PHYTOREMEDIATION OF PHARMACEUTICALS WITH *SALIX EXIGUA*

CARMEN FRANKS
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CARMEN G. FRANKS

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Dr. Stewart B. Rood, Supervisor, Department of Biological Sciences

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Dr. Alice Hontela, Thesis Committee Member, Department of Biological Sciences

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Dr. Bryan E. Kolb, Thesis Committee Member,
Department of Neuroscience - Cdn Ctr for Behavioural Neuroscience (CCBN)

Dr. David M. Reid, External Examiner, University of Calgary,
Department of Biological Sciences

Dr. Mathew G. Letts, Chair, Thesis Examination Committee, Department of Geography

Abstract

Municipal treated wastewater entering rivers contain biologically active pharmaceuticals capable of inducing effects in aquatic life. Phytoremediation of three of these pharmaceuticals and an herbicide was investigated using Sandbar willow (*Salix exigua*) and *Arabidopsis thaliana*. Both plants were effective at removing compounds from solution, with removal of 86% of the synthetic estrogen, 17α-ethynylestradiol, 65% of the anti-hypertensive, diltiazem, 60% of the anti-convulsant, diazepam (Valium®), and 51% of the herbicide atrazine, in 24 hours. Distribution of compounds within roots and shoots, in soluble and bound forms, differed among compounds. Uptake and distribution of pharmaceuticals within the study plants confirmed pharmaceutical behaviour can be predicted based on a physiochemical property, their octanol-water partitioning coefficients.

An effective method for detection of 17α-ethynylestradiol within surface water using solid phase extraction and gas chromatography-mass spectrometry was developed. Previously unreported breakdown of 17α-ethynylestradiol into another common estrogen, estrone, during preparative steps and gas chromatography was resolved.

Preface - Thesis Structure

This research-based MSc thesis includes an introductory chapter, three stand alone research chapters, and an integrative conclusion chapter.

Chapter 1, 'Introduction', provides background information on phytoremediation and the emerging issue of pharmaceuticals entering the water environment, as well as an introduction to the contents of this thesis.

Chapter 2, 'Phytoremediation of trace pharmaceuticals, diltiazem, diazepam and 17α-ethynylestradiol with sandbar willow (*Salix exigua*)', represents a stand alone research chapter. It describes the application of an abundant riparian willow species for the removal of trace pharmaceuticals from solution. The herbicide atrazine is included as a positive control, as it is already known to be taken up by plants.

Chapter 3, 'Phytoremediation of trace pharmaceuticals, diltiazem, diazepam and 17α-ethynylestradiol with *Arabidopsis thaliana*', is another stand alone research chapter. The model plant *Arabidopsis* is investigated not for its prospective field application, but for its possible future genetic inquiry.

Chapter 4, A 'Method for detection of 17α-ethynylestradiol in surface water,' is a methodology chapter. A method is outlined for extracting and measuring the synthetic birth control hormone ethynylestradiol from waste or surface water.

Chapter 5, 'Discussion,' integrates the information from the three prior research chapters.

Finally, Appendix A presents the statistical analyses conducted for the thesis, while Appendix B provides additional details of the experimental methods that are not provided in Chapters 2 or 3. Appendix C provides a figure of the nuclear magnetic resonance (NMR) analysis performed on 17α-ethynylestradiol to verify its purity. Further information on the pharmaceuticals and the herbicide used in this study are provided in Appendix D.

Acknowledgements

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Abbreviations

ANOVA = analysis of variance

ATZ = atrazine

BSTFA = *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide

C_{18} = octadecyl-functionalized silica

d_2-E2 = deuterated-17β-estradiol

DTZ = diltiazem

DZP = diazepam (Valium®)

E1 = estrone

E2 = estradiol

EDC = endocrine disrupting compound

EE2 = 17α-ethynylestradiol

fr. wt. = fresh weight

GC-MS = gas chromatography-mass spectrometry

hr = hour

IS = internal standard

Log K_{ow} = logarithm octanol-water partitioning coefficient

LSC = liquid scintillation counter

MeOH = methanol

RCF = root concentration factor

RT = retention time

SE = standard error

SIM = single ion monitoring

SPE = solid phase extraction

TIC = total ion chromatogram

TMCS = trimethylchlorosilane

TMS = trimethylsilyl

TSCF = transpiration stream concentration factor

WWTP = wastewater treatment plant

CHAPTER 1 Introduction

1.1 BACKGROUND: PHARMACEUTICALS IN THE WATER ENVIRONMENT

Water contaminants have been a growing concern since industrialization, as water bodies have been treated as a convenient place for disposing tailings waters, domestic and municipal sewage, industrial waste and other effluents. The developments of pharmacology have introduced a new range of water contaminants with some pharmaceuticals being chemically very stable and hence persisting and accumulating in surface waters and even potentially in ground water.

Recently, pharmaceuticals have been detected within treated wastewater, surface water, and dinking water, such as with the discovery of clofibric acid in surface and drinking water in Berlin (Germany) in 1991 (Stan and Linkerhägner, 1992, cited in Sengl and Krezmer, 2003). Germany and Switzerland have led the investigations of pharmaceuticals in surface and wastewater in the early 1990's (Loffler et al., 2005), with the rest of the world quickly following their lead. In 1999, researchers within the United States undertook a nationwide reconnaissance of 95 pharmaceutical and other organic wastewater contaminants in water resources, including anti-bacterial agents, hormones, personal care products, cleaners and others (Kolpin et al., 2002). Also in 1999, Ternes et al. (1999a and 1999b) reported on the occurrence of estrogens in water from municipal sewage treatment plants of Germany, Canada and Brazil, documenting detectable levels of natural and synthetic hormones. Cargouet et al., in 2004, examined and reported on endocrine disrupting compounds (EDC) in wastewater treatment plant (WWTP) influent, effluent and receiving waters in Paris, France and its suburbs. Servos et al., in 2005,

reported the presence of estrogens in municipal WWTP influent and effluent within Canada. Within the province of Alberta, Alberta Environment published a preliminary report on the presence of pharmaceuticals and EDCs in major cities' wastewater effluent and receiving waters in 2005 (Sosiak and Hebben, 2005). Scientists within the countries of China, Japan, New Zealand, Sweden, and others, have also reported on the levels of these compounds within their waters (Komori et al., 2004; Bendz et al., 2005; Hashimoto et al., 2005; Richardson et al., 2005; Sarmah et al., 2006).

Italy recently used the same techniques established for detecting and measuring pharmaceutical levels in water to quantify cocaine use through the river Po (Zuccato et al., 2005). Zuccato et al. (2005) estimated that 4 kg of cocaine flow down the river daily, a level much higher than determined by census reports.

WWTPs have varying effectiveness at removing pharmaceuticals from wastewater, as noted by the presence of these compounds in effluent, receiving waters, and even drinking water (Kuch and Ballschmiter, 2001). Designed to be stable for increased shelf- and biological-life, excreted pharmaceuticals are often still biologically active as metabolism in man or animals may be incomplete or results in an altered form that can become active again under certain conditions (Bendz et al., 2005). Environmental and WWTP processes such as microbial degradation, oxidation, UV degradation and partitioning to organic particulates, may help diminish the quantity of active compounds entering the environment (Ternes et al., 1999a and 1999b; Loffler et al., 2005).

Agro-chemical runoff is cited as another prominent source of surface water contamination. Included in this runoff are livestock natural hormone excretions, antibiotic and drug excretions, as well as crop pesticides and fertilizers. First-order streams and riparian corridors are considered effective means of removal and sequestering these contaminants (Angier et al., 2002). This runoff, when combined with wastewater, could add significantly to surface water contamination.

1.2 STUDY PHARMACEUTICALS

Three pharmaceuticals were chosen for this research project, including the synthetic birth control hormone 17α-ethynylestradiol (EE2), the antihypertensive diltiazem (DTZ), and the anticonvulsant diazepam (DZP) (Valium®). A fourth chemical, atrazine (ATZ) is a common herbicide that is effective in killing C-3 plants (Rao, 2000). ATZ was chosen as a positive control since it is known to be readily taken up by plants (Burken and Schnoor, 1996; Cherifi et al., 2001). Further information on the pharmaceuticals and herbicide used in this study can be found in Appendix D.

These pharmaceuticals are known to be only partially metabolized in humans with a percentage of the administered dose excreted as parent compound, lending them to entry into the environment. Disposal of unwanted drugs down sinks and toilets also contributes to the concentration of the active compounds entering wastewater. These drugs are administered worldwide and have been reported in the wastewaters and surface waters of many heavily populated regions (Ternes, 1998; Kolpin et al., 2002; Servos et al., 2005). The presence of atrazine within the water environment has been documented for many years and it has even been reported in rain water (Gfrerer et al., 2002).

Hormone and hormone mimics like EE2 are known as endocrine disrupting compounds (EDC) that interact with endogenous hormone systems in vertebrates. In aquatic ecosystems, these can influence fish and amphibians such as by inducing the production of vitellogenin (female egg proteins) in male fish and brown frog hepatocytes, as well as altering fish metallothionein, a metal binding protein whose levels reflect a toxic effect

(Werner et al., 2003; Gorshkov et al., 2004; Rankouhi et al., 2005). EE2 is a potent estrogen with even short term exposure to EE2 resulting in decreased fertility of sexually maturing male rainbow trout (Schultz et al., 2003). Purdom et al. (1994) reported altered vitellogenin production with concentrations of EE2 as low as 0.1 ng/ L and EE2 has been found in WWTP samples at levels between 1.0 and 3.2 ng/ L in Paris (Cargouet et al., 2004). Cargouet et al. (2004) suggested that EE2 appeared to resist biodegradation and accounted for 35-50% of estimated estrogenic activity in Parisian rivers.

Diltiazem, the antihypertensive, is of concern due to its Ca-channel blocking mechanisms. Calcium plays a very significant role in the physiology of many organisms and if a chemical was to disrupt this role, it could be detrimental for plants and animals. DTZ has been detected in surface water in the United States at a maximum level of 49 ng/ L and a median level of 21 ng/ L (Kolpin et al., 2002).

Diazepam, an anti-convulsant benzodiazepine substance, was originally thought to be a solely synthetic creation until the discovery of natural benzodiazpines in the 1960s in both plants and animals (Unseld et al., 1989; Kavvadias et al., 2000). Acting on the central nervous system, DZP has the potential to act on any organism with a nervous system (Wildmann, 1988). DZP has been detected in Belgium in wastewater influent and effluent, with levels as high as 59 and 118 ng/ L in influent and less than 10 ng/ L in effluent (van der Ven et al., 2004). Ternes et al. (1998) has also reported on DZP in sewage treatment plant effluent within Germany, with levels as high as 40 ng/ L, although it was not detected in the rivers and streams.

Atrazine is one of the most extensively used herbicides in the world. It acts on the photosynthetic electron transport system of plants. ATZ has been associated with endocrine disruption at low doses (Hayes et al., 2002). Hayes et al. (2002) found evidence of ATZ induced estrogen secretion and inhibited testosterone secretion, resulting in hermaphroditic and demasculinized frogs at levels between 10 ng/ L and 100 ng/ L. Developmental deformities in amphibian larvae have also been associated with ATZ with a dose-dependent increase in deformities with increasing herbicide exposure (Allran and Karasov, 2001).

ATZ also indirectly affects food webs and biodiversity by hindering the growth of primary producers such as algae, macrophytes, diatoms, phytoplankton and other microorganisms (Dewey, 1986; Tang et al., 1997; Detenbeck et al., 1996; DeNoyelles et al., 1982). Effects on food web foundations could be significant with prolonged and synergistic exposure to these compounds (Kolpin et al., 2002; Fent et al., 2006).

1.3 INTRODUCTION TO PHYTOREMEDIATION

Phytoremediation is not new, but has only been mentioned in the technical literature since about 1994 (Schnoor, 2002). As defined by Schnoor (2002), it is

> … the use of vegetation for *in situ* treatment of contaminated soils, sediments, and water. It is applicable at sites containing organic, nutrient, or metal pollutants that can be accessed by the roots of plants and sequestered, degraded, immobilized, or metabolized in place.

Remediation with plants has been used for inorganics such as nutrients, selenium and arsenic, metals such as lead, cadmium, nickel and zinc (Meagher, 2000, Deng et al., 2006; Fayiga and Ma, 2006; Pendergrass and Butcher, 2006; Shen et al., 2006). Phytoremediation has also been used for organic chemicals (Burken and Schnoor, 1998) such as pesticides (Henderson et al., 2006), polychlorinated biphenyls (Chekol et al., 2004), petrochemicals (Kassel et al., 2002), and explosives (TNT) (Thompson et al., 1998; Dzantor et al., 2000; Angier et al., 2002). Heavy metal clean-up has been a large area of study for phytoremediation. To date, there has been limited research into phytoremediation of pharmaceuticals from water.

In natural ecosystems, plants have been considered as the 'green liver.' As photosynthetic versions of mammalian livers, plants contain enzymes and metabolic processes that detoxify contaminants (Sandermann, 1994). Research into metabolism of plant-intended compounds and phytoremediation has examined the potential of many plants. Crop plants, such as barley, corn, sorghum, and vegetables, are commonly dosed with a pesticide or herbicide prior to harvest. Determining the outcome, effects, and methods of

'how it works' uncovered the vast ability of plants to survive in contaminated environments, from anthropogenic or natural sources.

Phytoremediation may involve uptake and metabolism, rhizosphere bioremediation, phytostabilization, phytoextraction, rhizofiltration, hydraulic control, phytovolatilization, vegetative caps, and constructed wetland (Schnoor, 2002). The type of technology used depends on the compound(s) involved and the location or environment. These areas are roughly defined and tend to overlap. The focus for this MSc study involved uptake and metabolism, for which the process is generalized below.

Uptake and metabolism – using metabolic capabilities of plants to metabolize compounds into less toxic or less biologically active forms upon uptake of the compound into the plant. This process has been studied extensively in poplar trees with atrazine (Burken and Schnoor, 1997) and the explosive TNT (Thompson et al., 1998).

Vegetation that thrives in riparian environments is a natural consideration for removing contaminants from surface and ground water. Common riparian vegetation includes phreatophytic trees and shrubs of the genera *Populus* and *Salix*, poplar and willow, respectively. These plants transpire large volumes of water, with trees using between 100 to 200 L/ day (Newman, 1997). Rapid growth is important in plants used for phytoremediation, ensuring rapid and continual uptake of the contaminant within the transpiration stream and possible storage in cell walls. Transpiration stream tension (pull)

may play an important role in the drawing of chemicals towards the root zone and their subsequent uptake, and this favours the use of trees that use large volumes of water.

Original reports focused on nutrient and metal remediation involving plants found to grow on or near contaminated sites or eutrophic surface waters. Aside from *Populus*, other plants include *Typha* (cattails), *Brassica* (mustard family), sunflowers, and many other genera (Lim et al., 2003; Barrera-Diaz et al., 2004; Nehnevajova et al., 2005; Nocito et al., 2006; Quartacci, 2006)..

Crop plants resistant to herbicides uncovered the ability of resistant plants to bind the herbicide or its metabolites to cell wall components as a form of detoxification or sequestering, preventing the herbicide from reaching its site of action (Mathew et al., 1998). The degree of binding and detoxification varies between plant species and individual resistant or susceptible plants, but has been found to apply to a variety of compounds (Langebartels and Harms, 1985; Scheunert et al., 1985; Dankwardt and Hock, 2001; Sapp et al., 2004; Weiss et al., 2004). Bound residues, not just of pesticides but of other chemicals, create a concern as to bioavailability after digestion or decay of the plant (Sandermann, 2004). Animals that graze or browse plants with bound residues have the potential to release the compounds (Sandermann et al., 1990 and 1992), especially ruminants with the bacteria that break down cellulose (Skidmore et al., 1998).

The formation of bound residues is primarily through processes resulting in covalent bonds or physical encapsulation within extracellular matrices (Skidmore et al., 1998).

Covalent bonds are most commonly formed by the reaction of electrophilic compounds with nucleophilic sites on proteins, nucleic acids or cell wall constituents such as lignin or cellulose. Encapsulation, or trapping of compounds, within spaces of the cell wall is another process proposed (Skidmore et al., 1998).

The occurrence of bound residues in food products, particularly of pesticides, has led to countries requiring Food and Drug Act regulations that set limits on the acceptable levels within imported and exported foods. In Canada, the Canadian Food Inspection Agency monitors pesticide residue levels on some domestic and imported foods to ensure the levels fall below set residue limits.

The costs of using plants to remediate contamination of soil or water is considered to be lower than conventional remediation technology that often involves removal and incineration of large quantities of earth, or the use of chelating chemicals. The long-term applicability of phytoremediation also provides environmental and aesthetic benefits and the *in situ* remediation makes it a technology worth further research and application.

New research directions in phytoremediation technology include genetic engineering of plants and examination of natural and induced mutants (Doty et al., 2000). Now that the poplar and *Arabidopsis* genome have been sequenced (Cobbett and Meagher, 2002), insight into the enzymes and the genes that code them should facilitate comparisons among plants and understanding of phytoremediation (underlying) mechanics (The Arabidopsis Genome Initiative, 2000; Sterky et al., 2004).

1.4 STUDY PLANTS: SALIX EXIGUA & ARABIDOPSIS THALIANA

Salix exigua. There are over 350 to 400 willow species, of the family Salicaceae, that are located primarily in the Northern Hemisphere, ranging from arctic through temperate latitudes. Most of these shrubs can be found in moisture rich regions, particularly riparian zones, with their roots obtaining water from the shallow, saturated or streamside water table below (phreatophyte). Commonly pioneer plants, the willows grow quickly on newly disturbed soil or newly formed streamside bars and banks.

Salix exigua Nutt.is known commonly as coyote willow, sandbar willow, basket willow, narrowleaf willow, slender willow, riverbank willow, acequia willow, long-leaved willow gray willow, dusky willow, and sometimes pussy-willow (Stevens et al., 2000; Nellessen, 2003), although, a more complete list of 34 synonyms exists in Kartesz (1994). Several characteristics that are uncommon for willow genus define *Salix exigua*, including stomata located on both upper and lower leaf surfaces (amphistomatous) and the ability to spread almost entirely clonally through creeping rootstock (Nellessen, 2003). The sandbar willow is quite tolerant of saturated soils for a prolonged period of time, but will begin to suffer if this regime is maintained for too long (Amlin and Rood, 2001).

The use of sandbar willow for phytoremediation has not been investigated, but other willow have been used in phytoremediation studies for cyanide (Larsen et al., 2005; Bushey et al., 2006 a and 2006 b), the anti-fouling agent tributylin (Trapp et al., 2004), and cadmium and copper (Kuzovkina et al., 2004).

Arabidopsis thaliana. Related to cabbage and mustard (family Brassicaceae), *Arabidopsis thaliana* is a small flowering plant used extensively in plant biology research. *Arabidopsis* has become the model plant due to its small, sequenced genome, small size, short life-cycle and easy manipulation. With the complete genome sequencing of *Arabidopsis thaliana* in 2000, identification of genetic diversity in terms of important, generalist metabolizing and detoxifying enzymes resulted in the discovery of over 270 members of cytochrome P450 monooxygenases, as well as numerous glutathione-S-transferases (The Arabidopsis Genome Initiative, 2000; Sterky et al., 2004). This reflects the immense genetic and encoded biochemical diversity and complexity found in plants.

Recognized as a tool for the discovery of genes in the role of transformation and phytoremediation, *Arabidopsis* has been used for phytotransformation studies. One such study examined the TNT metabolic pathways within *Arabidopsis* (Subramanian et al., 2006). Heavy metal uptake and *Arabidopsis* genes have been more extensively studied such as for arsenic (Geng et al., 2005) and the elements Zn, Co, Cu, Pb and Mn (Yang et al., 2005).

1.5 PLANT PHYSIOLOGY: ROOT UPTAKE AND TRANSLOCATION

The very physiology that enables plants to live (accumulate resources and water via a functional aqueous transportation system) is the same that can result in the uptake and accumulation of anthropogenic or natural compounds and elements. Plants have existed for millions of years and evolved hundreds of metabolic enzymes to deal with environmental compounds and contaminants. These adaptations allow for the exploitation of plants for remediation.

Plant roots. Plant root structure is quite well understood with the basic structure composed of the epidermis, cortex, endodermis (with Casparian strip), and vascular tissue containing xylem and phloem (Figure 1.1) (Taiz and Zeiger, 2002). The epidermis is the external layer of protective cells including those that form root hairs. Below the epidermis are loosely packed cells, the cortex. The next layer consists of a single encircling wall of cells termed the endodermis, sealed extracellularly with the Casparian strip. The vascular tissue is composed primarily of xylem (water transportation tissue from roots to shoot) and phloem (tissue that transports products of photosynthesis). Within this tissue structure there are two barriers to plant uptake: cell plasma membranes and the Casparian strip.

Plasma membranes of root cells are the primary barrier for ions within soil water. Membranes are made up of a bilayer of phospholipids, creating a hydrophilic exterior and hydrophobic interior to the membrane. Depending on a molecule's characteristics, it may be capable of diffusing across the membrane into the root cells and travel to the xylem

where it will be transported to the shoots. Diffusion across the membrane is primarily a function of a physiochemical property relating to the polarity of the compound, the octanol-water partitioning coefficient, expressed as the logarithm (log K_{ow}), a similar property to lipophilicity (Tracy, 2004).

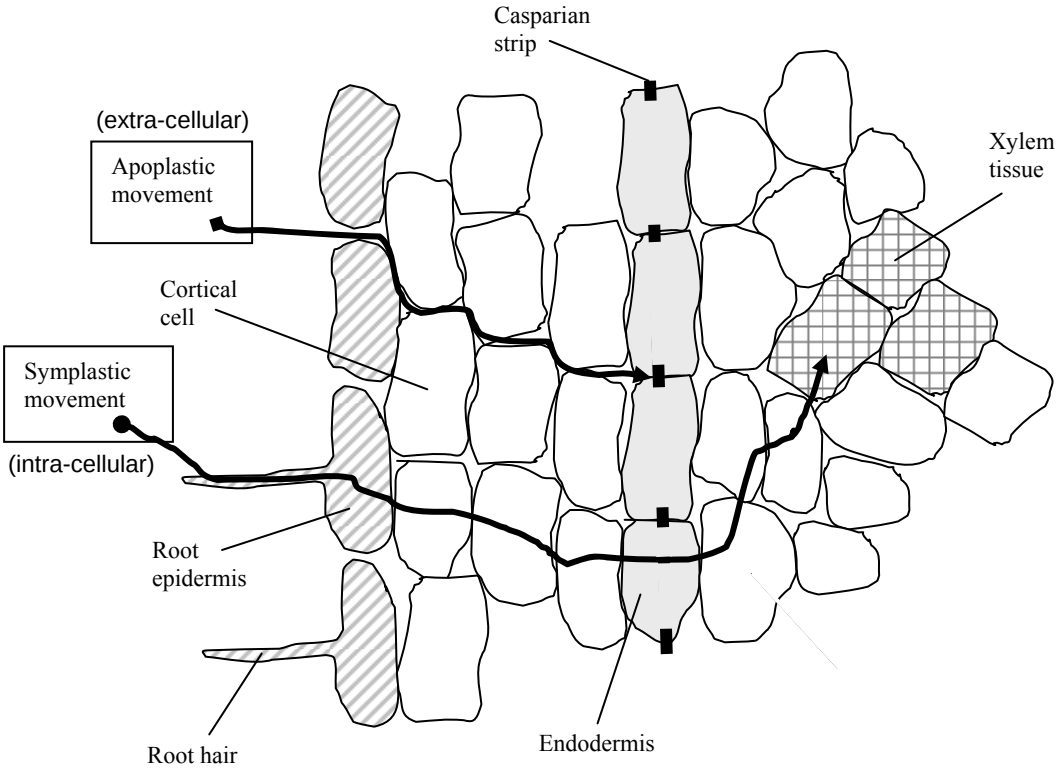

Figure 1.1. Diagram of basic plant root physiology and two water transport pathways.

The Casparian strip forms a seal around the endodermal cells and is comprised of a waxy material, suberin, which seals the extracellular space of the endodermis, somewhat similar to bricks and mortar. This seal limits the bulk flow of an external solution traveling through the extra-cellular spaces of root cells from flowing into the conducting xylem vessels to enter the transpiration stream. Bypassing this seal requires the compounds to cross the cell plasma membranes and travel within the cells, across the endodermis, to the xylem tissue.

There are two pathways for water and solute uptake into the root: apoplastic and symplastic. The apoplastic pathway involves movement through cell walls, extra-cellular air spaces, and the xylem vessels, without crossing any membranes (Taiz and Zeiger, 2002). Bulk flow moves through this space, successfully increasing the surface area for ion uptake and water contact with cell membrane surfaces. Movement through this space is generally inhibited at the Casparian strip. Inability to pass through the Casparian strip may result in sorption to root components such as cell wall, either in a relatively permanently bound form, covalently bonded or possibly encapsulated, or in a soluble form, more reversibly bound.

A proposed symplastic transport route includes entering epidermal or cortical cells by crossing the cell membranes and traveling within the cells, bypassing the Casparian strip and then exiting the symplast and entering the xylem vessels (part of the apoplast) into the transpiration stream (Taiz and Zeiger, 2002). Cell to cell transport would be via plasmodesmata, tubular structures connecting cytosol of adjacent cells, and crossing through adjacent cell membranes.

Aquaporins are trans-membrane channels that allow for the selective movement of water across membranes. The presence of these transmembrane, proteinaceous channels within plants was discovered in 1990 (Wayne and Tazawa, 1990) and although they are not fully understood, they may function similarly to animal aquaporins as water-selective channels or relatively non-selective channels for water and other small non-electrolytes (Tyerman et al., 2002). Relative to pharmaceuticals, the aquaporins small channel size and selectivity (currently poorly understood) likely prevent compound uptake through these pores (Tyerman et al., 2002).

Active uptake pathways require energy to move a solute across a membrane against a concentration gradient. This requires specific transport proteins, although some transport proteins may be more generalized and may transport compounds with similar molecular binding sites and size to the normal target compound (Buchanan et al., 2000).

Ionization, the process of dissociation of a compound into constituent ions, may also occur depending on their pKa (the negative log of the acid dissociation constant, Ka) and their surrounding pH. Lipophilicity and membrane solubility are related to the charge of molecules. If a molecule becomes ionized by releasing a proton (H^+) its charge becomes negative and hence more hydrophilic. Conversely, non-ionized compounds are generally more lipophilic. The pKa of a compound determines at what pH it will release or gain a proton and this influences solubility in soil or surface water. After a compound crosses a

lipid membrane, the pH within the cell and cell compartments differ, potentially altering ionization of the compound.

Charged, ionized, particles can also move across membranes at different rates than neutral compounds due to electrochemical charges within the cells and the associated electrochemical gradient (Taiz and Zaiger, 2002). Cell cytosol typically has a net negative charge, therefore, if a compound becomes positively charged while in the apoplast, it will be attracted to the negative charge within the cell and aid in its movement across the cell membrane. At the varying physiological pHs the pharmaceuticals of study, DZP, EE2 and the herbicide ATZ will remain primarily non-ionized, and therefore effectively neutral. Conversely, the pharmaceutical DTZ was obtained as DTZ-hydrochloride, lending this compound to positive ionization at physiological pH, and therefore the electrochemical gradients may influence its movement across cell membranes.

Uptake and transport of weak electrolytes is typically considered a more complex process to explain mathematically. Weak electrolyte uptake typically takes into consideration pH of different compartments, compound pKa and log K_{ow} (the logarithm of the octanol-water partitioning coefficient), as well as membrane permeability to ions and neutral compounds, and the potential for ion-trapping movement within the phloem (Trapp, 2004).

Neutral compound uptake and distribution often follow a relatively simple equation when compared to the more complex equation for weak electrolyte uptake. Neutral compound uptake is primarily dependant on diffusion across cell membranes into the symplast, which is typically a function of a physio-chemical property of the compounds, the octanol-water partitioning coefficient (expressed as the logarithm K_{ow}) (Shone and Wood, 1974; Briggs et al., 1982).

Octanol-water partitioning coefficient logarithm (Log K_{ow}). This physiochemical measure is often determined by two immiscible phases, water and an organic solvent (typically octanol), and the addition of a concentration of the compound to be determined. The phases are shaken and allowed to come to equilibrium. The concentration of the compound in both phases is determined and an octanol-water coefficient (K_{ow}) is determined (typically expressed as the logarithm):

K_{ow} = [compound] in octanol / [compound] in water

The preference of a compound for the lipid-like phase (octanol) is expressed as a large K_{ow} value, and is similar to hydrophobicity of the compound. Compounds with high K_{ow} values will move out of the water phase and into the membrane lipid phase. If the K_{ow} is large, the compound may thus not pass through the membrane, but will remain within the lipid core. Substances with very low K_{ow} values are very hydrophilic and these compounds may not enter the cell membranes at all and will thus remain within the water of the apoplast.

Plants. Documentation of plant uptake was provided in 1956, by Crowdy and Jones, who established that low rates of compound translocation within plants were associated with strong binding of the compound within the roots. The connection between a compound's K_{ow} and subsequent uptake by plants, similar to drug movement within humans, was made in 1982 by Briggs et al..

Initial stages of plant uptake involve the creation of equilibrium between the external solution concentration and the root. The ratio of the concentration within the root to the concentration in the external solution is termed the root concentration factor (RCF) (Shone and Wood, 1974). This equilibrium is met due to, and at a rate set by, the log K_{ow} of the compound and the concentration gradient. Diffusion across cell membranes can thus occur and subsequent movement into the transpiration stream and the shoots, lends to a transpiration stream concentration factor (TSCF), a reflection of the compounds diffusion rate (Briggs et al., 1982). The transpiration stream is considered the primary channel for movement of compounds from the root to the shoot. Phloem transport is generally slight, but may occur through ion-trapping movement, much as with phloem transport of sugars and other carbohydrates (Kleier, 1988; Zebrowski et al., 2004).

Root concentration factor (RCF). The root concentration factor (RCF) was introduced in 1974 by Shone and Wood as the equilibrium partitioning between soil/solution concentration and sorption root concentration:

RCF = concentration in the root (g/g) / concentration in external solution (g/mL).

Since RCF is a partition ratio, the values will not vary with the amount of root mass or volume used (assuming the concentration is not saturated), but the quantity of compound taken up will vary with the root mass or volume (Figure 1.2 A). Briggs et al. (1982) empirically defined the relationship between RCF and log K_{ow} for barley roots (Figure 1.2 B):

$$\log (RCF - 0.82) = 0.77 \log K_{ow} - 1.52.$$

In 1998, Burken and Schnoor, published a similar empirical relationship for hybrid poplar roots (Figure 1.2 B):

$$\log (RCF - 3.0) = 0.65 \log K_{ow} - 1.57.$$

Although equations have been developed to predict RCF values for hybrid poplar and barley, it is likely that equations from other experiments will differ as RCF values are dependent on specific factors, such as the plant species used, environmental conditions, compound characteristics and physiochemical properties in those environmental conditions. Dietz and Schnoor (2001) discuss how roots of hybrid poplars differ in their absorption rates depending on their environment as roots grown in the field had higher lipid contents than roots grown in a hydroponic system. For example, Thompson et al. (1998) examined the uptake of TNT by hybrid poplars, determining the RCF to be

approximately 49 mL/ g versus the calculated 1.7 and 3.5 mL/ g, using Briggs et al. (1982) and Burken and Schnoor's (1998) equations, respectively. Conversely, Briggs et al. (1982) reported that RCF values from other experiments fit their equation relatively well.

In dilute solutions, RCF is independent of concentration, behaving more as a partitioning reaction (Briggs et al., 1982). RCF has been shown to increase with increasing compound log K_{ow} in a non-linear fashion (Briggs et al., 1982; Burken and Schnoor, 1998). Compounds with log K_{ow} values > 3.0 are considered to sorb strongly to roots based on the equations developed by Briggs et al. (1982), and Burken and Schnoor (1998).

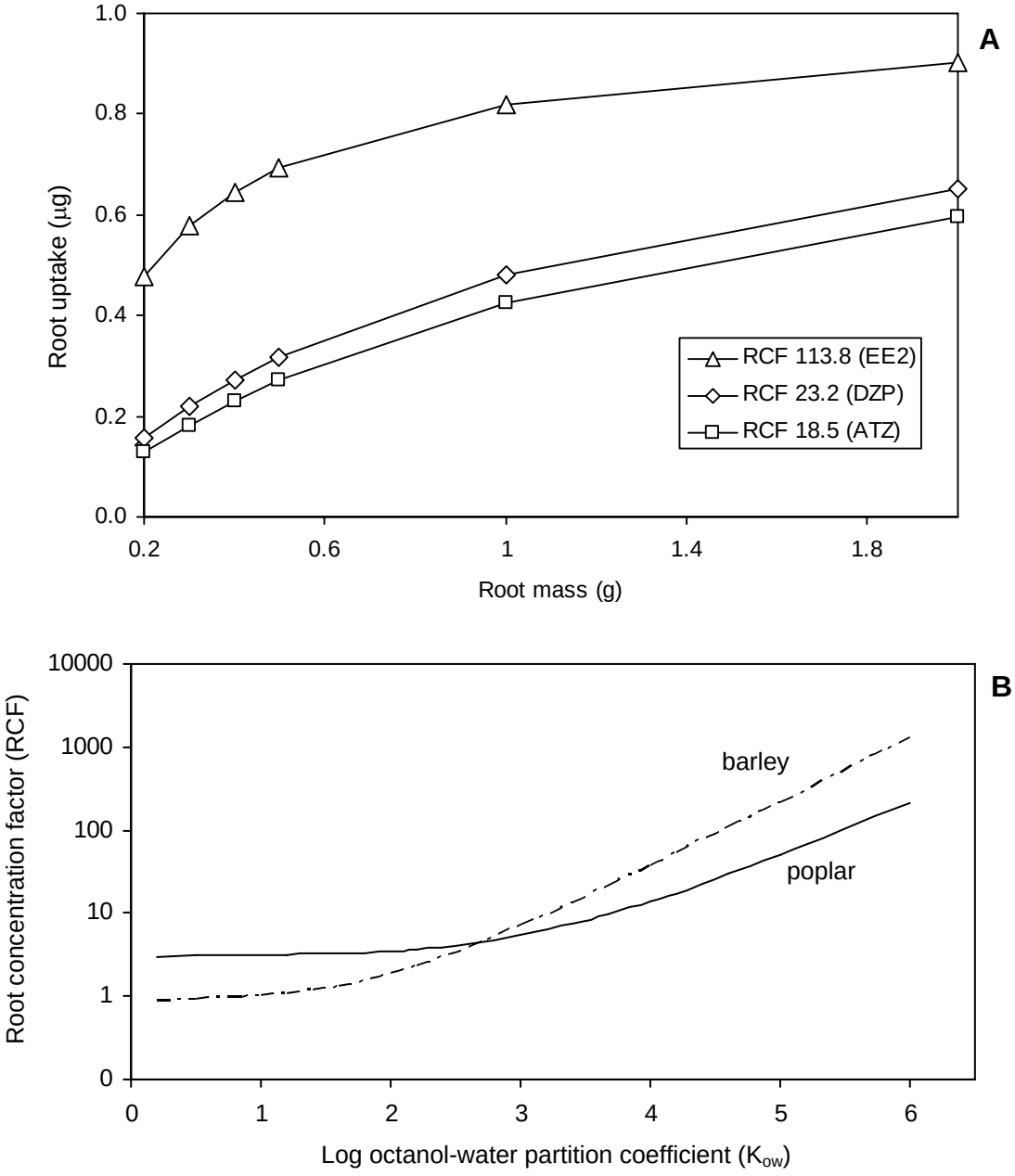

Figure 1.2. Plotted empirical relationships determined for A, root uptake with root mass varying with root concentration factor values; B, barley (Briggs et al., 1982) and hybrid poplar (Burken and Schnoor, 1998) relationship between log K_{ow} values and root concentration factor.

Transpiration stream concentration factor (TSCF). The transpiration stream concentration factor (TSCF) is defined as the concentration within the transpiration stream (μg/ mL) relative to the concentration in external solution (μg/ mL) (Briggs et al., 1982). The concentration within the transpiration stream is often measured as the amount of compound within the shoot, per volume of water transpired over the average of initial and final external solution concentrations (Briggs et al., 1982). Since transpiration is somewhat continuous, the mean of initial and final external solution concentrations are used (Briggs et al., 1982).

TSCF = (concentration in the shoot (μg) / volume transpired (mL)) / ((external solution concentration $(\mu g/mL)_{initial}$ + external solution concentration $(\mu g/mL)_{final}$)/ 2)

The TSCF versus log K_{ow} relationship is typically a bell-shaped, or normal curve, with the peak representing optimum uptake into the transpiration stream that is typically at a log K_{ow} value around 2, although this varies across plants (Figure 1.3). TSCF generally ranges from 0 to 1, with 1 implying passive uptake that follows the transpirational flow. Values less than 1 imply the compound is less readily moved into the transpiration stream and values greater than 1 imply active uptake, such as is typical for nutrients (Orchard et al., 2000; Dietz and Schnoor, 2001). Briggs et al. (1982) empirically defined the relationship between TSCF and log K_{ow} for barley roots (Figure 1.3):

$$\log TSCF = 0.784 \exp[-(\log K_{ow} - 1.78)^2 / 2.44].$$

In 1998, Burken and Schnoor, published a similar empirical relationship for hybrid poplar roots (Figure 1.3):

$$\log TSCF = 0.756 \exp[-(\log K_{ow} - 2.50)^2 / 2.58].$$

Analyses of RCF and TSCF allow for insight into a compound's distribution within a plant, the relationships between log K_{ow}, uptake and distribution, and thus enables comparisons between compounds and plants involved in phytoremediation. Predictable, deterministic behaviours are useful for considering the fate of pharmaceuticals in the environment.

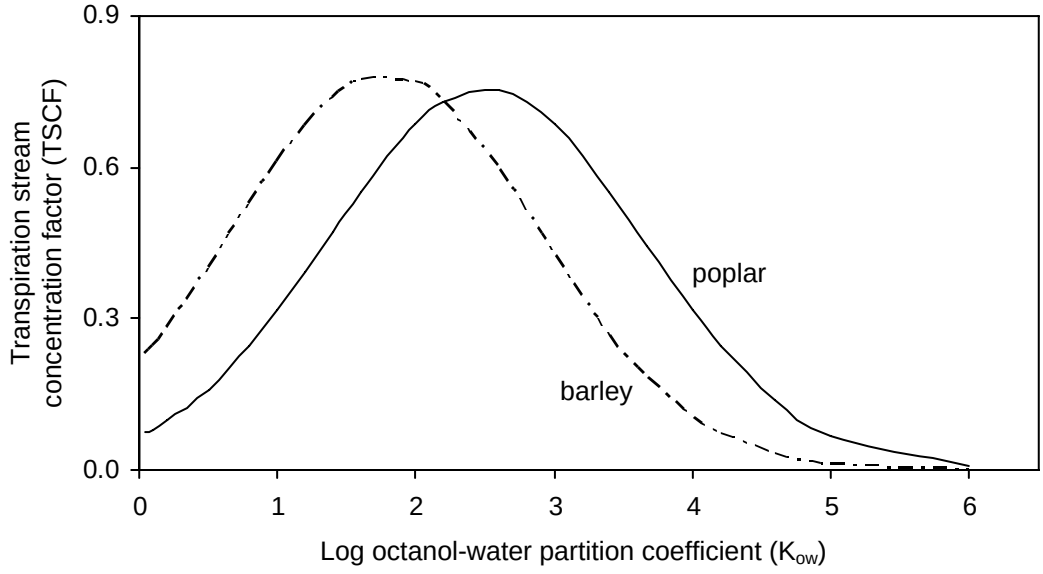

Figure 1.3. Plotted empirical relationships determined for barley (Briggs et al., 1982) and hybrid poplar (Burken and Schnoor, 1998) between log K_{ow} values and transpiration stream concentration factor. Optimum uptake into the transpiration stream is represented by the peak of the curve.

1.6 MSc Project summary

This MSc project investigated the potential application of a riparian plant for the removal of environmental levels of pharmaceuticals from water.

The primary focus was on phytoremediation of pharmaceuticals from solution using the riparian shrub, *Salix exigua*. *Salix exigua* was chosen as it is a common riparian plant that is able to survive within the wet conditions of a river bank. Willow species also possess other important traits, such as rapid growth and colonization, transpiration of large volumes of water, and extensive geographic distribution. Levels of the chosen pharmaceuticals used for this study are equivalent to 40 ng/ L. This level could be considered high when compared to some reported environmental levels found within streams and rivers, although a few have reported levels equivalent to this at the high end of detected values. When compared to wastewater influent or effluent, this concentration may be considered a median to high value.

Initial investigations answered the question as to whether these compounds are taken up, and at what rate they are taken up by plants, particularly willow. Willow's ability to remove these compounds from solution was then compared to predicted behaviours of the compounds based on an important physiochemical property, the octanol-water partitioning coefficient (K_{ow}, expressed as the logarithm). This examination included a compound distribution analysis within the plant of the compounds taken up, including the plant components of roots, wood stem, and green shoot (including leaves and green stem). Again, the distribution was compared to predicted behaviours of the compounds

from their log K_{ow}. Distribution within the plant was differentiated into soluble versus bound forms.

Secondary focus was the ability of *Arabidopsis thaliana* to remove these compounds from solution. A similar investigation into uptake, rates of uptake, and distribution within the roots and shoots of *Arabidopsis* was performed, as for willow. Investigation into *Arabidopsis* is seen as less of a field application of phytoremediation than as an important source for broadening the understanding of plant uptake. A model plant, *Arabidopsis* can provide insight into the enzymes and genes that play important roles in the outcome of pharmaceuticals within plants. *Arabidopsis* as well, proves that diverse ranges of plant genera are capable of 'mopping up' these environmental contaminants. A second plant genus also reaffirms that pharmaceuticals behave, to a degree, according to their log K_{ow} values, an important tool for predicting their relationship within plants and the environment.

Development of a method for the detection of 17α-ethynylestradiol in surface water by gas chromatography-mass spectrometry (GC-MS) originated from the interest as to whether this potent compound was present in local river water. In developing this method for detecting estrogens within environmental water samples it was discovered that EE2 can degrade into another commonly detected estrogen if analyzed improperly. This had not been reported in the literature at the time of this method development. A method for the analysis of EE2 minimizing degradation and optimizing its detection through GC-MS was developed. No environmental samples, unspiked, were analyzed since an

investigation by Alberta Environment was published reporting no detectable levels of EE2 within the local river.

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CHAPTER 2 Phytoremediation of trace levels of pharmaceuticals, diltiazem, diazepam and 17α-ethynylestradiol, and the herbicide atrazine, with sandbar willow (*Salix exigua)*

2.1 INTRODUCTION

The discovery that many biologically active compounds leave our wastewater treatment plants and enter the environment (Ternes et al., 1999; Kolpin, et al., 2002; Sosiak and Hebben, 2005), and occur in our drinking water (Stan and Linkerhägner, 1992, cited in Sengl and Krezmer, 2003; Kuch and Ballschmiter, 2001), has led to environmental and human health concerns. The environmental fate of these compounds, particularly pharmaceuticals, is poorly understood.

Phytoremediation, the use of plants to remediate environmental contaminants, is not new, but the term was only introduced in the scientific literature in about 1994 (Schnoor, 2002). Currently used for the removal of hydrocarbons, heavy metals and pesticides, phytoremediation may provide a natural and cost-effective method of removing wastewater pharmaceuticals from our waterways and thus minimizing their ecological and health impacts. The prospective phytoremediation of pharmaceuticals represents a new direction for research and application that should be considered, particularly as the quantities of contaminants are only increasing with increasing and aging populations and the proliferation of drug-treatments for human health.

Willow species, members of family Salicaceae and genus *Salix*, are rarely reported for phytoremediation projects, but they satisfy many requirements of a plant for phytoremediation. They are easy to propagate and grow rapidly and have abundant water usage with roots extending into water tables and may thus be especially suitable for phytoremediation of contaminants in ground and surface waters, including streams that often provide the primary water sources for municipal use. Willow also has the ability to survive prolonged periods of inundation, a characteristic useful when the areas to be remediated are riparian zones along river banks or lake shores. *Salix exigua*, commonly referred to as sandbar willow, was chosen for this project due to its local availability, extensive distribution and abundance, and preference for moist zones immediately adjacent to streams.

The present investigation studied the potential of this prominent riparian shrub to uptake and transport three common wastewater pharmaceuticals: diltiazem (DTZ), a calcium channel blocker; diazepam (Valium®) (DZP), an antianxiety drug; and 17α-ethynylestradiol (EE2), a synthetic birth control hormone that is a common component of the contraceptive pill. The herbicide atrazine (ATZ) was used as a positive control for this project, since it is known to be readily taken up by plants and is another prominent water pollutant that has been extensively investigated relative to phytoremediation.

Uptake and transport studies were undertaken, and subsequently root concentration factor (RCF) analyses were performed, to enable a comparison of *Salix exigua* root and

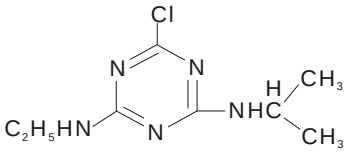
pharmaceutical relationships with other documented compound and plant root relationships, particularly for uptake studies with barley and hybrid poplar.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

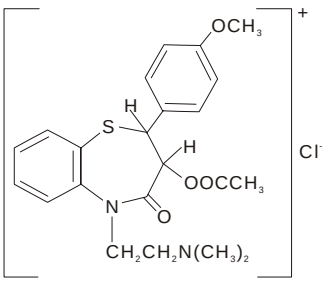
The compounds chosen for this study included the synthetic hormone 17α-ethynylestradiol (an EDC) (EE2), the anti-hypertensive agent diltiazem (DTZ), the anticonvulsant diazepam (DZP) and the herbicide atrazine (ATZ) (Figure 2.1). Cis-(+)-[N-methyl-^{3}H]-diltiazem (specific activity: 74.5 Ci mmol^{-1}; radiochemical purity > 97% to HPLC analysis), [methyl-^{3}H]-diazepam (specific activity: 86.0 Ci mmol^{-1}; radiochemical purity > 97% to HPLC analysis), and 17α-[6,7-^{3}H(N)]-ethynylestradiol (specific activity: 40.0 Ci mmol^{-1}; radiochemical purity >97% to HPLC analysis) were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). [Ring-U-^{14}C]-atrazine (specific activity: 10.35 mCi mmol^{-1}; radiochemical purity > 95% to HPLC analysis) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Non-labeled DTZ-hydrochloride, DZP, ATZ, and EE2 were obtained from Sigma-Aldrich Canada Ltd. Standards were dissolved in ethanol, since methanol is toxic to plants, and to maintain consistent experimental conditions since EE2 and DZP are insoluble in water but DTZ and ATZ are water-soluble. Some chemical properties of the compounds are listed in Table 2.1.

Atrazine



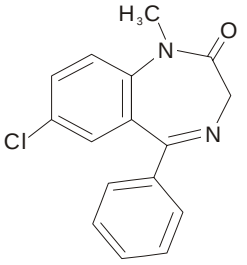
2-chloro-4(ethylamino)-6-(isopropylamino)-s-triazine

Diltiazem HCl



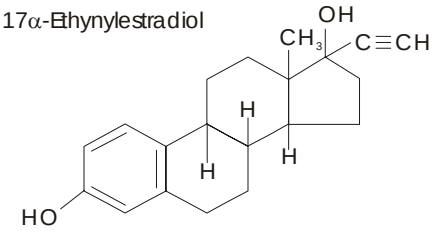
1,5-Benzothiazepin-4(5H)-one,3-(acetyloxy)-5[2-(dimethylamino)ethyl]
-2,-3- dihydro-2(4-methoxyphenyl)-, mono-hydrochloride, (+)-cis

Diazepam (Valium®)



7-chloro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one

17α-Ethynylestradiol



17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17
-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol

Figure 2.1. Chemical structures of the three pharmaceuticals and the herbicide used in the present phytoremediation study.

Table 2.1. Chemical properties of the three pharmaceuticals and the herbicide used in the present phytoremediation study.

	CAS Number	*M.W. (g/ mol)*	*Log K_{ow}**	*Water Solubility*
17α-ethynylestradiol	57-63-6	296.4	3.67	Slightly Soluble
diazepam	439-14-5	284.8	2.82	Slightly Soluble
diltiazem	42399-41-7	451.0	2.70	Soluble
atrazine	1912-24-9	215.7	2.61	Slightly Insoluble

* Log K_{ow} values estimated from ECOSAR™
(online demo source: http://www.syrres.com/esc/est_kowdemo.htm).

2.2.2 Plants, hydroponics and treatments

Sandbar willow (*Salix exigua* Nutt.) stem cuttings were collected from riparian zones in late winter and early spring along the Oldman River, Lethbridge, Alberta. The cuttings were about 0.5 cm in diameter and 10 cm long, and placed in water to allow adventitious rooting. Once rooting had begun the cuttings were transferred to a hydroponic system in a growth chamber.

The hydroponic system was adapted from Gibeaut et al. (1997) and used 37.9 L opaque tubs measuring 60.1 L x 47.0 W x 22.2 H cm for the reservoir. Forty 1.5 cm holes were drilled in the opaque plastic lid. A continuous aeration system used an aquarium pump (Petcetera Air Pump AP-3800) and 12.7 cm long diffusing stone. Reverse osmosis purified water was used enabling the pH of the hydroponic solution to drop to slightly acidic upon addition of Dutch Nutrient Formula Gro A & B solution, at 2.5 mL/ L. The rooted cuttings were inserted through the holes drilled into the reservoir lid and a strip of horticultural Rock Wool made from basalt fibers (FibrGrow Horticultural Products, Sarnia, Ontario), was wrapped around the cutting to provide support and block light from the reservoir (Figure 2.2).

Growth chamber (Conviron Model E15, Controlled Environments Ltd., Winnipeg, MB, Canada) conditions for willow were set at 16 hour day/ 8 hour night photoperiod, temperature was maintained at 20 °C, relative humidity was 70%, and photosynthetically active radiation at plant height was approximately 306 μmol $s^{-1}m^{-2}$ provided by a mixture of halogen 400W bulbs (Sylvania Metalarc and Philips). Willow cuttings approximately

20 days old with an approximate shoot length range of 8 to 12 cm were used for the uptake studies.

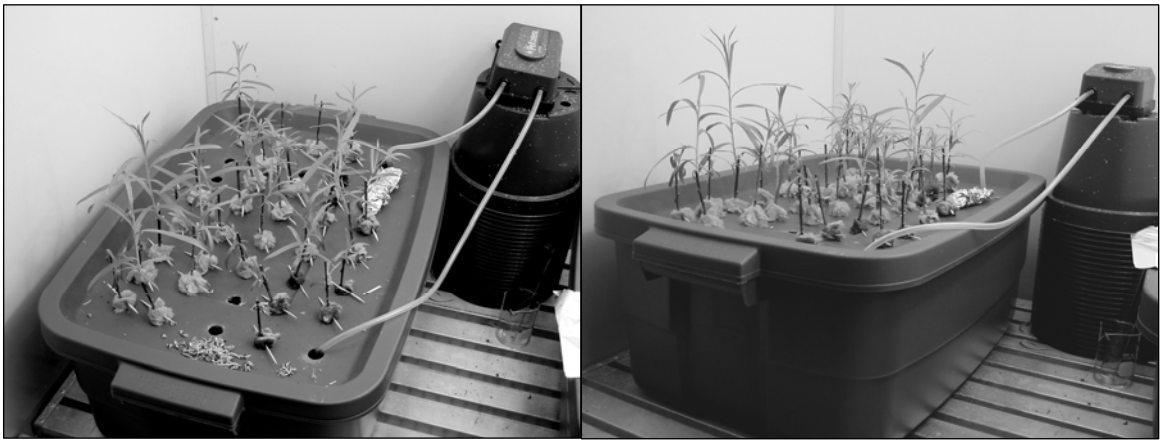


Figure 2.2. Hydroponic system with willow cuttings.

2.2.3 Uptake studies

Uptake studies were performed for 24 hours with solutions in 30 mL glass culture tubes using the hydroponically grown willow cuttings. This duration was sufficient for considerable uptake for analysis and to allow the measurement of rate of uptake. The 30 mL culture tubes were sufficient in size for the root mass of the willow cuttings and transpiration.

For each chemical replicate, 1 μg of the unlabeled compound plus 16,667 Bq of the radiolabeled compound was added to each culture tube (in ethanol solution resulting in 4.2 μL/ mL). Nutrient solution (24 mL) was taken from the active hydroponic reservoirs and added to the culture tubes for a concentration of 0.04 μg/ mL or 40 ng/ mL. At this time (t_0) a sample was taken and analyzed by a liquid scintillation counting (LSC).

Similar sized plants were chosen and inserted into the culture tubes. A rooted cutting was inserted into the culture tube at a level maintaining the stem cutting out of solution, with plant shoots supported, so that most of the root mass was submerged (Figure 2.3). The tubes were wrapped in foil and the study was run with the hydroponic growth chamber settings described above. Samples taken directly from the culture tube solution were analyzed with LSC at 2, 4, 8, and 24 hours to determine uptake, represented as loss of radioactivity from the solution. At harvest (24 hr) the cuttings were removed from solution, the roots were rinsed in nutrient solution to remove surface solution on the roots, and divided into green shoot (leaves and green stem), stem cutting, and roots. Each was weighed fresh and stored at -20 °C until analysis. Replicate plant numbers were 6 for

EE2, DZP, and ATZ, and 4 for DTZ. The experiment was repeated twice for EE2, DZP and ATZ, but not for DTZ as the DTZ standard was severely degraded by the time of the second experiment.

Transpiration volumes were monitored at each sampling time to check for changes in transpiration rates (a possible sign of phytotoxicity) and to provide a comparison between plants and compounds, as well as for estimating the transpiration stream concentration factors (TSCF). Just prior to sampling, nutrient solution was added to the culture tube to replace water lost through transpiration. The volume added was recorded as the volume transpired over the sampling period. The solution was mixed and allowed to sit for several minutes to ensure mixing of original and new solutions.

Other. Blank runs were monitored for the 24 hour period to determine evaporation, volatility and binding of the compounds to the glassware. For this control, compounds were added to nutrient solution, the tubes were covered with foil and the solution sampled at the same time points to measure remaining radioactivity.

Testing for compound binding to the culture tubes involved drying down the remaining solution after the termination of the study and rinsing the tubes with methanol. The concentrated solution plus methanol rinse was then resuspended and analyzed by LSC.

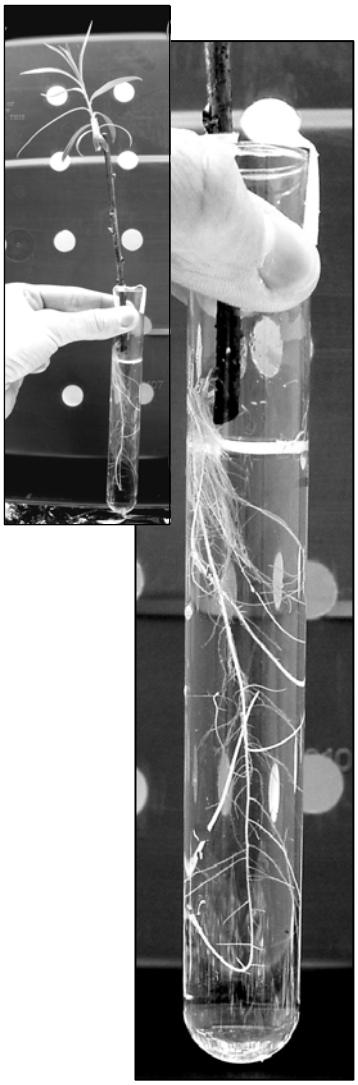


Figure 2.3. Culture tube set up for analysis of pharmaceuticals by willow.

2.2.4 Root concentration factors and transpiration stream concentration factors

To determine root concentration factors (RCF) for *Salix exigua*, 24 hour studies were performed using excised roots and roots attached to the cutting but without a shoot. RCF studies were carried out in nutrient solution in 30 mL glass culture tubes using hydroponically grown willow cuttings. For each chemical replicate, 1 μg (0.04 μg/ mL) of the unlabeled compound plus 8333 Bq of the radiolabeled compound was added to each culture tube (in ethanol solution). RCF values were only determined for EE2, DZP and ATZ, as the radioactive DTZ stock was degraded at the time of this study. Nutrient solution (24 mL) was taken from the active hydroponic reservoirs and added to the culture tubes. At this time (t_0) a sample was taken and analyzed by LSC.

For these analyses, willow plants were chosen based on apparently similar root masses. The roots were severed from the plant and submerged in the test tube solutions. For the roots that were left attached to the cutting the green shoot was removed and the roots submerged in the culture tube solution while maintaining the cutting out of the solution, as with the uptake studies. The test tubes were covered with foil to block out light and these studies were performed under the same environmental conditions as the uptake studies described in section 2.2.3. The experiment was performed with three replicates of excised roots and three replicates of roots attached to the cutting, for each of EE2, DZP and ATZ.

As with the uptake studies, the solution was sampled and analyzed by LSC to determine uptake, represented as removal of radioactivity from solution. The solutions were

sampled at 1, 2, 4, 8 and 24 hours and each sampling was followed by the addition of nutrient solution to replace the volume removed due to sampling. Evaporative losses were negligible.

At 24 hours the study was terminated. The excised roots were rinsed in fresh nutrient solution from the active hydroponic systems, blotted dry and weighed fresh. The same was carried out for roots attached to the cutting, with roots and wood being separated for individual weighing. Roots and cutting were stored at -20°C until solvent extraction and oxidation could be carried out. RCF values were calculated using results from excised roots and roots attached to the cutting, and estimated using results from whole plant uptake studies, and the equation (Shone and Wood, 1974):

root uptake = RCF (μg/mL) x root mass (g) x external solution concentration (μg/mL).

RCF for the compounds were also calculated from log K_{ow} values using equations developed by Briggs et al. (1982) for barley roots:

$\log (RCF - 0.82) = 0.77 \log K_{ow} - 1.52$,

and by Burken and Schnoor (1998), for hybrid poplar roots**:**

$\log (RCF - 3.0) = 0.65 \log K_{ow} – 1.57$.

Transpiration stream concentration factor (TSCF) for the four compounds in willow were estimated using results from whole plant uptake studies and the equation (Briggs et al., 1982):

T**SCF = [concentration in the shoot (μg) / volume transpired (mL)] / [(external solution concentration (μg/mL)$_{initial}$ + external solution concentration (μg/mL)$_{final}$)/ 2]**.

Although transpiration may not have been continuous, the means of initial and final external solution concentrations were used to estimate overall TSCF (Briggs et al., 1982).

TSCF for the compounds were also calculated from log K_{ow} values using equations developed by Briggs et al. (1982) for barley shoots:

log TSCF = 0.784 exp[-(log K_{ow} – 1.78)^2 / 2.44],

and by Burken and Schnoor (1998), for hybrid poplar shoots:

log TSCF = 0.756 exp[-(log K_{ow} -2.50)^2 / 2.58].

2.2.5 Soluble fractions

For half of roots and shoots harvested at the end of the uptake studies, except for DTZ in which all roots were used, a methanol solvent extraction was used to separate the soluble fraction from the non-extractable fraction that may have been contained within the cell walls. The determination of soluble versus bound fractions provides insight into the fate of the compounds once they are in the plant. Soluble fractions may still have the potential to be transported or metabolized, while bound fractions may be immobile, with minimal degradation after binding.

For roots, the frozen sample was ground in a mortar to a fine pulp with 80% aqueous methanol (MeOH:H_2O 80:20), centrifuged, and the supernatant decanted. The residue was resuspended with 100% methanol, centrifuged, decanted and the extraction was repeated a third time. The supernatants were pooled, aliquots taken and bleached using commercially available sodium hypochlorite (5.25% chlorine), and analyzed by LSC.

The solvent extraction procedure was repeated for the green shoot following a similar extraction and bleaching procedure. Depending on the mass of the shoot and leaves an extra volume of methanol was added and the steps were repeated a fourth time until the pigmentation was removed from the residue.

Initially, the cutting of the willows was ground to a powder in a mortar with sand and liquid nitrogen. Additional grinding was performed using 80% aqueous methanol. The sample was then cut and homogenized with sonication using a Polytron (Kinematica CH-

6010, Brinkmann Instruments, Westbury, NY). The homogenized sample was then subjected to a similar extraction procedure as the shoots. The extract was sampled and bleached for analysis by LSC, but it was found to contain concentrations of lignin that could not be bleached and which interfered with LSC. Subsequently, the cuttings were oxidized to determine total radioactivity that included both bound and soluble fractions.

Initially, the purification procedure involved a solid phase extraction (SPE) step using octadecyl-functionalized silica (C_{18}) to remove pigments from coloured samples. The supernatants were pooled and adjusted to 80% aqueous methanol and pH 7 using dilute sodium hydroxide. The extract was then run through a 2 g - C_{18} column to remove a large percentage of the pigments that interfere with LSC. Aqueous methanol (80%) was used to elute the compounds of interest, typically requiring less than 10 mL to remove it from the column, but this volume varied with compounds. The eluant was then sampled and analyzed using LSC.

Results from this purification procedure suggested there might be losses of radioactivity within either the C_{18} or from binding to particulate matter, notably with DTZ and EE2. The procedure was modified, removing the C_{18} clean-up step. Instead, the pooled supernatant was collected green and aliquots taken to be bleached prior to LSC as described above. Addition of bleach to the sample was sufficient to remove the color while not affecting the counting efficiency. Appendix B provides some of the results from the purification procedure that suggest the loss of radioactivity with the SPE procedure.

2.2.6 Bound fractions

Some of the compounds taken into the plants undergo a process that bind them irreversibly to cell wall constituents and these are then typically termed as 'bound.' This bound fraction is characterized by the inability to extract it using an extensive solvent extraction. Such bound radioactivity that could not be removed with organic solvent was quantified through oxidation that was performed on willow residues remaining after solvent extraction. As well, several replicate plants were analyzed whole without the prior solvent extraction to confirm the recovery levels from combination of the soluble extract fractions and the bound residue oxidation fractions.

Whole plant fractions and plant residues containing bound radioactivity were combusted in an R.J. Harvey Instrument Corporation Biological Material Oxidizer OX-500. Trapping of $^{14}CO_2$ from the oxidizer was done using the CO_2 absorber Carbo-Sorb E and the LSC cocktail Permafluor E+ obtained from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, USA). Trapping of $^{3}H_2O$ from the oxidizer was done using the scintillation cocktail Ecolite manufactured by MP (formerly ICN). CarboSorb E was combined with Permafluor E+ in a ratio of 1:2 in a trapping volume of 15 mL. Fractions of the whole plant of sufficient size (0.50 – 0.75 g fresh weight for roots and shoots, 0.20 g fresh weight for cutting) were oxidized to determine entire sample radioactivity that was analyzed by LSC. Plant residues were weighed dry (< 0.25 g dry weight) and oxidized after the addition of 0.2 to 0.5 mL of water.

Prior to oxidation, two procedures, cell wall fractionation and cell wall dissolution, were attempted to extract the bound residue for LSC. Both procedures are outlined in Appendix B. Neither procedure was able to provide results that could be reliably analyzed by LSC due to extensive pigmentation.

2.3 RESULTS

2.3.1 Uptake studies time course

Uptake study experiments were replicated twice, excluding DTZ due to degraded samples. Similar results were obtained from both replicate experiments and only one data set is presented for DZP, ATZ and EE2.

Percentage uptake, or removal from solution over time for EE2, DZP and DTZ followed a very similar pattern although maximum values varied between 10 and 30% (Figure 2.4). ATZ uptake followed a flatter curve than the other compounds, but uptake at 24 hours was 6% lower than for DZP (Figure 2.4). Uptake within the first 2 hours was generally consistent among the three pharmaceuticals, reaching approximately 37 - 50% removal from solution. ATZ was removed more slowly than the pharmaceuticals with about 13.5% uptake within the first 2 hours (Figure 2.4).

Salix exigua was thus very effective in removing EE2, DTZ and DZP from solution with removal of 88, 77, and 56%, respectively, in 24 hours. About 50% of the herbicide ATZ was removed within the 24 hours with the same treatment conditions.

Among compounds, the plants' weights (root, cutting, shoot and total weight) and total volume transpired, did not vary significantly (Table 2.2) (Table A2.1, ANOVA, $p > 0.05$) except for the cutting (ANOVA, $p = 0.004$); thus, replicate plants were of similar size and apparently similar health.

No plants showed signs of phytotoxicity during the period of study and with the concentrations used. There was no observable discoloration of the leaves or significant alteration in transpiration rates that were approximated by straight lines of cumulative transpiration up to 8 hours (Figure 2.5). A slight reduction in transpiration rate between 8 and 24 hours probably reflects reduced transpiration during the night phase of the study.

Similar cumulative transpiration trends over the 24 hour period were observed for all replicate plants for all compounds (Figure 2.5), but cumulative volumes transpired at t = 2, 4, and 8 did vary significantly between some of the compounds (Table A2.2, ANOVA, $p < 0.05$). At t = 2, volumes transpired varied significantly between DTZ or ATZ and EE2, but not between ATZ and EE2 (Table A2.3, Dunnett's C, $p < 0.05$). At t = 4 and t = 8, volumes transpired varied significantly between ATZ and DTZ (Table A2.3, Dunnett's C, $p < 0.05$).

A correlation analysis (Table A2.4, Spearman's rho) for uptake with fresh weight and volume transpired at each sampling time, found no consistent significant correlation between these factors and uptake for the compounds. ATZ uptake was only correlated with root and shoot fresh weight, and cumulative transpiration at t = 24 (Spearman's rho, $p < 0.05$). DZP uptake was correlated with shoot or total fresh weight, and cumulative volume transpired at t = 8 and t = 24 (Spearman's rho, $p < 0.05$). DTZ uptake appeared to be correlated to shoot, root and total fresh weight, and cumulative volume transpired at t = 8 and t = 24 (Figure A2.1). EE2 uptake was significantly correlated with root fresh weight at t = 4 and t = 24 (Spearman's rho, $p < 0.05$).

Cumulative volume transpired was consistently correlated with shoot and total fresh weights for DZP and EE2 over the study period (Table A2.5, Spearman's rho, $p < 0.05$). Cumulative volume transpired for ATZ was consistently correlated with total fresh weight across the sample times ($p < 0.05$).

Volatility of the compounds under the growth chamber conditions was negligible for the four compounds in the plant-free control conditions. Similarly, testing for compound binding to the culture tubes revealed no remaining glassware-bound radioactivity up to the t = 24 sampling time. It would be assumed that binding to the glassware would have occurred rapidly and therefore would not account for the observed uptake curves.

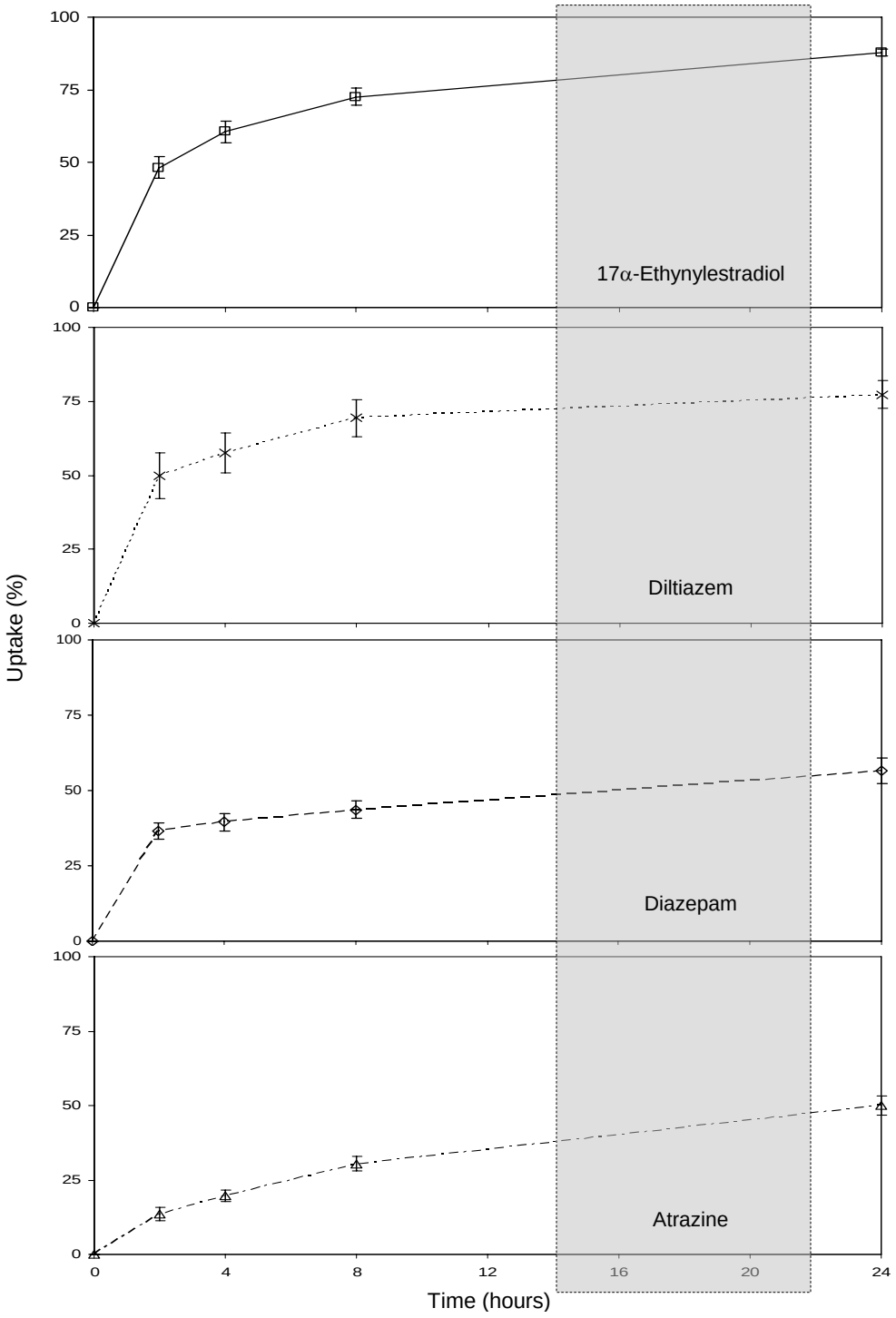


Figure 2.4. Percentage uptake from solution of 0.04 µg/ mL for 17α-ethynylestradiol (n = 6), diltiazem (n = 4), diazepam (n = 6), and atrazine (n = 6), by *Salix exigua*. Mean ± SE are plotted for the 24 hour period. The 16 hr day and 8 hr night (shaded) are indicated.

Table 2.2. *Salix exigua* summary plant information from uptake study. Mean ± SE are shown for plant fresh weights and total volume transpired.

	n	*Root fr. wt. (g)*	*Cutting fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total Volume Transpired (mL)*
17α-Ethynylestradiol	6	0.47 ± 0.05	0.91 ± 0.05	0.71 ± 0.05	10.42 ± 0.58
Diltiazem	4	0.41 ± 0.13	0.61 ± 0.05	0.64 ± 0.08	9.25 ± 0.46
Diazepam	6	0.53 ± 0.10	0.76 ± 0.09	0.93 ± 0.16	13.67 ± 2.39
Atrazine	6	0.48 ± 0.08	1.23 ± 0.15	1.00 ± 0.09	14.25 ± 1.23

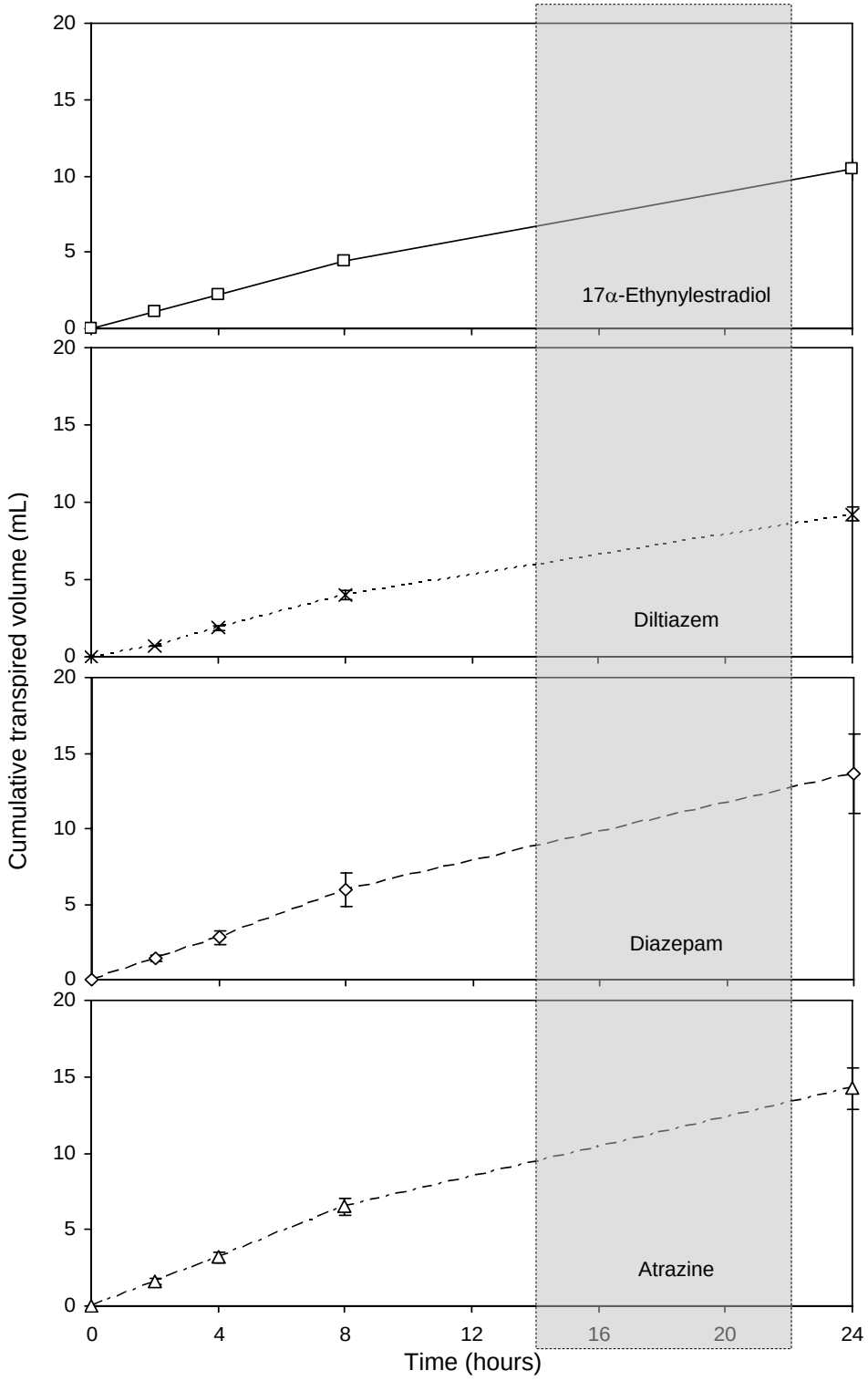


Figure 2.5. Cumulative transpired volumes measured for *Salix exigua* at each sampling time for the four compounds. Mean ± SE are shown. The 16 hr day and 8 hr night (shaded) are indicated.

2.3.2 Distribution

Plant components underwent extensive solvent extraction to retrieve the soluble fraction and the remaining residue was oxidized to retrieve the bound fraction. Recovery levels of radioactivity removed from solution were somewhat variable across the replicates and especially across compounds. Final percent recoveries for EE2, DTZ, DZP and ATZ were 88, 94, 80 and 37%, respectively (Figures 2.6 and 2.7).

EE2 recovery was mostly as a bound fraction within the roots with a small soluble fraction (10%), making up 92% of recovered radioactivity (Figures 2.6 and 2.7). Shoot soluble and bound fractions made up 6% of recovered with the bound fraction making up less than 1% of this value. Approximately 2% of EE2 was within the cutting. DTZ was recovered entirely as a soluble fraction within the roots (Figures 2.6 and 2.7). DZP remained primarily soluble with proportion of recovered radioactivity at 50% in the shoot, 14% in wood and 38% within the roots (Figures 2.6 and 2.7). The bound fraction represented less than 1% of total recovered radioactivity.

ATZ was not significantly bound to roots or shoots of willow. The recovery of radioactivity after extraction of shoots was low (Figure 2.7), but from oxidation the largest portion of recovered radioactivity was within the shoots (68%) (Figure 2.6). The cutting proportion of the recovered radioactivity was 20% and the roots contained 12%.

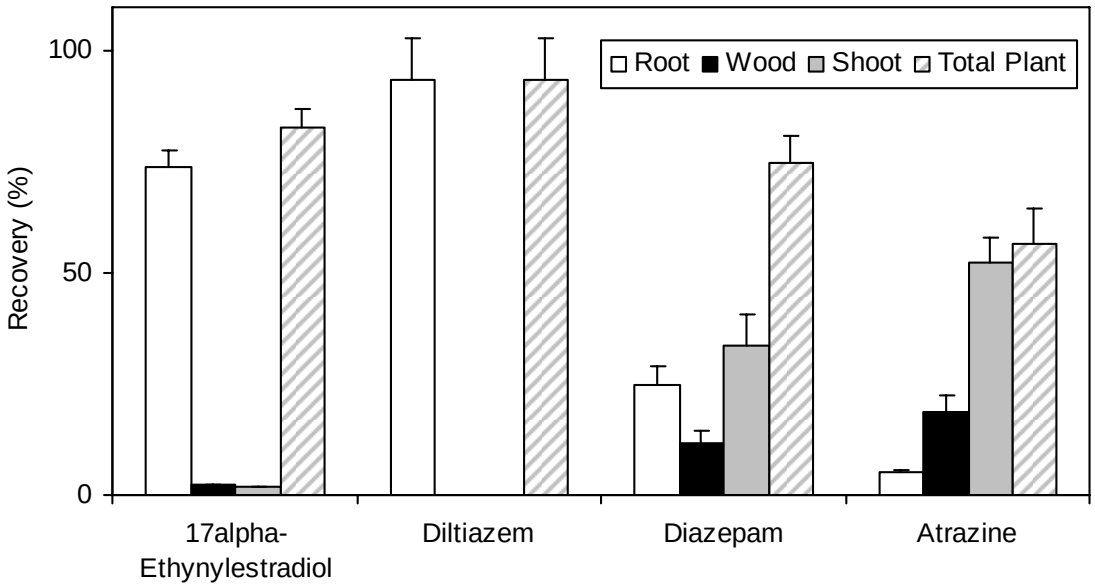


Figure 2.6. Distribution of recovered radioactivity from oxidation of whole root, shoot and cuttings and total plant recovery for radiolabeled compound added to *Salix exigua* for 17α-ethynylestradiol, diazepam, and atrazine (n = 3 for each) (mean ± SE). For diltiazem, n = 4, root value is from the soluble fraction recovery as analysis of shoot, wood and bound fractions recovered no measurable amounts of radioactivity.

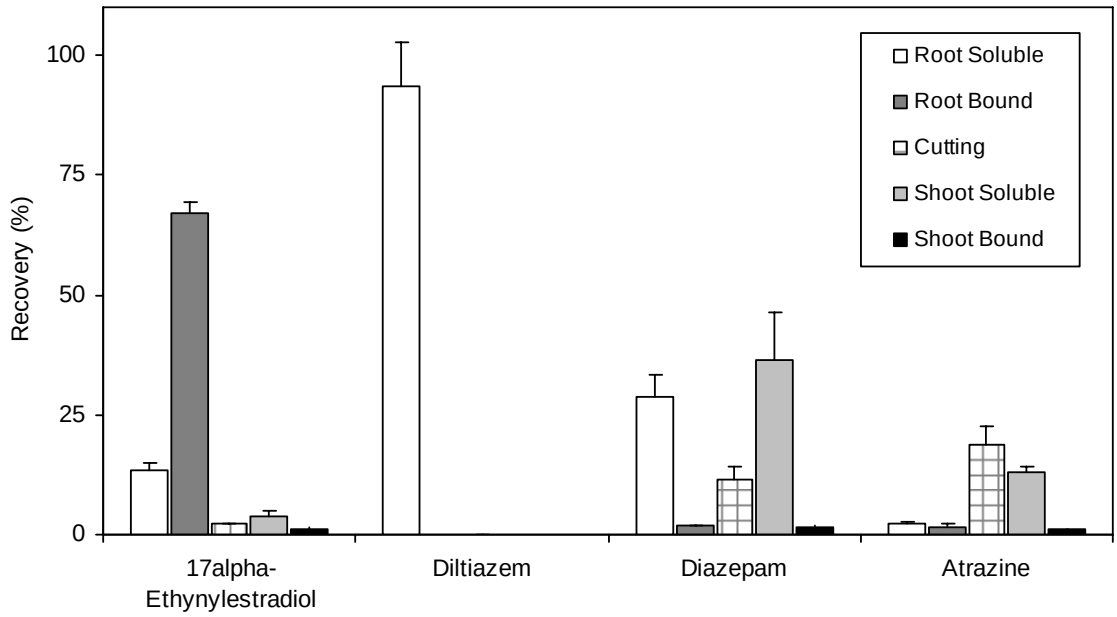


Figure 2.7. Distribution of recovered radioactivity among soluble and bound fractions in roots and shoots and cutting for radiolabeled compound added to *Salix exigua* (mean ± SE). For 17α-ethynylestradiol, diazepam, and atrazine, n = 6; 3 whole plant oxidized, 3 soluble + bound oxidized. For diltiazem, n = 4, soluble + bound oxidized.

2.3.3 Root concentration factors and transpiration stream concentration factors

Root concentration factor (RCF) experiments were carried out for *Salix exigua* to provide a comparison with other plants and to establish the relationship between RCF and pharmaceutical log K_{ow} values for willow roots. The experiment was terminated at 24 hours when there was apparently compound equilibrium between roots and solution. In prior studies, equilibrium apparently occurred quickly, within hours (Burken and Schnoor, 1998) or up to 24 hours (Briggs et al., 1982).

Fresh weights of the two types of roots used for determination of RCF values were similar, allowing comparison of uptake results between the two (Table A2.6, ANOVA, $p > 0.05$). Root uptake with excised roots followed similar trends for EE2, DZP and ATZ. Final root uptakes for EE2, DZP and ATZ were 79, 42 and 31%, respectively. Root uptake with roots attached to the cutting for the 3 compounds also followed similar patterns of uptake, although final root uptakes were slightly higher, at 88, 54 and 41%, respectively. The roots attached to the cuttings had uptake curves similar to excised roots, but uptake was faster for roots attached to the cutting and significantly different with 7 of 15 sampling pairs (Figure 2.8; Table A2.7, ANOVA, $p < 0.05$).

Average root uptake of results combined for the two root types varied significantly between compounds for all sampling times except $t = 1$ (Table A2.8, ANOVA, $p > 0.05$). At $t = 2$, EE2 and DZP root uptake varied significantly (Table A2.9, Tamhane, $p = 0.000$). By $t = 4$, EE2 and ATZ varied significantly (Tamhane, $p = 0.029$). At $t = 8$ and t

= 24, all compounds were significantly different in their root uptake except for ATZ and DZP.

RCF values for excised roots and roots attached to the cutting were calculated separately, but were similar enough that one equation was sufficient to explain the relationship between roots and log K_{ow} (Table A2.7, ANOVA). DTZ was excluded from this equation since the RCF value was calculated using whole plant uptake values. The equation developed from these calculations with an $R^2 = 1.00$ for willow RCF was (Figure 2.9):

$\log (RCF) = 0.93 \log K_{ow} - 1.10$.

RCF values were also calculated for the compounds using their log K_{ow} values and the equations for barley (Briggs et al. 1982) and poplar roots (Burken and Schnoor, 1998) (Figure 2.9).

RCF plants underwent similar solvent extraction and oxidation as for the whole plant uptake studies. The average percent recoveries and distributions between the excised roots and roots attached to the cutting varied among replicates and between compounds (Figure 2.10). Average percent recovery for EE2, DZP and ATZ were 54, 40 and 10%, respectively, for combined results from excised roots and roots attached to cuttings (Figure 2.10). The distribution ratios within the roots and wood were generally similar to those observed from the roots of whole plant uptake studies. Thus, soluble fractions contained more than 85% of recovered radioactivity for DZP and ATZ, while recovered

radioactivity for EE2 was more than 70% in the bound fraction (Figure 2.10). For all compounds, proportion within the cutting was small.

The RCF values for the four compounds and willow were estimated using results from the whole plant uptake studies and the equation for RCF (Shone and Wood, 1974). Calculated RCF values were not significantly different from experimentally derived RCF values except for ATZ (t-test, $t = -3.25$, $df = 5$, $p = 0.023$).

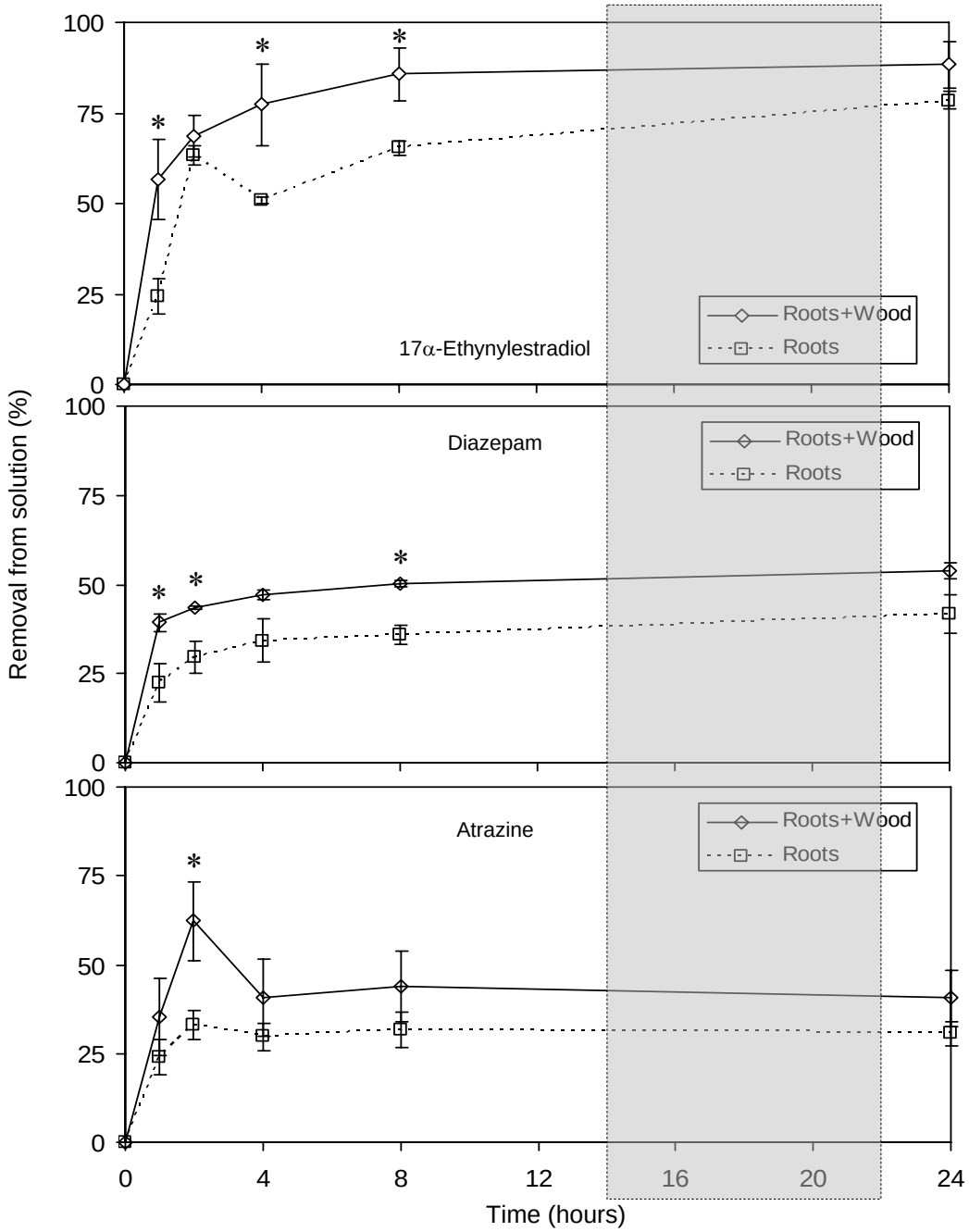


Figure 2.8. Uptake or equilibrium curves for willow roots following the addition of radiolabeled compounds. Percent removal of radioactivity over time for root concentration factor studies (mean ± SE plotted, n = 3). The 16 hr day and 8 hr night (shaded) are indicated. ***** Significantly different means between Roots versus Roots+Wood ($p < 0.05$) from Table A2.7 ANOVA analysis.

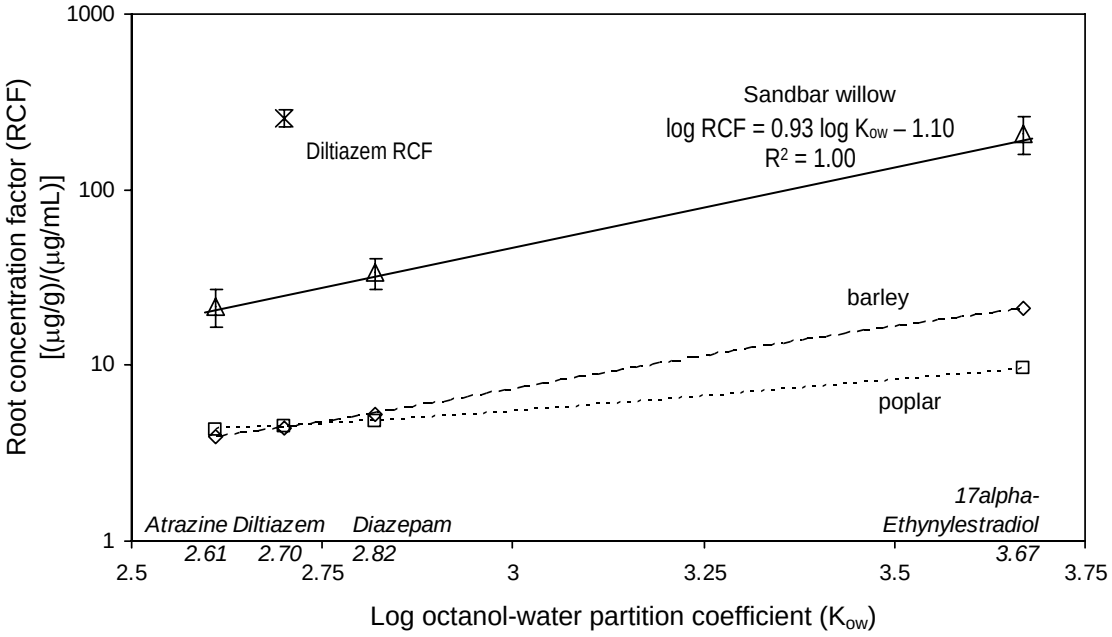


Figure 2.9. Calculated and experimentally determined root concentration factor values (mean ± SE) for *Salix exigua*. Values also calculated using compounds' log octanol-water partition coefficients and the equations for barley roots (Briggs et al., 1982) and hybrid poplar roots (Burken and Schnoor, 1998). Diltiazem RCF value was not calculated using excised roots, but from whole plant uptake studies and the proportion of the compound within the root, therefore DTZ was excluded from this equation development.

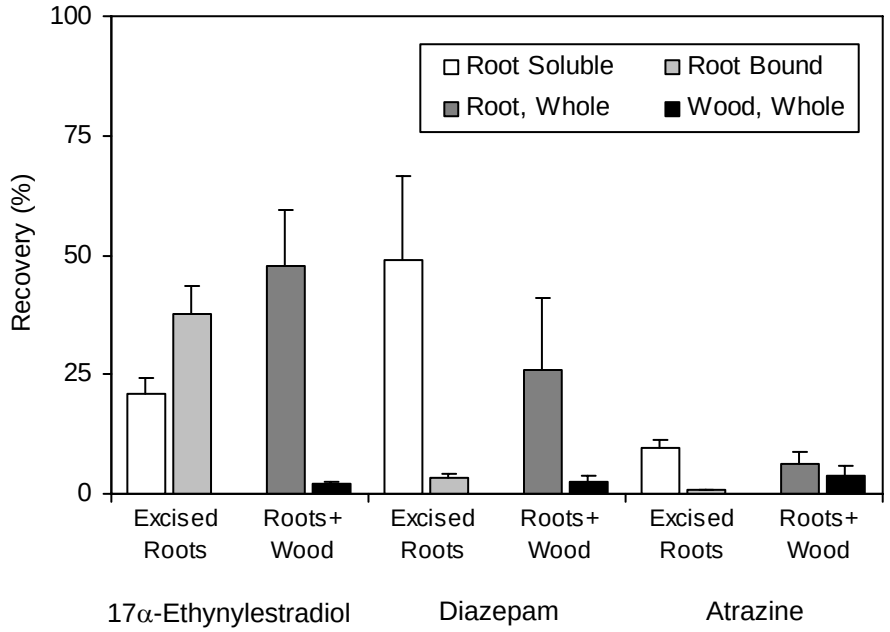


Figure 2.10. Distribution of recovered radioactivity within the roots and cuttings of root concentration factor experiment plants for *Salix exigua*. Mean ± SE are shown.

Estimated transpiration stream concentration factor (TSCF) values were not significantly different when calculated using percent recovery or percent distribution of recovered (t-test, ATZ, t = 0.520, df = 5, p = 0.625; DZP, t = 1.896, df = 5, p = 0.116; EE2, t = -2.287, df = 5, p = 0.071), therefore the data shown in Figure 2.11 are calculated from percent recovery. DTZ values were not used in this equation development as there was no movement into the shoot. The equation developed from these calculations with an R^2 = 0.822 for *Salix exigua* TSCF was (Figure 2.11):

$\log TSCF = 0.949 \exp[-(\log K_{ow} - 2.90)^2 / 0.321]$.

TSCF values for willow are less than 1 and decline in the order ATZ > DZP > EE2, with ATZ's value occurring between calculated TSCF values determined using Briggs et al. (1982) and Burken and Schnoor's (1998) equations and the compounds' log K_{ow} values (Figure 2.11). The TSCF value for EE2 was just below the value calculated for barley roots. The experimentally derived TSCF value for DZP was approximately 1.5x greater than the calculated TSCF value.

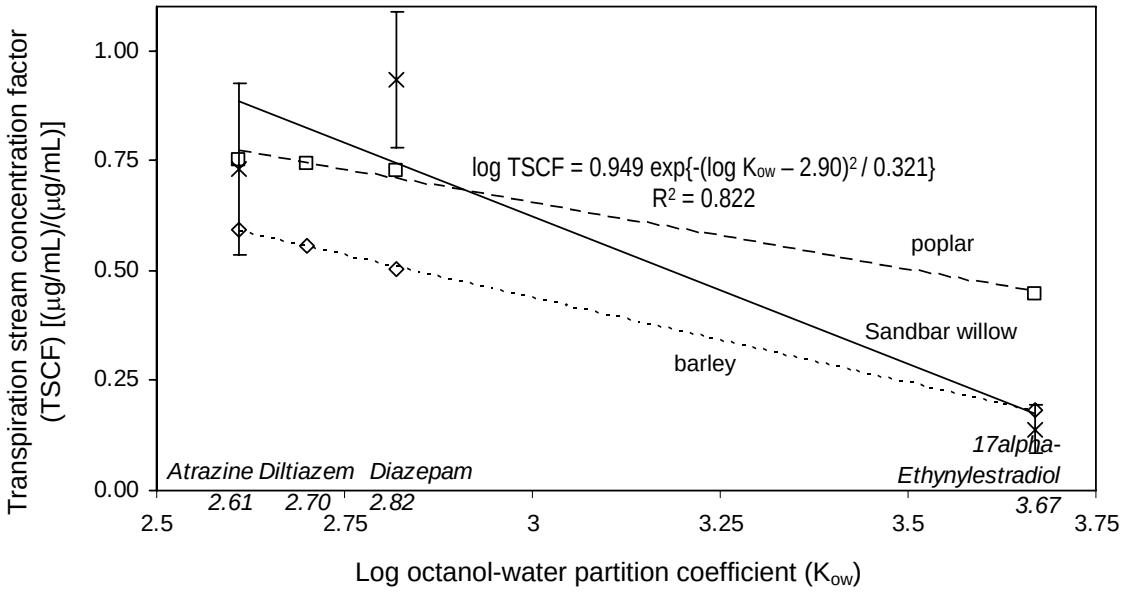


Figure 2.11. Calculated transpiration stream concentration factor values (mean ± SE) for *Salix exigua*. Diltiazem was excluded from this equation development. Values also calculated using compounds' log octanol-water partition coefficients and the equations for barley shoots (Briggs et al., 1982) and hybrid poplar shoots (Burken and Schnoor, 1998).

2.4 DISCUSSION

Uptake. *Salix exigua*, a common riparian plant, was examined for its ability to remove trace levels of three pharmaceuticals: EE2, DZP, and DTZ, and the herbicide ATZ, from solution. Hydroponically grown *Salix exigua* was very effective at removing EE2, DZP and DTZ from solution with percent removal of 88, 56 and 77% removal, respectively, in 24 hours. The herbicide ATZ, was also removed with 50% taken up in the same conditions and interval.

The uptake curves for EE2, DTZ and DZP were similar, while ATZ had had more gradual uptake (Figure 2.4). The final uptake values between compounds are generally consistent with predicted behaviour based on the compound's logarithm octanol-water partitioning coefficient (log K_{ow}). In order from highest to lowest log K_{ow} values the compounds were sequenced: EE2 (3.67) >> DZP (2.82) > DTZ (2.70) > ATZ (2.61). This order was largely reflected in uptake, thus the compounds were sequenced EE2 > DTZ > DZP > ATZ (Figure 2.4).

A higher octanol-water partitioning coefficient would reflect a compound's preference or attraction for the organic phase versus water. Consequently, a larger log K_{ow} value suggests the compound would sorb to root cells at a faster rate than a compound with a lower log K_{ow} value. This principle is reflected in the distribution of the different compounds. EE2, with its approximate 10-fold larger K_{ow}, apparently had a greater preference by the root cells than the other 3 compounds and this is reflected in the faster uptake curve (Figures 2.4 and 2.6). EE2 uptake was expected to be correlated with root

weight given its binding preference to the roots (RCF > 100) and its log K_{ow} value, but this was not consistently the case across the study period, likely due to the limited variation in root weights of the experimental plants.

The reversal of DTZ and DZP for uptake, may be suggesting processes are increasing the uptake of DTZ. As DTZ in this study was in the form DTZ-hydrochloride, it was not a neutral compound as the other 3 are expected to be, and would therefore become highly ionized, at external solution pH and physiological pH. DTZ uptake would then become a function of the proportion ionized and electrochemical gradients. ATZ uptake was expected to be correlated to shoot mass or transpired volumes given that it has a low log K_{ow} value and that its an herbicide with an action site in the shoot, and this was the case at t = 24.

Plant fresh weights and total volumes transpired did not vary significantly between compounds, except for wood weight, indicating unequal stem sizes across the treatments (Table 2.1 and A2.1). Fortunately, the differences are unlikely to affect uptake significantly since the wood contained low levels of the compounds. The effects of wood weight would be expected to be negligible, unless uptake is correlated to transpiration, which it generally was not (Table A2.5). The similar fresh weights and transpiration volumes of the replicate plants among compounds allows for comparisons between the compounds for uptake and distribution, as uptake is primarily a function of root mass.

The lack of consistent correlation between uptake and plants' fresh weights and volume transpired (Table A2.4) is likely due to the limited range of willow weights used for the replicates for each compound and across compounds. It would be expected that there would be a positive relationship between plant weight and uptake, as well as transpiration and uptake if the compound moved freely into the transpiration stream. The relationship between uptake and root mass/volume was positive, indicating that increasing the amount of root area for sorption increased the uptake. As well, if a larger volume was moving within the transpiration stream, there would be a larger carrier base for freely moving compounds.

Cumulative transpired volumes were correlated with shoot mass for DZP and EE2 (Table A2.5, Spearman's rho, $p < 0.05$). For all compounds, cumulative transpired volumes were correlated with total plant weight (Table A2.5, Spearman's rho, $p > 0.05$). This correlation is likely evident due to the sample sizes and the variance in plants' weights (Table 2.1).

No plants showed signs of phytotoxicity that would be evidenced by discoloration of the leaves or alterations in transpiration (Trapp et al., 2000). Since ATZ is an herbicide, there was the potential for an observable effect. As well, DTZ, the calcium-channel blocker, might have had an effect if it was impeding the plant's calcium transport.

Uptake and distribution of the herbicide ATZ in hybrid poplar trees have been studied extensively by Burken and Schnoor (1996, 1997 and 1998). The findings of the present

study correspond to those prior results with levels being highest in the shoots, followed by the cutting and then roots. Translocation from roots to shoots was apparently rapid when compared to the other compounds (Figures 2.6 and 2.7) and is also consistent with Burken and Schnoor (1997).

Root uptakes for the root concentration factor (RCF) experiments were lower for excised roots than for roots attached to the cuttings among the compounds over the sampling period (Figure 2.8; Table A2.7, ANOVA). Differences in root uptake between excised roots and roots attached to the cutting may be explained by the travel of the compound into the cutting by diffusion or slight evaporation from the severed shoot node. It was expected that uptake would be equivalent between excised roots and roots attached to the cutting as the root masses were similar (root mass and root uptake have a positive relationship), but this appears to not be the case.

Examination into distribution of the compounds within the roots and wood in the RCF plants, in terms of soluble and bound fractions, returned low and somewhat variable recovery levels, especially for ATZ. Recoveries for EE2, DZP and ATZ were 54, 40 and 10%, respectively. Proportions within the roots generally reflected the root proportions observed from the whole plant uptake studies. This suggests that the proportions observed with whole plant uptake can be largely accounted for by this initial equilibration between root and external solution concentrations (represented as RCF).

Calculated RCF values using whole plant uptake data were compared to experimentally derived RCF values from the root uptake experiments and were consistent except for ATZ (t-test, $t = -3.25$, $df = 5$, $p = 0.023$). Briggs et al. (1982) found that using the percentage of radioactivity within the roots from whole plant uptake studies to calculate RCF resulted in values similar to those determined experimentally using excised roots. Similar results may be represented here for willow. Based on the log K_{ow} value for DTZ, it was expected to behave similarly to ATZ and DZP with some of the compound moving into the shoot, but this did not occur suggesting DTZ may not follow the same relationship.

The relationship between TSCF and log K_{ow} is generally expressed as a bell shaped or normal curve, with maximum uptake corresponding to log K_{ow} values around 2, although this value varies across plants. The log K_{ow} values used in the present study ranged from 2.61 to 3.67, and therefore only the decreasing half of the normal curve is represented and the maximum uptake log K_{ow} could not be determined for willow. All estimated TSCF values fell below 1 indicating the compounds are not easily taken up into the transpiration stream.

The studies for barley (Briggs et al., 1982) and hybrid poplar (Burken and Schnoor, 1998) involved multiple chemicals with a range of log K_{ow} values enabling the determination of optimum uptake. Optimal uptake occurs with moderately hydrophobic compounds (log $K_{ow} = 1.0 – 3.5$), with values greater than 3 being highly sorbed to roots (Briggs et al., 1982; Burken and Schnoor, 1998). The range of log K_{ow} values used in this

study was 2.61 – 3.67. Uptake from solution and movement into the transpiration stream followed this suggested optimum range for uptake for willow.

A preliminary investigation into the uptake and time course distribution of EE2 with willow was performed prior to the experiments discussed in this chapter and reported in Appendix B. Three willow plants were treated with 3 concentrations of EE2 (0, 1 and 10 μg EE2 + 16,667 Bq ^{3}H-EE2) in the same manner as the uptake studies described here but the plants were harvested after 8 hours. Recovery was lower for 2 of the 3 plants from the extraction procedures (soluble, bound and whole cutting oxidation), but distribution of recovered radioactivity suggests concentration has minor influences on distribution and uptake. As well, 3 plants were exposed to a single concentration of EE2 (0 μg EE2 + 16,667 Bq ^{3}H-EE2) and one plant harvested at 8, 24 and 48 hours to discern any differences in distribution over time. There appears to be a change in distribution of EE2 within willow over time, with an increasing proportion becoming bound to the root (and hence less remaining soluble within the root).

Conclusions. *Salix exigua* was effective at uptake of the three pharmaceuticals in 24 hours. The synthetic estrogen EE2 was removed most effectively, with most of the compound becoming bound to the roots and little transport to shoots. DTZ, the anti-hypertensive, was removed from solution at an intermediate rate and remained within the soluble component within the root. The anti-anxiety drug DZP remained primarily soluble with over half of the compound taken up being transported to the shoots. The herbicide ATZ distribution was similar to that previously reported for poplars.

Predictive relationships between the pharmaceuticals' physio-chemical property, the octanol-water partitioning coefficient, and willow roots and shoots were developed and found to be similar to previously reported relationships for other compounds for hybrid poplar roots. However, DTZ uptake and distribution were different then predicted indicating that aspects other than partitioning apply. Based on the results for EE2 and DZP, it appears that pharmaceuticals behave similarly to other chemicals and this should allow for predictable behaviour with respect to phytoremediation.

I thus conclude that phytoremediation appears to be a promising solution for remediating environmental pharmaceuticals in water. This treatment may be seasonally limited, however, and prospective phytoremediation would be far less effective during the winter season when willow and other plants are leafless and often dormant. A concern may also be the release of these compounds back into the environment with winter leaf drop or the decay of fallen plants. Sequestration of these compounds within a bound form may inhibit re-entry into the environment. The more potent and influential compound on aquatic life, the synthetic estrogen EE2, is also significantly bound within willow. The herbicide ATZ is most likely to return to the environment through leaf drop or plant decay as it remains primarily in the soluble form within the leaves. As well, the results observed here with willow are consistent with prior research with poplar, suggesting poplar research may be applicable to willow, and vice versa.

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CHAPTER 3 Phytoremediation of trace levels of pharmaceuticals, diltiazem, diazepam and 17α-ethynylestradiol, and the herbicide atrazine, with *Arabidopsis thaliana*

3.1 INTRODUCTION

This present investigation studied the potential capability of *Arabidopsis thaliana* to uptake and transport three pharmaceuticals that represent common classes of substances that have been detected in municipal wastewater, including diltiazem (DTZ), a calcium channel blocker, diazepam (Valium®) (DZP), an anti-anxiety drug; and 17α-ethynylestradiol (EE2), a synthetic birth control hormone (a common component of the contraceptive pill). Although *Arabidopsis* is not a practical field plant for phytoremediation, its genome has been sequenced and it can provide useful insight into the genes involved in uptake, translocation and metabolism of pharmaceuticals within other plants (Cobbett and Meagher, 2002).

This study of uptake and distribution of these compounds within *Arabidopsis* provides a comparison to *Salix exigua*'s uptake and distribution of these compounds. As well, a second plant can help to confirm the behaviour of pharmaceuticals within plants and the properties that guide their uptake and distribution.

Phytoremediation of pharmaceuticals is not widely published in the literature, but is a new direction for research that should be considered, particularly as the quantities of detectable contaminants are only increasing as technology and methodologies change.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

The pharmaceuticals and other chemicals used for this study are described in Chapter 2.

3.2.2 Plants, hydroponics and treatments

Arabidopsis thaliana (var. Columbia) was started from seed and seedlings were grown using a hydroponic setup in a controlled environment growth chamber (Figure 3.1). The hydroponic system was described in Chapter 2, with adaptation for *Arabidopsis*. To provide a germinating and support medium for *Arabidopsis* seeds, horticultural Rock Wool made from basalt fibers (FibrGrow Horticultural Products, Sarnia, Ontario), was cut into 1.5 x 3.0 cm cubes and inserted into the drilled holes. Toothpicks supported the Rock Wool above the water surface allowing submersion about 2 cm into solution. Two seeds were placed on the top of each exposed end of Rock Wool for later thinning to one plant. Water level was kept between 1.5 to 2 cm below seed level to promote germination and proper growth, as was suggested by Gibeaut et al. (1997). Growth chamber and chamber conditions for *Arabidopsis* were the same as described in Chapter 2. *Arabidopsis* plants approximately 4 weeks old and approaching the flowering stage were used for the uptake studies.

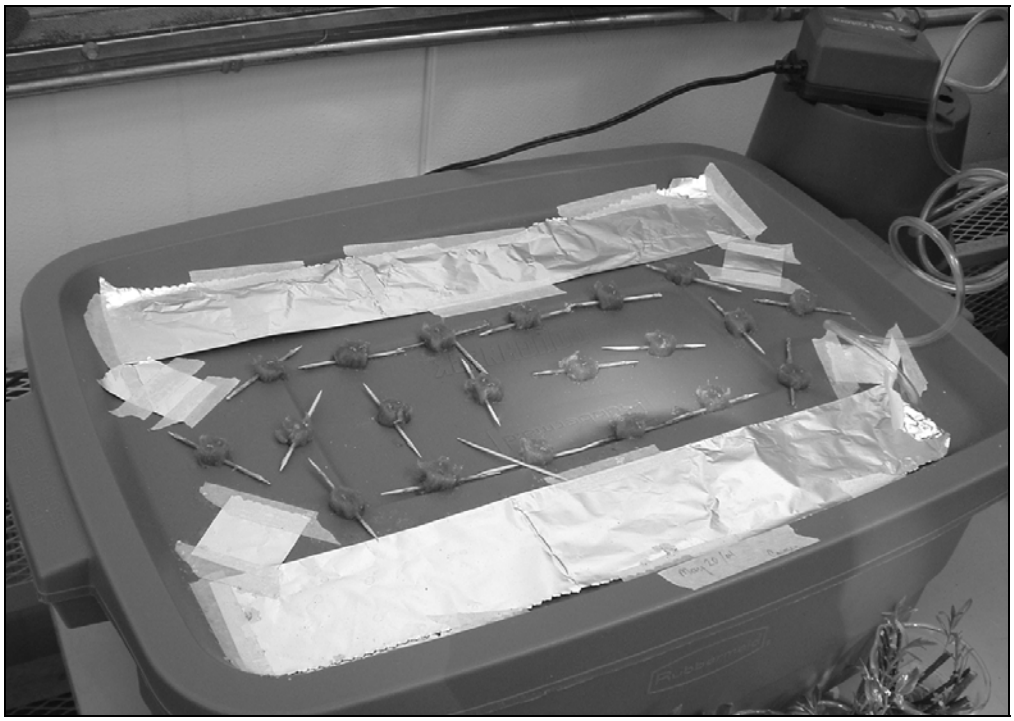

Figure 3.1. Hydroponic system for *Arabidopsis* seed germination and growth.

3.2.3 Uptake studies

The uptake study set-up is described in Chapter 2. The uptake experiments were replicated twice with similar results and one data set is presented. The second data set not presented involved n = 4 for each compound.

Similar sized plants were chosen and inserted into the culture tubes. Plant shoots were supported so that only the roots were submerged. At the time of harvest (24 hr) the plants were removed from solution, the roots were rinsed in nutrient solution to remove surface solution on the roots, and divided into shoots and roots. Each was weighed fresh and stored at -20 °C until analysis. For each compound, replicate plants numbered 10, 6, 6 and 5 for EE2, DTZ, DZP and ATZ for the data set presented, respectively. Transpiration volumes were monitored as described in Chapter 2.

3.2.4 Soluble fractions

A methanol solvent extraction procedure is described in Chapter 2 and was carried out on some of the replicate plants. For EE2, n = 4; DTZ, n = 4; DZP, n = 5; ATZ, n = 3. However, due to initial problematic experimental procedures using SPE to remove pigments from the shoot extracts (see Appendix B for some results and method changes), the numbers of results considered reliable decreased. Numbers of replicates from solvent extraction that were reliable became: EE2, n = 4; DTZ, n = 2; DZP, n = 4, ATZ, n = 1.

3.2.5 Bound fractions

Quantification of bound radioactivity that may have been within the cell walls that was not removed with organic solvent was performed on *Arabidopsis*. Residues that had undergone solvent extraction were oxidized to extract bound tritium or carbon-14, as described in Chapter 2. Some whole replicate plants that had not undergone solvent extraction underwent oxidation to confirm levels of radioactivity being recovered through combination of the solvent extraction and oxidation of residues. For whole plants that underwent oxidation: DTZ, n = 2; DZP, n = 1; EE2, n = 6; ATZ, n = 2.

3.2.6 Root concentration factor and transpiration stream concentration factor

The root concentration factors (RCF) for the four compounds and *Arabidopsis* were estimated using results from the whole plant uptake studies and the equation (Shone and Wood, 1974):

root uptake = RCF (μg/mL) x root mass (g) x external solution concentration (μg/mL).

RCF values were also calculated for the four compounds using log K_{ow} values and the equations developed by Briggs et al. (1982) for barley roots:

log (RCF - 0.82) = 0.77 log K_{ow} - 1.52,

and by Burken and Schnoor (1998), for hybrid poplar roots**:**

$\log (RCF - 3.0) = 0.65 \log K_{ow} - 1.57$.

Transpiration stream concentration factor (TSCF) estimates for the four compounds in *Arabidopsis* were estimated using results from whole plant uptake studies and the equation (Briggs et al., 1982):

TSCF = [concentration in the shoot (μg) / volume transpired (mL)] / [(external solution concentration $(\mu g/mL)_{initial}$ + external solution concentration $(\mu g/mL)_{final}$)/ 2].

Although transpiration may not have been continuous, the means of initial and final external solution concentrations were used to estimate overall TSCF (Briggs et al., 1982).

TSCF were also calculated for the four compounds using log K_{ow} values and the equations developed by Briggs et al. (1982) for barley shoots:

$\log TSCF = 0.784 \exp[-(\log K_{ow} - 1.78)^2 / 2.44]$,

and by Burken and Schnoor (1998), for hybrid poplar shoots:

$\log TSCF = 0.756 \exp[-(\log K_{ow} - 2.50)^2 / 2.58]$.

3.3 RESULTS

3.3.1 Uptake studies time course

Arabidopsis was capable of removing EE2, DZP and DTZ with removal at 85, 59 and 57%, respectively, in 24 hours. The herbicide ATZ was employed as a positive control as it is known to be readily taken up by plants. *Arabidopsis* removed 52% of ATZ within the 24 hour period.

The percentage uptake, or removal from solution for DTZ, ATZ, and DZP over time followed a very similar pattern and maximum values at 24 hours (Figure 3.2). Rapid uptake within the first 2 hours was consistent among the compounds, reaching approximately 25% removal from solution for DZP, DTZ and ATZ. *Arabidopsis* removed 74% of the synthetic estrogen within the first 2 hours.

The order of highest to lowest uptake amount is a reflection of the compounds' log K_{ow}: EE2 (85% uptake, log K_{ow} 3.67) >> DZP (59% uptake, log K_{ow} 2.82) > DTZ (57% uptake, log K_{ow} 2.70) > ATZ (52% uptake, log K_{ow} 2.61).

Among compounds, the plants' weights (root, shoot and total weight) and total volume transpired did not vary significantly (Table 3.1 and Table A3.1, ANOVA, $p > 0.05$), thus, replicate plants were of similar size and apparently similar health.

No plants showed signs of phytotoxicity during the period of study and with the concentrations used. There was no observable discoloration of the leaves or significant

alteration in transpiration rates, which were approximated by the straight lines of cumulative transpiration up to 10 hours (Figure 3.3). A slight reduction in transpiration rate between 10 and 24 hours probably reflects reduced transpiration during the night phase of the study.

Similar cumulative transpiration trends over the 24 hour period were observed for all replicate plants for all compounds (Figure 3.3 and Table A3.2, ANOVA). Transpiration volume at $t = 2$ varied significantly between all compounds except between DZP or DTZ and EE2 but did vary between DTZ and EE2 (Table A3.3, LSD, $p < 0.05$). At $t = 4$ transpiration volume varied significantly between EE2 and all other compounds but not between ATZ, DTZ and DZP (Table A3.3, LSD, $p < 0.05$).

A correlation analysis (Table A3.4, Spearman's rho) for uptake with fresh weight and volume transpired at each sampling time, found no significant correlation between these factors and uptake for DTZ or DZP (Spearman's rho, $p > 0.05$). There was no significant correlation between uptake and volume transpired for any of the compounds (Spearman's rho, $p > 0.05$). ATZ uptake was correlated with total fresh weight at time $t = 10$ and $t = 24$ (Spearman's rho, $p = 0.037$ and $p = 0.037$). EE2 uptake was significantly correlated with root, shoot and total fresh weight at $t = 2$ (Spearman's rho, $p < 0.05$). Cumulative volume transpired over time was not consistently correlated to plant fresh weights (Table A3.5, Spearman's rho, $p > 0.05$), except for EE2 ($p < 0.05$).

Volatility of the compounds under the growth chamber conditions was negligible for the four compounds in the plant-free control conditions. Similarly, testing for compound binding to the culture tubes revealed no remaining glassware-bound radioactivity up to the t = 24 sampling time.

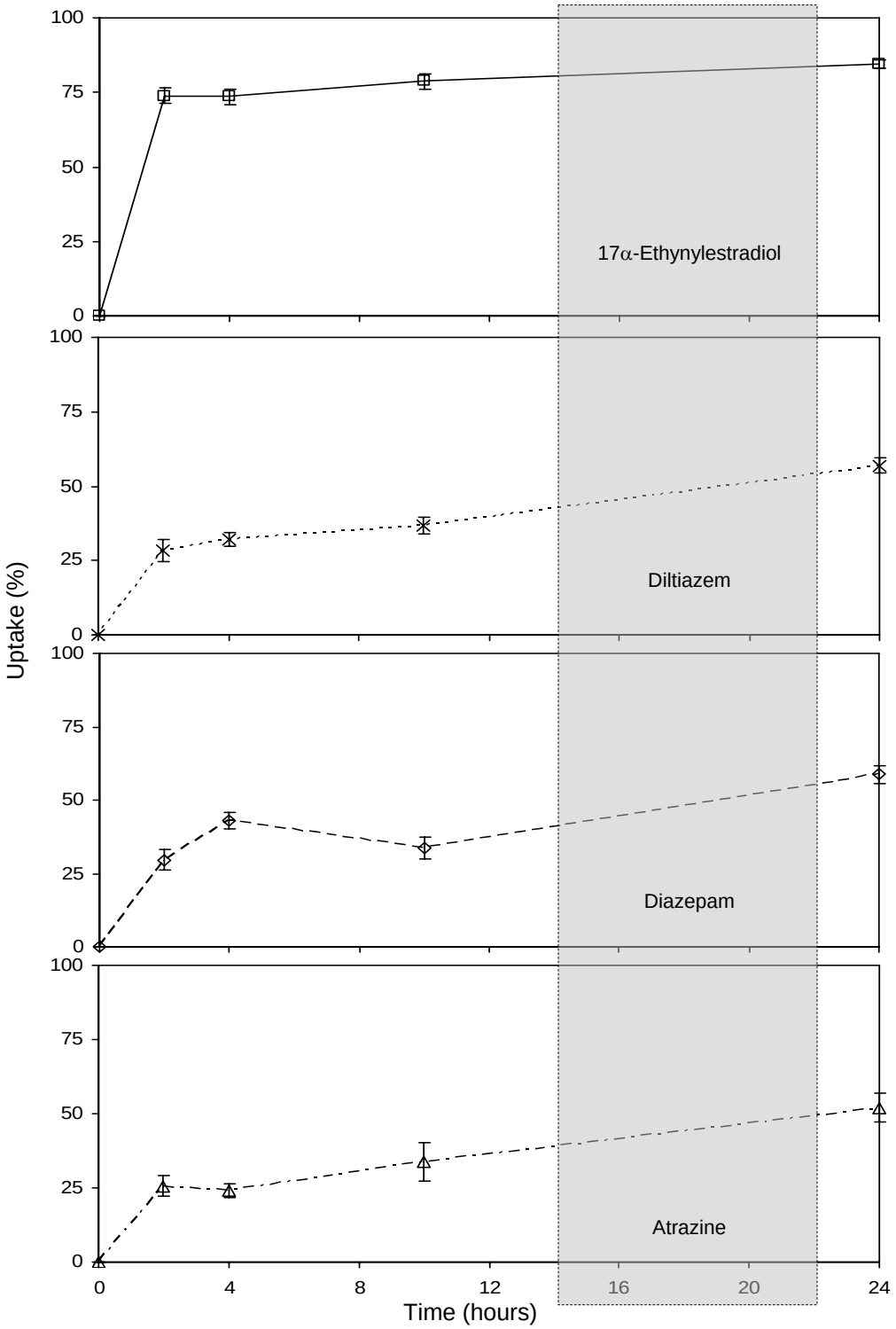

Figure 3.2. Uptake from solution of 0.04 μg/ mL of 17α-ethynylestradiol (n = 10), diazepam (n = 6), diltiazem (n = 6), and atrazine (n = 5), by *Arabidopsis*. Mean ± SE are plotted for the 24 hour period. The16 hr day and 8 hr night (shaded) are indicated.

Table 3.1. Summary information for *Arabidopsis* plants' fresh weight and total volume transpired in the 24 hour study. Mean ± SE are shown.

	n	*Root fr.wt. (g)*	*Shoot fr.wt. (g)*	*Total Volume Transpired (mL)*
17α-Ethynylestradiol	10	0.79 ± 0.12	1.85 ± 0.25	9.65 ± 0.81
Diltiazem	6	0.73 ± 0.10	1.65 ± 0.21	9.13 ± 0.97
Diazepam	6	0.70 ± 0.10	1.47 ± 0.21	8.83 ± 0.90
Atrazine	5	0.67 ± 0.18	1.75 ± 0.17	10.85 ± 1.40

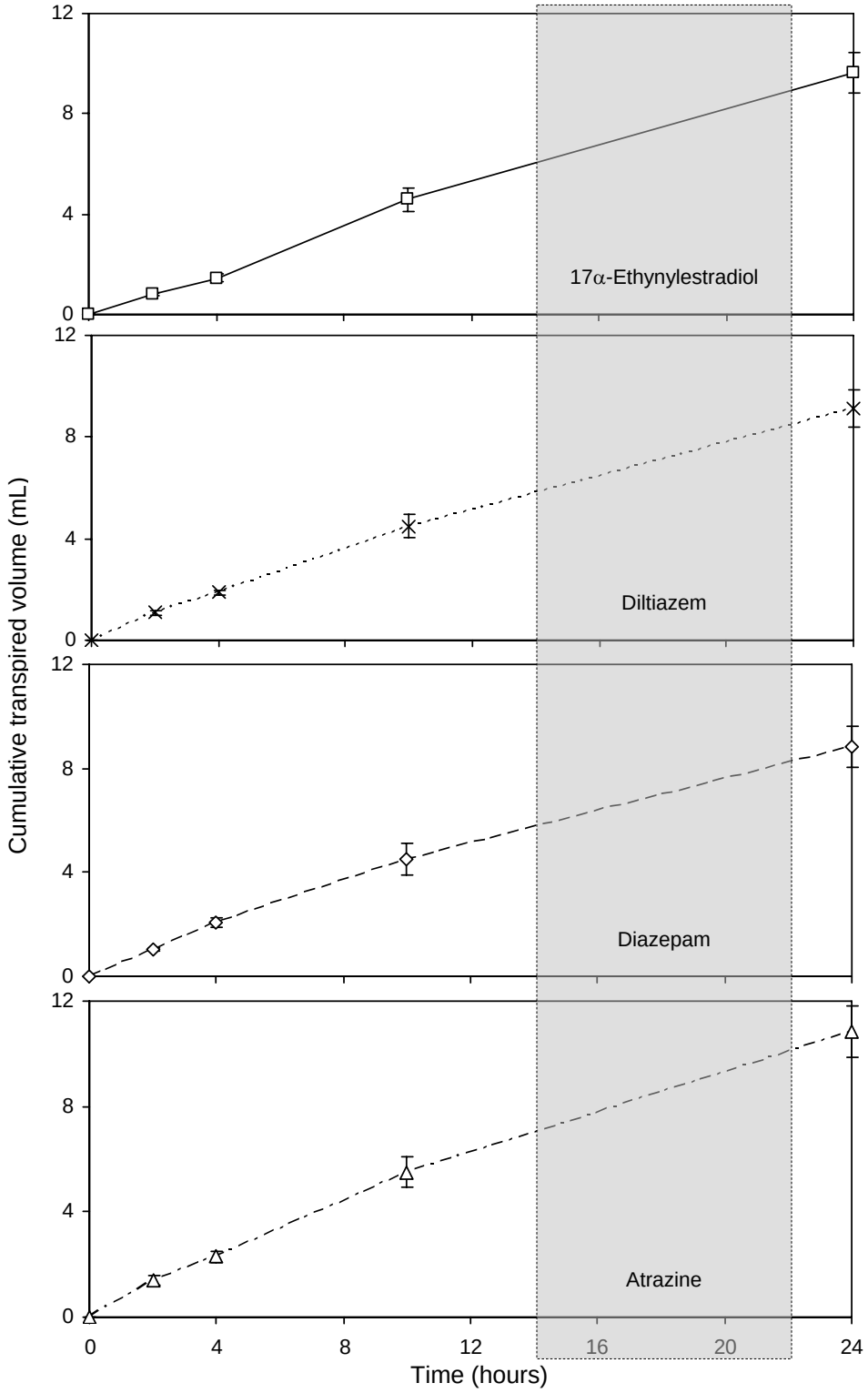

Figure 3.3. Cumulative transpired volumes measured for *Arabidopsis* at each sampling time for the four compounds. Mean ± SE are shown. The 16 hr day and 8 hr night (shaded) are indicated.

3.3.2 Distribution

Plant components underwent solvent extraction to retrieve the soluble fraction, and the remaining residue was oxidized to analyze the bound fraction. Recovery levels varied across the compounds. Final percent recoveries for EE2, DTZ, DZP and ATZ were 90, 55, 35, and 38%, respectively (Figures 3.4 – 3.9). Values for root and shoot are combined extraction methods; soluble, bound and whole plant oxidation. Note that the value of n for ATZ, DTZ, and DZP have changed from those shown in Figures 3.2 and 3.3 and Tables 3.1, A3.1 – A3.5, as the original methods resulted in unreliable values for recovery for some of the replicate plants (See Appendix B for discussion on methods changes).

Distribution ratios for *Arabidopsis*, root:shoot, from combined results (soluble solvent extract, bound residue oxidation and whole plant oxidation) were: EE2, 19:1; DTZ, 4:3 (1.3:1); DZP, 1:10; and ATZ, 1:10. Separate results for root:shoot ratios were EE2, 15.5:1 (soluble + bound) and 20:1 (oxidized whole plant); DTZ, 2:1 (soluble + bound) and 1:1.4 (oxidized whole plant); DZP, 1.2 (soluble + bound) and 1:2.5 (oxidized whole plant); ATZ, 1:9 (soluble + bound) and 1:9 (oxidized whole plant).

As recovery was varied and relatively low for the 4 compounds, but consistency with distribution of recovered radioactivity among replicates was evident, the data is presented both ways; as percentage recovery and as distribution of recovered radioactivity (Figures 3.6 – 3.9). The consistency within distribution of recovered radioactivity among replicates allows for discerning of trends that might otherwise not be as evident.

EE2 had the highest recovery levels and most consistent proportion distribution of recovered (due to high recovery). EE2 became largely bound within the root, with greater than 80%, with a small portion remaining soluble in the root. Shoot soluble fractions did occur and were approximate in proportion to shoot bound fractions, making up less than 10% of recovered radioactivity (Figures 3.5 and 3.6). DTZ remained primarily soluble within the root, making up approximately 50% of recovered radioactivity. A similar proportion moved into the shoots and also remained soluble as less than 45% of recovered. Root and shoot bound fractions were comparatively small, but did occur. The highest degree of variability in recovery was observed with DTZ, which extended into the proportion distribution of recovered (Figures 3.5 and 3.7).

DZP was found in the largest proportion in the shoot as a soluble form, greater than 60% of recovered radioactivity. Bound fractions in both the root and shoot did occur, but in comparatively small proportions. The root soluble fraction made up the second largest fraction with less than 40% (Figures 3.5 and 3.8). ATZ did not become extensively bound to root or shoot components, with the largest portion remaining soluble in the shoots, making up over 90% of the recovered radioactivity (Figures 3.5 and 3.9).

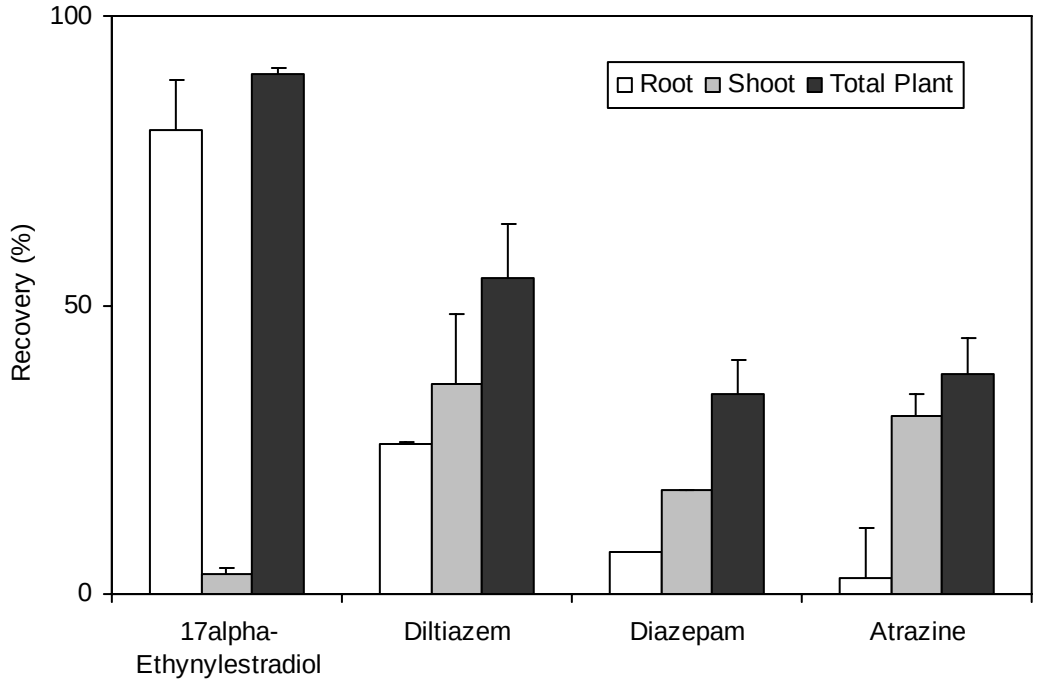

Figure 3.4. Distribution of recovered radioactivity from oxidation of whole root and shoot and total plant recovery for radiolabeled compound added to *Arabidopsis* (mean ± SE). For 17α-ethynylestradiol Root and Shoot, n = 6; diltiazem, n = 2; diazepam, n = 1; atrazine, n = 2. For Total Plant, 17α-ethynylestradiol, n = 10; diltiazem, n = 4; diazepam, n = 5; atrazine, n = 3.

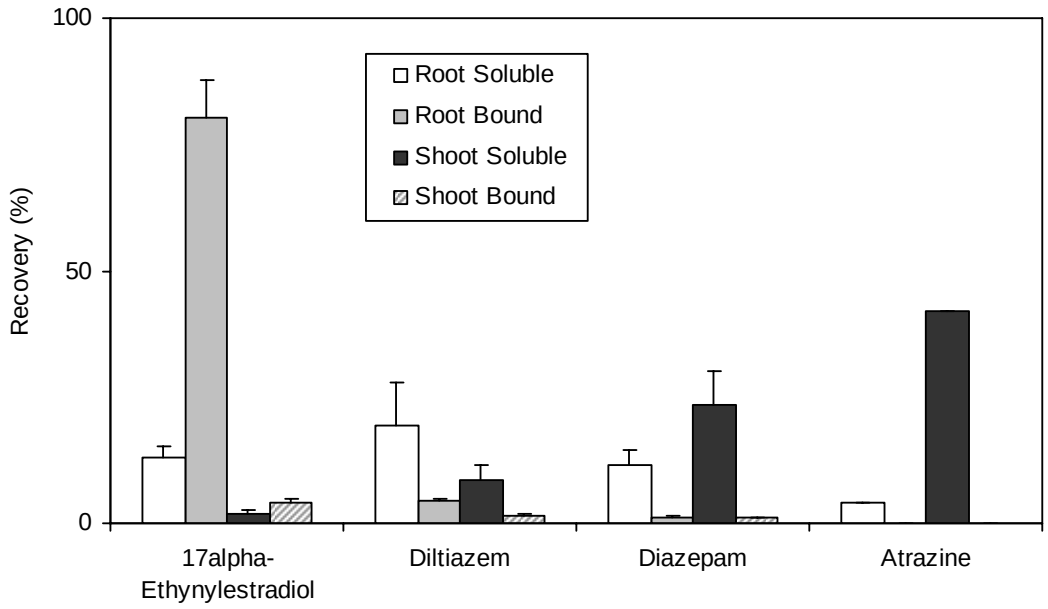

Figure 3.5. Distribution of recovered radioactivity among soluble and bound fractions in roots and shoots for radiolabeled compound added to *Arabidopsis* (mean ± SE). For 17α-ethynylestradiol, n = 4 soluble + bound; diltiazem, n = 2 soluble + bound; diazepam, n = 4 soluble + bound; atrazine, n = 1 soluble + bound.

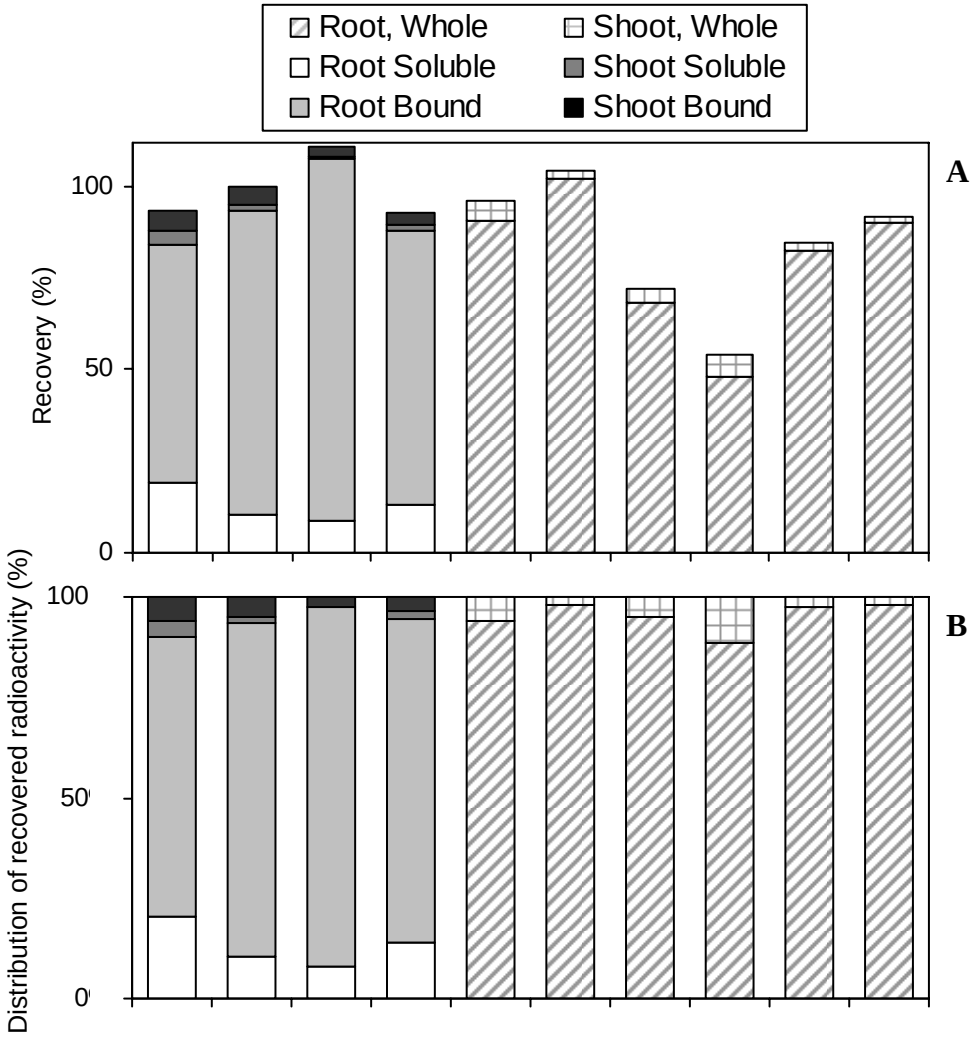

Figure 3.6. *Arabidopsis* individual replicate plant percent recovery of radioactivity for the synthetic estrogen 17α-ethynylestradiol (n = 10). A, actual percent recovered radioactivity for root and shoot components; B, percent distribution of recovered radioactivity from A. Note y-axis exceeds 100%.

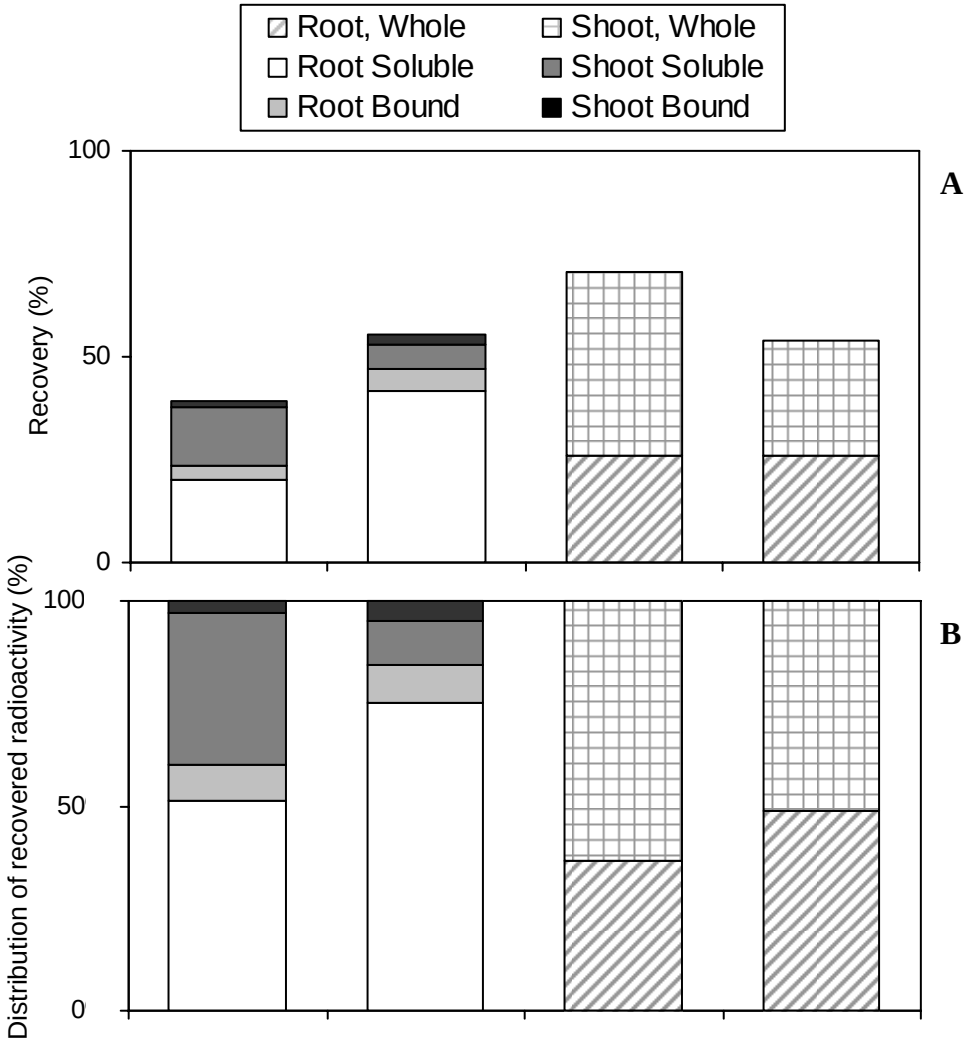

Figure 3.7. *Arabidopsis* individual replicate plant percent recovery of radioactivity for the anti-hypertensive diltiazem (n = 4). A, actual percent recovered radioactivity for root and shoot components; B, percent distribution of recovered radioactivity from A.

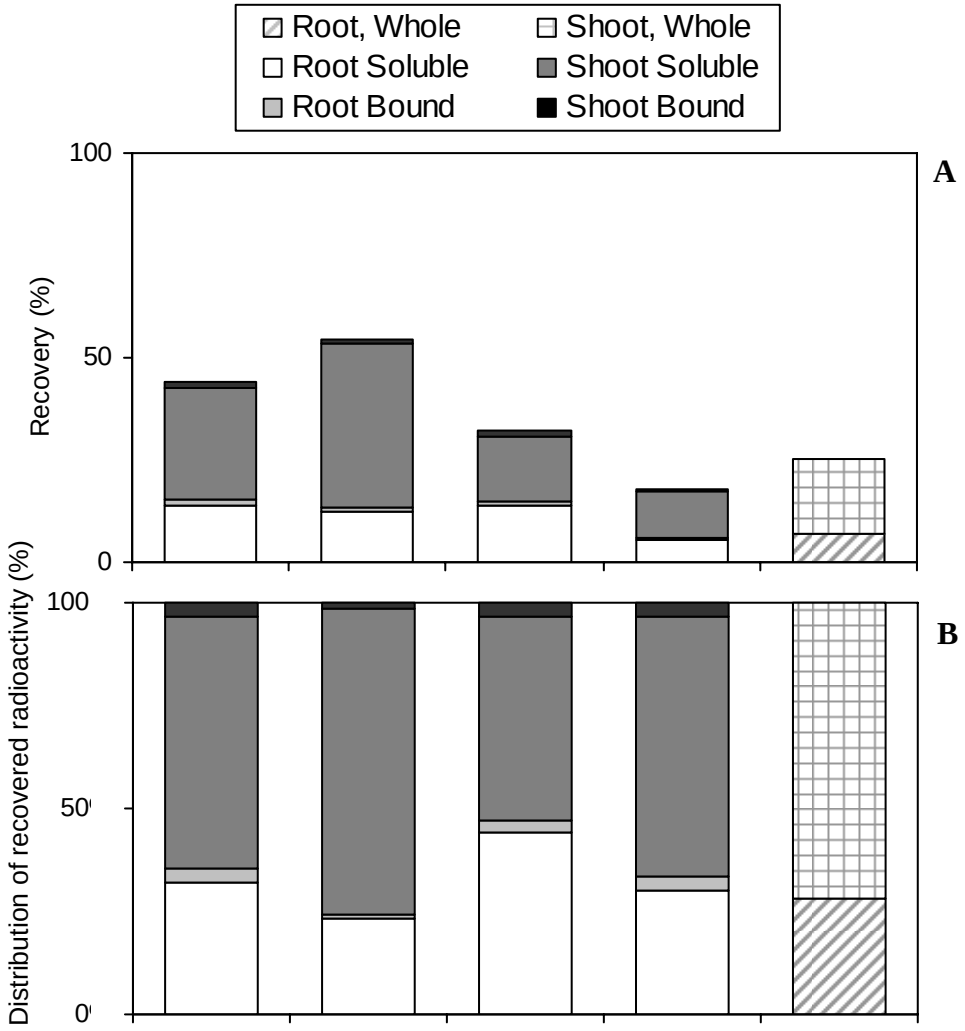

Figure 3.8. *Arabidopsis* individual replicate plant percent recovery of radioactivity for the anti-anxiety drug diazepam (n = 5). A, actual percent recovered radioactivity for root and shoot components; B, percent distribution of recovered radioactivity from A.

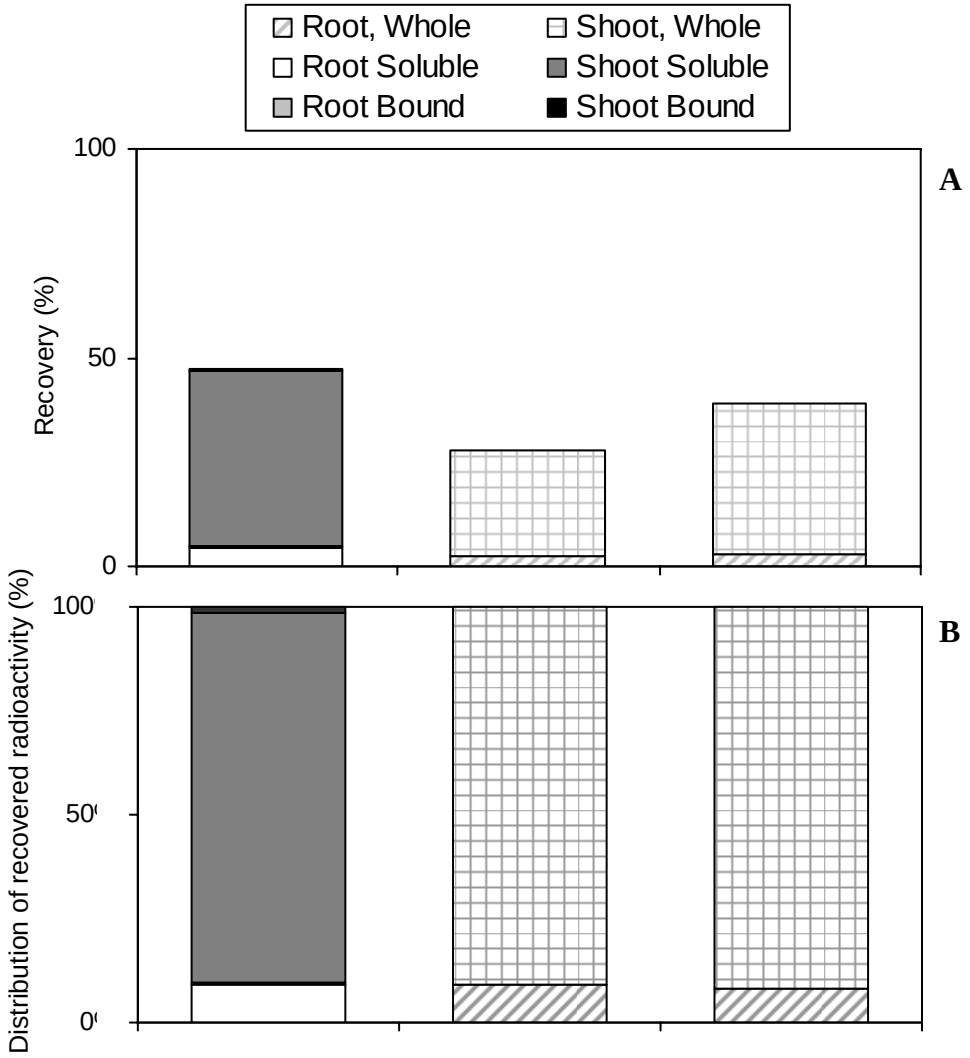

Figure 3.9. *Arabidopsis* individual replicate plant percent recovery of radioactivity for the herbicide atrazine (n = 3). A, actual percent recovered radioactivity for root and shoot components; B, percent distribution of recovered radioactivity from A.

3.3.3 Root concentration factor and transpiration stream concentration factor

Root concentration factors (RCF) for the four compounds and *Arabidopsis* were estimated using results from the whole plant uptake studies and the equation (Shone and Wood, 1974):

root uptake = RCF (μg/mL) x root mass (g) x external solution concentration (μg/mL).

Estimated RCF values were not significantly different when calculated using percent recovery or percent distribution of recovered (t-test, $p>0.05$), therefore RCF was calculated using percent recovery (t-tests RCF, EE2, $t = -0.663$, $df = 9$, $p = 0.524$; DZP, $t = -2.269$, $df = 4$, $p = 0.086$; DTZ, $t = -0.898$, $df = 4$, $p = 0.420$; ATZ, $t = -2.174$, $df = 4$, $p = 0.095$).

The equation developed from these calculations and using percent recovery, with an $R^2 = 0.802$, for *Arabidopsis* RCF was (Figure 3.10):

$\log (RCF) = 1.38 \log K_{ow} - 2.71$.

Also presented are the RCF values obtained from Briggs et al. (1982) and Burken and Schnoor (1998) equations.

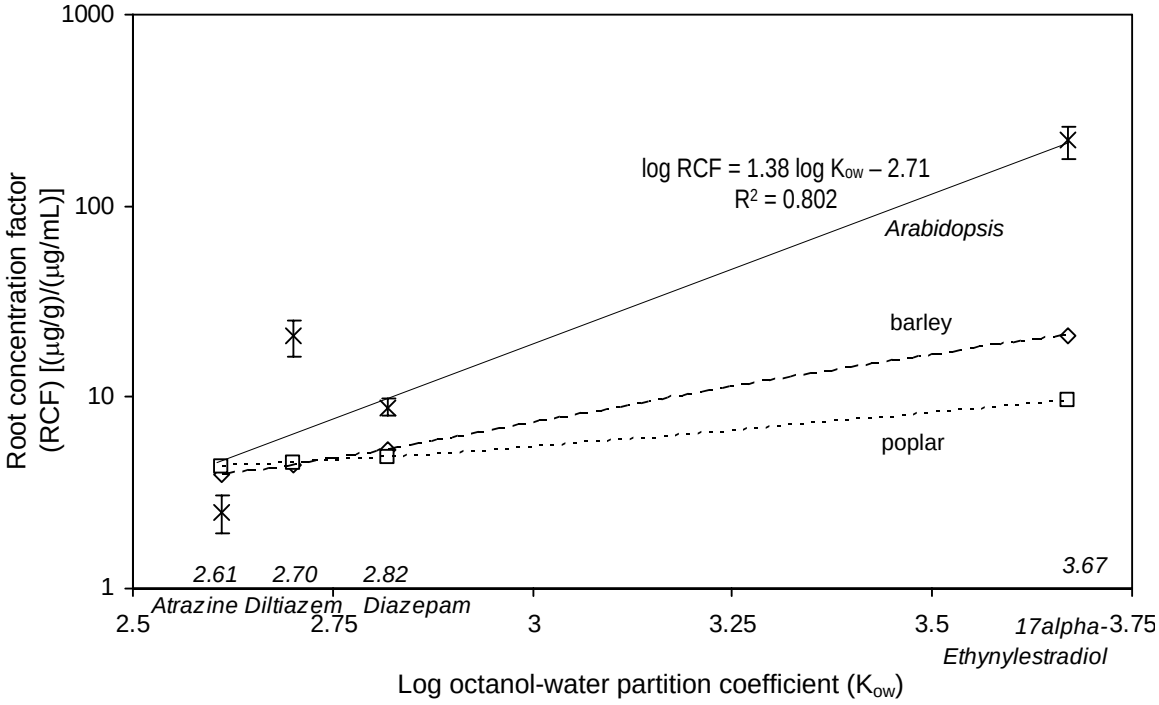

Figure 3.10. Calculated root concentration factor values (mean ± SE) for *Arabidopsis*. Values also calculated using compounds' log octanol-water partition coefficients and the equations for barley roots (Briggs et al., 1982) and hybrid poplar roots (Burken and Schnoor, 1998).

Transpiration stream concentration factors (TSCF) for the four compounds in *Arabidopsis* were estimated using results from whole plant uptake studies and the equation (Briggs et al., 1982):

T**SCF = [concentration in the shoot (μg) / volume transpired (mL)) / [(external solution concentration (μg/mL)$_{initial}$ + external solution concentration (μg/mL)$_{final}$)/ 2]**.

Estimated TSCF values were not significantly different when calculated using percent recovery or percent distribution of recovered (t-test, $p>0.05$), therefore TSCF values were calculated using percent recovery (t-tests TSCF, EE2, $t = 1.367$, $df = 9$, $p = 0.205$; DZP, $t = -2.450$, $df = 5$, $p = 0.058$; DTZ, $t = -0.862$, $df = 4$, $p = 0.437$; ATZ, $t = -1.365$, $df = 4$, $p = 0.244$).

The equation developed from these calculations using percent recovery, with an $R^2 = 0.997$, for *Arabidopsis* TSCF was (Figure 3.11):

$\log TSCF = 0.966 \exp[-(\log K_{ow} - 2.63)^2 / 0.694]$.

Also presented are the TSCF values calculated using the equations developed by Briggs et al. (1982) and Burken and Schnoor (1998).

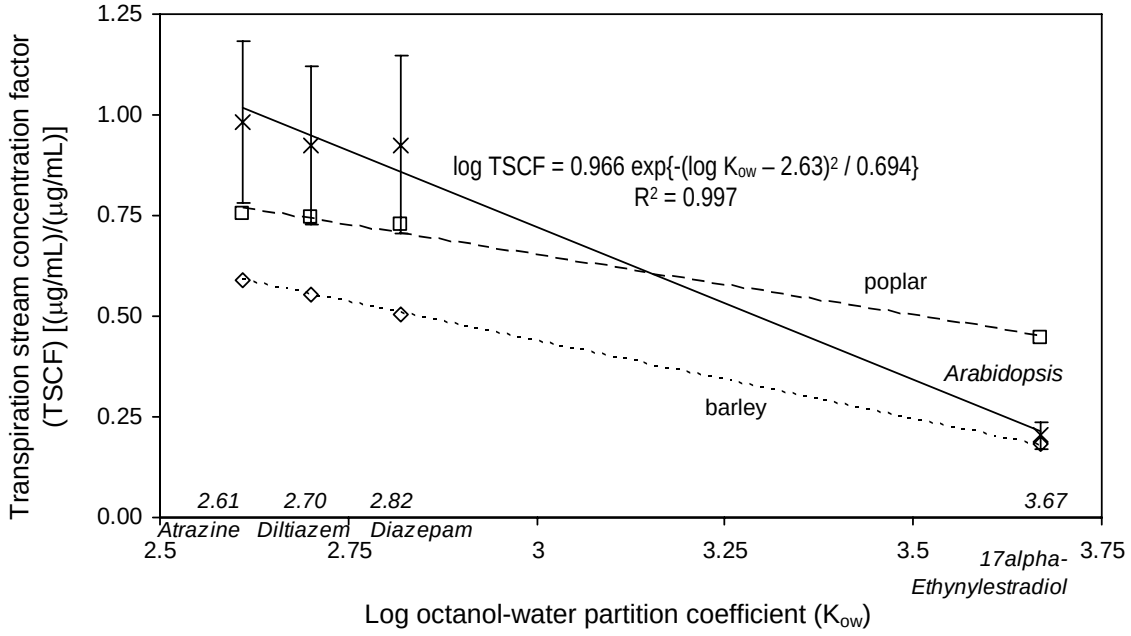

Figure 3.11. Calculated transpiration stream concentration factor values (mean ± SE) for *Arabidopsis*. Values also calculated using compounds' log octanol-water partition coefficients and the equations for barley shoots (Briggs et al., 1982) and hybrid poplar shoots (Burken and Schnoor, 1998).

3.4 DISCUSSION

Uptake. *Arabidopsis thaliana*, the international model plant for scientific research, was examined for its ability to remove trace levels of three pharmaceuticals, EE2, DZP, and DTZ, and the herbicide ATZ, from solution. *Arabidopsis* was effective at removing EE2, DZP and DTZ in a period of 24 hours with removal at 85, 59 and 57%, respectively. The herbicide was employed as a positive control as it is known to be readily taken up by plants. *Arabidopsis* removed 52% of ATZ within the 24 hour period.

Comparisons among compounds are possible due to the similar fresh weights and transpiration volumes among replicate plants across compounds (Table 3.1 and A3.1), as uptake is primarily a function of root mass.

Uptake curves for DTZ, ATZ, and DZP follow a very similar pattern and maximum values at 24 hours (Figure 3.2). EE2 was removed from solution more quickly and at a greater amount than the other 3 compounds (Figure 3.2). Similar uptake observed between DZP, DTZ and ATZ is likely a function of the compounds similar log K_{ow} values (with a range of 2.61 – 2.82) and the rates of transpiration, weights of plants (Table 3.1 and A3.1) and lipid content of the plants used. EE2 has a log K_{ow} of 3.67 which correlates to a greater uptake amount after 24 hours (Figure 3.2). The rate of uptake for EE2 may have slowed due to the decrease in external solution concentration to near 25% remaining within the first 2 hours, effectively reducing the available compound to a minimum. As well, hormones and hormone mimics are actively taken up from solution by plants (Geuns, 1978; Bhattacharya and Gupta, 1981; Hayat et al., 2001;

Janeczko et al., 2003), suggesting the efficient uptake observed for EE2 could partially be due to a role of active uptake.

Comparing final uptake values between compounds, they are consistent with predicted behaviour based on the compound's logarithm octanol-water partitioning coefficient (Log K_{ow}). In order of highest to lowest log K_{ow} values and greatest to lowest uptake: EE2 >> DZP > DTZ > ATZ. Generally, a larger octanol-water partitioning coefficient would reflect a compound's preference or attraction for an organic phase over water. Therefore, a larger log K_{ow} value would suggest the compound is sorbing to root cells at a faster rate than a compound with a lower log K_{ow} value. This process is reflected in the distribution of the different compounds; EE2, with its approximate 10-fold larger K_{ow}, has a greater preference to the root cells than the other 3 compounds and is reflected in the uptake curve (Figure 3.2 and 3.6).

The lack of correlation between plant weights, transpiration volumes and uptake is likely due to the limited range of *Arabidopsis* weights used for the replicates for each compound and across compounds. It would be expected that there would be a positive relationship between plant weight and uptake, as well as transpiration and uptake if the compound moved relatively freely into the transpiration stream. The relationship between uptake and plant mass/volume is a positive one, as increasing the amount of root area to sorb to is expected to increase the amount taken up. As well, if a larger volume was moving within the transpiration stream, it would suggest a larger carrier base for freely moving compounds to the shoot.

Transpiration is expected to be correlated to shoot mass (Ray and Sinclair, 1998), but no correlation between transpired volume and plant weight (Table A3.5; Spearman's rho, $p > 0.05$) was observed for these experiments except for EE2 (Table A3.5; Spearman's rho, $p < 0.05$), which is likely a function of a larger sample size ($n = 10$) and a wider range of weights across replicates than for the other compounds (Table 3.1).

No plants showed signs of phytotoxicity, during the period of study and at the concentrations used, as measured by discoloration of the leaves or significant alteration in transpiration rates (Trapp et al., 2000) (Figure 3.3, Tables 3.1, A3.1 and A3.2). As ATZ is an herbicide, there is the potential for an observable effect, but none were evident for the period of the study. As well, DTZ, the calcium-channel blocker, could have had an effect if it was acting upon the plant's calcium-channels.

Distribution ratios for *Arabidopsis*, root:shoot, from combined results (soluble solvent extract, bound residue oxidation and whole plant oxidation) were EE2, 19:1; DTZ, 4:3 (1.3:1); DZP, 1:10; and ATZ, 1:10. Separate results for root:shoot ratios, comparing oxidized whole plant and soluble + bound fractions were very similar to the combined results listed above.

Comparing the proportions of distributed radioactivity across compounds, they again follow the order of the compounds' log K_{ow} values, with the highest recovered proportion in the root coinciding with the largest log K_{ow} and vice versa, except for DTZ and DZP

which are switched in their expected order for proportions within the root (Figure 3.4). Movement into the shoot followed the order of the compounds' log K_{ow} values with the lowest values coinciding with the largest proportion of recovered radioactivity within the shoot. ATZ is designed as an herbicide that acts on the photosynthetic chain within pigmented plant cells, therefore it would be expected to be transported to the shoots, which it was (Figures 3.5 and 3.6). The synthetic estrogen EE2 was strongly sorbed to the roots, and taken up at a greater amount than the other compounds (Figure 3.3, 3.5 and 3.9) with the potential for some of this uptake to be accounted for active uptake of hormones by plants (Geuns, 1978; Bhattacharya and Gupta, 1981; Hayat et al., 2001; Janeczko et al., 2003). DTZ and DZP did not appear to induce any observable effects and behaved as predicted from their log K_{ow} values for both uptake and distribution.

Examination of the distribution of total recovered radioactivity shows consistency across replicates, suggesting the oxidation process is variable in terms of recovery, but it is not understood why. Each replicate shows consistent proportions in distribution likely due to their oxidation in sequence, i.e. replicate A's root and shoot were oxidized before moving onto the next replicate, possibly allowing for the same margin of error in each replicate, but not across the replicates. Oxidizer may not be the sole problem of poor recoveries, but it is not understood what other factors are contributing, particularly when EE2 recoveries were 90% and the others were below 55%.

Calculations of RCF and TSCF values for *Arabidopsis* derived using the percentage of radioactivity within the roots and the shoots from whole plant uptake studies. This

proposed method was suggested by Briggs et al. (1982) and found to result in values similar to those determined experimentally when using excised roots. Similar results may be represented here for *Arabidopsis*. RCF and TSCF are typically calculated taking into account degradation of the compound (Thompson et al., 1998), but as this was not determined for these studies RCF and TSCF were calculated assuming no degradation of the compound with all recovered radioactivity in parent form.

For comparison, RCF values and TSCF values were calculated two ways. One method was using the percentage recovery within the roots and shoots, including soluble and bound fractions. And the other method: as percent recovery was relatively low but percent distribution of recovered radioactivity was consistent across replicates, an assumption was made that the percent distribution of recovered radioactivity was accurate. The total uptake radioactivity was therefore multiplied by the fraction of radioactivity expected to be within the root or shoot based on the root and shoot distribution fraction of recovered radioactivity.

Estimated RCF and TSCF values were not significantly different when calculated using percent recovery or percent distribution of recovered (t-test, $p>0.05$), therefore RCF and TSCF values were calculated from percent recovery. Determined equations for *Arabidopsis* are:

$\log RCF = 1.38 \log K_{ow} - 2.71$,

and **$\log TSCF = 0.966 \exp[-(\log K_{ow} - 2.63)^2 / 0.694]$.**

Arabidopsis RCF values followed a similar trend as was found for barley roots (Briggs et al., 1982) and hybrid poplar roots (Burken and Schnoor, 1998), but were at a steeper slope (Figure 3.10). EE2 RCF values were 10x greater than those predicted using published equations. The trend was similar to other documented relationships for other compounds, therefore neutral pharmaceuticals may follow the same relationships as has been found for other compounds based on their log K_{ow} values.

TSCF values followed a similar trend as other documented relationships, but were approximately 1.2 - 1.5x larger for ATZ, DTZ and DZP with values nearing 1. EE2 calculated TSCF was similar to those predicted by Briggs et al. (1982) and Burken and Schnoor's (1998) equations (Figure 3.11). TSCF values of 1 imply passive uptake following the transpiration stream. Values < 1 suggest the compounds are not easily moved into the stream, and values > 1 suggest active uptake into the transpiration stream, such as for nutrients K, P, and N (Orchard et al., 2000; Dietz and Schnoor, 2001). The relationship between TSCF and log K_{ow} is expressed as a bell shaped curve, with maximum uptake corresponding to log K_{ow} values around 2, but this value varies with the plant of study. The log K_{ow} values used in this experiment ranged from 2.61 to 3.67, and therefore only the decreasing half of the bell is represented and the maximum uptake log K_{ow} could not be discerned for *Arabidopsis*.

The studies for barley (Briggs et al., 1982) and hybrid poplar (Burken and Schnoor, 1998) involved multiple chemicals with a range of log K_{ow} values enabling the determination of optimum uptake. Optimum uptake is suggested to occur with moderately hydrophobic compounds (log K_{ow} = 1.0 – 3.5) (Briggs et al., 1982; Burken and Schnoor, 1998), with values greater than 3 being highly sorbed to roots. The range of log K_{ow} values used in this study was 2.61 – 3.67. Uptake from solution and movement into the transpiration stream followed this suggested optimum range for uptake for *Arabidopsis*. EE2, with a log K_{ow} greater than 3, was highly sorbed to roots.

Conclusions. *Arabidopsis* was effective at uptake of 3 pharmaceuticals EE2, DZP and DTZ from solution in a period of 24 hours, with percent removal of 85, 59 and 57%, respectively. The synthetic estrogen EE2 was removed at the greatest amount, with most of the compound becoming bound to the roots and little transport to shoots, during the 24 hour period. The anti-anxiety drug DZP was removed from solution at the second greatest amount remained primarily soluble with over half of the taken up compound being transported to the shoots. DTZ, the anti-hypertensive, and also remained primarily soluble with over half recovered within the root. The positive control, ATZ, was also taken up by *Arabidopsis*, with removal at 52% and distributed mostly to the shoots as a soluble fraction, with minimal binding within roots or shoots. As many enzymes and genes of *Arabidopsis* are known, the potential exists for predicting the degradation pathways within plants.

Predictive relationships between pharmaceuticals' physio-chemical property, octanol-water partitioning coefficient, and *Arabidopsis* roots and shoots were developed and found to be similar to previously reported relationships for other compounds and hybrid poplar and barley roots. This suggests that pharmaceuticals behave as other chemicals, allowing for predictive behaviour in the environment and in plants.

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CHAPTER 4 Method for detection of 17α-ethynylestradiol in surface water

4.1 INTRODUCTION

The synthetic hormone 17α-ethynylestradiol (EE2) is a known, potent endocrine disrupting compound (EDC) and is a key contributor to the levels of estrogen and endocrine disruptors found within wastewater (Desbrow et al., 1998). Small concentrations of this hormone have been known to induce endocrine disruption effects in male trout (Routledge et al., 1998). Levels as low as 0.1 ng/ L have been reported to induce vitellogenin (precursor to yolk, a female-specific protein) production in male fish, as well as other sexual dysfunctions in fish (Purdom et al., 1994). Mammalian hormones that are also found within waste and surface waters are estrone (E1) and estradiol (E2). Although not as potent as EE2, E1 and E2 are known EDC and are typically found in higher concentrations than EE2 in waste and surface waters (Desbrow et al., 1998). Across Canada, mean concentrations of E2 and E1 in wastewater influent were 15.6 ng/ L and 49 ng/ L, and in final effluent they were reduced to 1.8 ng/ L and 17 ng/ L, respectively (Servos et al., 2005).

Reports of EE2 in the environment and wastewater have occurred around the world. A nationwide survey within the United States detected a maximum level of 83.1 ng/ L and a median level of 7.3 ng/ L of EE2 in streams (Kolpin et al., 2002). In Germany and the U.K. levels of up to 17 ng/ L and 7 ng/ L, respectively, of EE2 have been reported in wastewater effluent (Belfroid et al., 1999). Cargouët et al., (2004) reported EE2 levels ranging from 1.0 to 3.2 ng/ L in wastewater treatment plant effluent and river surface

waters of the Paris area, France. Coastal surface waters of the German Baltic Sea were examined for levels of estrogens, with levels of EE2 detected in a populated bay at 3.0 ng/ L in 2003 and 17.2 ng/ L in 2000 (Beck et al., 2005). Trace levels of EE2 have been reported in the Waikato region of New Zealand (Sarmah et al., 2006). Not only is EE2 being detected in waste and surface waters, but also in drinking water. In 2001, Kuch and Ballschmiter reported levels of up to 0.5 ng/ L of EE2 in tap water from a region of southern Germany that obtains its drinking water from a ground source.

Increasing concern over this compound, and other hormones and endocrine disrupting compounds, has led to the development of methods of detection in wastewater influent and effluent, as well as surface waters. Interest in the presence of this synthetic hormone in local waters led to the development of a method for detection of EE2 specifically using gas chromatography-mass spectrometry (GC-MS). GC-MS is a commonly used method for the detection of pharmaceuticals and hormones in water and entails a relatively simple stepped process of purification involving solid phase extraction (SPE) with C18 and silica gel, and compound preparation involving derivatization, prior to GC-MS analysis (Kelly, 2000; Kuch and Ballschmiter, 2000; Mol et al., 2000; Jeannot et al., 2002; Hernando et al., 2004; Shareef et al., 2004).

Silylation and GC-MS can result in the degradation of EE2 into E1 and E2 if performed improperly. This was not previously reported in the literature at the time of this method development. Recovery levels of EE2 after improper silylation or GC-MS analysis has the potential to result in low recovery levels of EE2. This degradation was noted during

this method development and a procedure was found that resulted in negligible degradation. Improper preparation of EE2 is particularly a concern when a composite detection method is employed, where more than one hormone is being detected in one sample, leading to elevated levels of estrone and possibly undetectable levels of EE2. This led to attempts at developing a method for the sole detection of EE2 to insure minimal degradation. The method outlined here minimizes the degradation of EE2 into E1 and optimizes the percentage of completely trimethylsilylated (TMS), di-TMS, versus partially trimethylsilylated, mono-TMS, derivatives of EE2 for GC-MS analysis.

A method for detection and measurement of EE2 in river water was developed and initially tested using river water samples and EE2 standard. Recoveries were low during this procedure, possibly due to binding of EE2 to particulate matter resulting in losses during clean-up steps. Improvement of this method was not concluded due to a publication by Alberta Environment in late 2005 with preliminary analyses of the Oldman River water for pharmaceuticals, including EE2, and other common wastewater compounds (Sosiak and Hebben, 2005). This report found no detectable levels of EE2 within the Oldman River downstream of the City of Lethbridge wastewater treatment plant.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

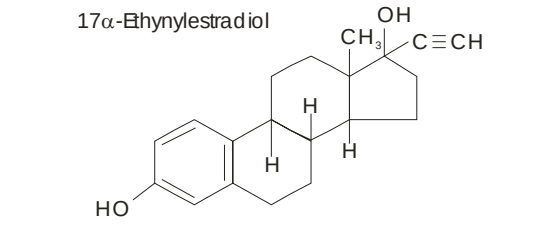
Tritium-labeled ethynylestradiol, 17α-[6,7-^{3}H(N)]-ethynylestradiol (^{3}H-EE2) (specific activity: 40.0 Ci mmol^{-1}; radiochemical purity >97% to HPLC analysis), was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Unlabeled EE2 was obtained from Sigma-Aldrich Canada Ltd. The chemical purity of EE2 was verified by nuclear magnetic resonance (NMR) analysis (performed by Dr. Peter Dibble, Chemistry Department, University of Lethbridge. See Appendix C for NMR results). The chemical structure of EE2 is presented in Figure 4.1. Inclusion of ^{3}H-EE2 was for tracing and quantification purposes during the purification steps.

An internal standard was chosen that had a similar retention time (RT) and chemical structure as EE2. The internal standard (IS), deuterated-17β-estradiol, (d_2-E2) (98 atom % D), was obtained from Sigma-Aldrich Canada Ltd. The chemical structure is presented in Figure 4.1. d_2-E2 was chosen as an IS as levels of E2 within the surface water were not known and may have been present. d_2-E2 would also serve as a quantitative standard for any E2 present.

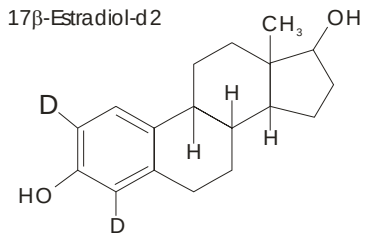
The silylation reagent, BSTFA (*N*,*O*-bis(Trimethylsilyl)trifluoroacetamide) with 1% TMCS (Trimethylchlorosilane) and the solvent pyridine were used for creation of trimethylsilyl (TMS) derivatives of the EE2 and d_2-E2 (Pierce Biotechnology, Inc., Rockford, IL, USA).

All standard solutions were prepared in methanol due to poor solubility in water. Ethyl acetate was found to be a suitable solvent for transfer to GC-MS.

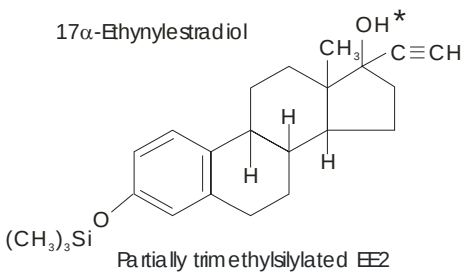
Solid phase extraction used octadecyl-functionalized silica gel (C_{18}) (Sigma-Aldrich Co.) and activated silica gel, 32-100 μm (Scientific Adsorbents Incorporated).



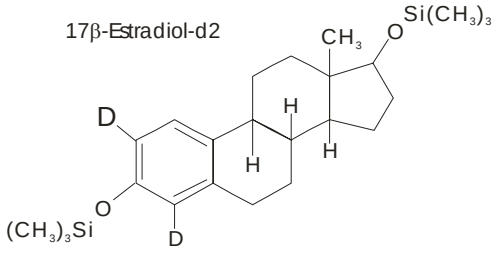
17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17
-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol



1,3,5[10]-estratriene-2,4-d_2-3,17β-diol



Partially trimethylsilylated EE2



Completely trimethylsilylated E2-d2

Figure 4.1. Chemical structures of 17α-ethynylestradiol (EE2) and the IS 17β-estradiol-d2 (d_2-E2) and their trimethylsilylated derivatives. The structure of partially trimethylsilylated EE2 is shown. The asterisk denotes other possible binding site for the TMS group on EE2. For partial TMS derivatives, either site may be used. Completely trimethylsilylated EE2 would involve TMS binding to both of the hydroxyl oxygens. Completely trimethylsilylated d_2-E2 is shown.

4.2.2 Trimethylsilyl derivatives

Silylation derivatives, replacement of a hydroxyl hydrogen with a trimethylsilyl (TMS) group ($Si(CH_3)_3$), were created in 3 mL reacti-vials. Addition of pyridine in a 1:1 volume ratio with the silylation reagent aided derivative formation. Figure 4.1 presents the chemical structures of the TMS derivatives of EE2 and d_2-E2.

A test standard of EE2 was prepared and dried down under nitrogen and low heat (35 °C). The vial was then removed from heat, 15 μL each of pyridine and BSTFA+1%TMCS were added, the vial sealed, and heated to 60 °C for 30 minutes. The derivatives were removed from the heat and allowed to cool to room temperature for 2 hours prior to GC-MS analysis.

Initial GC-MS analysis was conducted with injection of the silylation reagents on-column using the parameters described below. This method resulted in chromatograms containing the derivatives of EE2 and also, unexpectedly, of E1. Figure 4.2 presents the chemical structures of E1 and of TMS-E1 derivative. Discussion with Dr. P. Dibble suggested the fluorine present in BSTFA is a reactive species capable of facilitating the breakdown of EE2 into E1 during GC-MS analysis (personal communication with Dr. P. Dibble, Chemistry Department, University of Lethbridge). Consequent removal of this reagent as the GC-MS injection solution assisted in negligible breakdown of EE2 during the GC run. The methods were modified so that the silylated sample was dried under nitrogen and low heat (35 °C) to remove the silylation reagents. Derivative samples were resuspended in 15 μL of ethyl acetate.

Different combinations of temperature and time for creating complete TMS derivatives of EE2 were attempted to determine the optimal conditions. Manufacturer's suggested forcing conditions (150 °C for 12 hours) for complete silylation were also attempted at the early stages of method development, but these conditions were found to be extremely ineffective with degrading effects.

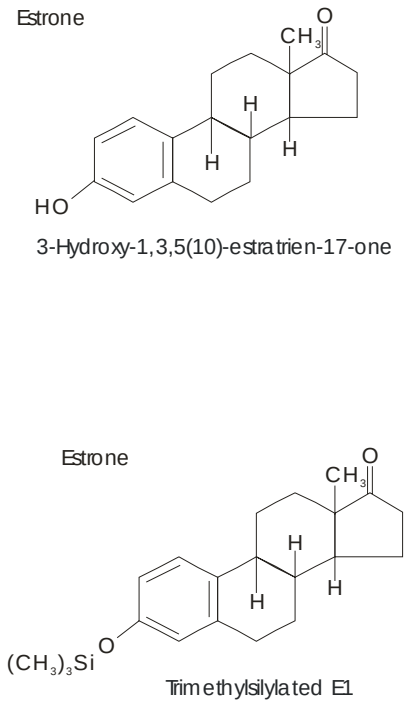


Figure 4.2. The chemical structure of estrone (E1) and of trimethylsilylated E1 is shown.

4.2.3 Gas chromatography-mass spectrometry

GC-MS determination was carried out on a HP5890A gas chromatograph coupled to a HP5970B MSD in the electron ionization mode (EI). Derivative mixtures were injected cold 'on-column.'

Column:	DB5MS (J & W Scientific) 15 m; 0.25 mm i.d.; 0.25 um film thickness; head pressure 4 psi helium
Precolumn:	50 cm; 0.53 um
GC parameters:	60 °C – (0.50 min) - 20 °C/min – 200 °C – 5 °C/min – 300 °C – (5 min)
MS parameters:	transfer line at 280 °C; EI-mode (70 eV)

4.2.4 Calibration curve

For quantification of EE2 by GC-MS a calibration curve for EE2 and d_2-E2 was created using single ion monitoring (SIM). Calibration standards ranged from 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng for EE2 to 10 ng of IS. The EE2 standards and IS were dried down under nitrogen and low heat (35 °C). Silylation was carried out as described in section 4.2.2. Standard mixtures were analyzed by GC-SIM. Characteristic ions and RTs are listed in Table 4.1. Integrated area under the curve for SIM of EE2 and IS characteristic ions at *m/z* 425/418 were used to calculate ratios. The 3 to 4 other characteristic ions for each compound were monitored to confirm identity of the EE2 and d_2-E2, and to check for the presence of E2 and E1.

Table 4.1. SIM monitoring for EE2, E2, d_2-E2, and E1 (TMS derivatives) characteristic ion *m/z* and retention times.

	SIM m/z			
	EE2	*E2*	d_2-E2	*E1*
RT (min)	15:90	14:20	14:57	14:30
Molecular ion	440	416	418	342
	425	401	403	257
	368	326	328	327
	285	344	287	218
		285	346	244

Note: EE2 and E2 both contain *m/z* 285

4.2.5 Preparative purification of Oldman River water

Sample collection and purification. The City of Lethbridge, Alberta, Canada, is located along the Oldman River. This river provides a source for drinking water and wastewater disposal, for not only Lethbridge, but other communities located along the river's reach. River water was collected during the month of July using sterilized containers. The containers were rinsed with river water prior to sample collection to neutralize active binding sites. Water was collected downstream of the drinking water intake diversion, but upstream of the wastewater outflows. The water was stored at 4 °C until its use in the experiments. As this preliminary method was not to detect levels of EE2 within the river water, but to prepare an analytical technique for the detection of hormones in surface waters, no preservative was added to the collected samples used to reduce the degree of microbial degradation (Labadie and Budzinski, 2005). The method outlined here was modified from previously reported methods (Kelly, 2000; Kuch and Ballschmiter, 2000; Jeannot et al., 2002; Hernando et al., 2004; Petrovic et al., 2004).

Initial sample preparation. The water sample was allowed to warm to room temperature and suction filtered to remove large particulates using filter paper (Whatman No.1). Five litres were filtered and the pH adjusted to 8 using a 1% HCl solution.

Solid phase extraction (SPE). Two - 1 g C_{18} columns were prepared in 4 mL glass SPE tubes. The C_{18} was suspended in methanol, the column was packed and a thick, glass microfibre filter (GF/D) circle was gently placed on top of the column (to provide another

layer of filtration, as well as protection for the C_{18} column). The column was conditioned with water.

A solution of EE2 was prepared (16,667 Bq ^{3}H-EE2 + 10 ng EE2 in 10% aqueous methanol as described in section 4.2.2) and divided between the two columns by addition to the top of the column and allowed to move into the C_{18} (gravity movement).

Figure 4.3 provides the schematic diagram of the solid phase extraction system used in this research. The system was sealed and under suction. Two and a half litres were allowed to run through each C_{18} column and collected.

The C_{18} columns were each then eluted with 3 mL 80 % aqueous methanol, the 2 eluants pooled and checked again for radioactivity using a liquid scintillation counter (LSC) to confirm recovery levels prior to the next purification step.

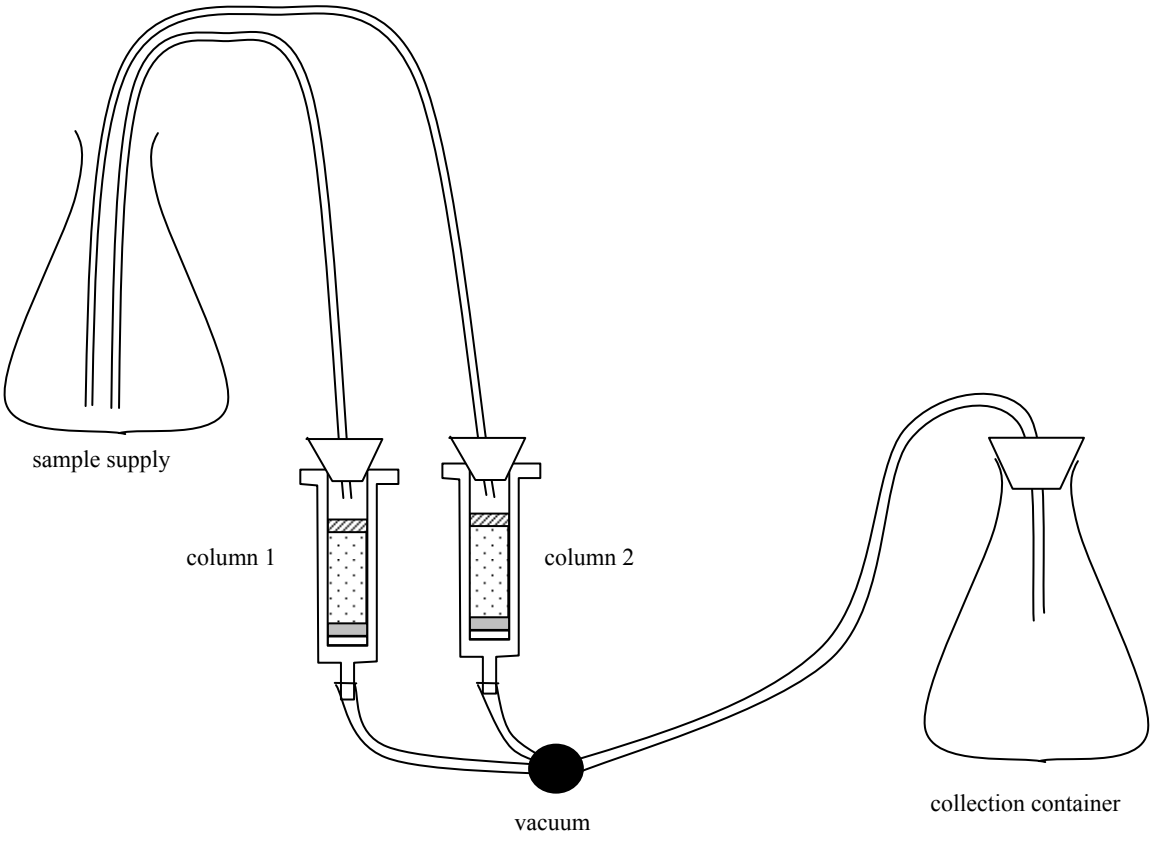


Figure 4.3. Schematic diagram of the solid phase extraction system for surface waters.

A 1-g silica gel column was setup like the C_{18} columns. The silica was equilibrated with hexane. The C_{18} eluant was dried under vacuum and low heat (35 °C). The sample was resuspended in 25 μL ethyl acetate, then 225 μL of hexane was added to create a 90:10 solvent ratio. This was added to the top of the silica column, and the vial rinsed with another 25 μL of ethyl acetate, to resuspend any residue, and the 90:10 solvent ratio created with 225 μL of hexane. Three solvent ratios were found to be sufficient to remove the compounds of interest from the silica column, with the analytes coming off primarily within the second solvent ratio (50:50% hexane : ethyl acetate):

90:10% hexane : ethyl acetate,	6 mL
50:50% hexane : ethyl acetate,	6 mL
100% ethyl acetate,	6 mL

The eluant was dried under partial vacuum and low heat (35 °C), the sample transferred to a 3 mL reacti-vial. At this stage the d_2-E2 was added to serve as a semi-quantitative IS for the subsequent GC-MS step. The combined eluant and IS were dried down under nitrogen and low heat in preparation for silylation (section 4.2.2).

GC-MS analysis. The procedure for GC-MS analysis of the river water samples is described in section 4.2.3. A full scan and an SIM analysis (*m/z* listed in Table 4.1) were performed for the prepared river water samples.

The experiment was carried out twice. The IS d2-E2 was available only for the second trial and was therefore not present in the first trial.

4.3 RESULTS

Trimethylsilyl derivatives and gas chromatography-mass spectrometry. Initial attempts at GC-MS analysis of EE2 showed the presence of estrone (E1) and partial and complete TMS-EE2 derivatives (Figure 4.4). The mass spectra of TMS-E1 and mono- and di-TMS-EE2 are shown in Figure 4.4a and 4.4b.

Temperatures higher than 60 °C resulted in degradation of EE2 into E1 (Table 4.2). Temperature and time of 60 °C for 30 minutes with 1:1 BSTFA+1%TMCS and pyridine ratio and an injection solvent of ethyl acetate, resulted in a high percentage of complete TMS derivatives for both EE2 and the IS, and low degree of degradation (Figure 4.5).

Table 4.2 presents some of the temperature, time and GC-MS injection solution combinations tested for creating TMS derivatives of EE2. Percentages of total area of the TIC peaks for the product of EE2 (E1) and TMS EE2 derivatives are presented.

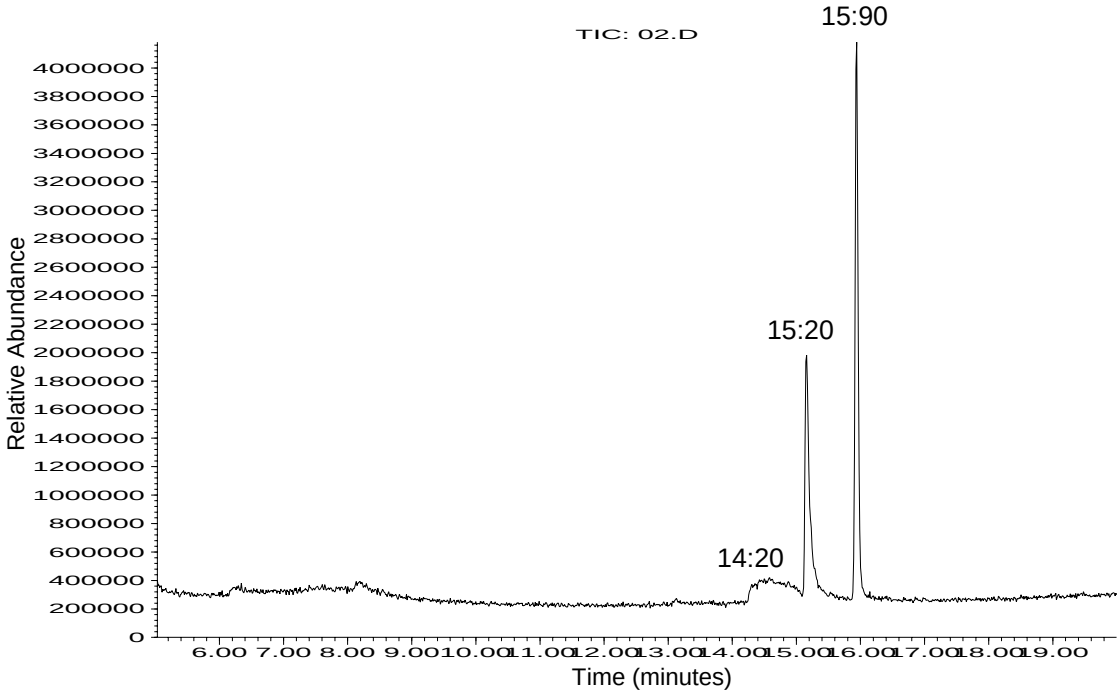


Figure 4.4. TIC of the products of EE2 produced during the silylation process and GC-MS analysis. EE2 was silylated at 100 ° C for 60 minutes and allowed to sit for 2 hours prior to GC-MS analysis in ethyl acetate. RTs: mono-TMS-EE2, 15:20; di-TMS-EE2, 15:90; TMS-E1, spreading peak at 14:20-15:00.

Table 4.2. Results from different temperature and time combinations for silylation of EE2 presented as the percentage of total integrated area for TMS-E1 and mono-/di-TMS-EE2 from the TICs.

Combination	*GC-MS Injection*	*% of Total*		
		E1 TMS	*EE2 mono-TMS*	*EE2 di-TMS*
100 °C, 60 min	Silylation solution	74	17	9
100 °C, 60 min	Ethyl acetate	22	33	45
100 °C, 60 min	Ethyl acetate	23	45	31
100 °C, 30 min	Silylation solution	53	39	8
100 °C, 30 min	Ethyl acetate	36	63	1
68 °C, 30 min	Ethyl acetate	3	28	70
60 °C, 60 min	Ethyl acetate	7	3	90
60 °C, 30 min	Ethyl acetate	0	2	98

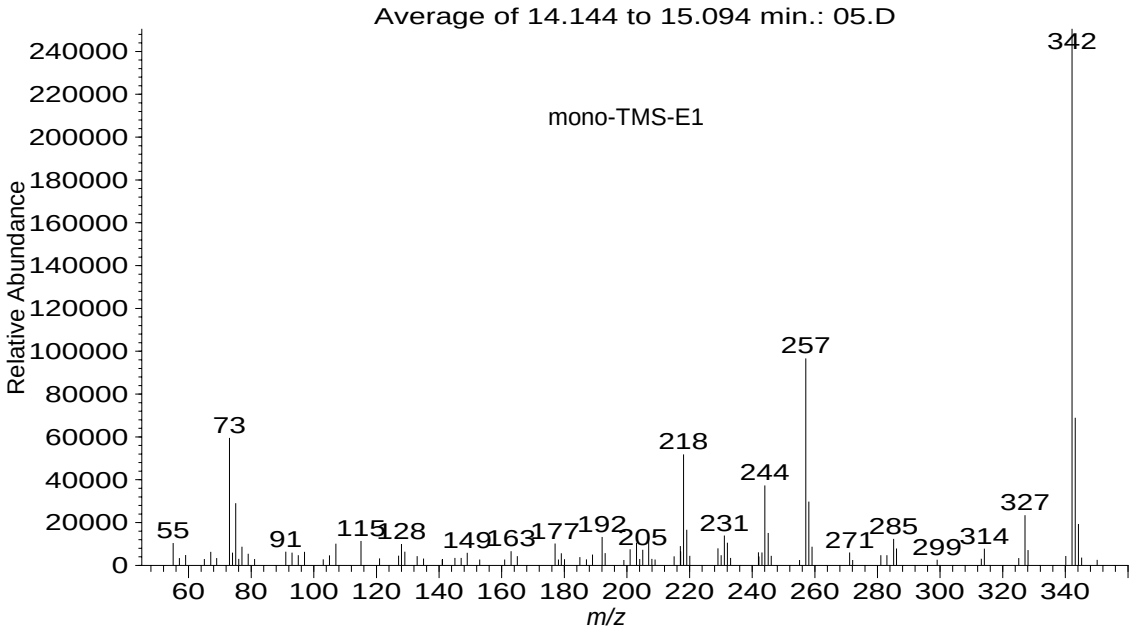


Figure 4.4a. Mass spectrum of TMS-E1 at 14:20-15:00 minutes from Figure 4.4.

Molecular ion 342.

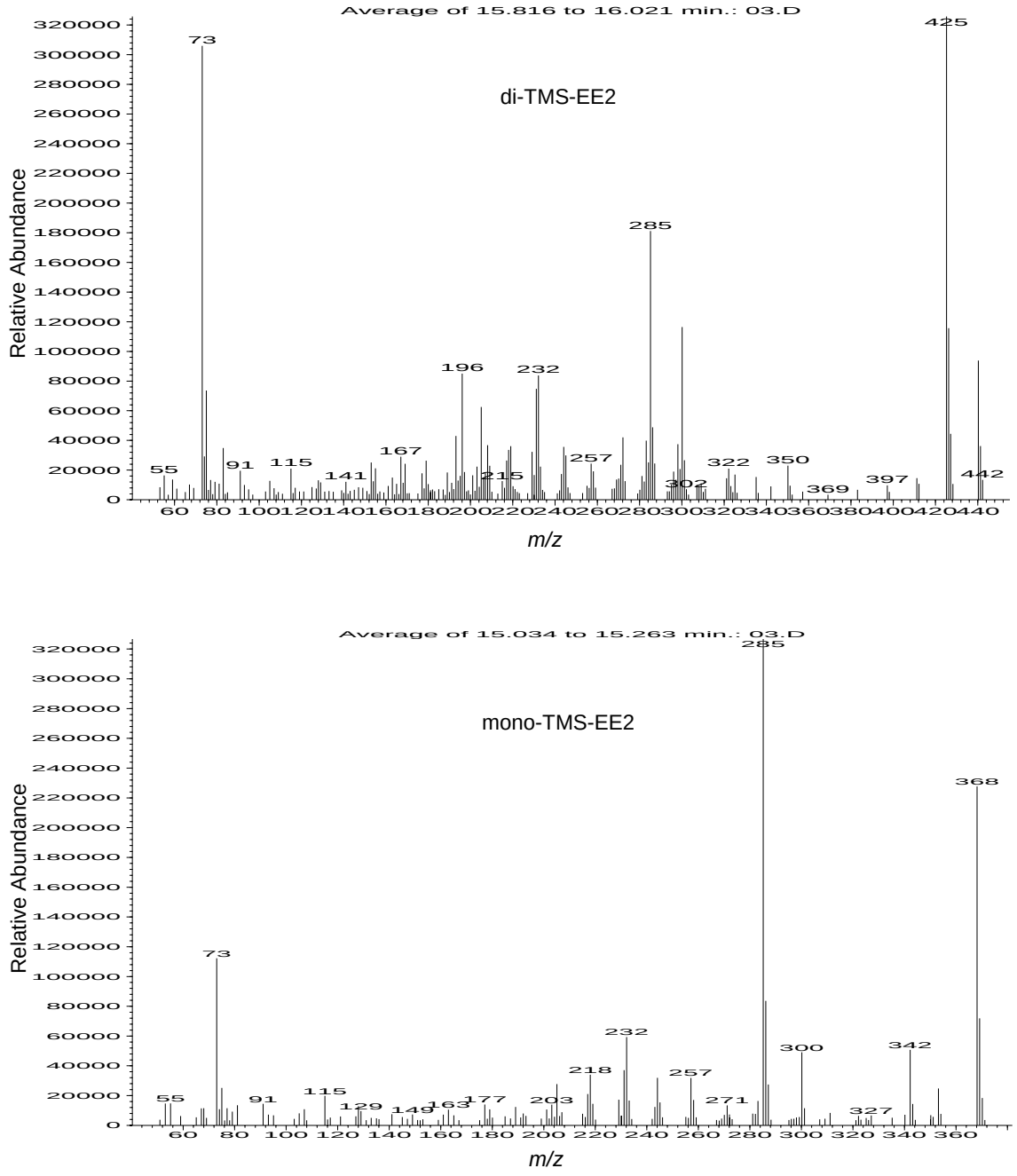


Figure 4.4b. Mass spectra of mono- and di-TMS-EE2 from Figure 4.4. Molecular ion 440 for di-TMS-EE2 and 368 for mono-TMS-EE2.

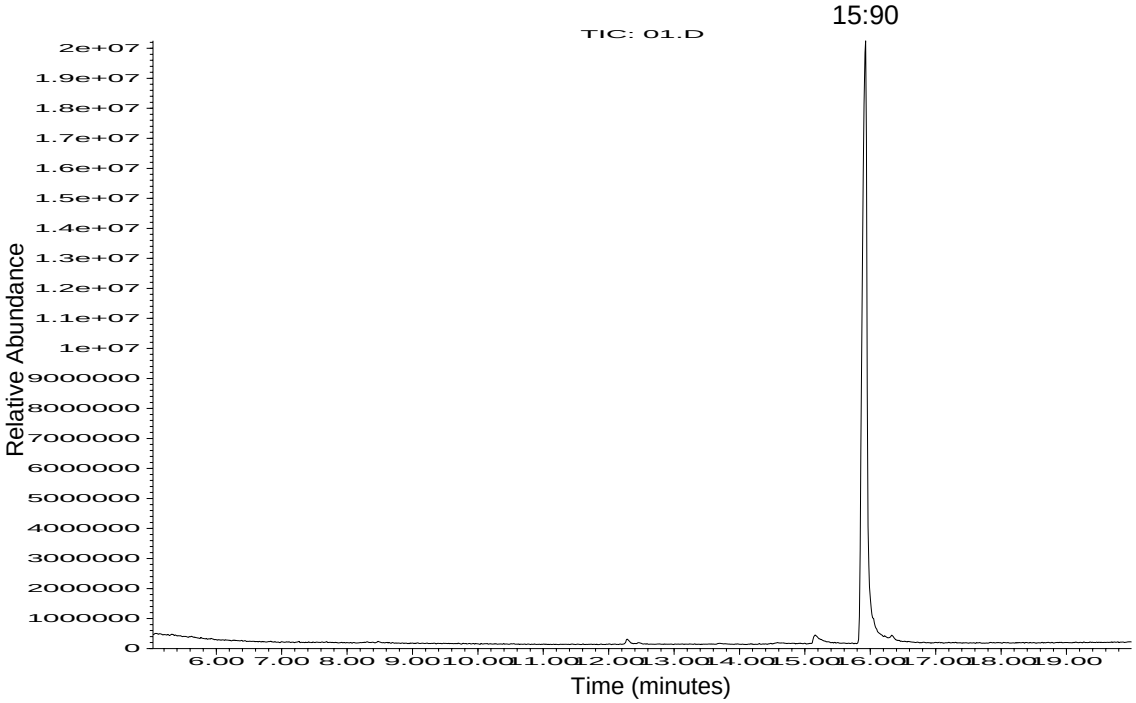


Figure 4.5. TIC of di-TMS-EE2 derivative. EE2 TMS derivatives were prepared at 60°C for 30 minutes and GC-MS analysis was in ethyl acetate.

Calibration curve. Ions for use in the calibration curve were selected from the full mass spectra of TMS-EE2 (Figure 4.4b) and TMS-d_2-E2 (IS) (Figure 4.7a). The calibration curve for EE2 to d_2-E2 was created using the area ratio of ions at *m/z* 425 to *m/z* 418, respectively. An example of single ion chromatograms used for the area calculations is shown in Figure 4.6. Figure 4.7 presents the TIC for 10 ng IS to 10 ng EE2. The mass spectrum of di-TMS-d_2-E2 is presented in Figure 4.7a. Figure 4.8 displays the area ratio calibration curve for EE2 to IS using the characteristic masses 425/418, EE2 and d_2-E2, respectively, with a linear regression R^2 of 0.997.

In regards to the calibration of EE2 and the IS, initial thoughts were that EE2 to d_2-E2 would be close to the same in relative abundance at the 1:1 ratio (10 ng/ 10 ng) in full scan analysis, but this was not the case. The TIC in Figure 4.7 shows the differences in peak heights for EE2 and d_2-E2 at the 1:1 ratio. Consequently, standards were prepared a second time and the calibration curve repeated with similar results.

There were no problems creating di-TMS-d_2-E2 derivatives when using the optimal temperature and time for di-TMS-EE2 derivatives determined in section 4.2.2.

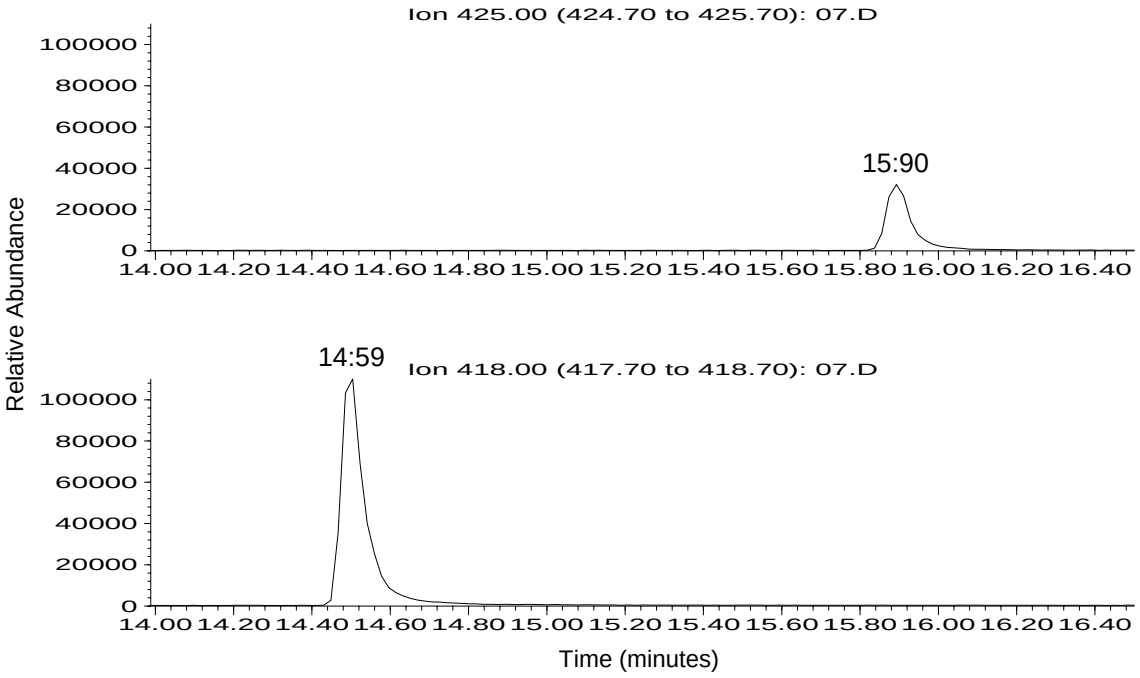


Figure 4.6. Single ion chromatograms from GC-SIM analysis of 10 ng di-TMS-EE2 (*m/z* 425, RT 15:90) to 10 ng di-TMS-d_2-E2 (*m/z* 418, RT 14:59).

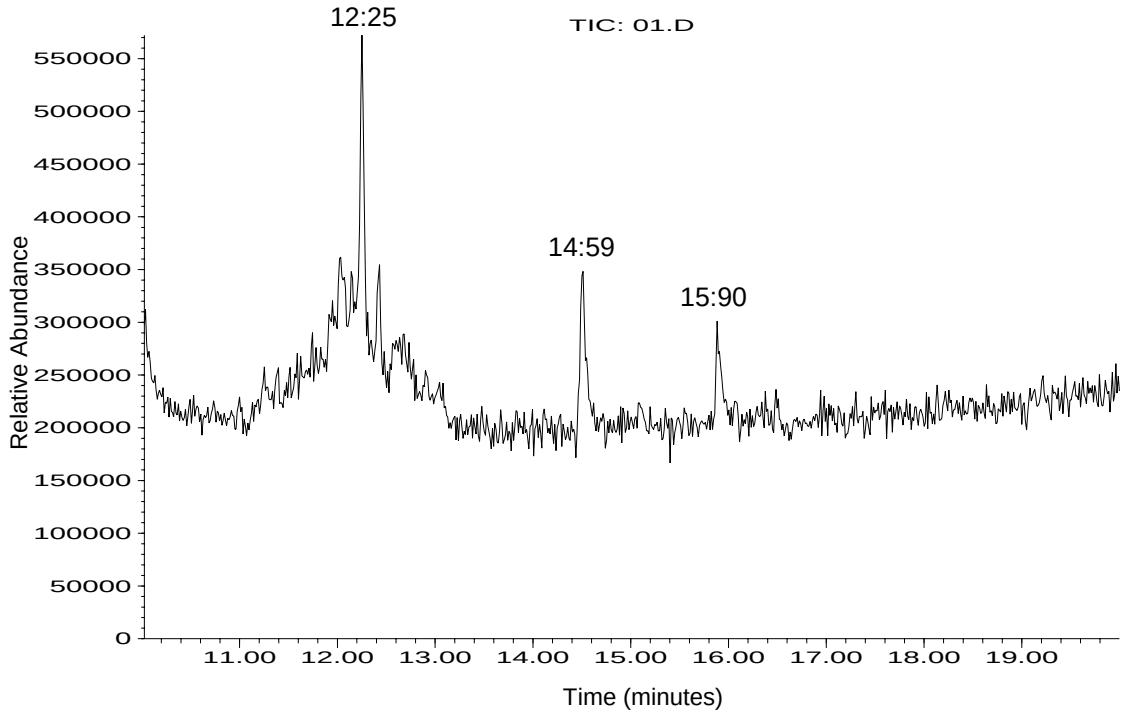


Figure 4.7. TIC from GC-MS (full scan) analysis of 10.0 ng di-TMS-EE2 (RT 15:90) and 10.0 ng IS (di-TMS-d_2-E2) (RT 14:57). Note: Peak at ~ 12.25 min corresponds to the mass spectrum of impurities found to be within the pyridine.

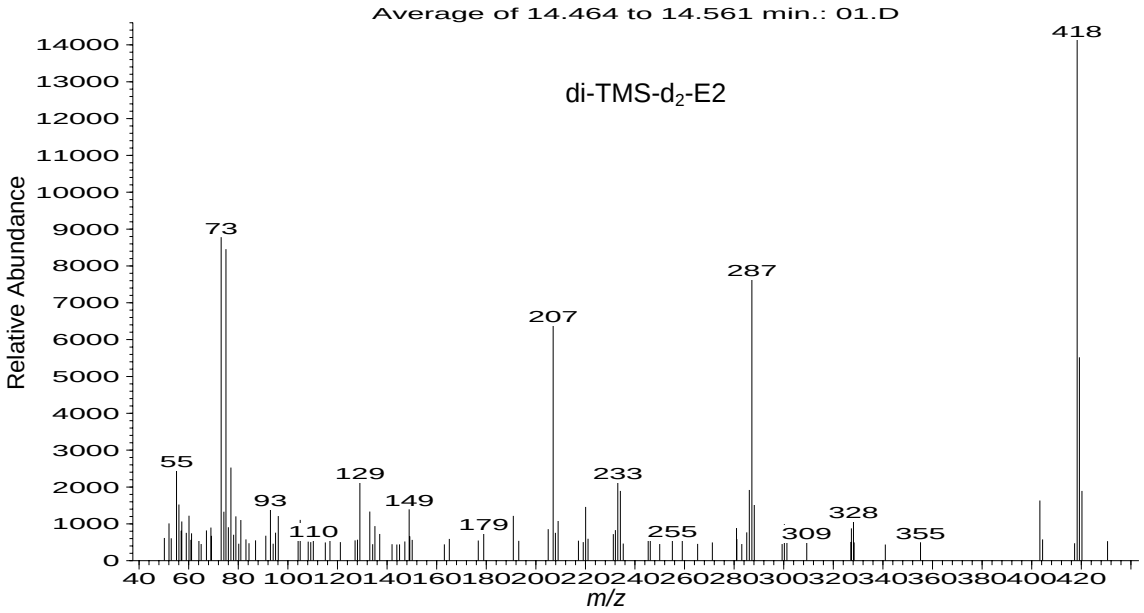


Figure 4.7a. Mass spectrum for the IS di-TMS-d_2-E2 from Figure 4.7. Molecular ion 418.

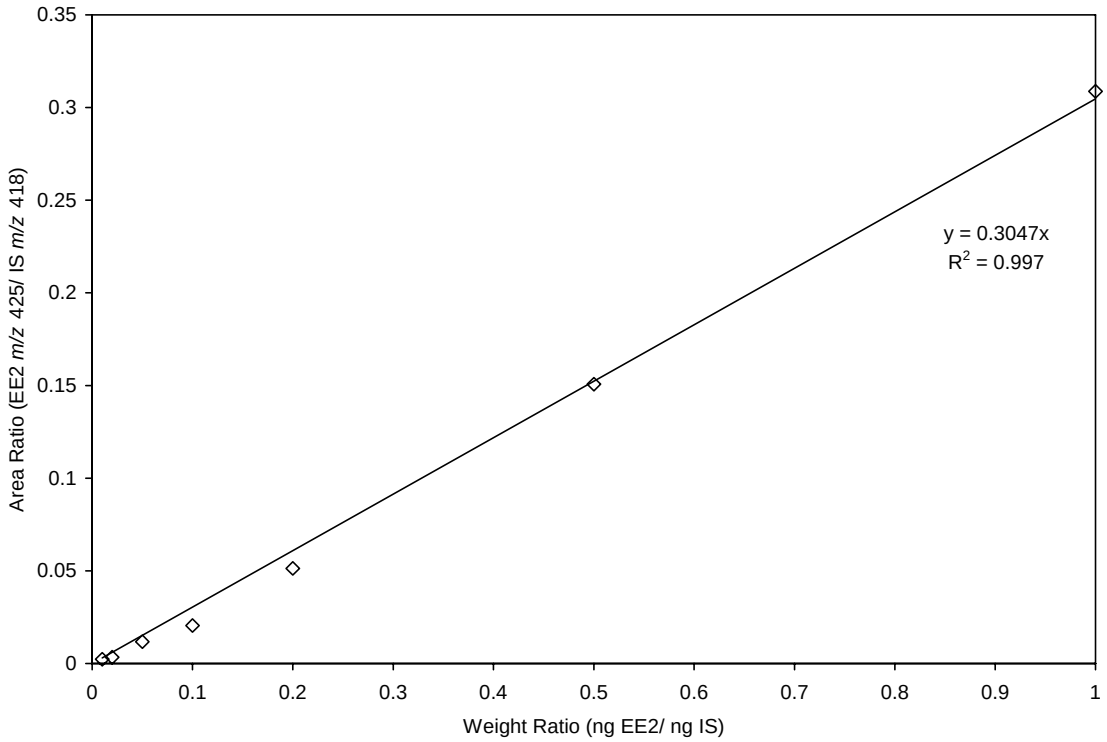


Figure 4.8. GC-MS calibration curve for EE2 and the IS d_2-E2 using characteristic masses 425/418, respectively.

Preparative purification of Oldman River water. Two experimental runs with spiked river water were performed.

The initial GC-MS run had no IS, but the characteristic ions for EE2 were present. Figure 4.9 presents the TIC of the first run with the extracted ion chromatograms for EE2 and IS also shown. Table 4.3 presents the recoveries for EE2 during the purification steps as quantified by the proportion of ^{3}H-EE2 recovered at each stage. For the first run, final recovery of EE2 was estimated by the amount of ^{3}H-EE2 present in the sample prior to GC-MS analysis, as the IS was not present. The second run was more successful with the IS notably present in the GC-MS analysis allowing for quantification (Figure 4.10).

After each clean-up step involving C_{18} and silica a fraction of the eluant was analyzed with LSC to check for radioactive losses during these steps, with the assumption that radiolabeled-EE2 would behave similarly to non-labeled EE2 and be lost in similar proportions following similar mechanisms. In both trials initial river water clean-up through C_{18} resulted in complete recovery of the added radioactivity once the columns were stripped (Table 4.3). The C_{18} eluant was dried and resuspended prior to silica gel purification. The first experimental run noted significant losses after the silica gel step, so attempts to track the losses during the second run involved checking the radioactivity prior to the silica stage after the C_{18} eluants had been dried and resuspended in a known volume. Losses were noted after this dry down step. Losses were again noted after the silica gel step.

The final recovery results acquired by GC-MS for the second experimental run were quantified using the IS and the calibration curve (Figure 4.10). Final recovery of the 10 ng of EE2 added in the initial stages of the second preparatory run was 2 ng (Table 4.3). Recovery from the first run was suggested to be 3 ng prior to GC-MS analysis by quantity of radio-labeled EE2 present in the sample after the silica gel step. GC-MS analysis of the first trial showed the presence of the characteristic ions for EE2 (Figure 4.9).

Table 4.3. Spiked river water recovery. Percentage recovery of EE2 after each river water purification step based on quantity of radio-labeled EE2 present and by the ratio of IS to non-labeled EE2 from the GC-MS analysis.

Run	*% Recovery after C_{18} clean-up*	*% Recovery after drying & resuspending C_{18} fractions*	*% Recovery after silica gel clean-up*	*Final % Recovery after GC-MS*
1	100	N/A	34	N/A*
2	100	68	49	20

*EE2 was present in the GC-MS analysis, but the IS was not added.

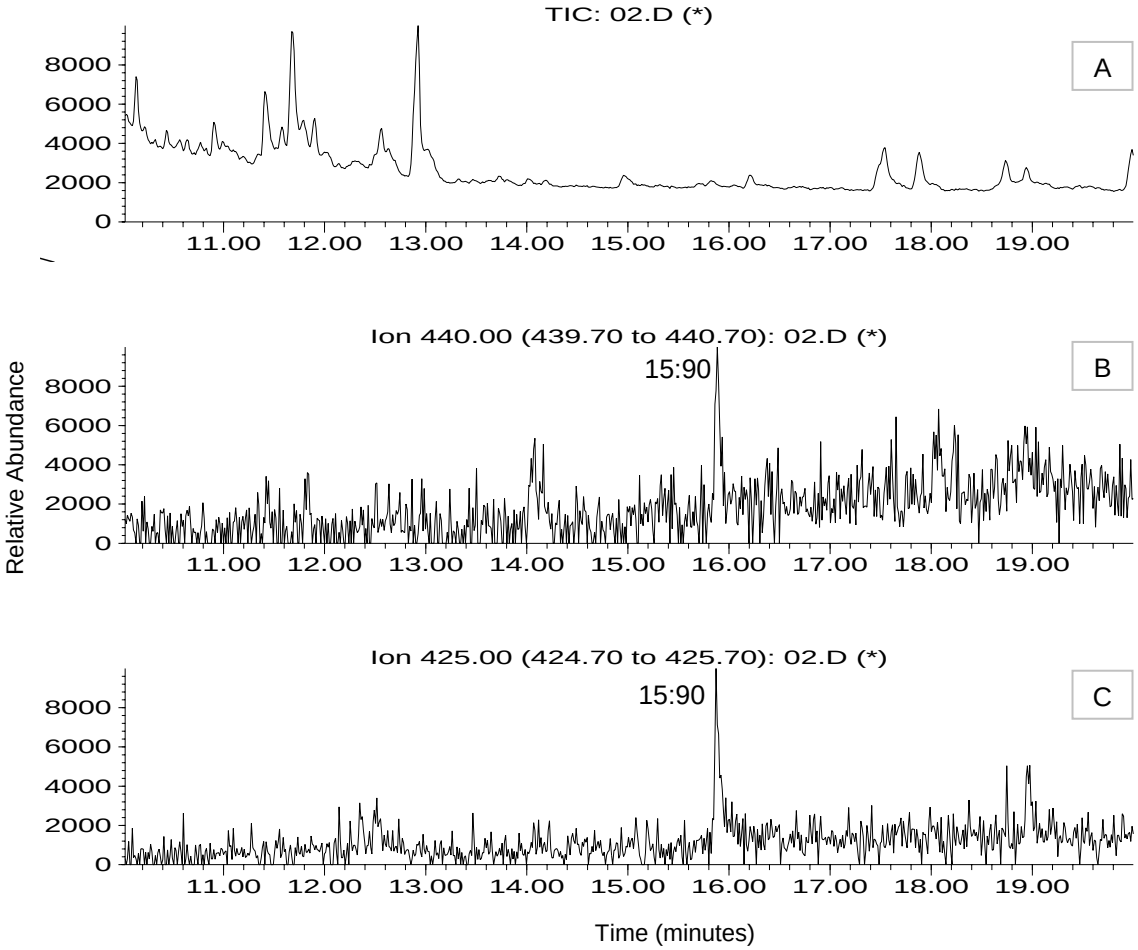


Figure 4.9. GC-MS (full scan) analysis for the first spiked river water trial. A, TIC; B, C, extracted ion chromatograms for EE2 (*m/z* 440 and 425, RT 15:90) are shown. Note the relative abundances for each plot have been normalized so that the largest peak is shown at full scale in each case.

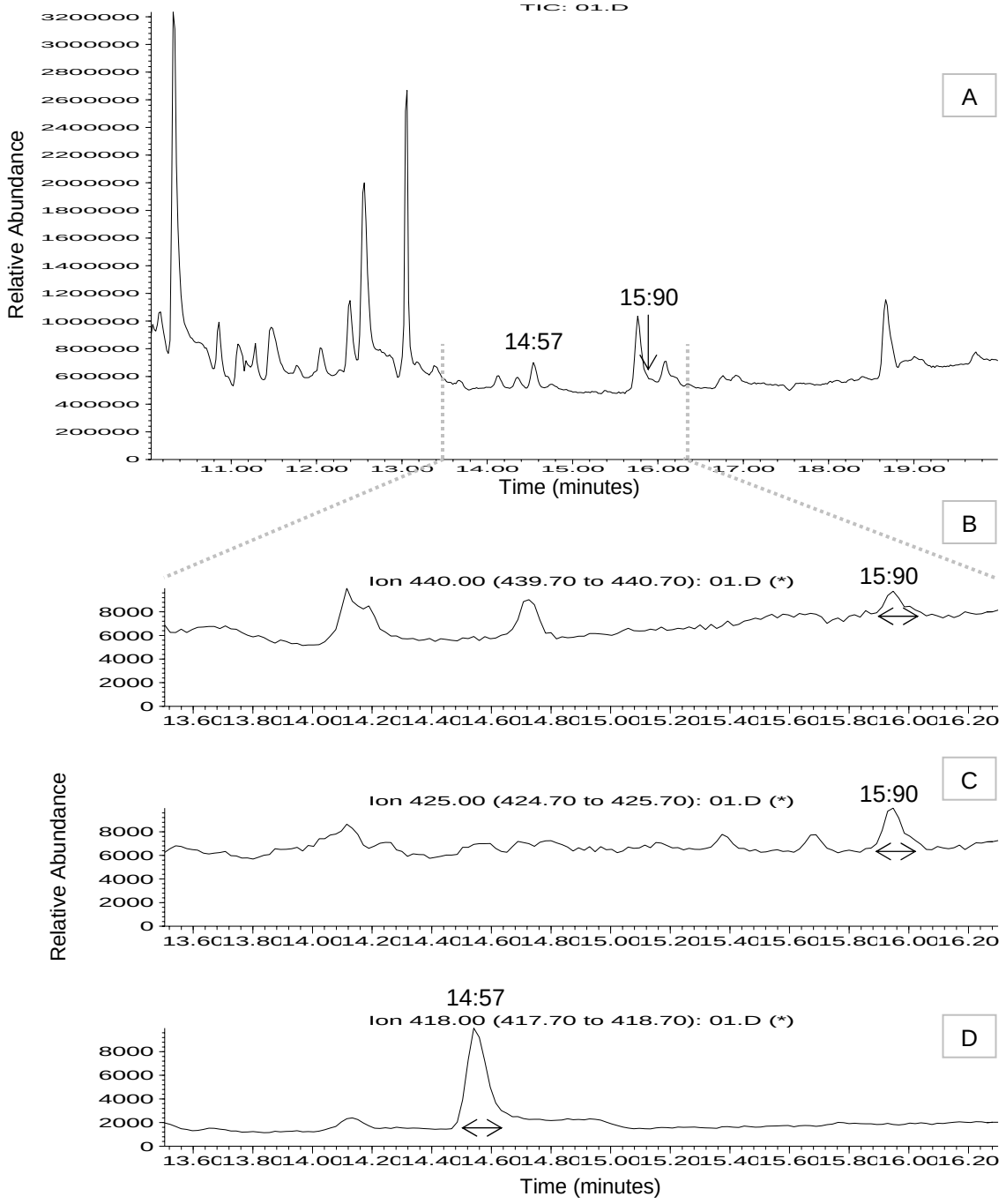


Figure 4.10. GC-SIM analysis of second spiked river water trial, with IS evident. A, TIC; B, C, D, single ion chromatograms for EE2 (*m/z* 440 and 425, RT 15:90) and the IS (*m/z* 418, RT 14:57). Note the different time ranges for single ion chromatograms and the TIC, as well relative abundances for each plot have been normalized so that the largest peak is shown at full scale in each case.

4.4 DISCUSSION

The methods outlined here are designed to selectively detect and measure the synthetic hormone EE2 in surface waters. Previously reported methods tended towards detection of a complex mixture of natural and synthetic estrogens, such as EE2, E1, and E2 (Kuch and Ballschmiter, 2000; Jeannot et al., 2002; Kolpin et al., 2002; Petrovic et al., 2004; Vethaak et al., 2005). This broad detection method likely resulted in previously undocumented degradation of EE2 into E1, with the potential for an underestimation of EE2 and overestimation of E1 in sampled waters.

A method for preparation of EE2 for GC-MS analysis minimizing degradation processes was developed. This process resulted in near 100% completely trimethylsilylated (di-TMS) EE2 derivatives with no breakdown into E1 (Figure 4.5). Creating the TMS derivatives was optimized when using the solvent pyridine at a ratio of 1:1 with BSTFA+1% TMCS. Heating at 60 °C for 30 minutes optimized complete TMS-EE2 (di-TMS) derivatives (Figure 4.5 and Table 4.2). Removal of the silyl reagent BSTFA prior to the GC-MS run aided in reducing the breakdown of EE2, as fluorine present in the BSTFA may be reactive enough to facilitate the breakdown (Dr. P. Dibble, personal communication).

The purification process of river water for the quantification of the synthetic hormone EE2 appears to be sufficient, as evidenced by the relatively clean TIC in Figure 4.9. SPE involving two-1g C_{18} columns, with a volume of 2.5 litres of river water per column, did not exceed the capacity of the column (and therefore not allowing EE2 to pass through

the column) as suggested by 100% recovery of the EE2 test standards off the C_{18} columns (Table 4.3). Final recovery was less than 70% of the added 10 ng/ L EE2 (Table 4.3 and Figure 4.10).

There is some evidence that EE2 adsorbs to particulate matter during purification with SPE and dry-down steps. This has been reported by Xiao et al. (2001) suggesting estrogen has capabilities of adsorbing to suspended particulate matter that may be removed during SPE, but not desorbed simply by rinsing with organic solvents. Lai et al. (2000) found synthetic estrogens to sorb to sediments and suspended particulates at a greater percentage than natural estrogens, with a general dependence on organic carbon content and particle size. Losses of radio-labeled EE2 tracer presented in Table 4.3 are consistent with these findings.

A method published by Ingrand et al. (2003) using LC-MS-MS ion trap system also noted losses during each preparation step for spiking mineral water, including SPE on C_{18}, liquid-liquid separation, and a clean-up step, ending with a total recovery of 87%. When using spiked wastewater effluent EE2 losses were substantial, resulting in levels lower than limits of quantification (10 ng/ L) when spiked with 10 or 20 ng/ L.

EE2 is typically reported at levels lower than detection, presumably due to the higher lowest level of detection and the smaller concentration being released into the wastewater. Natural estrogens are commonly found, with E1 typically at higher quantities than other natural estrogens. Also, degradation of these hormones during wastewater

treatment could result in E1 and the resulting higher quantities being detected. E2 is known to degrade into E1 by using nitrifying activated sludge (Ternes et al., 1999a and 1999b; Shi et al., 2004).

In 2004, Shareef et al. also noted the degradation of EE2 into E1 during certain experimental procedures. In the fall of 2005, Zhang and Zuo published an article on the degradation of EE2 into E1 during the process of creating derivatives using BSTFA + TMCS, as well as the problem of incomplete TMS-EE2 derivatives. Suggested methods for optimizing creation of TMS derivatives of EE2 are the same as those determined by the methods presented here.

In the time since this method was started, an improved method for the detection of EE2 in surface waters was published. Noppe et al., 2005, developed a method with GC-EI-MS-MS with full recoveries of standards. Similar to other methods, the initial purification of the water was performed with SPE extraction. Noppe et al. (2005) preferred the use of discs (using Bakerbond Speedisk octadecyl-bonded silica (C18XF)) over cartridges to limit the amount of clogging from colloidal material and suspended particles in the environmental samples. Clogging was found to not be a problem for the SPE methods used here. The pH of their water samples were adjusted to 7 to minimize ionized organic acids, although they found a pH range of 2 - 7 was optimal for recovery. The methods developed here used an adjusted pH of 8 to ensure the analyte and organic acids were non-ionized. It may be that a neutral pH was needed to minimize adsorption of EE2 to particulate matter.

At the time this method was being developed, Alberta Environment published a preliminary report examining pharmaceuticals and wastewater contaminants in the province of Alberta, including EE2 (Sosiak and Hebben, 2005). The Oldman River, downstream of the City of Lethbridge's wastewater treatment plant, was tested. Levels of EE2 were below levels of detection which ranged from 0.022 to 0.094 ng/ L across standards and between laboratories performing the analyses. The publication checked major Alberta cities wastewater effluent for 105 compounds and isomer mixtures. EE2 was not detected at other major cities, except for the City of Calgary with one wastewater treatment plant's effluent at a level of 8 ng/ L.

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CHAPTER 5 Discussion

5.1 PHYTOREMEDIATION OF PHARMACEUTICALS AND HERBICIDE

Pharmaceuticals are designed to be chemically stable to increase shelf life, and provide persistence in the metabolic environments. Consequently, it then should be no surprise that pharmaceuticals have been found, in both parental and metabolite form, in urine and feces making their way through wastewater treatment facilities to be disposed of often in surface waters. This deliberate drug stability therefore increases the prospective impact on non-target organisms and their ecosystems.

Natural, aesthetic, and cost efficient, phytoremediation with aquatic or phreatophytic plants may provide a method for removing pharmaceuticals from wastewater and the water environment. Using the log of the octanol-water coefficient (log K_{ow}) of the pharmaceuticals, estimates can be made into whether the compounds will be taken up by plants, how much could be taken up, and where they will be distributed within the plants. Each plant species can vary in the amount of water uptake and phytoremediation capabilities. Also, each pharmaceutical will have a different log K_{ow} and be affected differently by the environment, plants and chemical interactions with other compounds. Compounds with log K_{ow} values generally above and below 2 will be less likely to be taken into and transported within the plants.

Members of the Salicaceae family, particularly of the genus *Populus* commonly referred to as poplar trees, have been the plant of choice for most phytoremediation research and

field applications. Offering ample geographic distribution, fast growth, deep roots and extensive transpiration, these trees have been the focus in several areas of phytoremediation. Research into their application has been reported with oils and hydrocarbons (Widdowson et al., 2005), herbicides (Burken and Schnoor, 1996 and 1997), pesticides (Karthikeyan et al., 2004) and explosives (Thompson et al., 1998), synthetic compounds (Kassel et al., 2002), and various organic pollutants (Burken and Schnoor, 1998).

This plant family also includes the genus *Salix*, the willows. Although often ignored in the field of phytoremediation, this genus shares physiological characteristics of *Populus*. These shared traits, and family, would suggest willow to behave similarly to poplars in their abilities to remediate. Further insight into the genes and metabolic processes involved in *Populus* phytoremediation can now be investigated as a model for the processes within willow since the *Populus* genome has been sequenced (Sterky at al., 2004). The willows are just now beginning to be viewed as potential candidates for phytoremediation. Recent studies involving uptake of contaminants and willow include cyanide (Larsen et al., 2004; Larsen and Trapp, 2006), the 'antifoulant' tributyltin (Ciucani et al., 2004), cadmium (Lewandowski et al., 2006), as well as increased levels of polychlorinated biphenyl-degrading bacteria in the root soil of willows (Leigh et al., 2006). These studies revealed willow to be a promising remediation plant, especially for water contaminants since willows are exceptionally inundation tolerant and typically the woody plants closest to the streams in riparian zones.

Arabidopsis thaliana is a plant that would not normally be considered for a remediation project. A member of the *Brassica* family, *Arabidopsis* has been extensively studied and its genome sequenced. Determining the physiological genetics of uptake, transport and metabolism enzymes involved in detoxification of pharmaceuticals using *Arabidopsis* can be an important step towards better understanding phytoremediation, the processes at work and which plants might be more suited to certain remediation projects. For example, *Arabidopsis* has been used in transgenic studies with the insertion of a bacterial enzyme gene, mercuric ion reductase, improving the plants tolerance to mercuric ions and its abilities to convert it into less toxic elemental mercury (Dietz and Schnoor, 2001).

This MSc project set out to investigate the abilities of *Salix exigua* and *Arabidopsis thaliana* at uptake of three common pharmaceuticals from solution, followed by subsequent analyses examining the transport and apparent forms with which the pharmaceuticals were distributed within the plants. Both plants were effective at removing and transporting these compounds from solution. Of the compounds taken into the plants, bound and soluble forms were evident. An empirical relationship between compound octanol-water partition coefficient and uptake, distribution and the plant species was able to be developed. The determined empirical relationships were found to be similar to reported empirical relationships for barley and poplar and supported the theory that predictable behaviours for neutral pharmaceuticals can be based on their octanol-water partition coefficient.

Although there are major ecophysiological differences, it was interesting to note some of the similarities between willow and *Arabidopsis*. Particularly because some of the parameters measured (uptake, RCF, TSCF) depend on root and shoot masses of the experimental plants, a valid comparison of these parameters requires that root and shoot masses be similar. *Arabidopsis* shoot weights were significantly larger than for willow across compounds (Table 5.1 and Table A5.1, ANOVA, $p < 0.05$). Root fresh weight was similar for *Arabidopsis* and willow except for DTZ in which willow root weight was less than for *Arabidopsis* ($p = 0.048$). These similarities in fresh root weight allow for some comparisons among *Arabidopsis* and willow.

In comparing final cumulative transpiration between the plants and for each compound (Table 5.2 and Table A5.2, ANOVA) there were no significant differences. Even though shoot mass for *Arabidopsis* was significantly greater than for willow for all of the compounds, the final volumes transpired did not vary.

In comparing the percentage uptake over time between the plants and for each compound (Table A5.3a-d, ANOVA) a few differences occur. For DTZ, *Arabidopsis* uptake was significantly lower than with willow at each sampling time (ANOVA, $p < 0.05$). DZP uptake between plants was not significantly different except at $t = 8$ with *Arabidopsis* uptake significantly lower than with willow (ANOVA, $p = 0.036$). Uptake of ATZ varied between plants only at $t = 2$ with willow uptake significantly lower than with *Arabidopsis* (ANOVA, $p < 0.05$). Uptake over time barely differs among the plants and three of the

compounds, excluding DTZ, which is likely a function of root mass for the relatively short 24 hour period of time these studies were carried out.

The final percentage uptake, or removal from solution after the 24 hour study period between *Arabidopsis* and willow were similar except for DTZ, which was significantly lower for *Arabidopsis* (Table 5.2 and Table A5.1, ANOVA, $p < 0.05$). These similar final uptake values are likely a function of the similar root masses among plants (Table A5.1). The difference noted in DTZ is difficult to explain. It is unclear why DTZ would apparently behave as a neutral compound with one plant and an ionized compound with the other.

Uptake from solution did follow the predicted order based on the compounds log K_{ow} values. EE2, having the highest log K_{ow} of the four compounds was removed at a higher percent than the other compounds. Next was DTZ, DZP, then ATZ, with corresponding log K_{ow} of 2.70, 2.82, and 2.61. If the compounds were passive, and not ionized, then one might assume removal would correspond to the K_{ow}. DTZ, though, can be ionized at environmental and physiological pH and would become hydrophobic in the apoplast and pass the membrane easily (likely explaining its higher percentage of uptake over the higher K_{ow} DZP within willow).

Table 5.1. *Salix exigua* and *Arabidopsis* root and shoot fresh weight and total volume transpired over the 24 hour study period for three pharmaceuticals and the herbicide (mean ± SE). EE2, 17α-ethynylestradiol; DTZ, diltiazem; DZP, diazepam; and ATZ, atrazine.

Salix exigua				*Arabidopsis thaliana*		
Root fr. wt. (g)	*Shoot fr. wt. (g)*	*Total Vol. Transp. (mL)*		*Root fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total Vol. Transp. (mL)*
0.47 ± 0.05	0.71 ± 0.05	10.42 ± 0.58	EE2	0.79 ± 0.12	1.85 ± 0.25	9.65 ± 0.81
0.53 ± 0.10	0.93 ± 0.16	13.67 ± 2.39	DZP	0.70 ± 0.10	1.47 ± 0.21	8.83 ± 0.90
0.41 ± 0.13	0.64 ± 0.08	9.25 ± 0.46	DTZ	0.73 ± 0.10	1.65 ± 0.21	9.13 ± 0.97
0.48 ± 0.08	1.00 ± 0.09	14.25 ± 1.23	ATZ	0.67 ± 0.18	1.75 ± 0.17	10.85 ± 1.40

Table 5.2. Percentage final uptake of three pharmaceuticals and an herbicide (0.04 μg/ mL) by *Salix exigua* and *Arabidopsis* after 24 hours (mean ± SE), with listed log K_{ow} values.

		Uptake (%)	
	Log K_{ow}	*Salix exigua*	*Arabidopsis thaliana*
17a-Ethynylestradiol	3.67	87.8 ± 1.12	84.5 ± 0.01
Diazepam	2.82	62.5 ± 3.89	58.8 ± 0.03
Diltiazem	2.70	77.3 ± 6.12	56.9 ± 0.03
Atrazine	2.61	50.1 ± 3.24	52.0 ± 0.05

Salix exigua and *Arabidopsis* plants underwent the same separation into components and extraction procedures to determine distribution of the compounds within the plants. Recovery levels were acceptable for willow, although ATZ recovery was poor, while *Arabidopsis* recovery levels were significantly lower than for willow, except for EE2.

The reasons for the low and diverse recovery values are not understood, especially since a high degree of consistency for the proportions recovered for each replicate are evident while recoveries were more variable. There were procedural variations associated with oxidation and a possible explanation for the consistency in distribution among replicates may be because samples are oxidized in sequence (i.e. DZP replicate plant A root and shoot would be oxidized before replicate B). The same losses may have occurred on the entire replicate resulting in more accurate proportions.

Although *Arabidopsis* recoveries were low, replicate plant recoveries were similar enough in proportion distribution to provide an idea of general distribution within the plant. Sufficient consistency was achieved to enable comparison between *Arabidopsis* and willow.

Distribution between roots and shoots among *Arabidopsis* and willow were comparable between EE2, DZP and ATZ (Figures 2.6 and 2.7 and Figures 3.4 and 3.5). There were also similarities in the proportions of bound and soluble fractions between the plants. For EE2, most of the compound was found within the roots in a bound form in both willow and *Arabidopsis*. DZP and ATZ were both transported to the shoots with small

proportions becoming bound in roots and shoots. This again suggests that these pharmaceuticals behave as predicted by their log K_{ow} values. As the percentage distributions between the compounds were similar (Figures 2.6 and 2.7 and Figures 3.4 and 3.5), the relationship between transpiration and uptake may exist and occur as expected for neutral compounds. This correlation may be explained as TSCF. A correlation between uptake and cumulative transpiration trends for the individual plants was also not observed, except for *Arabidopsis* and EE2, likely due to the larger sample size and greater range of plant sizes.

DTZ distribution and uptake were significantly different between the plants. In *Arabidopsis*, DTZ appeared to behave as predicted by its log K_{ow} value (that of a neutral compound), with > 50% remaining within the root as soluble and bound fractions. In willow, DTZ apparently behaved more as an ionized compound and remained entirely soluble within the root.

Calculated RCF values for *Arabidopsis* were similar to those experimentally determined for willow (Table 5.1). EE2 RCF values were not significantly different, whereas DTZ and DZP were. *Arabidopsis* RCF to log K_{ow} values fit a linear regression equation at a similar, but more steeply increasing slope to willow (Table 5.3 and Figures 2.9 and 2.10). This consistent trend suggests that the uptake of relatively neutral pharmaceuticals is guided by their physiochemical properties, particularly their log K_{ow}. Following similar trends reinforces the idea of log K_{ow} being important in predictions of chemical behaviour in the environment. Uptake or equilibrium curves from the 24 hour uptake studies and the

RCF studies follow a similar curve, verifying that similar processes are taking place and that RCF plays a role in whole plant uptake values (Figures 2.4 and 2.8 and Figure 3.2).

Empirical relationships between TSCF and log K_{ow} for *Arabidopsis* and willow were very similar in their downward trend with increasing log K_{ow} values (Table 5.4 and Figures 2.11 and 3.11). The determined relationships for willow and *Arabidopsis* decline at a faster rate with increasing log K_{ow} than relationships determined for barley and poplar (Table 5.4). TSCF values for DZP and EE2 are equivalent between willow and *Arabidopsis*, with ATZ and DTZ values being significantly higher for *Arabidopsis* than willow. Although, the estimated TSCF value for willow and DTZ may be significantly different due to the observed difference in behaviour of DTZ among the study plants.

If a TSCF of 1 is equivalent to passive uptake following the transpiration stream, then the values determined for DZP, DTZ and ATZ with *Arabidopsis* suggest these compounds move almost passively with water. The TSCF value for DZP with willow is also close to the value of one. These values seem high, particularly when their log K_{ow} values, when compared with other compounds, would suggest a more restricted transport.

Comparing the RCF and TSCF values for willow and *Arabidopsis* to their uptake and distribution, the proportions of distribution of EE2, DZP and ATZ are almost identical (Figures 3.4 and 3.5 and Figures 2.6 and 2.7). Considering the RCF values of willow for ATZ and DZP were larger than for *Arabidopsis* (Table 5.3), it could be expected that a larger portion of the compound would be found within the roots of willow than in

Arabidopsis. This is not the case. The proportions of recovered radioactivity within the roots and shoots for both ATZ and DZP are equivalent between the two plants.

Table 5.3. Calculated and experimentally determined root concentration factor values (mean ± SE) for *Salix exigua* and *Arabidopsis*. Also shown are the root concentration factor values obtained for the four compounds using their log K_{ow} values and Briggs et al. (1982) equation for barley, and Burken and Schnoor's (1998) equation for hybrid poplar.

		Root concentration factor			
	Log K_{ow}	*S. exigua*	*Arabidopsis*	*barley*	*poplar*
17a-Ethynylestradiol	3.67	210 ± 50	219 ± 44	21.05	9.54
Diazepam	2.82	34 ± 7	8.9 ± 0.9	5.30	4.83
Diltiazem	2.70	257 ± 24	21 ± 5	4.44	4.53
Atrazine	2.61	22 ± 5	2.5 ± 0.57	3.91	4.34

Table 5.4. Calculated and experimentally determined transpiration stream concentration values (mean ± SE) for *Salix exigua* and *Arabidopsis*. Also shown are the transpiration stream concentration factor values obtained for the four compounds using their log K_{ow} values and Briggs et al. (1982) equation for barley, and Burken and Schnoor's (1998) equation for hybrid poplar.

		Transpiration stream concentration factor			
	Log K_{ow}	*S. exigua*	*Arabidopsis*	*barley*	*poplar*
17α-Ethynylestradiol	3.67	0.15 ± 0.06	0.20 ± 0.03	0.18	0.44
Diazepam	2.82	0.93 ± 0.15	0.93 ± 0.22	0.50	0.73
Diltiazem	2.70	0.00 ± 0.00	0.93 ± 0.20	0.55	0.74
Atrazine	2.61	0.73 ± 0.19	0.98 ± 0.20	0.59	0.75

Summary discussion. This study suggests that phytoremediation could be an effective option for removing pharmaceuticals within water environments. As both *Salix exigua* and *Arabidopsis* removed between 50 and 90% of the compounds from solution, it is probable that other plants are also capable of phytoremediation of pharmaceuticals. Both plants affirmed that uptake and transport of pharmaceuticals can be predicted based on their chemical properties, particularly their octanol-water partitioning coefficient.

Although willow and *Arabidopsis* both performed well within this experimental setup, a hydroponic system can be far from what is actually found within the environment. Field applications, such as monitoring wastewater treatment wetland vegetation for the presence of these compounds, need to be performed to further confirm the usefulness of willow in the field environment.

Further examination into the fate of these compounds once they have entered plants needs to be considered to determine the metabolites and their potency and possible release rates upon ingestion or decay. This process of determining if metabolism occurs, and potential metabolites, could involve high performance liquid chromatography (HPLC) analysis of the solvent-extracted soluble fractions followed by gas chromatography-mass spectrometry (GC-MS) analysis (the methods outlined in Chapter 4 could provide a framework for this process, particularly for EE2). Fate of these compounds within plants over time could be analyzed with time course experiments and longer periods of uptake prior to analysis (much as the preliminary investigation for *Salix*

exigua and EE2 in Appendix B was performed). The effect of different concentrations of these pharmaceuticals could be determined as well as the toxic levels.

Differences in environmental temperature, type of plants, age of plants and synergistic pharmaceutical effects could also be investigated. Not only does further research into phytoremediation of pharmaceuticals exist, but also into the methodologies for working with the pharmaceuticals. As was found in these experiments, the behaviour of pharmaceuticals with SPE, preparative steps prior to GC-MS analysis and GC-MS analysis, and losses incurred during dry-down steps during basic laboratory procedures suggests that methods of working with specific pharmaceutical needs to be determined prior to field inventories and experiments.

I thus conclude that willow is very effective at removing pharmaceuticals from solution and could provide sequestration of these compounds in a bound form, limiting their re-entry into the environment. Since willow is a member of the Salicaceae family and performed similarly to prior research with poplar, poplar research may be applicable to willow and vice versa. This study also confirms the predictable behaviour of pharmaceuticals in the environment and plants based on their octanol-water partitioning coefficient. The support of *Arabidopsis* confirming these predictions only affirms the potential of plants for phytoremediation of the water environment.

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APPENDIX A Statistical analyses

Chapter 2

Table A2.1. *Salix exigua* ANOVA comparison among compounds for mean total volume transpired, root, shoot, wood and total fresh weight of replicate plants.

		Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
Root fr. wt. (g) * Compound	Between Groups (Combined)	.037	3	.012	.298	.826
	Within Groups	.747	18	.042		
	Total	.784	21			
Wood fr. wt. (g) * Compound	Between Groups (Combined)	1.111	3	.370	6.330	**.004**
	Within Groups	1.053	18	.058		
	Total	2.163	21			
Shoot fr. wt. (g) * Compound	Between Groups (Combined)	.470	3	.157	2.479	.094
	Within Groups	1.139	18	.063		
	Total	1.609	21			
Total fr. wt. (g) * Compound	Between Groups (Combined)	2.827	3	.942	2.961	.060
	Within Groups	5.729	18	.318		
	Total	8.556	21			
Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	91.924	3	30.641	2.404	.101
	Within Groups	229.417	18	12.745		
	Total	321.341	21			

Table A2.2. Salix ANOVA comparison among compounds for cumulative transpiration volume at each sampling time. Table A2.3 for subsequent analysis.

Time (hours)			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
2	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	2.000	3	.667	5.333	**.008**
		Within Groups	2.250	18	.125		
		Total	4.250	21			
4	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	4.975	3	1.658	3.534	**.036**
		Within Groups	8.448	18	.469		
		Total	13.423	21			
8	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	22.663	3	7.554	3.266	**.045**
		Within Groups	41.635	18	2.313		
		Total	64.298	21			
24	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	91.924	3	30.641	2.404	.101
		Within Groups	229.417	18	12.745		
		Total	321.341	21			

Table A2.3. Dunnett C Post Hoc analysis for *Salix exigua* of cumulative transpiration between compounds and over time ANOVA analysis in Table A2.2

Time (hrs)	*(I) Cmpd.*	*(J) Cmpd.*	*Mean Diff. (I-J)*	*Std. Error*	*95% Confidence Interval*	
					Lower Bound	*Upper Bound*
2	**ATZ**	**DTZ**	**.8333(*)**	.16667	.2183	1.4483
		DZP	.1667	.26874	-.8250	1.1583
		EE2	.5000	.17480	-.1450	1.1450
	DTZ	**ATZ**	**-.8333(*)**	.16667	-1.4483	-.2183
		DZP	-.6667	.21082	-1.4446	.1112
		EE2	**-.3333(*)**	.05270	-.5278	-.1389
	DZP	ATZ	-.1667	.26874	-1.1583	.8250
		DTZ	.6667	.21082	-.1112	1.4446
		EE2	.3333	.21731	-.4685	1.1352
	EE2	ATZ	-.5000	.17480	-1.1450	.1450
		DTZ	**.3333(*)**	.05270	.1389	.5278
		DZP	-.3333	.21731	-1.1352	.4685
4	**ATZ**	**DTZ**	**1.2917(*)**	.32543	.0363	2.5470
		DZP	.3750	.50861	-1.5017	2.2517
		EE2	.9167	.32702	-.2900	2.1234
	DTZ	**ATZ**	**-1.2917(*)**	.32543	-2.5470	-.0363
		DZP	-.9167	.42898	-2.5410	.7076
		EE2	-.3750	.17970	-1.1368	.3868
	DZP	ATZ	-.3750	.50861	-2.2517	1.5017
		DTZ	.9167	.42898	-.7076	2.5410
		EE2	.5417	.43020	-1.0457	2.1291
	EE2	ATZ	-.9167	.32702	-2.1234	.2900
		DTZ	.3750	.17970	-.3868	1.1368
		DZP	-.5417	.43020	-2.1291	1.0457
8	**ATZ**	**DTZ**	**2.5000(*)**	.61237	.0472	4.9528
		DZP	.5417	1.13361	-3.6413	4.7246
		EE2	2.0833	.57615	-.0426	4.2093
	DTZ	**ATZ**	**-2.5000(*)**	.61237	-4.9528	-.0472
		DZP	-1.9583	1.05755	-5.9725	2.0558
		EE2	-.4167	.40654	-2.2078	1.3745
	DZP	ATZ	-.5417	1.13361	-4.7246	3.6413
		DTZ	1.9583	1.05755	-2.0558	5.9725
		EE2	1.5417	1.03699	-2.2847	5.3681
	EE2	ATZ	-2.0833	.57615	-4.2093	.0426
		DTZ	.4167	.40654	-1.3745	2.2078
		DZP	-1.5417	1.03699	-5.3681	2.2847
24	ATZ	DTZ	5.0000	1.31339	-.0265	10.0265
		DZP	.5833	2.68845	-9.3368	10.5035
		EE2	3.8333	1.36117	-1.1893	8.8559
	DTZ	ATZ	-5.0000	1.31339	-10.0265	.0265
		DZP	-4.4167	2.43299	-13.4914	4.6581
		EE2	-1.1667	.73786	-4.2100	1.8767
	DZP	ATZ	-.5833	2.68845	-10.5035	9.3368
		DTZ	4.4167	2.43299	-4.6581	13.4914
		EE2	3.2500	2.45911	-5.8239	12.3239
	EE2	ATZ	-3.8333	1.36117	-8.8559	1.1893
		DTZ	1.1667	.73786	-1.8767	4.2100
		DZP	-3.2500	2.45911	-12.3239	5.8239

* The mean difference is significant at the .05 level.

Table A2.4. Spearman's rho correlation analysis for *Salix exigua* between uptake and either fresh weights (root, shoot, total) or cumulative transpiration volumes over the 24 hour study period.

Compound	*Time (hours)*		*Root fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total fr. wt. (g)*	*Cumulative Transpiration (mL)*
ATZ	2	Corr. Coe.	.143	.429	-.029	-.203
		Sig. (2-tailed)	.787	.397	.957	.700
	4	Corr. Coe.	-.143	.086	-.314	-.371
		Sig. (2-tailed)	.787	.872	.544	.468
	8	Corr. Coe.	.429	.429	-.086	-.086
		Sig. (2-tailed)	.397	.397	.872	.872
	24	Corr. Coe.	**.886(*)**	.943(**)	.771	**.943(**)**
		Sig. (2-tailed)	.019	.005	.072	.005
DTZ	2	Corr. Coe.	**1.000(**)**	.600	.600	.
		Sig. (2-tailed)	.	.400	.400	.
	4	Corr. Coe.	.800	.800	.800	.775
		Sig. (2-tailed)	.200	.200	.200	.225
	8	Corr. Coe.	.800	.800	.800	**1.000(**)**
		Sig. (2-tailed)	.200	.200	.200	.
	24	Corr. Coe.	**1.000(**)**	.600	.600	.800
		Sig. (2-tailed)	.	.400	.400	.200
DZP	2	Corr. Coe.	.371	.086	.116	-.213
		Sig. (2-tailed)	.468	.872	.827	.686
	4	Corr. Coe.	.087	-.116	-.088	-.265
		Sig. (2-tailed)	.870	.827	.868	.612
	8	Corr. Coe.	.754	**.986(**)**	**.956(**)**	**.899(*)**
		Sig. (2-tailed)	.084	.000	.003	.015
	24	Corr. Coe.	.600	**.886(*)**	**.812(*)**	**.943(**)**
		Sig. (2-tailed)	.208	.019	.050	.005
EE2	2	Corr. Coe.	.725	.714	.771	.414
		Sig. (2-tailed)	.103	.111	.072	.414
	4	Corr. Coe.	**.812(*)**	.600	.714	.759
		Sig. (2-tailed)	.050	.208	.111	.080
	8	Corr. Coe.	.754	.657	.771	.551
		Sig. (2-tailed)	.084	.156	.072	.257
	24	Corr. Coe.	**.882(*)**	.638	.754	.500
		Sig. (2-tailed)	.020	.173	.084	.312

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table A2.5. Spearman's rho correlation for *Salix exigua* between cumulative transpiration and fresh weights across the study period.

Compound	*Time (hours)*		*Root fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total fr. wt. (g)*
ATZ	2	Corr. Coe.	.522	.551	**.899(*)**
		Sig. (2-tailed)	.288	.257	.015
	4	Corr. Coe.	.543	.771	**.943(**)**
		Sig. (2-tailed)	.266	.072	.005
	8	Corr. Coe.	.714	**.829(*)**	**1.000(**)**
		Sig. (2-tailed)	.111	.042	.
	24	Corr. Coe.	**.943(**)**	**.886(*)**	**.886(*)**
		Sig. (2-tailed)	.005	.019	.019
DTZ[a]	2	Corr. Coe.	.	.	.
		Sig. (2-tailed)	.	.	.
	4	Corr. Coe.	.258	.775	.775
		Sig. (2-tailed)	.742	.225	.225
	8	Corr. Coe.	.800	.800	.800
		Sig. (2-tailed)	.200	.200	.200
	24	Corr. Coe.	.800	.800	.800
		Sig. (2-tailed)	.200	.200	.200
DZP	2	Corr. Coe.	.638	**.941(**)**	**.893(*)**
		Sig. (2-tailed)	.173	.005	.016
	4	Corr. Coe.	**.812(*)**	**.986(**)**	**.971(**)**
		Sig. (2-tailed)	.050	.000	.001
	8	Corr. Coe.	.771	**.943(**)**	**.928(**)**
		Sig. (2-tailed)	.072	.005	.008
	24	Corr. Coe.	.771	**.943(**)**	**.899(*)**
		Sig. (2-tailed)	.072	.005	.015
EE2	2	Corr. Coe.	.630	**.828(*)**	**.828(*)**
		Sig. (2-tailed)	.180	.042	.042
	4	Corr. Coe.	.739	**.941(**)**	**.880(*)**
		Sig. (2-tailed)	.093	.005	.021
	8	Corr. Coe.	.647	**.986(**)**	**.899(*)**
		Sig. (2-tailed)	.165	.000	.015
	24	Corr. Coe.	.647	**.986(**)**	**.899(*)**
		Sig. (2-tailed)	.165	.000	.015

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

[a] Due to the small sample size for DTZ, the correlation analysis could not be done. See Figure A2.1.

Figure A2.1. Scatter plots of DTZ treated plants with plant shoot, root and total fresh weight for each sampling time.

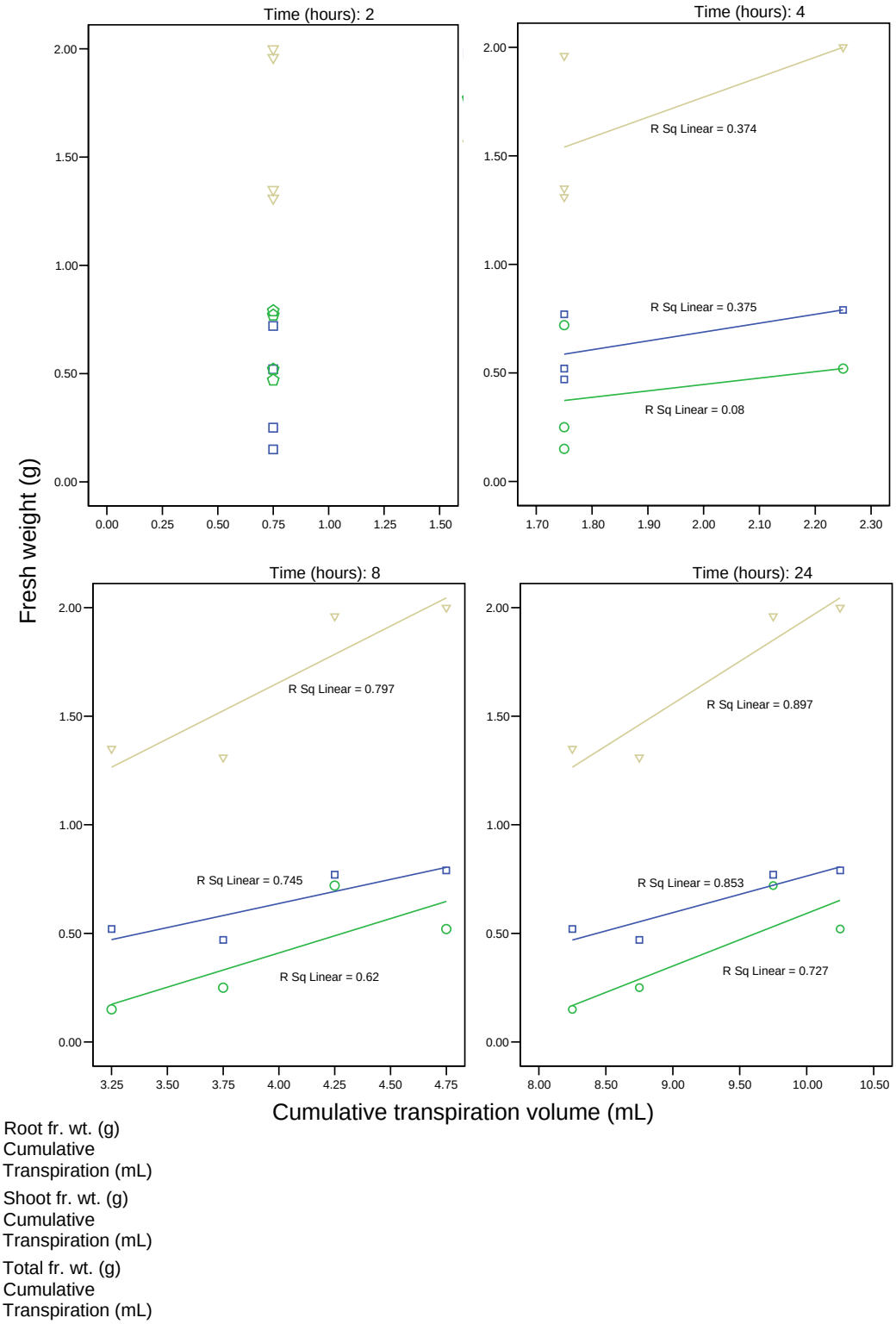


Table A2.6. ANOVA analysis root fresh weight for *Salix exigua* RCF plants among type, excised roots and roots attached to the cutting.

			Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
ATZ	Root fr. wt. (g) * Type	Between Groups (Combined)	.037	1	.037	.925	.391
		Within Groups	.159	4	.040		
		Total	.196	5			
DZP	Root fr. wt. (g) * Type	Between Groups (Combined)	.035	2	.017	.224	.812
		Within Groups	.232	3	.077		
		Total	.267	5			
EE2	Root fr. wt. (g) * Type	Between Groups (Combined)	.002	1	.002	.158	.711
		Within Groups	.061	4	.015		
		Total	.063	5			

Table A2.7. ANOVA analysis of root uptake in experiments with shoots removed from *Salix exigua* between excised roots and roots+wood for ATZ, DZP and EE2. Type, roots excised or roots attached to wood stem.

	Time (hrs)			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
ATZ	1	Percent Uptake * Type	Between Groups (Combined)	193.802	1	193.802	1.356	.309
			Within Groups	571.633	4	142.908		
			Total	765.435	5			
	2	Percent Uptake * Type	Between Groups (Combined)	1284.807	1	1284.807	9.214	**.039**
			Within Groups	557.753	4	139.438		
			Total	1842.560	5			
	4	Percent Uptake * Type	Between Groups (Combined)	184.815	1	184.815	1.349	.310
			Within Groups	547.953	4	136.988		
			Total	732.768	5			
	8	Percent Uptake * Type	Between Groups (Combined)	218.407	1	218.407	1.776	.253
			Within Groups	491.907	4	122.977		
			Total	710.313	5			
	24	Percent Uptake * Type	Between Groups (Combined)	147.015	1	147.015	1.239	.328
			Within Groups	474.800	4	118.700		
			Total	621.815	5			
DZP	1	Percent Uptake * Type	Between Groups (Combined)	421.682	1	421.682	11.948	**.026**
			Within Groups	141.167	4	35.292		
			Total	562.848	5			
	2	Percent Uptake * Type	Between Groups (Combined)	285.660	1	285.660	13.712	**.021**
			Within Groups	83.333	4	20.833		
			Total	368.993	5			
	4	Percent Uptake * Type	Between Groups (Combined)	245.760	1	245.760	6.415	.064
			Within Groups	153.233	4	38.308		
			Total	398.993	5			
	8	Percent Uptake * Type	Between Groups (Combined)	308.167	1	308.167	36.078	**.004**
			Within Groups	34.167	4	8.542		
			Total	342.333	5			
	24	Percent Uptake * Type	Between Groups (Combined)	220.827	1	220.827	6.695	.061
			Within Groups	131.933	4	32.983		
			Total	352.760	5			
EE2	1	Percent Uptake * Type	Between Groups (Combined)	1561.707	1	1561.707	10.935	**.030**
			Within Groups	571.253	4	142.813		
			Total	2132.960	5			
	2	Percent Uptake * Type	Between Groups (Combined)	38.002	1	38.002	.953	.384
			Within Groups	159.507	4	39.877		
			Total	197.508	5			
	4	Percent Uptake * Type	Between Groups (Combined)	1053.375	1	1053.375	7.950	**.048**
			Within Groups	529.973	4	132.493		
			Total	1583.348	5			
	8	Percent Uptake * Type	Between Groups (Combined)	622.202	1	622.202	10.657	**.031**
			Within Groups	233.547	4	58.387		
			Total	855.748	5			
	24	Percent Uptake * Type	Between Groups (Combined)	143.082	1	143.082	3.068	.155
			Within Groups	186.567	4	46.642		
			Total	329.648	5			

Table A2.8. ANOVA analysis of root uptake in experiments with shoots removed from *Salix exigua* among compounds over time. Subsequent Tamhane post hoc analysis in Figure A2.9.

Time (hrs)			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
1	Percent Uptake * Compound	Between Groups (Combined)	417.528	2	208.764	.905	.426
		Within Groups	3461.243	15	230.750		
		Total	3878.771	17			
2	Percent Uptake * Compound	Between Groups (Combined)	2611.963	2	1305.982	8.132	**.004**
		Within Groups	2409.062	15	160.604		
		Total	5021.025	17			
4	Percent Uptake * Compound	Between Groups (Combined)	2813.521	2	1406.761	7.772	**.005**
		Within Groups	2715.110	15	181.007		
		Total	5528.631	17			
8	Percent Uptake * Compound	Between Groups (Combined)	5032.588	2	2516.294	19.778	**.000**
		Within Groups	1908.395	15	127.226		
		Total	6940.983	17			
24	Percent Uptake * Compound	Between Groups (Combined)	7408.528	2	3704.264	42.603	**.000**
		Within Groups	1304.223	15	86.948		
		Total	8712.751	17			

Table A2.9. Tamhane post hoc analysis for ANOVA analysis of root uptake in experiments with shoots removed from *Salix exigua* among compounds presented in Table A2.7.

Time (hrs)	*(I) Cmpd.*	*(J) Cmpd.*	*Mean Diff. (I-J)*	*Std. Error*	*Sig.*	*95% Confidence Interval*	
						Lower Bound	*Upper Bound*
1	ATZ	DZP	-1.167	6.6540	.998	-20.284	17.951
		EE2	-10.750	9.8292	.665	-40.121	18.621
	DZP	ATZ	1.167	6.6540	.998	-17.951	20.284
		EE2	-9.583	9.4795	.717	-38.582	19.416
	EE2	ATZ	10.750	9.8292	.665	-18.621	40.121
		DZP	9.583	9.4795	.717	-19.416	38.582
2	ATZ	DZP	11.033	8.5859	.561	-15.800	37.867
		EE2	-18.183	8.2463	.194	-45.087	8.720
	DZP	ATZ	-11.033	8.5859	.561	-37.867	15.800
		EE2	**-29.217(*)**	4.3455	.000	-41.870	-16.564
	EE2	ATZ	18.183	8.2463	.194	-8.720	45.087
		DZP	**29.217(*)**	4.3455	.000	16.564	41.870
4	**ATZ**	DZP	-5.383	6.1421	.787	-23.251	12.484
		EE2	**-28.800(*)**	8.7866	.029	-54.603	-2.997
	DZP	ATZ	5.383	6.1421	.787	-12.484	23.251
		EE2	-23.417	8.1288	.066	-48.374	1.540
	EE2	**ATZ**	**28.800(*)**	8.7866	.029	2.997	54.603
		DZP	23.417	8.1288	.066	-1.540	48.374
8	**ATZ**	DZP	-5.200	5.9235	.787	-22.552	12.152
		EE2	**-37.783(*)**	7.2251	.001	-58.483	-17.084
	DZP	ATZ	5.200	5.9235	.787	-12.152	22.552
		EE2	**-32.583(*)**	6.3195	.002	-51.321	-13.846
	EE2	**ATZ**	**37.783(*)**	7.2251	.001	17.084	58.483
		DZP	**32.583(*)**	6.3195	.002	13.846	51.321
24	**ATZ**	DZP	-12.250	5.6996	.167	-28.796	4.296
		EE2	**-47.833(*)**	5.6316	.000	-64.240	-31.427
	DZP	ATZ	12.250	5.6996	.167	-4.296	28.796
		EE2	**-35.583(*)**	4.7694	.000	-49.228	-21.939
	EE2	**ATZ**	**47.833(*)**	5.6316	.000	31.427	64.240
		DZP	**35.583(*)**	4.7694	.000	21.939	49.228

* The mean difference is significant at the .05 level.

Chapter 3

Table A3.1. *Arabidopsis* ANOVA analysis among compounds for averaged cumulative transpiration volume, root, shoot and total fresh weight of replicate plants.

		Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
Root fr. wt. (g) * Compound	Between Groups (Combined)	.059	3	.020	.250	.860
	Within Groups	1.802	23	.078		
	Total	1.861	26			
Shoot fr. wt. (g) * Compound	Between Groups (Combined)	.567	3	.189	.638	.598
	Within Groups	6.814	23	.296		
	Total	7.382	26			
Total fr. wt. (g) * Compound	Between Groups (Combined)	.873	3	.291	.473	.704
	Within Groups	14.160	23	.616		
	Total	15.033	26			
Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	12.700	3	4.233	.980	.420
	Within Groups	99.402	23	4.322		
	Total	112.102	26			

Table A3.2. *Arabidopsis* ANOVA comparison among the different compounds of the mean transpired volume at each sampling time. Subsequent LSD post hoc analysis presented in Table A3.3.

Time (hours)			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
2	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	1.231	3	.410	6.576	.002
		Within Groups	1.435	23	.062		
		Total	2.667	26			
4	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	2.941	3	.980	6.533	.002
		Within Groups	3.452	23	.150		
		Total	6.394	26			
10	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	3.832	3	1.277	.810	.501
		Within Groups	36.256	23	1.576		
		Total	40.088	26			
24	Cumulative Transpiration (mL) * Compound	Between Groups (Combined)	12.700	3	4.233	.980	.420
		Within Groups	99.402	23	4.322		
		Total	112.102	26			

Table A3.3. LSD post hoc analysis for *Arabidopsis* cumulative transpiration among compounds for each sampling time. Extension of Table A3.2.

Time (hrs)	*(I) Cmpd.*	*(J) Cmpd.*	*Mean Diff. (I-J)*	*Std. Error*	*Sig.*	*95% Confidence Interval*	
						Lower Bound	*Upper Bound*
2	**ATZ**	**DTZ**	**.3167(*)**	.15127	.048	.0037	.6296
		DZP	**.3583(*)**	.15127	.027	.0454	.6713
		EE2	**.6000(*)**	.13683	.000	.3169	.8831
	DTZ	**ATZ**	**-.3167(*)**	.15127	.048	-.6296	-.0037
		DZP	.0417	.14423	.775	-.2567	.3400
		EE2	**.2833(*)**	.12901	.038	.0165	.5502
	DZP	**ATZ**	**-.3583(*)**	.15127	.027	-.6713	-.0454
		DTZ	-.0417	.14423	.775	-.3400	.2567
		EE2	.2417	.12901	.074	-.0252	.5085
	EE2	**ATZ**	**-.6000(*)**	.13683	.000	-.8831	-.3169
		DTZ	**-.2833(*)**	.12901	.038	-.5502	-.0165
		DZP	-.2417	.12901	.074	-.5085	.0252
4	**ATZ**	DTZ	.4250	.23459	.083	-.0603	.9103
		DZP	.2167	.23459	.365	-.2686	.7020
		EE2	**.8500(*)**	.21220	.001	.4110	1.2890
	DTZ	ATZ	-.4250	.23459	.083	-.9103	.0603
		DZP	-.2083	.22367	.361	-.6710	.2544
		EE2	**.4250(*)**	.20006	.045	.0111	.8389
	DZP	ATZ	-.2167	.23459	.365	-.7020	.2686
		DTZ	.2083	.22367	.361	-.2544	.6710
		EE2	**.6333(*)**	.20006	.004	.2195	1.0472
	EE2	**ATZ**	**-.8500(*)**	.21220	.001	-1.2890	-.4110
		DTZ	**-.4250(*)**	.20006	.045	-.8389	-.0111
		DZP	**-.6333(*)**	.20006	.004	-1.0472	-.2195
10	ATZ	DTZ	1.0000	.76026	.201	-.5727	2.5727
		DZP	1.0000	.76026	.201	-.5727	2.5727
		EE2	.9250	.68768	.192	-.4976	2.3476
	DTZ	ATZ	-1.0000	.76026	.201	-2.5727	.5727
		DZP	.0000	.72488	1.000	-1.4995	1.4995
		EE2	-.0750	.64835	.909	-1.4162	1.2662
	DZP	ATZ	-1.0000	.76026	.201	-2.5727	.5727
		DTZ	.0000	.72488	1.000	-1.4995	1.4995
		EE2	-.0750	.64835	.909	-1.4162	1.2662
	EE2	ATZ	-.9250	.68768	.192	-2.3476	.4976
		DTZ	.0750	.64835	.909	-1.2662	1.4162
		DZP	.0750	.64835	.909	-1.2662	1.4162
24	ATZ	DTZ	1.7250	1.25884	.184	-.8791	4.3291
		DZP	2.0167	1.25884	.123	-.5874	4.6208
		EE2	1.2000	1.13866	.303	-1.1555	3.5555
	DTZ	ATZ	-1.7250	1.25884	.184	-4.3291	.8791
		DZP	.2917	1.20025	.810	-2.1912	2.7746
		EE2	-.5250	1.07354	.629	-2.7458	1.6958
	DZP	ATZ	-2.0167	1.25884	.123	-4.6208	.5874
		DTZ	-.2917	1.20025	.810	-2.7746	2.1912
		EE2	-.8167	1.07354	.455	-3.0375	1.4041
	EE2	ATZ	-1.2000	1.13866	.303	-3.5555	1.1555
		DTZ	.5250	1.07354	.629	-1.6958	2.7458
		DZP	.8167	1.07354	.455	-1.4041	3.0375

Table A3.4. Correlation analysis between uptake from solution, plant fresh weight (root, shoot and total) and cumulative transpired volume, at each sampling time. Spearman's rho.

	Time (hours)		*Root fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total fr. wt. (g)*	*Cum. Trans.(mL)*
ATZ	2	Correlation Co.	.667	-.300	.700	.527
		Sig. (2-tailed)	.219	.624	.188	.361
	4	Correlation Co.	.667	-.300	.700	.527
		Sig. (2-tailed)	.219	.624	.188	.361
	10	Correlation Co.	.667	.100	**.900***	.800
		Sig. (2-tailed)	.219	.873	.037	.104
	24	Correlation Co.	.667	.100	**.900***	.800
		Sig. (2-tailed)	.219	.873	.037	.104
DTZ	2	Correlation Co.	.086	-.143	-.143	-.131
		Sig. (2-tailed)	.872	.787	.787	.805
	4	Correlation Co.	-.371	-.429	-.429	.507
		Sig. (2-tailed)	.468	.397	.397	.305
	10	Correlation Co.	.029	-.086	-.086	-.319
		Sig. (2-tailed)	.957	.872	.872	.538
	24	Correlation Co.	-.143	-.200	-.200	-.657
		Sig. (2-tailed)	.787	.704	.704	.156
DZP	2	Correlation Co.	.257	.143	.086	.655
		Sig. (2-tailed)	.623	.787	.872	.158
	4	Correlation Co.	.371	.486	.314	-.638
		Sig. (2-tailed)	.468	.329	.544	.173
	10	Correlation Co.	-.257	.086	-.086	-.429
		Sig. (2-tailed)	.623	.872	.872	.397
	24	Correlation Co.	-.200	-.086	-.143	-.143
		Sig. (2-tailed)	.704	.872	.787	.787
EE2	2	Correlation Co.	**.721***	**.673***	**.685***	.436
		Sig. (2-tailed)	.019	.033	.029	.208
	4	Correlation Co.	.491	.418	.442	.430
		Sig. (2-tailed)	.150	.229	.200	.215
	10	Correlation Co.	.552	.467	.491	.245
		Sig. (2-tailed)	.098	.174	.150	.496
	24	Correlation Co.	.588	.539	.552	.535
		Sig. (2-tailed)	.074	.108	.098	.111

* Correlation is significant at the 0.05 level (2-tailed).

Table A3.5. *Arabidopsis* correlation analysis of cumulative transpired volume at each sampling time correlated to plant fresh weights (root, shoot and total). Spearman's rho.

	Time (hours)		*Root fr. wt. (g)*	*Shoot fr. wt. (g)*	*Total fr. wt. (g)*
ATZ	2	Correlation Co.	.108	-.369	.316
		Sig. (2-tailed)	.863	.541	.604
	4	Correlation Co.	.108	-.369	.316
		Sig. (2-tailed)	.863	.541	.604
	10	Correlation Co.	.564	.400	**.900***
		Sig. (2-tailed)	.322	.505	.037
	24	Correlation Co.	.564	.400	**.900***
		Sig. (2-tailed)	.322	.505	.037
DTZ	2	Correlation Co.	-.393	-.393	-.393
		Sig. (2-tailed)	.441	.441	.441
	4	Correlation Co.	.541	.541	.541
		Sig. (2-tailed)	.268	.268	.268
	10	Correlation Co.	.783	.725	.725
		Sig. (2-tailed)	.066	.103	.103
	24	Correlation Co.	.771	**.829***	**.829***
		Sig. (2-tailed)	.072	.042	.042
DZP	2	Correlation Co.	.655	.393	.393
		Sig. (2-tailed)	.158	.441	.441
	4	Correlation Co.	.232	.290	.464
		Sig. (2-tailed)	.658	.577	.354
	10	Correlation Co.	.314	.371	.543
		Sig. (2-tailed)	.544	.468	.266
	24	Correlation Co.	.486	.600	.714
		Sig. (2-tailed)	.329	.208	.111
EE2	2	Correlation Co.	**.658***	**.658***	**.703***
		Sig. (2-tailed)	.039	.039	.023
	4	Correlation Co.	**.823****	**.898****	**.860****
		Sig. (2-tailed)	.003	.000	.001
	10	Correlation Co.	**.826****	**.924****	**.887****
		Sig. (2-tailed)	.003	.000	.001
	24	Correlation Co.	**.906****	**.967****	**.942****
		Sig. (2-tailed)	.000	.000	.000

***** Correlation is significant at the 0.05 level (2-tailed).
****** Correlation is significant at the 0.01 level (2-tailed).

Chapter 5

Table A5.1. ANOVA comparison of root and shoot fresh weight for *Arabidopsis* and willow among compounds.

Cmpd			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
ATZ	Root * Plant	Between Groups (Combined)	.101	1	.101	2.106	.181
		Within Groups	.431	9	.048		
		Total	.532	10			
	Shoot * Plant	Between Groups (Combined)	1.549	1	1.549	29.857	**.000**
		Within Groups	.467	9	.052		
		Total	2.016	10			
DTZ	Root * Plant	Between Groups (Combined)	.241	1	.241	5.458	**.048**
		Within Groups	.353	8	.044		
		Total	.593	9			
	Shoot * Plant	Between Groups (Combined)	2.477	1	2.477	26.919	**.001**
		Within Groups	.736	8	.092		
		Total	3.213	9			
DZP	Root * Plant	Between Groups (Combined)	.078	1	.078	1.594	.235
		Within Groups	.492	10	.049		
		Total	.570	11			
	Shoot * Plant	Between Groups (Combined)	.859	1	.859	5.340	**.043**
		Within Groups	1.608	10	.161		
		Total	2.467	11			
EE2	Root * Plant	Between Groups (Combined)	.380	1	.380	4.179	.060
		Within Groups	1.273	14	.091		
		Total	1.653	15			
	Shoot * Plant	Between Groups (Combined)	4.871	1	4.871	13.261	**.003**
		Within Groups	5.142	14	.367		
		Total	10.013	15			

Table A5.2. ANOVA comparison of total volume transpired among *Arabidopsis* and willow for the four compounds from the 24 hours uptake studies.

			Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
ATZ	CumTrans * Plant	Between Groups (Combined)	31.527	1	31.527	4.636	.060
		Within Groups	61.200	9	6.800		
		Total	92.727	10			
DTZ	CumTrans * Plant	Between Groups (Combined)	.038	1	.038	.018	.897
		Within Groups	16.719	8	2.090		
		Total	16.756	9			
DZP	CumTrans * Plant	Between Groups (Combined)	70.083	1	70.083	3.739	.082
		Within Groups	187.417	10	18.742		
		Total	257.500	11			
EE2	CumTrans * Plant	Between Groups (Combined)	2.204	1	2.204	.486	.497
		Within Groups	63.483	14	4.535		
		Total	65.687	15			

Table A5.3a. ANOVA comparison for percent uptake between *Arabidopsis* and willow across each sampling time for 17α-ethynylestradiol. Note that sampling time for willow was 0, 2, 4, 8, 24 and *Arabidopsis* was 0, 2, 4, 10, 24, therefore times t = 8 and t = 10 are considered the same sampling time for these comparisons.

	Time			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
17α-ethynylestradiol	2.00	Uptake * Plant	Between Groups (Combined)	2478.958	1	2478.958	39.795	**.000**
			Within Groups	872.114	14	62.294		
			Total	3351.072	15			
	4.00	Uptake * Plant	Between Groups (Combined)	641.280	1	641.280	10.738	**.006**
			Within Groups	836.049	14	59.718		
			Total	1477.329	15			
	8.00	Uptake * Plant	Between Groups (Combined)	139.416	1	139.416	2.680	.124
			Within Groups	728.338	14	52.024		
			Total	867.754	15			
	24.00	Uptake * Plant	Between Groups (Combined)	40.788	1	40.788	2.689	.123
			Within Groups	212.332	14	15.167		
			Total	253.120	15			

Table A5.3b. ANOVA comparison for percent uptake between *Arabidopsis* and willow across each sampling time for diltiazem. Note that sampling time for willow was 0, 2, 4, 8, 24 and *Arabidopsis* was 0, 2, 4, 10, 24, therefore times t = 8 and t = 10 are considered the same sampling time for these comparisons.

	Time			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
diltiazem	2.00	Uptake * Plant	Between Groups (Combined)	1107.165	1	1107.165	7.468	**.026**
			Within Groups	1186.008	8	148.251		
			Total	2293.174	9			
	4.00	Uptake * Plant	Between Groups (Combined)	1554.282	1	1554.282	15.521	**.004**
			Within Groups	801.135	8	100.142		
			Total	2355.417	9			
	8.00	Uptake * Plant	Between Groups (Combined)	2551.928	1	2551.928	26.128	**.001**
			Within Groups	781.349	8	97.669		
			Total	3333.277	9			
	24.00	Uptake * Plant	Between Groups (Combined)	996.664	1	996.664	15.777	**.004**
			Within Groups	505.366	8	63.171		
			Total	1502.030	9			

Table A5.3c. ANOVA comparison for percent uptake between *Arabidopsis* and willow across each sampling time for diazepam. Note that sampling time for willow was 0, 2, 4, 8, 24 and *Arabidopsis* was 0, 2, 4, 10, 24, therefore times t = 8 and t = 10 are considered the same sampling time for these comparisons.

	Time			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
diazepam	2.00	Uptake * Plant	Between Groups (Combined)	143.037	1	143.037	3.422	.094
			Within Groups	417.994	10	41.799		
			Total	561.031	11			
	4.00	Uptake * Plant	Between Groups (Combined)	34.544	1	34.544	1.002	.340
			Within Groups	344.753	10	34.475		
			Total	379.297	11			
	8.00	Uptake * Plant	Between Groups (Combined)	300.400	1	300.400	5.882	**.036**
			Within Groups	510.712	10	51.071		
			Total	811.112	11			
	24.00	Uptake * Plant	Between Groups (Combined)	17.017	1	17.017	.289	.603
			Within Groups	589.079	10	58.908		
			Total	606.096	11			

Table A5.3d. ANOVA comparison for percent uptake among *Arabidopsis* and willow across each sampling time for atrazine. Note that sampling time for willow was 0, 2, 4, 8, 24 and *Arabidopsis* was 0, 2, 4, 10, 24, therefore times t = 8 and t = 10 are considered the same sampling time for these comparisons.

	Time			*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
atrazine	2.00	Uptake * Plant	Between Groups (Combined)	398.992	1	398.992	11.070	**.009**
			Within Groups	324.390	9	36.043		
			Total	723.382	10			
	4.00	Uptake * Plant	Between Groups (Combined)	47.530	1	47.530	2.559	.144
			Within Groups	167.169	9	18.574		
			Total	214.699	10			
	8.00	Uptake * Plant	Between Groups (Combined)	29.305	1	29.305	.311	.591
			Within Groups	848.619	9	94.291		
			Total	877.925	10			
	24.00	Uptake * Plant	Between Groups (Combined)	9.598	1	9.598	.133	.724
			Within Groups	648.682	9	72.076		
			Total	658.280	10			

APPENDIX B Other experimental methods

Preliminary investigation: *Salix exigua*

Introduction. An initial investigation involving EE2 and several willow plants examined the effect of different concentrations, as well as uptake time, on the distribution of the EE2 with willow. As the number of plants is small, n = 1, the results presented here have not been affirmed, but may still suggest the effect of concentration and time on uptake and distribution of neutral pharmaceuticals.

Materials and methods. Three concentrations of unlabeled EE2 were added to three culture tubes, 0, 1, and 10 ug, with 16,667 Bq of ^{3}H-EE2 and 24 mL of hydroponic nutrient solution. Willows were inserted into each culture tube (with the cutting maintained out of the solution), the tubes covered with foil and harvested after 8 hours. The roots were rinsed with nutrient solution, the plants separated into roots, shoot and cutting, weighed fresh, and stored at -20°C until time of solvent extraction and oxidation.

As well, two other culture tubes were set up with 0 ug unlabeled EE2, 16,667 Bq ^{3}H-EE2, and 24 mL nutrient solution. Willows were inserted into the tubes and left for 24 and 48 hours before harvesting. At experiment termination, the roots were rinsed with nutrient solution and the plants separated into components, weighed fresh, and stored at -20°C until time of analysis.

Plant components were analyzed as described in section 2.2.5 and 2.2.6, including soluble extraction, and oxidation of residues for bound fractions and only oxidation of the cutting.

Results and discussion. Results from the preliminary investigation with EE2, although not replicated, still present useful information concerning what happens over time and uptake of different concentrations. Levels of recovery vary between the plants, but a general trend can still be extrapolated.

Uptake trends and values for the different concentrations were similar, except for the 8 hour 0 μg EE2 value (Figure B1.1).

Distribution of recovered radioactivity for the 3 plants exposed to 3 different concentrations of EE2 and harvested after 8 hours appear to be similar (Figure B1.2). It appears though, that lower concentrations are taken up at a faster rate than higher concentrations. Proportions of root soluble ^{3}H-EE2 appear to increase with decreasing concentrations. If uptake and distribution varies with concentration, it does not appear to be significant with these results.

Distribution of recovered radioactivity for the 3 plants exposed to one concentration of EE2 (0 ug + 16,667 Bq ^{3}H-EE2) and one plant harvested at 8, 24, and 48 hours clearly show differences (Figure B1.3). At 8 hours there is a considerably higher proportion of root soluble ^{3}H-EE2 than at 24 and 48 hours, with 48% versus 7% for both 24 and 48 hours. By 24 hours, the proportion of root bound ^{3}H-EE2 has increased to 75% and at 48

hours 98%, compared to the 48% at 8 hours. Proportions within the shoot are close to the same, and therefore difficult to determine if there are differences over time.

Conclusions. Considering the data presented, there appears to be little effect of concentration on uptake and distribution for EE2 and willow, but a definite effect of time on distribution within the plants. With increased in-plant time, the proportion of compound becoming bound to the root also increased (with the coinciding decrease in soluble compound). Although the results have not been replicated, they do provide suggestions into the effect of concentration and time on the uptake and distribution of EE2, and possibly other neutral pharmaceuticals, within *Salix exigua*.

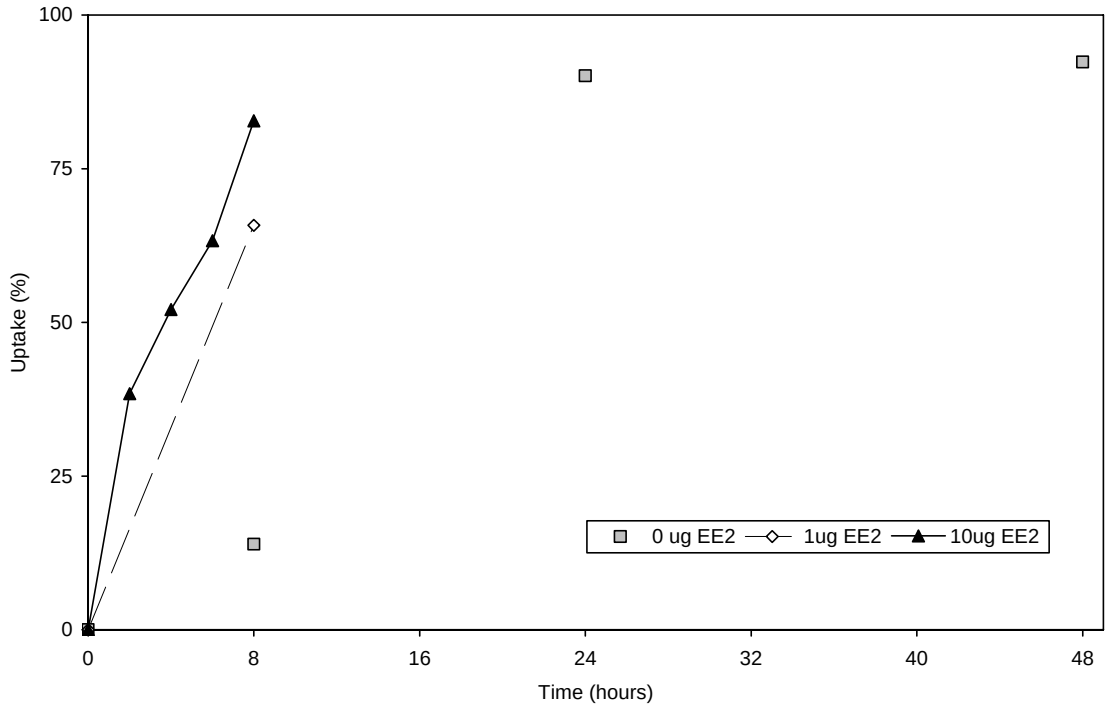
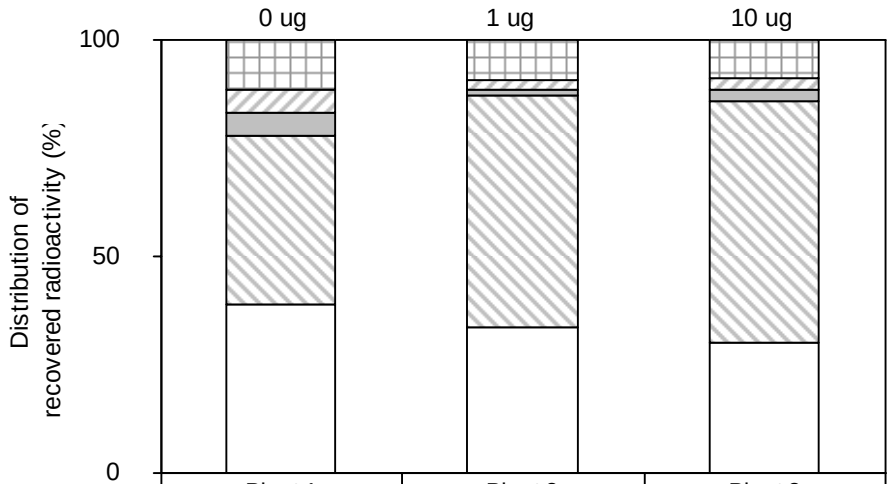
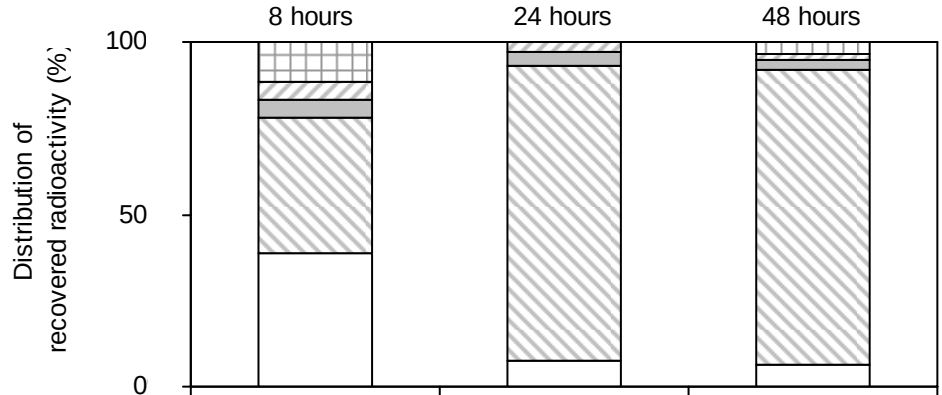


Figure B1.1. Uptake of 3 concentrations of 17α-ethynylestradiol (EE2) (0, 1, and 10 μg EE2 + 16,667 Bq ^{3}H-EE2) from 24 mL of solution. 0 μg EE2, n = 3, one plant harvested at 8, 24 and 48 hours; 1 μg EE2, n = 1, plant harvested at 8 hours; 10 μg EE2, n = 1, solution sampled at 2, 4, 6, and 8 hours with harvest at 8 hours.



	Plant 1	Plant 2	Plant 3
Wood, Whole, Oxidized	14.2	6.7	5.8
Shoot Bound, Oxidized Residue	6.5	1.6	1.7
Shoot Soluble, Solvent Extract	6.6	0.8	1.8
Root Bound, Oxidized Residue	48.3	37.8	36.7
Root Soluble, Solvent Extract	48.1	23.9	19.8

Figure B1.2. Distribution of recovered radioactivity from 3 *Salix exigua* cuttings exposed to 16,667 Bq ^{3}H-17α-ethynylestradiol and 3 concentrations of 17α-ethynylestradiol (EE2); 0, 1 and 10 μg, in 24 mL of nutrient solution. All three plants were harvested after 8 hours. Recovery values are presented in the table.



	Plant 1	Plant 2	Plant 3
Wood, Whole, Oxidized	14.2	n/a	4.0
Shoot Bound, Oxidized Residue	6.5	2.7	2.0
Shoot Soluble, Solvent Extract	6.6	3.3	3.1
Root Bound, Oxidized Residue	48.3	75.0	97.7
Root Soluble, Solvent Extract	48.1	6.6	7.2

Figure B1.3. Distribution of recovered radioactivity from 3 *Salix exigua* cuttings exposed to one concentration of 17α-ethynylestradiol (EE2) (0 μg) containing 16,667 Bq ^{3}H-17α-ethynylestradiol in 24 mL solution. One plant was harvested at each of 8, 24 and 48 hours. Recovery values are presented in the table. Plant 2 Wood, Whole, Oxidized value is not zero, but oxidation was not performed on the cutting as the cutting had previously undergone solvent extraction and oxidation was not possible.

Cell wall fractionation and cell wall dissolution

Several methods were attempted in an effort to release the bound compounds for quantification, but neither achieved the desired results. A process of step-wise cell wall fractionation was adapted from Laurent and Scalla (2000)[1] and cell wall dissolution adapted from Lu and Ralph (2003)[2] were attempted. Both processes result in samples that are permanently pigmented primarily due to lignin, which interferes with LSC.

Cell wall fractionation. A step-wise procedure to break down the cell wall into 5 constituent parts was attempted. The steps are outlined below in order of procedure, each commencing with a period of sonication prior to heating or cooling. Between each step, neutralizing the solution, filtration through glass fiber discs (GF/D) and rinsing with water were performed. Both the remaining residue and the glass fiber were carried into the next fractionation step to ensure no losses of cell wall particulates.

1. Pectins – water, 4 °C, 1 h + 100 °C, 2 h, then EDTA, 0.5%, 100 °C, 1 h
2. Hemicellulose I – KOH, 4%, 3 x 6 h
3. Lignin – $NAClO_4$ (sodium perchlorate), 1%, 80 °C, 3 x 3 h
4. Hemicellulose II – KOH, 24%, 3 x 6 h
5. Cellulose – H_2SO_4, 72%, 20 °C, 1 h, then 3%, 120 °C, 1h

This method was not suitable for extracting and measuring bound radioactivity. Removal of fractions involving an acid or base resulted in pigmented samples. The transfer of residue and glass fiber into the next step resulted in a large mass of glass fiber by the end of the procedure which made it difficult to ensure a sufficient volume was being used to rinse the fraction of interest from the fiber (also resulted in large volumes of solution with small amounts of radioactivity). As well, there was no way to ensure that the glass fiber was successfully preventing the flow of non-step specific cell wall constituents into the collected fraction. Neutralization of acidic and basic fractions with addition of dilute bases and acids also resulted in a large final volume to radioactivity ratio that made it difficult to measure the radioactivity.

Cell wall dissolution. A method combining dimethylsulfoxide (DMSO) and tetrabutylammonium fluoride (TBAF) was reported for use of dissolution of cell walls in the examination of constituents for high-resolution solution-state NMR spectroscopy, resulting in a transparent solution. This method was intriguing as it offered a means of complete dissolution of large particulates that could block tritium beta energy. A ratio of dried, ground residue to volume of DMSO and weight of TBAF was determined to be 225 mg: 6 mL DMSO: 1 g TBAF. The mixture was heated to 60 °C for 30 to 60 min. The result was a transparent, brown solution, with fine particles that readily settled out. This method was not suitable for removal and measuring of bound radioactivity. The coloration was not removable (and interfered with LSC) and the ratio of dried sample to DMSO:TBAF volume was large and therefore not cost effective and could result in a

[1] Laurent, F and Scalla, R. 2000. Phenoxyacetic acid residue incorporation in cell walls of soybean (*Glycine max.*). *J. Agric. Food Chem.* 48: 4389-4398.

[2] Lu, F and Ralph, J. 2003 Non-degredative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *The Plant Journal* 35: 535-544.

small amount of radioactivity within a large volume of solution, making it difficult to measure.

SPE of Pigmented Plant Extract Soluble Fractions

Examination of distribution of pharmaceuticals within *Arabidopsis* initially involved a purification step of pigmented extracts of shoots prior to LSC of the soluble fraction. The pigmented extracts were run through a 1 g – C18 column and stripped with 80 % aqueous methanol (methanol : water 80 : 20). Counts of the purified extracts appeared low. Concern that losses were occurring during this step, an alternative method involving sodium hypochlorite (bleach) was used to create samples that could be reliably analyzed by LSC (method discussed below). Commercially available bleach (5.25 % chlorine) was added to pigmented samples, heated until clear, and then analyzed by LSC. Comparison of recovery results for *Arabidopsis* DTZ and DZP replicates show marked increase in recovery with the addition of bleach over purification (Figure B1.4).

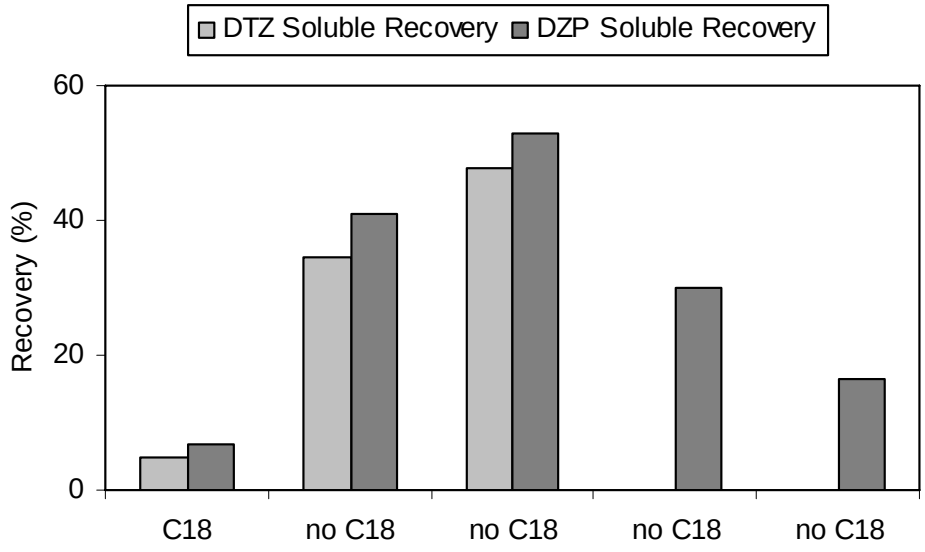


Figure B1.4. Total recovered soluble radioactivity for two methods of sample preparation prior to LSC for DTZ and DZP shoots. C18, soluble fractions underwent C18 purification to remove pigments; no C18, the soluble fractions were bleached to remove pigments. 5 individual examples are shown for DZP and 3 for DTZ.

LSC Pigmented Plant Samples – the effects of bleach

To determine the quantity of bleach necessary to achieve full recovery of radioactivity, fresh *Arabidopsis* leaves were extracted, aliquots taken and radioactive standard added. Bleach was added in increasing amounts and the samples analyzed by LSC. The process and final suggested procedure for bleaching is outlined below.

Methods. Fresh *Arabidopsis* leaves were solvent extracted as described in section 2.2.5. Following sampling techniques, 2 x 1.5 mL and 2 x 2.0 mL green samples were taken, radiolabeled standard was added to the pigmented extract (2000 Bq of ^{14}C) and counted by LSC.

Another set of aliquots were taken (1.5 mL and 2.0 mL volumes) and radiolabeled standard was added (2000 Bq ^{14}C). Sodium hypochlorite (chlorine bleach, pH 12.42) was used in 5 µL increments from 10 to 35 µL and at 50 µL for the 1.5 mL aliquots and 10, 15, 25 and 30 µL for the 2.0 mL aliquots. The vials capped, shaken, heated to 60 °C for 2 hours and dark adapted prior to LSC analysis.

Results. Recovery of the radioactive standard improved with increasing bleach quantities up to approximately 30 µL (Figure B1.5) for both the 1.5 and 2.0 mL aliquot sizes.

Suggested procedure based on above results:

1. Place 1.5 mL* green sample in scintillation vial.
2. Add 50 µL of sodium hypochlorite solution (5.25% household bleach).
3. Cap tightly, shake, and heat to 50 - 60°C for 1-2 hours (time is based on removal of pigmentation).
4. Cool to room temp and vent chlorine gas into fume hood. Blow out any remaining in the vial with N2 stream.
5. Add 5 mL scintillation cocktail. Shake well. Wipe vial with anti-static sheet. Dark adapt for at least an hour before counting.

The quantity of bleach may vary depending on the degree of pigmentation. It may be necessary to add as much as 200 uL of bleach to create a clear sample, but care must be taken to ensure that phase separation does not occur upon the addition of scintillation cocktail. Bleach quantity may also decrease the pH of the sample past the capabilities of the cocktail. As well, the volume of bleach added may result in chemoluminescence and interfere with counting efficiency that can be noted by changes in recovery of the same sample over time.[3]

* This volume was found to be a near maximum that could be counted in 7 mL scintillation vials upon the addition of 5 mL cocktail for a sample containing aqueous MeOH with around 6% H2O.

[3] Smith, IK and Lang, AL. 1987. Decoloration and solubilization of plant tissue prior to determination of ^{3}H, ^{14}C, and ^{35}S by liquid scintillation. *Anal. Biochem.* 164: 531-536.

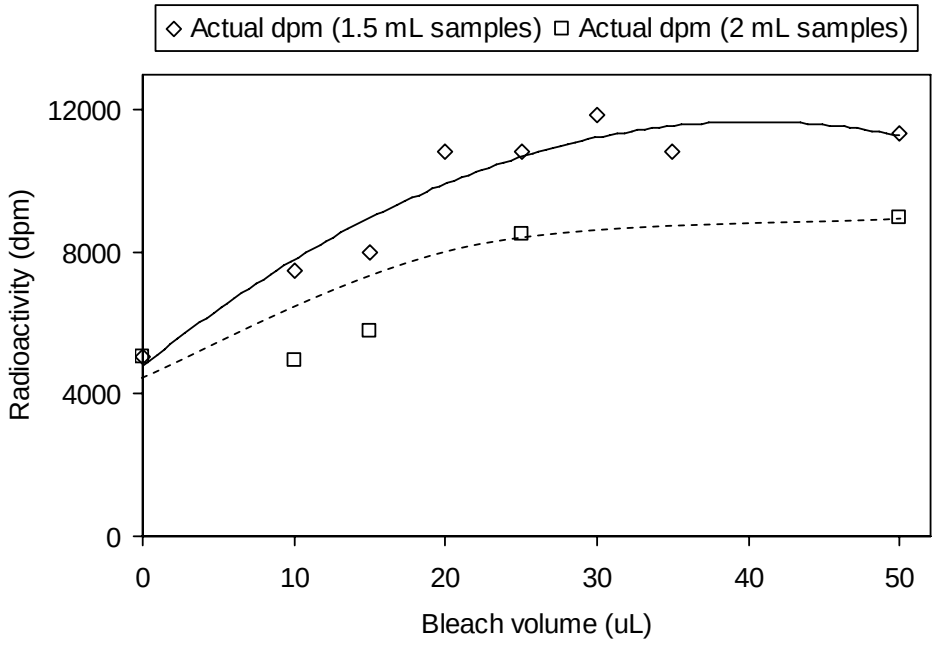


Figure B1.5. Effects of bleach on LSC analysis of pigmented plant extracts. Two pigmented sample volumes (1.5 mL and 2 mL) with the addition of approximately 2000 Bq ^{14}C were treated to increasing volumes of bleach (sodium hypochlorite). Bleached samples were heated to 60 °C for 2 hours and allowed to dark adapt prior to LSC analysis.

APPENDIX C NMR analysis of 17α-ethynylestradiol

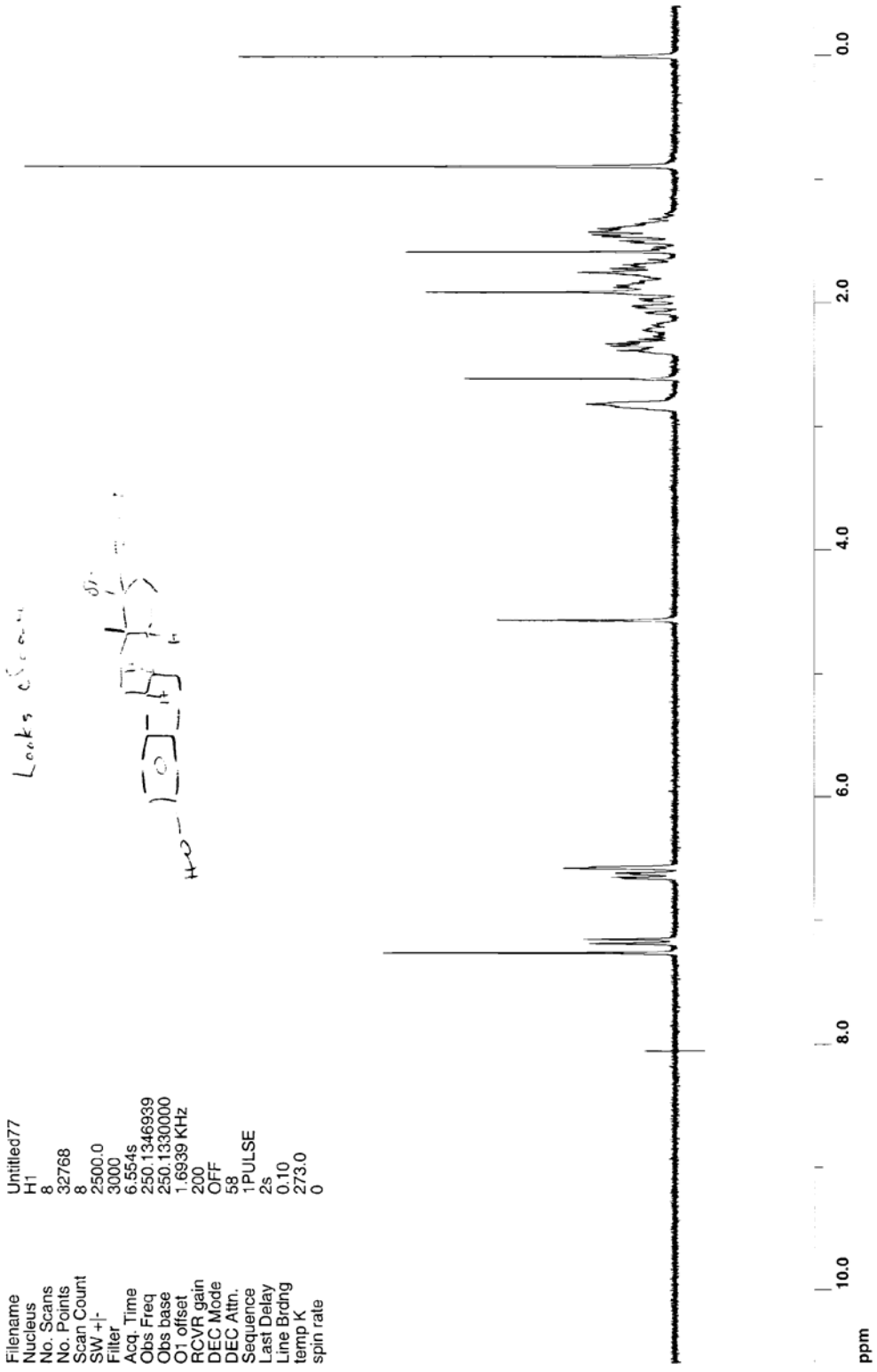


Figure C1.1. NMR purity analysis (clean standard) of 17α-ethynylestradiol performed by Dr. P. Dibble.

APPENDIX D The pharmaceuticals and herbicide: digging deeper

17α-ethynylestradiol. A synthetic estrogen, 17α-ethynylestradiol (EE2), is a common oral contraceptive used in birth control pills. Although the estrogenic effects of EE2 were known in the 1940's, and it was used widespread as a contraceptive by the 1960's, the first report on its metabolism in man was not until 1969 (Reed et al., 1972).

Synthetic estrogens are more stable in biological environments, lending to their widespread use. They are not metabolized easily and remain in active form for longer periods of time than natural estrogens.Due to the concerns generated by EE2 and its presence in the environment, research into wastewater and sewage treatment plant methods for degrading this estrogen into less active compounds has increased. Chlorination of EE2 resulted in rapid consumption, but the chlorinated products remained estrogenic and stable for long periods of time in the chlorine environment (Moriyama et al., 2004).

Evidence suggests that some plants have their own endogenous levels of mammalian sex hormones during peak times of reproduction and development. The role of these hormones is not yet understood and neither are the biochemical pathways. If these hormones are produced endogenously and play an important role in flower and embryo development, then is it possible that exogenous hormones, proven to be readily taken up, may exhibit some effect on the development, timing, and endogenous levels produced? If animals taking in phytoestrogens through the food they eat, or as pharmaceutical products, exhibit effects of the hormones on their own systems, why would plants not have the same effects on them in the uptake of our excreted hormones?

There are currently 6 primary phytohormones (hormones specifically found within plants); auxins, cytokinins, gibberellins (GA), abscisic acid, jasmonats, and ethylene (Kimball, 2006). Another group, the brassinosteroids (BR), is more recently emerging and characterized as influential growth regulators (Halliday, 2004). Generally, all phytohormones play diverse roles in growth and development, and even adaptations to stress. It has even been suggested that plants contain the more mammalian hormones estrone, estradiol, and estriol (Khaleel et al., 2003). Examining the chemical structure of 4 phytohormones, auxin, cytokinin, GA and brassinolind (Figure 5.1), and the structure of mammalian hormones, estrone, estradiol, progesterone and the synthetic estrogen EE2 (Figure 5.1), there are notable similarities. Not only are there structural similarities, but octanol-water coefficients for the hormones are similar, encompassing a range that allows them to diffuse across plasma membranes (Kimball, 2006). BRs are the most structurally similar to mammalian steroids and have been found to influence cell division and elongation of cells, including root cells (Amzallag and Goloubinoff, 2003). Their presence in the roots may allow for an interaction of the structurally similar EE2 with the BR receptor, assuming it has a relatively low degree of specificity.

Hormone signaling in both plants and animals is typically initiated by binding of the ligand to a receptor protein that may be located within the plasma membrane, or is found soluble within the cytosol or nucleus of the cell. Current understanding of plant hormone signaling is limited. Evidence for specific plant hormone receptors is just being documented. In 2005, it was proposed that a soluble GA receptor is present in the nucleus

that once GA binds to, induces a chain of events that still remain unclear (Ueguchi-Tanaka et al., 2005). Cytokinins bind to a trans-membrane protein that then induces a chain of signaling (Ferreira and Kieber, 2005). Recent work has linked auxin receptors to a soluble protein (Dharmasiri et al., 2003 and 2005). BR signaling is linked to a membrane bound surface protein (Halliday, 2004; Vert et al., 2005). Research points to the existence of structurally similar animal G-proteins, responsible for transducing the action of a wide variety of extracellular signaling molecules, in plants that may play a role in hormone signaling (Hooley, 1999; Jones and Assmann, 2004). Studies involving *Arabidopsis* uptake of mammalian estrogens have no problems with the removal of the hormones from the growing medium (Janeczko et al., 2003).

Not only do mammalian hormones affect other animals, but they have been noted to induce effects in plants. When some of these hormones have been applied exogenously to plants cell division was stimulated, pollen germination, and growth and flowering (Geuns, 1978; Bhattacharya and Gupta, 1981; Hayat et al., 2001). As EE2 is a synthetic hormone with its precursor compounds estrone and 17β-estradiol, it could be suggested that EE2 would readily be taken into the plant just as natural hormones are and induce similar effects. In 2003, Janeczko et al. examined the influence of common sex hormones estrone, estriol, 17β-estradiol, androsterone, androstenedione, and progesterone on the flowering induction of *Arabidopsis*, with the male hormones inducing flowering and the female hormones generally inhibited generative plant numbers. There is some debate that mammalian sex hormones are naturally occurring within plants (Simons and Grinwich, 1989; Milanesi et al., 2001; Khaleel et al., 2003; Milanesi and Boland, 2004).

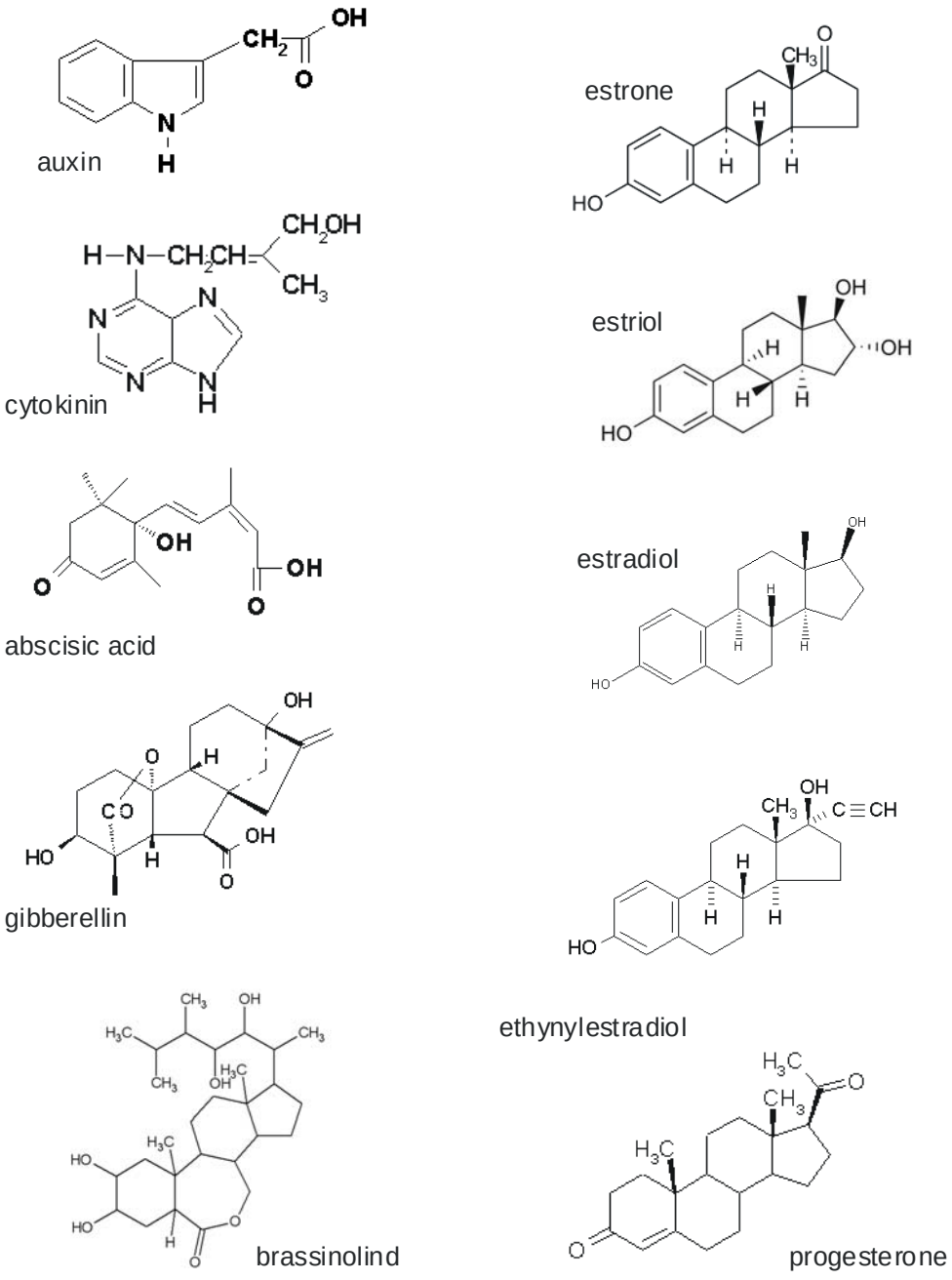


Figure D1.1. Plant steroid structures: auxin, cytokinin, abscisic acid, gibberellin and brassinolind and mammalian estrogen steroids and progesterone chemical structures: estrone, estriol, estradiol, ethynylestradiol and progesterone.

Diltiazem. Diltiazem (DTZ), or commonly known as Cardizem®, is a calcium-channel blocker used as an antihypertensive and muscle relaxant. DTZ is a member of benzothiazepine antihypertensive drugs, commonly grouped as a calcium channel blocker or calcium antagonist. Calcium antagonists were discovered in 1963 by chance, with their own drug designation given in 1969. DTZ joined this group when it was introduced in 1975 by Japanese pharmacologists (Yousef et al., 2005).

DTZ is a member of benzothiazepine antihypertensive drugs, commonly grouped as a calcium channel blocker or calcium antagonist. In animals, it is an L-type calcium channel blocker. Evidence suggests that plants contain Ca^{2+} channels structurally similar to animal L-type channels, likely providing binding sites within plant root plasma membranes for the pharmaceutical DTZ, without inhibiting Ca^{2+} uptake.

Mobilization of Ca2+ for signaling requires an initiator. The protein NAADP, has been identified as a potent mobilizer (Genazzani et al., 1997). In higher plants, diltiazem was found to inhibit NAADP (nicotinic acid adenine dinucleotide phosphate (a metabolite of NADP)) signaled release of Ca^{2+} suggesting structural similarities to animal L-type channels (Genazzani et al., 1997; Navazio et al., 2000). In plant root cells, the structurally similar L-type channels were relatively insensitive to DTZ with the influx of Ca^{2+} into the cytosol not inhibited by DTZ (Plieth, 2005).

These structurally similar L-type Ca^{2+} channels within plants could provide a site of action for DTZ and may result in a toxic effect for the plant.
Diazepam. Diazepam (DZP) is a benzodiazepine used as antianxiety agents, muscle relaxants, sedatives, hypnotics, and sometimes as anticonvulsants in both humans and animals. The specific chemical DZP is sold under the Valium trademark (Engelberg, 1982). DZP method of synthesis was developed in 1961, with market approval of Valium® granted in 1963 and patented in the United States in 1968 (Mune, 1990).

The environmental fate of DZP was investigated in a water/sediment system by Loffler et al. (2005) and was found to sorb to sediment at an elevated level, with persistence of > 365 days as near 100% parent compound. DZP is considered extremely stable in soils and during sewage treatment and relatively stable in surface waters excluding light-induced degradation (Loffler et al., 2005).

Benzodiazepine substances were originally thought to be a synthetic creation until their discovery in plant and animals in the mid-1980's (Kavvadias et al., 2000). Now endogenous benzodiazepines, including DZP, have been found in almost every organism tested (Unseld et al., 1989). Found to interact primarily with GABA receptors to exhibit their effect on the CNS of higher animals, now these receptors are being found within plants, bacteria and animals (Kinnersley and Lin, 2000; Papadopoulos, 2004).

Invertebrates form the bottom of many food chains therefore any drug that may impact population numbers is of concern. As DZP functions within the nervous system it can be potentially expected to induce effects in any organism with a nervous system, vertebrate

and invertebrate alike (Wildmann, 1988). Studies on nematodes have shown it to induce both relaxant and muscle activating responses via GABA receptors (Richmond and Jorgensen, 1999). Although in crayfish no modulation of the receptors was found (Pearstein et al., 1997). Receptors have been found in the CNS, peripheral systems, and in mitochondria of animals and bacteria (Slocinska et al., 2004).

The presence of GABA receptors in plants has been determined, but their location is still unresolved (Kinnersley and Lin, 2000). The association of GABA binding sites with G-proteins lends another receptor avenue, found within the plasma membrane (Jones and Assmann, 2004). Benzodiazepines have been identified within untreated animals and humans, with some speculation that it came from ingested plants (Kavvadias et al., 2000). It could be conceived that these similar receptors in plants could be places for the benzodiazepines to bind, as they do in animals, including DZP.

There is strong evidence that the roles of these receptors include steroid biosynthesis, control of mitochondrial respiration, cell proliferation, flow of calcium ions, cellular immunity, malignancy, and apoptosis (Slocinska et al., 2004). This variety of roles provides rationale for the presence of these compounds in plants. It could be possible for these natural benzodiazepines enhancing properties to assist these processes in plants and particularly in germination (Wildmann, 1988).

In potato, benzodiazepines and benzodiazepine receptors have been identified (Kavvadias et al., 2000; Corsi et al., 2004). It has been suggested that the existence of benzodiazepines and their receptors occurred before the divergence of the kingdoms and has been conserved across species (Papadopoulos, 2004). The exact role of these compounds and receptors in plants is not known, but evidence suggests roles in ion control (Kinnersley and Lin, 2000). In 1988, it was reported that levels of natural benzodiazepines increase by five-fold in seedlings during germination of wheat and potato, than of non-germinating tissue, suggesting a role of these compounds in the development of higher plants (Wildmann, 1988).

DZP has been reported to be oxidized by Cytochrome P450s (CYPs), particularly CYP3A4 to temazepam (hydroxylation) and CYP2C19 and CYP3A4 to *N*-demethyl-diazepam (*N*-demethylation) in man (Niwa et al., 2005). CYPs are ubiquitous enzymes capable of catalyzing metabolic reactions in plants and animals. It is possible that similar metabolites could be formed in plants, as in man.

Atrazine. Atrazine (ATZ) is one of the mostly widely used broad leaf herbicides (Hayes et al., 2002). ATZ is applied as both a pre- and post-emergence spray primarily in corn, soybean and sorghum. Originally developed in Switzerland in 1952 and patented in 1958, it was registered for commercial use in the United States by 1959 (Solomon et al., 1996). The use of atrazine has since spread to most of the world. Efforts are in effect to reduce the quantity used after environmental concerns were registering.

A member of the s-Triazine family of herbicides, ATZ acts to interrupt photosynthesis resulting in oxidative stress leading to death. ATZ does not 'starve' a plant to death, as previously believed. As ATZ binds specifically to certain transfer proteins within the photosynthetic chain, resistance can be developed through mutations in the genes coding for those proteins, or via naturally resistant plants possessing different transfer proteins. Corn, soy and sorghum are some of those naturally resistant plants. In the United States alone, approximately 800 million lb (363 million kg) was applied between 1980 and 1990 (Mandelbaum et al., 1995).

As a positive control, ATZ is known to be taken up by plants, therefore if it was removed from solution during the study then the experiment was set up to work at least for ATZ. Also, ATZ is known to be taken up and metabolized by hybrid poplar trees, of which their physiology could be described as close to willow physiology (they are from the same family Salicaceae), providing a basis for what could be occurring in willow (Burken and Schnoor, 1997 and 1998).

Water is the primary dispersing force for atrazine. Due to varying solubility, the parent compound is commonly found within runoff, while some degradation products such as deethylatrazine and deisopropylatrazine are more soluble and commonly found within ground water (Angier et al., 2002).

Reports have provided differing rates of degradation in both aerobic and anaerobic environments for atrazine. Seybold et al. (2001) reported a half-life of 38 days in aerobic wetland soil, and 86 days in the aqueous phase above the soil. Chung et al. (1995), also in anaerobic wetland soils, reported a half-life of 38 weeks (266 days). Other studies in an anaerobic aquatic environment, reported overall atrazine half-life of 608 days, a water half-life of 578 days, and in sediment 330 days (EPA, 2002).

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