



## Characterization and performance of local organic materials for passive treatment systems of mine drainage in Yukon



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## EXECUTIVE SUMMARY

Passive treatment systems (PTS) present a potential low-cost solution to treating mine-impacted waters (MIW) and impeding release of contaminants, such as metals and sulphate, into receiving waters in remote mine sites in Yukon. Nevertheless, the traditional carbon substrates used to maintain efficient functioning of these systems (i.e., molasses) are not readily available and can be expensive in remote locations. Moreover, the extensive periods of cold temperatures experienced in Yukon may impede the biological functioning of PTS. The goal of this study is to characterize a selection of local organic materials that may be used as complex carbon substrates for PTS and evaluate their potential performance as carbon substrates in the Yukon environment. In the first part of the study, the physiochemical properties of six local complex carbon substrates: willow leaves (*Salix* sp.), sedges (*Carex* sp.), peat, peat moss (*Sphagnum*), poplar (*Populus* sp.), wood chips, and spruce (*Picea* sp.) wood chips were characterized. In the second part, bench-scale bioreactors containing organic substrates, were used to evaluate carbon substrate degradation and the performance of bioreactors for sulphate and metal reduction from synthetic mine water at low temperatures for approximately five months.

Sulphate reduction in the carex, moss, and willow bioreactors was reduced 100%, and overall, these substrates appear to support microbial-mediated metal sulfide precipitation at low temperatures (<10°C). During the time-frame of the study, spruce was not found to be effective in sulphate reduction. With some exceptions, the overall metal reduction was found to be high in all bioreactors, including controls. Thus, most of the metal removal was attributed to abiotic processes.

Basic characterization of substrates, such as carbon content (DOC, TC) and nitrogen (TN) may be indicative of substrate performance, however a more in-depth analysis of the chemical properties of these components is warranted. This study is the first phase of a multiphase research project and is intended to inform future studies.

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## LIST OF ABBREVIATIONS

AMW	Acidic Mine Water
As	Arsenic
Cd	Cadmium
CEAEQ	Centre d'Expertise en Analyse Environmental du Quebec
CEC	Cation Exchange Capacity
C/N	Carbon/Nitrogen ratio
CH <sub>2</sub> O	Small organic carbon molecule
COD	Chemical Oxygen Demand
Cu	Copper
DOC	Dissolved Organic Carbon
Fe	Iron
H <sup>+</sup>	Hydrogen Ion
HCO <sub>3</sub> <sup>-</sup>	Bicarbonate
H <sub>2</sub> S	Hydrogen Sulfide
HS <sup>-</sup>	Bisulfide
M <sup>2+</sup>	Dissolved Cationic Metals
MIW	Mine-Impacted Water
NMW	Neutral Mine Water
ORP	Oxidation Reduction Potential
NO <sub>2</sub> /NO <sub>3</sub>	Nitrite/Nitrate
PTS	Passive Treatment System
QC	Quality Control
S <sup>2-</sup>	Sulfide
SO <sub>4</sub> <sup>2-</sup>	Sulfate
Se	Selenium
SMW	Synthetic Mine Water
SRB	Sulfate Reducing Bacteria
TC	Total Carbon
TOC	Total Organic Carbon
TN	Total Nitrogen
TVS	Total Volatile Solids
Zn	Zinc

## 1.0 INTRODUCTION

The process of mining sulphidic ore deposits often leaves a legacy of wastewater with low pH, elevated concentrations of sulphates, and dissolved heavy metals such as zinc, cadmium, copper and selenium (Waybrant, 1998). The resulting quality of the mine-impacted water (MIW) is extremely deleterious to the aquatic environment, and therefore, must be treated before being released (Neculita and Zagury, 2008). Many mining operations are located in remote areas thus the cost of materials and labor for maintaining active remediation activities can be substantial.

Passive treatment systems, such as bioreactors and constructed wetlands, may present a low maintenance, cost effective solution to these issues. These systems rely on native anaerobic sulphate-reducing bacteria (SRB) to remediate the impacted water. SRB-coupled sulphate reduction with organic carbon oxidation can result in increased pH and immobilization of heavy metals through precipitation (Neculita and Zagury, 2008).

In Yukon, the use of passive treatment systems (PTSs) may offer a cost-effective solution to treating MIW in remote locations (Janin and Harrington, 2015). Nevertheless, the low temperatures in the northern climate may impede microbial metabolic activity resulting in their reduced efficiency. SRB primarily use simple carbon molecules, such as ethanol and lactate, for the improved performance in passive treatment systems (Neculita and Zagury, 2008). The use of these carbon sources, however, can be costly. Previous research has focused on characterizing the complex reactive mixtures that contain substrates like hay, compost, agricultural wastes (manure), sawdust, molasses, etc. and their efficacy for sulphate reduction and metal precipitation (Waybrant, 1998; Neculita and Zagury, 2008; Schmitova and Baldwin, 2011). Unfortunately, because of the climate and the location of mine sites in Yukon, these typical substrates are not readily available. Thus, local, inexpensive organic substrate materials need to be identified for effective long-term use. Research findings that characterize the physiochemical properties of local, inexpensive organic substrates and their efficacy in microbially-mediated metal precipitation at low temperatures is expected to assist operators in developing affordable and effective PTSs in Yukon.

The goals of this project were to characterize the physiochemical properties of six potential local complex carbon substrates using laboratory methodologies and to monitor the carbon degradation and the ability of the substrates to support sulfide reduction and metal precipitation in cool temperatures using batch scale bioreactors. The experimental results are intended to be used as preliminary data for future studies using flow-through column bioreactors and carbon degradation modeling.

The specific objectives of this study were to: 1) characterize the physiochemical properties of six local carbon substrates, including decaying willow leaves (*Salix* sp.), decaying sedges (*Carex* sp.), peat, peat moss (*Sphagnum*), poplar (*populus* sp.), wood chips, and spruce (*Picea* sp.) wood chips; 2) monitor the degradation of these substrates using bench-scale bioreactors containing synthetic mine water and local creek sediment as a source of microbial inoculum (incubated at 5°C for a period of approximately 5 months); 3) assess the bioreactor performance for developing conditions favorable for SRB and reducing the sulphate and metal concentrations of the effluent over time; and 4) assess the impact of mine water pH on substrate bioreactor performance.

Due to the remote locations of mine sites in Yukon, local, inexpensive sources of carbon substrates are needed to support microbial remediation of MIW. The principal benefit of the project is the development of an inventory of physiochemical properties of local organic material that could be used in passive bioreactor systems for treating MIW in Yukon. In addition, the generated data may be used to assist the development of predictive models in the planning and designing of bioreactor systems.

Developing a more robust understanding of the relationships between substrate composition and degradation dynamics for a compendium of organic substrates will assist operators of PTSs in the design, planning, and predicting functions for their effective, long-term use in northern climate conditions. This knowledge may significantly reduce the labor requirements and the costs for operators, while attenuating the impact of MIW to host aquatic ecosystems.

## 2.0 LITERATURE REVIEW

The process of mining sulphidic ore deposits typically releases contaminants that are harmful to both aquatic life and human health, and therefore, must be treated before being released into the environment. Mining impacts fresh water in several ways, such as the heavy use of water in processing ore, precipitation, infiltration and seepage through tailings and waste rock impoundments, and through groundwater re-emergence into mine sites once mining activities have ceased (INAP, 2014). MIWs can be characterized by their low pH, elevated concentrations of sulphates, and their dissolved heavy metals such as iron (Fe), zinc (Zn), cadmium (Cd), copper (Cu), and metalloids\* ; selenium (Se), and arsenic (As) (Waybrant, 1998).

Treatment processes for MIW can be active or passive. Active processes may be abiotic or biotic or a combination, typically involving treatment with chemicals, such as limestone or soda ash to increase the pH of the water and facilitate metal precipitation as carbonates (INAP, 2014). Besides needing chemicals for neutralization, active treatment processes require energy and continuous monitoring, and thus, increase the labor requirements over long treatment periods, which may make these methods prohibitively expensive. The large quantities of metal-bearing sludge that are produced will eventually need to be disposed of, which contributes to the downstream management costs (Neculita and Zagury, 2008).

### 2.1 TREATMENT SYSTEMS FOR MINE-IMPACTED WATER

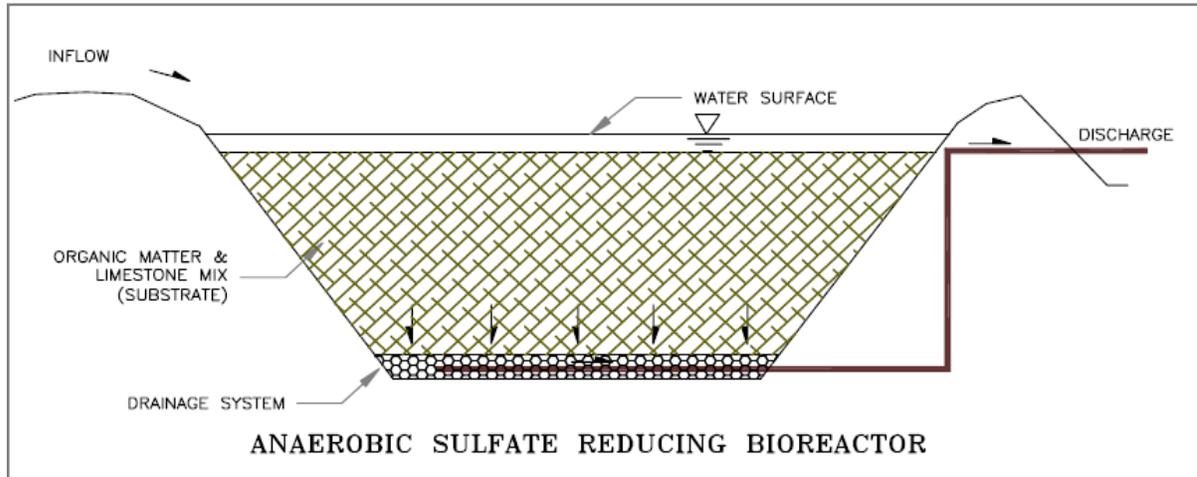
Passive treatment methods, such as constructed wetlands and bioreactors, rely on biological and geochemical processes that occur naturally in anoxic or aerobic environments to neutralize pH, reduce sulphate levels, and precipitate the dissolved metals to less bioavailable forms (Figure 1). These treatment methods can be engineered to emulate the attenuation of contaminants that has been observed to occur for centuries in natural wetlands and bogs. Constructed wetlands, consisting of an excavated pond, lined with an impermeable barrier consisting of either geotextile or clay materials, and slightly sloped, can allow a slow, gravity flow of water through the system. The pond can be first filled with porous substrates like sand or peat, which may also act as an adsorptive material, followed by native wetland plants. Such systems rely on the nutrient uptake by plants, microbial degradation of plant material, precipitation, and the adsorption of metals to the substrates in the system. These processes impede the release of contaminants from the effluent to both ground water and receiving surface waters (Ness et al., 2014).

Passive bioreactors generally consist of tanks or excavated ponds that are filled with porous substrate like sand and/or gravel, wetland or stream sediments as a source of microbial

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\*For clarity, the metalloids As and Se will be referred to as metals in this report.

inoculum, and organic matter like sawdust, compost, or hay as the source of carbon. If the mine effluent is acidic, a neutralizing agent, like limestone, may be added to the bioreactor. The effluent may percolate either vertically or horizontally through the system before discharge (Gusek and Wildeman, 2002; Schimdtova and Baldwin, 2011).



**Figure 1.** General diagram of a passive bioreactor design. (Gusek and Wildeman, 2002)

Anaerobic or anoxic passive biological treatment is accomplished through the action of a consortium of microbes that exist naturally in the organic substrates of bogs, wetlands, and streams. Ultimately, a diverse group of microbes, collectively termed sulphate-reducing bacteria (SRB), reduce sulphates through carbon oxidation, resulting in increased alkalinity and the immobilization of dissolved metals through precipitation.

The advantages of using passive treatment methods is that once in place, they require little intervention and therefore reduce labor, energy, and chemical costs associated with active treatment methods. The key to maintaining sustainable, consistent contaminant remediation in PTSs is to understand how the characteristics of the organic carbon substrates impact the performance of the system. Recent research has been aimed at elucidating the relationship between carbon substrate composition and degradation dynamics and the ability to sustain sulphate reduction over time (Waybrant et al., 1998; Zagury et al., 2006; Neculita and Zagury, 2008; Schimdtova and Baldwin, 2011).

Mine sites in Yukon (Figure 2) are located in remote locations that experience extreme winter conditions, such as short days and freezing temperatures. In addition, the limited access to power makes active treatment systems more difficult, making the passive treatment option become more attractive. Moreover, the extreme winter conditions also impede the biological functions of the passive systems through reduced biomass availability and reduced microbial activity in terms of biomass degradation and sulphate-reducing ability.



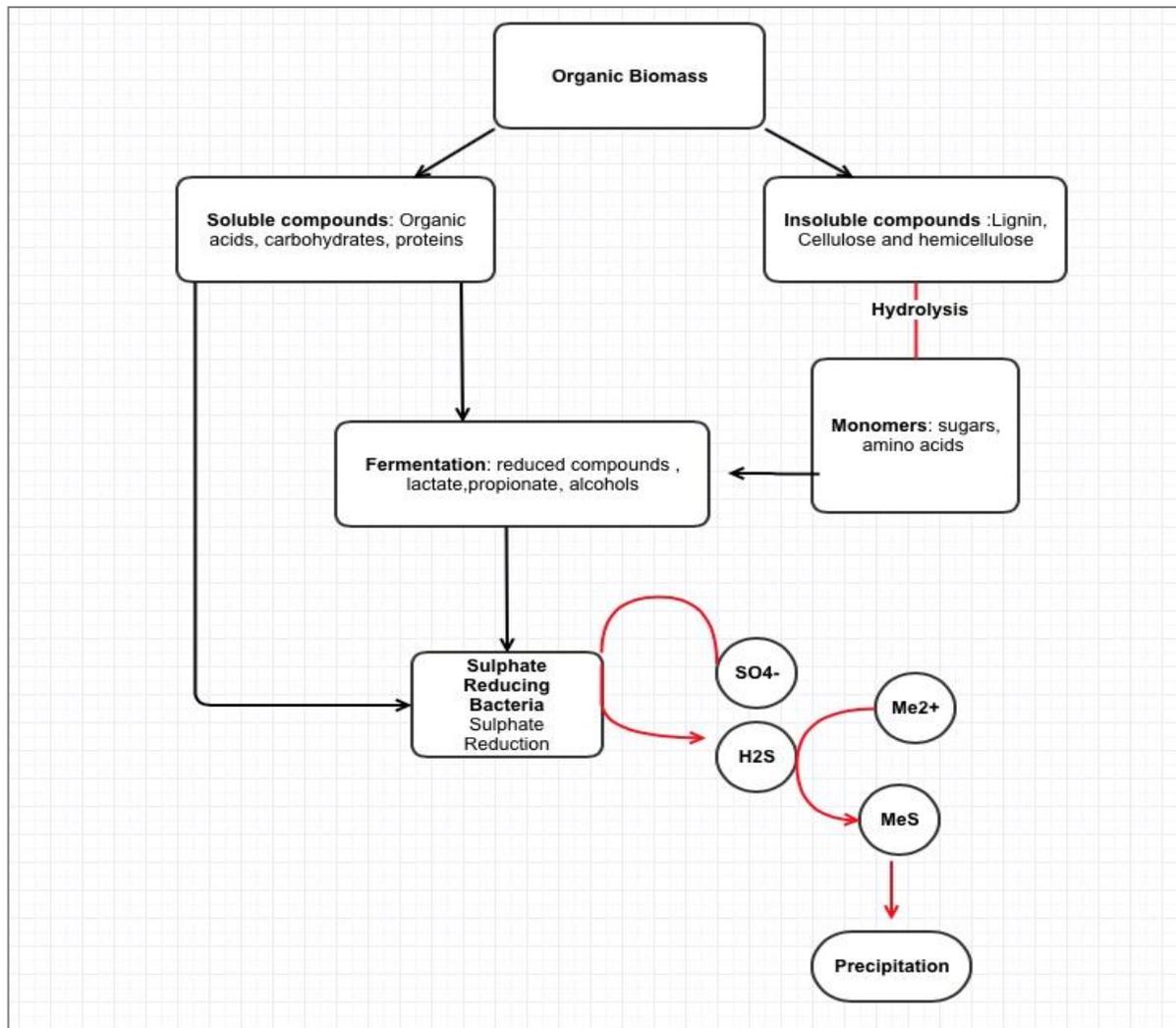
**Figure 2.** Project location map, Keno Hill District, Yukon. (From Janin and Harrington, 2015)

## 2.2 MICROBIAL DEGRADATION OF ORGANIC SUBSTRATE IN PTS

As discussed above, passive treatment systems rely on microbial communities, in particular, SRB that occur naturally in the environment, to reduce sulphate and precipitate metals from contaminated water (Figure 3). SRB use soluble, low molecular weight molecules like simple sugar, organic acids, amino acids, and alcohols as carbon sources. The continuous addition of these forms of carbon to the PTSs would be costly and time-consuming, thus local complex organic materials that are inexpensive and easily obtained are sought for use in these systems.

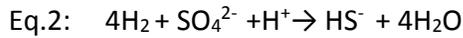
Plant biomass is comprised of a variety of constituents, including lipids, proteins, sugars, and larger complex carbohydrates like cellulose, hemicellulose, and lignin. When the available, low molecular weight molecules are used, the hydrolyzing and fermenting bacteria can degrade

complex, recalcitrant, insoluble organic materials into molecular forms that can be used by the SRB.



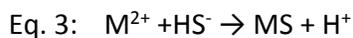
**Figure 3.** Schematic diagram of a PTS showing the degradation of complex carbon materials resulting in metal precipitation.

SRB use sulphate as an electron acceptor during carbon oxidation (Eq. 1) and (Eq.2):



Where CH<sub>2</sub>O, representing a small organic carbon molecule (i.e., lactate, etc.), or H<sub>2</sub> serves as the electron donor. This process consumes hydrogen ions and produces bicarbonate ions, thus increasing pH and alkalinity (Johnson and Halleberg, 2005)

The produced sulfide reacts with dissolved cationic metals (M<sup>2+</sup>) to form the metal sulfide precipitate (Eq. 3) (Prasad, 1999; Neculita and Zagury, 2008):



SRB are a diverse group of microorganisms, with members from both domains of archeal and bacterial prokaryotes (Muyzer and Stams, 2008). Prokaryotic organisms are unicellular organisms that lack membrane-bound nuclei or organelles. Despite having a diverse phylogeny and thus having a range of temperature requirements and conditions, they have an optimal laboratory culture temperature and pH of 30°C and 5-9.5, respectively (Postgate, 1979). Some taxa of SRB can also use NO<sub>2</sub>/NO<sub>3</sub> and O<sub>2</sub> as electron acceptors, while some are inactivated by the presence of oxygen. Therefore, these require anoxic conditions for optimal sulphate reduction. A redox potential of -100 mV to -200 mV is conducive to sulphate reduction (Postgate, 1979; Muyzer and Stams, 2008).

As sulphate reduction depends on a continuous supply of appropriate low molecular weight compounds for the SRB, much research has attempted to identify suitable organic materials for use in PTSs, including reactive barrier walls and bioreactor systems. A diverse consortium of possible complex organic materials, including compost, sheep and chicken manure, wood chips and sawdust, hay, leaf mulch, molasses, and pulp mill bio-solids, etc., have been tested in bench-scale bioreactor systems. Research has mainly focused on creating reactive mixtures of the above components to produce a substrate for short-term use by SRB and slower degrading materials to sustain longer-term reduction.

Using laboratory-based batch tests and flow-through column bioreactors, researchers have endeavored to find correlations between the organic substrate characteristics, like TOC and DOC content, carbon to nitrogen (C/N) ratios, etc., and the efficiency of sulphate reduction over a specified time course (Waybrant et al., 1998; Zagury et al., 2006; Neculita and Zagury, 2008; Schmidtova and Baldwin, 2011).

Although the results of the research studies have not been conclusive, some of the characteristics, such as COD/SO<sub>4</sub>, the C/N ratio, and the permeability/surface area of substrate have been shown to affect the ability of materials to support sulphate reduction. Fresh materials, such as woodchips, may contain resin acids that prevent degradation by microorganisms, thus pointing to partially degraded organic materials as a better choice.

Mine sites in Yukon (Figure 2) are located in remote locations that experience extreme winter conditions such as short days and freezing temperatures. The limited access to power also presents a hurdle to implementing active treatment systems, making passive treatment a more attractive option. Moreover, the extreme winter conditions also impede the biological functioning of passive systems by reducing the available biomass and reducing microbial functioning in terms of biomass degradation and sulphate reduction. The use of bench-scale testing may help in characterizing various organic materials and reactive mixtures at low temperatures and provide insight into the potential use of PTSs in these conditions.



## 3.0 MATERIALS AND METHODS

### 3.1 SITE DESCRIPTION AND LOCATION

The Keno Hill District is located 330 km north of Whitehorse, Yukon, in the traditional territory of the Na-Cho-Nyak Dun First Nation. The district is approximately 233 km<sup>2</sup> and contains 35 mine sites. This district has more than 100 years of recorded history of mining activity (Alexco Resources, 2016).

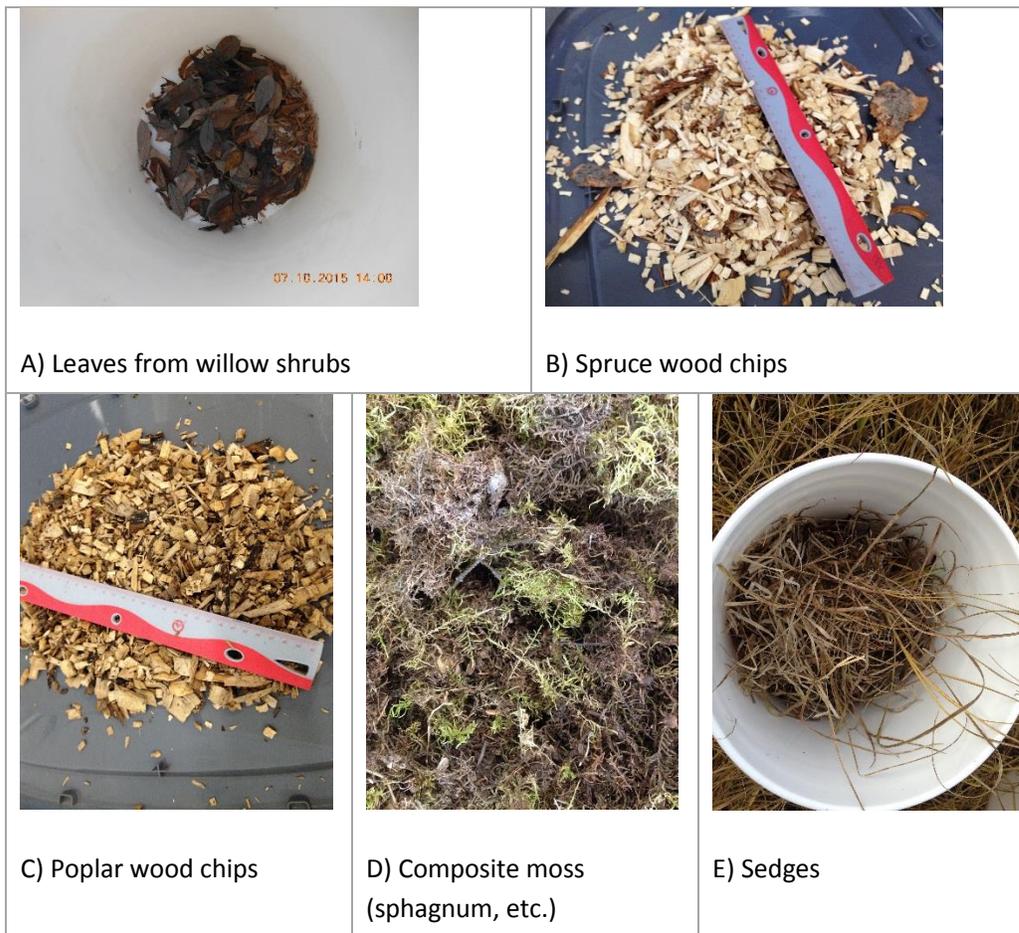
### 3.2 COLLECTION OF MATERIALS

Sediments used as a source of microbial inoculum were collected from creek beds located within the Keno Hill region of Yukon (Figure 2) and they were maintained in cold storage prior to use in bioreactors. A composite sample of inoculum was produced by sieving equal parts of sediments from three different creek beds to a maximum diameter of 2 cm, and the parts were thoroughly mixed together.



**Figure 4.** Inoculum sediments collected from the Keno Hill region.

Six carbon substrates were analyzed in this project, including leaves of willow shrubs (mixed species common in Yukon), decaying sedges, peat, composite moss samples (sphagnum, etc., including miscellaneous plant and organic soil material), spruce chips, and poplar chips. Senesced willow leaves (leaf litter) and decaying sedges were collected from a local wetland area close to the Yukon College campus, Whitehorse, Yukon. The materials were air-dried in an indoor facility and stored until used. Similarly, local sources of composite moss substrate samples were obtained from local areas and air-dried prior to use. Samples of peat, dried spruce, and poplar wood chips had been collected previously by Yukon Research Centre staff. Further details pertaining to the collection of materials and the processing are described in Appendix 1.



**Figure 5.** Locally collected substrate materials used in project.

### 3.3 CHARACTERIZATION OF ORGANIC MATERIALS

The physiochemical properties of organic materials were characterized as described below. Each analysis was performed in triplicate, unless otherwise stated.

The pH of carbon substrate was determined following standard methods (Method D 4972-95a, ASTM) for soils, with some modifications. Briefly, 2 g of each substrate sample was homogenized and suspended in deionized water to a final volume of 20 mL (1:10). Paste samples were measured using an Oakton PCD 650 multi-parameter meter (Vernon Hills, IL). The Oakton double junction pH electrode was calibrated with Fisher certified buffer solutions (Fisher Scientific, Canada) prior to measurements.

The total volatile solids (TVS) content of each carbon substrate was assessed following Method 1684 EPA with modifications. Briefly, samples were dehumidified at 60°C for 24 hrs and then ignited at 500°C in a muffle furnace. Following each drying step, the mass of the sample was used to determine fixed (or ash) and the volatile solids of each sample.

The metal content of the inoculum sediment and carbon substrate samples were determined following a Yukon Research Centre protocol developed from CEAEQ MA205-As 1.0, with modifications. Each extraction procedure included blanks and the spikes contained single element standards (SCP Sciences, Quebec). Extracts were analyzed using a PinAAcle 900 Atomic Absorption Spectrophotometer (Perkin Elmer, USA) by a Yukon Research Centre staff member. Cd, As, Se, Cu were analyzed by Grafite Furnace (GFAA) and Zn and Fe by Flame (FAA). The quantification limits are defined for each element as 0.8 µg/L As, 0.1 µg/L Cd, 0.6 µg/L Cu, 2.0 µg/L Se, and 6.0 µg/L Zn and 35 µg/L Fe. The instrument was calibrated using single element standards (SCP Science, Quebec). Mixed verification standards (Perkin Elmer, USA) and blanks were analyzed per 15 samples. Analyses were performed in accordance with Perkin Elmer protocols which meet EPA requirements.

The total carbon content of each substrate was determined using a Primacs TOC Analyzer (Skalar, Netherlands) following Yukon Research Centre protocols developed from the Primacs user manual. Briefly, substrate materials were dried, milled, and sieved to 412 µm, prior to analysis. The instrument was calibrated using oxalic acid dihydrate standards (ACS reagent, Sigma-Aldrich Canada), as described in the Primacs user manual.

The initial DOC content of each substrate was determined following methods from Schmidtova and Baldwin (2011). Briefly, 2 g of each substrate was suspended in 40 mL of deionized water, placed in a reaction vessel and shaken at a high velocity for approximately 2 hours. The supernatant was filtered using a 0.45 µm filter, and analyzed for carbon content using a Formacs TOC/TN Analyzer, following Yukon Research Centre protocols developed from the

Formacs user manual (Skalar 2012). The instrument was calibrated using Accuspec carbon and nitrogen standards (SCP Sciences, Quebec). QC standards including blanks were analyzed per 20 samples. The TOC/DOC/TN analyses were performed in accordance with Skalar protocols, which meet international standards including EPA and ASTM.

Quantification of total nitrogen (TN) of each carbon substrate was contracted out to the ALS Group Environmental Division as resources were not available for these analyses.

### 3.4 BIOREACTOR DESIGN AND OPERATION

The carbon degradation of each substrate and the corresponding reduction of sulphate and metal precipitation at low temperatures was assessed using bench-scale bioreactors operating over a 150-day period.

The bench-scale bioreactors were constructed from 4 L glass jars. Sampling ports were fashioned from rubber bungs, silicon tubing, and sterile syringes, which enabled the un-intrusive sampling of effluent. Bioreactors were vented with 0.2 µm filters to allow gas to escape, but preventing the intrusion of possible contaminants.

Duplicate bioreactors for each of the six substrates were prepared for slightly acidic (pH 5.1) and neutral synthetic mine wastewater (pH 6.7), for a total of 24 experimental bioreactors. Bioreactors were composed of 10% (v/v) inoculum, 40% (v/v) sand, and 50% (v/v) carbon substrate, as shown in Table 1. After assembly, the bioreactors were filled with ~2.4 L of synthetic mine water, and topped off with an additional volume of deionized water (up to 240 mL) to reduce air spaces and create relatively anoxic conditions that would be present in a passive bioreactor treatment system. Final weights of substrates and materials in each bioreactor is presented in Table 7 (Appendix 2).

Synthetic mine water was made using deionized water and metals from reagent-grade metal sulphate salts of As, Fe, Cd, Cu, Se, and Zn. The target concentrations of the metals were 5 mg/L for Cu, Fe, and Zn, and 0.5 mg/L for As, Cd, and Se. These concentrations reflect moderate to high values, as observed at water monitoring stations at mine sites in the region of interest, and/or high potential model-predicted values (Janin, 2014). Target sulphate concentrations of 500 mg/L reflect high value ranges, as previously observed in the region (A. Janin, personal communication). The pH of the synthetic mine water was adjusted with H<sub>2</sub>SO<sub>4</sub> or NaOH. The actual measured values of the acidic and neutral synthetic mine water are presented in Table 6 (Appendix 2). Ethanol is a soluble, low molecular weight molecule that is directly usable as a carbon source by SRB, and thus was used as a carbon substrate control in both acidic and neutral conditions. Bioreactors containing mine water, sand and ethanol (no sediments) were used as 'inoculum controls' to determine impacts of the microbial inoculum. Bioreactors used as controls for the effluent monitoring were: deionized water and sand only; deionized water, sand, and inoculum; acidic mine water; neutral mine water;. The deionized water controls were used specifically to monitor background presence or concentrations of sulphate and TOC in sand and sediments. The acidic and neutral mine water controls were used to monitor changes in metal and sulphate concentrations over the duration of the experiment due to precipitation, etc. Specific design and operation details are summarized in Table 1. The bioreactors were incubated in an outdoor facility during winter months that was heated to 5°C. Effluents were sampled at the time of filling (T=0) and at days 60, 105, and 150.

The bioreactor effluent was analyzed for pH, oxidation-reduction potential (ORP), sulphate and dissolved metal concentrations, TOC, and TN concentrations.

**Table 1. Summary of bioreactor experimental design and operating conditions**

Parameter	Operating Conditions
Reactor	4 L bottle; no flow; plugged, vented with 0.22 µm filter to maintain anoxic conditions. Syringe attached to sampling port to withdraw samples.
Reactor filling	10% (v/v) inoculum, 1536 mL (40% v/v) sand, 1920 mL (50% v/v) organic material (carbon substrates)
Synthetic mine water	Acidic SMW pH 5.1; Neutral SMW pH 6.7
Duration	150 day totals (sampling at 0, 60, 105, and 150 days)
Effluent volume	2.4 L + DI water to top-up bottle (avoid air space)
Temperature	Outdoor shed, heated to 5°C.
Replicates	Duplicate bioreactors for each of the six substrates with acidic and neutral effluents (24 total).
Ethanol control	One bioreactor containing sand, inoculum, and 1.34 mL ethanol with acidic and neutral effluents (2 total).
Inoculum control	One bioreactor containing sand and 1.34 mL ethanol with acidic and neutral effluents (2 total).
DI water controls	One bioreactor containing sand, inoculum, and distilled water; one bioreactor containing sand and distilled water.

After the *in situ* treatment, bioreactors were dismantled. Substrate samples were air dried and analyzed for DOC and TVS, as previously described. Sediment samples were obtained by scooping up the top ~2 cm layer of the sand-inoculum matrix using a 1 mm sieve and refrigerating it at 4°C for future analysis.



**Figure 6.** Assembled bioreactors.

### 3.5 BIOREACTOR MONITORING

Approximately 60 mL of effluent sample was withdrawn from bioreactors at each interval. Samples were collected using syringes attached to the sampling ports, as described in Section 3.4, so that the bioreactors incurred minimal disturbance. Samples were separated and treated according to the requirements of each assay.

The pH and ORP of each bioreactor effluent was determined using an Oakton PCD 650 multi-parameter meter. The pH probe was calibrated as described in Section 3.3. The Oakton ORP probe was calibrated using the Orion ORP standard (Fisher Scientific Canada).

Sulphate concentrations were determined spectroscopically, using a SmartChem<sup>®</sup> 170 Analyzer (Unity Scientific, USA) following Yukon Research Centre protocols, which were developed from the SmartChem<sup>®</sup> 170 Analyzer user manual. The instrument was calibrated using Accuspec sulphate standards (SCP Sciences, Quebec). QC standards at lower and upper detection limit concentrations of 10 mg and 80 mg, respectively, were analyzed per 10-15 samples.

Dissolved metal concentrations were determined using a PinAAcle 900 Atomic Absorption Spectrophotometer (Perkin Elmer, USA) and the TOC/TN content was determined using a Formacs Total Carbon Analyzer (Skalar, Netherlands), as described above.

After approximately 150 days, the bioreactors were disassembled and the remaining substrate was analyzed for its metal concentration and TVS, following methods indicated in Section 3.3.

## 4.0 RESULTS AND DISCUSSION

### 4.1 SUBSTRATE CHARACTERISTICS

Prior to the *in situ* treatment, metal extractions were performed on all carbon substrate materials to determine the baseline concentrations of each of the six metals analyzed in this study. No leaching tests were performed, and the post-treatment concentrations were not determined, due to time constraints. Data is tabulated in Appendix 2.

The characteristics of the organic substrates being tested for possible use in Yukon varied in terms of content and proportion of dissolvable and recalcitrant carbon constituents, nutrients, and acidic components. The results of the analyses are summarized in Table 2.

The pH values of the substrates were all slightly acidic, with carex being the most neutral (6.6) and spruce (4.4) and moss (4.9) substrates being the most acidic. Although the total carbon composition ranged from approximately 37% to 48%, the more herbaceous materials (carex, willow, and moss) had a higher DOC and a slightly lower TVS, compared to the wood substrates. Presumably, these substrates have a higher proportion of easily degradable substances and a lower content of the more recalcitrant materials (i.e., lignin and cellulose) compared to the woody substrates. The C/N ratios of carex, moss, and willow were also much lower than those of the woody substrates (i.e., spruce).

**Table 2. Physiochemical Properties of Organic Substrates**

Results are expressed as mean  $\pm$  standard deviation from n=3, except TC, where n=2 and total nitrogen, where n=1; DOC=dissolved organic carbon, TC=total carbon, TVS=total volatile solids, TN=total nitrogen.

Parameter	Carex	Willow	Spruce	Peat	Moss	Poplar
pH	6.6 $\pm$ 0.1	6.3 $\pm$ 0.0	4.4 $\pm$ 0.1	6.0 $\pm$ 0.0	4.9 $\pm$ 0.1	5.0 $\pm$ 0.1
Moisture (%)	8.1 $\pm$ 0.2	13.1 $\pm$ 0.1	2.6 $\pm$ 0.2	25.9 $\pm$ 2.1	8.12 $\pm$ 0.5	3.4 $\pm$ 0.4
DOC (mg/L)	514.2 $\pm$ 17.1	971.1 $\pm$ 58.1	250.9 $\pm$ 15.4	27.4 $\pm$ 4.6	835 $\pm$ 98.9	223.5 $\pm$ 41.7
TC (g/Kg dry wt)	402.7 $\pm$ 7.6	477.2 $\pm$ 4.8	477.6 $\pm$ 24.9	371.8 $\pm$ 10.8	411.9 $\pm$ 5.3	482.5 $\pm$ 79.3
TVS (% dry wt)	89.0 $\pm$ 0.2	91.4 $\pm$ 0.1	99.4 $\pm$ 0.1	35.8 $\pm$ 3.5	85.8 $\pm$ 1.4	98.3 $\pm$ 0.4
TN (% dry wt)	0.937	0.71	0.073	0.396	0.696	0.454
C/N ratio	50	78	762	109	69	124

Peat had the lowest TC, DOC, and TVS content, indicating a relatively high mineral/organic ratio. As expected, DOC decreased in all substrates during the bioreactor treatment, except for peat (Table 3). Larger losses of DOC were seen from the herbaceous substances, with decreases of approximately 67%, 73% and 77% for carex, willow and moss respectively, compared to decreases in poplar of approximately 63% and Spruce of approximately 44%. The change in TVS for the substrates was not significant, except for poplar and peat. However, the post treatment TVS analysis of peat may be inaccurate, as it was difficult to separate the peat from the sand matrix.

**Table 3. Changes in composition before and after treatment**

Results are expressed as the average  $\pm$  standard deviation from  $n=3$  samples. Post in situ treatment analysis was performed on materials from the neutral treatment bioreactor for each substrate.

Time (days)	DOC (mg/L)		TVS (%w/w)	
	0	150	0	150
Carex	514.2 $\pm$ 17.1	171.8 $\pm$ 15.0	89.0 $\pm$ 0.2	88.1 $\pm$ 1.0
Willow	971.1 $\pm$ 58.1	261.0 $\pm$ 49.5	91.6 $\pm$ 0.1	91.4 $\pm$ 0.1
Poplar	223.5 $\pm$ 41.7	82.9 $\pm$ 15.1	98.3 $\pm$ 0.4	95.0 $\pm$ 0.8
Spruce	250.9 $\pm$ 15.4	140.6 $\pm$ 13.2	99.4 $\pm$ 0.1	98.4 $\pm$ 0.4
Moss	835.0 $\pm$ 98.9	190.9 $\pm$ 15.7	85.8 $\pm$ 1.4	85.2 $\pm$ 1.7
Peat	27.4 $\pm$ 4.6	27.5 $\pm$ 5.0	35.8 $\pm$ 3.5	25.1 $\pm$ 0.5

Researchers have attempted to predict or correlate substrate degradability with bulk properties of substrates or reactive mixtures used in PTS (Prasad, 1999). Criteria include DOC, easily available substances (EAS), more recalcitrant substances (lignin), and TVS as an indicator of the total organic material available (Vasquez et al., 2015). Although the trend indicating that lower lignin content (Gilbert et al., 2004, etc.) and higher herbaceous content has higher DOC (Schmidtova and Baldwin, 2011; Lefticariu et al., 2015), favouring SRB growth and contaminant sequestration, the results of these studies have not been conclusive.

Agricultural research uses C/N ratio for solid organic materials as a metric to assess whether or not the soil or compost macronutrients are optimal for microbial decomposition. For decades, researchers have used the ratio in assessing the potential for organic substrates and mixtures to support sulphate reduction and the subsequent metal precipitation in PTSs. To date, the results have been inconclusive. While some authors have reported that ratios below  $\sim 10$  perform better (Prasad, 1999), others have not found any relationship between the ratio and performance (Zagury et al., 2006). Schmidtova and Baldwin (2011) make two interesting points: 1) very different C/N ratios have been reported for similar materials depending on their “nature, location and decomposition state”; and 2) influent waters may have sufficient concentrations of nitrate and ammonium to provide a nitrogen source for bacteria, thus allowing high C/N ratio materials to be more effective.

In their study, Schmidtova and Baldwin (2011) found a correlation between the C/N ratio and SBR abundance and sulphate reduction rates in a column bioreactor treatment of MIW. C/N ratios of the substrates ranged between 20 and 60, and the C/N ratio of 60 (silage) resulted in the highest SRB abundance and sulphate reduction rate.

In our study, substrates with lower C/N ratios (i.e., carex, moss, and willow), with ratios of 50, 69, and 78, respectively, had more rapid and greater overall rates of reduction of sulphate concentrations, compared to those with higher ratios (i.e., peat, poplar, and spruce), with ratios of 109, 124, and 762, respectively. Nevertheless, given the effects from the specific properties of each substrate (e.g., the very low carbon content of peat), it would be difficult to determine the impact that the C/N had on the bioreactor results.

## **4.2 BIOREACTOR MONITORING**

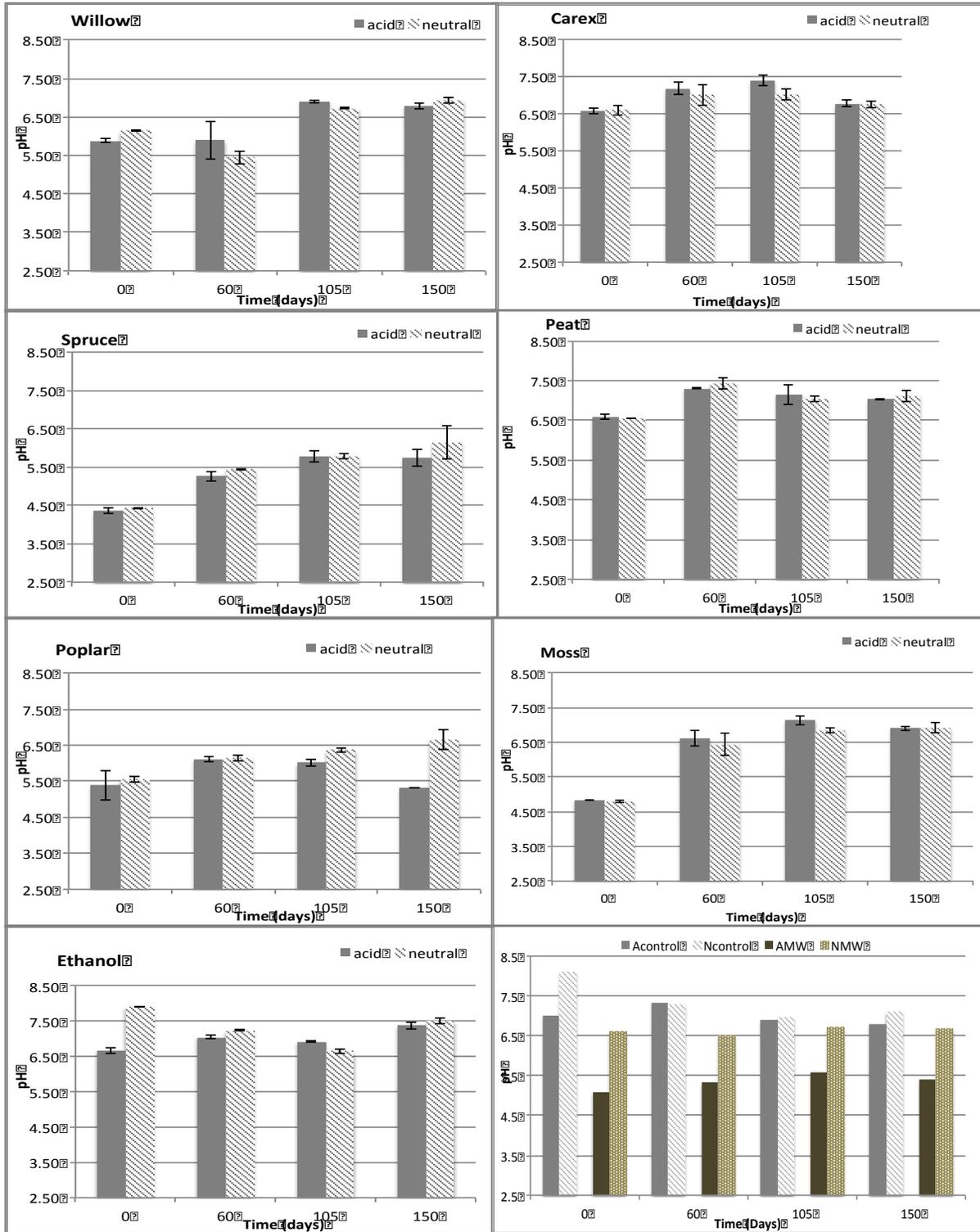
### **4.2.1 Temperature**

One of the objectives of this study was to observe the effect of low water temperatures, as experienced in northern climates, on the rates of degradation of organic substrates in PTS. During the first period (day 0 to 60) of this experiment, the average temperature in the outdoor shed was 5.2°C. However, the shed had no refrigeration capacity, and the unusually warm temperatures from February through April resulted in average temperatures of 8.2°C and 9.4°C during day 60 to 105, and day 105 to 150, respectively. Nevertheless, these temperatures are relatively low compared to similar batch or column bioreactor experiments where temperatures are typically ~21°C (Zagury et al., 2006; Neculita et al., 2008; Vasquez et al., 2016).

### **4.2.2 Impact of Substrate Composition on Effluent pH**

Another objective of this study was to determine the impact of the mine water pH on the degradation of carbon substrates and the microbially-mediated sulphate reduction. Nevertheless, the addition of 50% (v/v) organic substrate to each bioreactor caused the pH of the effluent to reflect the pH of the substrate, not the mine water treatment. Throughout the sampling period, the trend in pH values of the acidic or neutral treatments for each substrate was similar, as demonstrated by the standard deviation of the measurements in Figure 7.

To reduce the repetition, only the results for the neutral treatments of the substrates are displayed in the graphs of this report. Acid treatment data is available in Appendix 2.



**Figure 7.** Comparison of pH values of effluents from AMW and NMW treatments for each substrate during the sampling period.

### 4.2.3 DI Control Data

As described in the Materials and Methods section, DI water control bioreactors were used to assess the possible contributions of sand and inoculum to the overall concentrations of metals, sulphate, TOC, and TN and to provide a baseline for the bioreactor monitoring measurements (Table 4). Both controls contained small but measurable levels of carbon, nitrogen, sulphate, and metals. With the exception of sulphate, all measured constituents decreased after T=0.

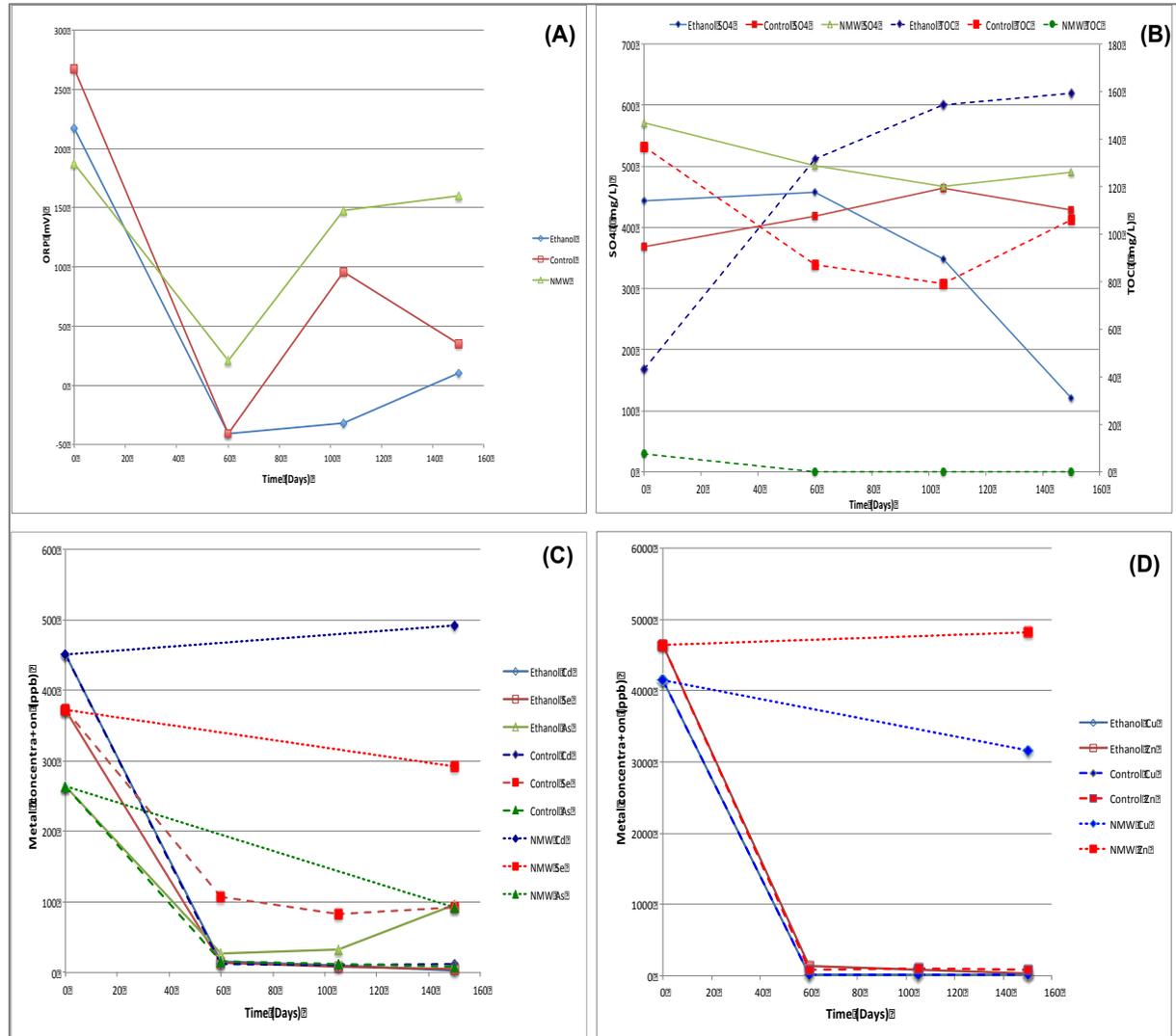
**Table 4. Results of the Deionized Water Control Bioreactor Monitoring**

Control	Time (days)	pH	ORP (mV)	TOC (mg/L)	TN (mg/L)	SO <sub>4</sub> (mg/L)	Cd (ppb)	Cu (ppb)	Fe (ppb)	Se (ppb)	Zn (ppb)	As (ppb)
DI-S	0	8.1	73.8	13.5	2.7	3.8	0.56	17.6	12885.5	4.0	61.5	4.7
	60	7.1	-30.9	8.5	0.23	55.0	<0.05	<12.3	558.2	3.5	14.3	1.5
	105	7.5	30.0	N/A	N/A	32.2	<0.05	<12.3	464.0	3.0	22.0	1.0
	150	7.3	35.0	8.5	0.26	35.8	<0.05	<12.3	130.6	2.5	6.8	<0.8
DI-S-Inoc	0	7.4	33.0	31.9	4.8	3.7	3.36	163.3	22630.2	2.6	317.6	56.9
	60	7.5	-48.6	18.1	0.61	72.5	<0.05	<12.3	197.8	3.2	21.2	6.0
	105	7.2	31.0	N/A	N/A	89.7	<0.05	<12.3	141.7	4.0	16.4	5.5
	150	7.3	38.0	24.6	0.66	85.6	<0.05	<12.3	58.4	3.6	6.0	5.4

### 4.2.4 Inoculum Controls

As described in the Materials and Methods section, an acid treatment control and a neutral treatment control, comprised of synthetic mine water, sand, and ethanol (no inoculum) were used to determine the impacts of the inoculum sediments on the measured parameters for the bioreactors. Containers with either acidic synthetic mine water (AMW) or neutral synthetic mine water (NMW) were also monitored during the experiment. These controls were compared to 'ethanol control' bioreactors, which contained inoculum in addition to sand. Effluent sulphate concentrations in both the acidic and neutral ethanol controls, with inoculum, decreased by just over 70%, from T=0 to T=150. In contrast, the acid control, the neutral control, and the AMW and NMW remained relatively stable throughout the experiment (Figure

8). Although the TOC appeared to increase in the ethanol bioreactor, this may be due to sampling error at T=0, or to changes in measured concentrations due to interactions between ethanol and the sand or sediments. All controls showed decreases in ORP from T=0 to T=60. Ethanol ORP remained negative until T=150. Measurements were taken from the samples after being withdrawn from the bioreactors. Although the measurements were recorded immediately, oxygen was likely being introduced to the effluent, altering the ORP values. For greater accuracy, the ORP should be measured at or below the sediment surface.



**Figure 8.** Temporal variation of ORP (A), sulphate concentrations (B), Cd, Se, and As concentration (C), and Cu and Zn concentration (D), of synthetic mine water, ethanol, and no inoculum control bioreactors.

Within the first 60 days of bioreactor operation, Zn, Cu, Cd, and Se effluent concentrations in the ethanol bioreactors (sand, inoculum, and ethanol) and the controls were reduced by up to 90-100% of the initial neutral mine water concentrations (Figure 8). In contrast, the ethanol bioreactor displayed a reduction in the sulfate concentration, a negative ORP, and a reduced DO (data not shown), suggesting that physiological changes may have been mediated by microbial activity. In addition, Figure 9 shows the substantial formation of a black precipitate, presumed to be partly a metal sulfide precipitate, by the end of the sampling period. In contrast, the effluent of the control bioreactor remained relatively clear.



**Figure 9.** Comparison of ethanol bioreactor (containing inoculum and ethanol)(left) and control bioreactor (containing ethanol, but no inoculum)(right) at T=150. The ethanol bioreactor had a substantial black precipitate, whereas the control had very little (a clear effluent).

Although sand contains a small amount of carbon and nitrogen and microbes are likely present, a metal precipitation is not likely due to microbial sulfate reduction.

Moreover, sandy soils are generally described as having a low cation exchange capacity (CEC), and the surface functional groups of silicates in sand provide sites for absorption of cations such as the metals described in this study (Korestsky, 2000). Sand also has a small particle size, compared to other substrates like pebbles or small gravel, and the large surface area of sand may retain electrostatically-bound metals in the static environment. Research has demonstrated that slow sand filters can be an effective mechanism for filtering low concentrations of heavy metals from water (Awan et al., 2003). The analysis of MIW commonly reveals significant concentrations of cations, such as Ca, Mn, Na, K, and Mg. Ideally, mine water obtained from mine locations in the area under investigation should be used in the bench-scale passive treatment studies. Alternatively, however, many researchers have composed synthetic mine water, as reflected by the inclusion of the above ions (Waybrant et al., 1998; Vasquez et al., 2016; Neculita and Zagury, 2008, Lefticariu, 2015, among others). These cations may impact the precipitation of metals in the water by competing for binding sites on substrates, sediments, and sand, or through additional geochemical processes. The synthetic mine water used in this study was composed of deionized water, metal sulfate salts, and metal oxides, as described in the Materials and Methods section. The omission of other components could have an impact on

the physiochemical qualities of the bioreactors, ultimately leading to the metal sequestration observed in this study.

Thus, even though the ethanol and control bioreactors appeared to be physiologically different, the reduction in metal concentrations observed in both are likely the result of abiotic processes due to the specific experimental conditions.

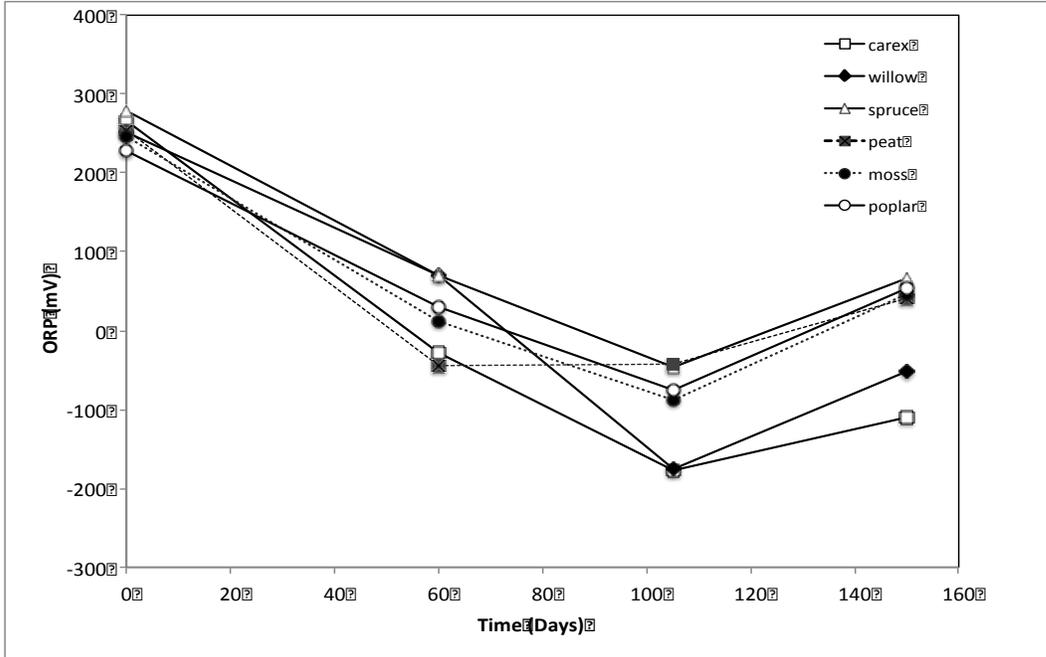
#### **4.2.5 pH and ORP**

As discussed above, the pH of the effluent at T=0 mirrored the substrate pH. The pH of more acidic substrates (i.e., spruce moss and poplar) (Figure 7) increased toward more neutral values, and by T=60, all bioreactor effluents were within a suitable range for SRB growth (Postgate, 1979). Generally, the effluent pH of the four bioreactors for each substrate was similar at each time point, regardless of treatment. Interestingly, the effluent from the acidic poplar bioreactors returned to the starting pH of ~5.5, in contrast to the neutral treatment that had a final average value of ~6.5.

None of the bioreactors attained ORP values of -100 mV or below, which is typically considered as a suitable reducing condition for bacterial sulphate reduction (Postgate, 1979) until after T=60 (Figure 10). By T=105, the effluents of the carex and willow bioreactors were below -100 mV and the poplar, moss, and ethanol values approached -100 mV. The effluent of the spruce and peat bioreactors did not reach conditions suitable for reduction at any time during the sampling.

Based on the development of suitable ORP and overall sulphate concentration reduction exhibited by substrate bioreactor effluents, the herbaceous substrates (moss, carex, and willow leaves) were more effective than the woody substrates (poplar, spruce) at low temperatures.

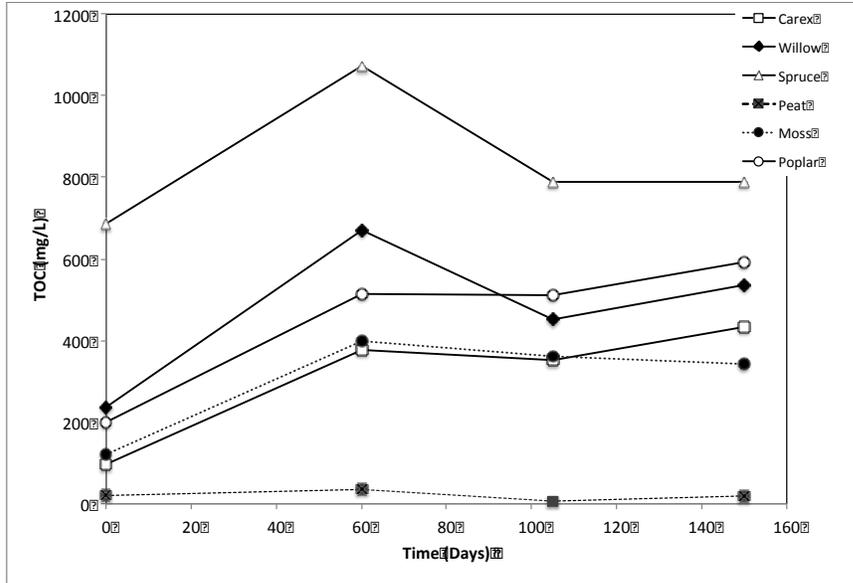
To determine why the more herbaceous substrates appeared to be more efficient under these conditions, the properties of substrate materials and the resulting effluent characteristics were examined. Presumably, substrate effluents with a higher TOC (i.e., higher concentration of lower molecular weight carbon compounds) would provide a more immediate carbon source for both fermenting bacteria and SRB. Peat, with the lowest TOC content was completely ineffective based on the above criteria. Spruce, however, had the highest TOC concentration, but also performed poorly compared to the herbaceous substrates and poplar during the time-frame of the experiment.



**Figure 10.** Temporal variation of ORP in neutral substrate bioreactors. Results are expressed as average of duplicates.

#### 4.2.6 TOC and Nitrogen

Substrates were added to the bioreactors on a percent (v/v) basis, therefore, the total weights of substrates added varied significantly between the different substrate bioreactors. The differences are generally reflected in the TOC concentrations of the effluents (Figure 11).



**Figure 11.** Temporal variation of TOC concentration in neutral substrate bioreactors. Results are expressed as the average of duplicates.

With the exception of spruce, the TOC in the effluent at T=0 was lower than DOC values determined for each substrate (Table 2). By T=60; however, the TOC concentrations in the effluent reached that of the TOC that would be predicted based on the DOC characterization, without the additional degradation of recalcitrant substance. After T=60, spruce and moss effluents had a slight decline in concentration, though the general trend was a stable TOC. Peat effluent TOC remained very low throughout the sampling duration with concentrations little above those of the DI-S inoculum controls (Table 2). The dissolved nitrogen values are presented in Table 9. Nitrogen concentrations steadily increased in all bioreactors except in the acidic treatment of poplar, which did not increase after T=150.

TOC concentrations in the effluents generally remained stable throughout the experiment. Similar results were observed by Zagury et al. (2006). As mentioned above, spruce and moss TOC concentrations declined most substantially from peak concentrations at T=60. Dissolved organic matter may bind to metals and sediments and precipitate from solution. Although some reduction of TOC may be due to microbial assimilation and metabolism, loss of TOC is not an effective parameter for determining microbial growth. The microbial assimilation of carbon is very low and soil anaerobes may incorporate approximately 1-5% and aerobes up to 10% of the carbon used (Alexander, 1991). Carbon can be lost through volatile CO<sub>2</sub> and CH<sub>4</sub> produced through microbial metabolism; however, in complex microbial systems these components can be re-used as carbon sources.

The TOC concentration in the ethanol control bioreactor does not appear to decrease, but rather, increases slightly. Depending on the consortia of bacteria present, ethanol may be reduced only to acetate, not fully to CO<sub>2</sub> (Velasco et al., 2008), and therefore little TOC is lost. Previous studies that focused on optimizing COD/SO<sub>4</sub> ratios for SRB bioreactors, were designed to monitor the changes in substrate concentrations; e.g., ethanol, lactate, acetate (Velasco et al., 2008; Nagpal et al., 2000), not TOC, as indicators of metabolic activity. In bioreactor treatment systems with constant flow, TOC may be assessed by monitoring the availability of carbon; however, TOC loss is not a useful indicator of microbial metabolism in batch reactor systems.

Since the identifying and monitoring of particular DOC constituents that may be used as the substrate or produced as metabolic products in PTS containing complex organic materials would be challenging, chromatographic techniques could also be useful.

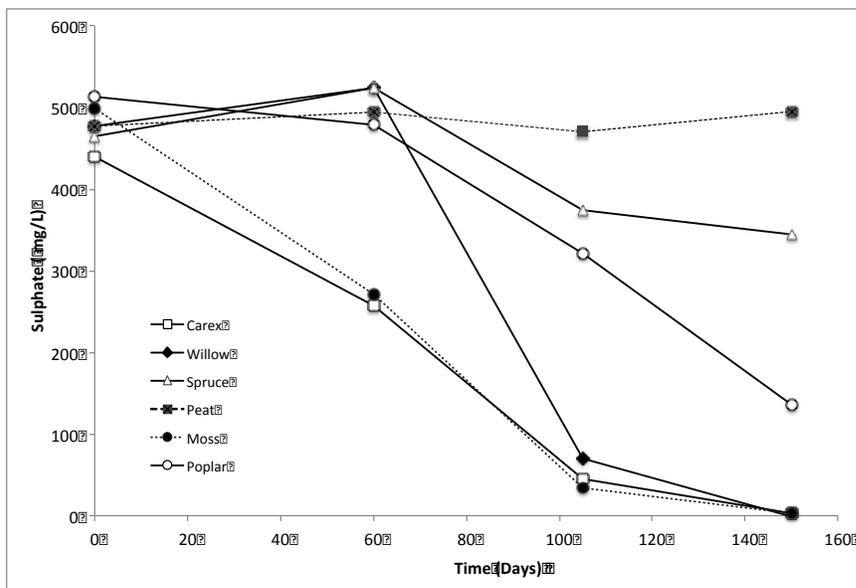
To study DOC cycling dynamics in northern ecosystems, Wickland et al. (2007) used resin-based chromatographic fractionation of DOC components of both pore waters and leachate of the dominant vegetation of the ecosystem, including black spruce twigs and bark and sphagnum moss, to quantify the components of the DOC, including hydrophilic and hydrophobic organic content. To assess biodegradability of DOC leachate from each sample type, the leachate was incubated with local soil materials as a source of native inoculum. Overall, the researchers found a strong positive correlation between hydrophilic DOC content and biodegradability. In particular, they found that spruce materials (twigs and bark) had a high hydrophobic DOC content and very low degradability (11%), whereas DOC extracted from mosses had very low hydrophobic content and higher hydrophilic content, and was rapidly metabolized by up to 93%. They concluded that the chemical nature of the DOC, not the rate of supply, had a greater impact on microbial mineralization.

Conifer tree outer bark tissues function to protect the tree from water loss and pathogen entry, and are composed of compounds, like lignin and waxes, that resist decay. In our study, poplar and spruce wood chips with very small amounts of outer bark materials were used. In any case, the in-depth analysis of qualities of DOC may be a useful indicator of substrate performance in PTS.

#### **4.2.7 Sulphate Reduction**

From T=0 to T=60, with the exception of the moss and carex bioreactors, effluents had no reduction in sulphate concentration and the concentration appeared to increase slightly in the peat, willow, and spruce bioreactors. This increase may be due to sulphate contributions from

inoculum and sand (Table 4). Moss and carex had the most rapid reductions in sulphate concentration, followed by willow, and by the final time point, the sulphate concentrations of these bioreactor effluents were below detection levels (Figure 12). A reduction of approximately 73% of the initial concentration was achieved in the poplar bioreactors, and an approximate 26% reduction in sulphate occurred in the spruce bioreactor effluent by the final time-point. Sulphate concentrations in peat bioreactors; however, remained relatively constant throughout the sampling period. The trends for reduced sulphate concentration values were consistent for each substrate in the acid treatment conditions with the exception of poplar. Interestingly, sulphate was reduced by only 28% in the acidic poplar bioreactor effluent.



**Figure 12.** Temporal variation of sulphate concentration in neutral substrate bioreactors. Results are expressed as the average of duplicates.

To explore the suggestion that mine water influent nitrogen concentrations may override the substrate C/N ratio, the C/N ratio of effluents were compared to the overall reduction of sulphate concentration (Table 5). As discussed earlier, the synthetic mine water used in this study did not contain any nitrogen, so all of the nitrogen in the effluent was the result of nitrogenous compounds dissolved from substrates, sediment, and sand in the bioreactors, or through the microbial degradation of substrates.

C/N effluent ratios follow similar trends to the substrate C/N ratios. Carex and moss effluents, followed by willow, have consistently lower C/N ratios, which are correlated with the complete reduction of sulphate concentration. C/N ratios are similar for acidic and neutral treatments at

each time-point, with the exception of poplar, where higher C/N ratios in the acidic bioreactors correspond to substantially lower sulphate reduction, in comparison to the neutral treatment.

Despite having high concentrations of DOC, bioreactors containing spruce chips had less of a decrease in sulphate concentrations, compared to other substrates, and casual observation revealed little or no microbial activity. Zagury et al. (2006) conducted a similar batch bioreactor analysis of single substrates, including leaf compost, sphagnum, and composted spruce chips and substrates mixed with poultry manure. The substrate C/N ratios were comparable to those found in this study. After 70 days, the authors found little sulphate reduction in sphagnum, leaf, and spruce compost bioreactors and concluded that they were not good substrates. Their sphagnum and leaf compost results appear to be in contrast to the results of this study, though the results for spruce chips are similar. Zagury et al. (2006) suggested that toxic or inhibitory compounds in the conifers may play a role in the lack of reduced sulphate concentrations.

Spruce and other woody tree species produce diterpene resin acids as part of their oleoresin defense system. These compounds have been shown to have antimicrobial effects, possibly by damaging cell walls and interfering with the proton transport across cell walls (Jokinen and Sipponen, 2016).

To assess the potential risk to aquatic ecosystems, Rex et al. (2016) performed experiments designed to emulate potential leachate from wood chip piles generated from road side biomass operations in British Columbia. Microtox™ assays clearly demonstrated that toxic leachate can be produced from wood chips of several conifer species. Although the toxicity did not correlate well with levels of known toxic compounds, the authors' conclusions, in addition to poor performance in bioreactor studies, suggested that using conifers as a sole substrate would not likely be beneficial or perhaps even environmentally sound. If conifer chips are an attractive option, because they are inexpensive and readily available, pretreatment options should also be examined.

Finally, the mechanisms for sulphate concentration reduction observed in this study are not known. Besides microbial sulphate reduction, abiotic factors, like sorption to substrates (Lefticariu et al., 2015) and sorption to ferric(oxy)hydroxides and subsequent precipitation (Waybrant et al., 1998) may also be responsible for the observed sulphate reduction.

**Table 5. C/N ratios of effluent over time and total sulphate reduction**

Substrate	ACID treatment effluent		NEUTRAL treatment effluent	
	C/N Molar ratio	Sulphate reduction % total	C/N Molar ratio	Sulphate reduction % total
<b>Poplar</b>				
T=0	175.7		145.7	
T=60	228.5		169.5	
T=105	190.6		165.6	
T=150	194.0	28.2	130.8	73.5
<b>Spruce</b>				
T=0	N/A		324.9	
T=60	636.5		533.9	
T=105	583.8		471.2	
T=150	274.4	33.4	229.8	25.9
<b>Peat</b>				
T=0	17.0		N/A	
T=60	32.3		37.6	
T=105	12.3		5.9	
T=150	14.9	13.0	11.0	0.0
<b>Carex</b>				
T=0	30.3		23.0	
T=60	42.0		40.5	
T=105	52.6		50.8	
T=150	44.0	100.0	43.2	100.0
<b>Moss</b>				
T=0	55.8		45.1	
T=60	38.1		35.5	
T=105	35.9		39.1	
T=150	27.8	100.0	26.7	100.0
<b>Willow</b>				
T=0	109.8		90.1	
T=60	137.7		119.2	
T=105	152.1		140.4	
T=150	109.4	100.0	115.5	100.0
<b>Ethanol</b>				
T=0	215.4		216.4	
T=60	359.1		401.1	
T=105	290.6		321.2	
T=150	111.3	72.0	91.7	73.3
<b>Control</b>				
T=0			N/A	
T=60	271.3		251.5	
T=105	126.0		279.7	
T=150	1012.0	0.0	1073.7	0.0

C/N ratio is calculated from the average effluent TOC and nitrogen concentrations from duplicate bioreactors. Final sulphate measurements BDL are considered to have 100% reduction.

#### 4.2.8 Metals Reduction

T=0 metal effluent concentrations for all substrates are expressed as initial values of NWM concentrations. Varying amounts of DI water (up to 200 mL) were added to the bioreactors to reduce air space at time of filling; thus, actual concentrations may vary by approximately 5-10%.

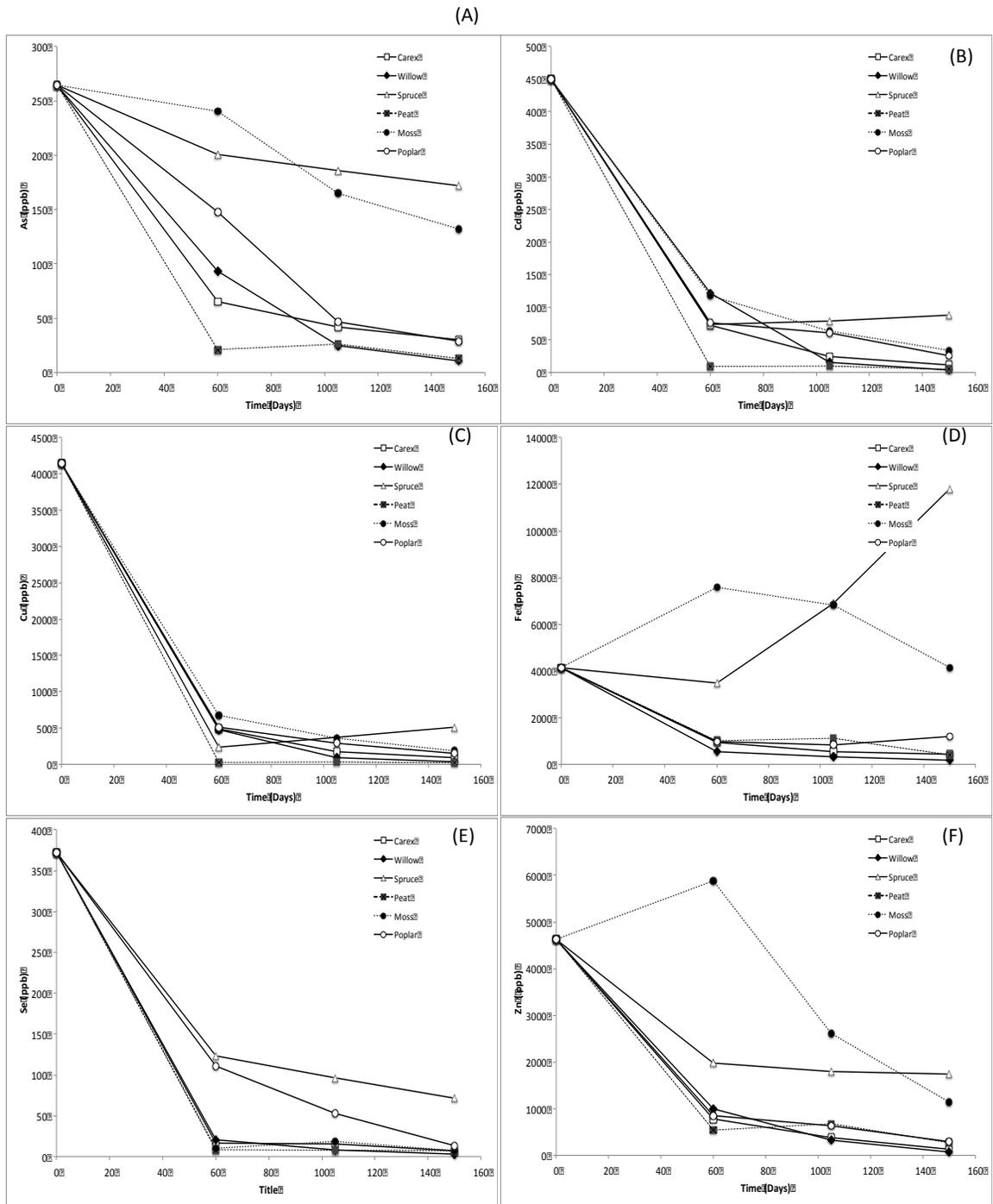
Overall, all bioreactors had large reductions (80-99%) in the effluent concentrations of Cd, Cu, and Se. Carex, willow, and poplar bioreactors also had reductions of 88% or more in Zn and As.

Like the controls containing ethanol as a carbon source, all six metals declined rapidly in the peat bioreactors. By the final time-point, with the exception of As, all metals were reduced by at least 94%.

Spruce and moss effluents have lower levels of reduction of Zn (62% and 75%, respectively) and As (35% and 50%, respectively). Interestingly, Fe concentrations increased substantially above NWM concentrations over time in the spruce bioreactor effluent in both neutral and acidic treatments (Figure 13 (D) and Figure 17 (D) (Appendix2)). This result may be due to the release of Fe from materials at the lower pH in spruce bioreactors at increasing temperatures.

Metals can be removed from effluent through multiple mechanisms. During initial the phases, metals are likely removed primarily through abiotic processes, like adsorption to substrates (Janin, 2014), and (oxy)hydroxide and carbonate mineral precipitation (Neculita et al., 2008). Changes in pH and temperature can also influence the solubility and precipitation of individual metals. In addition, metals can bind to DOC in aquatic environments, with an impact to the transport and partitioning of metals in these environments (McElmurray et al., 2010).

In this study, a large decrease in the concentrations of most metals analyzed in all bioreactors was generally observed, including the controls with no inoculum or organic substrate. To accurately assess the impact of substrate materials on metal removal, either through biotic or abiotic processes, additional analyses should be performed to determine the fate of metals.



**Figure 13.** Neutral treatment bioreactors. Change of concentrations of dissolved As (A), Cd (B), Copper Cu (C), Fe (D), Se (E), and Zn (F) in the organic substrate. T=0 value is equivalent to NWM T=0.

The objective of a PTS is to immobilize metals, preventing them from entering receiving waters on a long-term basis. Therefore, it is important to know the mechanism of sequestration in the system and the potential for the mobilization. Methods for elucidating the fate of dissolved metals in PTS include: geochemical models such as VMINTEQ to assess abiotic factors like pH and temperature that affect solubility and precipitation, and other geochemical processes like complexation and co-precipitation; and sequential extraction procedures (SEP) (Zagury et al., 1997) for the bioreactor content post-operation to determine each fraction of metal in a water soluble/exchangeable state, bound to organic matter, as carbonate or precipitated as insoluble metal sulfides, etc.; the acid volatile sulfide (AVS) (Leonard et al., 1996) analysis of sediments; and scanning electron microscopy (SEM) to quantify and visually examine metal sulfide precipitates on substrate surfaces (Neculita et al., 2008).

To characterize the biogeochemical processes taking place in a PTS, multiple lines of evidence are required, ideally including microbiological characterization. Researchers have used *in vitro* cultivation, including most probable number (MPN) techniques to demonstrate the presence and relative abundance of heterotrophic bacteria (specifically SRB) for decades (Postgate 1979; Neculita and Zagury, 2008; McQueen et al., 2016, etc.). Although these methods are limited, since the potential cultivable soil bacteria is estimated to be approximately 1%, when used with additional lines of evidence, this method may shed light on the microbial processes in PTS.

More recently, molecular techniques like 16S rRNA sequencing have been used to profile the complex microbiomes of MIWs and PTS and assess the relative abundance of specific taxonomic groups in these communities (Schmidtova and Baldwin, 2011; McQueen et al., 2016, among others). Monitoring the changes in a community profile can give valuable insight into the observed processes.

Besides direct evidence, effluent measurements of metabolic indicators of SRB activity, such as sulfide generation ( $\text{HS}^-$ ) and alkalinity ( $\text{HCO}_3^-$ ) (Eq. 1), are useful in studies of PTS.

## 5.0 CONCLUSIONS

This study is the first phase in a multiphase project investigating biodegradation and performance of organic substrates for possible use in a PTS in Yukon. The results are not intended to be conclusive, but may be used to inform future phases of this project. Prior to implementing pilot -scale testing, the next step of this study could be to employ larger, flow-through column bioreactors with experimental conditions as suggested in Section 6.0 of this report. A more thorough investigation may help address deficiencies and data gaps of this study.

### 5.1 GENERAL CONCLUSIONS

- Inoculum controls containing ethanol as a carbon source, but no inoculum sediments showed no significant decrease in sulphate concentration over the duration of the study, whereas bioreactors containing ethanol and inoculum sediments showed significant decreases in sulphate concentration by the final time point. Peat substrate bioreactors, which had very low carbon concentrations, also demonstrated little change in sulphate concentrations. These results suggest that both a minimal concentration of usable carbon source and inoculum sediments are necessary for sulphate reduction, and suggest that microbial sulphate reduction occurred. However, the inoculum controls and peat bioreactors generally showed similar decreases in metal concentrations compared to both ethanol and organic substrate bioreactors. These results indicate that metals were removed primarily by abiotic mechanisms, not by microbially -mediated metal sulfide precipitation.
- The CEC of sediment and inorganic matrices (e.g., sand) may have substantial effects on the chemistry of metals, particularly in static bench-scale bioreactor conditions.
- This preliminary study indicates that the more herbaceous substrates (carex, moss, and willow leaves) appear to support or promote sulphate reduction and microbial-mediated metal sulfide precipitation at low temperature (<10°C).
- DOC or TOC concentrations in effluents do not correlate with sulphate reduction; however, the C/N ratios of effluents may be useful predictors of sulphate reduction and should be investigated further.
- Spruce chips and peat did not perform well as organic substrates based on the parameters of this study. These results confirm the findings of previous studies.

## 6.0 RECOMMENDATIONS

- In future studies, a closer attention to experimental design may lead to more useful findings. The experimental conditions, both physiochemical and operational, including mine water composition and hydraulic retention times, must accurately reflect the particular remediation sites. Components of the impacted water may influence abiotic factors, such as competition for ligand binding with both organic matter and the mineral surfaces of sediments. The nitrogen and phosphorus content of MIW may also have an impact on the microbial degradation of substrates.
- If changes to metal concentrations (i.e., reduction) are used as a measure of substrate efficiency, then the properties of the experimental materials and the final fate of metals should be examined. The cation exchange capacity and Langmuir absorption curves should be established for sediments and materials to better understand the experimental results.
- The mechanisms of sulphate and metal sequestration and the removal from PTS need to be closely examined. Research has established that during the initial phases and at cooler temperatures, metals are sequestered in PTS primarily through abiotic processes. Much more research should be focused on determining the fate of metals in these systems and their potential for remobilization. Organic constituents that may serve as ligands will degrade over time. It would be useful to understand these processes for the sake of long-term planning. Techniques like SEP, AVS, and SEM could be used to characterize the partitioning of metals in PTSs.
- To establish the efficacy of substrates supporting microbial-mediated metal precipitation, multiple lines of evidence are needed for the microbial activity. Useful techniques would include AVS, DO, alkalinity, and examination of microbial community development with 16S rRNA sequencing, dsr qPCR, and MPN, etc.
- Many previous studies have focused on the DOC content and ratios for readily available vs. recalcitrant materials, indicating the ability of organic substrates or mixtures to support microbial sulfate reduction and metal precipitation. While having sufficient DOC is clearly important, research indicates that the quality, not quantity and availability of additional nutrients (like nitrogen) may be more informative. Studies establishing the relationships between microbial community development, PTS performance and chemical qualities of DOC may also be useful.
- Further studies on the particular chemical attributes of spruce chips and the potential pretreatment options are necessary for the effective use of spruce as a carbon substrate in PTS.

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## **APPENDIX 1: COLLECTION AND PROCESSING OF MATERIALS**

### **Carex, willow and moss**

- A tub of moss was collected 5-10 m from a roadside near the old town site of Elsa, Yukon, October 20, 2015. Moss, collected with a shovel, was a composite of moss sp., with a small amount of organic soil and misc. plant matter.
- ~ 18 L of carex was collected from the marsh area on the college property on October 7, 2015. In particular, dead blades were collected from the ground surrounded by new shoots.
- ~16 L of willow leaves (most likely mixed species) was collected from willow bushes growing beneath power lines near the creek on the Yukon College property on October 7, 2015. Most of the leaves (99%) were collected from the litter, surrounding the mostly denuded bushes. Some (the remaining 1%) were collected from the branches. The leaves were in various states of decay; some were very dry, and some were heavily infested with fungi (black and rotting).
- The moss, willow leaves, and carex were spread out into large plastic containers to air -dry in the storage unit until time of use.

### **Poplar, spruce and peat**

- Poplar was obtained from km 8.5 Hot Springs Road in Whitehorse. It had been cut in early-July 2013, chipped on July 9, 2013; it was dried and stored in cold storage.
- Spruce was from km 4.2 Fish Lake Road. It had been cut in June 2013, chipped on July 9, 2013 and dried and stored in cold storage.
- Peat was from 5-10 m from Cash Creek in the Keno Mining Site, Yukon. Samples were taken on July 21, 2013; and dried and stored at room temperature.

### **Inoculum preparation**

- Approximately 15 L of sediments was collected from Chrystal Creek, near Elsa, Yukon (location, UTM 08 (zone) V (latitude band) 0483901, 7088321). These sediments were mixed with sediments collected previously by Yukon College staff members from Minto Creek and Eagle Creek, in the same region.

- Sediments from each bucket were turned out onto plastic sheets and then mixed with a shovel.
- Each batch of sediments was sieved with a 1.25 x 1.25 mm soil sieve, and equal parts of each were thoroughly mixed together to form a composite sample.
- The Minto Creek sediments were completely dehydrated (and generally fine grained).
- The Eagle Creek sediments were still wet and comprised of clay.
- The Crystal Creek sediments were mixed and comprised of pebbles and coarse grains.

**Table 6. Synthetic mine water**

Component	Calculated ( $\mu\text{g/L}$ )	Measured Concentration ( $\mu\text{g/L}$ )		
		Acidic MW	Neutral MW	Source
Cd	500	447.2	450.1	CdSO <sub>4</sub>
Cu	5000	5311.6	4138.9	CuSO <sub>4</sub> (5H <sub>2</sub> O)
Fe	5000	4191.3	4154.3	FeSO <sub>4</sub> (7H <sub>2</sub> O)
Se	500	404.0	372.4	SeO <sub>2</sub>
Zn	5000	5367.4	4633.6	ZnSO <sub>4</sub> (7H <sub>2</sub> O)
As	500	304.4	264.8	As <sub>2</sub> O <sub>5</sub>
SO <sub>4</sub>	500(mg/L)	510.3 (mg/L)	571.6 (mg/L)	Na <sub>2</sub> SO <sub>4</sub>

**Table 7. Substrate weights**

Acidic SMW	OM weight (g)	Neutral SMW	OM weight (g)
AC1	55	NC1	55
AC2	49.2	NC2	53
AW1	60	NW1	62
AW2	68	NW2	65
ASP1	250	NSP1	247
ASP2	250	NSP2	250
APE1	410	NPE1	430
APE2	404	NPE2	420
AM1	90	NM1	92
AM2	96	NM2	90
APOP1	210	NPOP1	225
APOP2	215	NPOP2	220
AET1	1.34 mL	NET1	1.34 mL
AET2	1.34 mL	NET2	1.34 mL
ACON1	1.34 mL	NCON1	1.34 mL

**Ethanol addition**

- A COD/SO<sub>4</sub> ratio of 0.67 is suggested to be optimal for ethanol oxidation for HS production in flow-through bioreactor systems. Velasco et al. (2008) found that ratios up to 1.5 might be more effective in reactors where the metal content is over 10 mg/L.
- To 500 mg/L SO<sub>4</sub> (=5.2x10<sup>-3</sup> moles sulfate), add 2.4 L of effluent to each reactor (=0.0125 moles). Add 1.34 mL of 85% ethanol (density is 0.789) = 0.9389 g (~0.0953 mol) ethanol, to get a COD/SO<sub>4</sub> ratio of ~1.55.
- The carbon content in ethanol is ~52%. The total fluid volume is ~3 L, so ~313 mg/L ethanol ~163 mg/L carbon.

## APPENDIX 2: SUPPLEMENTAL DATA

**Table 8. Substrate metal concentrations**

Substrate	Cd (ug/kg)		Cu (ug/kg)		Fe (ug/kg)		Se (ug/kg)		Zn (ug/kg)		As (ug/kg)	
Carex	800	±25	9828	±327	534084	±28114	442	±58	34719	±1553	4427	±108
Willow	3559	±174	8083	±629	121959	±5520	491	±38	138527	±12195	2121	±147
Spruce	660	±11	3246	±634	15679	±1209	308	±6	15805	±1438	2338	±50
Peat	16864	±778	37812	±3708	18231542	±2290746	1233	±433	1054747	±106482	446759	±66173
Moss	10088	±821	16739	±1135	3601622	±244474	722	±246	539968	±33625	304292	±20636
Poplar	875	±21	7638	±498	75266	±7306	587	±214	42742	±5190	2885	±92

**Table 9. Effluent nitrogen concentration (mg/L)**

Results are expressed as the average ± the standard deviation of duplicate bioreactors.

### Acid

Time	Carex	Willow	Spruce	Poplar	Moss	Peat	Ethanol
T=0	2.13 ± 0.06	1.04 ± 0.52	N/A ± N/A	1.08 ± 0.42	1.41 ± 0.06	2.01 ± 0.16	0.64 ± 0.24
T=60	10.09 ± 0.74	5.62 ± 0.85	2.38 ± 0.15	2.85 ± 0.26	7.22 ± 1.36	2.63 ± 2.05	0.50 ± 0.01
T=105	7.73 ± 0.74	3.90 ± 0.53	1.94 ± 0.01	4.12 ± 0.02	9.10 ± 1.05	3.31 ± 2.10	0.72 ± 0.25
T=150	10.5 ± 0.07	5.60 ± 0.35	4.23 ± 0.39	3.93 ± 0.18	11.63 ± 0.25	5.25 ± 2.26	1.68 ± 0.11

### Neutral

	Carex	Willow	Spruce	Poplar	Moss	Peat	Ethanol
T=0	4.91 ± 0.33	3.06 ± 0.08	2.46 ± 0.46	1.60 ± 0.12	3.12 ± 0.34	N/A ± N/A	0.23 ± 0.06
T=60	10.82 ± 2.14	6.57 ± 1.09	2.34 ± 0.19	3.52 ± 0.11	13.12 ± 2.62	1.08 ± 0.10	0.38 ± 0.02
T=105	8.10 ± 1.50	3.76 ± 0.69	1.95 ± 0.09	3.59 ± 0.71	10.72 ± 1.16	1.25 ± 0.23	0.56 ± 0.05
T=150	11.68 ± 1.73	5.40 ± 0.21	4.00 ± 0.21	5.28 ± 0.04	14.95 ± 1.13	2.03 ± 0.11	2.03 ± 0.04

Section B. Acid Bioreactor Data

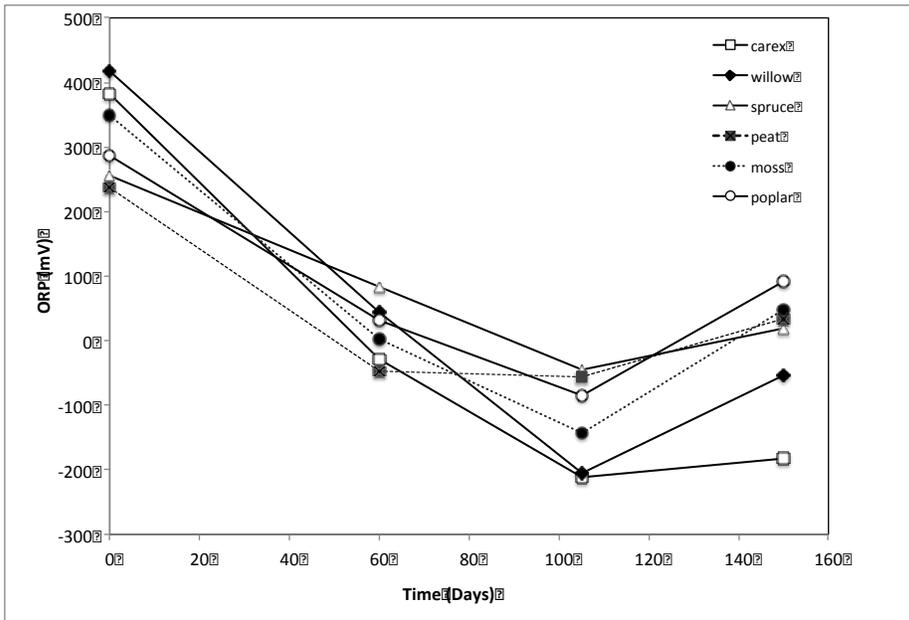


Figure 14. Temporal variation of ORP in acid substrate bioreactors.

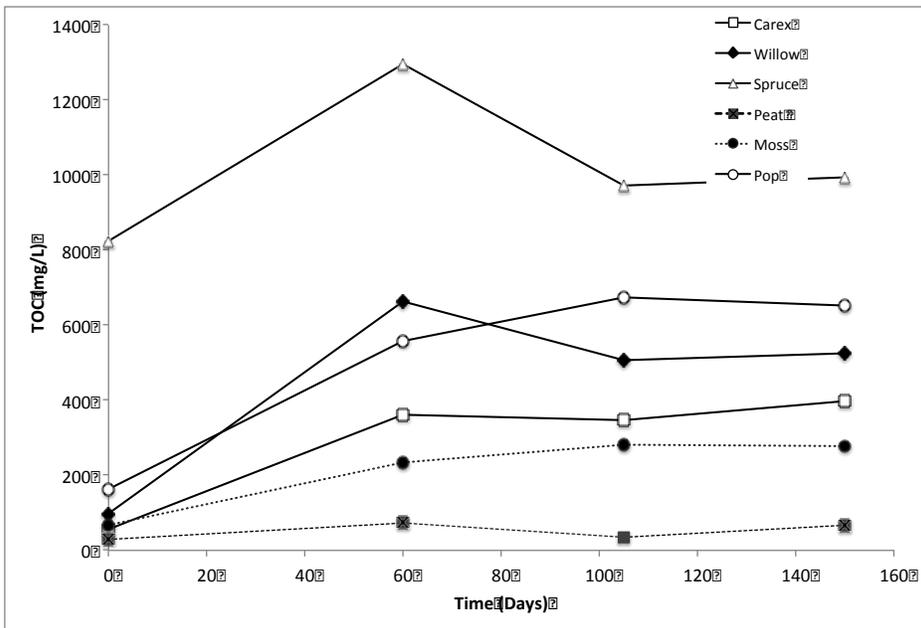
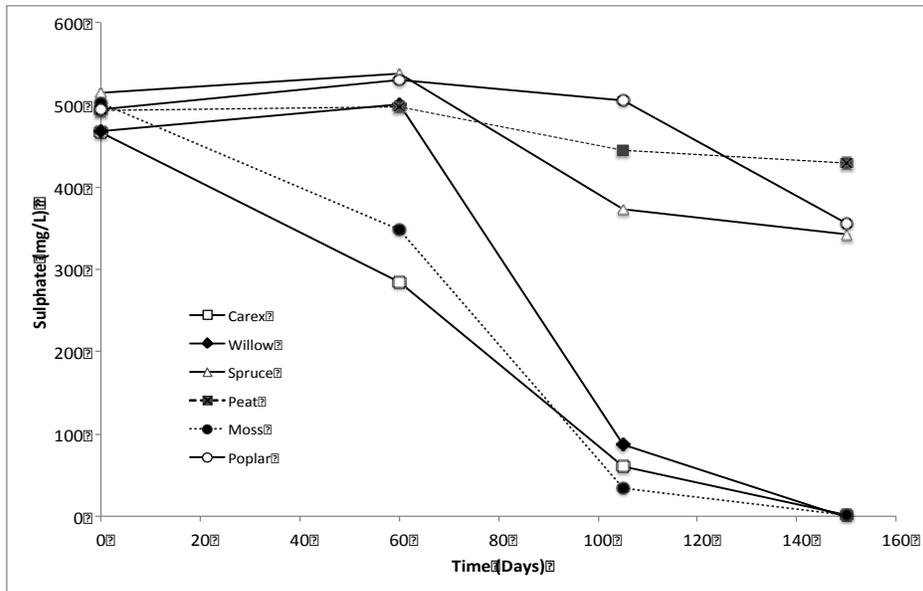
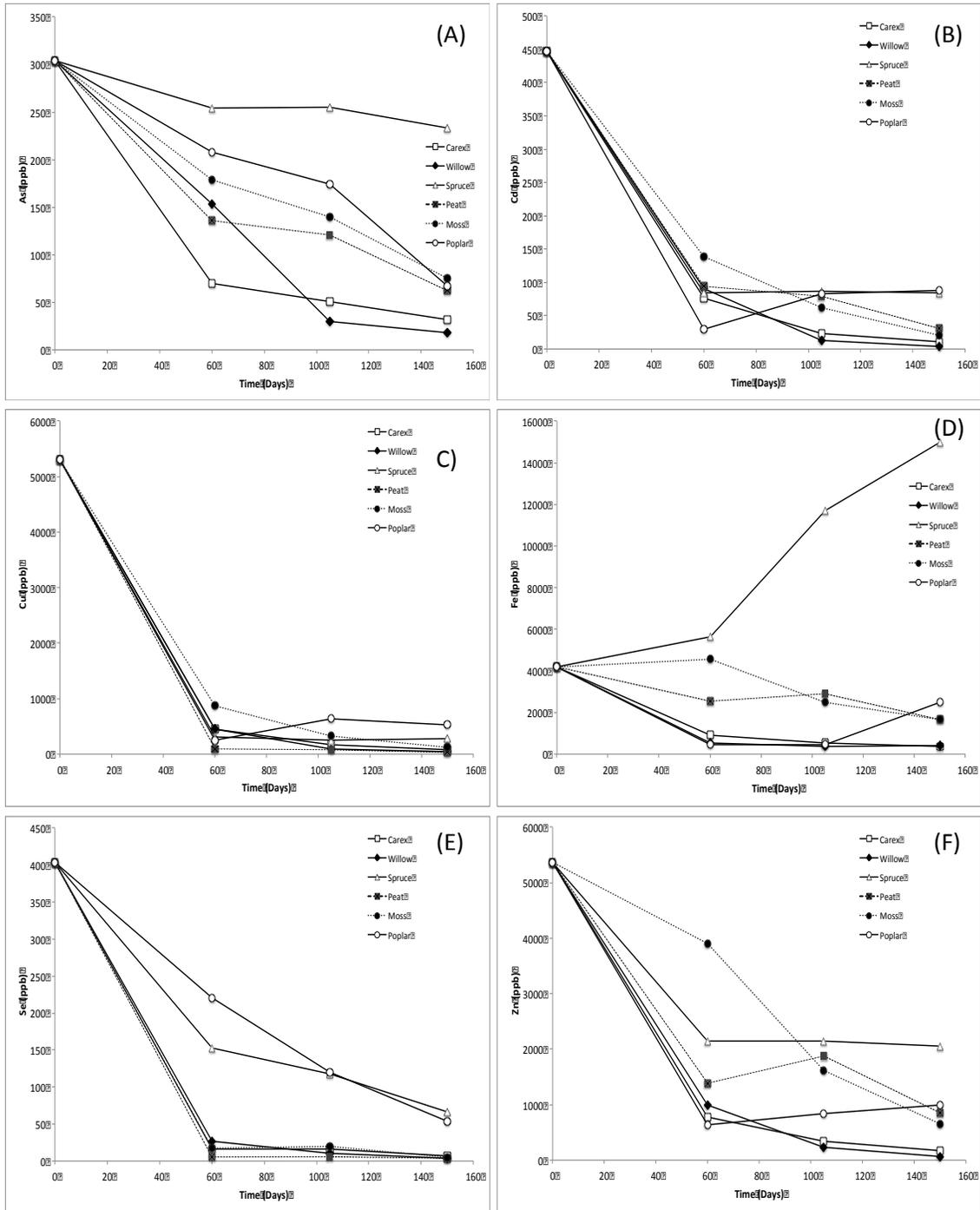


Figure 15. Temporal variation of TOC concentration in acid substrate bioreactors.



**Figure 16.** Temporal variation of sulphate concentration in acid substrate bioreactors.



**Figure 17.** Acidic treatment bioreactors. Change in concentration of dissolved As (A), Cd (B), Cu (C), Fe (D), Se (E), and Zn (F) in organic substrate. T=0 value is equivalent to AMW T=0.