Lansing, rust colored floculant | **Isolation** | Gel-stabilized (0.15% agarose) MWM medium buffered with 10nM bicarbonate |
| | | **[Fe(II)]** | 3 - 12 µM | **pH** | 6.4 |
| | | **Temperature** | 10°C | **Oxygen** | Opposing gradient of $Fe^{2+}$ and $O_2$ |
| | | **pH** | 7.1 | | |
| | Emerson and Moyer,1997 | **Location** | Groundwater from a basement tile drain in East Lansing, rust colored floculant | **Isolation** | Agarose Medium |
| | | **[Fe(II)]** | 3 - 12 µM | **pH** | 6.2 - 6.4 |
| | | **Temperature** | 10°C | **Temperature** | 21 ± 1°C |
| | | **pH** | 7.1 | **Note** | Growth in the dark |
| ***Gallionella capsiferriformans (ES-2)*** | Emerson and Moyer,1997 | **Location** | Groundwater from a basement tile drain in East Lansing, rust colored floculant | **Isolation** | Gel-stabilized (0.15% agarose) MWM medium buffered with 10nM bicarbonate |
| | | **[Fe(II)]** | 3 - 12 µM | | |
| | | **Temperature** | 10°C | **pH** | 6.4 |
| | | **pH** | 7.1 | **Oxygen** | Opposing gradient of $Fe^{2+}$ and $O_2$ |
| | Neubauer et al., 2007 | **Location** | Rhizosphere of *Typha Latifolia* in created marsh in Maryland | **pH** | ~6 |
| | | | | **Temperature** | 28°C |
| | | | | **[Fe]** | 1200 µmol/L |
| | | | | **note** | 14h light/ 10h dark |
| ***Sideroxydans paludicola*** | Weiss et al., 2007 | **Location** | Middle Atlantic, Roots of *Magnolia Virginia,* Fredericksburg, VA | **Isolation** | Gradient tubes and gradient plates, thin agarose-stabilized layer FeS in a Petri plate overlaid with liquid MWMM medium |
| | | **[Fe(II)]** | 453 µmol.$gdw^{-1}$ | | |
| | | **Temperature** | 10°C | | |
| | | **pH** | 4.5 | | |
| | | **Organic Matter** | High (13%) | **pH** | 4.5 - 7 |
| | | **Note** | soil [Fe(II)] = 72 µmol.gdw-1 | **Temperature** | 19 - 37 °C |
| ***Ferrotriphicum radicicola*** | Weiss et al., 2007 | **Location** | Middle Atlantic, Roots of Juncus effusus in the riparian strip of Contrary Creek Mineral,VA. Former pyrite mine | **Isolation** | Gradient tubes and gradient plates, thin agarose-stabilized layer FeS in a Petri plate overlaid with liquid MWMM medium |
| | | **[Fe(II)]** | 850 - 1230 µmol.gdw-1 | | |
| | | **pH** | 4.0 | **pH** | 4.5 - 7 |
| | | **Note** | soil [Fe(II)] = 72 µmol.gdw-1 | **Temperature** | 19 - 37 °C |

## 6. Focus on Gallionella

The Genus *Gallionella* is a group of β-proteobacteria. *Gallionella ferruginea* specifically, is a chemolithotroph that thrives at the interface of oxic-anoxic conditions in aquatic systems. This organism microaerobically oxidizes iron present in water as an energy source under circum-neutral conditions. The product of this metabolism is an insoluble ferric hydroxide that forms with excreted polymers a helical, stalk-like filament formed by the kidney-shaped cells (Emerson and Moyer, 2002). Unlike *Leptothrix ochracea*, bacteria discussed later, *Gallionella ferruginea* can be cultured in the laboratory. These organisms are found in various aquatic environments and isolation methods as well as growth conditions are well documented (Emerson and Moyer, 1997; Emerson and Moyer, 2002; Neubauer et al., 2007).

## 7. Focus on Leptothrix

The genera *Leptothrix* belongs to the *Sphaerotilus-Leptothrix* group (Van Veen et al., 1978). The literature classifies this group as the third of iron oxidizing bacteria after the autotrophic, obligatory aerobic, acidophilic *Thiobacillus ferrooxidans* and the microaerophilic autotrophic *Gallionella ferruginea*. *Leptothrix* is a gram-negative and autotrophic betaproteobacterium occurring in slowly running ferrous iron-containing water poor in readily decomposable organic material. Nothing is known yet concerning the mobility mechanisms of the cells. However, a gliding movement can not be excluded. As most of the bacteria from this group, *Leptothrix* is known to produce a sheath of extracellular iron oxides. These bacteria are considered to take energy from the oxidation of soluble $Fe^{2+}$ cations into insoluble $Fe^{3+}$ cations. They likely utilize a great variety of organic carbon compounds as a carbon source such as glucose, fructose, maltose, lactose, methanol, ethanol, acetate, or butyrate.

*Leptothrix* bacteria use also ammonia and nitrate as sole nitrogen source. Then, they precipitate the iron oxide sheath such as twisted stalk or curved sheaths (Hashimoto et al., 2007). This study stated that the sheath has an aspect ration (diameter-length) of 10-200. The hollow sheath thickness is in average 150 nm (Figure 15). Thus, nanoparticles are involved in the sheath composition. Those observations are shown by several pictures from the high-magnification scanning electron microscopy. The chemical composition was finally Fe:Si:P = 80:15:5 with the exception of oxygen, distributed uniformly. A study on the structure of the *Leptothrix discophora* SP-6 added the hypothesis that this layer is covered by a diffuse outer capsular layer of variable thickness. This second layer is a heteropolymer made of 6.5 nm diameter heteropolysaccharide fibrils tightly associated (uronic acids and amino sugars) and covalently linked to excreted protein involved in oxidation of both iron and manganese (Emerson and Ghiorse, 1992). In addition, this layer has a high cationic metal-binding affinity due to the net negative charge of the uronic acid residues (Emerson and Ghiorse, 1993). This sheath has beneficial functions such as accumulation of nutrients, attachment, and regulation of positioning in the environment.

*Gallionella ferruginea* and *Leptothrix ochracea* share metabolism similarities. The environmental interest is on the potential of these biogenic iron oxides to adsorb phosphorus (Rentz et al., 2008).

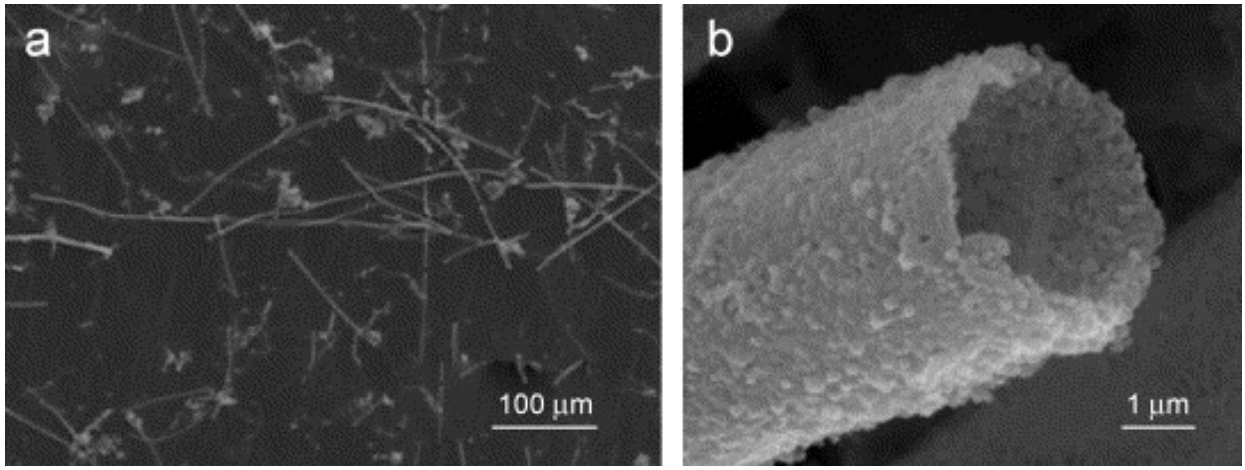


**Figure 15.** (a) Low- and (b) high-magnification of *Leptothrix Ochracea* sheath by scanning electron microscopy (from Hashimoto et al., 2007).

# APPENDIX C
# STANDARD OPERATING PROCEDURES

WASHINGTON STATE UNIVERSITY CIVIL AND ENVIRONMENTAL ENGINEERING

# Iron Oxide Characterization

# Standard Operating Procedures (SOPs)

**Washington State University**

**Center for Environmental, Sediment, and Aquatic Research (CESAR)**

**C/o Jeremy A. Rentz**

**Sloan Hall**

**Pullman, WA, 99164**

**Created by:** **Antoine Cordray, Graduate Research Assistant**
**Date: May 08, 2008**

**Adapted from: Ian Turner, Graduate Research Assistant**
**Date: November 30, 2007.**

**Reviewed by: Jeremy A. Rentz, Assistant Professor**
**Date: May 20, 2008**

## A. Scope and Application:

The procedures used here apply to microbial iron mat field collection and laboratory activities for the CESAR group of Washington State University. Procedures outlined include field sample collection, transportation, and sample characterization.

## B. Objectives:

The objective of this SOP is to provide consistent, well defined methods for use in multiple research applications.

## C. Field Sampling & Data Collection:

### I. Site Location:

Biogenic iron-oxides are typically located where $Fe^{2+}$ sources are met with an aquatic interfacial zone. Possible attributes include:

- Mountainous areas
- Streams and seeps
- Orange flocculent indicative of oxidized iron

### II. Equipment:

The following equipment is to be considered for each sampling trip although each item may not be necessary depending on the situation.

- Copy of SOP
- FOX Record Sheets
- Backpack
- Field Notebook
- Clipboard
- Labeling tape & Marker
- Pencil/Pen
- Automatic Pipetter, 5mL and 50mL Pipettes
- Method or apparatus for scooping sample
- Jars for transporting and storing samples
- Thermometer & Field pH meter
- 15 mL Falcon Tubes & FerroVer Pillows for Iron Test
- Scissors
- Ice chest with ice
- Waders
- Sunscreen
- Bug repellant

## III. Field Site Characterization:

Specific data needs to be collected at the sampling site in order to improve characterization of the iron-oxides. This data is to be recorded on the FOX sheets with one sheet used per sample.

Record the following on the FOX sheet for each sample:

- Sample name, date, & time
- Site description including specific location & weather.
- Names of those on the sampling trip

Label each container with the following FOX sheet information:

- Location prefix and number (ex. Y2B for Yakima 2, sample B)
- Date
- Sampler's initials

Record the following site characterizations on each FOX sheet:

- Field pH level
- Field temperature

## IV. Field Sampling Procedures:

Field samples collected from a body of moving water should be performed starting at the downstream end of the site and working upstream. This method is employed to ensure that samples are not impacted by previous sample disturbances. It should be noted that 1L of sample is required to perform one desired characterization analysis. If the three experiments (Batch equilibrium, PABE, and phosphorus recovery) are being investigated, three liters of sample are needed.

- **Field pH:**
    - Place field pH meter in water and allow to equilibrate
- **Field Temperature:**
    - Place thermometer near collection site and allow for equilibration
- **Field aqueous Fe concentration (n=3):**
    - Pipette 5mL of near collection site water into 15mL reaction tube
    - Add 1 FerroVer powder pillow to each tube and shake (Hach#8008)
    - Cap tube for transportation with analysis to be performed in the lab
- **Iron sample collection:**
    - Use 50 mL pipette to withdraw aqueous iron sample from site
    - Do not pipette sediments. Try to disturb iron oxide biofilm as less as possible
    - Transfer sample to 1L sample container
    - Repeat process until the desired 1L is achieved
    - Allow sample to settle, decant, and add more raw sample.
    - Place samples on ice for transport to the laboratory refrigerator

### D. Laboratory Data Collection

**Material**

- **Sample Characterization**

- 1L jar of freshly sampled material
- 15 x 50 mL tubes
- 20 x 15mL tubes
- 3 x Phosphorus vials and pillow for HACH method 8048
- 5mL, 10mL, 25mL and 50mL Pipettes
- 0-20µL, 0-200µL, 0-1000µL tips for micropipettes
- DNA microtubes
- Slides and cover glass for microscope study
- 1mL of 10 µg/L Acridine Orange solution (2µL of AO solution in 20mL DI water)
- 300 mL of 0.25M Oxalic Acid solution(Add 11.03g of Oxalic acid powder in 500 mL DI water)
- 20 mL of 1M $NaHCO_3$ solution (20.985g of powder $NaHCO_3$ in 250 mL DI water)
- FerroVer powder pillow for HACH method 8008
- 8 aluminum dishes

- **Sample Washing**

- 2 x 50ml tubes
- 700mL of 0.1M saline wash (700 mL DI water + 4.09 g NaCl + autoclave) or,
- 700 mL buffered saline wash (700mL of 0.1M saline wash + 0.588g of $NaHCO_3$ powder)

- **Batch Equilibrium**

- 800 mL of washed sample at leat
- 40 x 50mL tubes
- 1 x 2mL microtube
- 40 x Phosphorus vials and pillow for HACH method 8048
- 35 mL of 1g/L $PO_4$ solution (Add 0.4584g of $K_2HPO_4$ powder to 250mL DI water)
- 5mL, 10mL, 25mL and 50mL Pipettes
- 0-20µL, 0-200µL, 0-1000µL tips for micropipettes

- **pH Adujsted Batch Equilibrium (PABE)**

- 800 mL of washed and buffered sample at least
- 30 x 50mL tubes
- 30 x Phosphorus vials and pillow for HACH method 8048
- 6 x 250 mL Erlenmeyers
- 100 mL of 1g/L $PO_4$ solution (Add 0.4584g of $K_2HPO_4$ powder to 250mL DI water)
- 50 mL of 0.1M $HNO_3$ solution (665µL of nitric acid in 150 mL DI water)
- 5 mL of 0.1M NaOH solution (0.6g of NaOH powder in 150mL DI water)

- **Phosphorus Recovery**

- 800 mL of washed sample at least
- 16 x 50mL tubes
- 80 x Phosphorus vials and pillow for HACH method 8048
- 16 x 250 mL Erlenmeyers

- 55 mL of 1g/L $PO_4$ solution (Add 0.4584g of $K_2HPO_4$ powder to 250mL DI water)
- 500 mL of 0.01M NaOH solution (0.4g of NaOH powder in 1L DI water)
- 500 mL of 0.01M KCl solution (0.7445g of KCl powder in 1L DI water)
- 500 mL of 0.1M NaCl saline solution (700 mL DI water + 4.09 g NaCl + autoclave)
- 20mL of 0.3M MES buffer solution (5.856g of MES powder in 100mL DI water)

Upon returning to the lab, be careful to label all experimental containers in a manner consistent with the original field samples. Perform the following experiments sequentially on each sample in order to characterize the samples. Record all experimental results on the corresponding FOX record sheet. Batch equilibrium, PABE, and phosphorus recovery analysis may be performed in conjunction with characterization experiments.

## I. Pre-Wash Sample Characterization:

Perform the following laboratory characterizations prior to washing to obtain information about the natural sample. Transfer 100mL of natural sample to 2 x 50mL Falcon tubes for pre-wash characterization (tube 1 and 2). Experience on tube 1 can be made directly since the sample has to be well mixed. Let settle tube 2 overnight. Allow remaining sample to settle.

- **Tube 1: Mixed sample**

  **1: pH:**

  - Use pH meter to analyze.

  **2: Total Fe (n=3):**

  - Add 1mL mixed sample and 49mL of 0.25M oxalic acid to 50mL tubes
  - Allow oxalic acid digests to sit overnight

- Slowly add 2mL $NaHCO_3$ & 5mL oxalic acid digest to 15mL tubes
- Read pH to ensure it has been adjusted to the required 3-5 pH.
- Dilute samples by adding 1mL pH digest and 4mL DI water to 15mL tubes
- Add FerroVer pillows to new tubes (Hach method 8008)
- Transfer final solutions to square cuvettes for analysis
- Use preprogrammed spectrophotometer to determine total Fe
- If concentration is out of range, try another dilution keeping total volume at 5mL
- Account for dilution factors in determining Total Iron concentration
- x2.5 for difference between pre-programmed vial size and used cuvettes
- x50 for initial oxalic acid digest
- x1.4 for pH adjustment
- x*D* for final 5mL dilution with FerroVer pillow

**3: Dry Density (n=4):**

- Scribe/label and weigh aluminum evaporating dishes (n=4)
- Transfer 10mL of mixed sample to each dish
- Evaporate water in oven (110°C) overnight
- Weigh evaporating dishes with dried solids
- Dry density = (final dried weight - initial weight) / 10mL

**4: Organic Content (n=4):**

- Use the dried samples from the dry density characterization
- Place samples in muffle furnace (440°C).

- Allow samples to heat for 24 hours
- Place samples in dessicator to cool for 20 minutes
- Weigh samples to determine inorganic weight

- **Tube 2: Settled sample**

**5: Aqueous Phosphorus Concentration (n=1):**

- Withdraw 5mL of supernatant from the settled sample solution
- Analyze P concentration using Hach method 8048

**6: DNA/RNA Extraction:**

- Perform immediately
- Extract 500µL of solids from sample by filling a 2mL microtube, centrifuging, and getting rid of the supernatant multiple times
- Use the MO Bio Kit and procedure to extract DNA from sample solids
- Freeze extractions at -85°C

**7: Photographs of Iron-oxides:**

- Add 50 to 100µL of the specimen to a clean glass slide
- Add a drop of Acridine Orange (10 to 20µg/mL) to the specimen drop
- Mix to make it spread homogeneously using the pipette tip extremity
- Add a cover glass. Let one side of the cover glass touch the slide first. The solution is going to spread on this side. Then, let the cover slide drop to avoid air bubbles.

- Turn on microscope (Arclamp power supply, LEP Ltd)(Figure 1. A), camera (RT power supply, SPOT) (Figure 1. B), polarized light (Figure 1. C) and computer
- Place glass slide on the microscope
- Rotate numerated disk and select the position 3 (fluorescence) (Figure 1. D)
- Localize an appropriate view using 10x and 40x objectives
- Add a drop of immersion oil on the cover slide and select the 100x objective
- Increase or decrease light intensity to allow more or less fluorescence (Figure 1. F)
- Find appropriate settings to produce a desired image
- Set the vision screw either on "camera" (image just on camera) or on "50/50" (image for user and camera) to allow camera to get the image (Figure 1. E)
- Open the software "SPOT advanced". The shortcut is on the desktop
- On the bottom right corner, select the mode "ACAO" (Figure 2. H)
- Click on the button "get image" on the right side of the screen (Figure 1. G) or push F9
- The settings might change during the capture of the picture. Make sure that the clarity of the image stays the same during the capture

## II. Sample Washing:

Washing may be performed on a field sample in order to remove uncertainties associated with the composition of the *in situ* water.

- Allow sample to settle for a period of 24 hours
- Remove supernatant by pipetting without removing any solids

- Save 2 x 50mL of supernatant in reaction tubes at 4°C.
- Fill sample container back up with:
- Saline solution to total volume of 1L for batch equilibrium or phosphorus recovery analysis
- Buffered saline solution to total volume of 1L for PABE analysis
- Transfer 100mL of mixed washed sample in two Falcon 50mL tubes

## III. Post-Wash Sample Characterization:

After washing the sample, perform laboratory procedures 1-5 again with the two previous tubes and record results on the FOX record sheet.

## IV. Batch Equilibrium (n=5)

- Add 20ml of washed sample in 40 x 50 mL tubes
- Add the amount of DI water and 1g/L $PO_4$ solution given by the FOX sheet
- Place reactor tubes on the inversion rack for a period of 24 hours on setting #3
- Centrifuge reactor tubes at 4100 rpm during 10 minutes
- Test phosphorus in supernatant content using Hach method 8048

## V. pH Adjusted Batch Equilibrium (PABE) (n=5)

- Record pH of the buffered and washed sample
- Add 110mL to 6 clean Erlenmeyer
- Realize the following adjustment:

  Erlenmeyer 1: add 2 mL of 100mM NaOH buffer solution

  Erlenmeyer 2: add 1.5 mL of 100mM $HNO_3$ buffer solution

Erlenmeyer 3: add 3.1 mL of 100mM $HNO_3$ buffer solution

Erlenmeyer 4: add 8.1 mL of 100mM $HNO_3$ buffer solution

Erlenmeyer 5: add 11 mL of 100mM $HNO_3$ buffer solution

Erlenmeyer 6: add 15.4 mL of 100mM $HNO_3$ buffer solution

- Shake and measure pH of each Erlenmeyer
- With each Erlenmeyer, add 5 times 20 mL in 50mL tubes
- Add 3.2 mL of Phosphorus stock solution and 26.8 mL of DI water in every tube
- Place reactor tubes on the inversion rack for a period of 24 hours on setting #3
- Centrifuge reactor tubes at 4100 rpm during 10 minutes
- Record pH of each tube
- Test phosphorus content in supernatant using Hach method 8048

### VI. Phosphorus Recovery (n=4)

- Add 20mL of washed sample in 16 x 50 mL tubes
- Add 26.8mL of DI water and 3.2mg/L of1g/L $PO_4$ solution in each tube
- Place reactor tubes on the inversion rack for a period of 24 hours on setting #3
- Centrifuge reactor tubes at 4100 rpm during 10 minutes
- Take off 30 mL of supernatant
- Test phosphorus content in supernatant using Hach method 8048
- Add 30mL of DI water in 4 tubes (1.1 to 1.4)
- Add 30mL of 0.1M NaCl saline solution in 4 tubes (2.1 to 2.4)
- Add 30mL of 0.01M KCl solution in 4 tubes (3.1 to 3.4)
- Add 30mL of 0.01M NaOH solution in 4 tubes (4.1 to 4.4)
- Place reactor tubes on the inversion rack for a period of 24 hours on setting #3
- Centrifuge reactor tubes at 4100 rpm during 10 minutes
- Take off 30 mL of supernatant
- Place NaOH (4.1 to 4.4) tube supernatants to 4 erlenmeyers and add 1mL od 0.3M MES buffer to each. Mix and make sure that pH is between 5.5 and 8.5
- Test phosphorus content in supernatant using Hach method 8048
- Repeat this cycle 4 times for significant results

## E. Personnel Qualifications

Field sampling and laboratory analysis should be performed or supervised by someone having at least 1 experience performing the tasks required. Under special circumstances sampling and analysis procedures can be performed by inexperienced employees.

## F. Quality Control

### I. Duplicates:

Collect duplicate samples every fifth sampling trip in order to ensure that consistent sampling procedures are being performed. The duplicates should be collected at the same time as the actual sample and all typical analysis should be performed on it as well.

### II. Sample Frequency:

For specific field and laboratory characterization tests, multiple samples will be run to produce more acceptable and precise results. Some such tests will be run with n=4, others with n=5 samples. Each sample frequency is to be assigned its own number in order to keep the data organized on the FOX record sheet (4.1, 4.2, etc.).

### III. Standards:

Make standards for iron and phosphorus spectrophotometry methods. Construct blanks with the same water used to wash the samples. Make a 2 mg Fe/L sample using a dilution of a 100mg/L stock solution.

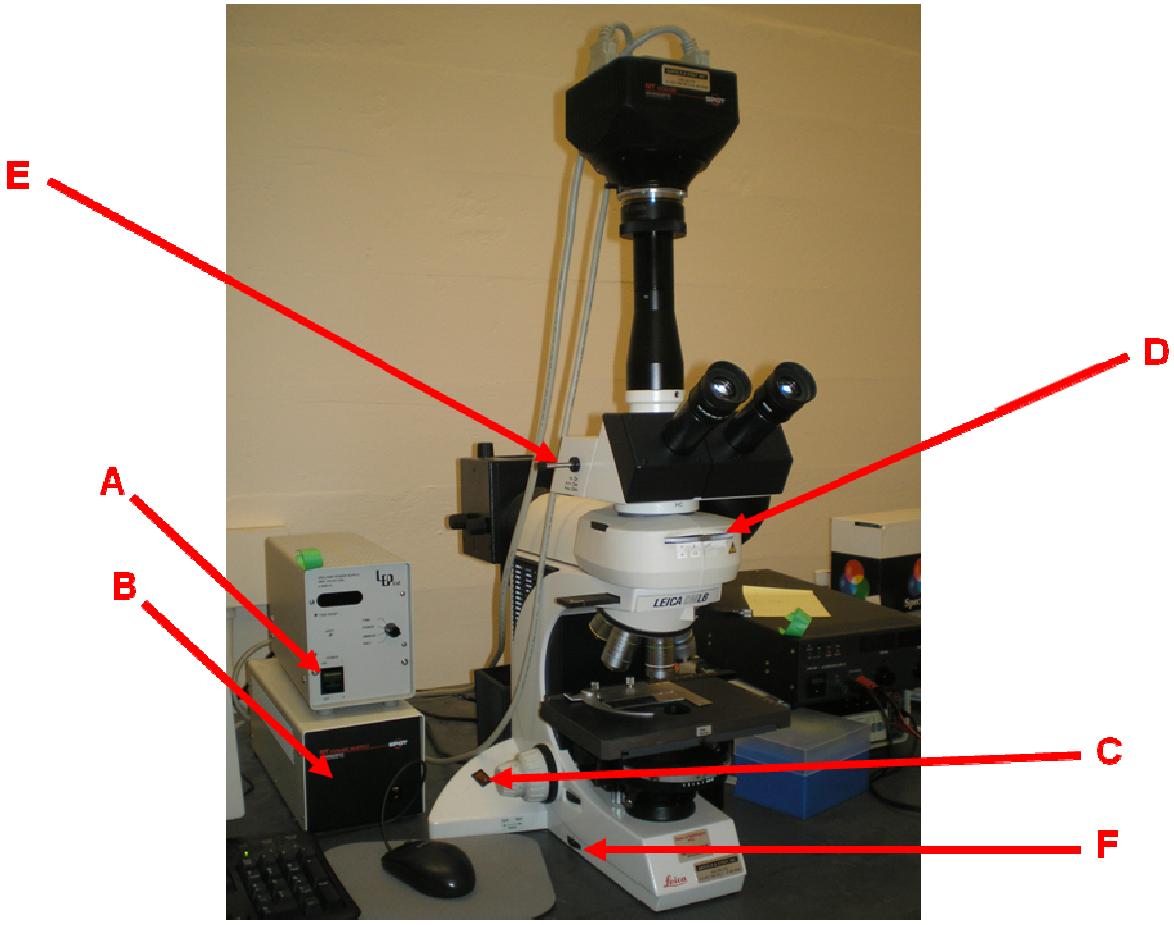


**Figure 16**. Leica DMLB fluorescence microscope picture

## G. Additional Documents: Fe-Oxide (FOX) Record Sheet

### FOx Record Sheet (1)

**Designation:**

**Sample Name:**
**Date:**

**Sample Information/Description:**

**Field:**
pH:
Temp:

Aqueous [Fe]: (mg/L)
(n=3)

Mean: #DIV/0!
St.Dev. #DIV/0!

**DNA/RNA?:**
**Photomicrograph?:**
**Deep Freeze?:**

*Total Iron Standard:

**Pre-Wash:**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH: | | Dry Density: (g/L) | n=1 | n=2 | n=3 | n=4 | Mean: | St.Dev. |
| P conc: (mg/L) | | *Empty M: | | | | | #DIV/0! | #DIV/0! |
| | | *Dry M: | | | | | #DIV/0! | #DIV/0! |
| Total Fe: (mg/L) | | *Density: | 0 | 0 | 0 | 0 | 0 | 0 |
| (n=3) | | | | | | | | |
| Dilution: x | | Org. Content:(g/L) | n=1 | n=2 | n=3 | n=4 | Mean: | St. Dev. |
| Mean: | #DIV/0! | *Initial M: | 0 | 0 | 0 | 0 | 0 | 0 |
| St. Dev. | #DIV/0! | *Final M: | | | | | #DIV/0! | #DIV/0! |
| | | *Org. Content: | 0 | 0 | 0 | 0 | 0 | 0 |

**Post- Wash:**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH: | | Dry Density: (g/L) | n=1 | n=2 | n=3 | n=4 | Mean: | St.Dev. |
| P conc. (mg/L): | | *Empty M: | | | | | #DIV/0! | #DIV/0! |
| | | *Dry M: | | | | | #DIV/0! | #DIV/0! |
| Total Fe: (mg/L) | | *Density: | 0 | 0 | 0 | 0 | 0 | 0 |
| (n=3) | | | | | | | | |
| Dilution: x | | Org. Content:(g/L) | n=1 | n=2 | n=3 | n=4 | Mean: | St.Dev. |
| Mean: | #DIV/0! | *Initial M: | 0 | 0 | 0 | 0 | 0 | 0 |
| St. Dev. | #DIV/0! | *Final M: | | | | | #DIV/0! | #DIV/0! |
| | | *Org. Content: | 0 | 0 | 0 | 0 | 0 | 0 |

**Batch Equilibrium:**

**[Fe] used per Reactor (mL):**
**P Soln. per Reactor:** 1M

| Initial [P]: | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | |
|---|---|---|---|---|---|---|---|---|---|
| P Stock Reqd: | 25u | 50u | 100u | 200u | 400u | 800u | 1.6m | 3.2m | |
| DI Reqd: | 29.975m | 29.95m | 29.9m | 29.8m | 29.6m | 29.2m | 28.4m | 26.8m | |
| #1 | | | | | | | | | |
| #2 | | | | | | | | | |
| #3 | | | | | | | | | |
| #4 | | | | | | | | | |
| #5 | | | | | | | | | |
| Dilution: | | | | | | | | | |

## FOx Record Sheet (2)

**Designation:** _____

**PABE:**

**Initial [P]** 64 mg/L
**P Stock Reqd:** 3,2mL
**DI Reqd:** 26.2 mL
**pH**

**[Fe] used per Reactor (mL):**
**P Soln. per Reactor:** 1M

| Buffer | Initial pH | | #1 | #2 | #3 | #4 | #5 |
|---|---|---|---|---|---|---|---|
| 2mL NaOH | | pH | | | | | |
| | | [P] mg/L | | | | | |
| 1.5mL $HNO_3$ | | pH | | | | | |
| | | [P] mg/L | | | | | |
| 3.1mL $HNO_4$ | | pH | | | | | |
| | | [P] mg/L | | | | | |
| 8.1mL $HNO_5$ | | pH | | | | | |
| | | [P] mg/L | | | | | |
| 11mL $HNO_6$ | | pH | | | | | |
| | | [P] mg/L | | | | | |
| 15.4mL $HNO_7$ | | pH | | | | | |
| | | [P] mg/L | | | | | |

| Phosphorus Recovery | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Experiment:** | | | | | | | | |
| tube | **A (DI Water)** | | | | **B (0.1M saline solution)** | | | |
| N | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** |
| t=0 | | | | | | | | |
| t=24 | | | | | | | | |
| t=48 | | | | | | | | |
| t=72 | | | | | | | | |
| t=96 | | | | | | | | |
| tube | **C (0.01M KCl Solution)** | | | | **D (0.01M NaOH solution)** | | | |
| N | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** |
| t=0 | | | | | | | | |
| t=24 | | | | | | | | |
| t=48 | | | | | | | | |
| t=72 | | | | | | | | |
| t=96 | | | | | | | | |

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