BIOACCUMULATION OF TRICLOCARBAN, TRICLOSAN, AND METHYL-TRICLOSAN IN A NORTH TEXAS WASTEWATER TREATMENT PLANT RECEIVING STREAM AND EFFECTS OF TRICLOSAN ON ALGAL LIPID SYNTHESIS

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Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

August 2007

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Coogan, Melinda Ann. *Bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant receiving stream and effects of triclosan on algal lipid synthesis.* Doctor of Philosophy (Environmental Science), August 2007, 78 pp., 10 tables, 19 figures, 101 references.

Triclosan (TCS) and triclocarban (TCC), widely used antimicrobial agents found in numerous consumer products, are incompletely removed by wastewater treatment plant (WWTP) processing. Methyl-triclosan (M-TCS) is a more lipophilic metabolite of its parent compound, TCS. The focus of this study was to quantify bioaccumulation factors (BAFs) for TCS, M-TCS, and TCC in Pecan creek, the receiving stream for the City of Denton, Texas WWTP by using field samples mostly composed of the alga *Cladophora* sp. and the caged snail *Helisoma trivolvis* as test species.

Additionally, TCS effects on *E. coli* and Arabidopsis have been shown to reduce fatty acid biosynthesis and total lipid content by inhibiting the trans-2 enoyl- ACP reductase. The lipid synthesis pathway effects of TCS on field samples of *Cladophora* spp. were also investigated in this study by using [2-^{14}C]acetate radiolabeling procedures. Preliminary results indicate high TCS concentrations are toxic to lipid biosynthesis and reduce [2-^{14}C]acetate incorporation into total lipids. These results have led to the concern that chronic exposure of algae in receiving streams to environmentally relevant TCS concentrations might affect their nutrient value. If consumer growth is limited, trophic cascade strength may be affected and serve to limit population growth and reproduction of herbivores in these riparian systems.

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ACKNOWLEDGEMENTS

I would like to thank God for the abilities and resources to complete the task that has been placed before me. I would also like to thank my son Ben, daughter Jessica, and best friend, Mike, for their endless local and long-distance support as I pursued the completion of my doctoral studies.

My committee members have been invaluable in their continued direction and endless hours of guidance. I wish to thank Dr. Thomas W. La Point, my dissertation advisor, for taking a chance on this "atypical" graduate student. His passion for arid region water issues is certainly contagious, and he has tremendous abilities to inspire up-and-coming graduate students. Dr. Barney J. Venables has the patience of Job, as he has worked endless hours helping me understand the analytical chemical analyses and chemical applications of our research, and I can never thank him enough. I would also like to thank Dr. Kent D. Chapman for taking his personal time to train me on radioactive labeling procedures and problem-solving issues that arose during the course of our lipid analyses. Dr. Kenneth E. Banks provided the impetus for me to pursue the use of algal species in my bioaccumulation studies, and it certainly proved to be fruitful, for which I wish to express tremendous gratitude. I would also like to thank Dr. Michael A. Lewis for his long-distance support as I grappled with understanding why environmental assessments are conducted in specific and, what appeared to me at times to be, foreign manners. TRAC laboratories and its managers, Steve Junot and Thomas Venables, also supported me as I began my project and needed to use various equipment and supplies.

Several other UNT professors were instrumental in the success of this project, such as Dr. James Kennedy, Dr. Kevin Stevens, Dr. Bruce Hunter, and Dr. Tom Waller. Professors and others outside of UNT, such as Dr. Jerry Brand with UTEX, Dr. Geoff Scott with S. Carolina

NOAA Labs, Dr. Mike Hooper with Texas Tech, Dr. Bryan Brooks with Baylor University, and Dr. Robert Dillon with Charleston University, also provided valuable advice and direction. The following UNT graduate students, Regina Edziyie, Cheryl Waggoner, Dave Johnson, Gary Stover, Gopi, Vatsalla, and Swati all contributed to this project in various and greatly appreciated ways. Emily Merrill, a Texas Academy of Math and Science student, also volunteered numerous hours helping select the Pecan Creek Collection sites in the heat of August 2005 and deserves my tremendous thanks.

None of us would be where we are within the UNT Environmental Science Department without the unending support of the EESAT office staff, and I would particularly like to express my gratitude for the numerous hours of guidance and patience shown by Candy King and Glenda Cole. The family I have found within the UNT Environmental Science program is as precious to me as those to whom I am connected by DNA, and they will never be forgotten.

This research was funded by an EPA TEAM grant to the University of North Texas.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

Chapter

1. INTRODUCTION 1

    References 5

2. ALGAL BIOACCUMULATION OF TRICLOCARBAN (TCC), TRICLOSAN (TCS), AND METHYL-TRICLOSAN (M-TCS) IN A NORTH TEXAS WASTEWATER TREATMENT PLANT RECEIVING STREAM 7

    Introduction 7

    Materials and Methods 8

    Results 14

    Discussion 18

    References 19

3. SNAIL BIOACCUMULATION OF TRICLOCARBAN (TCC), TRICLOSAN (TCS), AND METHYL-TRICLOSAN (M-TCS) IN A NORTH TEXAS WASTEWATER TREATMENT PLANT RECEIVING STREAM 22

    Introduction 22

    Materials and Methods 24

    Results 32

    Discussion 37

    References 40

4. EFFECTS OF TRICLOSAN ON ALGAL LIPID SYNTHESIS 44

    Introduction 44

    Materials and Methods 47

    Results 55

    Discussion 58

    References 64

5. DISCUSSION AND PROJECTIONS 68

References 70

REFERENCES 71

LIST OF TABLES

Page

1. Quality control data for antimicrobials in water and algae. Spike additions were at 50 ng L^{-1} for water and 25 ng g^{-1} for algae 14

2. Algal bioaccumulation factors (BAFs) based on fresh weight 17

3. Quality control data for antimicrobials in water, algae, and snails. Spike additions were at 50 ng L^{-1} for water and 25 ng g^{-1} for algae and snails 32

4. Clean and caged snail replicate concentrations (ppb) at each collection site and calculated means for TCS, M-TCS, and TCC........ 34

5. Replicate aqueous and algal concentrations (ppb) and calculated means for TCS, M-TCS, and TCC 35

6. Snail bioaccumulation factors (BAFs) based on antimicrobial concentrations (ppb) 36

7. Quality control data for triclosan in algae. Spike additions were at 5.0 µg g^{-1} for algae 55

8. Algal concentrations (ppm) in 100 mg replicate samples and calculated means for each aqueous TCS concentration (0 ppm, 1 ppm, 10 ppm) after a 3-hour incubation period. (N.D. is < PQL of 1.0 ppm) 56

9. Algal bioaccumulation factors (BAFs) based on triclosan aqueous concentrations and algal concentrations (ppm) following 3-hour incubation period 56

10. [2-^{14}C]acetate labeling scintillation results according to aqueous TCS concentrations and algal accumulation following 3 hour (bleached) and 2 hour (non-bleached) incubation 57

LIST OF FIGURES

Page

1. Location of the four collection sites on Pecan Creek, the receiving stream for the City of Denton WWTP effluent discharge 10

2. Site distribution map showing where *Cladophora* and water samples were collected in relationship to Lake Lewisville, Denton County 10

3. Mean and standard errors (one-way ANOVA, alpha = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in water from three selected sites on Pecan Creek, TX........ 15

4. Mean and standard errors (one-way ANOVA, alpha = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in algae from three selected sites on Pecan Creek, TX 16

5. Algal bioaccumulation factors (BAFs), [algae]/[water], for TCC, TCS, and M-TCS at three selected sites on Pecan Creek, TX 17

6. Caged snail placement just below the outfall of the Denton Wastewater Treatment Plant 26

7. Photograph showing cage construction 27

8. Placement of cages containing algae and snails was approximately 6 cm below surface of water........ 27

9. Means and standard errors (α = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in Clean and Caged Snails from the Denton WWTP outfall on Pecan Creek, TX........ 33

10. Means and standard errors (n = 5) of TCC, TCS and M-TCS concentrations (ppb) in water samples from the Denton WWTP outfall on Pecan Creek, TX 34

11. Means and standard errors (n = 5) of TCC, TCS and M-TCS concentrations (ppb) in algal samples from the Denton WWTP outfall on Pecan Creek, TX 35

12. Caged snail bioaccumulation factors (BAFs) for TCS, TCC, and M-TCS at the Denton WWTP outfall on Pecan Creek, TX 36

13. Photograph of *Cladophora* spp. after 4 hour incubation period in 0 ppm [TCS]........ 49

14. Photograph of *Cladophora* spp. after 4 hour incubation period in 1 ppm [TCS]........ 49

15. Photograph of *Cladophora* spp. after 4 hour incubation period in 10 ppm [TCS]........ 50

16. GC/MS quality control percent recoveries for triclosan in algae following spike additions 55

17. [2-^{14}C]acetate labeling results in pmol acetate g^{-1} fresh weight algae with means and standard errors ($\alpha = 0.05$, $n = 10$) indicating algal lipid production following a three hour incubation period at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm 59

18. Algal TCS concentration results with means and standard errors ($\alpha = 0.05$, $n = 5$) following a three hour incubation period at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm... 59

19. Algal TCS bioaccumulation factors (BAFs) at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm... 60

CHAPTER 1

INTRODUCTION[*]

Population growth and the accompanying landscape changes within urban areas of the United States, and globally, present ongoing challenges for those interested in the ecological dynamics of receiving waters. Anthropogenic changes by the year 2015 as a result of urban land use are inevitable as predicted growth of metropolitan areas with populations greater than one million increase from 118 to 272 while cities greater than ten million are predicted to increase from 14 to 27 (Shah, 1996). In order to adequately monitor the effects of urban land use on global water systems, limnologists and aquatic ecologists will need to work closely with urban water managers (Walsh, 2000).

Lotic water systems have traditionally been sites of urban center development due to ease of transport and availability of food source. Growing populations eventually required the use of urban water systems as sewers and industrial dump sites, which lead to the development of sewage treatment facilities (Chadwick, 1842). The resultant effects of sewage treatment on the ecology of receiving waters continue to be challenges as effluent composition reflects watershed usage and urbanization. Pharmaceuticals and personal care products (PPCPs) are released as down the drain chemicals inadequately removed by wastewater treatment plant (WWTP) processes (Waltman et al., 2006). Trophic structures within the riparian zones of WWTP receiving streams will require better levels of understanding as increasing levels of urbanization place greater demands on receiving stream systems that are constantly being subjected to anthropogenic influences. Small urban receiving streams, such as Pecan Creek,

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Denton, TX, become important as responsive systems for evaluating urbanization effects on natural habitats.

Traditionally, biotic communities within aquatic systems have been known to express higher levels of degradation with increasing levels of urbanization due to pollution delivery to receiving streams (Walsh, 2000). In order to limit the impacts of urbanization on riparian zones, measures leading to their protection and restoration are required, along with associated riparian forests and wetlands (May et al., 1997).

[*]Municipal wastewater treatment plants (WWTPs) are a major source of anthropogenic chemical release to the environment. Contaminants of concern, (PPCPs), are not completely removed during wastewater processing and are released to the environment in the form of WWTP residual solids and effluent (Waltman et al., 2006). Enriched concentrations of contaminants in WWTP effluents in the southwestern United States are of special concern because effluent often dominates receiving stream flow, especially during dry low-flow conditions (Bradley et al., 1995; Reilly et al., 2000). An incomplete understanding of the fate of contaminants hampers evaluation of potential ecological and human health risks associated with effluent discharges after their release to receiving streams.

[*]The antimicrobials triclosan (5-chloro-2-[2,4-dichloro-phenoxy]-phenol]; TCS) and triclocarban (3,4,4'-trichlorocarbanilide; TCC), are released to the environment by WWTP processing at sub-ppb effluent concentrations (Singer et al., 2002; Halden and Paull, 2005; Waltman et al., 2006). Methyl triclosan (M-TCS) is a metabolite of TCS more lipophilic and environmentally persistent than the parent compound. Concentrations of M-TCS are generally higher in WWTP effluent than influent, indicating formation of this transformation product in the treatment process (Bester, 2003; Balmer et al., 2004). TCS has been used for more than

thirty-five years as a disinfectant in items such as textiles, plastics, soaps, dermatological creams, and dental hygiene products (Singer et al., 2002). The U.S. Geological Survey (USGS), during a 1999 and 2000 organic wastewater contaminant (OWC) concentration study among 139 streams within 30 states, discovered OWCs in 80% of the sample sites, TCS being one of the most frequently detected compounds (Kolpin et al., 2002).

*TCC has been included in personal care products and detergents since 1957 and has an environmental occurrence rate similar to TCS. Halden and Paull (2005) measured concentrations of up to 6750 ng l^{-1} in Maryland streams and estimated concentrations of up to 1550 ng l^{-1} for streams surveyed previously by the USGS. It has been suggested that TCS and TCC are likely among the most frequently occurring of organic wastewater contaminants behind such well-known WWTP markers as coprostanol, cholesterol, N,N-diethyltoluamide and caffeine (Halden and Paull, 2005). In spite of a use rate of 500,000 – 1,000,000 pounds per year in the U.S.A., TCC had received little attention until the recent development of a liquid chromatography electrospray ionization mass spectrometry (LC/ESI/MS) method allowed successful TCC environmental analyses at the ng l^{-1} level (Halden and Paull, 2004). TCC , TCS, and M-TCS have relatively high log K*ow* values of 4.9, 4.8 (at pH 7.0), and 5.2, respectively, with sufficient predicted environmental persistence to bioconcentrate (Boehmer et al., 2004; Halden and Paull, 2004). Thus, their environmental occurrence may serve as a general indicator of the extent of WWTP contaminant distribution exhibiting similar hydrophobicity and persistence.

*Conflicting definitions of bioconcentration and bioaccumulation, along with their resultant bioconcentration and bioaccumulation factors (BCFs and BAFs), are evidenced in various literature sources. Some have defined BCF as a ratio between biota and water

concentration measurements as a result of laboratory experimentation, while BAF is used as an expression of the same ratio in response to field measurements and food chain accumulation (Wright and Welbourn, 2002). The *Handbook of Ecotoxicology* indicates bioconcentration within aquatic systems involves net accumulation into and onto an organism (Adams and Rowland, 2003). Alternately, bioaccumulation refers to accumulation from any external place, such as water, food, or sediment. For the purpose of this study, we will be using the term "bioaccumulation" to indicate general accumulation from the surrounding environment based on field measurements.

[*]Aquatic toxicological bioaccumulation assessment methods are crucial in monitoring community effluent waters. As various concentrations of persistent pharmaceuticals, hormones, and other wastewater contaminants continue to infiltrate water resources, increasingly sensitive biological methods of investigation need to be developed. Bioaccumulation of lipophilic contaminants through aquatic food chains may result in significant body burdens at subsequent trophic levels.

[*]Trophic transfer of lipophilic compounds within aquatic environments may result in high exposure concentrations among humans and wildlife that consume aquatic organisms (Jabusch and Swackhamer, 2004). This study presents the first report on the bioaccumulation potential of TCS, TCC, and M-TCS as important components of WWTP receiving streams and the first report of TCC bioaccumulation in natural environments. With urbanization and water reuse becoming major factors in municipal water quality, understanding the fate and occurrence of PPCPs in water supplies becomes increasingly important (Kolpin et al., 2002; Fitzhugh and Richter, 2004). Investigating the bioaccumulation of these compounds and their impacts on

higher trophic levels in receiving streams will be required to meet the challenge of this urbanization process.

Studies have shown that aquatic organisms are capable of bioaccumulating environmentally persistent organic chemicals with relatively high [Kow] (Zaranko et al., 1997). If it can be shown that high [Kow] compounds exhibit an increase in body burdens with an increase in trophic status after lipid content correction, then the potential for biomagnifications effects also exists (Zaranko et al., 1997). This study, however, has solely considered bioaccumulation and algal lipid synthesis effects.

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CHAPTER 2

ALGAL BIOACCUMULATION OF TRICLOCARBAN (TCC), TRICLOSAN (TCS), AND METHYL-TRICLOSAN (M-TCS) IN A NORTH TEXAS WASTEWATER TREATMENT PLANT RECEIVING STREAM*

Introduction

*Algae comprise the greatest abundance of plant biomass in aquatic environments and are a logical choice for aquatic toxicological studies, yet have historically been underutilized in this capacity (Wetzel, 2001). As primary producers of unique qualities, algae provide valuable links in food webs. In this study, we selected two commonly used antimicrobials as WWTP marker-compounds to evaluate persistence, downstream distribution, and algal bioaccumulation of trace contaminants in an effluent-dominated receiving stream.

*Algal lipid concentrations range from 5 to 70% of dry weight, depending upon species considered and nutrient limitations; the most common range for lipid-storing species, however, is 15 – 30% (Olsen, 1999). (This study proposes a lipid mass of 19% for *Cladophora* spp. samples.) *Cladophora coelothrix*, a ubiquitous Black Sea alga, was analyzed and found to contain approximately 23.7 mg/g dry weight lipid, with the majority (60.2%) being triacylglycerol (Nechev et al., 2002). Polychlorinated biphenyl (PCB) uptake in phytoplankton is known to occur through cellular membrane diffusion; phytoplankton BAFs for PCBs with log K*ow* values < 6.0 to 6.5 have been reported to range from 4.7 to 5.2 (Stange and Swackhamer, 1994)

*Several algal species have been selected for bioaccumulation and aquatic toxicity tests. For example, the alga *Raphidocelis subcapitata* bioconcentrates xylene, benzene, toluene, and ethyl benzene at a rate which increases as the lipophilic nature of each compound increases

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(Haglund, 1997). *Cladophora* has been used to quantify polychlorinated biphenyl (PCB) biomagnification in a Pottersburg Creek, London, ON, Canada riverine study (Zaranko et al., 1997) while Larsson further identified the kinetics of PCB uptake in *Cladophora glomerata* (Larsson, 1987).

Cladophora glomerata, and accompanying compartmental epiphytic species, are especially well-suited for effluent studies since they grow well both in the field and laboratory settings. They are widely distributed and generally found growing on hard substrates of freshwater systems fed by urban sewage inflow and agricultural runoff (Dodds and Gudder, 1992). With excessive *Cladophora* growth during summer months in aquatic environments containing high nutrient concentrations, *Cladophora* compartments, also described as *Cladophora* mats, have the capability of becoming habitats for epiphytes, bacteria, aquatic macroinvertebrates, and small fish (Chilton et al., 1986). Under these circumstances, the macroalgal mat, becomes a direct food source for generalized grazers, and, due to the relatively high lipid content of certain algal species, may provide the entry point of lipophilic compounds into the food chain of riparian systems (Dudley et al., 1986; Zaranko et al., 1997).

Materials and Methods

Sources of Chemicals

[*]Labeled internal standards ($^{13}C_{12}$-TCS, $^{13}C_{12}$-M-TCS), TCS, and M-TCS were from Wellington Laboratories (Guelph, ON, Canada). The deuterated TCC (d7 TCC) internal standard was a gift from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), and TCC was from Absolute Standards (Hamden, CT, USA). All other reagents and solvents were from Fischer Scientific (Houston, TX, USA).

Collection and Experimental Design

*Five collection sites, one upstream reference (Site 1), three downstream (Sites 3, 4, and 5), and one at the WWTP (Site 2), were chosen along Pecan Creek, the receiving stream for the City of Denton, Texas, USA WWTP (Figure 1), along with the fifth site being the Trinity River access at the Lewisville Lake Environmental Learning Area (LLELA) (Figure 2). Site 1 was identified with GPS coordinates Lat 33.20879 Lon 97.11148, Site 2 Lat 33.19362 Lon 97.07140, Site 3 Lat 33.18867 Lon 97.06418, Site 4 Lat 33.18301 Lon 97.04791, and Site 5 Lat 33.066722 Lon 96.964166. The stream channel lengths from Site 1 to Site 2, Site 2 to Site 3, Site 3 to Site 4, and Site 4 to Site 5 were 6.2 km, 1.1 km, 2.7 km, and 30 km respectively. Seven 60 ml Nalgene bottles were used to collect *Cladophora* spp. replicate samples and seven one-liter amber bottles to collect replicate grab samples from the surface water at collection Sites 1 through 4 from 9/2/2005 through 9/5/2005. The same collection regime was performed at Site 5 on 2/5/2006. Algal and water samples were handled using latex gloves, transported on ice to the laboratory, and maintained refrigerated at 4 °C until analysis. Effluent water quality during collection dates was typical of normal operating conditions of the plant according to Denton WWTP records. Effluent pH averaged 7.4 (range 7.3 – 7.5), flow rates averaged 11.59 million gallons per day (range 10.86 – 12.95), total suspended solids averaged 1.2 mg l^{-1} (range 0.4 – 2.0), and no precipitation was reported for the week prior to collection (personal communication, Gary Stover, City of Denton WWTP operator).

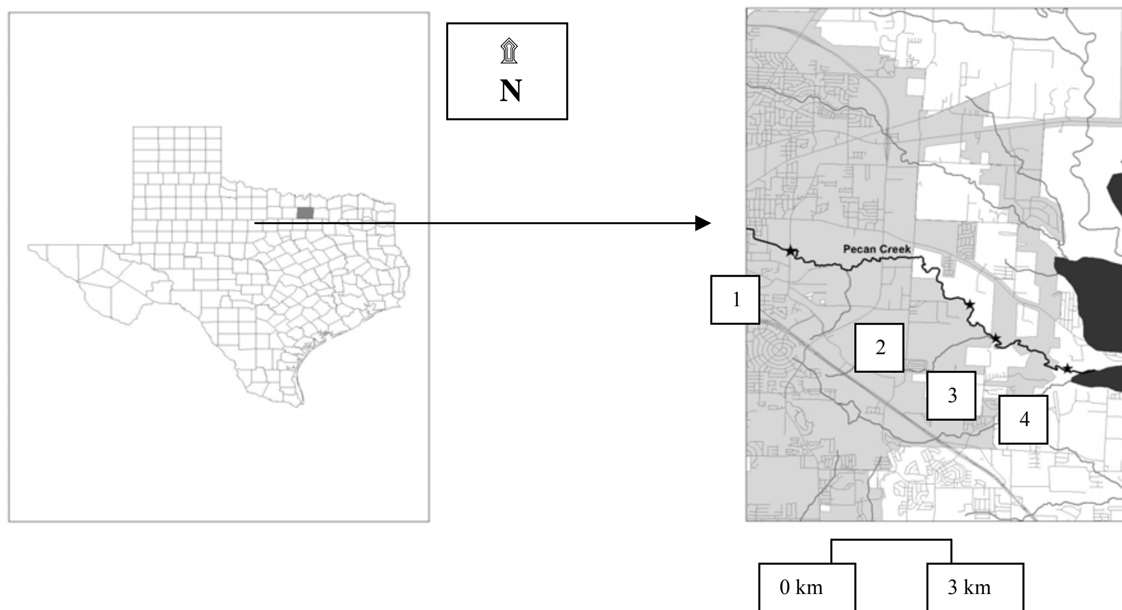

*Fig. 1 Location of the four collection sites on Pecan Creek (Denton, TX), the receiving stream for the City of Denton WWTP effluent discharge. Stream length distances from Site 1 to Site 2 were measured as being ~6 km and Site 2 to Site 4 as ~3 km.

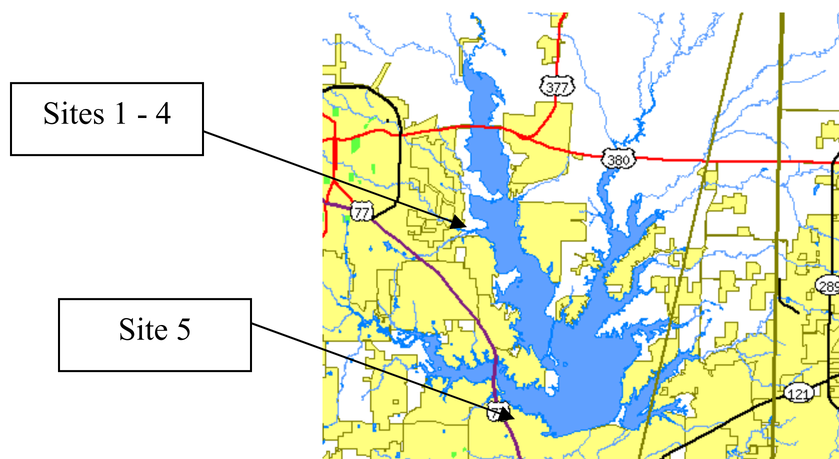

Fig. 2 Site distribution map showing where *Cladophora* and water samples were collected in relationship to Lake Lewisville, Denton County.

Algal Lipid Extraction and Analysis

*Algal samples were separated into 5.0 to 8.0 g sections after hand-washing the contents of each Nalgene bottle in two liter containers of Milli Q 18 MΩ deionized water and removing

visibly evident non-filamentous algal particles. The fractionated sections were then transferred to another two liter container of Milli Q 18 MΩ deionized water, hand-agitated for further removal of non-visible particles (Stevenson and Bahls, 1999) and blot-dried with paper towels. Hereafter, blot-dried will be referred to as fresh weight and will apply to all algal concentration references (ie. ng/g fresh weight). Random sections of each sample were removed and identified as *Cladophora* spp. by microscopic description at 10X magnification using G.W. Prescott's illustrated key (Prescott, 1964). For the purpose of this paper, a reference to *Cladophora* spp. samples and the resultant bioaccumulation is actually a reference to a more complex algal compartment (containing relatively small contributions of epiphytes and biofilm) of which the greatest biomass contribution is *Cladophora* spp.

[*]Approximately 2.0 g (fresh weight) of algae, prepared as described above, were dried with 35.0 g anhydrous Na_2SO_4 for each replicate analyzed. Algae and Na_2SO_4 were ground with mortar and pestle then placed in a 25 mm x 90 mm cellulose Soxhlet extraction thimble. Ten µl of 5 ng μl^{-1} $^{13}C_{12}$ TCS, $^{13}C_{12}$ M-TCS, and d7 TCC were added to each sample as internal standards. Soxhlets were heated to boiling for six hours and extracts (100 ml dichloromethane) were stored in amber bottles at 4 °C. Extracts were reduced to 5 ml by Kuderna Danish (KD) evaporation in a water bath at 60 – 70 °C. High molecular weight lipids were removed by gel permeation chromatography (GPC) with an ABC Laboratories (Columbia, MO, USA) Model SP-1000 GPC Processor according to manufacturer's recommended procedures. Samples were evaporated using a Labconco (Kansas City, MO, USA) RapidVap ™ nitrogen evaporator to a final extract volume of 100 – 1000µl and analyzed by gas chromatography-mass spectrometry (GC/MS) for TCS and M-TCS and by electrospray liquid chromatography-mass spectrometry (ESI-LC/MS) for TCC (see Section 2.5).

Water Analysis

*Unfiltered one liter water samples were fortified with 10.0 μl of 5 ng μl^{-1} internal standard mix immediately prior to solid phase extraction (SPE). Waters (Milford, MA, USA) Oasis HLB SPE cartridges (1.0 g) were conditioned with 10 ml each of dichloromethane, methanol, and Milli Q 18 MΩ deionized water. Cartridges were eluted with a 20 ml 90:10 (dichloromethane:methanol) solution and resultant extracts were evaporated and analyzed as described above.

Instrumental Analyses

*TCS and M-TCS analyses were conducted on an Agilent (Palo Alto, CA, USA) 6890 GC coupled with a 5973 mass selective detector MS (70-eV). An eight point standard curve was established with analyte concentrations from 5-1,000 pg μl^{-1} and internal standard concentrations at 500 pg μl^{-1}. The MS was operated in the single ion monitoring mode (SIM) with target and 3 confirmatory masses monitored (50 msec dwell time) for each compound. GC conditions were helium carrier gas at 480 hPa, inlet temperature at 260 °C (2μl, pulsed pressure at 1,700 hPa for 0.5 min, splitless injection), and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 μm film) temperature initially at 40 °C with a 1-min hold followed by a 50 °C min^{-1} ramp to 140 °C with a 5- min hold followed by a 10 °C min^{-1} ramp to 300 °C with a final 17-min bake-out. Transfer line temperature was 265 °C.

*TCC was analyzed using a modification of the recently reported LC-ESI-MS method (Halden and Paull, 2004; Halden and Paull, 2005). An Agilent 1100 LC/MS system was used with Model SL ion trap MS. The column was a C18 (monomeric, non-endcapped) Zorbax (2.1 X 150 mm) with a 5 μm particle size and 80 Å pore size. Samples were autoinjected (1-5 μl)

with a gradient program (300 μl min^{-1} flow throughout) that was initiated at 70% mobile phase B (95% acetonitrile and 5% water with 5 mM ammonium acetate) and 30% mobile phase A (95% water and 5% acetonitrile with 5 mM ammonium acetate), held for 1.0 min then ramped to 85% B at 10 min and 100% B at 10.1 min through 25 min, then returned to 70% B from 25.1 to 35 min. The ion trap was operated in the negative ion multireaction monitoring mode (MRM) isolating m/z 313 and 315 for native TCC and m/z 320/322 for d_7 TCC internal standard. These isolated pseudo-molecular ions ($[M-H]^-$) were fragmented (amplitude 0.8) to yield daughter ions at m/z 160 and 163 for native and d7 TCC, respectively. Five point standard curves were established for both the pseudo-molecular ions and the daughter ions with TCC concentrations from 10-1,000 pg μl^{-1} and d_7 TCC concentration of 50 pg μl^{-1}. The electrospray was operated with a nebulizer pressure of 2,100 hPa and nitrogen dry gas at eight l min^{-1} at 350 °C. TCC and d_7 TCC eluted at 7.5 min.

*Practical quantitation limits (PQL, Table 1) were established at approximately 10X the instrument detection limit, which was estimated as 3X S.D. of background noise levels for quantitation ions. Standard curves included PQL concentrations for each analyte.

2.6 Statistical analyses

*For each contaminant, concentrations among sites were compared by a one-way analysis of variance (ANOVA) using the Base SAS software, Version 8.2 (SAS Institute, Cary, NC, USA, 1999-2000). Supporting analyses included normality and a Student Newman Keul's (SNK) test, which was used to separate means if any significant differences were observed. The level of statistical significance for all analyses was $\alpha = 0.05$.

Results

Analytical Quality Control (QC) Results

*Recovery percentages for blank spike additions (50 ng/l for water samples and 25 ng/g for algal samples) ranged from 94% to 137%, with a mean of 113% for water analyses, and from 80% to 107%, with a mean of 98% for algal analyses (Table 1). Water column matrix spike and matrix spike duplicate recovery percentages ranged from 80% to 110%, with a mean of 96%, and 80% to 140% for algal analyses, with a mean of 112%. Overall, percent deviation for both water column and algal analyses of matrix spike duplicates ranged from 2.88% to 32.15%, with a mean of 11.2%. No blank values exceeded the PQLs.

*Table 1. Quality Control data for antimicrobials in water and algae. Spike additions were at 50 ng L^{-1} for water and 25 ng g^{-1} for algae.

	Blk (ppb)	**Blk Spk Rec (%)**	**Mat Spk Rec (%)**	**Mat Spk Dup Rec (%)**	**Dup Dev (%)**	**PQL (ppb)**
Water TCS	< 0.010	109%	98%	101%	2.9%	0.01
M-TCS	< 0.005	137%	110%	101%	7.9%	0.005
TCC	< 0.015	94%	80%	84%	3.7%	0.015
Algae TCS	< 10.0	106%	130%	140%	7.7%	10
M-TCS	< 5.0	107%	111%	80%	32.2%	5
TCC	< 10.0	80%	111%	98%	12.8%	10

(Blk = Blank, Spk = Spike, Rec = Recovery, Mat = Matrix, Dup = Duplicate, Dev = Deviation, PQL = Practical Quantitation Limits)

Water Analyses

*We measured statistically significant differences in aqueous TCS and M-TCS concentrations from the WWTP outfall at Site 2 to Site 4, ranging from approximately 0.12 ppb TCS at Site 2 to 0.06 ppb TCS at Site 4, and 0.08 ppb M-TCS at Site 2 to 0.05 M-TCS at Site 4 (Figure 3). TCC concentration reductions were also noted, ranging from approximately 0.20 ppb

TCC at Site 2 to 0.08 ppb TCC at Site 4. TCS, M-TCS, and TCC concentrations at the upstream reference site were less than the PQL and not reported. In general, TCS, M-TCS, and TCC water column concentrations showed a modest but consistent die-away from the WWTP outfall to the mouth of Pecan Creek.

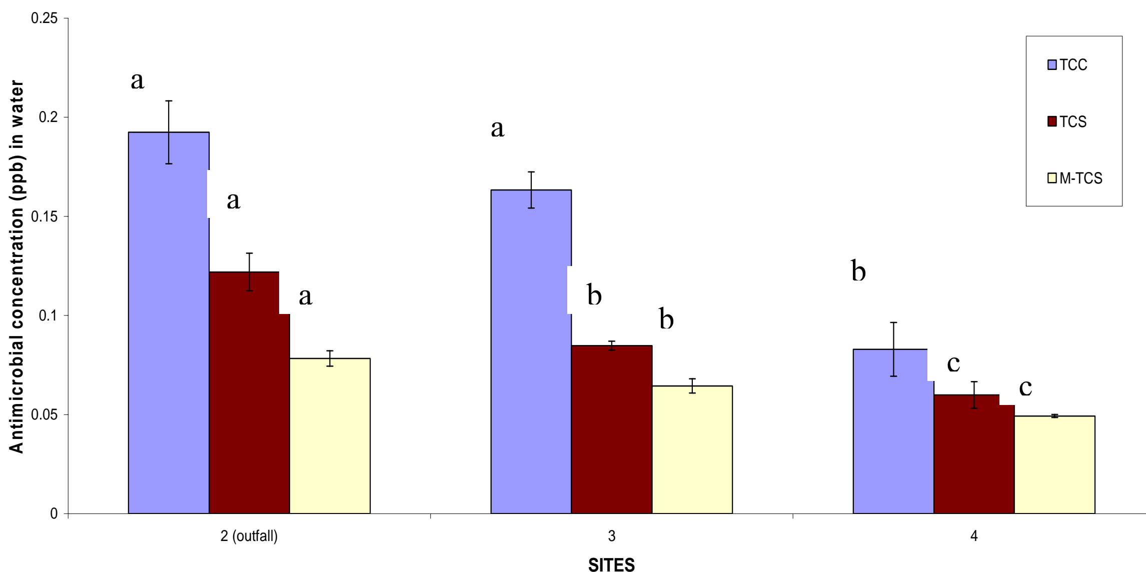

*Fig. 3 Mean and standard errors (one-way ANOVA, alpha = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in water from three selected sites on Pecan Creek, TX. Site means for a given antimicrobial (TCC, TCS, M-TCS) with different letters (a, b, c) are significantly different from the outfall. Concentrations from Site 1 (upstream from the outfall) and Site 5 (below the dam) were below detection limits (see Table 2).

Algal Analyses

*Only M-TCS concentrations in algae were statistically significantly different among collection sites (Figure 4). Concentrations for TCS, M-TCS, and TCC at the upstream reference site were less than the PQL and not included. For TCC, the mean levels range from approximately 200 to 400 ppb. Mean TCS algal concentrations were between 100-150 ppb. Mean M-TCS algal concentrations ranged from 50-90 ppb.

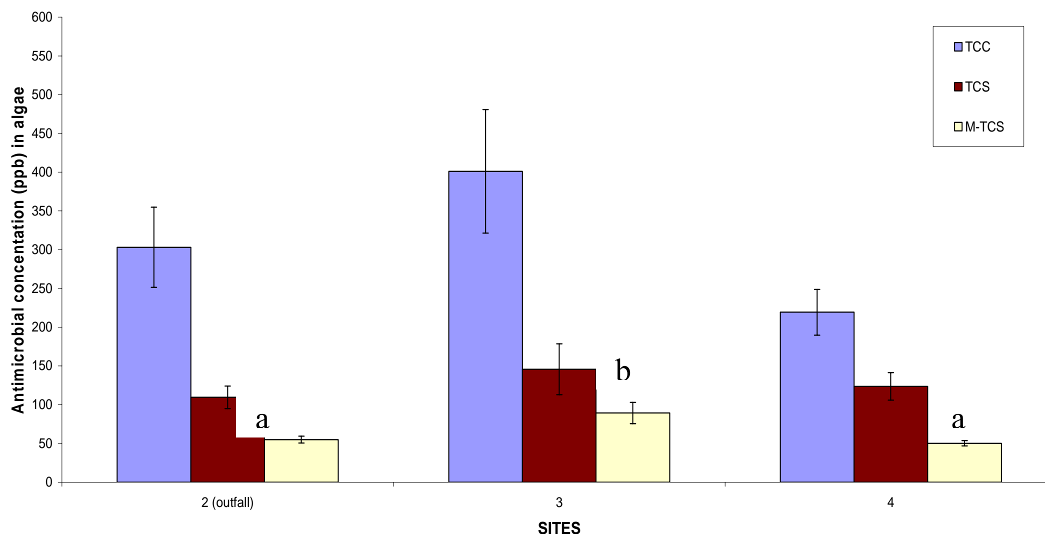

*Fig. 4 Mean and standard errors (one-way ANOVA, alpha = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in algae from three sites on Pecan Creek, TX. Site means for a given antimicrobial (TCC, TCS, M-TCS) with different letters (a, b) are significantly different from the outfall. Concentrations from Site 1 (upstream from the outfall) and Site 5 (below the dam) were below detection limits (see Table 2).

*The somewhat lower TCS, M-TCS, and TCC concentrations within the algal compartment at the outfall in the presence of increased aqueous concentrations may be a result of reduced lipid content within algae nearest the point source of municipal WWTP effluent (Stevenson and Stoermer, 1982). Depending upon nutrient and seasonal fluctuations, large amounts of epiphytic diatoms may be observed on *Cladophora* to the point of exceeding *Cladophora* biomass. Stevenson and Stoermer's investigation of *Cladophora* epiphyte population diversity and abundance supports the conclusion that undetermined factors near a WWTP point source may reduce epiphyte growth in spite of the presence of higher nutrient concentrations.

Antimicrobial Bioaccumulation

*We found mean TCS, M-TCS, and TCC bioaccumulation factors (BAFs) to be approximately three orders of magnitude greater than water concentrations (Table 2).

*Table 2. Algal bioaccumulation factors (BAFs) based on fresh weight.

Contaminant	MEDIUM	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5
TCC	WATER (ppb)	<0.015	0.19	0.16	0.08	<0.015
	ALGAE (ppb)	<10	303	401	219	<10
BAFs			1600	2500	2700	
TCS	WATER (ppb)	<0.01	0.12	0.08	0.06	<0.01
	ALGAE	<10	109	146	124	<10
BAFs			900	1800	2100	
M-TCS	WATER	<0.005	0.08	0.06	0.05	<0.005
	ALGAE	<5	55	89	50	<5
BAFs			700	1500	1000	

TCC BAFs ranged from 1600 at Site 2 to 2700 at Site 4, with a mean BAF value of about 2300. TCS BAFs ranged from 900 at Site 2 to 2100 at Site 4, with a mean BAF value of 1600. M-TCS ranged from 700 at Site 2 to 1500 at Site 3, with a mean BAF value of 1100. The relatively consistent algal concentrations of the three compounds, despite decreasing water concentrations, resulted in a general trend of increasing BAFs at downstream stations (Figure 5).

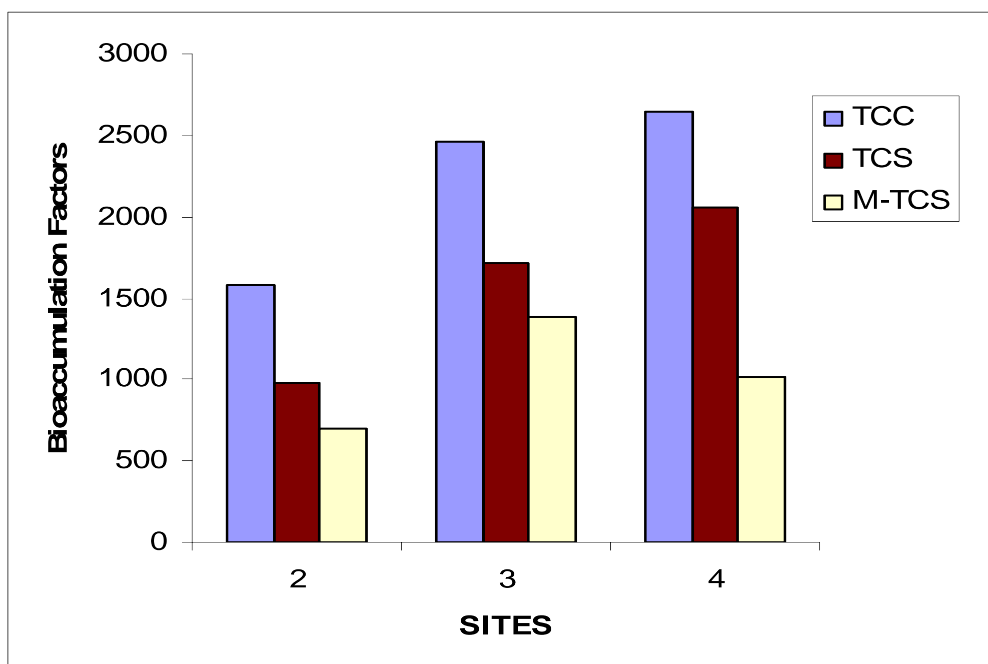

Fig. 5 Algal bioaccumulation factors (BAFs), [algae]/[water], for TCC, TCS, and M- TCS at three selected sites on Pecan Creek, TX.

Discussion

[*]This study explored whether TCS and TCC, along with the degradate M-TCS, bioaccumulate in a freshwater green algal compartment. Our analysis of this surface water downstream of the Denton WWTP confirms the co-occurrence of TCS and TCC previously reported (Halden and Paull, 2005), with TCC values consistently exceeding those of TCS. Bioaccumulation of hydrophobic compounds in phytoplankton has been previously investigated (Jabusch and Swackhamer, 2004). With the ability of certain algal species to produce triacylglycerols comprising up to 60% of their body weight, phytoplankton introduction of persistent bioaccumulative toxic compounds into aquatic food webs and their resultant trophic transfer presents the possibility of toxic exposure concentrations for humans and wildlife (Zaranko et al., 1997; Jabusch and Swackhamer, 2004).

Macroalgae as Biomonitoring Organisms for Bioaccumalative Contaminants

[*]Past studies have indicated significant losses of pharmaceuticals downstream from WWTP discharges, due to degradation, dilution, and sorption (Singer et al., 2002; Boyd et al., 2003). With algal BAF values approximating three orders of magnitude throughout this 3.8 km downstream reach of Pecan Creek (Sites 2 – 4), the potential contribution of antimicrobials to aquatic organism toxicity is worth further investigation. Seasonal water effects regarding receiving stream antimicrobial concentrations have indicated the highest concentrations are reported during summer months (Waltman et al. 2006), which is also the season of rapid growth for many aquatic organisms. Highest levels of trophic transfer for lipophilic compounds may also be seen during this critical time period, resulting in the greatest potential for chemical

movement through both aquatic and terrestrial biota that rely upon the aquatic organism as a food source.

Potential Risks to Algae of Chronic Releases of TCC, TCS, and M-TCS to the Environment

*Aquatic toxicity of TCS has been demonstrated at concentrations higher than that typically found in WWTP receiving streams. Algae are especially sensitive to antimicrobials such as TCS (Orvos et al., 2002; Wilson et al., 2003). The algal species *Scendedesmus subspicatus* was reported to have a 96-h effective concentration (EC50) for growth of 1.4µg l^{-1} and a 96-h no-observed-effect concentration (NOEC) of 0.69 µg l^{-1} (Orvos et al., 2002). With noticeable alterations in algal community structure at TCS concentrations of 0.12µg l^{-1}, further understanding of the effects of antimicrobials on community dynamics may also contribute to the use of algae as xenobiotic bioindicators (Wilson et al., 2003).

Although much less is known about the potential toxicity of M-TCS, information regarding TCC toxicity results can be found in unpublished industry reports. Based on existing data, indications are that algae, represented by *Selenastrum* sp., *Microcystis* sp., and *Navicula* sp., are unaffected at any ppt range (downloaded from TCC Consortium, 2002).

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CHAPTER 3

SNAIL BIOACCUMULATION OF TRICLOCARBAN (TCC), TRICLOSAN (TCS), AND METHYL TRICLOSAN (M-TCS) IN A NORTH TEXAS WASTEWATER TREATMENT PLANT RECEIVING STREAM*

Introduction

Helisoma trivolvis (Say) is an appropriate aquatic snail to use for bioaccumulation studies, and may elucidate the understanding of effluent antimicrobial bioaccumulation when investigated in conjunction with *Cladophora* spp. Along with providing substrate for epiphytic growth, *Cladophora* spp. provide a source of food and shelter for invertebrates such as aquatic snails (Chilton et al., 1986). As a freshwater pulmonate of ubiquitous existence throughout North America, and one that is easily maintained under standard laboratory conditions, *H. trivolvis*, also known as the "ramshorn" snail, is useful in ecological investigations (Thomas et al., 1975; Simonyi-Poirier et al., 2003). Past studies have identified the occurrence of *H. trivolvis* to be within a variety of eutrophic freshwater systems, such as lakes, creeks, swamps and canals (Russell-Hunter et al., 1984).

H. trivolovis contains a radula and gizzard that allow it to graze easily on both periphyton and macroalgal species such as *Cladophora*, which gives this snail a unique place in trophic studies (Smith, 1988; Pennak, 1989; Lombardo and Cooke, 2002). *H. trivolvis* tends to remain sedentary, and therefore has also been successfully used in artificial substrate studies (Simonyi-Poirier et al., 2003). The grazing activities of freshwater snails promote nutrient turnover within algal communities (Wallace and Hutchens, 2000), which may include associated concentrations of environmental pollutants such as triclosan (TCS), triclocarban (TCC), and methyl-triclosan (M-TCS).

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Traditionally, extrapolating invertebrate toxicological data to that of vertebrates has been difficult, due to differences in biological and physiological processes. Chemical fate differs among invertebrates when compared to vertebrates due to the morphology and anatomy of organs; xenobiotic excretion via urine or bile also plays a greater role in toxicant clearance among vertebrates (Lagadic and Caquet, 1998). Invertebrates have, however, been successfully implemented as environmental toxicological test species, leading to the development of various invertebrate bioassays used in addition to those obtained for vertebrates (Lagadic and Caquet, 1998).

As agents of possible bioaccumulation, aquatic organisms have also been shown to accumulate relatively high lipophilic compounds, such as polychlorinated biphenyls (PCBs), at greater concentrations than would be expected from water /tissue partitioning (Evans et al., 1991). Bioaccumulation studies using algae spiked with various concentrations of relatively high log Kow compounds, such as benzo(a)pyrene and polychlorinated biphenyl (PCB) congeners, indicate the Zebra Mussel, *Dreissena polymorpha*, may contribute to the trophic transfer of these environmental pollutants (Bruner et al., 1994).

Another study successfully used *Helisoma trivolvis* and periphyton as test species to investigate dissolved metal retention in Monahan Pond in Kanata, Ontario (Canada), a constructed treatment wetland built in 1995 (Simonyi-Poirier et al., 2003). Although metal retention and lipophilic accumulation are variable within treatment wetlands, further studies using aquatic organisms at the base of the food web may contribute to a better understanding of these dynamics. Chemical uptake among phytoplankton, along with that of macrophytes and filamentous algae such as *Cladophora*, follows first-order kinetics due to the rapid bioaccumulative nature; the movement of lipophilic compounds, such as PCBs, to higher trophic

levels is assisted by the grazing activities of aquatic macroinvertebrates such as chironomids (Zaranko et al., 1997) and possibly snails. If aquatic life, and those organisms that rely on aquatic life as sources of energy, are to be adequately protected, it is imperative that further studies investigating food web relationships and organismal bioenergetics be conducted (Zaranko et al., 1997). The objective of this study was to investigate the potential movement of TCC, TCS, and M-TCS into a trophic level of organisms involved in algal grazing activities at the outfall of the Denton WWWTP.

Materials and Methods

Sources of Chemicals

Labeled internal standards ($^{13}C_{12}$-TCS, $^{13}C_{12}$-M-TCS), TCS, and M-TCS were from Wellington Laboratories (Guelph, ON, Canada). The deuterated TCC (d7 TCC) internal standard was a gift from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), and TCC was from Absolute Standards (Hamden, CT, USA). All other reagents and solvents were from Fischer Scientific (Houston, TX, USA).

Collection and Experimental Design

Clean (non-detectable TCC, TCS, and M-TCS levels) *Helisoma trivolvis* and *Cladophora* spp.samples were collected on 7/09/2006 from the Lewisville Lake Environmental Learning Area (LLELA), Lewisville, TX with GPS coordinates Lat 33.066722 Lon 96.964166. The original site location of the *H. trivolvis* collection was the Lewisville Aquatic Ecosystem Research Facility (LAERF) Potamogeton pond. Snail identification was confirmed by Dr. Robert T. Dillon, Jr., (Department of Biology, College of Charleston, S.C.). *Cladophora*

samples were collected from the Trinity River below the Lewisville Lake dam (Site 5 in Chapter 1). This location was selected since previous site analyses indicated non-detectable levels of TCC, TCS, and M-TCS in samples collected below the Lewisville Lake dam (Coogan et al., 2007). Random sections of each sample were removed and identified as *Cladophora* spp. by microscopic description at 10X magnification using G.W. Prescott's illustrated key (Prescott, 1964). Three 60 ml Nalgene bottles were used to hold *Helisoma trivolvis* replicate samples and three 60 ml Nalgene bottles were used to hold *Cladophora* spp. replicate samples.

Two cages were constructed and placed at the WWTP outfall (Site 2 in Chapter 1) of Pecan Creek, the receiving stream for the City of Denton, Texas, USA WWTP (Figure 6). Cages were constructed from 11.4 cm PVC pipes cut into 30.5 cm lengths. Caps at each end held sections of 1.0 mm mesh in place. Holes were drilled through each cap for the purpose of attaching two one-meter long rebars while in the field in order to secure the cages to the substrate. Each cage contained a 10.2 cm by 20.3 cm "window" that was cut into the center of the 11.4 cm PVC pipe. The window was covered with sections of 1.0 mm mesh adhered with silicon to the PVC pipe (Figure 7). Roughly 40 grams of algae and 42 snails were placed in each cage and left for a two-week total exposure at a depth of approximately 6 cm below the water's surface before chemical analyses were performed (Figure 8). After two weeks, the algae and snails were removed from the cages and placed back in their respective Nalgene bottles. Seven one-liter amber bottles were used to collect replicate grab samples from the surface water. All samples were handled using latex gloves, transported on ice to the laboratory, and maintained refrigerated at 4 °C until analysis. Effluent water quality during collection dates was typical of normal operating conditions of the plant according to Denton WWTP records. Effluent pH averaged 7.4 (range 7.3 – 7.5), flow rates averaged 11.59 million gallons per day (range 10.86 –

12.95), total suspended solids averaged 1.2 mg l^{-1} (range 0.4 – 2.0), and no precipitation was reported for the week prior to collection (personal communication, Gary Stover, City of Denton WWTP operator).

Fig. 6. Caged snail placement just below the outfall of the Denton Wastewater Treatment Plant.

Fig. 7. Photograph showing cage construction.

Fig. 8 Placement of cages containing algae and snails was approximately 6 cm below surface of water.

Snail Lipid Extraction and Analysis

Linear shell measurements were made of maximum shell diameter (MD) in cm, with snail sizes varying from 1.5 cm in diameter to 2.0 cm in diameter. While in the laboratory setting, snails were maintained below 0 °C, following which the shell was broken away from the underlying body tissue using a scalpel, scissors, and forceps (Pennak, 1989). After separation from the shell, the animal was first weighed and once again maintained below 0 °C before further processing. It was determined that 6 to 8 snails provided adequate tissue for each 2.0 g (fresh weight) sample.

Approximately 2.0 g of snail tissue, prepared as described above, were used for each replicate sample analyzed. Eight 2.0 g samples, including a matrix, matrix spike, and matrix spike duplicate, were prepared and analyzed after collection from LAERF and will be referred to as the "Clean Snail" samples. Eight samples were also prepared and analyzed following Pecan Creek field exposure and will be referred to as the "Caged Snail" samples. Each replicate tissue sample was dried with 35.0 g anhydrous Na_2SO_4 by grinding with mortar and pestle then placed in a 25 mm x 90 mm cellulose Soxhlet extraction thimble. Ten µl of 5 ng μl^{-1} $^{13}C_{12}$ TCS, $^{13}C_{12}$ M-TCS, and d7 TCC were added to each sample as internal standards. Soxhlets were heated to boiling for six hours and extracts (100 ml dichloromethane) were stored in amber bottles at 4 °C. Extracts were reduced to 5 ml by Kuderna Danish (KD) evaporation in a water bath at 60 – 70 °C. High molecular weight lipids were removed by gel permeation chromatography (GPC) with an ABC Laboratories (Columbia, MO, USA) Model SP-1000 GPC Processor according to manufacturer's recommended procedures. Samples were evaporated using a Labconco (Kansas City, MO, USA) RapidVap ™ nitrogen evaporator to a final extract volume of 100 – 1000µl and analyzed by gas chromatography-mass spectrometry (GC/MS) for TCS and M-TCS and by

electrospray liquid chromatography-mass spectrometry (ESI-LC/MS) for TCC [see Instrument Analysis section, below].

Algal Lipid Extraction and Analysis

[*]Caged algal samples were separated into 5.0 to 8.0 g sections after hand-washing the contents of each Nalgene bottle in two liter containers of Milli Q 18 MΩ deionized water and removing visibly evident non-filamentous algal particles. The fractionated sections were then transferred to another two liter container of Milli Q 18 MΩ deionized water, hand-agitated for further removal of non-visible particles (Stevenson and Bahls, 1999), and blot-dried with paper towels. Hereafter, blot-dried will be referred to as fresh weight and will apply to all algal concentration references (ie. ng/g fresh weight). For the purpose of this paper, a reference to *Cladophora* spp. samples and the resultant bioaccumulation is actually a reference to a more complex algal compartment of which the greatest biomass contribution is *Cladophora* spp.

[*]Approximately 2.0 g (fresh weight) of algae, prepared as described above, was dried with 35.0 g anhydrous Na_2SO_4 for each replicate analyzed. Algae and Na_2SO_4 were ground with mortar and pestle then placed in a 25 mm x 90 mm cellulose Soxhlet extraction thimble. Ten µl of 5 ng μl^{-1} $^{13}C_{12}$ TCS, $^{13}C_{12}$ M-TCS, and d7 TCC were added to each sample as internal standards. Soxhlets were heated to boiling for six hours and extracts (100 ml dichloromethane) were stored in amber bottles at 4 °C. Extracts were reduced to 5 ml by Kuderna Danish (KD) evaporation in a water bath at 60 – 70 °C. High molecular weight lipids were removed by gel permeation chromatography (GPC) with an ABC Laboratories (Columbia, MO, USA) Model SP-1000 GPC Processor according to manufacturer's recommended procedures. Samples were evaporated using a Labconco (Kansas City, MO, USA) RapidVap ™ nitrogen evaporator to a

final extract volume of 100 – 1000µl and analyzed by gas chromatography-mass spectrometry (GC/MS) for TCS and M-TCS and by electrospray liquid chromatography-mass spectrometry (ESI-LC/MS) for TCC [see Instrument Analysis section, below].

Water Analysis

*Unfiltered one liter water samples were fortified with 10.0 µl of 5 ng μl^{-1} internal standard mix immediately prior to solid phase extraction (SPE). Waters (Milford, MA, USA) Oasis HLB SPE cartridges (1.0 g) were conditioned with 10 ml each of dichloromethane, methanol, and Milli Q 18 MΩ deionized water. Cartridges were eluted with a 20 ml 90:10 (dichloromethane:methanol) solution and resultant extracts were evaporated and analyzed as described above.

Instrumental Analyses

*TCS and M-TCS analyses were conducted on an Agilent (Palo Alto, CA, USA) 6890 GC coupled with a 5973 mass selective detector MS (70-eV). An eight point standard curve was established with analyte concentrations from 5-1,000 pg μl^{-1} and internal standard concentrations at 500 pg μl^{-1}. The MS was operated in the single ion monitoring mode (SIM) with target and 3 confirmatory masses monitored (50 msec dwell time) for each compound. GC conditions were helium carrier gas at 480 hPa, inlet temperature at 260 °C (2µl, pulsed pressure at 1,700 hPa for 0.5 min, splitless injection), and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 µm film) temperature initially at 40 °C with a 1-min hold followed by a 50 °C min^{-1} ramp to 140 °C with a 5- min hold followed by a 10 °C min^{-1} ramp to 300 °C with a final 17-min bake-out. Transfer line temperature was 265 °C.

*TCC was analyzed using a modification of the recently reported LC-ESI-MS method (Halden and Paull, 2004; Halden and Paull, 2005). An Agilent 1100 LC/MS system was used with Model SL ion trap MS. The column was a C18 (monomeric, non-endcapped) Zorbax (2.1 X 150 mm) with a 5 µm particle size and 80 Å pore size. Samples were autoinjected (1-5 µl) with a gradient program (300 µl min^{-1} flow throughout) that was initiated at 70% mobile phase B (95% acetonitrile and 5% water with 5 mM ammonium acetate) and 30% mobile phase A (95% water and 5% acetonitrile with 5 mM ammonium acetate), held for 1.0 min then ramped to 85% B at 10 min and 100% B at 10.1 min through 25 min, then returned to 70% B from 25.1 to 35 min. The ion trap was operated in the negative ion multireaction monitoring mode (MRM) isolating m/z 313 and 315 for native TCC and m/z 320/322 for d_7 TCC internal standard. These isolated pseudo-molecular ions ($[M-H]^-$) were fragmented (amplitude 0.8) to yield daughter ions at m/z 160 and 163 for native and d7 TCC, respectively. Five point standard curves were established for both the pseudo-molecular ions and the daughter ions with TCC concentrations from 10-1,000 pg μl^{-1} and d_7 TCC concentration of 50 pg μl^{-1}. The electrospray was operated with a nebulizer pressure of 2,100 hPa and nitrogen dry gas at eight l min^{-1} at 350 °C. TCC and d_7 TCC eluted at 7.5 min.

Practical quantitation limits (PQL, Table 3) were established at approximately 10X the instrument detection limit, which was estimated as 3X S.D. of background noise levels for quantitation ions. Standard curves included PQL concentrations for each analyte.

Statistical Analyses

Snail data collected before the two week exposure to effluent waters (Clean Snails) were compared to snail data collected after the two week exposure (Caged Snails) and analyzed by T-Test for statistical significance (Microsoft Excel, Microsoft Office XP, Copyright Microsoft

Corporation 1985-2001). The level of significance for all analyses was $\alpha = 0.05$.

Results

Analytical Quality Control (QC) Results

Recovery percentages for blank spike additions (50 ng/l for water samples and 25 ng/g for algal and snail samples) ranged from 87% to 127%, with a mean of 113% for water analyses, from 103% to 115%, with a mean of 108% for algal analyses, from 93% to 127% with a mean of 106% for Clean Snail analyses, and from 102% to 114% with a mean of 106% for Caged Snail analyses (Table 3). Water column matrix spike and matrix spike duplicate recovery percentages ranged from 91% to 110%, with a mean of 98%, from 73% to 140% for algal analyses, with a mean of 105%, from 88% to 143% for Clean Snail analyses, with a mean of 108%, and from 44% to 114% for Caged Snail analyses, with a mean of 91%. Overall, percent deviation for water column, algal, Clean Snail, and Caged Snail analyses of matrix spike duplicates ranged from 0.53% to 62.8%, with a mean of 14.1%. No blank values exceeded the PQLs.

Table 3. Quality control data for antimicrobials in water, algae, and snails. Spike additions were at 50 ng L^{-1} for water and 25 ng g^{-1} for algae and snails.

		Blk (ppb)	Blk Spk Rec (%)	M S Rec (%)	M S Dup Rec (%)	M S Dup Dev (%)	PQL (ppb)
Water	TCS	< 0.010	87	96.6	90.9	6.1	0.01
	M-TCS	< 0.005	96.8	96.4	92.7	3.9	0.005
	TCC	< 0.015	127.4	113.1	95.6	16.8	0.015
Algae	TCS	< 10.00	105.2	82.4	114.1	32.3	10
	M-TCS	< 5.000	103.1	100.7	121.3	18.6	5
	TCC	< 10.00	115.2	73	139.8	62.8	10
Clean Snails	TCS	< 10.00	93.2	142.9	119.8	1.8	10
	M-TCS	< 5.000	96.6	87.6	88.1	0.53	5
	TCC	< 10.00	127.1	108.3	100.3	7.6	10
Caged Snails	TCS	< 10.00	113.8	85.2	44.4	12	10
	M-TCS	< 5.000	102.2	109.1	114.1	4.5	5
	TCC	< 10.00	102.4	96.2	99	2.8	10

(Blk = Blank, Spk = Spike, Rec = Recovery, Mat = Matrix, Dup = Duplicate, Dev = Deviation, PQL = Practical Quantitation Limits)

Snail Tissue Analyses

Statistically significant differences for TCS, TCC, and M-TCS concentrations (ppb) in snail tissue occurred after being caged for two weeks in Pecan Creek at the Denton WWTP outfall (Figure 9). TCS Clean Snail concentrations were measured at 5.9 ppb net weight and increased to 58.7 ppb in Caged Snail tissue. TCC Clean Snail concentrations were measured at 9.8 ppb and increased to 299.3 ppb in Caged Snail tissue. M-TCS Clean Snail concentrations were measured at 0.8 ppb and increased to 49.8 ppb in Caged Snail tissue (Table 4).

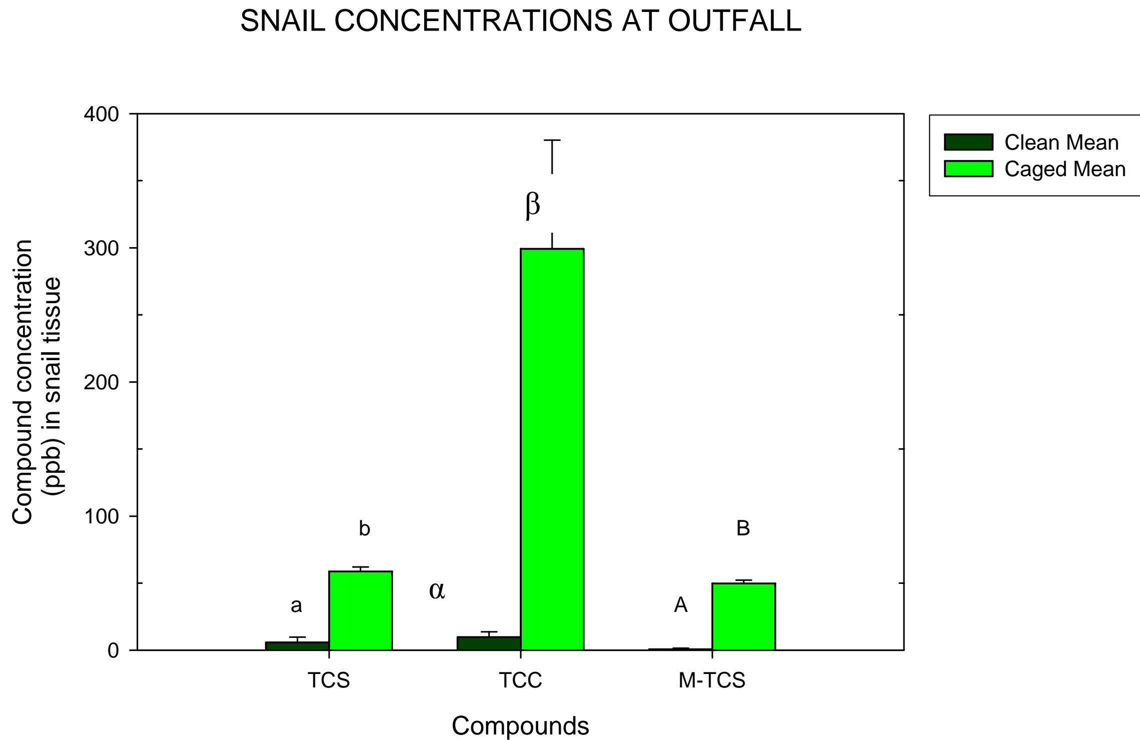

Fig. 9 Means and standard errors (α = 0.05, n = 5) of TCC, TCS and M-TCS concentrations in Clean and Caged Snails from the Denton WWTP outfall on Pecan Creek, TX. Histograms do not compare compounds, but compare means within each compound. Means for a given compound with different corresponding letters are significantly different (TCS – a, b; TCC – α, β; M-TCS – A, B).

Table 4. Clean and caged snails replicate concentrations (ppb) at each collection site and calculated means for TCS, M-TCS, and TCC.

Contaminant	Medium	A (ng/g)	B (ng/g)	C (ng/g)	D (ng/g)	E (ng/g)	Mean (ng/g)
TCS	Clean Snails	11.4	0	18.3	0	0	5.9
	Caged Snails	62.2	49	65.4	52.2	64.6	58.7
M-TCS	Clean Snails	3.9	0	0	0	0	0.77
	Caged Snails	53.5	45.4	53.8	54	42.2	49.8
TCC	Clean Snails	5.4	5.2	25.6	5.2	7.4	9.8
	Caged Snails	170.6	228.9	310.2	179.2	607.6	299.3

Water and Algal Tissue Analyses

TCS water concentrations from samples collected at the Denton WWTP outfall were measured at 0.11 ppb (Figure 10). A concentration of 162.5 ppb was detected in algal tissue that had been caged and grazed for two weeks at the same location (Figure 11). TCC water concentrations were measured at 0.19 ppb and increased to 367.3 ppb in algal tissue. M-TCS water concentrations were measured at 0.04 ppb and increased to 50.4 ppb in algal tissue (Table 5).

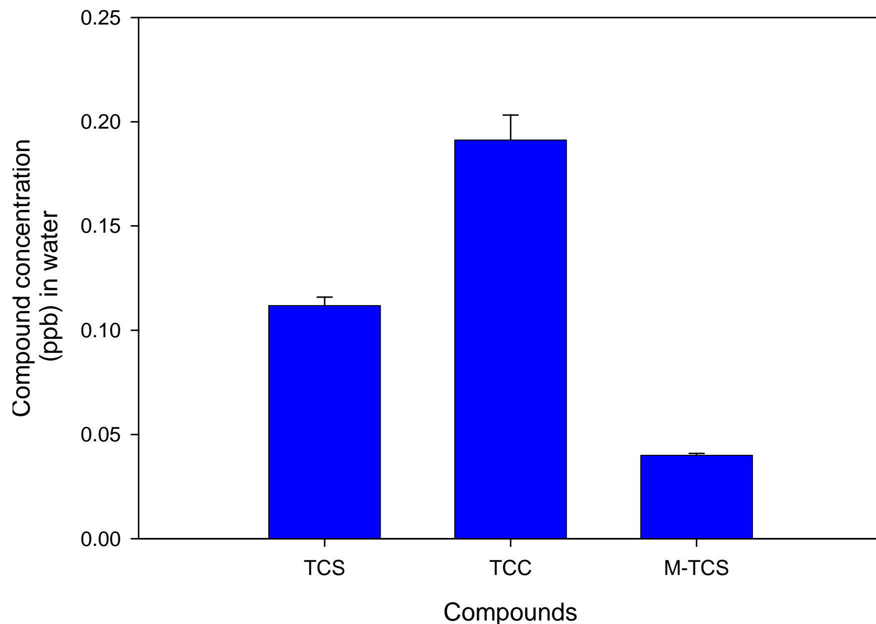

Fig. 10 Means and standard errors (n = 5) of TCC, TCS and M-TCS concentrations (ppb) in water samples from the Denton WWTP outfall on Pecan Creek, TX.

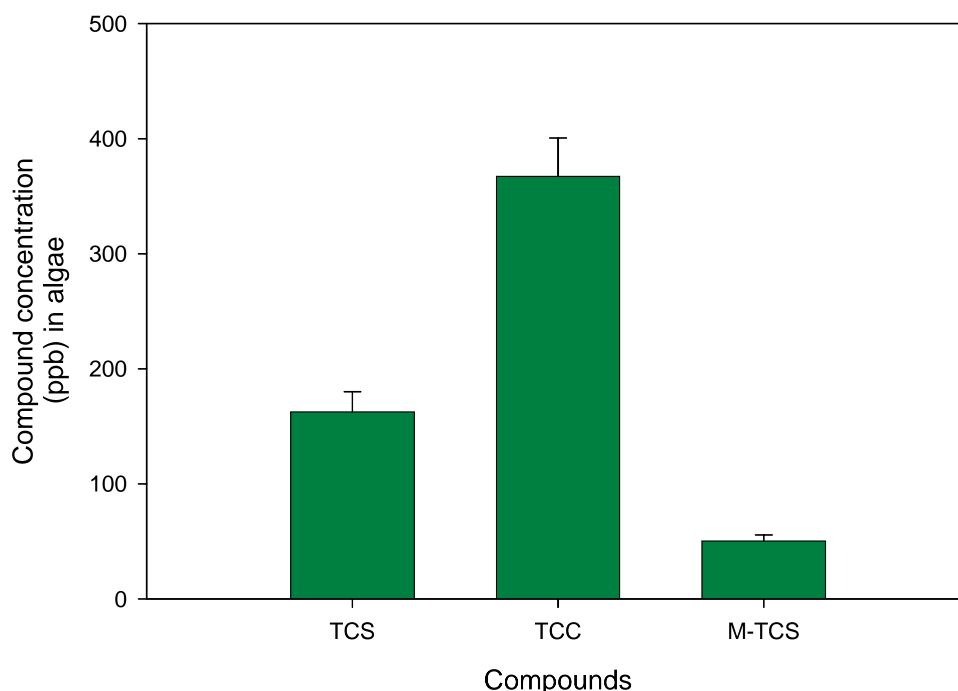

Fig. 11. Means and standard errors (n = 5) of TCC, TCS and M-TCS concentrations (ppb) in algal samples from the Denton WWTP outfall on Pecan Creek, TX.

Table 5. Replicate aqueous and algal concentrations (ppb) and calculated means for TCS, M-TCS, and TCC.

Contaminant	**Medium**	**A**	**B**	**C**	**D**	**E**	**Mean**
TCS	Water (µg/L)	0.117	0.118	0.102	0.12	0.102	0.112
	Algae (ng/g)	132.8	125.8	144	205	205	162.5
M-TCS	Water (µg/L)	0.042	0.042	0.039	0.037	0.04	0.04
	Algae (ng/g)	42	35.5	51.4	61.9	61.1	50.38
TCC	Water (µg/L)	0.194	0.189	0.18	0.16	0.233	0.191
	Algae (ng/g)	356.8	340.4	358.8	290	490.4	367.3

Antimicrobial Bioaccumulation

*We found mean TCC and M-TCS bioaccumulation factors (BAFs) to be approximately three orders of magnitude greater in the Caged Snail samples collected from the DWWTP outfall. The TCS BAF results were not as great as the other compounds of interest, but maintained a relatively high value of approximately 500. The TCC BAF result was 1600 and the

M-TCS BAF result was 1200 (Table 6). The relatively high concentrations of TCC measured in snail tissue and relatively low TCS concentrations measured in snail tissue resulted in the comparatively reduced BAF value of TCS. The relatively low aqueous concentrations of M-TCS measured resulted in a mid-range BAF value (Figure 12).

Table 6. Snail bioaccumulation factors (BAFs) based on antimicrobial concentrations (ppb).

Contaminant	Medium	Results
TCC	Water (ppb)	0.19
	Snails (ppb)	299.3
BAF		1600
TCS	Water (ppb)	0.11
	Snails (ppb)	58.7
BAF		500
M-TCS	Water (ppb)	0.04
	Snails (ppb)	49.8
BAF		1200

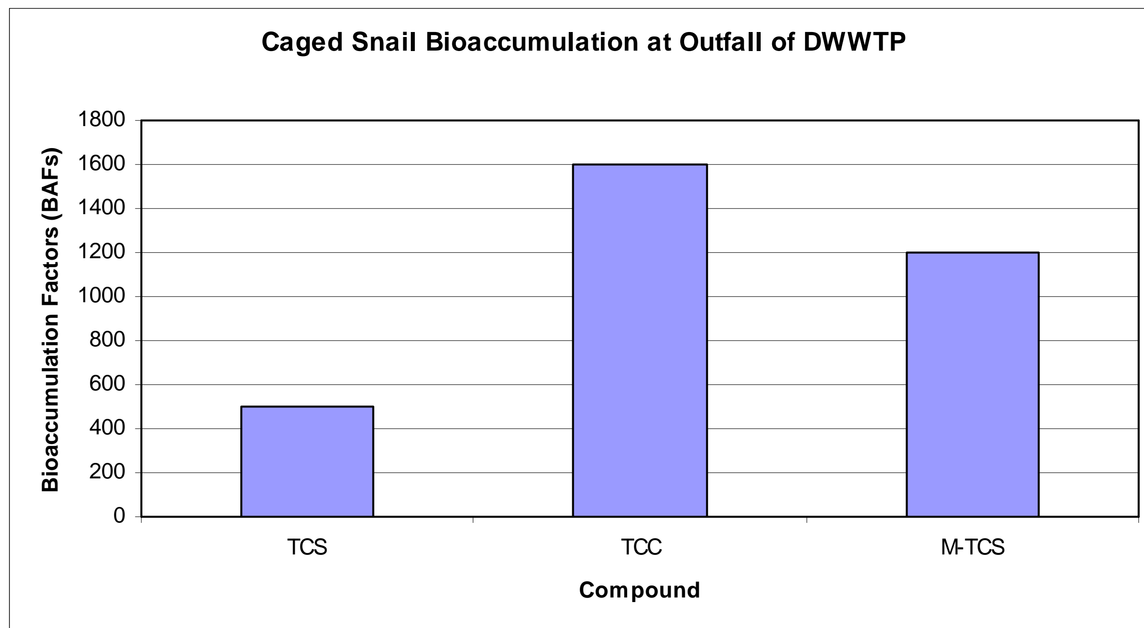

Fig. 12 Caged snail bioaccumulation factors (BAFs) for TCS, TCC, and M-TCS at the Denton WWTP outfall on Pecan Creek, TX.

Discussion

This study explored whether TCS and TCC, along with the degradate M-TCS, bioaccumulate in snail tissue. Our analyses of *H. trivolvis* and *Cladphora* spp. following a two week period of being caged and immersed in the WWTP receiving stream system confirm the co-occurrence of TCS and TCC previously reported (Halden and Paull, 2005), with TCC values consistently exceeding those of TCS. Snail BAFs were approximately three orders of magnitude for TCC, TCS, and M-TCS. The algal BAF for TCC was the same as was reported in Chapter 1, with TCS values reduced almost by half and M-TCS values almost doubled. Considering the biota measured in the snail bioaccumulation study had been caged, as opposed to being allowed to grow under normal field conditions, the results may have been affected. Grazing studies conducted by Hunter (1980) with *Lymnaea elodes* indicated grazing has the capability of altering algal standing crop community composition by removing large epiphytic diatoms and allowing smaller diatoms, such as *Gomphonema* and *Cocconeis*, to flourish (Hunter, 1980).

Aquatic Snails as Biomonitoring Organisms for Bioaccumalative Contaminants

The interactions between primary producers and their consumers provide an important foundation for bottom-up control of aquatic food webs (Lamberti et al., 1989). Since a large percentage of aquatic invertebrates (and fish) consume periphyton during some portion of their life stages (Lamberti, 1996), it would follow that studies investigating bioaccumulation of pollutants, such as hydrophobic organic contaminants (HOCs), at the base of the aquatic food web should be conducted. Better understanding of this entry point is needed, since factors influencing uptake are not well understood (Wallberg et al., 2001). How these pollutants are

partitioned among various plankton within aquatic systems probably influences water column residence time and potential trophic transfer (Wallberg et al., 2001).

Several aquatic snail bioaccumulation studies investigating uptake of various hydrophilic and hydrophobic compounds have been previously conducted. *Helisoma trivolvis*, in conjunction with periphyton, was successfully used in a constructed treatment wetland study investigating dissolved metal retention (Simonyi-Poirier et al., 2003). Cadmium and lead bioaccumulation potentials have been investigated by using the snail *Physaintegra* (Spehar et al., 1978). Arsenic and chromium bioaccumulation potentials in eipgean and hypogean freshwater macroinvertebrates were studied by using the freshwater snail *Physa fontinalis* (Canivet et al., 2001). Environmental studies using the snail *Helisoma* have also been conducted to determine the bioaccumulation potential of hexachlorobenzene in aquatic systems (Isensee et al., 1976). This study is the first to investigate the bioaccumulation potential of TCC, TCS, and M-TCS by using the aquatic snail *Helisoma trivolvis*.

Potential Risks to Animals of Chronic TCC, TCS, and M-TCS Releases to the Environment

As was indicated in Chapter 1, "With the ability of certain algal species to produce triacylglycerols comprising up to 60% of their body weight, phytoplankton introduction of persistent bioaccumulative toxic compounds into aquatic food webs and their resultant trophic transfer presents the possibility of toxic exposure concentrations for humans and wildlife (Zaranko et al., 1997; Jabusch and Swackhamer, 2004)." [1]TCS aquatic toxicity studies have indicated rainbow trout have a reported median EC50 of 350 $\mu g\ l^{-1}$ and a NOEC of 34 $\mu g\ l^{-1}$ (Balmer et al., 2004). TCS levels up to 47 $mg\ kg^{-1}$ fresh weight have been discovered in the bile of fish living in several Swedish WWTP receiving waters. The same study identified high TCS

levels among three of five randomly selected human milk samples, with one sample as high as 300 µg kg^{-1} lipid weight (Adolfsson-Erici et al., 2002). The mechanism of toxicity in metazoans has received little attention but a recent study has indicated TCS induces mitochondrial depolarization and impairment of energy metabolism in animals cells at concentrations greataer than 10 nmol mg^{-1} (Newton et al., 2005) as well as inhibition of sulfotransferases important in phase II detoxification mechanisms (Wang and James, 2006).

*Possible bacterial modes of action of TCS include the blockage of lipid synthesis. The trans-2-enoyl-ACP reductase in *E. coli*, known as FabI, regulates fatty acid synthesis and is inhibited by TCS (Sivaraman et al., 2004). This has led to concern that chronic exposure of natural bacterial populations in receiving streams might lead to development of strains cross-resistant to antibiotics. Strain-specific cross-resistance to chloramphenicol has been verified by TCS-adapted *E. coli* K-12, trimethoprim by *E. coli* O55, and chloramphenicol, tetracycline, amoxicillin, trimethorim, benzalkonium chloride, and chlorohexidine by *E. coli* O157:H7 (Braoudaki and Hilton, 2004).

*As was indicated in Chapter 1, much less is known about the potential toxicity of M-TCS. Environmental levels are similar to other persistent chlorinated trace organic pollutants. With a log K_{ow} value of 5.2, M-TCS persistence suggests a relatively high bioaccumulation potential in fish and other aquatic organisms (Balmer et al., 2004). Bioaccumulation of M-TCS was confirmed in a Lake Griefensee study in which fish tissue concentrations ranged from 165 – 300 ng g^{-1} lipid when compared to lake water concentrations of 0.8 -1.2 ng l^{-1} (Balmer et al., 2004). Our results indicating increased dominance of M-TCS in algal tissues were similar to those found in a retrospective study of fish tissues from German rivers contaminated by WWTP effluent (Boehmer et al., 2004).

[*]As was also indicated in Chapter 1, TCC toxicity results are limited to unpublished industry reports. For aquatic insects, the existing few data indicate no toxic effects in the ppt range for freshwater invertebrates, but chronic reproduction effects for estuarine mysid shrimp are reported in the environmentally relevant range of 60 to 125 ppt (downloaded from TCC Consortium, 2002). TCC toxicity has been reported for a variety of mammals. Reproduction and offspring survival rates decrease in rats and rabbits in response to elevated TCC levels. Additionally, TCC is known to cause methemoglobinemia ("Blue Baby" Syndrome) in humans (Johnson et al., 1963; Nolen and Dierckman, 1979). Cleavage of the carbon-nitrogen bonds and resultant release of *N*-hydroxylated metabolites at elevated pH and temperature results in primary aromatic amine production and increased incidence of methemolgobinemia (Johnson et al., 1963; Ponte, 1974). Mono- and dichlorinated anilines, environmentally persistent TCC breakdown products, are also known to express ecotoxicity, genotoxicity, and hematotoxicity (Gledhill, 1975; Boehncke et al., 2003). For aquatic insects, no toxic effects in the ppt range for freshwater invertebrates are indicted, but chronic reproduction effects for mysid shrimp are reported in the environmentally relevant range of 60 to 125 ppt (downloaded from TCC Consortium, 2002).

With multiple methods of stream analyses available, it could be difficult to determine which is most beneficial. Evolution has established a unique relationship between riparian vegetation and stream invertebrates (Cummins et al., 1989). A well-established relationship such as this could provide either a foundation or a complement to municipal receiving stream evaluations.

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CHAPTER 4

EFFECTS OF TRICLOSAN (TCS) ON ALGAL LIPID SYNTHESIS*

Introduction

Whole effluent toxicity (WET) tests, better known as "kill 'em and count 'em" tests, have been the traditional method of assessing aquatic system health among wastewater treatment plant (WWTP) receiving streams. Unfortunately, with the consistent application of pharmaceuticals and personal care products (PPCPs) into municipal receiving stream systems, these methods of assessment may produce results well after irreversible habitat damage and potential cascading effects have occurred. More sensitive evaluation methods, which require a better understanding of subtle biochemical or molecular biological effects, in conjunction with traditional WET tests are needed to help determine future health and to determine which regulatory measures are required to protect these delicate aquatic systems (Chapman, 2000).

The significance of this study is in the development of aquatic toxicological bioassay methods with algae, which could be beneficial to monitoring effluent quality in the presence of specific emerging contaminants such as triclosan (TCS). As primary producers with unique characteristics, algae are the base of most aquatic food webs and provide valuable food web links, along with lipophilic xenobiotic uptake capabilities (Haglund, 1997). Among all aquatic organisms used to study toxic effects of (TCS), algae and cyanobacteria appear to express the greatest responses at the lowest concentrations (Orvos et al., 2002). Results indicate that algae exposed to TCS at levels of 3.4, 6.3, and 13 $\mu g\ l^{-1}$ for 4 days result in impaired growth, but not death (Orvos et al., 2002). Further studies have confirmed that a specific green algal species,

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Scenedesmus subspicatus, is particularly susceptible to the presence of TCS in surface waters, with a no observed effect concentration of 500 ng l^{-1} (Singer et al., 2002).

*Possible bacterial modes of action of TCS include the blockage of lipid synthesis. The trans-2-enoyl-ACP reductase in *E. coli*, known as FabI, regulates fatty acid synthesis and is inhibited by TCS (Sivaraman et al., 2004). This has led to the concern that chronic exposure of natural bacterial populations in receiving streams might lead to development of strains cross-resistant to antibiotics. Strain-specific cross-resistance to chloramphenicol has been verified by TCS-adapted *E. coli* K-12, trimethoprim by *E. coli* O55, and chloramphenicol, tetracycline, amoxicillin, trimethorim, benzalkonium chloride, and chlorohexidine by *E. coli* O157:H7 (Braoudaki and Hilton, 2004).

Plants share similar fatty acid synthesis pathways with bacteria, and Arabidopsis has been used to verify that enoyl ACP reductase is also a possible TCS target with inhibitory effects reported at dose concentrations between 0.1 and 20 ppm (Serano et al., 2006). Plastids are the location for fatty acid synthesis in plants, and since plant plastids are thought to have originated from a photosynthetic symbiont, this shared pathway seems logical (Buchanan et al., 2000). It has, in fact, been shown that plant enoyl ACP reductase can successfully replace the rate-limiting FAS II complex subunit in *E. coli* (Kater et al., 1994).

Plant fatty acid synthesis involves two main enzyme complexes named acetyl-CoA carboxylase (ACCase) and the fatty acid synthase complex (FAS II) (Serano et al., 2006). ATP is required for the initial carboxylation step within fatty acid biosynthesis when acetyl-CoA forms malonyl-CoA, which then elongates the fatty acid by adding 2-carbon units to the acyl chain (Buchanan et al., 2000; Poghosyan et al., 2005). The free acetate needed for fatty acid synthesis is generated outside the chloroplast. Since acetyl-CoA is not able to cross membranes,

it is generally accepted that acetate is directly taken into the chloroplast by acetyl CoA synthetase for fatty acid production (Buchanan et al., 2000). When the *MOD1* gene, which encodes an enoyl ACP reductase in Arabidopsis, is substituted with the *mod1* mutant, fatty acid biosynthesis and total lipid content is reduced, resulting in abnormal development and spontaneous cell death (Mou et al., 2000). Using this information, Serrano et al. developed a study that confirmed the Arabidopsis *MOD1* gene as being the probable target of TCS (Serrano et al., 2006).

Similar TCS effects among algal species found within municipal receiving streams could potentially have detrimental consequences for food quality and growth among those consumers that rely upon algae as their primary source of nutrition. If consumer growth is limited due to poor nutrient concentrations among their prey, trophic cascade strength is greatly affected (Huxel, 1999). Food chain models have indicated system dynamics at the producer level have the capability of being limited by the availability of nutrients (DeAngelis, 1992; Holt et al., 1994). As a result, the concentrations of aquatic system nutrients, such as lipids, and their availability to biota may serve to regulate population growth and reproduction of herbivores (Boersma and Claus-Peter, 2000), with the overall effect being a potential alteration of system stability.

While the importance of algal food quality to an aquatic system has been verified (Sterner and Schulz, 1998), further studies are needed to determine what combinations of factors have the greatest impact on algal food quality within these aquatic systems (Brett, 1993; Hessen, 1993; Urabe and Watanabe, 1993; Muller-Navarra, 1995; Gulati and DeMott, 1997; Brett et al., 2000). The objective of this study was to investigate algal lipid inhibition effects of toxic TCS concentrations.

Materials and Methods

Sources of Chemicals for TCS Analyses

Labeled internal standard ($^{13}C_{12}$-TCS and $^{13}C_{12}$-M-TCS), TCS, and M-TCS were obtained from Wellington Laboratories (Guelph, ON, Canada). Chu *Cladophora* growth media was from UTEX; University of Texas at Austin Culture Collection (Austin, TX). All other reagents and solvents were from Fischer Scientific (Houston, TX, USA).

Sources of Chemicals for [2-^{14}C]acetate Radiolabeling

NEC-085D acetic acid, sodium salt, [2-^{14}C] (54 mCi mmol^{-1}) was ordered from NEN™ Life Science Products, Inc., Boston, MA. Chu algal culture media was ordered from UTEX; University of Texas at Austin Culture Collection (Austin, TX, USA), and DMSO was ordered from Fischer Scientific (Houston, TX, USA).

Collection and Experimental Design

Cladophora spp. field samples were collected on 3/14/2007 from the Lewisville Lake Environmental Learning Area (LLELA), Lewisville, TX with GPS coordinates Lat 33.066722 Lon 96.964166. The original site location was the Lewisville Aquatic Ecosystem Research Facility (LAERF) Potamogeton growth pond, which is filled daily with Lewisville Lake water. This location was selected since previous site analyses indicated non-detectable aqueous and algal concentration levels of TCS and M-TCS in samples collected below the Lewisville Lake dam (Coogan et al., 2007). Random sections of each sample were removed and identified as *Cladophora* spp. by microscopic description at 10X magnification using G.W. Prescott's

illustrated key (Prescott, 1964). Three 60 ml Nalgene bottles were used to hold *Cladophora* spp. replicate samples, which were kept on ice during transport.

Algal Lipid Extraction and Analysis

[*]Algal samples were field-collected prior to each analysis. Samples were combined after hand-washing the contents of each Nalgene bottle in two liter containers of Milli Q 18 MΩ deionized water and removing visibly evident non-filamentous algal particles. The fractionated sections were then transferred to another two liter container of Milli Q 18 MΩ deionized water, hand-agitated for further removal of non-visible particles (Stevenson and Bahls, 1999), and blot-dried with paper towels. Hereafter, blot-dried will be referred to as fresh weight and will apply to all algal concentration references (ie. ng/g fresh weight). For the purpose of this paper, a reference to *Cladophora* spp. samples and the resultant bioaccumulation is actually a reference to a more complex algal compartment of which the greatest biomass contribution (determined visually to be at least 90%) is *Cladophora* spp.

Stock solution of 1000 ppm [TCS] was prepared for exposures of algal field samples by dissolving 10 mg "neat" TCS into 10 ml dimethyl sulfoxide (DMSO). A pilot project was conducted to determine lipid mass, GC response, and M-TCS detection by using 100 mg fresh weight algal samples. Six algal samples, prepared as described above, were measured and separated into three [TCS] categories for separate dosing regimes (0 ppm, 1 ppm, and 10 ppm) to determine algal lipid bioaccumulation. The 0 ppm samples were placed in test tubes containing 2.0 ml Chu media and 20 µl DMSO as a control (Figure 13), 1 ppm samples in test tubes containing 2.0 ml Chu media and 2.0 µl TCS stock solution (Figure 14), and the 10 ppm samples in test tubes containing 2.0 ml Chu media and 20 µl TCS stock solution (Figure 15).

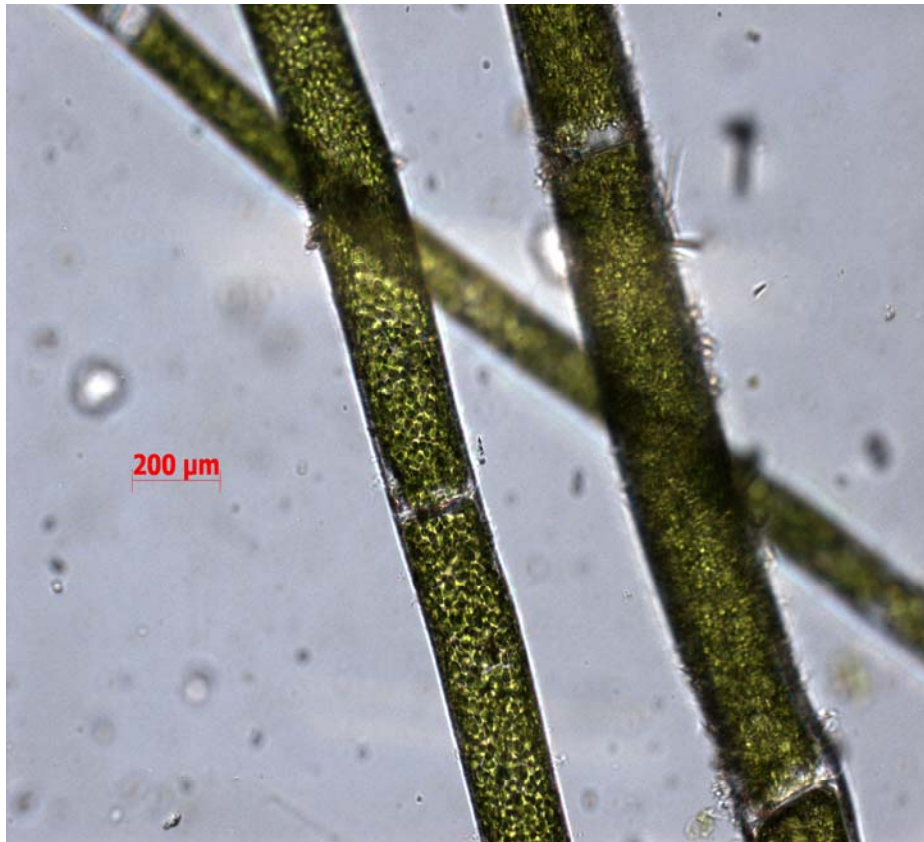

Fig. 13. Photograph of *Cladophora* spp. after 4 hour incubation period in 0 ppm [TCS].

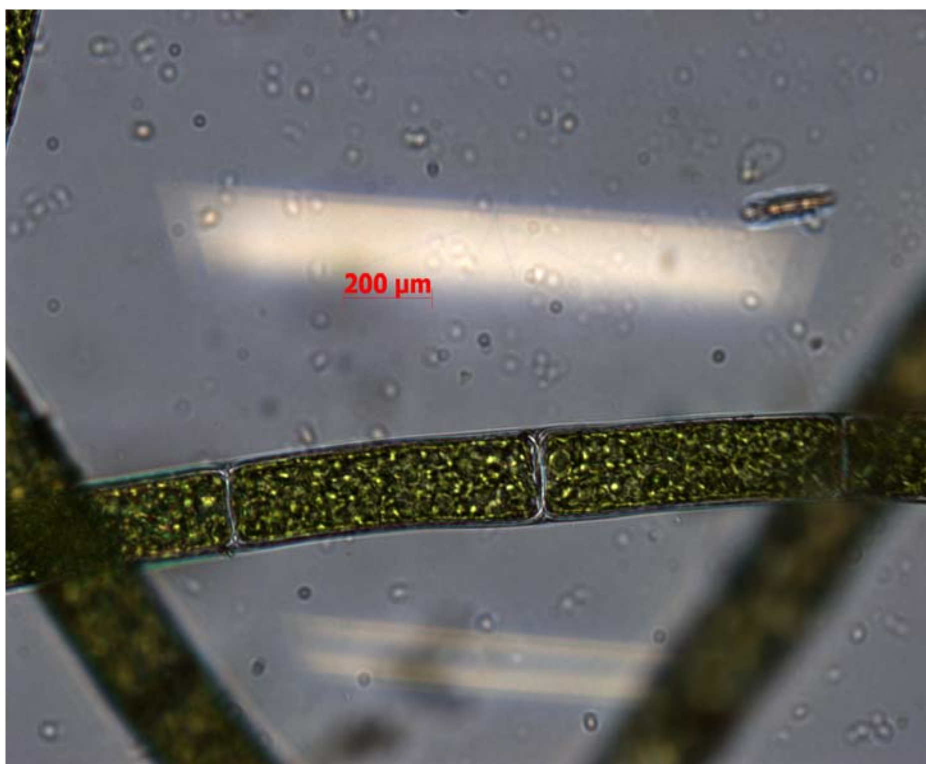

Fig. 14. Photograph of *Cladophora* spp. after 4 hour incubation period in 1 ppm [TCS].

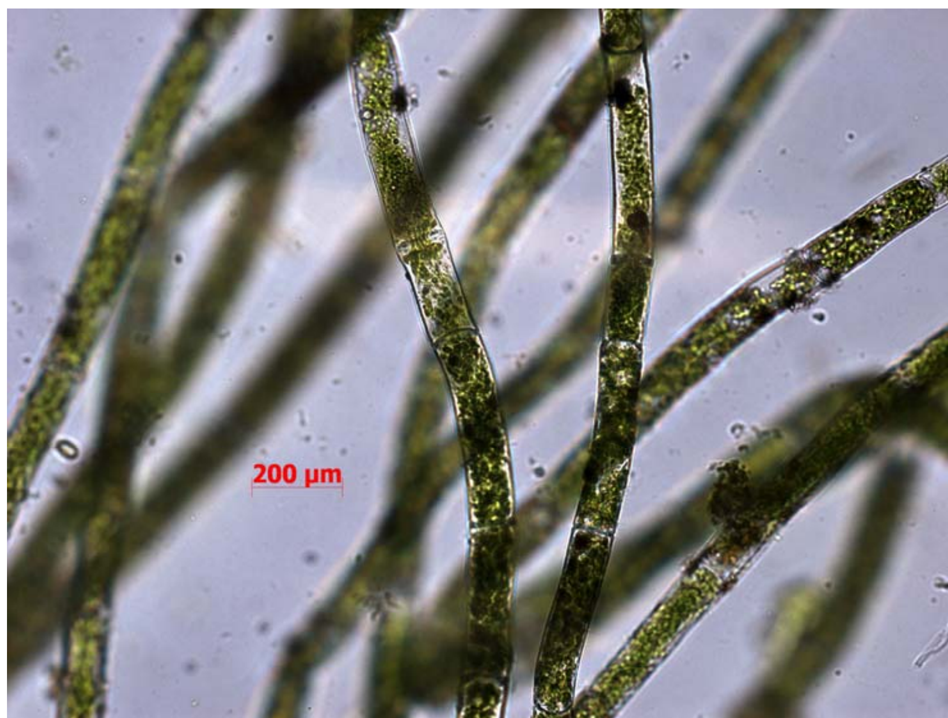

Fig. 15. Photograph of *Cladophora* spp. after 4 hour incubation period in 10 ppm [TCS].

Algal photographs and visual observations were made following incubation with use of a Zeiss Axio Imager.A1, Transmitted Light Microscope, 200X Magnification (Carl Zeiss, Germany). After vortexing, samples were allowed to incubate for three hours before further processing. Following incubation, algal samples were compressed against the inside of each test tube with a glass rod to remove excess solvent before being removed and placed in separate 5.0 ml Conical Screw Cap Microtubes (Quality Scientific Plastics). A three ml solution of 1:1, hexane:ethylacetate was added to each microtube along with 10 µl of 5 ppm TCS and 10 µl of 5 ppm M-TCS internal standards. Approximately 10 – 15 glass beads measuring 2.5 mm in diameter were poured into each tube. Tubes were then capped and placed in a Minibeadbeater™ (Biospec Products) at high speed for three minutes. Each homogenized product was poured into a Whatman Autovial 5 containing PTFE Filter Media with Polypropylene Housing and a 0.45 µm pore size, filtered into pre-weighed glass vials, and rinsed

with 100 µl solution of 1:1, hexane:ethylacetate. Samples were evaporated to dryness by nitrogen gas, weighed to obtain lipid masses, re-suspended in dichloromethane to a final extract volume of 100 µl, and analyzed by gas chromatography-mass spectrometry (GC/MS) for TCS and M-TCS.

Once results were confirmed from the pilot project, fifteen samples containing approximately 10.0 mg (fresh weight) algae, prepared from field samples as described in the Algal Lipid Extraction and Analysis section in chapter 2 (1st paragraph), were measured and separated into three [TCS] categories for separate dosing regimes (0 ppm, 1 ppm, and 10 ppm) to determine algal lipid bioaccumulation. Three additional samples were also processed for Quality Control analyses. The 0 ppm samples were placed in test tubes containing 2.0 ml Chu media and 20 µl DMSO as a control, 1 ppm samples in test tubes containing 2.0 ml Chu media and 2.0 µl TCS stock solution, and the 10 ppm samples in test tubes containing 2.0 ml Chu 2.0 µl TCS stock solution, and the 10 ppm samples in test tubes containing 2.0 ml Chu media and 20 µl TCS stock solution. After vortexing, samples were allowed to incubate for three hours before further processing. Following incubation, algal samples were compressed against the inside of each test tube with a glass rod to remove excess solvent before being removed and placed in separate 2.0 ml Conical Screw Cap Microtubes (Quality Scientific Plastics). A one ml solution of 1:1, hexane:ethylacetate was added to each microtube along with 100 µl of 5 ppm TCS as an internal standard. Additionally, 100 µl of 5 ppm native TCS was added to the blank spike, matrix spike, and matrix spike duplicate. Approximately 10 – 15 glass beads measuring 2.5 mm in diameter were poured into each tube. Tubes were then capped and placed in a Minibeadbeater™ (Biospec Products) at high speed for three minutes. Each homogenized product was poured into a Whatman Autovial 5 containing PTFE Filter Media with

Polypropylene Housing and a 0.45 μm pore size, filtered into pre-weighed glass vials, and rinsed with 100 μl solution of 1:1, hexane:ethylacetate. Samples were evaporated to dryness by nitrogen gas, weighed to obtain lipid masses, re-suspended in dichloromethane to a final extract volume of l ml, and analyzed by gas chromatography-mass spectrometry (GC/MS) for TCS.

Instrumental Analyses

*TCS and M-TCS analyses were conducted on an Agilent (Palo Alto, CA, USA) 6890 GC coupled with a 5973 mass selective detector MS (70-eV). An eight point standard curve was established with analyte concentrations from 5-1,000 pg μl^{-1} and internal standard concentrations at 500 pg μl^{-1}. The MS was operated in the single ion monitoring mode (SIM) with target and 3 confirmatory masses monitored (50 msec dwell time) for each compound. GC conditions were helium carrier gas at 480 hPa, inlet temperature at 260 °C (2μl, pulsed pressure at 1,700 hPa for 0.5 min, splitless injection), and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 μm film) temperature initially at 40 °C with a 1-min hold followed by a 50 °C min^{-1} ramp to 140 °C with a 5- min hold followed by a 10 °C min^{-1} ramp to 300 °C with a final 17-min bake-out. Transfer line temperature was 265 °C.

Practical quantitation limits (PQL, Table 7) were established at approximately 10X the instrument detection limit, which was estimated as 3X S.D. of background noise levels for quantitation ions. Standard curves included PQL concentrations for each analyte.

[2-^{14}C]Acetate Radiolabeling Procedure

To examine whether TCS could effectively reduce the rate of lipid synthesis from acetate in *Cladophora* spp., we followed a [2-^{14}C]acetate radiolabeling protocol modified from a tobacco

cell suspension study conducted by Hoang and Chapman (Hoang and Chapman, 2002). Since acetate is readily integrated into plant lipids, [2-^{14}C]acetate is used regularly as a radioactive tracer for *in vitro* experimentation investigating lipid biosynthesis (Qi et al., 1995; Roughan and Ohlrogge, 1996). Three sets of ten 100 mg algal samples were measured and placed in 125 X 16 mm test tubes with prepared solutions of 2.0 ml Chu algal culture media containing three concentrations of a TCS stock solution (0 ppm, 1 ppm, and 10 ppm) prepared with DMSO as the solvent. Following a thirty minute incubation period, 0.5 µCi of radiolabeled [2-^{14}C]acetate (54mCi mmol^{-1}) was pipetted into each sample, vortexed at low speed, and allowed to incubate for three hours at 24 °C with equal light intensity distribution on the surface of each tube adjusted to 2.9 µEinsteins using fluorescent lamps. Radioactive waste was pipetted from each sample tube and collected for quality control analysis. After adding 2 ml 70 °C isopropyl alcohol to each tube, foil caps were applied, then tubes were vortexed and maintained at 70 °C for thirty minutes. One ml chloroform was added to each tube, and after being vortexed, samples were kept covered at 4 °C overnight. After transferring the fluid to new tubes, 1 ml chloroform was added to each sample extract. Two ml of a 1 M KCl solution were then added to each extract and vortexed. Samples were then centrifuged at 1300 rpm for 5 minutes. The top aqueous layer was removed by aspiration, after which 2 ml of the 1M KCl was again added. Samples were centrifuged and aspirated two more times following the same procedure. Following the final aspiration, the organic layer was transferred to 6.5 ml polyethylene scintillation vials and evaporated to dryness with nitrogen gas. Once dried, 200µl ethanol and 100 µl bleach were added to each vial and left for at least 30 minutes to remove chlorophyll quenching effects from the scintillation counting. After bleaching, 5 ml scintillation fluid were added to each vial, vortexed, and placed in glass carriers for scintillation counting.

Our initial protocol did not include chlorophyll bleaching. Six samples were divided into sets of two, prepared by using the same TCS concentrations (0 ppm, 1 ppm, and 10 ppm), and following the same protocol, with three exceptions: bleaching was not applied, 200 mg algal samples were used, and scintillation analysis followed a two-hour incubation period.

To assess algal viability after incubation in 10 ppm TCS, plasma membrane integrity was investigated after a 24 hour incubation period by using Evans blue stain and protocol modified from a root cell viability study conducted by Yoko Yamamoto (Yamamoto et al., 2001). Three 200 mg *Cladophora* samples were cleaned and blot-dried. One sample was left on a piece of filter paper for 48 hours in a dark drawer, the second sample was exposed to 2.0 ml Chu media vortexed with 20 µl DMSO for 24 hours, and the third sample was exposed to 2.0 ml Chu media vortexed with 20 µl TCS stock solution for 24 hours. Following the allotted time periods, each sample was submerged in 2 ml of an Evans blue solution (0.025% [w/v] Evans blue in 100 µM $CaCl_2$, pH 5.8, temperature 21.8 °C) for 10 min. Algae were washed three times with 20 ml of 100 µM $CaCl_2$, pH 5.8 (Thermo Orion Model 720A pH meter, Cole-Parmer Instrument Company, Vernon Hills, IL), after which no further dye elution occurred from the algal samples. The algae stained with Evans blue were then observed under a light microscope (Zeiss Axio Imager.A1 Transmitted Light, Carl Zeiss, Germany) to compare cell viability.

[2-^{14}C]Acetate Analysis

Scintillation counting was performed by using the Beckman LS 600 IC. The units of measurement were counts per minute (cpm), with the correction factor being cpm/0.9 = decays per minute (dpm).

Statistical Analyses

Standard statistical analyses were performed using Microsoft Excel resulting in mean and standard error values for [TCS] in ppm and [2-^{14}C]acetate in pmol acetate g^{-1} fresh weight algae. Data were also analyzed by T-Test for statistical significance. The level of significance for all analyses was $\alpha = 0.05$.

Results

Analytical Quality Control (QC) Results

Recovery percentage for algal blank spike addition was recorded as being 109%. Matrix spike and matrix spike duplicate recovery percentage were recorded at 116% and 109%, respectively, with a mean of 112% (Figure 16). Percent deviation for algal analysis of matrix spike duplicate was recorded as being 6.2%. No blank values exceeded the PQLs (Table 7).

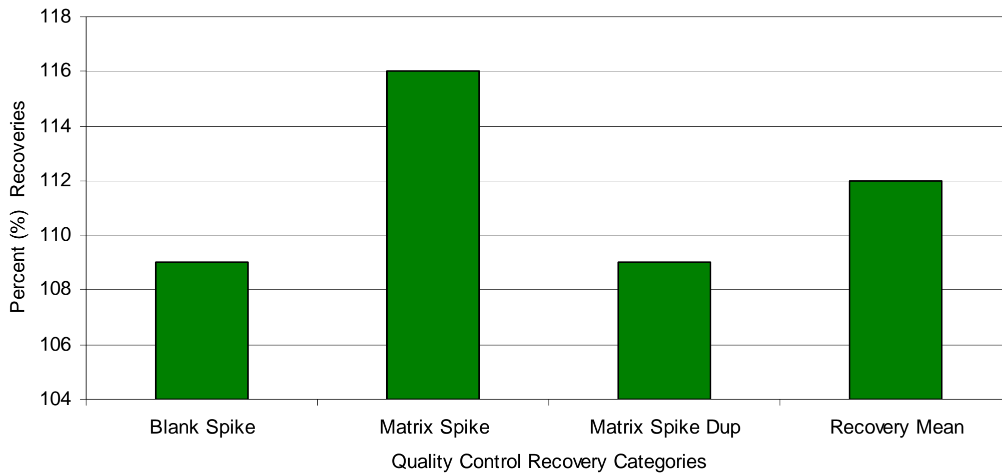

Fig. 16. GC/MS quality control percent recoveries for triclosan in algae following spike additions.

Table 7. Quality control data for triclosan in algae. Spike additions were at 5.0 µg g^{-1} for algae.

Blank (ppm)	Blk Spk Rec (%)	Mat Spk Rec (%)	Mat Skp Dup Rec (%)	Dup Dev (%)	PQL (ppm)
< 1.0	109	116	109	6.2	1.0

(Blk = Blank, Spk = Spike, Rec = Recovery, Mat = Matrix, Dup = Duplicate, Dev = Deviation, PQL = Practical Quantitation Limits)

Algal Lipid Bioaccumulation Results

TCS algal concentrations for each of five 10 mg samples exposed to three different dosing regimes are recorded in Table 8. For 0 ppm, algal concentration values for all five samples remained below the detection limits of 0.5 ppm. The 1 ppm algal concentration values ranged from 130 ppm to 226 ppm, with a mean value of 173 ppm. The 10 ppm algal concentration values ranged from 1210 ppm to 1793 ppm, with a mean value of 1398 ppm. Bioaccumulation for 1 ppm and 10 ppm aqueous concentrations of triclosan following a three hour incubation period resulted in algal lipid BAFs of 170 and 140, respectively (Table 9).

Table 8. Algal concentrations in 100 mg replicate samples (ppm) and calculated means for each aqueous TCS concentration (0 ppm, 1 ppm, 10 ppm) after a 3-hour incubation period. (N.D. is < PQL of 1.0 ppm).

Aqueous [TCS]	Algal [TCS] A (ng/mg)	Algal [TCS] B (ng/mg)	Algal [TCS] C (ng/mg)	Algal [TCS] D (ng/mg)	Algal [TCS] E (ng/mg)	Algal [TCS] Mean (ng/mg)
0 ppm	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1 ppm	226	176	170	130	161	173
10 ppm	1793	1416	1210	1142	1431	1398

Table 9. Algal bioaccumulation factors (BAFs) based on triclosan aqueous concentrations and algal concentrations (ppm) following 3-hour incubation period.

Aqueous [TCS]	Algal [TCS] Means	BAFs
0 ppm	< 1.0 ppm	NA
1 ppm	173 ppm	170
10 ppm	1398 ppm	140

[2-^{14}C]Acetate Labeling Results

The second part of this investigation to examine whether TCS may be involved in algal lipid biosynthesis following a three hour incubation using TCS concentrations of

0 ppm, 1 ppm, and 10 ppm involved the use of [2-^{14}C]acetate labeling. Algae treated with 1 ppm [TCS] produced a 32% reduction in lipid synthesis, while the 10 ppm [TCS] treatment resulted in an 89% reduction in lipid synthesis. Data for the [2-^{14}C]acetate labeling protocol, reported in pmol acetate incorporated into lipid per gram fresh weight algae, produced means of 671, 453, and 74.8 after a three-hour exposure period in 0 ppm, 1 ppm, and 10 ppm [TCS], respectively. Bleaching effects increased scintillation results 96% to 99% following application of 200 µl ethanol and 100 µl bleach (Table 10).

Table 10. [2-^{14}C]acetate labeling scintillation results according to aqueous TCS concentrations and algal accumulation following 3 hour (bleached) and 2 hour (non-bleached) incubation.

Aqueous [TCS]	Mean CPM (Bleached)	% Algal Lipid Inhibition	Mean CPM (Non-bleached)	% CPM Improvement
0 ppm	14476	0	202	99
1 ppm	9774	32	397	96
10 ppm	1614	89	48	97

(CPM = counts per minute)

Visual confirmation of algal plasma membrane integrity by using Evans blue stain resulted in the observation of nuclear uptake among dead algal cells from samples left in the dark for 48 hours. Samples exposed to 10 ppm TCS over a 24 hour incubation period and samples exposed to 0 ppm TCS over a 24 hour incubation period both expressed membrane integrity with no visual confirmation of Evans blue uptake.

An unexpected qualitative result was noticed during the microscopic observations of algal samples following the 4-hour incubation period in each aqueous TCS concentration (0 ppm, 1 ppm, and 10 ppm). The algae maintained in the 10 ppm aqueous TCS concentration experienced a “chlorophyll clumping” phenomenon not observed among algal samples incubated in the 0 ppm and 1 ppm aqueous TCS concentrations (see Figure 15).

Discussion

The results of our study support the assumption that high aqueous TCS concentrations are toxic to lipid biosynthesis among *Cladophora* spp. samples and reduce the acetate incorporation into total lipids. Although the indication of inhibition at the 1 ppm aqueous TCS concentration (453 pmol acetate g^{-1} fresh weight algae) was not statistically significant, the 3 hour algal incubation results at the 10 ppm aqueous TCS concentration (74.8 pmol acetate g^{-1} fresh weight algae) did indicate statistical significance following [2-^{14}C]acetate labeling (Figure 17). Overall, a 32% lipid biosynthesis reduction was observed at 1 ppm aqueous TCS concentration and 89% reduction at 10 ppm aqueous TCS concentration after a three hour incubation period. Algal TCS concentration results were statistically significant and were recorded as being 173 ppm for 1 ppm aqueous TCS concentrations and 1398 ppm for 10 ppm aqueous TCS concentrations (Figure 18). Although these algal TCS concentration values are orders of magnitude higher than environmentally relevant concentrations (approximately 100 ppb) within receiving streams of area wastewater treatment plants (Coogan et al., 2007), the results suggest the possibility that algal lipid inhibitory effects may be expressed *in situ* over longer exposure times at environmentally relevant concentrations. It has been speculated that lower algal TCS concentrations at the outfall of wastewater treatment plants in the presence of increased aqueous concentrations may be a result of algal lipid inhibition nearest the point source of municipal WWTP effluent (Coogan et al., 2007). The results of this study appear to support the potential of TCS inhibitory effects on algal lipid synthesis among organisms found in receiving streams of WWTPs. After three hours of exposure, the algal bioaccumulation factor (BAF) was reduced from 170 at aqueous TCS concentrations of 1 ppm to 140 at aqueous TCS concentrations of 10 ppm (Figure 19).

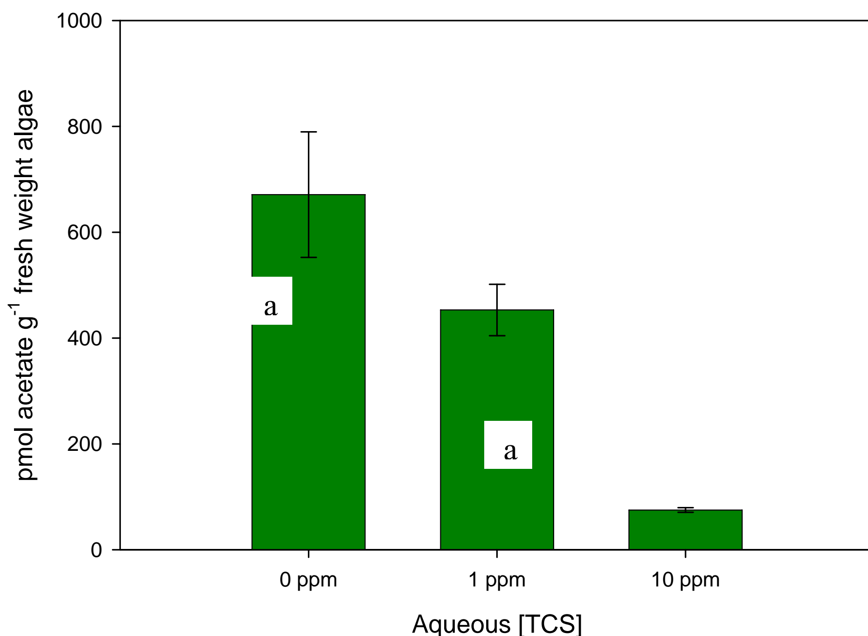

Fig. 17 [2-^{14}C]acetate labeling results in pmol acetate g^{-1} fresh weight algae with means and standard errors (α = 0.05, n = 10) indicating algal lipid production following a three hour incubation period at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm. Means for a given TCS concentration with different letters (a, b) are significantly different from the 0 ppm aqueous [TCS].

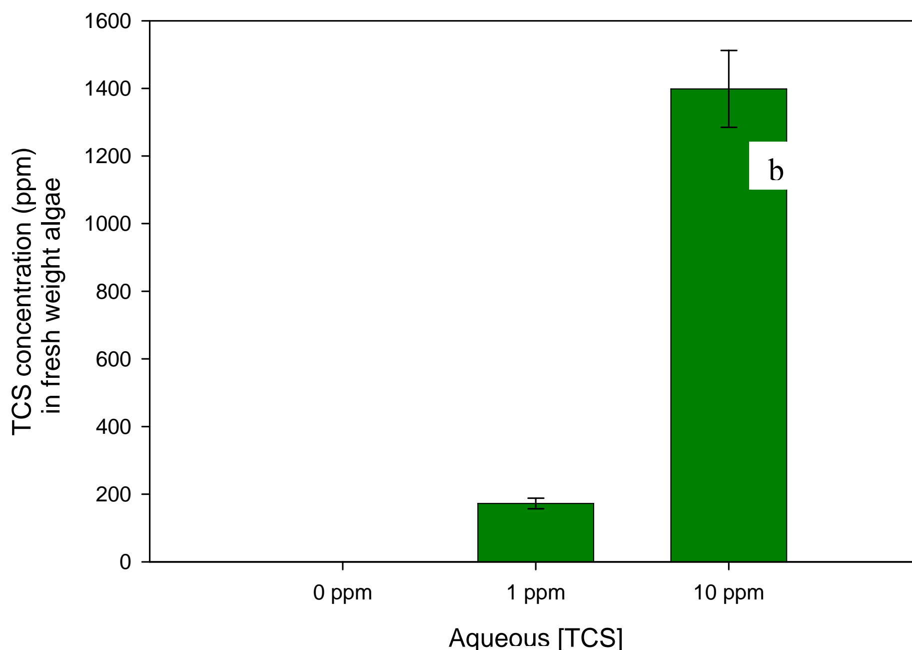

Fig. 18. Algal TCS concentration results with means and standard errors (α = 0.05, n = 5) following a three hour incubation period at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm. Means for a given TCS concentration with different letters (a, b) are significantly different from the 0 ppm aqueous [TCS].

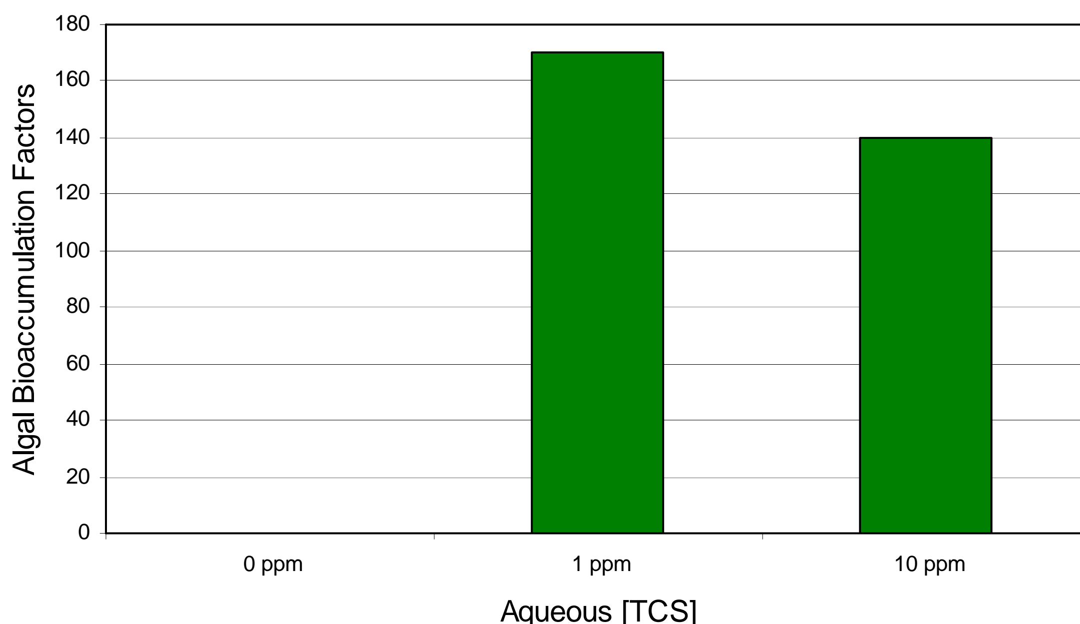

Fig. 19. Algal TCS bioaccumulation factors (BAFs) at aqueous TCS concentrations of 0 ppm, 1 ppm, and 10 ppm.

In spite of a 10 ppm increase in aqueous TCS concentration, the 89% algal lipid inhibition may have affected the ability of the algal cells to bioaccumulate TCS. Other possible explanations for the reduction in algal BAF at the 10 ppm aqueous TCS concentration might be a response to TCS uptake kinetics by *Cladophora* spp. or growth dilution (Gorski et al., 2006), both of which were not investigated in this study. In order to better understand TCS uptake in *Cladophora*, further studies would need to include investigations into such factors as time-dependent incorporation of [2-^{14}C]acetate into *Cladophora* total lipids (Hoang and Chapman, 2002) and binding site concentrations per cell, which would directly relate to cell wall properties that vary among algal species (Graham and Wilcox, 2000). Uptake rates for certain algae, such as *S. capricornutum* and *Cosmarium botrytis*, are known to be higher than cyanobacteria or diatoms (Miles et al., 2001), so the use of pure *Cladophora* cultures would be important for these types of investigations.

"Chlorophyll Clumping" at 10 ppm Aqueous TCS Concentrations

The "chlorophyll clumping" phenomenon observed among the algal samples during the 4-hour incubation period in the 10 ppm aqueous TCS concentration may have been a cellular response to senescence. A symptom of plant senescence, or chlorophyll catabolism, involves the production of substances catalyzed by the enzyme chlorophyllase, which is necessary for the conversion of chlorophyll to chlorophyllide.

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"Chlorophyll Clumping" at 10 ppm Aqueous TCS Concentrations

The "chlorophyll clumping" phenomenon observed among the algal samples during the 4-hour incubation period in the 10 ppm aqueous TCS concentration may have been a cellular response to senescence. A symptom of plant senescence, or chlorophyll catabolism, involves the

production of substances catalyzed by the enzyme chlorophyllase, which is necessary for the conversion of chlorophyll to chlorophyllide. One of these products is phytol. During the onset of senescence, phytol accumulates in lipid droplets known as plastoglobuli and may result in the appearance of "chlorophyll clumping." Further information indicates these changes can be differentiated from deterioration, which characterizes senescence as a programmed cellular response as opposed to a form of necrosis (Buchanan et al., 2000). How or if this response affects TCS bioaccumulation remains to be investigated.

Trophic Responses to TCS and Other Factors in WWTP Receiving Streams

Determining the bioavailability of TCS to algal species in receiving streams of municipal WWTPs may be important in understanding the movement of relatively high log Kow compounds into higher trophic levels within aquatic systems (Gorski et al., 2006). As human population numbers continue to increase and the prospect of water reuse becomes more of a reality, especially among arid climates within areas of the southwestern US, the potential exists for higher concentrations of PPCPs, such as TCS, to be found in natural water systems (Crook and Surampalli, 1996; Waltman et al., 2006). Could the environmentally relevant aqueous TCS concentration of 0.1 ppb ever reach the experimental concentrations of 1 ppm and 10 ppm used in this investigation? Probably not, but we are already finding TCS concentrations in human urine (127 ng ml^{-1}) have the capability of matching concentrations found in effluent systems (Ye et al., 2005). Bioaccumulation factors within biota of receiving streams at the base of the aquatic food web are already expressing TCS concentrations three orders of magnitude higher than aqueous concentrations (Coogan et al., 2007). Critical body burden analyses among the variety of organisms that live, reproduce, and grow within these systems will be required before a clear understanding of possible consequences can be established.

Even though fatality may not be a direct response to high concentrations of many aquatic pollutants, such as TCS, fecundity and longevity of many aquatic species have the potential to be negatively affected (Hutson, 1978). Biotic reproduction and growth rely heavily on the ability of organisms to obtain adequate supplies of fatty acids, which suggests the fundamental role these nutrients play in aquatic food webs (Arts and Wainman, 1999). Certain algal grazers have demonstrated deficiencies in specific long-chained poly-unsaturated fatty acids (PUFAs), since these consumers do not adequately synthesize PUFAs and must depend upon nutrient inputs through consumption (Hessen and Leu, 2006). Studies within Arctic ecosystems, which are characteristically rich in lipids, have substantiated the important role fatty acids play in membrane production, energy storage and use, and multiple other physiological responses (Arts and Wainman, 1999; Hessen and Leu, 2006). Hessen's studies investigated the ultraviolet radiation (UV-R) effect on phytoplankton nutrient quality in reference to fatty acid production; short-wave UV-R has been shown to play a vital role in affecting fatty acid profiles among autotrophs in response to biosynthesis effects or oxidation (Wang and Chai, 1994; Arts and Rai, 1997; Hessen et al., 1997). Another study investigated UV-exposure effects on PUFAs within freshwater Chlorophyceans and found small but notable impacts (Leu et al., 2007).

Individually, lipid inhibition by any one environmentally relevant factor may not be adequate to manifest negative system effects. Multiple stressor effects, however, may adequately result in synergistically-produced trophic degradation within receiving streams of wastewater treatment plants. Ozone depletion, global warming, and increased population growth within municipalities may, under optimal circumstances, multiply algal lipid inhibition effects and dramatically reduce food quality needs for healthy zooplankton growth and reproduction, and possibly the overall ecosystem dynamics.

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CHAPTER 5

DISCUSSION AND PROJECTIONS

This study investigated the use of freshwater algal and snail species to study the bioaccumulation potential of TCC, TCS, and M-TCS in surface waters of the Denton WWTP receiving stream. Additional research investigated algal lipid inhibition effects in response to toxic TCS aqueous concentrations. Results indicated a consistent reduction between water concentrations of TCS, TCC, and M-TCS from the DWWTP outfall to downstream sites. With elevated algal concentrations of the same antimicrobials remaining relatively similar among all sites, observed bioaccumulation results indicated BAFs approximately three orders of magnitude. Snail bioaccumulation results produced a similar pattern following a two-week exposure period at the outfall and produced BAFs of two to three orders of magnitude. Additionally, laboratory investigations indicated high aqueous TCS concentrations of 10 ppm resulted in approximately 90% algal lipid inhibition in response to TCS effects on enoyl ACP reductase.

Globally, communities continue to face increasing water demands. Water supply challenges are in direct response to factors such as population growth, contamination and depletion of groundwater, and drought conditions, especially within arid and semi-arid regions. Suggested solutions to these issues include water reuse and recycling, which in and of themselves provide additional challenges. Current reuse levels are small in comparison to the total production volume of industrial and municipal effluent (Miller, 2006). Methods of providing high-quality supplies of water (other than solely for the purpose of irrigating golf courses or industrial applications) in ways that will be beneficial for both the general public and the environment need to be further investigated. If general-purpose reuse is to become a reality, further research will need to be conducted to assure populations that their reclaimed water is safe

both chemically and biologically (Miller, 2006). Developing safe and sustainable water resources for our century will also require a better understanding of long-term impacts caused by environmentally relevant concentrations of emerging anthropogenic contaminants, such as TCS, M-TCS, and TCC, which are consistently introduced to the environment as a result of consumer activity.

As analytical chemical analyses continue to experience successes in measuring trace chemicals at continually lowered levels, our ability to assess meaningful environmental impacts may improve. Major and uncontrolled non-point pollution as a result of consumer activity has become a long-term source of environmental contamination (Daughton, 2004). Dealing with these impacts will require both traditional and novel approaches as water reuse becomes more of a reality. One of these approaches is found in the use of constructed treatment wetlands, which have the capability of transforming numerous common pollutants released from municipal wastewater treatment plants into biologically productive byproducts (Kadlec and Knight, 1996). Wetlands, and their associated wetland plants, have unique capabilities due to the high rate of biological activity found within these ecosystems and their ability to adapt to a variety of hydrological conditions. Additionally, wetlands provide a natural method of water treatment and require minimal energy input, in the form of fossil fuels, and no chemical treatments (Kadlec and Knight, 1996). Unfortunately, natural wetlands currently comprise less than 50 percent of their historical area in the continental United States. Globally, greater environmental awareness, especially within arid regions facing the potential for water shortages, high effluent concentrations, and extensive reuse, has begun to highlight the benefits of wetlands and resulted in increasing levels of restoration (Kadlec and Knight, 1996). Although analytical improvements in environmental analyses (lower detection limits, higher levels of reproducibility and accuracy)

present the possibility of enhanced plant monitoring methods for emerging contaminants within constructed treatment wetlands, difficulties still arise in the area of representative sampling (Markert, 1996). Additional research in sampling processes may result in higher levels of accuracy and reliability in plant analyses and provide greater credibility to the use of constructed treatment wetlands for water processing.

While the potential for evaluating the environmental risk associated with emerging contaminants continues to increase, the importance in communicating this risk to the general public also needs to be addressed. Daughton indicates the importance of effective risk communication in reference to two factors: newly-introduced emerging aquatic contaminants will continue to increase and water reuse pressures will expand as freshwater supplies decrease (Daughton, 2004). In light of these two factors, applicable research methods, such as those presented in our bioaccumulation/lipid biosynthesis study, need to be developed. Since safety factors based on observed water concentrations do not appear to present an immediate threat, long-term and synergistic affects need further investigation. The ultimate challenge, however, will be to communicate the significance of environmental exposure in a manner that results in both public action and ecological integrity.

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