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Biological Sulfate Removal Using Waste Organic Substrates in Continuous Flow-Through Columns Simulating Mine Water Vertical Flow Bioreactors

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Biological Sulfate Removal Using Waste Organic Substrates in Continuous Flow-Through
Columns Simulating Mine Water Vertical Flow Bioreactors

A THESIS APPROVED FOR THE

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ABSTRACT

In mine water passive treatment systems, biological sulfate (SO_4^{2-}) reduction in vertical flow bioreactors (VFBRs) is often utilized to precipitate trace metals as metal sulfides and to generate alkalinity. Sulfate removal is not typically targeted and is trivial in bioreactors compared to the removal of targeted metals. However, utilizing biological sulfate reduction in VFBRs to specifically remove sulfate can be an effective strategy to decrease elevated SO_4^{2-} concentration from mine drainage.

In this study, a laboratory bench-scale continuous flow-through column study simulating mine water vertical flow bioreactors was conducted over 370 days evaluating the effectiveness of three locally available waste organic substrates (Norman Aged Compost (NAC), Murphy Compost (MC), and Spent Mushroom Compost (SMC)), on biological SO_4^{2-} removal by sulfate reducing bacteria (SRB). The substrates were tested in triplicate columns, constructed in opaque PVC pipes (0.019 m^3), filled with a 2:1 mixture by volume of the organic substrate to washed river rock, and were fed with a solution containing 1000 mg SO_4^{2-} L^{-1} $\pm$ 10%. Conditions at the start and termination of the study were optimal for SO_4^{2-} reduction: circumneutral pH, reducing oxidation-reduction potential (ORP), and appropriate temperatures. During the first 305 days, SO_4^2 removal rates and percent removal of SO_4^2 decreased significantly ($p < 0.01$). On Day 306, the hydraulic retention time (HRT) was decreased from eight days to four days for 30 days and then again to two days on Day 336 for an additional 30 days in order to observe changes in SO_4^{2-} removal rates. At HRT = 8 days, the SMC treatment consistently produced the lowest effluent sulfate concentrations (median = 221 mg L^{-1}), greatest sulfate removal rates (mean = 548 mmol m^{-3} day^{-1}), percent removal of sulfate (median =77.3%), and effluent sulfide concentrations

(median = 123.6 mg L^{-1}) (p < 0.01). The SMC and MC treatments did not show significant differences in sulfate removal across all HRTs, however the NAC treatment had significantly lower sulfate removal with shorter HRTs (p < 0.05). Sulfide production decreased significantly with shorter HRTs in all treatments (p < 0.05).

The results of this study confirm that mine water passive treatment system VFBRs are appropriate for the removal of elevated sulfate given that optimal conditions for bacterial sulfate reduction are created and maintained. The selected waste organic substrates showed similar sulfate removal capabilities within a range of different organic carbon contents in the substrates and in the effluents. SMC has been demonstrated for its ability to serve as the media in VFBRs targeting metals removal and retention as metal sulfides and can also be utilized in those systems aiming to specifically remove sulfate.

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1. Introduction

Effective water quality improvement in properly designed and maintained mine water passive treatment systems (PTS) has been demonstrated for decades as a low-cost, more environmentally friendly alternative to active treatment, which has high costs and potential for secondary pollution (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Hedin, 2008; Neculita et al., 2008; Rakotonimaro et al., 2018). Most of these ecologically engineered ecosystems focus on removal of elevated concentrations of trace metals and acidity, which are ecologically devastating to receiving waters (Ayangbenro et al., 2018; Dvorak et al., 1992; Hedin, 2008; Huang et al., 2016; Neculita et al., 2007; Rakotonimaro et al., 2018; LaBar and Nairn 2017; Xu and Chen, 2020).

Sulfate-reducing compost process units, like vertical flow bioreactors (VFBRs), are common installments in passive treatment systems, effective for both alkalinity generation and retention of trace metals as sulfides (Ayangbenro et al., 2018; Dvorak et al., 1992; Hedin et al., 1994a; LaBar and Nairn, 2017; Neculita et al., 2007; Younger et al., 2002). VFBRs are designed to promote sulfate reducing bacteria (SRB) to microbially aid in production of sulfide, which later reacts to precipitate trace metals as shown in Equations (1) and (2) (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Huang et al., 2016; Neculita et al., 2011; Neculita et al., 2007; LaBar and Nairn, 2016; Rakotonimaro et al., 2018; Xu and Chen, 2020; Younger et al., 2002).

Equation 1

$$2\ CH_2O + SO_4^{2-}\ \rightarrow\ 2\ HCO_3^- + H_2S$$

Equation 2

$$H_2S + M^{2+} + 2\ HCO_3^-\ \rightarrow\ 2\ H_2O + 2\ CO_2 + MS\ (s)$$
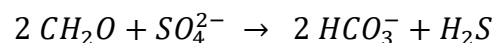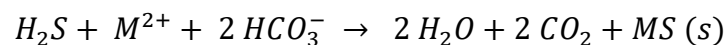
In this anaerobic treatment process, SRB utilize sulfate as a terminal electron acceptor and labile organic matter (OM) (represented as CH_2O in Equation 1) as an electron donor to harvest energy and, in the process, convert sulfate into sulfide (Edwards, 1998; Hedin et al., 1994a). Sulfide is acutely toxic to aquatic life by causing plant root suffocation and redox suppression, but as utilized in VFBRs, the excess soluble sulfide has the benefit of precipitating trace metals (Ayangbenro et al., 2018; Dvorak et al., 1992; Huang et al., 2016; Fernando et al., 2018; LaBar and Nairn, 2017; Orem et al., 2011; Song et al., 2012; Younger et al., 2002).

SRB are able to thrive in these anaerobic environments because VFBRs have relatively long hydraulic retention times (HRT), thus maximizing OM degradation and contact time of SRB with mine water (Xu and Chen, 2020). The OM is typically a mix of carbon types to maximize treatment efficiency and system longevity, and SRB must rely on other microbes to aid in the breakdown of recalcitrant carbon sources to utilize more labile forms (Edwards, 1998). These systems are designed to last for decades at a time, and thus the addition of more recalcitrant carbon is important for long-term success of the VFBR and the passive treatment system (Dvorak et al., 1992; Hedin et al., 1994a; Neculita et al., 2011; Song et al., 2012; Xu and Chen, 2020). Xu and Chen (2020) compiled a list of low-cost, previously tested substrates which varied regionally, but single substrates and mixes of labile and recalcitrant carbon sources, like compost and wood shavings, are often utilized to provide effective, long-term treatment.

Trace metal removal utilizing SRB has been demonstrated in the laboratory and in the field. Different studies have investigated the removal of various metals including zinc, nickel, cadmium, and lead among others with removal rates up to 99% in certain cases (Bowell, 2004; Dvorak et al., 1992; Hedin et al., 1994a; LaBar and Nairn, 2016; Song et al., 2012; Smyntek et al., 2017; Rakotonimaro et al., 2018; Wang et al., 2019; Xu and Chen, 2020). These studies

examined the different conditions necessary for sulfate reduction to occur including pH, temperature, oxidation-reduction potential (ORP), HRT, diversity and abundance of microbes, availability and type of carbon sources, chemical oxygen demand (COD) to sulfate ratio, sulfide and trace metal toxicity, and other synergistic and antagonistic effects in order to optimize performance (Xu and Chen, 2020). However, the removal of elevated sulfate in mine water is not typically targeted in VFBRs and removal is typically trivial in bioreactors when compared to targeted metals (Rakotonimaro et al., 2018; Runtti et al., 2018; Smyntek et al., 2017; Younger et al., 2002).

In recent years, total dissolved solids (TDS) and conductivity, driven by elevated sulfate concentrations in mine waters, have been shown to detrimentally impact streams (Fernando et al., 2018; Runtti et al., 2018; Smyntek et al., 2017; Armstead et al., 2016). With emphasis on the abatement of TDS and conductivity, and the contribution of sulfate to both parameters, effective sulfate removal warrants evaluation (Abdalla and Drohan, 2009; Lorax Environmental, 2003; Orem et al., 2011; Runtti et al., 2018; U.S. EPA, 2011). Optimization of PTS to remove sulfate is necessary because of the potential for negative water quality impacts from excess geogenic sulfate (Armstead et al., 2016; Orem et al., 2011; Neculita et al., 2011; Song et al., 2012; Xu and Chen, 2020). Other alternatives to treatment optimization include decreasing sulfur at the source and mitigating mine drainage with active treatment which can be prohibitively costly (Neculita et al., 2008; Neculita et al., 2011, Orem et al., 2011).

1.1. Mine Drainage and Passive Treatment Systems (PTS)

Discharge of polluted mine drainage (MD) into receiving water bodies causes aquatic habitat degradation (Armstead et al., 2016; Dvorak et al., 1992; Huang et al., 2016; Lorax

Environmental, 2003; Neculita et al., 2007; Orem et al., 2011; Song et al., 2012; Smyntek et al., 2017; Rakotonimaro et al., 2018; Xu and Chen, 2020). MD is typically characterized by elevated sulfate and metals concentrations due to its origin from sulfur bearing minerals like pyrite (FeS_2), sphalerite (ZnS), and galena (PbS) (Dvorak et al., 1992; Hedin et al., 1994a; Hedin et al., 1994b; Neculita et al., 2008; Neculita et al., 2011; Younger et al., 2002). MD can be net alkaline or net acidic, depending on the origin of geologic materials, aquifer composition, and soil types. The chemical composition of MD often changes when in contact with the atmosphere.

Passive treatment has been utilized as a successful remediation strategy to retain metals like aluminum, iron, manganese, arsenic, cadmium, nickel and zinc from mine drainage (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Hedin, 2008; Huang et al., 2016; LaBar and Nairn, 2017; Nairn et al., 2010; Nairn et al., 2020; Neculita et al., 2007; Orem et al., 2011; Rakotonimaro et al., 2018; Song et al., 2012; Wang et al., 2019; Xu and Chen, 2020). Passive treatment is effective for three primary reasons: it is low-cost, the processes are energy efficient, and it utilizes natural biogeochemical and physical processes that can remove a wide variety of different environmental stressors at acceptable removal rates (Ayangbenro et al., 2018; Dvorak et al., 1992; LaBar and Nairn 2016; LaBar and Nairn 2017; Nairn et al., 2010; Nairn et al., 2020; Neculita et al., 2007; Song et al., 2012; Smyntek et al., 2017; Wang et al., 2019; Xu and Chen, 2020). It is an alternative to active treatment which can involve costly and dangerous chemical additions to raise pH and precipitate certain metals, require energy to pump and treat the water, and potentially produce secondary pollution (Hedin et al., 1994b). Several factors affect passive treatment performance: location of the treatment area, geogenic origin of mine-water constituents, temperature and other seasonal variations, and flow rates (Ayangbenro et al., 2018; Miller, 2005). Passive treatment can also require large land areas (Bowell, 2004).

The most common process units in passive treatment systems include constructed wetlands, anoxic limestone drains, oxidation ponds, and VFBRs, each of which promote different functions aimed at removing different chemical constituents in the mine water (Hedin, 2008; Machemer et al., 1993; Nairn et al., 2010; Nairn et al., 2020). Passive treatment systems have been studied to determine their effectiveness and efficiency over time and can be uniquely tailored to specific discharge quality and quantity, and site-specific limitations (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Fernando et al., 2018; Neculita et al., 2008; Neculita et al., 2011; Rakotonimaro et al., 2018; Smyntek et al., 2017; Xu and Chen, 2020).

Limestone (predominantly calcite, $CaCO_3$) is commonly used in passive treatment, especially when the MD is net acidic, for production of alkalinity, and to increase overall hardness and pH (Fernando et al., 2018; Hedin et al., 1994b; Nairn et al., 2010). Limestone also serves as a potential source of electrical conductivity via dissolution of $CaCO_3$ so optimization of alkalinity production to minimize conductivity is necessary (Fernando et al., 2018; Runtti et al., 2018; Smyntek et al., 2017). Some systems utilize VFBRs so that sulfate reduction works two-fold: to remove sulfate (and thus conductivity) in addition to removing acidity by producing alkalinity (Smyntek et al., 2017).

1.2. Vertical Flow Bioreactors (VFBRs)

VFBRs are used for coal and hard rock mine drainage treatment (Armstead et al., 2016; Dvorak et al., 1992; Hedin et al., 1994a; Huang et al., 2016; Lorax Environmental, 2003; Machemer et al., 1993; Smyntek et al., 2017; Dvorak et al., 1992; Neculita et al., 2007; Song et al., 2012; Rakotonimaro et al., 2018; Xu and Chen, 2020). They are designed so that mine drainage flows vertically through organic substrates and subsequent drainage layers (and embedded perforated piping networks) before moving to the next process unit (Figure 1).
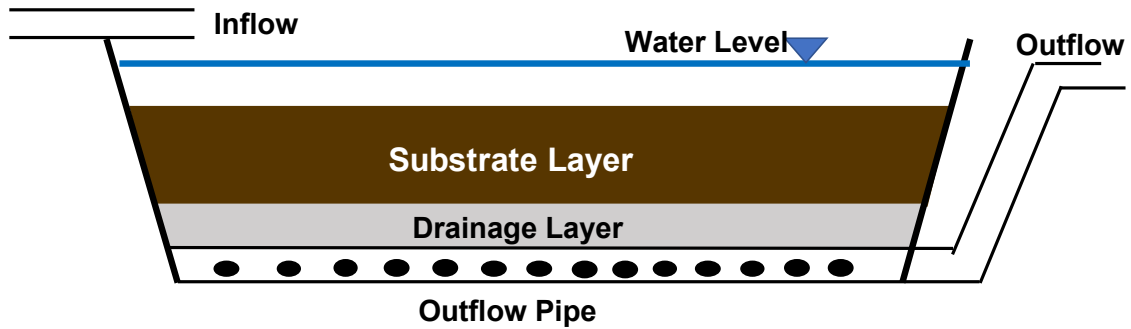
Figure 1: Cross-section schematic of typical VFBR

VFBRs are a preferred unit in passive treatment because they are relatively low maintenance and require no energy, they can increase alkalinity, and they can produce stable waste sludge (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Hedin et al., 1994a; Lorax Environmental, 2003; Neculita et al., 2007; Xu and Chen, 2020). VFBRs are designed to promote alkalinity generation and trace metal sulfide precipitation (Armstead et al., 2016; Dvorak et al., 1992; Huang et al., 2016; Lorax Environmental, 2003; Nairn et al., 2010; Neculita et al., 2007; Rakotonimaro et al., 2018; Song et al., 2012; Smyntek et al., 2017; Xu and Chen, 2020). They are also able to improve water quality by decreasing conductivity by removing sulfate and other dissolved solids (Fernando et al., 2018; Smyntek et al.,2017; LaBar and Nairn, 2017; Runtti et al., 2018). SRB are utilized in VFBRs to reduce sulfate to sulfide; without them, the treatment process is very slow or nonexistent (Younger et al., 2002). Excess sulfide bonds with divalent cations like cadmium, lead and zinc in solution to form precipitates as described earlier in equation (2) (Ayangbenro et al., 2018; Bowell, 2004; Dvorak et al., 1992; Edwards, 1998; Huang et al., 2016; Lorax Environmental, 2003; Nairn et al., 2010; Neculita et al., 2007; Song et al., 2012; Rakotonimaro et al., 2018; Xu and Chen, 2020). For the bioreactors to provide effective treatment, water levels are maintained well above the treatment media to prohibit plant

colonization which might introduce oxygen into the subsurface through rhizosphere oxygenation and ruin the necessary anaerobic conditions (Younger et al., 2002; Machemer et al., 1993).

In order for the sulfate reduction process to begin in these systems, the appropriate reducing conditions are caused by flooding the media, maintaining moderate temperatures, and promoting the presence of the preferred terminal electron acceptor (sulfate), and an electron donor (organic carbon in the treatment media) (LaBar and Nairn 2016; LaBar and Nairn 2017; Neculita et al., 2011). SRB need any longer-chain OM to be degraded by other microbes before being able to utilize lower molar mass carbon sources like glucose or lactate (Ayangbenro et al., 2018; Edwards, 1998; Huang et al., 2016; Xu and Chen, 2020). Therefore, a substrate having a mix of labile carbon readily degradable by SRB and recalcitrant carbon that other microbes can degrade slowly over time is optimal.

Bioreactors commonly use a labile waste organic material (e.g., spent mushroom compost) as a substrate, amended with more recalcitrant organic material like wood shavings (Ayangbenro et al., 2018; Hedin et al., 1994a; Huang et al., 2016; Machemer et al., 1993; Neculita et al., 2011; Rakotonimaro et al., 2018; Song et al., 2012; Smyntek et al., 2017; Xu and Chen, 2020; Younger et al., 2002). Jordan et al. (2006) documented variation in the composition of spent mushroom compost in Ireland to determine the best mixtures for mushroom yield, however, in general it is known for its typically high organic matter content, peat and calcium carbonate amendments, and availability of essential nutrients to plants. Outside of mining reclamation and use in bioreactors, spent mushroom compost is also used as a soil amendment and for horticulture (Jordan et al., 2006). The substrate can serve as a sorption and complexation site for trace metals, which aids in the overall removal of metals (LaBar and Nairn 2016; LaBar and Nairn 2017; Machemer et al., 1993). Lastly, the amount of available organic carbon from the

substrate can control the rate of sulfate reduction which directly affects treatment efficiency (Neculita et al., 2008; Neculita et al., 2011).

Longer HRTs (hours to days) are needed for promoting growth and activity of SRB by increasing contact time for microbial degradation which will stimulate sulfate reduction and metal removal (Genty et al., 2018; Neculita et al., 2008; Vasquez et al., 2016; Xu and Chen, 2020). Efficiency of metal and sulfate removal is limited by the HRT of the treatment system; a shorter contact time means less time for removal processes to occur.

Most SRB are neutrophilic, meaning they thrive in pH values from 7.0-7.8, reaching maximum sulfate reduction between 7.0 and 7.5. They are inhibited at pH greater than 9 and less than 5, having no activity at pH less than 2 (Ayangbenro et al., 2018; Xu and Chen 2020). However, some SRB are acidophilic and thrive in environments with pH less than 4 (Xu and Chen, 2020).

SRB are also mostly mesophilic and require temperatures from 20 to 40 °C with optimal metal removal near 37 °C (Xu and Chen, 2020). Although some SRB are known to be psychrophilic and some to be thermophilic, temperatures less than 35 °C are known to inhibit SRB activity due to bacterial inactivation and protein denaturation (Xu and Chen, 2020). In hotter conditions, less H_2S (g) is retained in the substrates and escapes the porewaters while the sulfate removal rates are greater (Machemer et al., 1993). Lastly, the SRB require conditions to be anaerobic and reducing, with ORP less than -100mV for optimal conditions for complete conversion to sulfide; ORP values greater than 100 mV are known to inhibit SRB activity (Ayangbenro et al., 2018; Connell and Patrick, 1968; Edwards, 1998; Runtti et al., 2018).

1.3. Conductivity, Total Dissolved Solids (TDS), and Sulfate

Conductivity is a measure of soluble ions in solution that can transmit electrical current; specific conductance is a measure of conductivity normalized to 25 °C which is used to compare waters of different temperature (Smyntek et al., 2017). In many mine waters, the major soluble ions tend to be sulfate, iron, bicarbonate, calcium, and magnesium, while chloride and potassium are also known to be contributors to conductivity in other waters. However, decreasing conductivity in general is often not a targeted design objective of mine water passive treatment (Hedin et al., 1994a; Smyntek et al., 2017). Some of these dissolved ions can serve as macronutrients to support aquatic ecosystems (magnesium and potassium), aid in osmoregulation (chloride), and serve as buffer agents to decrease metal toxicity (bicarbonate, calcium, and magnesium) (Armstead et al., 2016). The ions can also act synergistically to decrease toxicity in the presence of one another, allowing organisms to thrive in elevated conductivity environments (Armstead et al., 2016).

A review of current passive treatment systems to investigate the ability of PTS to remove conductivity was completed by Smyntek et al. (2017). It was determined that a theoretical maximum of 30-40% conductivity decrease could be achieved through sulfate reduction alone. This theoretical maximum reduction is based on the stoichiometry of waters in those systems that produce excess sulfide and alkalinity, both of which contribute to conductivity (Smyntek et al., 2017).

In the past decade, effluent regulatory requirements in mining impacted areas for TDS, conductivity, and sulfate have become prevalent, driven by U.S. EPA in-stream water quality criteria developed due to negative impacts to macroinvertebrate communities in mountaintop removal mining areas of the Appalachians (Armstead et al., 2016; U.S. EPA, 2011; Smyntek et

al., 2017; Runtti et al., 2018). TDS refer to dissolved matter (e.g., salts, organic matter, minerals) which can be toxic to aquatic life. Elevated conductivity and TDS are indicative of some disturbance and elevated TDS can come from mining, agriculture, urban development, oilfield operations, landfills, food processing, and wastewater treatment facilities (Abdalla and Drohan, 2009; Armstead et al., 2016). Conductivity is a known stressor on aquatic life and regulations are predicted to become stricter on conductivity or the specific contributing stressors (e.g., sulfate, calcium, magnesium, carbonate) involved (Armstead et al., 2016; Smyntek et al., 2017). Elevated conductivity has been shown to detrimentally impact aquatic life by decreasing abundance and diversity of benthic macroinvertebrates by increasing osmoregulatory stress (Armstead et al., 2016; Orem et al., 2011; Runtti et al., 2018; Smyntek et al., 2017). Calculating toxicity from cumulative measures like conductivity and TDS is difficult due to the variable toxicity of the individual ions (Armstead et al., 2016).

Sulfate is known to be a major component of inorganic TDS, along with magnesium, calcium, potassium, chloride, carbonate and bicarbonate (Armstead et al., 2016; LaBar and Nairn, 2016; LaBar and Nairn, 2017; Younger et al., 2002). Sulfate, although a dominant ion in conductivity and TDS, has a relatively low direct toxicity. The U.S. EPA established a Secondary Drinking Water Standard (SDWS) for TDS at 500 mg L^{-1}, for sulfate at 250 mg L^{-1} and a Health Reference Level for sulfate at 500 mg L^{-1}, because excess sulfate in drinking water can cause diarrhea and dehydration (Abdalla and Drohan, 2009; Smyntek et al., 2017; Younger et al., 2002). Restrictions on sulfate in drinking water are stricter in other parts of the world, like Finland which has a Drinking Water Standard (DWS) of 150 mg L^{-1} as a preventative measure for corrosion (Abdalla and Drohan, 2009; Reinsel 1999; Runtti et al., 2018). The established SDWS for sulfate and TDS are also in place as an aesthetic appeal to improve taste and reduce

odor (Bowell, 2004; Reinsel 1999). Increased sulfate, and by extension, TDS and conductivity, can lead to detrimental outcomes to warm water aquatic communities (Fernando et al., 2018; Orem et al., 2011; Runtti et al., 2018; Smyntek et al., 2017). Elevated TDS, sulfate, and conductivity can cause the release of nutrients bound to sediments, leading to internal eutrophication, organic matter degradation in soils, and impairment of benthic macroinvertebrates' abilities to osmoregulate (Armstead et al., 2016; Orem et al., 2011). Decreasing conductivity by decreasing the number of ions present in solution can improve water quality downstream. Thus, it is important to remove sulfate in discharges prior to release into the environment for benefit to downstream aquatic life, and for potential use as drinking water supplies.

There is concern that a lack of funds and direction exists to adequately investigate and monitor effluents from past and present mining operations (Smyntek et al., 2017). In section 303(d) of the Clean Water Act (CWA), a Total Maximum Daily Load (TMDL) is described to identify pollutant sources and require entities discharging into streams, rivers or lakes to have load reductions of the specific pollutants to bring the water within compliance (Smyntek et al., 2017). In West Virginia, Smyntek et al. (2017) noted that a TMDL was unable to be implemented for conductivity because it did not identify a reduction in the ionic stressor, which in this case was sulfate. Lastly, due to the unique inhibition caused by competing ions on TDS, and the positive result in decreasing bioavailability of metals by elevated hardness, the comparative effects of ions on downstream aquatic life needs to be studied extensively for region-specific reductions of ionic stressors (Nairn et al., 2010; Smyntek et al., 2017).

1.4.   Differences in VFBR Media and in Hydraulic Retention Time (HRT)

VFBRs have been studied and modeled in benchtop laboratory mesocosms to examine their effectiveness in optimizing metal removal and alkalinity production for several decades, but more recently there has been emphasis on their ability to remove sulfate, and by extension TDS and conductivity (Runtti et al., 2018). This is especially important to the Appalachian mountain region, which has its own conductivity discharge standard ($< 300\ \mu S\ cm^{-1}$) for streams as a benchmark for aquatic life (U.S. EPA, 2011).

Studies which have compared different substrates have been well documented by Neculita et al. (2007, 2008, and 2011). The mixtures of substrates tested were combinations of spent mushroom compost, cow manure compost, saw dust, wood chips, cut rice straw, creek sediment, and limestone. A mix of 40% cow manure compost, 40% saw dust, and 20% creek sediment was the most effective overall for removal of metals and sulfate in these studies. The mix consisting solely of spent mushroom compost was hypothesized to be the best substrate but showed an initial leaching of sulfate prior to any removal, consistent with a previous batch study (Neculita et al., 2011). Mixtures of substrates in mesocosms and field application have been shown to out-perform single substrates in sulfate and trace metal removal (Dvorak et al., 1992; Song et al., 2012; Xu and Chen, 2020).

It was determined in these studies that for all compositions of substrate (excluding those with greater than 40 percent rice straw) and varied HRTs (2.5, 5, 7.3 and 10 days), results were produced with similarly effective metals and sulfate removal (up to 99% for trace metals, and up to 77% for sulfate), as well as increases in pH and alkalinity. Vasquez et al. (2016) and Genty et al. (2018) found that the HRTs they tested (1, 2, and 4 days, and 5 and 7 days, respectively) had little influence on metal and sulfate removal. However, they noted that the major difference was

that longer HRTs (4 days from the Vasquez et al. (2016) study and 7 days from the Genty et al. (2018) study) produced more alkalinity and excess sulfide. Neculita et al. (2008) noted two potential controlling factors on sulfate removal. The first was iron precipitation occurring in the aerobic zone at the top of the columns and the other was fine particulates and metal sulfides collecting at the bottom of the columns. Iron precipitation ideally would not impact sulfate removal in field-scale applications, as oxidation ponds tend to be at the beginning of PTS and serve to remove iron first (Dvorak et al., 1992; Bowell, 2004; Fernando et al., 2018; Huang et al., 2016; Rakotonimaro et al., 2018; Xu and Chen, 2020). The second factor was determined during the HRT study performed by Neculita et al. (2008), which concluded that longer retention times (7.3 days and 10 days) were slightly more effective in metals and sulfate removal, however longer retention times could lead to decreased hydraulic conductivity and porosity from the effects of compaction, segregation of material, and creation of preferential flow paths. This result is consistent with a review conducted by Xu and Chen (2020) which found that HRTs greater than 4 days proved to be beneficial for degradation of organic nitrogen and carbon for use by SRB. They also noted a shorter HRT decreased growth of SRB from a constant, high influent input (Xu and Chen, 2020). Greater inflow led to increased oxygen entrapment and increased dissolved oxygen (DO) and ORP, therefore decreasing the anaerobic environment, and subsequently decreasing sulfate reduction and trace metal removal efficiency (Vasquez et al., 2016; Xu and Chen, 2020).

1.5.   Problem Statement

Although many studies have examined the performance of microbiologically mediated sulfate reducing systems to promote alkalinity and precipitate trace metals by altering substrates,

substrate composition, and HRT, a lack of studies exists which examine these systems specifically with respect to sulfate removal. Therefore, a laboratory bench-scale study was performed for 370 days from November 2018 to November 2019 to examine locally available waste materials as substrates in flow-through columns modelling VFBRs for the sole purpose of removing sulfate.

1.6. Hypotheses

The four hypotheses of this study are as follows:

1. Biological processes in the flow-through columns will decrease elevated sulfate concentrations (≈ 1000 mg L^{-1}) below the health reference level of 500 mg L^{-1} for a sustained period of time (> 6 months).

2. Sulfate removal (calculated as mmol SO$_4$ m^{-3} day^{-1} and as percent change in [SO$_4$]) will decline over time as organic matter is consumed by the microbial community (estimated as sulfate reducing bacteria populations).

3. Sulfate removal (calculated as mmol SO$_4$ m^{-3} day^{-1} and as percent change in [SO$_4$]) will decline with decreasing hydraulic retention time.

4. The waste organic substrate with the greatest initial organic matter content will result in the greatest sustained sulfate removal (estimated as mmol SO$_4$ m^{-3} day^{-1} and as percent change in [SO$_4$]).

1.7. Objectives

To assess these four hypotheses, four objectives were completed.

a. Objective 1: Evaluate the efficacy of sulfate removal by three organic substrates by observing sulfur species concentration changes over time in the aqueous influent and effluent, gaseous phase, and within the solid substrate.

b. Objective 2: Calculate removal rates and percent change of sulfur species over time and compare these results by substrate treatment.

c. Objective 3: Examine changes in sulfate removal rates by increasing flow rates, and thus decreasing HRT, and observing changes in sulfur species and removal rates.

d. Objective 4: Evaluate changes in organic matter content of the substrates at the beginning and the end of a long-term column experiment and compare them to sulfate removal.

2. Methodology

2.1. Treatment Media

Three locally available composts: Norman Aged Compost (NAC), Murphy Compost (MC) and Spent Mushroom Compost (SMC) were chosen as substrates for this continuous flow-through column study because of their known capabilities to remove sulfate by promoting SRB populations and by demonstrating the greatest sulfate removal rates in an initial preliminary batch reactor study. Batch reactors were prepared by adding 50 grams of air-dried substrate and approximately 700 mL of magnesium sulfate solution (1100 mg SO_4^{2-} L^{-1}) in one-liter mason jars, which created minimal headspace and submerged the substrate entirely. Sulfate concentrations were measured over the course of 125 days, weekly for the first 49 days and one

final measurement occurring on day 125. Seven different locally available organic substrates were examined including NAC, MC, SMC and four other substrates: Pure Leaf Compost (PLC), Norman Young Compost (NYC), Pine Bark Mulch (PBM), and cow manure. Each substrate was tested in triplicate, however one of the NYC replicate jars broke on day 14 of measurement. Removal rates were calculated by determining the difference in sulfate concentration in the batch reactors over time. A summary of these data is provided in Table 1.

Table 1: Sulfate Removal (mmol SO_4^{2-} m^{-3} day^{-1}) in Preliminary Batch Reactor Experiment

	Mean	Standard Deviation	Minimum	Maximum	N
NAC	191	161	63	461	21
MC	222	317	-241	811	21
SMC	55	334	-649	326	21
PLC	87	140	-79	601	18
NYC	205	196	-25	606	15
PBM	144	87	10	502	18
Manure	66	58	-21	152	21

NAC is compost obtained from the City of Norman, Oklahoma Compost Facility, which collects public yard waste, and is continuously turned and processed through a trommel screen throughout the aging process. This compost includes all "brown and green waste", including grass, tree, and hedge clippings. Sulfate removal in NAC during the preliminary study was promising as it was one of the only media examined which never leached sulfate back into solution, however the sulfate removal performance in the NAC treatment generally declined over time.

MC is a proprietary blend which includes combined and aged stable cleanings, cow manure, grass clippings, fruits and vegetables, leaves, recycled wood bits, and aged saw dust.

This compost blend is also pulverized and screened but only at the end of the aging process. Sulfate removal in MC was the greatest on average and had the highest maximum removal rate recorded of all the treatments occurring on measurement day 49. Between days 28 and 42, sulfate leached back into solution in all treatment replicates, producing a negative removal rate however this relatively higher sulfate concentration compared to the other substrates likely contributed to the greater removal rate calculated on day 49.

SMC was provided by JM Farms of Miami, OK. SMC is a growing media considered spent after mushrooms have been harvested. The media includes a wheat straw base, chicken litter, cottonseed meal, soybean meal, peat moss, sugar beet lime, and gypsum (LaBar and Nairn, 2017). SMC has been used in PTS design for decades and is known for its ability to perform well in VFBRs, but also for its potential to leach sulfate from gypsum (Neculita et al., 2011; LaBar and Nairn, 2017; Song et al., 2012). The mean sulfate removal rate of SMC was detrimentally affected by the leaching of sulfate that occurred during the first 2 weeks of measurement. Calculating the mean removal rate excluding the first two weeks where leaching was observed yielded a mean sulfate removal rate (211 mmol m^{-3} day^{-1}) comparable to the other two previously mentioned media.

The aforementioned substrates were the most effective in sulfate removal performance, for promoting the greatest SRB populations, and were readily available to the experimenter in Norman, Oklahoma at no cost. The material used for this study was collected in summer of 2018 and was stored in a walk-in cooler (approximately 4 $^{\circ}$C), prior to column construction. The different substrates were analyzed in triplicate to determine mean background concentrations of various elements.

2.2. Column Study Set up

The three composts were tested in triplicate in a set of continuous flow-through columns. The columns were approximately 38 cm in length, and 25.4 cm in diameter, and were constructed from opaque PVC pipe, with a glued PVC end cap on the bottom, and an unsecured end cap on the top. Volume of the vessel was calculated as the volume of a cylinder (radius = 12.7 cm, height = 38 cm) and was determined to be approximately 0.019 m^3.

The vessels were filled with a 2:1 mixture by volume of selected compost to washed river rock, with a 2.5-cm thick layer of washed river rock serving as drainage layer at the bottom with an approximately 1-mm opening mesh screen below (Figure 2). Each column was inoculated with 50 mL of a known active SRB solution from the previous batch study.
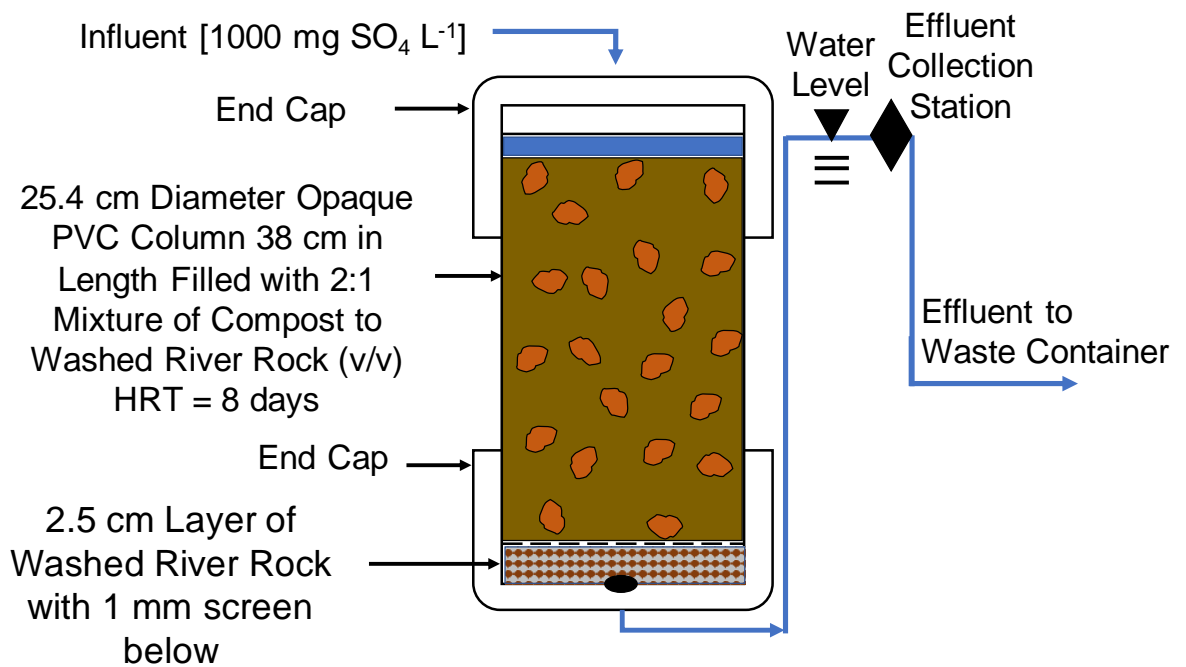
Figure 2: Schematic of Continuous Flow Through Column

The PVC end caps of the columns were fixed with small elbow adapters in the center to attach L/S size 16 tubing for distribution of the influent to and effluent from the vessels. L/S size

14 tubing was attached on the influent sections of the tubing at the pump in order to achieve the initial target flow rate of 0.5 mL min $^{-1}$, resulting in an approximate eight-day HRT.

The influent solution was fed from a 50-L carboy containing a magnesium sulfate solution [1000 mg SO_4^{2-} L^{-1} $\pm$ 10%] which was delivered to each column on a laboratory bench top via three Masterflex peristaltic pumps, each feeding influent solution to one of three replicates of each treatment (Figure 3). Fifty liters of influent solution were prepared weekly using magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and was mixed at 750 RPM for 30 minutes using a Southwest Science digital overhead stirrer. Since this study focused on sulfate removal and not metal sulfide formation, no other trace metals were included in the source water.
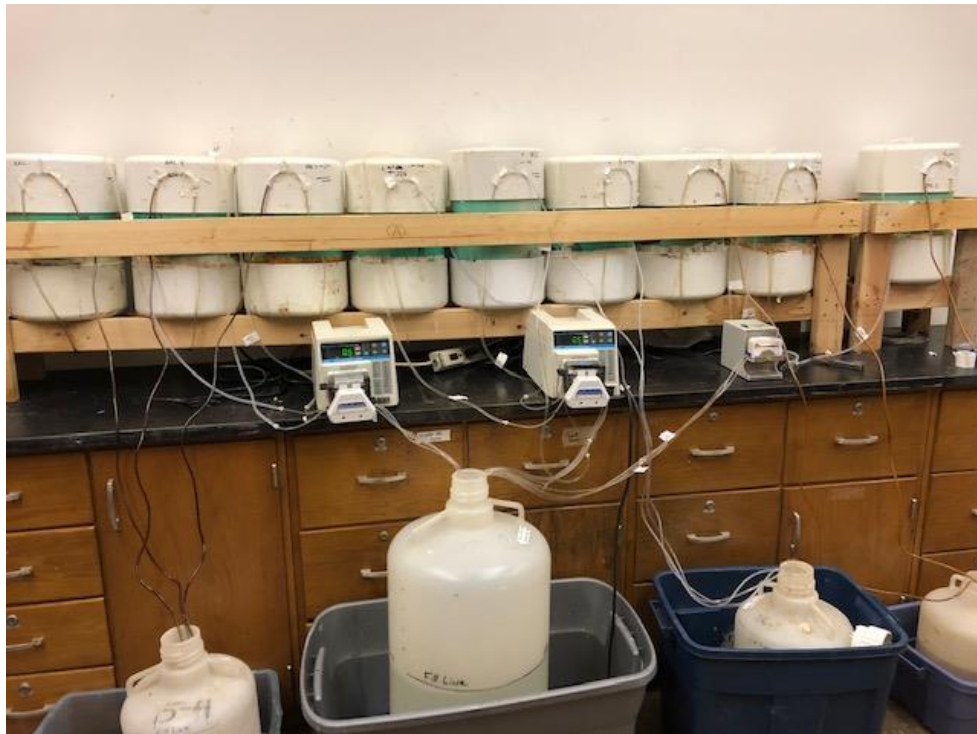
Figure 3: Layout of Study Setup on Benchtop

The effluent flowed out the bottom elbow of the vessel and back up to approximately 2 cm from the top of the column to maintain static water levels approximately 2.5 cm above the

substrate, and then down to a waste collection vessel. The effluent tubing was fastened to the vessel and included an effluent collection station at the desired water level which could introduce air to eliminate siphoning. Lastly, wastes from alike compost treatments (NAC, MC, and SMC) were collected in three 20-L carboys and properly disposed of weekly.

Prior to addition to the columns, a Biological Activity Reaction Test (BART) SRB population enumeration examination was performed on the inoculum to confirm the presence of SRB in August 2018. All tests showed greater than 2,000,000 colony forming units (CFU) mL^{-1}, which is the greatest value on the test, indicating abundant active bacteria. For 90 days, no influent or effluent samples were taken during an initialization phase where water levels were manipulated using the exterior effluent tubing to address siphoning of the water inside the column. During this time, the media was periodically inundated and flushed with the influent solution. To resolve the hydrologic issues, T-connectors were added to effluent tubing at the crest to allow for air to escape, waste disposal to continue, and to establish a point for effluent data collection. Once siphoning was addressed and before data collection began, another BART SRB population enumeration test was performed on the effluent from the columns, again confirming the presence of active bacteria (with all tests showing > 2,000,000 CFU mL^{-1}).

2.3.   Sampling Procedure

After the initialization phase was complete, samples were taken every 10 to 20 days for the first 120 days of the study. From then on, samples were taken every 21 to 28 days until the beginning of the flow/HRT manipulation portion of the study. During the flow/HRT manipulation, samples were taken every 7 to 10 days.

Influent samples were taken directly from the influent tubing of a randomly selected column using a random number generator. One sample was collected from each of the columns at the effluent collection station by first draining three effluent tubing volumes (approximately 60 mL) and then collecting roughly 100 mL of sample. Samples were unpreserved and analyzed immediately for total aqueous sulfate, total aqueous sulfide, pH, ORP, and temperature.

Samples for sulfate analyses were not filtered and, from November 2018 to March 2019, were analyzed via the HACH method 10227 adapted from Standard Methods for the Examination of Water and Wastewater, SM 4500-SO_4^{2-}E using TNTplus 865 for high range [150 to 900 mg L $^{-1}$] and TNTplus 864 for low range [40 to 150 mg L $^{-1}$] concentrations using a DR 3800 spectrophotometer. Afterwards, sulfate analyses were conducted using a Seal AQ300 Automated Discrete Analyzer following ASTM Method D516-90 (Seal Analytical, 2009).

Samples for determination of total aqueous sulfide concentrations were taken starting on day 55 and were colorimetrically determined every 10 to 20 days, and then every 21 to 28 days using a DR 3800 spectrophotometer according to HACH method 8131 (Baird and Bridgewater, 2017). Gaseous sulfide measurements were conducted using a GasBadge Pro Single Gas H_2S Detector and were taken at several locations. The detector was attached to the person conducting the sampling and was also deployed during sampling directly on top of the sampling vessel, at the tops of the open waste containers, and on top of the benchtop nearest the vessels.

Physical parameters were analyzed to determine if optimal conditions were present for sulfate reduction in the columns. Temperature, pH, and ORP were measured using an Accumet XL600 Multimeter according to USEPA-approved HACH method 8156 (Baird and Bridgewater, 2017) during the first phase of the study, and measurements were discontinued during the flow/HRT manipulation portion.

2.4. Flow/HRT Manipulation

At the beginning of the flow/HRT manipulation portion of the study, it was determined one of the MC treatment replicates (MC2) was not receiving flow, and therefore was removed from all future analysis regarding the effluent and analysis performed on the material after the autopsy of the columns. After 306 days, an adjustment to the influent flow rate, and subsequently column HRTs, was made to evaluate effects on sulfate removal capabilities. There were two HRTs tested during the flow manipulations, resulting from doubling and quadrupling the original influent flow rate of the columns. The influent solution flow rate was doubled to 1 mL min^{-1} for 30 days, resulting in a four-day HRT, and samples were taken for the following 30 days. The flow rate was changed again to 2 mL min^{-1}, resulting in an approximate two-day HRT in the columns and samples were taken for another 30 days. Samples for sulfate and sulfide analysis were collected weekly using the same sampling protocol as described previously. After 60 total days of flow manipulation, the vessels were returned to their original flow rate of 0.5 mL min^{-1} to operate until they could be autopsied.

2.5. Autopsy

The columns were dismantled and autopsied in order to determine sulfur, trace metals, and organic matter contents of the organic substrates. A common general autopsy procedure was conducted for the nine columns over four days.

Prior to autopsy, general observations were taken for each vessel, including depth to water and depth to substrate from the top of the columns. Water quality samples were then collected according to the general sampling procedure. One liter of sample was collected for analysis of physical parameters via a YSI 6920 v2 datasonde and for BART SRB population enumeration tests. Two 250-mL samples were collected; one left unpreserved for nutrient

analyses, Total Organic Carbon (TOC) (EPA 415.3, 2005), and sulfate (ASTM Method D516-90, 1995). The nutrients analyzed in the effluents included reactive phosphate (USEPA 600/R/93/100, Method 365.1, Rev 2.0 (1993)), and nitrate and nitrite (EPA 353.2 Rev. 2.0 (1993) and (Standard method 4500-NO3-F (2000)). Nutrients and sulfate were analyzed using a Seal AQ300 Automated Discrete Analyzer. Additional 250-mL samples were taken for sulfide analysis and were analyzed using the same DR 3800 spectrophotometer according to HACH method 8131 (2018). Total Carbon (TC) and Total Inorganic Carbon (TIC) contents of the effluents were measured using an Analytik Jena Multi N/C TOCN Analyzer according to EPA method 415.3. TOC was determined internally by the instrument from the difference between TIC from TC. Total Dissolved Carbon (TDC) species were determined by passing the samples through a 0.45-micron filter, and DOC (Dissolved Organic Carbon) was also determined internally from the difference of Dissolved Inorganic Carbon (DIC) from TDC. After sample collection was completed, the top end cap of the column was removed, and a falling head saturated hydraulic conductivity test was conducted according to the ASTM D 4511-11 (2020). The saturated hydraulic conductivity (K_{sat}) values were averaged by treatment, where the NAC and SMC treatments included 3 columns, and MC included only 2. Five measurements per column were obtained as per the method and were averaged by column and by treatment.

The substrate was then removed from the vessel carefully to maintain anaerobic conditions. Roughly 1/3 of the depth of the vessel was drained at a time via the effluent tubing at the bottom, leaving remaining portions saturated. Starting with the top 1/3, a depth of media was quickly removed using a hand shovel according to Figure 4. Organic substrate was collected from the top 5 cm and bottom 5 cm of each depth from zones A, B, and C (Figure 5) to create composite samples for each depth of the vessel. Therefore, three composite samples were

generated per vessel, one from the top 10 cm, one from the middle 10 cm, and one from the bottom 10 cm, composited from zones A, B, and C.
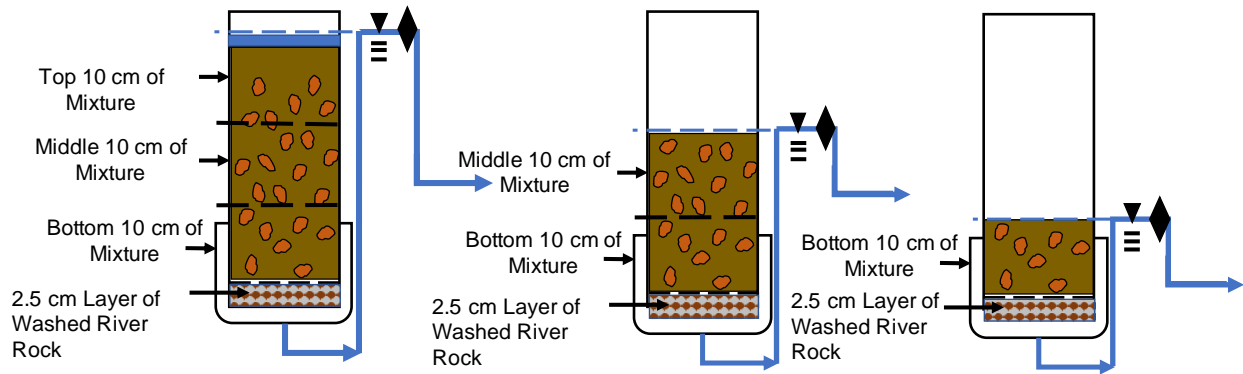
Figure 4: Schematic of Removal of Media from Columns
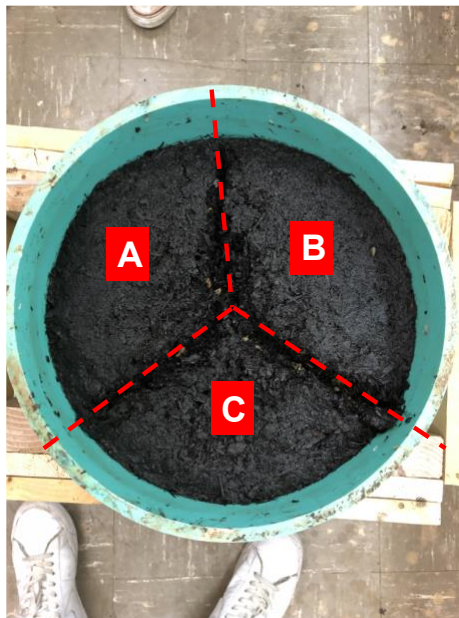
Figure 5: Zones A, B, and C of the Column for Autopsy

A total of 27 organic substrate samples were collected. Substrate samples were placed in sealed, low density polyethylene (LDPE) soil bags. Following the removal of the media, and hand removal of larger river rock in subsamples, gravimetric moisture content was determined following ASTM Method D4531-86 (2008). Substrate sub-samples were collected from the sealed soil bags and tested in triplicate.

After performing moisture content analysis, the remaining media was pulverized using a pestle and mortar before passing through a one-mm sieve (No. 18) for determination of organic matter content via Loss-on-ignition (LOI) and other analyses. Organic matter was estimated gravimetrically via LOI according to ASTM D 7348-13 (2013) (CREW, 2016). Elemental analysis, including sulfur, of the substrate was determined via analysis on a Varian Vista-PRO simultaneous axial inductively coupled plasma-optical emission spectrophotometer (ICP-OES) according to EPA method 6010c (2000) after first undergoing hot nitric acid digestion using a CEM Microwave Accelerated Reaction System (MARS) according to method EPA method 3051A (2007) for microwave assisted acid digestion of sediments, sludges, and soils. Particular elements of concern for this study include sulfur, macronutrients (calcium, iron, potassium, magnesium, and sodium), micronutrients (copper, manganese, nickel, and zinc) and other nonessential and potentially harmful trace metals (arsenic, aluminum, cadmium, chromium, and lead).

2.6. Statistics and Data Analysis

When data were normally distributed as determined by the Shapiro-Wilk Test ($\alpha = 0.05$), data were presented as the mean value and standard deviation by treatment. When not normally distributed, data were presented as the median value by treatment or by HRT. Volume adjusted removal rates (mmol m^{-3} day^{-1}), removal percentages by concentration, and mean concentrations were calculated for each column at each sampling time, and then averaged over the duration of the study for each treatment, and during different flow manipulation periods (Equations 3 and 4) (Machemer et al., 1993).

Equation 3

$Removal\ Rate$ (mmol $SO_4\ m^{-3}\ day^{-1}$)

$$= \frac{([\text{Influent SO}_4 \text{ mg L}^{-1}] - [\text{Effluent mg SO}_4 \text{ mg L}^{-1}]) * 1 \text{ g } 1000 \text{ mg}^{-1} * \text{Flow Rate L day}^{-1}}{(\{96.06 \text{ g SO}_4 \text{ mole}^{-1} * 1 \text{ mole } 1000 \text{ mmol}^{-1}\} * \text{Volume of Vessel (m}^3) * \text{Porosity (n} = 0.5))}$$

Equation 4

$$\text{Percent Removal SO}_4 \ (\% \ ) = \frac{|[\text{Influent SO}_4 \ (\text{mg L}^{-1})] - [\text{Effluent mg SO}_4 \ (\text{mg L}^{-1})]|}{[\text{Influent mg SO}_4 \ (\text{mg L}^{-1})]} * 100$$

Statistical analyses were done using Stats Plus PRO by AnalystSoft (2020) to assess the normality of the data and the statistical significance of differences between the treatments. The datasets were assessed for statistically significant differences between treatments using a one-way Analysis of Variance (ANOVA, $\alpha = 0.05$) and Tukey HSD Test ($\alpha = 0.05$). When normally distributed, two-tailed means comparison t-tests ($\alpha = 0.05$) were done to test for statistically significant differences between treatments at different stages of the flow adjustment. When examining trends over time, a two-tailed Pearson Product Moment Test ($\alpha = 0.05$) was performed to determine statistical significance of the trend.

3. Results and Discussion

3.1. Performance of Selected Organic Substrates

3.1.1. Physical Parameters

Effluent water quality from the columns is summarized in Tables 2 and 3, and Figure 6. Over the duration of the study, pH of the effluent remained circumneutral in all treatments. Mean ORP of the column effluents was negative at the start of the study and decreased until stabilizing between -300 and -350 mV in all treatments. This was lower ORP than expected, with values reported in the literature ranging between -100 mV and – 250 mV for optimal sulfate reduction conditions (Edwards, 1998). The temperature of the effluent remained near laboratory temperature (20 $^\circ$C) throughout the study. These physical parameters confirm optimal conditions for sulfate reduction to occur: circumneutral pH, negative ORP, and moderate temperatures.

Table 2: Summary statistics for column effluent pH from Different Treatment Groups

	Mean	Standard Deviation	N	Range
NAC	7.06	0.13	12	6.82 – 7.39
MC	7.22	0.12	12	6.93 – 7.41
SMC	7.10	0.14	12	6.82 – 7.41
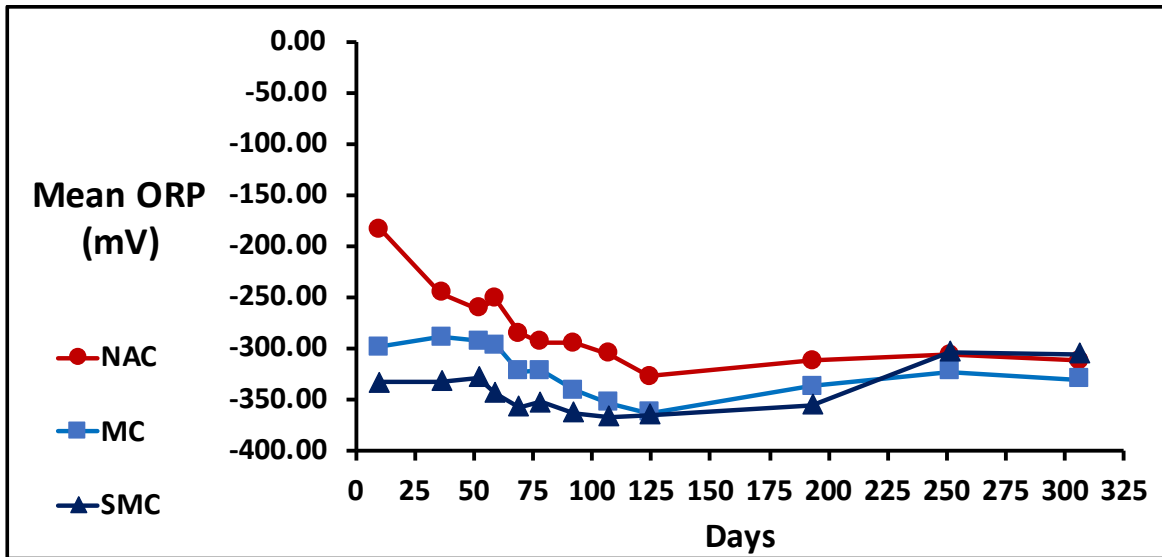
Figure 6: Mean ORP (mV) of Effluents from Different Treatment Groups (n = 12)

Table 3: Mean Temperature (°C) and Temperature Range of Column Effluents from Different Treatment Groups

	Mean	Standard Deviation	N	Range
NAC	19.90	0.56	12	18.3 – 20.7
MC	19.97	0.54	12	18.2 – 20.7
SMC	20.00	0.50	12	18.8 – 20.9

3.1.2. Changes in Sulfate and Sulfide Concentrations and Sulfate Mass Removal

Median sulfate and sulfide concentrations of the effluents from the different treatments are presented in Figure 7 and summarized in Table 4. The median concentrations of the effluents were chosen for this figure because effluent SMC sulfate concentrations, and NAC, MC, and SMC sulfide concentrations, were not normally distributed as determined by Shapiro-Wilk test for normality ($\alpha = 0.05$). The target concentration of the influent was designed within 10% of 1000 mg SO_4 L^{-1}, and the mean was 990.17 ± 69.7 mg L^{-1} (n = 17) for the study. Influent sulfide concentrations were below the detection limit of 5 μg L^{-1}. The sulfate and sulfide concentrations by treatment can be found in Appendix A and are presented individually by treatment.
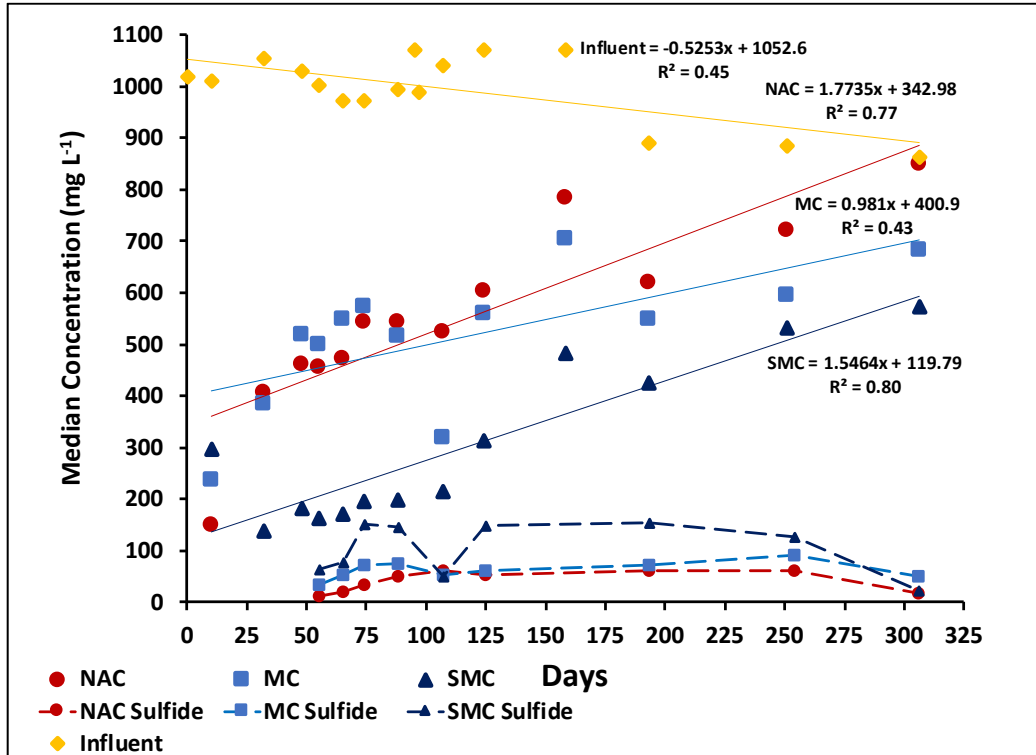
Figure 7: Median Concentration of Aqueous Sulfate and Sulfide in the Column Influent and Effluents prior to Flow/HRT Manipulation

Table 4: Summary of Sulfate and Sulfide Concentrations of Effluents prior to Flow/HRT Manipulation

| | Sulfate (mg L^{-1}) | | | Sulfide (mg L^{-1}) | | |
	Mean	Median	N	Mean	Median	N
NAC	512.9	542.4	39	36.03	34.7	27
MC	490.1	516.0	39	66.65	59.6	27
SMC	310.0	221.0	39	110.4	124	27

All treatments showed similar trends in effluent sulfate concentrations, and in sulfide generation, trending from low to high sulfate concentrations over time, while sulfide generation appeared to stabilize from Day 75 to Day 254. There was a strong linear trend in increased sulfate concentration (mg L^{-1}) as determined by Pearson product moment Test ($\alpha = 0.05$) for all three treatments over the first 305 days of the study (p < 0.001 for NAC and SMC, and p < 0.05 for MC). Effluent sulfate concentrations for the SMC treatment were considerably lower during

the majority of the study. SMC consistently produced the lowest median effluent sulfate concentration after Day 10. The SMC treatment also generated the most sulfide, followed by MC, and NAC. The sulfide generation in the SMC treatment was almost three times greater than in the NAC treatment, and almost twice as much as the MC treatment. Given that bacterial sulfate reduction was occurring, sulfide generation was ongoing, the dissolution of gypsum ($CaSO_4$) and subsequent release of sulfate over time potentially created a stabilizing effect of effluent sulfide concentrations even though effluent sulfate concentrations were increasing. It is also possible that the elevated sulfide concentrations in the effluent contributed to decreased sulfate removal. Vasquez et al. (2016) noted that increased concentrations of soluble sulfides (>100 mg L^{-1}) would decrease SRB cell size and growth, thus decreasing overall removal efficiency by having fewer active bacteria.

The influent solution concentration appeared to have decreased over time, yet the method of preparation had not changed, and this difference was not statistically significant. This apparent decrease is also accentuated by a smaller sample size near the end of the study where concentrations were lower than expected. During the flow manipulation portion of the study presented later, the concentration of the influent was greater than the target concentration on average.

Mean sulfate removal rates are presented in Figure 8 and summarized in Table 5. Removal rate (mmol m^{-3} day^{-1}) was determined to be normally distributed by Shapiro-Wilk test for normality ($\alpha = 0.05$) and thus the mean values were presented in the figure. Sulfate removal rates (mmol m^{-3} day^{-1}) by treatment are presented individually in Appendix A.
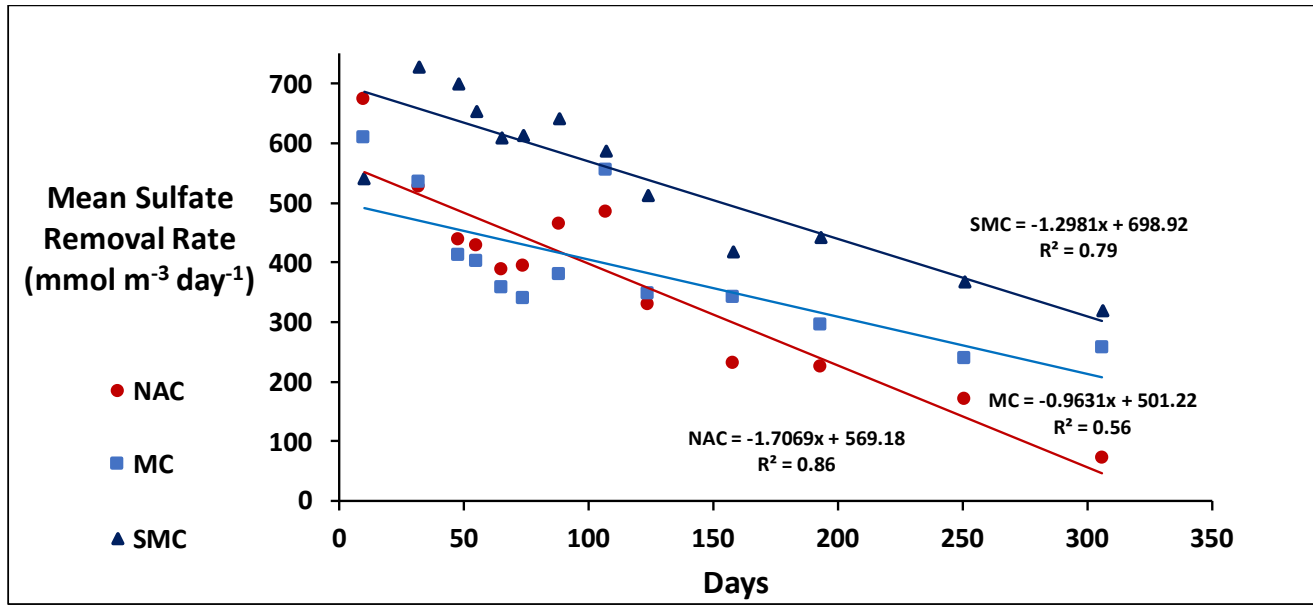
Figure 8: Mean Sulfate Removal Rate (mmol m^{-3} day^{-1}) prior to Flow/HRT Manipulation

Table 5: Summary of Mean Sulfate Removal (mmol m^{-3} day^{-1}) Prior to Flow Manipulation

	Sulfate Removal Rate (mmol m^{-3} day^{-1})		
	Mean	Standard Deviation	N
NAC	371	177	39
MC	389	140	39
SMC	548	143	39

NAC had the greatest decrease in sulfate removal performance, and the decline was significantly greater than both MC, and SMC ($p < 0.05$). There was a strong linear trend in decreased sulfate removal rate (mmol m^{-3} day^{-1}) in all three treatments ($p < 0.001$ for NAC and SMC, and $p = 0.003$ for MC). Maximum mean removal rates for NAC and MC were measured on Day 10 and found to be 674 mmol m^{-3} day^{-1} and 609 mmol m^{-3} day^{-1}, respectively. Maximum measured mean removal rate for SMC peaked on day 32 at 728 mmol m^{-3} day^{-1}. The SMC treatment produced the maximum rate of sulfate removal, as well as the greatest mean and median value of sulfate removal by a considerable margin, outperforming the two other

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treatments for the duration of the study, and consistently producing the lowest concentration of sulfate in the effluent waters.

Mean sulfate removal rates generally declined from the beginning of the study. At the start, the systems were likely at the end of the "honeymoon phase," where substrate sorption capacity for inert cations was much greater, ample time had been given for organic matter degradation, and sulfate removal rates were higher (Younger et al., 2002). Eventually this phase ended, and rates equilibrated after roughly 50 days, and then slowly declined over time. Vasquez et al. (2016) described what is likely happening: SRB consumed the easily available substances (EAS) sources at the beginning of the experiment and produced a resultingly high sulfate removal rate. As those EAS sources dwindled, SRB began to turn to other sources of labile C which are provided by, but also limited by, a finite rate of degradation by other microbes, and sulfate removal equalized over time to match that rate. With knowledge that SRB populations were very high ($<2,000,000$ CFU mL^{-1}) at all points measured during the experiment, this would suggest that the other microbes in these columns were rate-limiting for sulfate removal in the long-term.

Median sulfate removal was also plotted as percentage removal (Figure 9, Table 6). Removal rates (%) were not normally distributed and thus median sulfate removal is presented. Removal rates (%) presented by treatment are available in Appendix A.
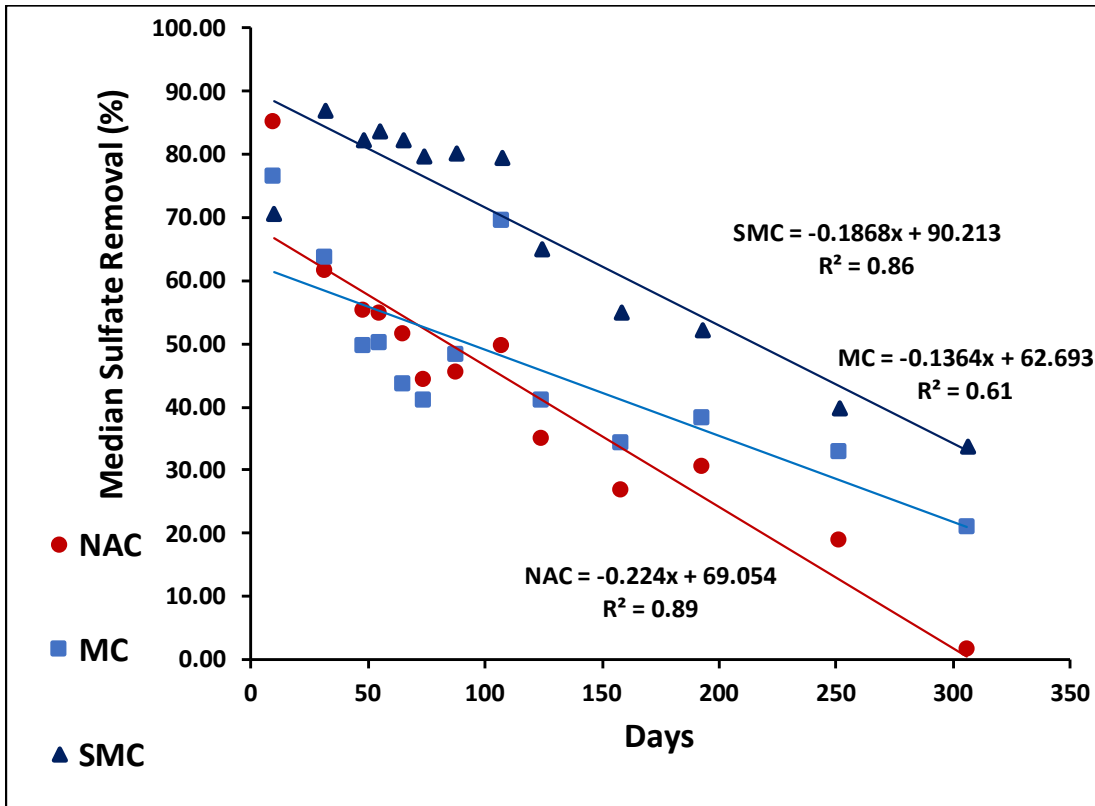
Figure 9: Median Sulfate Removal (%) prior to Flow/HRT Manipulation

Table 6: Summary of Median Sulfate Removal (%) Prior to Flow Manipulation

| | Sulfate Removal Rate (%) | | |
	Mean	Median	N
NAC	46.8	45.3	39
MC	49.4	48.1	39
SMC	67.7	77.3	39

Sulfate removal declined over time after peaking near 87% in NAC and SMC and 78% in MC at the beginning of the study. By the end of the study, the NAC columns were beginning to show signs of failure as percent removal grew closer to zero, and effluent sulfate concentrations increased to concentrations near the influent value. Although performance decreases were noted for MC and SMC treatments, they were still outperforming the NAC treatment in terms of sulfate removal at the end of the study. Again, there was a trend in decreased sulfate removal rate (%) in

all three treatments, the most significant in NAC, followed by SMC, and MC ($p < 0.001$ for NAC and SMC, and $p < 0.01$ for MC).

In summary, sulfate concentrations in the effluents were lowest in the SMC treatment throughout the study, however, they were significantly lower than the other two treatments only during these first 305 days (Tukey HSD, $p < 0.05$). Sulfate concentrations of the effluents increased significantly over the first 305 days as sulfate removal rates, and sulfate removal significantly decreased ($p < 0.05$). The SMC treatment produced significantly greater removal rates, percent removal, and effluent sulfide concentrations than other treatments (Tukey HSD, $p < 0.05$). Overall, even with the potential masking effect caused by additional dissolved sulfate from the source material, the SMC treatment had the best sulfate removal capability. It is suspected that the SMC had sufficient available OM during the 8-day HRT, and this could have allowed for SRB to maintain high levels of activity and remove sufficient sulfate. This compounded with minimal changes in OM in the substrate, shows that the OM in this substrate was very diverse and able to provide ample OM for consumption in the short-term and long-term.

3.2. Change in Hydraulic Retention Time (HRT)

3.2.1. Changes in Sulfate and Sulfide Concentrations, and Sulfate Mass Removal

During the flow/HRT manipulation period, no changes were made to the influent solution which maintained a target concentration of 1000 mg L^{-1} $\pm$ 10% during this study. First, the flow rates of the influent solution were doubled to 1 mL min^{-1} for 30 days and then afterwards, the flow rates were doubled again to 2 mL min^{-1} for another 30 days. The adjusted flow rates resulted in calculated HRTs of the vessels to be halved, and then halved again, to 4 and 2 days,

respectively. The median influent sulfate concentration during the four-day HRT and the two-day HRT were 972.6 mg $L^{-1} \pm 50.1$ (n = 4) and 1078.9 mg $L^{-1} \pm 48.5$ (n = 5), respectively. These values compare well to the influent concentration of 990.17 $\pm$ 69.7 mg L^{-1} (n = 17) for the first 305 days of operation with HRTs of 8 days.

After the initial change in flow rates, all treatments responded and stabilized in terms of sulfate removal and sulfide generation. The treatments also showed resilience and capability to adapt, by responding to the increased flow rate without leaching sulfate, with the exception of NAC at the conclusion of the study.

Beginning on day 310, and first measured on day 315, the 1 mL min^{-1} flow rate (4-day HRT) was tested for 30 days. Beginning at day 340, and first measured on day 343, the 2 mL min^{-1} flow rate (2-day HRT) was tested for 30 days. Effluent sulfate and sulfide concentrations were determined to be normally distributed at a four-day and two-day HRT (Shapiro Wilk, $\alpha = 0.05$). However, during the eight-day HRT, they were not and therefore mean and standard deviation are presented in Tables 7 and 8, but median sulfate and sulfide concentrations of the effluent are presented in Figures 10 and 11 and also included in Tables 7 and 8. Effluent sulfate and sulfide concentration during the flow manipulation period presented individually by treatment are available in Appendix A.
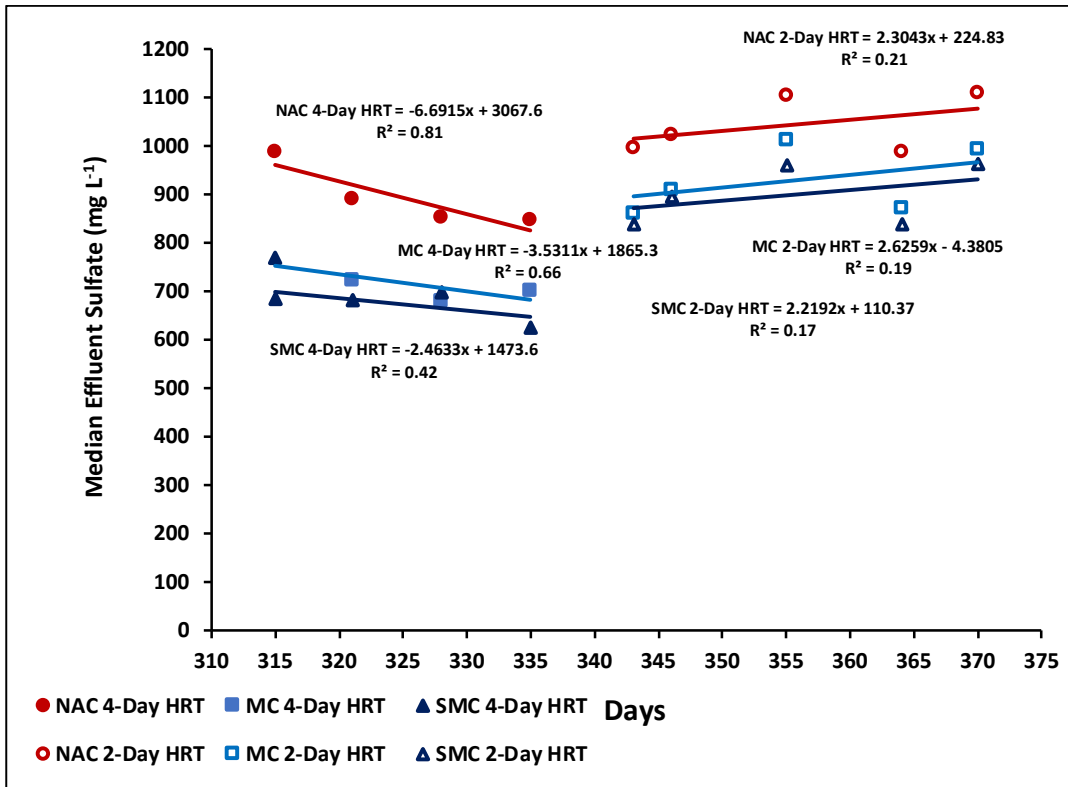
Figure 10: Median Effluent Concentration of Aqueous Sulfate during Flow/HRT Manipulation
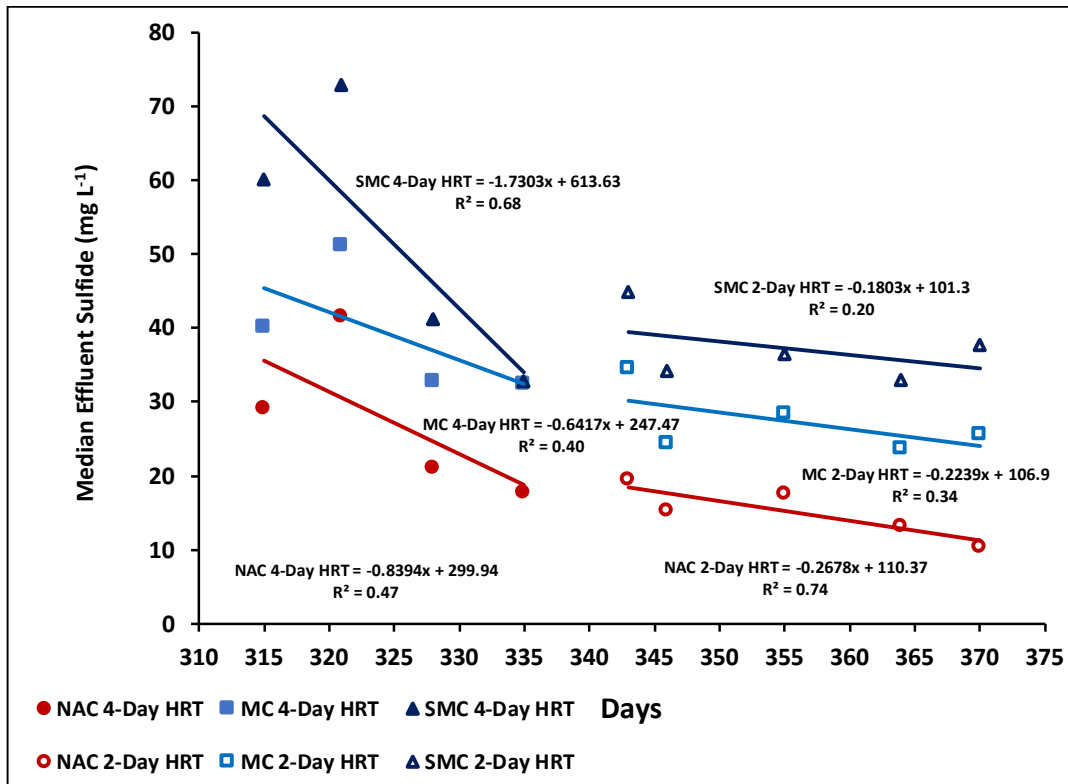

Figure 11: Median Effluent Concentration of Aqueous Sulfide during Flow/HRT Manipulation

Table 7: Effluent Sulfate and Sulfide concentrations during 4-day HRT

| | Sulfate (mg L^{-1}) | | | Sulfide (mg L^{-1}) | | | |
	Mean	SD	Median	Mean	SD	Median	N
NAC	894.3	65.3	873.7	29.1	13.2	26.8	12
MC	718.6	44.6	722.6	39.1	11.4	38.0	8
SMC	685.1	51.8	684.4	57.3	30.9	51.3	12

Table 8: Effluent Sulfate and Sulfide concentrations during 2-day HRT

| | Sulfate (mg L^{-1}) | | | Sulfide (mg L^{-1}) | | | |
	Mean	SD	Median	Mean	SD	Median	N
NAC	1047	54.1	1024	14.8	4.2	15.2	15
MC	929.4	67.4	910.8	27.3	5.1	25.6	10
SMC	901.8	69.6	894.9	36.8	8.6	36.4	15

The SMC treatment generated the most sulfide in comparison to the NAC and MC treatments at four-day and two-day HRTs. The effluent sulfate concentrations from the NAC treatment were significantly greater than the MC and SMC treatments (Tukey HSD, $p < 0.05$) at four-day and two-day HRTs. There were no significant trends in sulfate concentrations over time in either HRT during the flow manipulation period. Sulfate concentrations remained fairly stable during both HRTs tested, with the least effluent sulfate concentration measured in the SMC treatment, followed by MC and NAC. Effluent sulfide concentrations were significantly different (ANOVA, $p < 0.05$) between treatments, and at different HRTs. The SMC treatment generated the most sulfide, followed by MC, and NAC and more sulfide was generated in four-day HRTs than during two-day HRTs. There were no significant trends of sulfide concentration over time, and during the two-day HRT, sulfide concentrations appeared to stabilize in all treatments. The NAC treatment removed less sulfate at the lower HRTs and was found to be leaching sulfate in 1 of 12 samples (8%) during the four-day HRT, and 11 of 15 samples (73%) during the two-day HRT.

To investigate how sulfate removal capabilities changed over time at different HRTs, the mean sulfate removal rate (mmol m^{-3} day^{-1}) and median sulfate removal (%) were plotted in Figures 15 and 16. Sulfate removal rate (mmol m^{-3} day^{-1}) was determined to be normally distributed (Shapiro-Wilk, $\alpha = 0.05$), while sulfate removal rate (%) was not, and so to make valid comparisons, mean and median were plotted, respectively. Sulfate removal rates (mmol m^{-3} day^{-1}) and percent sulfate removal during the flow manipulation period presented individually by treatment are available in Appendix A.
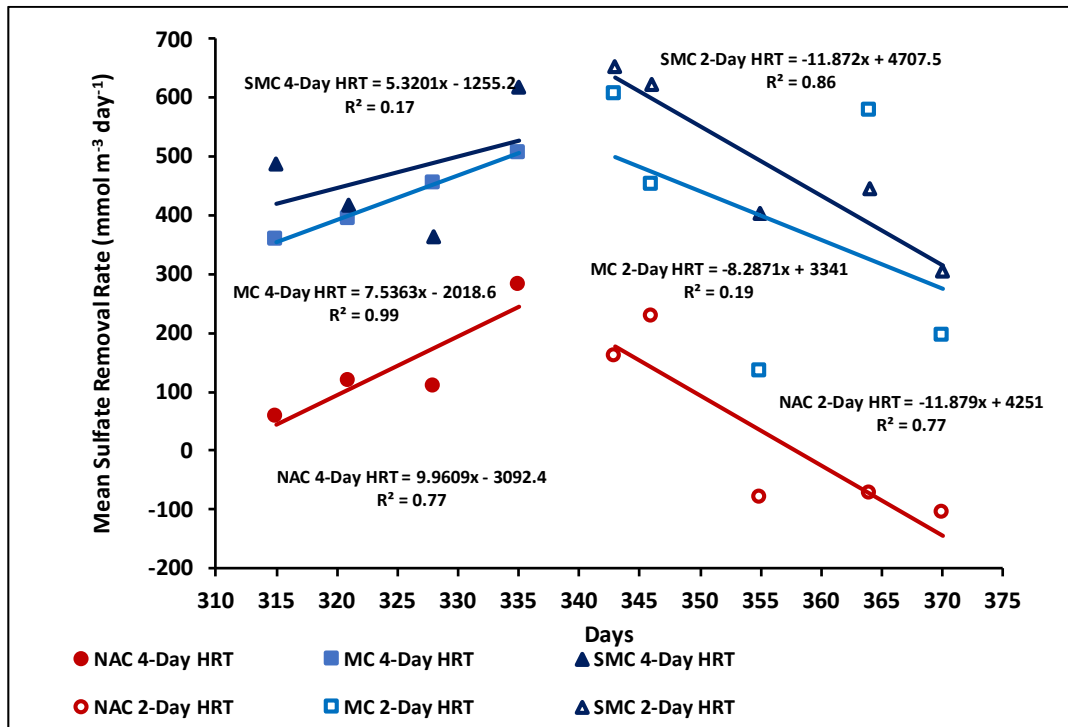
Figure 12: Mean Sulfate Removal Rate (mmol m-3 day-1) during Flow/HRT Manipulation
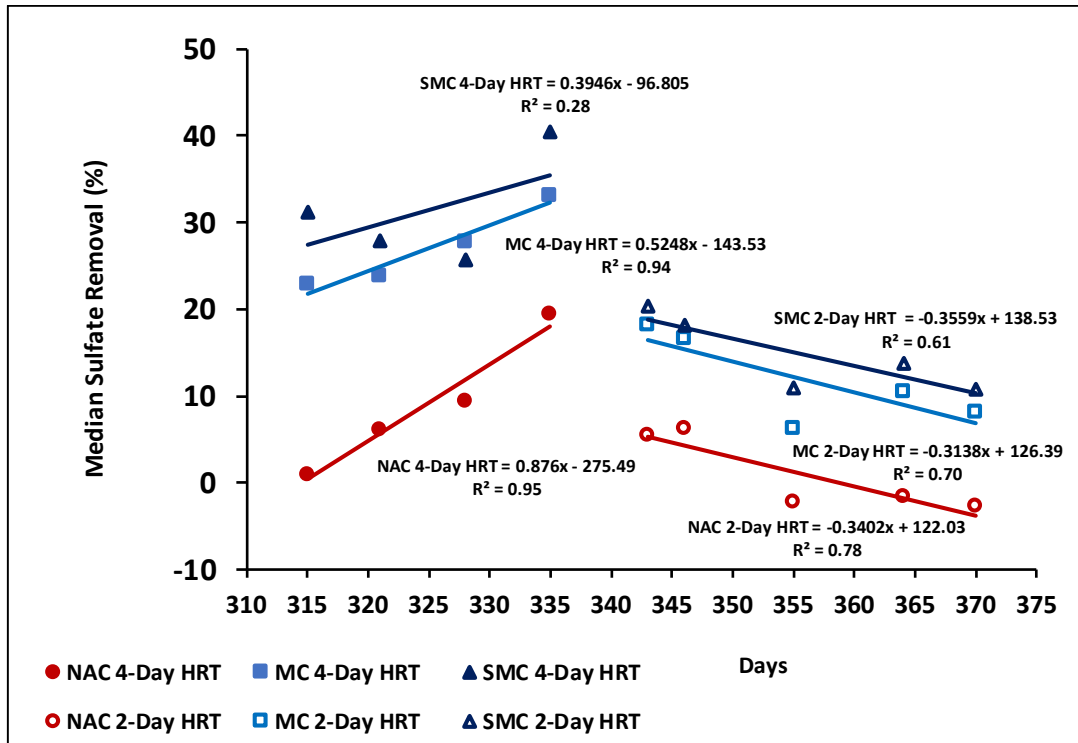
Figure 13: Median Sulfate Removal (%) during Flow/HRT Manipulation

During the four-day HRT, sulfate removal rates generally increased as the columns adapted to the increased flow rate until peaking prior to beginning the two-day HRT. There was a trend in increased sulfate removal rate (mmol m^{-3} day^{-1}) and percentage removal over time in the MC and NAC treatment ($p < 0.05$) but this trend was not seen in the SMC treatments. The SMC treatment had decreased removal until the final day of the 4-day HRT, where sulfate concentrations in the effluent were lower and thus produced an increased removal rate comparatively. The sulfate removal seen in the MC treatment during the 4-day HRT increased linearly over time ($r^2 = 0.99$ for removal rate and $r^2 = 0.94$ for percent removal).

During the two-day HRT, there was a dramatic decrease in the SMC and NAC treatment sulfate removal efficiency ($p < 0.05$). Removal rates plummeted by over 300 mmol m^{-3} day^{-1} over the course of the 2-day HRT in the NAC and SMC treatments, and became negative in the NAC treatment as sulfate began leaching from the columns. The MC treatment on the other hand

showed variable removal efficiency, and the removal efficiency did not significantly decline over time like the other two treatments. Although removal rates generally declined in all treatments at a 2-day HRT, the only statistically significant difference in percent removal of sulfate was in the NAC treatment ($p < 0.05$).

There was also a linear trend in increased sulfate removal (%) in the MC and NAC treatments during the four-day HRT ($p < 0.05$). The SMC treatment did have an increase in percentage removal; however, this change was not statistically significant.

Median sulfate removal (%) increased temporally after beginning the flow manipulation by changing from an eight-day to four-day HRT, peaking in all three treatments at the end of the four-day HRT at 19.37% for NAC, 33.12% for MC, and 40.43% for SMC. The mean removal rate (mmol m^{-3} day^{-1}) of the NAC treatment was found to be significantly different from the SMC and MC treatments in both the four-day HRT and two-day HRT, but the rates of sulfate removal were not statistically different between MC and SMC during flow manipulation (Tukey HSD, $p < 0.05$).

After peaking, the sulfate removal rate (mmol m^{-3} day^{-1}) and percentage sulfate removal both generally decreased and actually became negative in the NAC treatment as it began to leach sulfate after Day 355. To compare sulfate removal capability by treatment and by HRT, aqueous sulfate and sulfide, and sulfate removal rates (mmol m^{-3} day^{-1}) and sulfate removal (%) are summarized in Tables 9-11 and the distributions are plotted in Figures 14-17.

Table 9: Mean and Median Sulfate Removal Rate (mmol m^{-3} day^{-1}) of the Treatments at Different HRTs

HRT		Sulfate Removal Rate (mmol m^{-3} day^{-1})				
		Mean	Standard Deviation	Median	Maximum	N
8 days	NAC	371	177	362	759	39
	MC	389	140	396	730	39
	SMC	548	142	594	774	39
4 days	NAC	142	121	148	335	12
	MC	428	69.8	438	532	8
	SMC	472	125	469	699	12
2 days	NAC	26.8	149	-51.1	265	15
	MC	394	212	452	641	10
	SMC	485	175	505	774	15
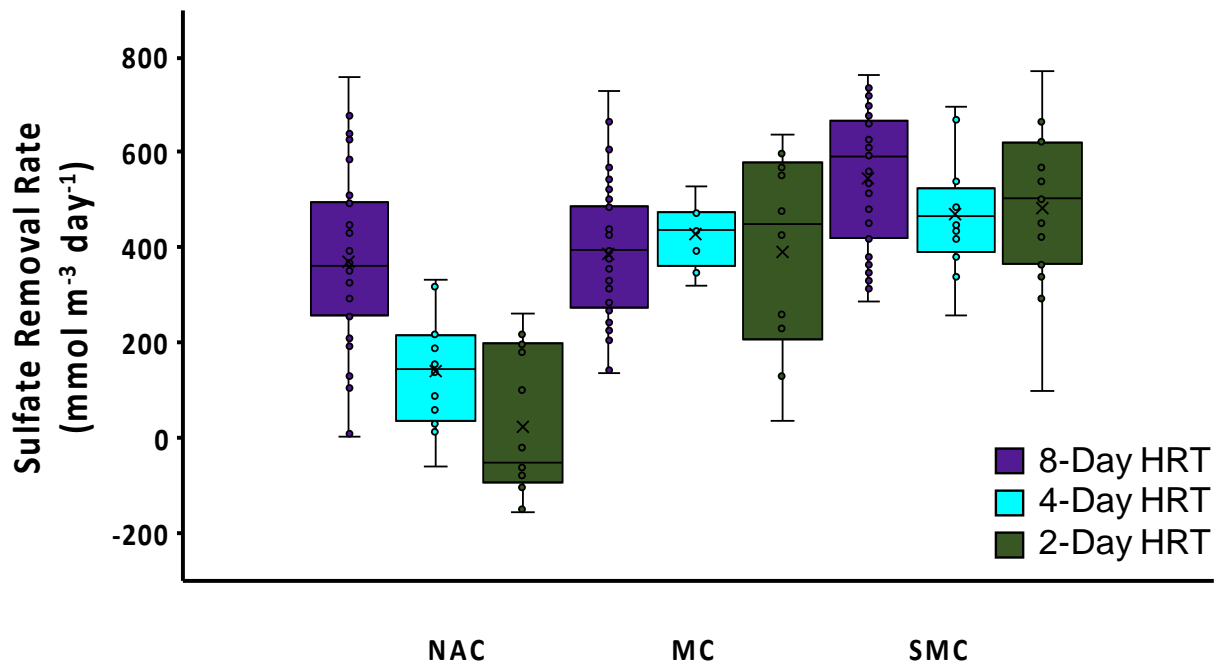
Figure 14: Distribution of Sulfate Removal Rates in All Treatments at Different HRTs

The MC and SMC treatments showed resilience and capability to adapt to a higher flow rate, and subsequently lower HRTs, having sulfate removal rates that were not significantly different over different HRTs (Tukey HSD, $p < 0.05$). The NAC treatment showed some resilience and improved sulfate removal performance from the end of the eight-day HRT to the

four-day HRT with increased sulfate removal over time during the four-day HRT, however performance drastically diminished after shortening the HRT to two days and the NAC columns began to leach sulfate.

The rates of sulfate removal in the NAC treatment were found to be significantly different from one another across the tested HRTs (Tukey HSD, $p < 0.05$). The overall mean removal rate throughout the entire study duration for NAC was 251.08 mmol m^{-3} day^{-1}, the lowest of the three treatments.

The rates of sulfate removal in the MC and SMC treatment were not found to be significantly different from one another, and both treatments continued to remove sufficient sulfate at shorter HRTs. The MC and SMC treatments sulfate removal rates were significantly greater than the NAC treatment rate at both HRTs of four and two days ($p < 0.05$). Only the SMC treatment sulfate removal rate was significantly greater than the other two treatments during the eight-day HRT ($p < 0.01$).

Overall, the MC treatment had a mean sulfate removal rate of 395.66 mmol m^{-3} day^{-1} over the entire study duration. The SMC treatment maintained the greatest mean sulfate removal at all HRTs but was only significantly greater during the eight-day HRT ($p < 0.01$). The SMC treatment had a mean sulfate removal rate of 520 mmol m^{-3} day^{-1} over the entire study duration. During the flow manipulation, the mean sulfate removal rate declined slightly in the SMC treatment, yet remained the greatest in comparison to the other treatments.
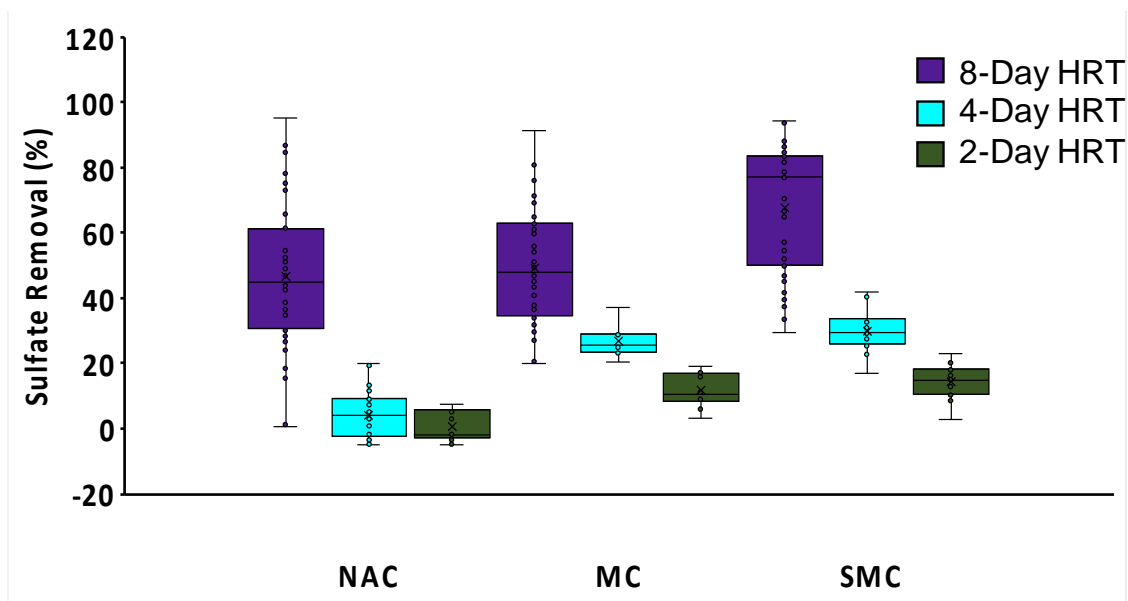
The rates of sulfate removal during the first 305 days without any flow manipulation were all greater than the recommended 300 mmol m^{-3} day^{-1} by URS (2003) and were comparable to those found by LaBar and Nairn (2016; 2017) that found removal rates up to 690 mmol m^{-3} day^{-1}. This greater removal rate can be partially attributed to a greater influent concentration of

sulfate (2100 mg L^{-1}) compared to this study (1000 mg L^{-1}) which can positively effect SRB population growth. It should also be discussed that another potential reason for the enhanced removal rates was that there were additional sulfate removal mechanisms at work including potential gypsum precipitation, sorption to organic matter, and formation of elemental sulfur, all of these which could have also been responsible for sulfate removal in this study. Formation of elemental sulfur was occasionally visible in the effluent tubing, but this should not be credited to what was happening in the columns because conditions in the tubing had increased dissolved oxygen due to the effluent collection station air release.

To look at overall removal from the influent, sulfate removal (%) was also measured during the flow manipulation period and compared across all treatments at all HRTs. Mean and median sulfate removals (%) of the different treatments at different HRTs are summarized in Table 10.

Table 10: Mean and Median Sulfate Removal Rate (%) of the Treatments at different HRTs

	Sulfate Removal Rate (%)			
HRT		Mean	Standard Deviation	Median	N
	NAC	46.8	10.9	45.3	39
8 days	MC	49.4	13.0	48.1	39
	SMC	67.7	5.85	77.3	39
	NAC	9.02	7.37	9.94	12
4 days	MC	26.9	5.10	25.6	8
	SMC	30.2	6.92	19.3	12
	NAC	0.74	4.43	-1.66	15
2 days	MC	12.0	5.25	10.6	10
	SMC	14.6	5.21	15.2	15

Figure 15:Distribution of Sulfate Removal (%) of the Treatments at Different HRTs

At an eight-day HRT, percent sulfate removal was significantly greater in SMC than the other two treatments (Tukey HSD, $p < 0.05$). During the first 305 days of the study, SMC had the greatest median removal of 67.72% while NAC and MC removals were 46.78% and 49.45%, respectively. During the four-day HRT and two-day HRTs, the NAC treatment was found to remove significantly less sulfate than both MC and SMC (Tukey HSD, $p < 0.05$).

Sulfate removal generally decreased throughout the first 305 days, and temporarily increased during the 4-day HRT, marked by a spike on day 335 as seen in Figure 16. During the two-day HRT, the mean removal of NAC, MC, and SMC were -1.62%, 10.61% and 15.19%, respectively. SMC had the greatest median percent removal in all cases, although this was not significantly greater than the MC treatment during either HRT during the flow manipulation period.

To fully understand the sulfate removal capabilities of the substrates, effluent sulfate and sulfide concentrations were examined over the course of both studies. A summary of these data is presented in Table 11.

Table 11: Mean and Median Effluent [Sulfate] and [Sulfide] of all Treatments at Different HRTs

| HRT		Mean	Standard Deviation	Median	N
		Effluent [Sulfate] mg L^{-1}			
8 days	NAC	513	192	542	39
	MC	490	151	516	39
	SMC	310	174	221	39
4 days	NAC	894	65.3	874	12
	MC	719	44.6	723	8
	SMC	685	51.8	684	12
2 days	NAC	1047	54.1	1024	15
	MC	929	67.4	911	10
	SMC	901	69.6	895	15
HRT		Mean	Standard Deviation	Median	N
		Effluent [Sulfide] mg L^{-1}			
8 days	NAC	36.0	23.0	34.7	27
	MC	66.7	28.9	59.8	27
	SMC	110.4	48.3	123.6	27
4 days	NAC	29.1	13.2	26.8	12
	MC	39.1	11.4	38.0	8
	SMC	57.3	30.9	51.3	12
2 days	NAC	14.8	4.2	15.2	15
	MC	27.3	5.1	25.6	10
	SMC	36.8	8.6	36.4	15
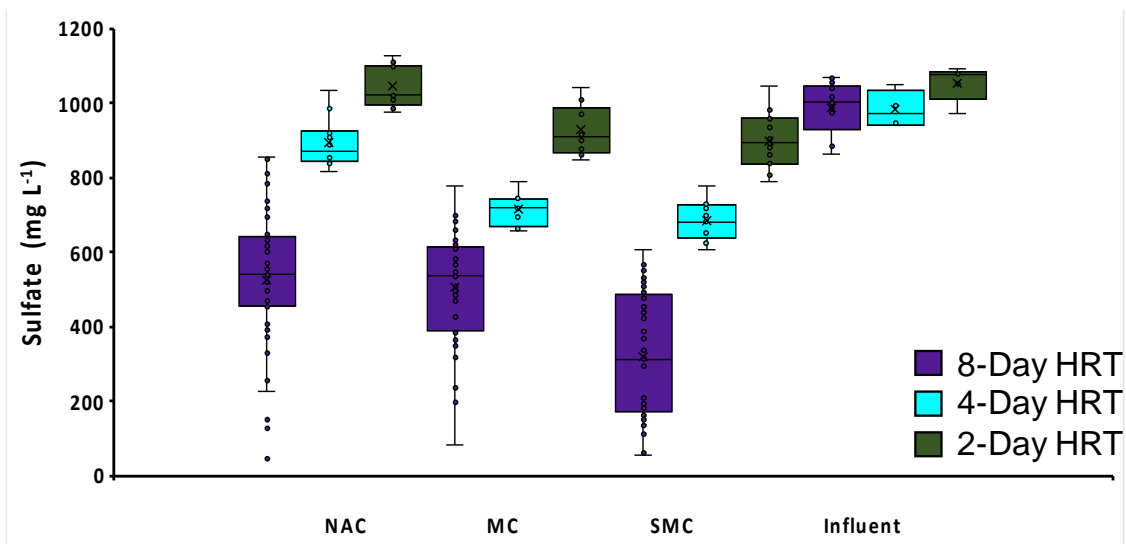
Figure 16: Distribution of Effluent Sulfate Concentrations of the Treatments at Different HRTs
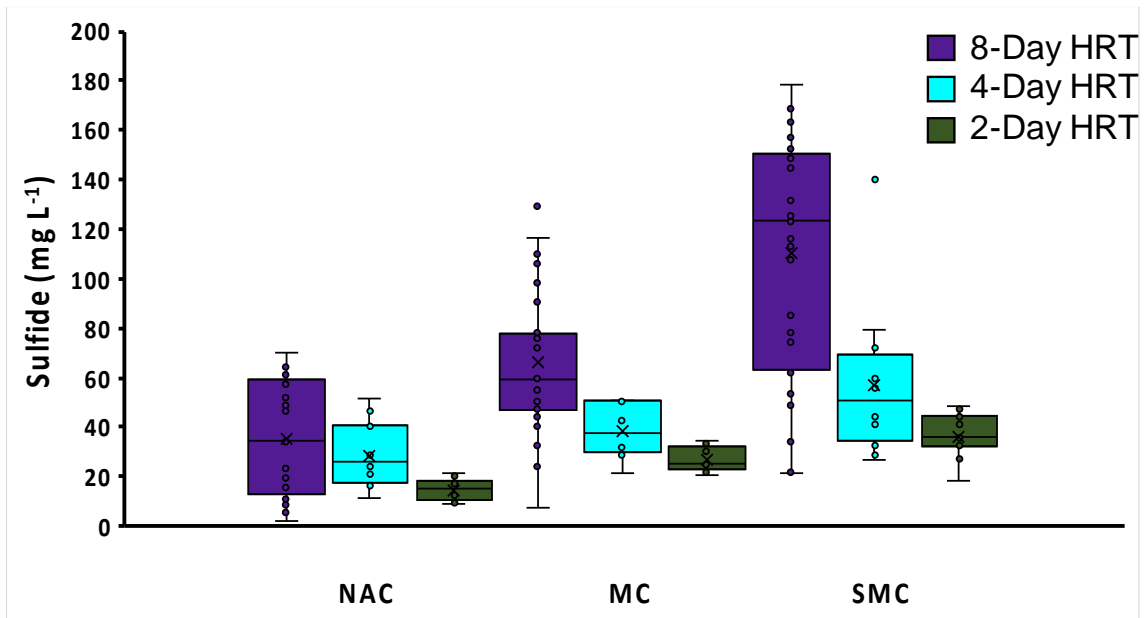
Figure 17: Distribution of Effluent Sulfide Concentrations in the Treatments at Different HRTs

As with the sulfate removal rate and percent removal, the SMC treatment produced effluent with significantly less sulfate, and significantly more sulfide than both NAC and MC (Tukey HSD, $p < 0.05$). The effluent sulfate concentrations during both HRTs from the NAC treatment were significantly greater than both of the MC and SMC treatments (Tukey HSD, $p < 0.05$).

Effluent sulfide decreased significantly (Tukey HSD, $p < 0.05$) for all HRTs in all treatments, wherein the 8-day HRT produced the most sulfide, followed by 4 days, and 2 days. This result was consistent with what was observed in field-scale applications of early VFBRs in a study by Machemer et al. (1993), that observed that at higher inflow rates, sulfide generation was the limiting factor to trace metals removal. In a column experiment conducted by Vasquez et al. (2016), they also found that sulfide generation was lower at shorter HRTs (2 days compared to 4 days), noting that there would be less time for oxidation of organic carbon of the substrates, and less reduction of sulfate by the SRB in the effluents. Lastly, effluent sulfide concentrations

were significantly greater (Tukey HSD, $p < 0.05$) in the SMC treatment followed by MC, and then the NAC treatment throughout all HRTs.

In summary, sulfate removal rates and sulfate removal (%) in the MC and SMC treatments were significantly greater than NAC during both of the tested HRTs during the flow/HRT manipulations (Tukey HSD, $p < 0.01$). Sulfate removal rates and sulfate removal percentages were not significantly different between HRTs for SMC and MC treatments but were significantly less in shorter HRTs for the NAC treatment (Tukey HSD, $p < 0.01$). During the four-day HRT, percent sulfate removal significantly increased over the short period of time in the NAC and MC treatments, and during the two-day HRT in the NAC treatment, significantly decreased (Pearson, $p < 0.05$). Sulfide production declined significantly with decreasing HRTs in all treatments (Tukey HSD, $p < 0.05$). The SMC and MC treatments both had unanticipated responses to shorter HRTs and produced similar sulfate removal capabilities across all HRTs tested. The enhanced sulfate removal capability of these substrates compared to the NAC treatment can likely be attributed to their diverse and reliable mix of carbon types. Both the composts which continued to remove sulfate have a larger variety of organic source materials, which helped to provide different types of carbon, as well as other essential nutrients which were beneficial to microbial growth. Due to similar initial bulk organic matter contents and in order to help understand the some of the differences in the substrates, the organic material was analyzed after the columns were autopsied to determine any changes in OM. Prior to analysis of the OM, some physical parameters of the effluent and substrate were investigated to determine evolution with time, and to ensure sulfate reducing conditions remained at the end of the study.

3.2.2 Physical Parameters

After column effluent collection ceased and the study was concluded, the columns were autopsied, and some general physical water quality parameters were recorded to conclude that proper conditions for bacterial sulfate reduction remained. A summary of relevant water quality data is reported in Table 12.

Table 12: Summary of Effluent and Influent Water Quality Parameters at the time of Autopsy

	Summary of Mean Water Quality at time of Autopsy				
	pH	ORP (mV)	Temperature (°C)	ODO (mg L^{-1}) and ODO saturation (%)	Turbidity (NTU)
NAC	7.55	-308.4	15.98	0.08, 0.83%	15.2
MC	7.42	-318.1	16.05	0.07, 0.70%	5.05
SMC	7.44	-342.7	15.74	0.08, 0.83%	8.5
Influent	6.07	132.5	15.58	8.66, 87.4%	2.3

ODO: Optical Dissolved Oxygen (Dissolved Oxygen concentration determined using an optical probe)

In conjunction with the negative ORP values less than -200 mV, anaerobic conditions were confirmed at the end of the study by low DO concentration and saturation. The pH values of the effluent at the time of the autopsy were circumneutral and were consistent with the pH values recorded prior to flow manipulation. Temperature values recorded in the effluents were lower by about four degrees (°C) than during the study, however this measurement was taken after the study had concluded and therefore is not seen as contributory towards sulfate removal rates. Turbidity values in all three treatments were low, confirming that the river rock drainage layer and mesh screen (≈1 mm opening) were preventing particulates from exiting the vessels in the effluent. At the time of autopsy, sulfate removal was ongoing, evident by active SRB populations determined by enumeration tests, sulfide odor, and sulfide production in all

treatments in conjunction with circumneutral pH, moderate temperatures, negative ORP, and low DO, providing more evidence of anaerobic conditions for ideal sulfate reduction.

There were different levels of compaction evident in the columns after they had settled, demonstrated by the differences in water depth and substrate depth in the columns (Appendix B), however all columns maintained similar operating conditions with roughly 2 cm of water ponded above the substrate. A summary of the saturated hydraulic conductivities is presented in the Appendix B.

The MC treatment was determined to have a statistically significant greater K_{sat} (Appendix B) than the NAC or SMC treatments (Tukey HSD, $p < 0.05$), but this difference was less than 10% and all measured values were within the same order of magnitude (10^{-3} cm sec^{-1}), and therefore the overall K_{sat} will be used when referencing the hydraulic conductivity of the columns. The K_{sat} values indicate that the columns were highly permeable, providing no evidence of preferential flow or clogging at the time of the autopsy. K_{sat} values typically decrease over time, especially for longer HRTs, however due to the fact that these columns had increased flow rates at the latter part of the experiment, this could have improved hydraulic conductivity and masked any potential decreases (Neculita et al., 2008; Rakotonimaro et al., 2018). The overall K_{sat} of the columns were an order of magnitude greater or more than those determined by Page (2016) in field-scale VFBRs, but on the same order of magnitude of those measured by Vasquez et al. (2016) in flow-through column reactors. K_{sat} values recorded are typically greater in laboratory applications than in field applications, and the K_{sat} of the columns tested in this experiment were amended with washed river rock in a 2:1 ratio by volume to increase porosity, and thus would increase the permeability of the material in the column (Neculita et al., 2008; Rakotonimaro et al., 2018).

3.3. Substrate Analysis

3.3.1. Organic Matter and Organic Carbon

Organic matter (OM) content was determined via LOI in 2018 for the three different substrates at the time of column construction. Each substrate was tested in triplicate at the beginning of the experiment. The columns were autopsied at the end of the study and OM content of the substrates were determined according to the autopsy procedure described in the methodology. One sample (SMC1 Bottom A) was compromised in the drying oven and was therefore removed from analysis. Mean percent change in OM was calculated by determining the difference in mean OM content of the starting material from the OM of the autopsied material (Table 13). Organic carbon of the substrates was also estimated using the commonly accepted ratio of organic carbon to organic matter (1:1.72) in soils utilized by the United States Department of Agriculture and National Resources Conservation Service which translates to roughly 58% of soil organic matter being organic carbon. It should be noted that organic matter and organic carbon in soils are not the same as what is found in compost, and this is only an approximation.

Table 13: Change in Mean OM Content (%) of Treatment Material from the Beginning and End of the Study

| | Organic Matter (%) in the Media | | | | | | |
| | Initial Material | | | Autopsied Material | | | Mean |
	Mean	SD	N	Mean	SD	N	Percent Difference
NAC	52.1	0.74	3	43.7	3.10	27	-8.4 %
MC	56.7	1.01	3	37.4	6.60	18	-19.4 %
SMC	57.3	1.85	3	51.8	2.34	26	-5.5%

The initial OM content of SMC and MC were determined to be significantly greater than the OM of NAC (Tukey HSD, $p < 0.05$). The OM content of the autopsied material was determined to be normally distributed (Shapiro-Wilk Test, $\alpha = 0.05$) in all treatments, and

therefore a one-way ANOVA and Tukey HSD test were conducted to determine significant differences. The OM contents of NAC, MC, and SMC were significantly different ($p < 0.01$) from one another post-experiment. No significant differences were found between the replicates of each treatment, or with regards to depth (top, middle, or bottom 10 cm of material) in each treatment. The mean OM content of each of the replicates with regard to depth can be found in Appendix B. Although there was no significant difference, there was an observed decrease in OM consistent in the NAC columns in the deeper material which was not observed in the other treatments. The difference in mean OM content in depth from the top to the bottom of the NAC treatment columns was only 3% and this difference can be explained in the variation of the OM content. There was even more variation found in the MC treatment OM content with regards to depth (6.6%), yet the difference was not statistically significant.

Jordan et al (2006) noted that variations in parameters such as OM in composts can be due to the different types of mixes, and ingredients utilized in the manufacturing of a specific compost. These composts were comprised of several different source materials. Unique to MC was manure and stable cleanings, while SMC included wheat straw base, chicken litter, soybean and cottonseed meal, sugar beet lime and gypsum. These two specific mixes are likely more consistent compared to NAC, which can be much more variable in material type. Although it is aged, screened, and amended with aged saw dust just like MC, the NAC source material is derived from publicly collected yard waste from the City of Norman, OK Composting Facility. This means that the overall organic matter content as well as type of organic matter depends largely on what is provided to the facility. Storms could contribute to larger pieces of woody debris being taken to the facility if trees are taken down. During the transition from fall to winter

seasons, and plant die-off occurs, there could be greater amounts of "green waste" as the public replaces their older, dying plants with newer, younger, and cold-resistant plants.

The SMC mix is derived for mushroom production, and MC is a commercially available product derived for many purposes including as a soil conditioner, fertilizer, natural pesticide, erosion control, and for wetland construction. The demand for a consistent material for the purpose of producing benefits (or producing results that customers approve of and will continue to buy) constitutes making a product (e.g., compost) which is reliable and consistent year-round. Thus, the MC and SMC substrates are both likely to have less impact from differences in source material seasonally and year to year, due to the fact the mixes of material are more consistent.

The change of OM was determined to be normally distributed in each of the treatments (Shapiro-Wilk, $\alpha = 0.05$), and therefore one-way ANOVA was conducted to determine differences. It was determined after using a Tukey HSD test ($p < 0.05$) that the change in OM in MC was significantly different from that of NAC and SMC.

MC had the most dramatic change in OM from the start of the experiment to the time of the autopsy. Compared to SMC, three times more OM was consumed over time in the MC treatment. The difference in the amount of change is likely attributable to the composition of the materials, and differences in amounts of labile and recalcitrant carbon types. MC is a blend of stable cleanings, grass clippings, fruits and vegetables, leaves, recycled wood bits, and aged saw dust, which is tumbled and sieved, leaving only smaller particles. MC provides a substrate readily available for microbes: it contains a large amount of labile carbon, and the material is smaller and has increased surface area, improving the ability for microbes to degrade the material.

The small change in OM content observed after the autopsy indicates that SMC likely has a larger fraction of recalcitrant carbon relative to the other two treatments. This relatively smaller change in OM could also indicate that the labile OM is not as accessible by the microbes. SMC is not tumbled or sieved so larger particles remain in the mix, thus less surface area is exposed for microbes to access.

NAC had the least amount of OM in the parent material, and did not change significantly more than SMC, only significantly less than MC (Tukey HSD, $p < 0.05$). Interestingly, NAC is a simple mix of yard waste, that was tumbled and sieved for smaller particles by Murphy Products. This type of mix is dominated by cellulosic plant material, more labile than lignin dominated mixes, which has been made more accessible from the tumbler, but there was not a significant difference in the change in OM from SMC, a vastly different mix. The SMC and MC mixes both likely have more lignin plant material in comparison. MC has stable cleanings (hay or straw), recycled wood bits, and aged saw dust while SMC has wheat straw, and soybean and cottonseed meal which contribute to lignin content. Lignin content directly relates to recalcitrant types of carbon and longevity of the material; thus, this is a potential reason why the MC and SMC mixes were able to perform better at shorter HRTs and maintained greater removal efficiencies over the entire study duration.

To improve understanding of the composition of the different materials, aqueous effluent TOC concentrations were measured at the time of the autopsy. Carbon species concentrations and percentages of carbon species of the effluents are presented in Tables 14 and 15. Almost all of the TC in the effluent was present in the dissolved phase (95-98%) and therefore the dissolved phase will be discussed. Carbon contents were normally distributed as determined by Shapiro-

Wilk test for normality ($\alpha = 0.05$) and therefore the mean value was chosen for representation of carbon content.

Table 14: Total and Dissolved Carbon Content (mg L⁻¹) of the Treatment Effluents at the Time of Autopsy

	TC (mg L^{-1})			TIC (mg L^{-1})			TOC (mg L^{-1})		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
NAC	115.6	7.6	15	75.3	7.2	15	40.3	0.4	3
MC	105.4	0.9	10	94.7	2.0	10	10.7	4.0*	2
SMC	160.9	13.2	15	126.7	9.3	15	34.2	4.7	3

	TDC (mg L^{-1})			DIC (mg L^{-1})			DOC (mg L^{-1})		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
NAC	110.1	9.0	15	74.0	7.0	15	36.0	2.0	3
MC	103.8	1.8	10	93.7	3.0	10	10.0	1.2*	2
SMC	154.8	12.6	15	124.9	10.3	15	29.9	3.2	3

(SD) = Standard Deviation, the asterisk (*) notes that in the MC treatment, there were only 2 replicates, and in place of SD, the difference in OC between the two replicates is presented.

Table 15: Ratios of Carbon Content of the Effluents in the Different Treatment Groups at the Time of Autopsy

	Total Carbon (%)		Dissolved Carbon (%)		Dissolved Fraction (%)		
	TOC/TC	TIC/TC	DOC/DC	DIC/DC	DC/TC	DOC/TOC	DIC/TIC
NAC	34.9	65.1	32.7	67.3	95.2	89.4	98.4
MC	10.2	89.8	9.7	90.3	98.4	95.4	99.0
SMC	21.2	78.8	19.3	80.7	96.2	87.5	98.6

In the substrates, the SMC had the most remaining organic matter after the autopsy (51.7%), which can explain the substrate having the greatest TC in the effluent (161 mg L^{-1}). However, TOC in the effluent did not follow the same trend and NAC had the highest % TOC in the effluent and amount of total organic carbon (mg L^{-1}), followed by SMC and then MC. The opposite was observed for inorganic carbon, where MC had the greatest amount and percentage, followed by SMC and then NAC. All treatment media effluents had similar amounts of organic

carbon in the dissolved phase (>87.5%) and the majority of the carbon was DC (> 95%). The

majority of the TIC was dissolved (>98%) and is assumed to be alkalinity byproducts (e.g.,

bicarbonate).

Although MC had the greatest change in OM, it also had the least DOC (mg L^{-1}) and

percent in effluent. Low substrate OM and low effluent OC at the time of the autopsy, when SRB

populations were still high, suggests that microbes which are responsible for the breakdown of

the recalcitrant and labile carbon were still active and capable of degrading the OM to support

the SRB (Neculita et al., 2011; Vasquez et al., 2016). Even with increased flow rates and shorter

HRTs, SRB were able to act on limited available carbon sources to remove sulfate at rates

comparable to longer HRTs, where there is more time for sulfate reduction to occur.

The NAC and SMC treatments both had similar amounts of DOC in the effluents, but

NAC had significantly more DOC by percentage in comparison to SMC (Tukey HSD, $p < 0.05$).

There was significantly higher DOC (%) in the effluents from NAC to those of SMC and MC,

but the NAC treatment showed little sulfate removal in comparison (Tukey HSD, $p < 0.05$).

After autopsy and examination of the amounts of DOC in the effluent, it is suggested that

microbes responsible for the breakdown of recalcitrant and labile carbon were effectively

breaking down carbon for the SRB. Vasquez et al. (2016) also noted that DOC leached

constantly and generally decreased in amount over time in a similarly constructed column

experiment and this corresponds with a decreasing removal efficiency over time, as less DOC is

available for microbes at the end of the experiment compared to the beginning. This result also

leads to the suspicion that DOC may have initially been greatest in the NAC treatment, yet initial

OC and OM was similar in the initial substrates. However, DOC in the NAC treatment effluent

was comparatively elevated, suggesting that the microbes which utilize this carbon (SRB) were

less active in this treatment. This result corresponds with a smaller relative amount of DIC, which is assumed to be alkalinity, another product of sulfate reduction. Less sulfate removal seen in the NAC treatment.

SMC had significantly greater amounts of DC and DIC in the effluent compared to NAC and MC, and significantly more DOC than MC. The SMC effluent data suggest that the microbes responsible for degradation of organic carbon and the SRB that consume it were still working in unison to remove sulfate from solution. The large amounts of DIC (mg L^{-1}) in the effluent, in conjunction with the elevated sulfate removal in comparison to the other two treatments, suggest that SMC may have better capability to generate alkalinity than NAC and MC because of its elevated carbon content.

3.3.2. Nutrient and Metals Content

An initial elemental analysis was completed on the treatment media in order to examine differences in sulfur content, as well as differences in potential macro- and micronutrients. The sulfur content of the media was determined before and after the experiment to help understand the fate of sulfur in the columns. A summary of the sulfur content in the starting treatment media, and of the autopsied material is presented in Table 16.

Table 16: Change in Sulfur Content of the Different Treatments

	Sulfur Content (g kg^{-1}) in the Media						
	Initial Material			Autopsied Material			Mean
	Mean	SD	N	Mean	SD	N	Percent Difference
NAC	1.98	0.26	3	2.71	0.39	27	36.7 %
MC	3.04	0.21	3	2.23	1.16	18	-26.7 %
SMC	13.24	2.31	3	6.26	0.35	27	-52.8%

Sulfur content in NAC and MC were about 0.2 and 0.3%, respectively. SMC had roughly 1.3% sulfur, greatly elevated compared to its counterparts, but SMC is amended with gypsum

(CaSO$_4$), contributing to elevated sulfur and calcium. SMC can have about 1-2% or 10-20 g kg^{-1} S as determined by Song et al. (2012) and the SMC used in this study falls within this range. In all cases, there was a significant difference in resulting sulfur content in the autopsied material from the initial material, however the treatments responded differently (Tukey HSD, p < 0.05). In the NAC treatment, there was a 36.7% increase in sulfur concentration in the media over the course of the study, and a similar trend was seen with magnesium, the other element in the salt (MgSO$_4$) used in the influent solution to the columns, which increased about half as much (16.2%). The changes in molar concentration of sulfur and magnesium were not 1:1 as expected, and about 1.5x more magnesium than sulfur was gained in the substrate. It is likely because the sulfur being loaded into the system is soluble sulfate, and other removal pathways for sulfate exist, sulfate reduction is still ongoing in the treatment vessels evident by the measurable effluent sulfide concentrations. Magnesium can be a macronutrient to microbes and is consumed in small quantities, but is relatively inert, and thus the primary removal pathway in these systems is likely to be solely sorption (Armstead et al., 2016). Although sorption was the primary removal mechanism for magnesium, targeting the removal of magnesium or other base cations like calcium which contribute to conductivity in mine waters is not often sought. This is due to the inhibitory effect caused by these relatively harmless cations which compete with remaining toxic trace metals like zinc and aluminum in aquatic ecosystems (Armstead et al., 2016). The trend in increased sulfur and magnesium measured in the NAC column substrate, in addition to leached sulfate measured in the effluent, supports that the influent solution was responsible for the increase in sulfur and magnesium in the NAC treatment.

In the other two treatments there was substantial decrease in sulfur and in magnesium in the media. In the MC treatment, there was a 26.7% decrease in sulfur, and a 16% decrease in

magnesium. In the SMC treatment there was a 52.8% decrease in sulfur, and 26% decrease in magnesium. There was no evidence of leaching sulfate at any point during the studies, which would suggest that the decreases in sulfur or magnesium from the media in the NAC and SMC treatments were likely due to the dissolution of these elements from source materials over time. Gypsum, sugar beet lime, manure, and stable cleanings, are potential sources of sulfur, alkalinity, and hardness as well as other nutrients found in MC and SMC. The leaching of sulfur (likely dominated by SO_4^{2-}) over time would contribute to masking overall removal efficiency by adding to effluent sulfate concentrations, resulting in lower removal rates and percent removal of sulfate. As also previously mentioned, this result could have contributed to elevated sulfide concentrations seen in these treatments and the stabilizing effect observed in effluent sulfate concentrations because there was more sulfate in the MC and SMC treatments than in the NAC treatment. Thus, there was more sulfate available for sulfate reduction to sulfide. Another possibility is that a large portion of these elements remain in solution, but located in the porewater, which was not sampled, and thus a potential pathway of release for these elements was not determined.

In general, the SMC used in this study had greater amounts of macronutrients, and micronutrients compared to the other two treatments. A summary of the macronutrients in the composts is presented in Table 17. Calcium in composts is known to have high variability due to the varying mixes and types of ingredients used and the elevated calcium in SMC is likely from gypsum included in the source material (Jordan et al., 2006). The amount of calcium in these composts was similar to the amounts seen in other spent mushroom composts, but less than the 100 g kg^{-1} Ca found in the SMC used by LaBar and Nairn (2016; 2017) from the same provider.

Table 17: Initial Macronutrient Concentrations in the Media (g kg⁻¹)

Macronutrients (g kg⁻¹) in the Media					
NAC (n= 3)		MC (n=3)		SMC (n=3)	
Mean	SD	Mean	SD	Mean	SD
Calcium	45.2	5.24	18.2	2.04	53.3	6.77
Iron	4.9	0.31	5.82	1.09	1.08	0.15
Potassium	7.31	0.49	10.3	0.63	37.4	0.57
Sodium	0.39	0.04	1.43	0.11	6.25	0.09

Iron concentrations in composts are typically low, especially in those used for mushroom growth because elevated iron can be inhibitory (Jordan et al., 2006). The composts used in this study had more iron in reference to those tested by Jordan et al. (2006), however another study by Maher et al. (2000) found 2.15 g kg⁻¹ iron in spent mushroom compost, which is much more similar to the material used in this study. Iron in NAC and MC, which are yard composts, is higher because iron is vital to plant health and development. Iron is used in plants for the production of chlorophyll, it aids in plant respiration, and in the transport of other nutrients (Miller et al., 1995).

The SMC used in this study had greater amounts of potassium and sodium than both the other media being tested. For sodium, SMC contained 6.25 ± 0.09 g kg⁻¹ compared to 1.43 ± 0.11 g kg⁻¹ in MC and 0.39 ± 0.04 g kg⁻¹ in NAC. The SMC in this study had slightly greater amounts of sodium on average than what was found in the SMC used by LaBar and Nairn (2016; 2017) from the same provider. For potassium, SMC contained 37.4 ± 0.57 g kg⁻¹ compared to 10.3 ± 0.63 g kg⁻¹ in MC and 7.31 ± 0.49 g kg⁻¹ in NAC. Jordan et al. (2006) also noted that the amount of potassium they found in their SMC mixes were substantially greater than their references. The additional sodium and potassium could be detrimental in mine drainage remediation as an additional source of conductivity due to dissolution (Runtti et al., 2018).

For the micronutrients, SMC had relatively higher levels of copper, zinc, manganese and nickel as summarized in Table 18, other metals analyzed on the ICP-OES not discussed are presented in Appendix C. In relation to the SMC tested by LaBar and Nairn (2016; 2017), the SMC used in this study had elevated amounts of zinc, nickel and manganese, where the NAC and MC had more comparably similar amounts of manganese and nickel but less zinc.

Table 18: Initial Micronutrient Concentrations in the Media (mg kg^{-1})

	Copper		Nickel		Manganese		Zinc	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NAC	33.1	2.49	6.18	0.72	361	21.8	113.4	14.7
MC	34.4	4.67	7.62	0.81	259	13.5	134.3	40.0
SMC	328	34.4	12.4	1.06	641	82.3	349.9	43.8

Examining potentially harmful trace metals, the composts tested in this study had elevated lead concentrations compared to the SMC used by LaBar and Nairn (2016). The lead concentrations of the treatment media were 26.0 ± 3.33 mg kg^{-1} for NAC, 27.1 ± 13.26 mg kg^{-1} for MC, and 21.7 ± 0.24 mg kg^{-1} for SMC, whereas the concentration determined by LaBar and Nairn (2016; 2017) was 6.85 ± 0.4 mg kg^{-1}.

In summary, the SMC had elevated amounts of nutrients as well as potential harmful trace metals comparatively. These nutrients could help boost microbial growth and production, but the elevated trace metals could serve to detrimentally affect receiving water bodies in field applications. The latter is less likely because in PTS VFBRs, the available trace metals would precipitate as metal sulfides and remain sequestered as they are designed to (Armstead et al., 2016; Dvorak et al., 1992; Huang et al., 2016; Lorax Environmental, 2003; Nairn et al., 2010; Neculita et al., 2007; Rakotonimaro et al., 2018; Song et al., 2012; Smyntek et al., 2017; Xu and Chen, 2020). There was also a significantly greater decrease in sulfur content in the SMC media compared to the other two treatments (Tukey HSD, $p < 0.01$) likely from dissolution of gypsum.

Lastly, SMC had the greatest concentrations of nutrients in the starting material excluding iron, which is known to stunt mushroom growth (Jordan et al., 2006).

4. Conclusions

A comparison was made of different organic media types under different HRTs in model VFBR columns for the purpose of removing sulfate. Three different media (Norman Aged Compost, Murphy Compost, and Spent Mushroom Compost) were tested in triplicate under continuous flow-through conditions for over >300 days to evaluate their sulfate removal capabilities. Over an additional 60 days, column HRTs were manipulated twice to investigate the effect of shorter HRTs on sulfate removal. Conditions were confirmed optimal for sulfate reduction at the beginning, during, and at the conclusion of the study: lack of oxygen, negative and reducing ORP, circumneutral pH, and moderate temperatures.

Effluent sulfate concentrations were measured from the treatments over the course of the study to evaluate sulfate removal rates and percent removal over time. Effluent sulfate concentrations increased over time ($p < 0.05$ in all treatments) and were found to be greater than the HRL for sulfate (500 mg L^{-1}) by Day 251 in all treatments. Ample SRB populations were present (>2,000,000 CFU mL^{-1}) during column construction, at the beginning of study, and at the end of the study confirming that sulfate removal had happened biologically. Although it was determined that the SMC treatment removed significantly more sulfate during the first 305 days than the other two treatments (Tukey HSD, $p < 0.01$), it barely eclipsed the hypothesized timeline of removing more than 500 mg L^{-1} over a period greater than 6 months. The SMC treatment had the lowest effluent sulfate concentrations, which were on average below the HRL for sulfate, the highest effluent sulfide concentrations, and greatest percent removal and sulfate removal rate in all three treatments at an eight-day HRT.

The HRL is important to this study, not necessarily as a health concern for sulfate, but more importantly to show approximately 50% removal efficiency. These effluent waters would not be ingested by humans, and in properly implemented PTS, VFBRs are followed up by other treatment units which increase DO (Hedin, 2008; Machemer et al., 1993; Nairn et al., 2010; Nairn et al., 2020). The water produced by these treatments had elevated sulfide of > 100 ppm in the effluent waste containers, which can lead to damage in the sense of smell and in worst case scenarios, death (Orem et al., 2011).

Sulfate removal rates were analyzed over time, and also at varying HRTs. During the first 305 days of the study, sulfate removal decreased significantly ($p < 0.05$) over time in all treatments. The most significant decrease was found in the NAC treatment which was showing signs of failure at the end of the eight-day HRT and leached sulfate during the flow manipulation portion of the study. Percent removal of sulfate also significantly declined over time ($p < 0.05$). All treatments showed losses in initial OM content by the end of the study, and this, coupled with significantly decreased sulfate removal rates, supports the hypothesis that these sulfate removal rates decrease with time.

The MC and SMC treatments did have significantly more initial OM in the substrate compared to NAC (Tukey HSD, $p < 0.05$), however SMC and MC lost OM at significantly different rates (Tukey HSD, $p < 0.01$). The MC treatment lost almost 20% OM over the course of the study, while SMC lost only 5% OM. Although SMC only had marginally more initial OM than MC, it did have a significantly less change in OM over time, and did have significantly greater mean sulfate removal rates, and median % removal (Tukey HSD, $p < 0.05$) during the course of the study. Since the OM contents of all the treatment media were within 5%, and the resulting removal rates were greater in SMC columns than in MC or NAC columns, it would be

more beneficial in future studies to examine the classification of types of OM present. Given this information, it cannot be supported that initial OM was solely responsible for the greatest sulfate removal rates.

Sulfate removal rates were also investigated over three HRTs with the respective treatments to determine if longer HRTs produced better removal rates. With less contact time for sulfate reduction to occur, less sulfate was reduced, and less sulfide was produced. Percent removal of sulfate in the SMC treatment was significantly greater compared to NAC and MC at an eight-day HRT ($p < 0.01$). However, percent removal in the NAC treatment was significantly less ($p < 0.01$) than both MC and SMC during both shorter HRTs of the flow manipulation. The sulfate removal performance measured by removal rate (mmol m^{-3} day^{-1}) or by percent removal in the SMC and MC treatments was not significantly different at HRTs equal to four or two days. Sulfate removal was significantly less ($p < 0.01$) in NAC treatments with shorter HRTs, supporting the hypothesis that removal would decline with decreasing HRTs. However, in MC and SMC treatments, sulfate removal rates were not significantly different from one another with respect to HRT, and therefore would not support the hypothesis that sulfate removal declines with decreasing HRT.

With stringent requirements on effluent discharge criteria for sulfate becoming increasingly common across the globe it is important to identify potential cost-effective and energy efficient ways to remove sulfate (Fernando et al., 2018; Smyntek et al., 2017, Runtti et al., 2018). Passive treatment has been identified for removing trace metals using sulfate reduction, however removing sulfate is typically not the driving factor when remediating mine drainage. Mine drainage remediation technologies like VFBRs provide an excellent option for efficient sulfate removal given that optimal conditions are created. Efficient sulfate removal is

dependent on circumneutral pH, anaerobic conditions with reducing ORP, moderate temperatures, a variety of carbon sources for SRB and other microbes, and ample time for these reactions to occur. These studies suggest that VFBRs can be utilized in passive treatment to remove sulfate. Specifically, spent mushroom compost has been, and still is, an acceptable media for VFBR construction for metals removal, but also for sulfate removal. When designing VFBRs and selecting the media, it is most important to know how to create those optimal conditions and to have an appropriate mix of different carbon types as to prolong the life of the system.

5. Limitations and Future Work Ideas

A limitation of this study was that due to hydraulic problems in initial column operation, any treatment which happened prior to the "initialization phase" was not analyzed. This lack of data effectively neglected any baseline removal or leaching of sulfate that occurred during that time, which would have been valuable information to show changes in removal rates over the entire lifespan of the columns. As well as valuable nutrients, bacteria and dissolved OM may have been flushed during those siphoning events.

Another limitation is that the specific types of carbon present in the media were not known. Instead of simply examining the change in overall OM, knowing the amount of labile and recalcitrant carbon in the treatment media, as well as knowing the amount of organic and inorganic carbon, would allow for more valid assumptions regarding the behavior of the bacteria in response to the carbon types (Vasquez et al., 2016). Currently only bulk OM was investigated in the media over time, but the types of OM are important in overall success of the treatment.

For future work, three main ideas are presented. First, a thorough comparison of the VFBR substrate mixes currently deployed at the Mayer Ranch PTS and Southeast Commerce PTS operated by CREW specifically with regard to sulfate removal capability should be

performed. These mixes both contain SMC from JM Farms, however the composition of substrates is different: Mayer Ranch PTS has 45% SMC, 45% wood chips, and 10% limestone sand and the Southeast Commerce PTS has 80% SMC, and 20% wood chips. These mixes could be tested directly against one another as is, or also amended with washed river rock to increase porosity like what was seen in this experiment. Second, a similar study could be conducted where porewater samples are also collected and a better gas collection device used to collect more accurate gaseous sulfide measurements. The gaseous sulfide measurements taken in this experiment were taken to ensure safety of the experimenter, however volatilization of sulfide in these conditions happens quickly and gaseous sulfide measurements are necessary for a complete mass balance. Lastly, it would be beneficial in redesigning this experiment to include an addition of a short-chain carbon source to boost sulfate removal rates. Lactate and ethanol are common low-molecular weight compounds which can serve as the carbon source themselves or be added to the media in order to boost sulfate reduction rates (e.g., Xu and Chen, 2020). This addition could be measured against the media without addition or could be done after a set amount of time to see the resulting effect of addition of the amendment to sulfate removal rate.

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7. Appendix A: Sulfate, Sulfide, and Sulfate Removal Performance
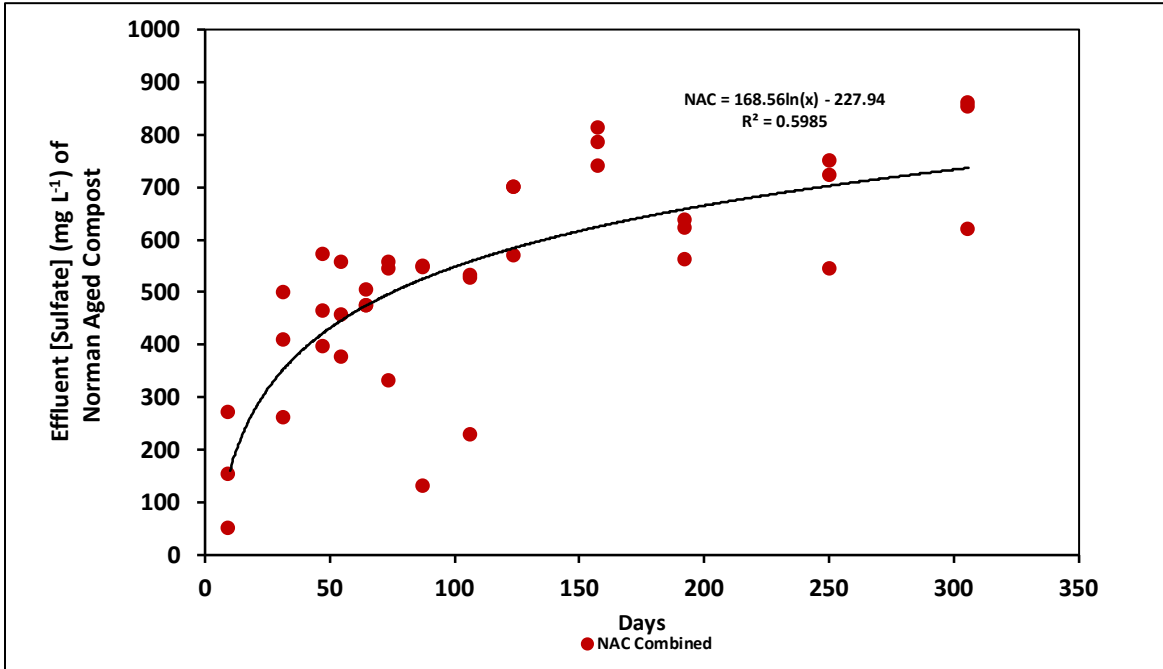
Figure A 1: Effluent Sulfate Concentration in NAC Treatment at 8-day HRT
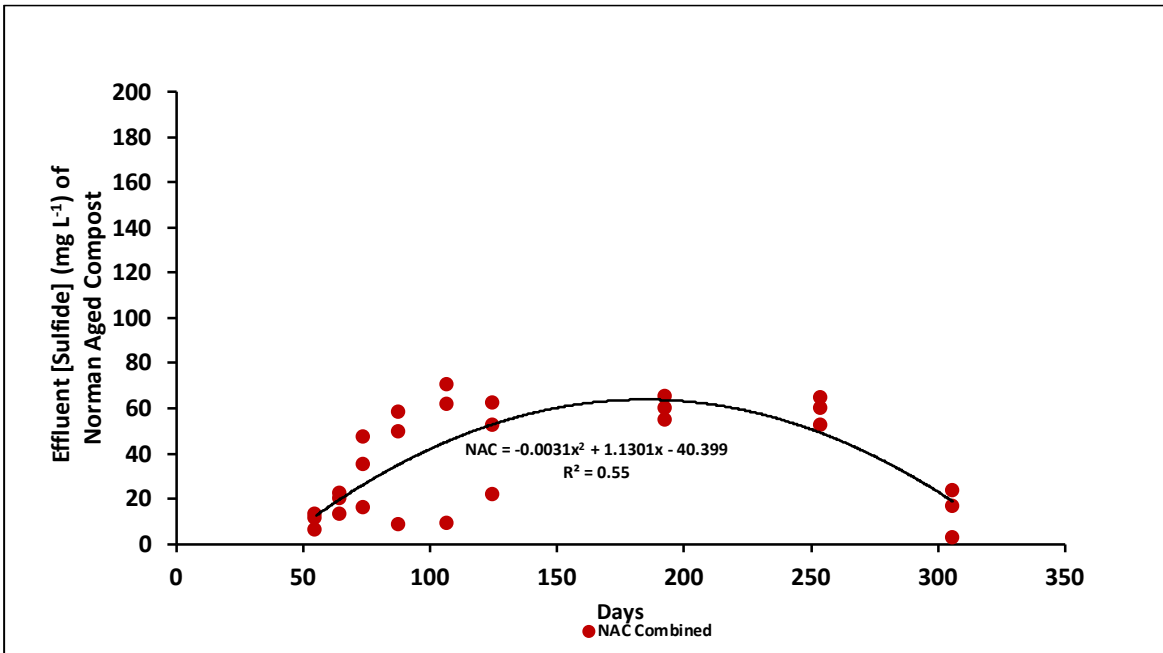
Figure A 2: Effluent Sulfide Concentration from NAC Treatment at 8-day HRT

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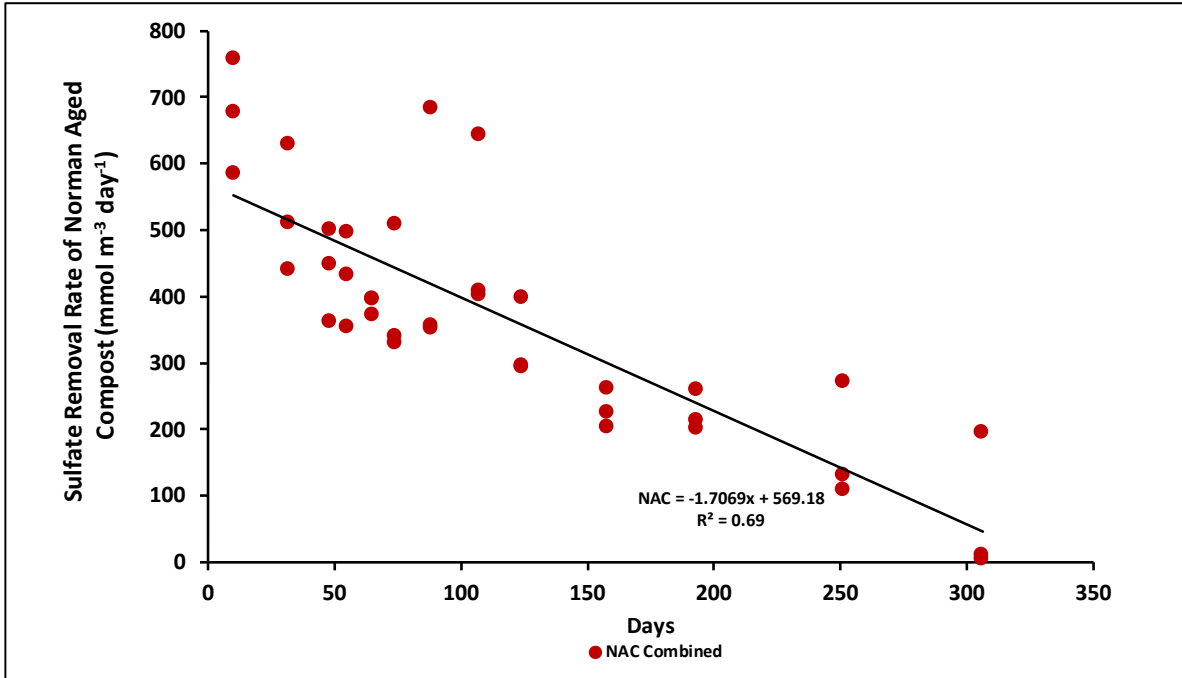
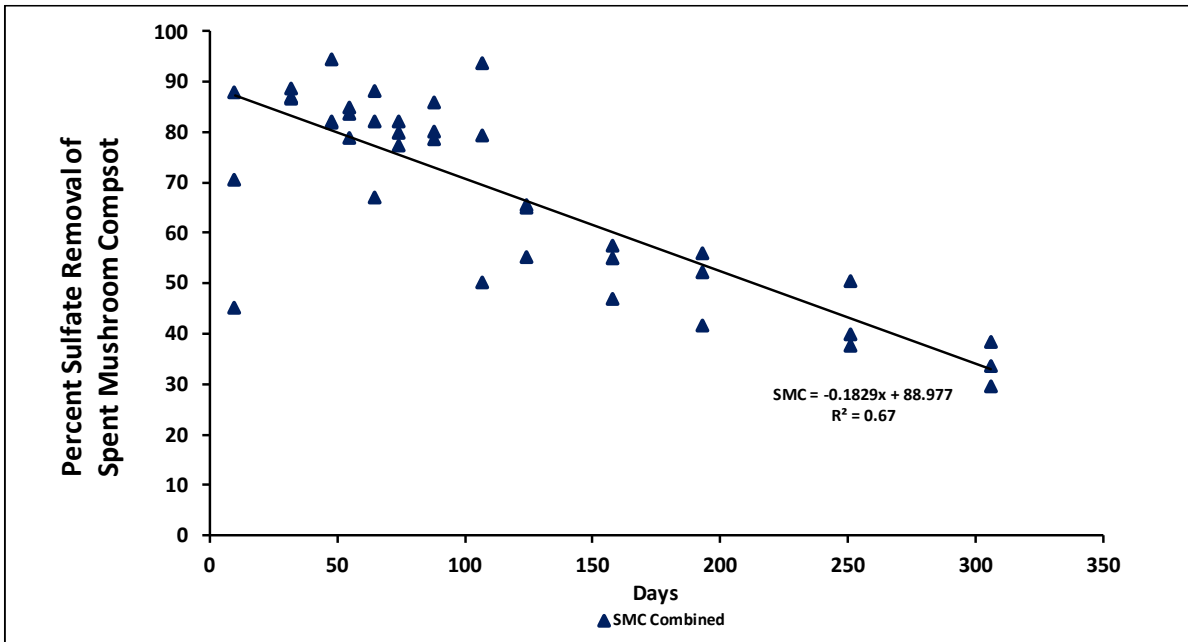*Figure A 3: Sulfate Removal (mmol m⁻³ day⁻¹) in NAC Treatment at 8-day HRT*
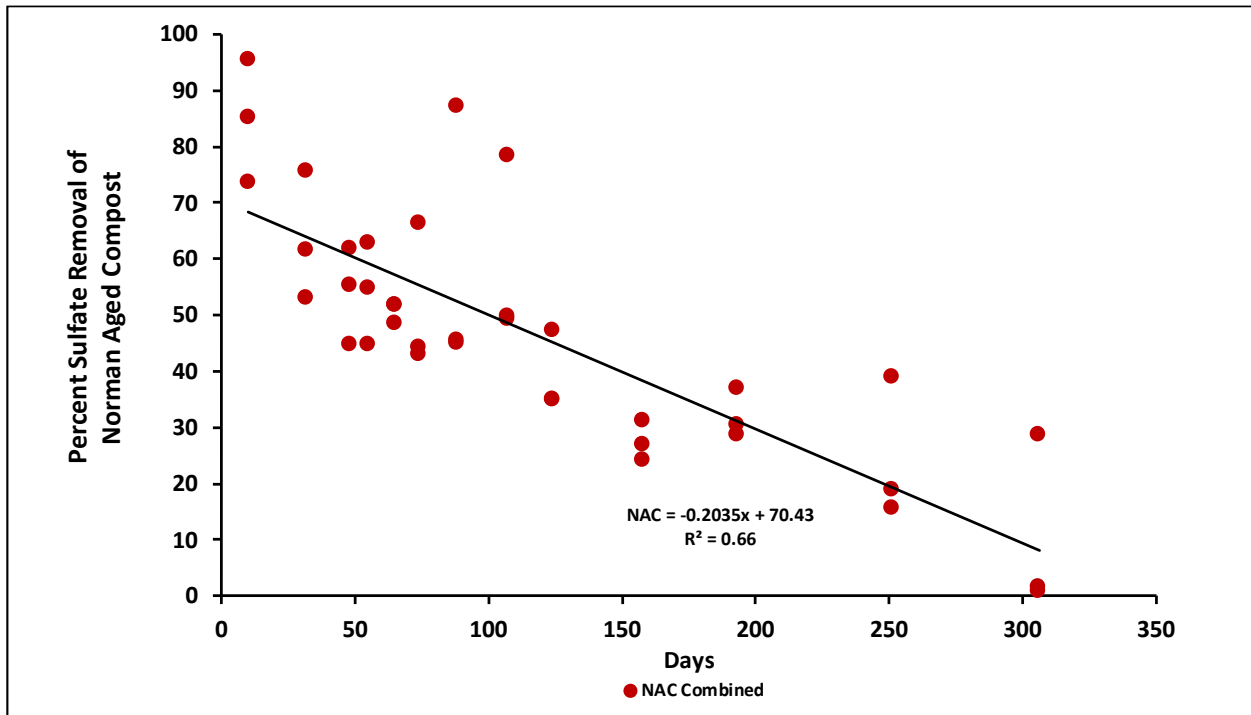
Figure A 4: Figure C 4: Sulfate Removal (%) in NAC Treatment at 8-day HRT
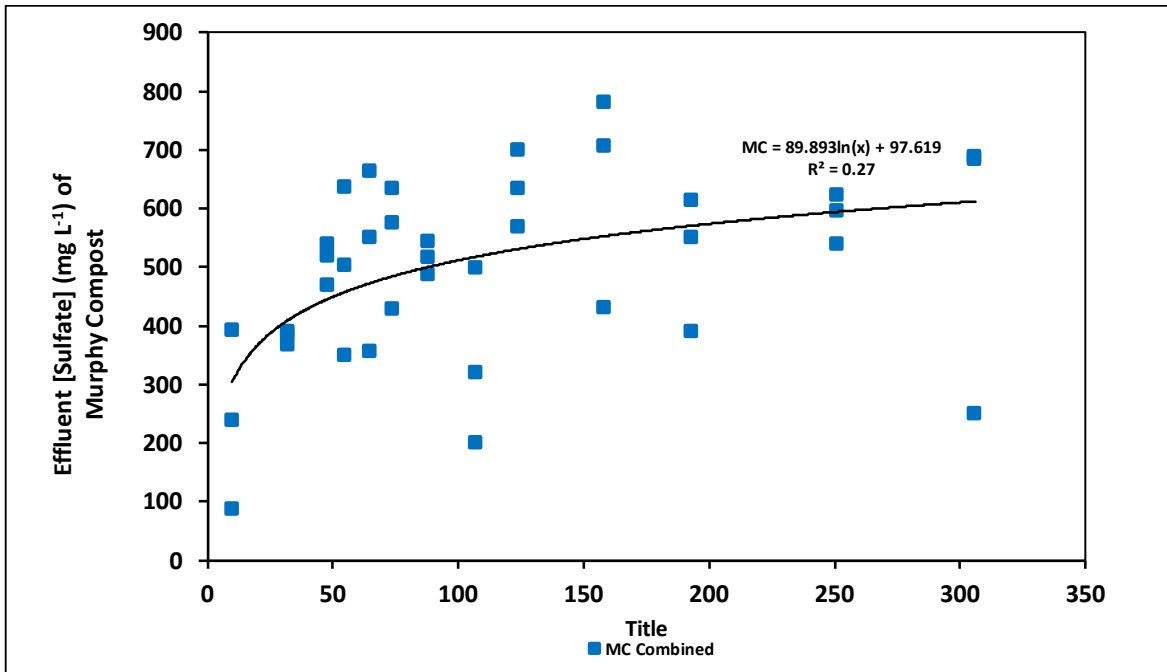
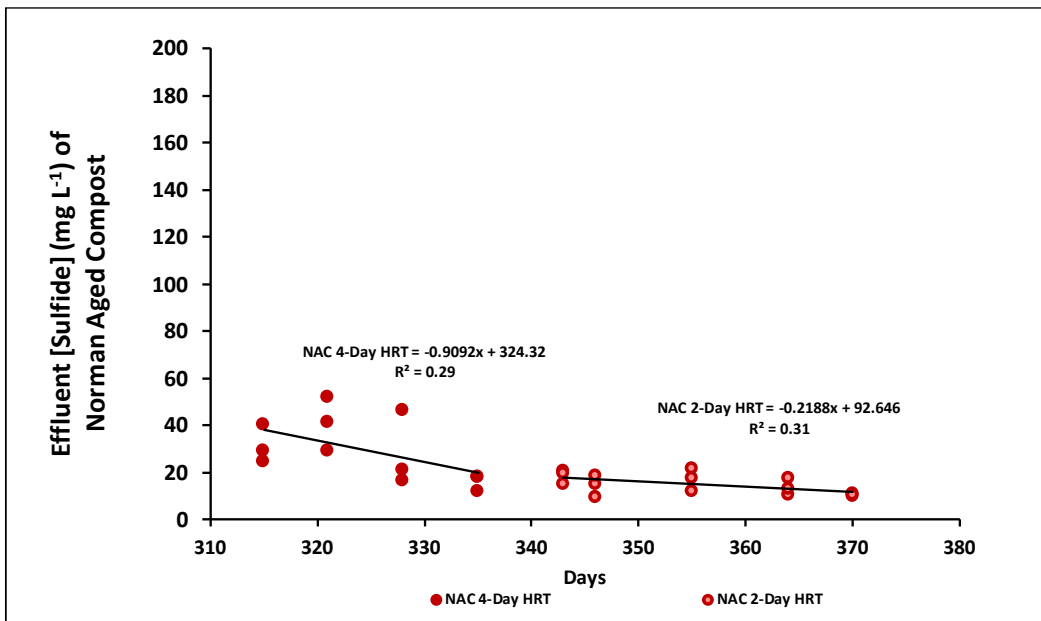*Figure A 5: Effluent Sulfate Concentration from MC Treatment at 8-day HRT*
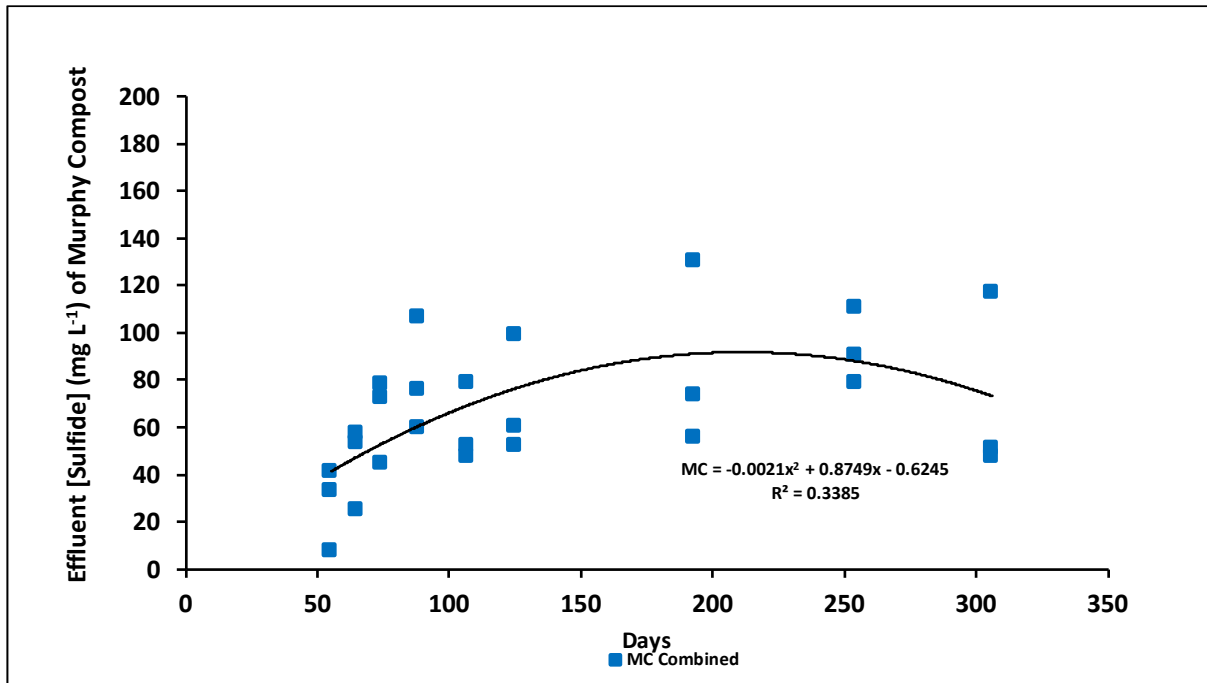
Figure A 6: Effluent Sulfide Concentration from MC Treatment at 8-day HRT
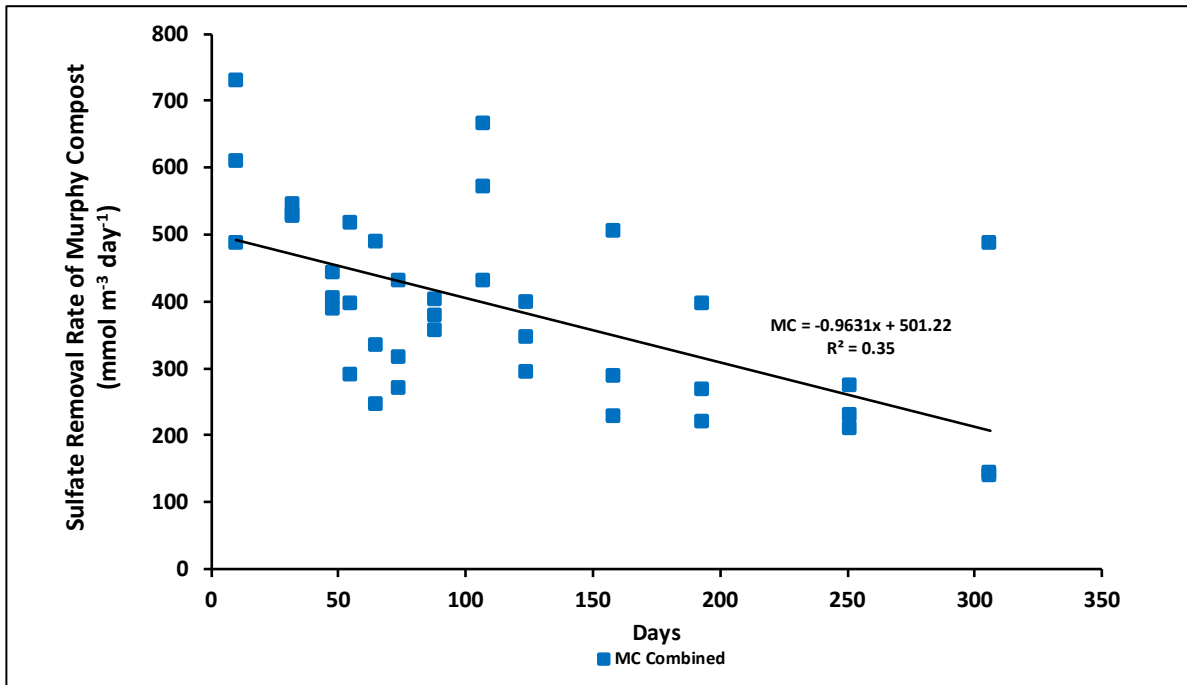
Figure A 7: Sulfate Removal (mmol m⁻³ day⁻¹) in MC Treatment at 8-day HRT
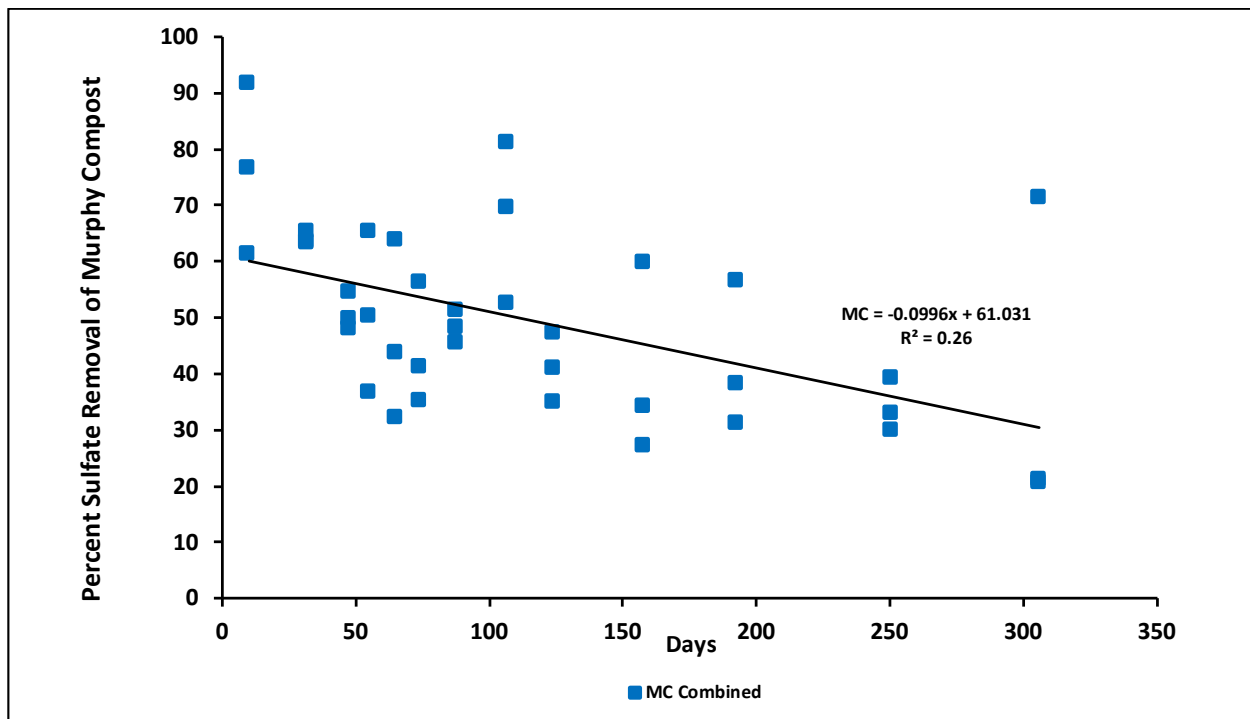
Figure A 8: Sulfate Removal (%) in MC Treatment at 8-day HRT
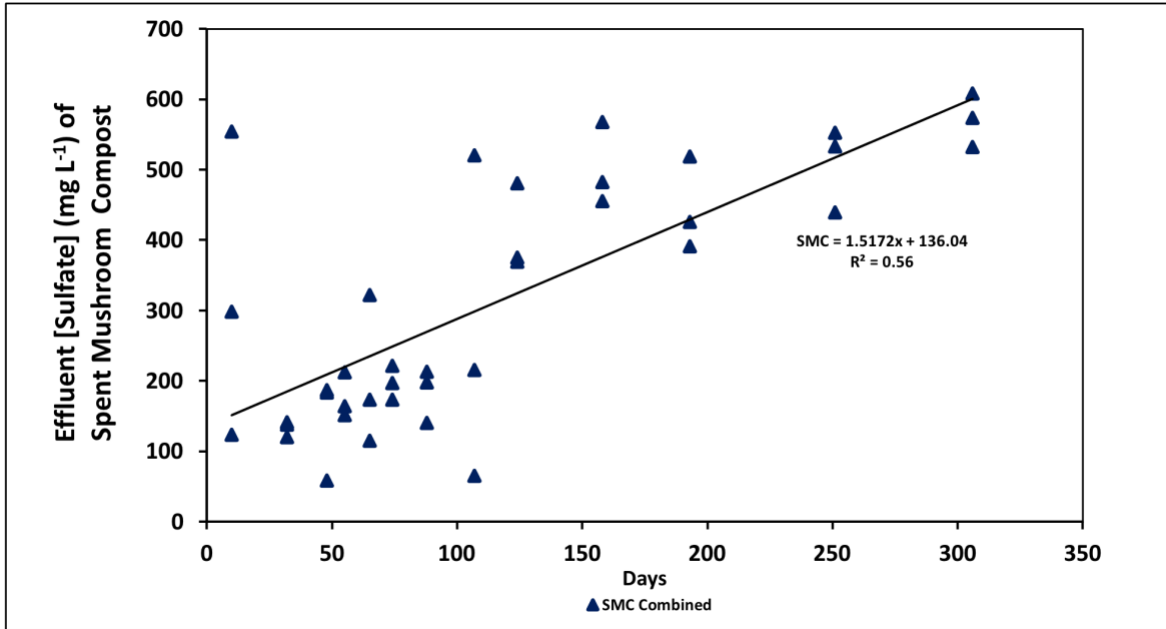
Figure A 9: Effluent Sulfate Concentrations from SMC Treatment at 8-day HRT
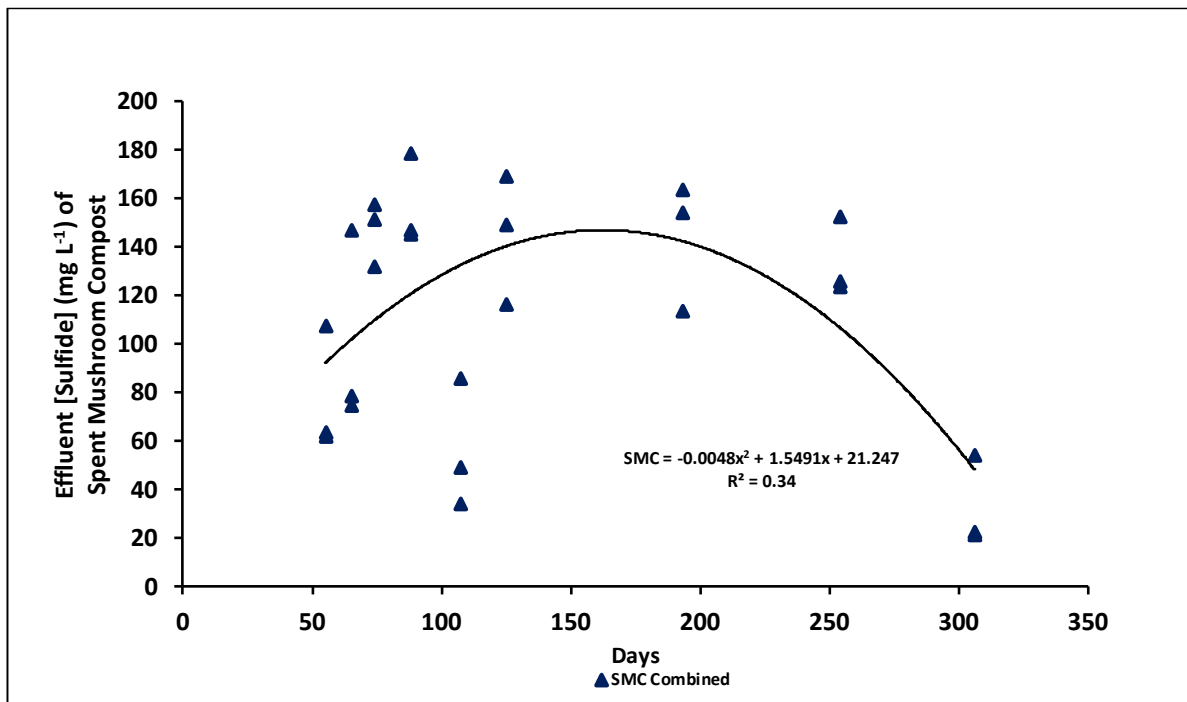
Figure A 10: Effluent Sulfide Concentrations from SMC Treatment at 8-day HRT

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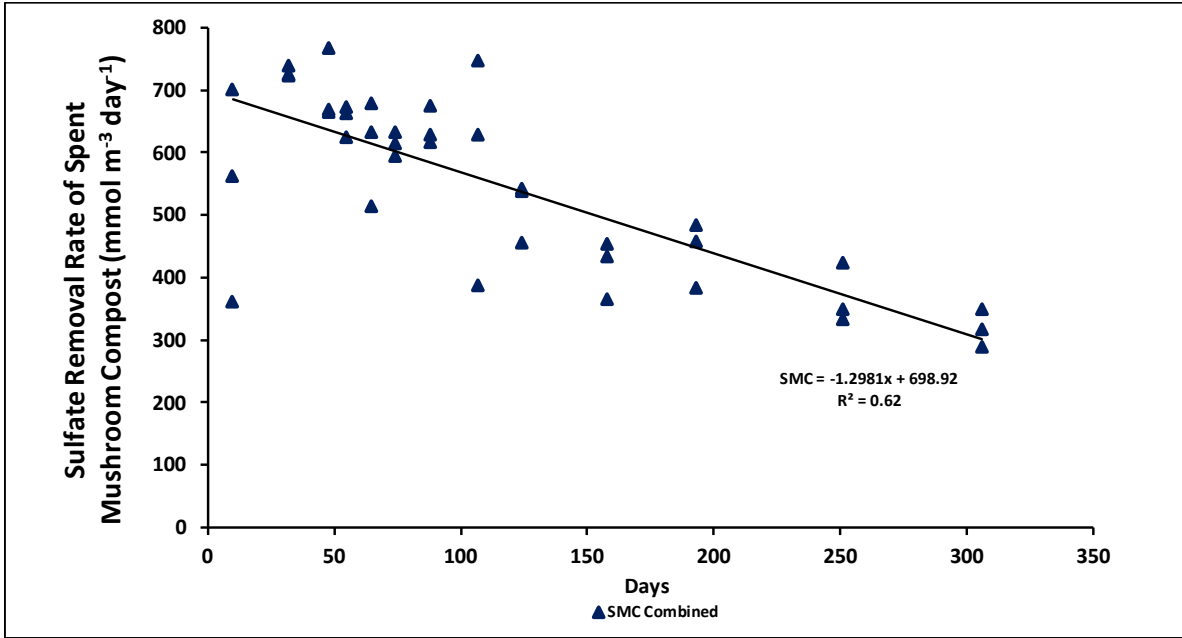
Figure A 11: Sulfate Removal (mmol m⁻³ day⁻¹) in SMC Treatment at 8-day HRT

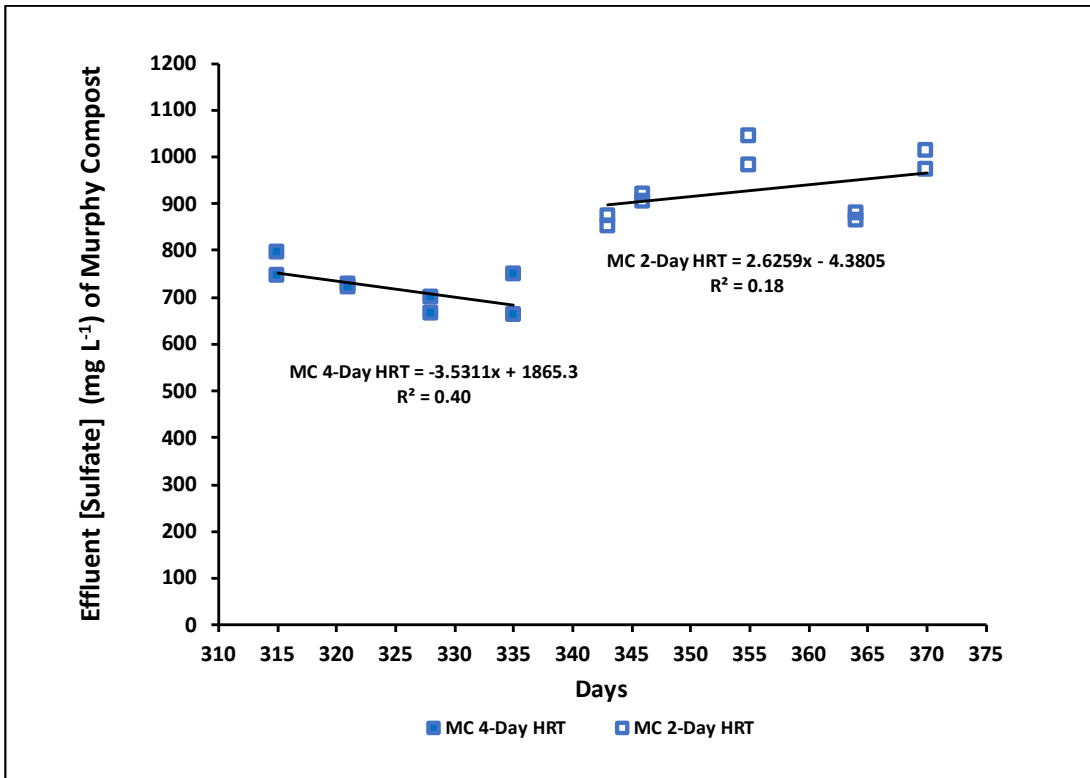*Figure A 12: Sulfate Removal (%) in SMC Treatment at 8-day HRT*
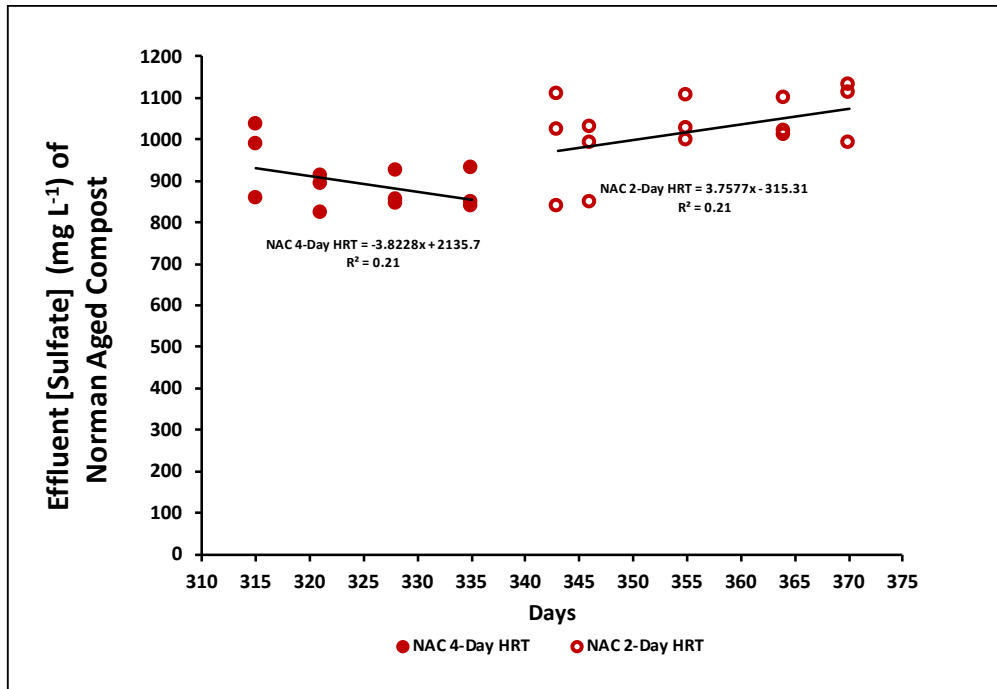
Figure A 13: Effluent Sulfate Concentration from NAC Treatment during Flow Manipulation

Figure A 14: Effluent Sulfide Concentration of NAC Treatment during Flow Manipulation
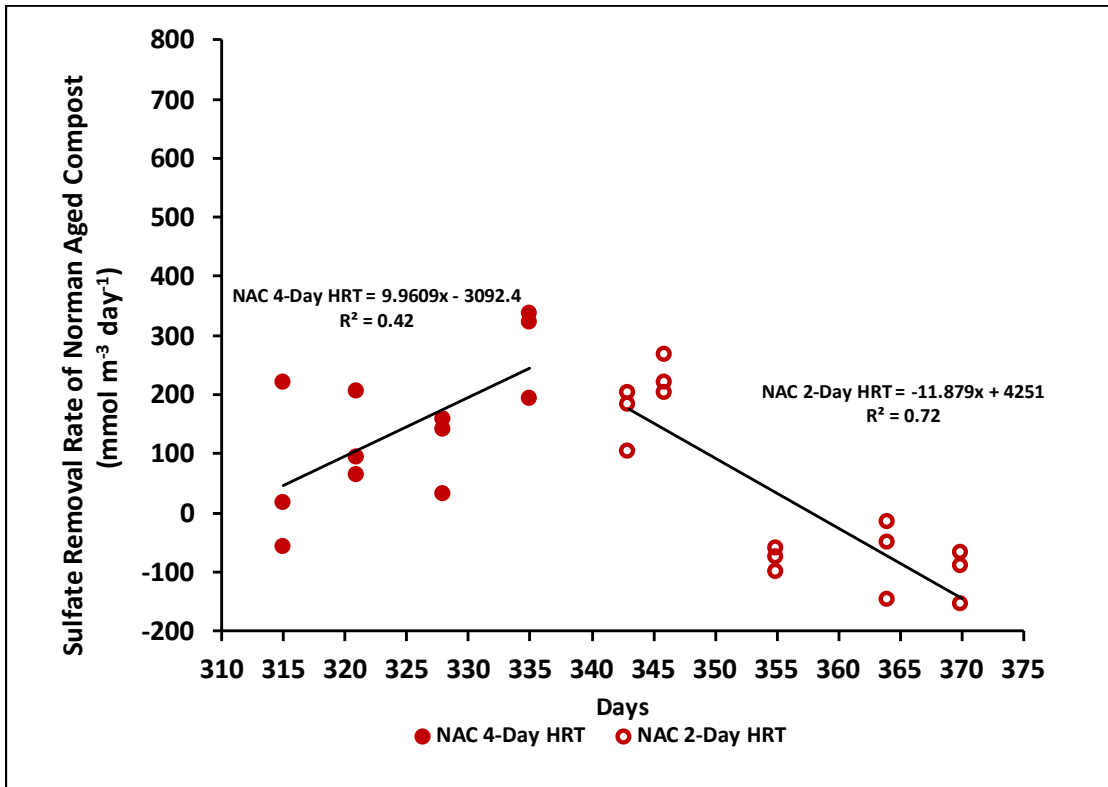
Figure A 15: NAC Sulfate Removal Rate (mmol m-3 day-1) during Flow Manipulation
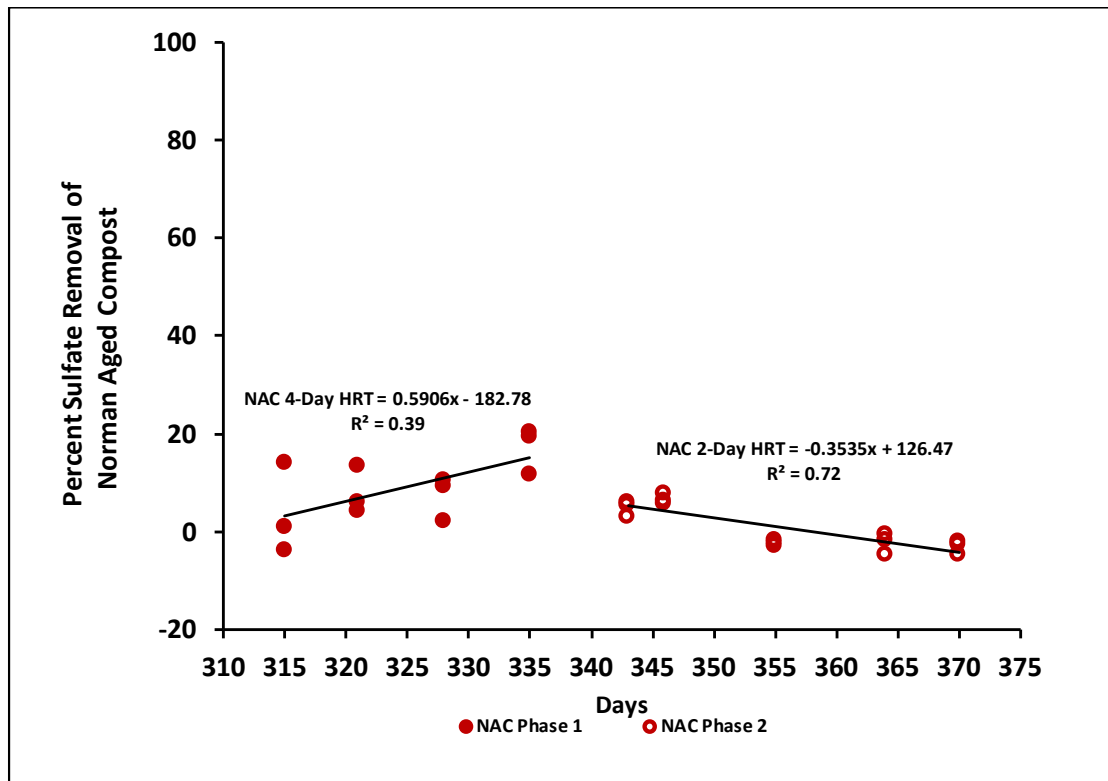
Figure A 16: NAC Sulfate Removal (%) during Flow Manipulation

Figure A 17: Effluent Sulfate Concentration from MC Treatment during Flow Manipulation
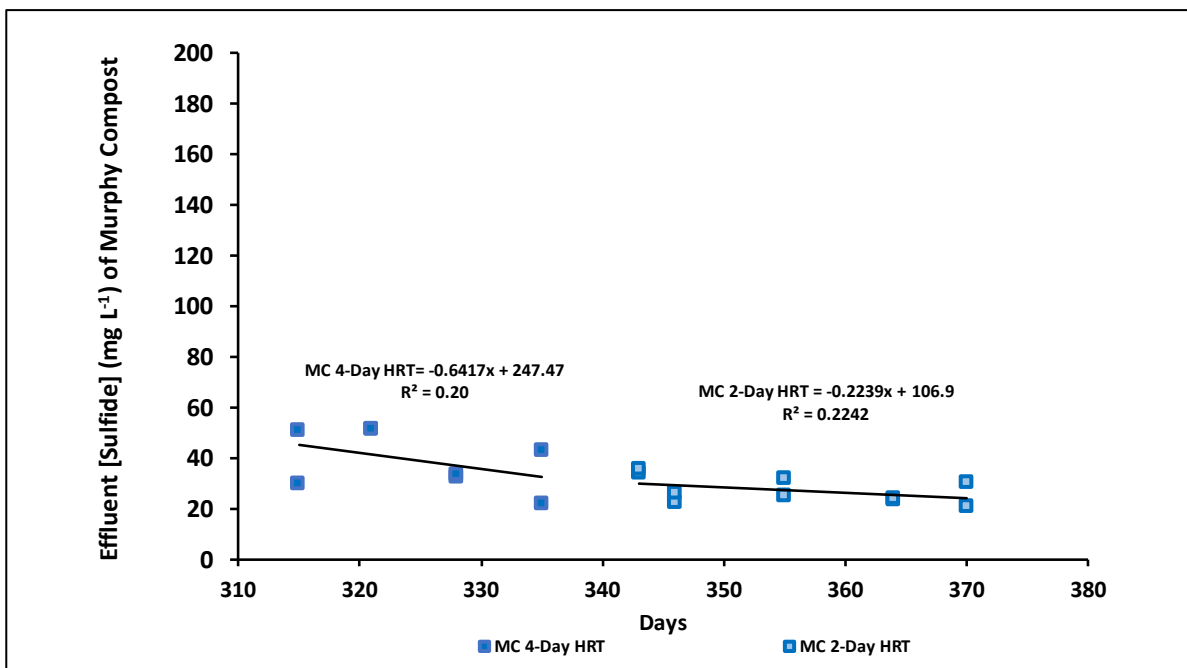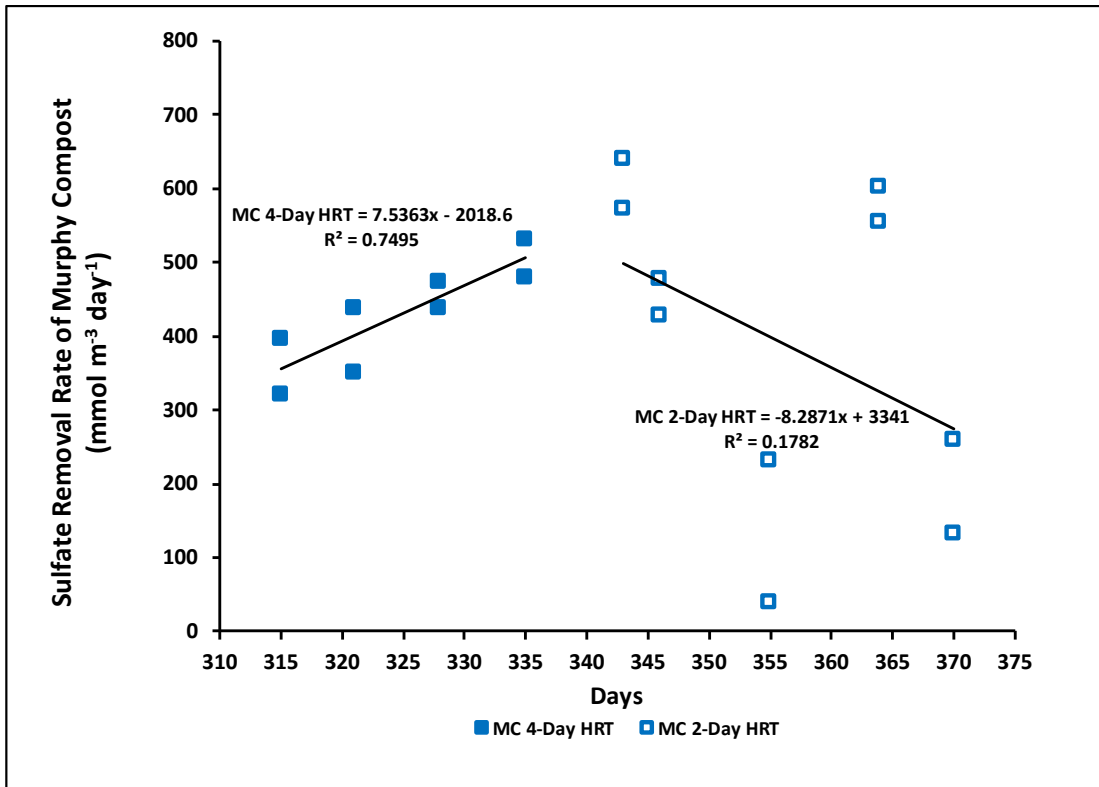

Figure A 18: Effluent Sulfide Concentration of MC Treatment during Flow Manipulation

Figure A 19: MC Sulfate Removal Rate (mmol m-3 day-1) during Flow Manipulation
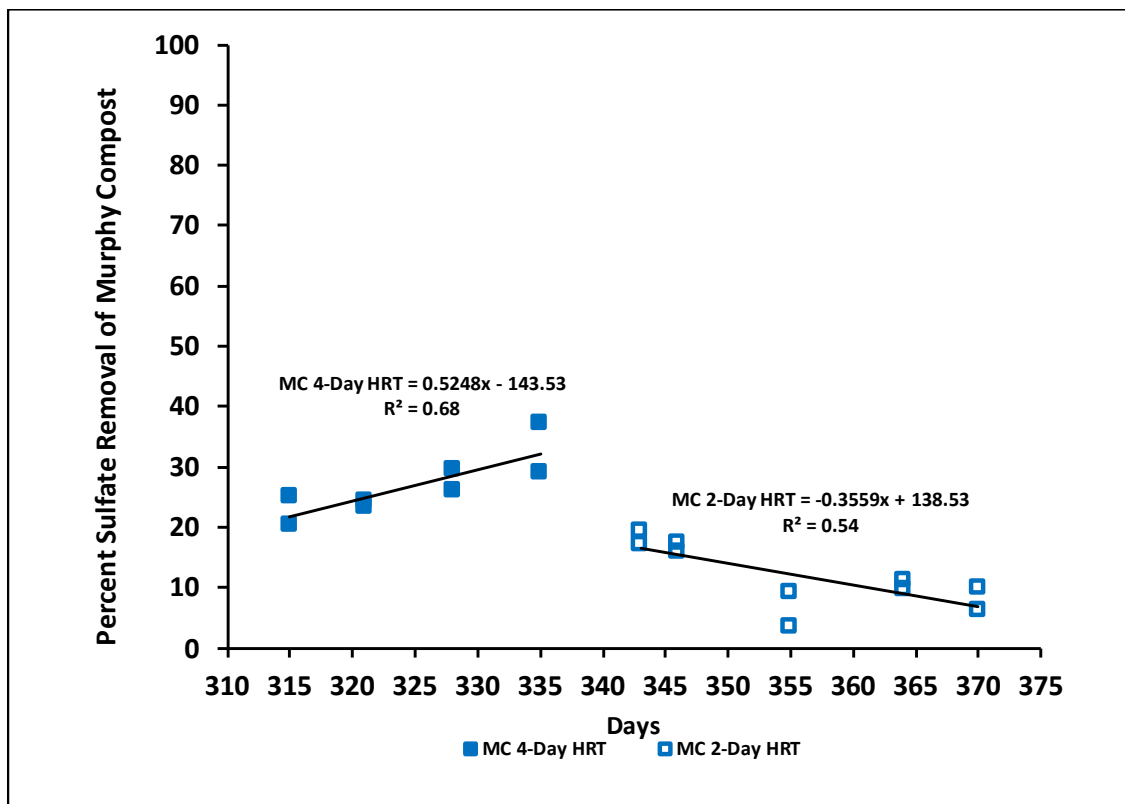

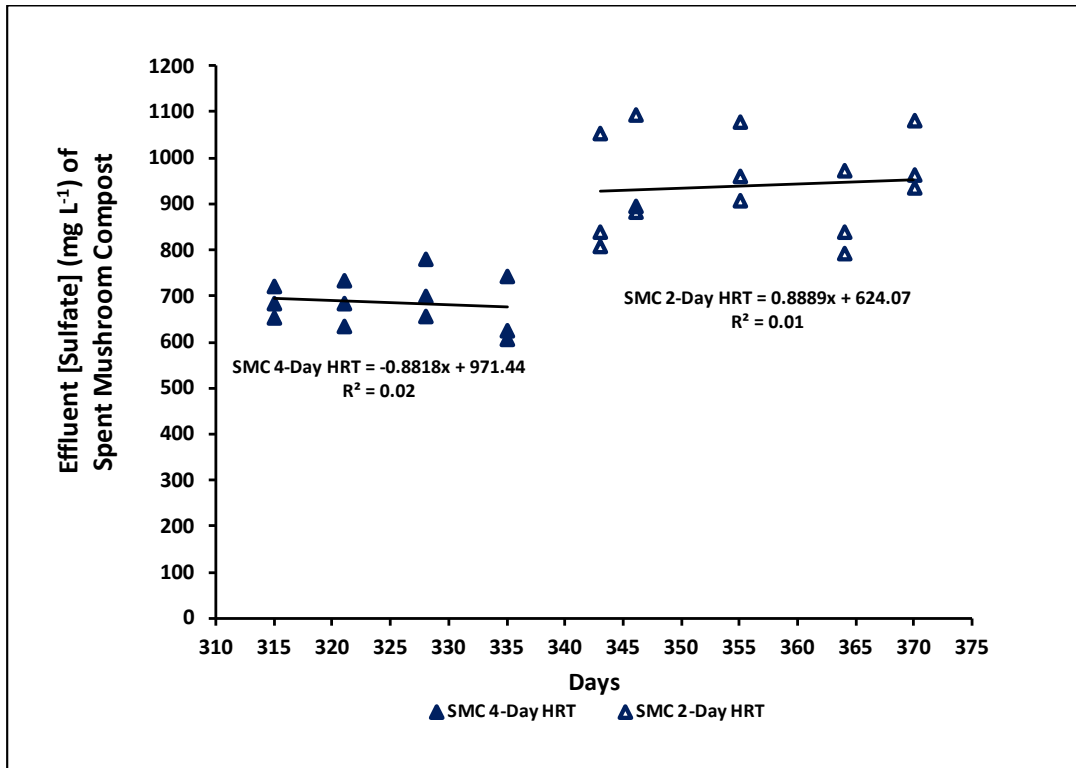*Figure A 20: MC Sulfate Removal (%) during Flow Manipulation*

Figure A 21: Effluent Sulfate Concentration from SMC Treatment during Flow Manipulation
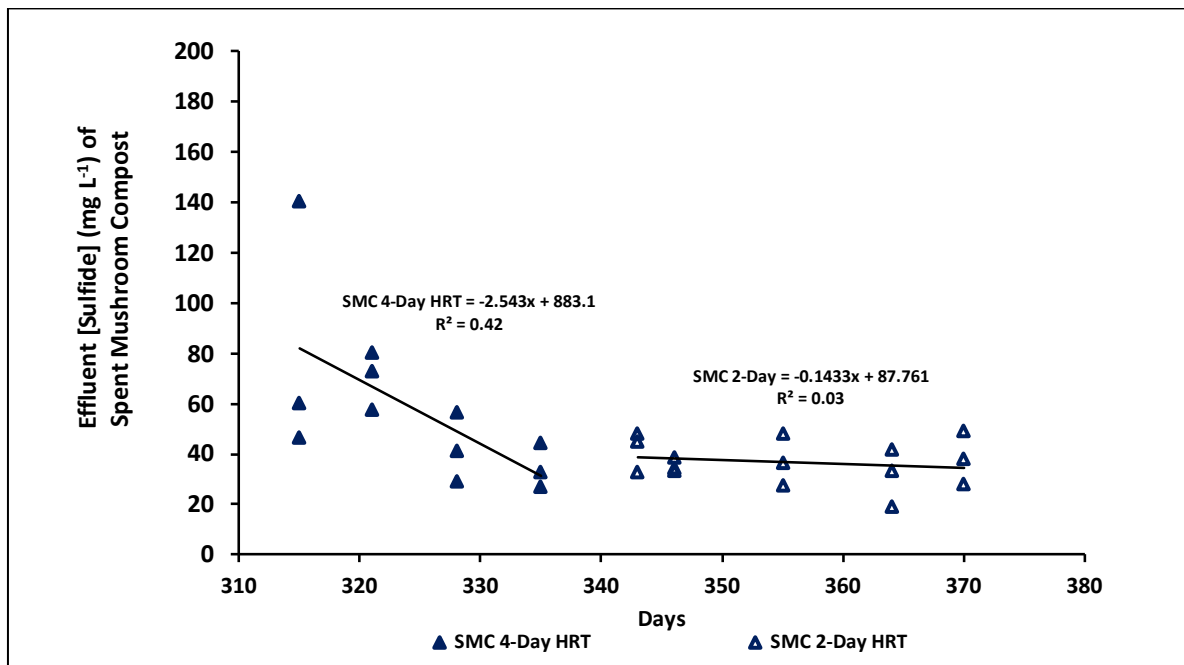

Figure A 22: Effluent Sulfide Concentration of SMC Treatment during Flow Manipulation
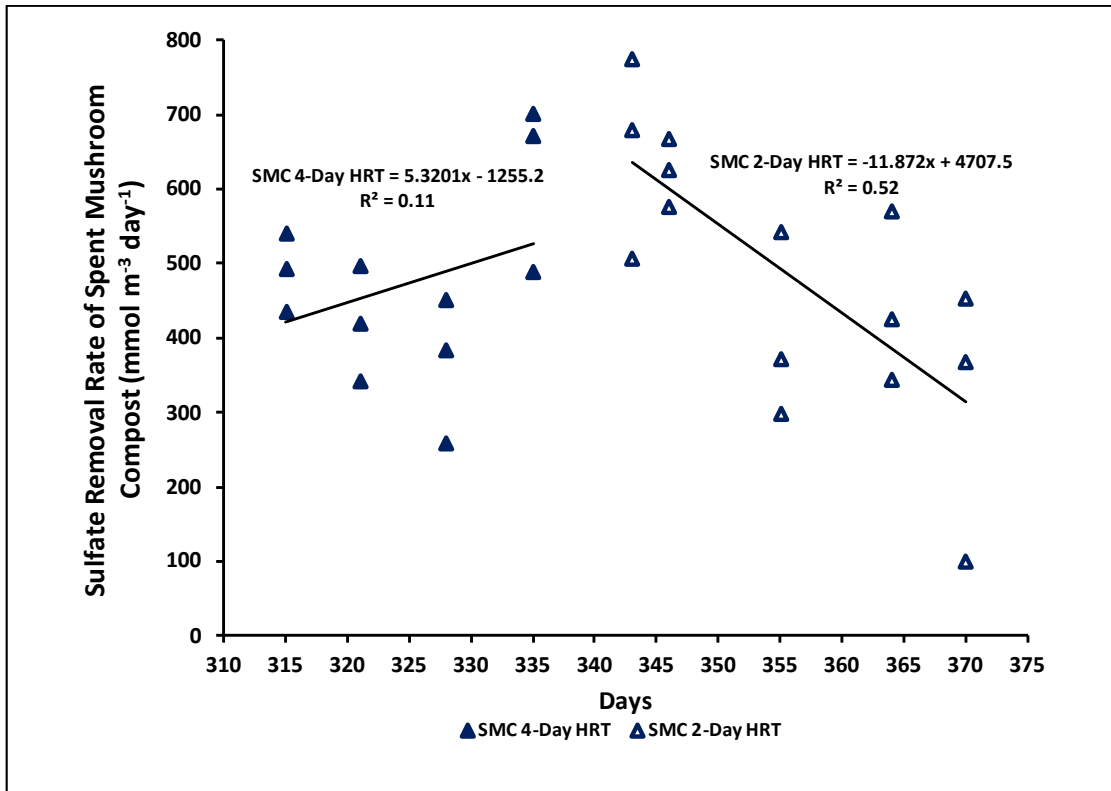
Figure A 23: SMC Sulfate Removal Rate (mmol m⁻³ day⁻¹) during Flow Manipulation
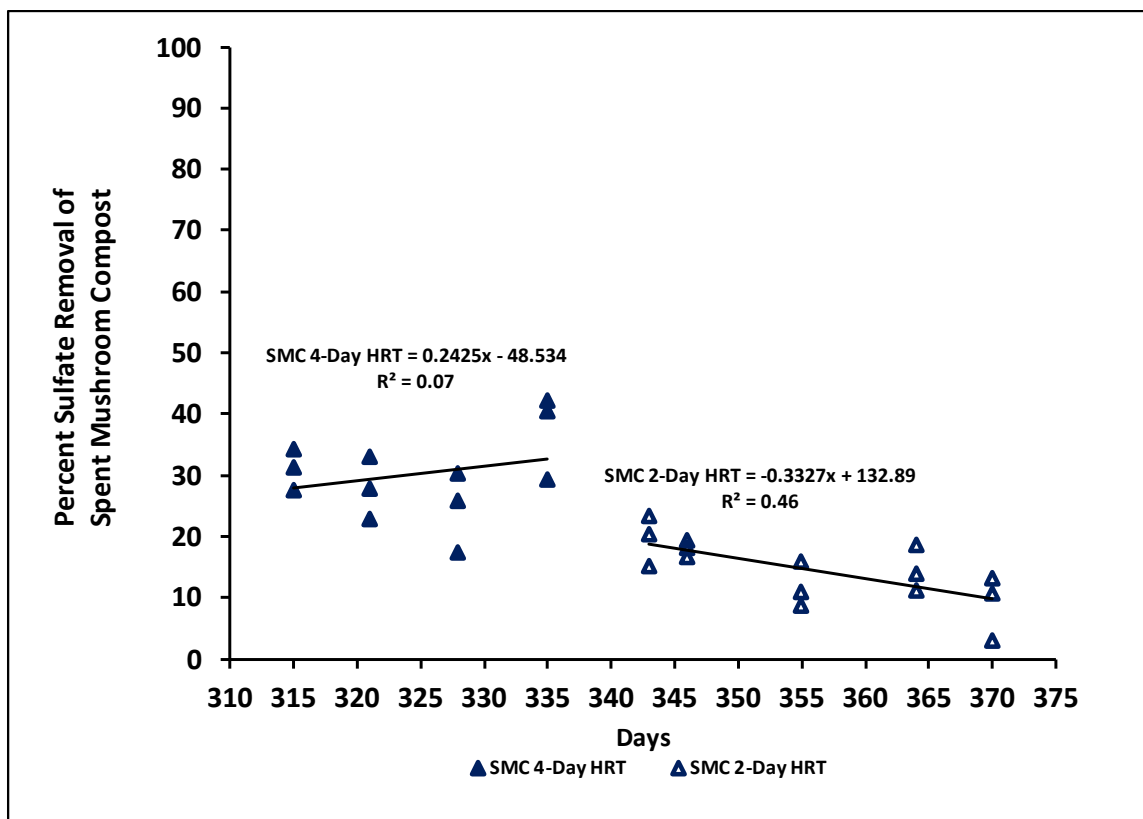

Figure A 24: SMC Sulfate Removal (%) during Flow Manipulation

8. Appendix B: Autopsied Material Data

Table B 1: Summary of Saturated Hydraulic Conductivity and Input Values by Treatment

		Water Depth and Substrate Depth after Autopsy			
Treatment	Replicate	Depth of Water (cm)	Depth of Substrate (cm)	Hydraulic Gradient (i)	Mean Saturated Hydraulic Conductivity (cm sec^{-1})
NAC	NAC 1	31.75	30.50	1.04	0.0126
	NAC 2	34.93	33.00	1.06	0.0139
	NAC 3	35.88	34.60	1.04	0.0147
MC	MC 1	34.29	32.70	1.05	0.0149
	MC 2*	35.56	31.43	1.13	0.0122
	MC 3	35.56	33.66	1.06	0.0149
SMC	SMC 1	34.29	31.75	1.08	0.0136
	SMC 2	33.34	31.12	1.07	0.0126
	SMC 3	36.20	33.02	1.10	0.0144
Overall		34.64	32.42	1.07	0.0139

*MC 2 was not included in any analysis as previously stated

Table B 2: Mean OM Content in Different Treatments with regard to Depth

	Organic Matter Content (%)		
	Mean	Standard Deviation	N
NAC Top 10 cm	45.2	2.98	9
NAC Middle 10 cm	43.9	2.93	9
NAC Bottom 10 cm	42.0	2.87	9
MC Top 10 cm	36.6	4.91	6
MC Middle 10 cm	39.6	8.58	6
MC Bottom 10 cm	35.9	6.42	6
SMC Top 10 cm	52.8	2.76	9
SMC Middle 10 cm	51.3	1.82	9
SMC Bottom 10 cm	51.3	2.03	8

9. Appendix C: Initial Substrate Data

Table C 1: Various Trace Metal Concentrations of Initial Materials

Mean Concentrations of Various Trace Metals from Initial Materials (mg/kg) (n= 3)

	Ag	Al	As	Ba	Co	Li	Se	Si
NAC	0.10	BDL	1.87	231.91	2.40	11.00	BDL	BDL
MC	0.11	BDL	2.00	123.59	3.06	12.05	1.51	BDL
SMC	0.13	BDL	2.11	138.40	1.76	5.80	2.47	BDL

BDL = Below Detection Limits