Mercury Methylation in Denitrifying Bioreactors: An Investigation in Pollution Swapping

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Abstract

Woodchip and corn cob filled bioreactors have been shown repeatedly to be effective at reducing nitrate pollution from agricultural fields by supporting denitrifying bacteria. Little attention has been paid, however, to other microorganisms that may also proliferate in these environments. Of particular concern are sulfate reducing bacteria and other organisms known to convert mercury to highly toxic methylmercury, since supporting such organisms could lead to increased levels of methylmercury in downstream rivers and lakes. To investigate this concern, we conducted two studies. We measured total and methylmercury concentrations and related parameters in upflow column bioreactors filled with either woodchips or corn cobs. The temperature of the water pumped into the column bioreactors was in the range of 1.8°C to 18.6°C to simulate cooler autumn and winter weather. There was no significant mercury methylation detected at these temperatures. The concentration of methylmercury flowing out of the column bioreactors filled with corn cobs showed greater variability than that of woodchip filled bioreactors. The second study was set up to pump water with low concentrations of nitrate into four edge-of-field woodchip bioreactors to produce a residence time of 24 hours or more. We measured total and methylmercury concentrations along with other indicators of water chemistry and biological activity. While the conditions monitored in the bioreactors were consistent with documented conditions that support mercury methylating bacteria, we did not find evidence of methylmercury being generated within the bioreactors. These studies indicate that the production of methylmercury is not likely in Minnesota edge-of-field woodchip bioreactors or in woodchip or corn cob bioreactors where water temperatures are below 18.6°C and nitrates are not completely reduced.

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Chapter 1 : LITERATURE REVIEW

The Dangers of Mercury

From Iraq to Japan, methylmercury has been implicated in mass poisoning incidents that were fatal for some people and caused permanent neurological damage for many others (Marsh et al. 2014; Maruyama et al. 2012; Bakir et al. 1973; Harada 1995; Yorifuji et al. 2009). In 1971, mercury poisoning came in the form of grain seeds that had been treated with a mercury containing fungicide and imported to Iraq. Farmers had already planted for the year and could not read the foreign warning labels, so they ground the seeds and used them for bread-making, exposing thousands to toxic levels of mercury (Marsh et al. 2014; Bakir et al. 1973). Other incidents have primarily affected people who consume fish as a main staple of their diet. In Minamata and Niigata, Japan, industrial contamination of nearby waters led to bioaccumulation of methylmercury in the aquatic food chain. Residents depend on fish for much of their protein, and were therefore affected in large numbers (Yorifuji et al. 2009; Maruyama et al. 2012; Harada 1995). Given the clear dangers of mercury exposure to humans, it is critical that we understand and minimize its sources to ensure the safe consumption of fish and waterfowl and prevent mercury poisoning in the future.

Mercury has also been shown to negatively impact ecological health. While one study found evidence of mercury transferring from a *Nerodia sipedon* (watersnake) mother to its offspring without a decrease in the size, quantity or short term survival of the offspring (Chin et al. 2013), numerous other studies have correlated high mercury levels in various organisms with increased infant mortality, immunosuppression, ataxia, paralysis and death (Wolfe, Schwarzbach, and Sulaiman 1998; Hopkins, Willson, and Hopkins 2013; Gehringer et al. 2013). Some organisms may be more resistant to the effects of mercury than others, but it is clear that mercury can hinder the survival and reproduction of many types of birds, mammals, fish and insects, potentially damaging the delicate balance of ecosystems.

Monomethyl Mercury

Mercury in the environment is present in three main forms: elemental mercury (Hg^0), ionic mercury (Hg^+ or Hg^{2+}) and organic mercury. Ionic mercury can form inorganic salts (*i.e.* $HgCl_2$, $Hg(NO_3)_2$) or can form ionic bonds with organic ligands while "organic mercury" refers to that which is covalently bonded to a carbon atom. In its most common organic form of monomethyl mercury (CH_3Hg^+ or MeHg), it collects in the tissues of organisms. Mercury concentrations in fish can be orders of magnitude higher than that of the surrounding water after biomagnification (Watras et al. 1998). All forms of mercury are toxic to humans, but most non-occupational exposure comes in the form of methylmercury, especially through the consumption of contaminated fish ("Mercury Study Report to Congress" 1997).

Ionic and elemental forms of mercury can be converted to MeHg, by a process known as methylation, through a variety of pathways. In 1985, *Desulfovibrio desulfuricans*, a sulfate-reducing bacteria (SRB), was identified as a key mercury methylator in anoxic saltmarsh sediments (Compeau and Bartha 1985). Researchers found an increase in mercury methylation when methanogen inhibitors were added to sediment slurries and a 95% decrease in mercury methylation when molybdate, a known inhibitor of SRB, was added. They isolated the bacteria of interest and further demonstrated its ability to methylate mercury, even in sterilized sediment. Several other species of SRB have since been shown to methylate mercury in other laboratories and environments as well (Dias et al., 2008; Ekstrom & Morel, 2008; King et al., 2001).

Mercury methylation can also be performed by other types of bacteria in addition to SRB. While molybdate, which inhibits SRB, does reduce the rate of mercury methylation in some environments, it does not always stop methylation completely. Fleming et al. (2006) explored this phenomenon further and isolated a strain of *Geobacter*, an iron-reducing bacterium (IRB), that was able to methylate mercury at a significant rate. In the same year, a study examined IRB of three genera. The authors

found that those genera more closely related to SRB, *Geobacter* and *Desulforomonas*, were able to methylate mercury while the *Shewanella* genus was not (Kerin et al. 2006).

Methanogens have also been implicated in mercury methylation. Methanogen inhibitors have been shown to both increase the rate of mercury methylation in some cases (Compeau and Bartha 1985), presumably by eliminating competition for electrons by SRB, and, conversely, to stop mercury methylation completely in other cases (Hamelin et al. 2011). The outcome depends on the environment and specific bacteria present. The complete cessation of mercury methylation in the presence of a methanogen inhibitor (Hamelin et al. 2011) implied, for the first time, that some methanogens are capable methylators of mercury. The researchers also discovered that sodium molybdate could increase mercury methylation in their incubated samples of periphyton mats, reinforcing the idea that SRB were not primarily responsible for methylation in this environment. The environment of a periphyton mat is comprised of many species of algae, autotrophic and heterotrophic bacteria, and detritus that have formed a coating on underwater surfaces.

More recent research has identified additional prokaryotes as mercury methylators. A gene cluster, *hgc*AB, was recently identified in two species that are known to methylate mercury. When either gene was removed, methylation was disabled (Parks et al. 2013). A subsequent study searched a wide variety of organisms for the gene cluster and tested their ability to methylate mercury. In testing 18 species, the researchers found the gene cluster to be a consistent predictor of methylation capability. All strains tested with *hgc*AB were shown to methylate mercury and the 3 control strains without it did not (Gilmour et al. 2013). This study expanded the current understanding of what types of organisms methylate. In addition to the established groups of SRB, IRB and methanogens, the researchers discovered some *Firmicutes*, including one isolated from wastewater and one from a termite's gut, could also methylate mercury. These included fermentative and acetogenic bacteria, further complicating the environmental conditions that can lead to mercury methylation.

Researchers are still actively exploring and debating the mechanism of bacterial mercury uptake and methylation. Schaefer et al. (2011) measured higher cellular uptake of mercury and subsequent methylation rates when adding a carbon source, thus demonstrating that for *G. sulfurreducens*, mercury seemed to be absorbed via active transport. Others have argued in favor of passive transport of HgS^0. Benoit, Gilmour, & Mason (2001) used a solid mercury source in bacterial cultures to conclude that methylmercury production was only weakly related to dissolved total mercury (THg) concentrations (r^2=0.18) and was more strongly correlated to the concentration of HgS^0 (r^2=0.80). This finding was consistent with earlier work in which the researchers created and validated a model for mercury complexation in two different ecosystems. Their model indicated that HgS^0 was the main form taken up by bacteria (Benoit et al. 1999). The pathway of mercury uptake by bacterial cells may remain unresolved for some time because the answer is complex. Some strains of mercury methylating bacteria lose their capacity to absorb and methylate mercury in the presence of certain types of thiols while others appear to be unaffected (Schaefer et al. 2011). Slight differences in physiology among organisms are likely to result in different transport mechanisms.

Once absorbed, the pathway for methylation of mercury within bacterial cells can also vary. In 1974, Wood suggested that bacterial mercury methylation involved a methylcorrinoid compound with a cobalt center. For some organisms, like *Desulfovibrio multivorans,* cobalt limitation can prevent mercury methylation which supports this theory, while for other organisms, no effect is seen (Ekstrom and Morel 2008). This discrepancy appears to be the result of differing carbon metabolisms. The *hgc*AB gene cluster in *D. africanus* proved to be necessary for MeHg production, but not for cell growth (Parks et al. 2013). This was the same species studied by Ekstrom and Morel (2008) that was not affected by cobalt limitation. These results indicate *D. africanus* does not methylate mercury using a methylcorrinoid compound with a cobalt center, nor is its mechanism directly related to its metabolism. It is unknown how many different pathways might exist for the bacterial methylation of mercury.

Schaefer et al. (2011) suggested that the function of bacterial uptake and methylation of mercury is connected to the organisms' need for other trace metals.

Uptake of some similar trace metals, such as selenium, might be especially beneficial in areas where such metals are scarce, but accidental uptake of mercury without some mechanism to quickly expel it from the cell could be lethal. Certain organisms may have evolved the ability to absorb metals bound to thiols and later evolved to methylate and export MeHg from the cell in response to the toxicity of excessive mercury accumulating in bacterial cells. There has been no evidence thus far to suggest that MeHg is beneficial to any living organisms. Mercury methylation as a defensive action to promote the bacterial cell's survival is not confirmed as most research is currently focusing on the mechanism and extent of methylation instead.

Methylation of mercury can also happen abiotically. In 2005, Siciliano et al. measured methylmercury in lake water and found a diurnal pattern in which concentrations of methylmercury increased during the day and decreased at night. The same pattern was evident when water was put into bottles to prevent deep water mixing, but not if the bottles were black to restrict solar radiation. The researchers implied solar radiation was responsible for mercury methylation in the water column, but they did not sterilize the lake samples for this study, so it is unclear if the solar radiation was stimulating biotic or abiotic methylation. To further investigate this question, they also studied the role of different sizes and concentrations of dissolved organic matter (DOM) fractions in mercury methylation. They passed lake water through a sterilizing filter and further diluted some of this water to limit the size and concentrations of DOM present. Some of these aliquots of water were exposed to solar radiation while others served as controls in dark bottles. They analyzed all samples for MeHg after 7.5 hours. The results confirmed that, despite sterilization, MeHg concentrations were higher in samples exposed to solar radiation as compared with controls. The concentrations were even higher in the presence of DOM between 4.4 or 7.1 mg L^{-1}, implying a function in the abiotic mechanism for DOM. Bacterial counts at the end of the study verified that biological activity could not explain the methylation. Yin et al., (2012) went a step further in identifying possible mechanisms by which abiotic methylation could be occurring in the presence of UV irradiation, but they point out that the lighting used in

their experiments differed from natural sunlight. While it is clear that abiotic mercury methylation exists, its significance in natural systems is still unclear.

Methylmercury production was established by Berman and Bartha (1986) to be predominantly carried out by microorganisms, with abiotic methylation playing a much smaller role. A review article later disputed this, arguing that this notion was based on laboratory studies in which high concentrations of Hg^{2+} were added (10 ppm $HgCl_2$) which would have selected for Hg resistant species that may not be so abundant *in situ* (Weber 1993). Weber (1993) also contested that studies of biotic mercury methylation often used molybdate (MoO_4^{2-}) as an inhibitor of sulfate reducing bacteria, but this addition could also be oxidizing HS^-, therefore changing the abiotic methylation potential as well. But this argument ignores Berman and Bartha's (1986) study, which demonstrated very little methylation in steam sterilized sediments without the addition of chemical inhibitors like MoO_4^{2-}. A more recent study also reinforced the importance of biological methylation by using highly sensitive ICP-MS methods which allowed stable isotope spikes close to environmental concentrations (Rodríguez Martín-Doimeadios et al. 2004). While there are disagreements in the literature, most subsequent studies have continued with the assumption that the majority of *in situ* mercury methylation was carried out by microorganisms and many have further explored specific organisms, cell uptake and methylation mechanisms (Ekstrom and Morel 2008; Barkay, Miller, and Summers 2003; Benoit, Gilmour, and Mason 2001; Choi, Chase, and Bartha 1994; Graham, Aiken, and Gilmour 2013; Fleming et al. 2006; Gilmour et al. 2013; Hamelin et al. 2011; Kerin et al. 2006; Achá, Pabón, and Hintelmann 2012).

Demethylation

To fully understand the methylation of mercury, demethylation must also be considered. Both processes happen simultaneously in nature, so it is actually the balance of the two, known as net methylation, which impacts methylmercury concentrations in aquatic systems. Like methylation, the decomposition of methylmercury can occur through biotic and abiotic pathways. In marine waters, the biotic pathways dominate,

while freshwaters tend to favor abiotic methods of demethylation (Sellers et al. 1996). Zhang and Hsu-Kim (2010) identified a probable cause for this tendency by incubating simulated seawater and freshwater with different concentrations of methylmercury and humic acid. They found that significantly more demethylation occurred when the estimated amount of reduced sulfur in the humic acid was greater than the amount of methylmercury present. This suggested that the methylmercury was forming complexes with the reduced sulfur as part of the process. This is consistent with a finding by Ni, et al. (2006) that bonding with thiols causes the carbon in methylmercury to become more electronegative and allows for increased reactivity with free radicals formed by sunlight. In seawater, it is generally thought that because of the higher Cl^- concentration, most methylmercury is in the form of CH_3HgCl. Since freshwater allows for CH_3HgSH to be the more prevalent form, it follows that freshwater methylmercury would be more susceptible to photodemethylation than that of seawater. Demethylation of mercury in sediments, like in ocean waters, is more likely to be biotic because sediments have less exposure to sunlight for abiotic methylation and they harbor a gradient of aerobic and anaerobic conditions to support a wide variety of bacteria.

Reductive biotic demethylation is controlled by the *mer* operon which contains several relevant genes. Some of the most well-known among them are the *mer*A and *mer*B genes present in some microorganisms (Jackson and Summers 1982; Huang 1999). It is generally assumed that they serve as a defense mechanism, allowing bacteria to withstand unusually high concentrations of mercury that would otherwise be toxic to them. In freshwater sediments, however, demethylation was inhibited by the addition of another one-carbon substrate (MeOH), indicating that demethylation may be associated with a metabolic pathway (Oremland, Culbertson, and Winfrey 1991). *Mer*B is the gene that codes for the organomercurial lyase enzyme that breaks the carbon-mercury bond in methylmercury, resulting in CH_4 and Hg^{2+}. MerA allows for the production of the mercuric reductive enzyme which converts Hg^{2+} into Hg^0. Since elemental mercury is volatile, it evolves from the water's surface and enters the atmospheric pool of mercury. Oxidative biotic demethylation instead yields CO_2 and Hg^{2+}. Without the second step of Hg^{2+} reduction, oxidative demethylation does not allow for the rapid escape of mercury.

It remains available to be methylated again and continue the cycle. This process is not as well understood as reductive demethylation, but has been shown to occur more readily in anaerobic sediments than does reductive demethylation (Oremland, Culbertson, and Winfrey 1991).

The Effect of Redox Potential

Assuming SRB are the main culprits behind mercury methylation, it is important to consider what environmental conditions support the proliferation of these organisms. Sulfate-reducing bacteria, as their name suggests, use sulfate (SO_4^{2-}) as their final electron acceptor. They do not thrive in aerobic environments. Since oxygen is a much more efficient electron acceptor, it allows aerobic organisms to dominate. Once oxygen has been depleted, nitrate (NO_3^-) is the next most energetically favorable electron acceptor. Therefore, organisms that can use nitrate will have an advantage over others until the nitrate is depleted. Ferric iron (Fe^{3+}) is another electron acceptor that is more efficient than sulfate (Sposito 1989). Thus, SRB are not likely to dominate an environment until most of the oxygen, nitrate and ferric iron have been depleted.

This concept is reinforced by the work of Todorova et al., (2009). In this study, a close negative relationship was found between the nitrate concentration of the hypolimnion of Lake Onondaga and its methylmercury production. It appears that mercury methylation was inhibited in Lake Onondaga during years of increased spring nitrate inputs. With sufficient nitrate concentrations, nitrate reducing bacteria are expected to dominate, obstructing the proliferation of SRB. Since SRB are known to be mercury methylators in several environments (Dias et al. 2008; Compeau and Bartha 1985; Ekstrom and Morel 2008; King et al. 2001), it follows that fewer SRB results in less mercury methylation.

Excessive Nitrate and Subsurface Drainage

While nitrate may have an inhibiting effect on the methylation of mercury, it is known more commonly for its own detrimental effects on the environment. Agricultural

production around the world has come to depend on external inputs of nitrogen to produce the food needed for a growing population. In the United States, the use of nitrogen fertilizer has risen from 62.29 to 70.73 tonnes of nitrogen per 1,000 hectares of arable land between 2002 and 2010 ("Food and Agriculture Organization of the United Nations" 2014). This increase has dramatic environmental costs. The nitrogen that is not absorbed by plants or soil is quickly oxidized to nitrate if not already applied in that form. The excess nitrate washes downstream contributing to hypoxia, eutrophication and pollution of drinking and recreational water sources (Camargo and Alonso 2006; Blann et al. 2009).

Nitrogen fertilizer is not the only source of the problem. As organic matter is decomposed in the soil, organic forms of nitrogen can also be mineralized to form soluble nitrates. A review of literature by Carlson, Vetsch, and Randall (2013) found that 20 lbs of nitrate-nitrogen per acre was being lost from fallow lands without the application of fertilizer.

The extensive network of subsurface drainage, also known as tile drainage, which exists throughout the Midwestern United States, exacerbates this problem. Much of the land that is currently being farmed in the Midwest would not be useful for agriculture without artificial drainage. A network of perforated plastic pipes is buried below the surface to allow water to drain out of the soil profile more quickly and be directed to ditches, streams and rivers. This allows the water table to drop, providing a more aerobic, suitable rooting zone for crops. While this practice has produced increases in productivity of 5-25% per year (Blann et al. 2009), it has an unintended side effect regarding nitrate. Since nitrate is highly soluble and is not sorbed or retained by soil particles, it travels quickly with moving water. By creating a fast track for water to leave agricultural fields, tile drainage also creates a fast track for nitrates to enter local streams, rivers and lakes. The extent of cropland that uses tile drainage has proven to be challenging to estimate (Jaynes & James, 1992). An estimate for the Midwest based on a 1987 survey suggested that up to 30% of croplands were tile drained at that time (Pavelis 1987). While the accuracy of this estimate may be unclear and many more miles of

drainage tiles have been added since then, there is little doubt that tile drainage is extensive enough to impact water quality downstream.

Part of the solution to nitrate pollution is to reduce nitrogen fertilizer use whenever practical. Unfortunately, even the most conservative recommendations cannot sufficiently ameliorate agricultural nitrate pollution because of limitations on the nitrogen uptake efficiency of corn and other commonly grown crops as well as the loss of mineralized nitrogen from organic matter in the soil. Usually less than 50% of the nitrogen applied to the soil will be harvested in a crop of corn (Fageria and Baligar 2005); an older estimate suggests the figure is closer to 33% (Raun and Johnson 1999). The remainder is lost through volatilization, plant release, denitrification in the soil, runoff and leaching. This has been a perplexing problem for agronomists. Raun and Johnson (1999) have suggested that the problem will persist without a globally coordinated, comprehensive package of recommended practices and continued research.

Denitrifying Bioreactors

While high nitrate concentrations appear to prevent methylmercury production in lakes, it is still not wise to overload rivers and lakes with nitrate. This can lead to algal blooms, polluting drinking water sources and contributing to dead zones downstream. According to Billen, Garnier, & Lassaletta (2013), 43 Tg of reactive nitrogen per year is carried to the oceans by rivers globally. That's almost 30% of all anthropogenically sourced reactive nitrogen.

One technique for treating nitrate polluted agricultural water is to direct it through a denitrifying bed, known as a bioreactor. This bed is usually composed of woodchips, sometimes mixed with soil, and is buried under ground (Figure A). The soil mixed in with the woodchips inoculates the bioreactor with naturally occurring bacteria. As drainage water fills the bioreactor, the oxygen is quickly consumed. Without oxygen available, the second most energy efficient electron acceptor, nitrate, allows the bacteria that depend on it to dominate the microbial community. Several different species participate in the denitrification of the nitrate (NO_3^-), first to nitrite (NO_2^-), then to nitric

and nitrous oxide (NO and N_2O) and finally to dinitrogen gas (N_2). It is crucial that this series of reactions is completed within the bioreactor to prevent export of N_2O, a potent greenhouse gas.

Bioreactors often include a simple control structure at the inlet and outlet of the bed to monitor and regulate how much water passes through. With sufficient rainfall and management of the control structures, anaerobic conditions can be maintained within the bioreactor. The outlet control structure must be kept restrictive enough to back up the flow, increasing the hydraulic residence time within the bioreactor. This allows the bacteria the time they need to consume all of the oxygen in the water and come in contact with the nitrate in the water. When provided with this anaerobic environment, rich in carbon from the woodchips, the bacteria reduce the nitrate at much higher efficiencies than would naturally occur in soil (Greenan et al. 2001; Jaynes et al. 2008; Warneke et al. 2011). Even after several years in operation, Schipper et al. (2010) noted several examples where at least 50% of the original nitrate reduction rates were maintained.

While bioreactors can be a very useful tool, it is important to consider that the flow rate and nitrate concentration of agricultural drainage water varies throughout the seasons (Greenan et al. 2001). If the flow is slow enough, or the concentration small enough, the denitrifying bacteria could consume all of the available nitrate. In this case, the anaerobic conditions and excessive carbon stocks from woodchips would support the growth of other bacteria, such as IRB, SRB and/or methanogens. All of these categories contain species capable of methylating mercury. It is important to examine bioreactors during times of low flow and low nitrate concentrations to determine if they could be supporting mercury methylation and increasing the concentration of MeHg in downstream rivers and lakes.

One study has examined this possibility in a streambed bioreactor (Shih et al. 2010). The researchers separated sampling points into "nitrate reducing conditions" and "sulfate reducing conditions." Nitrate reducing conditions were defined as times when the outlet nitrate concentration exceeded 0.5 mg/L, allowing for continued nitrate reduction throughout the length of the bioreactor. Sulfate reducing conditions were defined as times when the outlet nitrate concentration dropped below 0.5 mg/L, allowing

for observed reduction of sulfate. A suppression of methylmercury production was observed during nitrate reducing conditions, however an average of 15.2 μg MeHg per square meter of surface area per year were produced during sulfate reducing conditions. This average was calculated only based on the early fall and late spring, when sulfate reducing conditions were present, so it is misleading to convert and report it on an annual basis. Additional studies are needed to confirm this finding and determine whether it also applies in the context of other designs of bioreactors, such as those at the edges of farm fields directly collecting subsurface drainage water.

Chapter 2 : MODEL BIOREACTORS

Introduction

Denitrifying bioreactors are quickly proving to be a promising tool in the fight against nitrate pollution. In a review of bioreactor research, Schipper et al. (2010) failed to find an example of an ineffective bioreactor. The researchers concluded that the woodchip filled bioreactors can effectively reduce nitrate and clean agricultural drainage water for at least 15 years provided they remain saturated. Each bioreactor removed an average of 0.62-12.7 g NO_3^-N m^{-3} d^{-1}, often limited by the relatively low concentrations of nitrate entering the systems.

Many denitrifying bioreactor studies have been conducted above 20°C (Gibert et al. 2008; Greenan et al. 2006), but there has also been some evidence to show that they can be effective at colder temperatures as well. Healy et al. (2012) demonstrated appreciable denitrification while maintaining a temperature of 10°C throughout the study. Another recent study exploring the effects on denitrification rates of different types, particle sizes and volume ratios of wood substrates as well as the effects of nitrate concentration and temperature found measureable denitrification occurring even when groundwater temperature was 8°C (Schmidt and Clark 2013). However, they also found that changes in temperature accounted for about 50% of the variation in denitrification rates observed, much higher than the effects of other parameters tested. It is reassuring that denitrification can occur at cold temperatures, but the slower rates must be considered when designing and managing the systems.

While bioreactors have consistently exhibited their ability to enhance denitrification, the materials used to build them may not be the same for all regions. Due to the varying accessibility of large quantities of woodchips, many researchers have investigated the effectiveness of other carbon substrates for use in denitrifying bioreactors (Gibert et al. 2008; Greenan et al. 2006; Cameron and Schipper 2010). Some alternative carbon sources have proven to remove nitrates from drainage water at much higher rates than woodchips, but often with other negative consequences (Greenan et al. 2006; Cameron and Schipper 2010). For example, Cameron & Schipper (2010) observed

a 6.5 fold increase in nitrate reduction from experimental columns filled with maize cobs as compared with wood media at 14°C and a threefold increase at 23.5°C. Even after 10 months, however, the columns filled with maize cobs were leaching more NH_4-N than is allowed by regulatory standards. The rates of leaching were higher at the higher temperature, indicating that it may be due to microbial mineralization of the maize cobs. As new substrates are evaluated for their effectiveness, they must also be vetted with regard to potential pollution swapping.

As mentioned previously, the potential for mercury methylation in bioreactors is one of these bioreactor pollution risks that must be studied. While many studies have identified specific bacteria that methylate mercury in natural environments, most studies are conducted at relatively warm temperatures. Researchers have identified sulfate reducing bacteria (SRB) that methylate mercury at 25°C (Compeau and Bartha 1985), iron reducing bacteria at 30°C (Kerin et al. 2006) and methanogens in periphyton at 21-23°C (Hamelin et al. 2011). Dias et al. (2008) tested three additional SRB that methylate mercury and reported their optimal temperature to be 35°C. These temperatures are much warmer than agricultural drainage water during a typical Minnesota spring, suggesting the need for an investigation of mercury methylation in colder bioreactors.

The possibility of bacteria methylating mercury inside bioreactors has been scarcely studied and likely not studied at all using alternative carbon substrates. It is crucial that this relatively new topic of research not be neglected when determining the effectiveness of new carbon sources for bioreactors. This study aims to explore the potential of corn cobs, as compared with woodchips, to support mercury methylating bacteria during periods of cold temperatures.

Materials and Methods

Cylinders made of polyvinyl chloride (PVC) were converted into model denitrifying bioreactors by filling them with carbon substrate inoculated with soil. These model bioreactors, henceforth referred to as columns, were 6 inches (15.24 cm) in diameter and 49.5 cm long. They were each equipped with 3 sample ports for monitoring conditions inside the columns. The substrates investigated were

woodchips and corn cobs. They were soaked in reverse osmosis water for 48-72 hours before filling the columns. Each column was filled with 7-8 layers of soaked substrate in alternating layers with Waukegan silt loam soil from the plow layer of a University of Minnesota campus research and outreach center on the St. Paul campus. The total mass of saturated carbon substrate was 400 grams with an additional 140 grams of soil. These masses were determined based on previous porosity testing of similar columns with a goal of 60% porosity in the columns. The tops and bottoms of columns were fitted with 0.254 mm wire mesh and perforated PVC plates to hold the materials in place. They were closed off with PVC end caps and sealed with silicone sealant or welded in some cases to stop leaking. Each treatment was replicated 3 times.

Oxidation reduction potential (ORP) meters were installed into the center of each column top end cap and a reading was recorded once per hour with a data logger. One column of each treatment also had a ¾ inch schedule 40 PVC adapter with an o-ring and cover installed 2½ inches offset from the center of the end cap. Three days per week, the cover was removed so that a dissolved oxygen (DO) meter (Hanna 9142) could be inserted to record the concentration of DO.

Columns were positioned vertically while a peristaltic pump delivered water through the bottom of the columns and allowed it to exit through Tygon tubing attached to the top (Figure B). The pump was set to maintain an approximate flow rate of 5 ml/min throughout the study, resulting in an approximate hydraulic residence time (HRT) of 12 hours. Reverse osmosis water from the University of Minnesota greenhouse system was delivered to the columns in this manner to flush particulate and readily dissolvable C and to ensure anaerobic conditions before adding nutrients. After 11 days of conditioning, a suite of nutrients was added to the tank of water from which the pump filled the columns (influent). The concentrations of the chemicals and resulting nutrients can be found in Table 2.1and Table 2.2. They were selected to mimic the water chemistry entering field bioreactors in Minnesota (Ranaivoson et al. 2012) except for the nitrate and phosphate concentrations (50 mg L^{-1} and 0.3 mg L^{-1}, respectively) which were selected to ensure the ability to

distinguish between the effectiveness of the different treatments. Columns were kept in a temperature controlled room which was set to gradually decrease after nutrients were added until it reached 1.5°C (34.7°F). It stayed at this temperature for the remainder of the study (Feyereisen et al. *in review*).

Table 2.1: Concentrations of chemicals added to the 50 gallon (189 L) influent tank

Influent Solution with nutrients		
Formula	**g/100 liters**	**g/50 gal**
$CaCl_2*2H_2O$	29.40	55.57
KH_2PO_4	0.13	0.25
Na_2SO_4	1.04	1.97
H_3BO_3	0.01	0.02
$MgCl_2*2H_2O$	13.65	25.80
$Fe_2(SO4)_3$	0.03	0.06
KNO_3*	36.07	71.58
$CuSO_4$	0.01	0.01
$MnSO_4*H_2O$	$2.11*10^{-3}$	0.004
$ZnSO_4*7H_2O$	0.01	0.02

Note: We started with 68.17g KNO3, but switched to 71.58g on 1/31/13.

Table 2.2: Nutrients and their final concentrations in the influent

Nutrient	mol L^{-1}	Nutrient	mol L^{-1}
B	$2.16*10^{-4}$	Mn	$5.49*10^{-4}$
Ca	3.21	Na	0.09
Cl	7.66	NO3-N	0.70
Cu	0.00	PO4-P	0.01
Fe	0.01	SO4-S	0.09
K	5.46	Zn	$1.31*10^{-3}$
Mg	0.61		

Sample Collection

Samples were collected by connecting the Tygon tubing from the top of each column to a fluropolymer cap affixed to an acid washed, 250 ml borosilicate glass bottle. Bottles and caps were handled both before and after they were filled using the "clean hands – dirty hands" approach to reduce contamination ("Method 1669: Sampling Ambient Water for Trace Metals at EPA Water Quality Criteria Levels" 1996). This approach ensures that sample bottles and caps are only handled by someone wearing clean gloves while another person is available for touching the outside of containment bags and coolers. After filling, the bottles were capped, double bagged and placed in a cooler for transport to the mercury analysis laboratory across the street. In the lab, 1 ml of 12 M trace metal HCl was added to each bottle for preservation. The samples were then stored in a refrigerator until analysis.

Total Mercury Analysis and QA/QC

Total mercury (THg) concentration in samples was determined using EPA Method 1631: Revision E including oxidation, purge and trap, and cold vapor atomic florescence spectrophotometry. Samples were first treated with bromine monochloride (BrCl) and heated to 70°C overnight to oxidize all forms of mercury to a soluble, ionic form (Hg^{2+}). For every 125ml aliquot of sample, 2ml of BrCl was used. After heating, samples appeared yellow, indicating there was excess BrCl when the reaction was complete. In rare cases where the sample was not yellow, additional BrCl was added and these samples were heated another night to ensure that all organic compounds in the sample had been oxidized to solubilize the mercury present. Hydroxyl amine hydrochloride ($NH_2OH \cdot HCl$) was added to each sample shortly before analysis to pre-reduce the excess BrCl. For each milliliter of BrCl used, 0.2 ml of $NH_2OH \cdot HCl$ were used, thus most samples received 0.4 ml of $NH_2OH \cdot HCl$.

An aliquot of sample (approximately 50ml) was then mixed with 0.5ml of stannous chloride ($SnCl_2$) and purged with mercury-free dinitrogen gas (N_2) for 21 minutes. The purging vessel was connected to a mercury trap. As the $SnCl_2$ reduced all of the ionic mercury in the sample, it was converted to gaseous elemental mercury (Hg^0)

and pushed onto the trap by the flow of mercury-free N_2. The traps were constructed of glass tubes filled with glass beads that were coated in a layer of gold and held inside the tubes by tightly packed glass wool. When the Hg^0 came in contact with the gold-coated beads, it amalgamated to the gold and remained on the trap for analysis.

After purging and trapping, the trap was connected to the analyzer in series with an additional trap. With mercury-free argon flowing through, the first trap was heated to 450-500°C, allowing all mercury to desorb from the heated trap and attach to the next trap in series. This second "analytical" trap did not change between samples. It was then heated to 450-500°C, allowing the mercury to desorb again and flow directly into the analyzer. Using an analytical trap ensured that the release properties of individual gold traps did not interfere with the instrument response. The mercury analyzer used was a Brooks Rand Model III which utilizes cold vapor atomic fluorescence spectrophotometry.

When samples were being analyzed for THg, each day began with heating all sample traps while argon was pushed through them to flush any contamination. All vessels used for purging and trapping received approximately 100ml DDI water and 5ml of 12M HCl. Clean sample traps were attached to the vessels, 0.5ml of $SnCl_2$ was added to reduce any mercury present and the vessels were purged with mercury-free N_2. These traps were then connected to the analyzer and heated as described above to ensure all components of the process had been flushed of mercury. Next a calibration curve was run using a mercuric chloride ($HgCl_2$) standard. The R^2 of the calibration curves ranged from 0.9971 to 0.9995. For every 10 samples analyzed, there was an analytical blank, standard and sample duplicate analyzed to confirm a consistent response throughout the day. Once per day, a standard reference material (SRM) was tested to confirm accuracy and a sample spike was tested to determine if matrix effects were present. The SRMs used were 1515 (apple leaves) and 2976 (mussel tissue) from the National Institute of Standards and Technology and the average recovery was 104.4% of the expected. The average sample spike recovery was 101.1% of the calculated value. Each batch of results was accepted if the R^2 exceeded 0.995, standard recoveries remained within 90-110% of expected values and the spike and SRM recoveries were between 80-120% of expected

values. The method detection limit was determined to be 0.785 ng L^{-1} based on the analysis of ten low concentration standards.

Statistical significance was determined by t-tests ($\alpha = 0.05$) using JMP software (*SAS Institute Inc., 1989-2012*).

Monomethylmercury Analysis and QA/QC

Monomethylmercury (MeHg) concentration in samples was determined using a slightly adjusted version of EPA Method 1630. First, 35ml of each sample was combined with 156µl of ammonium pyrolithodicarbamide (APDC) in a fluoropolymer vial for distilling. They were distilled at 125°C into 40ml glass receiving vials with 5ml of DDI water in the receiving vials at the beginning. Distillation proceeded until the receiving vials reached a total volume of 30ml. Distillation of samples acted to pre-concentrate the mercury, as well as reducing the possibility of interference from more complex compounds in the sample with higher boiling points.

After distillation, 40µl of 2.5% ascorbic acid was added to each vial to react with any chloride ions that may be present and could interfere with analysis. Next, 263µl of acetate buffer was added to each vial to ensure the ideal pH of 4.9 for analysis. Finally, 30µl of sodium tetraethylborate ($NaBEt_4$) was added to each vial to ethylate the mercury present in the sample. The vials were shaken vigorously and allowed to rest so that the $NaBEt_4$ could react completely, volatilizing the ethylated mercury species present. In addition, a set of vials was prepared each day with a methyl mercuric chloride solution, DDI water, acetate buffer and $NaBEt_4$ to establish the instrument response and consistency throughout the testing period. Data was accepted if the R^2 for the calibration curve was a minimum of 0.995. Standards made with methyl mercuric chloride solution were distilled alongside samples and resulted in recoveries with an average of 95% expected. Sample duplicates averaged 96% of expected recoveries. The detection limit for methylmercury analysis of water was calculated to be 0.026 ng L^{-1} based on the analysis of eight low concentration standards.

All vials were analyzed by the Tekran 2700 Analyzer using a purge and trap method followed by GC column separation, a pyrolyzer to decompose all forms of

mercury and cold vapor atomic fluorescence spectrophotometry to produce peaks for each species of mercury. The accompanying software reports the MeHg peak area.

Results

During the month of December, the average total mercury concentration in the input waters was 11.24 ng/L (standard deviation = 2.99). The average for January through April was 8.01 ng/L (standard deviation = 3.07). Despite the presence of total mercury, methylmercury concentrations in both the influent water and all column output waters dropped below detection limit for all samples collected after the month of December. The temperature remained below 40°F (4.4ºC) after December, which likely inhibited most microbial activity including any possible methylation or demethylation of mercury (Figure C). There were modest reductions of nitrate; 1.6 g N m^{-3} d^{-1} for woodchips and 7.4 g N m^{-3} d^{-1} for corn cobs, but this was also much lower than what is seen at warmer temperatures (Feyereisen et al. *in review*). For this reason, the focus will be on results from the month of December 2012 only.

During the month of December, the temperature of the source water in the input tank fell from 65.5 °F (18.6°C) to 35.2 °F (1.8°C). Despite the saturated conditions within each column, the DO rose from 1.9-2.0 mg/L to 3.9-4.1 mg/L because of higher oxygen solubility at lower temperatures. The ORP remained positive throughout the month of December in all corn cob and woodchip filled columns. These data suggest that complete anaerobic conditions were not achieved during this time. There were, however, measurable changes in ORP and corresponding changes in the nitrate reduction occurring in each column, indicating some activity by anaerobic bacteria. Nitrate reduction in mg day^{-1} was defined as:

Eq. 1: *(Input NO_3^-N concentration – Column output NO_3^-N concentration) * Flow rate*

Figure D shows the ORP for all columns filled with corn cobs dropped below 200 mV between day 343 (December 8th) and day 353 (December 18th). Meanwhile, the same

columns demonstrated an increase in nitrate reduction that lasted the same amount of time. Columns filled with woodchips started December with ORP values below 200, but they quickly rose and then oscillated throughout the month (Figure D). As with the corn cob columns, the nitrate reduction of these woodchip filled columns increased when the ORP decreased and decreased when the ORP increased. This relationship between ORP and nitrate reduction can be described using equation 2. This equation predicts nitrate reduction based on ORP reading as seen in Figure E (R^2=0.43, p<0.0001). The average ORP value in corn cob filled columns for December was 199 mV and average nitrate reduction was 218.3 mg day^{-1} NO_3^--N. Those filled with woodchips averaged 260 mV and 55.8 mg day^{-1} NO_3^-N reduction in December. A t-test of the nitrate reduction for each substrate confirmed that corn cobs did reduce significantly more nitrate than woodchips ($p < 0.0001$) in the month of December.

Eq. 2: *Nitrate Reduction (mg day^{-1}) = -0.7782*ORP daily average (mV) + 318.33*

Table 2.3: Average total and methyl mercury concentrations and MeHg production of columns

	THg (ng L^{-1})		MeHg (ng L^{-1})		MeHg production (ng day^{-1})
	Inlet	Outlet	Inlet	Outlet	
Corn Cobs	11.243 (2.715)	5.352 (1.806)	0.105 (0.175)	0.252 (0.424)	1.125 (3.332)
Woodchips	11.001 (3.272)	4.087 (3.248)	0.018 (0.018)	0.069 (0.053)	0.371 (0.318)

Note: Standard deviations are in parenthesis.

Methylmercury production was defined as:

Eq. 3: *(Column output MeHg concentration - Input MeHg concentration) * Flow rate*

When the detection limit for methylmercury analysis of water in ng L^{-1} is multiplied by the number of liters flowing through a column in a given day, this yields 0.186 ng day^{-1} of methylmercury production. Methylmercury production ranged from -2.64 ng day^{-1} to 9.36 ng day^{-1} with an average of 0.69 ng day^{-1}. Columns filled with corn cobs had an average methylmercury production rate of 1.13 ng day^{-1} with a standard deviation of 3.33. The production rate for corn cob columns dropped below the detection limit one of the 9 sampling times and was negative twice, indicating demethylation or sorption of methylmercury. Those filled with woodchips had an average rate of 0.37 ng day^{-1} with a standard deviation of only 0.32. This rate was below the detection limit two times for woodchip filled columns, but was never negative. On December 17th (day 352), two out of three corn cob filled columns had MeHg production rates higher than 1.0 ng day^{-1}. Both the highest and lowest MeHg production rates were based on columns filled with corn cobs. The vast difference in variability between substrates seen in Figure G makes it difficult to differentiate between the mean production rates of each substrate. The non-parametric Wilcoxon test was performed to compare the mean of methylmercury production in corn cob filled columns with the same mean in woodchip filled columns. This Wilcoxon test resulted in a p-value of 0.9616 indicating no evidence to support a difference in means. Therefore it must be concluded that there is no significant difference in the MeHg production based on the carbon substrates tested in cold temperature settings. Furthermore, a one-tailed t-test (α=0.05) to compare the means of MeHg concentration at the outlet of the two types of columns also shows no significant difference with a p-value of 0.1182. All columns received water from the same source and their resulting output MeHg concentration were not significantly different. It is also useful to note that all averages of MeHg concentration and MeHg production shown in Table 2.3, including that of the source water, are within one standard deviation from their respective detection limits, making them indistinguishable from zero.

Discussion

These data suggest that when water temperatures are consistently below 40°F (4.4ºC), bioreactors do not support active mercury methylation by bacteria. Stagnant or low flow conditions are expected to allow for complete denitrification in bioreactors, which could give sulfate reducing bacteria and other anaerobic mercury methylators a competitive advantage over other bacteria. This study demonstrates the importance, however, of also considering the temperature of stagnant waters. When times of low flow occur during the winter in a Midwestern climate, the low water temperatures may allow for increased DO which favors aerobic bacteria instead. The higher levels of DO may also be a result of the reduced activity of aerobic bacteria which cannot consume as much oxygen from the water before it exits the bioreactor. Either way, low temperatures reduce the overall biologic activity within the bioreactor, including that of mercury methylators. This may have important implications for flows associated with snow melt or late autumn rainfall which could lie stagnant in bioreactors over the winter.

The data collected in December confirm that corn cobs, as a carbon substrate in bioreactors, have a higher nitrate reducing potential than do woodchips in the early stages of bioreactor operation. This is consistent with the findings of other scientists (Cameron and Schipper 2010; Feyereisen et al. *in review*) and has now been shown also to be true at relatively low temperatures (10-18°C). The nitrate removal rate of woodchip filled columns in this study averaged 6.18 g m^{-3} d^{-1} which is similar to rates found in a study by Warneke et al. (2011) that found 6.7 g m^{-3} d^{-1} NO_3^-N removed in April with an average temperature of 16.7°C. The longevity of corn cobs and their continued effectiveness were not examined in this analysis since most nitrate reduction was inhibited by low temperatures after December.

While their ability to support nitrate reducing bacteria clearly differs, corn cobs and woodchips did not show a significant difference in encouraging mercury methylating bacteria in this study. The output waters of corn cob filled columns had higher variability in terms of methylmercury concentration. It is possible that this difference in variability indicates that mercury methylating bacteria that survive in a corn cob filled bioreactor

have higher sensitivities to changes in environmental conditions. Both types of columns experienced similar shifts in temperature, DO, ORP and incoming nitrate concentrations. The MeHg production rate of woodchip filled columns remained between 0.0 and 1.0 ng day^{-1} throughout all sampling points. The corn cob filled columns, however, had negative production rates (-0.438 and -2.640 ng day^{-1}) measured on day 340 when the source water shifted from 37.2 to 48.6 mg NO_3^--N L^{-1} and the temperature reached a high of 18.6°C. Two out of three of the corn cob filled columns had unusually high MeHg production rates on day 352 (9.360 and 2.172 ng day^{-1}) when the DO levels of the source water and bioreactors dropped along with the ORP of the corn cob filled bioreactors. These extremes made it difficult to identify statistically significant methylation of mercury, but the details imply that corn cob filled bioreactors might induce mercury methylation under certain environmental conditions. They also imply, however, that corn cob filled bioreactors are likely to demethylate or absorb methylmercury under differing environmental conditions. Future studies are needed to pinpoint the net methylmercury production or loss given a series of conditions realistic for an intended bioreactor site.

The total mercury concentrations entering the columns were consistently higher than those measured by Shih et al. (2010). In their study, average total mercury concentrations at the inlet averaged less than 2 ng L^{-1}. Despite the increased Hg inputs in this study, with an average of 11.2 ng L^{-1}, the outlet methylmercury concentrations were sometimes lower. In the study by Shih et al. (2010), outlet methylmercury concentrations averaged 0.023 or 0.50 ng L^{-1} during nitrate-reducing and sulfate-reducing conditions respectively. In this study, outlet methylmercury concentrations had an average of 0.069 ng L^{-1} in woodchip filled columns and 0.252 ng L^{-1} in corn cob filled columns. The ORP measurements in this study rarely dropped below +100 mV, so the bioreactors were likely not reducing sulfate most of the time.

It is a positive outcome that there was no significant mercury methylation detected under these conditions. Future studies, however, should focus on warmer conditions that are more conducive to mercury methylation to ensure the safety of bioreactors filled with different carbon substrates. In addition to warmer temperatures, providing a longer HRT and lower nitrate concentrations should allow for nearly 100%

nitrate reduction which can lead to supporting SRB and other known mercury methylators. Conditions that could realistically be attained in Minnesota field bioreactors, but also maximize the potential for mercury methylation should consist of a 24 hour or longer HRT, nitrate concentrations at or near 0 mg L^{-1} and temperatures of approximately 15-20°C. In this study, there was sufficient total mercury present in the reverse osmosis water pumped through the columns, but depending on the water source of future studies researchers might also consider adding mercury at concentrations similar to that found in agricultural drainage water to observe whether or not it is transformed.

Chapter 3 : FIELD BIOREACTORS

Introduction

Woodchip bioreactors, as described in Chapter 1, have been installed as far north as Canada and as far south as New Zealand as a means of reducing nitrate entering surface and ground waters. The technology is growing in popularity, especially in areas where farmers are offered financial assistance to establish them. They are spreading with the best of intentions, but it is important to continue monitoring bioreactors in different locations and of different sizes and designs to ensure their continued efficacy and identify any unforeseen risks and consequences.

Several studies have examined possible pollution swapping in bioreactors through greenhouse gas emissions (Elgood et al. 2010; Greenan et al. 2001; Healy et al. 2012; Warneke et al. 2011; Feyereisen et al. (*in review*)), but these have not been shown to exceed the emissions from alternative forms of treating polluted water. Leaching of NH_4 and dissolved organic carbon (DOC) can be another unintended consequence of bioreactors, but most of this occurs in the early stages of use so it has been suggested that initial leachate be collected (Healy et al. 2012) or initial set-up of bioreactors occur during colder times of the year (Schipper et al. 2010) to reduce the effects downstream. A relatively new concern has been raised by Shih et al. (2010) regarding the methylation of mercury within bioreactors. Specifically, during conditions favoring sulfate reduction, researchers found increases in methylmercury concentrations at the outlet of a streambed bioreactor in comparison with its inlet. This study further investigated this risk in four active woodchip bioreactors on Minnesota farms. We attempted to determine the potential for mercury methylation during periods of low nitrate availability in edge-of-field bioreactors designed to treat agricultural waters.

The purpose of this study was to assess the risk of increasing methylmercury loads to the environment when using denitrifying bioreactors to mitigate nitrate pollution from agriculture. We worked on four southern and central Minnesota farms with active woodchip bioreactors. We intended to establish conditions within each system that would be optimal for the survival of mercury methylating bacteria. The main conditions

we targeted were: an approximate hydraulic residence time of 24-48 hours; water sources that were much lower in nitrate concentration than the typical drainage water; and reducing conditions as indicated by low ORP and DO concentrations. The long residence time and reducing conditions were designed to ensure that bacteria within the bioreactor would consume the nitrate in the water, resulting in outlet nitrate concentrations at or below 0.5 mg NO_3^--N/L. This concentration at the outlet of a bioreactor was defined as sulfate reducing conditions by Shih et al (2010), and was the level at which they found a pattern of mercury methylation. While we believe these conditions are not present during most of the year, it is likely that such long residence times and low nitrate concentrations could occur during warm, dry periods. We intended to develop this worst-case scenario to quantify the risk associated with it.

While creating these conditions, we planned to monitor each bioreactor for six-days. Our monitoring objectives included regular sampling for mercury and methylmercury at the inlet and outlet of the bioreactors. We analyzed these to determine if outlet concentrations of methylmercury were significantly higher than inlet concentrations, and if methylmercury, as a proportion of total mercury, was changing in the bioreactor. We also intended to collect and analyze samples for nitrate, sulfate, DOC and some key dissolved gas concentrations because of their associations with mercury methylating bacteria.

I hypothesized that the conditions we were creating would have different effects on different bioreactors because of the existing microbial communities present and the varying nutrients and respiration substrates available. Specifically, I hypothesized that bioreactors with low levels of sulfate would not be able to support bacteria that methylate mercury since many of those known to do so are dependent on sulfate as an electron acceptor. I hypothesized that some of the bioreactors would show signs of mercury methylation when maintained under these conditions, but that the overall quantity of methylmercury exiting the systems would be low.

Methods

This study aimed to establish conditions in field bioreactors that could potentially induce mercury methylation, such as: low flow, warm temperatures and low nitrate concentrations. After confirming that these conditions were reached, we monitored relevant parameters, including: flow rates, water temperature, ORP, DO concentrations and pH. During this monitoring period, we collected water samples from the inlet, outlet and central access hatch of the bioreactor to be analyzed for mercury, sulfate-S, nitrate-N, DOC and key dissolved gases. In doing so, we hoped to determine the threat, if any, of denitrifying bioreactors increasing the methylmercury load into nearby streams, rivers and lakes.

Field Procedures

To create a relatively steady, slow flow rate into each bioreactor for a six-day study, we used a pump to load water from a local source to a 500 gallon (1,893 L) tank near the bioreactor inlet. The source of water was different for each site and is listed in Table 3.3. For all bioreactors except the one at Morris, the source water was a type of surface water that was warmed by the sun during the day. This likely resulted in warmer temperatures within the bioreactors than would normally occur with tile drainage water. The hose drawing water from the source had several layers of mesh and wire wrapped around its end to filter out large particles. This crudely filtered water was pumped into the holding tank. We covered the tank to block sunlight to prevent algal growth. A PVC pipe attached to the tank directed water into the bioreactor inlet control structure. A paddlewheel flow gauge was inserted in the middle of the pipe. The water in the tank was gravity fed through the pipe and the paddlewheel was connected to a data logger to record the flow rate of water entering the system. There was a pressure transducer sitting near the bottom of the water tank connected to the same data logger. The data logger was programmed to turn the pump on anytime the water level in the tank dropped below a certain level and turn it off again when it reached a higher level, about 40 cm up. The

tank levels were selected to maintain enough hydraulic head in the tank to keep water moving into the bioreactor within a narrow range of flow rates. Flow rates were different for each site, but generally were selected to approximate the flow needed for a 24-48 hour hydraulic residence time in the bioreactor based on its estimated pore volume.

Monitoring and Sampling

We measured temperature, pH, and DO concentrations with probes inserted into the central access hatch of the bioreactor. A CR23X datalogger continuously recorded their values over the duration of the study. Manual measurements were also taken daily at the inlet and outlet of the bioreactors to assess pH and DO.

Table 3.1: Sampling schedule for chemical analyses

	Mercury & Methylmercury	Nitrate & Sulfate	Dissolved Gases (N_2O, CO_2, CH_4)	Dissolved Organic Carbon
Inlet	1 sample per 2 days: subsamples every 4 hours (ISCO 6700)	1 sample per 12 hours: subsamples every 1 hour (ISCO 3700)	1 sample per day: manually pumped	1 sample per day: manually pumped
Access Hatch	1 sample per day: manually pumped	1 sample per day: manually pumped		
Outlet	1 sample per 2 days: subsamples every 4 hours (ISCO 6700)	1 sample per 12 hours: subsamples every 1 hour (ISCO 3700)	1 sample per day: manually pumped	1 sample per day: manually pumped

The sampling schedule is outlined in Table 3.1. ISCO 3700 and 6700 autosamplers were used to collect samples for mercury, nitrate and sulfate analysis at the inlet and outlet of each bioreactor throughout the six-day study. Water subsamples (80 mL) for nitrate-nitrogen and sulfate-sulfur analyses were collected every hour into 2 bottles (one for NO_3^--N and one for SO_4^{2-}-S analysis) in an ISCO 3700 autosampler. Bottles were changed every 12 hours, producing 2 bottles for each analysis every day.

Bottles for nitrate analysis were preloaded with 1 ml of H_2SO_4 to preserve the sample. An ISCO 6700 autosampler was located next to each ISCO 3700 autosampler to collect water samples for mercury analysis. These samplers were equipped with 1L glass collection bottles that had been acid washed and preloaded with 4 ml of concentrated HCl for preservation. This 4 ml of HCl was diluted with DI water to a total volume of 20 ml for all sites except Windom. We did this to reduce the evaporative losses of acid noticeable at Windom.

We replaced external tubing with acid washed Teflon tubing and internal tygon tubing was also acid washed before beginning the experiment at each site. Subsamples (80 mL) were collected by the ISCO 6700 every 4 hours, resulting in a one liter sample for mercury analysis for every 2 days. Each bottle used for mercury analysis was covered with parafilm and only uncovered during the 2 days when that particular bottle was actively being filled. At the end of the six-day experiment, we screwed caps with acid washed Teflon lid liners onto all bottles.

We also pumped daily samples by hand from the access hatch for nitrate, sulfate and mercury analysis. The 1.0 liter bottles used for nitrate samples were preloaded with 1 ml of 18.4M H_2SO_4. We pumped mercury samples through Teflon tubing, a Teflon cap and into an acid washed 250 ml glass bottle. Immediately after collection, 1 ml of concentrated HCl was added to manually pumped mercury samples. We also pumped samples by hand from the inlet and outlet of the bioreactor for determination of DOC and dissolved gases (N_2O, CH_4 and CO_2). All hand-pumped samples were stored in coolers on ice until they could be transported back to a 4°C refrigerated storage room.

Bromide Tracer Test

The flow characteristics of all bioreactors were assessed with a bromide tracer test. Potassium bromide has been shown to be a conservative tracer in soil-water studies, including O soil horizons with relatively high organic matter concentrations (Levy and Chambers 1987). While this is not identical to the matrix of the bioreactors, the assumption was made that the amount of bromide lost to adsorption would be

insignificant. Other studies have also used potassium bromide as a tracer for hydraulic assessment of woodchip filled bioreactors (Cameron and Schipper 2012; Ghane, Fausey, and Brown 2015).

During the bromide tracer test, flow at all sites except Windom was established by pumping water into a tank and allowing it to be gravity fed to the bioreactor as described above. We measured flows with paddlewheels. At Windom, holes were drilled in between compartments of the inlet control structure to allow a slow delivery of drainage water as described in Site Specifics. While monitoring the outlet flow rates, we adjusted the flow into the bioreactors to produce a theoretical hydraulic residence time (HRT) of approximately 24 hours. Since leaking was occurring at some sites, establishing the inlet flow based on outlet measurements resulted in lower than desired HRTs at some sites.

Over the bioreactor inlet compartment, a bucket with a concentrated potassium bromide solution was hung and a tube from near the bottom slowly drained this bucket into a tipping rain gauge. The tips of the rain gauge measured the flow of concentrated bromide solution which then was diluted as it entered the water flowing in from the field inlet at Windom, or the tank at all other sites. The bromide solution was gradually added in this manner for 4 hours, except at Morris where it was poured in within 30 minutes because of precipitation of KBr.

Shortly after starting the input of bromide, ISCO 3700 autosamplers were set up at the access hatch and outlet of the bioreactor and programmed to collect a subsample every 6 minutes and change collection bottles after every 5 subsamples. This resulted in 24 samples, each representative of 30 minutes of flow. At the end of this 12 hour period, we reprogrammed the autosamplers to collect a subsample every 12 minutes resulting in 24 samples, each representative of 1 hour of flow. We transported these samples on ice and kept them in a 4°C refrigerated room until analysis.

They were analyzed for bromide concentration using an Orion bromide electrode (94-35) and double junction reference electrode (90-02) that were connected to an Orion EA940 ionAnalyzer. We adjusted the ionic strength of samples shortly before analysis by adding 1 ml of 5M $NaNO_3$ to 50 ml aliquots of sample. We continuously mixed

samples with magnetic stir bars during analysis. The flow measurements and bromide concentrations were used to calculate the total mass of bromide exiting the system.

The mass of bromide exiting the bioreactor was calculated based on the flow rates multiplied by the concentration of each sample and the time that sample represents (30 minutes or 60 minutes). For example, a sample representing 30 minutes of subsampling had a concentration of 70 mg L^{-1} bromide during which time the outlet flow was measured to be 62 L min^{-1}. The mass of bromide leaving the bioreactor at this time was calculated as follows:

30 min * 70 mg L^{-1} Br^- * 62 L min^{-1} = 130,200 mg Br^- = 130 g Br^-

To assess the flow characteristics more thoroughly, a series of calculations were made with the results of the bromide tracer test (Table 3.4). The theoretical hydraulic residence time (T) was determined based on inlet and outlet flow separately by multiplying the total volume of each bioreactor by an approximation of the porosity for woodchip filled media (0.52) (Feyereisen et al. *in review*) and dividing that by the average flow rate during the bromide test. The mean tracer residence time (t) was estimated as described in Christianson et al. (2013) and used to determine the effective volume (e). Next, the times associated with peak bromide concentration at the outlet (t_p), 10% (t_{10}), 16% (t_{16}), 50% (t_{50}) and 90% (t_{90}) of the bromide reaching the outlet were used to calculate additional indicators of flow as shown in Table 3.4 (Persson, Somes, and Wong 1999; Ta and Brignal 1998). These indicators are intended for use with a pulse input tracer test, in which all of the tracer solution is injected at time zero. While the four hour delivery of tracer makes it difficult to compare the results with previous studies, it is still useful for comparing each bioreactor to the others studied here.

Site Specifics

Table 3.2: A summary of bioreactor dimensions and characteristics

Site	County	Volume (m^3)	Length x Width (m)	Depth (m)	Contributing Area (ha)	Lined
Windom	Jackson, MN	128	22.86 x 3.05	1.83	23.47	Yes
Grand Meadow	Mower, MN	322	86.87 x 2.44	1.52	80.94	Yes
Granite Falls	Yellow Medicine, MN	148	22.86 x 3.05	2.29	8.09	No
Morris	Stevens, MN	200	18.30 x 6.10	1.80	8.09	Yes

Windom

The first bioreactor studied, near Windom, MN (Figure H), was the smallest of the four, but served the second largest area of contributing cropland (Table 3.2). The land that drains into it is in a corn-soybean rotation. It is equipped with a liner under the woodchips and two control boxes. The inlet control box includes a field input chamber, a bioreactor input chamber and a bypass chamber for unusually heavy flows (Figure I). The height of the boards between the field input and bioreactor input chambers can be adjusted to control how much water is allowed to enter the bioreactor. The height of the boards between the bioreactor input and bypass chambers can be adjusted to control how much water skips the bioreactor and exits the system directly. The output control box has two chambers. Water exiting the bioreactor fills the first chamber until it reaches the height of the boards between the chambers. At that point, the excess water flows over the top of the boards into the second chamber which allows for a direct path out of the system. Whether water exits through the input bypass or the output box, it all is directed downhill into Fish Lake.

During the experiment at this site, the water in the storage tank was gravity fed to the bioreactor input chamber of the control box with the flow rate continuously monitored by a 1 inch paddlewheel. Because the outlet pipe of the Windom bioreactor was difficult to access, we measured the outlet flow with a tipping bucket instead of a paddlewheel. We set the tipping bucket up at the outlet pipe leading to Fish Lake. Each

tip of the bucket was recorded by a CR3000 data logger and we combined these counts with manufacturer calibration data to determine the flow. The average flow rate exiting the system was 54% of the flow rate entering, indicating a significant loss of flow from the bioreactor. We suspect some of this water leaked below and to the sides of the bioreactor through rips in its liner and still more water may have been lost to evapotranspiration from the grasses planted above it. This leakage may be in part because it was the oldest of the bioreactors we studied, installed in 2009. We suspect that the bioreactor has completely dried and then been resaturated several times, accounting for the unusually fast degradation of the woodchips.

Because rain storms occurred before the bromide tracer test in Windom, there was a large quantity of water in the field ready to enter the bioreactor. To allow the field to drain, we used this water for the bromide test instead of filling the tank described previously. We drilled two holes into a water control board to allow water to pass from the field inlet to the bioreactor inlet chamber. The holes were 7/8 inches and ¾ inches in diameter. We installed two PVC pipes above the holes that allowed excess water to travel over the bioreactor inlet and into the bypass compartment (Figure J). The PVC pipes were each 2 inches in diameter. This excess water was drained to keep the head pressure from the field inlet compartment relatively steady to maintain consistent flow through the two lower holes. We measured the resulting flow at the outlet with a tipping bucket as described above. This system of water delivery, using holes and PVC pipes, was only used during the bromide tracer test and was not used at any other site.

Grand Meadow

The second site we studied is southwest of the town of Grand Meadow (Figure H). This was the largest bioreactor examined in this study in terms of volume and length, but it was not the widest or deepest (Table 3.2). Despite the large volume, this bioreactor had the lowest ratio of volume to contributing area since it received drainage waters from over 80 hectares of adjacent land. It was built in the shape of a semi-circle that wraps around a constructed wetland. The bioreactor outlet leads into the wetland for further treatment before the water exits into a drainage ditch. This helps to ensure substantial

denitrification. The bioreactor has a liner under the woodchips and two control structures, one at the field inlet and one at the outlet leading into the wetland. The inlet control box does not have a bypass compartment, so all tile drainage waters are directed through the bioreactor. The inlet and outlet flows were measured by 2 inch paddlewheels and were not significantly different from each other. This bioreactor was installed in 2011. With such a large contributing area and no bypass compartment, it is likely rare that this bioreactor is allowed to dry out completely.

Granite Falls

The bioreactor near Granite Falls, MN (Figure H) was designed to use one four-chamber control structure. Water extends away from the structure and then makes two turns to come back to different chambers within the same structure (Figure L). Flow rates were measured by 2" paddlewheels and monitored by data loggers. This bioreactor had the second highest ratio of volume to contributing area, meaning it would theoretically be one of the best candidates to consume all available nitrates and allow for the bacterial methylation of mercury. However, this bioreactor also had the most significant loss of flow through the bioreactor with the outlet flow rate only 34% of the inlet flow rate. We suspect that the lack of liner was a factor in this since it was several years newer than the other bioreactor with significant flow losses. It was installed in 2012. With a relatively small contributing area, however, it is more susceptible to drying during periods of low precipitation.

Morris

The final bioreactor tested was at the University of Minnesota West Central Research and Outreach Center in Morris, MN (Figure H). The general set-up was very similar to most other sites; however the outlet pipe was inaccessible. Consequently, we measured outlet flow with a pressure transducer that monitored the height of the water over a v-notch weir cut into the outlet control box. Inlet flow rate was monitored by a paddlewheel as with all other sites. Another unique characteristic of this site is a liner

over the top of the woodchips. While most of the bioreactors studied were installed with liners under the woodchips, this one also had one above to isolate the flow from precipitation and prevent evapotranspiration from within the bioreactor. Because of the lower temperatures at night we placed domes heated with a small space heater over the autosamplers at both the inlet and outlet to maintain temperatures above 0°C and prevent the freezing of samples. Average outlet flows were 87% of average inlet flows, indicating minor water losses. This bioreactor was installed in 2012 and is the widest of all that we studied. It has the largest ratio of volume to contributing area which could leave it vulnerable to dry out in the absence of sufficient rainfall or snowmelt.

Table 3.3: Dates of study, site specific water source and average flow rates.

Site	Dates of Study (2013)	Water Source	Inlet average flow (L min^{-1})	Outlet average flow (L min^{-1})
Windom	Aug. 7-13	Fish Lake	52.08 (4.79)	27.97 (2.26)
Grand Meadow	Aug. 29-Sept. 4	Drainage Ditch	121.95 (14.25)	119.57 (25.91)
Granite Falls	Sept. 24-30	Yellow Medicine River	122.89 (14.62)	41.38 (4.72)
Morris	Nov. 1-7	Well Water	65.05 (1.46)	56.44 (9.00)

Note: Standard deviation is in parenthesis.

Analytical Methods

Nitrate and sulfate analyses were performed by the University of Minnesota Soil Testing and Research Analytical Laboratory. Nitrate+nitrite concentrations were detected using a colorimetric technique (Henriksen and Selmer-Olsen 1970; ALPKEM Corporation 1986) on a Lachat 8500 flow injection analyzer after reduction to nitrite by a copper-cadmium column. A second measurement taken without the copper-cadmium column was subtracted to account for background nitrite concentrations, and nitrate concentrations were determined by difference. Sulfate was determined with a Dionex 120 ion chromatograph (U.S. EPA 1991).

Total organic carbon (TOC) and DOC were measured on a Phoenix-Dohrmann 800 carbon analyzer which removes inorganic carbon with nitrogen gas, oxidizes the

organic carbon and then measures the resulting CO_2 with non-dispersive infrared radiation (APHA, AWWA, WEF, 1992).

Dissolved gases, including nitrous oxide (N_2O), methane (CH_4) and carbon dioxide (CO_2) were measured using gas chromatography of headspace.

Samples collected for mercury determination were filtered with 0.7 micron glass microfiber filters (Whatman 1825-047) before analysis. They were analyzed for total and methyl mercury using the same methods described in chapter two.

Statistical significance was determined by t-tests ($\alpha = 0.05$) using JMP software (*SAS Institute Inc., 1989-2012*).

Results

Windom

Due to the alternate set-up at Windom during the bromide tracer test, the inlet flow did not come from the holding tank and was not measured by the paddlewheel as described previously. The holes used to deliver water to the bioreactor (Figure J) were drilled to establish the desired flow rate as measured by the outlet tipping bucket. It was later determined that there were significant losses of water between the inlet and outlet of this bioreactor. Therefore, it is likely that the inlet flow rate was much higher than intended since only the outlet was actually being measured during this test. It follows that the residence time was also much shorter. This discrepancy in flow rates must be considered when interpreting the results of the bromide test at Windom. The inlet flow rate was estimated by dividing the average outlet flow rate by the average water recovery (54%) from the later, six-day experiment (Table 3.4).

Only 65% of the bromide mass that was dripped into the bioreactor was measured at the outlet. The average water flow exiting the bioreactor during the tracer test was measured to be 59.6 L min^{-1} (standard deviation = 2.44). While it is possible that some of the apparent bromide losses could be the result of stagnant pockets of water that took more time to work their way through the bioreactor than the time for which it was being monitored, it is more likely that most of the lost bromide did not ever exit through the

control structure. Instead it likely leaked out of the bioreactor since the concentrations of bromide quickly dropped and did not show evidence of pulses later in the 36-hour monitoring cycle (Figure K). This theory is also supported by the fact that during the full six-day study, 46% of water was lost from the inlet to the outlet based on flow measurements.

The bioreactor at Windom had an effective volume, e, of 0.90 when calculated based on the estimated inlet flow. Since this value is close to one, it indicates that most of the volume of the bioreactor is being used. The Morrill Dispersion Index (MDI) of 4.67 shows that this bioreactor had the most dispersion of all those studied. None of the bioreactors studied exhibited an early peak which can be indicative of major short circuiting, but S values below 1 do suggest that some water may be travelling through preferential flow paths. Windom had the lowest S value of the four studied. These results describe a very leaky bioreactor that mixes solutes throughout its volume, while allowing some water to travel more quickly through preferential flow paths.

Table 3.4: Calculations from the bromide tracer tests

Site	$T_{in} = V*\rho/Q_{in}$	$T_{out} = V*\rho/Q_{out}$	$t = \frac{\sum t_i c_i \Delta t_i}{\sum c_i \Delta t_i}$	e = t/T (in, out)	$\lambda = t_p/T$ (in, out)	$S = t_{16}/t_{50}$	$MDI = t_{90}/t_{10}$
Windom	10.01*	18.54	8.98	0.90*, 0.48	0.60*, 0.32	0.64	4.67
Grand Meadow	22.68	22.20	16.58	0.73, 0.75	0.57, 0.59	0.78	2.22
Granite Falls	10.10	31.14	10.33	1.02, 0.33	0.79, 0.26	0.67	3.44
Morris	18.58	27.83	14.10	0.76, 0.51	0.81, 0.54	0.68	2.53

*Windom inlet flow was estimated. Variables are defined in Appendix C.

Table 3.5: Continuously measured conditions within the access hatch at Windom

Windom	Access Hatch		
	pH	DO (mg L^{-1})	Temp (°C)
Average	7.04	0.18	23.37
Min	7.03	0.16	22.32
Max	7.08	0.46	24.81
St Dev	0.01	0.02	0.68

After the bromide tracer test, a full six-day study was conducted as described previously. The control structure boards with holes were replaced with new ones so that the water entering the bioreactor going forward came from the holding tank only. During this time, the continuously measured pH averaged 7.04 (standard deviation = 0.01) in the access hatch. Daily pH measurements at the access hatch were slightly higher (avg = 7.16), but confirmed its circumneutral status. Daily pH measurements at the inlet (avg = 8.04) and outlet (avg = 6.87) suggest a decline in pH as water passes through the bioreactor. This increase may suggest fermentative bacteria within the bioreactor, since denitrification and other forms of anaerobic respiration tend to increase pH slightly.

The DO concentrations also declined as it passed through the bioreactor. Daily inlet measurements averaged 7.79 mg L^{-1} (standard deviation = 0.69). Daily access hatch measurements averaged 0.17 mg L^{-1} (SD = 0.10) with continuous access hatch measurements averaging 0.18 mg L^{-1} (SD = 0.02). This rapid decline indicates the consumption of most of the available oxygen between the inlet and access hatch of the bioreactor. At the outlet, the DO averaged just 0.05 mg L^{-1} (SD = 0.01). The average ORP in the access hatch was -544. These data clearly demonstrate that most biological activity in the bioreactor was anaerobic.

The temperature in the bioreactor was artificially raised by the source of water used in the experiment as mentioned previously. The average inlet temperature was 24.6°C (SD = 1.16). This cooled to an average of 22.5°C (SD = 0.45) at the outlet. The continuously monitored temperature readings from the access hatch averaged 23.4°C which agrees with the gradual, downward trend of temperatures across the bioreactor.

During the six-day experiment, the flow rates measured at the outlet were, on average, 54% lower than those at the inlet. When calculating the loads of the various analytes, the flow rate associated with the location from which the sample was taken (i.e. inlet or outlet) was used. The fact that flow rates were lower at the outlet, complicates load comparisons. Comparing concentrations at the inlet and outlet for this site assumes that analytes are lost proportionally with water seepage out of the bioreactor.

Table 3.6: Concentrations of dissolved organic carbon and dissolved gasses at Windom

Windom	Inlet (mg L^{-1})				Outlet (mg L^{-1})			
	DOC	N_2O	CH_4	CO_2	DOC	N_2O	CH_4	CO_2
Average	9.62	0.25	1.41	6582	9.66	0.28	408	7514
Min	7.19	0.02	1.26	2935	6.79	0.26	1.29	1182
Max	14.50	0.29	1.71	12264	12.94	0.31	2136	17417
St Dev	2.15	0.09	0.16	3262	1.72	0.01	709	5192

The inlet methane concentration averaged 1.41 ppm (SD = 0.16). The outlet was much higher in the beginning of the study with an average of 190.16 ppm (SD = 62.15) for the first four days, but it dropped sharply to an average of 1.44 ppm (SD = 0.21) for the last two days of monitoring. Methanogenesis appears to have been active at the start, but was suppressed, possibly by the addition of nitrate to the bioreactor during the last two days.

Dissolved nitrous oxide (N_2O) concentrations did not fluctuate as dramatically as methane. The average inlet concentration was 0.23 ppm (SD = 0.10) and the average outlet concentration was 0.28 ppm (SD = 0.02). There was no significant difference between these values to suggest production or consumption of dissolved N_2O.

There was not a clear pattern to the changes in carbon dioxide (CO_2) concentrations. The average inlet concentration was 6,582 ppm (SD = 3,262) and the average outlet concentration was 7,514 ppm (SD = 5,192). The samples collected at the outlet on the last two days of the study had concentrations of 1,182 and 1,195 ppm, much lower than the average for the first four days (7,779 ppm SD = 1,576). Since there was not a corresponding drop in the inlet concentrations, this implies CO_2 consumption within the bioreactor. The lower availability of CO_2 towards the end of the study may be part of the reason the methanogenesis activity appeared to decrease.

Table 3.7: Concentrations of mercury, nitrates and sulfates at Windom

Windom	Total Mercury (ng L^{-1})		Methyl Mercury (ng L^{-1})		Nitrate (mg N L^{-1})		Sulfate (mg S L^{-1})	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Average	3.33	5.37	1.50	3.14	0.55	0.03	8.96	7.72
Min	2.26	3.79	0.95	2.54	0.04	0.01	8.72	6.81
Max	4.41	8.26	2.10	3.61	2.15	0.09	9.21	8.74
St Dev	0.92	2.14	0.49	0.47	0.85	0.03	0.15	0.75

Sulfate-S concentrations consistently decreased from the inlet to the outlet of the bioreactor, but in the first 3 days of the study, that decrease was smaller than during the second half of the study (Figure M). On average, the inlet sulfate concentration was 8.96 mg S L^{-1} (SD = 0.15) and the outlet was 7.72 mg S L^{-1} (SD = 0.75). If sulfate reduction is defined as ((I – O)/I)*100% where I is the inlet concentration and O is the outlet concentration, the reduction for each day ranged from 2% to 22%. There was a gradual increase from 2% reduction on day 1 and it was consistently close to 20% for samples collected on days 4, 5 and 6.

Nitrate-N concentrations also decreased from the inlet to the outlet. During the first 4 days of the experiment, this decrease was significant ($p = 0.0061$, one-tailed t-test) despite the fact that nitrate concentrations were consistently below 0.1 mg N L^{-1} at the inlet, so the load of nitrate reduced was minimal. A solution of potassium nitrate was added to the inlet stream during the last 2 days of the study. As a result, the inlet concentrations were measured to be 0.89 and 2.15 mg N L^{-1} for the 5^{th} and 6^{th} days, respectively. The corresponding outlet concentrations were 0.02 and 0.09 mg N L^{-1}, indicating almost complete reduction of the nitrate added.

The total mercury concentration going into the bioreactor gradually decreased across the three sampling periods, as did the total mercury concentration at the outlet. Methylmercury concentrations, however, did not have a consistent upward or downward trend over time. The average methylmercury concentration at the inlet was lower than that of the outlet (1.50 ng L^{-1} compared with 3.14 ng L^{-1}), but it is also important to

consider the differences in flow at the inlet and outlet and how this impacts the resulting load of methylmercury entering and leaving the system.

When the concentration is multiplied by the flow to determine actual loads of methylmercury, there is no clear pattern or evidence to suggest methylmercury production. The average inlet load was 4,690 ng hr^{-1} (SD = 1,611) while the average outlet load was 5,254 ng hr^{-1} (SD = 739). A student's t-test (α=0.05) demonstrates that this difference is not significant (p=0.29). If we assume that the incoming methylmercury leaked out of the bioreactor proportionally with the water that leaked, we would expect an outlet load of 2,533 ng hr^{-1}, as compared to the measured load of 5,245 ng hr-1. Making this assumption does result in a significant difference between the methylmercury load at the inlet and outlet.

The ratio of methylmercury to total mercury concentrations at the outlet did rise from the beginning of the six-day study (0.44) to the end (0.86), but the inlet ratio also rose (0.22 to 0.64) and a similar t-test also revealed no significant difference between the inlet and outlet ratios (p=0.47).

Grand Meadow

The bromide tracer test at Grand Meadow resulted in the measurement of 71% of the bromide injected into the bioreactor exiting through the outlet. The flow rates at the inlet and outlet during the six-day study were much closer together than at Windom (122 and 120 L min^{-1} respectively). It did not appear that major losses of water were occurring throughout the bioreactor. This, combined with the fact that the concentration of bromide at the outlet did not decrease to the initial levels by the end of the monitoring period suggests that the bromide not detected was mostly the result of water held in stagnant pockets within the bioreactor gradually flowing out.

This bioreactor had the lowest hydraulic efficiency (0.57) when calculated based on inlet flows, described as "satisfactory" by Persson, Somes, and Wong (1999). It also had the lowest MDI of 2.22 indicating minimal dispersion and the highest value of S (0.78) indicative of less short circuiting (Table 3.4). The mean tracer residence time is 5-6 hours shorter than the theoretical residence time. This bioreactor clearly has portions of

its volume that are not effectively being used and are perhaps instead serving as areas of stagnation. Pockets of water trapped in these areas for longer periods of time would theoretically allow for complete microbial consumption of nitrates and possibly lead to redox conditions that promote mercury methylation.

Table 3.8: Continuously measured conditions within the access hatch at Grand Meadow

Grand Meadow	Access Hatch		
	pH	DO (mg L^{-1})	Temp (°C)
Average	7.03	0.17	19.39
Min	7.00	0.16	12.85
Max	7.05	0.18	24.60
St Dev	0.01	0.01	3.01

The pH was measured daily at the inlet, outlet and 3 access hatches along the length of the bioreactor. Every day showed a consistent decrease across the length of the bioreactor, but the range of pH values always remained within 6.47-7.36. The middle access hatch was also monitored constantly by a pH probe which recorded data to a data logger. This reading averaged a pH of 7.03 (SD = 0.01).

The daily DO measurements clearly indicate a rapid consumption of available oxygen within the bioreactor. The inlet concentration ranged from 7.8 – 10.9 ppm, while daily measurements for all three access hatches and the outlet were between 0.04 ppm and 0.17 ppm. The central access hatch was constantly monitored and had an average DO oxygen concentration of 0.17 ppm (SD = 0.006). The ORP monitored in the central access hatch remained below -300 every day of the study, confirming the presence of anaerobic conditions.

The water temperature in the bioreactor averaged 18.8°C across the inlet, outlet and all access hatches throughout the six-day study. A student's t test (α=0.05) revealed no significant difference in the average temperatures taken at different locations along the bioreactor, but there is a noticeable decline in temperature over time. The average temperature on the first day of the study was 22.6°C and on the last day was 16.1°C with a consistent decreasing trend in between.

Table 3.9: Concentrations of dissolved organic carbon and dissolved gasses at Grand Meadow

Grand Meadow	Inlet (mg L^{-1})				Outlet (mg L^{-1})			
	DOC	N_2O	CH_4	CO_2	DOC	N_2O	CH_4	CO_2
Average	1.62	0.40	1.38	1048.53	4.30	0.39	1.34	889.49
Min	0.42	0.36	1.34	547.08	2.26	0.38	1.29	660.24
Max	5.61	0.42	1.40	1501.85	6.17	0.41	1.42	1040.67
St Dev	1.66	0.02	0.02	358.60	1.32	0.01	0.05	134.63

This DOC concentration consistently increased across the length of the bioreactor. The average inlet concentration was 1.62 mg C L^{-1} while the average outlet concentration was 4.30 mg C L^{-1}. The added DOC may have leached from the woodchips in the bioreactor.

The dissolved gases measured showed no evidence of a significant trend across the length of the bioreactor. When inlet average concentrations were compared with outlet average concentrations, it resulted in a p-value of 0.22 for CH_4, p = 0.33 for CO_2 and p = 0.51 for N_2O. These gas data do not show clear evidence for the dominance of one type of bacteria or another within this bioreactor. They also confirm complete denitrification since there is not a significant production of N_2O throughout the bioreactor.

Table 3.10: Concentrations of mercury, nitrates and sulfates at Grand Meadow

Grand Meadow	Total Mercury (ng L^{-1})		Methyl Mercury (ng L^{-1})		Nitrate (mg N L^{-1})		Sulfate (mg S L^{-1})	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Average	5.72	13.13	2.13	1.73	3.46	BDL	6.29	5.48
Min	1.97	1.67	1.07	1.31	3.06	BDL	5.99	4.75
Max	12.13	35.02	3.31	2.07	4.05	0.02	6.88	6.76
St Dev	4.76	16.17	0.96	0.33	0.34	0.01	0.27	0.70

The concentration of sulfate entering the Grand Meadow bioreactor remained between 5.99-6.88 mg L^{-1} SO_4^{2-}S with a slight trend upward as time passed. Despite these consistent inputs, the amount of sulfate that was apparently reduced decreased over time (Figure M). The sulfate reduced was defined as inlet concentration minus outlet concentration. It peaked at 1.30 and 1.21 mg S L^{-1} based on the first two sets of samples collected on day 1 and declined to 0.01 and 0.13 mg S L^{-1} based on samples collected

during day 6. An analysis of variance of sulfate reduced by day confirmed the significant effect of time ($p = 0.046$).

The nitrate concentrations entering the bioreactor also remained relatively constant between 3.06-4.05 mg L^{-1} NO_3^--N. The samples collected from the outlet, however, all had concentrations below the detection limit except for one measured at 0.02 mg L^{-1}. This implies consistent, complete nitrate reduction across the length of the bioreactor.

The means of the total mercury (THg) concentrations at the outlet as compared to the inlet were not significantly different from each other ($p = 0.14$). The heterogeneous composition of water resulted in what appear to be outliers for THg concentration in two samples. One inlet sample had 12.13 ng L^{-1} THg and one outlet sample had 35.02 ng L^{-1} THg. The average of the other four inlet and outlet samples was 2.35 ng L^{-1} THg (SD = 0.64). Due to the small sample size, the outliers were not removed during analysis and contributed to the difficulty in detecting a significant difference.

Methyl mercury (MeHg) concentrations at the outlet were not significantly different from those at the inlet ($p = 0.19$). The concentrations were in the range of 1.07 – 3.31 ng L^{-1} MeHg with the inlet average (2.12 ng L^{-1}) being slightly higher than the outlet average (1.73 ng L^{-1}). There were no obvious outliers for MeHg concentration. The load of methylmercury was slightly higher at the inlet (15,218 ng hr^{-1}) as compared with the outlet (12,366 ng hr^{-1}), but with standard deviations of 7,277 and 4,176 respectively, these differences were not significant ($p = 0.25$). The ratio of MeHg to THg also did not change significantly across the bioreactor ($p = 0.96$).

Granite Falls

In the Granite Falls bioreactor, the concentration of bromide passing through the access hatch peaked after only 4 hours. It peaked at the outlet after 8 hours, but after 40 hours of monitoring there was still more than 30 mg min^{-1} of bromide passing through. In the end, only 24.55% of the injected bromide was measured exiting the bioreactor. The percentage measured at the access hatch was very similar, comprising 25.24% of the injected mass. These results indicate preferential flow paths in the bioreactor allowing

for a much shorter residence time than anticipated for some of the water while other portions may have been trapped temporarily in stagnant pockets. This is supported by a value of S = 0.67, lower than most other sites (Table 3.4).

In addition, the flow measured at the inlet and outlet of the bioreactor differed greatly. The average inlet flow during the bromide test was 128 L min^{-1} while the average outlet flow was only 41 L min^{-1}. Not only was bromide delayed by stagnant pockets, but this suggests that some also leaked out of the bioreactor. With only 32% of the inlet flow being measured at the outlet, this bioreactor leaked more than any other in the study. The unconventional u-shaped design might have contributed to this leakage.

The MDI of 3.44 shows that dispersion was prevalent. This is not surprising since water traveling around a bend would be expected to experience variations in velocity that would allow for greater mixing and dispersion.

Table 3.11: Continuously measured conditions within the access hatch at Granite Falls

Granite Falls	Access Hatch		
	pH	DO (mg L^{-1})	Temp (°C)
Average	6.22	0.07	16.57
Min	6.21	0.07	14.78
Max	6.23	0.07	18.25
St Dev	0.00	0.00	0.97

The daily measurements of pH averaged 7.24 with a slight decrease from inlet (7.41) to outlet (7.08). The pH probe constantly submerged in the access hatch recorded an average pH of 6.22.

The DO concentration measured daily averaged 9.17 mg L^{-1} at the inlet and quickly dropped to an average of 0.13 mg L^{-1} at the access hatch, indicating a rapid progression to anaerobic conditions. The DO and ORP probes that continuously monitored these variables in the access hatch confirmed the lack of available oxygen with average readings of 0.07 mg L^{-1} DO and -534 mV.

The average water temperature in the access hatch throughout the study was 16.57°C. The average of daily measurements was 16.72°C without any major changes from inlet to access hatch to outlet.

Table 3.12: Concentrations of dissolved organic carbon and dissolved gasses at Granite Falls

Granite Falls	Inlet (mg L^{-1})				Outlet (mg L^{-1})			
	DOC	N_2O	CH_4	CO_2	DOC	N_2O	CH_4	CO_2
Average	4.59	0.41	1.33	648.99	4.89	0.38	1.36	567.17
Min	4.10	0.39	1.30	567.88	4.21	0.35	1.27	486.71
Max	5.47	0.43	1.38	769.42	5.99	0.42	1.51	651.44
St Dev	0.56	0.02	0.04	66.52	0.66	0.03	0.09	68.71

The average DOC concentration was 4.74 mg L^{-1} with no significant difference between the inlet (avg = 4.59, SD = 0.56) and the outlet (avg = 4.89, SD = 0.66). There were no clear patterns to indicate net production or consumption of DOC within the bioreactor at this site. This is also true of all three dissolved gasses measured (N_2O, CH_4 and CO_2). Comparing the means of the inlet and outlet concentrations of each gas using a Student's t-test resulted in no significant difference for any of the gases.

Table 3.13: Concentrations of mercury, nitrates and sulfates at Granite Falls

Granite Falls	Total Mercury (ng L^{-1})		Methyl Mercury (ng L^{-1})		Nitrate (mg N L^{-1})		Sulfate (mg S L^{-1})	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Average	2.69	2.21	1.26	1.30	0.68	0.09	107.51	101.49
Min	2.20	1.75	0.99	0.99	0.13	BDL	94.65	85.80
Max	3.17	2.70	1.44	1.56	3.22	0.56	116.59	111.60
St Dev	0.42	0.41	0.20	0.24	0.91	0.16	6.26	6.31

The sulfate concentration in this bioreactor was much higher than that of the Windom and Grand Meadow sites. The average inlet concentration was 107.5 mg L^{-1} SO_4^{2-}S which was significantly higher than the average outlet concentration of 101.5 mg L^{-1} SO_4^{2-}S ($p = 0.0283$). This implies modest sulfate reduction within the bioreactor (Figure N).

Nitrate concentrations were low at this site with an inlet average of 0.68 mg/L NO_3^-N and an outlet average of 0.09 mg L^{-1} NO_3^-N. There were attempts to inject a potassium nitrate solution into the bioreactor slowly during the last two days to observe the effects, but because of the limited solubility of the solution and the colder

temperatures, it precipitated repeatedly and clogged the tube from which it was supposed to drip. The outlet nitrate concentration remained below 0.10 mg L^{-1} NO_3^-N for the first 3.5 days indicating nearly complete reduction of nitrate. It did rise after the attempted nitrate additions, but the outlet continued to be lower than the inlet. The samples with the largest difference were taken on day 5 with an inlet concentration of 3.22 mg L^{-1} NO_3^-N and an outlet concentration of 0.56 mg L^{-1} NO_3^-N.

The average total mercury (THg) concentration at the inlet of the bioreactor was 2.69 ng L^{-1}. The average outlet concentration was significantly lower at 2.21 ng L^{-1} ($p = 0.01$). This discrepancy implies that the bioreactor may have been filtering out some particulates which contained mercury or the mercury adsorbed to the wood chips. The MeHg concentration was not significantly different from inlet to outlet, and neither was the ratio of MeHg to THg ($p=0.19$). The load of MeHg was significantly higher at the inlet ($p < 0.0001$), but this can easily be accounted for by assuming that MeHg leaked out of the bioreactor proportionally with water. By multiplying the inlet load by the average water recovery at the outlet, we obtain an expected outlet load of 3,160 ng hr^{-1}. The actual outlet load averaged 3,163 ng hr^{-1} (standard deviation = 466), which is not significantly different from the expected load.

Morris

The bromide tracer test was monitored at the Morris location for only 23 hours because air temperatures fluctuating below 0°C put some equipment at risk. At this site, we also poured the KBr solution in within 30 minutes rather than dripping it slowly. During that time, 23.2% of the injected bromide was measured at the outlet. Possible reasons for this low recovery include precipitation of potassium bromide within the bioreactor due to the low temperature, leaking of bromide with water and stagnant pockets of water preventing it from flowing through within the monitoring period. By the end of 23 hours, the bromide concentration did not drop back down to the level it was at during the first few hours of monitoring, so it is likely that some stagnant pockets were preventing a portion of the bromide from flowing at a steady pace. Precipitation was observed within the bucket of potassium bromide solution that was being injected and

frequent stirring was required to keep the chemical suspended. It is therefore logical to assume that precipitation within the bioreactor was also a contributing factor to the bromide loss.

The effective volume (e) of this bioreactor (0.76) indicates some unutilized portions of the bioreactor which may be harboring stagnation. There does not seem to be as much dispersion as most of the other sites however, with a MDI of 2.53. This bioreactor had the highest hydraulic efficiency of all of those measured (0.81). These results suggest that most of the water travels smoothly through the bioreactor, but a significant amount leaks and another portion gets caught in eddies to be released later.

Table 3.14: Continuously measured conditions within the access hatch at Morris

Morris	Access Hatch		
	pH	DO (mg L^{-1})	Temp (°C)
Average	6.19	0.23	9.00
Min	6.18	0.21	8.65
Max	6.19	0.25	9.47
St Dev	0.00	0.01	0.17

The pH in the Morris bioreactor was consistently below 7. There was no significant change in the pH from the inlet to the outlet ($p = 0.75$). The daily averages of pH as measured continuously from the access hatch did gradually decrease over time, but remained within the range of 6.18-6.19.

The DO was quickly consumed within the Morris bioreactor. The average of daily inlet concentrations measured was 4.85 mg L^{-1} while the continuously monitored probe in the access hatch had average readings of 0.23 mg L^{-1} (SD = 0.01). Continuously monitored ORP readings stayed below -400 mV, although daily measurements were quite different and varied more than at other sites (range: -141 - +39 mV). Without knowing the full suite of chemical species present, it is difficult to fully interpret this discrepancy, but it is likely that reducing conditions sufficient to support SRB were present given the values recorded.

The water temperature within the bioreactor was near 9.0°C throughout the study. The continuously recorded temperature in the access hatch averaged 9.0°C during the six-

days. The average of daily measurements at the inlet, outlet and access hatch combined was slightly lower at 8.8°C. The minimum and maximum temperatures recorded daily both occurred at the inlet. The maximum was 9.2°C on day 2, and the minimum was 8.0°C on day 6.

Table 3.15: Concentrations of dissolved organic carbon and dissolved gasses at Morris

Morris	Inlet (mg L^{-1})				Outlet (mg L^{-1})			
	TOC	N_2O	CH_4	CO_2	TOC	N_2O	CH_4	CO_2
Average	9.33	0.33	1.37	1249	11.30	0.33	2.02	663
Min	8.58	0.30	1.25	531	9.24	0.28	1.41	569
Max	9.75	0.34	1.49	3748	15.95	0.36	3.61	761
St Dev	0.41	0.01	0.10	1234.43	2.40	0.03	0.91	68.94

The concentration of total organic carbon ranged from 8.58 to 15.95 mg C L^{-1}. The outlet concentrations were slightly higher on average, but not enough evidence was present to declare a statistical difference between the outlet and inlet ($p = 0.08$). The concentrations were, on average, higher than all other sites except Windom.

There is no evidence to suggest the net production or consumption of dissolved nitrous oxide in this bioreactor. The inlet and outlet concentrations both averaged 0.33 ppm, respectively. This confirms complete nitrate reduction to nitrogen gas.

Comparing the dissolved methane concentrations at the inlet and outlet requires inspection of their variability. The inlet average was 1.37 mg L^{-1} with a standard deviation of 0.10, while the outlet average was 2.02 mg L^{-1} with a standard deviation of 0.91. This difference was caused by two outlet measurements that were almost 1 or 2 ppm higher than the overall average concentration. It appears that some methanogenesis could have been taking place within the bioreactor during specific time periods.

Dissolved carbon dioxide concentrations followed a pattern nearly the opposite that of methane. The inlet and outlet concentrations were quite similar, except for one, much higher inlet measurement. This elevated carbon dioxide concentration could have been a source of electron acceptors for methanogens within the bioreactor since it occurred the day before the first elevated methane sample. Another possible explanation

is that it was an outlier, unrepresentative of the typical conditions within the bioreactor, since it only happened once.

Table 3.16: Concentrations of mercury, nitrates and sulfates at Morris

Morris	Total Mercury (ng L^{-1})		Methyl Mercury (ng L^{-1})		Nitrate (mg N L^{-1})		Sulfate (mg S L^{-1})	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Average	1.30	0.76	0.36	0.26	0.66	0.15	159.36	131.58
Min	0.59	0.51	0.24	0.08	0.01	0.00	126.98	110.50
Max	2.08	1.25	0.55	0.47	3.99	0.84	265.16	142.68
St Dev	0.64	0.36	0.14	0.17	1.44	0.26	49.59	7.93

The sulfate concentration was higher at Morris than at all other sites in this study. The average inlet concentration was higher (159 mg L^{-1} $SO_4^{2-}S$) than that at the outlet (132 mg L^{-1} $SO_4^{2-}S$) and this difference proved to be significant ($p = 0.0402$, one-tailed t-test). There were two measurements at the inlet over 100 mg L^{-1} higher than the overall average, so it is unclear if there is a pattern of sulfate reduction within the bioreactor or if these possible outliers are skewing the data (Figure N).

The well water pumped into this bioreactor during the study was very low in nitrate. For the first 4 days of the study, the average inlet concentration was 0.01 mg L^{-1} NO_3^-N. A potassium nitrate solution was added to the bioreactor during the last 2 days of the study, resulting in nitrate fluctuations between 0.01 and 3.99 mg L^{-1} $NO3^-N$ at the inlet. Before the nitrate additions, the outlet concentration remained below 0.01 mg L^{-1} $NO3^-N$, but during the last 2 days, the average was 0.45 mg L^{-1} NO_3^-N. The outlet nitrate concentration never reached 1 mg L^{-1} NO_3^-N, indicating that what little nitrate was present was being almost completely reduced within the bioreactor.

The total mercury (THg) concentrations at the inlet of the Morris bioreactor were consistently higher than those of the outlet with a notable significant difference ($p = 0.02$). The THg concentration at the inlet decreased over time, but never below the concentration at the outlet. It appears that particulates containing THg were being filtered by the bioreactor or the mercury was adsorbing to the woodchip surfaces. This is similar to the pattern seen in the Granite Falls bioreactor.

The methyl mercury (MeHg) concentrations at the outlet also decreased over time and were always lower than the concentrations at the inlet for the same and previous days. The difference between inlet and outlet concentrations was not statistically significant. The loads of MeHg were also higher at the inlet, but not significantly different between the inlet and outlet ($p = 0.08$). The difference remains insignificant even when the leaking of MeHg with water is accounted for ($p = 0.24$). The ratio of MeHg:THg remained stable across the bioreactor with an inlet and outlet average of 0.32.

Discussion

The purpose of this study was to create conditions within real, functioning bioreactors in Minnesota, that could theoretically support mercury methylating bacteria to determine if their activity would result in increased amounts of methyl mercury being produced and discharged into local waterways. Water was pumped slowly into each bioreactor to generate a 24-48 hour residence time. This created highly reducing conditions within the bioreactors. The water used for pumping was selected based on proximity, but generally resulted in warmer water temperatures within the bioreactor than would have been present under natural conditions. The nitrate concentrations of the water were very low since the water was not coming from the drainage of cropped fields as it normally would be. Warm temperatures, low levels of nitrate and intense reducing conditions at most sites would all theoretically support the growth of SRBs and methanogens, both groups of bacteria known to include mercury methylators. Despite all of these measures, only one of the four bioreactors studied showed evidence of possible mercury methylation.

Most of the bacteria that have thus far been implicated in the methylation of mercury belong to the *Deltaproteobacteria* class (Kerin et al. 2006; Yu et al. 2013). Most of them are anaerobic bacteria that use sulfate or iron as electron acceptors and therefore have a survival advantage when higher energy yielding electron acceptors like oxygen and nitrate are depleted. While the flow characteristics of each bioreactor in this study varied greatly, it is clear that anaerobic conditions were achieved in all of them. The average DO concentration present in the middle of the bioreactors based on

continuous measurements ranged from 0.07 mg L^{-1} at Granite Falls to 0.23 mg L^{-1} at Morris. The average DO concentration at the outlet was below 0.20 mg L^{-1} for all sites (Table 3.17). ORP readings were consistently below -300 mV. Connell & Patrick (1968) studied sulfate reduction while controlling redox potential in two waterlogged soils. They found a dramatic increase in sulfate reduction as the ORP dropped below -150 mV, confirming that -300 mV is low enough to allow for sulfate reduction to occur.

Table 3.17: Average dissolved oxygen concentrations measured daily

Average DO Concentrations		
Site	**Inlet**	**Outlet**
Windom	7.79 (0.69)	0.05 (0.01)
Grand Meadow	9.43 (0.99)	0.08 (0.03)
Granite Falls	9.17 (0.71)	0.18 (0.06)
Morris	4.85 (0.28)	0.11 (0.06)

Note: Standard deviations in parenthesis.

Dias et al. (2008) studied several strains of anaerobic, sulfate reducing bacteria that are known to methylate mercury. They found a common optimal temperature for 3 strains of *Desulfomicrobium* to be 35ºC. Another strain and close relative had a lower optimal temperature range of 25-30ºC. Other mercury methylating bacteria have been studied at 30ºC (Kerin et al. 2006) and 25ºC (Compeau and Bartha 1985). The water inside of a Minnesota bioreactor is not likely to reach these optimal temperatures, but the temperature may be warm enough to support some growth. The water temperature in the warmest bioreactor in this study averaged 23.4 ºC. This was surface water that had been warmed by sunlight before being artificially pumped into the bioreactor. Under natural drainage conditions, the water would usually be colder.

The study by Dias et al., (2008) identified an optimal pH of 7.2 for the same bacteria. All of the bioreactors in this study maintained pH values between 6 and 8. Windom and Grand Meadow were notably close to the optimum determined by Dias et al. at 7.04 and 7.03, respectively. Despite these circumneutral pH values capable of supporting known mercury methylating bacteria, methylation does not appear to be likely in most Minnesota bioreactors.

Since many known mercury methylating bacteria depend on sulfate as an electron acceptor, researchers have established a link between sulfate concentration and mercury methylation rates (Coleman Wasik et al. 2012; Gilmour, Henry, and Mitchell 1992). If sulfate concentrations are too low, SRB growth and activity can be limited, but excessively high sulfate concentrations have also been shown to reduce mercury methylation (Gilmour, Henry, and Mitchell 1992). The exact range of sulfate concentrations that allows for mercury methylation can vary depending on other environmental factors. Gilmour et al. (1992) found higher concentrations of methylmercury after incubating sediment under water with 2-100 μM sulfate added. In another study, 200-500 μM sulfate was identified as the optimal concentration (Gilmour and Henry 1991). The sulfate concentrations in the Windom and Grand Meadow bioreactors were within this range throughout the study (Table 3.16). The Granite Falls and Morris bioreactors, however, had much higher sulfate concentrations, generally above 1,000 μM sulfate. There was evidence of some sulfate reduction occurring in at least 3 out of the 4 bioreactors examined, including Granite Falls with concentrations of sulfate above 500 μM. It appears plausible that sulfate-reducing bacteria were active in the bioreactors studied.

Table 3.18: Minimum and maximum sulfate-S concentrations in μM.

Sulfate Concentration Ranges (μM)		
Site	**Min**	**Max**
Windom	70.89	95.88
Grand Meadow	49.45	71.62
Granite Falls	893.19	1213.72
Morris	1150.32	2760.36

The apparent reduction of sulfate in the Grand Meadow bioreactor seemed to decrease over time. As sulfate is reduced, the products include sulfides, which create a negative feedback for additional sulfate reduction. This may not be as evident at other sites due to other species present that react with the sulfides and take them out of

solution. If iron is present, for example, low redox conditions within the bioreactor would have allowed Fe^{3+} to be reduced to Fe^{2+}, making it available for bonding with S^{2-}. These species may react to form solid FeS. Oxidized iron was observed precipitating on tubing at the Morris site as indicated by its red color. This suggested that this site had enough iron to form FeS as described above. The Grand Meadow site likely did not have sufficient iron for this process and allowed a build-up of sulfides, inhibiting future sulfate reduction.

Both sites with high sulfate concentrations, Granite Falls and Morris, experienced an apparent loss of total mercury from inlet to outlet. Sulfides (S^{2-}) produced in sulfate reduction may have bonded to ionic and/or methyl mercury to form insoluble HgS or MeHgS and remained inside the bioreactors.

A previous study that examined the possibility of mercury methylation in bioreactors defined "sulfate-reducing conditions," which seemed to allow for mercury methylation, as a period of time in which the outlet nitrate-nitrogen concentrations were below 0.5 mg L^{-1} (Shih et al. 2010). Table 3.19 shows that in the four sites we studied, the average nitrate-nitrogen concentration was always below that threshold at the outlet. There was one sampling time out of twelve at Morris and one sampling time at Granite Falls for which it rose above 0.5 mg L^{-1}. These data clearly demonstrate that all four bioreactors had sufficiently low nitrate-nitrogen concentrations to support sulfate reduction. Despite this constructed advantage for bacteria that are known to methylate mercury, our results did not demonstrate the apparent methylation observed by Shih et al. (2010).

Table 3.19: Maximum and average measured $NO3^-$-N concentrations at the outlets

Outlet NO_3^--N Concentrations (mg L^{-1})		
Site	**Maximum**	**Average**
Windom	0.09	0.03
Grand Meadow	0.02	BDL
Granite Falls	0.56	0.09
Morris	0.84	0.15

Note: BDL = below detection limit

Comparing concentrations of methylmercury from the inlet of the bioreactor to the outlet can be deceiving. If the flow rate exiting a bioreactor is very slow, even a high concentration may not be adding a significant amount of methylmercury to the downstream waters. As soon as that water leaves the bioreactor, it is greatly diluted by the waterbody it enters. It is also important to consider a difference between flow rates at the inlet and outlet of a bioreactor. If a large percentage of water is lost as it passes through the bioreactor due to seepage or transpiration of plants above, the methylmercury may appear to be enriched at the outlet provided it is not lost in the same proportions as the water. To avoid misconceptions, we used the measured flow rates in conjunction with concentrations to calculate the actual load of methylmercury entering and exiting the bioreactors. We also compared the ratio of methylmercury to total mercury since it would be expected to increase if methylation were occurring. A two-tailed t-test was performed to compare the inlet and outlet loads of methylmercury as well as their ratios of methylmercury to total mercury (α=0.05). The only inlet-outlet combination that yielded a significant difference in either category was the load at Granite Falls. At this site, the average methylmercury load was found to be higher at the inlet, indicating a loss of methylmercury across the system.

Table 3.20: T-test results comparing outlet and inlet loads and ratios of methylmercury

T-test comparing outlet and inlet values	MeHg Loads (ng)	MeHg:THg Ratio
	p value (2 tailed)	p value (2 tailed)
Windom	0.29	0.47
Grand Meadow	0.25	0.96
Granite Falls	<0.01*	0.19
Morris	0.08	0.98
*Note: Inlet average MeHg load was higher than outlet.		

The bioreactor in this study with the highest percentage of water lost from inlet to outlet was at the Granite Falls site. This was also the only u-shaped bioreactor, and it had the second largest volume to contributing area ratio (18). I suspect that the u-shaped

design allows for unequal water velocities across the width of the bioreactor which makes stagnant pockets more likely. Combining this with a relatively small contributing area may lead to more frequent drying out of the woodchips when rainfall is sparse. As the woodchips wet and dry repeatedly, they are degraded at a faster rate resulting in a less effective bioreactor. While the bioreactor at Windom was not u-shaped and had a much smaller volume to contributing area ratio (5), it also had a bypass compartment. When water is allowed to bypass the bioreactor to help drain fields, the bioreactor is more likely to dry out before the next big rainfall event. This feature may be partially responsible for water losses observed at Windom. Grand Meadow was the site with the smallest volume to contributing area ratio (4) and also the site with little or no water lost. Its long, straight design with a relatively large contributing area and no bypass compartment may prove to be a more long lasting design choice.

Conclusions

There was no clear evidence found in this study to indicate the methylation of mercury in Minnesota bioreactors, although there is some suggestion that it might have been occurring at times in the Windom bioreactor. Based on what is known about mercury methylating bacteria and their optimal growth conditions, the conditions in the bioreactors during this experiment should have been more likely to stimulate mercury methylation than the conditions that typically exist in Minnesota bioreactors. Despite the elevated temperatures, reduced nitrate concentrations and long residence times, these still do not appear to be effective sites for producing methylmercury.

The inside of a bioreactor is designed to encourage microbial growth. As a result, they are likely filled with a complex community of bacteria that are well adapted to the nutrients and conditions normally present. While there may be periods of relatively warm, low-nitrate, and slow-flowing waters, the bacterial community has developed to take advantage of the nitrate-rich drainage water from adjacent farm fields. The bacterial community may not have enough time to adapt to the occasional conditions created in this study and allow for the dominance of SRBs and other types of mercury methylating bacteria. The denitrifying bacteria that ideally dominate the inside of a bioreactor may be

able to persevere despite fluctuations in available nitrate, and they appear to help in outcompeting possible mercury methylators.

Chapter 4 : CONCLUSIONS

Denitrifying bioreactors are a valuable tool in the quest for cleaner water. The studies herein confirm that they are unlikely to pose a significant risk of mercury methylation at the edge of farm fields in Minnesota. Times of the year when Minnesota farms experience low rainfall and produce drainage with low nitrate concentrations are theoretically the highest risk times of year with regard to mercury methylation. Since these conditions are most likely to occur in the winter or during an early spring snowmelt, the cold water temperatures in the drain tiles underground will likely negate the risk. DO levels in the water create circumstances unfavorable to the bacteria known to methylate mercury. The only bioreactor shown to produce methylmercury in these studies had average water temperatures above 23°C, higher than is expected from tile drainage especially during periods of low nitrate concentrations. In addition, the sulfate concentration of drainage waters in western Minnesota may be sufficiently high as to depress mercury methylation.

Corn cob filled bioreactors may be a viable alternative to woodchips, but they come with some uncertainty. The methylmercury concentration at the outlet of corn cob filled columns was much more variable than that of woodchip columns. While the overall trend did not reveal significant mercury methylation, the wide range of results indicates a need for additional monitoring. Monitoring of an active corn cob filled bioreactor in a farm field would provide the most useful perspective.

In addition to considering the temperature and DO concentration in a bioreactor at times of low nitrate loading, it may also be important to consider other species present. While sulfate reduction is often considered an important indicator of mercury methylation potential, it occurred in three out of four bioreactors studied without evidence of mercury methylation. In some cases, the sulfides produced in sulfate reduction may have inhibited further reduction and therefore limited the growth of mercury methylating SRBs. In other bioreactors, the sulfides may bind with available mercury creating insoluble HgS molecules. In either case, it may be possible that the

microbial communities did not have sufficient time to adapt since they have evolved to survive an environment that is often enriched with nitrate.

Woodchip bioreactors are generally a safe, relatively inexpensive method of improving water quality at the edge of farm fields. The bioreactor is this study with the highest S value, indicating less short circuiting, the lowest Morrill Dispersion Index, and the least water leakage was the Grand Meadow site. This was the longest, narrowest bioreactor with the smallest volume to contributing area ratio (3.98 m^3 ha^{-1}). This site did show evidence of some areas of stagnation, but it was not sufficient to result in the methylation of mercury. A long, narrow design such as this may lead to better hydraulic characteristics, but if it is too long for the area it serves, it may reduce all nitrates in a plug of water before it exits the bioreactor. This could theoretically allow for sulfate reducing bacteria to dominate in the later sections of the bioreactor, and possibly pave the way for mercury methylation. This is why it is crucial to combine a long narrow design with a low volume to contributing area ratio. This will help to ensure the woodchips remain saturated, degrade more slowly and encounter higher concentrations of nitrate throughout the year. If well designed, a woodchip bioreactor can effectively reduce nitrates for more than a decade with little to no risk of promoting mercury methylation.

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Appendix A

Chapter 1 Figure

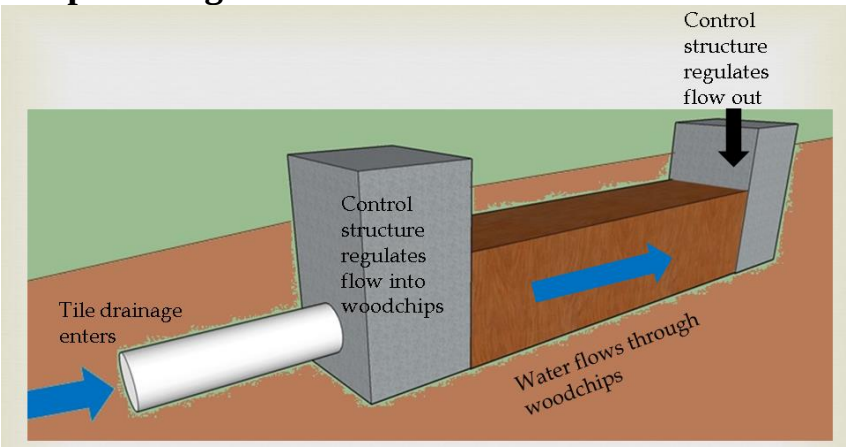

Figure A: Diagram of denitrifying bioreactor

Chapter 2 Figures

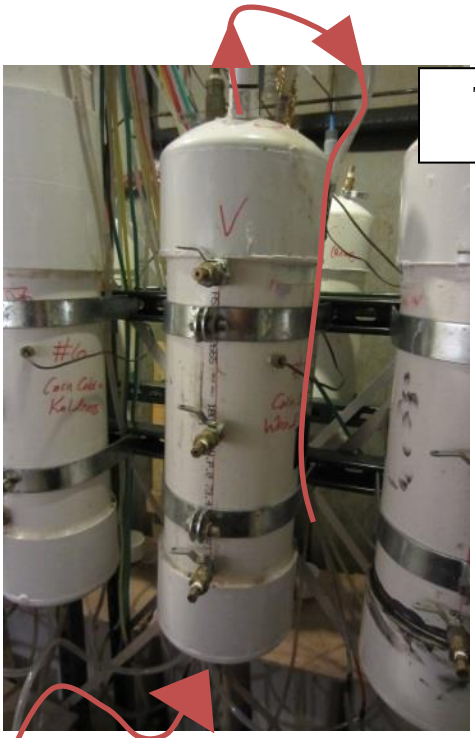

Figure B: PVC column filled with carbon substrate and connected to peristaltic pump

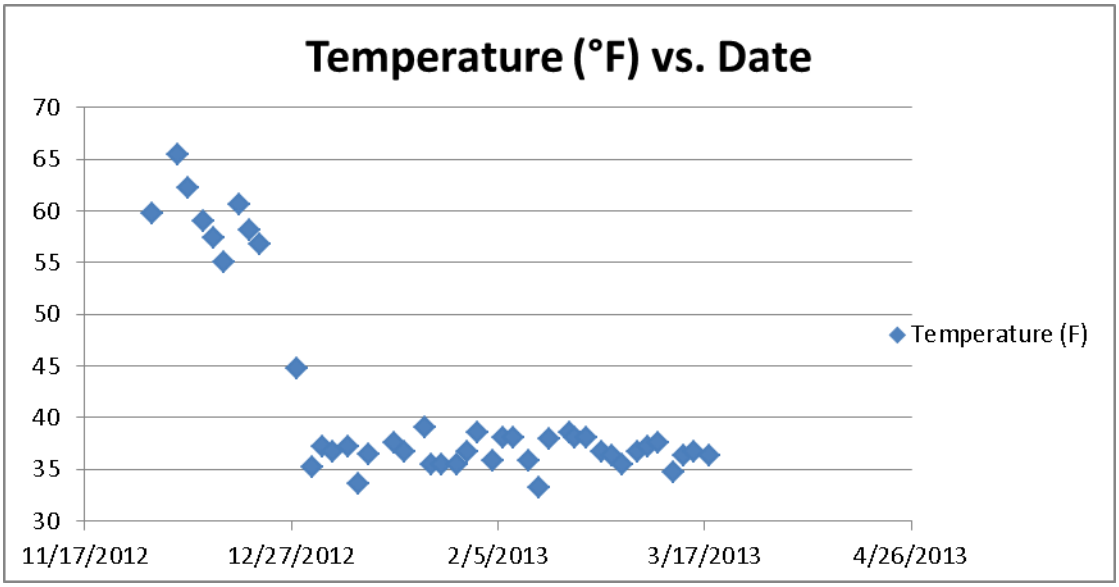

Figure C: Temperature in Fahrenheit of input water throughout the study period

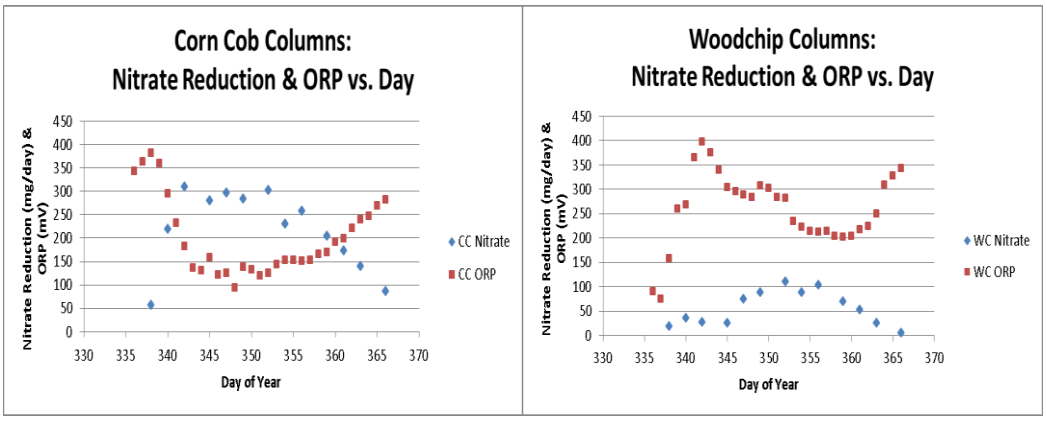

Figure D: Nitrate reduction and oxidative reduction potential within both types of columns throughout the study period

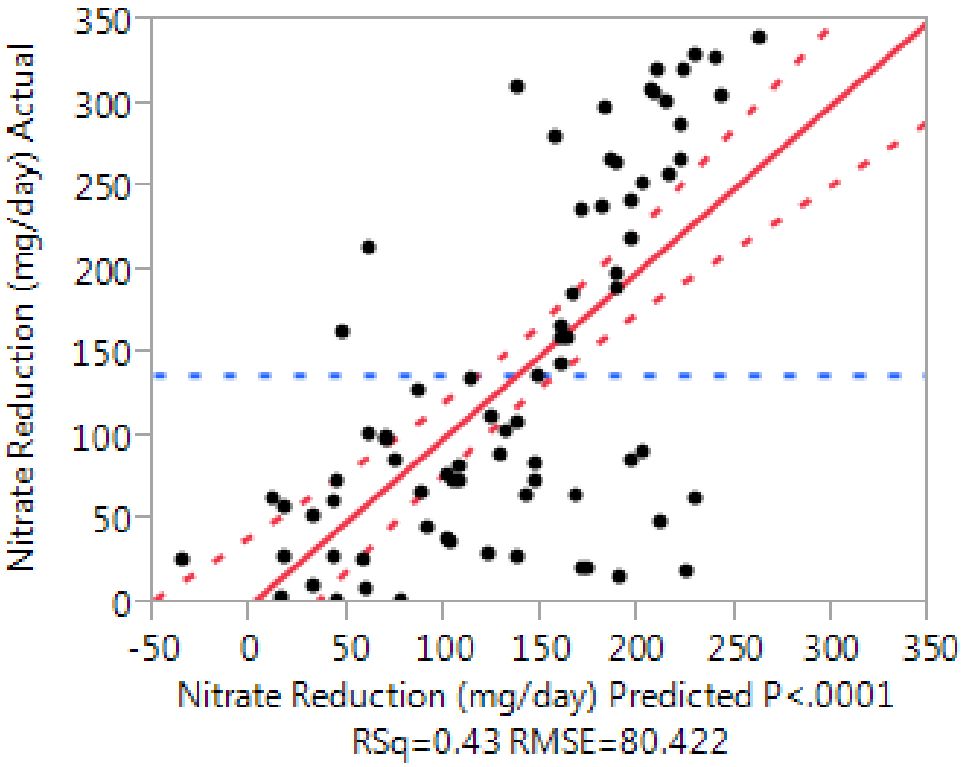

Figure E: JMP plot of nitrate reduction in all columns vs. nitrate reduction predicted by equation 2

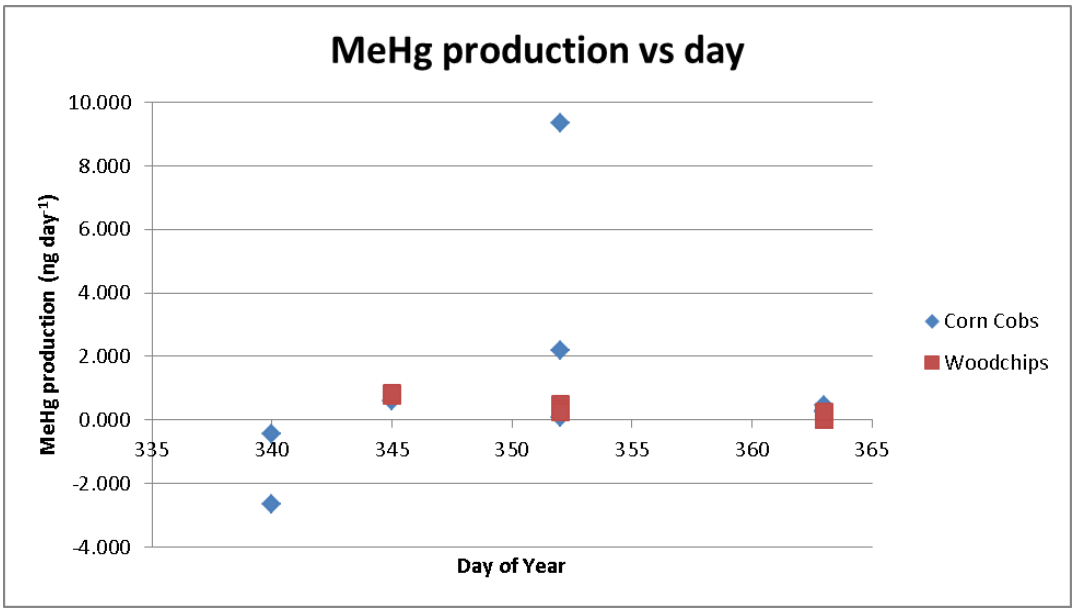

Figure F: Methylmercury production (equation 3) throughout the month of December

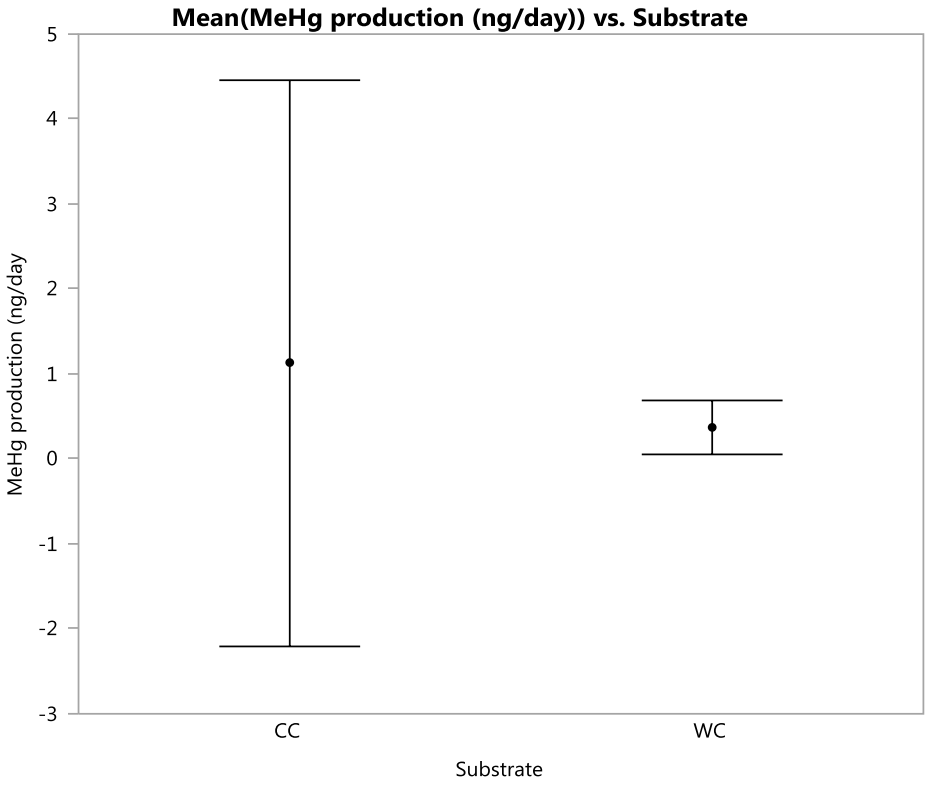

Figure G: Mean methylmercury production per day for each substrate. Each error bar is constructed using 1 standard deviation from the mean.

Chapter 3 Figures

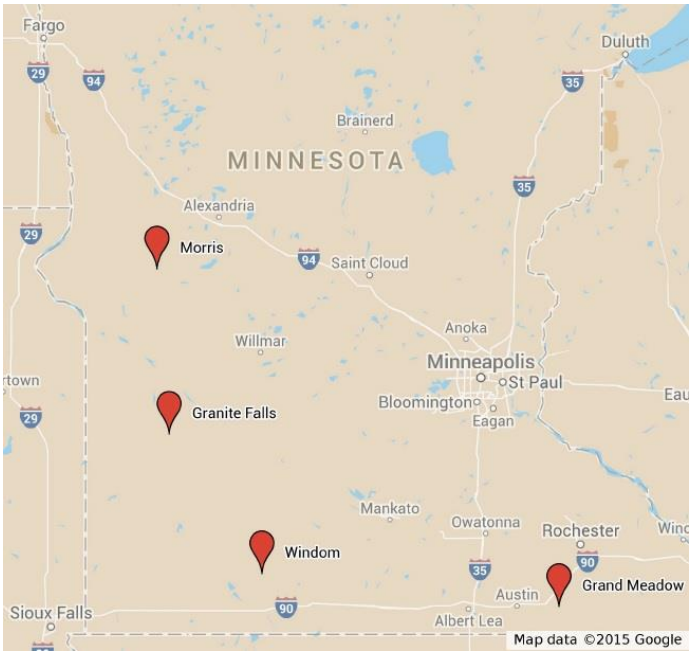

Figure H: Map of bioreactor sites

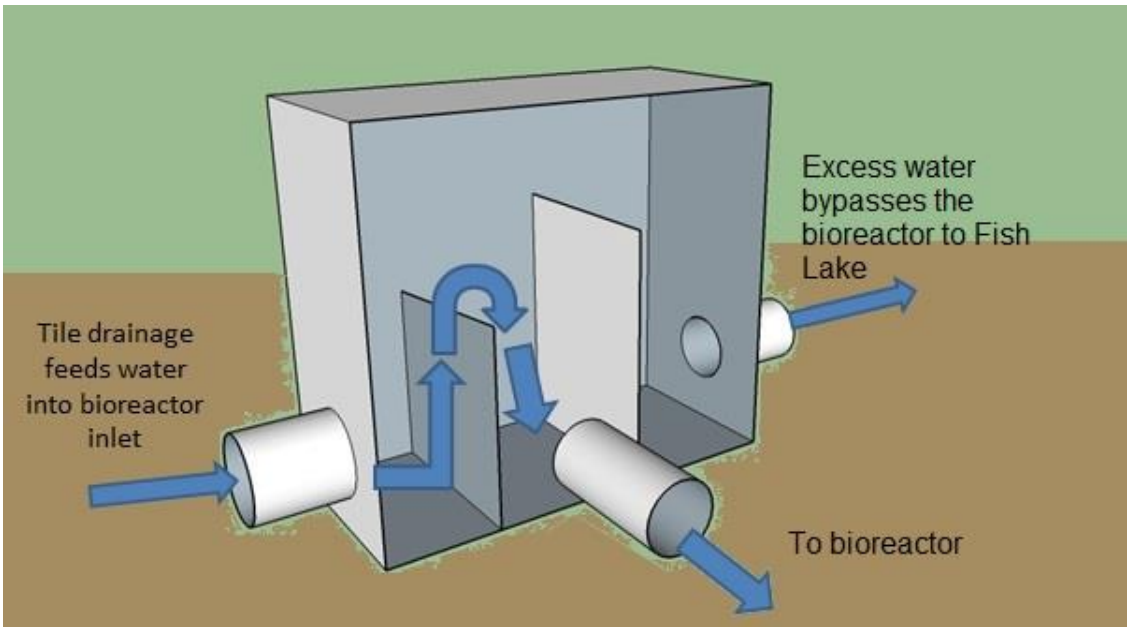

Figure I: Inlet control box for Windom bioreactor

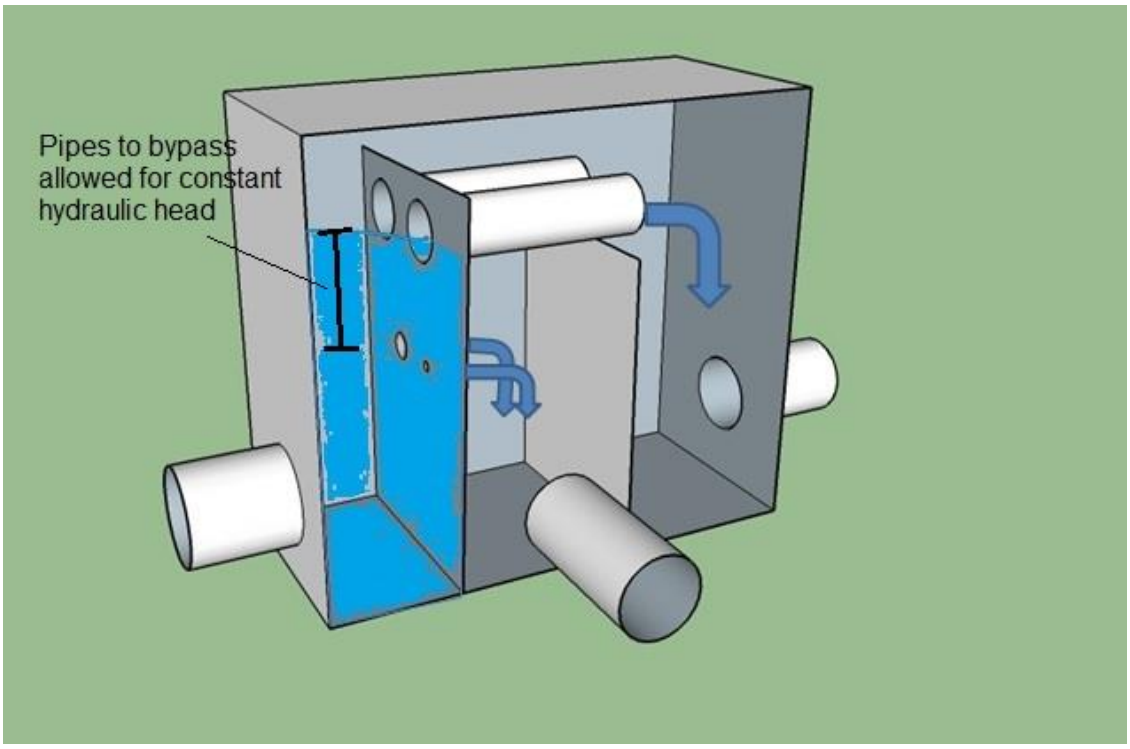

Figure J: Inlet control box for Windom bioreactor with holes drilled between the 1st and 2nd compartments and bypass pipes added to maintain a constant flow through the holes.

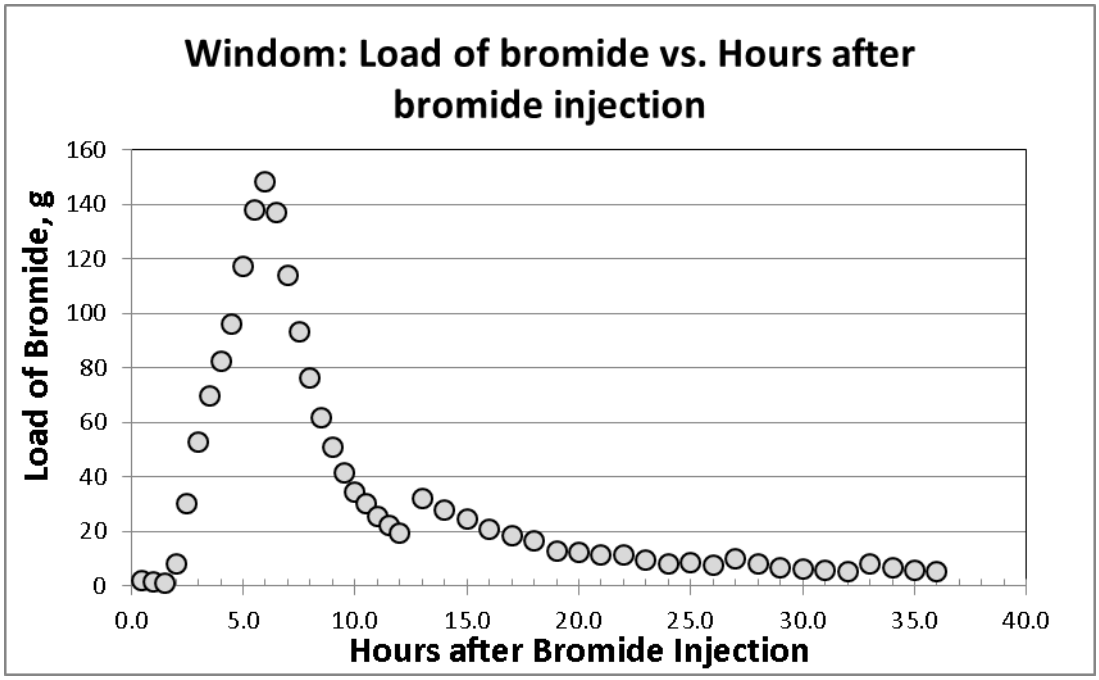

Figure K: Plot of bromide load as calculated from concentration and flow measurements at the outlet of the Windom bioreactor

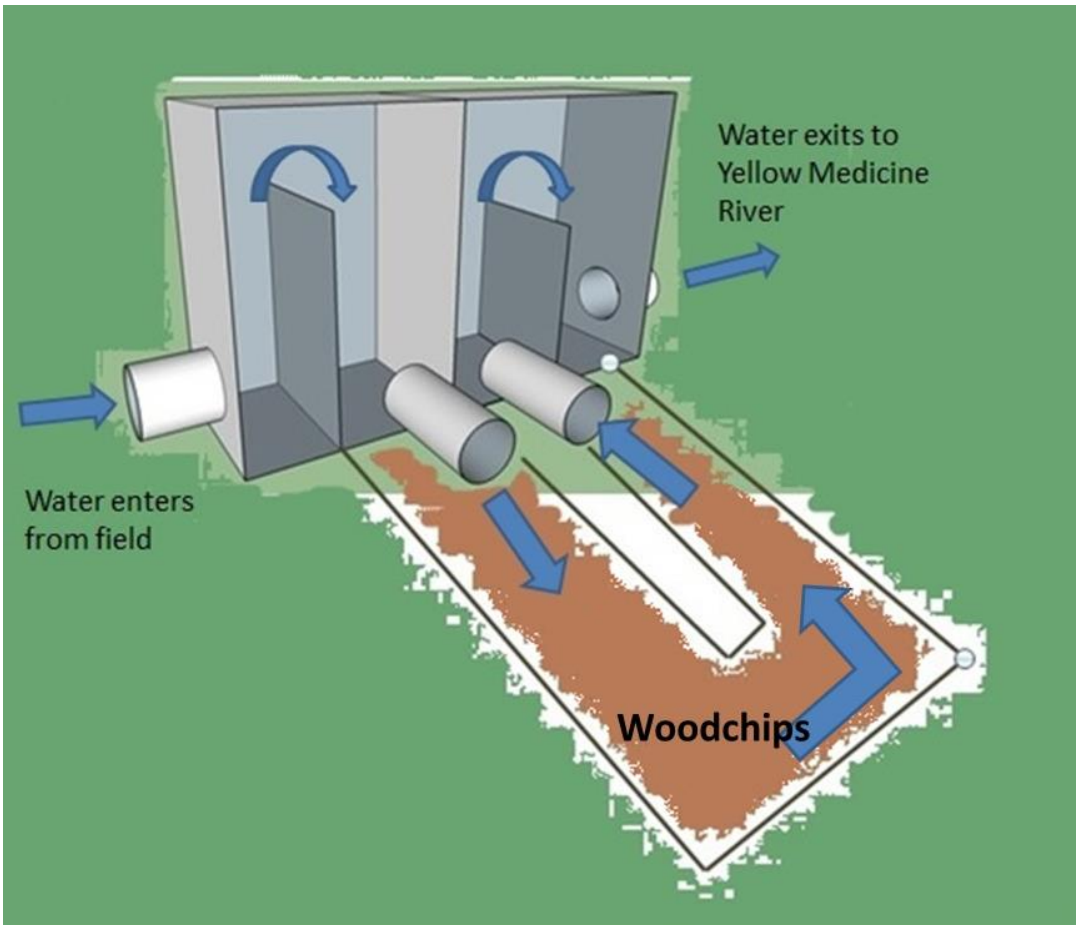

Figure L: Granite Falls loop design bioreactor

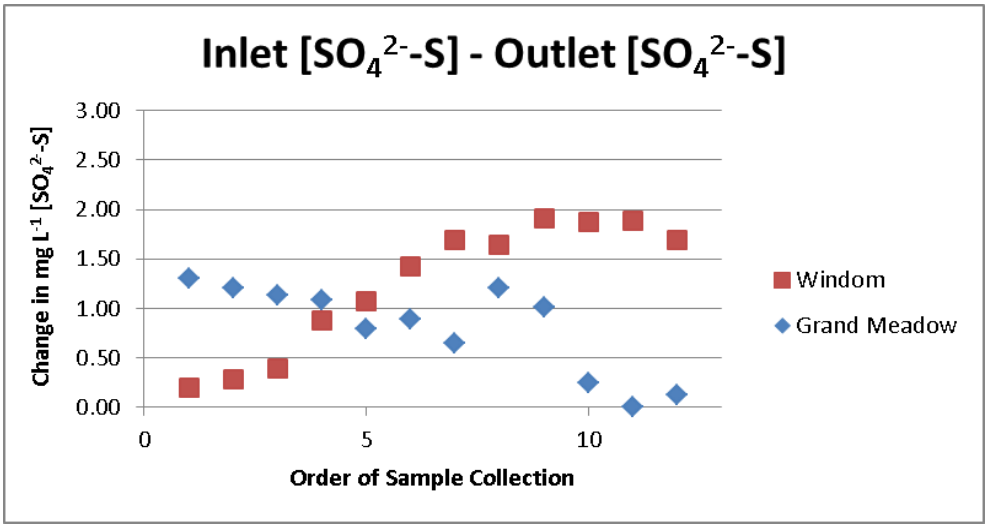

Figure M: Change in sulfate-sulfur concentration between the inlet and outlet of the Windom and Grand Meadow bioreactors

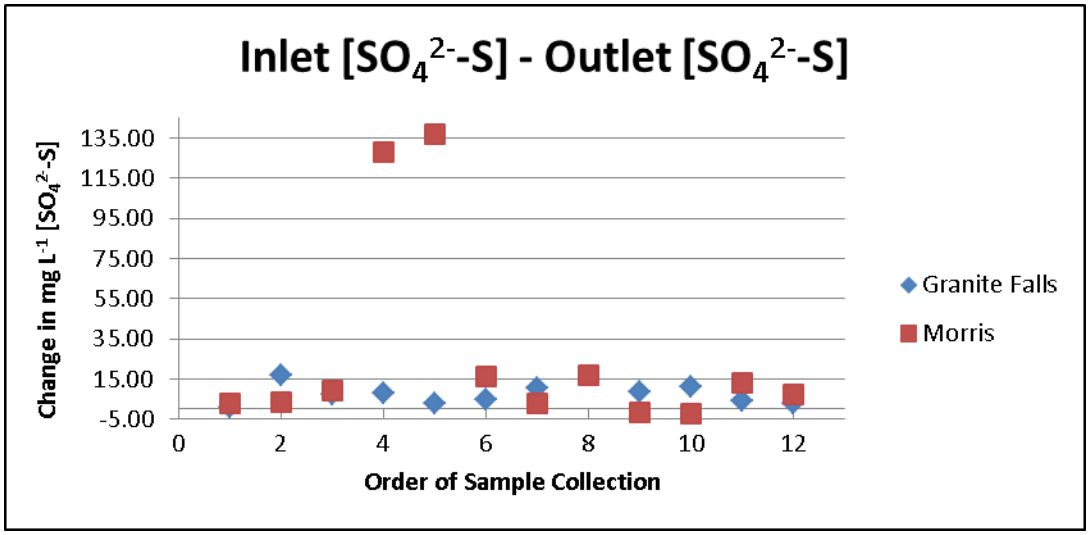

Figure N: Change in sulfate-sulfur concentration between the inlet and outlet of the Granite Falls and Morris bioreactors

Appendix B

Chapter 2 Raw Data

Source Water Raw Data (Input Tank)					
Date	Day	NO3--N (mg L^{-1})	DO (mg L^{-1})	Temp (°F)	Temp (°C)
11/30/2012	335	32.7	4.3	59.7	15.39
12/3/2012	338	37.2	4.5	No data	No data
12/5/2012	340	48.6	4.6	65.5	18.61
12/7/2012	342	48.3	7.1	62.2	16.78
12/10/2012	345	49.0	6.1	59.0	15.00
12/12/2012	347	47.4	6.6	57.4	14.11
12/14/2012	349	49.8	7.0	55.0	12.78
12/17/2012	352	48.3	5.9	60.6	15.89
12/19/2012	354	49.5	6.7	58.1	14.50
12/21/2012	356	50.7	6.7	56.8	13.78
12/24/2012	359	48.0	No data	No data	No data
12/26/2012	361	49.2	No data	No data	No data
12/28/2012	363	50.4	7.6	44.8	7.11
12/31/2012	366	49.8	8.5	35.2	1.78

Date	Day	Substrate ID	Column ID	Nitrate In-Out	Daily Average ORP (mV)	DO* (mg L^{-1})
12/3/2012	338	CC	2	7.2	366	2
12/5/2012	340	CC	2	22.5	347	2.1
12/7/2012	342	CC	2	42.96	232	3.3
12/10/2012	345	CC	2	37	169	2.7
12/12/2012	347	CC	2	45.38	99	2.8
12/14/2012	349	CC	2	42.38	97	2.7
12/17/2012	352	CC	2	47.15	71	2
12/19/2012	354	CC	2	36.9	123	2.9
12/21/2012	356	CC	2	39.8	123	2.2
12/24/2012	359	CC	2	35	149	No data
12/26/2012	361	CC	2	27.5	166	No data
12/28/2012	363	CC	2	22.1	202	2.6
12/31/2012	366	CC	2	15.6	250	4.1
12/3/2012	338	CC	8	8.7	394	2
12/5/2012	340	CC	8	38.81	206	2.1
12/7/2012	342	CC	8	41.35	175	3.3
12/10/2012	345	CC	8	36.7	166	2.7

12/12/2012	347	CC	8	33.6	157	2.8
12/14/2012	349	CC	8	33.1	176	2.7
12/17/2012	352	CC	8	32.8	190	2
12/19/2012	354	CC	8	23.2	202	2.9
12/21/2012	356	CC	8	25.8	195	2.2
12/24/2012	359	CC	8	19.9	204	No data
12/26/2012	361	CC	8	18.6	263	No data
12/28/2012	363	CC	8	13.8	319	2.6
12/31/2012	366	CC	8	8.4	353	4.1
12/3/2012	338	CC	14	8.1	387	2
12/5/2012	340	CC	14	29.7	331	2.1
12/7/2012	342	CC	14	44.6	139	3.3
12/10/2012	345	CC	14	42.78	142	2.7
12/12/2012	347	CC	14	44.37	122	2.8
12/14/2012	349	CC	14	42.58	141	2.7
12/17/2012	352	CC	14	45.71	113	2
12/19/2012	354	CC	14	35.7	131	2.9
12/21/2012	356	CC	14	41.85	134	2.2
12/24/2012	359	CC	14	30.4	157	No data
12/26/2012	361	CC	14	26.2	167	No data
12/28/2012	363	CC	14	22.17	199	2.6
12/31/2012	366	CC	14	12.3	244	4.1
12/3/2012	338	WC	9	2.1	164	1.9
12/5/2012	340	WC	9	4.2	252	2
12/7/2012	342	WC	9	3.9	353	2.7
12/10/2012	345	WC	9	1.3	332	2.5
12/12/2012	347	WC	9	9.3	295	2.7
12/14/2012	349	WC	9	12	313	2.6
12/17/2012	352	WC	9	13.5	318	2.2
12/19/2012	354	WC	9	10.2	271	2.7
12/21/2012	356	WC	9	11.4	270	2.7
12/24/2012	359	WC	9	10.2	274	No data
12/26/2012	361	WC	9	6.3	292	No data
12/28/2012	363	WC	9	3.6	334	2.6
12/31/2012	366	WC	9	1.5	367	3.9
12/3/2012	338	WC	15	2.7	119	1.9
12/5/2012	340	WC	15	5.1	277	2
12/7/2012	342	WC	15	3.9	386	2.7
12/10/2012	345	WC	15	9.1	194	2.5
12/12/2012	347	WC	15	11.7	221	2.7

12/14/2012	349	WC	15	10.8	279	2.6
12/17/2012	352	WC	15	15	231	2.2
12/19/2012	354	WC	15	12	157	2.7
12/21/2012	356	WC	15	12.6	149	2.7
12/24/2012	359	WC	15	8.7	113	No data
12/26/2012	361	WC	15	6.9	137	No data
12/28/2012	363	WC	15	3	185	2.6
12/31/2012	366	WC	15	0.3	352	3.9
12/3/2012	338	WC	16	3	189	1.9
12/5/2012	340	WC	16	5.4	277	2
12/7/2012	342	WC	16	3.6	453	2.7
12/10/2012	345	WC	16	0.4	389	2.5
12/12/2012	347	WC	16	10.2	351	2.7
12/14/2012	349	WC	16	14.1	330	2.6
12/17/2012	352	WC	16	17.7	298	2.2
12/19/2012	354	WC	16	14.4	239	2.7
12/21/2012	356	WC	16	18.9	219	2.7
12/24/2012	359	WC	16	10.2	220	No data
12/26/2012	361	WC	16	9	225	No data
12/28/2012	363	WC	16	3.9	232	2.6
12/31/2012	366	WC	16	0.3	308	3.9

Note: DO was measured for 1 column per substrate, but is displayed next to all columns of the same substrate in this table.

Date	Day	Substrate	Column ID	THg (ng L^{-1})	MeHg (ng L^{-1})	Tank THg (ng L^{-1})	Tank MeHg (ng L^{-1})	MeHg production (ng day^{-1})
12/17/2012	352	CC	2	6.550	1.335	7.730	0.035	9.360
12/28/2012	363	CC	2	3.120	0.040	11.000	0.000	0.291
12/5/2012	340	CC	8	6.705	0.306	11.969	0.367	-0.438
12/10/2012	345	CC	8	5.174	0.104	14.273	0.019	0.610
12/17/2012	352	CC	8	4.320	0.045	7.730	0.035	0.070
12/28/2012	363	CC	8	8.830	0.033	11.000	0.000	0.237
12/5/2012	340	CC	14	5.080	0.000	11.969	0.367	-2.640
12/17/2012	352	CC	14	5.170	0.337	7.730	0.035	2.172
12/28/2012	363	CC	14	3.220	0.065	11.000	0.000	0.465
12/10/2012	345	WC	9	8.730	0.127	14.273	0.019	0.774
12/17/2012	352	WC	9	5.185	0.102	7.730	0.035	0.481
12/28/2012	363	WC	9	1.290	0.000	11.000	0.000	0.000

12/10/2012	345	WC	15	8.752	0.136	14.273	0.019	0.845
12/17/2012	352	WC	15	3.040	0.087	7.730	0.035	0.371
12/28/2012	363	WC	15	0.970	0.000	11.000	0.000	0.000
12/17/2012	352	WC	16	3.910	0.070	7.730	0.035	0.248
12/28/2012	363	WC	16	0.820	0.034	11.000	0.000	0.245

Appendix C

Definition of variables from Table 3.4:

$T_{in} = V*\rho/Q_{in}$ and $T_{out} = V*\rho/Q_{out}$ where

T_{in} = theoretical hydraulic residence time based on inlet flows
T_{out} = theoretical hydraulic residence time based on outlet flows
V = volume of bioreactor
ρ = approximate porosity of woodchip filled bioreactor (0.52)
Q_{in} = average flow rate at the inlet
Q_{out} = average flow rate at the outlet

$t = \frac{\sum t_i c_i \Delta t_i}{\sum c_i \Delta t_i}$ where

t = calculated hydraulic residence time
t_i = sample collection time after potassium bromide injection started (h)
c_i = concentration of Br^- at time t_i (mg L^{-1})
Δt_i = time between t_i and next sample collection (h)

$e = t/T$ where

e = effective volume

$\lambda = t_p/T$ where

λ = hydraulic efficiency
t_p = time of highest Br^- concentration

$S = t_{16}/t_{50}$ where

S = short circuiting metric
t_{16} = time when 16% of total Br^- measured at outlet during study has passed through the outlet
t_{50} = time when 50% of total Br^- measured at outlet during study has passed through the outlet

$MDI = t_{90}/t_{10}$ where

MDI = Morrill Dispersion Index
t_{90} = time when 90% of total Br^- measured at outlet during study has passed through the outlet
t_{10} = time when 10% of total Br^- measured at outlet during study has passed through the outlet