BIOCONCENTRATION OF TRICLOSAN, METHYL-TRICLOSAN, AND TRICLOCARBAN IN THE PLANTS AND SEDIMENTS OF A CONSTRUCTED WETLAND

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Triclosan and triclocarban are antimicrobial compounds added to a variety of consumer products that are commonly detected in waste water effluent. The focus of this study was to determine whether the bioconcentration of these compounds in wetland plants and sediments exhibited species specific and site specific differences by collecting field samples from a constructed wetland in Denton, Texas. The study showed that species-specific differences in bioconcentration exist for triclosan and triclocarban. Site-specific differences in bioconcentration were observed for triclosan and triclocarban in roots tissues and sediments. These results suggest that species selection is important for optimizing the removal of triclosan and triclocarban in constructed wetlands and raises concerns about the long term exposure of wetland ecosystems to these compounds.

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CHAPTER 1

INTRODUCTION

Pharmaceutical and personal care products (PPCPs) are a class of emerging organic pollutants that have received increased attention from the scientific community due to their cosmopolitan distribution in surface waters (Kolpin et al., 2002). The incomplete removal of PPCPs from domestic sewage by conventional waste water treatment methods and subsequent discharge of waste water effluent into streams and rivers has been identified as the primary source of PPCPs contamination (Oulton et al., 2010). Over the last decade, new research suggesting that PPCPs may have adverse impacts on human health and concern over the potential ecotoxicological effects of PPCPs on aquatic ecosystems (Caliman and Gavrilescu, 2009) has prompted water resource managers to identify additional measures for the removal of PPCPs from waste water effluent.

Constructed wetlands have been identified in numerous studies as a cost-effective treatment option for the removal of PPCPs from both untreated (Conkle et al., 2008) and treated domestic waste water effluent (Hijosa-Valsero et al., 2010a; Llorens et al., 2009; Matamoros et al., 2008; Matamoros and Bayona, 2006). Previous studies have focused primarily on the removal efficiencies for different PPCPs using various constructed wetland designs (e.g. surface flow, subsurface flow) and, to a lesser extent, on potential mechanisms (e.g. sorption and photodegradation) related to the removal of different PPCPs. Recent studies have implicated the importance of wetland plants in the removal of PPCPs (Dordio et al., 2009; Dordio et al., 2010; Hijosa-Valsero et al., 2010b; Zhang et al., 2011) and the ability of wetland plants to bioaccumulate PPCPs (Park et

al., 2009) in constructed wetlands; however, there is little information concerning the species specific ability of wetland plants to bioconcentrate PPCPs. Since species selection is widely recognized as an important factor contributing to efficiency of constructed wetlands for the removal of pollutants (Brisson and Chazarenc, 2009) and the selection of appropriate phytoremediation strategies (Dhir et al., 2009), research on the ability of wetland plants to bioconcentrate PPCPs is needed to enhance the design of constructed wetlands for PPCPs attenuation.

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol; TCS) and triclocarban (3,4,4'-trichlorocarbanillide; TCC) are commercial bactericides added to a wide variety of commercial products and are among the most commonly detected PPCPs in surface waters (Halden and Paull, 2005; Kolpin et al., 2002) and biosolids (McClellan and Halden, 2010). Both TCS and TCC are lipophilic (Log *Kow* 4.8 and 4.9, respectively; Coogan et al., 2007), environmentally persistent (Ying et al., 2007), and readily bioaccumulate in aquatic organisms (Chalew and Halden, 2009; Coogan and La Point, 2008; Coogan et al., 2007). Due to their ubiquity, TCS and TCC have been suggested as general indicators of the distribution of Waste Water Treatment Plant (WWTP) contaminants with similar hydrophobicity and persistence in aquatic environments (Coogan et al., 2007). Detailed information on the occurrence of TCS and TCC in constructed or natural wetland environments is limited. Recent studies have documented the successful use of constructed wetlands to treat TCS in waste water effluent with removal efficiencies ranging from 60-100% (Park et al., 2009; Waltman et al., 2006). Furthermore, mesocosm and laboratory studies have documented the bioconcentration of TCS and TCC in aquatic plants (Adhikari, 2010; Stevens et al.,

2009) and demonstrated the inhibition of aquatic plant growth from TCS exposure (Stevens et al., 2009); however, these findings have yet to be confirmed in plants grown under field conditions. In contrast to the controlled environment of laboratory and mesocosm studies, the complexity of environmental factors in field studies, such as microbial activity and chronic exposure to contaminants in the water column, could influence the bioconcentration of TCS and TCC by aquatic plants. Given the wide distribution of TCS and TCC in the environment and the aforementioned examples of both bioconcentration and removal using constructed wetlands, TCS and TCC are optimal candidates for the study of the bioconcentration of PPCPs in constructed wetlands.

To address the paucity of information concerning the ability of constructed wetland macrophytes to bioconcentrate PPCPs, I conducted a study to examine the bioconcentration patterns of TCS and TCC in wetland plants from a pilot-scale, surface flow constructed wetland located at the City of Denton's Pecan Creek WWTP. Additionally, analysis of the triclosan metabolite methyl-triclosan (5-chloro-2-[2,4-dichlorophenoxy]; MTCS) was also included due to evidence of its bioconcentration in wetland plants (Adhikari, 2010; Stevens et al., 2009). The three main objectives of the study were the assessment of: 1) bioconcentration patterns among different wetland plant species, 2) analyte concentration patterns in plants and sediments at different locations in the wetland, and 3) the relationship between analyte concentrations in tissues and sediments. The justification, practical implications, and hypothesized outcomes for each objective are described below in further detail.

1.1 Bioconcentration Patterns among Different Species

Recent laboratory and mesocosm studies have indicated that the bioconcentration of TCS, MTCS, and TCC in wetland plants varies among species and within species tissues (i.e. roots and shoots) (Adhikari, 2010; Stevens et al., 2009). However, in both of these previous studies experiments were performed in laboratory and mesocosm studies using seedlings and potted plants, respectively. Under field conditions, the potential of different wetland plant species to bioconcentrate TCS, MTCS, and TCC may differ due to environmental factors, such as microbial activity, soil type, and species competition. Furthermore, wetland plants growing under field conditions may have a greater potential for the bioconcentration of target compounds due to longer exposure times. In order to determine the potential bioconcentration of TCS, MTCS, and TCC in different species growing under field conditions, I collected and analyzed the roots and shoots of three different wetland plants species from an operational constructed wetland. The practical implications of this research include: 1) the verification of species contaminant bioconcentration patterns under field settings and 2) the identification of potential “hyper-accumulator” species for the removal of the target contaminants. I hypothesized that the bioconcentration of TCS, MTCS, and TCC in the roots of different species would be greater than shoots. Based on the findings of Adhikari (2010), I also hypothesized that the bioconcentration of TCS, MTCS, and TCC by *P. cordata* would be greater than *S. graminea*.

1.2 Analyte Concentration Patterns in Plants and Sediment at Different Locations

The ability of constructed wetlands to effectively treat organic contaminants, such

as PPCPs, is generally related to the multiple destructive (e.g. phyto- and microbial degradation) and non-destructive (e.g. sorption, volatilization, plant uptake) processes that are simultaneously ongoing in constructed wetlands systems (Imfeld et al., 2009). Although the contribution of individual processes to overall treatment efficacy is hard to quantify, longer exposure of the pollutant load to the constructed wetland environment (i.e. hydraulic retention time) has been associated with increased removal efficiencies (Matamoros et al., 2008). Thus, the exposure concentrations and, by extension, the amount of contaminants available for bioconcentration in wetland plants and sediments would also be expected to decrease as the plug of effluent water travels through the constructed wetland. As part of my study, I conducted an experiment to test the theory that wetland plants and sediments are exposed to progressively lower concentrations of the target contaminants by comparing the bioconcentration of TCS, MTCS, and TCC in plant tissues and sediments at different locations within an operational constructed wetland. The practical implications of this research include 1) the documentation constructed wetland PPCPs removal efficiency via bioconcentration patterns in wetland plants tissues and sediments. I hypothesized that analyte concentrations in plant tissues and sediments at the wetland inflow would be greater than the wetland outflow.

1.3 Relationship between Analyte Concentrations in Tissues and Sediments

Existing models concerning the ability of plants to uptake organic contaminants is limited for compounds with high octanol-water partition coefficients (Log *Kow*), such as TCS, MTCS, and TCC (Briggs et al., 1982). Furthermore, fugacity models concerning the fate of TCS and TCC in the environment indicate these compounds are likely to

show a strong sorption capacity in anaerobic sediments and resist biodegradation (Ying et al., 2007). Terrestrial studies of TCS bioconcentration in plants indicate that uptake from the soil does occur (Wu et al., 2010); however, this has not been verified for wetland plants growing in anaerobic soils. The original plant uptake model described by Briggs et al. (1982) does not allow for the prediction of plant tissue concentrations of hydrophobic contaminants from sediment concentrations. As part of my study, I compared the bioconcentration of wetland plant tissues to sediment concentrations in an operational constructed wetland to determine if the uptake of TCS, MTCS, and TCC by wetland plants is related to sediment concentrations. The practical implications of the research include: 1) the determination of the influence of sediment concentration on TCS, MTCS, and TCC bioconcentration patterns in wetland plants, 2) the confirmation of the adherence of TCS, MTCS, and TCC bioconcentration patterns to existing models of plant uptake based on Log *Kow*, and 3) an increased knowledge on the ability of wetland plants to be used for the removal of TCS, MTCS, and TCC from anaerobic soils. I hypothesized that the bioconcentration of target analytes in plant tissues would be positively related to sediment concentrations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study Area

The study area is a pilot-scale, surface flow constructed wetland located at the Pecan Creek WWTP in Denton, Texas. The Pecan Creek WWTP uses conventional activated sludge treatment followed by ultraviolet light disinfection. The WWTP design capacity is 21 million gallon per day (MGD), however, the plant typically treats approximately 18 MGD (David Hunter, personal communication). The wetland was constructed in 1992 with a design treatment capacity of 1% (approximately 180,000 gallons) of the total daily effluent produced by the plant (David Hunter, personal communication). The wetland has four sites separated by three earthen berms and encompasses a total area of approximately 0.21 hectare (46 m x 46 m). The average storage volume of the wetland is estimated at 570,000 L with an average inflow rate of 2,968 L/h and an average retention time of 4.3 days (Hemming et al., 2001).

Wetland sites are dominated by a mixture of herbaceous emergent, submergent, and floating aquatic plant species. The first site at the wetland inflow contains the greatest diversity of species including broadleaf cattail (*Typha latifolia*), pickerelweed (*Pontederia cordata*), grassy arrowhead (*Sagittaria graminea*), switchgrass (*Paspalum spp*.), pondweed (*Potamageton spp*.), duckweed (*Lemna spp.*), coontail (*Ceratophyllum demersum*), buttercup (*Ranunculus spp*.), and azolla fern (*Azolla caroliniana*) (personal observation). The remaining three sites are dominated by *T. latifolia*.

The substrate in the constructed wetland is classified as a loam soil. Soil texture percentages range from 41.0 – 38.2 sand, 43.8 – 47.2 silt, and 14.6 – 17.3 clay. Total

organic carbon in the wetland ranges from 2.5 – 6.0 µg/mg dry weight (Frederick Zarate, unpublished data).

With the exception of operational maintenance (e.g. removal of accumulated debris from the outflow, repair of the effluent pump, and mechanized clearing of nuisance vegetation) the wetland has been in continuous operation since its construction. Previous studies have documented the capacity of the wetland to remove a variety of PPCPs, as well as the insecticide, diazinon (Baerenklau, 1996; Brooks et al., 2011; Hemming et al., 2001; Waltman et al., 2006). Fig. 1 below depicts the location of the Pecan Creek WWTP and the constructed wetland.

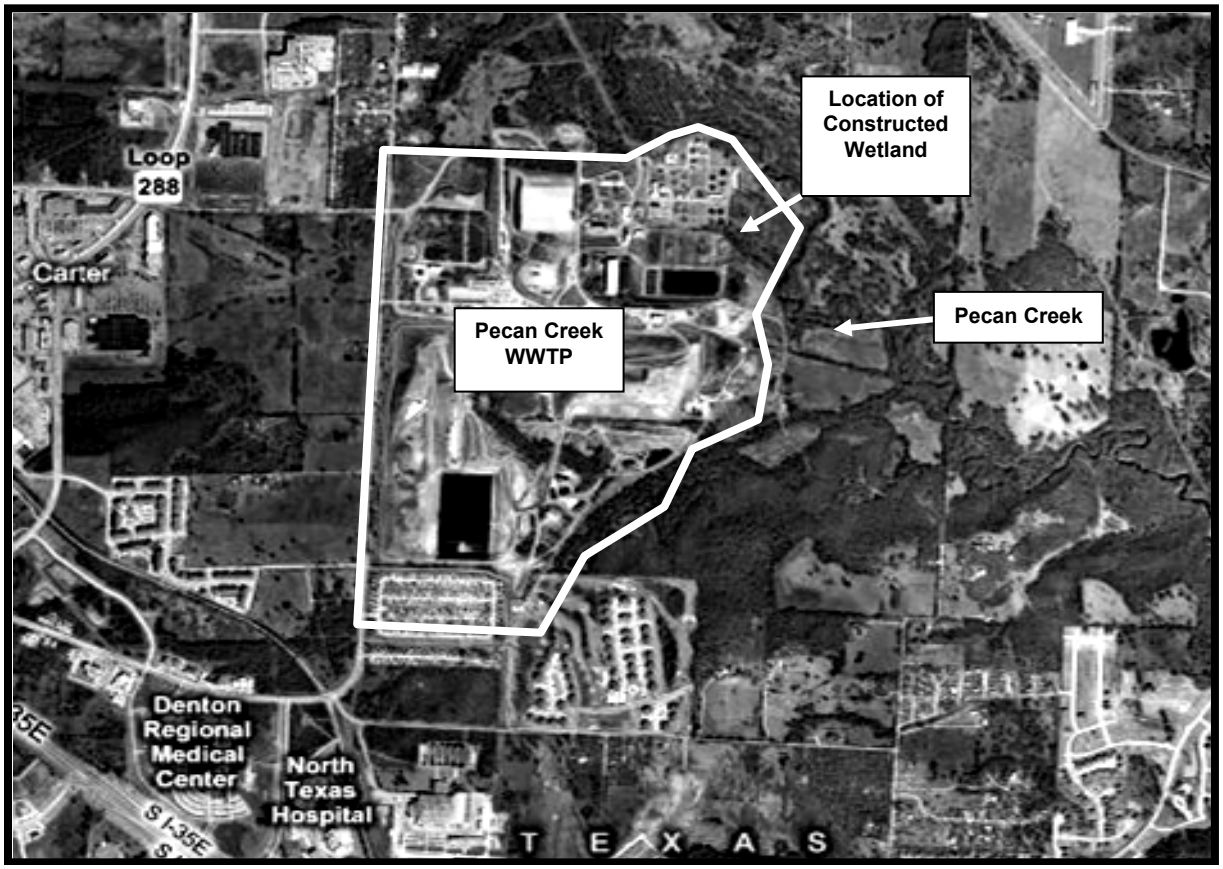

Fig. 1. Location map of the constructed wetland at the Pecan Creek Waste Water Treatment Plant in Denton, Texas.

2.2 Plants Studied

The three wetland plants species collected for the analysis of TCS, MTCS, and TCC tissue concentrations were broadleaf cattail (*Typha latifolia)*, pickerelweed (*Pontederia cordata*), and grassy arrowhead (*Sagittaria graminea*) (Fig. 2). All three species are classified functionally as emergent plants, which grow with basal and root portions of the plant submerged beneath water, while aerial and reproductive portions of the plant remain above the water surface. These species were selected based on their relative abundance within the constructed wetland and their widespread use in constructed wetland treatment systems.

Fig. 2. Site photographs of the three emergent species of wetland plants selected for study: pickerelweed (*P. cordata*), broadleaf cattail (*T. latifolia*), and grassy arrowhead (*S. graminea*).

2.3 Sampling

Collection sites were located at the inflow (Site 1), outflow (Site 3), and a third site (Site 2) representing the midpoint of the flow path between the inflow and outflow.

Each site consisted of a rectangular plot measuring 3.1m x 6.1m (10ft x 20ft) oriented parallel to the flow of water. Dominate flow patterns, inflow, outflow, and sampling sites are presented in Fig. 3.

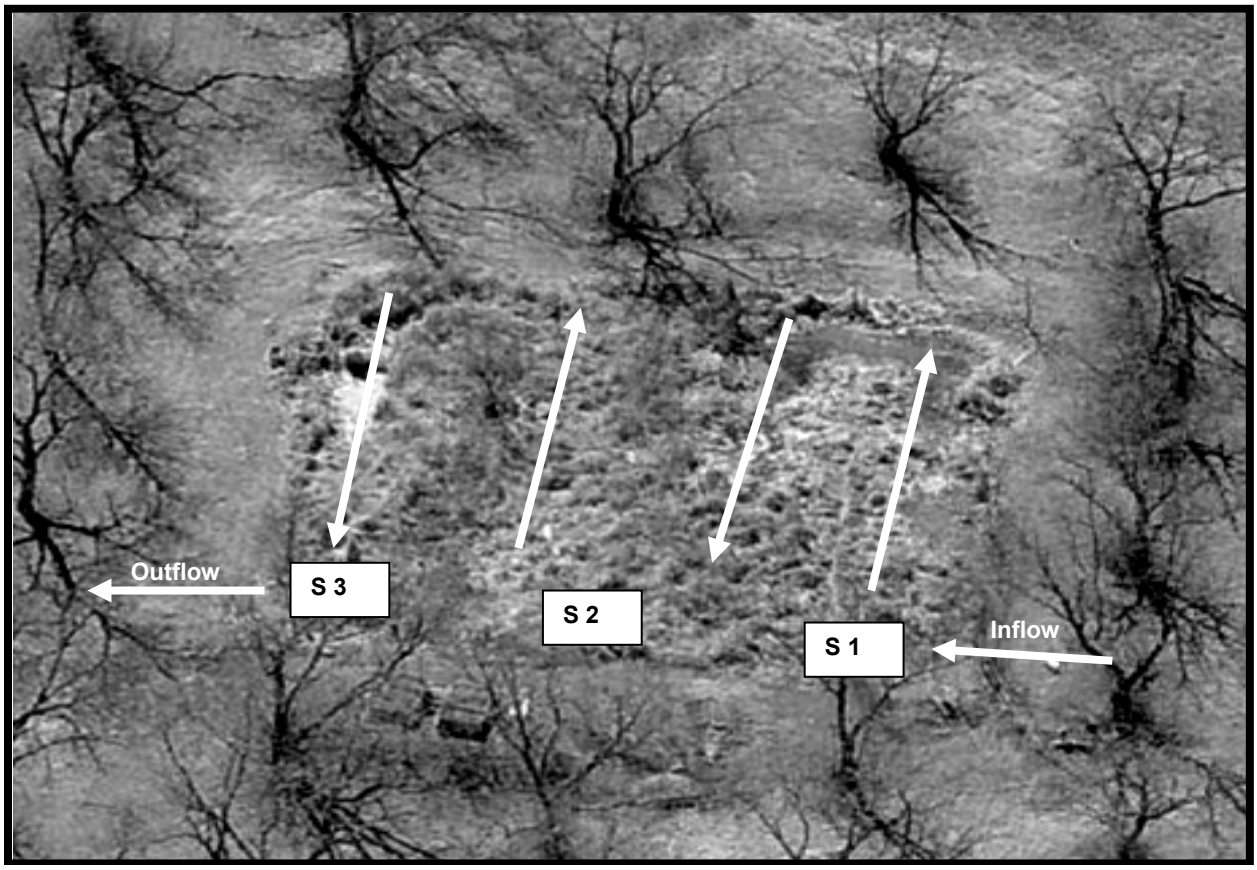

Fig. 3. Flow patterns and locations of the inflow, outflow, and sampling sites (S) in the Pecan Creek Waste Water Treatment Plant constructed wetland.

Sampling was conducted in the spring of 2010. To compare the bioconcentration patterns among species and within species tissues, two individuals of each species were obtained from five locations at Site 1. Additionally, to compare bioconcentration patterns in plant tissues across sites, two individuals of *T. latifolia* were obtained from five locations at Site 2 and Site 3. Individual specimens were uprooted by hand with care given to ensure the complete collection of the root system. The entire plant was then wrapped in aluminum foil for transport to the laboratory.

To compare sediment concentration patterns and to examine the relationship between sediment and tissue concentrations across sites, sediment samples were collected from five locations at each site. Samples were collected by inverting 50 ml polypropylene sample tubes into the sediment to a depth of approximately 7.6 cm (3 in) near the roots of the collected *T. latifolia* specimens. Sample tubes were capped and transported back to the laboratory for analysis.

2.4 Division of Tissues

In the laboratory, individual plants were rinsed with tap water to remove soil particles and epiphyton attached to plant surfaces. After rinsing, plants were separated into root and shoot tissues. To ensure adequate tissue mass for analytical procedures, roots and shoots from the two individuals collected from each of the five sampling locations within each plot were pooled. Only live tissues were used for the analysis. Root tissues included both fibrous and tap roots, while shoots included both leaves and stems; rhizomatous tissues were excluded from the analysis. Separated tissues from each pooled sample and sediment sample were stored until preparation for extraction and analysis.

2.5 Chemicals

Labeled internal standards $^{13}C_{12}$ TCS, $^{13}C_{12}$ MTCS, native TCS and MTCS were obtained from Wellington Laboratories (Guelph, ON, Canada). The dueterated TCC (d_7TCC) internal standard was obtained from Cambridge Laboratories and native TCC was obtained from Absolute Standards (Hamden, CT, USA). Hexane, ethyl acetate,

chloroform, acetonitrile, and N-Methyl-N-(trimethylsilyl) trifluoroacetamide were from Fisher Scientific (Houston, TX, USA).

2.6 Tissue Sample Preparation and Extraction

Tissue sample preparation and extraction procedures described here were modified from Stevens et al. (2009). Each pooled sample was finely macerated using a stainless steel razorblade, blotted dry using a disposable cloth, and homogenized. Shoot and root tissues, approximately 500 mg, were randomly sub-sampled from individual composite samples and added to 50 ml polypropylene vials with 7.5 ml of MilliQ water. Fresh tissue weights were recorded to the nearest 0.1 mg. Ten microliters of each internal standard was added to each sample vial at the following concentrations: 5 ng/µL $^{13}C_{12}$ TCS, 5 ng/µL $^{13}C_{12}$ MTCS, and 1 ng/µL d_7 TCC. Plant tissues were mechanically homogenized using a handheld Fisher Tissuemiser (Fisher Scientific, USA). Tissues were extracted with 30 ml of 1:1 hexane: ethyl acetate, vortexed for 2 min, and centrifuged for 12 min at 3,000 rpm. The supernatant was transferred to 30 ml glass vials and evaporated using a RapidVapTM nitrogen evaporator (Labconco, Kansas City, MO, USA). To ensure adequate extraction, the process was repeated a second time, extracts were combined, evaporated and transferred to pre-weighed 2 ml amber vials with 2 ml of 1:1 hexane ethyl: acetate and evaporated to dryness with N_2 gas. Extracts were weighed to the nearest 0.1 mg to estimate lipid content.

2.7 Sediment Sample Preparation and Extraction

Sediment sample preparation and extraction procedures were modified from

those used by Stevens et al. (2009) for tissues. Sediment sample vials were centrifuged at 3,000 rpm for 12 min and the supernatant from each soil sample, as well as large pieces of organic matter, were discarded. A sediment subsample (approximately 500 mg) from each vial was transferred into 4 ml polypropylene Biospec (Bartlesville, OK, USA) vials. Two milliliters of 1:1 hexane: ethyl acetate was added to each vial along with internal standard additions as described above. A mix of 2.5 mm and 1 mm glass beads sufficient to fill approximately one fourth of the vial was added to each sample. Vials were sealed and placed in a Biospec Mini Bead Beater machine for 3 min. The contents of polypropylene vials were syringe filtered (0.45 µm pore size) into pre-weighed 4 ml glass vials and evaporated to dryness using N_2 gas.

2.8 Lipid Cleanup in Extracted Samples

The lipid cleanup procedures described here are modified from those reported by Stevens et al. (2009). Evaporated extracts, both soils and tissues, were reconstituted with 200 µl of 1:1 hexane: ethyl acetate and transferred to 1.5 ml micro centrifuge tubes. Samples were evaporated using N_2 gas and reconstituted with 1 ml acetonitrile. Samples were then placed in a freezer at - 80° C for 10 min and centrifuged immediately at 14,000 rpm for 30 sec to facilitate the coagulation of lipids. The supernatant of each sample was transferred to 2 ml amber vials, evaporated to dryness using N_2 gas, and reconstituted to a final volume of 100 µl with acetonitrile.

From the 100 µl final volume, 20 µl of each extracted sample was transferred into a 100 µl conical bottom insert for TCC analysis with LC-MS/MS (see below). The remaining 80 µl of each extracted sample was evaporated to dryness using N_2 gas and

reconstituted with 50 µl of MSTFA and 50 µl of acetonitrile. Samples were derivatized for 2 h at 60°C. After derivatization, each sample was evaporated to dryness using N_2 gas and reconstituted to a total volume of 80 µl with 70 µl of dichloromethane and 10 µl of MSTFA and transferred to a 200 µl flat-bottom insert for TCS and MTCS analysis by GC-MS.

2.9 Quality Control

Quality control measures included the analysis of method blanks, spike blanks, and matrix spikes for each matrix sampled (i.e. sediments, roots, and shoots). Three replicates of each quality control sample were included for both root and shoot tissues, while only a single replicate was included for soil samples. Spikes, method blanks, and matrix spikes were amended with internal standards as described above as well as10 µl of target analytes at the following concentrations: TCS 5 µg/ml, MTCS 5 µg/ml, and TCC 1 µg/ml.

The method detection limit (MDL) for clean root and shoot samples was determined in a previous study using similar tissue preparation steps as those outlined above (Adhikari, 2010). The MDL study included seven replicate matrix spikes for both root and shoot tissues and was estimated as the standard deviation x 3.14 (standard methods). Because sediments and roots lack chlorophyll, the MDL for root tissue was applied to sediments. All of the samples in Adhikari (2010) were spiked with 10 µl of 1µg/ml $^{13}C_{12}$ TCS, $^{13}C_{12}$ MTCS, and d_7 TCC internal standard and 10µl of 1 µg/ml native TCS, MTCS, and TCC compounds. Method detection limits are presented in Table 1 below.

Table 1
Method detection limits (MDL) for triclosan (TCS), methyl-triclosan (MTCS), and triclocarban (TCC) rounded to the nearest whole number from Adhikari (2010). Spike additions were at 20 ng/g.

Analyte	Tissue	Matrix Spike Recovery (%, n=7)	MDL (ng/g, n=7)	Relative Standard Deviation (%)
TCS	shoot	138	17	7 (n=8)
MTCS	shoot	140	4	20 (n=8)
TCC	shoot	93	9	13 (n=7)
TCS	root	62	6	14 (n=6)
MTCS	root	119	6	4 (n=6)
TCC	root	124	11	11 (n=8)

Reference plant material used for matrix spikes was from *S. graminea* and was provided by Joe Snow Aquatic Plants, Inc., Denton, TX, USA. Reference materials were not certified contaminant free; however, prior to their use, reference materials were tested for concentrations of TCS, MTCS, and TCC and were determined to have tissue concentrations below the established MDL.

2.10 Instrumental Analysis

The instrumental analysis of TCS and MTCS in plant tissues and soils was conducted by isotope dilution gas chromatography-mass spectroscopy using methodology previously published (Coogan and La Point, 2008; Coogan et al., 2007; Stevens et al., 2009). TCS and MTCS analyses were conducted on an Agilent 6890 GC (Palo Alto, CA, USA) coupled with a 5973 mass selective detector MS (70-eV). GC operating conditions were helium carrier gas at 480 hpa, inlet temperature at 260 ^{0}C

and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d, 0.25 µm film). The starting temperature of the oven was 40 ^{0}C with 1 min hold followed by subsequent ramps; ramp 1 (0 min-hold, 50 ^{0}C /min, 220 ^{0}C), ramp 2 (0 min- 5 ^{0}C /min, 285 ^{0}C), ramp 3 (16 min-hold, 10 ^{0}C /min, 300 ^{0}C). Injection volume was 2 µl, pulsed pressure 25 psi and pulsed splitless mode.

TCC analyses were conducted by the LC-ESI-MS method using electrospray liquid chromatography MS/MS (Coogan et al., 2007) using an Agilent 1100 LC/MS system with a Model SL ion trap (Palo Alto, CA, USA). The column is C18 (monomeric, non-endcapped), Zorbax with 5 µm particle size and 80 Å pore size. A two microliter sample was autoinjected with a gradient program 300 µl/min (70% mobile phase B and 30% mobile phase A). Mobile phase B constitutes 95% acetonitrile and 5% water with 5 mM ammonium acetate, while mobile phase A includes 95% water and 5% acetonitrile with 5 mM ammonium acetate. The ion trap was operated in negative ion multireaction monitoring mode (MRM) isolating m/z 313-315 for native TCC and m/z 320-322 for d_7 TCC internal standard. These isolated pseudomolecular ions ($[M-H]^-$) were fragmented (amplitude 0.8) to yield daughter ions at m/z 160 and 163 for native TCC and d_7 TCC, respectively (Coogan et al., 2007). Five point standard curves were established for both the pseudo-molecular ions and the daughter ions with TCC concentrations from 16 – 1000 pg/µl and d_7 concentrations of 100 pg/µl.

2.11 Data Analyses

Two - way ANOVA was used to compare analyte concentrations in tissues (roots and shoots) among different species at Site 1and across sampling sites for *T. latifolia*.

One - way ANOVA was used to compare analyte concentrations in sediments across samplings sites. Multiple comparisons for the separation of means were analyzed using contrast statements in SAS (SAS Institute, Cary, NC, USA). Because multiple comparisons were determined *a priori* in the present study, contrast statements eliminate the potential overcompensation for Type I error introduced by standard multiple comparison tests. Differences between means were considered significant if $p < 0.05$.

Pearson correlation was used to determine if concentrations of the target analytes in the tissues of *T. latifolia* and sediments were significantly correlated. Bioconcentration factors (BCFs) were calculated as the ratio of the concentrations of target analytes in tissues to sediments and effluent water.

All statistical tests were conducted using SAS® software, Version 9.2. A value equal to the MDL was assigned to all tissue and sediment samples with analyte concentrations below the MDL. Because the MDL represents the greatest possible analyte concentration that could be detected using the current methods, the substitution of the MDL value represents a conservative estimate of actual tissue and sediment concentrations. Sample means which include three or more MDL substituted values are identified in all figures and tables.

To meet assumptions for normality and equality of variance TCS concentrations among species were square root transformed, while TCS concentrations in the tissues of *T. latifolia* across sites were log transformed. Additionally, TCC concentrations among species were log transformed, while the analysis of TCC concentrations in the tissues of *T. latifolia* across sites was performed on ranked data.

CHAPTER 3

RESULTS

3.1 Quality Control Data

The analysis of quality control samples revealed consistent recovery of internal standards and native compounds. Method blank concentrations of the target analytes were below the MDL reported by Adhikari (2010) for all samples. The results of this analysis are presented below in Table 2.

Table 2
Quality control data for triclocarban (TCC), triclosan (TCS), and methyl-triclosan (MTCS) in sediments, roots, and shoots. Spike recoveries indicated as recovery of 20 ng/g fresh tissue weight for TCC matrix spikes and 100 ng/g fresh tissue weight for TCS and MTCS matrix spikes or an equivalent amount spiked into method blanks. The method detection limits (MDL) are those reported previously by Adhikari (2010).

Medium	Method Blank (ng/g)*	Blank Spike Recovery (%)**	Matrix Spike Recovery (%)**
Sediment			
TCC	< MDL (n=1)	135 (n=1)	130 (n=1)
TCS	< MDL (n=1)	111 (n=1)	116 (n=1)
MTCS	< MDL (n=1)	109 (n=1)	103 (n=1)
Root			
TCC	< MDL (n=3)	88 (n=3)	142 (n=3)
TCS	< MDL (n=3)	133 (n=3)	113 (n=3)
MTCS	< MDL (n=3)	99 (n=3)	109 (n=3)
Shoot			
TCC	< MDL (n=3)	87 (n=3)	97 (n=3)
TCS	< MDL (n=3)	134 (n=3)	116 (n=3)
MTCS	< MDL (n=3)	99 (n=3)	120 (n=3)

* Tissue specific MDL values reported in Table 1

**Mean recovery values are presented for roots and shoots

3.2 Patterns of Tissue Concentrations Among Different Species

MTCS was not detectable in any of the tissues examined. In all species, the accumulation of TCS in roots was greater than shoots with the exception of *P. cordata* (Fig. 4 A). Measured shoot tissue concentrations were near the MDL for all species. In the case of *S. graminea* and *T. latifolia,* means represent MDL substituted values and not actual tissue concentrations. Mean root concentrations of TCS were measurable in all species, with *T. latifolia* roots (40.3± 11.3 ng/g) being significantly greater than *P. cordata* roots (15.0 ± 1.9 ng/g). The mean root concentration in *S. graminea* was not significantly different from the other species. Within species, root tissue concentrations of TCS were significantly greater than shoots (MDL substituted values) for *T. latifolia*; however, the concentration of TCS in roots was not significantly different from shoots for *S. graminea* or *P. cordata*.

TCC concentrations, like TCS, were highest in the root tissues (Fig. 4 B). Mean shoot concentrations of TCC for *S. graminea* (22.8 ± 9.3 ng/g) were significantly greater than *T. latifolia* (MDL substituted values), while shoot tissue concentrations of *P. cordata* were not significantly different from either species. Mean TCC concentrations in roots were not significantly different among species and were within the range of concentrations observed for TCS. Within species, the concentration of TCC in the roots (34.4 ± 5.3 ng/g) of *P. cordata* was significantly greater than shoots (15.4 ± 2.8 ng/g). Similarly, TCC concentrations in the roots (26.0 ± 3.6 ng/g) of *T. latifolia* were significantly greater than shoots (MDL substituted values); however, TCC concentrations in the roots of *S. graminea* were not significantly different from the shoots.

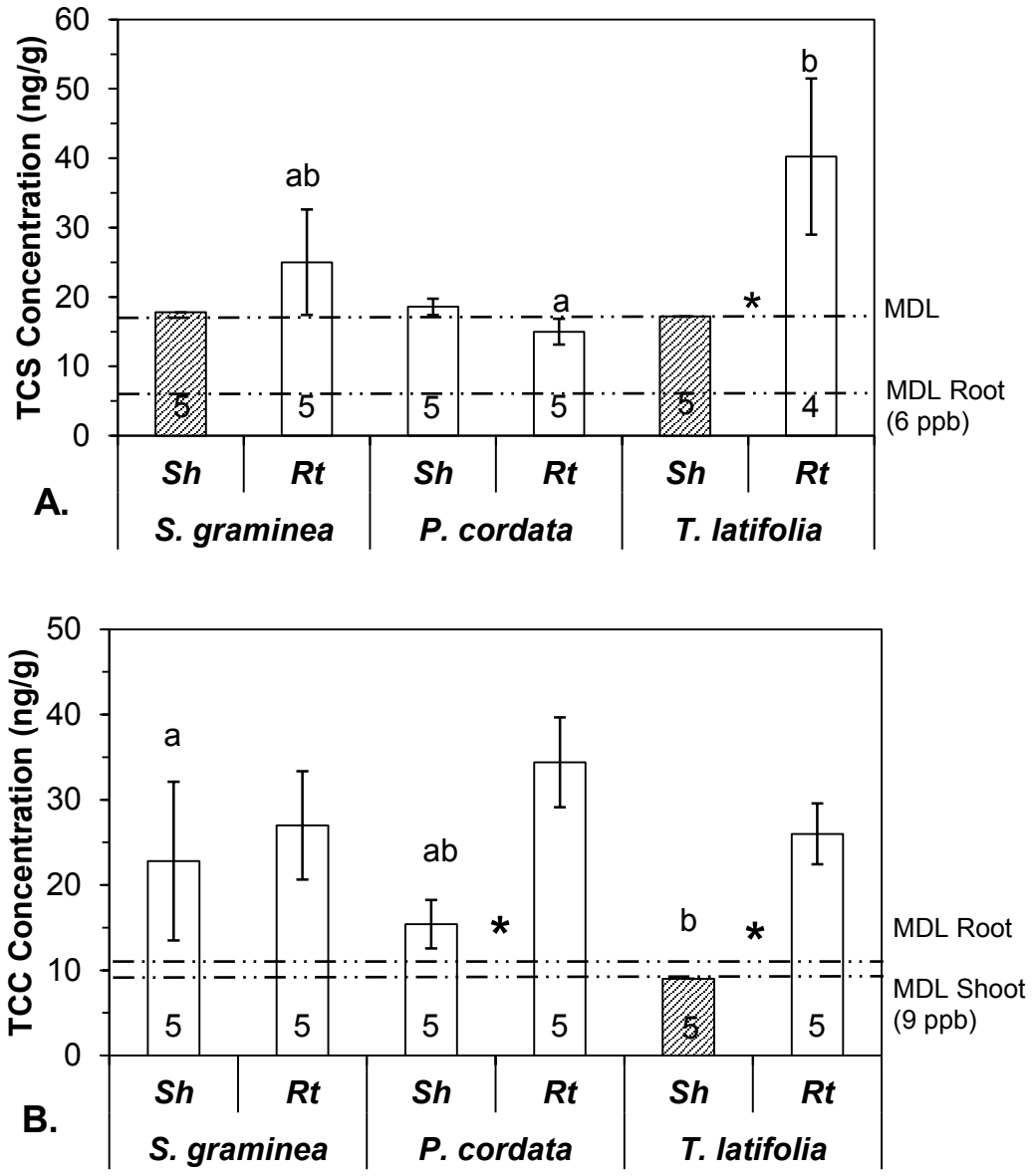

Fig. 4. (A.) Triclosan (TCS) and (B.) triclocarban (TCC) fresh weight concentrations in the roots (Rt) and shoots (Sh) of *S. graminea*, *P. cordata*, and *T. latifolia* at Site 1. For all figures means are presented with standard error. Numbers inside data series represent sample sizes. Different letters above the error bars identify mean analyte concentrations that differ significantly among species (p<0.05). Asterisks between data series identify mean analyte concentrations that differ significantly between the roots and shoots of a given species. Method detection limits (MDL) depicted as dashed lines. Shading identifies MDL substituted means.

3.3 Analyte Concentration Patterns in Plants at Different Locations

Across sites, the concentrations of TCS in roots were generally observed to decrease from inflow to outflow through the constructed wetland (Fig. 5). TCS concentrations in *T. latifolia* shoots were below the MDL for all sites. Consequently, the means depicted for shoots in Fig 3.2 represent MDL substituted values. Mean TCS concentrations in *T. latifolia* roots were significantly greater at Site 1 (40.3 $\pm$ 11.3 ng/g) and Site 2 (29.2 $\pm$ 6.1 ng/g) compared to Site 3 (12.4 $\pm$ 2.3 ng/g). This represents an approximately 69% decrease overall in TCS concentration in root tissues from Site 1 to Site 3. Additionally, TCS concentrations in the roots (40.3 $\pm$ 11.3 ng/g) of *T. latifolia* at Site 1 were significantly greater than the shoots (MDL substituted values).

Measurable concentrations of TCC were limited to *T. latifolia* roots (26.0 $\pm$ 3.6) at Site 1. Consequently, the TCC concentrations in shoots could not be compared across sites. When MDL values are substituted for shoots at Site 1, TCC concentrations in the roots of *T. latifolia* are significantly greater than shoots. Additionally, when MDL values are substituted for roots at Sites 2 and 3, TCC concentrations in root tissues at Site 1 are significantly greater than Sites 2 and 3, indicating an overall reduction of TCC in root tissues from inflow to outflow within the constructed wetland.

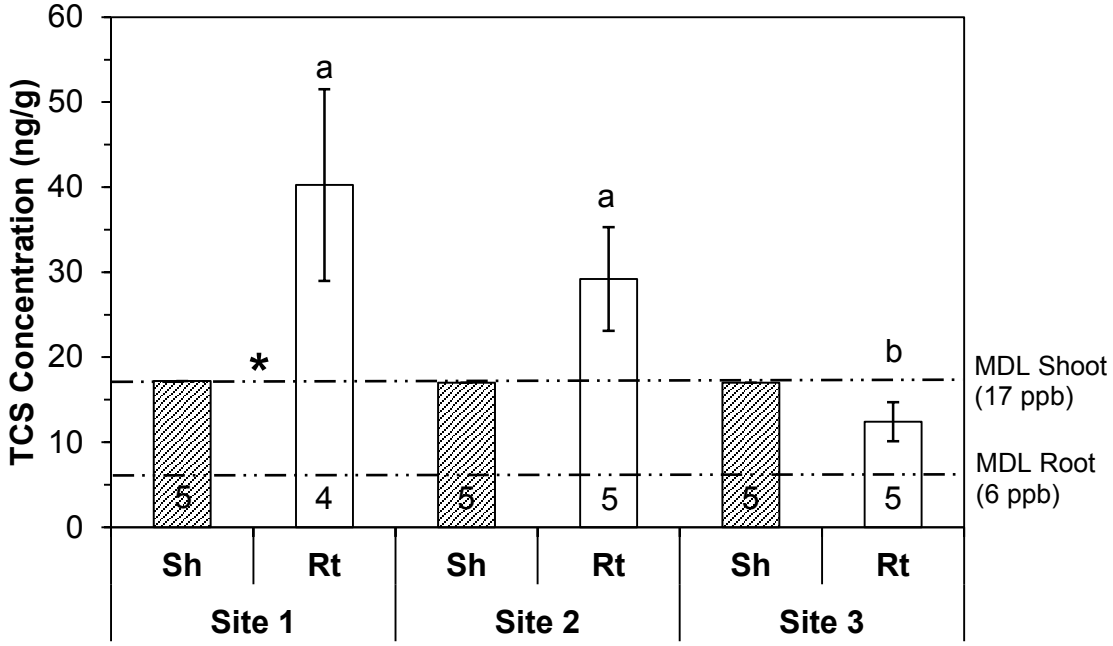

Fig. 5. Triclosan (TCS) fresh weight concentrations in the roots (Rt) and shoots (Sh) of *T. latifolia* from Sites 1 - 3. Means ± standard error are presented. Numbers inside data series represent sample sizes. Different letters above the error bars identify mean analyte concentrations that differ significantly across sites (p<0.05) for roots. Method detection limits (MDL) depicted as dashed lines. Shading indentifies MDL substituted means.

3.4 Analyte Concentration Patterns in Sediments at Different Locations

Similar to tissues, the pattern of analyte concentrations in sediments across sites was observed to decrease generally from inflow to outflow within the constructed wetland (Fig. 6). MTCS was not detectable in any of the sediments examined. Mean TCS concentrations in sediments were significantly greater at Site 1 (29.2 ± 8.5 ng/g) than Site 2 (7.8 ± 0.9 ng/g) representing an approximate 73% decrease. The mean TCS concentration in sediments at Site 3 did not differ significantly from the other sites. Mean TCC concentrations in sediments at Site 1 (50.3 + 13.1 ng/g) were significantly greater than Site 2 (11.8 + 2.4 ng/g) and Site 3 (17.0 + 4.3 ng/g), which represents an

approximately 65% decrease in sediment concentrations of TCC overall from Site 1 to Site 3.

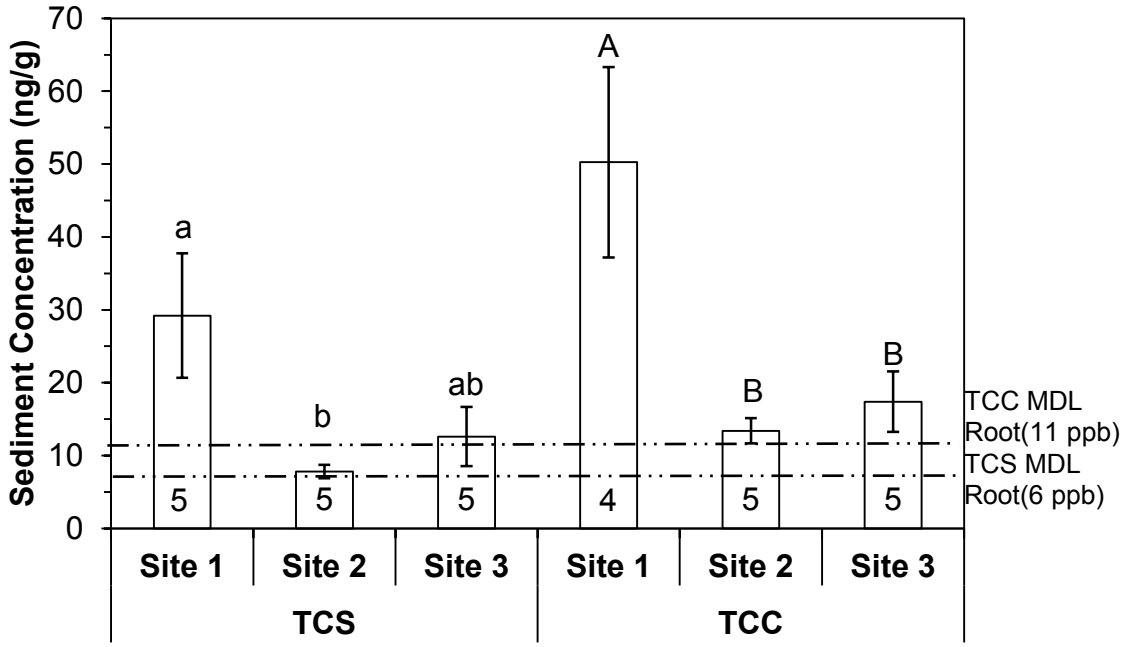

Fig. 6. Triclosan (TCS) wet weight concentrations in sediments from Sites 1 - 3. Means ± standard error are presented. Numbers inside data series represent sample sizes. Different letters above the error bars identify mean analyte concentrations that differ significantly across sites ($p<0.05$) for sediments. Method detection limits (MDL) depicted as dashed lines.

3.5 Reductions in Analyte Concentrations Across Sites

The reduction of analyte concentrations from inflow (Site 1) to midpoint (Site 2) and from inflow to outflow (Site 3) in the Pecan Creek WWTP constructed wetland are presented in Table 3 below. The percentage of reduction was observed to vary by contaminant and medium (i.e. sediments and plant tissues). The reduction of TCC and TCS concentrations in sediments was observed to peak from inflow to midpoint, while the peak reduction of TCS concentrations in *T. latifolia* root tissues was observed from inflow to outfall.

Table 3
Reduction of triclosan (TCS), methyl-triclosan (MTCS), and triclocarban (TCC) concentrations in sediments and *T. latifolia* root tissues at different locations within the Pecan Creek Waste Water Treatment Plant constructed wetland.

Contaminant	Medium	% Reduction (Inflow – Midpoint)	% Reduction (Inflow – Outflow)
TCC	Root Tissue	ND	ND
	Sediments	73	65
TCS	Root Tissue	27	69
	Sediments	73	57
MTCS	Root Tissue	ND	ND
	Sediments	ND	ND

ND = no data; more than three values are MDL substitutions

3.6 Relationship Between Analyte Concentrations in Tissues in Sediments

TCC concentrations in the root tissues of *T. latifolia* were significantly correlated with sediment TCC concentrations ($r = 0.54$, $p<0.05$). In contrast, TCS concentrations in the root tissues of *T. latifolia* were not significantly correlated with sediment TCS concentrations ($r = 0.19$, $p>0.05$).

3.7 Bioconcentration Factors

Bioconcentration factors (BCFs) were calculated as the ratio of mean analyte concentrations in species tissues to sediments and water concentrations. BCF values for water were estimated using mean waste water effluent concentrations of TCC and TCS at the Pecan Creek WWTP reported previously by Coogan et al. (2007) as 0.20 and 0.12 ppb, respectively. In both sediments and water, the greatest BCFs for TCC were observed in *P. cordata* roots, while for TCS, BCFs were highest in *T. latifolia* roots.

BCFs for water were at least one order of magnitude greater than sediments for all species studied. In general, BCFs were slightly higher for TCS than TCC. Table 4 presents the BCF values for TCC and TCS in the different species tissues for sediments and water.

Table 4
Sediment and water bioconcentration factors (BCFs) for measurable fresh weight concentrations of triclocarban (TCC) and triclosan (TCS) in the roots and shoots of *T. latifolia, P. cordata,* and *S. graminea* at the Pecan Creek Waste Water Treatment Plant constructed wetland.

		TCC			TCS	
Medium (ppb)	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Water *	0.20	0.20	0.20	0.12	0.12	0.12
Sediment	50.3	13.4	17.4	29.2	7.8	12.6
S. graminea root	27.0	NA	NA	25.0	NA	NA
S. graminea shoot	22.8	NA	NA	ND	NA	NA
P. cordata root	34.4	NA	NA	15.0	NA	NA
P. cordata shoot	15.4	NA	NA	18.6	NA	NA
T. latifolia root	26.0	ND	ND	40.3	29.2	12.4
T. latifolia shoot	ND	ND	ND	ND	ND	ND
BCF Sediment						
S. graminea root	0.54	NA	NA	0.86	NA	NA
S. graminea shoot	0.45	NA	NA	ND	NA	NA
P. cordata root	0.68	NA	NA	0.51	NA	NA
P. cordata shoot	0.31	NA	NA	0.64	NA	NA
T. latifolia root	0.52	ND	ND	1.38	3.74	0.98
T. latifolia shoot	ND	ND	ND	ND	ND	ND
BCF Water						
S. graminea root	135	NA	NA	208	NA	NA
S. graminea shoot	114	NA	NA	ND	NA	NA
P. cordata root	172	NA	NA	125	NA	NA
P. cordata shoot	77	NA	NA	155	NA	NA
T. latifolia root	130	ND	ND	335	243	103
T. latifolia shoot	ND	ND	ND	ND	ND	ND

* Measured concentrations of TCC and TCS in waste water effluent reported by Coogan et al. 2007; ND = no data; more than three values are MDL substitutions; NA = no samples collected for species at sampling location

CHAPTER 4

DISCUSSION

The present study explored the accumulation patterns of TCS, the degradate MTCS, and TCC in the plant tissues and sediments of an operational constructed wetland. Recent controlled studies have confirmed the potential of wetland plants to bioconcentrate TCS, MTCS, and TCC (Stevens et al., 2009; Adhikari 2010) and the importance of both plants and sediments for PPCPs removal in pilot-scale constructed wetlands (Dordio et al., 2009; Matamoros et al., 2010); however, the species specific ability of free-living wetland plants to bioconcentrate these compounds and contaminant concentration patterns under field conditions have received little attention. Here we report for the first time the uptake of TCS, MTCS, and TCC by three different species of free-living wetland plants. Uptake was consistently higher in roots than shoots and bioconcentration factor estimates for water were greater than sediments for all species studied. Furthermore, the bioconcentration of target analytes under field conditions showed species-specific differences. Finally, we found that the concentration of these compounds declined in both plant tissues and sediments from the inflow to the outflow, indicating that analyte concentration patterns vary with contaminant loading at different locations in the constructed wetland.

4.1 Bioconcentration Patterns in Plant Tissues

Existing plant uptake models have successfully predicted the uptake of contaminants in terrestrial crops using Log *Kow*. In their classic study of plant uptake of organic contaminants, Briggs et al. (1982) reported that the root concentration factor of

insecticides and herbicides in 6 day old barley plants was observed to increase steadily with Log *Kow*, while the transpiration stream concentration factor (i.e. shoot uptake) was limited to compounds with a Log *Kow* ranging from -0.5 – 3.5. Stephan (2000) has suggested that the decreased translocation of lipophilic compounds (Log *Kow* > 2) results from sorption to the endodermis in the central cylinder of plant roots. This has also been confirmed experimentally by Wild et al. (2005) who observed that the radial movement of the lipophilic contaminants, anthracene and phenanthrene, did not extend past the endodermal cell layer of wheat and maize roots.

The use of Log *Kow* to predict uptake in wetland species has been limited to controlled studies. Burken and Schnoor (1998) demonstrated that the bioconcentration of the hydrophobic contaminants 1,2,4-triclorobenze (Log *Kow* = 4.25) and pentachlorophenol (Log *Kow* = 5.04) in hybrid poplar trees poplar trees grown in hydroponic solution was concentrated in root tissues. More recently, a laboratory study by Stevens et al. (2009) using a continuous flow-through system found that the bioconcentration factors and fresh weight tissue concentrations of TCS in the shoots of two emergent species, *S. herbacea* and *B. frondosa*, were consistently lower than those observed in roots at exposure levels ranging from 0.4 to 1000 ppb. Additionally, Adhikari (2010) reported that TCS and TCC accumulation was limited to root tissues for specimens of *P. cordata* and *P. hydropiperoides* that were grown in mesocosms and continuously exposed to treated effluent from the same source as the Pecan Creek WWTP constructed wetland.

In the current study, the bioconcentration of TCS and TCC in free living wetland plants growing in an operational constructed wetland displayed greater concentrations

in root tissues compared to shoots. Given that the Log *Kow* of TCS and TCC are 4.8 and 4.9, respectively, the patterns of bioconcentration observed here corroborate existing models of plant uptake for hydrophobic contaminants proposed by Briggs et al. 1982. Consequently, the results of this study suggest that Log *Kow* might be a useful determinant of contaminant bioconcentration patterns in emergent wetland species grown under field conditions.

4.2 Bioconcentration Patterns Among Species

There is little information regarding the ability of different wetland plants species to bioconcentrate hydrophobic PPCPs, such as TCS and TCC. In constructed wetland design, species selection is typically under emphasized because the role of plants in contaminant removal (i.e. provisioning of carbon for microbial respiration and surfaces for biofilms and contaminant sorption) is primarily viewed as a passive process, which is augmented by plant productivity and surface area, rather than the direct uptake and removal of target contaminants (Brooks et al., 2011; Kadlec and Knight, 1996; Wallace and Knight, 2006). Recent controlled studies, however, have provided evidence that species specific differences exist for the uptake of TCS, MTCS, and TCC in emergent wetland plants. Stevens et al. (2009) exposed seedlings of *B. frondosa* and *S. herbacea* to TCS and MTCS for 28 days and reported greater concentrations of TCS in the roots of *B. frondonsa* (ca. 1,000-10,000 ng/g) compared to *S. herbacea* (ca. 100-1,000 ng/g), while *S. herbacea* roots (ca. 1,000-100,000 ng/g) were observed to have greater concentrations of MTCS compared to *B. frondonsa* roots (ca. 100-1,000 ng/g). In a recent mesocosm study at the Pecan Creek WWTP, Adhikari (2010) observed

significantly greater accumulations of TCS and TCC in the root tissues of *P. hydropiperoides* (55 ng/g TCS; 160 ng/g TCC) compared to *P. cordata* (9 ng/g TCS; 25 ng/g TCC) when potted specimens were exposed to treated effluent from the same source as the constructed wetland for a period of 60 days.

Similar studies in operational constructed wetlands are limited. Park et al. (2009) reported no difference in the accumulation of TCS in single whole plant samples of *Typha* spp. and *Acorus* spp. taken from a constructed wetland in Korea. These findings contrast with the present study, which observed that the bioconcentration of TCS in the root tissues of *T. latifolia* was significantly greater than *P. cordata* and that the bioconcentration of TCC in the shoot tissues of *S. graminea* was significantly greater than *T. latifolia*. Differences in the accumulation of target compounds are potentially attributable to anatomical differences among the different species in this study. Specifically, the greater accumulation of TCS in the roots of *T. latifolia* compared to *P. cordata* may be related to the presence of a complex, 4 -6 layered, hypodermis which has been described at length in the roots of *Typha* spp. (Peterson and Perumalla, 1990; Seago Jr. et al., 1998; Seago Jr. and Marsh, 1989). In contrast, the hypodermis of *P. cordata* roots is simple, typically containing only one to two layers (Seago Jr. et al., 2000). During root growth, the formation of specialized structures in the hypodermis (i.e. casparian bands, suberin lamellae, and secondary wall thickenings) results in the deposition of lipid rich compounds, which have been indicated as major sorption sites for the partitioning of lipophilic compounds in plant roots (Trapp, 2000; Wild et al., 2005). Consequently, the more complex hypodermis of *T. latifolia* roots, with their greater lipid content, may provide some explanation for the higher TCS accumulation

observed in this study. The complex hydodermis of *T. latifolia* roots may also limit the translocation of TCS and TCC to aboveground tissues, thus providing a possible explanation for the lack of accumulation of either compound in the shoots of *T. latifolia*. In the case of *S. graminea*, the greater accumulation of TCC in the shoots compared *T. latifolia* may also be explained by phenotypic differences in leaf form. Whereas *T. latifolia* possesses a single leaf form which emerges from a protected sheath at or near the water surface, the leaves of *S. graminea* possess submerged and emergent leaf forms (Wooten, 1970). During the current study, all specimens of *S. graminea* collected from the Pecan Creek WWTP constructed wetland displayed only submerged leaf forms. As a result, shoot tissue concentrations of TCC measured for *S. graminea* may have been elevated by fractions of TCC that were sorbed to the surface of submerged leaves. Clearly, in the case of shoot uptake, field studies pose experimental challenges that could be addressed in future studies via the use of radio-labeled tracers. Nevertheless, the findings of this study suggest that phylogenetic differences may exist which favor the accumulation of hydrophobic PPCPs by certain wetland plants. Although the anatomical differences described here were not directly compared in the specimens sampled, experimental comparisons of these traits in future studies may help to reveal the basis for potential phylogenetic genetic differences associated with the accumulation of hydrophobic PPCPs in emergent wetland plants.

Several studies have documented the ability of *T. latifolia* to remove moderately hydrophobic contaminants such as trichloroethylene, atrazine, and 2,4,6-trinitrotoluene (Haberl et al., 2003; Runes et al., 2001) from constructed wetland systems. From the results of this study, we know now that *T. latifolia* also has the ability to bioconcentrate

hydrophobic PPCPs, such as TCS and TCC, and could potentially be used to enhance the removal of the lipophilic contaminants from constructed wetlands. Furthermore, this study has demonstrated the potential of less well studied species (i.e. *S. graminea* and *P. cordata*) to bioconcentrate hydrophobic PPCPs.

4.3 Analyte Concentrations at Different Locations and the Relationship Between Tissue and Sediment Concentrations

The ability of constructed wetlands to remove contaminants, such as PPCPs, is related to the simultaneous occurrence of multiple destructive (e.g. phyto- and microbial degradation) and non-destructive (e.g. sorption, volatilization, plant uptake) processes (Imfeld et al., 2009). The influence of these processes on analyte concentration patterns in tissues and sediments at different locations within constructed wetlands is poorly understood. Numerous studies have documented the treatment efficiency of constructed wetlands by comparing changes in the concentration of target analytes in effluent samples collected from the inflow and outflow (Hijosa-Valsero et al., 2010a; Hijosa-Valsero et al., 2010b; Matamoros et al., 2009; Matamoros and Bayona, 2006). Presently it is not know whether changes in effluent concentration correspond with changes in analyte concentration in tissues and sediments.

Previous studies at the Pecan Creek WWTP constructed wetland have documented the significant reduction of TCS concentrations in effluent samples from the wetland inflow (0.09 µg/L) to the wetland outflow (0.04 µg/L) (Waltman et al., 2006). The current study demonstrated a similar pattern for the concentrations of TCS in tissues and TCC in tissues and sediments, whereby concentrations at the inflow were significantly reduced compared to the outflow. Therefore, the findings of the present

study provide evidence that analyte concentration in tissues and sediment at different locations in the constructed wetland vary with contaminant loadings at different locations within the constructed wetland. However, TCS sediment concentrations at the inflow were not significantly different from the outflow. More comprehensive sampling and greater recognition of site characteristics may unravel why these differences were not observed in TCS sediment concentrations.

In their partition-limited model, Chiou et al. (2001) indicate that the effective amount of any organic contaminant available for plant uptake is related to sediment concentration. Consequently, we would expect tissue concentrations to be related to sediment concentrations; however, only TCC sediment and tissues concentrations were significantly correlated. The poor association of TCS sediment and tissue concentrations is potentially attributed to the small sample size and low variability of the present study.

4.4 Lack of MTCS Accumulation

In the present study, accumulation of MTCS was not observed in the tissues of any species nor in the sediments of the Pecan Creek WWTP constructed wetland. The accumulation of MTCS reported by Stevens et al (2009) and Adhikari (2010) was limited to species not considered in the present study. Consequently, mechanisms of formation of MTCS, such as endogenous *O*-methylation, may be species specific. Given that the log Kow of MTCS (5.2; Coogan et al., 2007) exceeds that of TCS and TCC, the lack of MTCS accumulation in wetland sediments is not well understood. It is possible, that the low effluent concentration of MTCS (0.08 ppb) previously reported for the Pecan Creek

WWTP (Coogan et al., 2007) may limit sediment accumulation. Future studies utilizing techniques with increased sensitivity and greater quantities of sediment may help to reveal the pattern of MTCS concentration in constructed wetland sediments.

CHAPTER 5

CONCLUSIONS

Previously, controlled studies have demonstrated the bioconcentration of TCS and TCC in emergent wetland plants as well as the potential for negative impacts to root system development resulting from TCS exposure. To our knowledge this is the first study to consider the bioconcentration of TCS and TCC in an operational constructed wetland. The results of the present study have shown that TCS and TCC are readily accumulated in the root tissues of free living wetland plants and the bioconcentration of these compounds show species specific differences. Although the presence of reduced root systems was not confirmed in this study, the concentrations of TCS in root tissues are comparable to those shown to impact root system development in laboratory studies. The potential ecological concerns associated with decreased root systems in wetland plants include reduced nutrient uptake, decreased competitive ability, and increased potential for uprooting. This raises concerns for the long term exposure of wetland ecosystems, both constructed and natural, to wastewater effluent sources containing TCS. A growing body of research has demonstrated the efficacy of constructed wetlands for the removal of PCPP's from wastewater effluent. However, the sustainable long-term use of constructed wetlands will require additional research to determine the role of species selection for the optimization of PPCPs removal. Furthermore, field studies will also be necessary to evaluate potential negative impacts to wetland ecosystems from long term exposure to TCS and TCC.

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