SOLVENT EFFECTS AND BIOCONCENTRATION PATTERNS OF ANTIMICROBIAL COMPOUNDS IN WETLAND PLANTS

Sajag Adhikari

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APPROVED:

Kevin J. Stevens, Major Professor
Barney J. Venables, Committee Member
Thomas L. Beitinger, Committee Member
Art J. Goven, Chair of the Department of Biological Sciences
James D. Meernik, Acting Dean of the Toulouse Graduate School
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This study looked at effects of organic solvents dimethylsulfoxide, dimethylformamide and acetone at 0.01%, 0.05% and 0.1% concentration on germination and seedling development wetland plants. Even at 0.01% level, all solvents affected some aspect of seed germination or seedling growth. Acetone at 0.01% was least toxic. Root morphological characteristics were most sensitive compared to shoot morphological characteristics. This study also looked at bioconcentration patterns of antimicrobial compounds triclosan, triclocarban and methyl-triclosan in wetland plants exposed to Denton Municipal Waste Water Treatment Plant effluent. Bioconcentration patterns of antimicrobial compounds varied among species within groups as well as within organs of species. The highest triclocarban, triclosan and methyltriclosan concentration were in shoot of *N. guadalupensis*, root of *N. lutea* and in shoots of *P. nodous* respectively.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER I INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Problem Statement</td>
<td>2</td>
</tr>
<tr>
<td>Objectives</td>
<td>3</td>
</tr>
<tr>
<td>CHAPTER II EFFECTS OF CARRIER SOLVENTS ON SEED GERMINATION AND SEEDLING GROWTH AND DEVELOPMENT OF FOUR WETLAND PLANTS (Bidens frondosa, Eclipta prostrata, Cyperus acuminatus, and Rumex crispus)</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>7</td>
</tr>
<tr>
<td>Test Chemicals</td>
<td>7</td>
</tr>
<tr>
<td>Plants</td>
<td>8</td>
</tr>
<tr>
<td>Exposure Conditions</td>
<td>8</td>
</tr>
<tr>
<td>Exposure System</td>
<td>8</td>
</tr>
<tr>
<td>Seed Germination Study</td>
<td>9</td>
</tr>
<tr>
<td>Seedling Growth Study</td>
<td>11</td>
</tr>
<tr>
<td>Toxicity Test</td>
<td>11</td>
</tr>
<tr>
<td>Harvesting and Quantifying Seedling Growth and Performance</td>
<td>11</td>
</tr>
<tr>
<td>Statistical Analyses</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>13</td>
</tr>
<tr>
<td>Effects of DMSO on Seed Germination</td>
<td>13</td>
</tr>
<tr>
<td>Effects of DMF on Seed Germination</td>
<td>18</td>
</tr>
<tr>
<td>Effects of Acetone on Seed Germination</td>
<td>23</td>
</tr>
<tr>
<td>DMSO Effects on Seedling Growth</td>
<td>28</td>
</tr>
<tr>
<td>DMF Effects on Seedling Growth</td>
<td>34</td>
</tr>
<tr>
<td>Acetone Effects on Seedling Growth</td>
<td>40</td>
</tr>
<tr>
<td>Discussion</td>
<td>46</td>
</tr>
<tr>
<td>Seed Germination</td>
<td>46</td>
</tr>
</tbody>
</table>
Table 2.1. Quality control data for antimicrobials bioconcentration in wetland plants. Spike additions were at 20 ng/g.

Table 2.2. Tissue specific bioconcentration factors (BCFs) based on fresh weight. Concentrations of analytes below MDL were not included in calculation of BCFs. Empty space represents for tissue parts with Bioconcentration below MDL.
LIST OF FIGURES

Fig 1.1. Schematic representation of exposure system..........................................................9

Fig 1.2. Petri dish with filter paper wick. Filter paper wick passing through slit made in
bottom plate of Petri dish to provide continuous exposure solution to seeds..................10

Fig 1.3. Design of seed germination tray. Filter paper wick hanging from upper tray is in
contact with lower tray carrying exposure solution.........................................................11

Fig 1.4. Flow chart of materials and methods showing exposure of four species of
wetland plants (B. frondosa, C. acuminatus, E. prostrata and R. crispus) at three levels
(0.01%, 0.05% and 0.1%) of Dimethylsulfoxide, Dimethylformamide and Acetone to
monitor effects on seed germination and seedling growth.............................................12

Fig 1.5. Germination % of B. frondosa (a), C. acuminatus (c), E. prostrata (d) and R.
crispus (d), grown for 16 days under exposure to 0.01%, 0.05% and 0.1% DMSO. Data
shown are means ± one standard error, control (n = 14) and solvent (n = 8). The *
indicates significant difference between treatment and control......................................16

Fig 1.6. Germination% of B. frondosa, C. acuminatus, E. prostrata, R. crispus as
compared to their control grown for 16 days under exposure to 0.01% (a), 0.05% (b)
and 0.1% (d) DMSO. Data shown are means ± one standard error, n = 8. Similar letter
indicates no significant differences between treatment and controls.............................18

Fig 1.7. Germination % of B. frondosa (a), C. acuminatus (b), E. prostrata (c) and R.
crispus (d) grown for 16 days under exposure to 0.01%, 0.05% and 0.1% DMF. Data
shown are means ± one standard error, control (n = 14), solvent (n = 8). The * indicates
significant difference between treatment and control.....................................................21

Fig 1.8. Germination % of B. frondosa, C. acuminatus, E. prostrata and R. crispus as
compared to their control grown for 16 days under exposure to 0.01% (a), 0.05% (b)
and 0.1% DMF (c). Data shown are means ± one standard error, n = 8. Similar letter
indicates no significant between treatment and control..................................................23

Fig 1.9. Germination % of B. frondosa (a), C. acuminatus (b), E. prostrata (c) and R.
crispus (d) grown for 16 days under exposure to 0.01%, 0.05% and 0.1% acetone. Data
shown are means ± one standard error, control (n = 14) and solvent (n = 8). The *
indicates significant difference between treatment and control......................................26

Fig 1.10. Germination % of B. frondosa, C. acuminatus, E. prostrata and R. crispus as
compared to their control grown for 16 days under exposure to 0.01% (a),0.05% (b) and
0.1% (c) acetone. Data shown are means ± one standard error, n = 8. Similar letter
indicates no significant between treatment and control..................................................27
Fig 1.11. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of B. frondosa, C. acuminatus, E. prostrata and R. crispus grown for 22 days on exposure to three concentrations of DMSO (0.01%, 0.05% and 0.1%). Data shown are means ± one standard error, n = 10. The * indicates significant differences between treatment and control. ........................................................................................ 33

Fig 1.12. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of B. frondosa, C. acuminatus, E. prostrata and R. crispus grown for 22 days under exposure to three concentrations of DMF (0.01%, 0.05% and 0.1%). Data shown are means ± one standard error, n = 10. The * indicates significant differences between the treatment and control. .................................................................................. 39

Fig 1.13. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of B. frondosa, C. acuminatus, E. prostrata, R. crispus grown for 22 days under exposure to three concentrations of acetone (0.01%, 0.05%, and 0.1%). Data shown are means ± one standard error, n = 10. The * indicates significant differences between the treatment and control. .......................................................................................................................... 46

Fig 2.1. Eleven different species of wetland plants belonging to five functional groups (emergent, semi-emergent, submerged, floating leaved and free floating) of wetland plants. ................................................................................................................................ 58

Fig 2.2. Overview of the mesocosm facility at Denton Waste Water Treatment Plant. .58

Fig 2.3. Diagrammatic representation of plants used and locations of organs harvested related to the water level. ................................................................................................................................. 60

Fig 2.4. TCS concentration (mean ± SE) of P. hydropiperoides, P. cordata, B. monnieri, P. nodosus, V. americana, N. lutea and Nymphaea sp. belonging to four functional groups of wetland plants. Number inside data series represents sample size. Within an organ system (rt= root, st = shoot, rh= rhizome). Similar letter and number above error identify TCS means that do not significantly differ (Upper case = root TCS concentration within functional group and across species, st= shoot concentration within concentration within functional group and across species, number = organ specific TCS concentration within species. ................................................................................................................... 68

Fig 2.5. MTCS concentration (mean ± SE) in P. hydropiperoides, P. nodosus, N. guadalupensis, V. americana, N. lutea and Nymphaea sp. belonging to four functional groups of wetland plants. Numbers inside data series represents sample size. Within an organ system (rt = root, st = shoot, rh= rhizome). Similar letter and numbers above error bar identify MTCS means that do not significantly differ (Upper case = root MTCS concentration within functional group and across species, lower case = shoot concentration within functional group and across species, number = organ specific MTCS concentration within species. ........................................................................................................ 69
Fig 2.6. TCC concentrations (mean ± SE) in *P. hydropiperoides*, *P. cordata*, *B. monnieri*, *P. nodosus*, *H. dubia*, *N. guadalupensis*, *V. americana*, *N. lutea*, and *Nymphaea sp.* belonging to four groups of wetland plants. Within an organ system (rt = root, st = shoot, rh = rhizome). Similar letter and number above error bar identify TCC means that do not significantly differ (Upper case = root TCC concentration within functional group and across species, lower case = shoot TCC concentration within functional group and across species, number = organ specific TCC concentration within species. ..................70

Fig 2.7. Root concentration of TCS, MTCS and TCC (mean ± SE) in *P. hydropiperoides* (*n* = 3), *P. cordata* (*n* = 4), *P. nodosus* (*n* = 4), *N. guadalupensis* (*n* = 3) and *N. lutea* (*n* = 4). Similar letter identify means that do not significantly differ. ..................................71

Fig 2.8. Shoot concentration of TCS, MTCS and TCC (mean ± SE) in *B. monnieri* (*n* = 3), *P. nodosus* (*n* = 4), *N. guadalupensis* (*n* = 3), *V. americana* (*n* = 4) and *Nymphaea sp.* (*n* = 4). Similar letter identify means that do not significantly differ. ...............................72
CHAPTER I

INTRODUCTION

Increased population growth accompanied with lifestyle changes, like the wider application of pharmaceutical and personal care products (PPCPs) in everyday urban activities including human and veterinary drugs, antibiotics, fragrances, sunscreens, and antiseptics has changed the composition of municipal waste water. As the demand for quality water increases with escalating population growth, increase in household waste water will also occur (Bower, 2003). Pharmaceutical and personal care products belong to a relatively new group of contaminants referred to as unrecognized or emerging contaminants and have dominated urban waste water (Ellis, 2006).

Municipal waste water treatment plants (WWTPs) are the primary route of entry of PPCP’s to the environment. In a study by Kolpin et al., (2002) for a nationwide survey of occurrence of pharmaceuticals, hormones, and other organic waste water contaminants (OWCs) in water resources, OWCs were present in 80% of sampled U.S. streams. The antimicrobial compound triclosan was one of the most frequently detected compounds in the study. According to (Halden and Paull, 2005) another antimicrobial compound triclocarban (TCC) is also prevalent in aquatic environments and co-occurs with triclosan. Triclocarban (TCC) is commonly used in bar soaps, while triclosan (TCS) is used in wide range of products like toothpaste, soaps, deodorants and cosmetics (Halden and Paull, 2005). Based on their application and disposal TCS and TCC are commonly known as “down the drain contaminants” and enter the environment through municipal waste water. During activated sludge treatment processes, a large fraction (95 to 99%) of these compounds is removed. The remaining fraction of TCS and TCC
enters the environment in effluent water (Heidler and Halden, 2007; Waltman et al., 2006; Bester, 2005). The application of WWTP sludge as an agricultural soil amendment provides a second way for these compounds to enter the environment. Methyl triclosan (MTCS) is the biotransformation product of TCS and is reported to present effluent water and surface water along with TCS and TCC (Coogan et al., 2007).

**Problem Statement**

TCS, MTCS and TCC are known to cause toxic effects on aquatic organisms and aquatic toxicity assays have been conducted in organisms like algae, invertebrates, fish (Orvos et al., 2002; Farré et al., 2008; U.S. EPA, 2009). Wetland plants are equally exposed to these contaminants in aquatic environments and except for a few studies like the study by Stevens et al., (2009) that looked at effects of TCS in seed germination and seedling growth of wetland plants, toxicity effects of TCS, TCC and MTCS in wetland plants are not reported. Additionally, based on their log Kow values, TCS (4.8), TCC (4.9) and MTCS (5.2) have potential of bioaccumulation in exposed organisms and studies have shown their bioaccumulation in algae and snails (Coogan et al., 2007, Coogan and La Point, 2008). Bioconcentration of TCS and MTCS in wetland plants is reported by Stevens et al., (2009).

Effects of TCS, MTCS and TCC and bioconcentration patterns in wide range of wetland plants are still exploratory. TCS, MTCS and TCC have very low solubility and to conduct toxicity assays of these compounds preparation of stock solution in organic solvents is also required. Selection of the right solvent and its concentration is very
important to detect the toxicity effects of the test substance alone. U.S. Environmental Protection Agency (EPA) and Organization for Economic Co-operation and Development (OECD) recommended levels and types of carrier solvent are primarily based on results from animal and algal species assays (Hutchinson et al., 2006; Okumura et al., 2001) and are not representative of wetland plants. To prepare the foundation for conducting toxicity effects of OWCs including TCS, MTCS and TCC in wetland plants in future and to understand bioconcentration patterns of TCS, TCC, MTCS in broad range of wetland plants this study has taken two broad objectives.

Objectives

1) To look at the effects of carrier solvents on seed germination and seedling growth of wetland plants. Seed germination and seedling growth are commonly used tests in standard test guidelines for phytotoxicity test of terrestrial plants. This study will identify the best solvent and concentrations to be used in toxicity assays of various pollutants on wetland plants.

2) To look at bioaccumulation patterns of the antimicrobial compounds triclosan (TCS), triclocarban (TCC) and methyl-triclosan (MTCS) (metabolite of triclosan) on wetland plants exposed to WWTP’s effluent.
CHAPTER II

EFFECTS OF CARRIER SOLVENTS ON SEED GERMINATION AND SEEDLING GROWTH AND DEVELOPMENT OF FOUR WETLAND PLANTS (*Bidens frondosa*, *Eclipta prostrata*, *Cyperus acuminatus*, and *Rumex crispus*)

Introduction

The organic solvents acetone, N-N-dimethylformamide (DMF), and dimethylsulfoxide (DMSO) are recommended to dissolve water immiscible organic compounds in the standard toxicity test protocols of regulatory organizations including the United States Environmental Protection Agency (US EPA), the American Society of Testing Materials (ASTM), and the Organization for Economic Cooperation and Development (OECD). Acetone is a highly volatile, naturally occurring, organic compound produced as a metabolic byproduct of plants and animals and is emitted from volcanic eruptions and forest fires (Hallare et al., 2006). DMSO is a commercially manufactured dipolar aprotic solvent is also naturally occurring substance (Wiley Heyden Ltd, 1984). DMF, a polar solvent, is widely used in industrial applications including manufacturing of synthetic fibers, leather, and films (Gescher, 1993). In toxicity studies of water insoluble herbicides, pesticides or polycyclic aromatic hydrocarbons, these compounds are dissolved in organic solvents, diluted to achieve a target concentration, then test organisms are exposed to solution of these compounds to assess potential effects (Okumura et al., 2001). Since the goal of these studies is to assess the effects of various compounds, the solvent used to solubilize the water immiscible compounds should not affect the test organism.

Organic solvents may have toxic effects to organisms because they can affect cell membrane integrity and induce the formation of toxic metabolites (Robinson et al.,
Application of 2% acetone in soybean cell cultures led to a depletion of starch reserves in the amyloplasts, membrane abnormalities, and cytoplasmic degeneration in cultured cells. In the same study 3% DMSO resulted in cellular hypertrophy including enlargement of nuclei and reduction in cell wall thickness Davis et al., (1978). A study by Perata and Alpi (1991) showed the exogenously added ethanol was converted to acetaldehyde in the medium of suspension cultured carrot cells. Because of potential effects of carrier solvent on test organisms, it is important to select the best solvent and a non toxic concentration of the solvent in toxicity assays of water immiscible compounds. Studies on toxicities of common organic solvents include aquatic organisms like grass shrimp (Rayburn and Fisher, 1997), *Daphnia magna* (Leoni et al., 2008; Leblanc and Surprenant, 1983), common carp (Mashova et al., 2009), zebrafish (Hallare et al., 2006), microalgae (Okumura et al., 2001, Cho et al., 2009) and economically important crops like Red kidney beans, Alaskan peas, Himalayan barley and Tetratakus rye (Erdman and Hsieh, 1969). After an extensive review of available literature, it is concluded that wetland plants have not been included in studies of the effects of organic solvents on plant growth and development.

Seed germination, root elongation, and seedling growth are the commonly described phytotoxicity assays to study effects of test chemicals in terrestrial plants in the standard chemical test guidelines. An aquatic plant toxicity test using *Lemna* spp. is the most common aquatic toxicity tests utilizing vascular plants (US EPA 1996; ASTM, 2009; OECD, 2003). Recommended carrier solvents and the levels to use for toxicity assays are different among agency guidelines. EPA seed germination/root elongation toxicity test have not specified any particular solvent or its concentration but have
recommended it to be at minimum level and be non toxic to plants. ASTM standard guide for conducting terrestrial plant toxicity tests (2009) recommends the solvent concentration to be as low as possible with maximum concentration of 1%. EPA test guidelines for aquatic plant toxicity test using *Lemna* sp. have established the upper limit of carrier solvent to be 0.05% (US EPA, 1996). The maximum solvent concentration recommended by OECD in aquatic toxicity testing 0.01% (Hutchinson et al., 2006).

Wetland plants include species that normally grow in or on water, or low to high soil saturation and have the ability to develop anatomical and physiological adaptations to survive in low oxygen environments (Cronk and Fennessy, 2001). Wetland habitats are threatened by anthropogenic stressors including pollution, agriculture, hydrological alterations and increased urbanization which are often threatening to the wetland species growing in those habitats (Cronk and Fennessy, 2001). Of particular concern is the effect of PPCPs on wetland plants since this area has been rarely studied (Stevens et al., 2009), however, these compounds are pervasive and expected to increase in prevalence with increased urbanization. Furthermore, since WWTPs discharge to local watersheds, wetland plants are being exposed to these compounds with unrecognized consequences. As a first step, to developing a standard method of toxicity testing using wetland plants, this study looked at the effects of three carrier solvents (acetone, dimethylsulfoxide and n-n-dimethylformamide) on the germination and development on four wetland plants (*Eclipta prostrata*, *Bidens frondosa*, *Rumex crispus* and *Cyperus acuminatus*). These plants have been chosen since they represent species from a range of taxonomic groups and are species commonly found in our local watershed.
This study used a flow-through system to maintain a constant exposure concentration of the solvents during the study. This is a more realistic exposure system than static renewal or non-renewal studies since the discharge from WWTPs is generally continuous. The goal of this study is to understand the sensitivity of each species towards solvents, identify the best endpoints, best solvents and its concentrations for future toxicity tests of water immiscible organic compounds commonly released to the environment.

Hypotheses:

1) Germination of *B. frondosa*, *E. prostrata*, *C. acuminatus* and *R. crispus* exposed to three concentrations of DMSO, DMF and acetone will not differ from non-exposed controls.

2) Seedling growth and development of *B. frondosa*, *E. prostrata*, *C. acuminatus* and *R. crispus* exposed to DMSO, DMF and acetone will not differ from non-exposed controls.

Materials and Methods

A brief outline of materials and methods is provided in figure 1.4. Individual sections will be explained below.

Test Chemicals

Certified ACS grade Dimethylsulfoxide (99.9%) and N-N-dimethylformamide (99.9%) and HPLC grade acetone (99.8%) from Fisher Scientific were used (Houston, TX, USA).
Plants

Four wetland plant species, *Bidens frondosa*, *Eclipta prostrata*, *Cyperus acuminatus* and *Rumex crispus*, a native to North Central Texas and belonging to three different plant families (Asteraceae, Cyperaceae and Polygonaceae) were used. Seeds of *Bidens frondosa*, *Eclipta prostrata* and *Rumex crispus* were collected in the fall of 2007 (Greenbelt corridor, Denton TX) and stored in a refrigerator at 4°C until germination. Seeds of *Cyperus acuminatus* were collected from plants grown in a greenhouse on the UNT campus in 2010.

Exposure Conditions

For all solvent exposure treatments, de-ionized water (DI) was mixed with stock solvents to give final target solvent concentrations of 0.01%, 0.05% and 0.1%. DI water was used as the control treatment. In total ten treatments were used. To ensure plants had an adequate source of nutrients, 1/64th strength Long Ashton nutrient solution (Hewitt 1952) was mixed with DI water in all treatments.

Exposure System

The flow-through exposure system consisted of a series of syringe pumps, peristaltic pumps, dosing pump, nutrient water reservoir, mixing flasks, magnetic plates, magnetic stir bars, potting trays and different diameter sizes polyvinyl tubing (Figure 1.1). Plastic trays (54×28×6 cm, non-draining potting trays, Summit Plastic Company) were plumbed and used as seed germination exposure trays and seedling growth trays. Long Ashton nutrient media was mixed with DI water using a Dosmatic MiniDos pump.
then discharged into a 50 L reservoir. The delivery of DI water + nutrients was controlled with a float valve fitted into the reservoir. The nutrient media was pumped from the reservoirs using two 12-channel peristaltic pumps (Carter 12/6, cassette pump) and delivered to 25 ml mixing flasks placed on a 12-channel stirring plate. Stock solvents in 30 ml plastic syringes (BDscientific, Franklin Lakes, NJ, USA) were delivered to the mixing flasks using two 1-channel syringe pumps (kdScientific, Model 200series, Holliston, MA, USA). The positive pressure generated by the peristaltic and syringe pumps forced the exposure solution from the mixing flasks, delivering it to the exposure trays through 1/8” vinyl tubing at a flow rate of 2.68 ± 0.13 ml/min. There were two sets of exposure trays for each treatment. Exposure solutions were first delivered to an elevated seed exposure tray. At one end of each seed exposure tray an outlet (0.5 cm dia.) was plumbed at 1.5 cm height. This outlet was used to transfer exposure solution to seedling growth trays.

Fig 1.1. Schematic representation of exposure system.

Seed Germination Study

Seed germination studies were carried out in plastic petri dishes (Fisher brand, dia. 5 cm). Whatman, (dia. 47 mm) filter paper was placed at the bottom of each petri
Contact between the petri dish with seeds and exposure solution was made with a filter paper wick to prevent light penetration and subsequent algal growth in the system (Figure 1.2). About 1 cm slit was plumbed into the bottom of each of the petri dish and a filter paper wick was passed through this slit making contact with filter paper placed at the bottom of each petri dish. About 1.5 cm slits were also made at the bottom of germination trays. The slit in the tray was used to hold the petri dish in place and making a connection between the filter paper wick placed at the bottom of each petri dish and the exposure solution without direct exposure of the nutrient solution to light (Figure 1.3). In the control germination trays twenty eight slits were made to hold twenty eight petri dishes, in other treatment trays sixteen slits were made to hold sixteen petri dishes. Germination trays with petri dishes were then placed over the germination exposure tray separated by four spacers (PVC pipe, 3.5 cm height) placed at the four corners of each exposure tray. To prevent any light reaching the nutrient loaded water in the seed germination exposure tray, the space between the seed germination trays and seed germination exposure trays was enclosed in with duct tape. Delivery tubing from the mixing flasks to the exposure trays was passed through black tubing to prevent direct exposure to light and subsequent algal growth. The system was allowed equilibrate with exposure solution for three days.

![Diagram](image)

**Fig 1.2.** Petri dish with filter paper wick. Filter paper wick passing through slit made in bottom plate of Petri dish to provide continuous exposure solution to seeds.
Seedling Growth Study

Each seedling tray contained 40 individual pots (6×4×5 cm) to hold ten replicate plants for each of the four species. Individual pots were filled with sand. All the ten seedlings transplanted in control treatments were germinated in petri dishes with DI water exposure. Transplantation of seedlings was completed within two days. Seedlings were harvested after twenty two days.

Toxicity Test

The number of seeds germinating in each treatment was recorded on 4th, 8th, 12th and 16th days after exposure. Seeds displaying radicle emergence were considered “germinated”. The effects of solvents on seed germination and seedling growth were assessed as difference in germination percentage and seedling growth morphological characteristics in solvent exposure treatments compared to controls.

Harvesting and Quantifying Seedling Growth and Performance

Seedlings were harvested after twenty two days of exposure at three levels of DMSO, DMF and Acetone. Leaf number of each seedling was counted before their
Uprooting. Seedlings were carefully separated from growth medium, rinsed off with tap water and blotted dry with paper towels. Shoot height and total fresh mass of the seedlings were recorded. Seedlings were cut with scissors at the junction of root and shoot. Individual roots were scanned by using Epson Expression 10000XL scanner and analyzed with WinRhizo PRO (ver 2007c, Reagent Instruments) and root length, root surface area, and root volume were obtained. Shoot and root samples were oven dried for 48 hr at 80°C and dry mass was recorded.

Fig 1.4. Flow chart of materials and methods showing exposure of four species of wetland plants (B. frondosa, C. acuminatus, E. prostrata and R. crispus) at three levels (0.01%, 0.05% and 0.1%) of Dimethylsulfoxide, Dimethylformamide and Acetone to monitor effects on seed germination and seedling growth.
Statistical Analyses

Germination percentage of all four species at each level of exposure solvent and at each time of measurement was compared with controls using repeated measures ANOVA in SAS followed by Dunnett’s - Kramer. Additionally, germination percentage as relevant to control was derived by dividing germination percentage of each species in solvent treatment with germination percentage of individual species in control treatment. Using this derived variable the difference in germination patterns among species at each times of measurement was determined by using repeated measures ANOVA in SAS followed by Dunnett’s - Kramer. The difference in seedling growth parameters for each variable in each species exposed to solvent treatment was compared with seedlings in control treatment using factorial ANOVA in SAS followed by Dunnett’s – Kramer multiple comparison tests. For all analyses an alpha level ≤ 0.05 was considered to be significant.

Results

Effects of DMSO on Seed Germination

Comparisons within Species

Differences in germination rates within species at three levels of DMSO exposure treatments compared to control varied with concentration of solvent and exposure time. By the 8th day of exposure more than 80% of the seeds germinated for B. frondosa, E. prostrata and C. acuminatus at all three exposure concentration of DMSO. Only for R. crispus germination % in 0.01% DMSO was below 80 % (76.9 ± 7.4%). For B. frondosa significant differences in seed germination compared to control (92.5 ± 2.0%)
was observed on the 8th day of exposure in 0.05% (83.7 ± 3.2) and 0.1% (83.1 ± 4.0) DMSO. This difference in germination persisted in 0.1% DMSO on 12th day did not occur on 16th day of exposure (Figure 1. 5a). DMSO did not affect seed germination of *C. acuminatus* (Figure 1.5b). For *E. prostrata*, no initial difference in seed germination rate was observed in exposure to DMSO but a significant difference in seed germination rate was seen on 12th (81.9 ± 2.7%) and 16th (83.1 ± 2.7%) days of exposure in 0.1% DMSO (Figure 1.5c). A reduction in seed germination rate was seen for *R. crispus* (43.1 ± 8.3%) in 0.01% DMSO on the 4th day but the effect was not evident after the fourth day (Figure 1.5 d).

**Comparisons among Species**

On the 4th day of exposure to 0.01% DMSO, germination percentage relevant to control in *C. acuminatus* (146.8 ± 7.4%) was significantly greater than *B. frondosa* (93.4 ± 6.3%), *E. prostrata* (81.6 ± 8.6%) and *R. crispus* (68.2 ±13.2%). With increase in exposure time the significant differences were not evident on the 12th and 16th day (Figure 1. 6 a). A similar response to 0.01% DMSO was seen on the germination pattern among species exposed to 0.05% and 0.1% DMSO on 4th day of exposure but on the 8th, 12th and 16th days of exposure significant decrease in germination percentage of *E. prostrata* occurred in both exposure treatments (Figure 1. 6 b, c).
**B. frondosa**

![Graph showing germination percentages over days for B. frondosa](image)

**C. acuminatus**

![Graph showing germination percentages over days for C. acuminatus](image)
Fig 1.5. Germination % of *B. frondosa* (a), *C. acuminatus* (c), *E. prostrata* (d) and *R. crispus* (d), grown for 16 days under exposure to 0.01%, 0.05% and 0.1% DMSO. Data shown are means ± one standard error, control (*n* = 14) and solvent (*n* = 8). The * indicates significant difference between treatment and control.
Fig 1.6. Germination% of *B. frondosa*, *C. acuminatus*, *E. prostrata*, *R. crispus* as compared to their control grown for 16 days under exposure to 0.01% (a), 0.05% (b) and 0.1% (d) DMSO. Data shown are means ± one standard error, $n = 8$. Similar letter indicates no significant differences between treatment and controls.

Effects of DMF on Seed Germination

*Comparisons within Species*

A significant difference in germination rate compared to controls existed for all species at least at one exposure concentration of DMF and times of measurement. For *B. frondosa* there was no difference in seed germination compared to control (92.5 ± 2.0%) in 0.01% DMF but significant difference was seen in 0.05% (83.1 ± 4.2%) and 0.1% DMF (67.5 ± 8.6%) on 8th day of exposure. The difference in germination % continued in 0.1% DMF on 12th and 16th day of exposure (Figure 1.7a). For *C. acuminatus* significant differences in seed germination in 0.01% DMF (99.4 ± 0.6%) and 0.05 % DMF (81.2 ± 5.1%) were observed on 8th day but the effect did not continue on 12th and 18th day (Figure 1.7b). DMF in 0.1% had no effect on germination rate of *C.*
For *E. prostrata* compared to control (69.9 ± 5.2%) a significant difference in germination % in 0.05% DMF (43.75 ± 5.8%) was observed on 4th day and the effect persisted throughout the study. There was no initial difference in germination rates observed in 0.01% DMF for *E. prostrata* but with increased exposure time a significant difference in germination was observed (Figure 1.7c). Similarly for *R. crispus*, the difference in germination rates was found in 0.1% DMF (83.8 ± 4.5%) on the 4th day. This effect disappeared with increased exposure time (Figure 1.7d). Overall, 0.01% DMF had no effect on germination rate of *B. frondosa*. 0.1% DMF had no effect on germination of *E. prostrata*. 0.01% and 0.05% DMF had no effect on germination of *R. crispus*.

**Comparisons among Species**

Seed germination patterns among species exposed to three concentrations of DMF varied with exposure concentration and time. On the 4th day of exposure 0.01% DMF had stimulatory effect on *C. acuminatus* (158.1 ± 14.9%) and germination % of *C. acuminatus* relevant to control was significantly greater than *B. frondosa* (104.4 ± 8.2%) and *E. prostrata* (79.8 ± 10%) but not greater than *R. crispus* (122.6 ± 14.6%). With an increase in exposure time, differences existed only between *C. acuminatus* and *E. prostrata* (Figure 1.8a). On the 4th day of exposure germination % in *C. acuminatus* (36.7 ± 11.34%) and *E. prostrata* (63.5 ± 8.3%) exposed to 0.05% DMF was significantly lower than *B. frondosa* (104.4 ± 8.2%) and *R. crispus* (122.6 ± 14.7%), the similar differences between *E. prostrata* and *R. crispus* continued on the 8th, 12th and
16th day (Figure 1.8b). On the 4th day of exposure to 0.1% DMF *R. crispus* had a significantly higher germination compared to all other species the differences continued for *B. frondosa* and *E. prostrata* but not for *C. acuminatus* on 8th, 12th and 16th days.
Fig 1.7. Germination % of B. frondosa (a), C. acuminatus (b), E. prostrata (c) and R. crispus (d) grown for 16 days under exposure to 0.01%, 0.05% and 0.1% DMF. Data shown are means ± one standard error, control (n = 14), solvent (n = 8). The * indicates significant difference between treatment and control.
Fig 1.8. Germination % of *B. frondosa*, *C. acuminatus*, *E. prostrata* and *R. crispus* as compared to their control grown for 16 days under exposure to 0.01% (a), 0.05% (b) and 0.1% DMF (c). Data shown are means ± one standard error, n = 8. Similar letter indicates no significant difference between treatment and control.

**Effects of Acetone on Seed Germination**

*Comparisons within Species*

Similar to DMSO and DMF exposure treatments germination response of species varied with concentration of acetone and exposure time. No effect on germination rate was seen for *B. frondosa* on 4<sup>th</sup> day of exposure in all three exposure treatments of acetone, but on the 8<sup>th</sup> day of exposure a significant difference in germination was observed in 0.1% acetone (85 ± 4.4%) and the effect did not continue on the 12<sup>th</sup> and 16<sup>th</sup> days (Figure 1.9a). A significant lower seed germination percentage was seen in *E. prostrata* in 0.1% acetone at all four times of measurement (Figure 1.9c). Acetone had no effect on germination of *C. acuminatus* (Figure 1.9b). For *R. crispus*, compared to control (63.2 ± 8.3%), a significant lower germination percentage in 0.1% (45 ±
11.3%) and significant higher germination percentage was observed on the 4\textsuperscript{th} day. The effect continued in 0.05% on the 8\textsuperscript{th} and 16\textsuperscript{th} day of exposure (Figure 1.9d).

**Comparisons among Species**

On the 4\textsuperscript{th} day of exposure in 0.01% acetone, *R. crispus* germination percentage relevant to control (112.7 ±17\%) was significantly higher compared to *B. frondosa* (92.5 ± 6.4), *E. prostrata* (74.3 ± 9.1\%) and *C. acuminatus* (90.3 ± 14.1\%). Significant differences continued between *E. prostrata* and *R. crispus* in 8\textsuperscript{th}, 12\textsuperscript{th} and 16\textsuperscript{th} days (Figure 1.10a). Significant differences in seed germination were only seen between *E. prostrata* and *R. crispus* in 0.05% acetone on the 4\textsuperscript{th}, 8\textsuperscript{th} and 16\textsuperscript{th} days of exposure (Figure 1.10b). No initial difference in germination patterns of species were seen in 0.1% acetone but differences in germination percentage of *E. prostrata* and *R. crispus* were seen on 8\textsuperscript{th} and 16\textsuperscript{th} days (Figure 1.10c).
C. acuminatus

E. prostrata

Germination (%)

Days

Control
Aconite 0.01%
Aconite 0.005%
Aconite 0.005% Acidone
Aconite 0.005% Acidone 0.1%
Fig 1.9. Germination % of *B. frondosa* (a), *C. acuminatus* (b), *E. prostrata* (c) and *R. crispus* (d) grown for 16 days under exposure to 0.01%, 0.05% and 0.1% acetone. Data shown are means ± one standard error, control ($n = 14$) and solvent ($n = 8$). The * indicates significant difference between treatment and control.
Fig 1.10. Germination % of *B. frondosa*, *C. acuminatus*, *E. prostrata* and *R. crispus* as compared to their control grown for 16 days under exposure to 0.01% (a), 0.05% (b) and 0.1% (c) acetone. Data shown are means ± one standard error, *n* = 8. Similar letter indicates no significant between treatment and control.
DMSO Effects on Seedling Growth

Total dry mass, shoot dry mass, root dry mass, shoot height, total fresh mass, root length, root surface area, root volume for seedlings of *B. frondosa, E. prostrata, C. acuminatus* and *R. crispus* exposed to 0.1% and 0.05% DMSO were significantly different than the controls (Figure 1.11b, c, d, e, a, h, j, i). For *B. frondosa* leaf number in 0.05% DMSO and shoot/root ratio in 0.1% DMSO were not different than the controls (Figure 1.11f). For *R. crispus* and *E. prostrata* leaf numbers were significantly different than the controls in 0.05% and 0.1% DMSO but shoot/root ratio was not different. For, *C. acuminatus* leaf number was not different than the controls in 0.05% and 0.1% DMSO (Figure 1.11f, g). Total dry mass, root dry mass, total fresh mass was significantly different than the controls for *B. frondosa, E. prostrata* and *R. crispus* in 0.01% DMSO. Shoot dry mass was significantly different for *B. frondosa* and *R. crispus* but no different in *E. prostrata* (Figure 1.11b, d, a, c). For *B. frondosa* shoot height was not different but was significantly different for *E. prostrata* and *R. crispus in 0.01% DMSO* (Figure 1.11e). Leaf number was significantly different for *B. frondosa, E. prostrata* and *R. crispus* in 0.01% DMSO (Figure 1.11f). In 0.01% DMSO root length, root surface area, root volume was significantly different than the controls for *B. frondosa* and *R. crispus* but only root length and root surface for *E. prostrata* (Figure 1.11h, j, i). Total dry mass, shoot dry mass, root dry mass, shoot height, total fresh mass, root length, root surface area, root volume and leaf number was not different than the controls in 0.01% DMSO for *C. acuminatus* (Figure 1.11b, c, d, e, a, h, j, i, f).
Fig 1.11. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of *B. frondosa*, *C. acuminatus*, *E. prostrata* and *R. crispus* grown for 22 days on exposure to three concentrations of DMSO (0.01%, 0.05% and 0.1%). Data shown are means ± one standard error, *n* = 10. The * indicates significant differences between treatment and control.
DMF Effects on Seedling Growth

Total dry mass, shoot dry mass, root dry mass, shoot height, total fresh mass, root length, root surface area, root volume, shoot / root ratio was significantly lower than the controls for *B. frondosa*, *E. prostrata*, *C. acuminatus* and *R. crispus* in 0.1% and 0.5% DMF (Figure 1.12c, b, d, e, a, h, j, l g). For *B. frondosa*, leaf number was significantly lower than the controls in 0.1% DMF but had no effect in 0.05% DMF. For *E. prostrata*, *C. acuminatus* and *R. crispus* leaf number was significantly lower than the controls in 0.1% and 0.5% DMF (Figure 1.12f). Total dry mass for *B. frondosa* was significantly higher than the controls in 0.01% DMF but 0.01% DMF had no effect on total dry mass for *E. prostrata*. For *R. crispus* and *C. acuminatus* total dry mass was significantly lower than the controls in 0.01% DMF (Figure 1.12b). For *B. frondosa*, *E. prostrata* and *R. crispus* 0.01% DMF had no effect on shoot dry mass (Figure 1.12c). Root dry mass was significantly higher than the controls for *B. frondosa* and *E. prostrata* in 0.01% DMF and was significantly lower than the controls for *R. crispus* (Figure 1.12d). For *B. frondosa*, *E. prostrata* and *C. acuminatus* 0.01% DMF had no effect on shoot height but was significantly lower for *R. crispus* (Figure 1.12e). Total fresh mass was significantly higher than control for *B. frondosa*, significantly lower for *E. prostrata* and no effect on total fresh mass for *C. acuminatus* and *R. crispus* (Figure 1.12a). Root length was not affected For *B. frondosa* in 0.01% DMF. For *C. acuminatus*, *E. prostrata* and *R. crispus* root length was significantly lower than the controls (Figure 1.12f). Root surface area, root volume and leaf number was significantly higher than the controls for *B. frondosa*. 0.01% DMF had no effect on root surface area, root volume and leaf
number of *C. acuminatus*, *E. prostrata* and *R. crispus* root surface area, root volume and leaf number was significantly lower than the controls (Figure 1.12j, l, f).
Fig 1.12. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of *B. frondosa*, *C. acuminatus*, *E. prostrata* and *R. crispus* grown for 22 days under exposure to three concentrations of DMF (0.01%, 0.05% and 0.1%). Data shown are means ± one standard error, n = 10. The * indicates significant differences between the treatment and control.
Acetone Effects on Seedling Growth

For *B. frondosa* and *C. acuminatus* none of the acetone exposure solution had an effect on total dry mass (Figure 1.13b). *E. prostrata* and *R. crispus* had significantly lower total dry mass and shoot dry mass at 0.1% and 0.05% acetone but had no effect in 0.01% acetone (Figure 1.13a, c). For *B. frondosa*, root dry mass was significantly lower than the controls in 0.1% acetone but 0.05% and 0.01% acetone had no effect. For *E. prostrata* root dry mass was lower than control in 0.01%, 0.05% and 0.1% acetone. For *C. acuminatus* and *R. crispus* root dry mass was significantly lower than the controls but 0.01% acetone had no effect (Figure 1.13d). Shoot height was not affected for *B. frondosa* in 0.01% and 0.05% acetone but was significantly lower in 0.1% acetone. For *E. prostrata*, *C. acuminatus*, *R. crispus* 0.01% acetone had no effect but in 0.05% and 0.1% acetone shoot height was significantly lower than the controls (Figure 1.13e). Total fresh mass was significantly lower than the controls for *B. frondosa*, *E. prostrata* and *R. crispus*. For *C. acuminatus* 0.05% and 0.1% acetone had no effect in total fresh mass (Figure 1.13a). Root length and root surface area for *B. frondosa*, *E. prostrata* and *R. crispus* was significantly lower in 0.1% and 0.05% acetone. For *C. acuminatus* root length and root surface area was significantly lower than the controls in 0.1% acetone but 0.05% acetone had no effect (Figure 1.13h, j). For *B. frondosa*, *R. crispus*, *E. prostrata*, root volume was significantly lower than the controls in 0.1% acetone (Figure 1.13i). For *B. frondosa* leaf number was not affected in none of the acetone exposure solution. For *E. prostrata* leaf number was significantly lower than the controls in all three acetone exposure treatment. For *C. acuminatus* leaf number was not affected in 0.1% and 0.05% acetone solution, leaf number significantly increase in
0.01% solution than the controls (Figure 1.13f). For *B. frondosa* shoot/root ratio was not affected in 0.01% and 0.1% acetone and was significantly higher in 0.05% acetone solution. For *E. prostrata* none of the acetone exposure solution had an effect in shoot/root ratio. Shoot/root ratio of *R. crispus* was significantly lower in 0.1% and 0.05% acetone but had no effect in 0.01% acetone (Figure 1.13g).
Fig 1.13. Total fresh mass (a), total dry mass (b), shoot dry mass (c), root dry mass (d), shoot height (e), leaf number (f), shoot/root ratio (g), root length (h), root volume (i), root surface area (j) of B. frondosa, C. acuminatus, E. prostrata, R. crispus grown for 22 days under exposure to three concentrations of acetone (0.01%, 0.05%, and 0.1%). Data shown are means ± one standard error, n = 10. The * indicates significant differences between the treatment and control.

Discussion

Seed Germination

The null hypothesis that germination of B. frondosa, E. prostrata, C. acuminatus and R. crispus exposed to three concentrations of DMSO, DMF and acetone will not differ from non-exposed controls is rejected. Optimum requirements for seed germination like water, temperature and oxygen (Grappin, 2001) were provided equally to the system so any differences found in germination percentage of species compared to control can be related to effect of organic solvents used for study. The effects were stimulatory as well as negative and varied with species on observed time.
All three concentrations of DMSO and 0.01% DMF had stimulatory effect of in seed germination of *C. acuminatus* while DMF (0.01% and 0.1%) and acetone (0.01% and 0.05%) had promotive effect on seed germination of *R. crispus*. Stimulatory effect of acetone is reported by Rao et al., (1976). Their study showed an increase in germination of lettuce seeds in dark when these seeds were soaked in 100% acetone from 10min to 12hr but found reduction in germination in seeds that were soaked for 24hr. The promotion of dark germination of lettuce seeds in their study is related to the ability of the acetone to remove the restraining influence of endosperm on embryo growth. Seeds in our study were exposed to organic solvents mixed with water as opposed to soaking of dry seeds to organic solvent and germination was monitored for sixteen days and it is evident from the study that even a low concentration of continuous exposure of organic solvent is able to cause an effect on germination. The promotive effect of organic solvents in germination is also related to its ability to remove waxy layer of seed coat and thereby facilitating water imbibition (Subbaih, 1982). Variation in seed coat among species could be one of the factors to cause differences in seed germination pattern among species observed in our study.

The reduction in seed germination was evident on 4th day for *E. prostrata* and *C. acuminatus* in 0.05% DMF, for *R. crispus* in 0.1% acetone and 0.01% DMSO and the effect evened out with increase in exposure time. *B. frondosa* had lower germination in 0.1% DMF. The initial reduction in seed germination of species observed in our study is not clear. Very few studies have looked upon the effects of organic solvents on seed germination with a continuous exposure system and observation for longer duration. Most of the available literatures have monitored effects of organic in terms of in terms of
its ability to penetrate testa and ability to carry test compounds varied with organic
solvents (Tao et al., 1974; Anderson et al., 1973; Shortt and Sinclair, 1980). In our study
any supporting experiments to find out the probable reason for reduction in germination
was not conducted so the probable reason for reduction in seed germination observed
in our study is not known.

Effects of Organic Solvents on Seedling Growth

The null hypothesis that seedling growth of *B. frondosa, E. prostrata, C. acuminatus* and *R. crispus* exposed to DMSO, DMF and acetone is not different from control is rejected. The result of this study demonstrate that acetone, dimethyl formamide and dimethylsulfoxide in 0.1% concentration is most toxic to studied wetland plant seedlings followed by 0.05% and 0.01% concentration. Concentrations of solvents selected for this study includes the maximum acceptable values of organic solvents recommended in standard test guidelines by EPA, OECD and ASTM, and organic solvents seem to have an effect even at lowest recommended level. DMSO in 0.01% had no effect on *C. acuminatus* but for *B. frondosa, E. prostrata* and *R. crispus* no observed effect concentration (NOEC) of DMSO was below 0.01%. All three solvents at 0.1% concentration had a negative impact on seedling growth. Seedlings of all four species varied in their response to DMF, DMSO and acetone. Variation in solvents effects in this study is in agreement with result of some other studies like growth of algae (Okumuara et al., 2001), sensitivity of grass shrimp embryos (Rayburn and Fisher, 1997), algal photosynthetic activity (Cho et al., 2009), acute and chronic toxicity with *Daphnia magna* (LeBlanc et al., 1983).
The effect of DMSO, DMF and acetone in 0.1% concentration is related to a reduction in root and shoot morphological characteristics. Anderson and Dunford (1996) reported a detrimental effect of DMSO applied to tubers of purple nutsdege in terms of inhibition of root growth, suppression of shoot growth and they inferred that suppression of shoot growth could be due to reduction in root growth. Though no quantitative measurement of plant growth was done, in a study by Robinson et al., (2006) A. thaliana plants grown in 0.05% and 0.1% were of smaller size. Schnurr et al., 1996 used 1% DMSO for foliar application of growth regulators in pine seedlings. In their study, 1% DMSO had no effect on growth modification index measured in terms of shoot height, stem caliper, root and shoot dry weight and shoot:root ratio. In their study, application of solvent and growth regulators was done after successful establishment of seedlings, the mode of application was foliar spray and application of the solvent was done only once. Seedlings in this study were continuously exposed to exposure solution, a more realistic approach for organisms exposed to WWTP effluent.

Reduction in soybean cell suspension cultures with acetone and DMSO is reported by Davis et al., (1978). In their study, plant cell suspension of tissues varied with organic solvents. Their observations of cells from suspension cultures containing 0.5% and 2% acetone under transmission electron microscopic resulted in several cellular abnormalities like variations in shape and size of cells, decrease in number of starch grains in amyloplasts compared to control. In the same study 3% DMSO, resulted in increase in size and shape of cell and decrease in cell wall thickness (Davis et al., 1978). In my study these cellular abnormalities might have occurred in seedlings exposed to solvents but no anatomical study was carried out so no evidence could be
submitted. Bajaj et al., (1970) studied toxic effects of DMSO in terms of respiration in excised roots, stem callus tissue, leaf disks and enzymatically isolated cells of two cultivars of bean (*P. vulgaris*) in 0.1, 1 and 10 % DMSO. In this study an initial increase in respiration in 0.1% and 1% DMSO followed by slight decrease in 1% DMSO after 2-3hr was reported for leaf disk tissue but for other tissues all three concentrations of DMSO had inhibitory effect on respiration. Decrease in root respiration followed by negative effects on root and shoot growth might have occurred in my study that could impacted root and shoot growth.

All three solvents in 0.01% of had promotive effect for at least some of the measured morphological characteristics and the response varied among species and solvent. Stimulatory effects of low concentrations of DMSO have been reported previously for other organisms including an increase in average weight of larvae of common carp exposed to 0.02% DMSO (Machova et al., 2009) and an increase in heart rate of Zebra fish (Hallare et al., 2006). Kumar et al., (1976) showed an increase in dry weight of rice in 0.01 and 0.1% soil applied DMSO and 0.001 and 0.01% foliar applied DMSO. Low concentration of organic solvents had a stimulatory effect of seedling growth of wetland plants but the reason behind this stimulatory effect is not known.

Conclusions

1) Even the lowest recommended levels (0.01%) of organic solvents had an effect on seed germination and seedling growth of wetland plants. Recommended
levels of organic solvents from existing literature are not applicable for wetland plant toxicity tests using a flow through system.

2) Carrier solvents had stimulatory as well as inhibitory effect on seed germination and seedling growth of wetland plants with root morphology being the most sensitive.

3) DMSO at 0.01% affected seed germination of *R. crispus*, while DMF and acetone had no effect on seed germination at 0.01%. DMSO and DMF at 0.01% had an effect on seedling growth of all four species while 0.01% of acetone had an effect on seedling growth *C. acuminatus* and *E. prostrata*. Among solvents, acetone can be considered least toxic compared to DMSO and DMF.
CHAPTER III

BIOCONCENTRATION PATTERNS OF TRICLOSAN (TCS), METHYL TRICLOSAN (M-TCS) AND TRICLOCARBAN (TCC) IN TISSUES OF WETLAND PLANTS EXPOSED TO WASTEWATER EFFlUENT

Introduction

Triclosan (TCS) and triclocarban (TCC) are antimicrobial compounds commonly used in personal care products (PCPs) like toothpaste, medical skin creams, hand disinfecting creams, soaps, and household cleaners. Nearly, $1 billion/year is spent by US consumers on TCS and TCC containing liquid soaps and bar soaps (US EPA, 2007). Waste water treatment plants (WWTPs) are the main receiver of TCS and TCC which end up releasing these contaminants to the environment in the form of effluent. During the WWTP’s processing, TCS and TCC removal occurs through biological degradation, adsorption to sludge and some photodegradation. Studies on the efficiency of WWTTP’s to remove TCS and TCC have shown that these contaminants are not completely removed (Ying and Kookana, 2007; Waltman et al., 2006; Heidler and Halden, 2007) and removal efficiency of antimicrobials is dependent upon the technology applied in sewage treatment plants (Bester, 2005). In addition to WWTP effluent, TCC and TCS can enter the environment from discharge or from runoff from agricultural areas that receive WWTP’s sludge as a soil amendment (Ying et al., 2007; Heidler and Halden, 2007).

Considerable variability in reported values of TCC, TCS and methyl triclosan (MTCS; the biotransformation product of TCS) exists. Concentrations of TCS reported for some waste water effluents include 0.042 - 0.213 µg/L (Singer et al., 2002), 0.007-0.650 µg/L (Lindström et al., 2002), 0.07 ± 0.06 µg/L (Heidler and Halden, 2007), 0.022
µg/L - 0.434 µg/L (Ying and Kookana, 2007), 0.11 µg/L (Waltman et al., 2006), 0.12 µg/L (Coogan et al., 2007). Reported concentrations of TCC in effluent water include 0.084 ± 0.110 µg/L (Heidler and Halden, 2007), 0.20 µg/L (Coogan et al., 2007) 0.157 - 36.50 µg/L (Kumar et al., 2010). Reported concentration of MTCS in effluent water is 0.08 µg/L (Coogan et al., 2007), maximum 0.011 µg/L (Lindstrom et al., 2002).

The antimicrobial activity of TCS action is due to its inhibition of fatty acid synthesis. A similar biological pathway of fatty acid synthesis is shared by plants, and a similar mode of TCS action on plants is expected. This pathway of fatty acid synthesis in not shared by animals. Toxicity studies of TCS, MTCS are available for some organisms like *Vibrio fischeri* bioluminescence inhibition (Farre et al., 2008), *Daphnia magna*, fathead minnow (*Pimephales promelas*) and bluegill sunfish (*Lepomis macrochirus*), algae and duckweed (Orvos et al., 2002). Terrestrial plant toxicity studies have been conducted for *Oryza sativa* (rice) and *Cucumis sativus* (cucumber; Liu et al., 2009). Wetland plant toxicity studies of TCS have been conducted by Stevens et al., (2009) and included assessment of seed germination and seedling growth. In this study TCS had no effect on seed germination but had significant reduced root length and root surface area of TCS exposed plants compared to control. A transformation product of triclosan, methyl triclosan (MTCS) was detected in the system.

MTCS is the biotransformation product of TCS and is considered to be more hydrophobic and stable than the parent compound (Lindstrom et al., 2002; Delorenzo et al., 2008). TCS, MTCS and TCC have a relatively similar hydrophobicity with an estimated log octanol –water partition coefficient (Log Kow) at neutral pH of 4.8, 5.2 and 4.9 respectively. Based on Log Kow these compounds are lipophilic and studies have
shown their bioaccumulation in zebrafish (Orvos et al., 2002), snails (Coogan and La Point, 2008), and algae (Coogan et al., 2007). TCS and MTCS bioaccumulate in the root and shoot of S. herbacea and B. frondosa in exposure concentration of 10 - 100 ppb and the concentrations of these analytes differed in shoot and root within species and among species (Stevens et al., 2009).

In arid regions, the use of constructed wetlands for reuse and recycling of municipal waste water is one of the best techniques to meet the increased demand in quantity and quality of water. Waltman et al., (2006) showed that Denton WWTP’s constructed wetland was able to reduce TCS concentrations from 0.11 µg/L in the effluent to 0.04 µg/L in the wetland outflow. Although it is recognized that wetlands can reduce antimicrobial concentrations in WWTP effluent, little is known of the effectiveness of species from different functional or taxonomic groups to facilitate the removal. Wetland plants have been classified into functional groups including emergent, submerged, floating leaved and free floating. These classifications are based on exposure of root and shoots of wetland plant species to water (Sculthrope, 1967; Cronk and Fennessy, 2001). Understanding the bioconcentration patterns of wetland plants belonging to different taxa and functional groups will help to design a constructed wetland with increase removal efficiency of TCS, MTCS and TCC. Plants with high bioconcentration capacity could also be used as reference plants for the phytoremediation of lipophilic organic compounds entering into constructed wetlands. Understanding bioaccumulation pattern might also have ecological implications with regard to plant establishment, competitive ability and fitness in areas receiving WWTP effluent.
Hypotheses:

1) Organ specific bioconcentration of TCS, MTCS and TCC is not different among species within functional groups or among species across functional groups.

2) The degree of bioconcentration in a tissue will not differ among compounds.

Materials and Methods

Sources of Chemicals

Labeled internal standards $^{13}\text{C}_{12}$- TCS, $^{13}\text{C}_{12}$-M-TCS, native TCS and M-TCS were obtained from Wellington Laboratories (Guelph, ON, Canada). The dueterated TCC (d$_7$TCC) internal standard was obtained from Cambridge Laboratories (Andover, MA, USA) and TCC was obtained from Absolute Standards (Hamden, CT, USA). Organic solvents hexane, ethyl acetate, chloroform and acetonitrile were obtained from Fisher Scientific (Houston, TX, USA).

Study Plants

Twelve species of wetland plants representing five functional groups (Figure 3.1) were obtained from Joe Snow Wetland Plant Nursery (Denton, TX). Polygonum hydropiperoides, Pontederia cordata, Sagittaria graminea represented the emergent plants. This group includes plants with their root or basal portion in water while their stem and reproductive structure grow above water. Bacopa monnieri and Potamogeton nodosus represented the semi-emergent group. The root of semi-emergent grows under water while shoot extends above water surface. Vallisneria americana, Najas guadalupensis and Heteranthera dubia represented submerged plants. Submerged
plants usually have both the root and shoot under water. *Nymphaea* sp. and *Nelumbo lutea* represented floating leaved plants. Leaves of floating leaved plants float on the water surface while petiole and roots are under water. *Spirodella* sp. and *Lemna minor* represented free floating plants. In this group there the leaves of float at the water surface while roots, if present, hang in the water.

Experimental Design and Exposure of Plants to Effluent

The study was carried out in Pecan Creek Experimental Wetland Facility established at Denton’s waste water treatment plant (Pecan Creek, Denton, TX). The study facility is equipped with 8 mesocosms. Each mesocosm is fitted with an inlet system that supplies effluent from a reservoir (0.492L/min) to the mesocosm and an outlet system that drains out effluent from the mesocosm. Water depth was controlled with stand pipes in each mesocosm (Figure 2.2). Four plants of each species representing emergent, floating leaved and submerged groups were transplanted into one gallon plastic pots filled with a mixture of sand and gravel placed in the bottoms of the mesocosms. The floating plants, *Spirodella* sp. and *Lemna* sp. were directly released into the mesocosm. Before exposure of plants to effluent, plants were randomly assigned to the mesocosms. There were two replicate mesocosms for each functional group. Within each mesocosm, the locations of pots were randomized. Along with exposure of plants to effluent, non-exposed plant samples from each of the representative groups were obtained to analyze background levels of antimicrobials. These specimens were rinsed with tap water, blotted dry with paper towel, wrapped in aluminum foil and stored at -10°C for analytical study.
Harvesting and Storage of Plant Samples

Plants were harvested after two months of exposure. Harvesting, cleaning and storage of samples were completed within two days. To prevent contamination all plant material was handled using latex gloves. Whole plants of *Najas guadalupensis*, *Heteranthera dubia*, *Potamogeton nodosus*, *Vallisneria americana*, *Bacopa monnieri*, *Nymphaea sp.* and *Nelumbo lutea* were removed from pots, the soil attached to root was rinsed with effluent water, then the entire plant wrapped in aluminum foil and brought to laboratory. For *Polygonum hydropiperoides*, *Pontederia cordata* and *Sagittaria graminea* shoots and roots were separated upon harvest and wrapped separately in aluminum foil. In the laboratory samples were rinsed with tap water, blotted dry with paper towels and separated into root and shoots. Each tissue was wrapped in new aluminum foil, labeled and stored at -10°C for analytical study.
Fig 2.1. Eleven different species of wetland plants belonging to five functional groups (emergent, semi-emergent, submerged, floating leaved and free floating) of wetland plants.

Fig 2.2. Overview of the mesocosm facility at Denton Waste Water Treatment Plant.
Division of Tissue Parts for Analytical Studies

For *Polygonum hydropiperoides*, *Pontederia cordata*, *Sagittaria graminea*, *Bacopa monnieri*, *Potamogeton nodosus*, *Vallisneria americana*, *Najas guadalupensis* and *Heteranthera dubia* plants were divided into root and shoot tissues (Figure 2.3). For the floating leaved species, *Nymphaea* sp. and *Nelumbo lutea*, tissues were divided into leaf, root and rhizome (Figure 2.3). Mesocosms with the free-floating species (*Lemna* sp. and *Spirodella* sp.) were infested with the leaf-boring insect *Synclita obliteralis* eliminating these species from further analysis.
Fig 2.3. Diagrammatic representation of plants used and locations of organs harvested related to the water level.

Analytical Study of TCS, MTCS and TCC in Plant Tissue

*Tissue Sample Preparation and Extraction*

Plant tissues were removed from the freezer and defrosted. Moisture accumulated after defrost was removed with paper towel. Each tissue part was finely chopped with stainless razor blade. From this chopped sample, 2-5 g of tissue was grinded using IKA® A11 basic (IKA® Works, Inc.) mill. From this grinded sample, 500mg of subsample was weighed in a 30ml glass centrifuge tube. To the weighed sample 20 ml of 1:1 Hexane: Ethyl Acetate (HEX: EA), 10 ml of milliQ water, 10 µl of 5 ppm 13C12TCS, 13C12-M-TCS and 10 µl of ppm d7TCC internal standard were added. The sample was vortexed for 30 seconds and then homogenized with Fisher Scientific Powergen 1000 (Fisher Scientific, USA) tissue homogenizer. The homogenate was vortexed for 2 min and centrifuged at 300 rpm for 12 min. After centrifugation the liquid layer and solvent layer was separated and the solvent layer was transferred into a 30 ml test tube using Pasteur pipette. An additional 5 ml of 1:1 HEX: EA was added to the remaining layer of the water and tissue in the centrifuge vial. The sample was vortexed for 2 min, following similar steps as with the initial homogenate. The process was repeated one more time with 5 ml of solvent. The sample collected in 30 ml test tube was reduced to
approximately 1 ml using Labconco (Kansas, MO, USA) RapidVap™ nitrogen evaporator. From the 30 ml test tube each sample was transferred to 4 ml glass vials followed by its transfer into to 1.5 ml preweighed plastic centrifuge vials. During each transfer the test tube and 4 ml glass vials were rinsed three times with 1:1 HEX: EA. The sample in centrifuge vial was completely dried by nitrogen blow down and the mass of the centrifuge vial with dried sample was recorded. The initial mass of centrifuge vial without sample and mass of centrifuge after sample dried were used to calculate the lipid mass. For root samples, the sample in the vial was reconstituted using 500 µl of acetonitrile and clean up of lipids was done which will be explained separately. Shoot samples were reconstituted to 5 ml of 1:1 Hex: EA and proceeded for chlorophyll cleanup.

Chlorophyll cleanup in Shoot Samples

Due to interference of chlorophyll during GC/MS a cleanup step using Florisil to reduce chlorophyll concentrations was required. Florisil (mesh size 60-100 mm) from Fisher Scientific was conditioned at 150°C for 12 hr prior to its use. About 2g of conditioned florisil was taken in two 20 ml test tubes per sample. Shoot samples were reconstituted with 5 ml of 1:1 Hex: EA were added into first test tube, vortexed and centrifuged. The solvent layer from first test tube was transferred into the second test tube. The second test tube was also vortexed, centrifuged and the solvent layer in the second test tube was transferred into 30 ml glass test tubes. Then the first test was subsequently rinsed twice with 5 ml of 1:1 Hex: EA and twice with 5 ml of 1:1 chloroform: Acetonitrile. After each rinse of the first test tube solvent layer was
transferred into the second test tube. The second test tube was vortexed, centrifuged and solvent layer was collected in the 30ml glass test tubes. Samples collected in 30ml glass tubes were brought down to about 1 ml - 2 ml with Labconco (Kansas city, MO, USA) RapidVapTM nitrogen evaporator. Samples dried in 30 ml test tubes were transferred into 4ml glass vials and finally to 1.5 ml plastic centrifuge vial for further clean up.

Lipids Cleanup in Extracted Samples of Root and Shoot

Dried root and shoot samples in plastic centrifuge vials were reconstituted with 500 µl of acetonitrile. Samples were placed in a -80°C freezer for ten minutes followed by immediate centrifugation at 14,000 rpm for 30s. The supernatant was transferred to a 2 ml amber vial. To ensure complete extraction of all three analytes, centrifuge vials were rinsed twice with acetonitrile following similar freezing and centrifugation. The supernatant of each sample was blow down completely using nitrogen gas then reconstituted with 100 µl of acetonitrile. From this 100 µl final volume, 20 µl was transferred into 100 µl conical glass inserts for TCC analysis with LC/MS. The remaining 80ul was evaporated using N₂ gas. The dried sample was reconstituted to 100 µl, with 50 µl of MSTFA and 50 µl of acetonitrile, vortexed and kept in oven at 60°F for derivatization. After one hour of derivatization, the sample was taken out of oven and brought to room temperature. The sample was then vortexed and evaporated using N₂ gas. At this point, the dried sample was reconstituted to a final volume of 80ul by using 75 µl of dichloromethane and 5 µl of MSTFA. The sample was vortexed and transferred
into a 200 µl flat bottom insert placed in an auto sampler vial for TCS and MTCS analysis.

Quality Control (QC)

As a quality method, blanks and matrix blanks were included in each batch of samples. The method detection limit (MDL) was determined for clean root and shoot samples and followed the tissue preparation steps outlined above. MDL included 2 method blanks, 2 matrix blanks and seven replicate matrix spikes for each matrix type. MDL was calculated by using formula (MDL = SD \times 3.14) (APHA Standard Methods 1030E). All the samples were spiked with 10 µl of 5 µg/ml TCS, MTCS and 10 µl 1 µg/ml \text{^{13}C}{_{12}} TCS, \text{^{13}C}{_{12}}MTCS and \text{d_{7}}TCC of internal standard. 10 µl of 1 µg/ml of TCS, MTCS and TCC were added for matrix spike.

Instrument Analyses

TCS and MTCS analyses were done by using GC/MS, an Agilent (Palo Alto, CA, USA) 6890 GC coupled with a 5973 mass selective detector. GC conditions are helium carrier gas at 480 hpa, inlet temperature at 260{^0}\text{C} and column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 µm film). The starting temperature of oven was 40{^0}\text{C} with 1min hold followed by subsequent ramps; ramp 1 (0min-hold, 50{^0}\text{C} /min, 220{^0}\text{C}), ramp 2 (0min- 5{^0}\text{C} /min, 285{^0}\text{C}), ramp 3 (16 min-hold, 10{^0}\text{C} /min, 300{^0}\text{C}). Injection volume is 2 µl, pulsed pressure 25 psi and pulsed splitless mode. TCC was analyzed by using LC- ESI- MS method (Halden and Paull, 2004; Halden and Paull, 2005). An Agilent 1100 LC/MS system with Model SL ion trap MS was used. The
column was a C18 (monomeric, non-endcapped), Zorbax with a 5 µm particle size and 80Å pore size. 2 µl sample was autoinjected with a gradient program 300 µl/min (70% mobile phase B and 30% mobile phase A). Mobile phase B constituted 95% acetonitrile and 5% water with 5 mM ammonium acetate while mobile phase A constituted 95% water and 5% acetonitrile with 5 mM ammonium acetate. The ion trap was operated in negative ion multireaction monitoring mode (MRM) isolating m/z 313-315 for native TCC and m/z 320-322 for d7 TCC internal standard. These isolated pseudomolecular ions ([M-H]-) were fragmented (amplitude 0.8) to yield daughter ions at m/z at 160 and 163 for native and d7 TCC, respectively (Coogan et al., 2007). Five point standard curves were established for both the pseudo-molecular ions and the daughter ions with TCC concentrations from 16 to 1000 pg/µl and d7 concentration of 100 pg/µl.

Data Analyses

For measurements below method detection limit (MDL) a value of (1-p) × MDL was assigned where p is the proportion of measurements below MDL (Jaspers et al., 2006). Samples with more than 50% of measurements below MDL, non-detects and samples with only two replicates were not included in statistical analyses. All statistical analyses were done by using Instat version 3.06, Graphpad software. Sample size was limited to maximum four so differences in bioconcentration patterns of TCS, MTCS and TCC among species were done individually by parametric one way ANOVA if these compounds were detected in more than three species. If the compounds were present only in two species, differences in means were compared by unpaired t-tests. Differences between means were considered significant if α ≤ 0.05.
Results

Quality Control (QC) Results

TCS concentration in clean shoot samples were not detected (<17 ng/g). The MDL of TCS, MTCS and TCC varied with matrix and analyte. TCS and MTCS were not detected in method blanks while TCC were below MDL (8.654 ng/g). TCS in shoot and root matrix blanks were below MDL. MTCS was not detected in both matrixes. TCC in root matrix was below MDL.

Table 2.1. Quality control data for antimicrobials bioconcentration in wetland plants. Spike additions were at 20 ng/g.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Tissue</th>
<th>Matrix spike (% Recovery, n=7)</th>
<th>MDL (ng/g, n=7)</th>
<th>Percentage Relative Standard Deviation (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS</td>
<td>shoot</td>
<td>138.2</td>
<td>16.5</td>
<td>7.2 (n=8)</td>
</tr>
<tr>
<td>MTCS</td>
<td>shoot</td>
<td>139.7</td>
<td>4.4</td>
<td>20.1 (n=8)</td>
</tr>
<tr>
<td>TCC</td>
<td>shoot</td>
<td>93.5</td>
<td>8.7</td>
<td>13.0 (n=7)</td>
</tr>
<tr>
<td>TCS</td>
<td>root</td>
<td>61.8</td>
<td>5.8</td>
<td>14.3 (n=6)</td>
</tr>
<tr>
<td>MTCS</td>
<td>root</td>
<td>118.8</td>
<td>6.4</td>
<td>3.9 (n=6)</td>
</tr>
<tr>
<td>TCC</td>
<td>root</td>
<td>123.9</td>
<td>11.4</td>
<td>10.8 (n=8)</td>
</tr>
</tbody>
</table>

TCS Bioaccumulation Pattern

Tissue specific bioconcentration of TCS varied within functional groups. Root TCS bioconcentration of emergent species was significantly higher in *P. hydropiperoides* (55 ± 14.7 ng/g) compared to *P. cordata* (8.9 ± 1.4) (Figure 2.4). TCS was not detected in the roots of *S. graminea* or in the shoots of any emergent plant. Shoot and root TCS levels did not differ between either semi-emergent species. TCS
was not detected in the roots of *H. dubia* or the shoots of any submerged plant. There were no significant differences in TCS concentration in the roots of *N. guadalupensis* and *V. americana* (Figure 2.4). There were no differences in TCS concentration of roots or shoots of the floating leaved species, however concentrations in the roots of *N. lutea* were significantly greater that *Nymphaea* sp. In those species where TCS was detected *P. hydropiperoides* and *N. lutea* had the highest levels of TCS.

Organ specific bioaccumulation pattern of TCS within species varied among species. For *P. hydropiperoides, P. cordata, N. guadalupensis, V. americana* TCS above MDL was found only in one of the tissue part analysed and completely below MDL in *S. graminea* so no comparison between tissue parts within species could be made for these species. TCS bioconcentration in shoot of *B. monnieri* was significantly higher than root but no difference was found for TCS bioconcentration in root and shoot of *P. nodosus*. TCS in *N.lutea* differed among all three organs and was greatest in the roots and lowest in the rhizome. For *Nymphaea* sp. there were significant differences among all three tissues. Similar to *N. lutea* the lowest levels were found in the rhizomes but in contrast the highest levels were in shoots.

**MTCS Bioaccumulation Pattern**

MTCS above MDL was detected in few of oragan samples. Organ specific differences in MTCS bioconcentration among species of each functional group were compared for submerged and floating leaved group. Shoot MTCS concentrations in submerged as well as floating leaved species were not different.
Among species there was no significant difference in mean shoot MTCS concentration of *P. nodusus* and *N. guadalupensis*. *P. nodusus* shoot MTCS was significantly different than *V. americana*, *N. lutea* and *Nymphaea* sp. while shoot MTCS concentration of *N. guadalupensis* was significantly different than *N. lutea* and *Nymphaea* sp. Root MTCS concentration above MDL was detected only in *P. hydropiperoides* and *N. lutea* (Figure 2.5). MTCS concentration in root and shoot of *N. lutea* was significantly different (Unpaired t-test, α ≤ 0.05).

TCC Bioaccumulation Pattern

Shoot TCC concentrations in all emergent species were below MDL. Shoot TCC concentrations among species in semi-emergent and floating leaved groups were not different. In the submerged species, shoot TCC concentration of *N. guadalupensis* was significantly different than *H. dubia* and *V. americana* but was not different between *H. dubia* and *V. americana* (Figure 2.6). Likewise in the floating leaved groups, shoot TCC concentration in *N. lutea* was not different from *Nymphaea* sp..

TCC bioconcentration across species was different. Root TCC concentration in *P. hydropiperoides* and *N. lutea* was not different but was significantly different than *P. nodosus*, *N. guadalupensis*. Root TCC concentration in *P. nodosus* and *N. guadalupensis* were also not different (Figure 2.6). Shoot TCC concentration of *N. guadalupensis* (256.726±49.001ng/g) was significantly higher than *B. monnieri* (81.053 ± 29.298ng/g), *N. lutea* (53.96 ± 2.9), and *Nymphaea* sp. (59.85 ± 5.299ng/g) but there was no difference among these species.
Shoot TCC concentrations of *P. nodosus* and *N. guadalupensis* were significantly different than roots whereas for *N. lutea* root TCC concentration was significantly different than shoot and rhizome and shoot concentration was significantly higher than rhizome (Figure 2.6).

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**Fig 2.4.** TCS concentration (mean ± SE) of *P. hydropiperoides*, *P. cordata*, *B. monnieri*, *P. nodosus*, *V. americana*, *N. lutea* and *Nymphaea sp.* belonging to four functional groups of wetland plants. Number inside data series represents sample size. Within an organ system (rt = root, st = shoot, rh = rhizome). Similar letter and number above error identify TCS means that do not significantly differ (Upper case = root TCS concentration within functional group and across species, st = shoot concentration within concentration within functional group and across species, number = organ specific TCS concentration within species.)
Fig 2.5. MTCS concentration (mean ± SE) in *P. hydropiperoides*, *P. nodosus*, *N. guadalupensis*, *V. americana*, *N. lutea* and *Nymphaea sp.* belonging to four functional groups of wetland plants. Numbers inside data series represents sample size. Within an organ system (rt = root, st = shoot, rh = rhizome). Similar letter and numbers above error bar identify MTCS means that do not significantly differ (Upper case = root MTCS concentration within functional group and across species, lower case = shoot concentration within functional group and across species, number = organ specific MTCS concentration within species.)
Fig 2.6. TCC concentrations (mean ± SE) in *P. hydropiperoides*, *P. cordata*, *B. monnieri*, *P. nodosus*, *H. dubia*, *N. guadalupensis*, *V. americana*, *N. lutea*, and *Nymphea sp.* belonging to four groups of wetland plants. Within an organ system (rt = root, st = shoot, rh = rhizome). Similar letter and number above error bar identify TCC means that do not significantly differ (Upper case = root TCC concentration within functional group and across species, lower case = shoot TCC concentration within functional group and across species, number = organ specific TCC concentration within species.

TCS, MTCS and TCC in Root Tissue

TCS and MTCS concentration in root of *P. hydropiperoides* were not different but were significantly different than root TCC (Figure 2.7). For *N. lutea*, root TCS and
TCC concentrations were not different but were significantly different from MTCS concentrations (Figure 2.7).

![Graph](image)

**Fig 2.7.** Root concentration of TCS, MTCS and TCC (mean ± SE) in *P. hydropiperoides* (*n* = 3), *P. cordata* (*n* = 4), *P. nodosus* (*n* = 4), *N. guadalupensis* (*n* = 3) and *N. lutea* (*n* = 4). Similar letter identify means that do not significantly differ.

**TCS, MTCS and TCC Shoot Tissue**

Shoot TCS and MTCS of *P. nodosus* were not different but were significantly different from shoot TCC. For, both *N. lutea* and *Nymphaea* sp. shoot TCS, TCC and MTCS were significantly different from each other.
Fig 2.8. Shoot concentration of TCS, MTCS and TCC (mean ± SE) in *B. monnieri* (*n* = 3), *P. nodosus* (*n* = 4), *N. guadalupensis* (*n* = 3), *V. americana* (*n* = 4) and *Nymphaea* sp. (*n* = 4). Similar letters identify means that do not significantly differ.

Tissue Specific Bioaccumulation Factors (BCFs) of TCS, MTCS and TCC

Mean TCS concentrations in effluent samples from Pecan Creek Waste Water treatment are reported as 0.11 ppb (Waltman et al 2006). TCS, TCC, MTCS from the same site is reported as 0.12, 0.20 and 0.08 ppb respectively (Coogan et al 2007). The calculation of mean BCFs is based on these reported values. The highest root TCS BCF’s were in *N. lutea* root followed by *P. hydropiperoides*, while shoot TCS BCFs was higher in *Nymphaea* sp. For MTCS the highest BCF’s was in *P. nodosus* shoot. For TCC, the highest BAF was in *N. guadalupensis* (Table 2).
Table 2.2. Tissue specific bioconcentration factors (BCFs) based on fresh weight. Concentrations of analytes below MDL were not included in calculation of BCFs. Empty space represents for tissue parts with Bioconcentration below MDL.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue</th>
<th>Emergent</th>
<th>Semiemergent</th>
<th>Submerged</th>
<th>Floating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. hydropiperoides</em></td>
<td><em>P. cordata</em></td>
<td><em>B. monnieri</em></td>
<td><em>N. guadalupensis</em></td>
</tr>
<tr>
<td>TCS</td>
<td>shoot</td>
<td>175.0</td>
<td>143.5</td>
<td></td>
<td>253.4</td>
</tr>
<tr>
<td></td>
<td>root</td>
<td>458.6</td>
<td>74.3</td>
<td>207.5</td>
<td>64.6</td>
</tr>
<tr>
<td></td>
<td>rhizome</td>
<td>90.525</td>
<td>68.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTCS</td>
<td>shoot</td>
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<td>286.3</td>
<td>148.1</td>
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</tr>
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<td></td>
<td>rhizome</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC</td>
<td>shoot</td>
<td>699.2</td>
<td>426.6</td>
<td>1351.2</td>
<td>576.3</td>
</tr>
<tr>
<td></td>
<td>root</td>
<td>835.2</td>
<td>154.0</td>
<td>92.5</td>
<td>387.8</td>
</tr>
<tr>
<td></td>
<td>rhizome</td>
<td></td>
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</tr>
</tbody>
</table>
Discussion

Tissue specific bioconcentration patterns of TCS, MTCS and TCC in *P. hydropiperoides*, *P. cordata*, *S. graminea*, *B. monnieri*, *P. nodosus*, *H. dubia*, *V. americana*, *N. guadalupensis*, *N. lutea* and *Nymphaea* sp. varied with species across functional group, among species within functional group and among tissue within species. The discussion will be focused on each functional group.

For emergent plants, TCS, MTCS and TCC were detected only in the root of *P. hydropiperoides* and *P. cordata* and were below MDL for effluent water exposed shoot as well as unexposed shoot for all three species. Root BCF of *P. hydropiperoides* and *P. cordata* are consistent with the concentrations reported in WWTP effluent. Considering Log Kow of TCS (4.8), TCC (4.9) and MTCS (5.2) higher bioconcentration of MTCS can be expected but the concentration of these microbial compounds in effluent water was higher for TCC followed by TCS and MTCS. Similar bioconcentration pattern of TCS, MTCS and TCC was recorded for algal species collected from different sites of Pecan Creek receiving Denton WWTP’s effluent water (Coogan et al., 2007). Lipid content is also one of the factors affecting bioconcentration but no difference in root lipid mass was seen between emergent root systems (data not shown), so it can be concluded that factors beyond lipid mass are playing a role in different root bioaccumulation pattern of antimicrobials between species. The transpiration stream is considered as one of the major pathways for the transport of pollutants from roots to shoots of terrestrial plants (Briggs et al., 1998; Trapp, 2000; Simonich and Hites, 1995). The transport of chemicals to transpiration stream is dependent on several other factors like physicochemical properties of chemicals (log Kow, solubility, temperature,
concentration of chemicals) and plant properties like lipid content (Simonich and Hites, 1995). Low concentration of TCS, MTCS and TCC in shoot of all three emergent plants in this study is not clear.

Submerged plants are in direct contact with water or exposure medium, so bioaccumulation patterns in these species might be related to uptake from root as well as surrounding medium rather than transpiration dependent bioconcentration in emergent plants (Turgut, 2005). High concentrations of TCC in the shoots of submerged plants can be related to higher concentration of TCC in effluent water. Studies on organ specific bioconcentration pattern or uptake of pollutants in submerged plant is varied among contaminants with some studies showing higher bioconcentration in foliar portion (Hinman and Klaine, 1992; Hopple and Foster 1996; Turgut, 2005) and some studies in root portion (Liu and Schnoor, 2008). In the present study, roots of *H. dubia* were not analysed but for *N. guadalupensis* and *V. americana* MTCS and TCC were detected in shoot and TCS in root. Higher BAF’s of *N. guadalupensis* might be related to species specific ability to bioconcentrate organic compounds.

*P. nodosus* and *B. monnieri* were included as semi-emergent plants in this study because some portion of the plant was completely inside water and some apical portion of shoot was above water. No similarities in bioconcentration pattern within the group were observed; rather it was similar to *Nymphaea* sp for *P. nodosus* and similar to *N. lutea* for *B. monnieri*. Patterns of bioconcentration in these compounds might involve direct uptake from medium as well as from transpiration stream as upper apical portion of plant is exposed to water.
For floating leaved plants higher BAFs in root compared to shoot of the *N. lutea* is in agreement with expected low transport of these compounds in shoots based on their log Kow ranging from 4.8 to 5.2. Higher concentrations of TCS and MTCS in root compared to shoot is reported for seedling exposed to TCS in growth room condition (Stevens et al 2009). Higher BAFs of TCS, MTCS and TCC in *Nymphaea* shoot might have resulted due to differences in root anatomy and transpiration efficiency between these species. Lipid content in shoots of both species was higher than in root and rhizome so it could be that high transpiration of water from leaf surface left TCS and TCC to bioconcentrate in shoot (leaf +petiole).

The difference in bioconcentration patterns observed among tissues of plants among species and across groups exposed to effluent water might have occurred due to several reasons like individual plant characteristics, physicochemical properties of each compound, exposure time and exposure condition. Understanding the anatomy and physiology of wide range of wetland plants will help to answer why there are differences in bioconcentration patterns of these species.

**Conclusions**

Highest TCS bioconcentration in was detected in root of *N. lutea* (76.±7.7 ng/g) followed by *P. hydropiperoides* (55 ± 14.7 ng/g) while TCC bioconcentration was in shoot of *N. guadalupensis* (256.726±49.001 ng/g) followed by root of *P. hydropiperoides* (158.68 ± 9.019 ng/g). MTCS bioconcentration was highest in shoot of *P. nodous* (24.5 ± 2.1 ng/g) followed by shoot of *N. guadalupensis* (22.9 ± 4.2 ng/g). These differences in bioconcentration patterns of among species have practical applications as follows:
1) Understanding bioconcentration patterns of each wetland plant is necessary if it is going to be a part of a constructed wetland.

2) The bioconcentration patterns of these plants can be used as reference plants to study the removal efficiency of other lipophilic compounds present in waste water effluent.

3) It will also help to understand the competitive ability and fitness of these plants in natural ecosystem.
CHAPTER IV

SUMMARY

Literature available recommended levels of organic solvents, 0.01%, 0.05% and 0.1% had toxic effects on seed germination and seedling growth of wetland plants using flow through system. The effects varied with species. It is concluded that use of the literature available recommended levels of organic solvents is not applicable for wetland plants to be used for toxicity test of water immiscible organic contaminants.

Bioconcentration patterns of TCS, MTCS and TCC varied with species among and across functional groups of wetland plants. This study shows that it is necessary to emphasize the role of wetland plants during a design of a constructed wetland. Differences in bioconcentration patterns of wetland plants not only help to select wetland plants with increase the removal efficiency of interested compounds but also will assist in the design of constructed wetland.
REFERENCES


LeBlanc GA, Surprenant DC. 1983. The acute and chronic toxicity of acetone, dimethyl formamide and triethylene glycol to Daphnia magna (Straus). *Arch Environ Contam Toxicol* 12:305-310


