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Minto Mine Constructed Wetland Treatment Research Program – Pilot Scale

Prepared for

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1. Abbreviations and Definitions

CWTS(s) – Constructed Wetland Treatment System(s).

Dissimilatory reduction – is a process utilized by specific types of bacteria where an electron donor material (e.g. organic carbon) is oxidized, producing energy for the bacteria and reducing another material (e.g. sulphate, or selenium) as part of the microbial metabolism.

DNA (deoxyribonucleic acid) – is the hereditary material in almost all living organisms. This can be used to identify organisms.

DO – Dissolved Oxygen.

Heterotroph – an organism deriving its nutritional requirements from complex organic substances.

Metagenomics – the study of genetic material recovered directly from environmental samples, related to a community rather than an individual organism.

Microbes – microscopic organisms which may be uni- or multi-cellular. This includes algae, bacteria, fungi, viruses, and yeast.

MPN (Most Probable Number) – a statistical value representing the viable population of microbes in a sample through use of dilution and multiple inoculations.

Oxidation – is the loss of electrons or increase in oxidation state by a molecule, atom, or ion. Can be driven by microbes. Process is complementary to chemical reduction.

Redox – Oxidation-reduction potential, measured in millivolts.

Reduction (chemical) – is the gain of electrons or a decrease in oxidation state by a molecule, atom, or ion. Can be catalyzed by microbes. Process is complementary to chemical oxidation.

RRC (Removal Rate Coefficient) - a numerical value, expressed as the variable *k,* representing a first order rate of removal, considering removal extent and the time required for removal.

SeIV broth or agar – laboratory growth medium that contains selenite (SeIV) and indicates reduction of selenite to elemental selenium through a visible colour change.

Species – one of the basic units of biological classification and a taxonomic rank. Rank in the classification of organisms below genus and above strain.

Taxonomy – classification, identification, and naming of organisms.

TSS – Total Suspended Solids.

YTS250 (Yeast, Tryptone, Starch, 250g each) – a type of laboratory growth medium used for environmental microbes.

2. Introduction

Constructed Wetland Treatment Systems (CWTSs) have been proposed as a method for improving the quality of site runoff water in the post-closure period for the Minto Mine (Minto Phase V/VI Expansion Project, YOR Project Number 2013-0100). Once established, wetlands can become self-sustaining ecosystems with the plants providing yearly renewal of carbon to fuel microbial activity. As such, they possess the desirable potential to remediate contaminated mine drainage for as long as it is generated. In order for CWTSs to be effective, however, they must be designed, piloted, optimized, implemented, and maintained in a site-specific manner.

Wetlands provide a natural environment in which unique biogeochemical reactions can occur. CWTSs can be designed to have desirable conditions similar to those in natural wetlands, and should be designed specifically for each site. Having a site-specific approach allows for the appropriate set of operational conditions to be achieved, where complex coupled reactions can take place for treatment of targeted constituents. This can be done with complex and challenging waters, where problematic constituents are transferred or transformed to less bioavailable forms. The benefits of CWTSs include:

- low operational cost,
- low maintenance,
- effective and robust treatment,
- increased effectiveness over time,
- aesthetically pleasing,
- tolerance to changes in flow rate and contaminant load, and
- simultaneous treatment of multiple constituents more effectively than chemical or physical treatment processes.

CWTSs have been used to mitigate risks to aquatic receiving environments from a variety of aqueous contaminants, including copper (Cu) and selenium (Se). The biogeochemical reactions that take place in a wetland environment are many, but there are specific reactions that may decrease the aqueous and more bioavailable concentrations of some contaminants (e.g. Cu, Se), thus lowering risk to the receiving environmental systems by decreasing exposure to contaminants. A process called dissimilatory sulphate reduction can chemically reduce available aqueous sulphate to sulphide through the metabolism of specific bacteria. Once dissimilatory sulphate reduction has occurred, it provides a mechanism to remove dissolved cations such as Cu from water by complexation with the sulphide ion to produce copper sulphide, an insoluble precipitate. Selenium treatment in a CWTS is a similar process, but with slightly different results. The mechanism for Se treatment is more direct, through dissimilatory selenium reduction that is also microbially mediated, often by some of the same bacteria that undergo dissimilatory sulphate reduction. The most common forms of aqueous Se are selenite and selenate, with valence states of IV and VI, respectively. When these are reduced in a wetland to an insoluble state, Se precipitates as elemental Se(0).

In both cases, the Cu and Se precipitates are removed from the water through filtration mechanisms inherent in wetlands and are sequestered into the sediments over time as an accretive process. Accretion is the naturally occurring process of accumulation of wetland sedimentary material (soil, minerals, decaying plant material, etc.) over time. Once an accreting CWTS is established and mature, targeted constituents are sequestered into the sediment and covered over time by newly generated sediments and detritus. This essentially seals away the treated constituents under layers of sediment, rendering them less bioavailable and less susceptible to re-entry into the water column. There is therefore no need to dredge or harvest wetland plants for the Minto site; in fact, this type of activity would disrupt the treatment functions and re-expose the previously sequestered constituents. This process mimics what occurs in naturally created wetlands; therefore, this process is often the best option for long-term, low-maintenance and effective treatment.

3. Background

At the request of Minto Explorations Ltd, Contango Strategies has undertaken a pilot-scale study to inform the design of site-specific passive treatment technologies for the Minto site during post-closure. These pilot-scale trials build upon a site assessment that was conducted and reported on in 2013. The overriding goal of this pilot study is to develop, optimize, and test site-specific pilot-scale CWTSs for post-closure water treatment at the Minto site.

4. Pilot System Operations

4.1. Design and assembly

In October 2013 three pilot CWTSs were constructed, each in duplicate, to evaluate the performances of different designs for selenium and copper treatment (Figures 1 and 2). These were planted with either Sedge (*Carex aquatilis*), or Sedge and aquatic moss as follows:

- System 1 and 2: *Carex aquatilis*
- System 3 and 4: *Carex aquatilis* + Moss
- System 5 and 6: *Carex aquatilis* + Moss, with biochar amendment in soil

The pilot CWTS systems were built with two cells per series, each cell having a 41 cm diameter and a height of 57 cm. To establish the plants, soil was filled to a depth of 20 cm at the bottom of each cell, and submerged under 10 cm of tap water. The water depth was changed throughout the pilot testing to evaluate effects on performance, and develop strategies for optimization.

4.1.1. Hydrosoil

Hydrosoil is a specially selected soil blend to aid in achieving the targeted hydraulic conductivity required to meet the CWTS objectives. In this case, the hydrosoil mixture was the same for all pilots; sand amended with 2% woodchips (v/v) and 5% peat, except that the third system design (system 5 and 6) also included a 5% pine biochar amendment. The sand and amendments were tested for initial metal and nutrient contents (Table 5). This specific type of biochar was chosen as an amendment as previous studies conducted by Contango have demonstrated that addition of biochar to the hydrosoil can result in faster establishment and growth of newly planted wetland plants (SETAC Presentation #593, Haakensen 2013). The biochar selected was low in soluble salts, had a high surface area, low metals content, and moderate available organic carbon and phosphorous (Appendix 1). The peat borrow site at the Minto Mine was selected as it provided a sufficient quantity for a full-scale wetland. It was tested for metals content and other relevant parameters such as nutrients (N, P, K), and total organic carbon before use in the pilot CWTS system.

4.1.2. Plants

Two plant species were of interest for the pilot CWTS based on data collected during the August/September 2013 site assessments (reports 2013-0100-256 and 2013-0100-257 on the YESAB registry). *Carex aquatilis* (sedge) was chosen, as it is readily available at the Minto site and was one of the first plants to colonize cleared areas, indicating that it may be a good candidate for quickly establishing the CWTS. Moss was also used in the second and third pilot CWTS designs (systems 3-6), as locations at Minto with shallower water had *Carex* and Moss coexisting. As such, we wanted to assess whether the Moss contributes positively or negatively to treatment, and also whether succession from *Carex* to Moss as the dominant plant can be prevented or enhanced through modification of water depth.

It is well known that Moss has a high uptake rate of cations (such as copper and selenium) and is also a relatively benign sink for these elements (i.e., is not a food source for invertebrates or higher animals and as such does not contribute greatly to bioaccumulation (Haines & Renwick, 2009; Longton, 1997; Suren & Winterbourn, 1991)). This was also confirmed in the site assessment in 2013, which produced data indicating that Moss at the Minto site has a high uptake of elements such as copper, and could therefore be an effective means of removing elements from seepage through uptake. During the September 2013 site visit, Moss and *Carex* were collected from the Minto site and these were subsequently used to vegetate all pilot CWTS in this study. A total of 10-12 *Carex* were planted per cell, with approximately a 250ml volume of Moss (compressed) added to systems 3-6.

4.1.3. Water and Testing Periods

The CWTS(s) were designed for treatment of water that would be present at the Minto site after closure. As such, this water does not yet exist and had to be synthesized as an approximation based on water quality modeling. The worst-case post-closure water chemistry was used based on the month of October, as it had the highest concentrations of key elements for any month with free-flowing water. The synthetic water underwent five iterations of design, testing, and optimization to ensure the composition and method of formulating the water resulted in analytical chemistry that matched the water quality predicted to exist after closure and reclamation of the Minto mine. The final recipe and resultant water chemistry is provided in Tables 1 and 2.

Design of the synthetic water chemistry focused on constituents requiring treatment and elements/compounds that are expected to affect treatment rates or capacity based on previous experience and scientific literature. For the first period (High N) of the pilot-phase work, the predicted maximum worst-case ammonia and nitrate concentrations were used, which reflected residuals of these compounds from blasting. The ammonia concentrations measured on site are lower than would be expected based on the chemical composition of ammonia nitrate used for blasting, suggesting that the ammonia has already undergone oxidation to nitrate prior to the seepage exiting the waste rock pile. Nitrate can affect compounds such as selenium that are removed from water through coupled biogeochemical reactions and dissimilatory reduction, as nitrate has a high affinity for available electrons and is preferentially reduced before other compounds. However, it is known that over time in closure, the concentrations of ammonia and nitrate will decrease dramatically, and this may affect removal rates of other compounds. As such, the pilot-scale CWTSs were subjected to a 6-week trial of synthetic water of the same max worst-case post-closure water quality, but with depleted ammonia and nitrate concentrations to simulate the chemistry expected in long-term closure (Low N period).

It is possible to shift the functionality of a CWTS to stimulate additional microbial dissimilatory selenium reduction and enhance overall treatment of this element. The resultant CWTS would be a hybrid bioreactor-CWTS and is a “passive care” option. This means that the hybrid-CWTS would require more maintenance and operational intervention than a normal CWTS, with periodic dosing of an organic amendment needed (once or a few times annually) in order to achieve the targeted decreased dissolved oxygen and redox levels. However, depending on actual inflow and target outflow concentrations, this approach may be a necessary compromise.

The third and final stage of pilot testing therefore included the addition of an organic carbon amendment (alfalfa hay and straw) to stimulate reducing conditions for improved selenium treatment. Alfalfa hay and straw were chosen as they would provide both short and long-term carbon sources compared to a metered dosing of more available electron donors such as ethanol. This hybrid bioreactor-CWTS period was conducted as an 8-week trial to test how the CWTS functions once converted from conventional CWTS operation to the hybrid configuration. A total of 1.4 g of alfalfa pellets were added per liter of water in each A cell. Straw was added at 10 cm^3 per L to A cells, and 5 cm^3 per L to B cells. The alfalfa pellets and oat straw were tested to ensure suitability for use (Appendix 1). A summary of the periods and timelines is provided in Table 3.

Over the course of the study, a total of 45,000 L of synthetic water (influent) was received by the pilot CWTS (in 41 batches), approximately 1,200 L of synthetic water per week in a pilot CWTS footprint of 1.56 m^2. The system operated with a flow rate of 20 mL/min, resulting in a hydraulic retention time (HRT) ranging from 34 to 94.3 hours, depending on the period of testing and water depth (Table 3).

4.1.4. Routine Monitoring of Explanatory Parameters

Explanatory parameters are characteristics of a specific environment that describe conditions in the context of how these parameters can affect the chemical reactions taking place. These parameters, when within specific ranges of values, can be used to predict and/or promote certain reactions in the aforementioned environment. Accordingly, dissolved oxygen (DO; mg/L and %), temperature (°C), pH, conductivity (µS/cm), specific conductivity (SPC; µS/cm), and oxidation-reduction potential (ORP; mV) were measured on a routine schedule with a YSI Professional Plus handheld unit.

Hydrosoil reduction-oxidation (redox) potential was measured using inert electrodes (copper wire probes with platinum tips) that were permanently installed in the hydrosoil of the cells and remained there for the duration of the project to ensure accurate readings (Faulkner et al., 1989). On testing days, a reference electrode (Accumet Calomel) was suspended in the water column above the inert electrode. The redox potential was then measured in millivolts by a voltmeter.

4.1.5. Flow Rates

Constant flow metering pumps were used to set the flow rate of water into the system. To confirm flow rates, the influent to cell A was collected into a graduated cylinder over a timed period of several minutes allowing a flow rate to be calculated in milliliters per minute (mL/min). Flow rates were measured regularly (at minimum weekly) and adjusted as necessary to within +/-10% of the desired flow, to achieve the desired HRT. The rate of water outflow from a CWTS cell was measured using the same procedure. Outflow rates for a series were typically measured every 2 weeks.

4.1.6. Water sampling

Water samples were collected from the outflow port of each CWTS cell. Care was taken to avoid collecting plant matter, invertebrates, or other debris in the sample. Samples were collected from downstream to upstream to ensure the water sampling did not affect sample collection from the next cell (e.g., disrupting flow rates or suspending solids). All samples were collected according to the schedule in Table 4, with parameters outlined in Table 5.

4.1.7. Stem Counts and Height Measurements

Carex aquatilis stems were counted in each CWTS cell, and 10 stems were randomly chosen in each cell for height measurement. The height of the tallest leaf of each stem was measured to the nearest 0.5 cm from the soil surface to the tip of the leaf. Stem counts and heights were taken monthly during the High N period, as well at the end of the Low N and hybrid bioreactor-CWTS periods (Table 4).

4.1.8. Sediment Sampling

Sediment/hydrosoil samples were collected at key intervals as per Table 4. Sediment was collected by carefully taking a grab sample of a small section of the top 6 cm of hydrosoil in each CWTS cell into a 50 mL centrifuge tube, pouring off any excess water after collection. In addition to the analyses listed in Table 5, sediment was also collected for microbial profiling according to the schedule in Table 4 and as described in Section 4.3.

4.1.9. Plant Sampling

Both *Carex* and Moss were collected for metals analysis at the beginning of the study, as well as at end of the Low N period. This schedule was chosen as it was uncertain if the Moss would survive through the hybrid bioreactor-CWTS period. For sample collection at the end of the Low N period, 4 stems of *Carex* (~15% of total stems) were randomly selected from each cell and trimmed at the surface of the water. An approximate 250 ml volume of compressed Moss was also collected from each cell in series 3-6. Plants from replicate series (e.g., series 1&2, 3&4, 5&6) were combined and sent for metals analysis as a representative sample for each system type. For the final sampling point (i.e., at pilot system takedown) all remaining *Carex* plants in each cell were trimmed at the soil level and rinsed with water prior to sending for metals analysis.

4.1.10. Freeze-Thaw Trial

In addition to routine analysis for metals in plants, a replicate of each plant sample collected at the end of the low N period was used in a freeze-thaw trial to determine if the plants will leach constituents of concern such as Cu and Se after freezing. In this trial, 200 mL of water from the corresponding CWTS cell was added to the plant sample to completely cover the plant material. The samples were frozen at -20°C for 5 days, and then thawed at room temperature (~22°C) overnight. The plant samples were removed and lightly squeezed to return residual water to the container, and the plant tissue was sent for metals analysis to compare with pre-freeze concentrations.

4.1.11. Organic Carbon Leach Test

To determine the constituents that could leach from the alfalfa and straw that were added for organic carbon during the hybrid bioreactor-CWTS period, a leach test was performed. In this test, 2 L of outflow from system 1 was collected and alfalfa and straw were added to mimic the concentrations that were added to the system. The water was tested for total metals 3 weeks after the organic carbon addition to determine if any metals had leached from the alfalfa and straw into the water.

4.2. **Methods for microbial analyses**

4.2.1. Growth-based analyses (MPN)

The most-probable number (MPN) of bacteria was determined for all micro samples. The MPN test allows for an estimation of the number of bacteria that can grow in a specific laboratory medium. The media tested in this project were specific for bacteria that can reduce nitrate, selenite, and sulfate, respectively, as well as the total number of heterotrophic bacteria for comparative purposes (i.e., a growth-permissive test for all bacteria capable of growing on organic carbon as an energy source). MPN tests for sulphate (Active Standard ASTM D4412) were conducted under anaerobic conditions, whereas selenite (Siddique et al., 2006) and nitrate (Nitrate Reduction Test, supplied by Sigma-Aldrich) were tested under aerobic conditions. Total heterotrophs (grown with YTS250 medium; (Lefrançois et al., 2010) were quantified in both aerobic and anaerobic conditions.

In brief, sediment samples were diluted 1:100 with a 0.1% peptone solution. This starting dilution was then used for a serial dilution along a sterile 96-microwell round-bottom plate containing the respective growth media. Sediment dilutions tested in the MPN plate ranged from 1/400 to 1/419,430,400. At minimum, all tests were conducted in duplicate. Wells were incubated at +30°C without light and assessed for visible growth (formation of a bacterial pellet) and/or colour change specific to the type of media after 27 days. A colour change to black or red/orange indicated sulphate or selenite reduction, respectively. Nitrate reduction was tested as per the kit manufacturer's protocol by addition of reagents to differentiate between reduction to nitrite or nitrogen gas. The most probable number of bacteria capable of each metabolism was then calculated as described in the FDA Bacteriological Analytical Manual, Appendix 2: Most Probable Number from Serial Dilutions: (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm). The MPN of organisms in each pilot system was compared over time using the non-parametric Kruskal-Wallis test.

4.2.2. DNA-based analyses

DNA was extracted from all samples using the MO BIO Powersoil Powerlyzer DNA extraction kit, with the addition of phenol during cell lysis as per the manufacturer's alternative protocol. Targeted sequencing was used to identify bacteria present in each sample via polymerase chain reaction (PCR) amplification of the v3/v4 region of the 16S ribosomal RNA gene (Klindworth et al., 2013). Library preparation and sequencing was performed as per the manufacturer's instructions for MiSeq v3 paired-end 300 bp sequencing (Illumina). All raw sequences were filtered to remove low quality reads, and the forward and reverse primers were truncated. Bioinformatics pipelines consisting of internal scripts and selected QIIME scripts (Caporaso et al., 2010; Edgar, 2010) were used to process the reads. Similar sequences were clustered into groups called Operational Taxonomic Units (OTUs) using a 97% identity threshold. Taxonomic classification of the OTU's was performed according to the Greengenes database (DeSantis et al., 2006; McDonald et al., 2012). Non-metric multidimensional-scaling was used to compare microbial communities across samples using the Bray-Curtis dissimilarity metric. The percentage of the microbial community classified as organisms of interest (e.g., sulphate-reducing bacteria) was also compared across samples. The most abundant OTU's were compared to the Cu, Se, and S concentrations in the sediment to calculate the Spearman correlation coefficient and associated p-value.

5. Results and Discussion

5.1.1. Outflow concentrations of copper and selenium

The pilot systems were operated for 37 weeks with three distinct trial periods of High Nitrogen, Low Nitrogen, and Hybrid Bioreactor-CWTS, with the average influent and outflow water chemistry provided in Tables 6, 7, and 8, respectively. The minimum, maximum, and average measurements of selected parameters and copper and selenium concentrations are summarized in Table 9. While copper treatment was relatively stable through both the High N and Low N periods, selenium treatment improved during the High N period (Figure 3), and then stabilized through the Low N period. For both the High N and Low N periods, the systems that were planted with Moss and *Carex* had significantly better copper treatment than Moss only. There was no statistically significant difference found among the three pilot system designs for selenium removal (i.e., Moss or biochar did not show a significant effect on selenium treatment during these periods).

The hybrid bioreactor-CWTS configuration was effective for both copper and selenium treatment. The purpose of the bioreactor-CWTS configuration is to serve as a contingency in case greater treatment is needed than is being achieved by the CWTS at a given point in time (e.g., the influent water quality or outflow objectives change). The tests here show that the CWTS can be repurposed as a hybrid bioreactor-CWTS with improved selenium and copper treatment within the same footprint, and without need for added construction. However, operating the system as a hybrid bioreactor-CWTS would require greater operational maintenance than it would in its original CWTS configuration.

In the case of selenium, the reconfiguration resulted in a statistically significant decrease in outflow concentration compared to that prior to the reconfiguration (Figure 3). This result for selenium was expected based on the design targeting more favourable conditions for selenium reduction, such as lower dissolved oxygen and ORP resulting from the additional electron donors from the microbial decomposition of the alfalfa and straw. In terms of copper, the lowest outflow concentration of the study was measured during the hybrid bioreactor-CWTS phase (7.5µg/L); however, prior to this there was a brief decrease in treatment effectiveness in all systems (Figure 3, June 19th data point). However, even during this brief disruption, 86% of the total copper entering the system was treated.

It is not known whether this brief change in treatment effectiveness was due to a short-term loss of treatment capacity during the transition, or if it might have been caused by a release of copper and other constituents that were sorbed to the moss or other organic matter during the transition. This decrease in treatment effectiveness coincided with low dissolved oxygen and moderately reducing conditions in the water column being attained in all cells (DO < 0.5 mg/L, ORP < -80 mV); however, it was not clear if the additional copper in the outflow was released from the alfalfa and straw that were added, or if constituents were briefly released from the sediment or moss and detritus after the system transitioned into more reducing conditions. As a result, a leach test was conducted on the alfalfa and straw and determined that there was no discernible release of copper from these organic materials. The origins of the decrease in treatment effectiveness could not be determined definitively, with the disturbance being registered during a single sampling point, with a maximum potential disturbance period of 4 weeks.

While the hybrid bioreactor-CWTS configuration improved selenium treatment, and demonstrated the lowest outflow concentration of copper of the entire trial, the short upset of treatment performance during the transition is meaningful in terms of monitoring during site implementation. It should be emphasized that if a transition from a typical CWTS design to a hybrid bioreactor-CWTS style is performed, careful monitoring of the CWTS is required as some elements may have decreased treatment effectiveness for a brief period. During this timeframe, if concentrations of elements exceed regulatory guidelines, water may need to be recycled through the wetland, or a contingency water treatment plan should be put in place for the outflow over the period of treatment disturbance.

5.1.2. Removal Rate Coefficients

An important factor for wetland design is the rate of treatment, also known as the removal rate coefficient (RRC). The RRC is based on treatability of a specific compound and the hydraulic retention time of the system. Once the RRC is known, the equation is rearranged and the RRC can be used to calculate the HRT needed to meet outflow objectives (and accordingly, the size of the wetland). It can also be used to predict the expected outflow concentrations of a specific element in a wetland of the same design based on inflow rates, concentrations, and footprint. RRCs vary with the concentrations of an element, and therefore, a sequential cell approach was used in this pilot study to allow for the RRC to be calculated over a broader range of inflow and outflow concentrations than if the system were only considered as a single treatment unit. Together these can then be used to estimate the size needed for a full-scale CWTS to meet outflow concentration performance objectives for a given influent concentration and flow rate. It should be noted that a RRC is specific to each given constituent respective to a particular wetland design, and can change based on the water chemistry and/or system design. The RRCs presented in this paper are a representative approximation of the Minto Mine site in a closure scenario and accounts for High N, Low N, and the use of a hybrid bioreactor-CWTS operational period (Figure 4, Table 10). When comparing the RRC of different designs of pilot systems in which the retention time and inflow concentrations are the same, a higher RRC indicates increased removal of the constituent. In the equation below, the first order RRC has been reconfigured to calculate *t* (hydraulic retention time), allowing the use of *Ci* (initial concentration), *Cf* (final, desired concentration), and *k* (removal rate constant, RRC) to be used to size the wetlands accordingly.

$$k = \frac{-ln(C_f/C_i)}{t}$$

$$t = \frac{-ln(C_f/C_i)}{k}$$

Equation: Removal rate coefficient (RRC) calculation, and rearranged to solve for HRT

When comparing the copper RRC of cells A and B within a series during the High N period, the RRC of the first cell is very high, while the RRC of the second cell is negligible (Figures 5 and 6). This phenomenon is frequently observed in treatment wetlands, where the treatment rate is faster at higher concentrations than at lower concentrations (Horner et al., 2012; Murray-Gulde et al., 2008; Spacil et al., 2011). As such, increasing the HRT of these systems by adding additional cells to the series would not result in a linear decrease in the final copper outflow concentration.

In contrast, the Low N and hybrid bioreactor-CWTS periods do not have this same degree of difference between copper RRC between cells A and B within a series (Figure 5). This change in cell-to-cell difference of RRC still results in similar or improved outflow concentrations. Generally speaking, the RRC of the first cell in the system has decreased, while that in the second cell has improved. This observation can be attributed to a variety of factors. Based on previous experience, the most likely explanation is that there was an abundance of sorption sites available in all cells at pilot initiation. As the study progressed, the sorption sites became occupied and removal via this mechanism was no longer available. This same phenomenon is also often seen when setting up an on-site demonstration-scale or full-scale treatment wetland. As such, both pilot and demonstration scale studies should be conducted for a sufficiently long period of time so as to realize the actual treatment capacity of the system beyond this initial sorption phase. This phenomenon must also be taken in the context of the duration over which the system must operate within a year, and the knowledge that the reaction front will move downstream within a wetland through this time. Therefore, an increased wetland size might not significantly improve outflow concentrations, but will aid in maintaining treatment performance through the year, so long as the added size does not result in concentration of the elements due to evaporation of the water caused by increased size.

A second possibility is that since the High N conditions coincided with an algal bloom (both visible, but also confirmed by community profiling that there was an abundance of cyanobacteria; Figure 7), the greater concentrations of cyanobacteria and algae during the High N period could have also been responsible for increased sorption and uptake of copper in the A cells. In either case, it is not to say that the B cells were not capable of a high removal rate, but rather that the concentration of copper in the A cell effluent left very little copper available for treatment in the B cell, regardless of capacity for treatment.

It is important to note that the RRCs for copper decreased during the hybrid bioreactor-CWTS period (Figure 4). This can be attributed to the longer HRT created by the increased water depth in this period despite similar outflow concentrations. Through all periods, the outflow concentration of copper for any given system did not vary significantly, indicating that regardless of the cause of the difference in RRC at different points in the system operations (e.g., sorption, algal uptake, increased water depth), the thermodynamic minimum for copper removal has likely been approached within the system, with an average outflow concentration of 18 ± 5 μg/L (Figure 6). That is to say, making the wetland bigger using any of the tested designs would not result in a further significant decrease in outflow copper concentration, but rather, design alterations would need to be made in order to attempt to achieve lower outflow concentrations. During the High and Low N periods when the Moss was actively growing, copper removal rates were greater in systems containing Moss than in systems with only *Carex*. It is likely that as the Moss continues to grow over time and increase in total mass, the RRC would also improve in these systems. Finally, the addition of biochar did not have any significant effect on performance.

Selenium tells a different story than copper in regards to the RRC. Selenium had a fairly similar removal rate in both the A and B cells during the Low N period (Figures 5 and 6). This could be because the selenium treatment is microbially mediated, or because the inflow concentrations are already so low that both the inflow and outflow concentrations of all cells still fall within the same RRC range. A test of the metals composition in the plants and subsequent mass balance of the systems confirmed that the system was operating as designed to target selenium mineralization to the sediment, as plant uptake was responsible for only 1-3% of the total selenium that was removed from the water during the entire course of the study (Figure 10). Based on the RRCs for selenium in the Low and High N periods, an increased HRT is expected to result in a lower outflow concentration of selenium (Figure 6). This could be achieved by increased footprint, or water depth, so long as the depth does not exceed that in which the *Carex* and Moss will grow.

Conversely, the hybrid bioreactor-CWTS time period had a significantly higher RRC for selenium in the A cells compared to the B cells (Figure 5). It should be noted that while the HRT is longer during the hybrid bioreactor-CWTS period than it was during the Low or High N periods due to an increase in water depth, it can be calculated using the RRC from the previous periods that this alone does not account for the degree of improvement in outflow concentration of the bioreactor-CWTS design. If the improvement in outflow concentration could be attributed solely to the increased HRT, the RRC would remain the same. Instead, we observe an improvement of not just the outflow concentration, but also in improvement in the RRC for selenium in all systems when converted to the bioreactor-CWTS configuration. This higher RRC is presumably due to the more favourable conditions that have been targeted for selenium reduction, including lower dissolved oxygen and a lower ORP resulting from the additional electron donors available through the microbial decomposition of alfalfa and straw. Through all time periods, there was no discernible benefit measured by the addition of biochar into the soils of the systems; rather, the systems that were planted with *Carex* and Moss performed similarly regardless of whether biochar had been included in the soil or not.

As would be expected based on the RRC data for systems containing *Carex* and Moss, the final sediment concentrations of copper are higher in the A cells than the B cells, with the B cells being only slightly higher in concentration than the starting levels (Figure 8). The same trend is evident for selenium; however, the starting concentration and concentrations in most of the B cells are below detection limits. It is of note that the copper and selenium sediment concentrations are positively correlated to an increase of sulphur in the sediment, while no discernible change was found in iron concentrations (Figure 8). This correlation is important to understand the long-term stability of copper within the system, as copper is highly stable when mineralized in a sulphide form and maintained under reducing conditions such as those in the wetland soils. In this case, the sulphides are being formed by microbes living in the soil and associated with the plant roots (see black areas in soil and around roots in Figures 14 and 15). This is supported by the finding of an initial increase of sulphate-reducing bacteria in the sediment, followed by stability over time at a most probable number of approximately 10,000-100,000 microbes per gram of sediment that are capable of reducing sulphate to sulphide (Figure 17). In the case of selenium, the correlation with sulphur in sediment is presumably due to the ranges of explanatory parameters found within the system (dissolved oxygen, ORP, soil redox, pH) which are favorable to both microbially-mediated selenium and sulphur reduction, suggesting the sedimentary selenium is likely in an elemental form, or sorbed to organic materials such as biofilms. While, the stability of the copper and selenium mineralizations were not tested in this study, it is planned for testing to be performed during the upcoming on-site demonstration-scale trial by using sequential extractions on the sediment paired with ICP analysis.

5.1.3. Fate and distribution of copper and selenium

Mass balances were calculated for each pilot system to assess the fate and distribution of elements removed from the water during the course of testing (i.e., the full 37 week period). For systems that were planted with *Carex* and Moss, 64-75% of the copper removed from the water was sequestered into the top 6 cm of sediment (Figure 9). In contrast, systems with only *Carex* planted and no Moss had only 33% of the copper ending up in this top sediment fraction (Figure 9). For systems planted with both *Carex* and Moss, 68-83% of the treated selenium was sequestered to this top portion of the sediment, but less than 52% was in the sediment of the system planted with only *Carex* (actual percentage cannot be calculated as the selenium was below the sediment detection level; Figure 10). For both elements, only 1-3% of the total load removed from the water was found in the above water vegetation (*Carex*) at the end of pilot testing.

It was not possible to determine the concentrations of elements in Moss, as the majority did not survive in the hybrid bioreactor-CWTS period conditions. However, in anticipation of this potential issue, samples of Moss were sent for analysis prior to the start of the hybrid bioreactor-CWTS period, allowing for a bioconcentration factor (BCF) to be calculated.

5.1.4. Plant uptake and overall performance

The bioconcentration factor (BCF) is the ratio of the amount of an element taken up into or sorbed onto the surface of a plant, compared to the amount of the element in the water, and is expressed as the ratio of mg of chemical per kg of organism to mg of chemical per liter of water. The amount of an element sorbed onto the surface of a plant was included in the BCF calculation to give a better representation of the amount that would be present in and on a plant in the wetland (i.e., for plant death/degradation as well as consumption perspectives). As would be expected based on samples collected during the site visit in 2013 and supported by scientific literature, we found in this study, and also in the site visit that *Carex* had a much lower BCF than Moss for both selenium and copper (Table 11). This finding is expected, as moss is known to have a high sorption capacity and therefore, high BCF (Aldrich & Feng, 2000; Gstoettner & Fisher, 1997). For some elements, bioconcentration factors within a plant species are higher when the element itself is at a lower concentration. This held true in the pilot systems when comparing cells A and B in a series, as the BCF for copper in *Carex* was significantly higher in B cells (which had lower aqueous concentrations) than in A cells (with higher influent concentrations) for all three pilot system designs tested (Figure 11). However, for selenium, the A cells had a similar BCF as B cells planted with both *Carex* and Moss (not statistically different), but a significantly higher BCF in the A cells planted with only *Carex* (Figure 11).

Selenium bioconcentration in *Carex* in the pilot systems was very low, resulting in average final tissue concentrations of only ~1.9 mg/kg wet weight. The BCF of selenium was greater in the Moss than the *Carex* in the pilot systems (Table 11). However the Moss BCF recorded here is still relatively low overall based on the United States Environmental Protection Agency Toxic Substances Control Act which considers BCF less than 1000 as non-bioaccumulative. It should also be noted that although selenium is generally known to bioaccumulate, Moss is not a food source for higher animals or invertebrates; as such, the selenium in the Moss should not be contributing to bioaccumulating pathways (Haines & Renwick, 2009; Longton, 1997; Suren & Winterbourn, 1991).

It is important to the overall treatment system design to determine if elements may be released by the plants by freezing and thawing in the water, as would occur through natural seasonal progression. A freeze-thaw trial was conducted in which *Carex* and Moss were collected and sent for metals analysis both before and after being submerged in water from the wetland and freezing to -20°C followed by thawing to room temperature. It was promising to find that *Carex* released only a small percentage of the selenium (18-38%) and copper (0-13%) as noted by the decrease in tissue concentrations after freezing and thawing (Figure 12). Moreover, Moss was found to have a significant increase in concentration of these elements after being frozen and thawed (Figure 13). It appears that through the freeze-thaw process, the Moss has an enhanced sorption of these compounds, removing an additional amount from the water.

Based on the pilot system design and plant densities, *Carex* would theoretically release on average 0.14 mg of copper and 0.27 mg of selenium per square meter of wetland area, while Moss would theoretically bind 511 and 11.8 mg/m^2, respectively. This finding is important, as it demonstrates that despite some release of constituents by *Carex* through freeze-thaw, the Moss will provide uptake in excess of what is released by the *Carex* and also assist with treatment during initial spring thaw before biogeochemical processes are at full capacity.

During the pilot CWTS takedown, it was noted that the *Carex* had an extensive root structure throughout the entire hydrosoil (Figures 14 and 15), making it an ideal candidate for construction of stable wetlands on site. Additionally, it should be noted that no significant difference in plant health or growth (i.e., number of stems, plant height, weight, and percent moisture at end of pilot) was found for systems with or without biochar or Moss (Figure 16). Based on the data collected during the pilot-phase testing, we can conclude that the combination of *Carex* and Moss is the ideal CWTS design for long-term and sustainable copper and selenium treatment. In this design the *Carex* provides structure to soil and sediment of the wetland with extensive root structure, additional treatment capacity based on the roots drawing water down into the sediment, renews organic carbon in the sediment and contributes to accretion through detritus decomposition, and aids in the production of sulphate reducing zones (as indicated by black areas in Figure 15). Meanwhile, the Moss provides sorption and uptake of elements even through freeze-thaw events.

5.1.5. Microbially-mediated treatment

In order to fully understand the function of biogeochemical processes such as the mineralization of copper and selenium, it is critical that the microbial populations performing these functions are well characterized and understood. Therefore, the microbial communities populating the sediment of each pilot CWTS cell were analyzed by both genetic and growth-based methods. The genetic method identifies and comparatively quantifies all of the bacteria in a sample without requiring growth, which is very important because a large proportion of environmental organisms will not grow in a laboratory setting. The genetic-based profiling is therefore used to assess which microbes are present and at what abundance, giving an idea of the robustness of the community and allowing for potential function to be inferred. The second method is called the most-probable number (MPN) assay, which is a dilution-based growth method that can determine the number of selenite-reducing, sulphate-reducing, and nitrate-reducing organisms per gram of sediment. The MPN method is therefore a means to evaluate the biogeochemical cycling potential of a sample; however, because the analysis is based on growth in laboratory media, it is presumed that not all organisms are able to grow and that only a partial profile of the microbial community is provided. Both methods allow for comparisons to be made of the microbial populations across samples, treatments, or time series.

With the microbial analyses, we were interested in three key questions:

- How does the microbial community adapt and change with different influent water chemistries and system designs?
- Are microbes that contribute to selenium, copper, or nitrate treatment present in the pilot wetland cells, and if so, are these same organisms present at the Minto site?
- Is selenium or copper treatment mediated by microbes present in the sediment?

As Figure 7 shows, the microbial population adapted rapidly and consistently to the changes in influent chemistry (i.e., High N, Low N), as well as the addition of organic matter. The nMDS plot of Figure 7 can be interpreted simply as samples that cluster together have more similar microbial communities, while those that are farther apart have distinct differences in their populations (such as identity or relative abundance). At the beginning of the pilot system operation, the microbial community had a proportionately high number of *Actinobacteria*, which then transitioned to *Cyanobacteria* during the High N period corresponding to a visible algal bloom. The Low N and hybrid bioreactor-CWTS phases had similar microbial populations, indicating that either the microbes present in the Low N phases were also well suited to the bioreactor-CWTS conditions, or that the community was still in flux at the final sampling, and treatment would have continued to improve over time in the hybrid system as more anaerobic organisms flourished. Overall, Figure 7 shows that as expected, the microbial populations were both adaptive and robust, shifting to respond to changes in the water chemistry and explanatory parameters such as dissolved oxygen and redox.

The results of the MPN testing indicate that the High N period had an abundance of organisms capable of denitrification including the reduction of nitrate to nitrite, and nitrate to N_2 gas (Figure 17). As expected based on the elevated water concentrations of nitrate, the High N period had a large number of organisms present that could reduce nitrate to N_2 gas. Although in decreased abundance, denitrifying bacteria were present through the Low N period and the hybrid bioreactor-CWTS period, despite having much lower nitrogen concentrations during this timeframe. As such, it is anticipated that fluctuations in ammonia and nitrate concentrations over time would be rapidly met with millions of microbes per gram of soil that are able to perform denitrification reactions.

Sulphate-reducing bacteria consistently increased in abundance over time in all system designs, starting out with a most probable number of approximately a thousand per gram of sediment and increasing by two orders of magnitude through the study (Figure 17). This increase is consistent with trends observed in the soil redox measurements becoming more negative over time in this study. The presence and abundance of sulphate-reducing bacteria were positively correlated with final sulphur concentrations in sediment, according to both the genetic and growth-based profiling methods. Several groups of known sulphate-reducing bacteria (e.g., *Desulfobacterium, Desulfosarcina, Desulfotomaculum)* were found to also correlate with copper concentrations in the sediment, suggesting that their presence leads to reduction of sulphate to sulphide, which then binds to copper and precipitates into the sediment. This can also be demonstrated by the black areas surrounding the *Carex* roots and black precipitates on the sediment surface observed during system takedown (Figures 14 and 15). This copper-sulphide mineralization mechanism is ideal for treatment as it provides a non-bioavailable and stable form of copper in the sediment.

We can tell from the genetic sequencing that these same types of sulphate-reducing bacteria were found in samples that were collected during the August 2013 site visit, specifically associated with *Carex* roots and sediment at the W10 location, as well as in the sediment at W15. It is likely that these were brought to the pilot system along with the plant roots as these *Carex* were harvested from the Minto W10 area. This indicates that despite having different sediment and environmental conditions at the site in comparison to the pilot CWTS, these microbes are robust and can be further encouraged to thrive and perform beneficial functions by having the ecological niches that they prefer (e.g., *Carex* roots, low ORP and DO).

The most probable number of selenite-reducing bacteria ranged from hundreds of thousands to tens of millions per gram of soil and was similar between the different system designs, having similar variance through the three testing periods (Figure 17). There was a general increase in selenite-reducing bacteria during the High N and Low N periods, followed by a decrease in their numbers during the hybrid bioreactor-CWTS period similar to denitrifying organisms discussed above. It was not possible to calculate a correlation between the number of selenite-reducing bacteria and the selenium concentration in the sediment due to the generally low levels (i.e., non-detectable) of selenium in the sediment of most cells. An abundance of selenite-reducing organisms in the pilot systems as well as at the Minto site indicates that these organisms are a robust and diverse group, and are present with a latent capacity to perform microbially-mediated selenium treatment given the proper conditions (e.g., electron donors, targeted ORP and DO levels).

The importance of these microbial results is two-fold: first, the treatment of selenium, copper, or nitrate can be mediated and sustained in a very passive manner by microbes if provided the right environment. Second, this necessary environment can be targeted through system design and adjustments in operation as have been done through this study, resulting in stimulation of these microbes in abundance, diversity, and activity.

5.1.6. Range of operational parameters

In order to be able to effectively implement a full-scale passive treatment system such as a CWTS, the targeted explanatory parameters must be predefined. As such, one of the goals of this pilot-scale work was to confirm or optimize the ranges of these parameters from what can be derived from publications and previous experience. These explanatory parameters can then be used to predict the functionality of a wetland and efficacy of treatment. Moreover, well-defined explanatory parameters allow for adaptive management plans to be developed in order to maintain optimal treatment effectiveness and predictability. The pilot systems tested here performed well not only across a variety of water chemistries and induced conditions, but also over a wide range of water pH, DO, ORP and soil redox. Table 9 includes the minimum, maximum and average recorded values of some selected explanatory parameters for the different periods of testing. Most importantly, this table shows that the range of conditions needed for effective copper and selenium treatment were achieved for this site-specific CWTS design, highlighting the robustness and adaptability of the design and providing a range of conditions under which the system will function.

6. Conclusions and Recommendations

When designed and implemented in a strategic and scientifically guided manner, CWTSs can mitigate risks posed by many contaminants. A treatment plan that includes processes that precipitate insoluble species of these constituents for sequestration into the sediments of the wetland are very desirable, as this mechanism captures the contaminant and stores it with stability in the sediments, rather than transferring the contaminant to an indeterminate fate (e.g., plant uptake or volatilization). This study has addressed several important design considerations regarding implementation of a CWTS at the full scale for the treatment of copper and selenium at the Minto Mine. A synopsis of the conclusions and recommendations are:

- An effective and robust pilot-scale CWTS design was demonstrated and optimized using plants from the Minto site and predicted closure water chemistries.

- The pilot-scale CWTSs provided much of the information needed to effectively size full-scale CWTS for treatment at the Minto site, but sizing will also be dependent on other factors (hydrology, available area, and constructability considerations).

- The recommended CWTS design for long-term passive treatment of copper and selenium at the Minto site is a combination of *Carex* and Moss planted in a sand substrate supplemented with woodchips and peat.

- The selected design achieved average outflow concentration of 12 µg Cu/L (average influent 146 µg Cu/L), and 6 µg Se/L (influent 10.2 µg Se/L).

- Conversion of the CWTS to a hybrid bioreactor-CWTS design resulted in the lowest copper (7.5 µg/L) and selenium (1.9 µg/L) concentrations of the study, but was preceded by a brief disruption of treatment capacity. This configuration would require greater maintenance than the basic CWTS, but could be implemented if any changes in influent water quality are predicted or if outflow objectives change.

- If transitioning from the CWTS design to hybrid bioreactor-CWTS, close monitoring of CWTS and potential consideration of contingency treatment is recommended during the transition period, as there may be a brief and temporary disruption in treatment effectiveness.

- The majority of the copper and selenium treated by the system was sequestered to the top 6 cm of sediment, with only a small portion (1-3%) being taken up into the *Carex* (commonly known as sedge).

- After undergoing a freeze-thaw cycle, the *Carex* used in this study released only a small amount of copper and selenium; however, under the same trial the binding and sorption capacity of the Moss increased, resulting in a net removal of copper and selenium from the water through a freeze-thaw cycle.

- Fluctuations in nitrogen levels (as would be expected in early closure due to residue from blasting activities) are responded to by a diverse microbial community with a robust capacity to treat nitrate, but also continue to treat Cu and Se through this transition;

- There were thousands to billions of nitrate, sulphate, and selenium reducing bacteria present per gram of soil in the pilot CWTS, many of which were confirmed through genetic testing to be the same groups of organisms as found at the Minto site.

- It is recommended that the design be tested on-site at Minto with a demonstration-scale CWTS receiving seepage water.

7. Closure

We trust the information herein satisfies your present requirements. Should you have any questions, please contact the persons listed below. We appreciate the opportunity to provide the services detailed in this report, and look forward to discussing any comments you may have.

Respectfully submitted,

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9. Tables

Table 1: Synthetic water formulation.

Element	Compound	Concentration (mg/L)
Aluminum	$Al_2(SO_4)_3 \cdot 18H2O$	0.929
Ammonium	NH_4HCO_3	1.00[1]
Arsenic	$Na_2HAsO_4 \cdot 7H_2O$	0.012
Barium	$BaCl_2 \cdot 2H_2O$	0.817
Cadmium	$CdCl_2$	0.0005
Calcium	$CaCl_2$	11.68
	$Ca(NO_3)_2 \cdot 4H_2O$	197.59[1]
Chromium	CrO_3	0.013
Cobalt	$CoCl_2 \cdot 6H_2O$	0.017
Copper	$CuCl_2 \cdot 2H_2O$	0.239
Iron	$FeSO_4 \cdot 7H_2O$	15.29
Lead	$Pb(NO_3)_2$	0.0007
Magnesium	$MgSO_4 \cdot 7H_2O$	324.77
Manganese	$MnSO_4 \cdot H_2O$	8.10
Molybdenum	$Na_2MoO_4 \cdot 2H_2O$	0.026
Nickel	$NiCl_2 \cdot 6H_2O$	0.023
Potassium	KNO_3	28.34[1]
	KH_2PO_4	0.298
Selenium	$Na_2SeO_3 \cdot 5H_2O$	0.032
Silver	$AgNO_3$	0.0005
Sodium	Na_2SO_4	54.14
Strontium	$SrCl_2 \cdot 6H_2O$	8.73
Vanadium	V_2O_5	0.026
Zinc	$ZnCl_2$	0.056

[1] During Low N and hybrid bioreactor-CWTS periods of testing, NH_4HCO_3 and KNO_3 were not added, and the $Ca(NO_3)_2 \cdot 4H_2O$ was decreased to 1.976 mg/L

Table 2: Influent chemistry for pilot CWTS.

Element (total)	**unit**	**Influent**				
		Targeted	Acclimation Period	High N	Low N	Hybrid bioreactor-CWTS
Aluminum (Al)	mg/L	0.100	0.089	0.085	0.080	0.098
Antimony (Sb)	mg/L	0.00160	0.00060	0.00065	0.00061	0.0006
Arsenic (As)	mg/L	0.0030	0.0031	0.0033	0.0033	0.0037
Barium (Ba)	mg/L	0.500	0.153	0.284	0.192	0.224
Beryllium (Be)	mg/L	0.00078	<0.001	<0.001	<0.001	<0.001
Boron (B)	mg/L	0.315	0.0243	0.0262	0.0239	0.0243
Cadmium (Cd)	µg/L	0.3340	0.011	0.236	0.336	0.392
Calcium (Ca)	mg/L	82	70.33	73.90	40.73	48.22
Chromium (Cr)	mg/L	0.0065	0.0058	0.0064	0.0071	0.0075
Cobalt (Co)	mg/L	0.0039	0.0039	0.0042	0.0043	0.0044
Copper (Cu)	mg/L	0.13	0.14	0.15	0.15	0.18
Iron (Fe)	mg/L	3.1	3.1	3.1	3.2	3.5
Lead (Pb)	mg/L	0.00070	0.00044	0.00067	0.00059	0.00071
Magnesium (Mg)	mg/L	50	49	49	51	53
Manganese (Mn)	mg/L	2.6	2.5	2.5	2.6	2.7
Molybdenum (Mo)	mg/L	0.012	0.013	0.015	0.013	0.013
Nickel (Ni)	mg/L	0.0072	0.0077	0.0087	0.0076	0.0080
Phosphorus (P)	mg/L	0.082-0.27 [1]	0.333	0.236	0.082	0.082
Potassium (K)	mg/L	14	14.3	14.6	3	3
Selenium (Se)	mg/L	0.010	0.010	0.011	0.010	0.011
Sodium (Na)	mg/L	44	40	41	42	43
Strontium (Sr)	mg/L	2.9	2.5	2.5	2.5	2.6
Sulphur (S)	mg/L	85	85	84	86	88
Uranium (U)	mg/L	0.0051	0.0012	0.0012	0.0012	0.0014
Vanadium (V)	mg/L	0.016	0.015	0.013	0.014	0.018
Zinc (Zn)	mg/L	0.032	0.0517	0.037	0.040	0.042
Nutrients						
Total Ammonia (N)	mg/L	0.2-0.5 [2]	0.61	0.50	0.29	0.05
Dissolved Nitrate (N)	mg/L	0.8-28 [2]	29.33	29.67	0.86	0.84
Misc. Inorganics						
pH	pH	7.5-8.0	7.89	8.16	7.96	8.01
Anions						
Alkalinity (Total as CaCO3)	mg/L		107.33	107.00	100	100
Bicarbonate (HCO3)	mg/L		133.33	130.00	122	130

[1] Phosphorous was not modeled for closure, so the maximum concentration from W15 (2007-2012) was used (0.27mg/L), this was later decreased to 0.082mg/L (95th percentile).

[2] Ammonia and nitrate were not added to the synthetic water for the Low N and hybrid bioreactor-CWTS periods.

Table 3: Pilot CWTS testing periods.

Period	Timeline	# Weeks	Water Depth	HRT (hr) ± Std. Dev.	Description/Purpose
Pre-Influent	Nov 4, 2013 – Dec 12, 2013	6	10 cm	N.D.	Plant establishment
Acclimation	Dec 13, 2013 – Feb 26, 2014	10	10cm	37± 3	Acclimation of system to influent
High N	Feb 27, 2014 – Apr 12, 2014	7	10 cm, 17 cm[1]	37 ± 3 53 ± 3[1]	High ammonia and nitrate in influent (early closure)
Low N	Apr 13, 2014 – May 28, 2014	7	17 cm	53 ± 2.5	Low ammonia and nitrate (long-term closure)
hybrid bioreactor-CWTS	May 29, 2014 – Jul 16, 2014	7	30 cm	87 ± 3	Post alfalfa and straw addition (hybrid bioreactor-CWTS)

[1] 17 cm from March 13 to Apr 12, 2014 to test higher water depth during High N Period. This therefore results in a longer HRT for this timeframe.

Table 4: Monitoring schedule.

Water	
Temperature	Weekly
pH	
Dissolved oxygen	
Regulated metals water package [1]	Bi-weekly
Phosphorus	
Ammonia [2]	
Nitrate	
Flow rate	
Alkalinity [3]	Monthly
Hardness [3]	
Conductivity [3]	
Chemical oxygen demand (COD) [4]	
Total organic carbon [4]	
Total Kjeldahl Nitrogen (TKN) [4]	
Sulphate [3]	
Biological oxygen demand (BOD) [3]	Monthly (outflow only)
Total suspended solids [2]	
Soil	
Eh (redox)	weekly
Available NPK	Initial soil, beginning of influent, end of study
Regulated metals package	
Total organic carbon	Initial soil, end of study
Particle size analysis	
Conductivity	
Cation exchange capacity (CEC)	
Sodium adsorption ratio	
Plant	
Regulated metals	Start and end; Freeze-thaw trial
Stem counts and heights [4]	Monthly
Microbial	
Most probable number (growth-based)	Initial soil; beginning High N period; end Low N period; end of study
Genetic microbial community profiles	

[1] Dissolved metals was tested in addition to total metals for the Low N and hybrid bioreactor-CWTS periods

[2] Not performed during hybrid bioreactor-CWTS period, except at takedown of pilot

[3] Bi-weekly during hybrid bioreactor-CWTS period

[4] Not performed during Low N and hybrid bioreactor-CWTS periods, except at takedown of pilot

Table 5: Summary of water, soil, and plant analyses.

Water	Soil	Plant
ICP-MS Total or Dissolved (Al, Sb, As, Ba, Be, B, Cd, Cr, Co, Cu, Fe, Pb, Li, Mn, Mo, Ni, P, Se, Si, Ag, Sr, Tl, Sn, Ti, U, V, Zn, Ca, Mg, K, Na, S)	ICP-MS (Al, B，Sb, As, Ba, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, P, K, Se, S, Ag, Na, Sr, Tl, Sn, U, V, Zn,)	ICP-MS (Al, Sb, As, Ba, Be, Bi, B, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Mo, Ni, P, K, Se, Ag, Na, Sr, Tl, Sn, Ti, U, V, Zn)
Routine Water (Alkalinity, HCO_3, Ion Balance, Dissolved ions, Hardness, Nitrate/Nitrite)	Available Nitrogen, Phosphorous, and Potassium (NPK)	
Total Suspended Solids (TSS)	Cation Exchange Capacity (CEC)	
Chemical Oxygen Demand (COD)	Sodium Adsorption Ratio (SAR)	
Biological Oxygen Demand (BOD)	Total Organic Carbon (TOC)	
Total Kjeldahl Nitrogen (TKN)	Electrical Conductivity (EC)	
Total Organic Carbon (TOC)	Texture Analysis (Physical properties)	
Ammonia		

Table 6: Performance of pilot CWTS during High N period.

Elements (Total)	**Unit**	**Influent**	**Outflow Average**		
		Average	*Carex* only	*Carex* + *Sphagnum*	*Carex* + *Sphagnum* + Biochar
Aluminum (Al)	mg/L	0.085	0.012	0.008	0.009
Arsenic (As)	mg/L	0.0033	0.0010	0.0008	0.0007
Barium (Ba)	mg/L	0.284	0.039	0.036	0.040
Boron (B)	mg/L	0.0262	0.025	0.027	0.055
Cadmium (Cd)	µg/L	0.236	0.038	0.023	0.017
Calcium (Ca)	mg/L	73.9	78	78	77
Chromium (Cr)	mg/L	0.0064	0.0024	0.0023	0.0024
Cobalt (Co)	mg/L	0.0042	0.0008	0.0004	0.0003
Copper (Cu)	mg/L	0.150	0.018	0.013	0.012
Iron (Fe)	mg/L	3.10	0.08	0.08	0.08
Magnesium (Mg)	mg/L	49	50	51	51
Manganese (Mn)	mg/L	2.5	1.1	0.37	0.2
Molybdenum (Mo)	mg/L	0.0150	0.0139	0.0130	0.0128
Nickel (Ni)	mg/L	0.0087	0.0042	0.0027	0.0020
Phosphorus (P)	mg/L	0.236	<0.1	<0.1	<0.1
Potassium (K)	mg/L	14.6	14.9	15.3	15.1
Selenium (Se)	mg/L	0.0110	0.0076	0.0073	0.0067
Sodium (Na)	mg/L	41	43	45	42
Strontium (Sr)	mg/L	2.5	2.7	2.7	2.6
Sulphur (S)	mg/L	84	86	87	86
Uranium (U)	mg/L	0.0012	0.0011	0.0011	0.0011
Vanadium (V)	mg/L	0.0130	0.0023	0.0019	0.0017
Zinc (Zn)	mg/L	0.0370	0.0042	0.0044	0.0034
Nutrients					
Total Ammonia (N)	mg/L	0.50	0.05	0.05	0.05
Dissolved Nitrate (N)	mg/L	29.67	29.4	29.4	29.5
Explanatory Parameters					
pH	pH	8.16	7.47	7.61	7.80
DO (mg/L)			5.33	5.16	5.52
Water redox (mV)			154.5	144.2	136.0
Soil Redox (mV)			-148	-191	-220

Table 7: Performance of pilot CWTS during Low N period.

Elements (Total)	**Unit**	**Influent**	**Outflow Average**		
		Average	*Carex* only	*Carex* + Moss	*Carex* + Moss + Biochar
Aluminum (Al)	mg/L	0.080	0.008	0.006	0.007
Arsenic (As)	mg/L	0.0033	0.0007	0.0006	0.0006
Barium (Ba)	mg/L	0.192	0.028	0.027	0.030
Boron (B)	mg/L	0.024	0.023	0.024	0.022
Cadmium (Cd)	µg/L	0.336	0.070	0.027	0.023
Calcium (Ca)	mg/L	41	47	47	47
Chromium (Cr)	mg/L	0.0071	0.0034	0.0029	0.0030
Cobalt (Co)	mg/L	0.0043	0.0006	<0.0003	<0.0003
Copper (Cu)	mg/L	0.150	0.0210	0.0113	0.0125
Copper (Cu) Dissolved	mg/L	0.037	0.0123	0.0086	0.0086
Iron (Fe)	mg/L	3.20	0.16	0.06	0.09
Magnesium (Mg)	mg/L	51	55	55	55
Manganese (Mn)	mg/L	2.6	0.9	0.2	0.2
Molybdenum (Mo)	mg/L	0.0130	0.0110	0.0104	0.0105
Nickel (Ni)	mg/L	0.0076	0.0035	0.0018	0.0015
Phosphorus (P)	mg/L	0.11	<0.10	<0.10	<0.10
Potassium (K)	mg/L	3.0	3.2	3.4	3.4
Selenium (Se)	mg/L	0.0100	0.0061	0.0060	0.0063
Selenium (Se) Dissolved	mg/L	0.0080	0.0052	0.0053	0.0052
Sodium (Na)	mg/L	42	44	44	45
Strontium (Sr)	mg/L	2.5	2.7	2.7	2.7
Sulphur (S)	mg/L	86	93	94	93
Uranium (U)	mg/L	0.0012	0.0009	0.0010	0.0010
Vanadium (V)	mg/L	0.0140	0.0023	0.0016	0.0022
Zinc (Zn)	mg/L	0.0400	0.0082	0.0032	0.0031
Nutrients					
Total Ammonia (N)	mg/L	0.29	<0.05	<0.05	<0.05
Dissolved Nitrate (N)	mg/L	0.86	0.26	0.25	0.25
Explanatory Parameters					
pH	pH	7.96	7.58	7.76	7.61
DO (mg/L)			5.69	6.05	5.42
Water redox (mV)			179	170	205
Soil Redox (mV)			-148	-183	-209

Table 8: Performance of pilot CWTS during hybrid bioreactor-CWTS period.

Elements (Total)	Unit	Influent	Outflow Average [1]		
		Average	*Carex* only	*Carex* + Moss	*Carex* + Moss + Biochar
Aluminum (Al)	mg/L	0.098	0.010	0.013	0.009
Arsenic (As)	mg/L	0.0037	0.001	0.001	0.001
Barium (Ba)	mg/L	0.224	0.061	0.061	0.058
Boron (B)	mg/L	0.024	0.026	0.026	0.025
Cadmium (Cd)	µg/L	0.392	0.057	0.067	0.067
Calcium (Ca)	mg/L	48	49	48	48
Chromium (Cr)	mg/L	0.0075	<0.001	<0.001	<0.001
Cobalt (Co)	mg/L	0.0044	0.003	0.002	0.002
Copper (Cu)	mg/L	0.180	0.021	0.023	0.026
Copper (Cu) Dissolved	mg/L	0.0350	0.0122	0.0134	0.0168
Iron (Fe)	mg/L	3.50	0.41	0.33	0.22
Magnesium (Mg)	mg/L	53	54	54	55
Manganese (Mn)	mg/L	2.7	5.2	5.1	5.7
Molybdenum (Mo)	mg/L	0.013	0.015	0.020	0.021
Nickel (Ni)	mg/L	0.008	0.006	0.006	0.007
Phosphorus (P)	mg/L	0.12	0.11	0.11	0.10
Potassium (K)	mg/L	3.0	6.4	6.1	6.3
Selenium (Se)	mg/L	0.0100	0.0040	0.0040	0.0040
Selenium (Se)	mg/L	0.0119	0.0038	0.0039	0.0041
Sodium (Na)	mg/L	43	44	44	44
Strontium (Sr)	mg/L	2.6	2.6	2.6	2.6
Sulphur (S)	mg/L	88	87	87	87
Uranium (U)	mg/L	0.0014	0.0010	0.0010	0.0010
Vanadium (V)	mg/L	0.018	0.002	0.002	<0.001
Zinc (Zn)	mg/L	0.042	0.007	0.005	0.006
Nutrients					
Total Ammonia (N)	mg/L	0.05	0.06	0.05	0.05
Dissolved Nitrate (N)	mg/L	0.84	0.08	0.06	0.03
Explanatory Parameters					
pH	pH	8.01	7.16	7.20	7.19
DO (mg/L)			1.52	1.73	1.86
Water redox (mV)			-34	-91	-134
Soil Redox (mV)			-197	-218	-226

[1] The average outflow for the hybrid bioreactor-CWTS period includes data from June 19th, when there was a spike in several constituents when the system reached low dissolved oxygen and moderately reducing conditions in the water column.

Table 9: Summary of explanatory parameters and outflow concentrations of copper and selenium

Period	Parameter	Influent	System								
			Carex			*Carex* + Moss			*Carex* + Moss + Biochar		
		Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
High Nitrogen	Copper (µg/L)	146.4	16	20	18	11	16	13	9	14	12
	Selenium (µg/L)	10.2	5.8	9.7	7.6	6.0	9.5	7.3	5.8	8.5	6.7
	pH	8.00	6.99	7.94	7.49	7.19	8.29	7.64	7.39	8.28	7.84
	DO (mg/L)	-	4.08	6.69	5.36	2.59	7.55	5.35	3.50	8.70	5.75
	ORP (mV)	-	82	215	157	104	215	247	84	205	140
	Redox (soil,mV)	-	-229	-51	-144	-273	-177	-190	-301	-135	-218
Low Nitrogen	Copper (µg/L)	145.5	17	32	21	10	13	11.3	11	15	12.5
	Selenium (µg/L)	10.2	5.3	6.7	6.1	5.6	6.5	6.0	5.8	6.9	6.3
	pH	7.96	7.16	8.05	7.54	7.26	8.32	7.71	7.15	8.25	7.60
	DO (mg/L)	-	3.99	7.72	5.61	3.02	9.78	5.73	2.57	9.04	5.21
	ORP (mV)	-	126	220	168	113	188	159	106	374	182
	Redox (soil,mV)	-	-249	-39.5	-150	-246	-88	-185	-264	-123	-211
Hybrid Bioreactor-CWTS	Copper (µg/L)	175.6	7.5	45	21	8.3	44	23	8.3	64	26
	Selenium (µg/L)	11.9	1.9	5.6	4.0	2.5	5.4	4.0	2.8	5.4	4.0
	pH	8.01	6.91	7.62	7.16	6.95	7.82	7.20	6.96	7.71	7.19
	DO (mg/L)	-	0.02	3.23	1.52	0.11	5.61	1.73	0.03	4.71	1.86
	ORP (mV)	-	-175	-79	-129	-212	-101	-150	-195	-30	-134
	Redox (soil,mV)	-	-232	-170	-197	-268	-159	-218	-290	-150	-226

Table 10: Removal rate coefficients for each system and period of testing.

Element	**System**	**Period RRC (hr^{-1})**		
		High N	Low N	hybrid bioreactor-CWTS [1]
Copper	*Carex*	0.0465	0.0390	0.0250
	Carex + Moss	0.0519	0.0497	0.0236
	Carex + Moss + Biochar	0.0543	0.0462	0.0242
Selenium	*Carex*	0.0069	0.0103	0.0130
	Carex + Moss	0.0078	0.0102	0.0128
	Carex + Moss + Biochar	0.0095	0.0090	0.0123

[1] The calculation of RRC for the hybrid bioreactor-CWTS period included data from June 19th, when there was a spike in several constituents when the system reached low dissolved oxygen and moderately reducing conditions in the water column.

Table 11: Bioconcentration factor for each plant species at the end of the Low N period.

Plant Species	**Copper (L/kg)**	**Selenium (L/kg)**
Carex	23	113
Moss	1390	593

10. Figures

Figure 1: Pilot CWTS layout

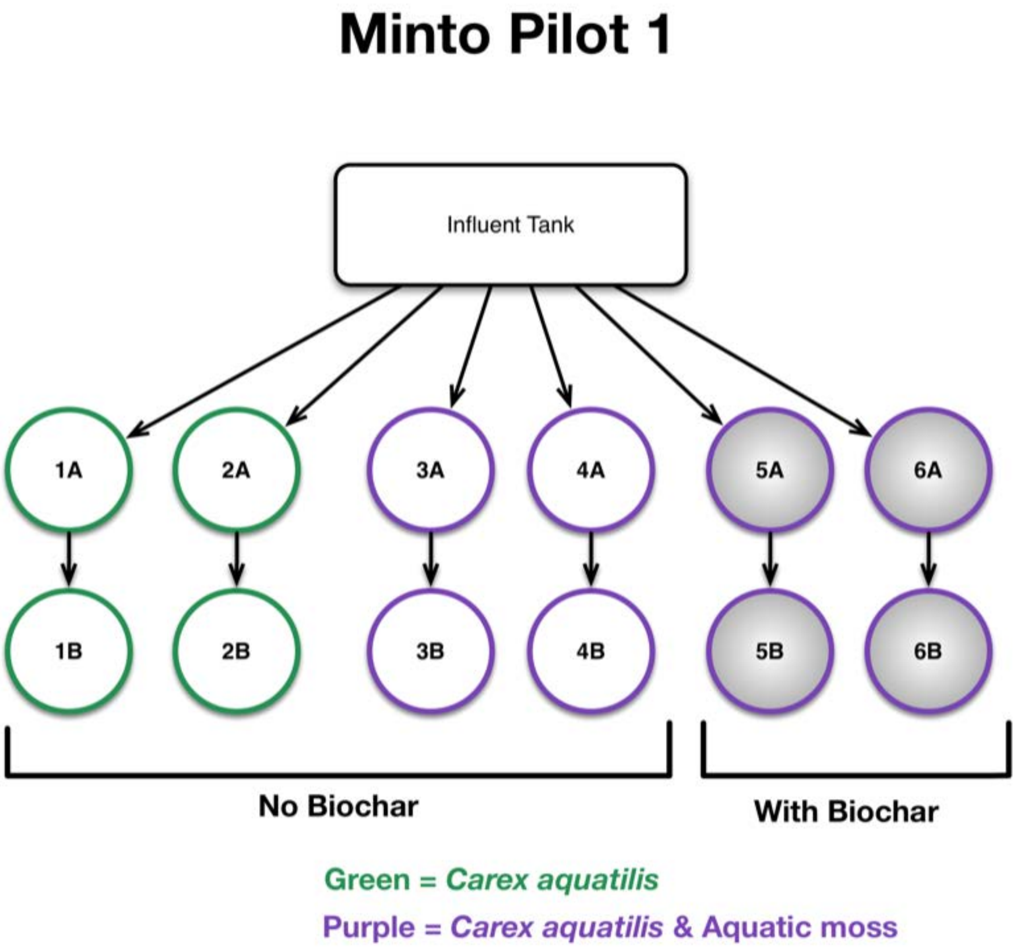


Figure 2: Photographs of pilot CWTS set up. Top is entire system, with 6 series of two cells; bottom is one cell.

Figure 3: Outflow copper and selenium water concentrations over time compared to influent.

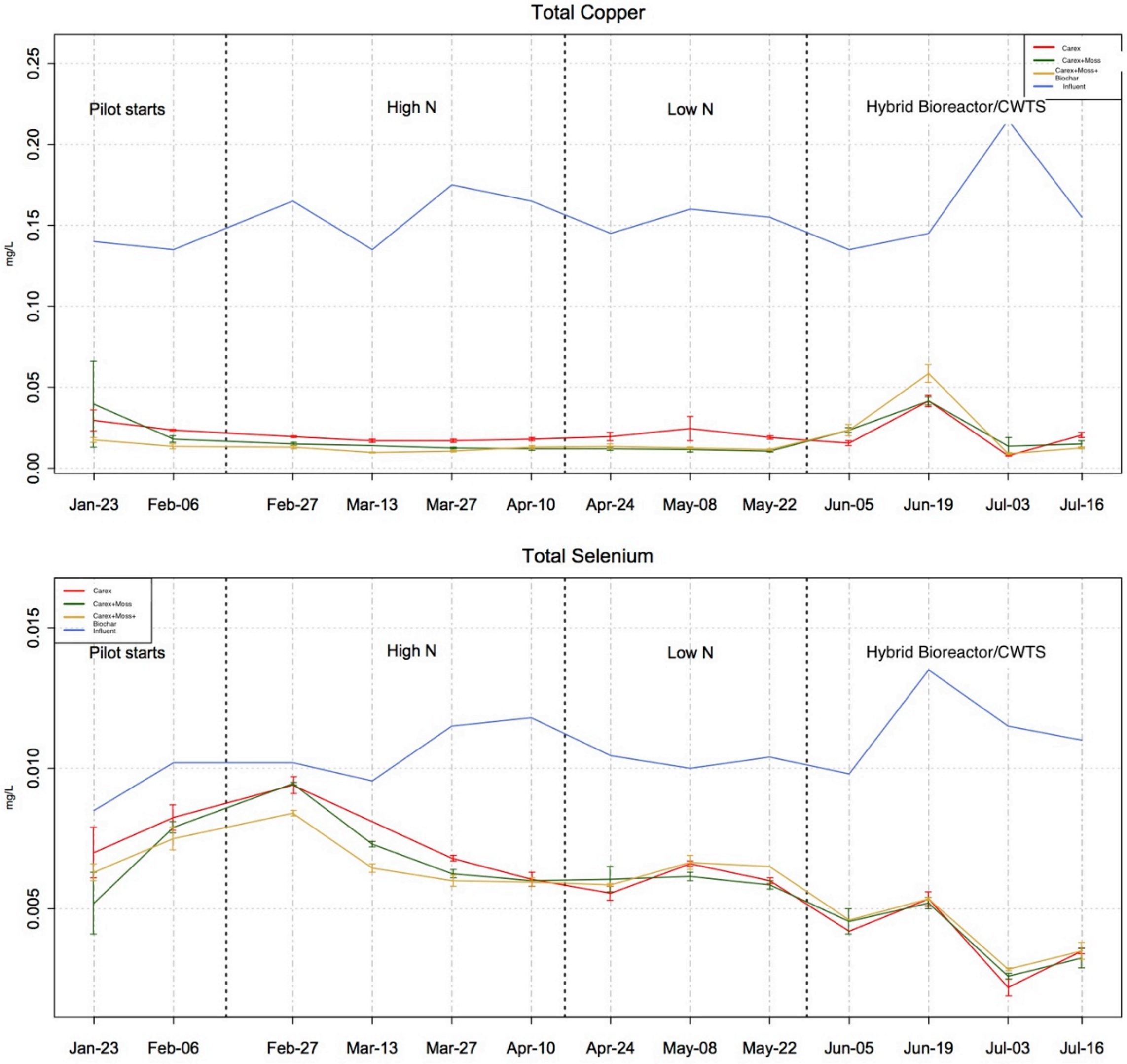


Figure 4: Copper and selenium removal rate coefficients during all periods of testing for the three system designs.

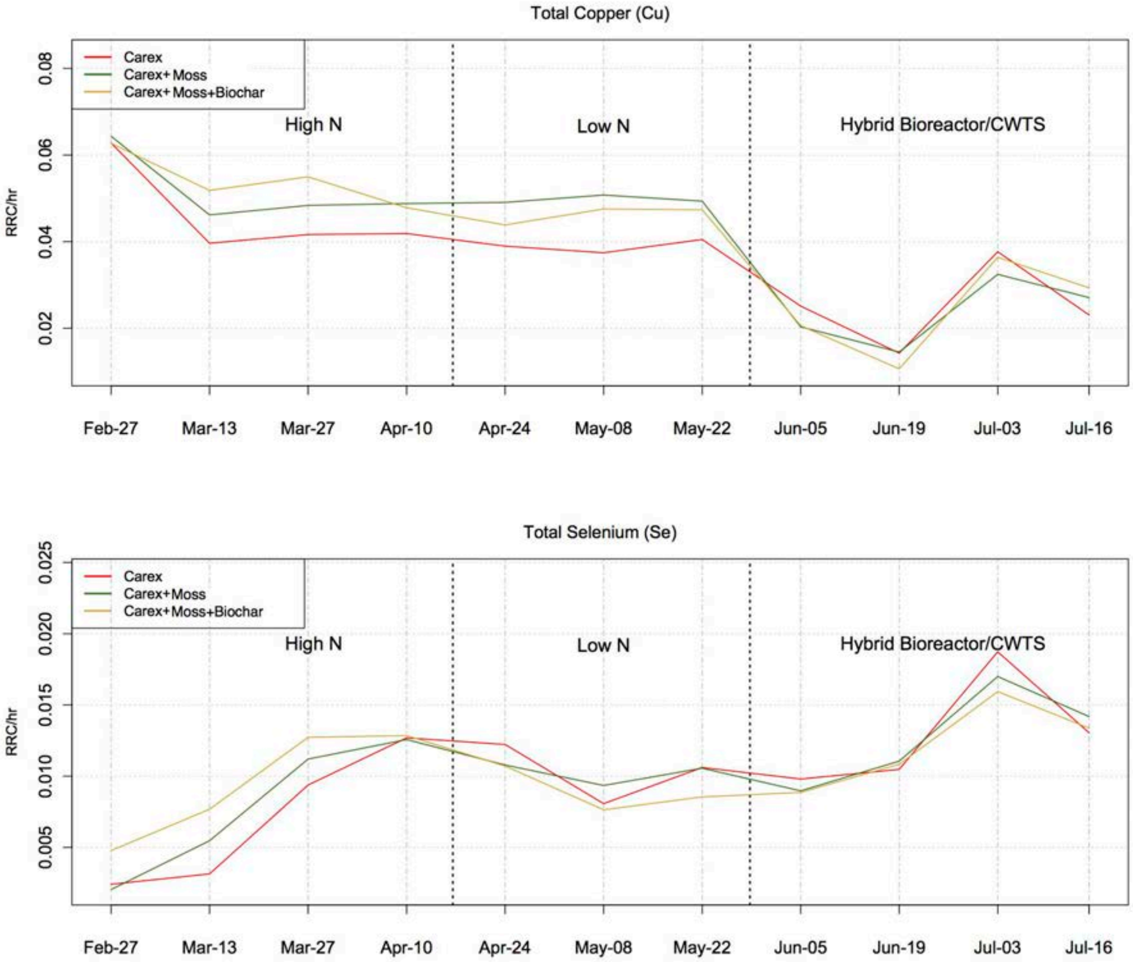


Figure 5: Copper (top) and selenium (bottom) removal rate coefficients for cells A and B in each system design. All three periods of testing are plotted separately (High N = Feb 27-April 12; Low N = April 13-May 28; hybrid bioreactor/CWTS = May 29-July 16). The average RRC is indicated with the bar chart, and error bars show the range. Note that the water depth was changed during the hybrid bioreactor/CWTS phase, which impacts the RRC.

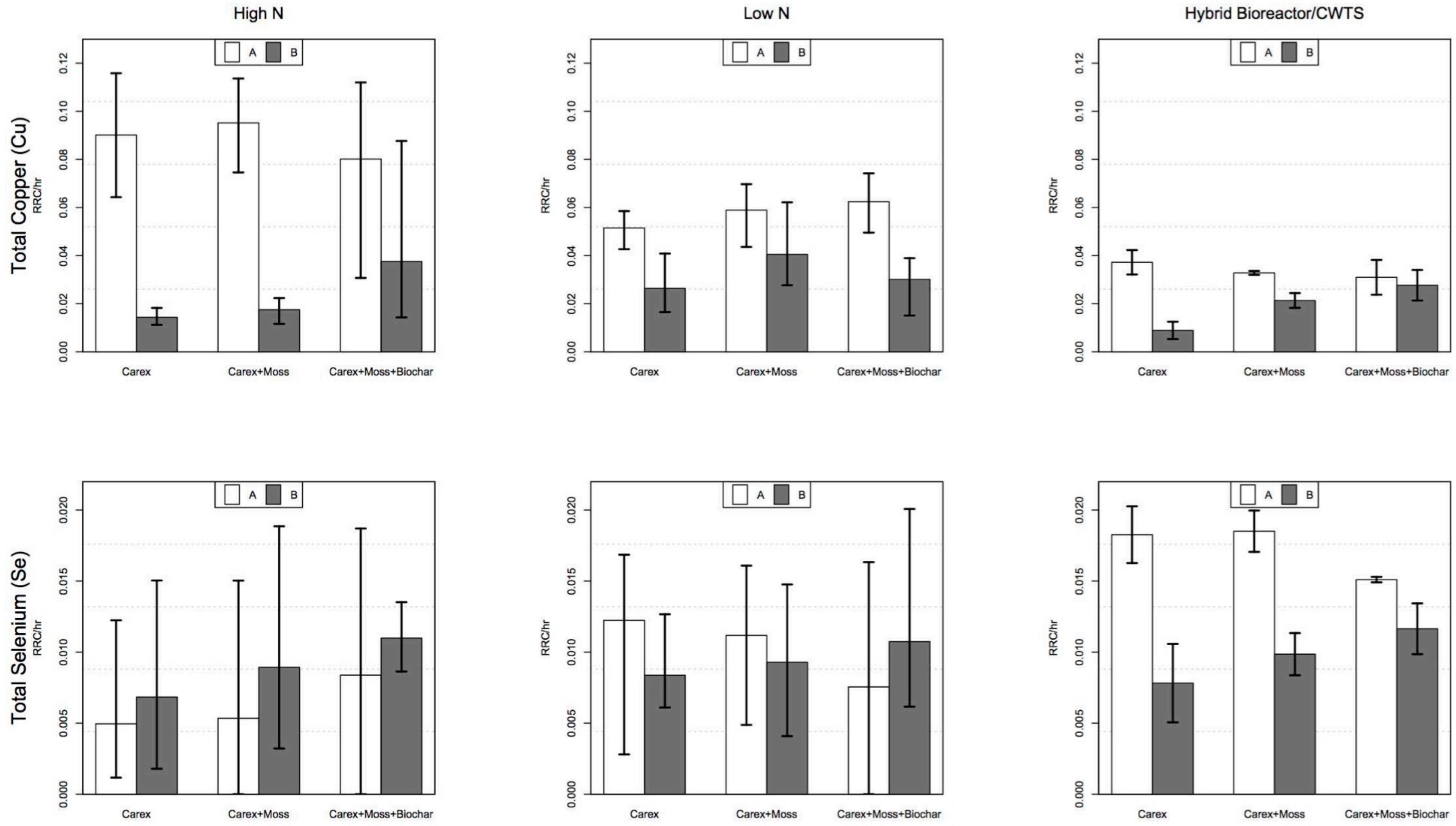


Figure 6: Outflow copper and selenium water concentrations from A and B cells over time compared to influent.

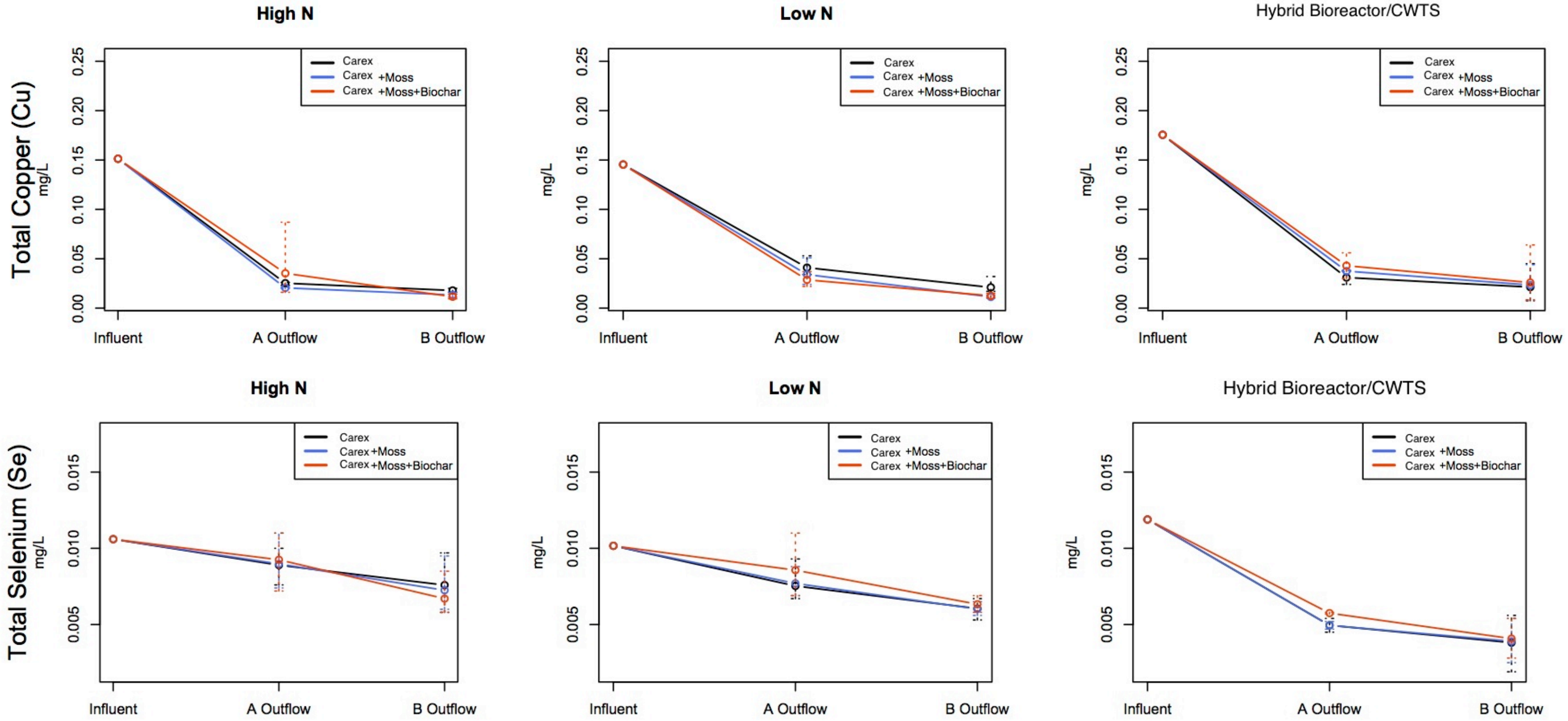


Figure 7: Non-metric multidimensional scaling (nMDS) analysis plot for bacterial community profiles of each pilot system design over time. Results are based on the genetic profiling method. Each time point during testing is shown with a different color, while the system design is indicated with shapes. All points that cluster together indicate similar bacterial communities. At the end of the pilot testing, we also determined the microbial population of the top root mass (orange outline), which was very similar to the microbial population in the sediment. The top 10 bacterial groups are indicated on the plot with dark blue numbers. The distance between a bacterial group label and a sample point depicts how high that group is in that sample.

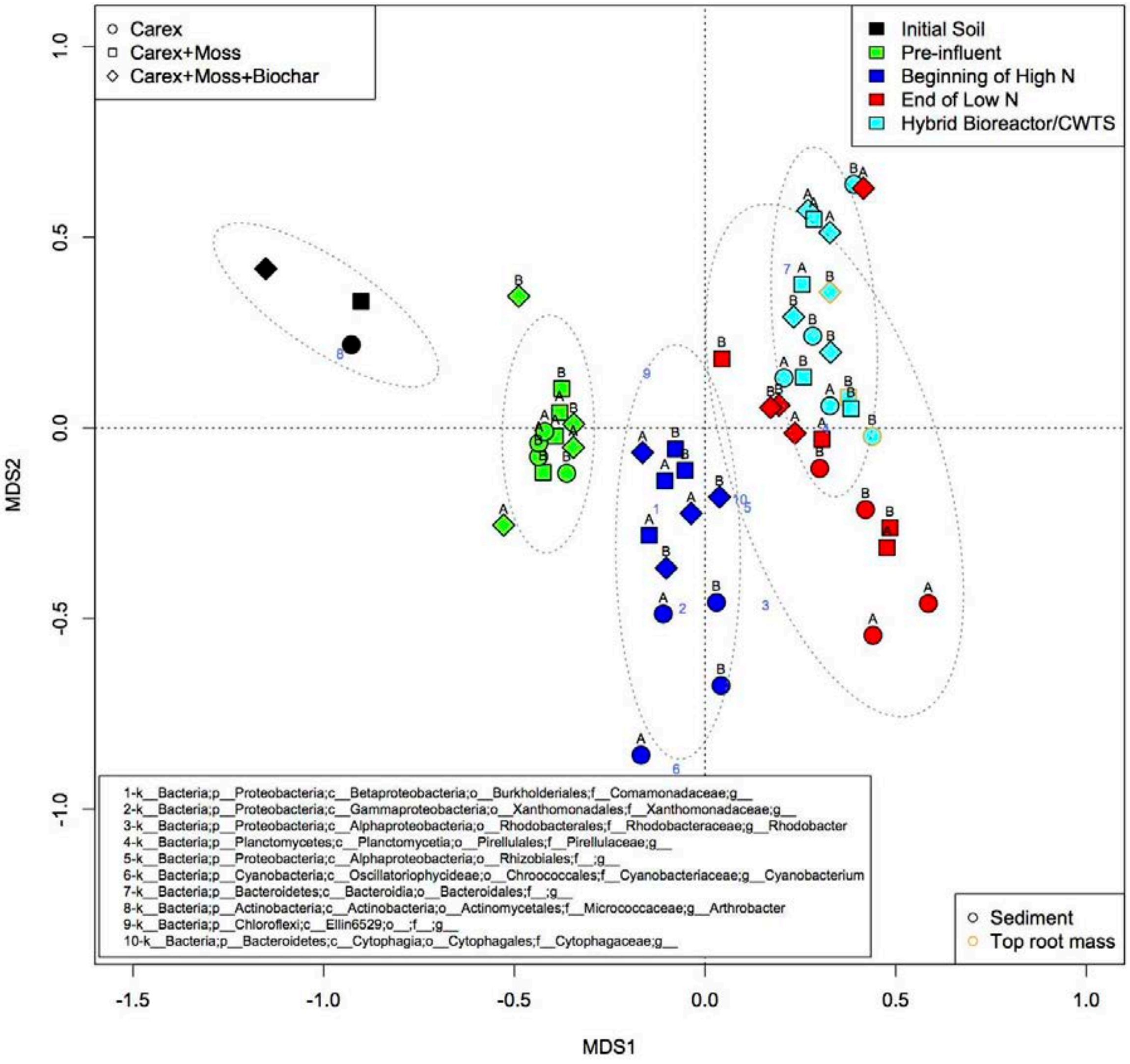
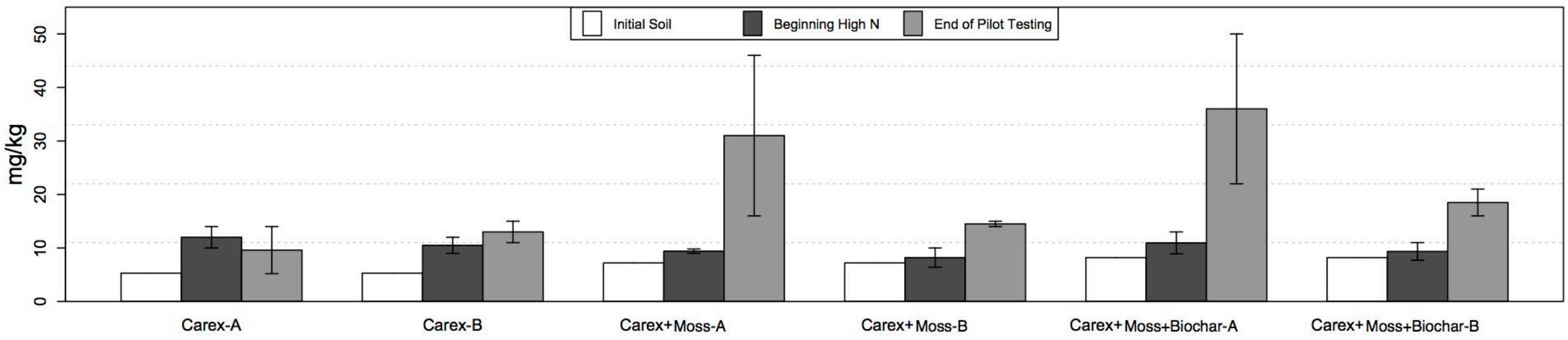
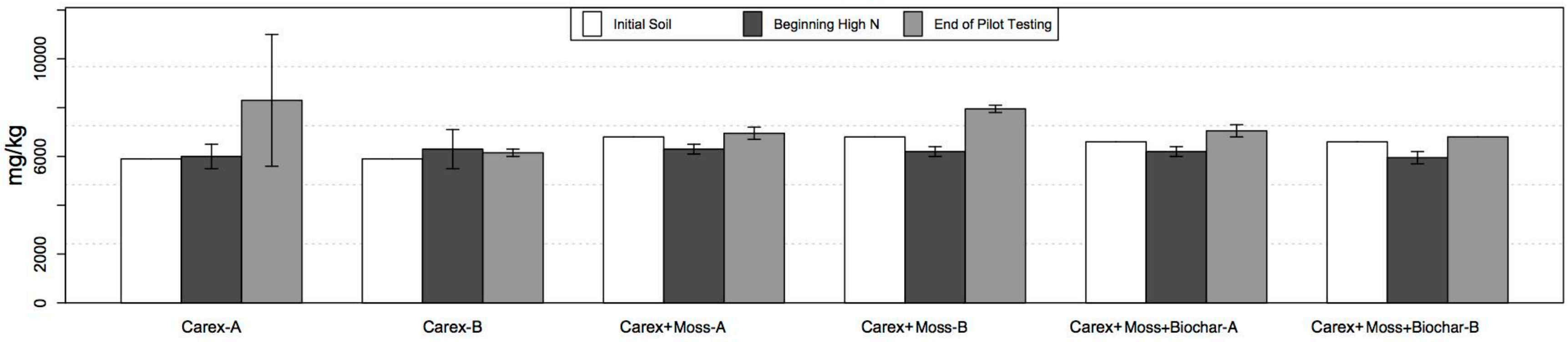


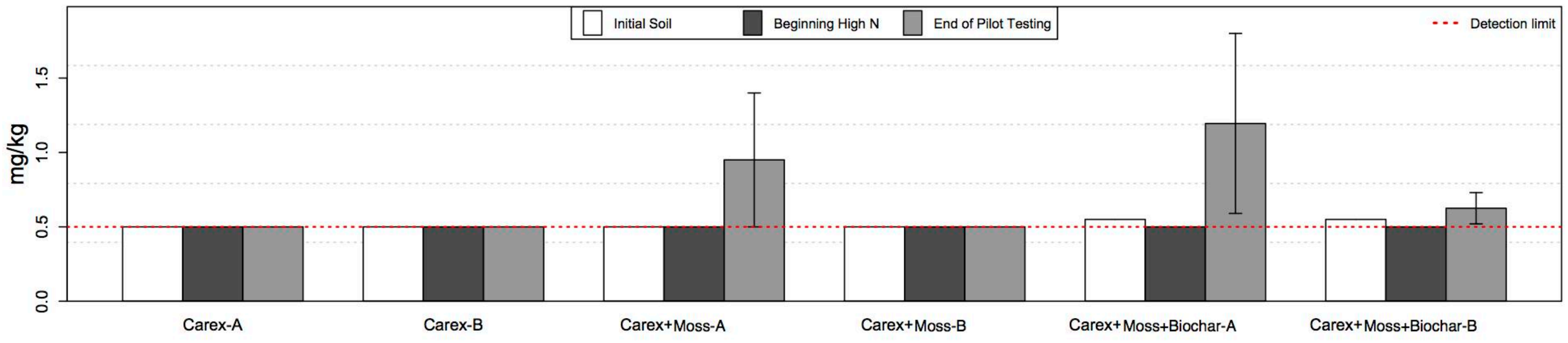
Figure 8: Copper, iron, selenium and sulphur concentrations in sediment throughout the pilot testing. Initial soil is the soil that was used to build the pilot systems, before any water was introduced. A and B cells for system designs are plotted separately, with error bars indicating the maximum and minimum values. The dotted red line indicates the selenium detection limit, as several samples were below this level. Selenium and sulphur plots are on the following page.

Total Copper (Cu)



Total Iron (Fe)




Total Selenium (Se)

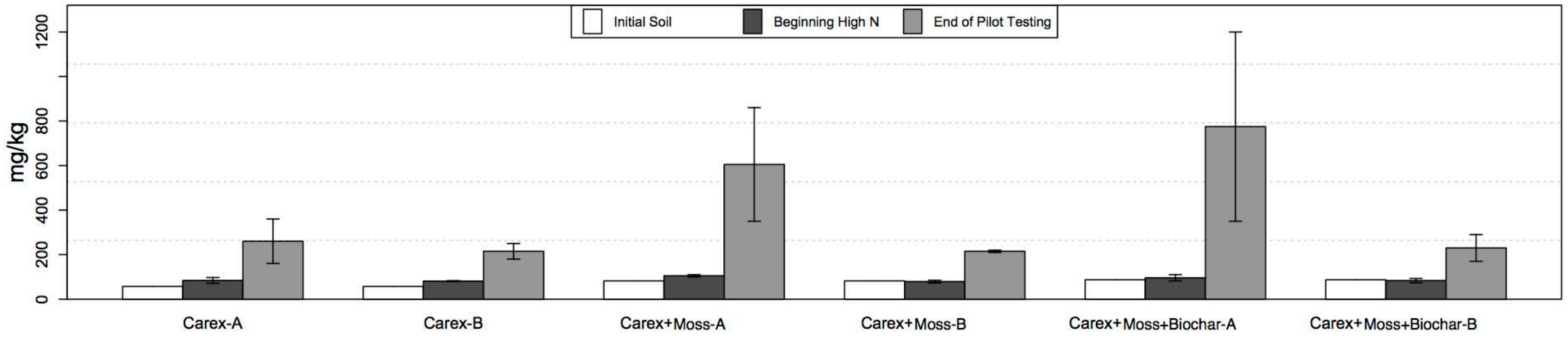

Total Sulphur (S)

Figure 9: Mass balance of copper in the pilot CWTS at the end of testing. The total amount of copper in the top 6 cm of sediment in A and B cells is shown for each system design, as well as the *Carex* in A and B cells. “Other” is the portion that was not analyzed for metals, and therefore cannot be differentiated. Examples of locations in the “Other” category would be plant roots, biofilms, and deeper sediment, as all of these were not analyzed. Additionally, it should be noted that the Moss did not survive the hybrid bioreactor-CWTS period, and therefore was not tested at the end of the pilot testing.

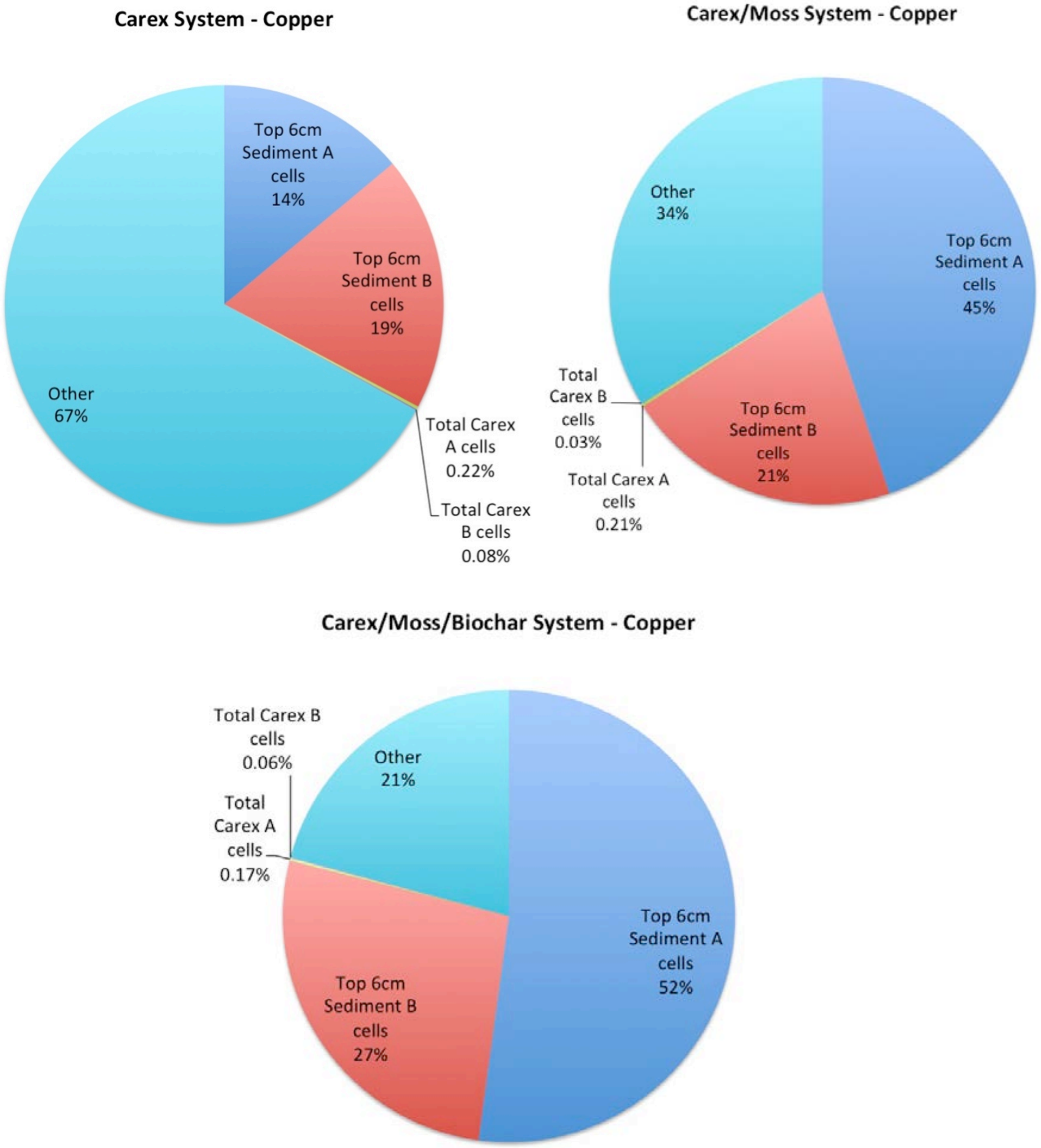


Figure 10: Mass balance of selenium in the pilot CWTS at the end of testing. The total amount of selenium in the top 6 cm of sediment in A and B cells is shown for each system design, as well as the *Carex* in A and B cells. “Other” is the portion that was not analyzed for metals, and therefore cannot be differentiated. Examples of locations in the “Other” category would be plant roots, biofilms, and deeper sediment, as all of these were not analyzed. Additionally, it should be noted that the Moss did not survive the hybrid bioreactor-CWTS period, and therefore was not tested at the end of the pilot testing.

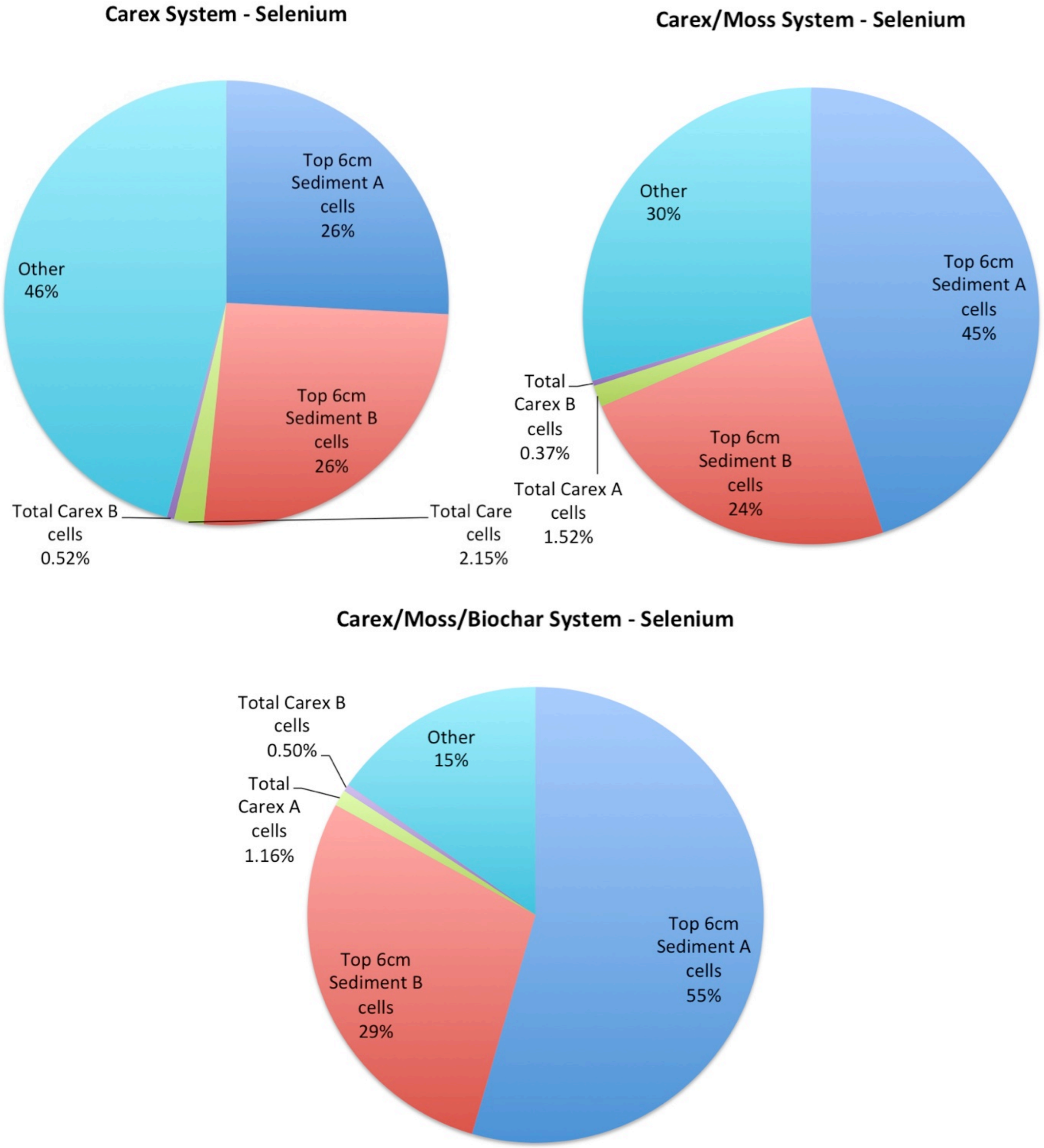
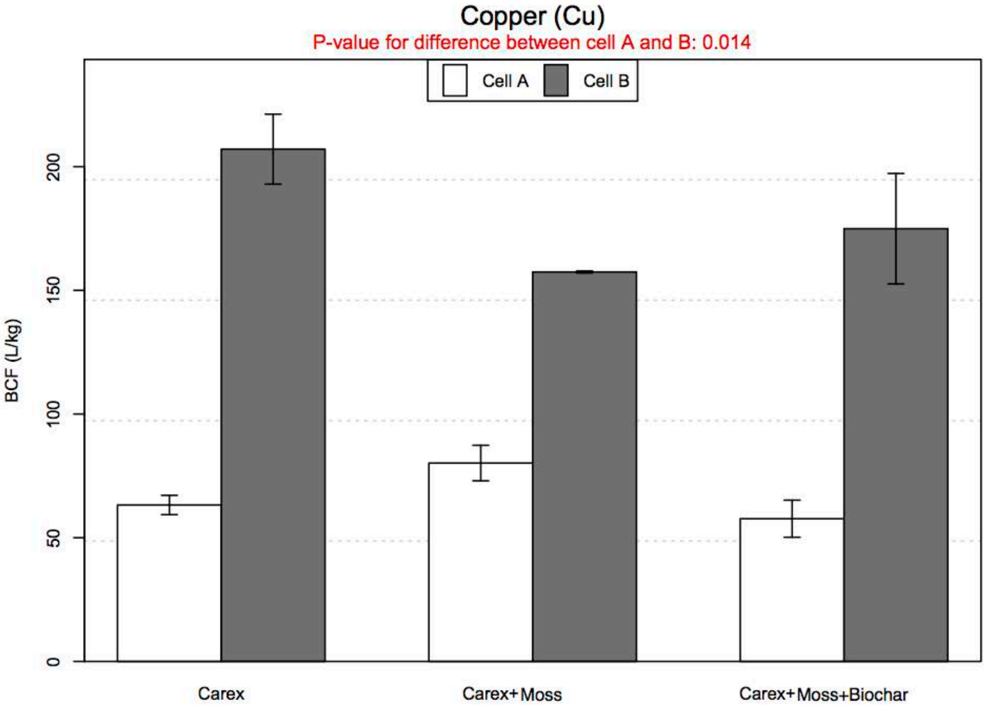


Figure 11: Comparison of bioconcentration factor for copper and selenium in *Carex* in A and B cells from each pilot system design. The p-value is calculated using Friedman test, with significant results shown in red. A significant test means that a difference between the BCF in A and B cells is found (e.g., A cells are always higher than B cells, or vice versa).



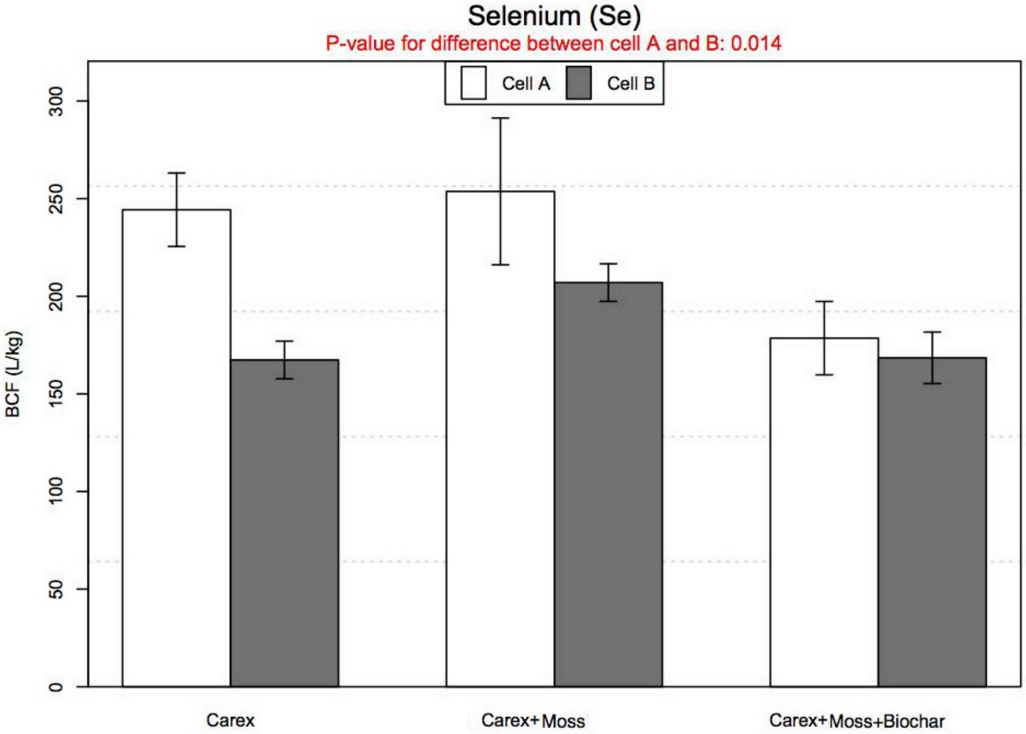


Figure 12: Copper and selenium concentrations in *Carex* before and after the freeze-thaw test, for all three system designs.

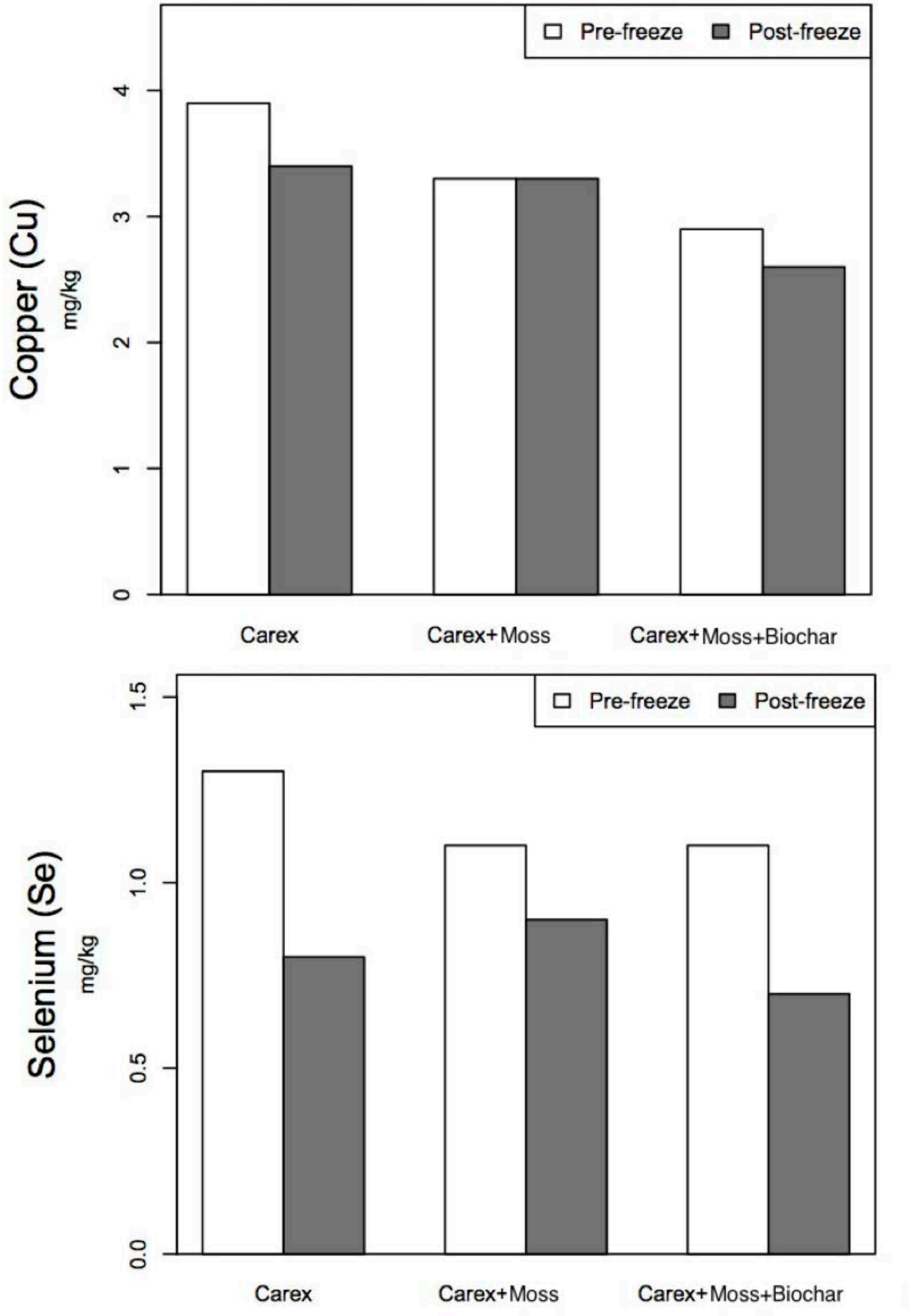


Figure 13: Copper and selenium concentrations in Moss before and after the freeze-thaw test, for the two system designs that had Moss.

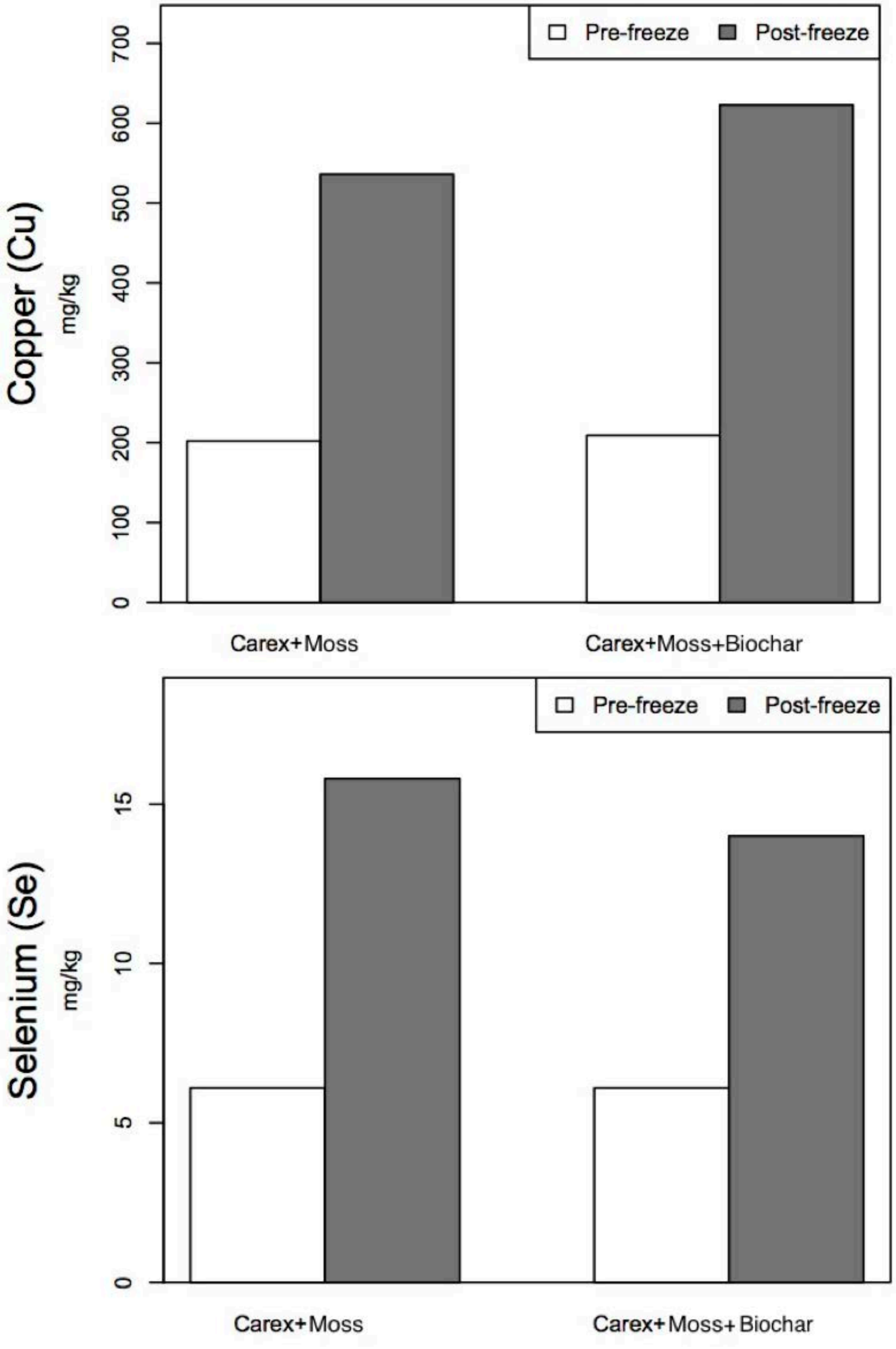
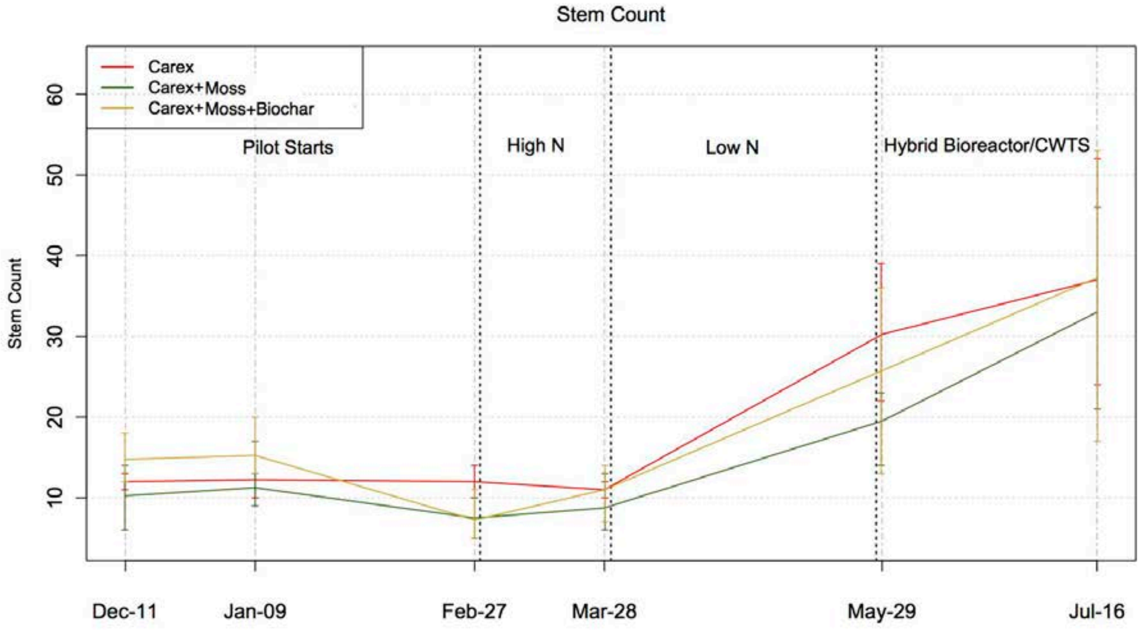


Figure 14: Photograph of *Carex aquatilis* (Sedge) roots at system takedown

Figure 15: Photograph of CWTS cell during takedown of the system. Orange-colored hydrosoil layer is approximately 18 cm, black detritus toproot zone is ~3 cm. Water depth was ~20 cm during the high and low nitrogen periods, and ~30 cm above the sediment during the hybrid bioreactor-CWTS period.

Figure 16: *Carex* stem counts (top) and height (bottom) of each system design, during all periods of testing.



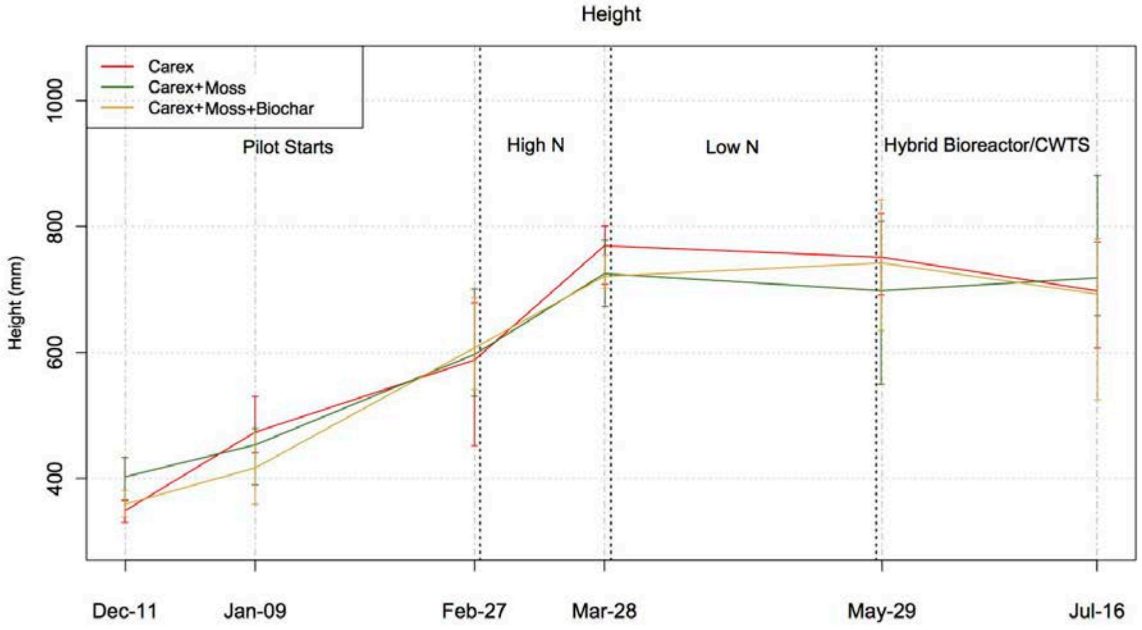
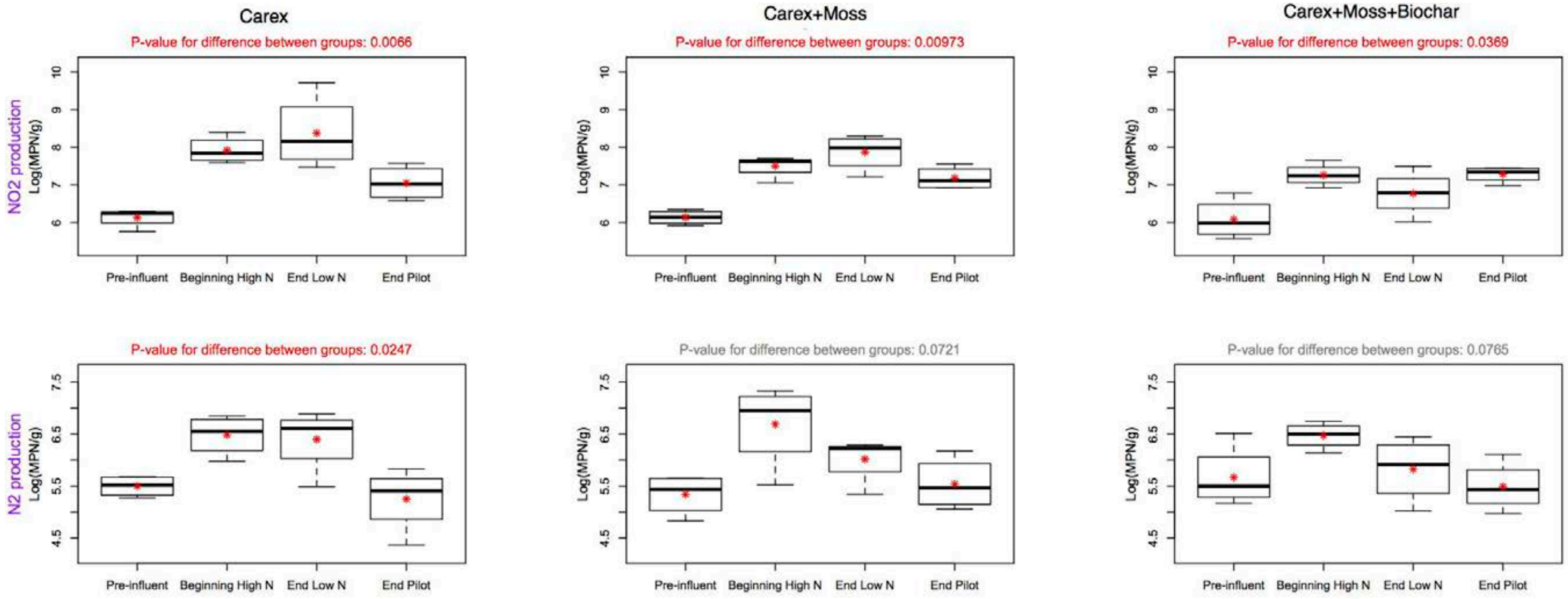
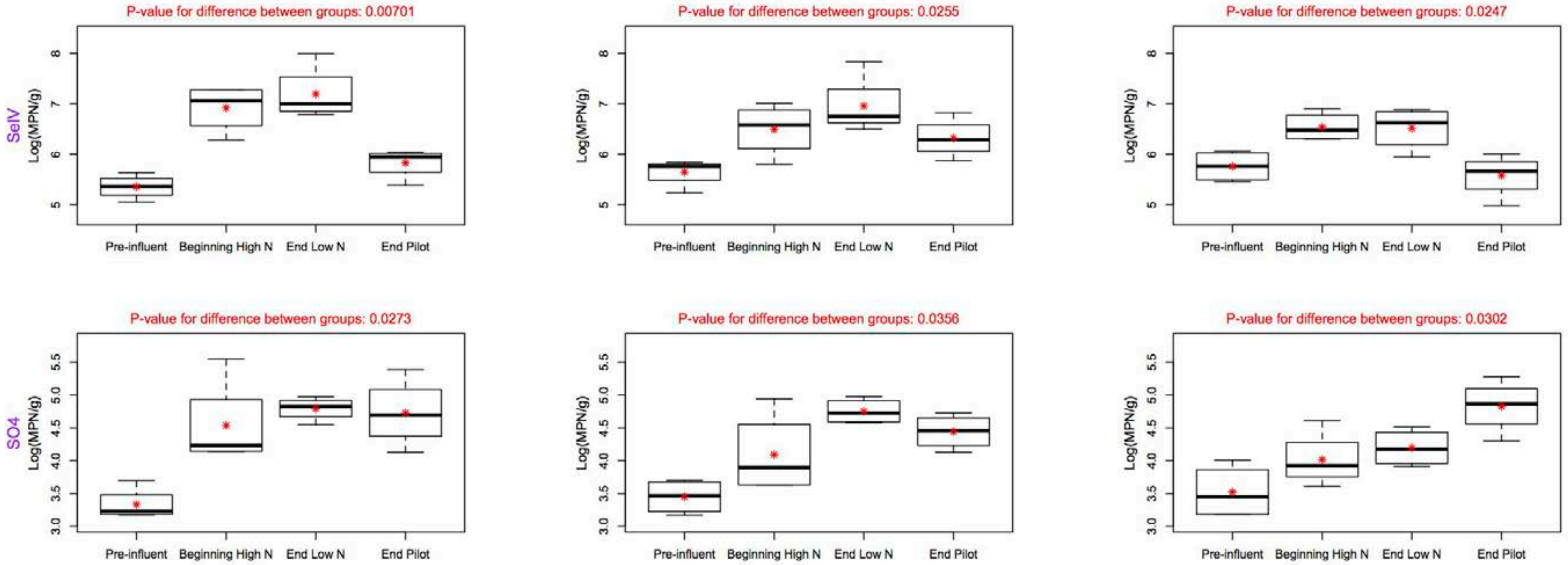


Figure 17: Comparison of the most probable number (MPN) of nitrate-, selenite-, and sulphate-reducing organisms in each system over time. NO_2 production indicates the MPN of organisms that are capable of reducing nitrate to nitrite. N_2 production shows the MPN of organisms that can reduce nitrate to nitrogen gas. The MPN was compared among the four time periods, and a P-value was calculated using the non-parametric Kruskal-Wallis test. The first plot represents systems with just *Carex*, the second plot is systems with *Carex* and Moss, while the third plot is systems with *Carex,* Moss, and biochar. Significant differences ($P<0.05$) are indicated in red, meaning a significant difference is found between the four testing periods in a given system design. Box plots are used to visualize the results; top and bottom edges of the box show the 25th and 75th percentile, respectively, while the thick black line depicts the median and the red asterisk shows the mean. The upper and lower whiskers provide the maximum and minimum non-outlier values. The remainder of the figure is provided on the following pages.

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P-value for difference between groups: 0.0255
P-value for difference between groups: 0.0247
SeIV
Log(MPN/g)
5
6
7
8
Pre-influent
Beginning High N
End Low N
End Pilot
P-value for difference between groups: 0.0273
P-value for difference between groups: 0.0356
P-value for difference between groups: 0.0302
SO4
Log(MPN/g)
3.0
3.5
4.0
4.5
5.0
5.5