FATE AND EFFECT OF TRICLOSAN AND SULFAMETHOXAZOLE WITHIN MESO-SCALE VERTICAL FLOW CONSTRUCTED WETLANDS

DEVENIR ET LES EFFETS DU TRICLOSAN ET LE SULFAMÉTHOXAZOLE DANS MÉSO-ÉCHELLE ZONES HUMIDES ÉCOULEMENT VERTICAL CONSTRUITE

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by

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To my parents Mark and Dayle Cosway

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ABSTRACT

The presence of pharmaceuticals and personal care products are increasing in our natural environment due to their incomplete removal within traditional wastewater treatment plants. Constructed wetlands are used as secondary or tertiary wastewater treatment and as such may receive both pharmaceuticals and personal care products with subsequent implications for their treatment efficacy. The aim of this study was to examine the fate and effects of antimicrobials in planted and unplanted vertical flow constructed wetlands. Twelve mesocosms were inoculated with activated sludge from the Cataraqui Bay Wastewater Treatment Plant. Six were planted with reed canary grass (*Phalaris arundinacea*) and the remaining six were left unplanted. The wetland mesocosms were assessed using a variety of parameters including water treatment (chemical oxygen demand removal rate), hydrological (porosity, evapotranspiration/evaporation) water quality (temperature, pH, specific conductivity, dissolved oxygen, redox potential, total dissolved solids), ecological (plant height and stem count), and microbial community function (community level physiological profiling).. Community-level physiological profiles were gathered for the wetland microbial community using Biolog Ecoplates^{TM}. The development phase was initially characterized for all mesocosms over a ninety-day period to establish ecological stability. The microbial communities were then subjected to *ex-situ*, dose-response exposures (0 – 1000 $\mu g \cdot L^{-1}$) for trimethoprim, triclosan and sulfamethoxazole to gain an understanding of the ecotoxicity of these antimicrobials. Following the *ex-situ* exposures, *in-situ* exposures were performed with triclosan and sulfamethoxazole at low (100 $\mu g \cdot L^{-1}$) and high (500 $\mu g \cdot L^{-1}$) concentrations. The low concentration was selected based on literature reviews of the levels found in water bodies and the high concentration was selected to represent a shock-loading scenario. Hydrological, ecological and microbial parameters were monitored before, immediately after and over a recovery period of four weeks following each exposure. During the developmental period both the planted and unplanted mesocosms developed similarly and ecological stability was established. However, a distinct microbial community profile was observed in the planted mesocosms. In the *ex-situ* dose-response experiments the effect of trimethoprim was negligible on the planted microbial communities but with some removal of microbial function for the unplanted microbial communities. Triclosan exposure led to a moderate decline in microbial function for both the planted and unplanted communities. Sulfamethoxazole exposure led to a severe decline in microbial function in both the planted and unplanted microbial communities, so much so that negligible activity was observed at 1000 $\mu g \cdot L^{-1}$. Following the *in-situ* low and high exposures of triclosan and sulfamethoxazole there was a significant removal of the compounds (>80%) from the mesocosm water column. The effects of both low and high triclosan and sulfamethoxazole exposures were minimal within the exposed mesocosms. There were no major changes observed with respect to the water treatment ability, hydrological or ecological parameters. Following the low triclosan exposure, the planted microbial communities showed some removal of microbial activity in the week following the exposure while the unplanted microbial communities were unaffected. No other microbial community effects were observed for any of the exposure scenarios. Following sulfamethoxazole exposures, the water treatment ability (COD removal rate) of the exposed mesocosm systems was reduced in some cases, however not consistently. No adverse effects on water treatment ability were observed for the triclosan exposures. Over the course of these experiments, the planted and unplanted mesocosms continued to develop distinct microbial community profiles. These findings suggest that whilst triclosan and sulfamethoxazole are potentially harmful to wetland microbial communities, based on *ex-situ* results, they have a limited effect within the wetland mesocosms (*in-situ*). Based upon these observations, vertical flow constructed wetland have shown to be robust and able to handle shock loads from these common pharmaceutical compounds. Further research should examine longer-term and multiple compound antimicrobial exposures to gain a better understanding the fate and effect of pharmaceuticals in constructed wetlands.

RÉSUMÉ

La présence de produits pharmaceutiques et de soins personnels sont en augmentation dans notre environnement naturel en raison de leur utilisation croissante et une élimination incomplète au sein des usines traditionnelles de traitement des eaux usées. Les marais artificiels sont de plus en plus utilisés comme traitement d'eaux usées secondaire ou tertiaire et en tant que tel peut recevoir à la fois des produits pharmaceutiques et de soins personnels qui a des répercussions subséquentes sur leur efficacité de traitement. Le but de cette étude était d'examiner le devenir et les effets des antibiotiques dans l'écoulement marais artificiels verticaux plantés et non plantés. Douze mésocosmes ont été inoculées avec des boues activées à partir de l'usine de traitement d'eau de Cataraqui Bay. Six ont été plantés avec l'alpiste roseau (Phalaris arundinacea) et les six autres ont été laissés en friche. Les mésocosmes de zones humides ont été évaluées en utilisant une gamme de paramètres, y compris le traitement de l'eau (taux de DCO), l'hydrologie (porosité, l'évapotranspiration), laqualité de l'eau (température, pH, conductivité spécifique, l'oxygène dissous, potentiel redox, les solides dissous totaux), l'écologie (hauteur de la plante et le nombre de tiges), et la fonction des communautés microbiennes (niveau de la communauté profilage physiologique. Profils physiologiques au niveau communautaire ont été recueillis pour la communauté des zones humides microbiennes à l'aide Biolog EcoplatesTM. La phase de développement a été initialement caractérisée pour tous les mésocosmes sur une période de quatre-vingt-dix jours pour établir la stabilité écologique. Les communautés microbiennes ont ensuite été soumis à une exposition ex-situ, dose-réponse (0-1000 µg•L-1) pour le triméthoprime, le triclosan et le sulfaméthoxazole d'acquérir une compréhension de l'écotoxicité de ces antimicrobiens. Après les expositions ex situ, des expositions in situ ont été réalisées avec le triclosan et le sulfaméthoxazole à faible (100 µg•L-1) et haute (500 µg•L-1) concentrations. La faible concentration a été choisi sur la base des analyses documentaires des niveaux trouvés dans les masses d'eau et la forte concentration a été sélectionné pour représenter un scénario choc de chargement. Paramètres hydrologiques, écologiques et microbiennes ont été surveillé avant, immédiatement après et pendant une période de récupération de quatre semaines suites à l'exposition. Au cours de la période de développement à la fois les mésocosmes plantés et non plantés ont évolué de façon similaire et la stabilité écologique a été créé. Cependant, un profil de la communauté microbienne distincte a été observé dans les mésocosmes plantés. Dans les expériences dose-réponse ex-situ l'effet de triméthoprime a été négligeable sur les communautés microbiennes plantées mais avec une certaine diminution de la fonction microbienne pour les communautés microbiennes non plantés. Exposition à tricolosana conduit à une baisse modérée de la fonction microbienne pour les communautés plantés et non plantés. Exposition à sulfaméthoxazole a conduit à une baisse sévère de la fonction microbienne dans les communautés microbiennes plantés et non plantés, de telle sorte que l'activité négligeable a été observée à 1000 µg•L-1. Après les expositions in situ faibles et élevées de triclosan et le sulfaméthoxazole il y avait une élimination significative des composés (> 80%) de la colonne d'eau de mésocosme. Les effets des deux expositions faibles et élevés de triclosan et sulfaméthoxazole étaient minimes dans les mésocosmes exposés. Il n'y avait pas de grands changements observés par rapport à la capacité de traitement de l'eau ou par rapport aux paramètres hydrologique ou écologiques. Suite à l'exposition triclosan faible, les communautés microbiennes plantées ont montré une réduction de l'activité microbienne dans la semaine suivant l'exposition, tandis que les communautés microbiennes non plantés ne sont pas affectées. Aucun autre effet des communautés microbiennes ont été observées pour tous les scénarios d'exposition. Après les expositions sulfaméthoxazole, la capacité de traitement de l'eau (taux de DCO) des systèmes de mésocosmes exposés a été réduit dans certains cas, mais pas toujours. Aucun effet néfaste sur la capacité de traitement de l'eau a été observée pour les expositions de triclosan. Au cours de ces expériences, les mésocosmes plantés et non plantés continué à élaborer des profils des communautés microbiennes distinctes. Ces résultats suggèrent que, bien que le triclosan et le sulfaméthoxazole sont potentiellement

dangereux pour des zones humides communautés microbiennes, en fonction des résultats ex-situ, ils ont un effet limité dans les mésocosmes des zones humides (in situ). Sur la base de ces observations, les experiences ont montré que lécoulement marais artificiels verticaux est robuste et capable de gérer les charges de choc de ces composés pharmaceutiques courants. D'autres recherches devraient examiner à plus long terme et de multiples expositions antimicrobiens composés afin de mieux comprendre le devenir et les effets des produits pharmaceutiques dans les zones humides artificielles.

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CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Modern Wastewater Treatment

Treatment of water resources has been, and continues to be necessary for thriving populations. Early civilizations used boiling and filtration to treat contaminated water (United States Environmental Protection Agency, 1999; Wiesmann, *et al*., 2009). During the industrial revolution wastewater volumes increased drastically to render previously employed treatment methods no longer effective. Direct discharge of raw wastewater into natural rivers and streams and application onto farmland soils became increasingly more common. Over time these natural landscapes become over-polluted and a new method of wastewater treatment arose. The wastewater treatment plant (WWTP) was first established in the late 1800s and quickly grew in popularity (Brix, 1994; Kadlec and Wallace, 2009; Wiesmann *et al*., 2009). Wastewater treatment plants (WWTP's) utilize methods of settling, biological and chemical breakdown of wastewater constituents for wastewater treatment. These systems are capable of handling large volumes of wastewater and are the major method of wastewater treatment within current society. There are several sources of wastewater including agricultural, domestic, industrial and storm-water runoff (Chambers, 1997; Metcalf and Eddy, 2003). Agricultural wastewater contains high concentrations of organic matter, nutrients and pesticides. Domestic wastewater contains high organic matter and synthesized compounds including antimicrobials, pharmaceuticals, personal care products and nanomaterials. Industrial wastewater can vary however most commonly contain high organic matter and metals. Storm-water runoff largely contains suspended solids, nutrients and metals. In city center areas wastewater is collected from these different sources and treated as one large volume within wastewater treatment plants (Metcalf and Eddy, 2003).

Wastewater treatment plants utilize four stages of treatment to produce cleaner water at the discharge point (FIGURE 1.1) preliminary, primary, secondary and tertiary. Preliminary treatment uses a series of rotating screens or grills to remove large solid debris (Metcalf and Eddy, 2003). This stage is important because large solids can clog piping or become lodged within moving parts of the WWTP. Debris is collected and taken offsite to be landfilled. Screened influent most commonly passes through a clarifier to dampen the flow velocity, before the influent is passed into the primary basin. Sludge produced from the clarifier is disposed of in a landfill.

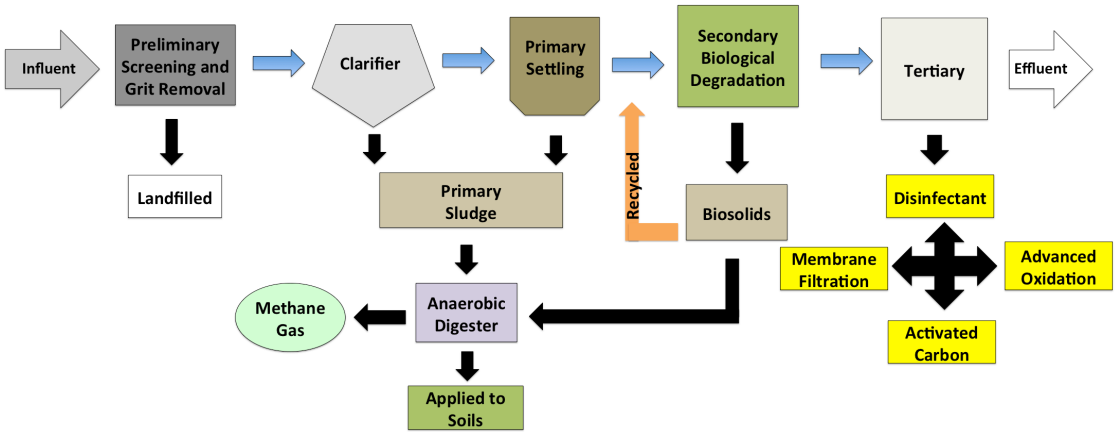

FIGURE 1.1: Wastewater treatment plant schematic [Adapted from Metcalf and Eddy, 2003; Environment Canada, 2006].

The primary basin(s) focus on flocculation and chemical coagulation, followed by settling of large organic particles within the incoming wastewater (Metcalf and Eddy, 2003). The average retention time of the primary basin is commonly one to two hours, depending on the basin size and flow rate. Settling of particles forms a sludge layer at the bottom of the primary basin that is collected and stabilized in an anaerobic digester prior to being disposed of offsite. The primary basin is effective at removing organics if the wastewater temperature and velocity are consistent. Eddying currents are necessary for flocculation to occur and the temperature gradient of the basin determines the presence of these currents. Short-circuiting of the wastewater occurs with high flow rates that can lead to lower settling rates and partially treated wastewater (Metcalf and Eddy, 2003).

The secondary basin acts to biologically degrade organic compounds within the wastewater (Metcalf and Eddy, 2003; Kadlec and Wallace, 2009). Activated sludge consisting of bacteria, protozoa, fungi and rotifer biofilm communities is added/promoted in the secondary basin to enhance microbial biodegradation rates. The amount of organic matter in the incoming wastewater, water temperature and water pH affect the microbial activity in the secondary basin. The majority of microorganisms found within the activated sludge function best at water temperatures between 20° C - 25 ° C and water pH between 6.5 -7.5. Organic compounds within wastewater act as energy for the growing microbial communities. Necessary oxygen is supplied to the basin through pumps or mechanical mixers.

The tertiary basin provides advanced treatment including the addition of disinfectants, activated carbon, membrane filtration, and advanced oxidation (USA EPA, 1999; Alonso, *et al.* 2001; Namasivayam and Kavitha, 2002; Metcalf and Eddy, 2003). Disinfectants such as chlorine, ozone and ultra violet radiation can be used to reduce the pathogenic population within the discharge water (Metcalf and Eddy, 2003). Filtration using synthetic membranes that come in a variety of filtration sizes (microfiltration, ultrafiltration, nanofiltration) are effective at removing fine particles from the wastewater (Alonso, *et al*. 2001) To filter effectively, the wastewater must be free of large particles which is done through a secondary clarifier after the secondary basin. Activated carbon is commonly used in developing countries due to its reasonable cost and high adsorption capacity for organics and pollutants within wastewater (Namasivayam and Kavitha, 2002). Activated carbon sources can include wood, fired clay, chitin, and silica. Advanced oxidation includes the use of ozone (O_3) that is produced onsite (due to its short half-life). Ozone

decomposes in water to form free radicals (hydrogen peroxide and hydroxyl radicals) that oxidize the cell wall of bacteria causing lysis (USA EPA, 1999). The majority of wastewater treatment plants utilize only one method of tertiary treatment due to high operational costs (Environment Canada, 2011).

1.1.2 Water Treatment within WWTPs

Wastewater treatment plants (WWTP's) perform well at removing the majority of organics, solids and nutrients from wastewaters (Metcalf and Eddy, 2003). The secondary stage greatly enhances the removal of organics and the tertiary stage allows for finer particles and pathogen removal. Longer retention time within the treatment plant enhances particle settling in the primary stage and organic removal in the secondary stage. Increasingly, wastewater contains synthetic compounds that are less likely to be reduced in the wastewater treatment plant. These synthetic compounds include antimicrobials, pharmaceuticals, personal care products and nanomaterials (Daughton and Ternes, 1999; Ternes *et al*., 1999; Herber *et al.*, 2001; Carballa *et al.*, 2004; Matamoros *et al.,* 2009). These "emerging contaminants" (ECs) have been reduced 40-90% within wastewater treatment plants, depending on the compound properties and the treatment stages used (Batt *et al.*, 2007; Conkle *et al.*, 2008; Onesios *et al.*, 2009). Due to the incomplete removal of these compounds, they are increasingly being found within the natural environment (Park and Kidd, 2005; Kidd *et al*., 2007; Weber *et al*., 2008; Weber *et al*., 2011; Helt *et al*., 2012). The effects of such biota/EC exposure within the natural environment are not well understood, though new studies hope to shed light on the impact of emerging contaminants on sensitive aquatic ecosystems.

Over the past sixty years there have been new developments in wastewater treatment methods. The constructed wetland was first conceptualized Dr. Kathe Siedel in the 1950s (Brix, 1994; Kadlec and Wallace, 2009). Constructed wetlands are man-made wetlands having similar physical and ecological functions to natural wetlands. Constructed wetlands have recently been shown to remove emerging contaminants beyond the ability of traditional wastewater treatment plants (Gross *et al*. 2004; Matamoros *et al*. 2006; Matamoros *et al*. 2007; Conkle *et al*., 2008; Matamoros *et al*., 2008; Faulwetter *et al*., 2009; Onesis *et al*., 2009; Song *et al*., 2009; Garcia *et al*., 2010; Kadlec and Wallace, 2009).

1.2 Constructed Wetlands

1.2.1 Background

Wetlands are described as low-elevation areas within a natural landscape where groundwater and surface runoff collect to form a saturated soil medium (Brix, 1994; Kadlec and Wallace, 2009). The saturated medium contains flood-resistant vegetation and an active microbial community within the rhizosphere and surrounding media. Wetlands are unique due in their ability to form only in select regions of the world. Water is received within the wetland through incoming subsurface flows, precipitation and surface runoff. In nature, water is naturally treated through biological and physical processes before being released from the wetland through outgoing flows, groundwater infiltration and evapotranspiration. Wetlands were first documented for water treatment in the early 1900s (Brix, 1994). The use of natural wetlands for wastewater treatment continued through the 1980s until environmental groups began to force legislation to protect these ecosystems (Kadlec and Wallace, 2009). The use of constructed wetlands for water treatment and associated research has increased immensely followings these actions.

The constructed wetland, described as a man-made unit, replicates a natural wetland environment (Brix, 1994; Kadlec and Wallace, 2009). Constructed wetlands include a saturated bed medium, flood-resistant vegetation and an active microbial community (FIGURE 1.2). The saturated bed medium can contain a variety of substrates including soil, sand or gravel, though gravel is the most common due to its low tendency to clog (Kadlec and Wallace, 2009). Water flows within these systems either on the surface or below the surface of the wetland (FIGURE 1.2A). Subsurface water flows can be either horizontal (FIGURE 1.2B) or vertical (FIGURE 1.2C) through the bed medium. The first subsurface constructed wetland (horizontal subsurface) was built in the early 1980's in Germany (Brix, 1997). This system was built over 22 hectares and contained clay and silt soil and the treatment area required was 3-5 $m^2 \cdot person^{-1} \cdot day^{-1}$. The major problem with this design was the low soil permeability resulting in unwanted surface water flow. Over time horizontal subsurface flow constructed wetlands increased in popularity across Europe, with the bed medium changing from clay soils to the more common medium sized gravel (Kadlec and Wallace, 2009). Vertical flow constructed wetlands were implemented in the late 1980's and included a gravel bed medium with pulse-fed designs to increase the treatment effectiveness (Kadlec and Wallace, 2009). The treatment area required for these systems is 1-3 $m^2 \cdot person^{-1} \cdot day^{-1}$. Hybrid designs including both horizontal and vertical flow constructed wetlands have been built, though their popularity has remained only moderate over the years (Kadlec and Wallace, 2009).

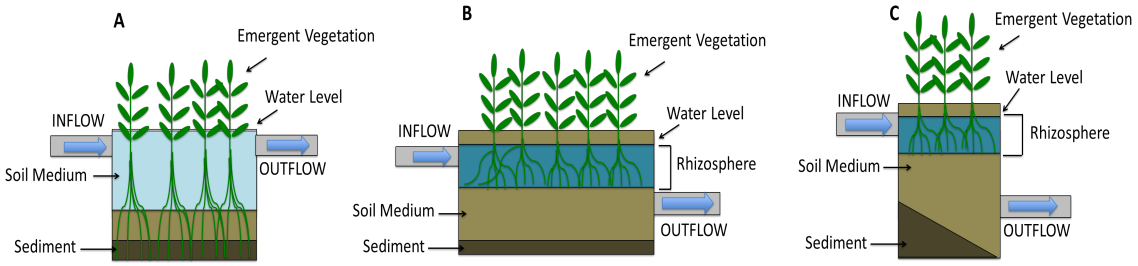

FIGURE 1.2: Constructed wetland schematic. A) Surface flow; B) Horizontal subsurface flow; C) Vertical subsurface flow.

1.2.2 Constructed Wetland Design

Constructed wetlands can be surface flow, horizontal subsurface flow or vertical subsurface flow (Brix, 1994; Kadlec and Wallace, 2009). Surface flow constructed wetlands are the most similar to natural wetlands. These systems are usually built on a large scale using the natural landscape as the basin, installing bottom liners to eliminate infiltration and water seepage, and installing inflow and outflow pipes to move wastewater to the discharge point. Natural wetland vegetation is established within the basin and given time to fully develop (sometimes taking several years to reach mature height). Water flows directly at the surface of the wetland with oxygen diffusion occurring at the surface (Brix, 1994). The rate of oxygen diffusion depends upon environmental conditions such as air temperature, water temperature and water level. The surface of this system is considered aerobic due to oxygen diffusion, though deeper within the bed oxygen rapidly decreases due to biological demands (Kadlec and Wallace, 2009). Surface flow constructed wetlands were most popular during the early years of wetland research and have

become less popular as smaller systems (subsurface flow wetlands) have been developed in Europe over the past 20-30 years.

Subsurface flow constructed wetlands operate with horizontal or vertical subsurface flow (Brix, 1994; Kadlec and Wallace, 2009). The horizontal subsurface flow constructed wetland is largely anaerobic due to limited oxygen diffusion ability into the bed medium (Brix, 1994). The atmospheric oxygen diffusion ability is impeded by water flowing beneath the surface and has been estimated to be $0.11 g \cdot m^{-2} \cdot d^{-1}$ (Tanner and Kadlec, 2003). The horizontal subsurface flow system requires a longer bed to ensure treatment performance. The vertical flow subsurface constructed wetland is largely aerobic due to its high oxygen diffusion ability (from diffusion during spreading, and the venturi effect). The venturi effect occurs when water is forced through a small-diameter opening and its velocity increases to cause a decrease in pressure (Kadlec and Wallace, 2009). The venturi effect occurs within vertical subsurface flow constructed wetlands as the surface water is drawn vertically down through pore spaces, causing a suction of oxygen into the depth of the bed medium. This greater oxygen concentration within the bed medium allows for a more active aerobic microbial community, which has been shown to increase treatment performance, especially for organic compounds (Kadlec and Wallace, 2009).

The physical properties of the constructed wetland (CW) are important to consider. The selected bed medium (sand, soil, gravel) influences the porosity of the system. Gravel is by far the most common bed medium chosen for its larger size (Sauter *et al*., 1997; Garcia *et al*., 2004; Kadlec and Wallace, 2009; Nivala, 2012). The feeding method of the constructed wetland alters the oxygen concentration of the system (Stein *et al*., 2003; Sklzar, *et al*., 2009; Faulwetter *et al*., 2009; Kadlec and Wallace, 2009). The batch-fed system operates with the constructed wetland being drained prior to the addition of feed to allow for oxygen to penetrate through the bed medium. The intermittent-fed system operates with the constructed wetland being fed without prior draining. The batch-fed method is the more popular of the two, given its allowances for greater oxygen penetration into the bed medium. Early constructed wetland systems used low permeability soil (like clay-based mediums) and had intermittent feeding which resulted in lower treatment performances (Kadlec and Wallace, 2009).

1.2.3 Nutrient Cycling within Constructed Wetlands

Carbon, nitrogen and phosphorus are the three major nutrients cycled within natural wetland systems (Faulwetter *et al*., 2009; Kadlec and Wallace, 2009; Garcia *et al.*, 2010). These nutrients are important for healthy ecosystem function within the constructed wetland (CW) environment. Autotrophic and heterotrophic microorganisms exist within the wetland environment and play important roles in organic compound removal. Autotrophs include photoautrophs, utilizing solar radiation for energy, and chemoautotrophs, utilizing inorganics for energy. Heterotrophs utilize organic molecules for energy thus play the most important role within CWs consuming the majority of incoming organic constituents in the wastewater (Kadlec and Wallace, 2009). Vegetation assimilates some nutrients though generally their role in nutrient cycling is considered minimal (Brix, 1997).

Carbon is present within incoming wastewater and decomposing organic matter. Carbon is assimilated within CWs by microorganisms and vegetation (Kadlec and Wallace, 2009). Carbon is necessary in several aerobic and anaerobic biological reactions including respiration, fermentation, denitrification, iron removal, sulfate removal and methanogenesis. The rate of carbon utilization within a CW is based upon water temperatures with increased temperature

increasing the activity of the microorganisms present (Faulwetter *et al*., 2009; Kadlec and Wallace, 2009).

Nitrogen is present within incoming wastewater as organic nitrogen, ammonium and nitrate (Zhu and Sikoria, 1995; Green *et al*., 1997; Green *et al*., 1998; Tanner *et al*., 1999; Tanner and Kadlec, 2003). Different sources of wastewater have varying levels of nitrogen compounds. Organic nitrogen can be mineralized into ammonium (NH_4^+) through the ammonification reaction and then aerobically reduced into nitrate (NO_3^-) through the nitrification reaction occurring in an aerobic environment. Nitrate (NO_3^-) is reduced into dinitrogen gas (N_2) through the denitrification reaction occurring within an anaerobic environment. Organic nitrogen can also be assimilated within vegetation (Kadlec and Wallace, 2009). In subsurface horizontal flow CWs nitrogen transformation occurs largely within the rooted zone (rhizosphere) of the wetland where aerobic and anaerobic microsites are present (Kadlec and Wallace, 2009. In vertical flow CWs oxygen is plentiful throughout and therefore nitrification can be completed outside the rhizosphere as well, although denitrification is challenging due to a lack of anaerobic regimes.

Phosphorus is present within incoming wastewater mostly as inorganic orthophosphates (Stein *et al*., 2003; Garcia *et al*., 2004). Orthophosphates, though utilized in small quantities by organisms, are not readily removed within the environment. The major removal mechanism for phosphorus within the wetland environment is sedimentation. Orthophosphates bind to organic matter and particulates within the wetland then fall to the bottom sections. Due to its difficult removal, orthophosphates are closely monitored in incoming wastewater. Excessive phosphorus within wastewater are commonly removed in a sedimentation basin prior to the wastewater flowing into the constructed wetland.

Wastewaters can have a variety of inorganic constituents present depending on their source (Kadlec and Wallace, 2009). Industrial wastewaters can contain hydrocarbons, halogens, metals and sulfur compounds amongst others. Hydrocarbons are produced from incomplete combustion of fossil fuels (Tian *et al.*, 2012) and bind strongly to suspended solids within wetland environments. These bound hydrocarbons eventually settle to the bottom of the basin where they do not undergo further biodegradation (Tian *et al.*, 2012). Common halogens include bromide, chlorine and fluoride. Chlorine is toxic to microorganisms and is regularly added to drinking water as a disinfectant, this residual chlorine can often find its way into CW treatment systems. Fluoride has the greatest capacity to bind to biomass and soil particles within CWs with the consequences still largely unstudied (Kadlec and Wallace, 2009). Metals are found naturally within sediments or produced from a variety of industries including metal shops, car washes, and dentistry (Sorme and Lagerkvist, 2002). Metals can be used by microorganisms and vegetation or become bound to particles within the wetland environment (Wang *et al.*, 2006; Kadlec and Wallace, 2009; Barakat, 2011). Metal binding is affected by water pH with increased ability to bind at higher water pH (Wang *et al.*, 2006). Sulfate and sulfide are common within agricultural and industrial wastewaters. Sulfate is prominent in aerobic environments and sulfide present in anaerobic environments (Kadlec and Wallace, 2009). Sulfate has been found within groundwater between 100 - 3000 $mg \cdot L^{-1}$ (Wu *et al*., 2013). Within agricultural and industrial wastewaters the sulfate concentrations can be 4-10x the concentration of regular groundwater. The major sources of sulfate are wineries, metal shops, road runoff and peroxide production (Wu *et al*., 2013). Wetlands are able to convert sulfate via sulfate-reducing bacteria into hydrogen sulfide within the rhizopshere (Kadlec and Wallace, 2009).

1.2.4 Constructed Wetland Vegetation

Vegetation is an important component within the wetland environment. Vegetation provides physical structure to the wetland and increased surface area for attachment of microbial communities (Dordio *et al*., 2008; Kadlec and Wallace, 2009; Xian *et al*., 2010; Dordio, et al, 2011). Vegetation facilitates oxygen diffusion into the wetland through the roots (Brix, 1997). Each species of wetland vegetation has a unique oxygen release rate that allows for microsites of aerobic environments to be produced (Zhu and Sikora, 1995; Tanner and Kadlec, 2003; Bezbaruah and Zhang, 2005; Gagnon *et al*., 2007; Faulwetter *et al*., 2009; Wu *et al*., 2011). Wetland vegetation is diverse and classified as emergent, floating, submerged or woody species (FIGURE 1.3). Shown in FIGURE 1.3, Emergent vegetation grows from the soil medium above the water surface including reed (*Phragmites australis*), canary grass (*Phalaris arundinacea*) and cattail (*Typha*) species. Floating vegetation grows at the water surface of the wetland including algae, water lily (*Nymphaea alba*) and water hyacinth (*Eichhornia crassipes*) species. Submerged vegetation grows entirely underwater including pondweed (*Potamogeton crispus*) and littorella (*Littorella uniflora*) species. Woody vegetation includes tree and shrub species. All of these wetland vegetation species are well adapted to living in flooded environments to have air sacs, shallow root penetration and large above ground biomass to ensure adequate survival (Kadlec and Wallace, 2009).

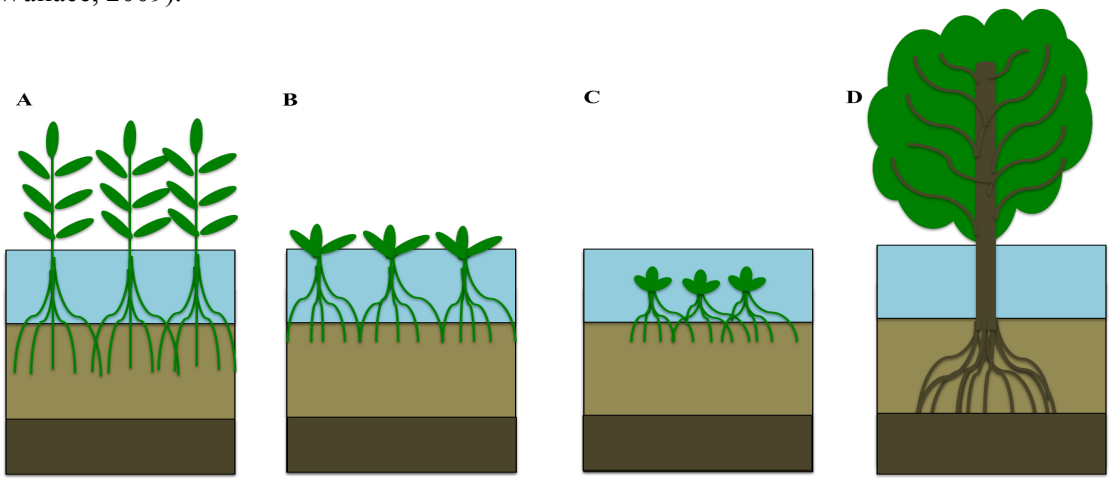

FIGURE 1.3: Wetland vegetation. A) Emergent; B) Floating; C) Submerged; D) Woody.

1.2.5 Intensified Designs of Constructed Wetlands

Intensified constructed wetland systems incorporate the introduction of air, nutrients and allow for fluctuating water heights (Green *et al*., 1997; Tanner *et al*., 1999; Stein *et al*., 2003; Tanner *et al*., 2003; Nivala, 2012). These methods have been shown to outperform passive (such as surface flow) constructed wetland systems. The introduction of air into the wetland system through pumps, fans or compressors allows for air to infiltrate the bed medium that increases the oxygen content (Green *et al*., 1997; Nivala, 2012). This allows for a more active microbial community. Water height fluctuations are performed by draining the system and allowing air to be pulled into the bed medium (Tanner *et al*., 2003; Nivala, 2012). This method works best on vertical flow constructed wetland systems. Shallow bed mediums with depths matching rhizosphere penetration are the most effective due to all wastewater flowing through the most

active region of the wetland (Nivala, 2012). All of these intensified designs are important to consider during construction as they play an important role in the capacity of water treatment.

1.2.6 Cold Climates

Constructed wetlands are able to function in both warm and cold climate regions (Werker, *et al*., 2002). In cold climate regions, low water temperatures have been thought to cease the treatment capacity of the wetland. While it is true that lower water temperatures have been shown to reduce the activity of microorganisms, treatment still occurs at a slower rate. Carbon has been shown to assimilate and biologically transform in cold climate wetlands. Nitrogen has been shown to be the most difficult to remove due to the reduced function of microorganisms at cold temperatures (Kadlec and Wallace, 2009). Pathogens have as well been shown to be removede through sedimentation, predation and disinfectants in cold climate wetlands (Werker, *et al*., 2002). To mitigate the effects of temperature in cold climates wetland water temperatures are kept above freezing through the use of a subsurface flow regime with an insulated surface (vegetation or mulch covering). Finally, more recent designs include compressed air input that increased the activity of the microorganisms within these systems (Werker, *et al*., 2002).

1.2.7 Design Limitations of Constructed Wetlands

Constructed wetlands have physical and hydrological design limitations (Sauter *et al.*, 1997; Scholes *et al*., 1998; Rash and Leir 1999). Physical limitations include the size of the wetland bed, depth of the bed and type of bed medium selected. CWs with deeper media beds have been shown to be less effective than shallower beds due to the limit of the vegetated rhizosphere (Kadlec and Wallace, 2009). Shallower beds allow for the incoming wastewater to flow directly through the rhizosphere as opposed to beneath it. Short-circuiting of wastewater can occur within deeper beds. Low permeability soil beds have been phased out of constructed wetlands, due to the tendency for water to pool at the surface (Kadlec and Wallace, 2009). In their place, gravel bed mediums have become increasingly popular.

The constructed wetland is considered a secondary or tertiary stage of water treatment since these systems cannot handle heavy wastewater loads (Kadlec and Wallace, 2009). Often pre-treatment of wastewater (in the form of settling ponds, solid waste removal and wastewater dilution) is necessary for the wetland to function properly. With the proper design, constructed wetlands can operate as effective wastewater treatment systems.

1.2.8 Constructed Wetland Modeling

Mathematical models have been used over the past twenty years to predict contaminant removal within wetland environments (Kadlec and Wallace, 2009). Compound removal can be generally described using zero order, first order or second order reactions.

Zero Order

$$\frac{dC}{dt} = -k \qquad (1)$$

First Order

$$\frac{dC}{dt} = -kC \quad (2)$$

Second Order

$$\frac{dC}{dt} = -kC^2 \quad (3)$$

k = first order aerial reaction rate constant ($m \cdot d^{-1}$)
C = concentration ($mg \cdot L^{-1}$)

The most common model applied to constructed wetlands is the first order plug flow model:

$$k = q \cdot ln\left(\frac{C_i}{C_o}\right) \quad (4)$$

k = first order aerial reaction rate constant ($m \cdot d^{-1}$)
q = hydraulic loading rate ($m \cdot d^{-1}$)
C_i = incoming concentration ($mg \cdot L^{-1}$)
C_o = outflow concentration ($mg \cdot L^{-1}$)

The major concern with the plug flow model is that the calculated outflow concentration (C_o) is lower then what is actually observed within the wetland (Kadlec and Wallace, 2009). This is due to the presence of background concentration (the fraction of compounds resistant to degradation). The background concentration is not accounted for within the plug flow model.

Also known as the "K-C-star" model, the first order tank-in-series model considers the background concentration:

$$\frac{C-C^*}{C_i-C^*} = \frac{1}{1+\frac{k}{Pq}^P} \quad (5)$$

C = outflow concentration ($mg \cdot L^{-1}$)
C_i = incoming concentration ($mg \cdot L^{-1}$)
C^* = background concentration ($mg \cdot L^{-1}$)
k = first order areal reaction rate constant ($m \cdot d^{-1}$)
P = number of constructed wetland systems
q = hydraulic loading rate ($m \cdot d^{-1}$)

The described models above estimate the reaction rate (k) over the entire surface area of the constructed wetland (Kadlec and Wallace, 2009). This estimation is not a perfect representation of CWs as microbial biofilm does not develop uniformly across the wetland, and depth is not considered. Despite these failings, first order models are still favoured due to their simplicity of use (Kadlec and Wallace, 2009).

1.2.9 Water Treatment within Constructed Wetlands

Constructed wetlands are effective at removing organics and nutrients from wastewater. In a very general sense the vertical subsurface constructed wetland is considered the most efficient at reducing such wastewater constituents (60 - 99%) due to its highly oxygenated environment (Matamoros, *et al*., 2007; Conkle *et al*., 2008; Huang *et al*., 2009; Matamoros *et al*., 2009; Garcia *et al*., 2010). The surface flow and horizontal subsurface flow show moderate removals (50%) of such wastewater constituents.

Emerging contaminants are defined as compounds that have been previously undetected or unknown to cause harmful impacts on flora, fauna and microorganisms or the ecosystem (Daughton and Ternes, 1999). Emerging contaminants include antimicrobials, pharmaceuticals, personal care products and nanomaterials. Antimicrobials and pharmaceuticals are chemical compounds used to treat human bacterial infections and diseases (Daughton and Ternes, 1999). Personal care products include domestic goods such as laundry detergents, hand soaps and clothing (Daughton and Ternes, 1999). Nanomaterials are generally defined as any particle under 100 nm in dimension and are produced from combustion or released from products such as nano-silver embedded t-shirts and socks, or TiO_2 found in sunscreen (Daughton and Ternes, 1999; Klaine *et al*., 2009). The majority of these emerging contaminants have unknown ecological effects within aquatic environments (Ternes *et al*., 1999; Herber *et al.*, 2001; Carballa *et al.*, 2004; Matamoros *et al.,* 2009). However several scientific studies have emerged in recent years to define the impacts of such compounds on select aquatic organisms - which have in some cases been shown to be significant (Park and Kidd, 2005; Kidd *et al*., 2007; Proia *et al*., 2011; Weber *et al*., 2011; Helt *et al*, 2012). For example estradiol (synthetic estrogen found in birth control pills) has been shown to feminize fish populations (Kidd *et al*., 2007) leading to population crashes. Constructed wetlands have the ability to effectively remove emerging contaminants within wastewater (Huang *et al.*, 2004; Matamoros *et al.*, 2006; Matamoros *et al.*, 2007; Conkle *et al.*, 2008; Matamoros *et al.*, 2008; Matamoros *et al.*, 2009; Park *et al.*, 2009; Conkle *et al.*, 2010)(see TABLE 1.1).

TABLE 1.1: Summary of pharmaceutical removal efficiency within constructed wetlands.

	Percent Removal of Pharmaceutical (%)		
Pharmaceutical	Surface Flow CW	Subsurface Vertical Flow CW	Subsurface Horizontal Flow CW
Caffeine	99[3]		99[2]
Carbamazepine	47[5], 50[3]	0[6]	5[4]
Clofibric acid	32[5]		
Sotalol	30[3]		
Linear alkylbenzene sulfonate		71[1]	
Ibuprofen	95[5], 99[3]	89[6]	51[4], 62[2]
Naproxen	52[5], 99[2]	92[6]	80[2]
Salicylic acid		87[6]	92[2]
Gemfibrozil	64[3]		
Ketoprofen	97[5]	0[6]	45[2]

*Adapted from Huang *et al*., 2004[1]; Matamoros and Bayona, 2006[2]; Conkle *et al*., 2008[3]; Matamoros *et al*., 2008[4]; Matamoros *et al*., 2008c[5]; Matamoros *et al*., 2009[6].

1.2.10 Effect of Emerging Contaminants on Constructed Wetlands

The effect of emerging contaminants on constructed wetland environments is relatively unknown. Studies within the past decade have shed important light of these effects, which are centered on the wetland microbial community (Weber *et al.* 2011; Helt, *et al.* 2012). Exposures of $ng \bullet L^{-1}$ to $\mu g \bullet L^{-1}$ concentrations of antimicrobial agents have shown to reduce wetland microbial populations, decrease microbial activity (Weber *et al.* 2011) and cause structural damage (cell wall destabilization). Some of the wetland populations showed increased antibiotic resistance following these exposures (Constanzo *et al.* 2005; Helt, *et al.* 2012). Some of the studies showed the wetland microbial communities recovering in population numbers and function in weeks following exposure (Weber *et al*. 2011; Helt, *et al*. 2012). This would suggest that while wetland microbial communities are susceptible to emerging contaminant exposures, they are able to return to normal function given time. Importantly these studies have only selected a few antimicrobial compounds (ampicillin, erythromycin, ciprofloxacin, tetracycline, trimethoprim), which is only one class of the emergent contaminant group. Additional studies are required to understand the full picture of what is occurring to the microbial community of contructed wetlands following emerging contaminant exposures and multiple exposures over a long period of time. The future will potentially create more strain upon the natural environment. Perhaps emerging contaminants will increase, due to increased utilization and prescriptions which lead to greater concentration exposures within the natural environments. The effects of such large and potentially dangerous exposures have not been studied in detail in the natural environment or in wetland environments. Knowledge must be gained in the present to become fully aware of the concerns so that appropriate time is allowed to implement solutions.

1.3 Objectives

The overall objective of this study was to observe the effects of antimicrobial exposures on vertical flow constructed wetland environments.

Study Objectives:

A) Characterize the development period of planted and unplanted vertical flow constructed wetland mesocosms.

B) Quantify the effects of *ex-situ* exposures of trimethoprim, triclosan and sulfamethoxazole on interstitial wetland microbial communities.

C) Assess the fate of triclosan and sulfamethoxazole in vertical flow constructed wetland mesocosms.

D) Quantify the effect of *in-situ* low and high triclosan and sulfamethoxazole exposures within planted and unplanted vertical flow constructed wetlands.

1.4 Thesis Organization

This thesis consists of five chapters starting with an introduction (Chapter 1) and ending with conclusions (Chapter 5):

Chapter 1 provides an introduction to wastewater treatment and relevant background information on the role of constructed wetlands.

Chapter 2 describes the design and operation of the vertical flow constructed mesocosms used in this study. The quantification methods for the water treatment ability, ecological characteristics and microbial community dynamics of the mesocosms are also described.

Chapter 3 summarizes the microbial community, water treatment, and ecological characteristic dynamics during the development period of the wetland mesocosms.

Chapter 4 describes four different antibiotic exposure experiments where the CW mesocosms are exposed to differing concentration of triclosan and sulfamethoxazole. The water treatment ability, ecological characteristics and microbial community dynamics are characterized prior to the exposure, during the exposure and after the exposure periods in all cases generating an account of the exposure effect in each case.

Chapter 5 summarizes the principle outcomes of the study. Future work and recommendations are presented in this chapter.

Appendix A summarizes supporting hydrological and ecological data for Chapter 3.

Appendix B summarizes supporting hydrological and ecological data for the low concentration triclosan exposure section of Chapter 4.

Appendix C summarizes supporting hydrological and ecological data for the high concentration triclosan exposure section of Chapter 4

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Appendix D summarizes supporting hydrological and ecological data for the low concentration sulfamethoxazole exposure section of Chapter 4.

Appendix E summarizes supporting hydrological and ecological data for the high concentration sulfamethoxazole exposure section of Chapter 4.

1.5 Research Timeline

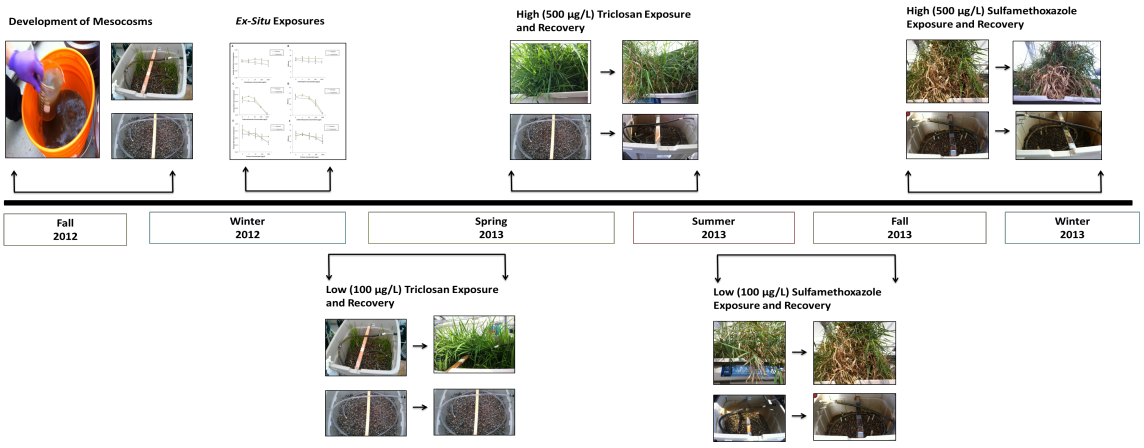

FIGURE 1.4: Research Timeline.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

Constructed wetlands can operate as large or small scale systems. The large scale system is inefficient for scientific research purposes due to their long development period, variable environmental conditions and lack of reproducibility (Kadlec and Wallace, 2009). The small scale constructed wetland is effective for scientific research due to its shorter development period, ability to control experimental conditions and reproducibility. The systems used in this study operate as mesocosms which are singular units containing properties of full-scale natural wetland systems. Wetland mesocosms, though not entirely representative of full-scale constructed wetlands, are effective for understanding physical and ecological properties that could be theoretically applied to larger scale systems. Wetland mesocosms have been shown to effectively reduce excessive organics, nutrients and pathogens within received wastewaters (LeChevallier *et al*., 1988; Green *et al*., 1998; Tanner *et al*., 1999; Stein *et al*., 2003; Tanner and Kadlec, 2003; Wahid and Tanaka, 2012). Recent studies have shown their ability to reduce emerging contaminant loads through adsorption, metabolism and sedimentation (Garcia *et al*., 2004; Matamoros *et al*., 2008; Marchard *et al*., 2010; Xian *et al*., 2010; Reyes-Contreras *et al*., 2011; Weber *et al*., 2011; Proia *et al*., 2013).

2.2 Experimental Design

The experimental design of the study was based on a factorial scheme. The 2^2 factorial design was implemented for this study where there were two types of mesocosms (planted and unplanted) exposed to two different treatments (exposure to antibiotics and no exposure). For this study, a total of twelve vertical flow constructed wetland mesocosms were built; six were planted and six were unplanted. Within these planted and unplanted groups, three mesocosms were randomly selected for exposure to antimicrobial compounds (trimethoprim, triclosan and sulfamethoxazole) over varying periods, with the other three left as control systems. Prior to these exposures all twelve mesocosms were allowed to naturally develop over a ninety day period. The ninety day development period was based on previous work by Weber and Legge (2011), which indicated ecological stabilization of treatment wetland mesocosm systems after this time period. The antimicrobial exposures were performed in low (100 $\mu g \cdot L^{-1}$) and high (500 $\mu g \cdot L^{-1}$) concentrations for each of the antibiotic compounds. Following the exposures, the mesocosms were allowed a four-week recovery period which was characterized for water treatment, ecological and microbial changes.

2.3 Mesocosm Set Up

Twelve constructed wetland mesocosms were built using 180 L plastic rain barrels (Suncast Model RB502PK) filled to 30 cm with washed Hillview limestone gravel (FIGURE 2.1). A sampling port was constructed from 2'' black PVC pipe and connected to the side of each mesocosm for easy access. All mesocosms had a starting void volume of 30 L which decreased overtime as biofilm developed. Water was continuously circulated through the mesocosms using a small rotary pump and distributed atop the surface using perforated 5/8'' OD clear tubing (which circled the width of the mesocosm surface). The outlet was constructed from ½'' OD black tubing and connected to a ball-valve which controlled the water height within the sampling

port. For the planted systems reed canary grass (*Phalaris arundinacea*) was seeded using a planting ratio of 1g per mesocosm. Microbial community seeding was performed by adding 800 mL of undiluted activated sludge from the Cataraqui Bay Water Treatment Plant at the gravel filling stage. Chlorinated tap water was used to fill the mesocosms to the gravel surface.

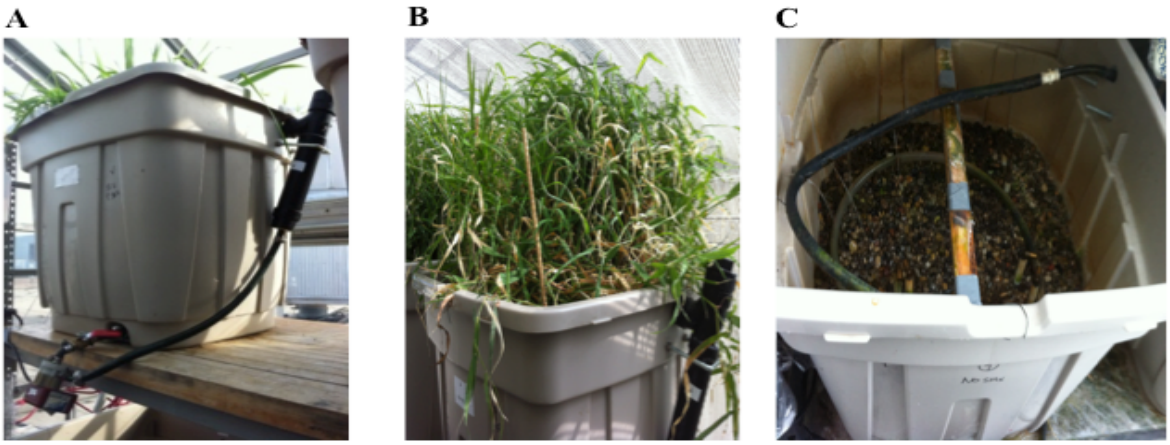

FIGURE 2.1: Vertical flow constructed wetland mesocosms. A) Exterior; B) Planted; C) Unplanted.

2.4 Mesocosm Maintenance

The mesocosms were maintained under laboratory conditions with relative air humidity of 20-80% and air temperature of 10-40°C. Plants were sprayed daily with chlorinated tap water to reduce drying. Mesocosms were completely drained once a week. Following the draining the mesocosms were refilled with a simulated wastewater solution described in Weber *et al.* (2008). The simulated wastewater solution was prepared using tap water and essential plant nutrients. The simulated wastewater solution contained 1 $g \cdot L^{-1}$ molasses, 28.75 $mg \cdot L^{-1}$ $NH_4H_2PO_4$, 151.5 $mg \cdot L^{-1}$ KNO_3, 236 $mg \cdot L^{-1}$, $Ca(NO_3)_2 \cdot 4H_2O$, 123.25 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 9.175 $mg \cdot L^{-1}$ FeNaEDTA, 0.715 $mg \cdot L^{-1}$ H_3BO_3, 0.4525 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$; 0.055 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$; 0.0125 $mg \cdot L^{-1}$ $CuSO_4$ and 0.005 $mg \cdot L^{-1}$ $(NH_4)6Mo_7O_{24} \cdot 4H_2O$. The molasses contributed 500 $mg \cdot L^{-1}$ of chemical oxygen demand (COD) giving the simulated wastewater a COD:N:P ratio of 100:5:1. This drain/feed operation also allowed for hydrological parameters such as porosity (drainable volume of mesocosms) and evapotranspiration (daily water volume lost) to be measured accurately. The mesocosms were not disassembled during the study in order to keep established biofilm within the gravel medium intact. The interstitial microbial communities were examined during the experimental periods, which in systems employing complete recirculation have been shown to be representative yet not exactly the same as biofilm communities (Weber and Legge, 2013).

2.5 Water Treatment Methods

2.5.1 Chemical Oxygen Demand

Chemical oxygen demand is defined as the number of oxygen equivalents used in the oxidation of compounds within a water sample (Eaton *et al.,* 1995). The COD of the simulated wastewater solution was 500 $mg \cdot L^{-1}$ at the time of each refilling. Standard Method #5520 D was adapted for the mesocosms to include 1.5 mL of digestion solution (5.13g potassium dichromate

dried at 103 Celsius for 1 hr, 84 mL of sulfuric acid, 16.67 g of mercury sulfate, 500 mL of deionized water), 3.5 mL of sulfuric acid solution (5.10 g of silver sulfate, 500 mL of deionized water), 2.5 mL of interstitial water (Eaton et al., 1995). Interstitial water was collected from the sampling port of all twelve mesocosms immediately after each weekly feed and three hours following the feed. This time selection was based upon the observed COD ($mg \cdot L^{-1}$) removal trends (FIGURE 2.2A) and was chosen to reduce the number of samples each week to 24 total (2 per mesocosm) rather than 240 total (20 per mesocosm as shown in FIGURE 2.2A). The interstitial water samples were kept refrigerated (at 4˚C) for 24 hrs prior to being analyzed. Within a 10 mL test tube, 1.5 mL of digestion solution, 3.5 mL of sulfuric acid solution and 2.5 mL of interstitial water was added. These prepared vials were shaken and heated at 150 ˚C on a block heater for two hours. Following this incubation, the vials were allowed to cool and shaken once more. The cooled vials were read using a Thermo-scientific colorimeter set to 610 nm. The readings were recorded and then transformed via the linear calibration curve (y = mx +b) into COD concentration ($mg \cdot L^{-1}$) using a prepared standard curve (FIGURE 2.2B). The COD standard curve was created using potassium hydrogen phthalate (KHP) concentrations that were read using a Thermo-scientific colorimeter set to 610 nm.

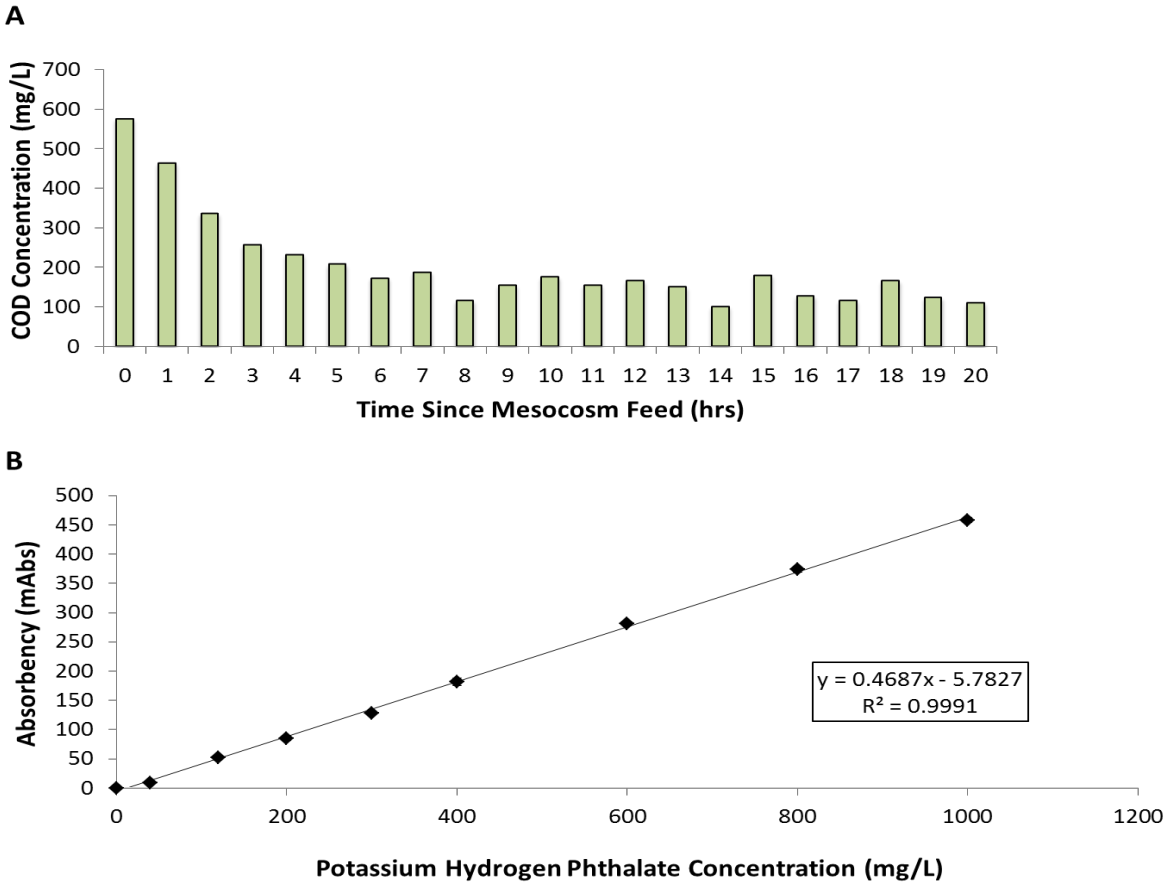

FIGURE 2.2: A) Chemical oxygen demand removal ($mg \cdot L^{-1}$); B) Standard curve using potassium hydrogen phthalate.

2.5.2 Water Chemistry

YSI Professional Plus probes were used to collect daily measurements of water quality. Water quality variables included ammonium (NH_4^+, $mg \cdot L^{-1}$), conductance ($\mu S \cdot cm^{-1}$), dissolved oxygen ($mg \cdot L^{-1}$), nitrate (NO_3^-, $mg \cdot L^{-1}$), pH, redox potential (mV) and water temperature (˚C). The YSI probes were inserted into each mesocosm sampling port and water quality readings recorded once the variables stabilized. The recorded data was downloaded within the YSI Data Manager software and exported into a Microsoft Excel file for data analysis.

2.6 Ecological Methods

2.6.1 Vegetative Mass

Six mesocosms were planted with reed canary grass (*Phalaris arundinacea*) at 1g per mesocosm at the start of the development period. Plant height and stem count were measured weekly within these mesocosms.. Plant height was measured using ten randomly selected and representative reed stems.

2.7 Hydrological Methods

2.7.1 Evapotranspiration

Evapotranspiration is a measure of water loss from a surface based upon air movement, air temperature and transpiration of vegetation (Kadlec and Wallace, 2009). Each mesocosm had a reference height marked at 4 cm below the gravel surface. Water was added daily to reach the top of the marked height. The volume added each day was representative of the last days water loss and is discussed here as evapotranspiration ($L \cdot d^{-1}$).

2.7.2 Porosity

Porosity was calculated from the volume of the bed medium and the volume of the pore space. The volume of the medium (dimensions which both the gravel and water occupied) was said to be the volume of the bed medium. The volume of the pore space is represented here as the drained volume of the bed medium (i.e. drainable porosity). The mesocosms were drained weekly prior to feeding and this volume was used to calculate porosity.

$$\phi = \frac{\text{Volume of pore space}}{\text{Volume of medium}} \quad (1)$$

2.8 Microbiological Methods

2.8.1 Community Level Physiological Profiling

Community level physiological profiling characterizes heterotrophic microbial function based on carbon utilization (Weber and Legge, 2010). BIOLOG™ microplates have 96 wells (31 carbon wells and one blank well, all in triplicate) providing 3 replicated carbon source utilization patterns (CSUPs). Each well contains a different carbon substrate and a tetrazolium violet dye. Interstitial water, containing a mixed microbial community was collected from each wetland mesocosm at various times throughout the studies. Each microplate well was inoculated with 100 µL of a mixed microbial community sample and allowed to develop over time. Microbial activity within each well was signified by NADH production that reduced the tetrazolium violet to formazan resulting in a purple colour. The purple colour was detected photometrically using a BIORAD iMark ™ Microplate Reader at 595 nm wavelength. The inoculated BIOLOG™ microplates were read at selected times post-inoculation e.g. (18 hrs, 24 hrs, 42 hrs, 48 hrs, 66 hrs, 72 hrs, 90 hrs, 96 hrs). A representative time point was then selected (Weber and Legge, 2010) and the data was analyzed by examining the average well colour development, substrate diversity, richness, and carbon source utilisation patterns (CSUPs) (Weber and Legge, 2010). Average well colour development expresses the activity of the microbial community. Greater microbial activity is indicated with a deeper colour development in the wells. Substrate diversity is a metric used to describe the overall microbial community functional capacity. Substrate richness expresses the number of different carbon sources utilized by the microbial community (in the context of water treatment this can be said to be a measure of microbial community potential). Greater richness is indicated with a greater number of developed wells.

Average well colour development (AWCD):

$$\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0) \quad (2)$$

A_i = absorbance reading of well *i*
A_0 = absorbance reading of blank well (no carbon source)

Substrate Diversity (Shannon Index):

$$H_{CLPP} = -\sum p_i \ln (p_i) \quad (3)$$

$$p_i = \frac{\text{Activity of a particular substrate}}{\text{Sum of activities of all substrates}} \quad (4)$$

Activity = absorbance value at 595 nm

Substrate Richness:

$$\#\text{ of wells where } (A_i - A_0) \geq 0.25 \quad (5)$$

Principle component analysis (PCA) was performed using the covariance (n-1) matrix of CSUP data to further assess for differences between mesocosms. PCA is used to ordinate a large amount of data onto a two dimensional plane. Datasets were subjected to Taylor transformation

based on assessment of normality and homoscedasticity following the recommendations of Weber *et al* (2007).

2.8.2 Interstitial Microbial Community Sampling

Interstitial water (containing a mixed microbial community) was collected from all twelve wetland mesocosms two days prior to the feed. These samples were also collected weekly following each antimicrobial exposure for a total of four-weeks. The black sampling port was unscrewed and 40 mL of water was collected with a 10 mL Eppendorf Research® Plus pipette. The water samples were left at room temperature prior to CLPP analysis to prevent shock to the microorganisms.

2.8.3 Inoculation of Microplates

Twelve BIOLOG™ microplates were left on the bench top to heat to room temperature while the interstitial microbial communities were sampled. The microplate inoculation occurred using aseptic techniques inside a clean hood that was washed with a 70% ethanol/water solution prior to its use. The twelve interstitial samples, twelve labelled BIOLOG™ microplates, one stack of sterile petri dishes, one box of sterile 200 µL pipette tips and one 30-300 µL Eppendorf Research® multi-channel pipette were placed inside the clean hood. One sterile petri dish was taken out of the package and separated into its top and bottom halves. Each half of the petri dish was put face down to prevent contamination. One interstitial sample was gently shaken and one half of the petri dish was up-righted. In the up-righted petri dish 20 mL of the interstitial sample was emptied. Eight sterile 200 µL pipette tips were attached to the 30-300 µL Eppendorf Research® multi-channel pipette. Pipette tips were first rinsed with the interstitial sample before each inoculation. Each well was inoculated with 100 µL of the interstitial sample. New pipette tips were attached for every sample to avoid cross contamination. Once all twelve BIOLOG™ microplates were inoculated they were incubated in the dark at room temperature (20˚C) with gentle agitation (VWR Minishaker at RMP 100). Microplates were read photometrically at defined time intervals using a BIO RAD iMark Microplate Reader. The microplates were read individually at 595 nm following a 5 second shake at medium setting to ensure each well was well mixed. The resulting absorbance readings were exported as Microsoft Excel files for later data analysis.

CHAPTER 3 – MESOCOSM DEVELOPMENTAL PERIOD

3.1 Introduction

There is a lack of understanding when it comes to the developmental period of constructed wetland systems (Kadlec and Wallace, 2009). The developmental period is defined as the time required for microbial and vegetative structures to stabilize within a constructed wetland system. Recently, planted vertical flow constructed wetland mesocosms were shown to require 90 days to reach an ecological equilibrium (Weber and Legge, 2011). Throughout the development period, there are changes to the entire wetland system that are often not characterized. Objective A is to characterize the development period of the planted and unplanted vertical flow constructed wetlands. A variety of metrics were measured to record the ecological, hydrological and microbial community changes throughout this period.

3.2 Materials and Methods

Refer to Chapter 2 for a full description of materials and methods.

3.3 Results and Discussion

3.3.1 Water Treatment

Water treatment in treatment wetlands is largely based upon microbial activity within the bed medium (Kadlec and Wallace, 2009). COD removal and nitrogen removal are common measures of water treatment in aquatic environments. The nitrogen cycle is a multi-step process that requires the nitrification cycle and de-nitrification in order to convert organic nitrogen into volatile di-nitrogen gas (Lee, 2009).

The planted and unplanted mesocosms showed similar chemical oxygen demand removal rates throughout the start-up period (FIGURE 3.1). The chemical oxygen demand ($mg \cdot L^{-1}$) removal was calculated on a weekly basis to provide the removal rate ($mg \cdot L^{-1} \cdot h^{-1}$). During the start-up phase there were no antibiotic exposures, therefore there were 6 replicates for each of the planted and unplanted systems. Throughout the entire start-up period there was no statistically significant difference between planted and unplanted systems ($p<0.05$). Within the early weeks of the development period the unplanted mesocosms seemed to have a greater capacity for the removal of organics. This could be due to a faster microbial community development within the unplanted systems. The activated sludge was collected in 5 gallon pails, and although well mixed before adding to the mesocosms did have a significant solids fraction which could settle and therefore bias certain 800 mL fractions. The planted mesocosms caught up to the unplanted mesocosms at the fourth week. Following the fourth week, the chemical oxygen demand removal rates for both systems gradually increased towards the end of the period. Although not significantly different (mostly due to large standard deviations) the planted systems had a greater capacity for COD removal than the unplanted in the latter part of the development period. This is likely due to the presence of the rhizosphere in the planted systems, which provided more area for microbial attachment and enhanced microbial activity (Stottmeister, *et al*. 2003; Vacca, *et al*. 2005; Zhao *et al*. 2012). This is consistent with the observations of Weber and Legge (2013) where the rhizosphere microbial community was shown to be 10 times more active than biofilm communities in mesocosm treatment wetland systems. The ammonium and nitrate

removal/generation capacity was also measured throughout this experimental period. The data was not conclusive (attributed to poor data quality collected by the YSI probes) and is available in Appendix A for further consideration.

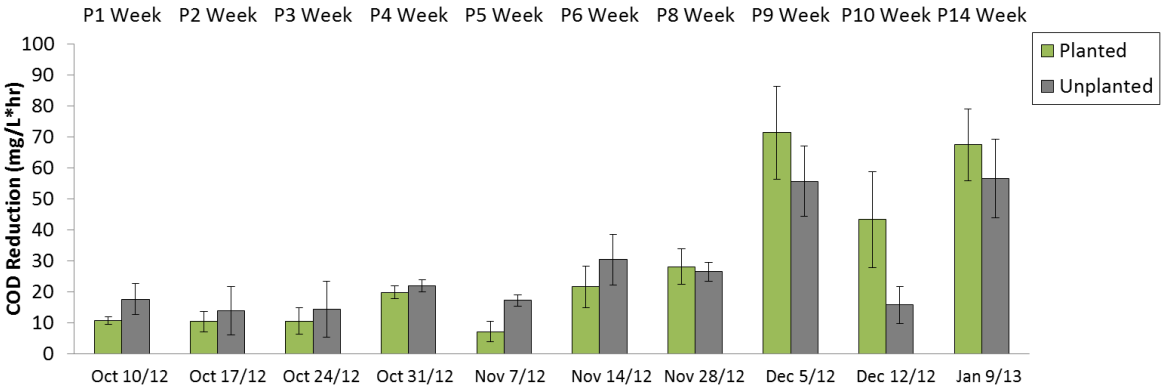

FIGURE 3.1: Chemical oxygen demand removal rate ($mg \cdot L^{-1} \cdot hr^{-1}$) during start-up period.

3.3.2 Water Quality

Water quality parameters were measured using the YSI Professional Plus probes daily. The parameters measured were conductivity, dissolved oxygen concentration, redox potential, water temperature and pH. The planted and unplanted systems had different total dissolved solid concentrations at the outset of the start-up period (FIGURE 3.2). Additional nutrients, which the unplanted systems did not receive, were added to the planted systems for the first 10 weeks to promote an observed slow plant development. This lack of nutrients could explain the differences observed between the mesocosm types. The planted systems had significantly ($P < 0.05$, ANOVA) greater total dissolved solids compared to the unplanted systems throughout the majority of the development period (FIGURE 3.2). Both planted and unplanted systems received the same nutrient solution (described in Chapter 2) after week 10. Following this, the unplanted mesocosms approximately four weeks to reach the total dissolved solids concentration of the planted systems.

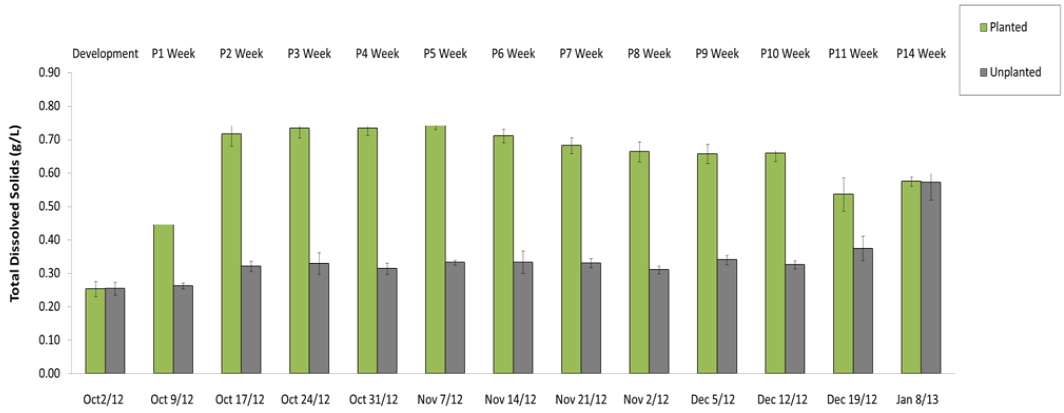

FIGURE 3.2: Total dissolved solids ($g \cdot L^{-1}$) during start-up period.

The unplanted systems had higher dissolved oxygen compared to the planted systems (FIGURE 3.3A). This was unexpected as the unplanted systems had high COD removal rates during this period. The dissolved oxygen levels for unplanted systems were between 1-4 $mg \cdot L^{-1}$ higher than the planted systems depending on the day, with samples obtained from the sampling port (FIGURE 3.3A). The dissolved oxygen dropped during the first two weeks of the experimental period, more so for the planted systems (8 $mg \cdot L^{-1}$ to 2 $mg \cdot L^{-1}$) than the unplanted (8 $mg \cdot L^{-1}$ to 6 $mg \cdot L^{-1}$). This drop may signify an enhanced microbial activity that consumed the available oxygen within the wetland system during metabolism (Kadlec and Wallace, 2009). The planted systems had a greater microbial population, perhaps due to the presence of the rhizosphere, and thus had a greater loss of dissolved oxygen in the water phase during the first weeks of the experimental period. Cycles within each mesocosm were also observed, the dissolved oxygen for both unplanted and planted systems increased after the feed day, indicating re-aeration occurring from the draining process.

Redox potential is a measure of oxidation/reduction potential within the environment with positive values indicating an aerobic environment and negative values indicating an anaerobic environment (Kadlec and Wallace, 2009). Within the first week of the study the redox potential dropped drastically (+350 mV to +75 mV) for both the unplanted and planted systems, perhaps due to the addition of activated sludge that caused oxygen consumption. The redox potential decrease one week post start-up would indicate a declining oxygen environment, which was also shown in FIGURE 3.3A. At the second week the planted mesocosms had similar redox potential (+100 mV) as compared to the unplanted systems (+75 mV) (FIGURE 3.3B). The unplanted and planted systems had similar redox potential patterns, which decreased directly after the feed-day and increased several days after the feed day, being consistent with expectations based on the DO profiles.

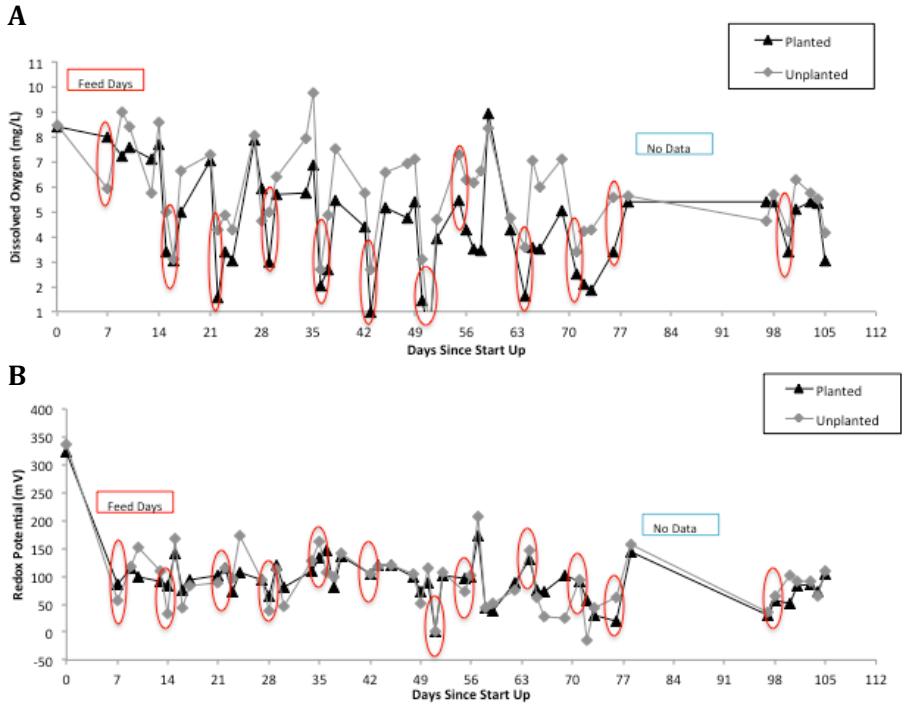

FIGURE 3.3: A) Dissolved oxygen ($mg \cdot L^{-1}$); B) Redox potential (mV) during start-up period.

Water temperature (FIGURE 3.4A) had a range of 15- 23 Celsius for the planted systems and 14-22 Celsius for the unplanted. The planted systems had significantly ($P < 0.05$, ANOVA) greater water temperatures compared to the unplanted systems for half of the observed weeks. The planted mesocosms most likely had greater water temperature (and enhanced microbial activity) due to being on the top shelf of the greenhouse (the unplanted mesocosms were on the bottom shelf and were therefore partially shaded). The water pH (3.4B) for the unplanted and planted systems was consistent throughout the experimental period. This was due to the mesocosm beds being filled with limestone gravel, which acted as a buffer. The pH for both systems was between 7.5 and 8.0 during this period.

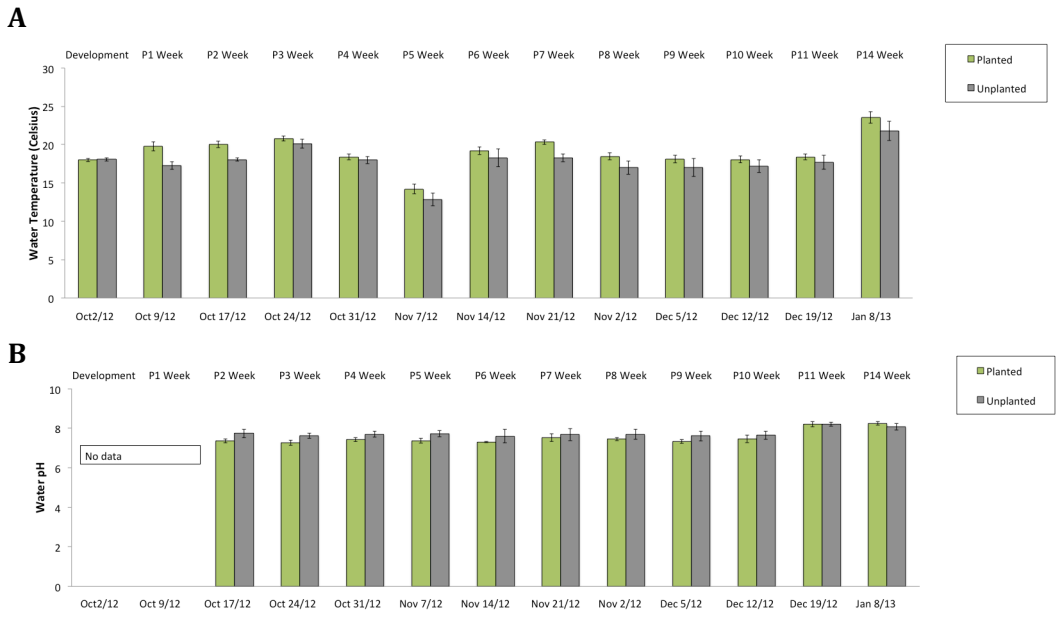

FIGURE 3.4: A) Water temperature (Celsius); B) Water pH during start-up period.

3.3.3 Ecological and Hydrological Characteristics

The hydrological characteristics were similar between the planted and unplanted systems. The porosity of the planted systems was between 0.31 – 0.35 and the unplanted between 0.30 – 0.35 (considering standard deviation bars). The porosity remained consistent over the development period for both the planted and unplanted mesocosms (FIGURE 3.5A). Over time biofilm is produced by the microbial communities within the bed medium of the constructed wetland and sheared from the medium by water velocity (Kadlec and Wallace, 2009). The exact quantities of the biofilm were not directly measured. This observation is consistent with other published data (Weber *et al.,* 2011).

The evapotranspiration rates within the planted mesocosms were similar to the evaporation rates in the unplanted mesocosms (FIGURE 3.5B). The large fluctuation of evapotranspiration over the development period does not correlate with water temperature (FIGURE 3.4A) that remained close to 20 Celsius for the majority of the period (save a small decrease in water temperature during the fifth week). The evapotranspiration and evaporation

rates were large during the first few weeks of the experimental period. The planted evapotranspiration rate was significantly ($P<0.05$) greater than the unplanted evaporation rate during the second week. Though beyond this, the two systems experienced similar water loss trends. The evapotranspiration was low for the majority of the experimental period, followed by large increases during the seventh, eighth and fourteen weeks. Although the data was not collected, incident solar radiation did seem to generally correlate with the observed evapotranspiration trends.

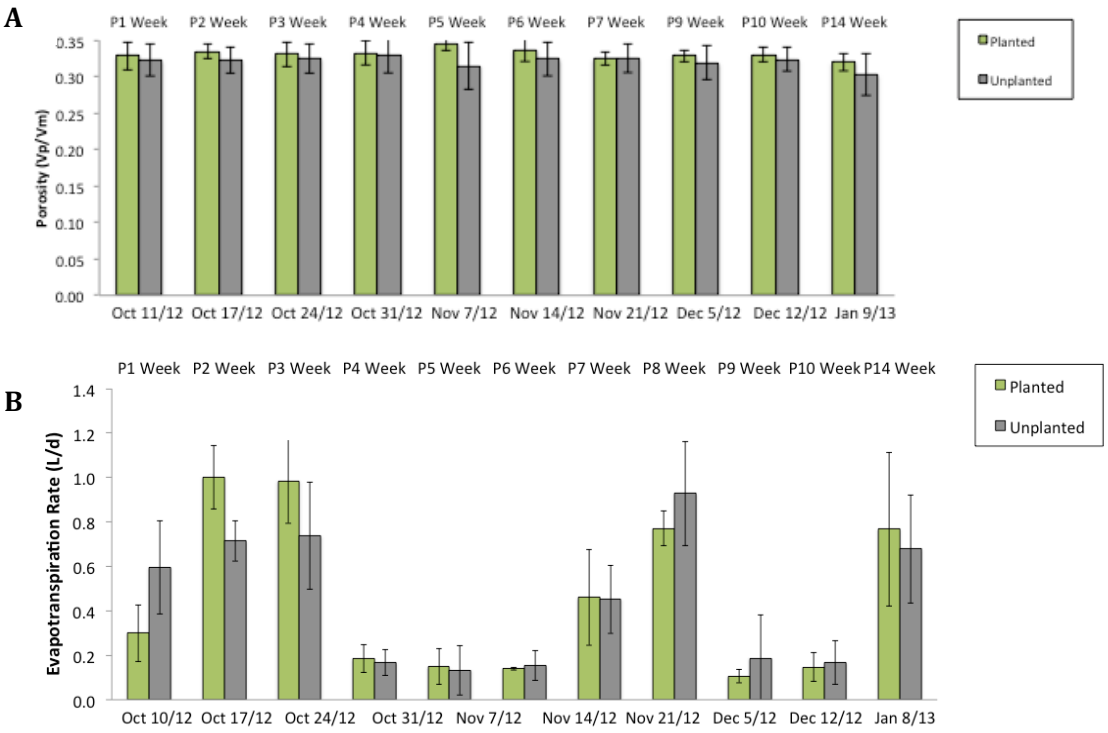

FIGURE 3.5: A) Porosity (volume of pore space/volume of bed medium); B) Evapotranspiration rate ($L \cdot d^{-1}$) during start-up period.

The planted systems were seeded with 1 g of reed canary grass seed per mesocosm. During the fifth week post-inoculation the vegetation experienced a die off (FIGURE 3.6A) perhaps due to cold air temperatures. A small decrease in water temperature can be seen in FIGURE 3.4A, however this does not describe the cold air temperatures that were observed (5 degrees C due to a building heating malfunction). Despite this die back of plant mass, plant height was relatively unchanged and growth increased steadily over the development period (FIGURE 3.6B). The rhizosphere zone was not directly characterized in each mesocosm in this study, though the increasing height of the vegetation would indicate a healthy ecosystem and developing rhizosphere zone.

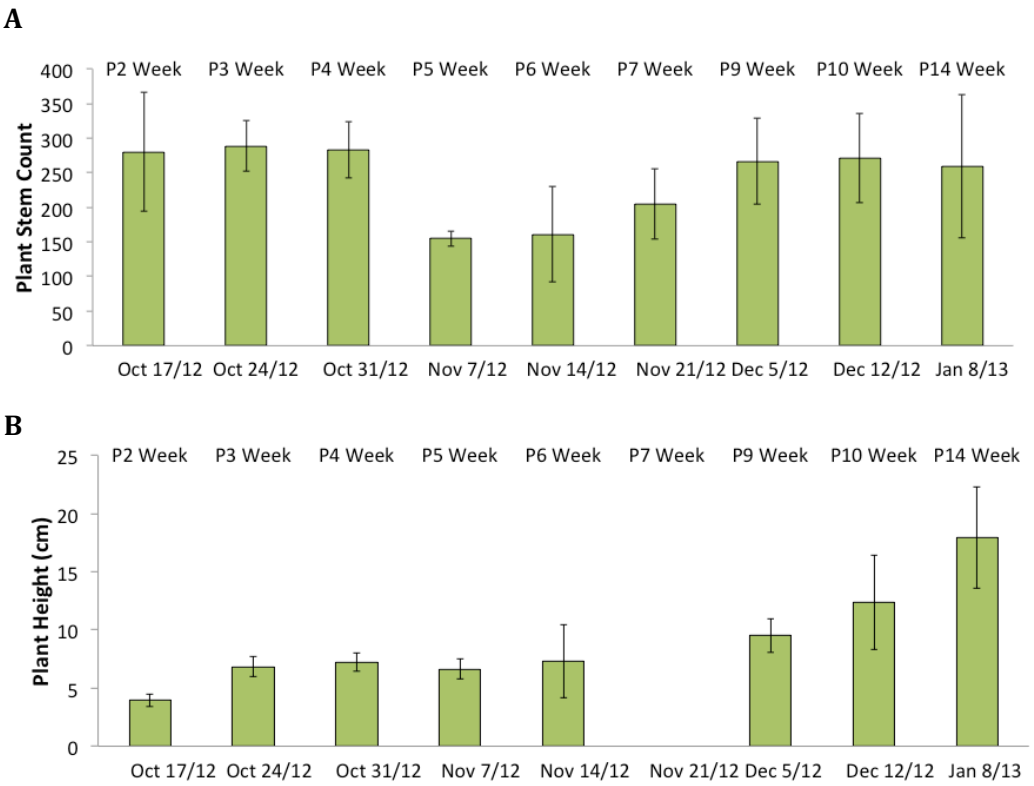

FIGURE 3.6: A) Plant count; B) Plant height (cm) during start-up period.

3.3.4 Microbial Characteristics

Microbial development was quantified using community level profiling data, with metrics being average well colour development, richness and diversity (Chapter 2 Section 2.8). Previous studies have used the community level profiling method to evaluate microbial community function within constructed wetland environments (Weber *et al*., 2007; Weber *et al*., 2008; Weber and Legge, 2009; Weber *et al*., 2010; Weber and Legge, 2011). As opposed to looking at overall averages (AWCD) or single carbon sources, carbon utilization can also be examined by grouping the carbon sources into guilds (TABLE 3.1) giving an indication of the range of function for a microbial community for a specific carbon source type.

TABLE 3.1: Biolog Ecoplate™ carbon source guilds.

Well Number	Label	Carbon Source	Guild
1	c0	Water (blank)	
2	c1	Pyruvic acid methyl ester	Carbohydrate*
7	c6	D-cellobiose	Carbohydrate
8	c7	Alpha-D-lactose	Carbohydrate
9	c8	Beta-methyl-D-glucoside	Carbohydrate
10	c9	D-xylosea[a]	Carbohydrate
11	c10	I-erythritol	Carbohydrate
12	c11	D-mannitola[a]	Carbohydrate
13	c12	N-acetyl-D-glucosaminea[a]	Carbohydrate
15	c14	Glucose-1- phosphatea*[a]	Carbohydrate
16	c15	D,L-alpha-glycerolphosphate*	Carbohydrate
14	c13	D-glucosaminic acid	Carboxylic & acetic acids
17	c16	D-galactonic acid-gamma lactone	Carboxylic & acetic acids
18	c17	D-galacturonic acid	Carboxylic & acetic acids
19	c18	2-Hydroxy Benzoic acid	Carboxylic & acetic acids
20	c19	4-Hydroxy Benzoic acid	Carboxylic & acetic acids
21	c20	Gamma-hydroxybutyric acid	Carboxylic & acetic acids
22	c21	Itaconic acid	Carboxylic & acetic acids
23	c22	Alpha-Ketobutyric acid	Carboxylic & acetic acids
24	c23	D-malic acida[a]	Carboxylic & acetic acids
3	c2	Tween 40	Polymers
4	c3	Tween 80	Polymers
5	c4	Alpha-cyclodextrin	Polymers
6	c5	Glycogen	Polymers
25	c24	L-arginine	Amino acids
26	c25	L-asparagine	Amino acids
27	c26	L-phenylalaninea[a]	Amino acids
28	c27	L-serine[a]	Amino acids
29	c28	L-threoninea[a]	Amino acids
30	c29	Glycyl-L-glutamic acid	Amino acids
31	c30	Phenylethylamine	Amines & amides
31	c31	Putrescinea[a]	Amines & amides

* adapted from Weber and Legge, (2011)

[a] root exudates listed by Hodge (1998).

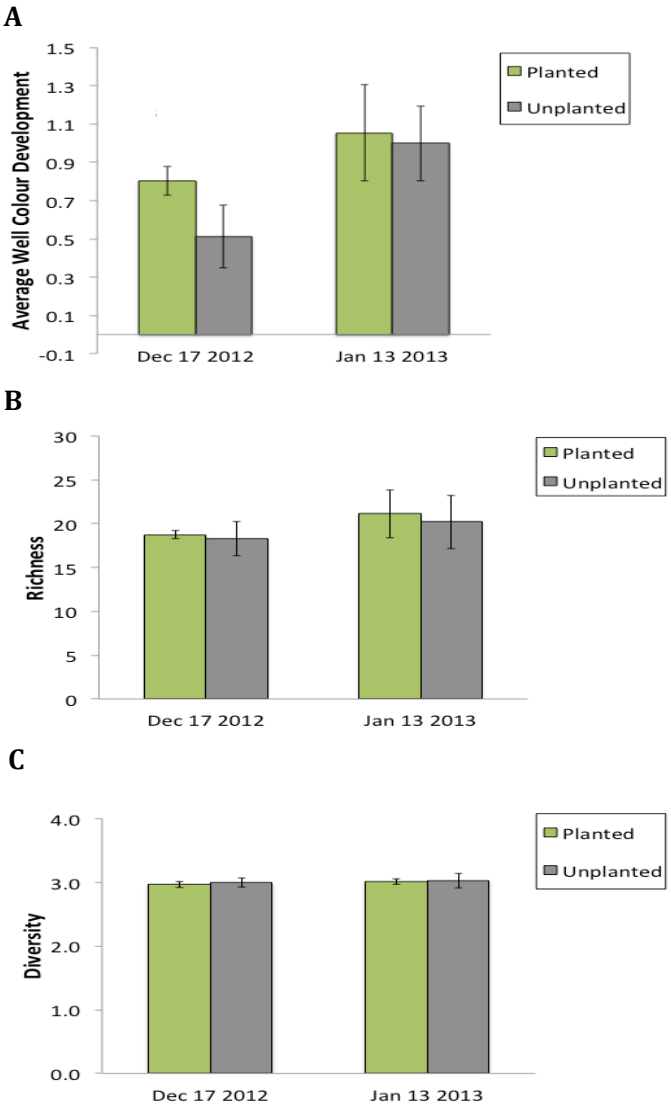

FIGURE 3.7: A) Microbial community metabolic activity (AWCD); B) Richness (number of carbon sources utilized); C) Diversity (metabolic capacity) during start-up period.

All mesocosms had increased average well colour development through the experimental period (FIGURE 3.7A). The planted systems increased from 0.70 to 1.3 and unplanted systems 0.50 to 1.2 (considering standard deviation bars). The planted systems had significantly ($P<0.05$, t-test) greater average well colour development at the start of the development period (December 2012) compared to the unplanted (FIGURE 3.7A). This could be due to the planted systems benefiting from rhizosphere influences. As described by Weber and Legge (2013) the rhizospheric region has been shown to be 10 times more active than biofilm regions in wetland mesocosms. Both richness and diversity parameters remained unchanged in the planted and unplanted systems over the development period indicating that although activity increased the relative number and proportions of carbons sources utilized did not change over this time period.

Principle component analysis (PCA) was used to examine the carbon source utilization patterns (CSUPs) of the planted and unplanted systems. Prior to performing the PCA, the community level profiling dataset was transformed via the Taylor function to increase the normality and homscedasticity of the dataset (Weber *et al*., 2007). The planted and unplanted mesocosms grouped differently which indicated different carbon utilization patterns (FIGURE 3.8). PCA as shown here incorporates the relative utilization of all carbon sources on an individual basis. The planted mesocosms were more tightly grouped (more so in the centre and top section of the ordination) than the unplanted, most likely due to the release of common root exudates by the rhizosphere (Vacca, *et al*. 2005). The planted systems were defined by C9, C11, C14, C23, C26 and C27 carbon sources, which are known root exudates (TABLE 3.1). There was a greater spread of data within the unplanted systems, which indicates greater differences in carbon sources utilized between systems. The unplanted systems obviously do not have a common rhizosphere zone to help regulate the microbial community function.

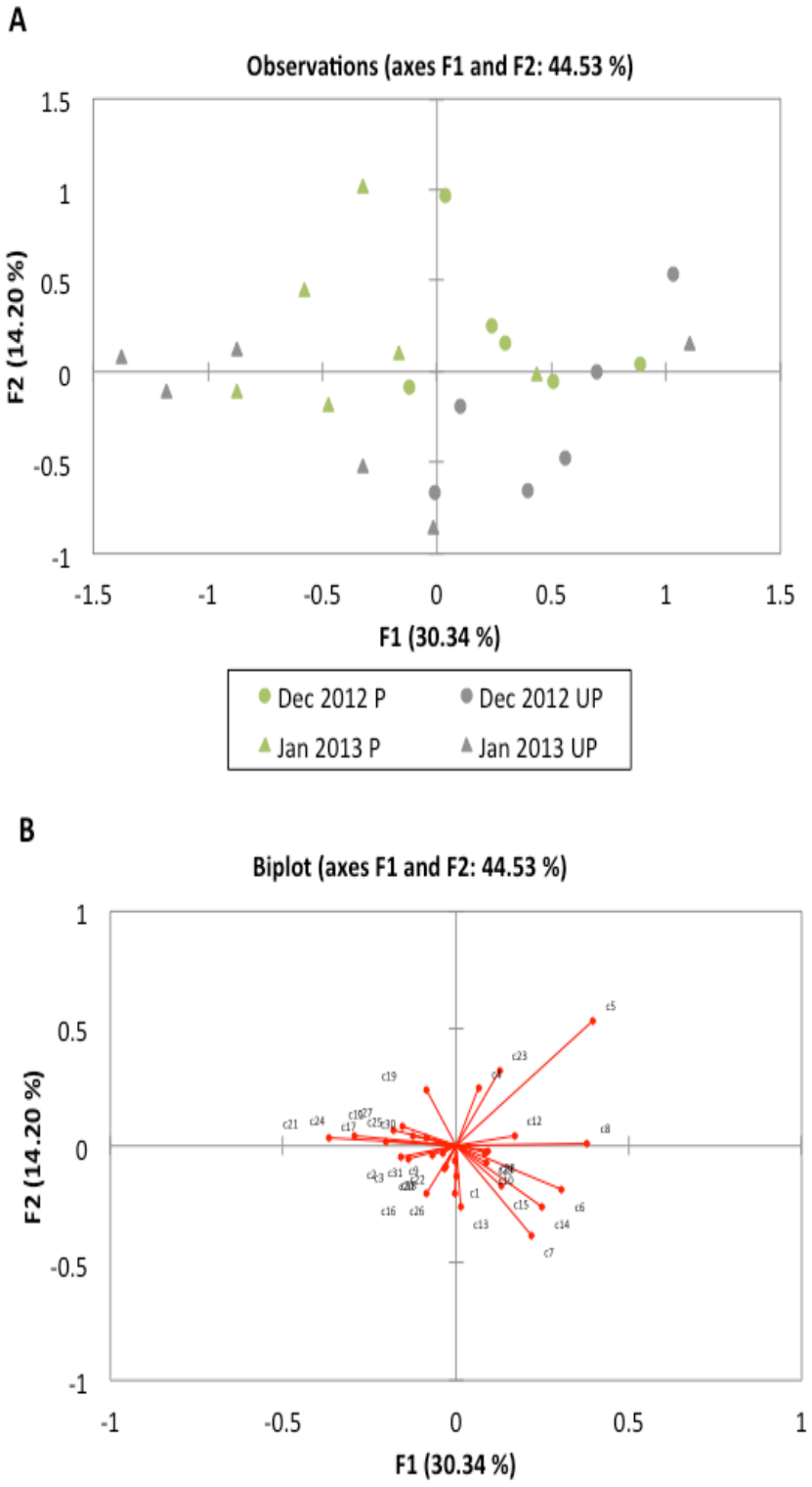

FIGURE 3.8: A) Principle component analysis plots based upon Taylor transformed carbon source utilization patterns (CSUPs) during start-up period. Mesocosms are P (planted) and UP (unplanted). B) Carbon source utilization bioplot.

3.4 Conclusions

The development period is an important stage to consider in constructed wetland systems. This study examined the development period for both planted and unplanted vertical flow constructed wetland mesocoms over a ninety day period. The planted systems had enhanced water treatment abilities (chemical oxygen demand removals) compared to the unplanted systems. The two systems had similar hydrological and water quality (porosity, evapotranspiration, water temperature and pH) properties. The planted systems had a more tightly knit microbial community function compared to the unplanted systems considering the principle component analysis plots. The carbon guild utilizations (CSUPs) were different between the two systems with the planted systems utilizing root exudates not found in the unplanted systems. The results observed give insight into the development period of what could be expected for full-scale systems. Future constructed wetland studies should consider characterizing the development period to a) understand the changes occurring within the systems and to b) have an accurate baseline of activity within the system prior to any experimentation.

CHAPTER 4 – FATE AND EFFECTS OF TRICLOSAN AND SULFAMETHOXAZOLE ON VERTICAL FLOW CONSTUCTED WETLAND MESOCOSMS

4.1 Introduction

4.1.1 Background

Ecological risks imposed by anthropogenic activities are present in current society. Terrestrial and aquatic landscapes are affected by habitat fragmentation, elimination of species and the introduction of chemical species in the environment (Daugthon and Ternes, 1999). Of these, the most concerning ecological risk is arguably the introduction of compounds within the natural environment (Constanzo, *et al*. 2005; Clarke and Smith, 2011; Chen, *et al.* 2011). The major classes of compounds concerning ecological systems are pharmaceuticals and personal care products (collectively referred to as PPCPs) and nanomaterials (Klaine, *et al.* 2009). These chemicals are continuously being added into the environment through inadequate wastewater treatment and surface runoff (Ternes, 1999; Heberer, 2002). Some of these compounds are known to be persistent over time leading to chronic exposures to aquatic organisms. Pharmaceuticals are compounds used to treat physical ailments of humans and other animals (Caliman and Gavrilescu, 2009). Pharmaceuticals include antibiotics, lipid regulators, anti-inflammatories, beta-blockers, antineoplastics, retinoids, impotence drugs and tranquilizers. The release of pharmaceuticals into the environment is predominantly through the release of human body waste into our sewer systems. Some chemical species bio-transform within the body and are excreted as different species, which can contain properties varying from the parent compound. Personal care products are compounds manufactured for human use (Caliman and Gavrilescu 2009). These include cleansers, soaps, oral hygiene products, hair care products, sunscreens and perfumes. These compounds are directly released into the environment through wash-off within recreational waters or volatilization into the air. Selected pharmaceuticals have been shown to be moderately effectively (33-99%) removed in wastewater treatment plants having aerated basins, long solid retention times (over 4 hrs), and a secondary treatment stage containing activated sludge (Batt, *et al*. 2007). These incoming pharmaceuticals are biodegraded in the secondary treatment stage or adsorbed onto particles that eventually settle to the bottom of the basins where they then become part of the biosolids stream, with consequent uncertainty in fate and behaviour during processing/final disposal. Wastewater treatment plants can face varying concentrations of pharmaceuticals and personal care products from incoming wastewater depending on the season (Conkle, *et al*. 2008). The incomplete removal of PPCPs in wastewater treatment plants allows some amount ($\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$) of these products to enter the aquatic environment (Daughton and Ternes, 1999). Over the past decade the analytical detection limit of emerging contaminants has been improved from $\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$ using advanced methods of detection such as liquid chromatography/mass spectrometry/mass spectrometry (Haack, 2009). With these new detection methods established the accurate quantification of emerging contaminants within wastewater effluent and the natural environment are becoming better known (Kolpin, *et al.* 2002; Gomez, *et al*. 2006).

Constructed wetlands are utilized for wastewater treatment in a variety of countries (Matamoros *et al*., 2006; Matamoros *et al*., 2007; Conkle *et al*., 2008; Matamoros *et al*., 2008; Dordio *et al*., 2009; Matamoros *et al*., 2009; Park *et al*., 2009; Garcia *et al*., 2010). Denmark for example has over 10,000 single home vertical flow subsurface CW system installations

augmenting septic tanks in both rural and suburban regions (approximately 4 m^2 for a single home).

The constructed wetland is a man-made system containing varying redox regions, vegetation regions with highly developed and active microbial communities.. Due to a greater efficacy following filtration for solids CWs are generally used for secondary or tertiary water treatment (Kadlec and Wallace, 2009). The treatment ability of constructed wetlands depends on the incoming chemical compounds, the environmental conditions within the wetland and the type of wetland used (surface or subsurface flow). The vertical flow constructed wetland is a popular system, having high aeration and thus lending itself to enhanced removal of select pharmaceuticals and personal care products (Huang, *et al*., 2004; Matamoros, *et al*., 2009). The microbial communities within wetlands are the major treatment mechanism for organic compounds (Faulwetter *et al*. 2009; Kadlec and Wallace, 2009, Weber and Gagnon, 2014). With microbial communities playing such an important role, the effect of PPCPs like antibiotics is of particular concern to CW scientists, engineers, and regulators. The effect of the common antibiotic ciprofloxacin on vertical flow constructed wetlands has been recently studied (Weber, *et al*. 2008; Weber, *et al*. 2010; Weber *et al*. 2011; Helt, *et al*. 2012). The chemical exposures were shown to initially decrease CW microbial community activity and diversity and increase microbial resistance to other antibiotic compounds, however, owing to the robust nature of CW systems a fast recovery occurred 2 weeks after the exposure period. In addition the planted mesocosms used in the study showed more resilience to the exposure compared to the unplanted mesocosms, perhaps due to the presence of the rhizosphere. These studies were the first of their kind and did not track water treatment efficiency during the same period. Although it is generally understood that a reduction in microbial activity likely indicates a reduction in organic removal rates, this has never been explicitly studied.

The objectives (B, C, D) of this study were to evaluate and quantify the effect of selected antimicrobial compounds on vertical flow constructed wetland systems. Over the course of one year, four antimicrobial exposure experiments involving triclosan and sulfamethoxazole were completed using planted and unplanted vertical flow constructed wetland mesocosms. The antimicrobials selected were introduced at higher concentrations than found within aquatic environments to represent worst-case scenarios or "shocks" to the vertical flow constructed wetlands. This ‘shock’ is representative of a situation where a small community is prescribed the same antibiotic during a community-wide infection (sulfamethoxazole) and therefore higher than normal concentrations can be found entering water treatment facilities such as CW systems, or during the same event copious amounts of hand sanitizer are used (triclosan). Over the past twenty-five years, pharmaceutical prescriptions have been on an increasing trend in North America and Europe (Canadian Institute for Health Information; Silwer, 2007). Increasing demands on health care systems have led to increased prescriptions and increased expenditures for pharmaceutical compounds. This pattern of pharmaceutical usage increases in times of population illness. The effect of such high exposures to antimicrobials within vertical flow constructed wetlands (as studies have focused on removal, not effect of PPCPs) (Matamoros *et al*., 2006; Matamoros *et al*., 2007; Conkle *et al*., 2008; Matamoros *et al*., 2008; Dordio *et al*., 2009; Matamoros *et al*., 2009; Park *et al*., 2009; Garcia *et al*., 2010). Water treatment abilities, ecosystem characteristics and microbial community properties were monitored throughout each antimicrobial experiment in this study.

4.1.2 Trimethoprim

Trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) is an antimicrobial used to treat urinary tract infections (Straub, 2013). Trimethoprim is a bacteriostatic that prevents fatty acid synthesis in the cell, which is required for DNA replication. Trimethoprim is not considered carcinogenic and is not lipophilic in nature (Straub, 2013). Trimethoprim is not readily removed (0-40%) in aerobic environments (Ternes, *et al*. 1999; Batt, *et al*. 2006; Lindberg, *et al.* 2006), though it is readily removed (> 98%) in anaerobic environments containing nitrifying bacteria (Göbel *et al.* 2005; Batt, *et al*. 2006; Mohring *et al.* 2009). Considering the majority of wastewater treatment facilities rely on an aerobic secondary treatment stage, the majority of trimethoprim remains persistent within the discharged wastewater effluent. The concentration of trimethoprim detected in European wastewater effluents is between 100 - 200 $\mu g \cdot L^{-1}$ (Straub, 2013). Trimethoprim is considered toxic at $mg \cdot L^{-1}$ concentrations for activated sludge (18 $mg \cdot L^{-1}$) *D. magna* at (123 $mg \cdot L^{-1}$) and green algae (100 $mg \cdot L^{-1}$) (Halling – Sorensen, *et al*. 2000). The effect of trimethoprim on wetland microbial communities has not been extensively characterized.

4.1.3 Triclosan

Triclosan (2,4,4'-Trichloro-2'-hydroxydiphenyl ether phenol) is an antimicrobial additive in fabrics, plastics, paints, sealants and cleaning products (EPA, 2006). Triclosan acts as biocide and inhibits the fatty acid synthesis. Considered non-carcinogenic and having a short half-life in the environment, triclosan has not previously been considered toxic within the environment (Lindstrom, *et al*., 2002; Aranami, 2007). The concentrations of triclosan detected within natural waters of European and North America rivers are between of 0.1 – 5 $\mu g \cdot L^{-1}$ (Lindstrom *et al*., 2002). Triclosan has been shown to preferentially bind to soil medium within the environment (Ying *et al*., 2009; Wick *et al*., 2011). Triclosan has been shown to be toxic at very low ($ng \cdot L^{-1}$ ~ $\mu g \cdot L^{-1}$) concentrations to algae and fish, having the ability to degrade into further toxic compounds such as methyl-triclosan (Lindstrom, *et al*., 2002; Aranami, 2007; Riva *et al*., 2012). Following a 60 $\mu g \cdot L^{-1}$ exposure of triclosan biofilm bound microbial communities from rivers faced a population decline that was persistent until two weeks post exposure (Prioa, *et al.* 2009; Prioa, *et al*. 2013), after which the microbial communities were shown to recover normal functions. The effects of triclosan have only been studied in river systems, not in constructed wetland environments and therefore its impacts to the wetland or constructed wetland microbial communities remain unknown (Prioa, *et al.* 2009; Ricart, *et al*. 2010; Prioa, *et al*. 2013).

4.1.4 Sulfamethoxazole

Sulfamethoxazole (N^1-(5-Methyl-3-isoxazolyl) sulfanilamide) is an antimicrobial used to treat respiratory and urinary tract infections (World Health Organization, 2001; Drilla *et al*., 2005). Sulfamethoxazole is a bacteriostatic that interferes with the folic acid synthesis of the cell, which is required for DNA replication. Sulfamethoxazole is considered a carcinogen with exposures leading to the development of acute lung, ovarian and leukemia cancers within mice (World Health Organization, 2001). Biodegradation of sulfamethoxazole is thought to be rapid within the environment due to the compound containing carbon and nitrogen species, which are utilized in biological metabolism (Drilla *et al*., 2005). However sulfamethoxazole has been detected in the natural environment at $ng \cdot L^{-1}$ ~ $mg \cdot L^{-1}$ concentrations (Senta *et al*., 2012) and has

been shown to be toxic to alga at low concentrations (0.027 $mg \cdot L^{-1}$), bacteria at moderate concentrations (78 $mg \cdot L^{-1}$) and fish at high concentrations (560 $mg \cdot L^{-1}$) (Galan *et al*., 2005). The effect of sulfamethoxazole has not been expressly studied in wetland environments and therefore its impacts upon the wetland microbial community are unknown.

4.2 Materials and Methods

4.2.1 Experimental Design

Twelve vertical flow constructed wetland mesocosms were built and allowed to naturally develop over a ninety-day period. The top six mesocosms were planted with reed canary grass (*Phalaris arundinacea*) and the bottom six mesocosms were left unplanted (FIGURE 4.1). All twelve mesocosms were inoculated with activated sludge during the development period. Following the developmental period, three planted and three unplanted mesocosms were randomly selected to be exposed sequentially to the low (100 $\mu g \cdot L^{-1}$) and high concentration (500 $\mu g \cdot L^{-1}$) of triclosan and sulfamethoxazole (FIGURE 4.1). The same three exposed planted and unplanted mesocosms received the sequential doses of low and high triclosan, sulfamethoxazole compounds. The low concentration was selected based upon literature reviews of Canadian wastewater effluent. The high concentration was chosen to mimic a shock load of antimicrobials into the wastewater systems, which would occur in a sick population.

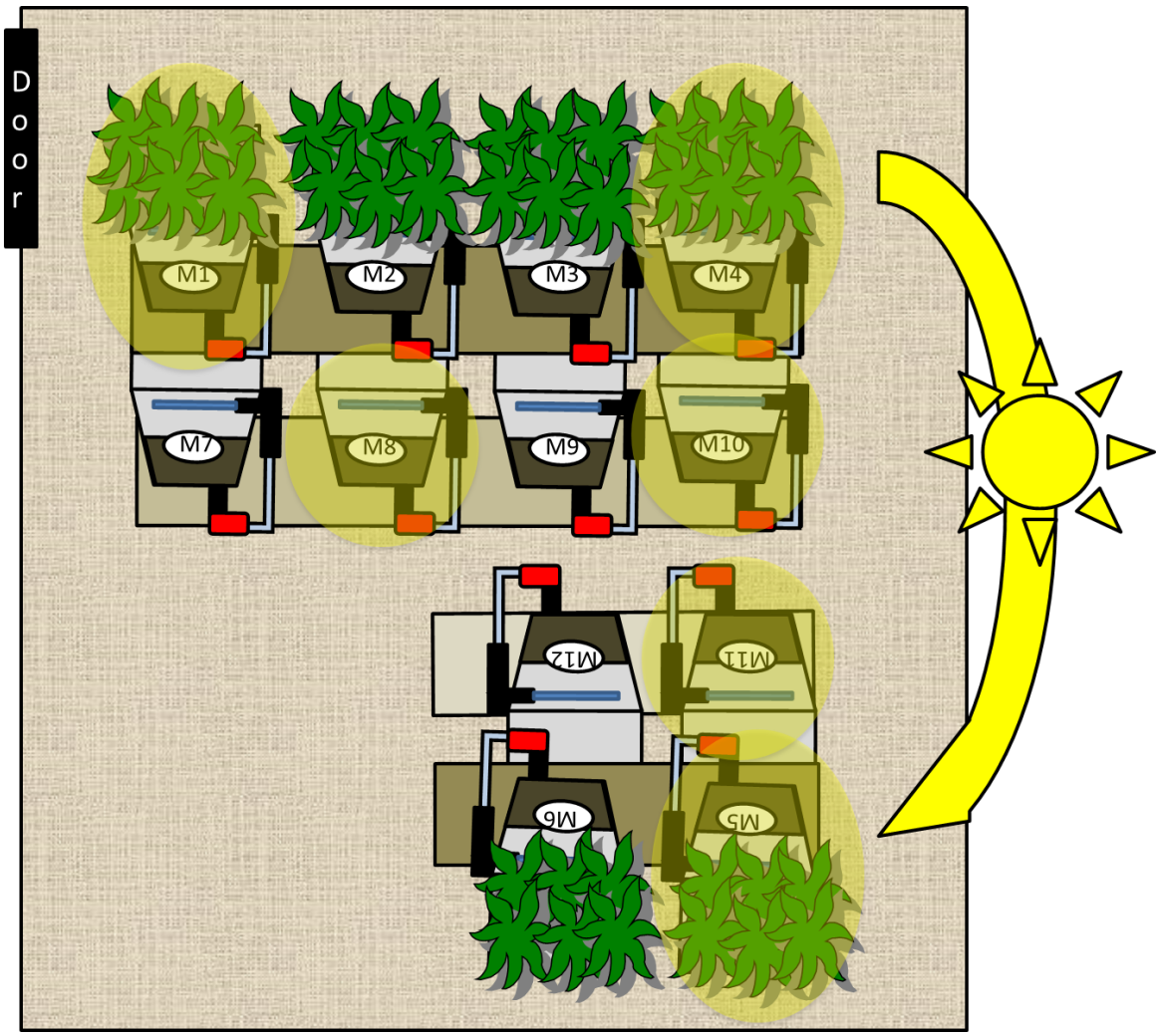

FIGURE 4.1: Greenhouse layout (exposed mesocosms highlighted).

4.2.2 Preparation of Triclosan and Sulfamethoxazole Stock Solutions

For each antimicrobials exposure, the stock solution was prepared the day prior to the mesocosm feed. The stocks were prepared near the solubility limit of each compound (triclosan 0.010 $g \cdot L^{-1}$ and sulfamethoxazole 0.50 $g \cdot L^{-1}$). The volume of stock required per mesocosm to create the low (100 $\mu g \cdot L^{-1}$) and high (500 $\mu g \cdot L^{-1}$) concentrations was calculated and the appropriate stock solution volumes added for the exposures.

To prepare for the low and high triclosan exposure the appropriate amount of Irgasan (Sigma, ≥ 97.0% HPCL - common name for triclosan) was weighed in a small plastic weight boat and added to a 2 L flask that was filled with tap water. Chlorinated tap water was chosen over distilled water, as tap water would be more representative of the conditions within wastewater treatment plants and natural wetland environments. The triclosan stock was heated at 30 Celsius overnight to allow for complete dissolution. The stock solution was wrapped in aluminum due to

triclosan preferentially degrading when exposed to sunlight (EPA, 2006) and the solution was refrigerated overnight.

To prepare for both the low and high sulfamethoxazole exposures, 0.100 g of Sulfamethoxazole (Fluka Analytical) was weighed in a small plastic weight boat and added to a 200 mL flask that was filled with tap water. The solution was heated at 30 Celsius for 30 minutes and 400 µL of 0.012 M hydrochloric acid was added to enable complete dissolution. The stocks solutions were wrapped in aluminum due to sulfamethoxazole producing photo-toxins when exposed to sunlight (Drillia, 2005). The solutions were refrigerated overnight.

4.2.3 *Ex-Situ* Trimethoprim, Triclosan and Sulfamethoxaole Exposures

To gain an initial understanding of the potential effects of the selected antimicrobial compounds on the CW microbial communities an *ex-situ* exposure based on the recent methods developed by Weber et al. (2014) was performed. The wetland interstitial microbial communities were exposed *ex-situ* to trimethoprim, triclosan and sulfamethoxazole separately. Microbial community samples were collected from the wetland mesocosm sample ports two days before the feed day to gain a sample comparable to those collected during the planned *in-situ* exposures.

Serial dilutions (0, 1, 10, 100, 1000 $\mu g \cdot L^{-1}$) of the trimethoprim, triclosan and sulfamethoxazole stock solutions were prepared in a 20 mL vials using the sampled mesocosm water (which contained the interstitial microbial communities). These prepared samples were shaken to ensure the solutions were well mixed prior to the sample inoculation. Prior to the inoculation, Biolog™ plates were left at room temperature for 30 minutes. A Eppendorf Multichannel Pipette 30 – 150 µL was used to inoculate the room-temperature Biolog™ plates. The pipette tips were wetted prior to the inoculation with the sample solution to ensure a consistent inoculate volume. Once all plates were inoculated, they were incubated at room temperature on a VMR Shaker (100 RPM). At selected time intervals post-inoculation the incubating Biolog™ plates were read using an iMark Bioplate Reader (595 nm wavelength, 5 second orbital shake). The resulting absorbance values were exported into Microsoft Excel 2010 and used for further data analysis.

4.2.4 Triclosan and Sulfamethoxazole Concentration Calculations

Triclosan is found within a variety of personal care products including deodorants, soaps, cosmetics, textiles, toothpaste, mouthwash and sunscreens (Health Canada Environment Canada, 2012). The concentration of triclosan within each of these products varies from <1% (soaps and cleansers) to 20% (textiles). The most common route of exposure to humans is through oral or dermal contact (Health Canada Environment Canada, 2012). Triclosan is excreted from the body through urination, mostly in its original form (85%) though can metabolize into glucuronide and sulphate compounds (25%). The average concentration of triclosan excreted by a sampled adult population was 0.0029 mg•kg of body $weight^{-1} \bullet d^{-1}$ (Health Canada Environment Canada, 2012)

1) Triclosan excreted in human urine

$$= \frac{0.0029 \text{ mg}}{\text{kg of body weight} * \text{d}}$$

2) Triclosan concentration per body weight

Average person weighs 63 kg (140 lbs)

$$= \frac{0.0029 \text{ mg}}{\text{kg of body weight} * \text{d}} * \frac{63\ kg}{person} = \frac{0.183\ mg}{d * person}$$

3) Triclosan concentration per L of water consumed

Each person utilizes 200 – 400 L water per day

$$= \frac{0.183\ mg}{d*person} * \frac{1000\ \mu g}{mg} * \frac{person*d}{200\ L\ water} = \frac{0.913\ \mu g}{L}$$

$$= \frac{0.183\ mg}{d * person} * \frac{1000\ \mu g}{mg} * \frac{person * d}{400\ L\ water} = \frac{0.457\ \mu g}{L}$$

Sulfamethoxazole is prescribed for acute urinary tract and respiratory infections. The common oral dose is 400 mg every 12 hrs (IARC, 1987). Often sulfamethoxazole is paired with trimethoprim to treat urinary tract infections. Sulfamethoxazole is not stored in the body and the majority (85%) is excreted in the urine (30% as sulfamethoxazole and 55% as its metabolite acetylsulfamethoxazole) (IARC, 1987).

1) Sulfamethoxazole oral adult dose

$$= \frac{400\ mg}{12\ hr} * \frac{24\ hr}{d} = \frac{800\ mg}{d}$$

2) Sulfamethoxazole excreted in urine

$$= \frac{800\ mg}{\text{d}} * 0.85 = \frac{680\ mg}{\text{d}} * 0.30 = \frac{204\ mg}{\text{d}}$$

3) Sulfamethoxazole concentration per L of water consumed

Each person utilizes 200 – 400 L water per day

$$= \frac{204\ mg}{d*person} * \frac{1000\ \mu g}{mg} * \frac{person*d}{200\ L\ water} = \frac{1020\ \mu g}{L}$$

$$= \frac{204\ mg}{d * person} * \frac{1000\ \mu g}{mg} * \frac{person * d}{400\ L\ water} = \frac{510\ \mu g}{L}$$

4.2.5 *In-Situ* Triclosan and Sulfamethoxazole Exposures

Triclosan and sulfamethoxazole were sequentially introduced into selected vertical flow constructed wetlands. Triclosan is less toxic to aquatic organisms compared to sulfamethoxazole and was therefore selected as the first compound to be used for the exposure studies (Lindstrom,

et al., 2002; Aranami, 2007). First a low (100 $\mu g \cdot L^{-1}$) concentration triclosan exposure was performed followed by a recovery period. After the recovery period, a high (500 $\mu g \cdot L^{-1}$) concentration triclosan exposure was completed, followed by another recovery period. Sulfamethoxazole exposures followed in a similar manner. The exposed mesocosms were extensively characterized during the exposure and recovery periods (see Chapter 2 for details). All mesocosms were completely drained and fed with the simulated wastewater and nutrients stock prior to the exposure (as per weekly operation previously described). The prepared stock solution was added to the center of the exposed mesocosms surface during filling. The stock solutions were added after the feed solution to prevent the compound from binding to the organics in the feed. All wetland mesocosms had a retention time of seven days, after which the water in the mesocosm was completely drained and filled with fresh simulated wastewater. Mesocosm systems were topped up daily with tap water to compensate for and quantify evapotranspiration losses.

4.2.6 Analytical Methodology for the Quantification of Triclosan and Sulfamethoxazole in Water

Sulfamethoxazole samples were analyzed by LC-MS/MS using an Agilent 1290 Infinity HPLC coupled with 6460 Triple Quadrupole Mass Spectrometer. HPLC separation was performed with an Agilent ZORBAX Eclipse Plus C-18 2.1 mm x 100 mm, 1.8 µm column with a mobile phase flow rate of 0.2 $mL \cdot min^{-1}$. The sample injection volume was 15 µL. The mobile phase composition consisted of two eluents which were (A) 0.1% HCOOH in H_2O and (B) 0.1% HCOOH in ACN using a binary gradient program over 10min. The relative flow of 0.1% HCOOH in ACN was 90% for 0.5 min and 100% at 10 min. MS/MS analysis was undertaken using electrospray ionization in positive ionization mode (+ESI) using multiple reaction monitoring (MRM) mode. The transaction used for SMX quantitative analysis was 254.1>108.

Triclosan samples were analyzed using the same instrument, column and mobile phase flow rate. Sample injection volume was also 15µL. The mobile phase composition consisted of two eluents which were (A) 0.1% Ammonium formate pH=5.5 in H_2O and (B) Ammonium formate pH=5.5 in MeOH using a binary gradient program over 10min. The relative flow of Ammonium formate pH=5.5 in MeOH was 90% for 0.5min and 100% at 10min. MS/MS analysis was undertaken using electrospray ionization in negative ionization mode (ESI-) using multiple reaction monitoring (MRM) mode. The transaction used for TCS quantitative analysis was 286.9>35.2.

4.3 Results and Discussion

4.3.1 *Ex- Situ* Trimethoprim, Triclosan and Sulfamethoxazole Exposures

The ecotoxicological effects of trimethoprim, triclosan and sulfamethoxazole were examined with respect to the interstitial wetland microbial communities of the exposed mesocosms. A range of concentrations (0 – 1000 $\mu g \cdot L^{-1}$) was examined during this study to quantify the full potential of effects to the interstitial microbial communities. Based upon these results, triclosan and sulfamethoxazole were chosen to be exposed *in*-situ due their moderate effects on the interstitial microbial community.

Trimethoprim had a negligible effect on the microbial communities from planted mesocosms and only a small effect on the microbial communities from unplanted wetland

mesocosms. Microbial activity (AWCD) was steady (within error bars) for the planted mesocosms with a small (within error bars) increase at the 10 $\mu g \cdot L^{-1}$ dose (FIGURE 4.2A). Microbial activity (AWCD) decreased with increasing trimethoprim concentrations for the unplanted mesocosms. The microbial community richness followed a similar trend (FIGURE 4.2B). The planted mesocosms had a steady richness for all concentrations, while the unplanted microbial richness decreased as trimethoprim concentrations increased. Interestingly, the above observations show that trimethoprim had non-lethal effects on planted and unplanted wetland microbial communities from the vertical flow CW mesocosms. This was not expected.

Sulfamethoxazole had a prominent effect on both the planted and unplanted mesocosms. The planted mesocosms had higher AWCD compared to the unplanted mesocosms at lower sulfamethoxazole concentrations (FIGURE 4.2C). Following the 10 $\mu g \cdot L^{-1}$ exposure, the planted and unplanted mesocosms experienced a drastic decrease in microbial community activity (FIGURE 4.2C). The microbial community richness followed a similar trend. The planted mesocosms had higher richness compared to the unplanted mesocosms at all concentrations (FIGURE 4.2D). At 100 $\mu g \cdot L^{-1}$ exposure the planted and unplanted mesocosms drastically decreased in richness. Both the activity and richness of the planted and unplanted microbial communities ceased at the 1000 $\mu g \cdot L^{-1}$ exposure. The observations show that sulfamethoxazole can be acutely toxic to planted and unplanted wetland microbial communities at concentrations beyond 10 $\mu g \cdot L^{-1}$.

Triclosan had a measurable effect on the planted and unplanted mesocosms (FIGURE 4.2E). The activity decreased for the planted mesocosms microbial communities as the triclosan concentrations increased. The unplanted mesocosm microbial community activity also decreased over the same concentration increase. Trends for richness were similar (FIGURE 4.2F). In general the observed microbial activity and richness of the planted systems can be seen to be higher most likely due to the presence of rhizospheric microbial communities (Tillman, *et al.* 1996). The rhizosphere increases surface area for biofilm growth and increases the diversity of the microbial population (Weber and Gagnon, 2014). This enhanced microbial community is often said to be more resilient to environmental stress (Pearson, 1998; Picard, *et al.* 2004; Bias, *et al*. 2006).

Ex-situ exposure results helped to outline the effects of the different antimicrobial compounds on CW mesocosm microbial communities. The effects of the compounds to the rhizosphere were not quantified. Sulfamethoxazole had a clear acute toxic effect and was able to reduce microbial activity to a negligible level at 1000 $\mu g \cdot L^{-1}$, whereas triclosan had a negative effect (approximate 25% reduction in microbial activity) at 1000 $\mu g \cdot L^{-1}$. The effects of trimethoprim were small or negligible at all concentrations.

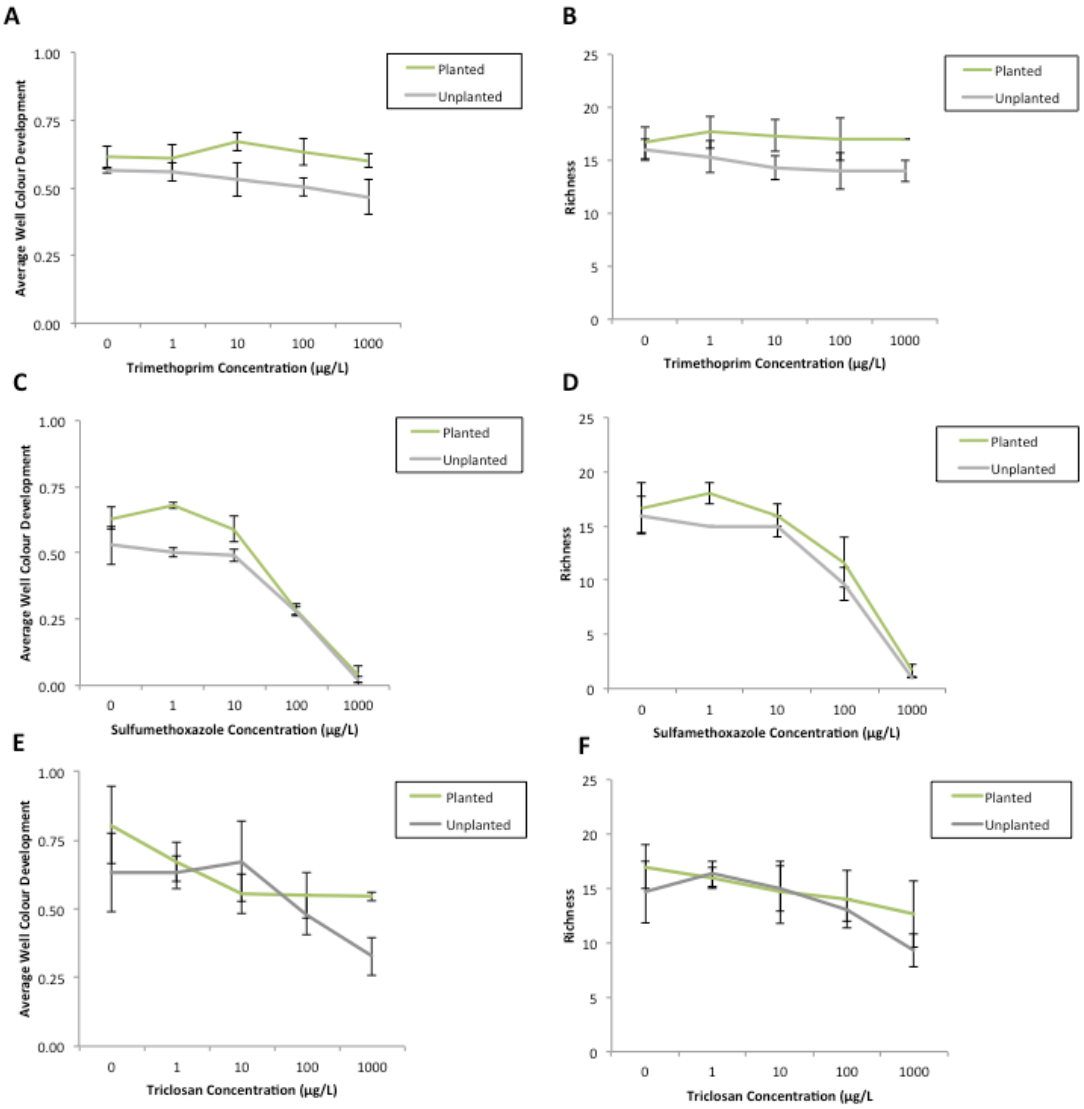

FIGURE 4.2: Microbial activity (AWCD) and richness of mesocosms following *ex-situ* trimethoprim, sulfamethoxazole and triclosan exposures.

4.3.2 *In-Situ* Low Triclosan Exposure

4.3.2.1 Triclosan Fate in Exposed Mesocosms

Triclosan concentrations were measured in the mesocosm water directly (1hr) after the exposure and approximately one week (150 hr) after the exposure. Samples were collected from each mesocosm sampling port in a sterilized test tube and analyzed in the laboratory. The one week mesocosm samples (150 hr) were sampled prior to the drain/feed procedure. 100% of the triclosan was removed from the water column 1hr post exposure (TABLE 4.1). The transformation of triclosan was not examined within the exposed mesocosms and it could be possible that instead of binding, the triclosan was metabolized into a different form. Further

investigations to determine the concentration of triclosan and other metabolites within the biofilm, above ground biomass and root biomass is undergoing.

TABLE 4.1: Antimicrobial concentrations in mesocosms following exposure

	Low TCS 100 µg/L			
Mesocosm Type	1 hr (µg/L)	% Removal	150 hr (µg/L)	% Removal
Planted	0	100	0	100
Unplanted	0	100	0	100
	High TCS 500 µg/L			
Mesocosm Type	1 hr (µg/L)	% Removal	168 hr (µg/L)	% Removal
Planted	76	85	0	100
Unplanted	99	80	0	100
	Low SMX 100 µg/L			
Mesocosm Type	1 hr (µg/L)	% Removal	168 hr (µg/L)	% Removal
Planted	1	99	0	100
Unplanted	1	99	0	100
	High SMX 500 µg/L			
Mesocosm Type	1 hr (µg/L)	% Removal	168 hr (µg/L)	% Removal
Planted	12	98	0	100
Unplanted	6	99	0	100

Triclosan has very low water solubility and volatilization, preferably binding to organic matter (soil and sludge) within the environment (Ying *et al*., 2009; Wick *et al*., 2011). Triclosan was not detected in the exposed mesocosms following the low concentration exposure at either 1h or 150h (TABLE 4.1). Based on triclosan's documented binding properties triclosan was most likely bound to biofilm or other organic particles within the simulated wastewater solution. No re-suspension occurred in the water column (150h observations) again re-emphasizing the preference for non-reversible binding to organic matter. Triclosan removal in VF CWs has never been studied. This study shows that triclosan is completely removed from the water column in these vertical flow CW systems within a single hour. This is a significant finding in itself and warrants further study. Although adsorption is generally not a reliable long term (30yr +) mechanism for pollutant removal in CW systems, triclosan may be further degraded once bound in the biofilm and accessible to potentially tolerant microbial communities. It is recommended that when these systems are decommissioned the biofilm is investigated for triclosan and perhaps triclosan degradation products. In addition antibiotic resistance studies should also be performed on the biofilm communities.

4.3.2.2 Water Treatment Effectiveness following Low Triclosan Exposure

Water treatment ability was measured by chemical oxygen demand removal rates. Chemical oxygen demand is a measure of the oxidizable organic and inorganic matter within a water sample (Eaton, 1995). There was no statistically significant ($p < 0.05$) difference among the mesocosms in FIGURE 4.3. The removal rate of chemical oxygen demand was affected by the low triclosan exposure (FIGURE 4.3) in some cases though. The exposed mesocosms showed

reduced removal rate of chemical oxygen demand compared to the control mesocosms, directly following the low triclosan exposure. The unplanted mesocosms were affected more by this exposure than the planted mesocosms. The exposed planted mesocosms were able to recover to the ability of the control in the later weeks, which was not observed in the exposed unplanted. The removal rate of organic matter in CWs is dependent on microbial community activity (Weber and Gagnon, 2014). Enhanced organic removal comes from a more active microbial environment, which break down organic molecules for growth and energy (Kadlec and Wallace, 2009). Planted mesocosm biofilms have been shown to have higher activity than unplanted communities (Weber and Legge, 2013), especially within the rhizospheric regions. The rhizosphere offers root exudates, microenvironments and enhanced surface area for microbial biofilm attachment (Grayson and Jones, 1996; Hodge, *et al*. 1998; Weber and Gagnon, 2014), perhaps explaining this better recovery compared to the unplanted systems

The nitrogen data collected (ammonium and nitrate removals) was inconclusive due to a malfunctioning probe and is located in Appendix B for further consideration

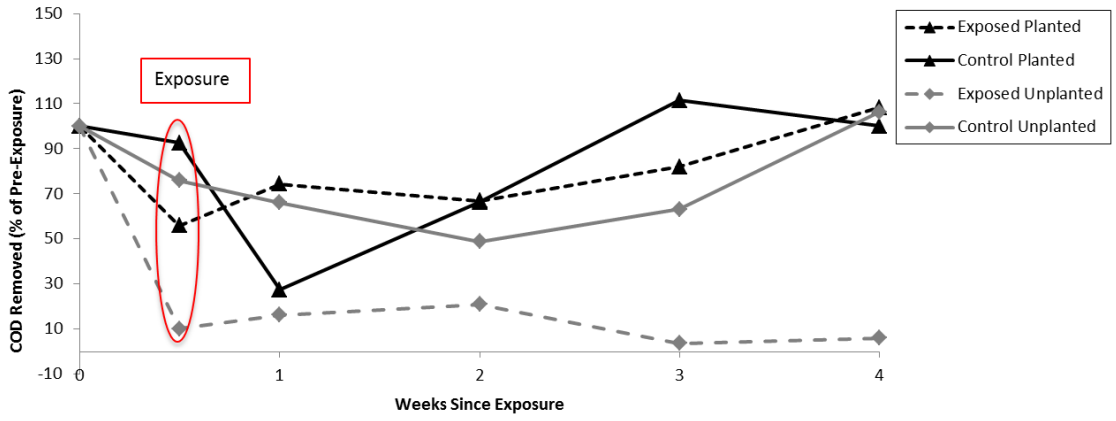

FIGURE 4.3: Chemical oxygen demand removal rate (% of pre-exposure) following low triclosan exposure.

4.3.2.3 Water Quality Changes following Low Triclosan Exposure

Water quality data was measured using the YSI Professional Plus probes on a daily basis. The parameters measured included conductivity, dissolved oxygen, redox potential, water temperature and water pH. Supporting data can be found in Appendix B for further consideration.

Total dissolved solids are the measure of filterable inorganic and organic compounds within a water sample (Kadlec and Wallace, 2009). There were no measurable differences of total dissolved solids between the exposed and control mesocosms (FIGURE 4.4). There was some weekly variation in the total dissolved solids that could be related to variation in tap water quality.

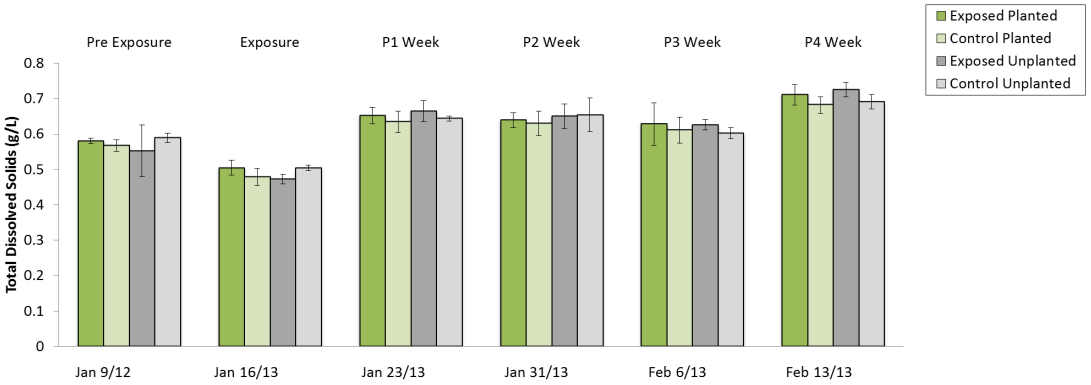

FIGURE 4.4: Total dissolved solids ($g \cdot L^{-1}$) following low triclosan exposure. P = post.

Daily dissolved oxygen followed a similar trend for all mesocosms (FIGURE 4.5A). The wetland microbial community had an initial decline in oxygen following the weekly addition of the simulated wastewater and nutrients due to the presence of organic matter. The unplanted mesocosms had higher dissolved oxygen compared to the planted mesocosms, most likely due to their less populated microbial community. There was weekly variation of dissolved oxygen concentration within the planted and unplanted mesocosms due to fluctuating air temperatures in the greenhouse.

Redox potential is the measure of the reducing potential in an environment (Kadlec and Wallace, 2009). Redox potential closely followed the dissolved oxygen trend with little difference between each of the systems (FIGURE 4.5B). Lower dissolved oxygen concentrations on the feed days led to lower redox potentials. The redox potential was slightly higher at some time points for the unplanted mesocosms due to their higher dissolved oxygen concentrations. Overall, all mesocosms had around 50-150 mV redox potential through the experimental period.

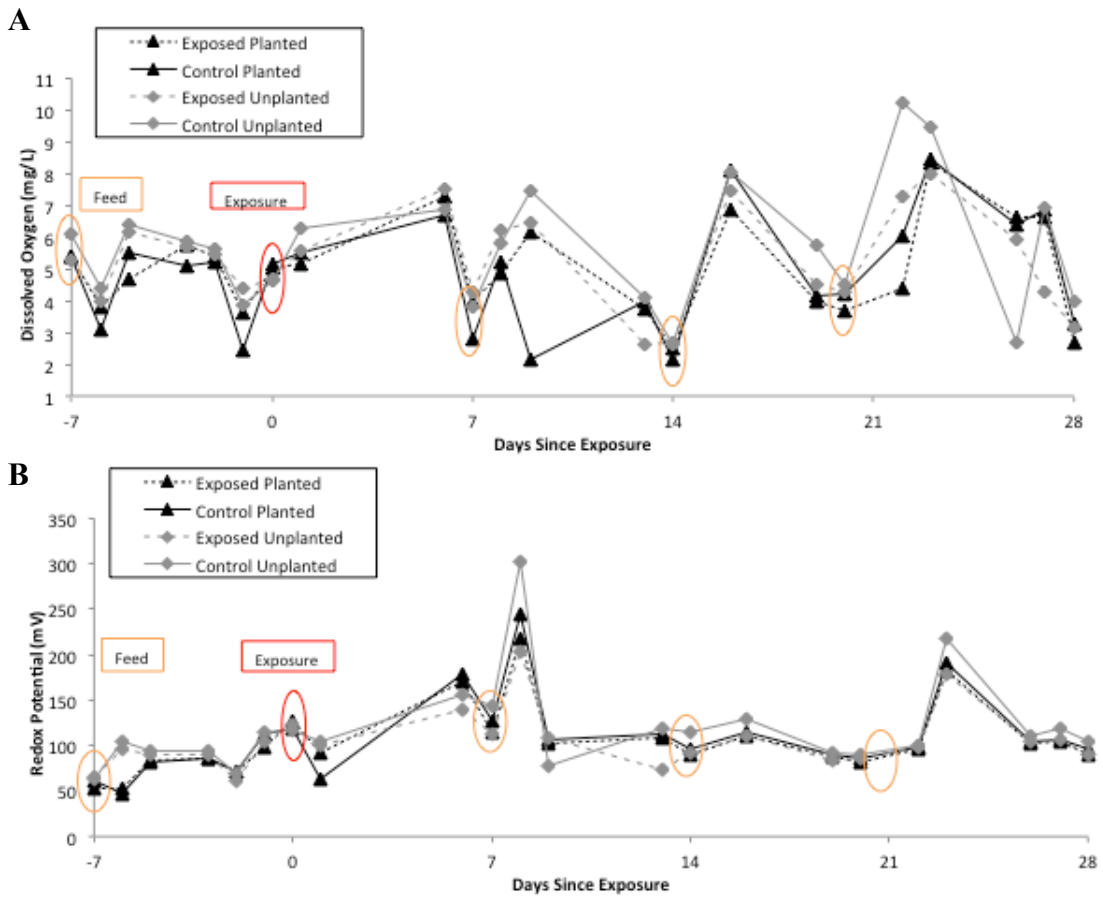

FIGURE 4.5: Dissolved oxygen ($mg \cdot L^{-1}$); B) Redox potential (mV) following low triclosan exposure.

The water temperature of the mesocosms fluctuated throughout the experiment (FIGURE 4.6A). The planted mesocosms displayed slightly higher (1-2 Celsius) water temperatures than the unplanted mesocosms. The planted mesocosms were located on the top shelf and exposed to more direct sunlight than the unplanted. The water pH (FIGURE 4.6B) fluctuated only slightly throughout the experiment for all mesocosms. All of the twelve mesocosms had a limestone gravel bed medium that acted as a buffer to keep the water pH near neutral. The planted and unplanted mesocosms had similar water pH values between 7.50 – 8.25. The low triclosan exposure seemed to have a negligible effect on the water temperature and water pH of the exposed mesocosms.

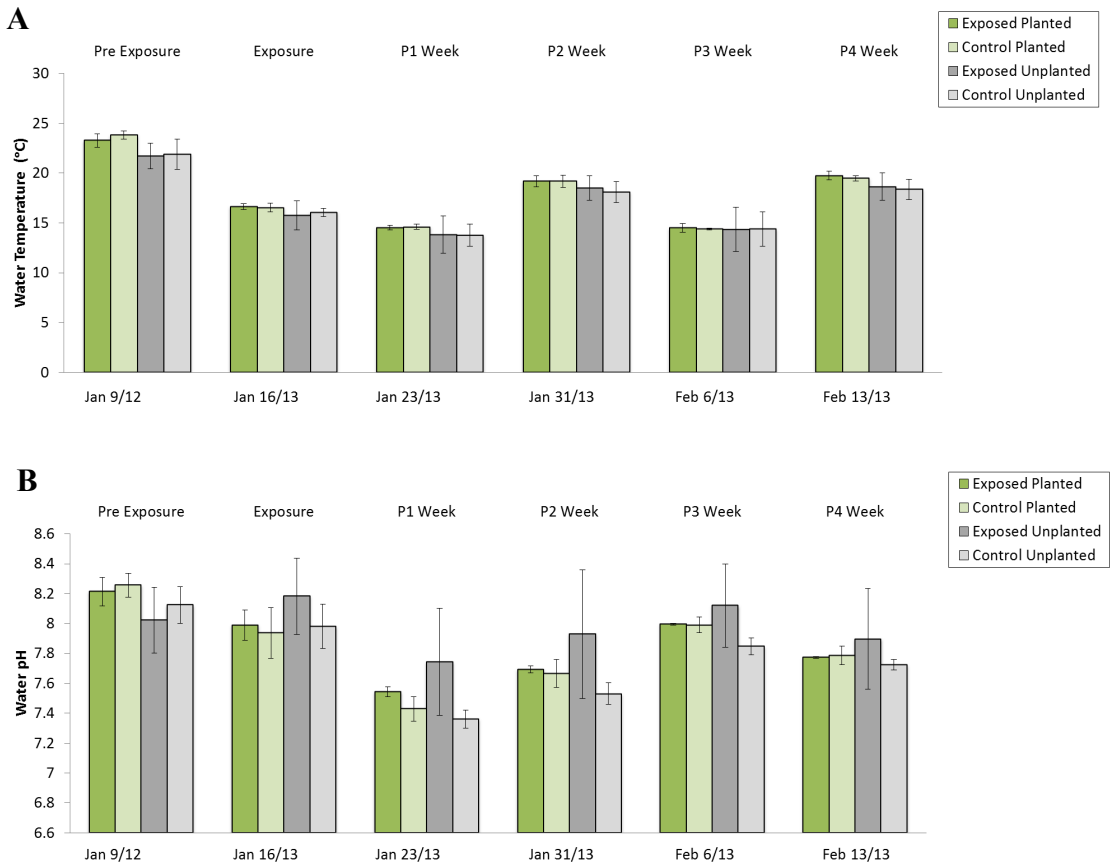

FIGURE 4.6: A) Water temperature (Celsius); B) Water pH following low triclosan exposure. P = post.

4.3.2.4 Ecological and Hydrological Changes following Low Triclosan Exposure

The mesocosms were assessed for ecological and hydrological changes over the experimental period. The plant health (stem counts and stem height), evapotranspiration and drained volume (porosity) of the mesocosms were recorded weekly. These metrics are important for maintaining and evaluating CW operations (Kadlec and Wallace, 2009).

Porosity remained fairly consistent within all mesocosms over the experimental period. The exposed planted and unplanted mesocosms did not show an increase in porosity that would have indicated that the triclosan was causing biofilm or root death (FIGURE 4.7A). The planted and unplanted mesocosms had similar porosities throughout the experiment.

Evapotranspiration/evaporation within the exposed mesocosms seemed to be unaffected by the triclosan exposure (FIGURE 4.7B). Evapotranspiration is a function of air temperature, wind conditions and the surface conditions (Kadlec and Wallace, 2009). Due to their vegetated surface, planted mesocosms can have greater evapotranspiration rates from the water loss occurring at the leaf surface. The planted mesocosms had similar evapotranspiration rates compared to the unplanted at this stage (January 2013) as they were still developing. The unplanted mesocosms had similar evaporation rates throughout this experimental period.

At this stage of the experiment there were no observed differences in stem count or plant height within the planted mesocosms (FIGURE 4.8A and B respectively). The low triclosan exposure did not affect the above ground biomass of the exposed mesocosms.

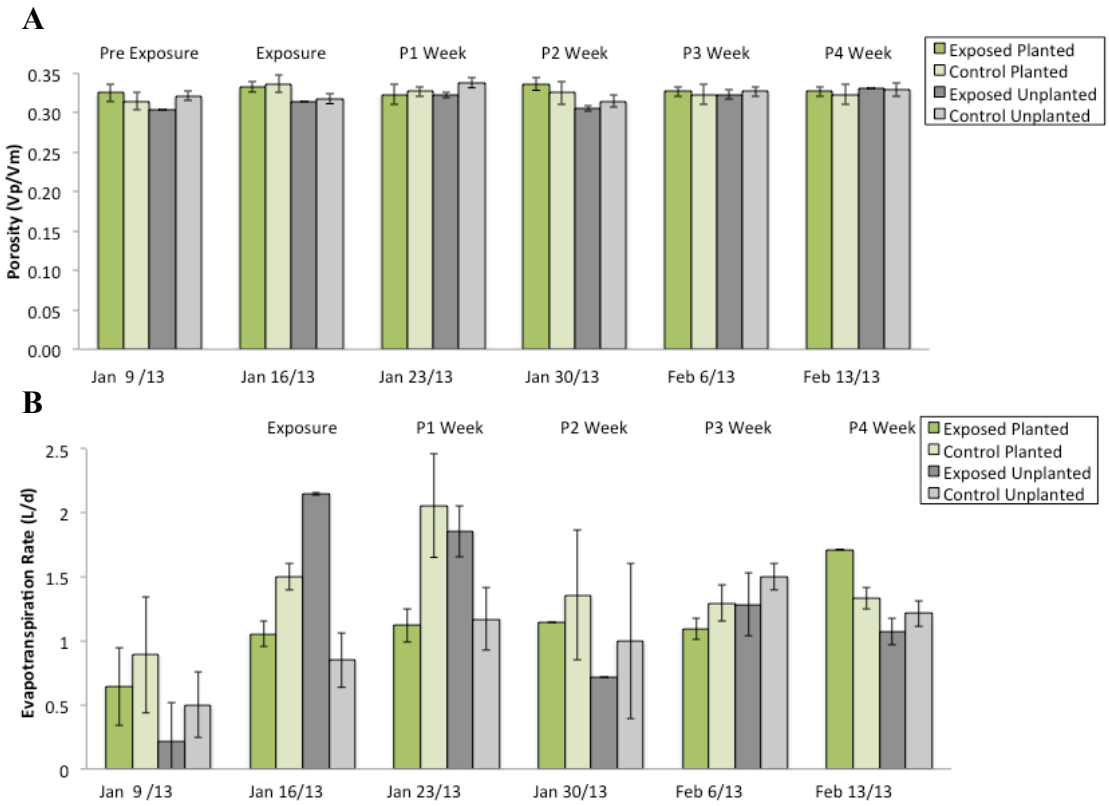

FIGURE 4.7: A) Porosity (volume of pore space/volume of bed medium); B) Evapotranspiration rate ($L \cdot d^{-1}$) following low triclosan exposure. P = post.

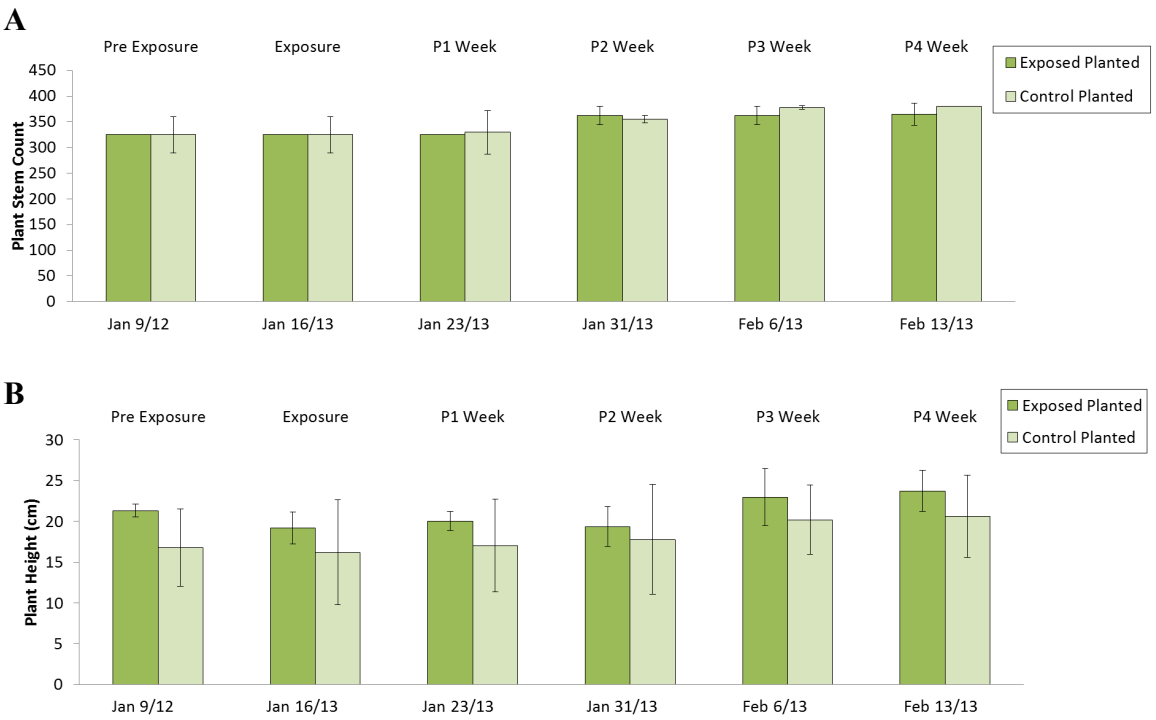

FIGURE 4.8: A) Plant count; B) Plant height (cm) following low triclosan exposure. P = post.

Plant root development as a function of time is outlined in FIGURE 4.9. A microcosm (46 cm x 46 cm x 6 cm) was constructed from plexiglass, filled with limestone pea gravel and planted with reed canary grass to facilitate visualization of root development. This microcosm was fed the representative volumes of simulated wastewater and nutrients. No antibiotics were added to this microcosm. Timeline references to exposure periods are for correlation and relation purposes only. The microcosm rhizosphere developed gradually over the course of the experiment. Shallow root depth and thin scattered roots were present during the low triclosan exposure in January 2013 (FIGURE 4.9A). Roots began to thicken and become dense, growing longer during the high triclosan exposure period in July 2013 (FIGURE 4.9B). The rhizosphere was well established during the low and high sulfamethoxazole exposures in September and November 2013 (FIGURE 4.9C and D respectively) having thick, long roots with many branches. Reed canary grass can thrive in most environments that has made it an invasive species in North America (Lavergne and Molofsky, 2004).

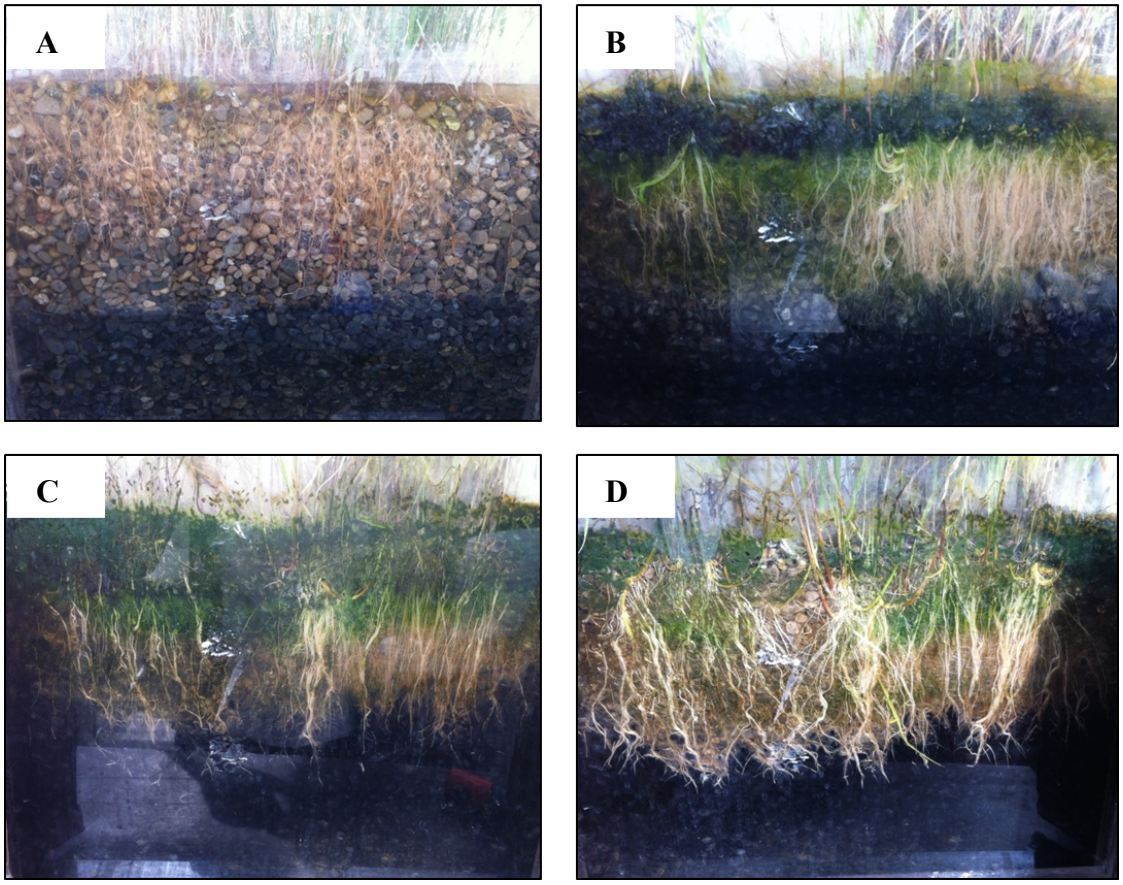

FIGURE 4.9: Photos depicting rhizosphere development through exposure. A) January 2013; B) July 2013; C) September 2013; D) November 2013.

4.3.2.5 Microbial Community Changes following Low Triclosan Exposure

Wetland microbial community activity and function were analyzed using the community-level physiological profiling method (described in Chapter 2). The method examined the activity (average well colour development), functional richness and functional diversity of microbial communities. Average well colour development (AWCD) is the average corrected absorbance readings of all wells and can be associated and described as an overall microbial community activity measurement. Richness is the number of carbon source wells utilized and is calculated as the number of wells with a corrected absorbance ($A_i - A_o$) greater than 0.25. Carbon sources utilized within each plate are grouped into five major guilds: carbohydrates, carboxylic acids, polymers, amines/amides, amino acids (TABLE 3.1). Principle component analysis (PCA) with a covariance (n-1) matrix was used to examine the carbon source utilization patterns (CSUPs) within the exposed and control mesocosms. The CSUP data was first subjected to a Taylor transformation to correct for normality and homoscedasticity prior to the PCA analysis (Weber, *et al*. 2007).

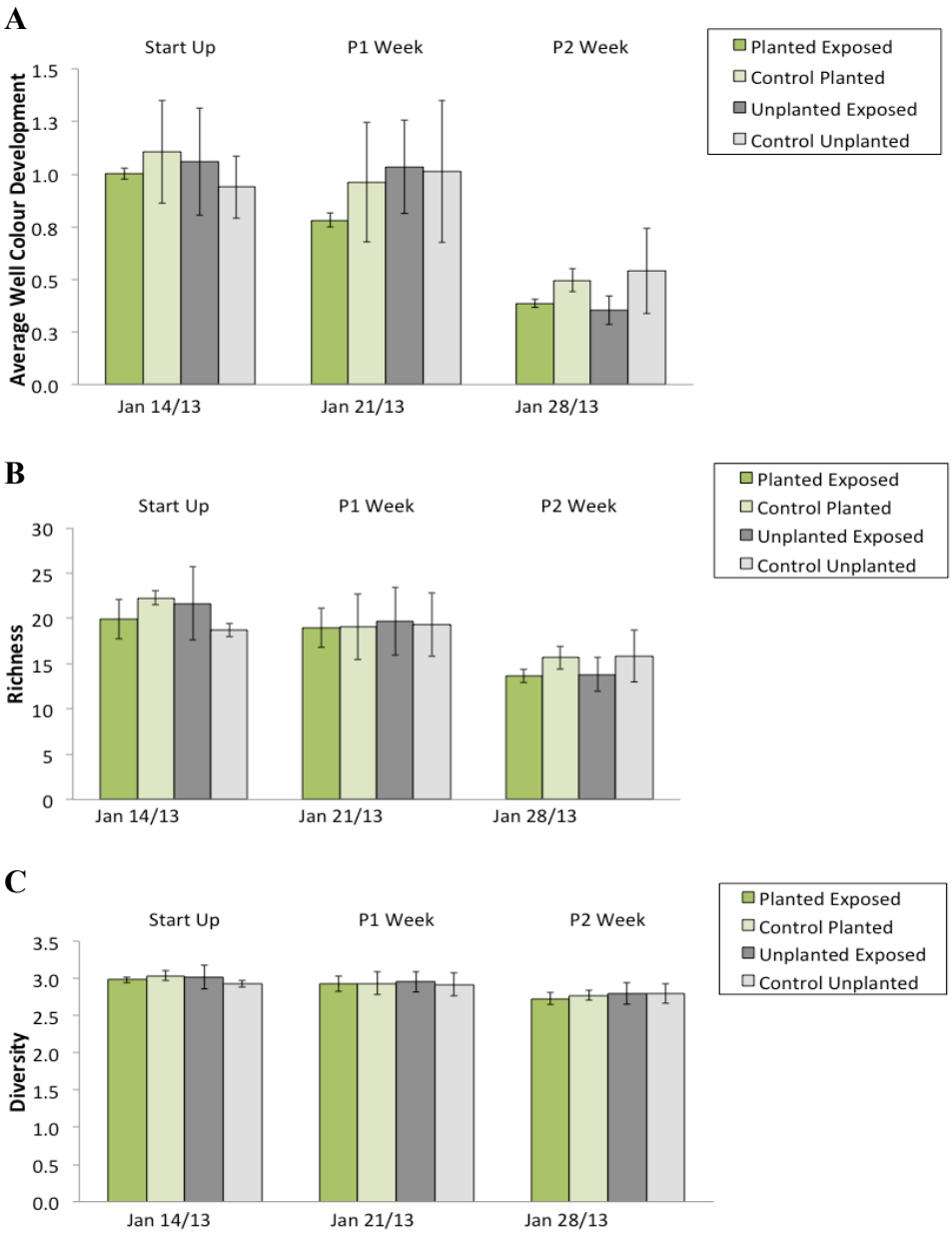

FIGURE 4.10: A) Microbial community activity; B) Richness; C) Diversity following low triclosan exposure. P = post.

The low triclosan exposure did not negatively affect the microbial community in terms AWCD, richness or diversity (FIGURE 4.10 A, B, C respectively) with no significant differences observed between the exposed and control groups. There was a small decrease in AWCD in the planted exposed by comparison to the control at 1 week post exposure which diminished at 2 weeks post exposure. A decrease in both activity (AWCD) and richness was observed in all systems 2 weeks post exposure and is therefore most likely attributable to natural variance. Whilst the *ex-situ* exposure experiment indicated a moderate toxic effect at 100 $\mu g \cdot L^{-1}$ (FIGURE 4.2E) this *in-situ* experimental result is not surprising due to the observation that the triclosan concentration was reduced by 100% 1 hour after exposure (TABLE 4.1).

4.3.3 *In-Situ* High Triclosan Exposure

4.3.3.1 Triclosan Fate in Exposed Mesocosms

Triclosan was detected in the exposed mesocosms following the high concentration exposure (TABLE 4.1). Between 80-85% of the triclosan was bound within the wetland matrix, leaving upwards of 25% unbound in the water column after one hour. Triclosan was not detected in the exposed mesocosms one-week (168 hr) following the exposure (TABLE 4.1), the remaining triclosan in the water column was most likely bound onto organic matter over the seven days. Even at this high concentration, triclosan was effectively removed one hour after exposure within these mesocosms. Again this is an interesting observation worthy of further study.

4.3.3.2 Water Treatment Effectiveness Following High Triclosan Exposure

The COD removal rate for the exposed planted mesocosms declined compared to the control at one-week post exposure (FIGURE 4.11). A different trend was observed for the unplanted mesocosms where both the exposed and control showed similar overall trends and recovery four weeks after exposure. No significant differences were observed between exposed and control mesocosms (FIGURE 4.11). The exposed mesocosms showed recovery to pre-exposure levels in the second week where the planted systems had greater recovery. As suggested previously this could be due to the rhizosphere zone enhancing the microbial communities and providing resistance to environment perturbations (Pearson, 1998; Picard, *et al.* 2004; Bias, *et al*. 2006). Due to the controls behaving similarly to the exposed mesocosms, the high triclosan exposure had minimal affects upon the unplanted exposed mesocosms.

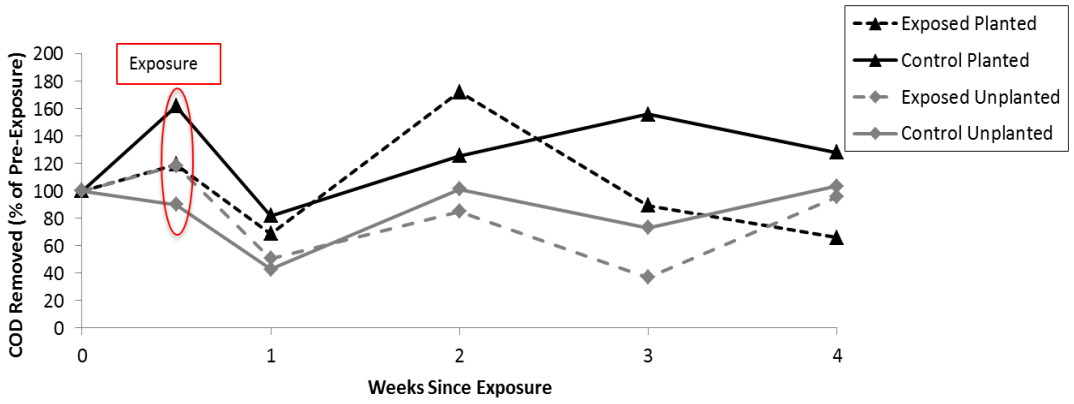

FIGURE 4.11: Chemical oxygen demand removal rate (% of pre-exposure) following high triclosan exposure.

4.3.3.3 Water Quality Changes following High Triclosan Exposure

As observed in the low triclosan exposure there were no measurable differences in total dissolved solids between the exposed and control mesocosms for the high triclosan exposure (FIGURE 4.12). None of the differences between exposed and control mesocosms were statistically significant ($p<0.05$). Again, the small amount of weekly variation within the total dissolved solids was most likely related to variation in tap water quality. Daily dissolved oxygen and redox potential were similar in trend to that of the low triclosan exposure with no discernible effects from the high triclosan exposure. The relevant figures are included in Appendix C.

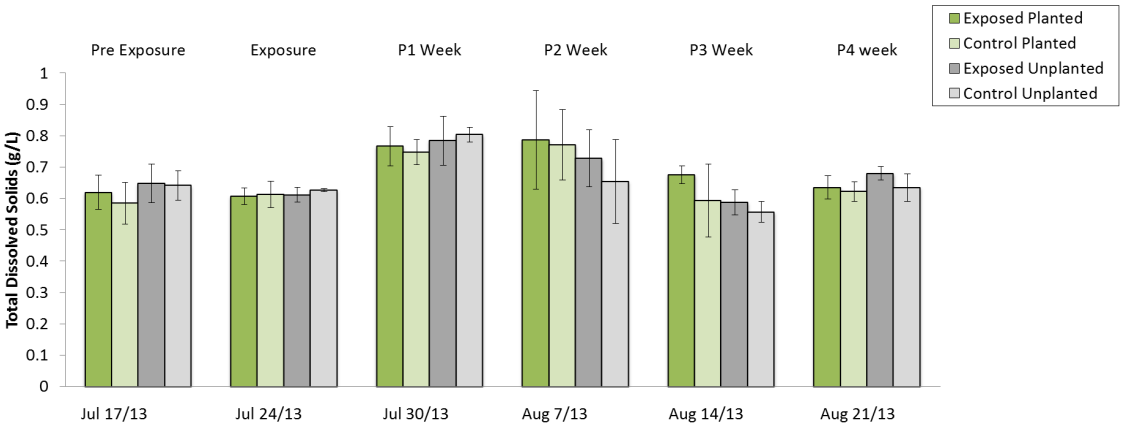

FIGURE 4.12: Total dissolved solids ($g \cdot L^{-1}$) following high triclosan exposure. P = post.

The water temperature was stable for the majority of the experimental period during the high triclosan exposure from July 17 to August 21 2013 (FIGURE 4.13A). Water pH (FIGURE 4.13B) remained fairly constant at around 8 with some minor week to week fluctuation as was observed in the earlier stages of the experimental work.

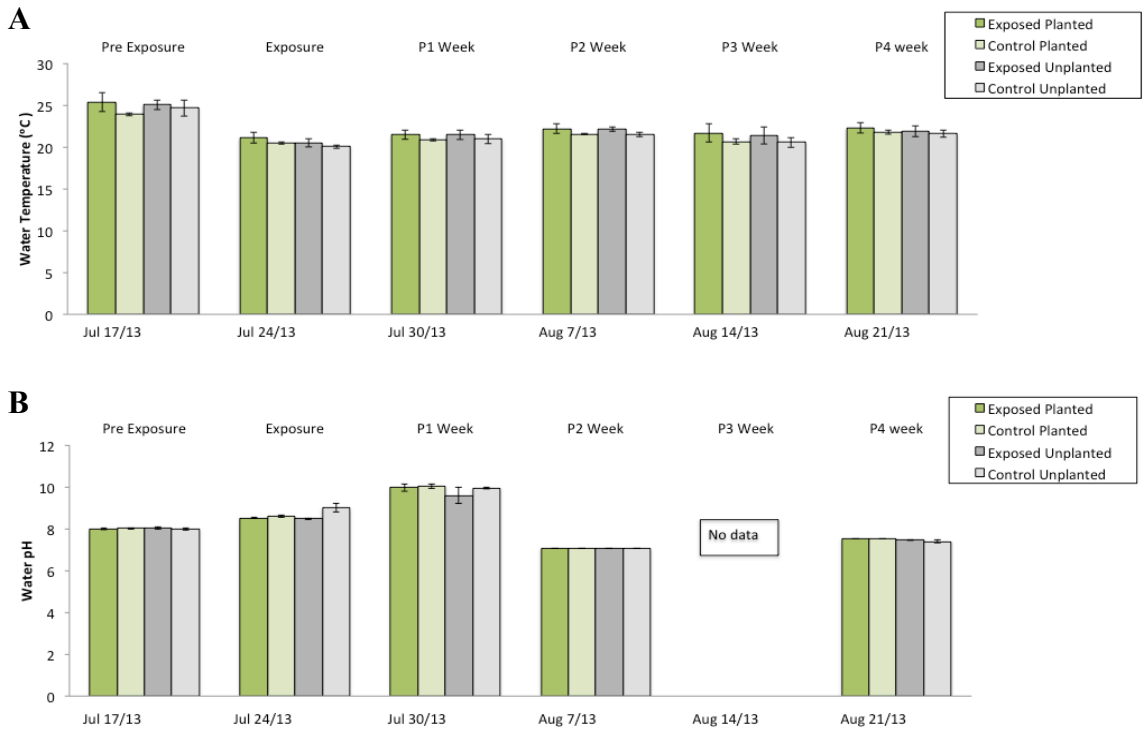

FIGURE 4.13: A) Water temperature (Celsius); B) Water pH following high triclosan exposure. P = post.

4.3.3.4 Ecological and Hydrological Changes following High Triclosan Exposure

Porosity fluctuated slightly in the weeks following the exposure for all mesocosms (FIGURE 4.14A) but no significant differences were observed between the control and exposed mesocosms. In general porosity showed a trend of stabilization following the 2-week post exposure time point. The porosity was expected to change with the higher dose exposure however as with the low triclosan exposure, the high triclosan exposure had no obvious effect on the porosity of the exposed planted and unplanted mesocosms.

Due to the well-developed rhizosphere and healthy above ground biomass at this stage of the experiment (see FIGURE 4.9B), the planted mesocosms had greater evapotranspiration rates compared to the unplanted mesocosms (FIGURE 4.14B). The exposed planted mesocosms had similar evapotranspiration rates compared to the control. The unplanted mesocosms had low evaporation rates, due to their non-vegetated surfaces and the exposed were similar to the control. Potentially due to the sequential exposure of the low concentration followed by the high concentration, the exposed systems could have gained resilience to the triclosan over time (which would have caused these limited results). The ecological parameters (stem count and plant height) were constant during this experiment period and figures are in Appendix C for further consideration.

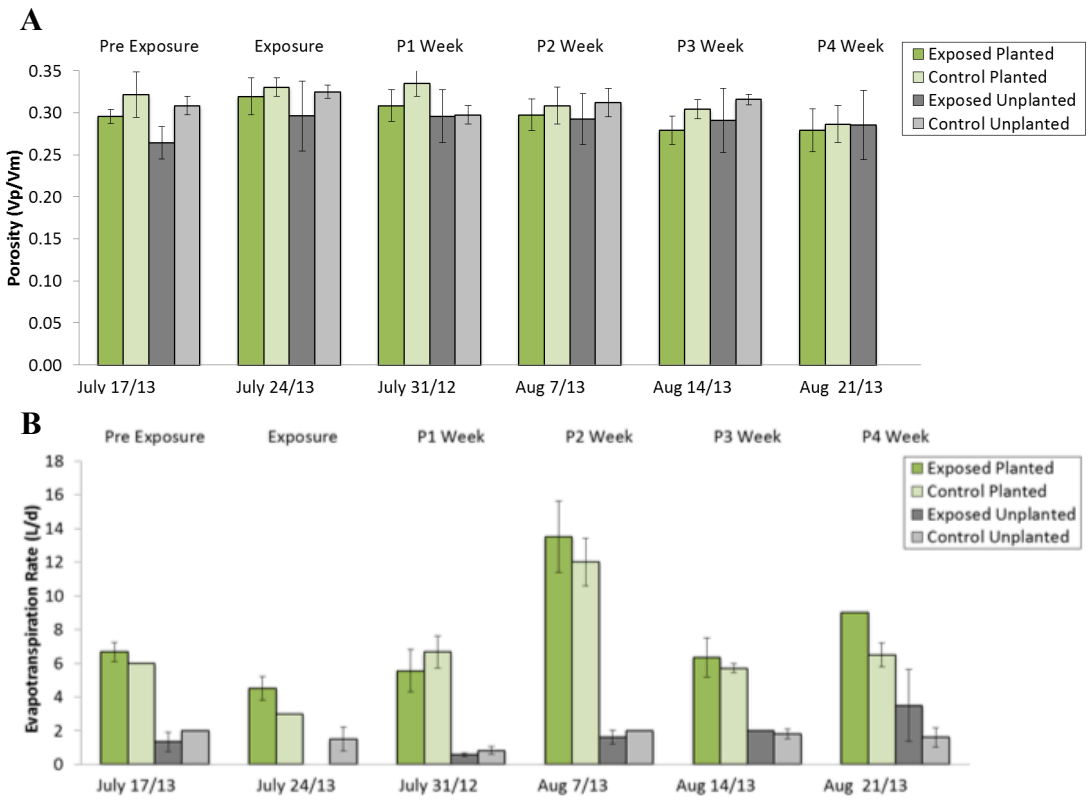

FIGURE 4.14: A) Porosity (volume of pore space/volume of medium); B) Evapotranspiration rate ($L \cdot d^{-1}$) following high triclosan exposure. P = post.

4.3.3.5 Microbial Changes following High Triclosan Exposure

No clearly observable effects occurred in the microbial community following the high triclosan exposure. No significant differences ($p<0.05$) were observed between the exposed and control groups for activity (AWCD), richness or diversity (FIGURE 4.15 A-C respectively). There was a small decrease in AWCD and richness over the two weeks following the exposure but this occurred for all mesocosms and cannot therefore be attributed to the triclosan exposure. The trends observed here are comparable to those of the low triclosan exposure with little or no clearly discernible impacts of the pharmaceutical on the microbial community. For the high exposure the removal rate after 1 hr was less than for the low exposure at 80-85 % meaning that higher levels of triclosan, between 76-99 ($\mu g \cdot L^{-1}$) were present in the water column and therefore accessible to the microbial community. Despite these levels the microbial community was unaffected suggesting a certain degree of resilience to this compound when exposed *in-situ*.

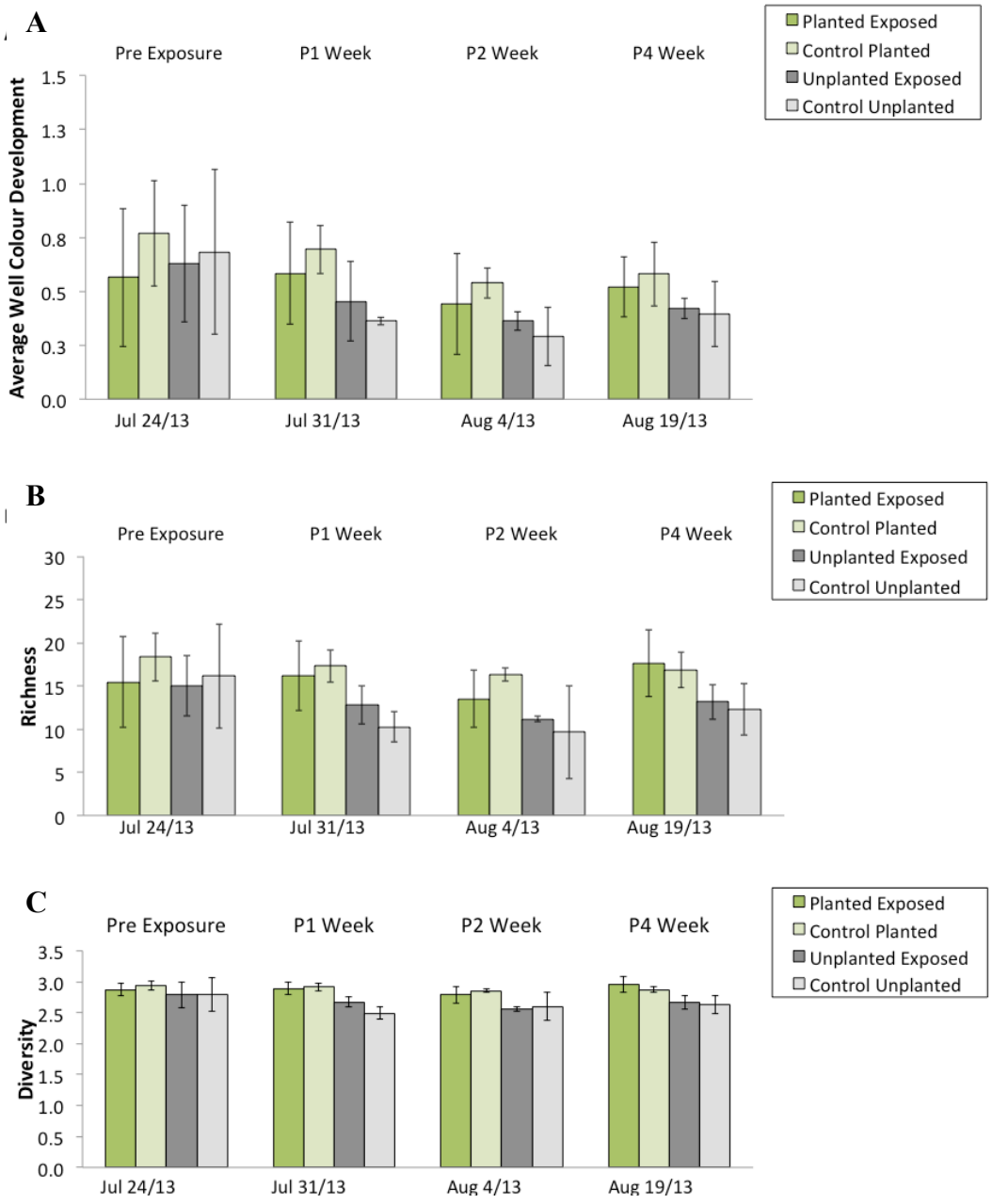

FIGURE 4.15: A) Microbial community activity (AWCD); B) Richness; C) Diversity following high triclosan exposure. P = post.

4.3.4 *In-Situ* Low Sulfamethoxazole Exposure

4.3.4.1 Sulfmethoxazole Fate in Exposed Mesocosms

Low levels (1 $\mu g \cdot L^{-1}$) of sulfamethoxazole were detected in the exposed mesocosms following exposure to 100 $\mu g \cdot L^{-1}$ (TABLE 4.1). Removal rates were comparable to triclosan at this stage with nearly all (99%) of the sulfamethoxazole bound within the wetland matrix after one hour. Sulfamethoxazole was not detected in the exposed mesocosms approximately one week (168 hr) following the exposure (TABLE 4.1) meaning that the remaining 1% of sulfamethoxazole in the water column was successfully bound onto organic matter over the seven days. As seen with the triclosan exposure experiments, sulfamethoxazole as well binds strongly to organics and can be effectively removed within wetland systems. The transformation of sulfamethoxazole was not examined within the exposed mesocosms and it could be possible that instead of binding, the sulfamethoxazole was metabolized into a different form. Further investigations are undergoing to determine the concentration of sulfamethoxazole and other metabolites within the biofilm, above ground biomass and root biomass.

4.3.4.2 Water Treatment Effectiveness following Low Sulfamethoxazole Exposure

Differences in COD removal rate observed between the control and exposed mesocosms were not statistically significant and no clear impacts of the low sulfamethoxazole exposure were evident (FIGURE 4.16). The exposed unplanted COD removal rate was lower than the control, whilst the exposed planted COD removal rate was higher. This was due to a low pre-exposure COD removal rate within the exposed planted mesocosms, which when compared to the weeks following the exposure caused skewed percent removals. The dataset was left it its original state and no values were removed in order to provide a fair assessment. There were great fluctuations in the COD removal rate data during this experimental period.

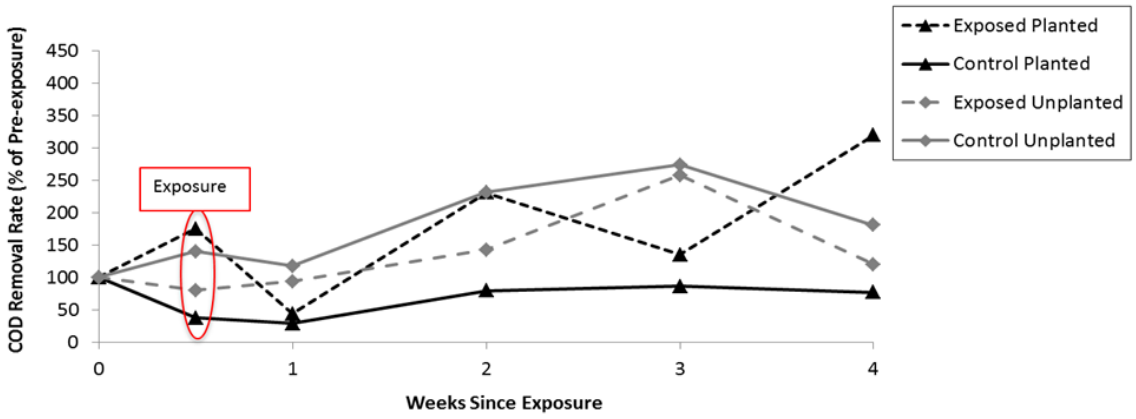

FIGURE 4.16: Chemical oxygen demand removal rate (% of pre-exposure) following low sulfamethoxazole exposure.

4.3.4.3 Water Quality Changes following Low Sulfamethoxazole Exposure

The total dissolved solids were consistent for all mesocosms throughout the low sulfamethoxazole experimental period, as was seen for all other *in-situ* exposures. The low sulfamethoxazole did not seem to affect the total dissolved solids within the exposed mesocosms. Daily dissolved oxygen and redox potential were similar in trend to that of both the low and high triclosan exposures with no discernible effects of the low sulfamethoxazole exposure. The relevant figures are included in Appendix D.

Water temperature was again fairly consistent at around 20 Celsius throughout the low sulfamethoxazole experimental period (FIGURE 4.17A). The exposed mesocosms had similar water temperatures to the control mesocosms. The water pH was constant throughout the experimental period (FIGURE 4.17B). The low sulfamethoxazole exposure had no effect on either of these water quality parameters.

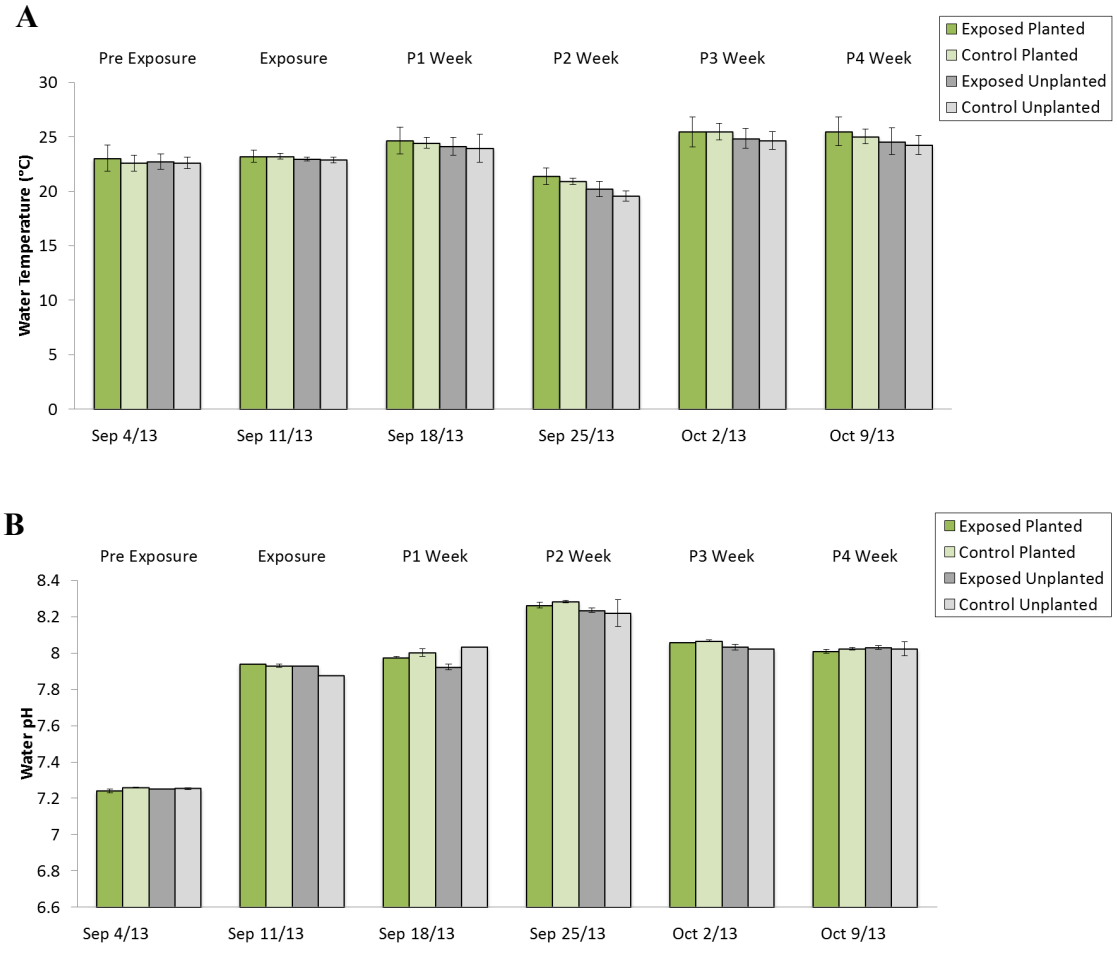

FIGURE 4.17: A) Water temperature (Celsius); B) Water pH following low sulfamethoxazole exposure. P = post.

4.3.4.4 Ecological and Hydrological Changes following Low Sulfamethoxazole Exposure

Porosity during the low sulfamethoxazole experimental period fluctuated slightly in the weeks following the exposure for all mesocosms (FIGURE 4.18A) but no significant differences were observed between the control and exposed mesocosms, as was also observed for triclosan. In general porosity did not show increasing stabilization over time. As with the triclosan exposures, the low sulfamethoxazole had no obvious effect on the porosity of the exposed planted and unplanted mesocosms.

At this stage of the experimental process both the above and below ground plant biomass had increased markedly (FIGURE 4.9 C). This was evident in the increasing difference in evapotranspiration between the planted and unplanted mesocosms (FIGURE 4.18B). For several of the unplanted mesocosms zero evaporation of water was recorded, most likely due to their positioning within the greenhouse that provided shelter from direct sunlight. The exposed planted mesocosms had similar evapotranspiration rates compared to the control and there was no effect from the low sulfamethoxazole exposure. The ecological parameters (stem count and plant height) remained constant during this experimental period, as in previous exposure period. The relevant figures are included in Appendix D for further consideration.

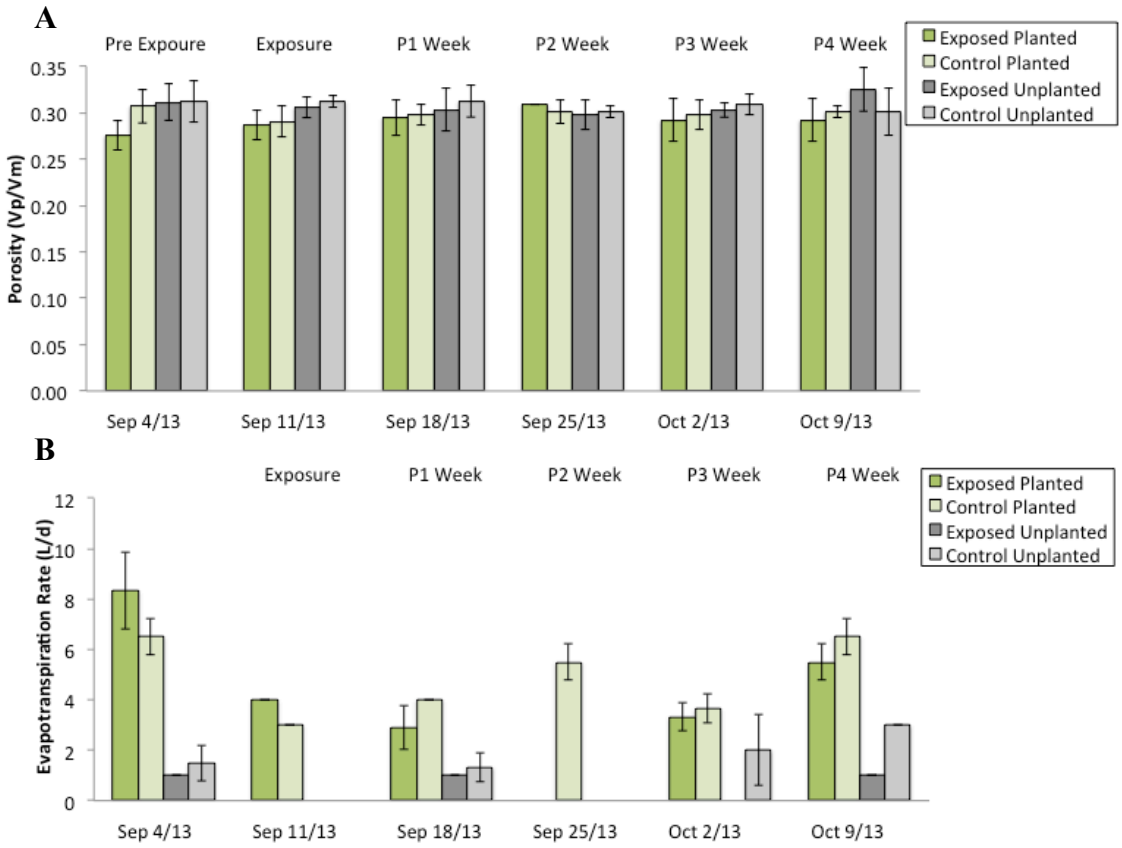

FIGURE 4.18: A) Porosity (volume of pore space/volume of bed medium); B) Evapotranspiration rate ($L \cdot d^{-1}$) following low sulfamethoxazole exposure. P = post.

There were no clearly evident effects of low sulfamethoxazole on the microbial communities, which was similar to the previous exposure experiments. A small decrease in AWCD and richness for the exposed mesocosms was noted at 1-week post exposure compared to the control, however differences between the exposed and control were not statistically significant ($P < 0.05$) for activity (AWCD), richness or diversity (FIGURE 4.19 A-C respectively). As in the high triclosan exposure, there was a decrease in AWCD and richness over the two weeks following the exposure within all mesocosms. This decrease was equally pronounced in both the control and exposed mesocosms and is therefore not attributable to the low sulfamethoxazole exposure. The lack of any effect is supported by the fact that the low sulfamethoxazole was nearly completely removed (99%) in the water column after 1 hr (TABLE 4.1). The concentration of sulfamethoxazole in the water column (1 µg/L) was comparable to the low triclosan exposure (0 µg/L) and was much lower than the high triclosan exposure. In the *ex-situ* exposures the planted and unplanted mesocosms showed microbial activity and richness decline at 100 µg/L that was expected for the *in-situ* experiment. The results indicate the *in-situ* exposures do not affect the exposed wetland microbial communities to the same extent as the *ex-situ* exposures and are likely due to increased resilience of the microbial community within the mesocosm environment.

4.3.4.5 Microbial Changes following Low Sulfamethoxazole Exposure

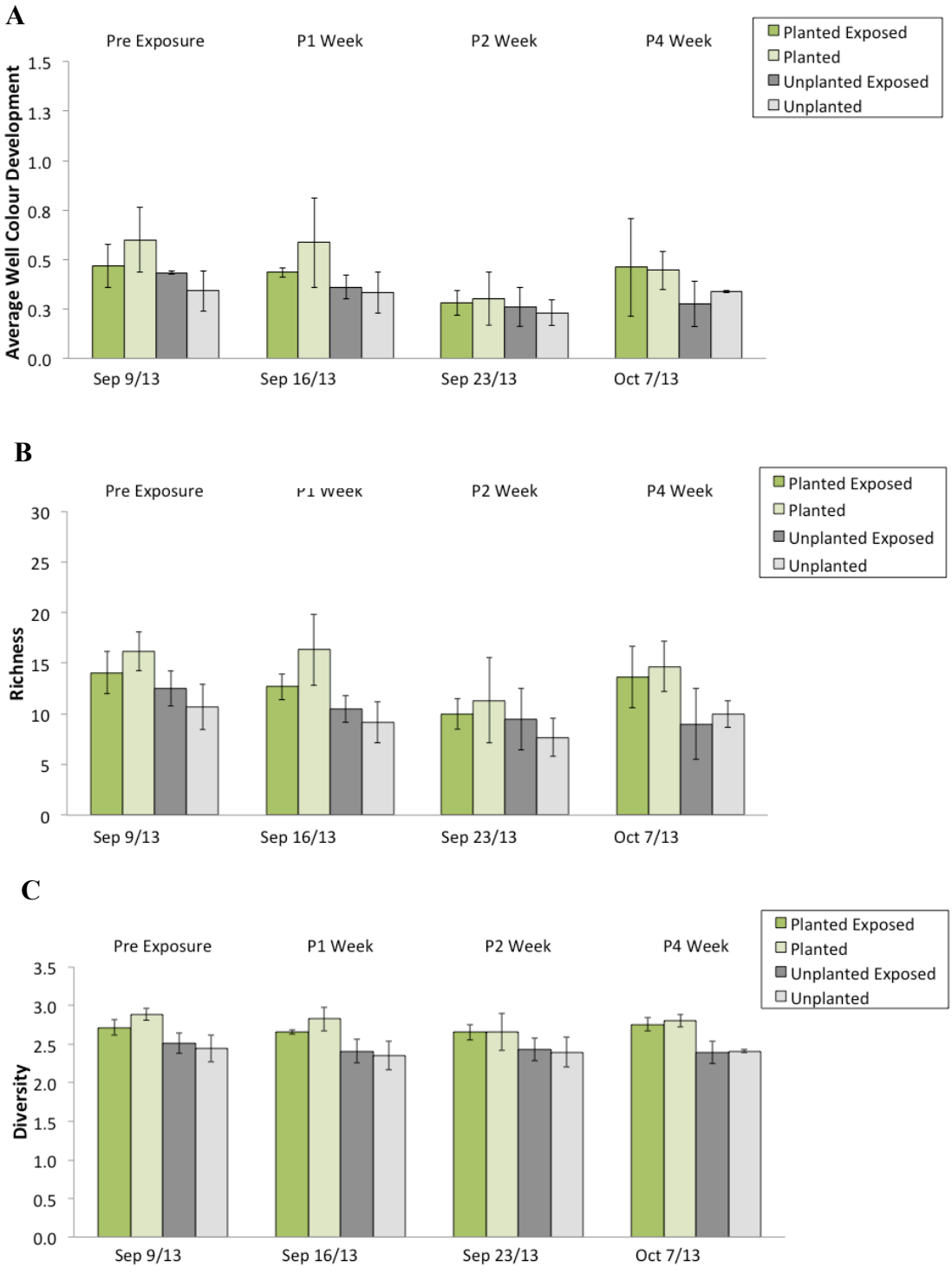

FIGURE 4.19: Microbial community activity (AWCD); B) Richness; C) Diversity following low sulfamethoxazole exposure. P = post.

4.3.5 *In-Situ* High Sulfamethoxazole Exposure

4.3.5.1 Sulfamethoxazole Fate in Exposed Mesocosms

Low levels of sulfamethoxazole were detected in the planted and unplanted exposed mesocosms (12/6 $\mu g \cdot L^{-1}$ respectively) following the 500 $\mu g \cdot L^{-1}$ exposure (TABLE 4.1). Removal rates of the high sulfamethoxazole were comparable to low sulfamethoxazole with nearly all (98-99%) bound within the wetland matrix after one hour. As with the previous exposures, sulfamethoxazole was not detected one week (168 hr) following the exposure (TABLE 4.1). Even at this high concentration of 500 ($\mu g \cdot L^{-1}$), sulfamethoxazole is effectively removed in constructed wetland environments with the likely mechanisms being the process of binding to organics.

4.3.5.2 Water Treatment Effectiveness following High Sulfamethoxazole Exposure

The COD removal rate for the exposed unplanted mesocosms was lower compared to the control throughout the experimental period (FIGURE 4.20). The exposed planted mesocosms had similar or higher COD removal rates compared to the control and were unaffected by the exposure which could be due to a more resilient microbial community (Pearson, 1998; Picard, *et al.* 2004; Bias, *et al*. 2006). The exposed mesocosms followed similar general trends to the controls over the entirety of the experimental period (FIGURE 4.20). These observations were comparable to the low sulfamethoxazole exposure.

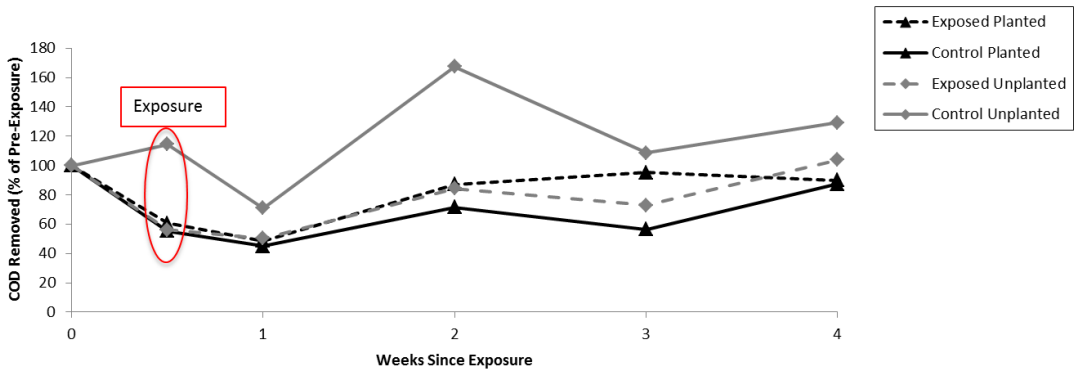

FIGURE 4.20: Chemical oxygen demand removal rate (% of pre-exposure) following high sulfamethoxazole exposure.

4.3.5.3 Water Quality Change following High Sulfamethoxazole Exposure

The total dissolved solids were similar for all mesocosms in the high sulfamethoxazole experimental period. The total dissolved solids of the exposed mesocosms were not affected by the introduction of the high sulfamethoxazole. Daily dissolved oxygen and redox potential were similar in trend to that of all previous exposure periods. Supporting relevant figures are included Appendix E.

Water temperature declined slightly at the beginning of the high sulfamethoxazole exposure and then remained consistent at around 17 Celsius (FIGURE 4.21A). The exposed

mesocosms had similar water temperatures to the control mesocosms. The water pH was constant throughout the experimental period (FIGURE 4.21B). As with the previous exposures, the high sulfamethoxazole exposure had no effect on water temperature or water pH.

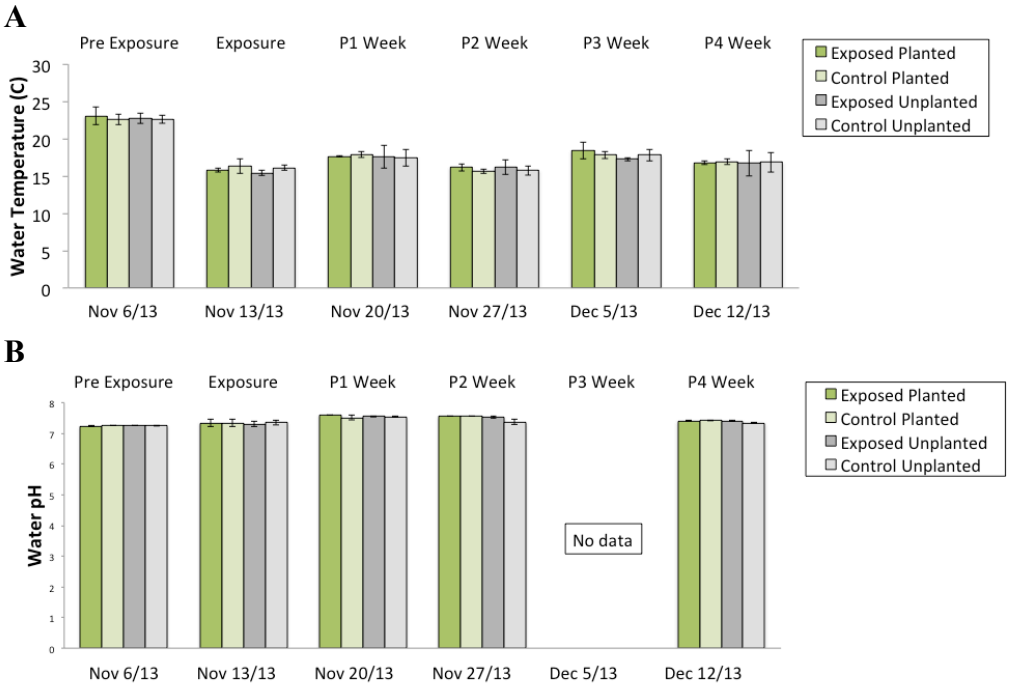

FIGURE 4.21: A) Water temperature (Celsius): B) Water pH following high sulfamethoxazole exposure. P = post.

Porosity for the planted mesocosms was lower than the unplanted mesocosms during the high sulfamethoxazole experimental period (FIGURE 4.22A). No significant differences in porosity were observed between the exposed and control mesocosms The lower porosity in the planted mesocosm was most probably a function of thick roots occupying the pore-space in the bed medium as was observed in the separate experiment on root development (FIGURE 4.9D). Porosity fluctuated slightly from week to week through the high sulfamethoxazole exposure period. The high sulfamethoxazole did not affect the porosity of the exposed mesocosms that was similar to the previous exposures.

The planted mesocosms had higher evapotranspiration compared to the unplanted mesocosms, which was expected due to large above ground biomass (FIGURE 4.22B). In the latter portion of the experimental period, some unplanted mesocosms had zero evaporation. As with the previous exposures, there was no effect of high sulfamethoxazole on the evapotranspiration/evaporation of the exposed mesocosms. The ecological parameters (stem count and plant height) once again remained constant and the relevant figures are included in Appendix E.

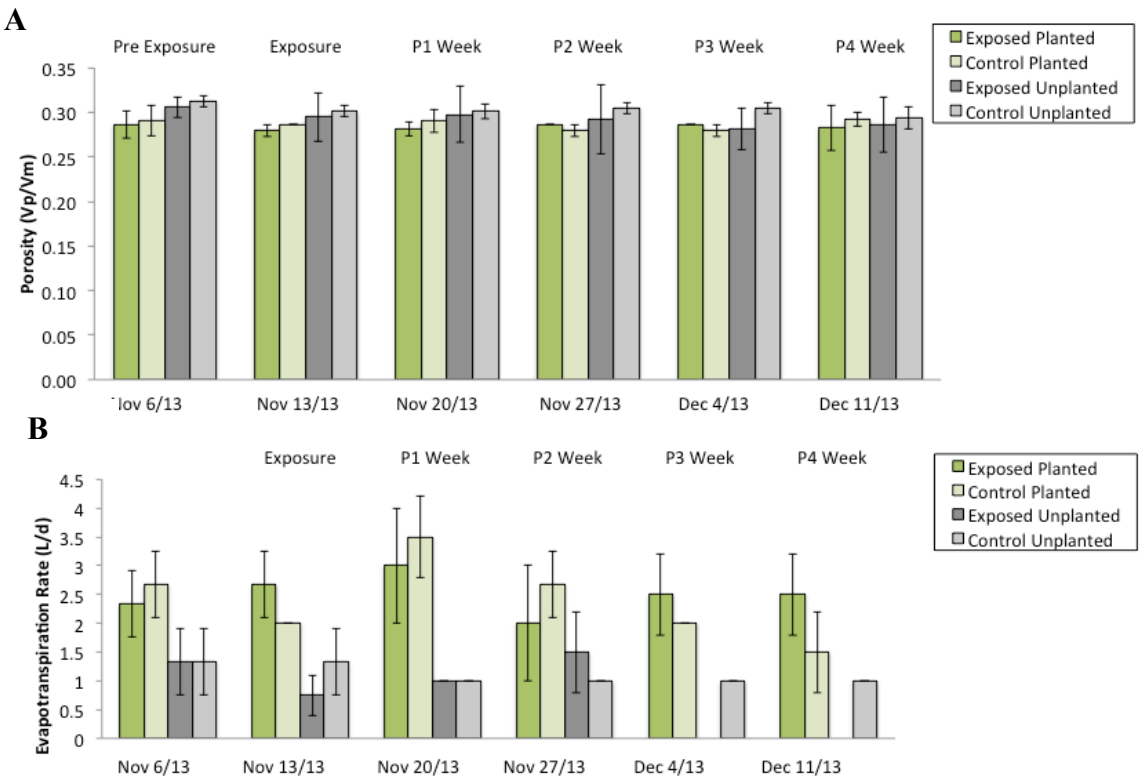

FIGURE 4.22: A) Porosity (volume of pore space/volume of bed medium); B) Evapotranspiration rate ($L \cdot d^{-1}$) following high sulfamethoxazole exposure. P = post.

4.3.5.4 Microbial Changes following High Sulfamethoxazole Exposure

As seen with previous exposures, the high sulfamethoxazole had no clearly observable effect on the exposed microbial communities in terms of activity (AWCD), richness and diversity (FIGURE 4.23 A-C respectfully). Differences between the exposed and control mesocosms were not significant ($p<0.05$) for any of the metrics although the unplanted exposed tended to be lower than the unplanted control for AWCD and richness. Activity and richness decreased for all mesocosms one-week post exposure and recovery was seen in latter weeks. The planted mesocosms in the 2nd week and 4th week post exposure had higher activity and richness compared to the unplanted which was a function of the rhizosphere. As seen in all previous exposures, the high sulfamethoxazole was also nearly completely removed (98-99%) in the water column after 1 hr (TABLE 4.1) that helps explain the lack of observable effect on the microbial community. The microbial communities were affected severely in the *ex-situ* exposures at concentrations over 100 $\mu g \cdot L^{-1}$, again suggesting that within the wetland environment the microbial communities are more resilient to sulfamethoxazole toxicity compared to the *ex-situ* experiments.

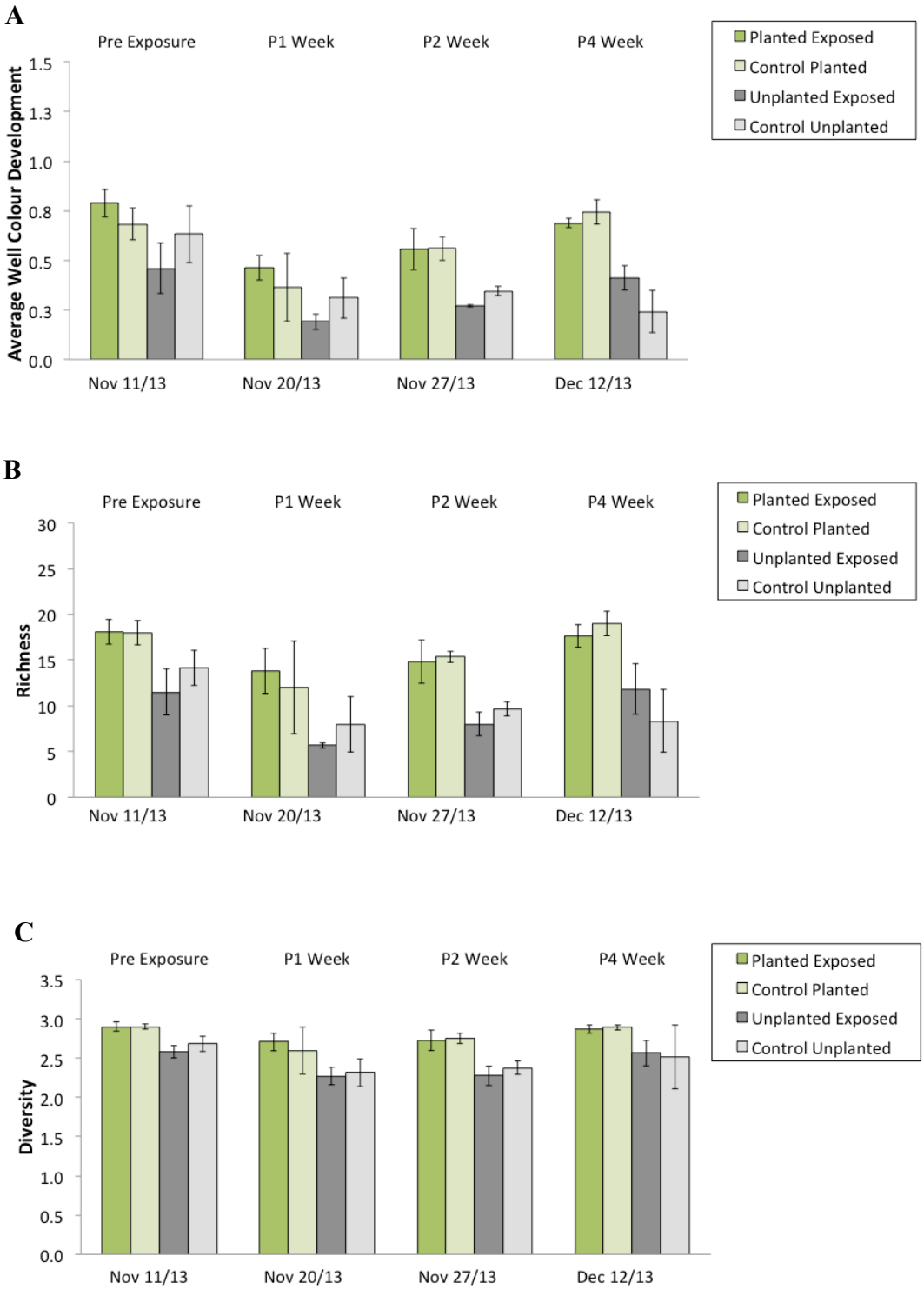

FIGURE 4.23: Microbial community activity (AWCD); B) Richness; C) Diversity following high sulfamethoxazole exposure. P = post.

4.3.6 Microbial Community Functional Dynamics through Exposure Periods

Principal component analysis of the CSUP data was performed on the combined dataset for the entire duration of each exposure period to assess shifts in microbial community function. During the low triclosan exposure period (FIGURE 4.24A) there was no clear trend in carbon utilization for the planted or unplanted mesocosms. During this experimental period the rhizosphere was early in its development period (FIGURE 4.9A) and the microbial communities were still being established. The low triclosan exposure occurred after the development period, which had a scattered PCA plot as well (Chapter 3) indicating a developing microbial community for both the planted and unplanted mesocosms.

Following the high triclosan exposure (FIGURE 4.24B) the planted and unplanted mesocosms began to form distinct carbon utilization patterns although the groupings for each, as indicated by the circles on FIGURE 4.24B, were fairly close. This indicates the beginning of microbial community divergence between the planted and unplanted mesocosms. The planted mesocosms were more closely grouped compared to the unplanted, which could be due to common root exudates being released from the developing rhizosphere (Tillman, *et al.* 1996; Pearson, 1998; Picard, *et al.* 2004; Bias, *et al*. 2006). The exposed mesocosms had no clear trend of changing carbon utilization over the weeks following the exposure adding more evidence to previous findings that this high triclosan exposure was not harmful to the microbial community.

After the low sulfamethoxazole exposure (FIGURE 4.24C), the planted and unplanted mesocosms diverged further as indicated by the more clearly separate groups on FIGURE 4.24C. The microbial communities for the planted and unplanted mesocosms were therefore continuing differentiate over the experimental period. The planted mesocosms at this point had a well-developed rhizosphere which is the most likely factor causing the continued divergence.

Some interesting differences were noted in the PCA analysis of the high sulfamethoxazole exposure CSUPs (FIGURE 4.24D). The CSUPs of the planted and unplanted mesocosms continued to diverge but some clustering based on weeks post exposure became evident. In particular the exposed planted mesocosms showed some distinct grouping of points based on the week following exposure. The high sulfamethoxazole was the most toxic exposure and thus could have caused this shift within the exposed planted mesocosms.

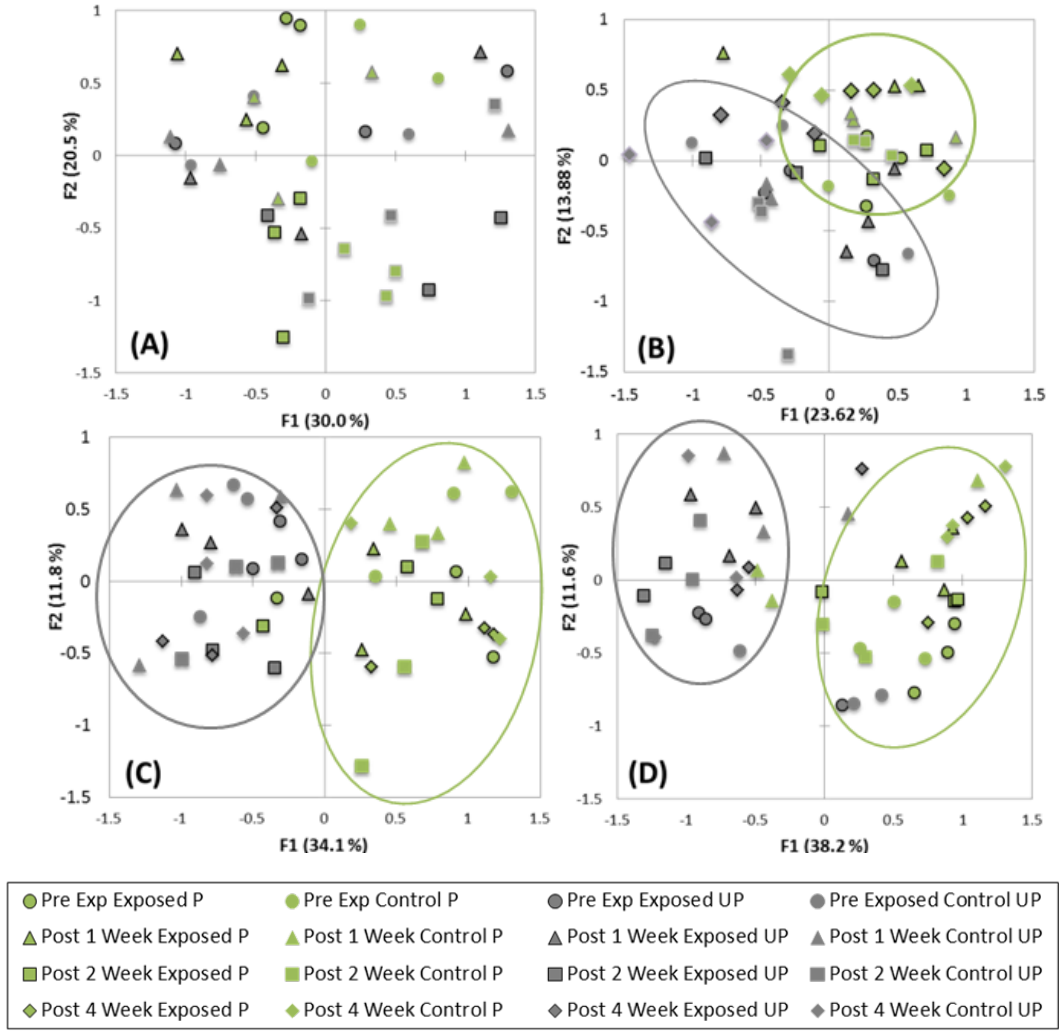

FIGURE 4.24: Principle component plots based on Taylor transformed carbon source utilization patterns (CSUPs) following A) low triclosan; B) high triclosan; C) low sulfamethoxazole; D) high sulfamethoxazole exposures. Green circles indicate planted mesocosms and grey circles indicate unplanted mesocosms.

4.4 Conclusions

The potential toxic effect of the pharmaceuticals triclosan and sulfamethoxazole were assessed *ex-situ* and *in-situ* within the vertical flow constructed wetland mesocosms. The effects of triclosan *ex-situ* to interstitial wetland microbial communities were minimal. The effect of sulfamethoxazole *ex-situ* to interstitial wetland microbial communities was severe, causing population collapse in moderate to high $\mu g \bullet L^{-1}$ concentrations. The *in-situ* exposures of triclosan and sulfamethoxazole resulted in minimal changes to the wetland microbial communities. Small decreases in water treatment ability (chemical oxygen demand removals) were observed in some cases. The exposed mesocosms were able to recover close to pre-exposure levels after four weeks for all of these exposures. Microbial community functions decreased the two weeks following the

exposure for the low and high sulfamethoxazole though no significant differences were observed between the control and exposed mesocosms making it unclear as to whether the exposure was the cause of the decrease. The robustness of the mesocosms was evident through these *in-situ* studies. This recovery ability of vertical flow constructed wetland microbial communities has previously been observed after a moderate ciprofloxacin exposure (Weber *et al.*, 2011; Helt, *et al.* 2012). The enhanced recovery ability of the exposed planted and unplanted mesocosms seen during the sulfamethoxazole exposures could be due to these microbial communities gaining resistance overtime and adapting to pharmaceutical induced stress. Interestingly no vegetation biomass or hydrological changes were observed within the low or high triclosan and sulfamethoxazole exposures. The planted mesocosms showed no deterioration of the reed canary grass through any of the exposures, only deterioration from harsh air temperatures within the greenhouse. The planted mesocosms overall seemed to handle the antimicrobial exposures better than the unplanted mesocosms. The rhizosphere zone is an important component within planted constructed wetlands and this study showed the rhizosphere plays an important role in buffering the microbial community from toxic effects of introduced compounds (Tillman, *et al.* 1996; Pearson, 1998; Picard, *et al.* 2004; Bias, *et al*. 2006). Impressive triclosan and sulfamethoxazole removal rates were observed in this study and warrant further investigation.

Improvements to this study would include having all mesocosms on the same height level and located within a light and temperature controlled environment. Future work should consider long-term exposures (lasting months) and multi-compound exposures to understand their effects on wetland environments. Given the outcome, this study indicates that antimicrobial exposures do not significantly harm vertical flow constructed wetlands and recoveries within the wetland microbial communities occur with time. Constructed wetlands have been shown to be effective and robust systems able to tolerate high concentrations of potentially toxic emerging contaminants.

CHAPTER 5 – PRINCIPLE OUTCOMES AND RECOMMENDATIONS

5.1 Main Objectives

The main objective of this study was to observe the effects of antimicrobials within planted and unplanted vertical flow constructed wetland mesocosms.

Within this main objective were the following aims:

A) Characterize the development period of planted and unplanted vertical flow constructed wetland mesocosms.

B) Quantify the effect of *ex-situ* exposures of trimethoprim, triclosan and sulfamethoxazole on interstitial wetland microbial communities.

C) Assess the fate of triclosan and sulfamethoxazole in vertical flow constructed wetland mesocosms.

D) Quantify the effect of *in-situ* low and high triclosan and sulfamethoxazole exposures within planted and unplanted vertical flow constructed wetlands.

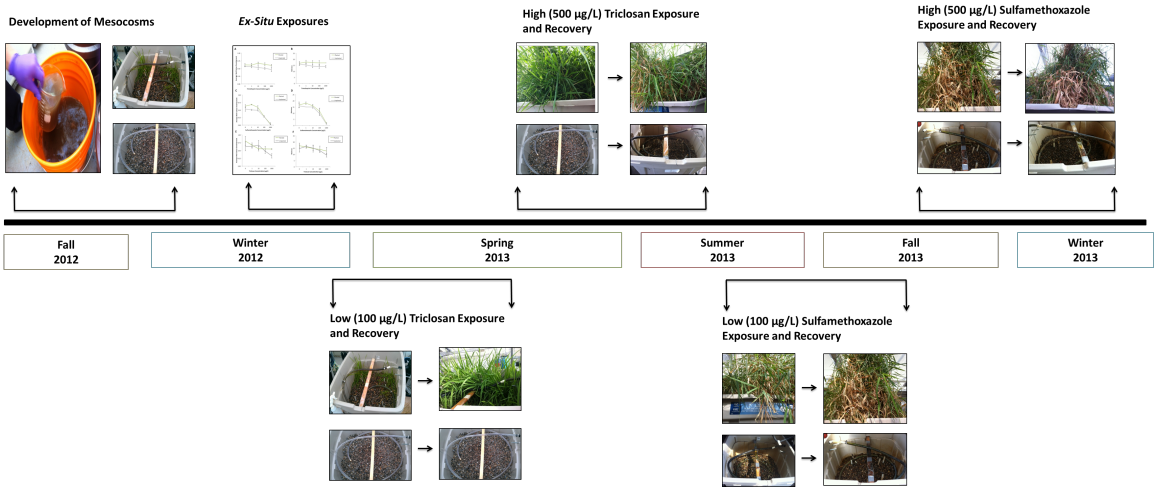

FIGURE 5.1: Research timeline.

5.1.1 Objective A – Characterize the development period of planted and unplanted vertical flow constructed wetland mesocosms

Twelve vertical flow constructed wetland mesocosms were built and inoculated with activated sludge from a local wastewater treatment plant. These mesocosms were observed for 90 days following the inoculation. The water treatment ability of the planted and unplanted systems was similar and increased over the development period. Dissolved oxygen was greater in the

unplanted systems compared to the planted, perhaps due to a smaller microbial population, the lack of a rhizosphere, or due to a greater venturi effect. The hydrological parameters measured (porosity and evapotranspiration) were similar between the planted and unplanted systems. Porosity decreased slightly over the development period indicating biofilm and/or root growth. The microbial activity and richness increased over time over the development period within both the planted and unplanted mesocosms. This experimental period highlighted the slight differences of development within planted and unplanted vertical flow constructed wetland mesocosms and showed some level of equilibrium was reached within the mesocosm systems.

5.1.2 Objective B – Effect of *ex-situ* trimethoprim, triclosan and sulfamethoxazole exposures on interstitial wetland microbial communities

Ex-situ studies were employed to gain a better understanding of the potential ecotoxicological effects of pharmaceutical compounds within the exposed mesocosms. Environmental factors (light, temperature) were controlled in *ex-situ* studies and results are purely based upon the introduced chemical species and the wetland microbial population. Interstitial wetland microbial communities were exposed to three antimicrobial compounds (trimethoprim, triclosan and sulfamethoxazole) at varying concentrations (0 $\mu g \cdot L^{-1}$ to 1000 $\mu g \cdot L^{-1}$). Trimethoprim and triclosan had minimal effects on the planted and unplanted interstitial microbial populations. Sulfamethoxazole had significant effects on the planted and unplanted mesocosms. Microbial activity and richness declines were shown in the planted mesocosms at 10 $\mu g \cdot L^{-1}$ and for the unplanted mesocosms at 1 $\mu g \cdot L^{-1}$. As seen within the trimethoprim and triclosan studies, the planted mesocosms had higher values of activity and richness compared to the unplanted. The rhizosphere within the planted mesocosms is believed to enhance the microbial population and increase population resilience to environmental changes. Through these experiments, the planted mesocosms seem to be the more robust system.

5.1.3 Objective C – Assess the fate of triclosan and sulfamethoxazole in vertical flow constructed wetlands

The removal of triclosan and sulfamethoxazole was assessed within the wetland mesocosm environments. Water samples were collected from the mesocosms 1 hour and approximately 168 hrs (7 days) following the exposures. The samples were analyzed by LC-MS/MS using a direct injection (15 µL) method utilizing formic acid, acetonitrile, ammonium formate mobile phases and electrospray ionization detection. Triclosan concentrations in the interstitial water after 1 hour were reduced by 100 % in the low exposure experiment and by around 85% for the high exposure. In both cases triclosan was completely absent from the interstitial water approximately 7 days following exposure. Similar removal trends were also seen for both of the sulfamethoxazole exposures with the vast majority (approximately 99%) removed 1 hr after exposure and complete removal of the compound observed after around 7 days. These findings are significant and warrant further investigation to both confirm this removal rate and explore further the possibilities of re-suspension into the water column with longer-term continuous exposure as might be encountered in a larger system.

5.1.4 Objective D – Effect of *in-situ* triclosan and sulfamethoxazole exposures in vertical flow constructed wetland mesocosms

The low concentration (100 $\mu g \cdot L^{-1}$) exposure of triclosan began in January 2013 and the recovery period of the exposed mesocosms was characterized. The water treatment ability of the exposed mesocosms was not significantly affected by the introduced of triclosan. The exposed mesocosms had similar dissolved oxygen and redox potential profiles compared to the control, though the unplanted mesocosms were slightly lower than the control. The measured hydrological and ecological parameters of the exposed mesocosms were similar to that of the controls. Triclosan did not affect the activity, richness or diversity of the exposed microbial communities. PCA analysis showed scattered CSUPs for both the planted and unplanted mesocosms which indicated developing microbial communities. There was little divergence between the planted and unplanted mesocosms with no weekly trends observed in exposed mesocosms following the exposure.

High (500 $\mu g \cdot L^{-1}$) exposure of triclosan began in July 2013 and had a similar recovery period to the low triclosan exposure. The water treatment ability of the exposed mesocosms was minimally affected by the introduction of triclosan. The exposed mesocosms COD removal rate declined one week following the exposure. There was little change in the hydrological and ecological parameters throughout this exposure period. The exposed planted mesocosms had greater evapotranspiration rates compared to the unplanted due to developing above ground biomass. This high triclosan exposure did not affect the wetland microbial community function. The exposed mesocosms were similar to the control. The PCA of CSUPs showed a more diverging microbial community between the planted and unplanted mesocosms, though no clear differences were shown between the weeks following the exposure.

The low (100 $\mu g \cdot L^{-1}$) exposure of sulfamethoxazole was introduced in September 2013. The COD removal rates were not altered by the introduction of the low concentration sulfamethoxazole. As observed in the triclosan exposures, the differences between the hydrological and ecological parameters of the exposed mesocosms were similar to that of the control mesocosms. The low sulfamethoxazole did not affect the exposed wetland microbial communities, as previously observed in the triclosan exposures. In this case PCA analysis of CSUP data showed enhanced divergence between the planted and unplanted mesocosms.

The high (500 $\mu g \cdot L^{-1}$) exposure of sulfamethoxazole was introduced in November 2013. The water treatment ability, expressed as COD removal, of the exposed unplanted mesocosms was slightly affected by the sulfamethoxazole exposure, being lower than the control. As seen within the low sulfamethoxazole exposure, the hydrological and ecological parameters did not change for the exposed mesocosms. As with the previous exposures, the wetland microbial communities showed no change resulting from the high sulfamethoxazole exposure. According to PCA analysis the CSUPs were the most divergent in this exposure with clear groupings for the planted and unplanted mesocosms. The exposed planted mesocosms had clear groupings correlating to the weeks following the exposure suggesting some alteration in microbial function post exposure, which was not as apparent in any of the previous exposures.

5.2 Conclusion and Recommendations

The study focused on quantifying the effects of the antimicrobials trimethoprim, triclosan and sulfamethoxazole in vertical flow constructed wetland mesocosms. *Ex-situ* dose-response testing with the antimicrobial compounds demonstrated that all had the potential to induce harmful effects in the wetland microbial communities in the form of reduced activity and richness. Of the investigated compounds sulfamethoxazole was clearly the most toxic leading to dramatic reduction in microbial function at relatively low concentrations (100 $ug \cdot L^{-1}$). Different results

were observed when the exposure experiments were carried out *in-situ*. Both the low and high (100 and 500 $ug \cdot L^{-1}$) exposures of triclosan and sulfamethoxazole showed minimal effects upon the exposed mesocosms ecological parameters. The removal of these compounds from the water column was relatively quick and complete helping to explain this difference in the toxicity between *in-situ* and *ex-situ* exposures. Considering this, vertical flow constructed wetland mesocosms have been shown to be robust systems able to handle sequential loads of toxic pharmaceuticals.

Recommendations for future work include:

1) Observe the effects of pharmaceuticals and personal care products in vertical flow constructed wetlands over a long time period (months – years)

2) Quantify the effects of multiple antibiotic exposures to wetland microbial communities

3) Quantify the effects of triclosan and sulfamethoxazole within different wetland designs

4) Examine the concentrations of triclosan and sulfamethoxazole within the microbial biofilms and vegetative biomass

5) Examine the effects of antimicrobial removals within different vegetated mesocosms. Different vegetative species could promote different microbial communities which might be more effective at removing certain compounds

6) Locate the mesocosms in a temperature and light controlled environment to reduce variation in environmental conditions.

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APPENDICES

Appendix A: Chapter 3 - Development Period

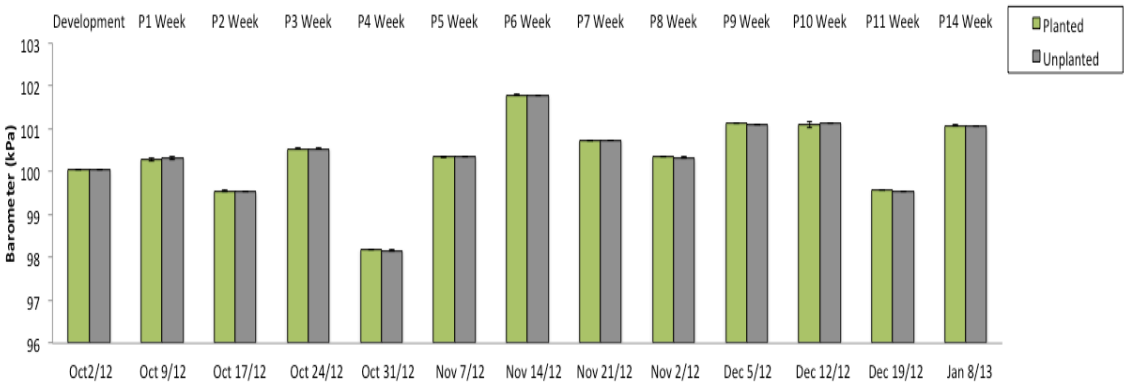

FIGURE 1: Barometer (kPa) through development period.

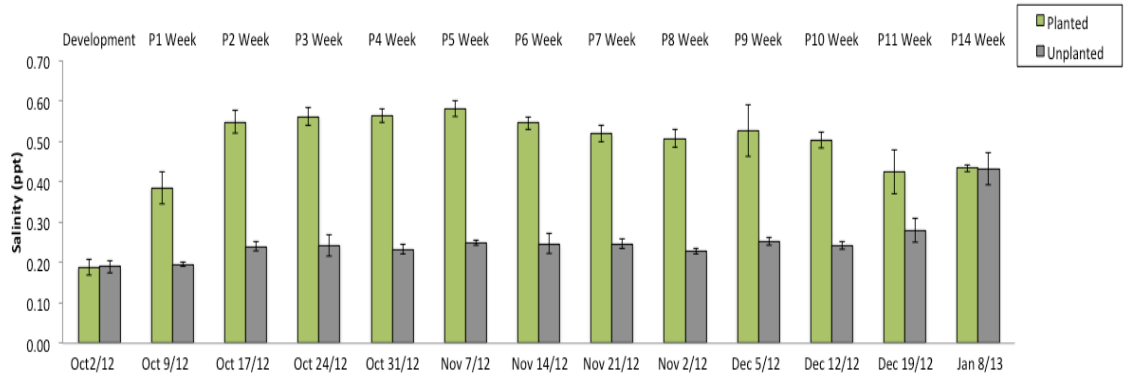

FIGURE 2: Salinity (ppt) through development period.

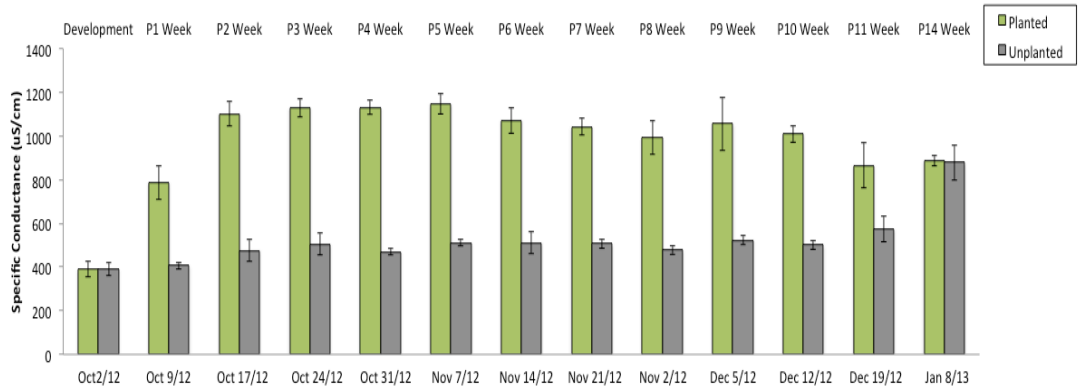

FIGURE 3: Specific conductivity ($\mu S \cdot cm^{-1}$) through development period.

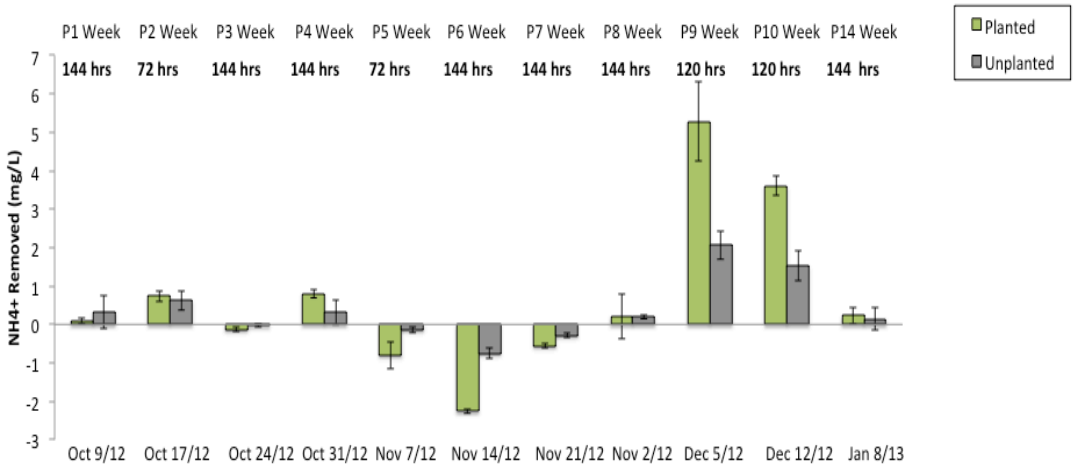

FIGURE 4: Ammonium removal ($mg \cdot L^{-1}$) through development period.

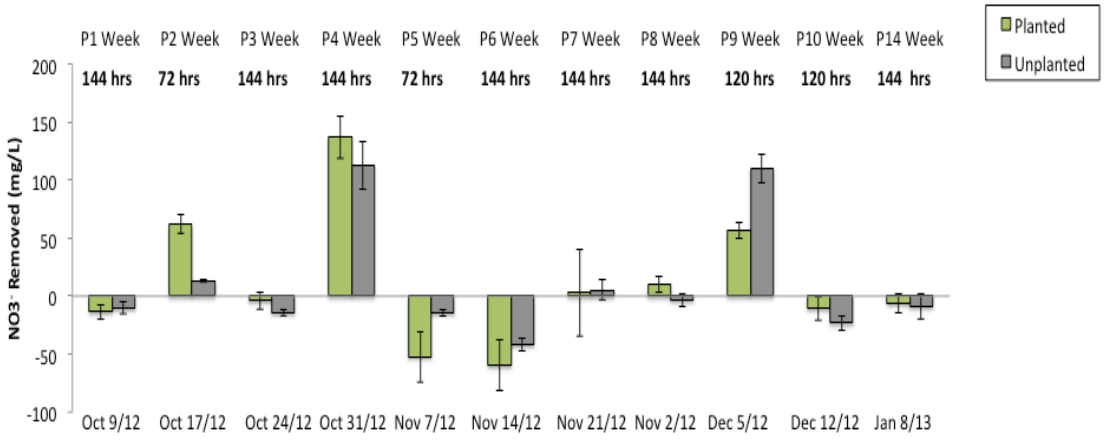

FIGURE 5: Nitrate removal ($mg \cdot L^{-1}$) through development period.

Appendix B: Chapter 4 - Low Triclosan Exposure

Molar Concentration of low (100 µg·L^{-1}) exposure:

$$= \frac{100\ \mu g}{L} * \frac{mg}{1000\ \mu g} * \frac{g}{1000\ mg} = \frac{0.0001\ g}{L}$$

$$= \frac{0.0001\ g}{L} * \frac{mol}{289.54\ g} = \frac{3.45 * 10^{-7} mol}{L}$$

FIGURE 6: Barometer (kPa) following low (100 µg·L^{-1}) triclosan exposure

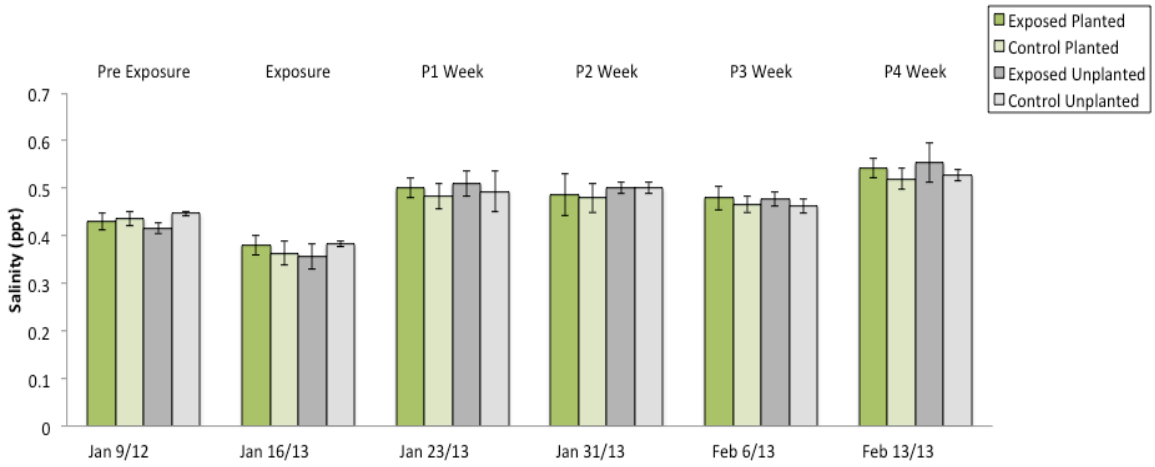

FIGURE 7: Salinity (ppt) following low (100 µg·L^{-1}) triclosan exposure.

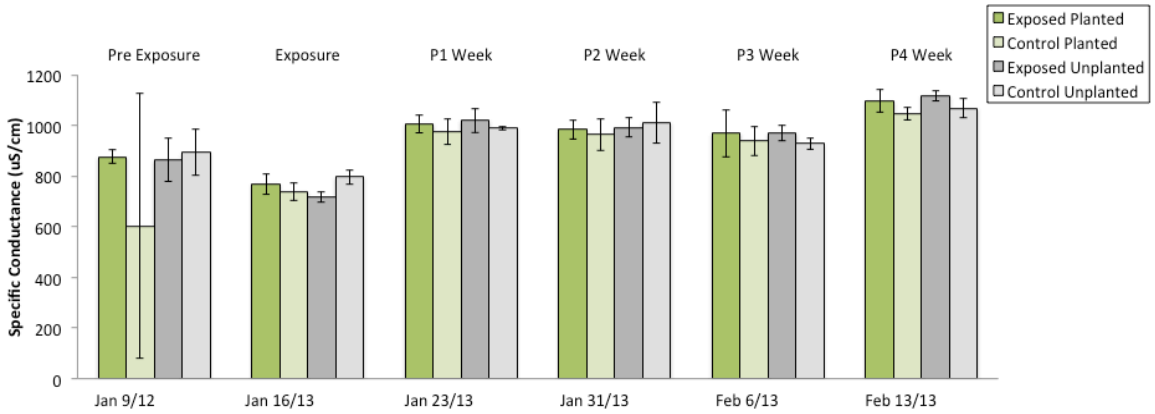

FIGURE 8: Specific conductivity ($\mu S \cdot cm^{-1}$) following low (100 $\mu g \cdot L^{-1}$) triclosan exposure.

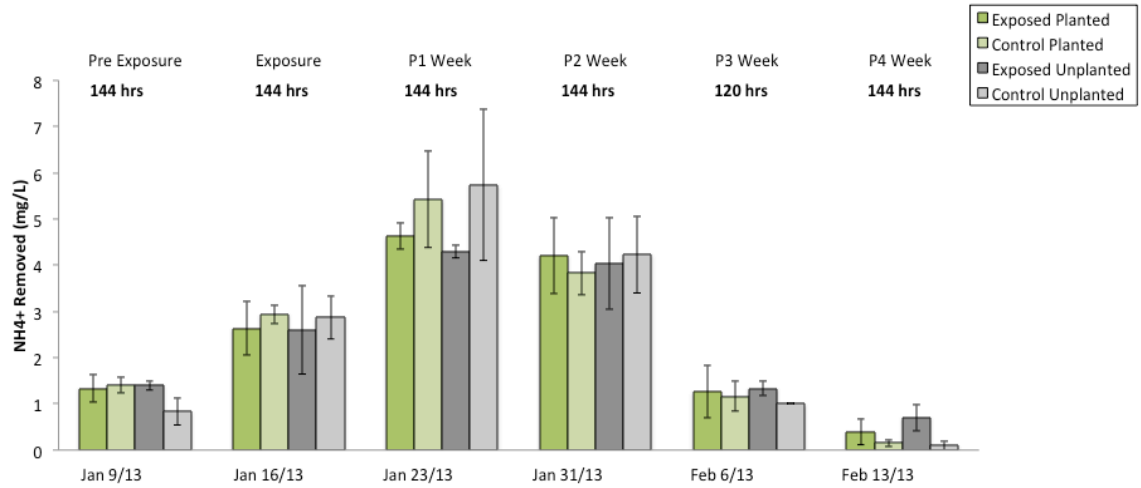

FIGURE 9: Ammonium removal ($mg \cdot L^{1}$) following low (100 $\mu g \cdot L^{-1}$) triclosan exposure.

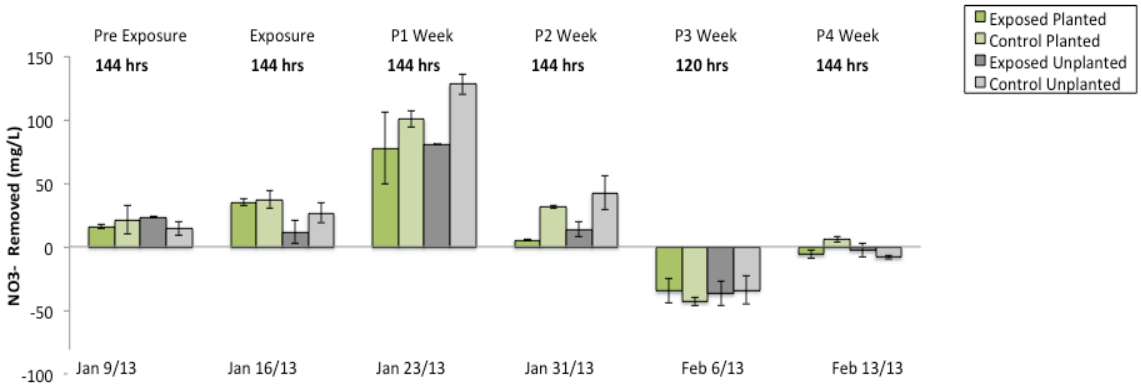

FIGURE 10: Nitrate removal ($mg \cdot L^{1}$) following low (100 $\mu g \cdot L^{-1}$) triclosan exposure.

Appendix C: Chapter 4 - High Triclosan Exposure

Molar Concentration of high (500 µg·L^{-1}) exposure:

$$= \frac{500\ \mu g}{L} * \frac{mg}{1000\ \mu g} * \frac{g}{1000\ mg} = \frac{0.0005\ g}{L}$$

$$= \frac{0.0005\ g}{L} * \frac{mol}{289.54\ g} = \frac{1.73 * 10^{-6} mol}{L}$$

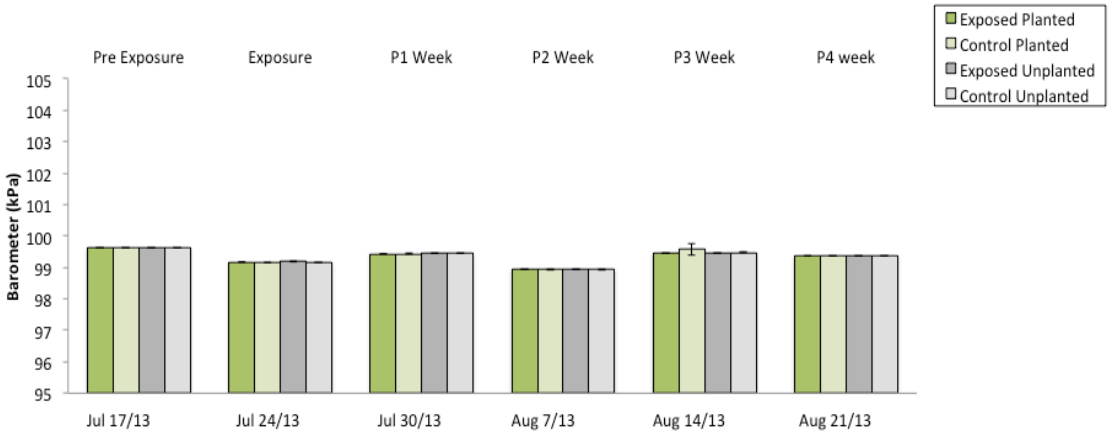

FIGURE 11: Barometer (kPa) following high (500 µg·L^{-1}) triclosan exposure.

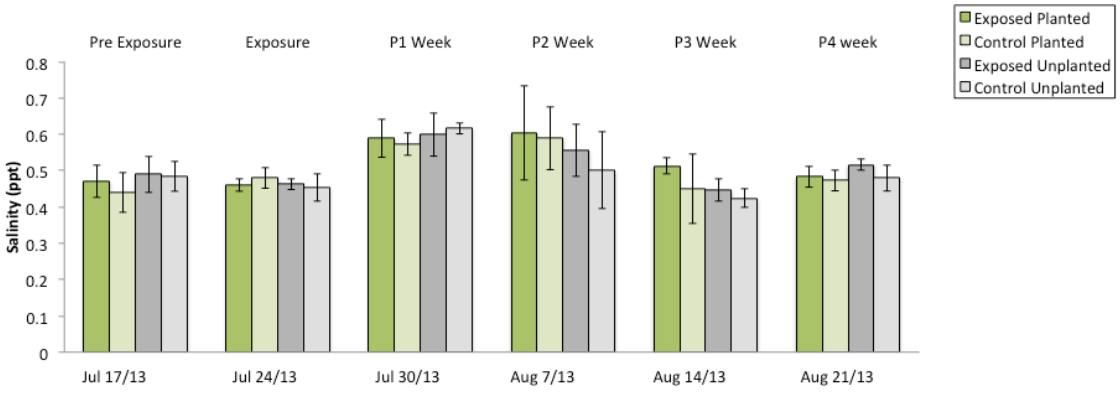

FIGURE 12: Salinity (ppt) following high (500 µg·L^{-1}) triclosan exposure.

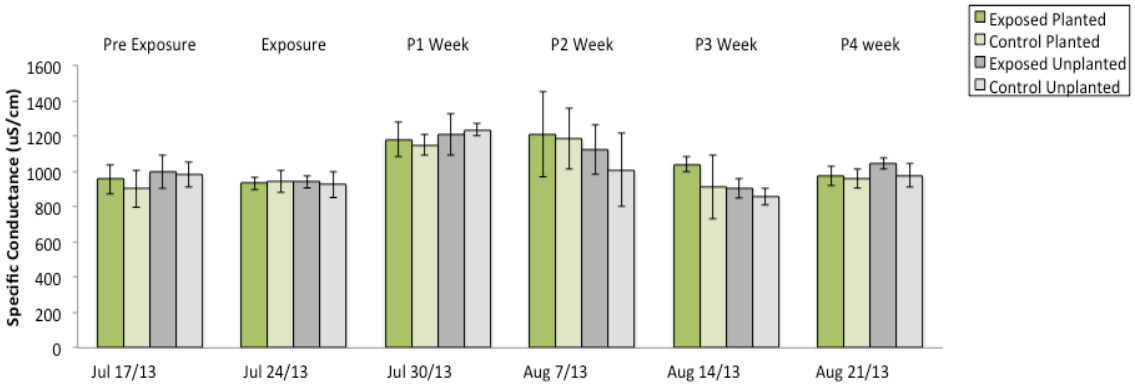

FIGURE 13: Specific conductivity ($\mu S \cdot cm^{-1}$) following high (500 $\mu g \cdot L^{-1}$) triclosan exposure

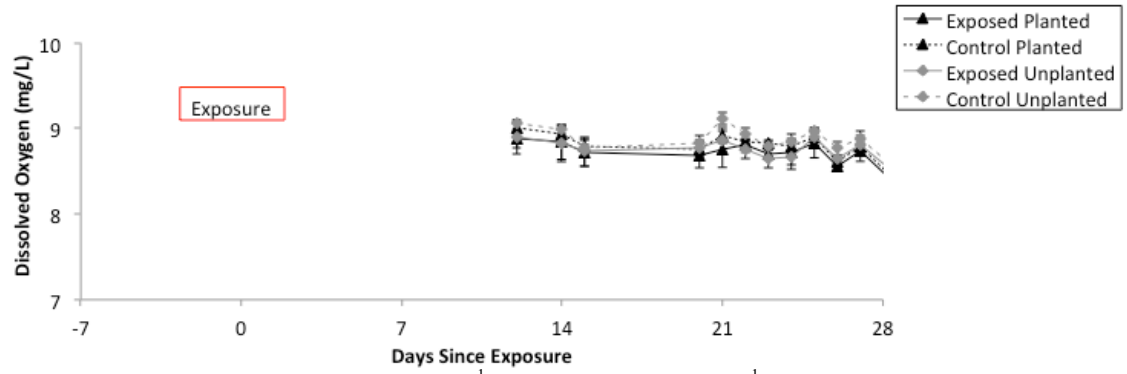

FIGURE 14: Dissolved oxygen removal ($mg \cdot L^{1}$) following high (500 $\mu g \cdot L^{-1}$) triclosan exposure.

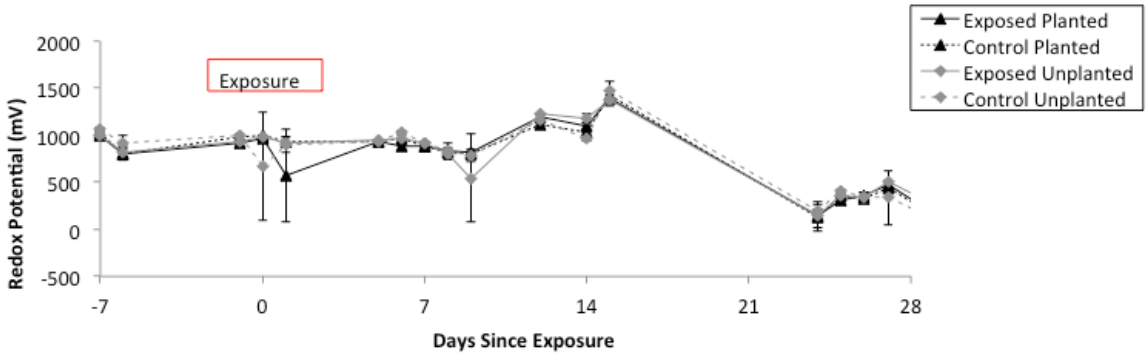

FIGURE 15: Redox potential removal ($mg \cdot L^{1}$) following high (500 $\mu g \cdot L^{-1}$) triclosan exposure.

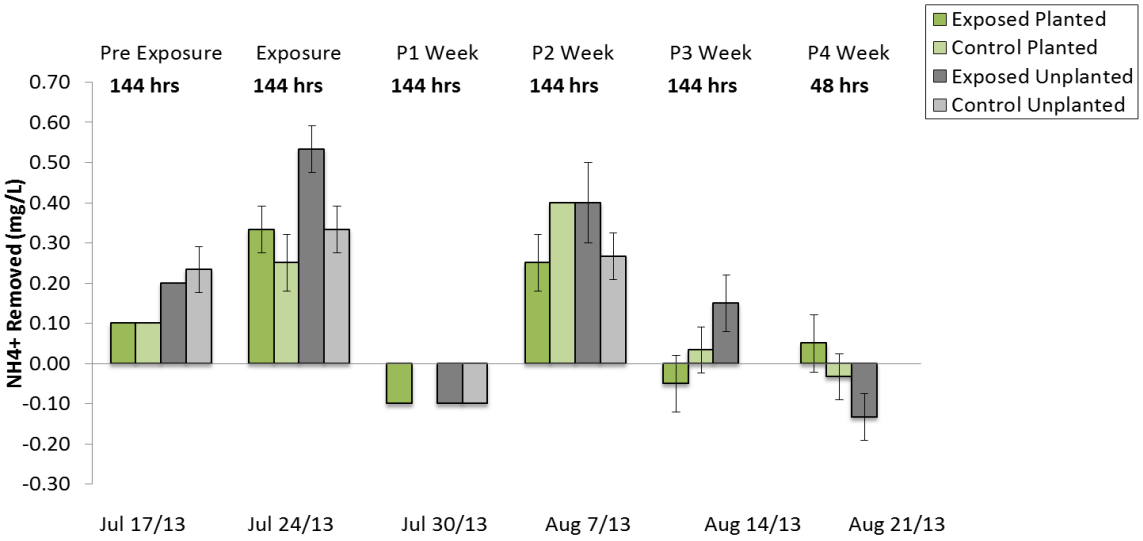

FIGURE 16: Ammonium removal ($mg \cdot L^{1}$) following high ($500\ \mu g \cdot L^{-1}$) triclosan exposure.

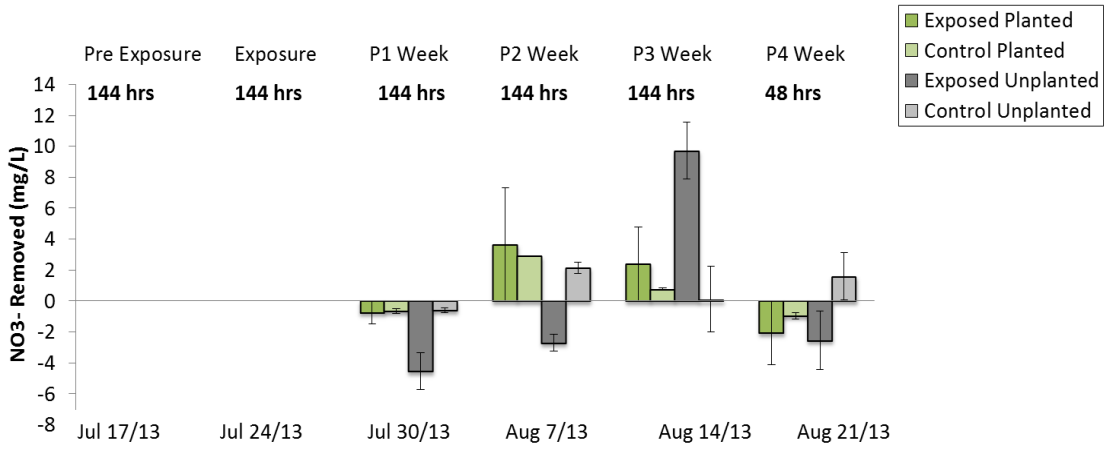

FIGURE 17: Nitrate removal ($mg \cdot L^{1}$) following high ($500\ \mu g \cdot L^{-1}$) triclosan exposure.

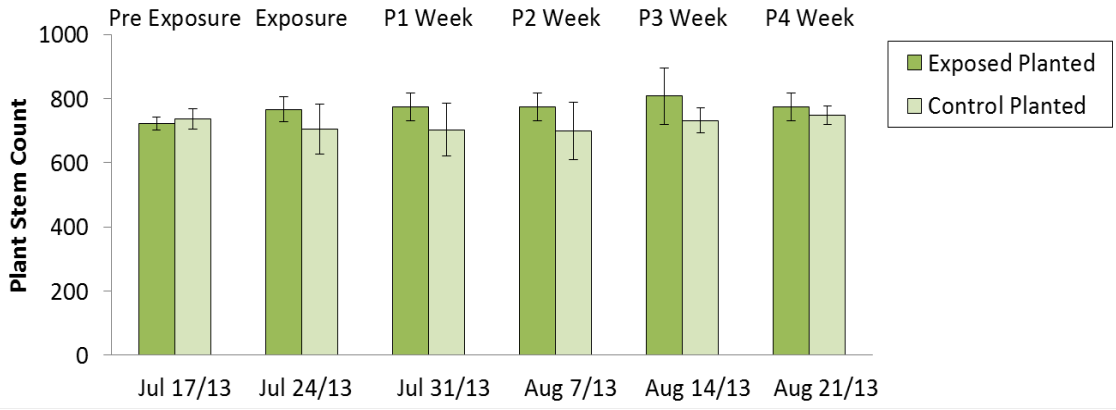

FIGURE 18: Plant stem count following high (500 $\mu g \cdot L^{-1}$) triclosan exposure.

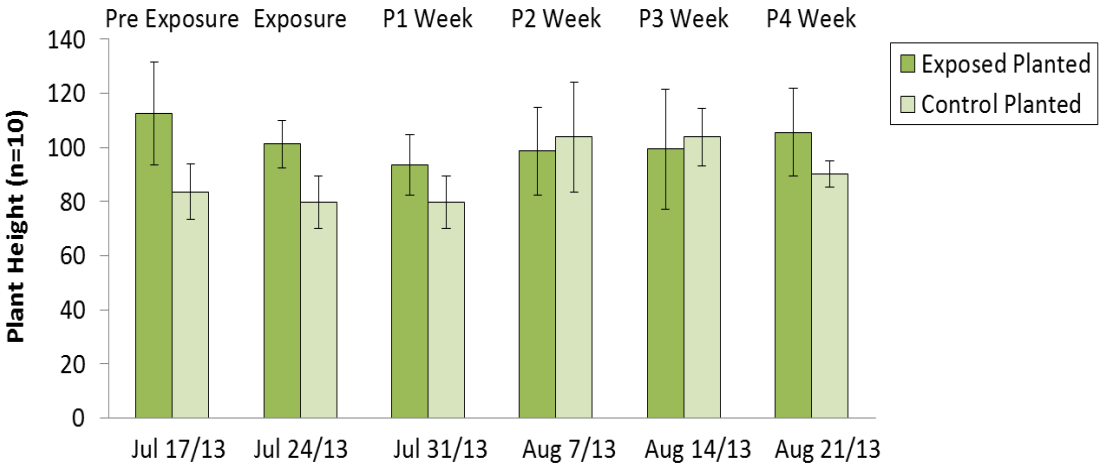

FIGURE 19: Plant height (cm) following high (500 $\mu g \cdot L^{-1}$) triclosan exposure.

Appendix D: Chapter 4 -Low Sulfamethoxazole Exposure

Molar Concentration of low (100 µg·L^{-1}) exposure:

$$= \frac{100\ \mu g}{L} * \frac{mg}{1000\ \mu g} * \frac{g}{1000\ mg} = \frac{0.0001\ g}{L}$$

$$= \frac{0.0001\ g}{L} * \frac{mol}{253.28\ g} = \frac{3.95 * 10^{-7} mol}{L}$$

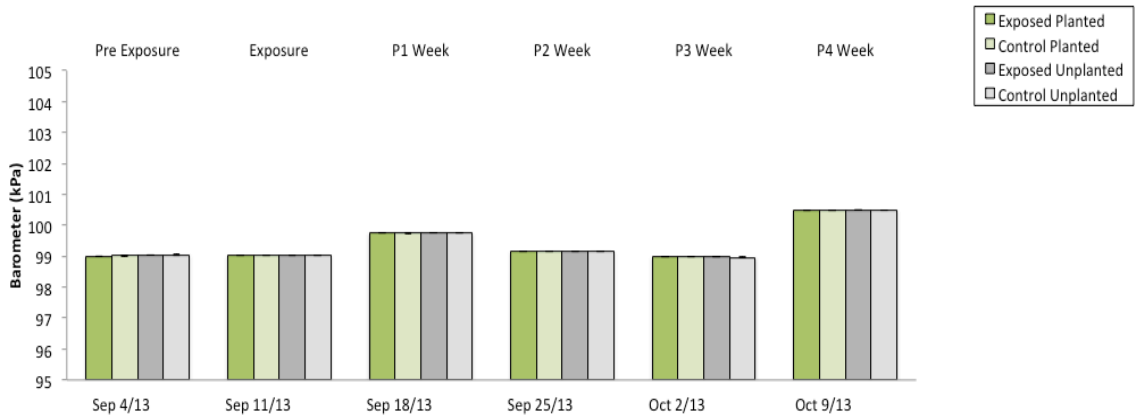

FIGURE 20: Barometer (kPa) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

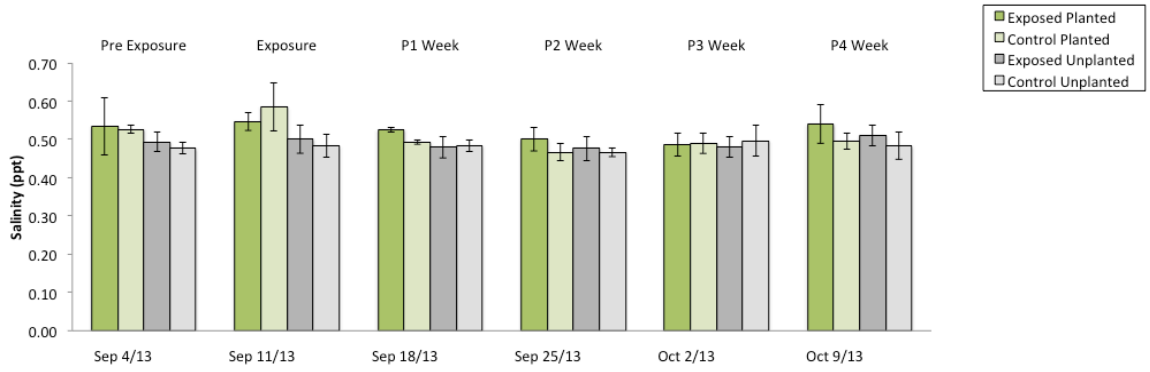

FIGURE 21: Salinity (ppt) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

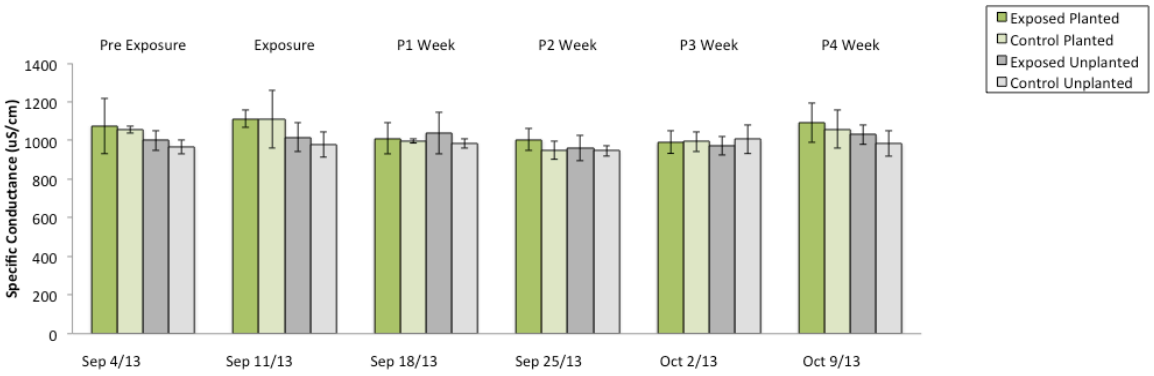

FIGURE 22: Specific Conductivity ($\mu S \cdot cm^{-1}$) following low ($100\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

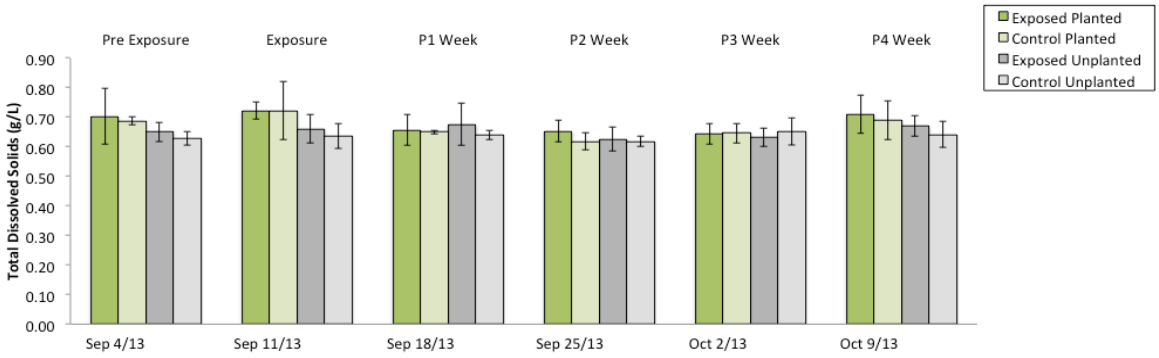

FIGURE 23: Total dissolved solids ($g \cdot L^{-1}$) following low ($100\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

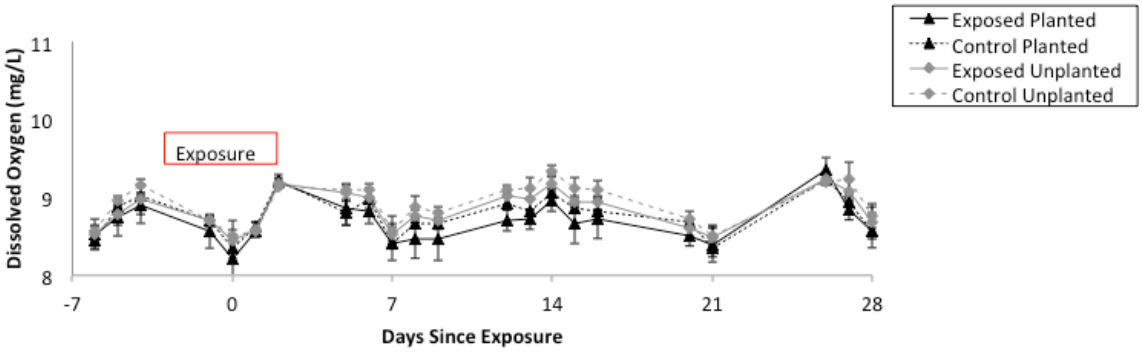

FIGURE 24: Dissolved oxygen removal (mg/L) following low ($100\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

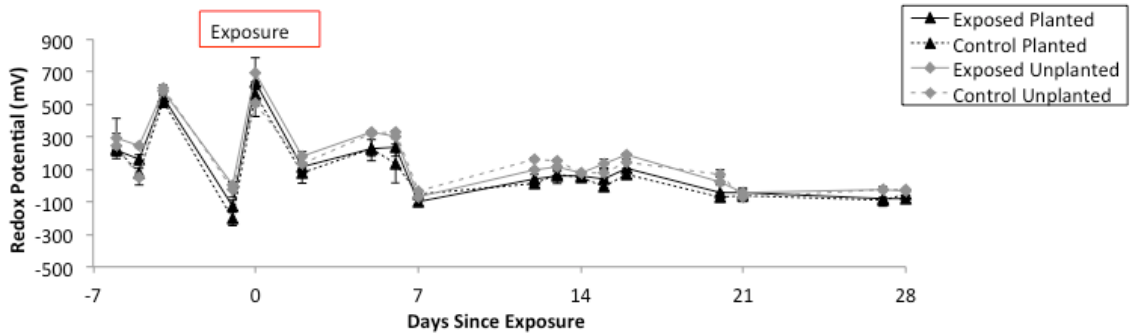

FIGURE 25: Redox potential removal (mg·L^{-1}) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

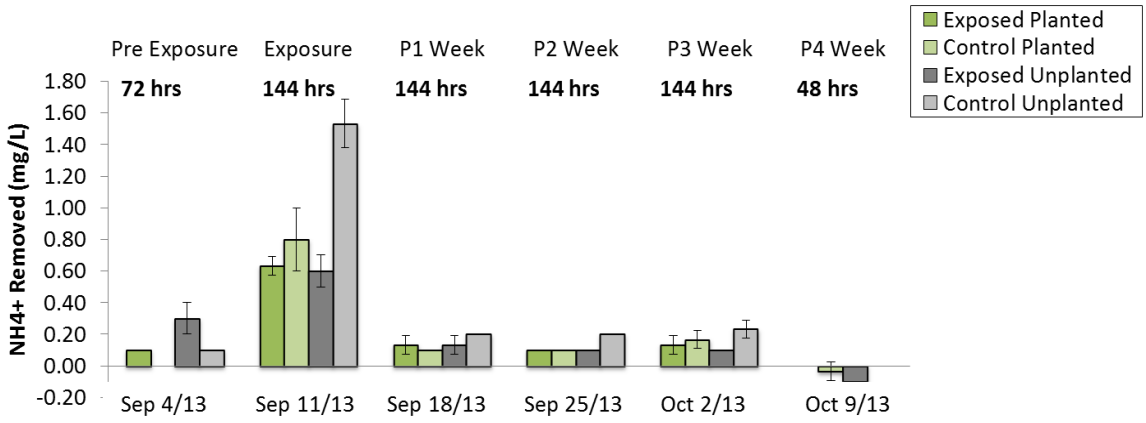

FIGURE 26: Ammonium removal (mg·L^{-1}) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

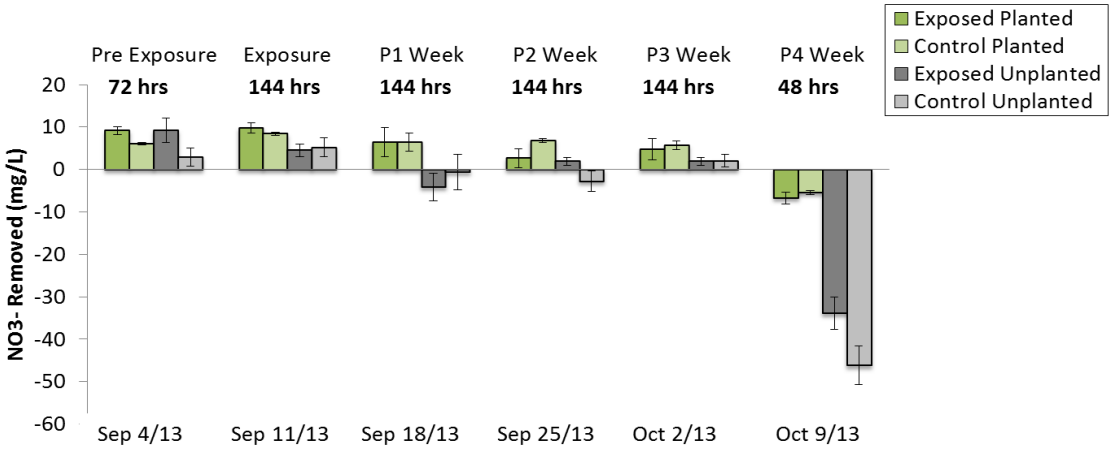

FIGURE 27: Nitrate removal (mg·L^{-1}) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

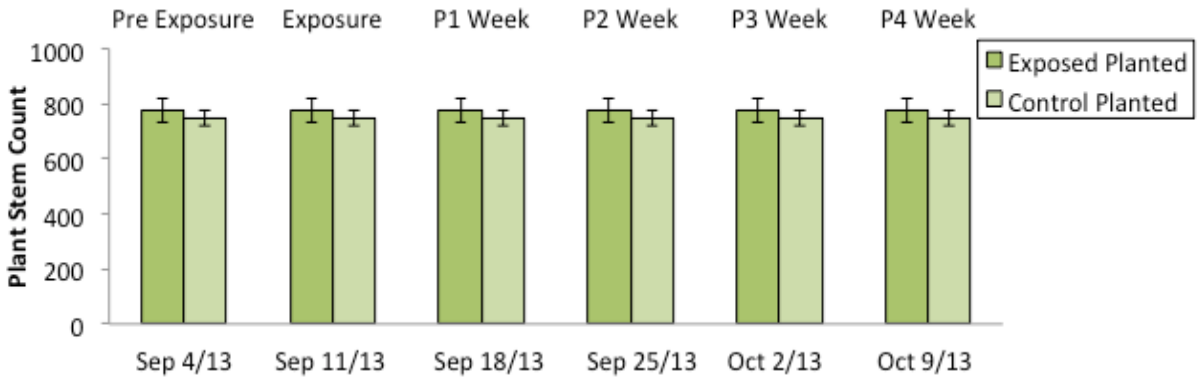

FIGURE 28: Plant stem count following low (100 µg·L^{-1}) sulfamethoxazole exposure.

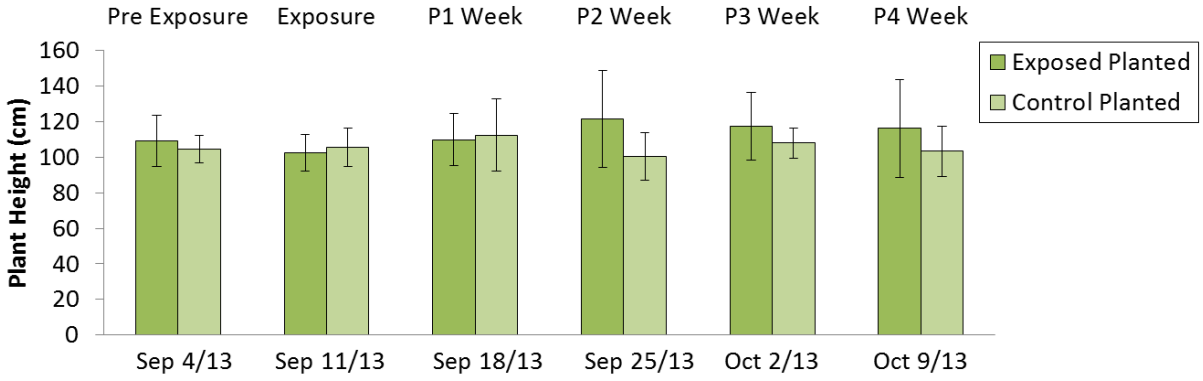

FIGURE 29: Plant height (cm) following low (100 µg·L^{-1}) sulfamethoxazole exposure.

Appendix E: Chapter 4 - High Sulfamethoxazole Exposure

Molar Concentration of high (500 µg·L^{-1}) exposure:

$$\frac{500\ \mu g}{L} * \frac{mg}{1000\ \mu g} * \frac{g}{1000\ mg} = \frac{0.0005\ g}{L}$$

$$= \frac{0.0005\ g}{L} * \frac{mol}{253.28\ g} = \frac{1.97 * 10^{-6} mol}{L}$$

FIGURE 30: Barometer (kPa) following high (500 µg·L^{-1}) sulfamethoxazole exposure.

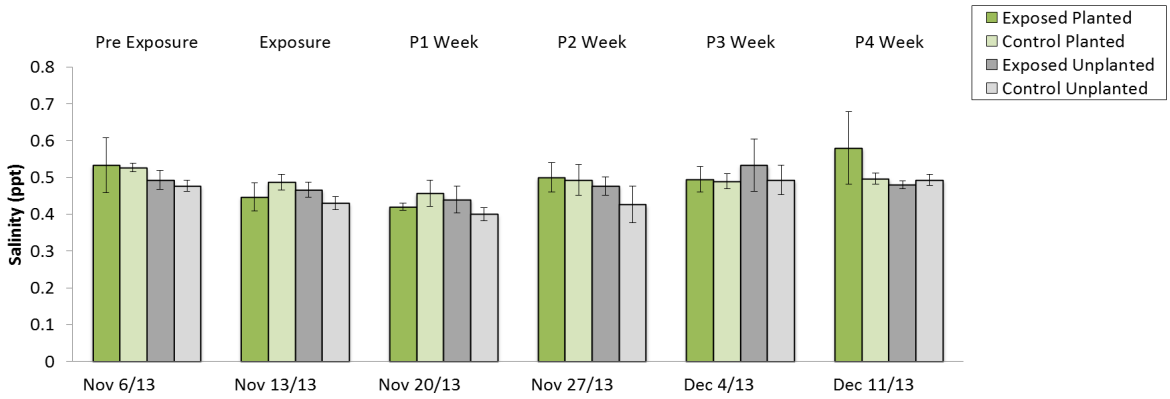

FIGURE 31: Salinity (ppt) following high (500 µg·L^{-1}) sulfamethoxazole exposure.

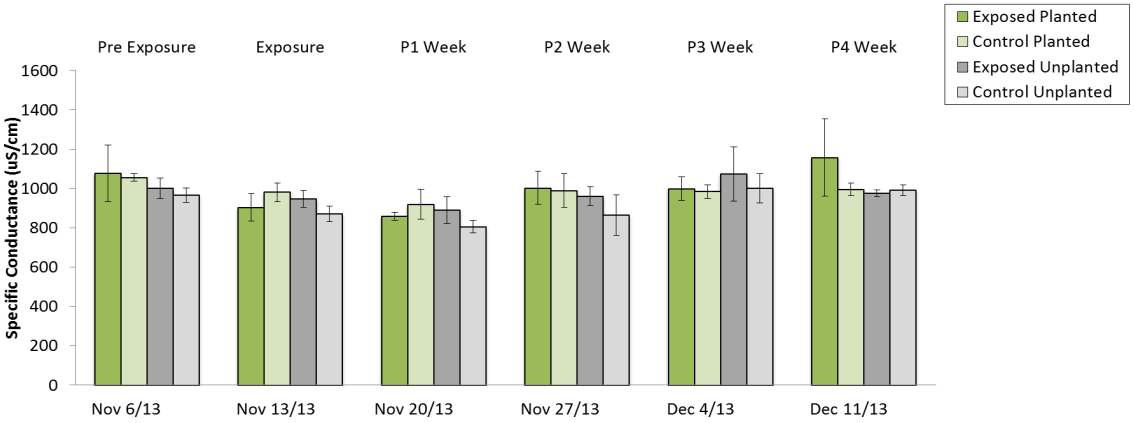

FIGURE 32: Specific conductivity ($\mu S \cdot cm^{-1}$) following high (500 $\mu g \cdot L^{-1}$) sulfamethoxazole exposure.

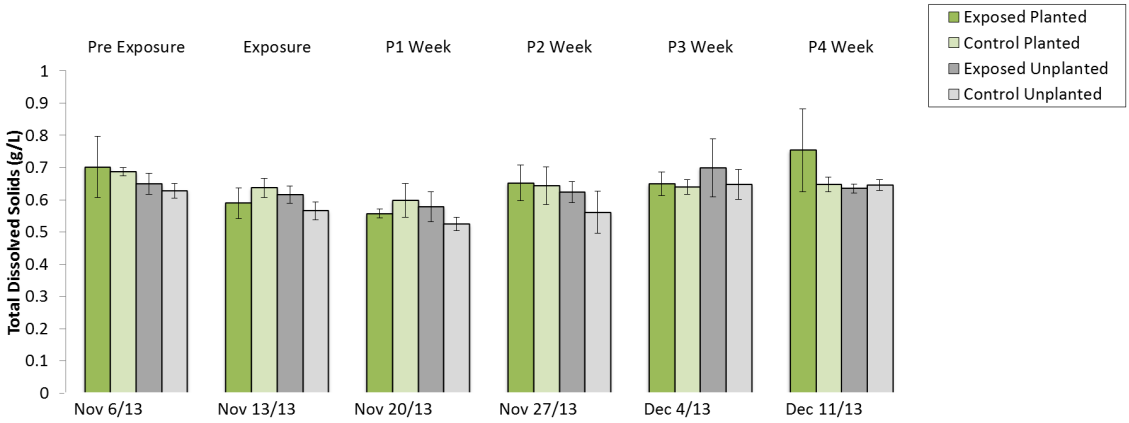

FIGURE 33: Total dissolved solids ($g \cdot L^{-1}$) following high (500 $\mu g \cdot L^{-1}$) sulfamethoxazole exposure.

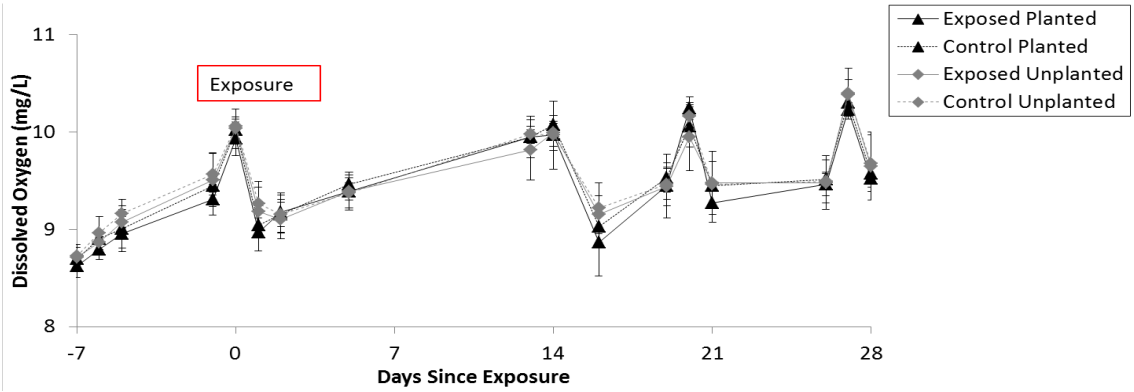

FIGURE 34: Dissolved oxygen removal (mg·L-1) following high (500 µg·L-1) sulfamethoxazole exposure.

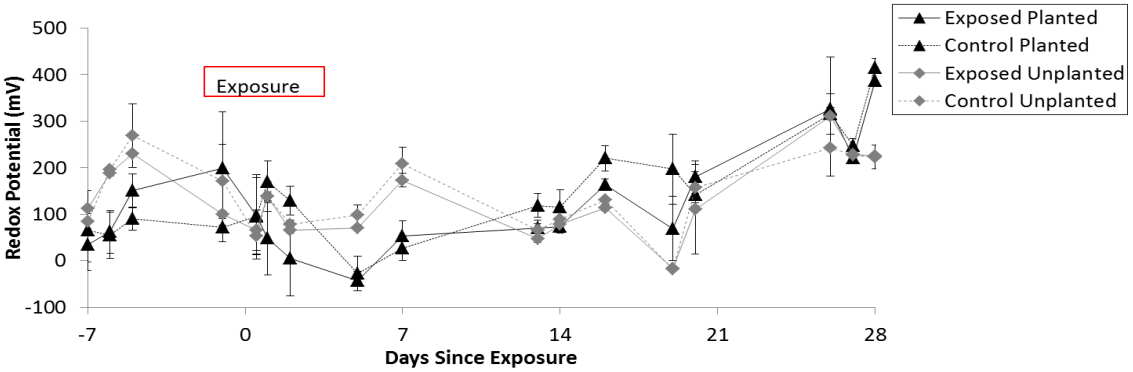

FIGURE 35: Redox potential removal ($mg \cdot L^{-1}$) following high ($500\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

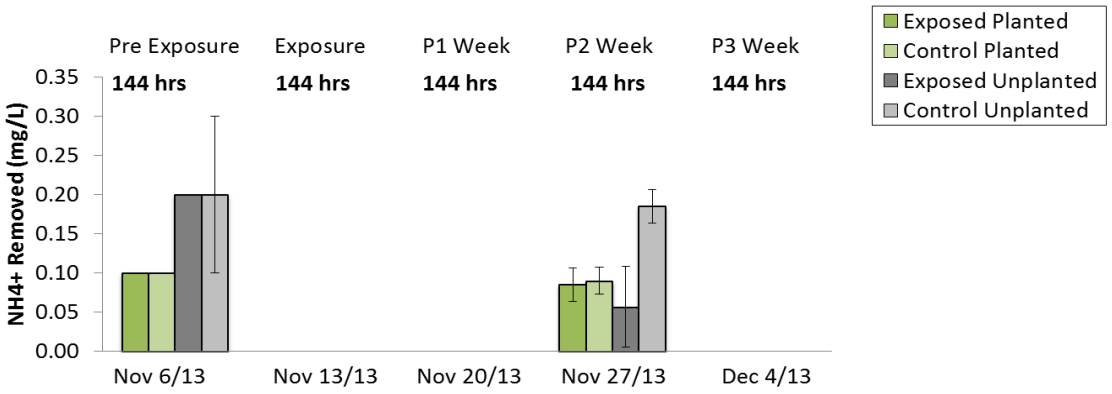

FIGURE 36: Ammonium removal ($mg \cdot L^{-1}$) following high ($500\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

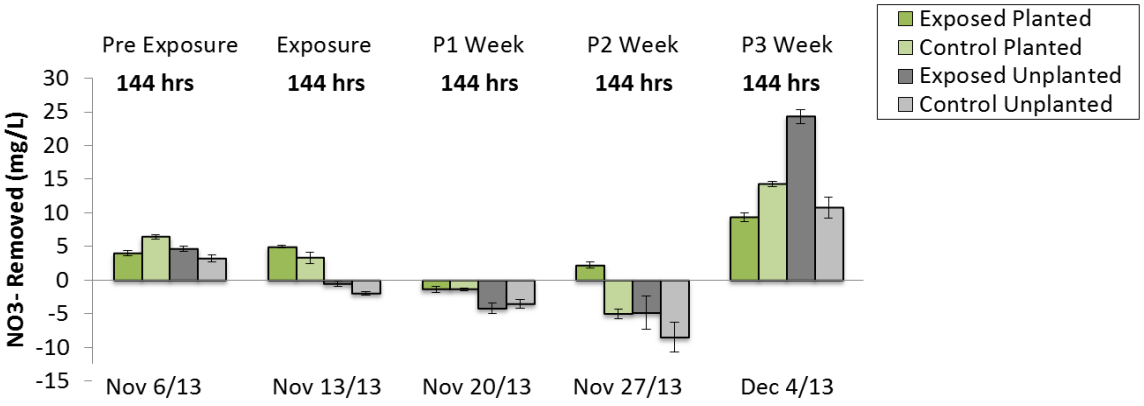

FIGURE 37: Nitrate removal ($mg \cdot L^{-1}$) following high ($500\ \mu g \cdot L^{-1}$) sulfamethoxazole exposure.

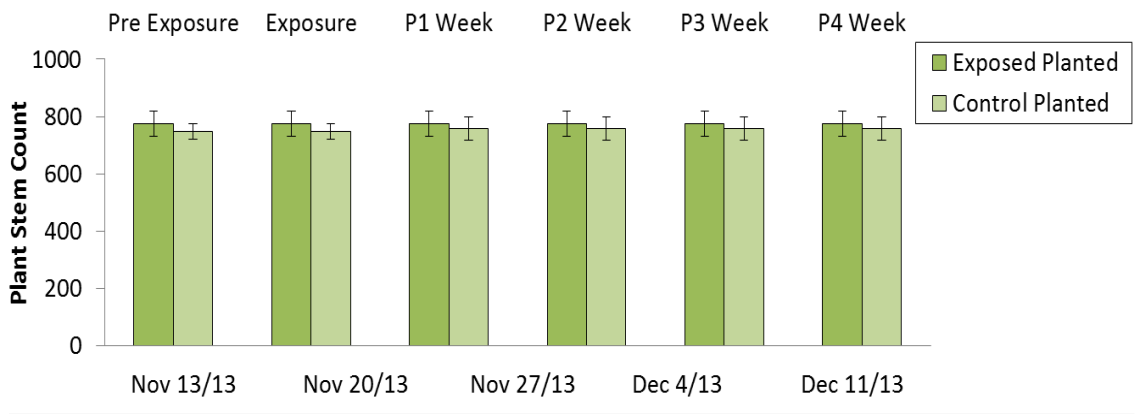

FIGURE 38: Plant stem count following high (500 µg·L^{-1}) sulfamethoxazole exposure.

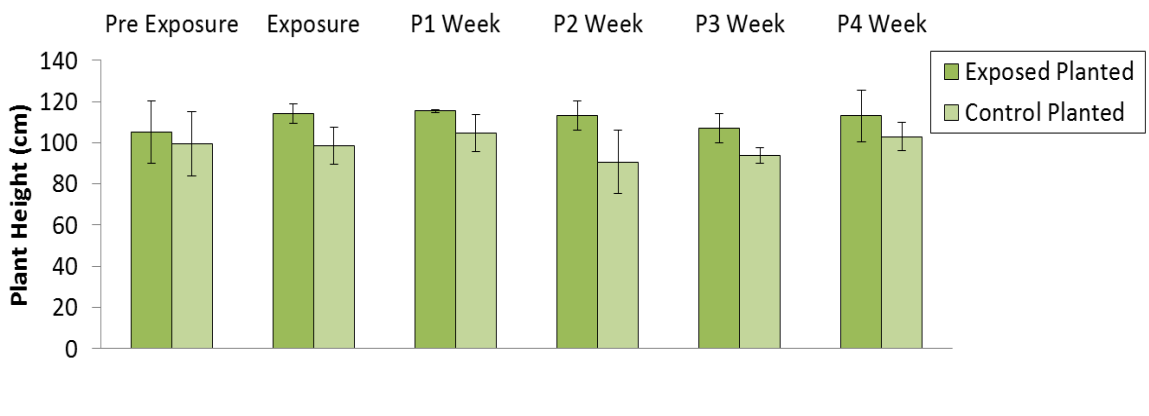

FIGURE 39: Plant height (cm) following high (500 µg·L^{-1}) sulfamethoxazole exposure.