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Phytoremediation of natural and synthetic steroid growth promoters used in livestock production by riparian buffer zone plants

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PHYTOREMEDIATION OF NATURAL AND SYNTHETIC STEROID GROWTH PROMOTERS USED IN LIVESTOCK PRODUCTION BY RIPARIAN BUFFER ZONE PLANTS

by

Sam Bircher

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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has been approved by the Examining Committee for the
thesis requirement for the Master of Science degree in
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To my family, friends and wife Haixin for their patience, caring and support in this endeavor

As scientists have delved deeper into the nervous, immune, and endocrine systems-the body's three great integrating networks they have encountered profound interconnections: between the brain and the immune system, the immune system and the endocrine system, and the endocrine system and the brain. The links sometimes seem utterly mystifying. How, for example, could a woman suffering from multiple personality disorder play with a cat for hours while she was one personality and suffer violent allergic reactions to cats when she took on another?

Theo Colburn (Our Stolen Future)

A truly healthy environment is not merely the responsibility to make it safe, but likewise stimulating

Adapted from William H. Stewart, ES&T 1968

In all things of nature there is something of the marvelous

Aristotle

One hundred years into the Industrial Revolution, we are only now opening our eyes and realizing our artificially constructed world is not isolated from the real one. It is enmeshed in a larger natural world that cradles and nourishes us, making all of our activities possible. Fouling this nest, a lesson other organisms learned long ago, can be a deadly business

Janine Benyus (Biomimicry)

Our goal is delightfully diverse: a safe, healthy and just world, with clean air, water, soil and power—economically equitably, ecologically and elegantly enjoyed

William McDonough

Let us be clear, the use of hormones for growth promotion purposes are of no benefit to the consumer (except by making the meat marginally cheaper), are of no benefit to the animals that are treated in this way and pose a potential risk to wildlife if excretion of the hormones contaminates pasture or waterways

Richard Sharpe

Apt risks grant contentment when succeeded and wisdom if unfulfilled…

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CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1 Background on Endocrine Disrupting Chemicals (EDCs)

A 2006 report by the International Joint Commission's *Expert Consultation On Emerging Issues of the Great Lakes in the 21st Century* identified steroid hormones and pharmaceuticals as emerging contaminants of concern. These substances belong to a class of compounds classified as endocrine disrupting chemicals (EDCs), which have been defined as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (epa.gov/endocrine/). Many of the EDCs stem from an anthropogenic group of compounds called Pharmaceuticals and Personal Care Products (PPCPs), which have become ubiquitous in our society since the onset of the industrial revolution. They are products of synthetic chemicals used as industrial solvents/lubricants and their byproducts, along with various kinds of pharmacologically active substances. Types of endocrine disruptors from PPCPs include antibiotics, steroids, antidepressants, narcotics, phthalates (used in industrial manufacturing of various consumer products like flooring, perfumes, lotions, cosmetics, varnishes, soaps, shampoos, sunscreen, fragrances, and timed release pharmaceuticals), diethylstilbestrol (DES), organochlorinated pesticides (dichlorodiphenyltrichloroethane (DDT), methoxychlor), herbicides (atrazine), mercury, polychlorinated byphenyls (PCBs), plasticizers (phthalates, bisphenol A (BPA), degradation products of detergents or alkylphenols (nonylphenol), polycyclic aromatic

hydrocarbons (PAHs), flame retardants (polybrominated diphenyl ethers (PBDEs)), fungicides (vinclozin), and Polychlorinated dibenzodioxins (PCDDs) or more simply dioxins.

Each of the synthetic EDC and PPCP compounds listed previously are considered xenoestrogens or false messengers that are biologically active, which act to mimic, repress, decrease, and alter thyroid hormones in wildlife and humans. Because these chemicals have become an integral part of the manufacturing of most of the common goods we consume, there is a real threat to our national waterways. In a national reconnaissance evaluation of 139 streams in 30 states performed by the United States Geological Survey (USGS) from 1999 to 2000, looking at 95 target compounds (including 14 steroids) from human or livestock sources, found EDCs in all and PPCPs in over 80% of the tested water bodies. The most common detected PPCPs were steroids, non-prescription drugs, insect repellent and detergent metabolites with the highest concentrations being steroids, plasticizers and detergent metabolites (Kolpin et al., 2002). Based upon these results and well publicized reports of chemical leaching, it is estimated that PPCPs get released into the environment at levels similar to agrochemical nutrients (thousands of tons per year). But unlike pollution by fertilizers, which are easily observed by eutrophication and fish kills, PPCPs and their estrogenic impacts are only noticed with mass reproductive disorders (the most famous being dichlorodiphenyltrichloroethane or DDT) and thus often difficult to make a direct link to reductions in health and fertility.

Research on endocrine disruption by xenoestrogenic compounds has in the past been somewhat obscure, due to deficiencies with instrumentation and more so the

complexity of the biologic systems that EDCs influence. After the release of the book *Our Stolen Future* in 1996 however, the scientific community has increasingly revealed that EDCs can undermine proper development and acknowledge the alarming and profound implications that threaten our fertility, intelligence, and survival. The means in which EDCs affect wildlife and humans biologically are very intricate, beginning with the overall command of the body's internal regulation via hormonal and chemical signals in the bloodstream. These biological messages are also critically responsible for coordinating proper development of organs and tissues needed to work in concert together to keep the body functioning properly, as well as directing sex and reproduction prenatally. To quote *Our Stolen Future*:

> Hormones, which get their name from the Greek word meaning "to urge on," are produced and released into the bloodstream by a variety of organs known as endocrine glands, including the testicles, the ovaries, the pancreas, the adrenal glands, the thyroid, the parathyroid, and the thymus. The thyroid, for example, produces chemical messengers that activate the body's overall metabolism, stimulating tissues to produce more heat. In addition to eggs, a woman's ovaries release estrogens-the female hormones that travel in the bloodstream to the uterus, where they trigger growth of the tissue lining the womb in anticipation of a possible pregnancy. Yet another endocrine gland, the pituitary, which dangles on a stalk from the underside of the brain just behind the nose, acts as a control center, telling the ovaries or the thyroid when to send their chemical messages and how much to send. The pituitary gets its cues from a nearby portion of the brain called the hypothalamus, a teaspoon-size center on the bottom of the brain that constantly monitors the hormone levels in the blood in much the way that a thermostat monitors the air temperature in a house. If levels of a hormone get too high or too low, the hypothalamus sends a message to the pituitary, which signals the gland that produces this hormone to gear up, slow down, or shut off. The messages travel back and forth continuously. Without this cross talk and constant feedback, the human body would be an unruly mob of some 50 trillion cells rather than an integrated organism operating from a single script.

There is perhaps no other physiological process more dependent on the proper functioning of hormones than sexual development, specifically with males, where simply possessing an XY chromosome is not sufficient. For the first six weeks of life the fetus is

sexually neutral, containing a pair of unisex gonads that can develop into either testes or ovaries. Near the seventh week a gene on the X chromosome sends out directives for the gonads to develop into male testicles and from that point whether the course of masculinization is successful or not is solely dependent on hormones. Specifically attuned hormonal signals are sent out at precise moments that will determine whether a fetus develops the rest of the male parts and turn into a boy.

During this period, hormones are also guiding the growth of an unborn baby's nervous and immune systems, and programming organs and tissues like the liver, blood, kidneys, and muscles, which function differently in men and women. Moreover, proper brain development is dependent on thyroid hormones that get cued at specific times to develop nerves to then migrate to the correct areas. It is also important to note that hormones do all this without altering genes or causing mutations. They only control the "expression" of genes an individual inherits from its parents. Hormones present in the womb during development determine which genes will be expressed for a lifetime as well as the frequency. For all of these integrated systems in the body, normal development depends on getting the exact hormone messages in the exact amount to the right place at the right time. Everything centers on timing and necessary signals and if something were to disrupt those signals during a critical phase of development, it can have severe lifetime outcomes for prenatal and early childhood offspring.

In the historical account of the biological effect EDCs can induce, seminal research studies were performed by Fredrick vom Saal, a biologist at the University of Missouri. He explored how hormones help make us who are we are by experimenting on mice, which share 99% of human genes (http://news.bbc.co.uk/2/hi/science/nature/253

6501.s tm), discovering that minute shifts in these chemical messengers before birth are extremely important and can indeed have lifelong repercussions. He discovered several significant cause and effect properties about EDCs that mimic estrogens using DES or diethylstilbestrol. For instance traditional toxicology assumes that there is some level of exposure or threshold, below which small amounts of a contaminant have no effect, but EDCs violate this assumption and have literally no threshold when they get added to a hormone system that is already active.

Endocrine disruptors can exhibit an "inverted U" or "non-monotonic dose response curve" shown below as the left graphic in Figure 1-1, where administering of low doses cause a greater response than high doses. At high EDC concentrations, at least for mammals, the immune and endocrine systems react to a barrage of a foreign biochemical as overly toxic, but very low concentrations in contrast (potentially more dangerous over a long time period especially for women) can cause greater consequences than higher doses for a specific response. An example of this phenomenon, from the graphic on the right in Figure 1-1, shows that prenatal exposure to low doses of DES produce significant postnatal enlargement of the prostate (which commonly cause adverse health effects in elder men) weight of adult mice compared to higher doses, where there is no enlargement and more so a decrease (vom Saal et al. 1997).

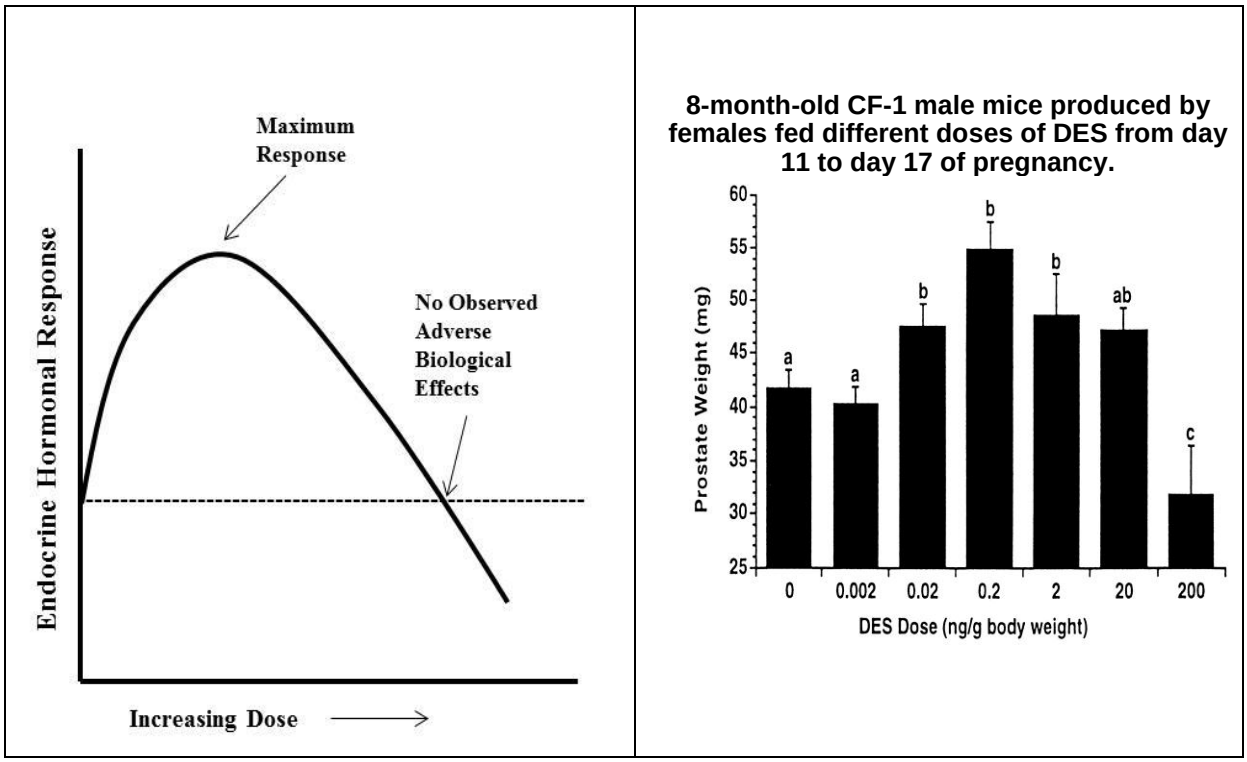


Figure 1-1 Example of a classic Inverted "U" Dose Response Curve (left) and results from a study involving prenatal exposures to DES with mice on adult prostate weight (right), adapted from vom Saal et al. (1997).

Equally as important, vom Saal discovered that the timing of the dose is usually more crucial than the dose amount as well as how little it takes to produce harmful effects. The endocrine system maintains an exquisite sensitivity, operating at concentrations as low as parts per trillion or ng/L, thus making EDCs capabilities exceptionally potent during the varying stages of development. Lastly, the studies revealed that developmental disorders especially with the reproductive system inherited by offspring while in the womb, were passed on and showed up in as far as the third-generation (Colborn et al., 1993). Susceptibility to biological systems from EDCs are likely due to the endocrine system being virtually unchanged in vertebrates over millions

of years of evolution, where, for example, the same estrogens circulating in a rattlesnake or a great white sharks bloodstream are exactly the same as those in a human (Trimel, 2001). With the progression of biologic research, scientists have been able to develope a good understanding of the mechanisms and actions of hormones, along with how chemical messages are sent and received and therefore how some synthetic chemicals can disrupt this communication.

Several well-known examples of human exposure to EDCs highlight the vulnerability to these types of substances that people can retain, with the most famous of these being the tragic saga of DES. In 1938 a "miracle drug," a man-made estrogen created by a team of British scientists, was prescribed to young women suffering from insufficient levels of natural estrogen widely believed to be causing premature births and miscarriages. Before long some five million women in the U.S. were given the drug at various stages of their pregnancies. Then in the late 1960's, doctors were puzzled by a sudden rash of extremely rare cancers in woman and girls between the ages of 15 and 22, who were later infamously referred to as DES daughters. After closer investigation, it was found that the mothers of these affected women were prescribed DES during the first trimester of their pregnancies. Soon a host of problems started showing up in DES babies, like abnormalities of the uterus and vagina, undescended testicles and malformed sperm in boys, along with immune system defects, sexuality issues, chronic depression, and other psychiatric disorders. For the first time, it was apparent that exposure in the womb to a synthetic EDC could be passed on by a mother to her offspring and these effects, which unlike typical birth defects obvious at birth, showed up years and even decades later. These results also illustrate that man-made chemicals like EDCs could

cause damage that was slow, insidious, and imperceptible (Colborn et al., 1996). Moreover, the same damage to the genitals and reproductive tracts were seen with mice in the studies done by vom Saal, further showing the common effect of EDCs on mammals and humans during prenatal development.

Another well-known study by Fein et al. in the early 1980's, investigated the potential contamination of eating fish after reports of heavy pollution in the Great Lakes. They found that children of mothers who ate two to three meals of fish per month were born sooner, weighed less, and had smaller heads than mothers who were not eating the fish. The culprit, PCBs, which was also determined that the greater the concentrations in umbilical cord blood, the more poorly a child scored on intelligence tests (Fein et al., 1984, Jacobson et al., 1985). A more unusual account of the possible effects on humans over a half a century of exposure to EDCs are the growing reports of a decline in sperm counts of young men. A landmark article published in the *British Medical Journal* in 1992, based on over 60 studies from 20 countries on every continent, revealed that the average male sperm count had decreased by 45%, as well as a rise in extremely low sperm counts from 6% in 1940 to 18% in 1990 (Carlsen et al., 1992). The studies also uncovered that most of the decline was among the youngest men in the sample group, pointing towards environmental factors as the most likely cause. This was further supported by a paper in the prestigious medical journal, *The Lancet*, suggesting that prenatal exposure to elevated levels of synthetic estrogens was very likely to blame for the surge in reproductive problems among men (Sharpe and Skakkebaek, 1993).

Other reasons why EDCs have the potential to be so harmful to overall human health is their physical-chemical properties and how the body reacts in response to them.

A key feature of EDCs is their relative insolubility in water. Since they do not readily dissolve or breakdown in water, they are usually not effectively excreted from the body. They instead get stored in the body's fatty tissue, due to being fat soluble, where they persist and build up to very high concentrations during a lifetime of exposure. This is also why humans that eat at the top of food chain are especially susceptible to EDCs like PCBs because they bioaccumulate within the food web, increasing to concentrations millions of times more than what is typically found in water bodies (Pringle, 2003).

Every person on earth for example currently carries traces of hundreds of synthetic chemicals that have accumulated in our fat cells through water, air, and food over our lifespan. Previous research also illustrates that synthetic hormone mimics are able to trick the body's receptors that readily accept them in the customary "lock and key" mechanism, inducing a biological response that can disrupt proper cellular activity. Unlike the body's ability to repair damaged DNA for say cancer, there is no analogous repair mechanism to cope with the hormone disrupting effects of synthetic chemicals. This is because cells are primed to receive hormone messages, and respond to imposters as legitimate messengers, allowing them to bind to hormone receptors, not distinguishing their action as damage that needs to be repaired (Dumanoski et al., 1996). Similarly, the addition of estrogens to biologically active systems can be dangerous, especially for pregnant mothers. A study investigating the effects of hormones on pregnant Rhesus monkeys, found that 96% of the naturally occurring estrogen 17β-estradiol (E2) was metabolized to the less potent estrogen estrone (E1), while in contrast a synthetic estrogen did not undergo placental metabolism, and entered the fetal circulation in the parent compound form (Slikker et al., 1982). From these results it is thought that

mammalian developmental systems have found evolutionary methods that attempt to deal with the unprompted addition of certain naturally occurring hormones, but is unequipped to handle synthetic versions that operate in a similar manner.

In the effort to regulate EDCs and limit the exposure to people, an official Endocrine Screening Program was established in 1996 by the EPA (Environmental Protection Agency). The EPA is continually bogged down with issues relating to testing methodologies, protocols for screening, and determining which chemicals need to be screened; and thus legislative action regarding water quality standards for EDCs is far behind what it should be. The delays in part may be due to a widespread preoccupation with carcinogens instead of impairment wrought by EDCs, which ominously act in a subtle and cumulative nature. The biggest threat EDCs pose to humans is not to adults, which have no observable effects, but vitally instead with exposure during prenatal development and during early childhood including breast-feeding.

Farm runoff is the leading source of water pollution in the U.S., with sediment as the most prevalent source (www.epa.gov/owow/NPS/Ag_Runoff_Fact_Sheet.pdf); including over 90% of the total estrogenic compounds released into the environment via spillover and runoff from concentrated animal feeding operations (CAFOs) waste lagoons and manure applied to fields as fertilizer (Maier et al., 2000). Little is known about the environmental fate of EDCs and their effects on aquatic ecosystems and human health, but there is evidence that estrogenic compounds may have serious detrimental impacts. For example, concentrations as low as 10 ng/L have been found to feminize male fish and reduce fertility in wild fish populations (Jobling et al., 2006). For the purposes of this research project, the following introduction and background material will focus on the

hormones featured in contraceptives and steroid growth promoters given to livestock, in an attempt to assess their risk and environmental impact.

1.2 Uses of Target Hormones and Environmental Fate

Oral contraceptives, which are consumed by 11.6 million women of reproductive age in the U.S. (Guttmacher Institute 2008), have gained more attention due to incomplete degradation by waste water treatment plants (WWTPs) and low levels of estrogens found in surface waters downstream, causing concern about drinking water contamination. This has to do with pharmaceuticals designed to be chemically stable to increase shelf life, and as a result are persistent in the environment. 17α-ethinylestradiol (EE2), the main estrogenic component in birth control pills and a known potent EDC, is the most widely used synthetic estrogen with an average daily dose of 30-35 µg per pill. It is a biologically-active estrogen derived from estradiol, which inhibits several hormones, leading to prevention of ovulation, and gets released as a xenoestrogen EDC in urine and feces.

EE2 enters the water treatment system mainly as domestic sewage, where it has been estimated that women fully metabolize EE2 at only 20-48% of the daily ingested dose (Reed et al., 1972), while the rest is excreted in either original form or as EE2 sulfate or glucuronide conjugate forms. Additionally, of the roughly 60% of EE2 that is excreted, most of it is deconjugated back to the original EE2 form after entering into sewage treatment plants (Johnson et al., 2004). Disposal of unwanted medications down

sinks and toilets is another major source contributing to the concentration of the bio-active compounds entering centralized wastewater systems. Due to high costs of advanced WWT along with the lack of toxicological data and knowledge of the fate of estrogens, municipal plants are ineffective at degrading highly estrogenic compounds such as EE2 to low enough concentrations, thus reports of varying detections in the environment have occurred around the world for this estrogen. In the previously mentioned USGS survey, EE2 was detected at a maximum concentration of 83.1 ng/L and a median level of 7.3 ng/L in streams (Kolpin et al., 2002). Reports of wastewater effluent in Germany had levels of up to 17 ng/L, in the U.K. up to 7 ng/L, and a range of 1.0 to 3.2 ng/L in the Paris, France area for effluent and surface water (Belfroid et al., 1999, Cargouet et al., 2004). Even in surface waters of a coastal bay area of the Baltic Sea, EE2 levels were reported to be 17.2 ng/L in 2000 and 3.0 ng/L in 2003 (Beck et al., 2005). Besides WWTP effluent and surface waters, EE2 has even been found in drinking water, with measured levels up to 0.5 ng/L in tap water from sourthern Germany with a groundwater source (Kuch and Ballschmiter, 2001). These finding are noteworthy for the health of aquatic ecosystems; due to reports of levels of EE2 as low as 0.1 ng/L inducing vitellogenin production (biomarker for estrogen exposure as well as a female protein and precursor to yolk) in male fish along with other sexual dysfunctions (Purdom et al., 1994).

Although there is a consistent influx of estrogenic compounds entering WWTPs, basic treatment with sludge retention times (SRT) of 5-15 days has been found to remove 80% or more of the available EDCs on average, mostly due to sorption by sludge and biodegradation, enhanced by longer retention times and exposure to nitirifiers (USEPA: Treating Contaminants of Emerging Concern, 2010). A greater risk though to ground

water sources and surface water bodies is not sewage effluent, but rather from WWT biosolids or sludge along with CAFO manure applied to land as fertilizer. There are an estimated 16,583 WWT facilities in the U.S. and of the 7,189,000 dry tons of biosolids produced in 2004, an estimated 2,925,920 tons of that total was used on farmland (New England Biosolids and Residuals Association, 2007). Moreover, U.S. policy does not require manure and biosolids to be treated as long as it is not being discharged directly into surface waters.

There are three major categories of CAFOs that use or produce EDCs, which are cattle, poultry, and swine. Cattle CAFOs employ a mixture of growth promoting estrogens, poultry CAFOs use natural estrogens E2 and E1 as well as testosterone, while swine CAFOs don't use growth promoters, but do produce natural estrogens and testosterone. There are obvious potential hazards to water bodies in the U.S. where growth promoters are commonly used. It was estimated in 2002 that overall hormone excretion from farm animal facilities per year was at least 330 tons (http//hill.beef.org/ft/fsgph.htm, http://usda.mannlib.cornell.edu/reports/nassr/livestock /pgg-bb).

The main source of growth promoting compounds comes from use in the beef industry and cattle CAFOs. According to the 2011 semi-annual cattle inventory report, the United States Department of Agriculture (USDA) estimates there are 92.6 million cattle being raised in the U.S. (usda.mannlib.cornell.edu) with 80% receiving steroid hormones (as either subcutaneous implants or in feed) to accelerate weight gain and increase the efficiency of conversion of feed to muscle mass (Raloff, 2002). The driving force behind the development and use of growth promoters has been and continues to be

return on economic investment, where the incentive for farmers to use hormones can amount to a 40 fold return on initial capital investment. It costs farmers only 1-3 dollars per head to treat their livestock through either feed or control released implants, which can increase a cow's growth by 20%, translating into a typical gain of 3 pounds per day (Raloff, 2002).

The U.S. Food and Drug Administration (USFDA) has approved 25 different exogenous growth promoting implants for use in beef cattle (two thirds of the total industry being transplanted) most commonly containing one or at most two of the following steroidal hormones: the synthetic estrogen mimic zeranol (zearalanol or ZAL) as a 36 or 75 mg single dose, a synthetically derived testosterone trenbolone acetate (TBA) as a 140-200 mg single dose and natural estrogens E2 as a 24 mg single dose, progesterone (P), melengestrol acetate (MGA), and testosterone (T) (Kolok and Sellin, 2008). Of the highly potent implants, the most effective has been found to be a combination of 120-200 mg TBA and 24-28 mg E2, which most steers, heifers, and calves are likely to receive in finishing lots before slaughter (Siemens, 1996). Other countries in the developed world however do not employ the same "innocent until proven guilty" policies like the U.S. for the approved release of chemicals into the market. In 2006 for example, due to potential health risks and the uncertainty of exposure limits associated with many of these substances, the European Union has banned the use of all drugs used for growth promoting purposes.

Although some of the common growth promoting hormones are typically not tested for, there is still evidence of widespread detections of estrogens in surface water. In the USGS national evaluation found that all tested water bodies contained EDCs, with

E2 and its metabolite E1 at concentrations as high as 200 and 112 ng/L; respectively (Kolpin et al., 2002). Furthermore, a nationwide study in Canada found mean concentrations of E2 and E1 in wastewater effluent to be 1.8 ng/L and 17 ng/L respectively (Servos et al., 2005). Studies on U.S. livestock producers have shown total national daily discharges of E2 and E1 from dairy and swine to be between 10 and 30 kg and 20 to 80 kg respectively (Fine et al., 2003). Raman et al. (2004) found concentrations of E1 in well water near a swine farm to be 4.5 ng/L, suggesting that estrogens from agricultural practices can make their way into groundwater sources.

To pinpoint sources and quantities of growth-promoting compounds from agricultural fields and manure piles, studies have examined rates at which hormones are being excreted from livestock and subsequently wash into rivers and streams. One such study found that 80% of radiolabeled TBA was excreted in feces 24 hours after injection (Pottier et al., 1981) and Krzeminski et al. (1981) showed that although the liver excreted approximately 87% of the total injected radiolabeled MGA injected, 10%-17% administered in the feed passed through the heifers unabsorbed in the parent form. In flow-interrupted transport experiments, E2 concentrations in the effluent were found to decrease while E1 increased. Once flow resumed and additional estrogen was added to the system, the E2 returned to elevated levels (Das et al., 2004). From this it was concluded that because feedlots experience a continuous reloading of E2 in manure, periodic rainstorms can easily release the newly deposited estrogens into runoff.

A simulated rain and runoff study on pasture amended with poultry litter by Nichols et al. (1997) revealed that in the first storm runoff concentrations of E2 increased from 1.28 μg/L to 198 μg/L as application of poultry litter increased. Moreover, during a

second storm event E2 was still detected seven days later (losses were 66%-69% less than the first runoff event). Thus, it was suggested by the authors that E2 may persist in the field as it aggregates in the chicken manure, which can hinder photo- and microbial degradation. Additionally, a soil sorption experiment showed after adding labeled steroids to 1 kg of heavily irrigated soil that 56% of E2 and 52% of E1 washed out while the remaining portion was tightly bound (Shore et al., 1993).

In another field runoff trial from fescue-planted land amended with litter from broiler chickens, measured concentrations of E2 increased 10-fold from 55 ng/kg before application to 675 ng/kg afterward. They also found that levels of E2 were still elevated in soils 14 days after application and overall E2 from the fields in runoff increased from background levels of 50-150 to 20-2530 ng/L, depending on application rate and time between treatment and runoff (Finlay-Moore et al., 2000). In an experiment tracking the fate of TBA and MGA after being administered to steers and heifers, trace amounts of unmodified MGA were reported after 195 days in soil and solid dung. Additionally, trace amounts of trenbolone (TBOH), the main metabolite of TBA, were found after 58 days in solid dung, 260 days in liquid manure and several months later in soil after fertilization (Schiffer et al., 2001).

To date, there is only one known study focused on feedlot runoff estrogenic effects on downstream aquatic wildlife. In this experiment, the researchers collected adult fathead minnows (*Pimephales promelas*), considered an aquatic sentinel species, from three sites including a convergence site between a major feedlot drainage ditch and the Elkorn River in Nebraska. They found males from the contaminated and intermediate sites with significantly smaller testes, diminished secondary sexual characteristics, and a

decrease in T synthesis as well as females with a significantly lower estrogen:androgen ratio, which translates to the male fatheads becoming feminized and females becoming masculinized (Orlando et al., 2004).

Cattle feedlot runoff typically contains a suite of growth promoting compounds and interpreting the biological effects of exposure to complex mixtures containing androgenic, estrogenic, and progestogenic chemicals will continue to be an important area of study. A potential source of EDCs getting released into the environment is from CAFO waste lagoons, however there is a lack of data representing in the literature. One study by Hutchins et al. (2007) sampled three CAFO locations in south-central U.S. for lagoon effluent concentrations of varying estrogens that included suspended solids used for land application. They reported total free estrogen concentrations (E1, E2, 17α-estradiol, and E2 metabolite estriol (E3)) of 1000-21000 ng/L swine primary, 1800-4000 ng/L poultry primary, 370-550 ng/L dairy secondary, and 22-24 ng/L beef secondary whole lagoon samples. They also found several estrogen conjugates estrone-3-sulfate, 17β-estradiol-3-sulfate, 17α-estradiol-3-sulfate, and 17β-estradiol-17-sulfate at concentrations of 2-91 ng/L, 8-44 ng/L, 141-182 ng/L and 72-84 ng/L, respectively, which contribute significant amounts to the overall estrogen load across different types of CAFO lagoons.

Because little is known about Zeranol, a principle compound for the focus of this project, describing some of the specifics of the environmental fate and general facts of this commonly utilized growth promoting compound is valuable. ZAL is used extensively across the U.S. and is prepared commercially from zearalenone (sold as the product Ralgro®) and most closely mimics many of the effects of the natural estrogen E2.

It is a powerful estrogenic chemical as demonstrated by its ability to stimulate growth of human breast tumor cells in vitro at doses similar to E2 and the carcinogen DES (Leffers, 2001). Moreover, a series of studies examining the estrogenic activity in normal breast epithelial cells and breast cancer cells treated with ZAL discovered that abnormal cell growth was significant even at levels about 30 times lower than the FDA established limit in beef (Liu and Lin, 2004). Additionally, data indicates that serum from ZAL-treated beef cattle can stimulate the transformation of tumor cells in vitro (Xu et al., 2009).

In a field study of the environmental fate of ZAL done by Dixon and Mallinson (1986), several important properties of the compounds were uncovered. They found that ZAL exhibited a half-life in a variety of soils between 49-91 days and about 60 days in feces as well as adsorption/desorption tests revealing that 44% to 58% of the ZAL was adsorbed into soil after application. In a trial, in which 27 steers were implanted with 36 mg of ZAL, they also discovered a mean maximum concentration in feces of 5.8 ng/g after 15 days and declining to 1.67 ng/g on day 34. Risks of water contamination from runoff after fields have been applied with manure containing this substance are obvious, but in addition as much as 30% of the compound may remain in the ear subcutaneous injection point at slaughter, making the disposal of the tissue is a notable concern (Raloff, 2002).

1.3 Properties of Target Hormones

Table 1-1 Properties of target parent hormones compounds and their metabolites.

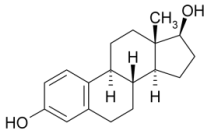
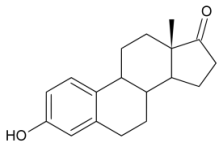
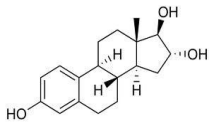
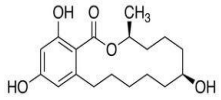
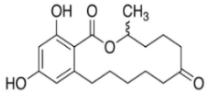
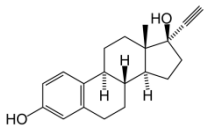
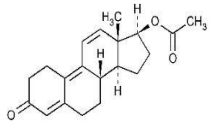
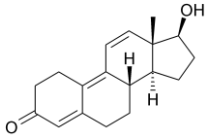
Hormone Compound	**Acronym**	**Chemical Structure**	**MW (g/mol)**	**Sw (mg/L) (20°C)**	**LogK$_{ow}$**	**pKa**	**VP (kPa)**	**Ref**
17β-Estradiol (natural hormone)	E2		272.4	13.3	3.94	10.4	3 x 10^{-8}	1
Estrone (metabolite of E2)	E1		270.4	13	3.43	10.3	3 x 10^{-8}	1
Estriol (metabolite of E1)	E3		288.4	13.3	2.81	10.4	9 x 10^{-13}	1
Zeranol (α-zearalanol)	ZAL		322.4	5.14	4.04	8.44, 11.42	3.9x10^{-9}	2
Zearalanone (metabolite of ZAL)	ZAN		320.38		3.86			3
17α-Ethinylestradiol (synthetic estrogen, birth control)	EE2		296.4	4.8	4.15	10.4	6 x 10^{-9}	1
Trenbolone Acetate	TBA		312.4		4.35			4
17β-Trenbolone (metabolite of TBA)	TBOH		270.37		3.08		8 x 10-^{11}	5

Table 1-1 Continued-

MW: molecular weight; Sw: water solubility; $LogK_{ow}$: octanol-water partition coefficient; pKa: $-log_{10}$acid dissociation constant; VP: vapor pressure; Ref: reference.

1: Lai et al. (2002), 2: Sigma-Aldrich, 3: Sigma-Aldrich

4: Estimated using the Sparc On-Line Calculator (accessed July 18, 2011)

5: Blackwell et al. (2011)

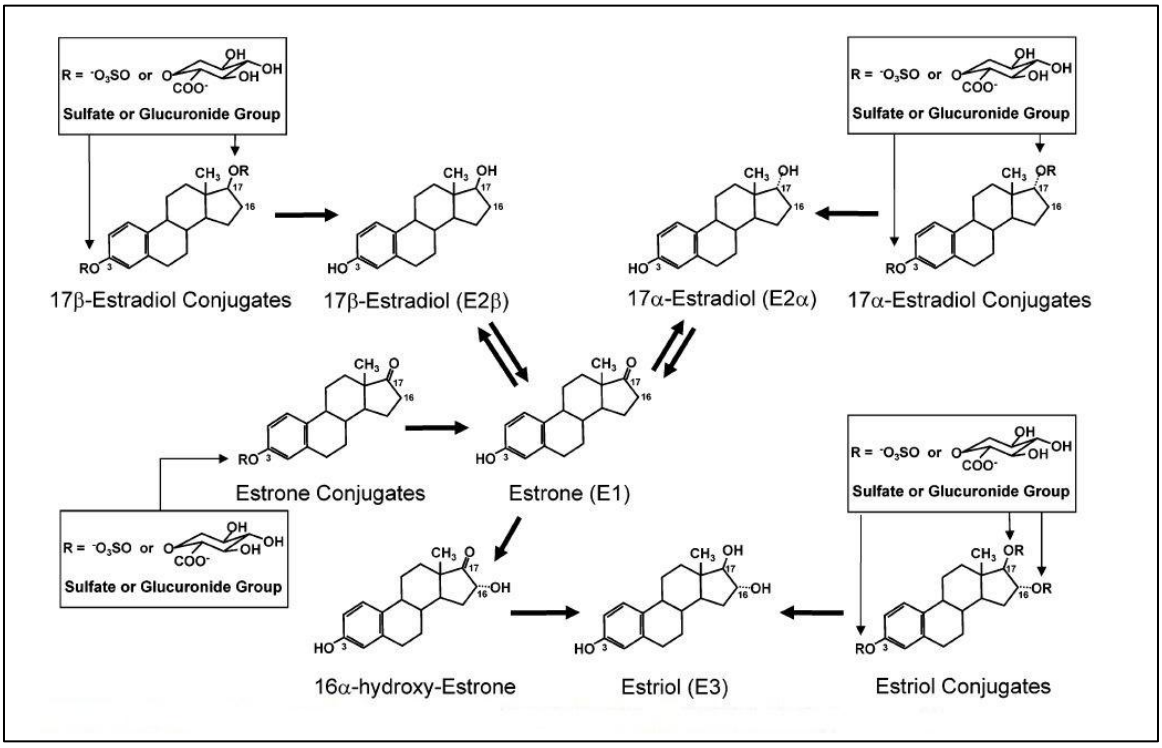


Figure 1-2 17β-estradiol (E2) metabolic pathway including metabolites Estrone (E1) and Estriol (E3), adapted from Hutchins et al. (2007).

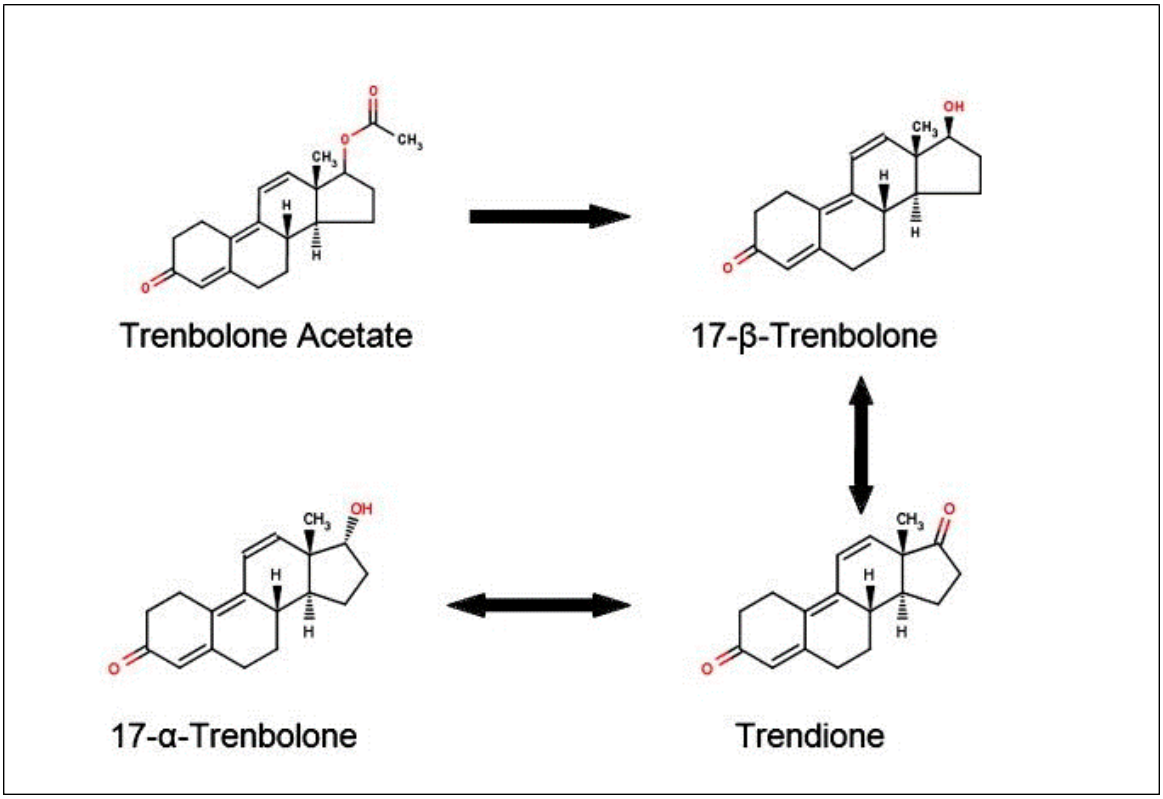


Figure 1-3 Trenbolone acetate (TBA) metabolic pathway including metabolite 17β-Trenbolone (TBOH), adapted from Blackwell et al. (2011).

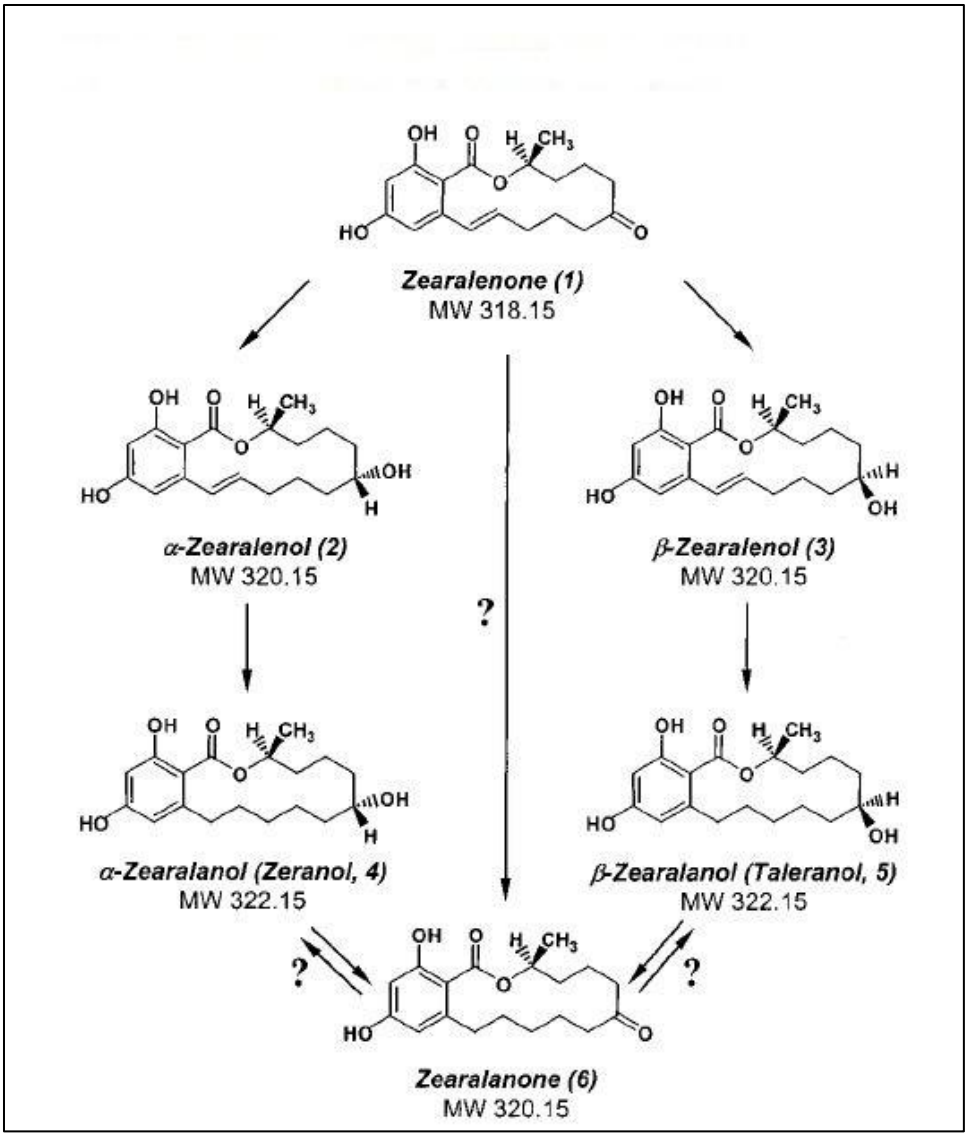


Figure 1-4 Zeranol metabolic pathway including metabolite Zearalanone (ZAN), adapted from Kleinova et al. (2002).

1.4 Phytoremediation of Steroid Growth Promoters and EDCs

Phytoremediation is defined by McCutcheon and Schnoor (2003) as "the use of vascular plants, algae, and fungi either to remove and control wastes or to spur waste breakdown by microorganisms in the rhizosphere." An important function that makes this process work is due to vascular plants having the remarkable ability to regulate *in situ* the local rhizosphere biogeochemistry along with water and nutrient availability. There are six major mechanisms that enable various phytotechnologies to remove, transfer, degrade, or stabilize contaminants: phytoextraction, phytotransformation, rhizosphere bioremediation, phytostabilization, rhizofiltration, and phytosequestration (Sengupta and Dalwani, 2008). This project will focus on phytodegradation, phytotransformation and phytosequestration of synthetic and natural steroid growth promoters that are commonly used for prescribed birth control and by livestock producers.

There have been very few studies looking at the role of plants to degrade EDCs, and more specifically only one that measured the impact of a common growth promoter used in CAFOs and animal husbandry. In one study Imai et al. (2007) investigated the removal of phenolic EDCs by *Portulaca oleracea* known commonly as the garden purslane. 17β-Estradiol along with bisphenol A (BPA), a well-known estrogenic EDC and other suspected EDCs octyphenol (OP), and nonylphenol (NP), were rapidly removed from water at initial concentrations of 50 µM OP, 40 µM NP, 25 µM E2, mostly disappearing within 24 hours, while removal did not occur in the absence of the plants. Even at the highest concentration of 500 µM BPA, 95% was removed after 24 hour time

period. These results are intriguing considering that annual production worldwide of BPA has been estimated at more than 500,000 tons (Staples et al., 1998) and that levels as high as 74 µM were detected in waste landfill leachates (Yamamoto 2001).

A study, performed by C. Franks (2006) examined the uptake and degradation potential of three common wastewater pharmaceuticals: diltiazem (DTZ), a calcium channel blocker, diazepam (Valium®) (DZP), an antianxiety drug, and EE2, using sandbar willow (*Salix exigua)*, a riparian shrub. The herbicide atrazine (ATZ) was used as a positive control, since it is known to be readily taken up by plants. He found that 20 day old willow cuttings with an average length of 10 cm were able to remove EE2, DZP, DTZ, and ATZ from solution at 88%, 56%, 77%, and 50% respectively after 24 hours, with an initial concentration of 40 ng/L for each compound. The sole study by Card and Chin (2011) investigating the role of plants on the growth promoter ZAL, its metabolite zearalanone (ZAN) and other estrogens, used maize seedlings in hydroponic solution. They found that with initial concentrations o6.27 mg/L E2, 8.11 mg/L E1, 13.54 mg/L ZAL, and 10.57 mg/L ZAN, after 8 days of exposure E2 and E1 were undetectable, while ZAL and ZAN had decreased by 53% and 91%, respectively, from the solution while glassware controls showed insignificant losses.

Plants, also known as "green livers", contain analogous metabolic processes and enzymes to mammalian livers, and are able to detoxify pollutants and xenobiotics through phytotransformation (Sandermann, 1994). This is achieved through transformations of parent compounds to less toxic metabolites. The technology of phytoremediation has seen an increase in acceptance and popularity due to its low energy demands (being solar powered), lower costs than conventional remediation treatment

methods, aesthetically pleasing, and eco-friendly nature. In light of this increase in general interest for phytoremediation as a remediation approach, environmental consulting firms are progressively offering services regarding this technology (Pilon-Smits, 2005).

The specific method of remediation employed depends on several factors, such as the physical-chemical properties of the contaminant, the substrate and the regionally appropriate plant species. Phytoremediation has been successfully applied to a wide range of both organic and inorganic compounds. Although inorganic compounds cannot be fully degraded or transformed, they can be sequestered in the plant tissues or stabilized in the root zone. Organic contaminants including herbicides metochlor and atrazine, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and explosives like TNT and RDX, have been studied extensively and found to be effectively degraded by plant technologies (Henderson et al., 2006; Gfrerer et al., 2002; Dzantor et al., 2000; Burken and Schnoor, 1998).

The most effective plant species studied, include riparian vegetation composed of phreatophytic trees and shrubs, notably the genera *Populus* and *Salix* (poplars and willows). These plants are able to transpire large volumes of water between 100 to 200 L/day in trees (Newman et al., 1997). These species are also fast growing and deep rooted, which is important in the efficacy of phytoremediation, to ensure rapid and continuous uptake of pollutants within the transpiration stream and potential storage in the cell walls of the plant. The productive uptake of contaminants depends heavily on the influence of the transpiration stream tension (or pull) to draw chemicals towards the root zone and subsequently be metabolized into less toxic or less biologically active forms

along with sequestration and stabilization. Another important feature regarding a plants physiology and the potential uptake of chemicals depends on the $\log K_{ow}$ (log octanol-water coefficient) of the contaminant, which can be used to estimate whether the compounds will be taken up by plants, how much could be taken up, and where they will be distributed within the plants. Optimal uptake has been found to occur with moderately hydrophobic compounds ($\log K_{ow}$ 1.0- 3.5), with values greater than 4 highly sorbing to roots (Burken and Schnoor, 1998). Each plant will vary in the amount of water it can uptake and its subsequent phytoremediation capabilities, as well as contaminants having a varying degree of $\log K_{ow}$ values, which will each behave uniquely depending on the plant and chemical interactions occurring in that specific environment.

Since there are an estimated 238,000 working farms and ranches across the U.S. considered to be animal feeding operations that generate approximately 500 million tons of manure each year (http://www.epa.gov/owow/NPS/Ag_Runoff_Fact_Sheet.pdf), the quantity of commonly used steroid hormones excreted in urine and feces that eventually get discharged into surface waters are a serious environmental and health hazard. To limit the biological impact of these steroid hormones, it is essential that residual traces of these chemicals are rendered inactive before reaching adjacent water bodies. They may be considerably attenuated due to interactions with plants in agriculture fields and riparian buffer strips; thus the phytoremediation of ZAL, E2, TBA and EE2 will be tested with hybrid poplar *(Populus deltoids nigra)* and soft-stem bulrush (*Scirpus validus)* plants due to being commonly used within riparian buffer strip systems. To date, phytoremediation of hormonally-active growth promoters has not been explored, but this study may show that common plants are capable of attenuating and degrading these

compounds. The results of this study may provide additional support for the application of biotechnologies such as phytoremediation to protect the quality of aquatic habitats, groundwater, and ultimately human health. Moreover, utilizing natural biochemical reactions, such as plant and associated microbial metabolic processes, is less invasive, aesthetically pleasing and is significantly more cost effective and eco-friendly than conventional remediation techniques.

1.5 Objectives of Research Study

The main objective of this project is to determine whether phytoremediation can effectively reduce environmental contamination of biologically active steroid growth promoters that get released into water bodies. Since plants have been widely known to remediate many kinds of organic pollutants, this research will focus on the potential of riparian buffer zone plants, through phytoaccumulation and phytodegradation, to remove ZAL, EE2, TBA, and E2 from soils and runoff prior to flowing into waterways that eventually drain into the Great Lakes. I will test the hypothesis that this pharmaceutical compound and these hormones can be degraded and intercepted by these plants and their associated microbial communities. My results may demonstrate that chemicals regularly used in animal husbandry operations and birth control can be removed passively by upland buffer zone plants, wetland plants and their associated rhizosphere bacteria. Furthermore, this thesis research will test whether these plant systems can both accumulate and transform such substances to less toxic metabolites.

A series of hydroponic experiments have been designed to test the hypothesis, which is based upon several specific objectives:

1. Evaluate the potential for the upland riparian plant hybrid poplar *(Populus deltoids nigra)* and an emergent wetland plant softstem bulrush (*Scirpus validus)* to uptake and translocate zeranol, 17β-estradiol, trenbolone acetate and 17α-ethinylestradiol
2. Determine if these compounds bio-accumulate within the target plant tissues; and

3. Analyze the efficacy by which these plants and associated rhizosphere bacteria metabolize and biodegrade the target natural and synthetic steroid growth promoters

Based on the uncertain environmental fate of these "emerging" contaminants in aquatic ecosystems and given that the target substances affect the endocrine system, their release to surface waters could be extremely precarious to human health and aquatic organisms. Our approach, if demonstrated successfully, could provide watershed managers further incentive to preserve and develop riparian buffer zones on streams and rivers and could exhibit additional "built in" protection through uptake and degradation by plants and rhizosphere bacteria and their metabolic processes.

CHAPTER 2

BIODEGRADATION OF SYNETHIC AND NATURAL STERIOD GROWTH PROMOTERS BY SOFTSTEM-BULRUSH (*SCHOENOPLECTUS VALIDUS*)

2.1 Materials and Methods

Chemicals: E2 (1, 3, 5(10)-Estratrien-3,17β-diol-2,4,16,16-d4 [50-28-2]) (98%), **E1** (1, 3, 5(10)-Estratrien-3-ol-17-one-2,4,16,16-d4 [53-16-7]), (99%), **zearalanone (ZAN)** (2,4-Dihydroxy-6-(10-hydroxy-6-oxoundecyl)benzoic acid μ-lactone [5975-78-0] (98%), were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). **EE2** (1, 3, 5(10)-Estratrien-17α-ethynyl-3, 17β-diol-2,4,16,16-d4 [57-63-6] (98%) was obtained from U.S. Pharmacopeia (Rockville, MD). **ZAL** (6-(6,10-Dihydroxyundecy)-β-resorcylic acid-lactone [26538-44-3]) was extracted and purified from Ralgro Magnum (Schering-Plough Animal Health Corp., Union, NJ). Other chemicals, including LC/MS solvents, were purchased from Fisher Scientific (Pittsburgh, PA).

Hydroponic Uptake Experiment: Uptake of E2, ZAL, and EE2 were evaluated using vigorously growing softstem bulrush plants (*Schoenoplectus validus,* Ion Exchange Inc, Harpers Ferry, IA) originally received as small plugs in soil and grown for 64 days. The experiment was conducted by growing the softstem bulrush in hydroponic solution prepared as ½-strength Hoagland's nutrient solution (pH 6.8) buffered with 1.0M NaOH. The softstem bulrush plugs were received and initially watered within the soil plug matrix for 10 days, followed by a washing away of the soil from the roots with deionized water and placed in flasks with the hydroponic solution and refilled based on

evapotranspiration rates. After 64 days of growth, as shown in Figure 2-1, the plants were placed in autoclaved 500 ml Erlenmeyer flasks and 300 ml of hydroponic solution with treatment 1) spiked with initial concentrations of 6 mg/L EE2, 2 mg/L E2, and 3 mg/L ZAL and treatment 2) with only 3 mg/L ZAL. Large concentrations of these parent compounds were desired compared to much lower environmental sample concentrations so they could be detected in the plant tissues. There were three replicates per sampling period with 4 separate exposures (only 3 exposures in treatment 2) including: full plant, autoclaved glassware controls with no plants, negative control with dead plants and excised plants with solely roots present (used in treatment 1 only). All flasks were wrapped in aluminum foil and placed in a plant growth chamber (Percival Scientific PVG-40, Perry, IA) with a16:8 hour light:dark photoperiod (150 μmol s^{-1} m^{-2}) at 23^{o}C and 48% relative humidity. To maintain initial water levels, flasks were refilled daily with Milli-Q-water and measured the evapotranspiration.

Plants were destructively sampled at 2, 7, and 14 day time points. Four 12ml aliquots (totaling 48ml) were collected from the well mixed aqueous phase, combined with a 1:1 ratio of acetonitrile (to stop any further biodegradation during storage) and filtered using HVLP type Durapore® fiber filters with 0.45 μm pore size (Millipore Corp.) and collected for analysis by a liquid chromatography and mass spectrum (LC/MS) instrument. Roots were removed from the flasks, dried overnight at 100^{o}C and weighed. The material was then ground up using a mortar and pestle with liquid nitrogen and shaken in glass centrifuge vials with 10ml acetonitrile overnight. The vials were then centrifuged at 3000 rpm (Beckman Coulter, Brea, CA, Model J2-21M) for 30 min and

filtered using the same Durapore® fiber filters mentioned above, and collected for analysis on the LC/MS.

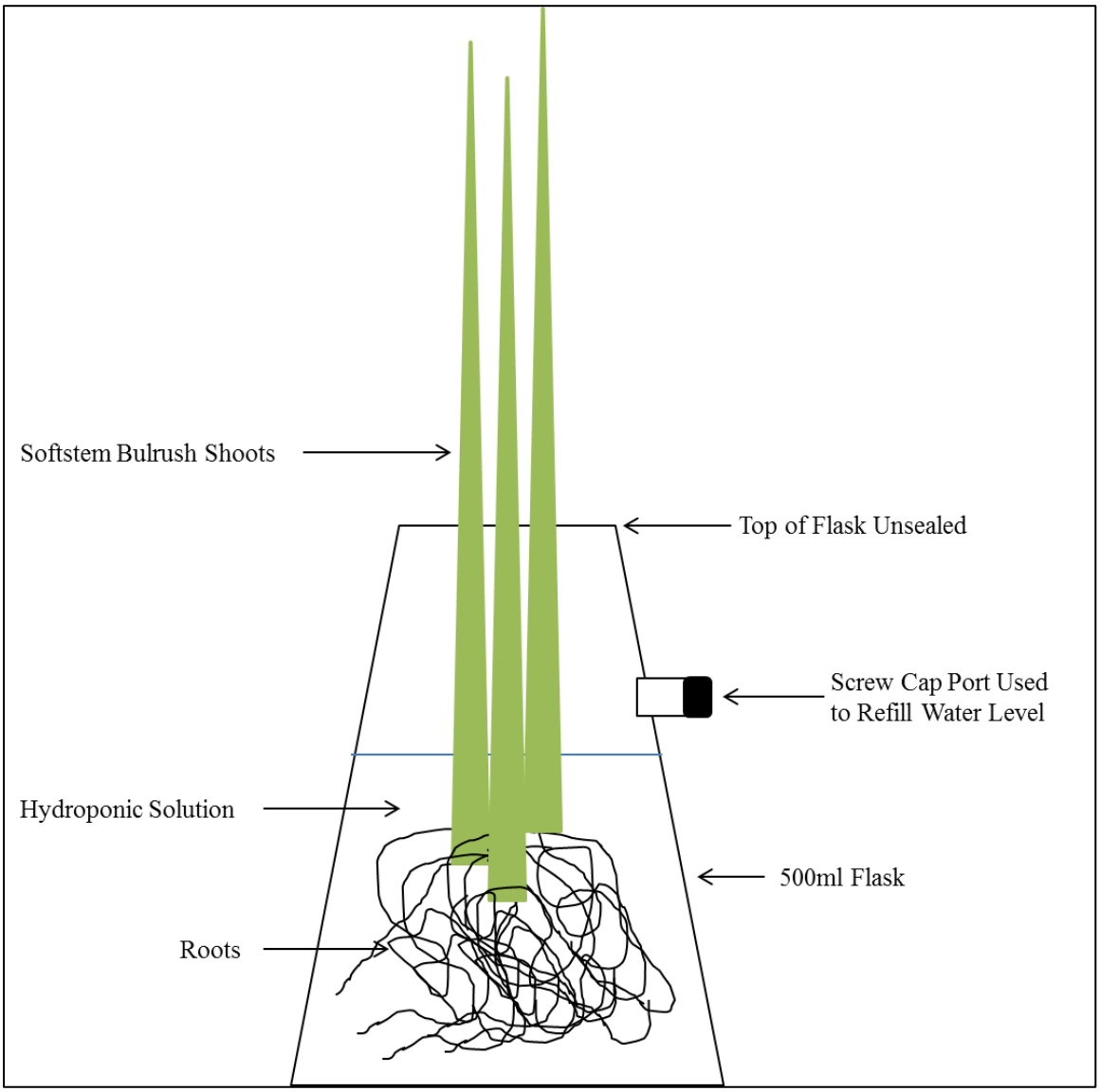


Figure 2-1 Hydroponic reactor flask schematic for the softstem bulrush experiment.

LC/MS Analysis of steroid hormones: Quantification of the steroid growth promoter concentrations from the aqueous solution and plant samples were performed using liquid chromatography-mass spectrometry (LC/MS). An Agilent 6140 Quadrupole LC/MS was used with an Acclaim 120 Å C_{18} column (2.1 x 150 mm, 5 µm; Dionex). The mass spectrometer was operated in negative-ion electrospray mode. An injection volume of 20 µL and mobile phase consisted of acetonitrile:water 50:50 v/v at a flow rate of 0.4 ml/min. Calibration standards and blanks were analyzed before and after sample runs to ensure quality control. The parameters for each compound analyzed by the LC/MS are listed below in Table 2-1.

Table 2-1 LC/MS parameters for target hormone compounds in the softstem bulrush experiment.

Hormone Compound	EE2	E2	ZAL	E1 [1]	ZAN [2]
Mass To Charge Ratio (*m/z*)	295	271	321	269	319
Retention Time (min)	3.7	3.1	2.9	4.2	5.1

1: Metabolite of E2, 2: Metabolite of ZAL

2.2 Results and Discussion

It was hypothesized that EE2, E2, and ZAL would be bioavailable to plants and associated microbes, due to being moderately hydrophobic with $\log K_{ow}$ values of 3.13, 3.94, and 4.15 respectively listed in Table 1-1 (Dietz and Schnoor 2001). This prediction was confirmed in the first two experiments when these synthetic and natural hormones were exposed to mature softstem bulrush, being quickly removed from spiked hydroponic solutions. In the full plant exposure for treatment 1, after 14 days EE2, E2, and ZAL in the aqueous solution were all undetected with only low concentrations for E1 (metabolite of E2) of 0.069 mg/L and ZAN (metabolite of ZAL) at 0.249 mg/L, while the excised (only roots present) plant exposure had somewhat higher concentrations of 0.053 and 0.207 mg/L, respectively (Figure 2-4, Figure 2-5, Figure 2-6 and Table 2-2). In treatment 2, similarly after 14 days, the ZAL was undetectable and ZAN was 0.175 mg/L in the aqueous solution (Figure 2-10 and Table 2-4). Additionally, from Figure 2-2 the cumulative evapotranspiration rates show that the full plants exposures were transpiring steadily during the entire course of the experiment, compared to the dead plant and excised plant exposures, which were not.

Within the plant root material for treatment 1, E1 and ZAL were undetectable and EE2, E1, and ZAN had low concentrations of 0.063, 0.028, and 0.111 mg/L in the full plant exposures, while the excised exposures were somewhat higher at 0.156, 0.108 and 0.032 mg/L, respectively after 14 days (Figure 2-7, Figure 2-8, and Figure 2-9). In treatment 2 with ZAL solely spiked, the parent compound was undetectable and ZAN was only found to be 0.064 mg/L after 14 days (Figure 2-11 and Table 2-4). There were still major decreases of EE2, E2, and ZAL in the "dead plant controls," suggesting that

there was some microbial biodegradation even when the plants were dead and autoclaving may have been necessary to eliminate bacterial growth and subsequent biodegradation.

As seen from Figure 2-3 and listed in Table 2-2 in the blank glassware controls, losses of parent compounds EE2, E2, and ZAL initially spiked at 6, 2, and 3 mg/L were reduced by 11%, 39%, and 24% (flasks were uncapped, open to the environment) respectively while metabolites E1 and ZAN only increased to 0.12 and 0.18 mg/L (Table 2-2). These losses of the parent compounds under controlled conditions are likely due to chemical hydrolysis and/or oxidation in the presence of water at pH 6.8. The natural estrogen E2 was the most reactive (39% loss in 14 days). Losses of the parent compounds can also be attributed to photochemical transformation by direct and indirect light, where steroids such as E2 and E1 have been found to readily degrade (Liu et al. 2004). Formation of metabolites E1 and ZAN were insufficient to fully explain the loss of E2 and ZAL, respectively; in the glassware controls other metabolites likely were also formed. Subsequent experiments will therefore remove those potential non-plant associated degradation pathway by sealing and capping the reactor flasks.

It is clear from Figure 2-4, Figure 2-5, and Figure 2-6 that the full plant exposures caused a more rapid loss of parent compounds EE2, E2, and ZAL, and also resulted in more rapid production of metabolites E1 and ZAN, and the subsequent loss of these metabolites by day 14 compared to the dead exposures. In full plant exposures, E2 was undetectable after only 2 days while ZAL and EE2 disappeared after 14 days. Metabolites E1 and ZAN were formed and reached a maximum at day 7, and then decreased presumably due to further biodegradation.

Table 2-2 Total aqueous concentrations and mass fraction as a percentage for each sampling time point for treatment 1.

Aqueous Concentrations (mg/L)						
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**ZAN**	**E1**
Blank Control	0	5.763	1.743	2.807	0.000	0.000
Blank Control	2	5.550	1.490	2.710	0.050	0.020
Blank Control	7	5.410	1.130	2.440	0.120	0.050
Blank Control	14	5.120	1.050	2.120	0.180	0.120
Excised	2	1.154	0.141	0.660	0.220	0.382
Excised	7	0.557	0.000	0.325	0.438	0.482
Excised	14	0.093	0.000	0.000	0.207	0.053
Full Plant	2	0.845	0.000	0.720	0.293	0.472
Full Plant	7	0.302	0.000	0.265	0.580	0.571
Full Plant	14	0.000	0.000	0.000	0.249	0.069
Dead	2	2.088	0.697	1.253	0.122	0.040
Dead	7	1.295	0.177	0.664	0.223	0.119
Dead	14	0.528	0.000	0.415	0.309	0.183
Mass Fraction (% of Blank Aqueous Control)						
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**ZAN**	**E1**
Excised	2	20.78	9.49	24.36	8.12	25.63
Excised	7	10.30	0.00	13.32	17.95	42.66
Excised	14	1.82	0.00	0.00	9.78	5.07
Full Plant	2	15.22	0.00	26.58	10.81	31.66
Full Plant	7	5.59	0.00	10.86	23.75	50.53
Full Plant	14	0.00	0.00	0.00	11.74	6.61
Dead	2	37.62	46.78	46.23	4.48	2.70
Dead	7	23.93	15.67	27.19	9.12	10.55
Dead	14	10.30	0.00	19.59	14.59	17.39

Table 2-3 Total root concentrations and mass fraction as a percentage for each sampling time point for treatment 1.

Root Concentrations (mg/L)						
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**ZAN**	**E1**
Excised	2	0.381	0.000	0.494	0.206	0.362
Excised	7	0.228	0.000	0.100	0.289	0.343
Excised	14	0.156	0.000	0.000	0.108	0.032
Full Plant	2	0.428	0.057	0.623	0.245	0.312
Full Plant	7	0.280	0.000	0.121	0.357	0.401
Full Plant	14	0.128	0.000	0.000	0.038	0.028
Dead	2	0.862	0.394	0.938	0.022	0.167
Dead	7	0.553	0.248	0.375	0.077	0.103
Dead	14	0.414	0.124	0.163	0.119	0.096
Mass Fraction (% of Blank Aqueous Control)						
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**ZAN**	**E1**
Excised	2	6.87	0.00	18.24	7.60	24.28
Excised	7	4.22	0.00	4.10	11.86	30.40
Excised	14	3.05	0.00	0.00	5.11	3.02
Full Plant	2	7.72	3.83	23.00	11.54	29.76
Full Plant	7	5.18	0.00	4.95	14.63	35.47
Full Plant	14	2.50	0.00	0.00	1.78	2.68
Dead	2	15.54	26.42	34.62	0.92	14.79
Dead	7	10.23	21.99	15.39	3.15	9.12
Dead	14	8.08	11.83	7.67	5.63	9.11

In table 2-2, the mass fraction data for treatment 1, which was calculated by dividing the average concentration of the three replicate samples by the concentration of the aqueous blank controls, and then multiplying by 100 to get a percentage. This was done to investigate how much of the initially spiked compounds can be accounted for

throughout the experiment as well as the proportion of metabolites that were generated from the initially spiked parent compounds. As seen in Table 2-2 and Table 2-3, the mass fraction results differed between the dead plant control and the live plant exposures. On day 14 in the aqueous samples, EE2, E2, ZAL, ZAN, and E1 in the dead controls represented 10, 0, 19.6, 14.6, and 17.39 percent of the spiked glassware controls. The excised plant exposures had 1.8, 0, 0, 9.8, and 5 percent remaining and the full plant exposure contained 0, 0, 0, 11.7, and 6.6 percent respectively of the glassware controls over the same 14 day period. In the root material for the same order of compounds over the same 14 day period, the dead controls possessed 8, 11.8, 7.7, 5.6, and 9.1 percent, while the excised exposures retained 3, 0, 0, 5.11, and 3 percent and the full plant exposures encompassed 2.5, 0, 0, 1.8, and 2.7 percent of the initially spiked parent compounds.

Table 2-4 Total aqueous and root concentrations for treatment 2.

Aqueous Concentrations (mg/L)			
Exposure	**Day**	**ZAL**	**ZAN**
Blank Control	0	2.806	0.000
Blank Control	2	2.640	0.050
Blank Control	7	2.550	0.160
Blank Control	14	2.180	0.240
Dead	2	1.383	0.107
Dead	7	0.903	0.191
Dead	14	0.718	0.288
Full Plant	2	0.318	0.563
Full Plant	7	0.101	0.817
Full Plant	14	0.000	0.175
Root Concentrations (mg/L)			
Exposure	**Day**	**ZAL**	**ZAN**
Dead	2	0.427	0.082
Dead	7	0.354	0.152
Dead	14	0.211	0.169
Full Plant	2	0.113	0.673
Full Plant	7	0.031	0.271
Full Plant	14	0.000	0.064

In treatments 1 and 2, when comparing the blank and dead controls to the plant groups, there are clear differences in the degradation rates of the parent steroid hormones and the formation of metabolites. In treatment 1, E2 is reduced to basically zero in plant and aqueous samples after only two days in the excised and full plant treatment groups, while the blank controls shows little decline and dead plants still have E2 present after 14 days. ZAL in the same plant treatment groups reacted in the same fashion, although

slower, with zero detections after 14 days and considerable reductions in the first 7 days. EE2 in the excised and full plant groups behaved similarly to ZAL, with 97% and 99% decreases in the aqueous solution. Additionally, the rapid losses of parent steroid hormones from solution in the dead plant controls, as well as decreases in the blank controls can most likely be attributed to microbial contamination and photolysis since the flasks were uncovered and the dead plants were not autoclaved with the flasks. The production of ZAN and E1 metabolites are also much different in the plant treatment groups compared to the blank and dead controls. Both are generated much more quickly and at much higher concentrations between the 2 and 7 day sampling periods and then significantly reduced by the 14 day sampling, compared with the blank and dead plant controls that are slowly increasing linearly throughout the experiment.

These results clearly show that metabolism by the live plant and associated microbes treatment groups are playing a major role in the reduction of EE2, E2, and ZAL as well as the formation and subsequent loss of ZAN and E1. In treatment 2, the transformation of ZAL and ZAN in the live plant exposed samples functioned analogously to the results treatment 1, again demonstrating the metabolism of parent steroid hormones and their transformation products by live plants. These findings also correspond to a study done by Card and Chin (2011) where E2 and E1 were undetectable and ZAL and ZAN were significantly reduced after 8 days after being spiked into hydroponic solution and exposed to maize seedlings.

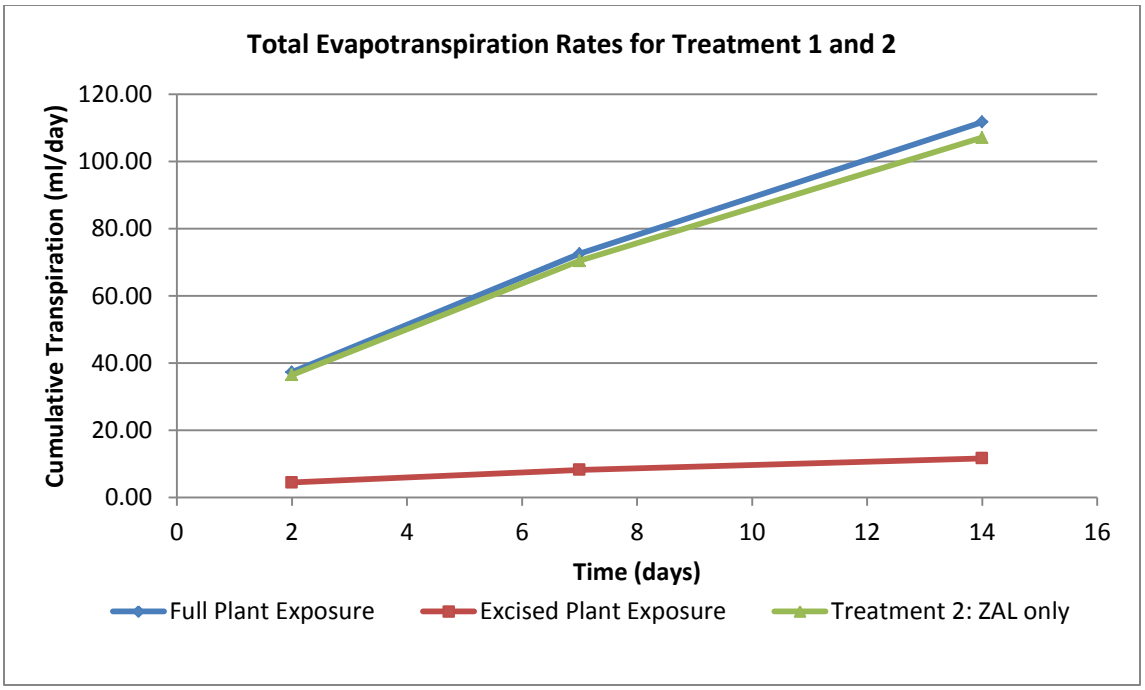


Figure 2-2 Cumulative evapotranspiration rates for live plant exposures in treatment 1 and 2 over the 14 day experiment.

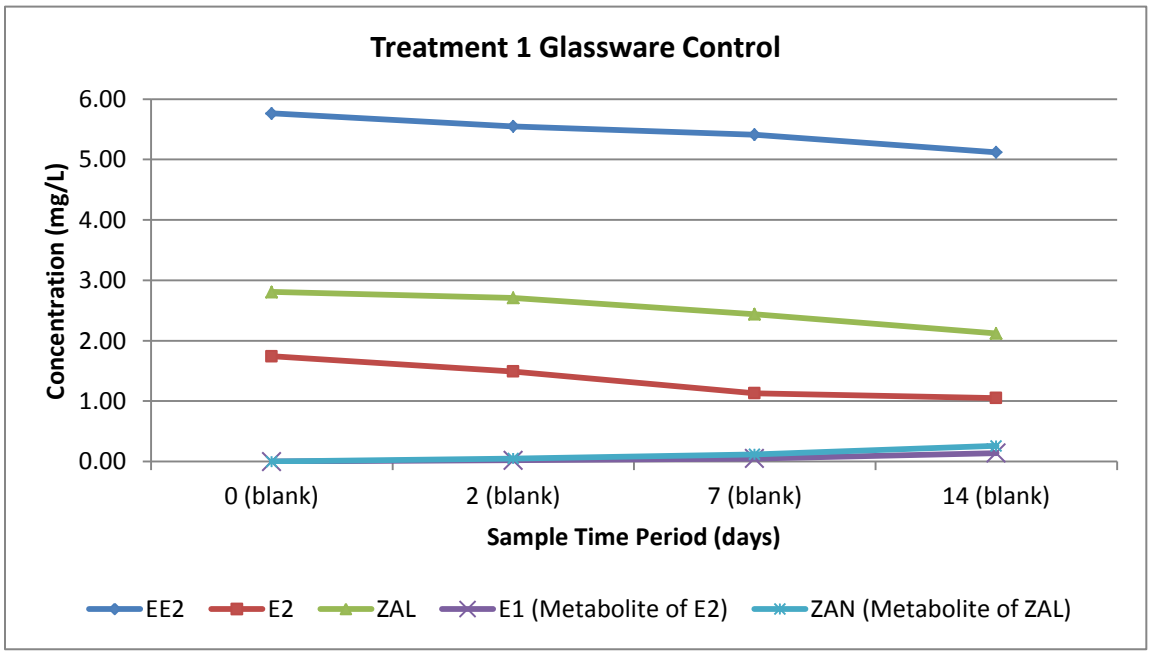


Figure 2-3 Glassware control concentrations of target compounds for treatment 1 over 14 day sampling period.

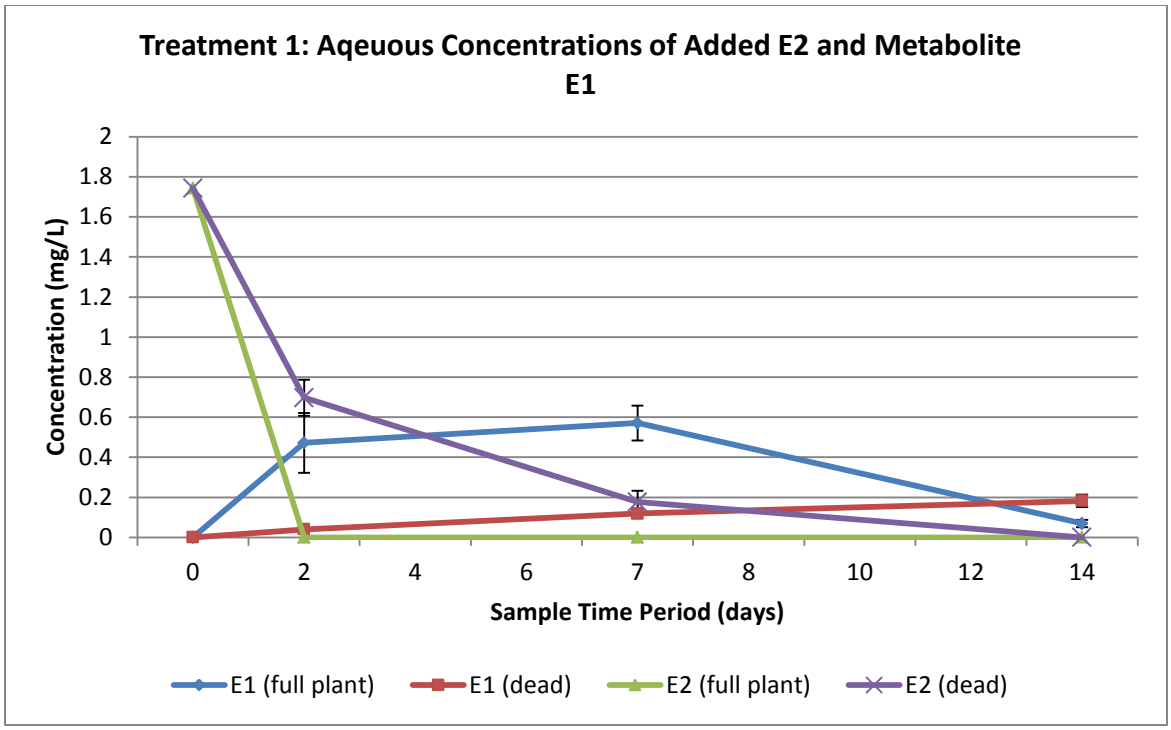


Figure 2-4 Aqueous concentrations of added E2 and formation of metabolite E1 in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

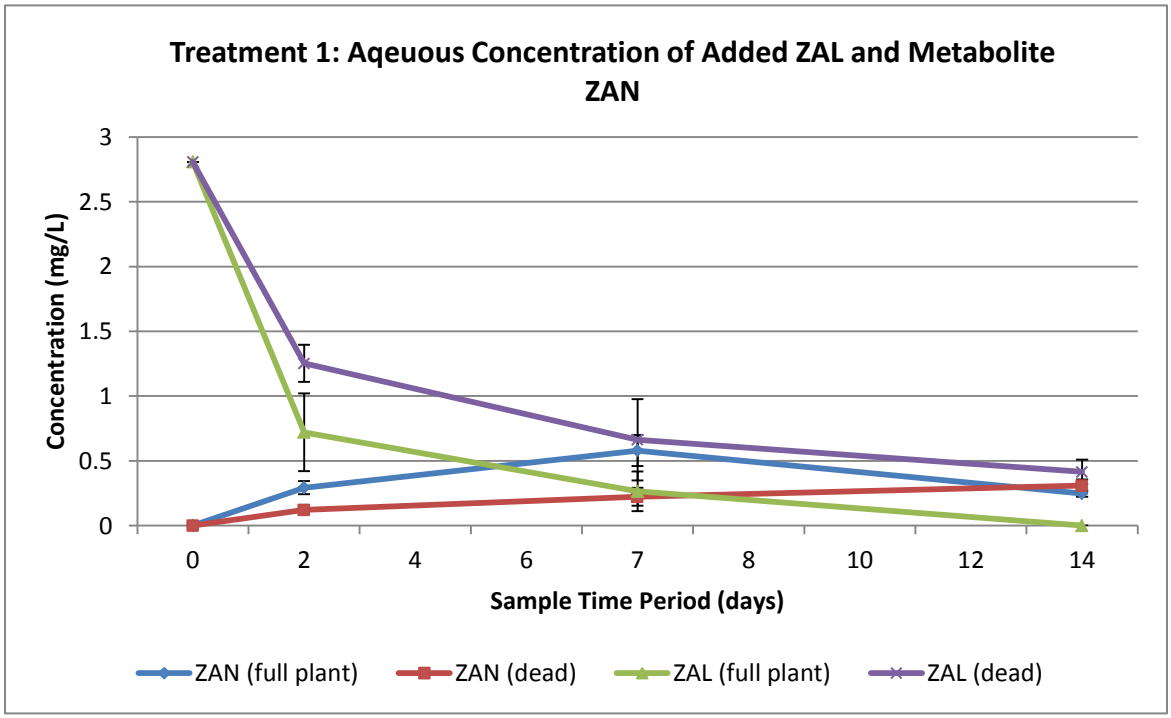


Figure 2-5 Aqueous concentrations of added ZAL and formation of metabolite ZAN in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

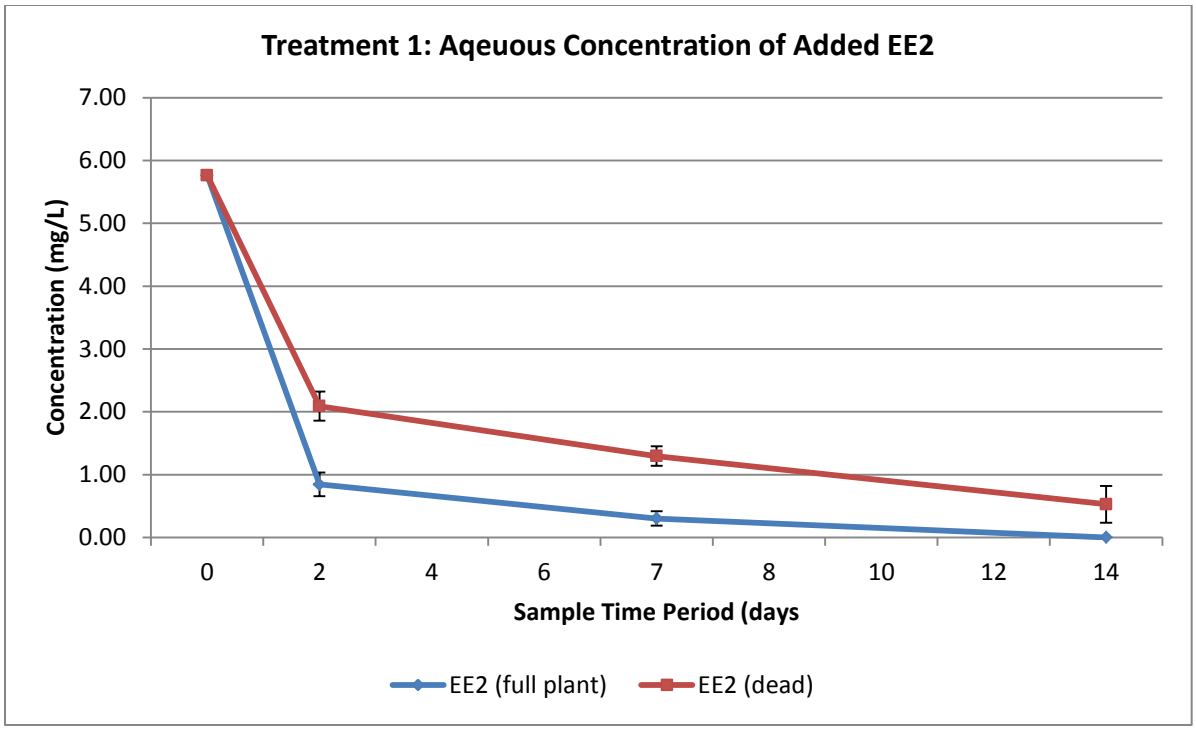


Figure 2-6 Aqueous concentrations of added EE2 in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

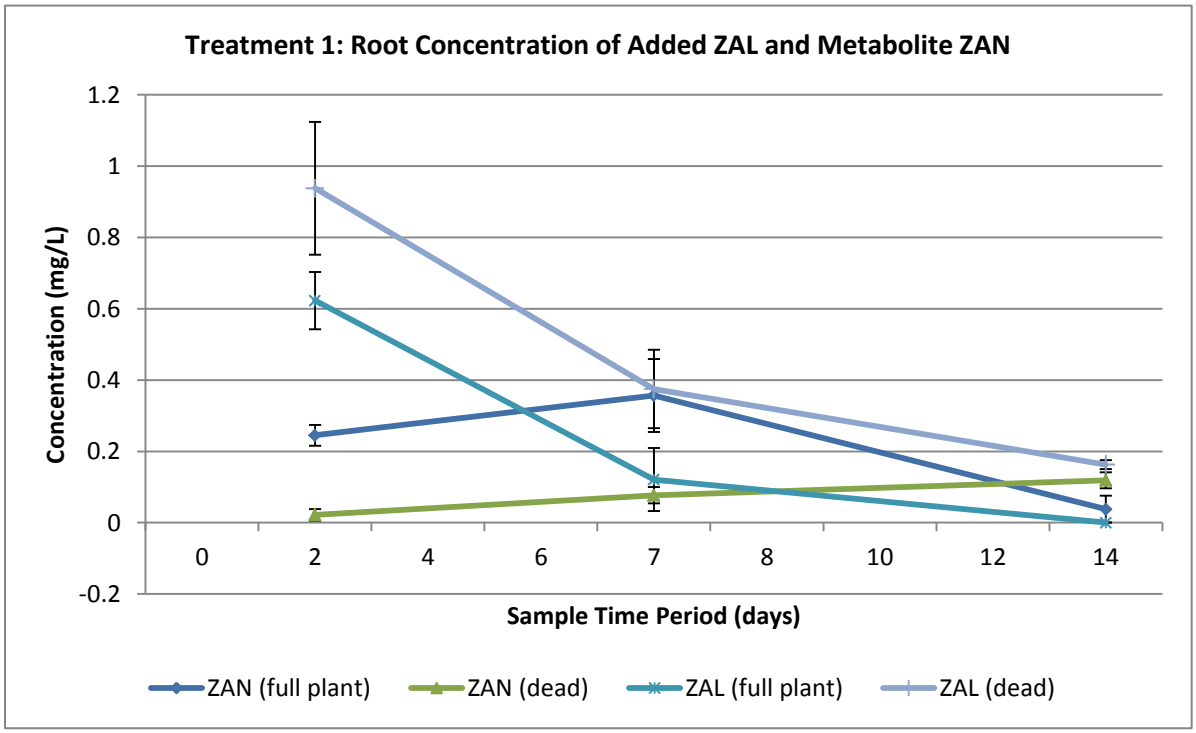


Figure 2-7 Root concentrations of added ZAL and formation of metabolite ZAN in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

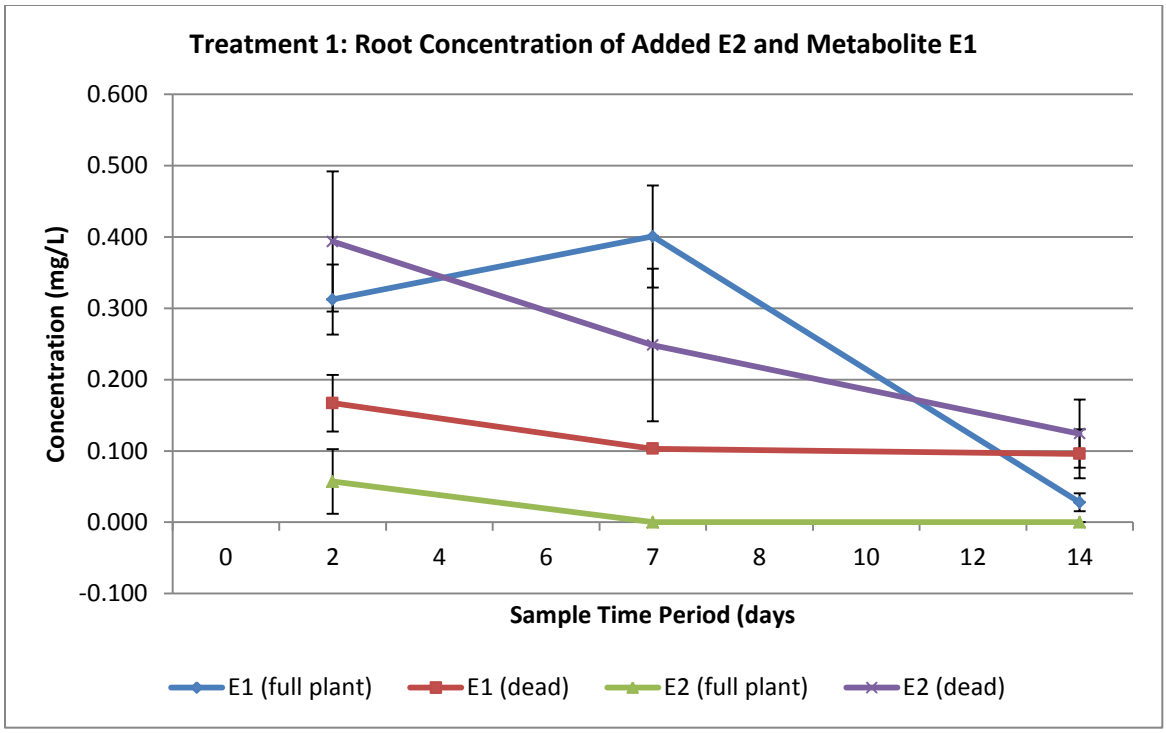


Figure 2-8 Root concentrations of added E2 and formation of metabolite E1 in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

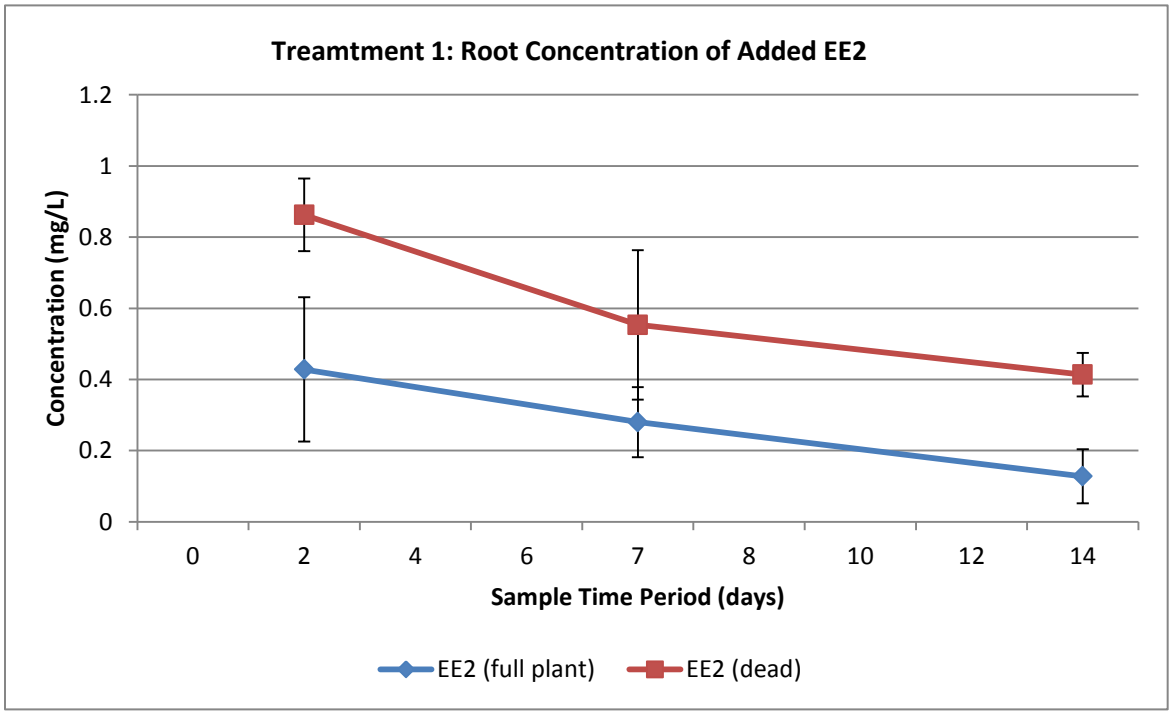


Figure 2-9 Root concentrations of added EE2 in Treatment 1 for full plant vs. dead plant exposures over the 14 day experiment.

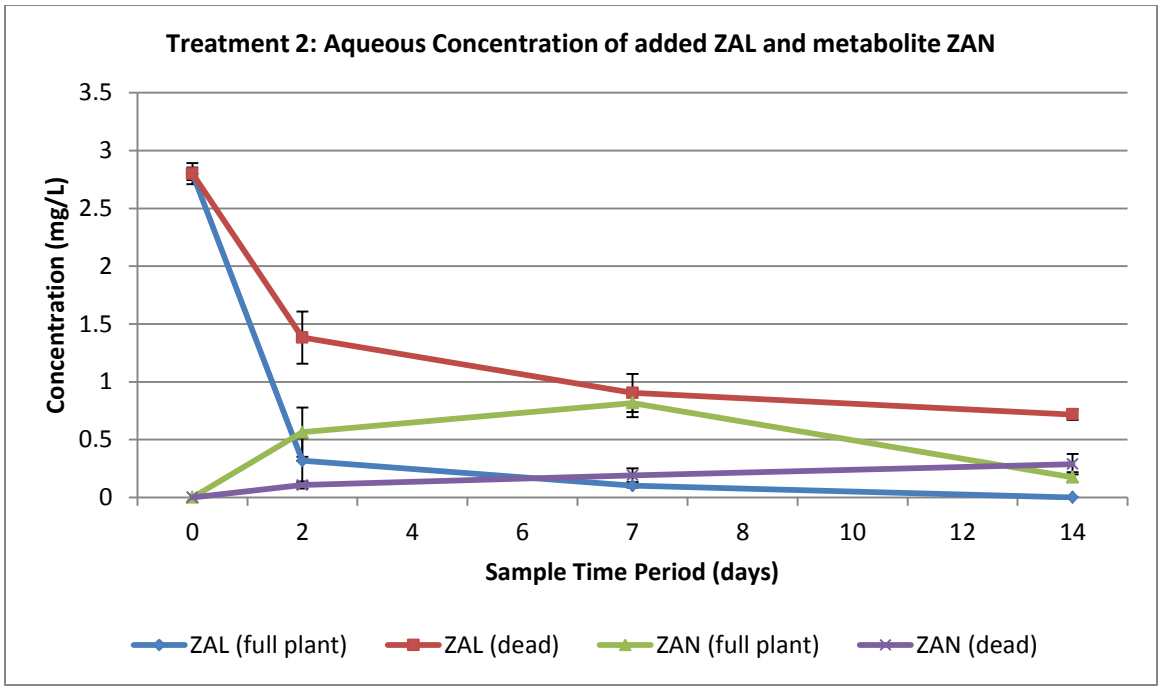


Figure 2-10 Aqueous concentrations of target compound ZAL and formation of metabolite ZAN for treatment 2 over the 14 day experiment.

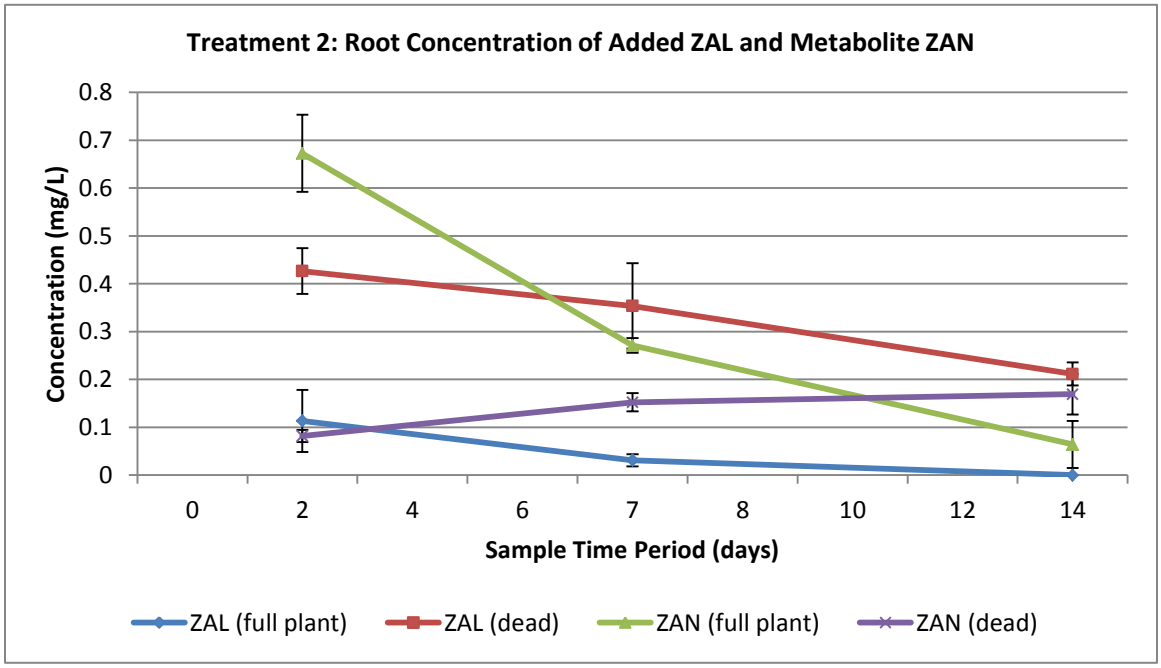


Figure 2-11 Root concentrations of target compound ZAL and formation of metabolite ZAN for treatment 2 over the 14 day experiment.

CHAPTER 3

BIODEGRADATION OF SYNETHIC AND NATURAL STERIOD GROWTH PROMOTERS BY HYBRID POPLAR (*POPULUS DELTOIDES NIGRA*, DN-34*)*

3.1 Material and Methods

Chemicals: E2 (1, 3, 5(10)-Estratrien-3,17β-diol-2,4,16,16-d4 [50-28-2]) (98%), **E1** (1, 3, 5(10)-Estratrien-3-ol-17-one-2,4,16,16-d4 [53-16-7]), (99%), **zearalanone (ZAN)** (2,4-Dihydroxy-6-(10-hydroxy-6-oxoundecyl)benzoic acid μ-lactone [5975-78-0] (98%), were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). **EE2** (1, 3, 5(10)-Estratrien-17α-ethynyl-3, 17β-diol-2,4,16,16-d4 [57-63-6] (98%) was obtained from U.S. Pharmacopeia (Rockville, MD). **ZAL** (6-(6,10-Dihydroxyundecy)-β-resorcylic acid-lactone [26538-44-3]) was extracted and purified from Ralgro Magnum (Schering-Plough Animal Health Corp., Union, NJ). **Estriol (E3)** (16α-Hydroxy-17β-estradiol[50-27-1]), **Trenbolone Acetate (TBA)** (4, 9, 11-Estratrien-17β-ol-3-one acetate [10161-34-9](> 99%) and **17β-Trenbolone (TBOH)**, (17β-Hydroxyestra-4,9,11-triene-3-one [10161-33-8] (>99%), were obtained from Steraloids Inc. (Newport, RI). Other chemicals, including LC/MS solvents, were purchased from Fisher Scientific (Pittsburgh, PA).

Hydroponic Uptake Experiment: Uptake of E2, ZAL, EE2 and TBA were evaluated using vigorously growing hybrid poplars (*Populus deldtoides* x *Populus nigra*, DN-34). The experiment was conducted by growing hybrid poplar cuttings (DN-34) in hydroponic solution prepared as ½-strength Hoagland's nutrient solution (pH 6.8) buffered with 1.0M NaOH. The poplars were placed in flasks with the hydroponic

solution and refilled based on evapotranspiration rates. As seen in Figure 3-1, after 48 days of growth, the plants were placed in autoclaved 500 ml Erlenmeyer flasks and 300 ml of hydroponic solution. The solution was spiked with initial concentrations of 2 mg/L EE2, 2 mg/L E2, and 2 mg/L ZAL and 2 mg/L TBA. Large concentrations of these parent compounds were desired compared with much lower environmental sample concentrations so they could be detected in the plant tissues. There were three replicates per sampling period with 4 separate exposures including: full plant, autoclaved glassware controls with no plants, negative control with dead plants and excised plants with solely roots present. The quantified concentrations of the three replicates for each exposure were averaged and listed as the measured amount for each sampling event along with standard deviations shown on the graphs, using the concentration data from the three replicates. All flasks were wrapped in aluminum foil and placed in a plant growth chamber (Percival Scientific PVG-40, Perry, IA) with a16:8 hour light:dark photoperiod (150 μmol s^{-1} m^{-2}) at 23^{o}C and 42% relative humidity. To maintain initial water levels, flasks were refilled daily with Milli-Q-water and measured the evapotranspiration.

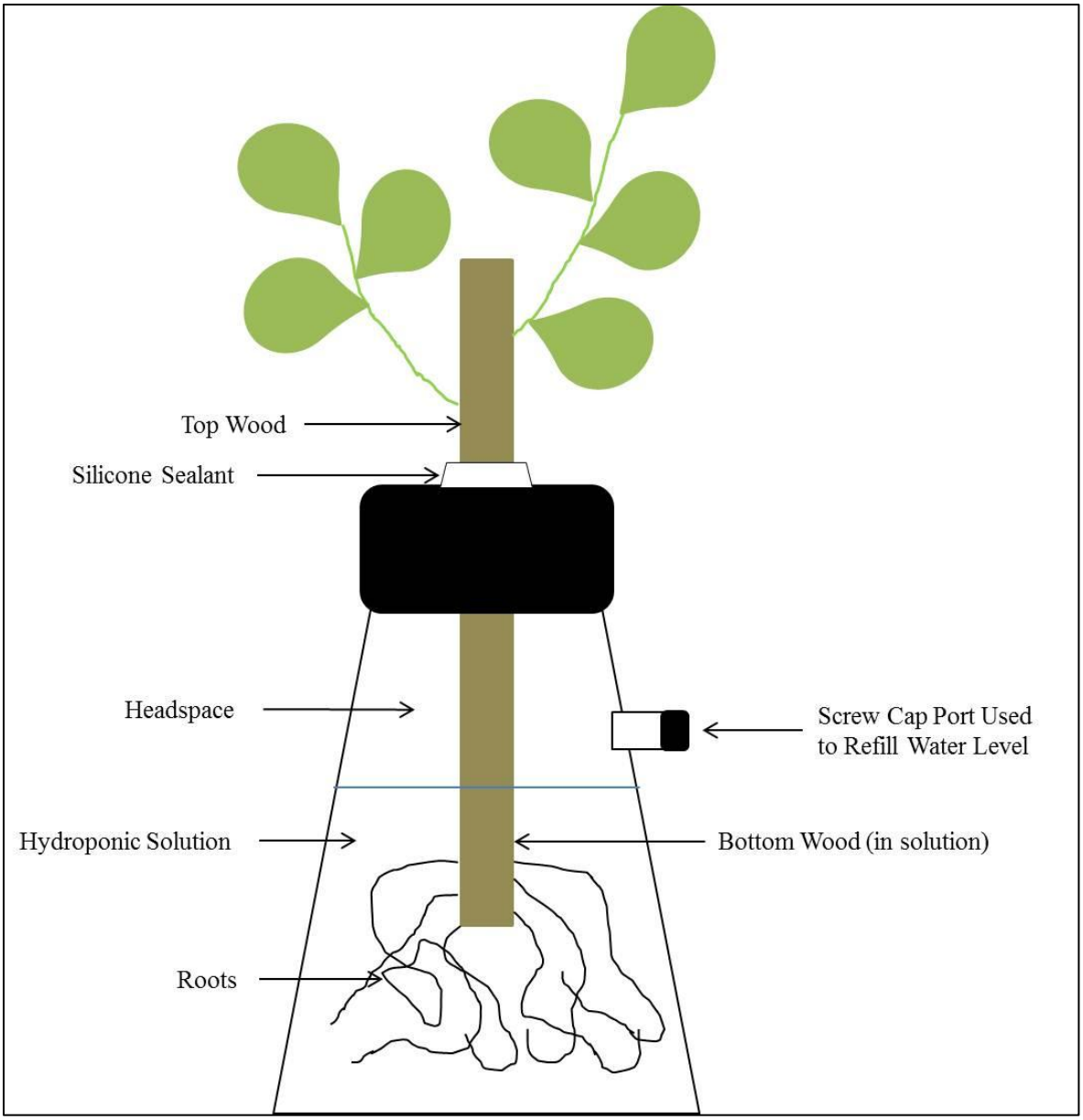


Figure 3-1 Hydroponic reactor flask schematic for the poplar experiment.

Plants were destructively sampled at 1, 2, 5 and 10 day time points. Four 12ml aliquots (totaling 48ml) were collected from the well mixed aqueous phase, combined with a 1:1 ratio of acetonitrile (to stop any further biodegradation during storage) and filtered using HVLP type Durapore® fiber filters with 0.45 µm pore size (Millipore

Corp.) and collected for analysis by a liquid chromatography and mass spectrum (LC/MS) instrument. Roots were removed from the flasks, dried overnight at 100^{o}C and weighed. The material was then ground up using a mortar and pestle with liquid nitrogen and shaken in glass centrifuge vials with 10ml acetonitrile overnight. The vials were then centrifuged at 3000 rpm (Beckman Coulter, Brea, CA, Model J2-21M) for 30 min and filtered using the same Durapore® fiber filters mentioned above, and collected for analysis on the LC/MS. The woody parts of the poplars were sampled and separated into two sets: 1) bottom wood, which was the section of the cutting permanently exposed to the aqueous solution and 2) the top wood, which was located outside the flask. The wood and bark for each sample set were chopped up, saturated in acetonitrile and shaken in glass centrifuge vials overnight, and then the solute was filtered with the same 0.45 µm fiber filters and collected for analysis on the LC/MS.

LC/MS Analysis of steroid hormones: Quantification of the growth promoter concentrations from the aqueous solution and plant samples were performed using liquid chromatography-mass spectrometry (LC/MS). An Agilent 6140 Quadrupole LC/MS was used with an Acclaim 120 Å C_{18} column (2.1 x 150 mm, 5 µm; Dionex). The mass spectrometer was operated in negative-ion electrospray mode for EE2, E2, ZAL, E1, E3 and ZAN while TBA and TBOH were operated in positive-ion electrospray mode. An injection volume of 20 µL and mobile phase consisted of acetonitrile:water 50:50 v/v at a flow rate of 0.4 ml/min. Calibration standards and blanks were analyzed before and after sample runs to ensure quality control. The parameters for each compound analyzed by the LC/MS are listed below in Table 3-1.

Table 3-1 LC/MS parameters for target hormone compounds in the poplar experiment.

Hormone Compound	EE2 [1]	E2 [1]	ZAL [1]	TBA [1]	E1 [2]	E3 [3]	ZAN [4]	TBOH [5]
Ion-Mode	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Pos.
Mass To Charge Ratio (*m/z*)	295	271	321	311	269	287	319	271
Retention Time (min)	3.9	3.1	2.9	4.1	4.4	1.4	5.1	2.4

1: Initially spiked parent compounds, 2: Metabolite of E2, 3: Metabolite of E1,

4: Metabolite of ZAL, 5: Metabolite of TBA

3.2 Results and Discussion

Just as in the first two experiments, the target steroid hormone compounds EE2, E2, and ZAL were theorized to be bioavailable to plants and associated microbes due to being moderately hydrophobic with logKow values ranging from about 3-4. In this experiment, a new plant was examined, the hybrid poplar (DN-34), as well the same parent compounds used with one additional parent compound, TBA, and two additional metabolites, E3 and TBOH. The bioavailability hypothesis was again verified in this third experiment when the synthetic and natural hormones were exposed to hybrid poplar trees, being readily removed from the spiked hydroponic solution and confirmed subsequent uptake into the roots and woody main stem of the poplar. As shown in

Table 3-3 after 10 days with an initial spiked concentration of 2 mg/L into the aqueous solution for each parent compound, EE2, E2, ZAL, and TBA were measured at 0.008, 0.003, 0.060, 0.033 mg/L, respectively, in the full plant exposures and 0.003, 0.011, 0.033, and 0.047 mg/L, respectively, in the excised plant exposures. Compared

with those, the dead plant exposures contained somewhat higher concentrations of 0.424, 0.301, 0.322, and 0.498 mg/L, respectively remaining after 10 days. From Figure 3-2 the cumulative evapotranspiration rates show that the full plants exposures were transpiring steadily during the entire course of the experiment, compared to the dead plant and excised plant exposures, which were not. Additionally from Figure 3-3, the blank glassware controls showed little losses of the parent compounds and very minimal amounts of metabolites formed, revealing that the reactors were set up appropriately to control for any non-plant related degradation of the initially spiked hormones.

Root concentrations listed in Table 3-5 showed that after 10 days EE2, E2, ZAL, and TBA were measured at 0.015, 0.063, 0.014, and 0.068 mg/L respectively for the excised plant exposures while the full plant exposure had 0.008, 0.036, 0.019, and 0.039 mg/L respectively.

Concentrations in the main stem of the poplars, (shown in Figure 3-12, Figure 3-13, Figure 3-14, Figure 3-15, Figure 3-16, Figure 3-17, Figure 3-18, Figure 3-19, Figure 3-20 and Table 3-7) after 10 days, the bottom wood concentrations of EE2, E2, ZAL, and TBA were 0.030, 0.037, 0.085, and 0.220 mg/L and top wood concentrations were 0.015, 0.001, 0.049, and 0.022 mg/L, respectively, in the full plant exposures. Excised plant exposures for the same order of compounds and duration were measured to be 0.073, 0.054, 0.122, and 0.054 mg/L for the bottom wood and 0.073, 0.072, 0.068, and 0.026 mg/L respectively for the top wood. The dead plant exposures after 10 days measured 0.887, 0.392, 0.759, and 0.660 mg/L in the bottom wood and 0.076, 0.253, 0.100, and 0.095 mg/L, respectively, in the top wood.

Table 3-2 Initial reaction rates for the initially spiked parent hormone compounds within the aqueous solution.

Initial Reaction Rates (after 24 hours)					
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**
Dead	1	1.215	1.206	1.120	0.960
Excised	1	1.931	1.782	1.740	1.690
Full Plant	1	1.891	1.876	1.788	1.700
Blank	1	0.083	0.062	0.088	0.072

Table 3-3 Aqueous concentrations of each parent compound and their respective metabolites for all exposures on each sampling event over the 10 day experiment duration.

Aqueous Concentrations (mg/L)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Blank Control	0	2.037	2.008	2.015	2.002	0.000	0.000	0.000	0.000
Blank Control	1	1.954	1.947	1.927	1.930	0.000	0.000	0.000	0.003
Blank Control	2	1.887	1.862	1.899	1.843	0.000	0.000	0.000	0.003
Blank Control	5	1.873	1.782	1.822	1.812	0.000	0.000	0.005	0.002
Blank Control	10	1.735	1.626	1.774	1.739	0.000	0.001	0.007	0.002
Dead	1	0.822	0.803	0.895	1.042	0.001	0.003	0.020	0.027
Dead	2	0.756	0.726	0.622	0.795	0.014	0.007	0.031	0.044
Dead	5	0.604	0.484	0.419	0.580	0.026	0.062	0.044	0.064
Dead	10	0.424	0.301	0.322	0.498	0.055	0.095	0.049	0.076
Full Plant	1	0.106	0.226	0.275	0.311	0.028	0.022	0.027	0.042
Full Plant	2	0.074	0.080	0.076	0.155	0.058	0.044	0.050	0.089
Full Plant	5	0.008	0.049	0.013	0.054	0.127	0.089	0.081	0.134
Full Plant	10	0.008	0.003	0.060	0.033	0.058	0.012	0.131	0.029
Excised	1	0.146	0.133	0.227	0.301	0.018	0.006	0.038	0.040
Excised	2	0.012	0.077	0.080	0.075	0.043	0.009	0.046	0.079
Excised	5	0.004	0.026	0.032	0.076	0.100	0.097	0.070	0.165
Excised	10	0.003	0.011	0.033	0.047	0.016	0.007	0.110	0.071

Table 3-4 Mass fraction of the aqueous solution each parent compound and their respective metabolites for each plant exposure on each sampling event over the 10 day experiment duration.

Aqueous Mass Fraction (% of blank aqueous controls)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Dead	1	42.05	41.24	46.42	53.98	0.04	0.17	1.05	1.39
Dead	2	40.07	38.97	32.74	43.12	0.76	0.40	1.62	2.38
Dead	5	32.24	27.16	23.02	32.03	1.43	3.47	2.41	3.52
Dead	10	24.42	18.53	18.15	28.67	3.09	5.85	2.75	4.35
Excised	1	5.42	11.62	14.27	16.12	1.46	1.13	1.42	2.17
Excised	2	3.93	4.32	4.00	8.41	3.08	2.36	2.62	4.81
Excised	5	0.44	2.73	0.70	2.96	6.98	4.98	4.43	7.40
Excised	10	0.45	0.20	3.36	1.91	3.27	0.71	7.41	1.68
Full Plant	1	7.47	6.81	11.76	15.61	0.94	0.28	1.99	2.07
Full Plant	2	0.65	4.14	4.22	4.10	2.26	0.46	2.41	4.31
Full Plant	5	0.20	1.46	1.74	4.17	5.52	5.45	3.82	9.08
Full Plant	10	0.19	0.70	1.86	2.70	0.91	0.44	6.17	4.11

The mass fraction, which was calculated by dividing the concentration for each compound at each sampling event by the blank aqueous glassware controls and multiplying by 100 to get a percentage, represents the proportion remaining from what was originally spiked into solution. As listed in

Table 3-4 there is quite a difference when comparing the live plant exposures to the dead plant exposure. After 10 days for the parent compounds EE2, E2, ZAL, and TBA there was 24.4%, 18.5%, 18.2%, and 28.7% respectively remaining in the dead exposures, while the full plant exposure only had 0.2%, 0.7%, 1.9%, and 2.7%

respectively, and the excised plant exposures contained only 0.45%, 0.2%, 3.4%, and 1.9% respectively.

It is also clear from Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7and

Table 3-3 in the aqueous solution that the live plant exposures caused a more rapid loss of parent compounds EE2, E2, ZAL, and TBA and also resulted in more rapid production of metabolites E1, E3, ZAN, and TBOH as well as the subsequent loss of these metabolites by day 10 compared to the dead exposures.

Table 3-5 Root concentrations of each parent compound and their respective metabolites for the live plant exposures on each sampling event over the 10 day experiment.

Root Concentrations (mg/L)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Excised	1	0.724	0.402	0.618	0.651	0.070	0.073	0.082	0.036
Excised	2	0.356	0.299	0.344	0.203	0.114	0.119	0.213	0.184
Excised	5	0.117	0.086	0.094	0.133	0.187	0.205	0.135	0.220
Excised	10	0.015	0.063	0.014	0.068	0.033	0.014	0.012	0.051
Full Plant	1	0.664	0.639	0.787	0.464	0.052	0.054	0.099	0.069
Full Plant	2	0.533	0.227	0.342	0.245	0.157	0.075	0.188	0.196
Full Plant	5	0.090	0.041	0.087	0.106	0.146	0.133	0.087	0.294
Full Plant	10	0.008	0.036	0.019	0.039	0.047	0.015	0.011	0.099

Table 3-6 Mass fraction in the roots of each parent compound and their respective metabolites for both live plant exposures on each sampling event over 10 days.

Root Mass Fraction (% of blank aqueous controls)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Excised	1	37.03	20.64	32.07	33.75	3.63	3.76	4.24	1.85
Excised	2	18.84	16.07	18.13	11.01	6.00	6.40	11.21	9.97
Excised	5	6.25	4.80	5.18	7.33	10.29	11.51	7.43	12.16
Excised	10	0.84	3.85	0.81	3.94	1.84	0.83	0.69	2.91
Full Plant	1	34.00	32.83	40.86	24.06	2.70	2.75	5.13	3.56
Full Plant	2	28.24	12.18	18.03	13.27	8.27	4.03	9.92	10.63
Full Plant	5	4.82	2.29	4.77	5.83	7.99	7.47	4.76	16.24
Full Plant	10	0.43	2.19	1.07	2.22	2.67	0.93	0.60	5.71

As seen in Table 3-5, and Table 3-6 as well as Figure 3-8, Figure 3-9, Figure 3-10, and Figure 3-11 in the live plant exposures a large proportion of the parent compounds were detected in the root tissue on day 1 and by day 10 had mostly gone away. For the compounds EE2, E2, ZAL, and TBA in the full plant exposure there was a mass fraction of 34%, 32.8%, 40.9% and 24.1%, respectively, measured in the roots after 1 day of exposure and 37%, 20.7%, 32.1%, 33.8%, respectively, in the excised plant root samples. There was also evidence of the metabolites E1, E3, ZAN, and TBOH being formed, at the highest concentrations by day 5 and then degrading by day 10, revealing there was a high degree of metabolism going on in the roots over the course of the 10 day experiment. It appears that most of the metabolism is occurring in root tissues, but there is also evidence of translocation and metabolism in the bottom wood, and to a lesser extent in the top wood.

Table 3-7 Wood concentrations of each parent compound and their respective metabolites for each plant exposure on each sampling event over the 10 day experiment.

Wood Concentrations (mg/L)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Bottom Dead	1	0.405	0.558	0.198	0.238	0.002	0.023	0.005	0.003
Bottom Dead	2	0.687	0.328	0.344	0.315	0.002	0.025	0.012	0.004
Bottom Dead	5	0.778	0.224	0.557	0.419	0.005	0.044	0.012	0.011
Bottom Dead	10	0.887	0.392	0.759	0.660	0.054	0.078	0.011	0.026
Top Dead	1	0.000	0.054	0.023	0.037	0.000	0.005	0.006	0.007
Top Dead	2	0.008	0.124	0.045	0.052	0.000	0.003	0.010	0.024
Top Dead	5	0.055	0.161	0.090	0.088	0.000	0.006	0.005	0.012
Top Dead	10	0.076	0.253	0.100	0.095	0.003	0.016	0.012	0.028
Bottom Excised	1	0.464	0.237	0.200	0.330	0.015	0.065	0.102	0.050
Bottom Excised	2	0.581	0.283	0.388	0.232	0.042	0.084	0.211	0.057
Bottom Excised	5	0.394	0.190	0.403	0.373	0.064	0.202	0.166	0.066
Bottom Excised	10	0.073	0.054	0.122	0.054	0.039	0.097	0.147	0.148
Top Excised	1	0.029	0.011	0.036	0.091	0.002	0.039	0.029	0.008
Top Excised	2	0.033	0.069	0.064	0.070	0.000	0.114	0.102	0.010
Top Excised	5	0.058	0.125	0.043	0.118	0.013	0.068	0.240	0.052
Top Excised	10	0.073	0.072	0.068	0.026	0.005	0.034	0.109	0.047
Bottom Full Plant	1	0.417	0.107	0.214	0.262	0.057	0.044	0.321	0.096
Bottom Full Plant	2	0.754	0.566	0.286	0.688	0.054	0.170	0.484	0.161
Bottom Full Plant	5	0.027	0.216	0.255	0.322	0.159	0.281	0.291	0.225
Bottom Full Plant	10	0.030	0.037	0.085	0.220	0.049	0.131	0.244	0.333
Top Full Plant	1	0.087	0.041	0.059	0.084	0.000	0.036	0.150	0.038
Top Full Plant	2	0.050	0.143	0.115	0.159	0.000	0.058	0.205	0.033
Top Full Plant	5	0.094	0.186	0.056	0.165	0.011	0.013	0.136	0.047
Top Full Plant	10	0.015	0.001	0.049	0.022	0.002	0.016	0.078	0.024

It is apparent from that the live plant exposures took up more of the parent compounds EE2, E2, ZAL, and TBA after 2 days and also resulted in more rapid production of metabolites E1, E3, ZAN, and TBOH as well as the subsequent loss of these metabolites by day 10 compared to the dead exposures. Throughout the experiment duration, the dead exposures increased in linear increments for the parent compounds and very small amounts of metabolites without showing losses or metabolism by day 10.

Additionally from Table 3-8, the mass fraction of the parent compounds EE2, E2, ZAL, and TBA in the woody material after day 10 in the dead plant exposure represented a combined value of 55.5%, 56.1, 48.4%, and 43.5% of the initially spiked concentrations of parent compounds; indicating a large amount of uptake by diffusion/sorption, but little production of metabolites. In contrast, in the excised plant exposure for the same order of parent compounds there was a combined mass of 15.3%, 7.7%, 11.7%, and 10% left after 10 days, while in the full plant exposure for the same compounds and duration had 7.5%, 2.4%, 7.4%, and 7.6% remaining in the main stem material. Reactors with live plant material, thus, showed more metabolism of the parent compounds, especially the live, whole plants.

Table 3-8 Mass fraction in bottom and top wood of each parent compound and their respective metabolites for each plant exposure on each sampling event over the 10 day experiment.

Wood Mass Fraction (% of blank aqueous controls)									
Exposure	**Day**	**EE2**	**E2**	**ZAL**	**TBA**	**ZAN**	**E1**	**E3**	**TBOH**
Bottom Dead	1	20.71	11.34	10.26	12.33	0.12	1.20	0.27	0.17
Bottom Dead	2	36.41	24.76	18.14	17.11	0.12	1.33	0.64	0.22
Bottom Dead	5	41.54	27.57	30.56	23.14	0.27	2.49	0.70	0.62
Bottom Dead	10	51.11	40.47	42.78	37.98	3.05	4.77	0.65	1.47
Top Dead	1	0.00	2.77	1.21	1.92	0.00	0.26	0.31	0.34
Top Dead	2	0.41	6.69	2.38	2.84	0.00	0.15	0.56	1.28
Top Dead	5	2.94	9.03	4.97	4.87	0.00	0.32	0.28	0.65
Top Dead	10	4.36	15.58	5.64	5.45	0.16	1.00	0.76	1.59
Bottom Excised	1	23.77	12.18	10.38	17.10	0.77	3.33	5.24	2.59
Bottom Excised	2	30.81	15.23	20.41	12.60	2.21	4.53	11.33	3.11
Bottom Excised	5	18.87	10.66	22.11	20.59	3.53	11.36	9.29	3.66
Bottom Excised	10	11.11	3.33	6.89	8.47	2.18	5.95	9.03	8.51
Top Excised	1	1.48	0.58	1.85	4.72	0.08	2.01	1.49	0.42
Top Excised	2	1.77	3.71	3.40	3.80	0.02	6.10	5.48	0.52
Top Excised	5	3.08	7.01	2.33	6.51	0.69	3.81	13.45	2.88
Top Excised	10	4.18	4.42	3.81	1.52	0.29	2.11	6.69	2.72
Bottom Full Plant	1	32.02	8.27	16.64	20.38	4.40	3.39	6.30	7.47
Bottom Full Plant	2	39.96	30.40	15.05	37.35	2.86	9.15	15.24	8.71
Bottom Full Plant	5	21.13	12.14	13.98	17.77	8.72	15.76	20.10	12.40
Bottom Full Plant	10	6.64	2.26	4.79	6.31	2.78	8.03	15.03	19.18
Top Full Plant	1	2.90	2.12	3.08	4.36	0.00	1.83	7.72	1.97
Top Full Plant	2	3.71	7.70	6.04	8.63	0.00	3.11	11.01	1.77
Top Full Plant	5	5.00	10.44	3.05	10.22	0.59	0.71	7.64	2.61
Top Full Plant	10	0.85	0.05	2.74	1.27	0.09	0.98	4.78	1.40

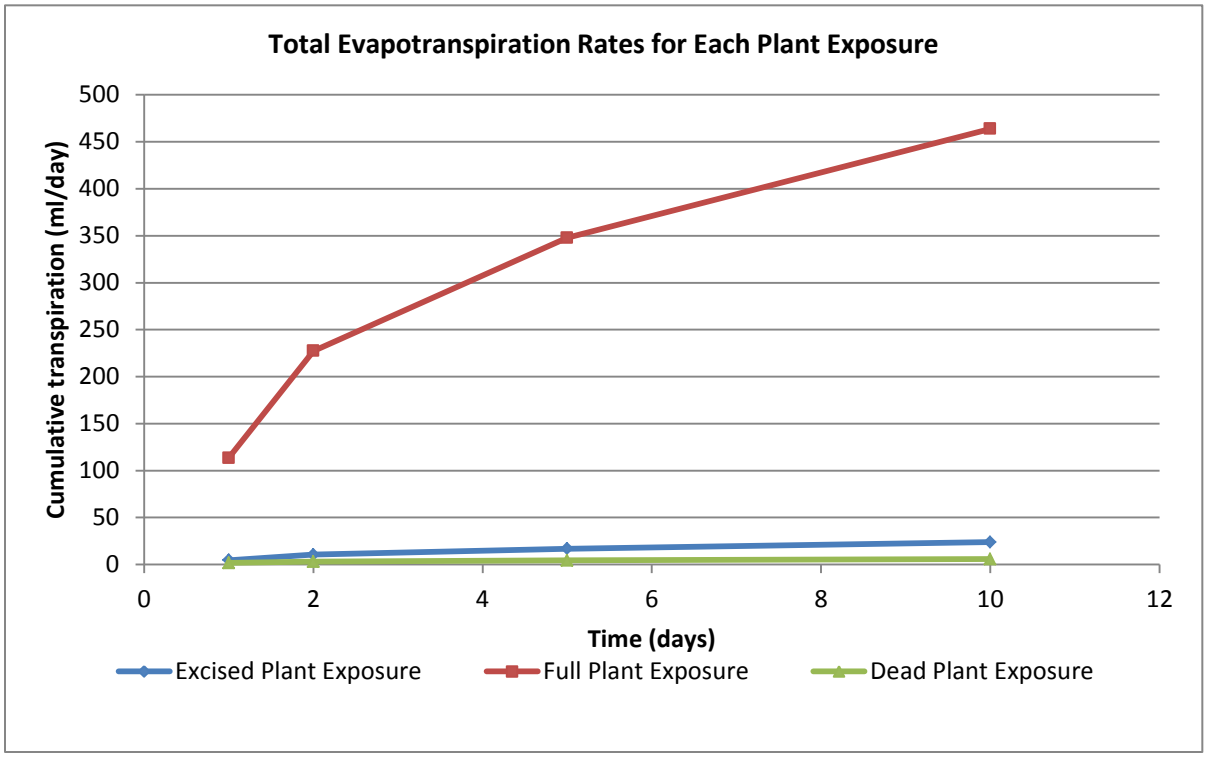


Figure 3-2 Cumulative evapotranspiration rates for live and dead plant exposures over the 10 day experiment.

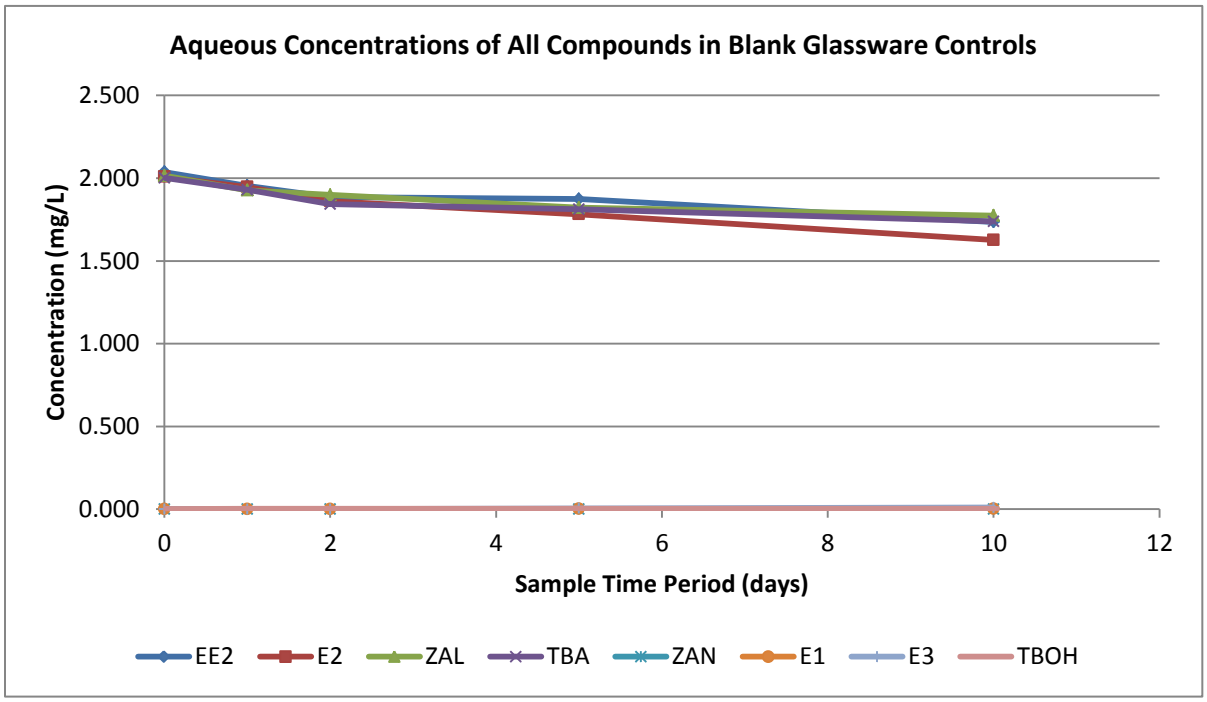


Figure 3-3 Aqueous concentrations for blank glassware controls during the 10 day experiment.

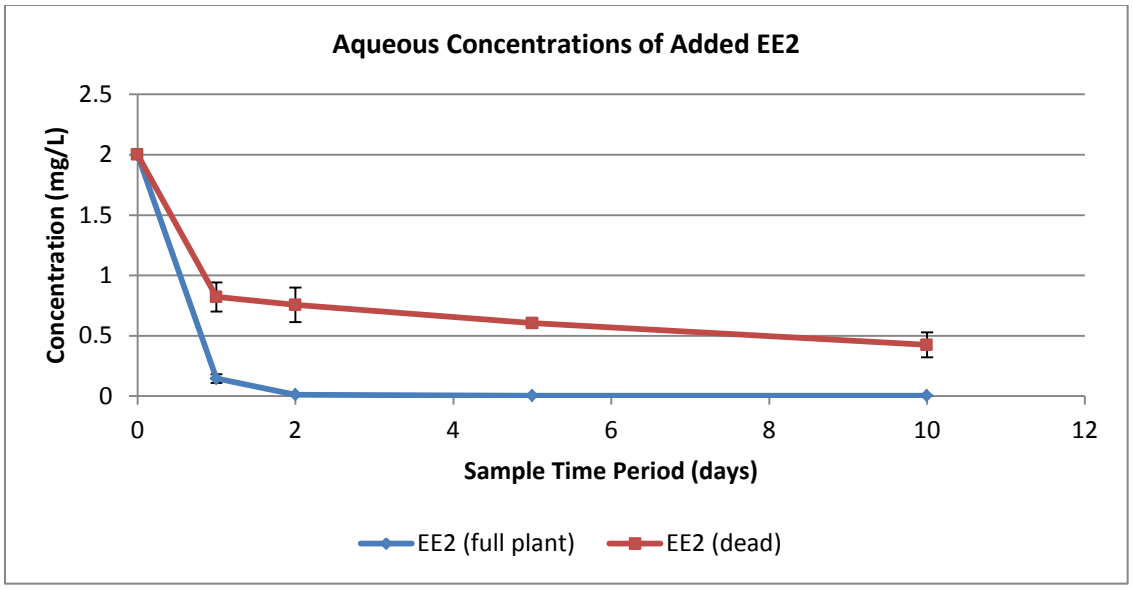


Figure 3-4 Aqueous concentrations of added EE2 for full plant vs. dead plant exposures throughout the 10 day experiment.

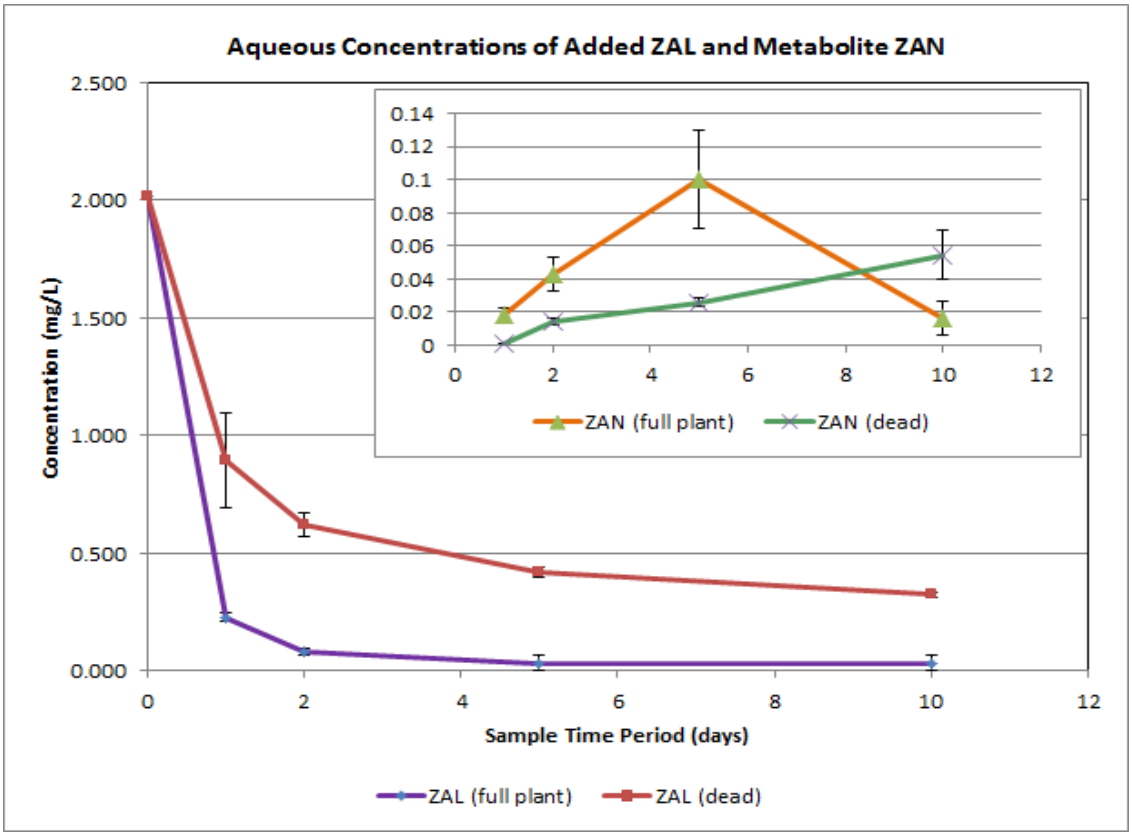


Figure 3-5 Aqueous concentrations of added ZAL and formation of metabolite ZAN duration for full plant vs. dead plant exposures throughout the 10 day experiment.

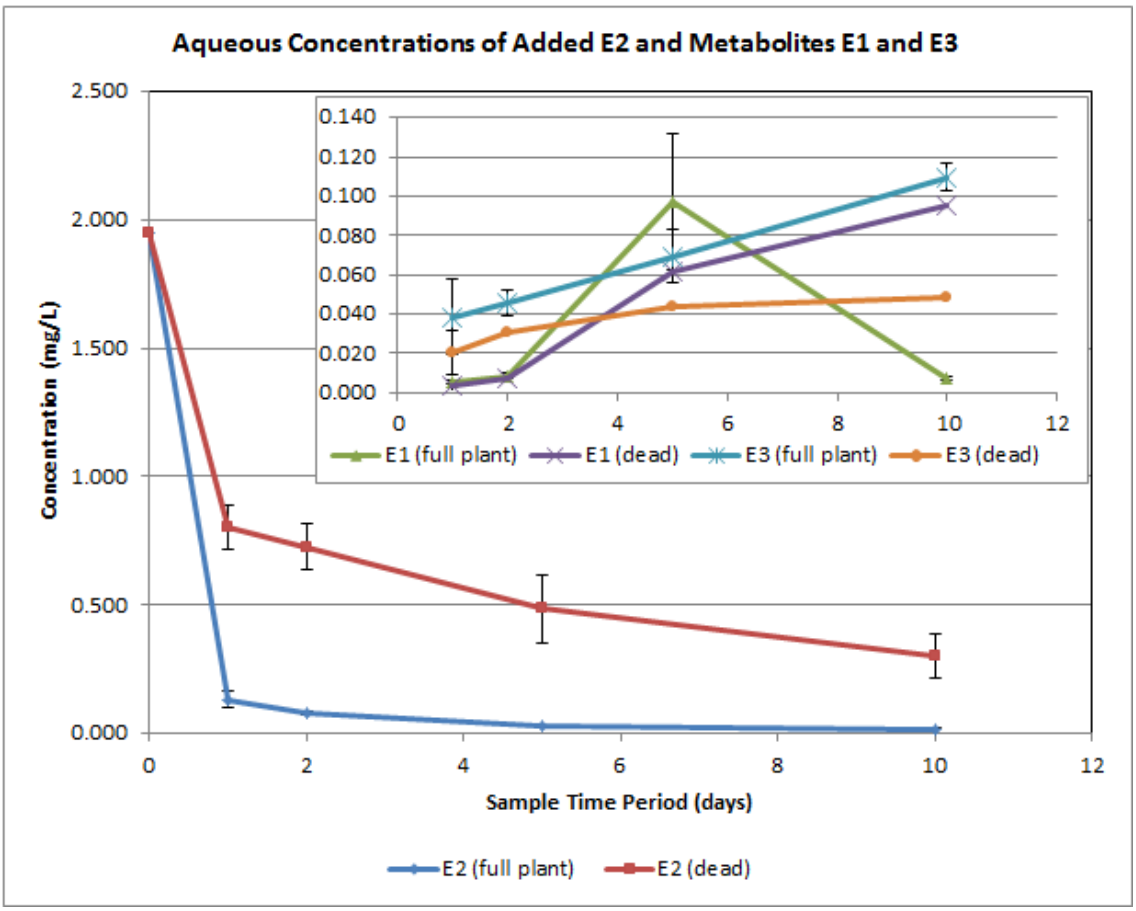


Figure 3-6 Aqueous concentrations of added E2 and formation of metabolites E1 and E3 for full plant vs. dead plant exposures throughout the 10 day experiment.

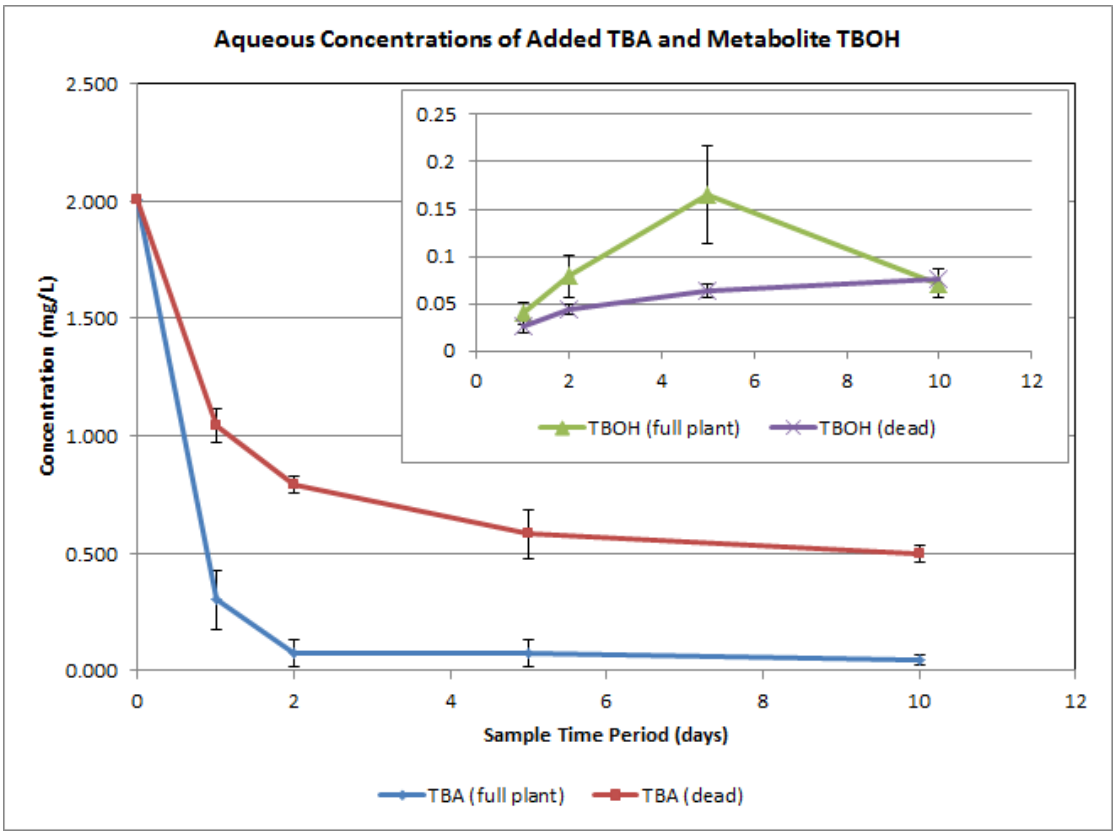


Figure 3-7 Aqueous concentrations of added TBA and formation of metabolite TBOH for full plant vs. dead plant exposure throughout the 10 day experiment.

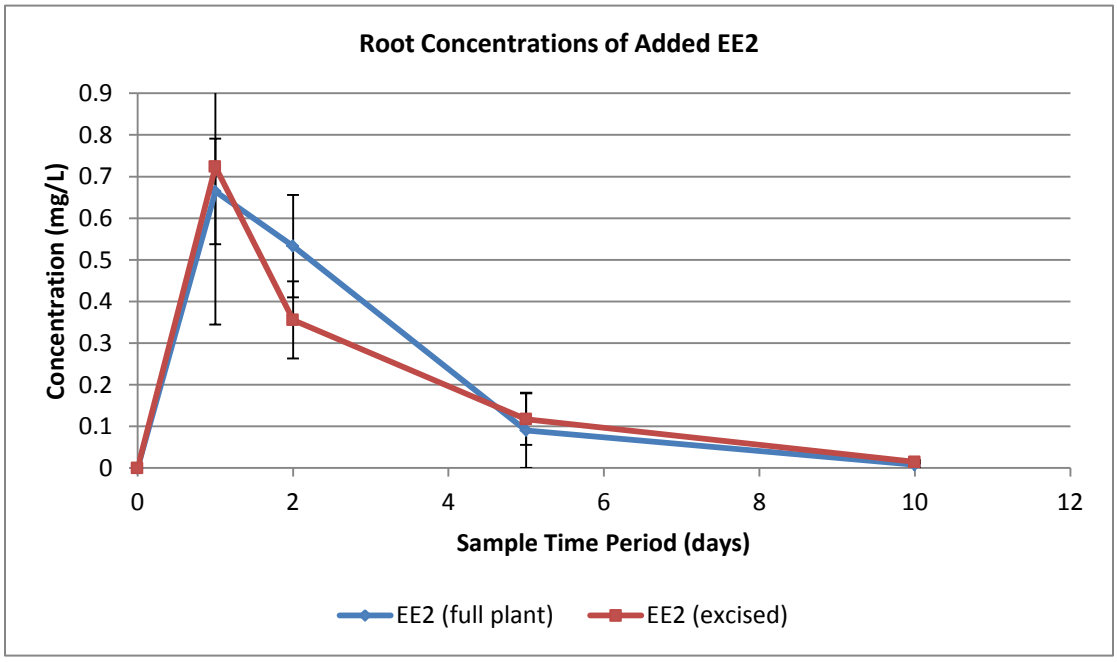


Figure 3-8 Root concentrations of added EE2 for full plant vs. excised plant exposures throughout the 10 day experiment.

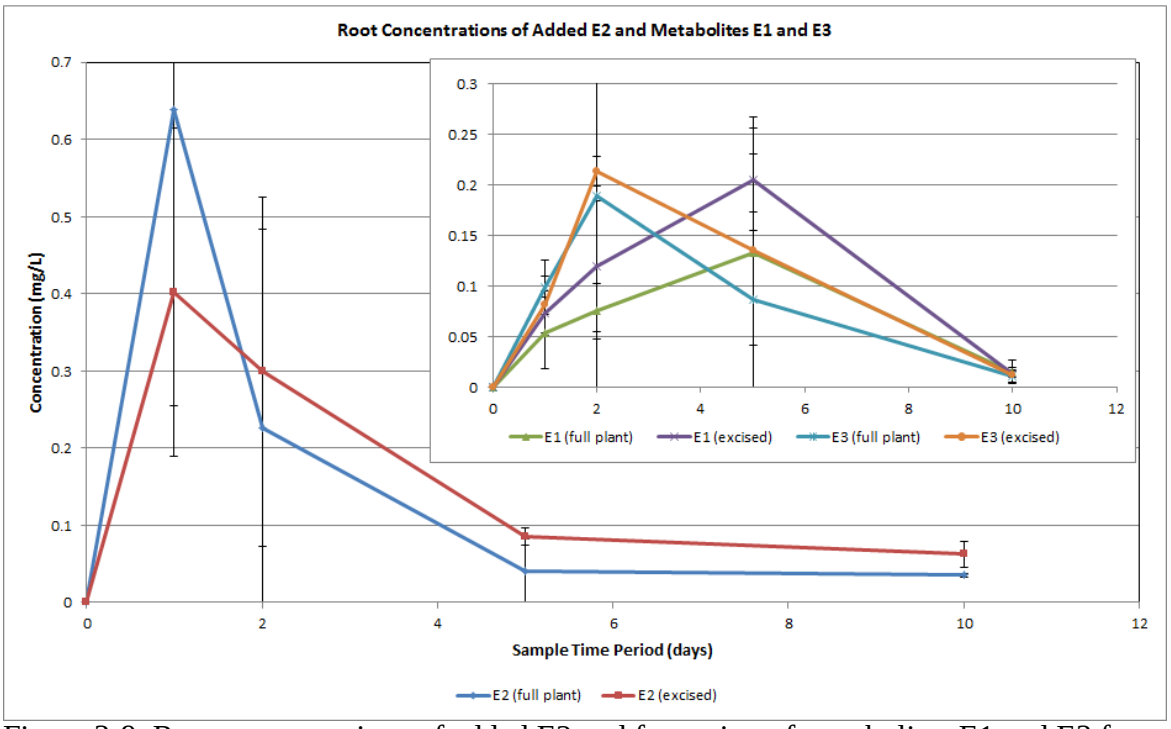


Figure 3-9 Root concentrations of added E2 and formation of metabolites E1 and E3 for full plant vs. excised plant exposures throughout the 10 day experiment.

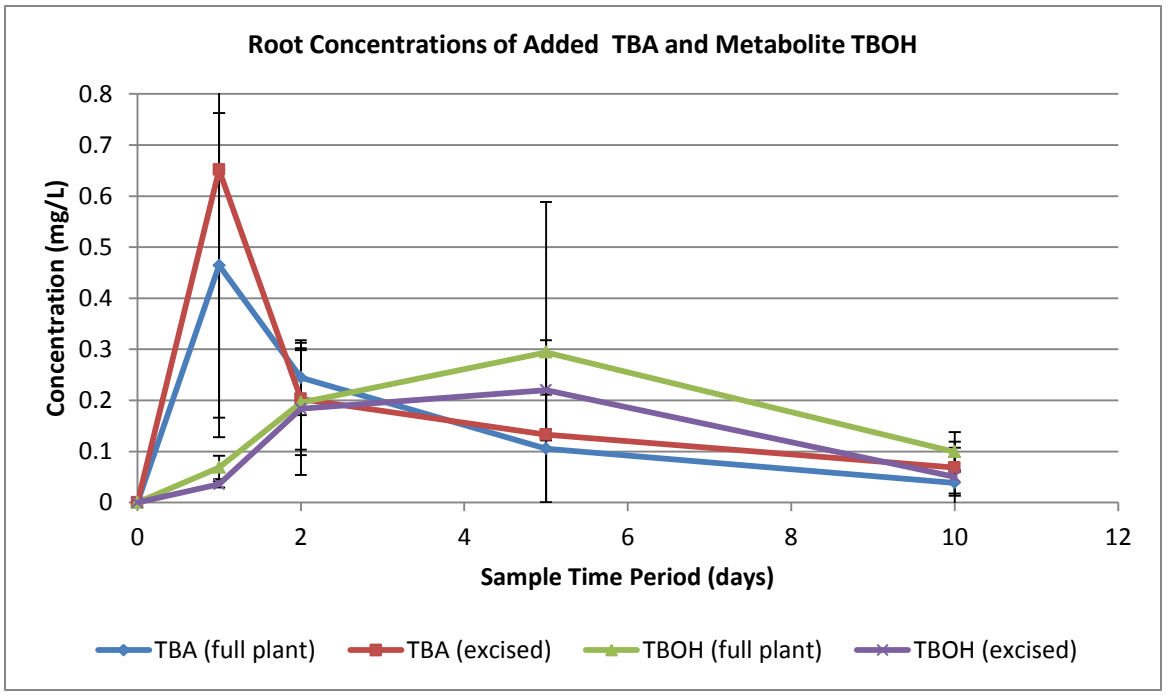


Figure 3-10 Root concentrations of added TBA and formation of metabolite TBOH for full plant vs. excised plant exposures throughout the 10 day experiment.

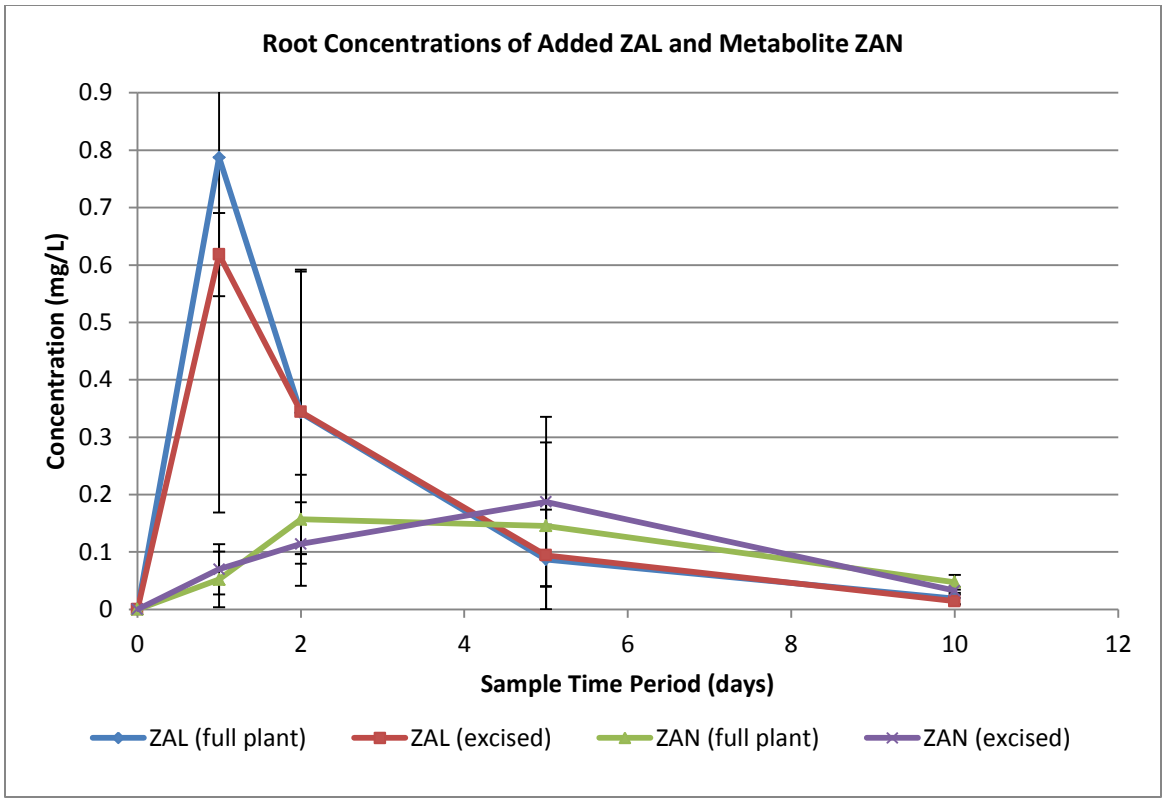


Figure 3-11 Root concentrations of added ZAL and formation of metabolite ZAN for full plant vs. excised plant exposures throughout the 10 day experiment.

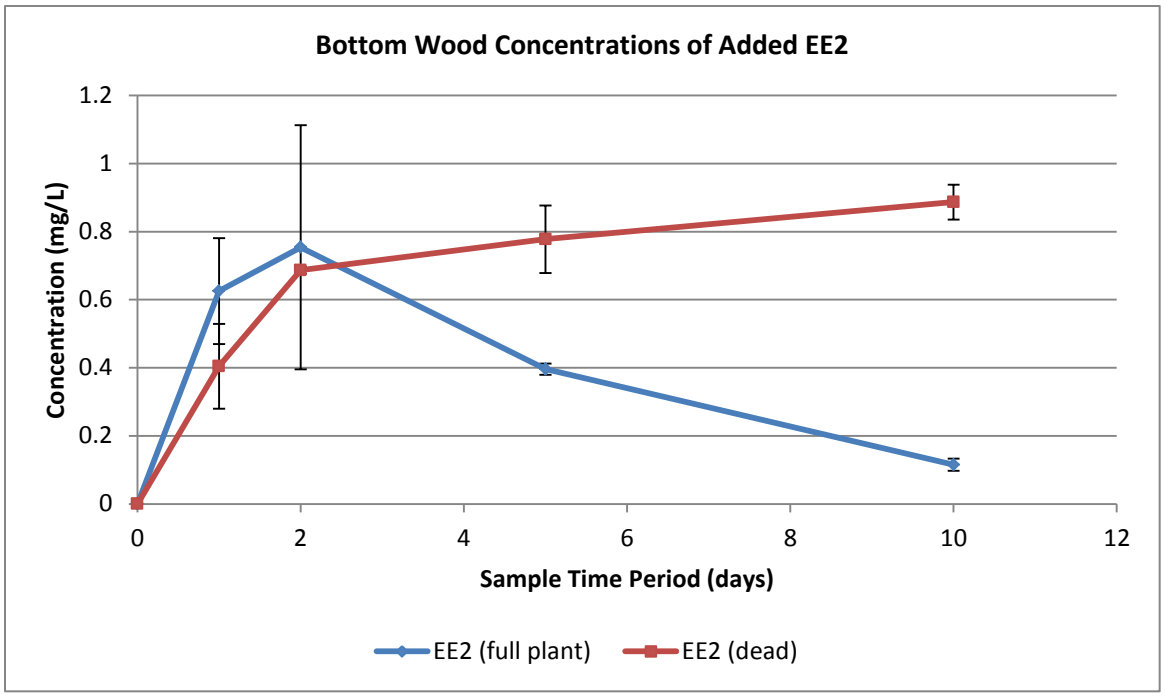


Figure 3-12 Bottom wood concentrations of added EE2 duration for full plant vs. dead plant exposures throughout the 10 day experiment.

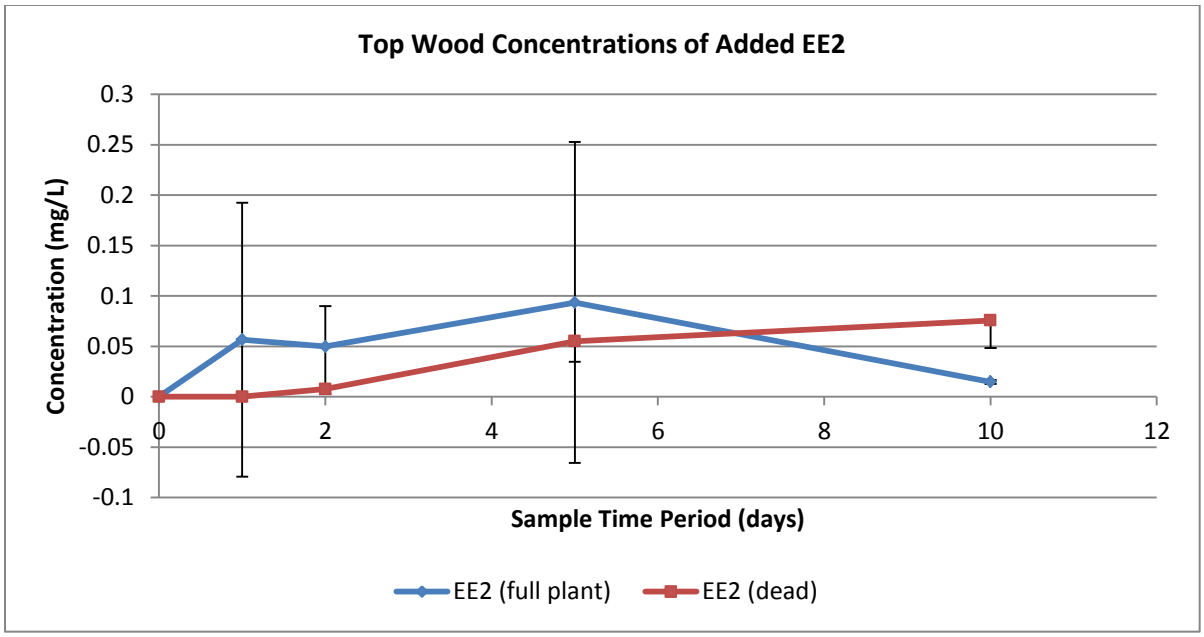


Figure 3-13 Top wood concentrations of added EE2 for full plant vs. dead plant exposures throughout the 10 day experiment.

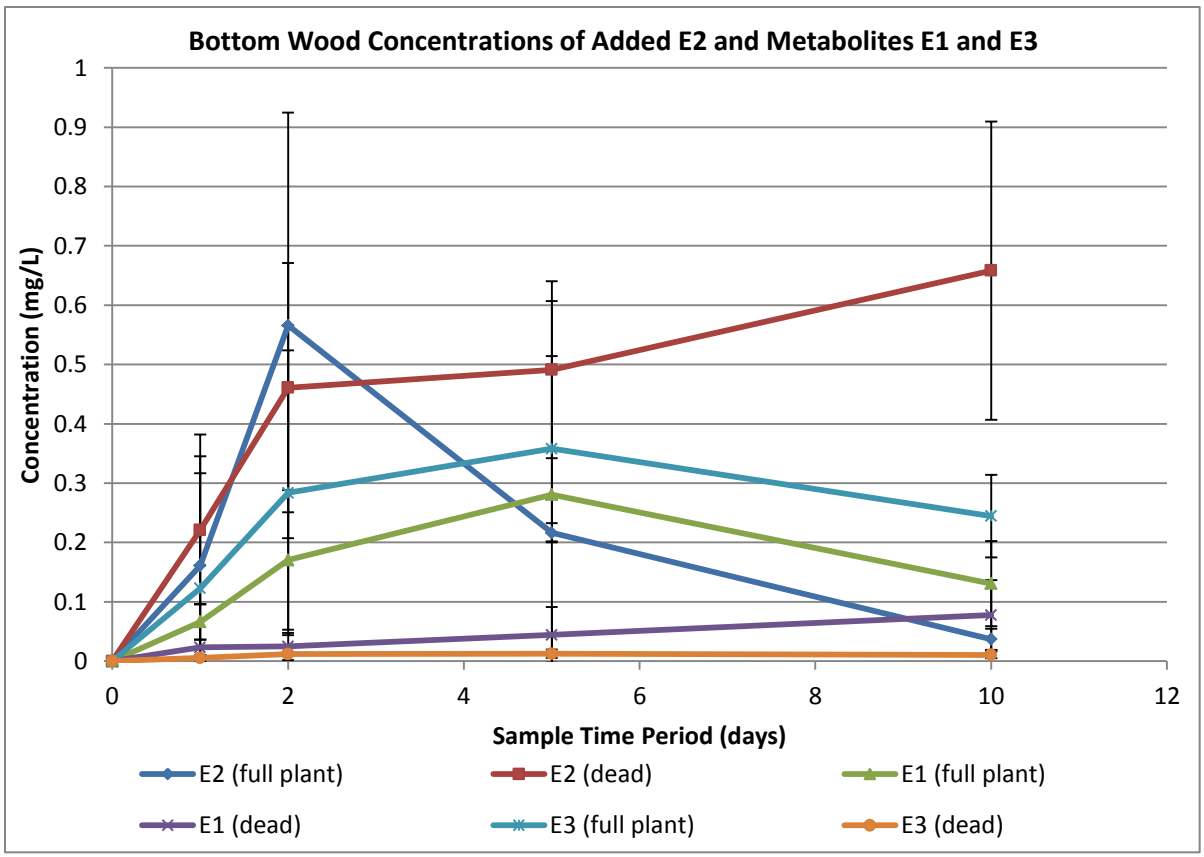


Figure 3-14 Bottom wood concentrations of added E2 and formation of metabolites E1 and E3 for full plant vs. dead plant exposures throughout the 10 day experiment.

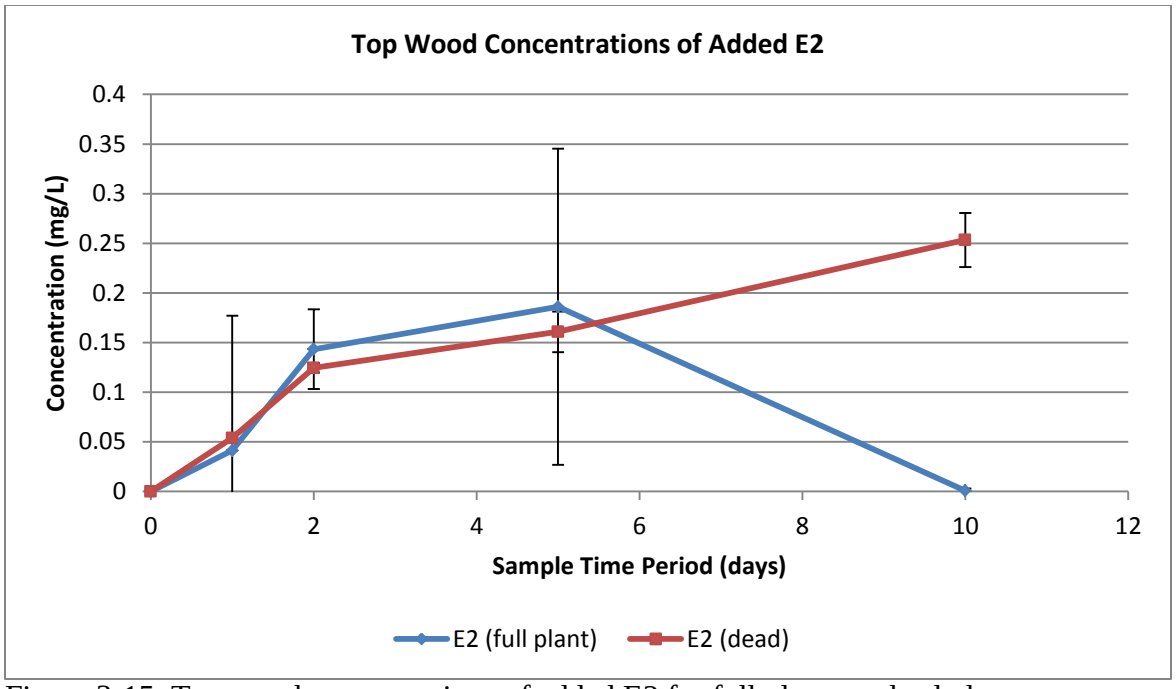


Figure 3-15 Top wood concentrations of added E2 for full plant vs. dead plant exposures throughout the 10 day experiment.

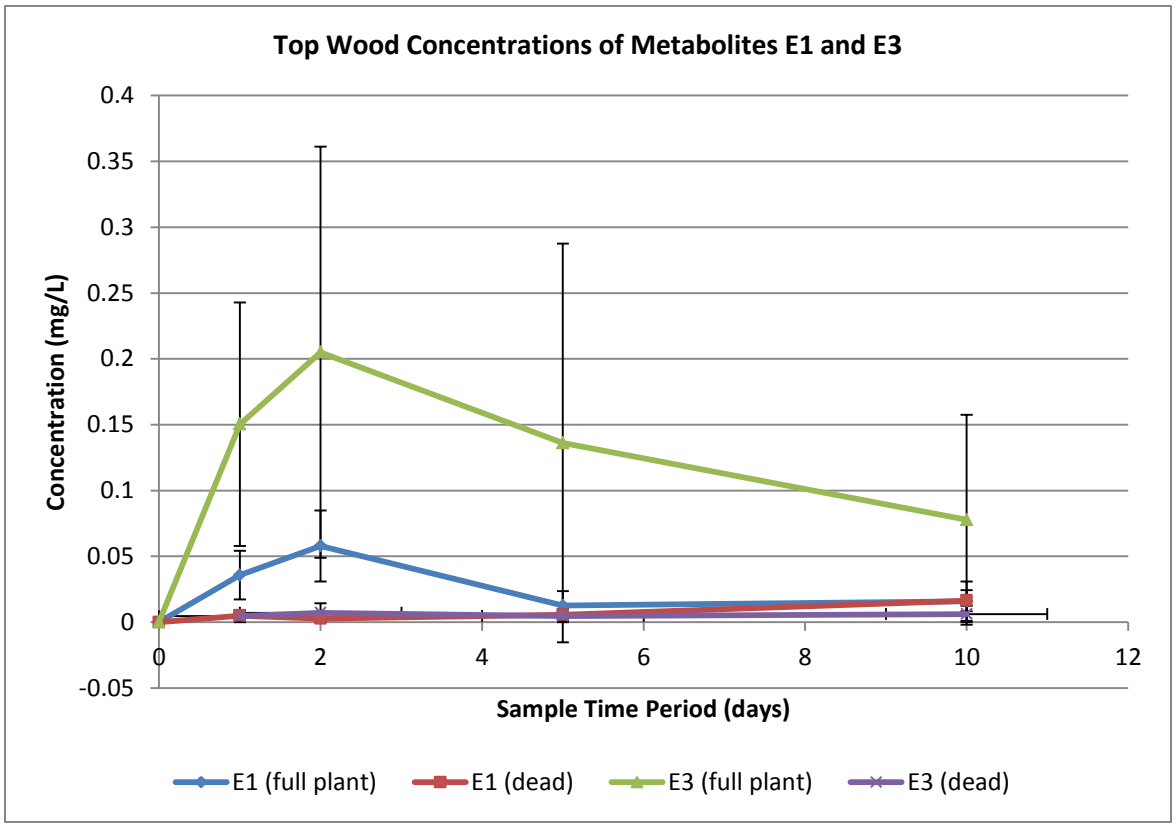


Figure 3-16 Top wood concentrations of the formed metabolites E1 and E3 for full plant vs. dead plant exposures throughout the 10 day experiment.

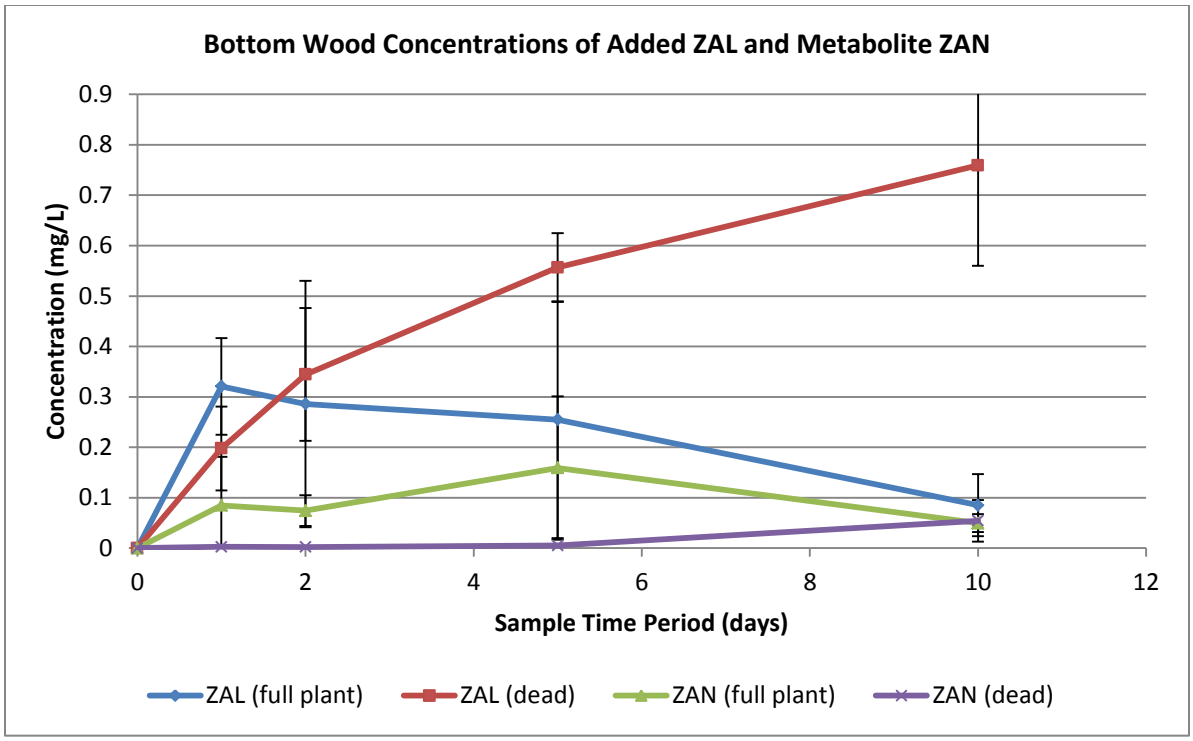


Figure 3-17 Bottom wood concentrations of added ZAL and formation of metabolite ZAN for full plant vs. dead plant exposures throughout the 10 day experiment.

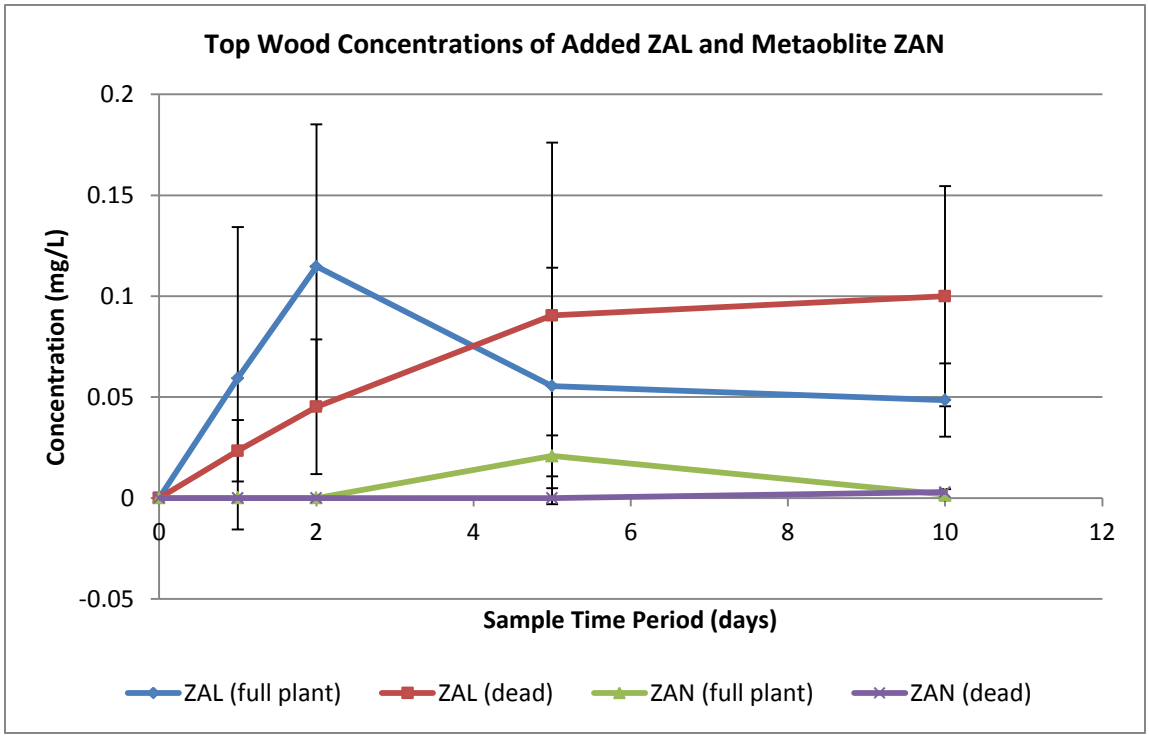


Figure 3-18 Top wood concentrations of added ZAL and formation of metabolite ZAN for full plant vs. dead plant exposures throughout the 10 day experiment.

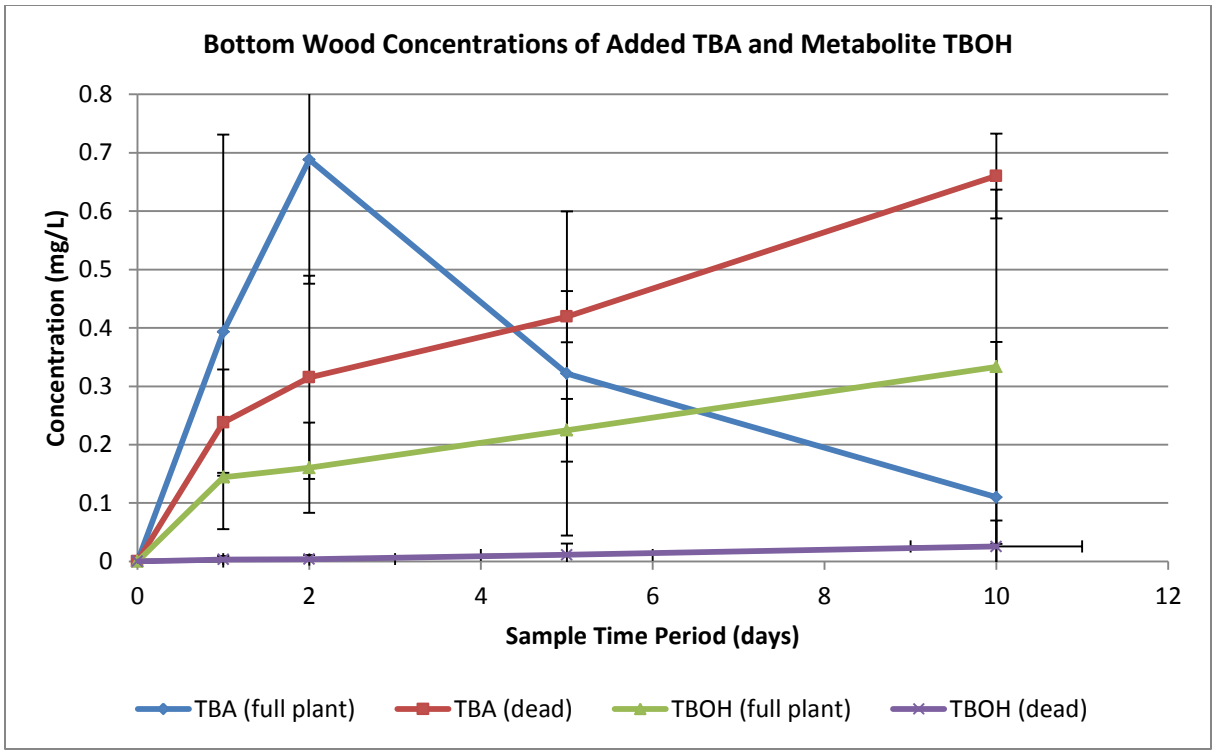


Figure 3-19 Bottom wood concentrations of added TBA and formation of metabolite TBOH for full plant vs. dead plant exposures throughout the 10 day experiment.

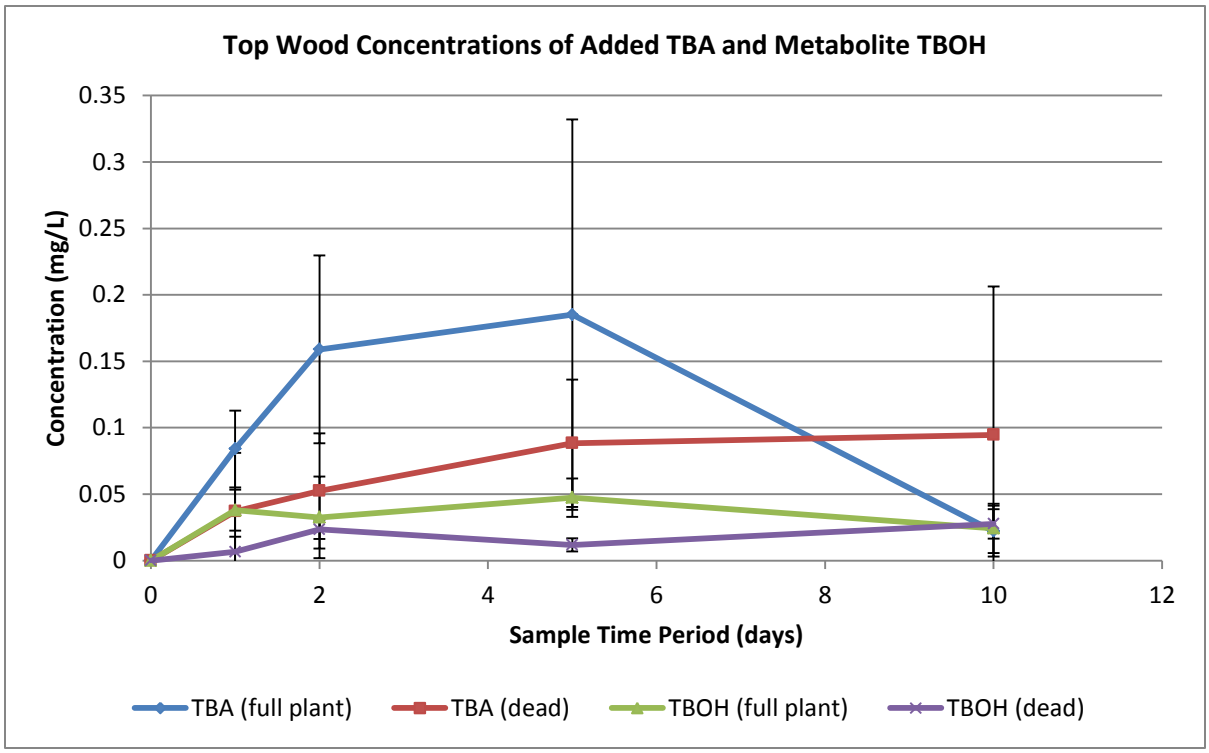


Figure 3-20 Top wood concentrations of added TBA and formation of metabolite TBOH for full plant vs. dead plant exposures throughout the 10 day experiment.

CHAPTER 4

PROJECT CONCLUSIONS

During the course of studies with steroid growth promoter spiked hydroponic solutions exposed to live plant treatments of softstem bulrush and hybrid poplar, the project objectives and basic hypothesis were successfully addressed:

1. *To evaluate the potential for an upland riparian plant (hybrid poplar) and a emergent wetland plant (softstem bulrush) to uptake and translocate zeranol, 17β-estradiol, trenbolone acetate and 17α-ethinylestradiol*

The ability of these plants to uptake the initially spiked target parent compounds was proven with the growth promoters being found in the initial transpiration stream of both species of plant through evidence of measurements of concentrations in the roots, although it was not possible to distinguish between sorption to the outside of the roots and that material taken inside. In the softstem bulrush experiment, it is most likely that translocation into the shoots did not occur due to metabolism by the large dense root system present after 64 days of growth. These results also correspond with the hybrid poplars, where no detections of the spiked parent compounds were found in either the secondary stems or leaves. The target compounds each contain very reactive hydroxyl groups shown below in Figure 4-1 and Figure 4-2 where transformation reactions can be quite rapid, thus inhibiting the compounds from entering into the transpiration stream past the roots. In the experiments with the hybrid poplar plants, it was clear that uptake

and translocation was occurring with corresponding metabolism in root tissues and bottom wood.

2. *To determine if these compounds bioaccumulate within the target plant tissues*

In each of the experiments, no bio-accumulation of the initially spiked target compounds within any plant tissues was measured by the end of the experimental durations, but did observe trace amounts especially in the woody main stem tissue of the poplar plants samples, which can be attributed to sorption and diffusion. With both species of plants, the target steroid hormone compounds were uptaken into roots and the main stem (poplar only) after the first couple sampling events, but by the end of the duration of the experiments, there were apparent losses with no observed storage or accumulation of the growth promoting compounds.

3. *To analyze the efficacy of these plants and associated rhizosphere bacteria to metabolize and biodegrade the target natural and synthetic growth promoters*

In both experiments with each species of plant, there is clear evidence that the live plant exposures were able to metabolize and biodegrade zeranol, 17β-estradiol, trenbolone acetate (poplar experiment only) and 17α-ethinylestradiol to a much greater extent than dead controls. Proof of this was through enhanced losses in the aqueous solution (to almost zero with each plant species exposures) and greater increases of the compounds in the plant material during the early sampling events including more rapid

production of metabolites, followed by losses of those parent compounds and metabolites by the end of the experiments compared to dead plant exposures, which did not follow those rising and diminishing trends and instead only increased at small linear incremental levels.

For the biodegradation of individual compounds, the natural estrogen E2 was the fastest to be removed from solution and had the highest metabolite concentrations while also being metabolized more readily after the midpoint of the experiments. The synthetic steroids ZAL, EE2 and TBA were reduced at slower rates, with lower generated metabolite concentrations and less total degradation by the end of the experiments. Parallel metabolism to the other compounds not circled shown in Figure 4-1, Figure 4-2, and Figure 4-3 could not be measured due to a lack of standards.

When comparing plants to microbes in the capability to biodegrade growth promoters, the results show that the plants are mostly responsible for the degradation because without live plants, the metabolites have much lower yields. There was also no significant differences between the excised plant and full plant exposures in removing the parent steroid compounds even with average evapotranspiration rates of 3.5 ml/day vs. 36 ml/day (Figure 2-2 and Table A-2) in the softstem bulrush experiment and 6.5 ml/day vs. 116.8 ml/day (Figure 3-2 and Table B-2) in the poplar experiment, revealing that the live roots and associated microbial populations were primarily responsible for the uptake and transformation of the initially spiked growth promoters.

Moreover, the biodegradation results with the live plants for each experiment are very encouraging especially considering that the metabolite ZAN is a weak estrogen, only containing approximately 13% of the estrogenic activity of ZAL (Shier et al. 2001).

Additionally the metabolite E1 comprises between 10-20% of the activity of E2 as well as the second metabolite E3, being a significantly less potent estrogen than the original parent compound and only contains 2% of the estrogenic activity of E2 (Lai et al., 2002). The overall project findings demonstrate that softstem bulrush and hybrid poplars as well as their associated microbes may have a substantial impact on the environmental fate of natural and synthetic growth promoters, through experimentally confirmed alcohol←→ketone (Figure 4-1 and Figure 4-2) and dealkylation (Figure 4-3) transformations of the spiked parent compounds shown below with red circles.

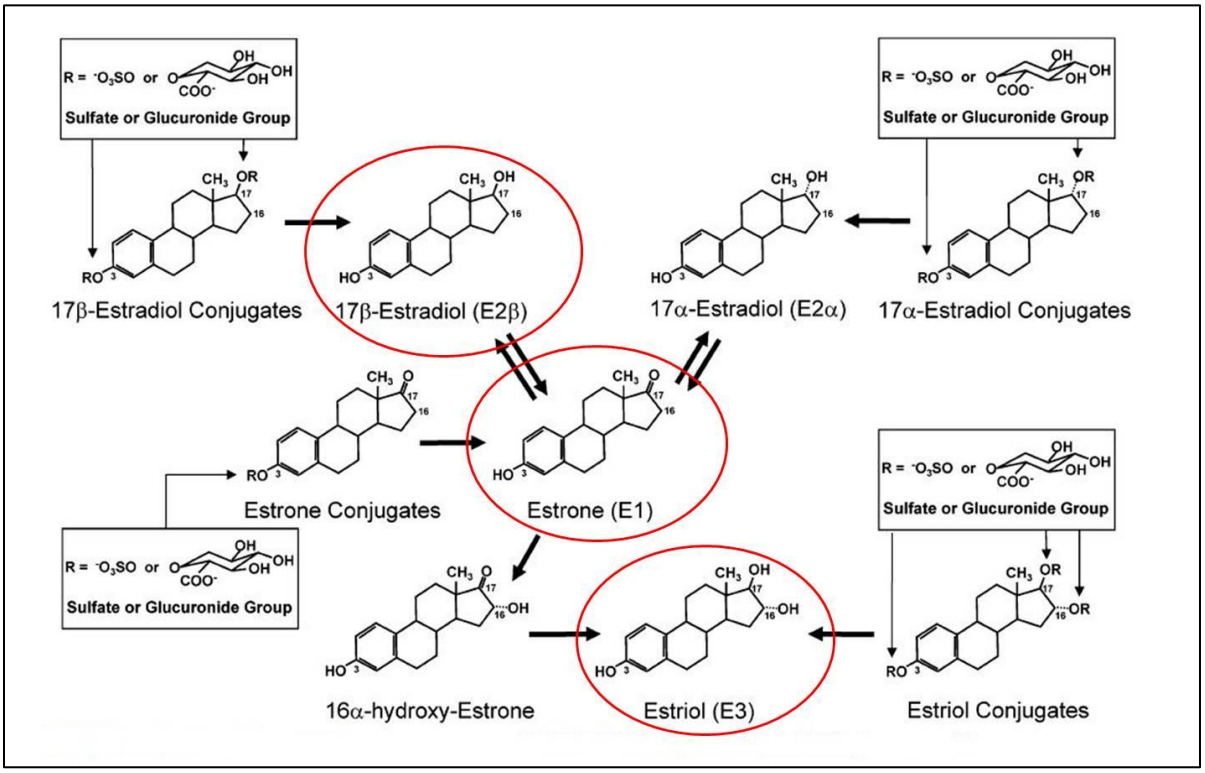


Figure 4-1 17β-estradiol (E2) metabolic pathway including metabolites estrone (E1) and estriol (E3), adapted from Hutchins et al. (2007). Circled compounds are those analyzed and confirmed in this research.

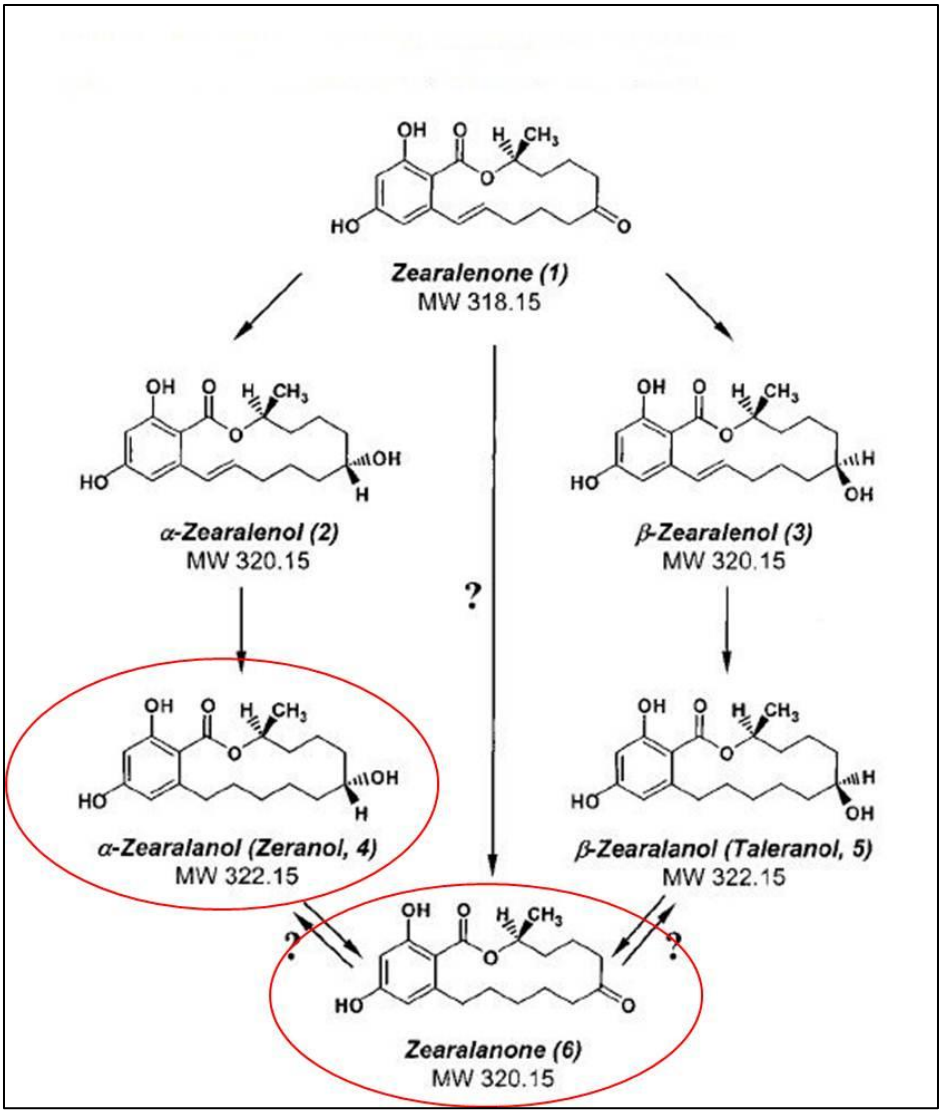


Figure 4-2 Zeranol metabolic pathway including metabolite zearalanone (ZAN), adapted from Kleinova et al. (2002). Circled compounds are those analyzed and confirmed in this research.

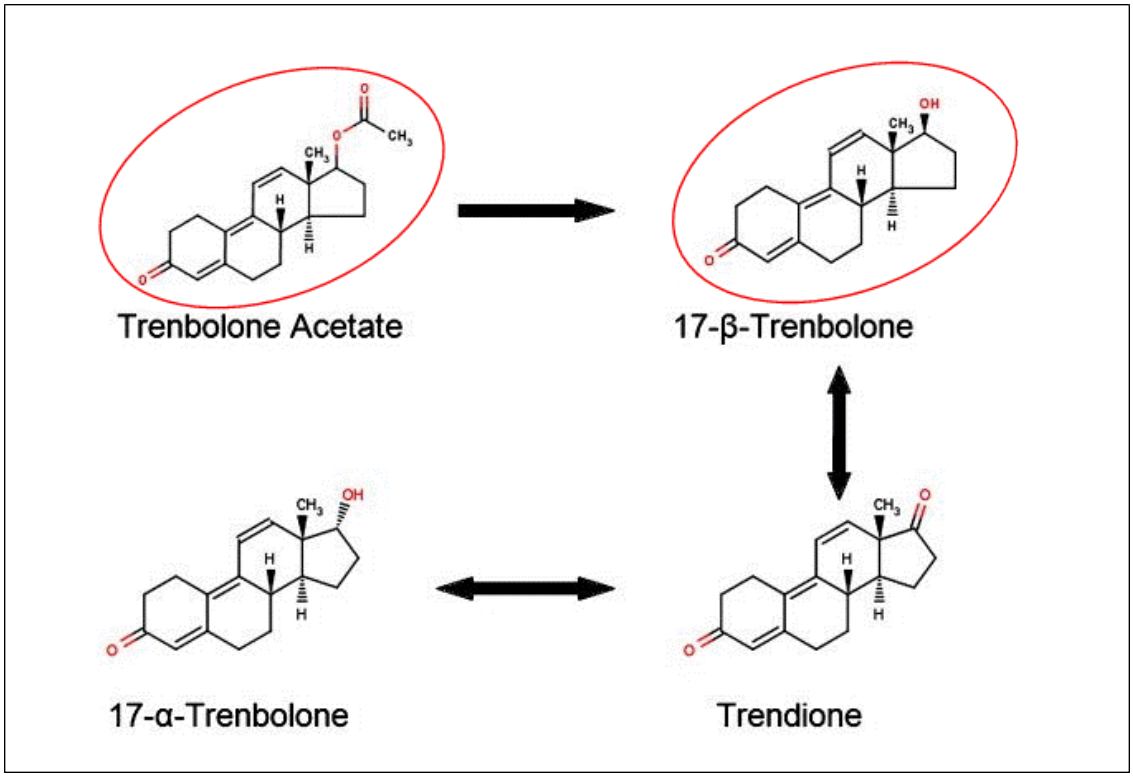


Figure 4-3 Trenbolone acetate (TBA) metabolic pathway including metabolite 17β-trenbolone (TBOH), adapted from Blackwell et al. (2011). Circled compounds are those analyzed and confirmed in this research.

Given these findings; it is suggested that wetland and upland riparian plants (within riparian buffer strip systems, which actively work to reduce sheet flow runoff water and provide a critical barrier against relatively unhindered deposition of chemicals into adjacent water bodies) may be capable of attenuating and biodegrading compounds such as hormonally-active growth promoters and other EDCs present in runoff water and sediment linked to CAFO waste and agriculture, which are commonly found in our waterways, exposing wildlife and humans to biologically active contaminants which could potentially undermine proper prenatal and postnatal development at concentrations as low as ng/L or parts per trillion.

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APPENDIX A

SUPPLEMENTAL MATERIAL FOR THE SOFTSTEM BULRUSH EXPERIMENT

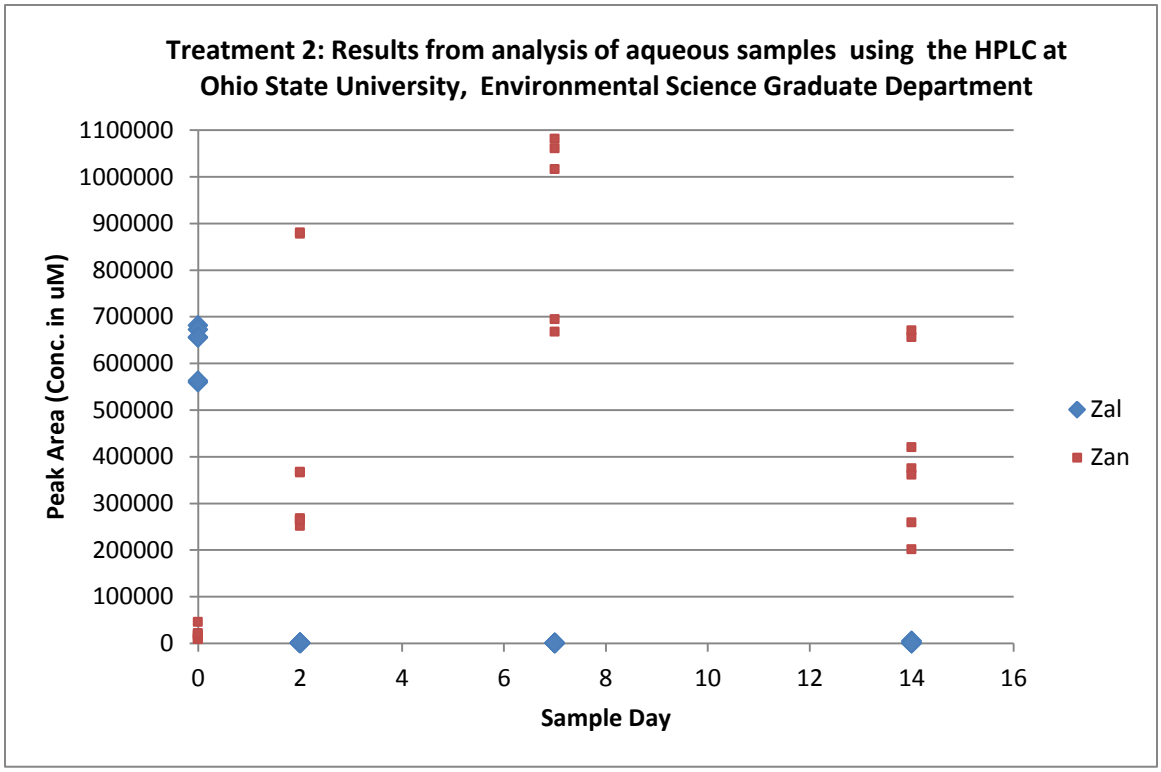

Figure A-1 QA/QC aqueous solution results for treatment 2 with spiked ZAL only using HPLC instrument and working with graduate researcher Marcy Card at Ohio State University, Environmental Science Graduate Department.

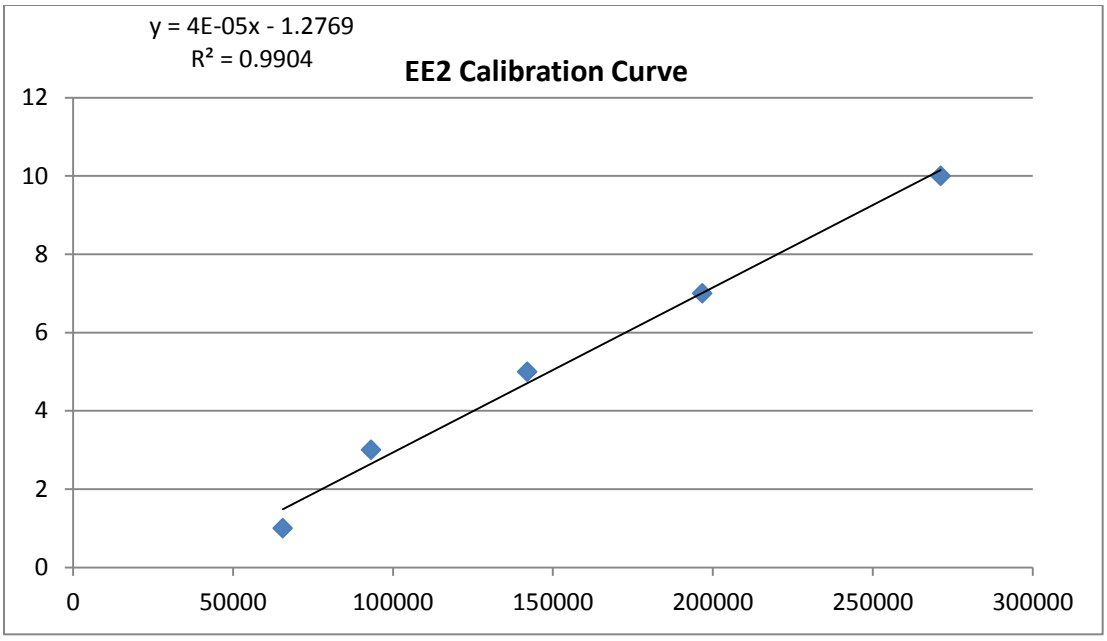

Figure A-2 Standard curve based on measured peak areas from LC/MS analysis for parent EE2 in the softstem bulrush experiment.

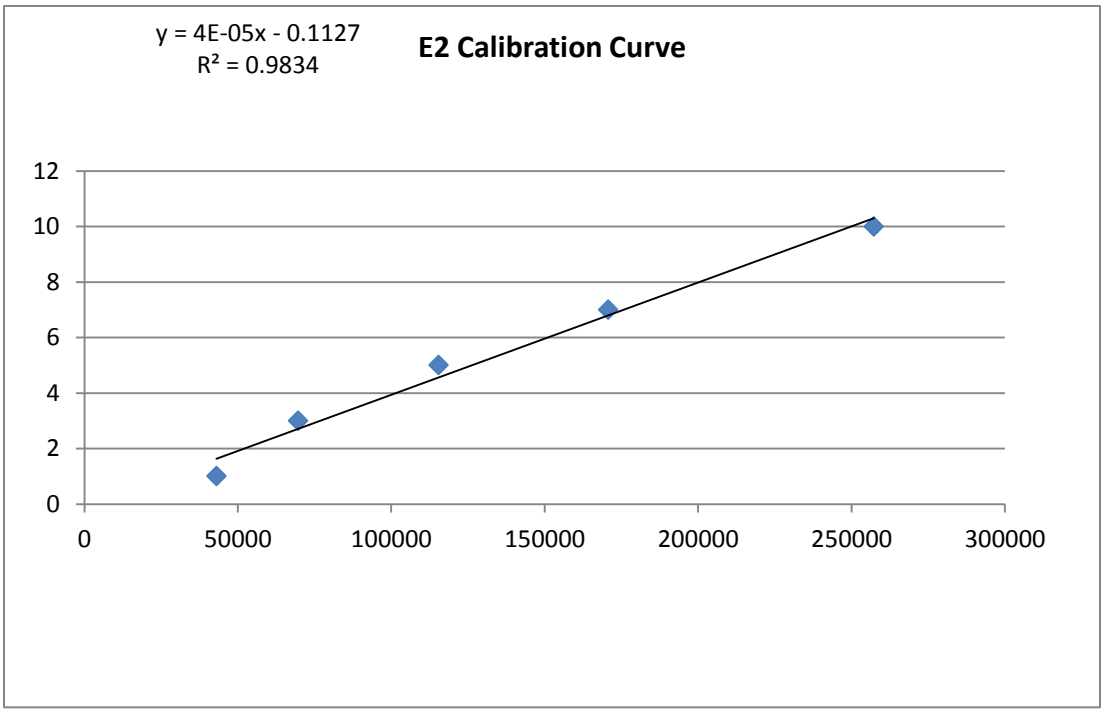

Figure A-3 Standard curve based on measured peak areas from LC/MS analysis for parent E2 in the softstem bulrush experiment.

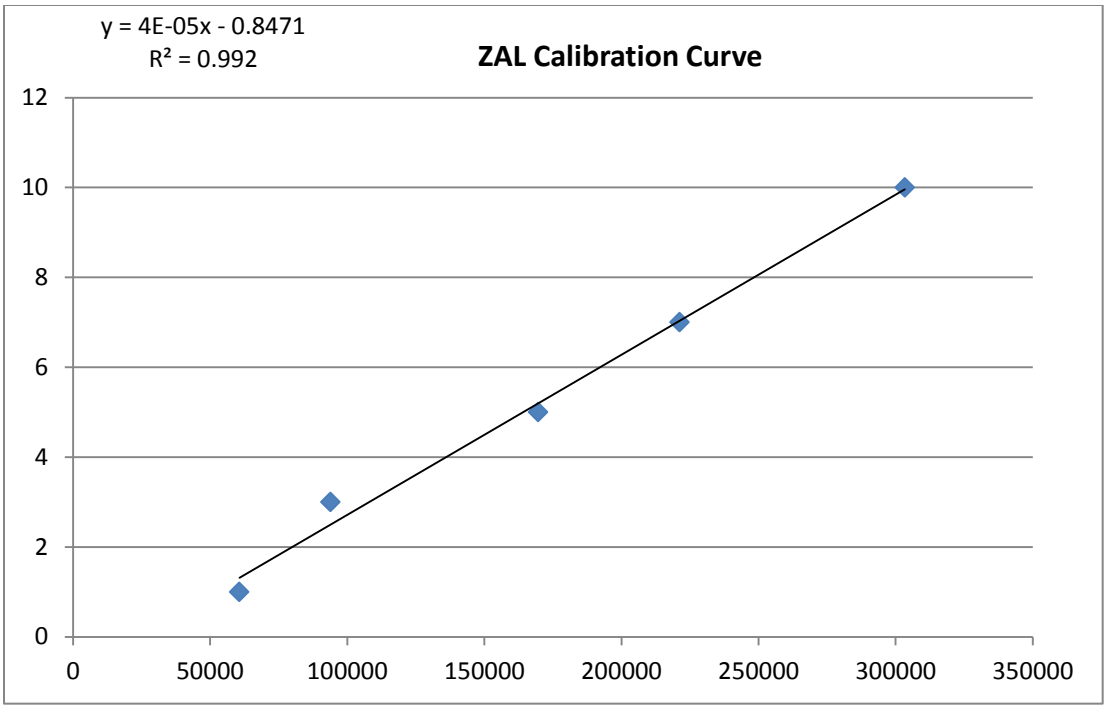

Figure A-4 Standard curve based on measured peak areas from LC/MS analysis for parent ZAL in the softstem bulrush experiment.

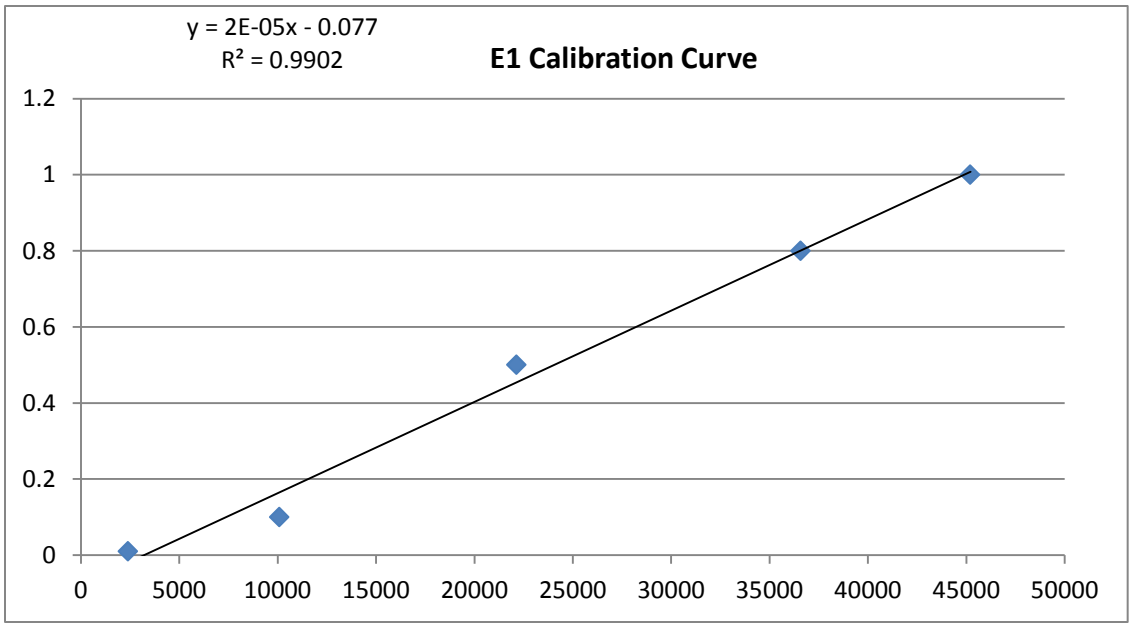

Figure A-5 Standard curve based on measured peak areas from LC/MS analysis for metabolite E1 in the softstem bulrush experiment.

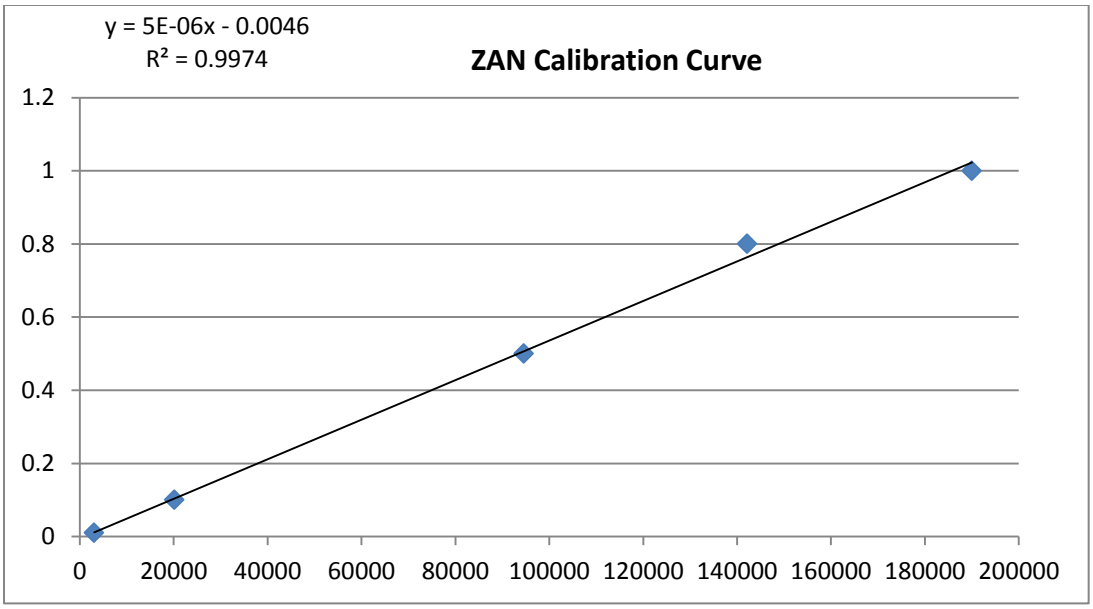

Figure A-6 Standard curve based on measured peak areas from LC/MS analysis for metabolite ZAN in the softstem bulrush experiment.

Method detection limits (MDLs), found using the chromatogram peaks on the LC/MS analysis was calculated using the following equation:

$$MDL = \frac{\text{Lowest concentration of detected compound}}{\text{compound peak height}/3*\text{noise peak height}}$$

Table A-1 Method Level Detection Limits in the softstem bulrush experiment for each parent compound and their respected metabolites using the equation shown above.

Hormone Compound	EE2	E2	ZAL	E1 [1]	ZAN [2]
Mass To Charge Ratio (*m/z*)	295	271	321	269	319
Retention Time (min)	3.7	3.1	2.9	4.2	5.1
MDL (µg/L)	8.12	6.55	0.167	0.732	0.484

1: Metabolite of E2, 2: Metabolite of ZAL

Table A-2 Evapotranspiration rates for each live plant exposure in treatment 1 and 2 over the 14 day experiment.

Treatment 1: Full Plant Evapotranspiration (ml/day)														
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
2	39.6	41.2												
5	39.6	41.2												
12	29.9	32.5												
4	46.3	48.2	47.1	42.3	36.5	41.2	40.8							
7	32.6	31.5	35.4	29.5	28.8	31.8	27.3							
10	35.2	36.5	33.8	29.5	25.2	30.5	29.5							
3	54.6	55.2	53.2	47.5	46.5	48.3	41.5	44.5	38.2	29.4	36.5	35.2	29.5	31.2
6	35.6	36.2	38.9	32.3	35.6	28.5	27.3	35.4	29.4	28.6	27.6	31.3	27.9	26.3
1	52.2	51.3	53.4	49.6	47.5	45.2	39.6	43.3	41.2	38.2	36.6	41	39.5	37.2
												Average = 36.4		
Treatment 1: Excised Evapotranspiration (ml/day)														
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
8	4.6	4.3												
9	4.8	4.6												
23	4.3	4.1												
22	4.8	4.6	4.8	3.3	3.2	2.8	2.5							
27	4.8	4.9	4.8	3.5	3.3	3.2	3.3							
11	4.3	4	4.8	2.7	2.8	3.2	3.6	3.8	3.1	2.2	2.6	2.2	2.3	2.5
24	4.6	4.5	4.8	3.1	3	3.2	3.2	2.8	2.9	3	2.7	2.6	2.6	2.8
25	5	4.4	4.8	3.6	3.5	3.9	2.8	2.7	2.8	2.9	2.9	3	2.8	2.9
26	4.4	4.6	4.8	3.5	3.6	3.6	3.9	3.7	3.3	3.2	2.8	3.6	2.5	2.2
												Average = 3.47		
Treatment 2: ZAL Only Evapotranspiration (ml/day)														
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
19	42.6	48.2												
20	29.5	33.5												
16	32.1	33.2												
17	34.2	33.9	34.1	30.2	28.6	26.8	26.5							
18	38.2	37.7	37.4	32.5	31.3	28.4	25.9							
15	46.2	42.2	41.3	36.9	36.6	35.6	28.2							
13	40.2	45.3	46.8	41.5	42.6	40.8	45.2	39.5	48.2	38.5	32.4	31.5	29.5	32.5
14	41.2	41.5	39.9	38.7	39.6	37.5	26.5	38.5	36.2	35.5	28.5	26.6	35.6	36.2
21	42.4	40.2	41.1	32.3	36.5	38.4	33.5	29.1	29.6	33.6	36.3	32.8	27.2	32.1
												Average = 35.8		

Figure A-7 Experimental set up for hydroponic exposure experiment with softstem bulrush in growth chamber.

Figure A-8 Picture of a full live softstem bulrush plant including root system.

Figure A-9 Picture of an excised softstem bulrush plant including root system.

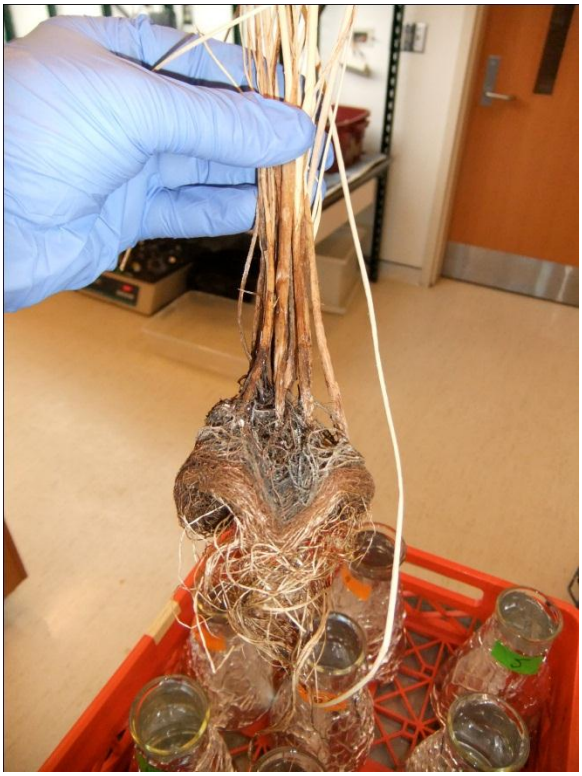

Figure A-10 Picture of a dead softstem bulrush plant including root system.

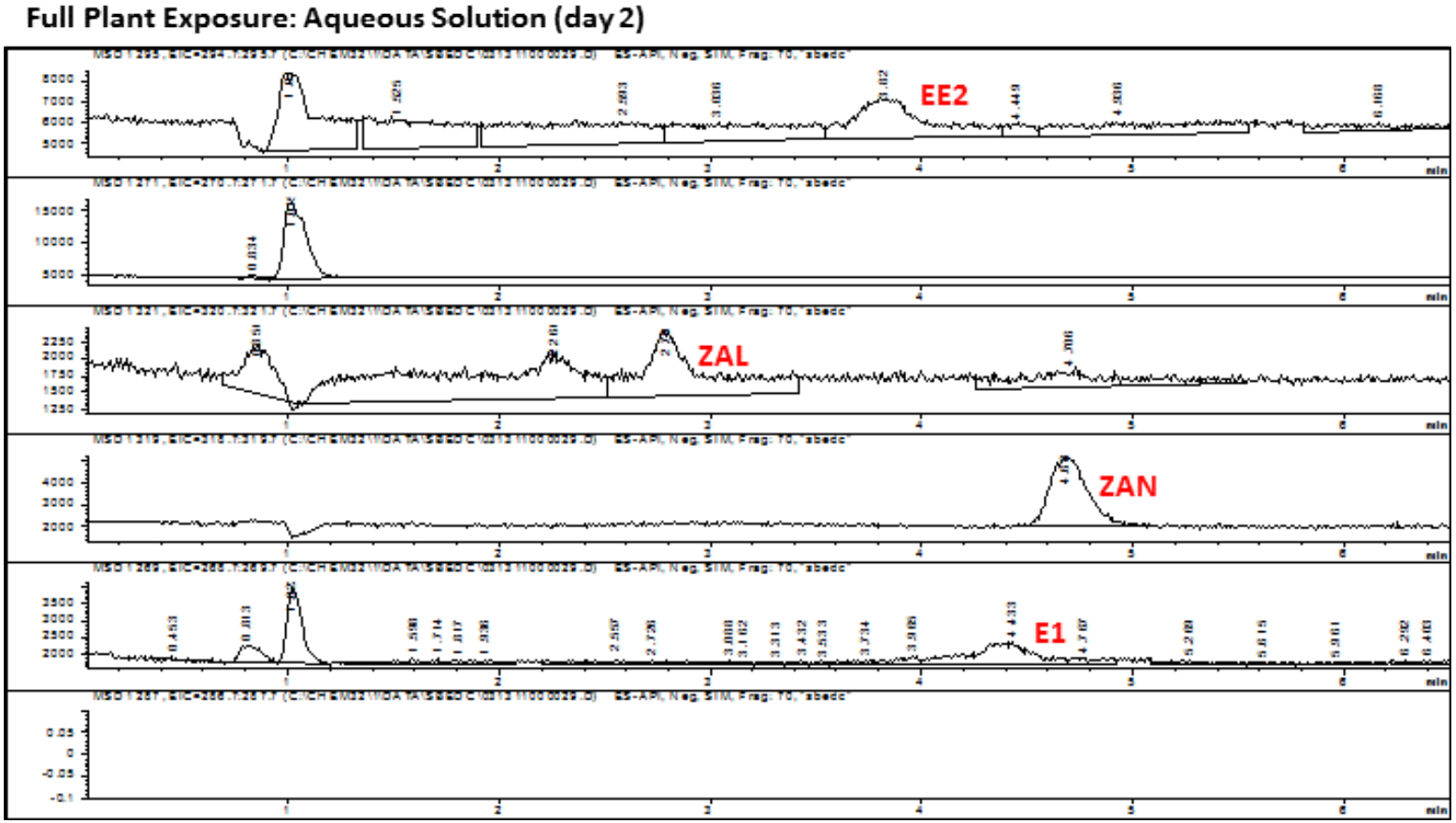

Figure A-11 Example chromatogram from the aqueous solution of the full plant exposure on day 2 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

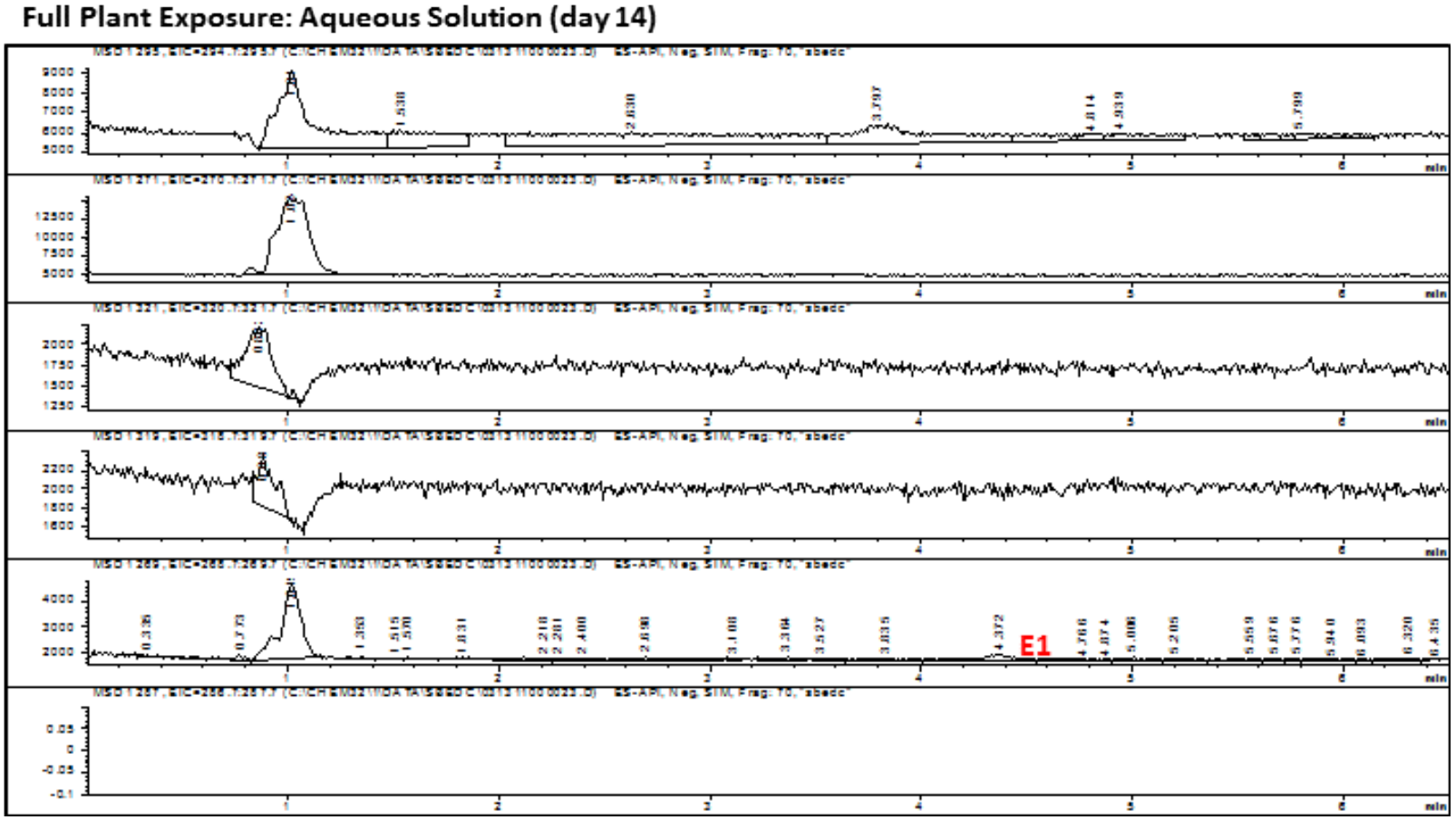

Figure A-12 Example chromatogram from the aqueous solution of the full plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

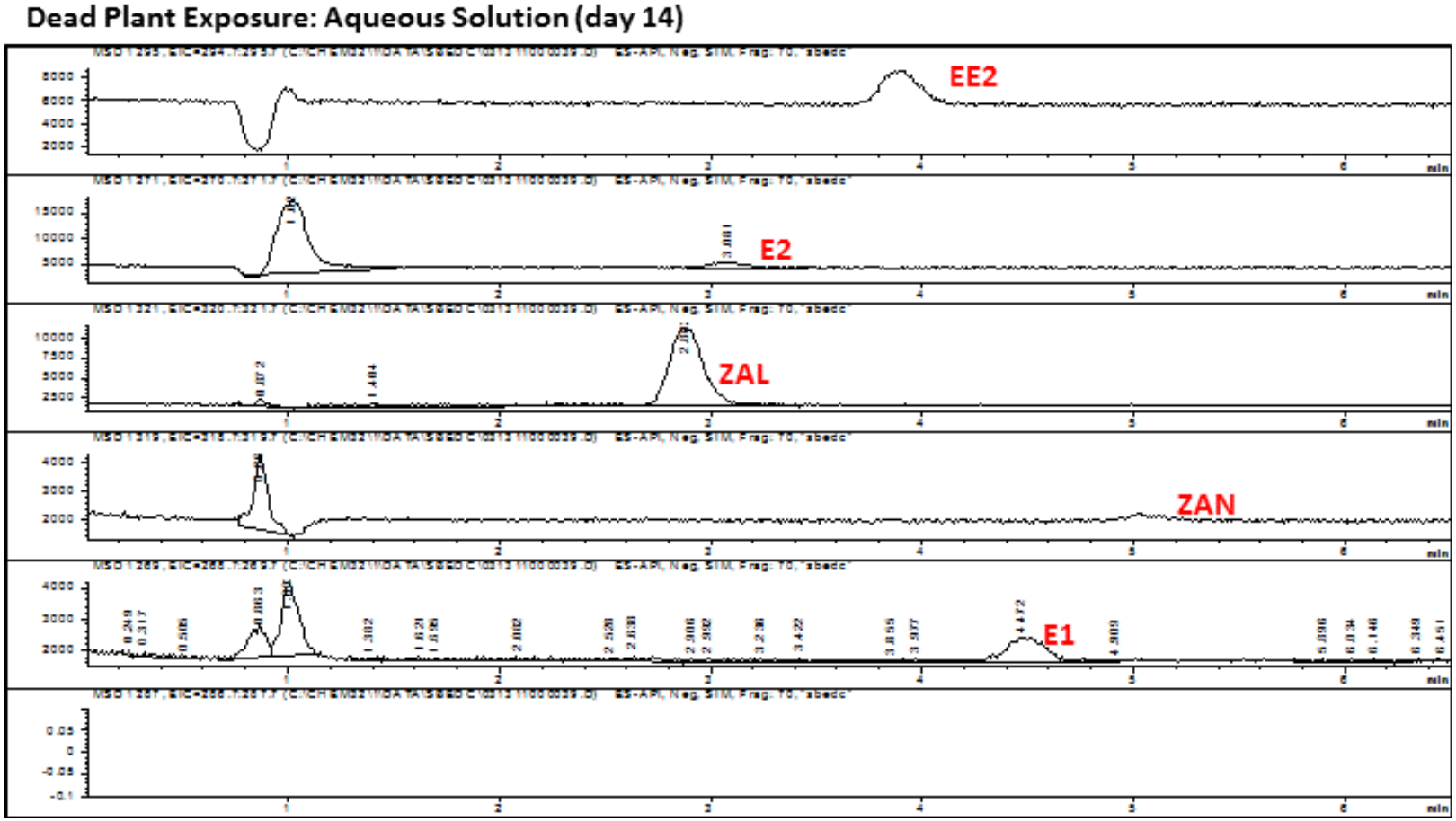

Figure A-13 Example chromatogram from the aqueous solution of the dead plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

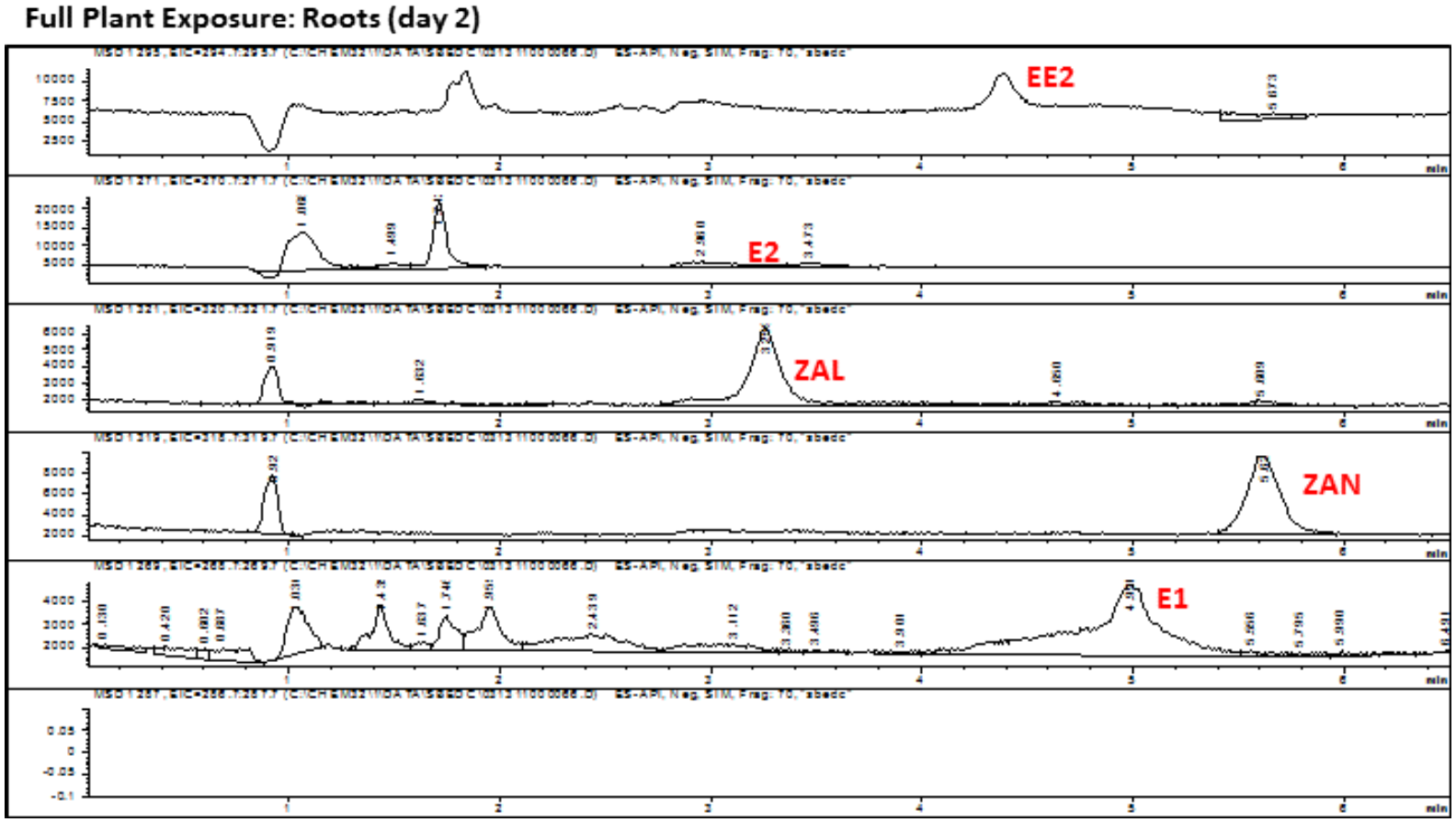

Figure A-14 Example chromatogram from the roots of the full plant exposure on day 2 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

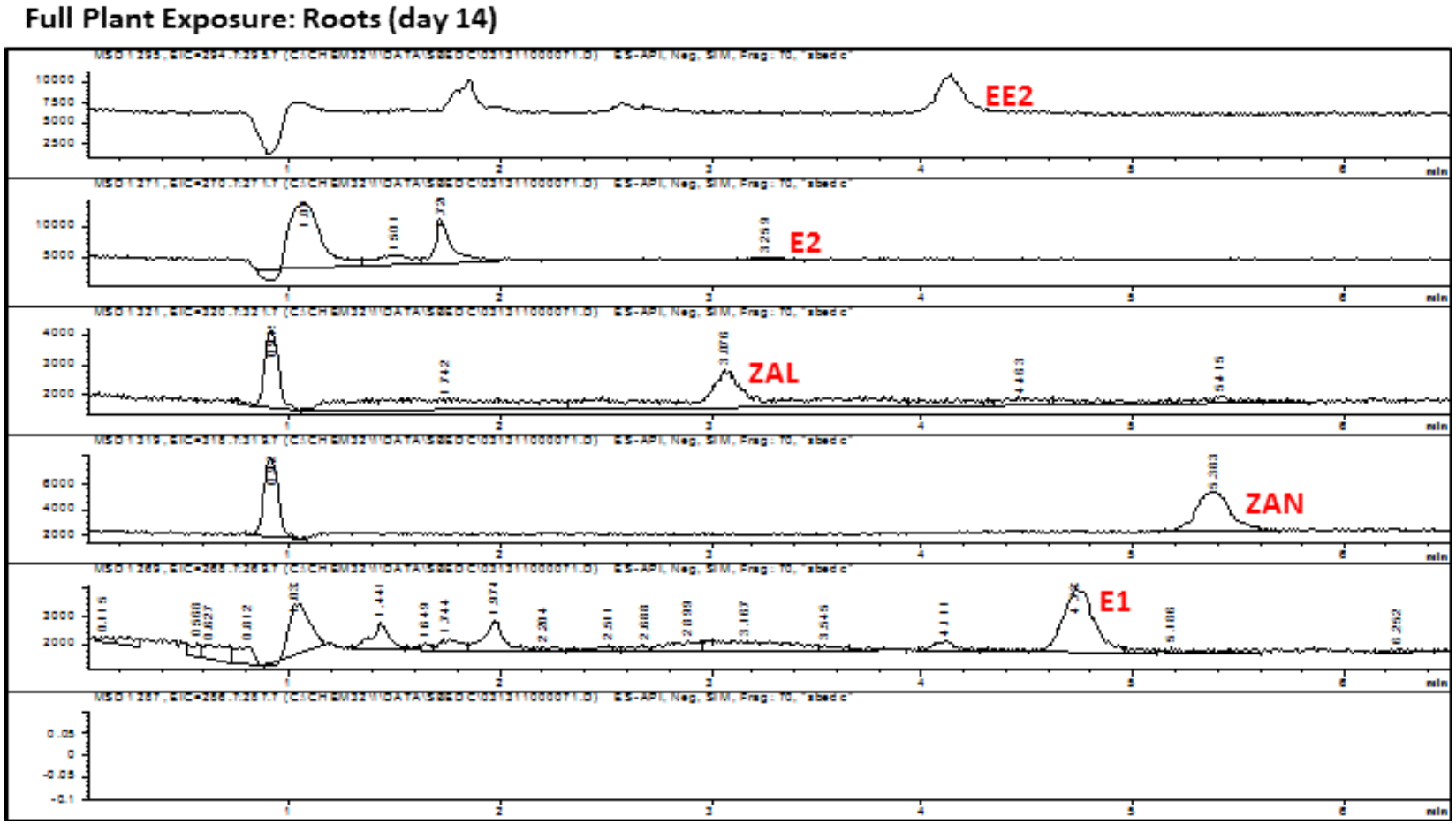

Figure A-15 Example chromatogram from the roots of the full plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

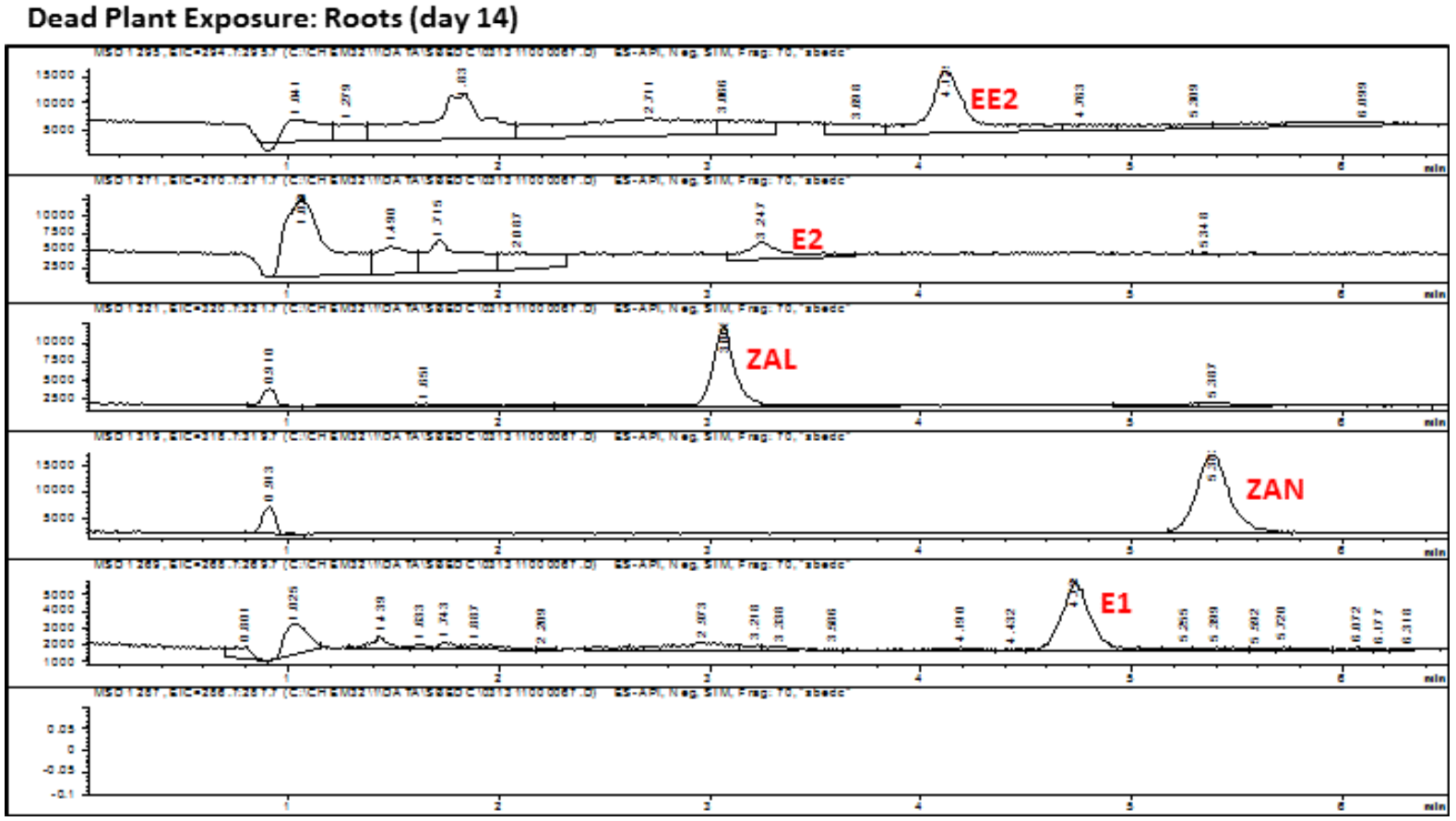

Figure A-16 Example chromatogram from the roots of the dead plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

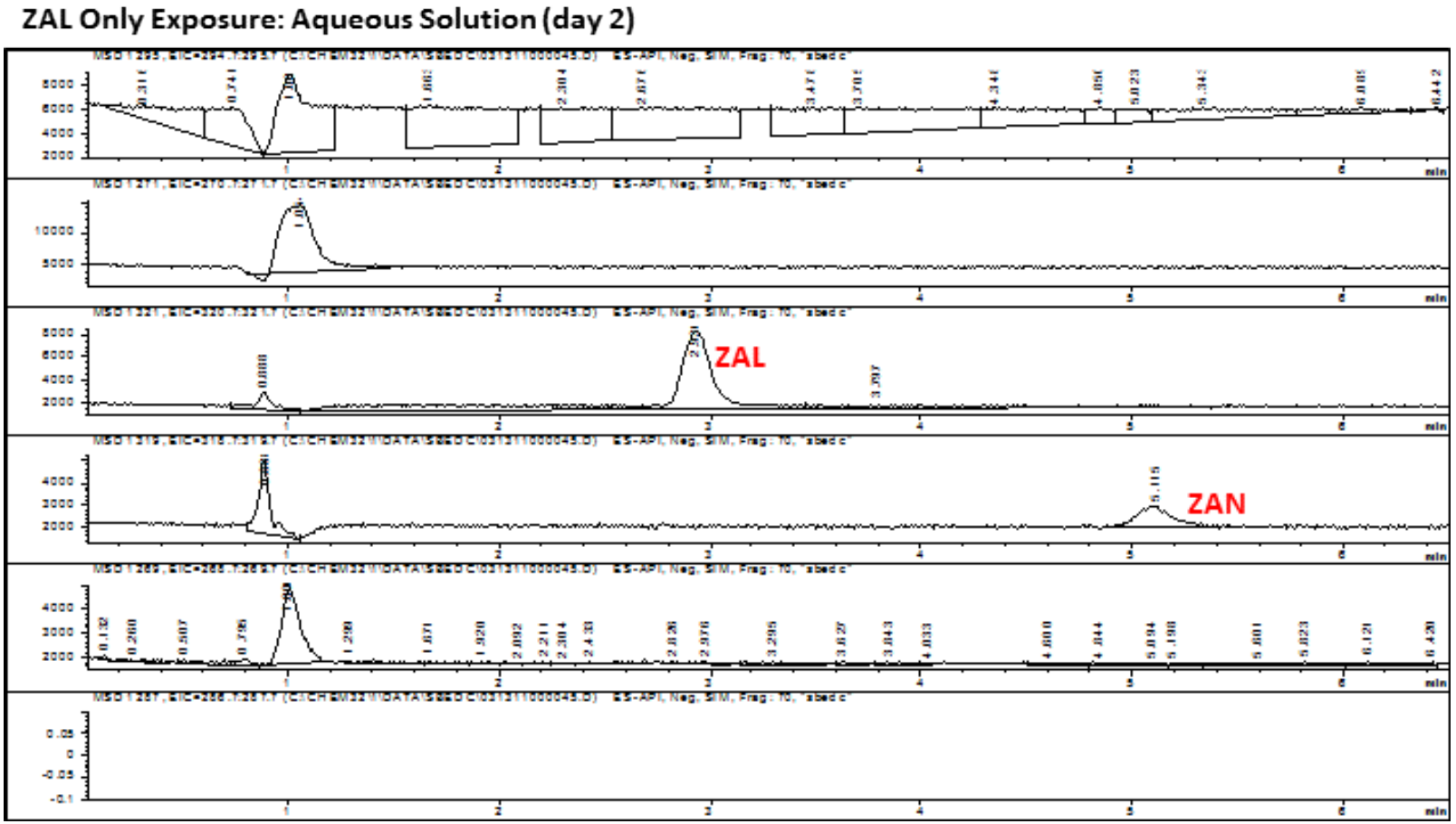

Figure A-17 Example chromatogram from the aqueous solution of the ZAL only full plant exposure on day 2 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

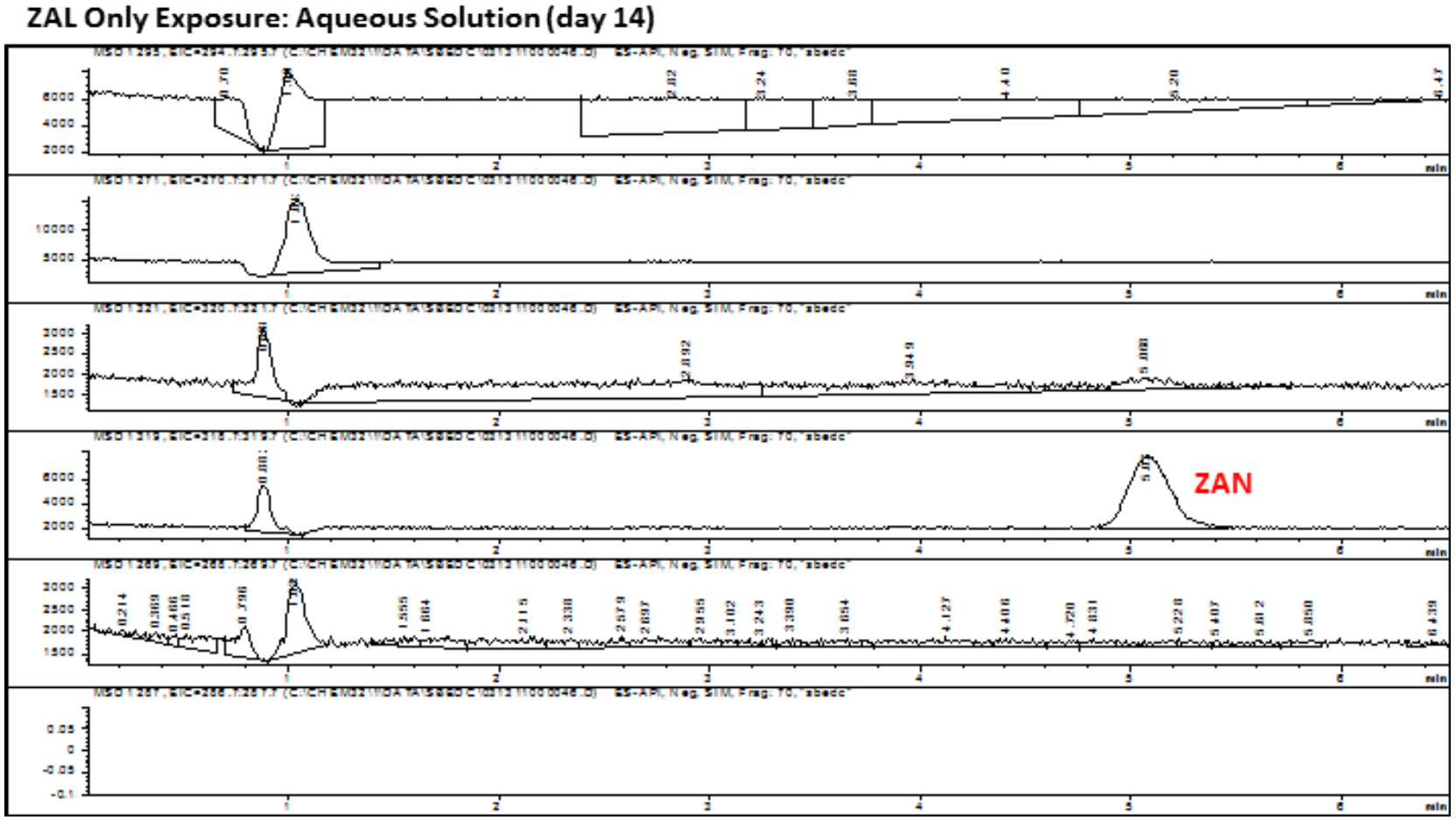

Figure A-18 Example chromatogram from the aqueous solution of the ZAL only full plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

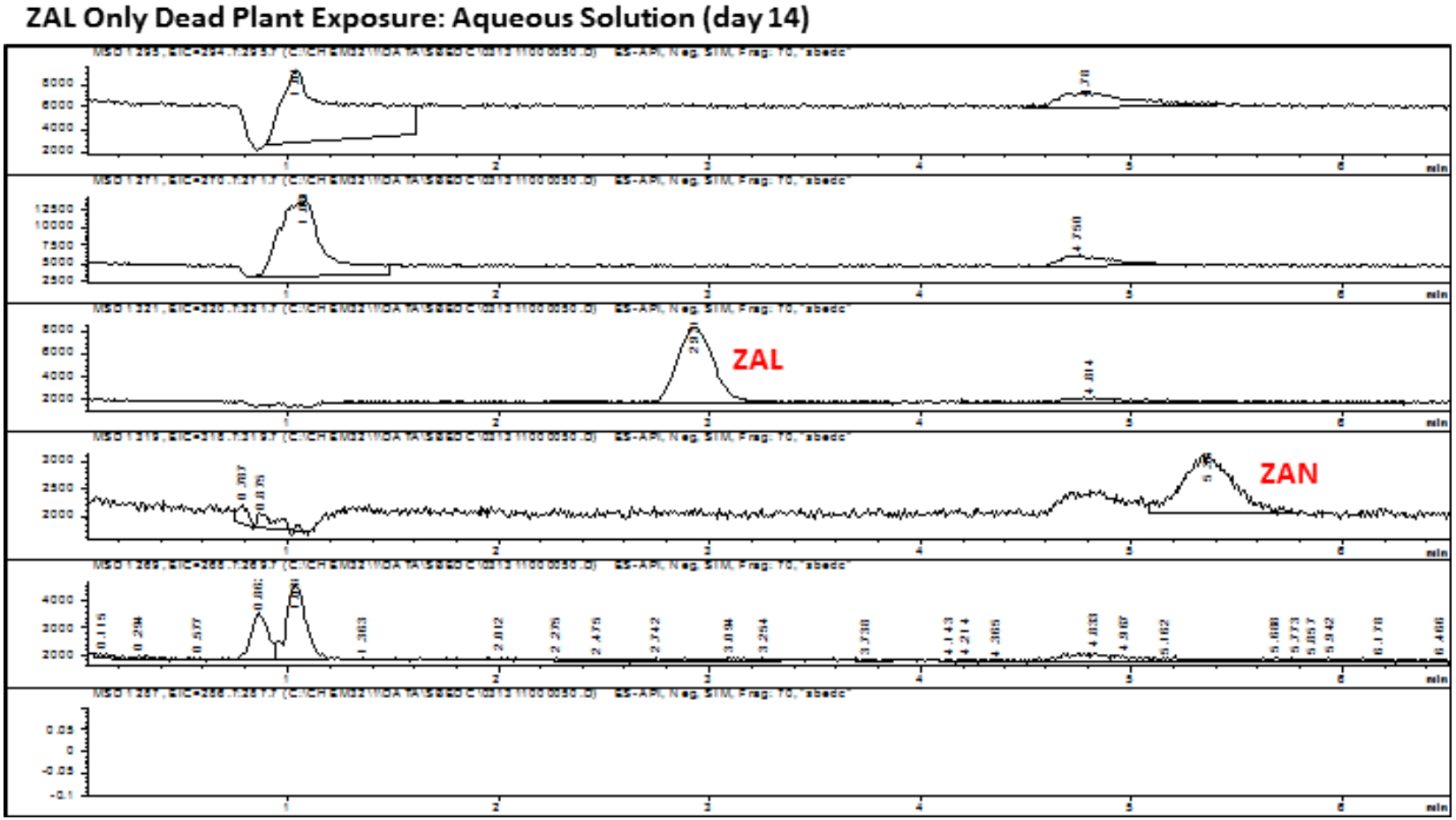

Figure A-19 Example chromatogram from the aqueous solution of the ZAL only dead plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

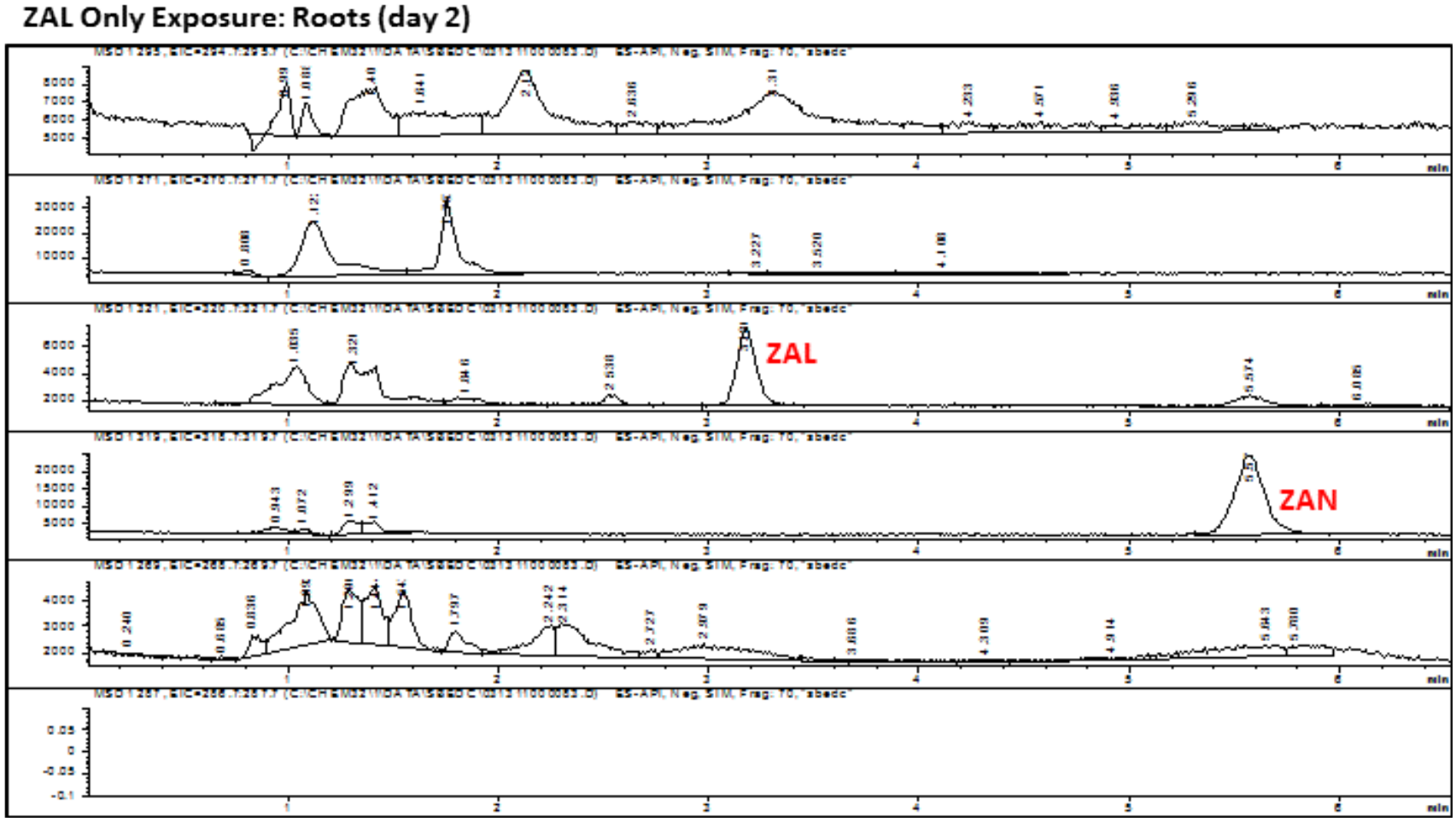

Figure A-20 Example chromatogram from the roots of the ZAL only full plant exposure on day 2 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

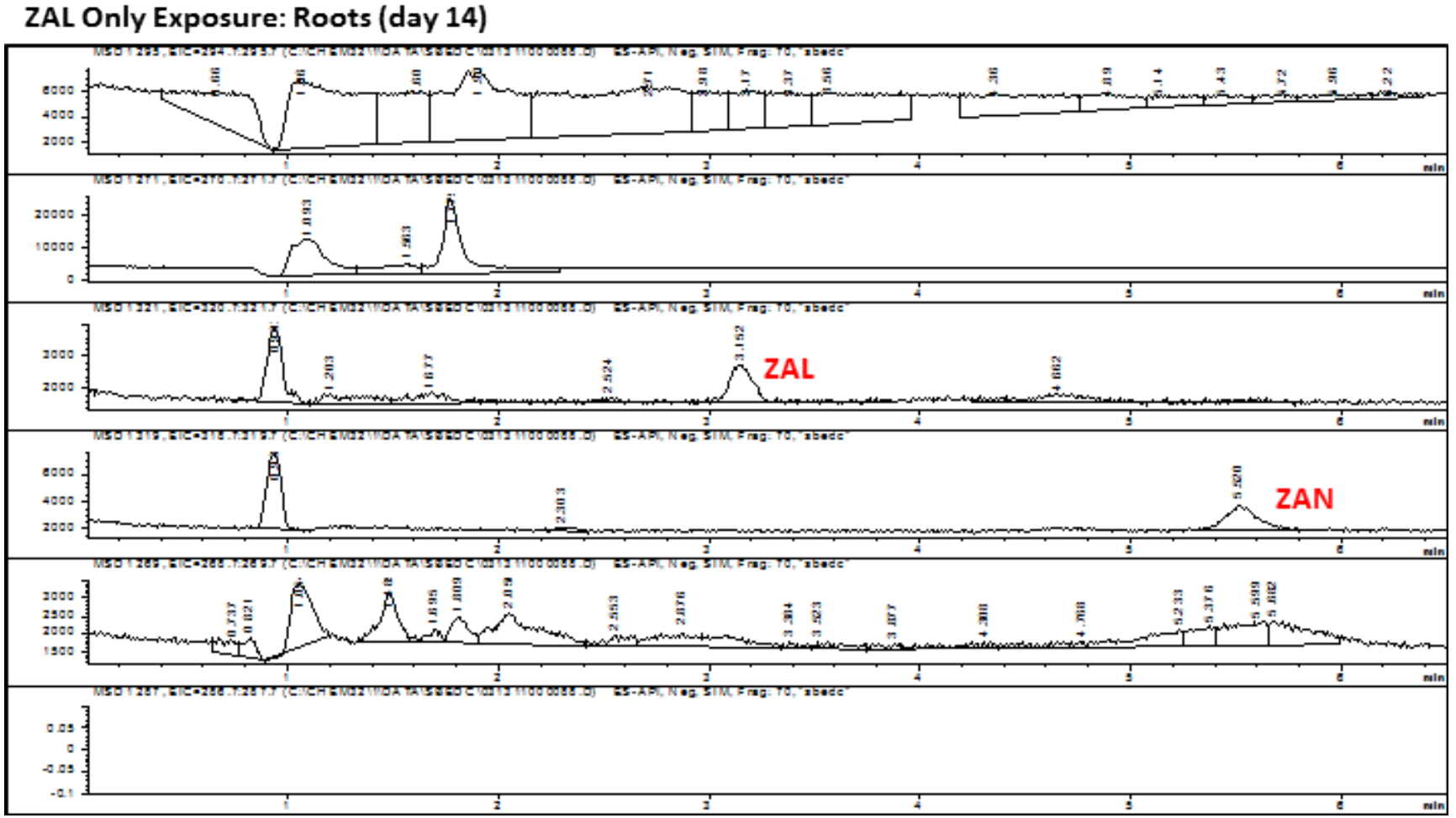

Figure A-21 Example chromatogram from the roots of the ZAL only full plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

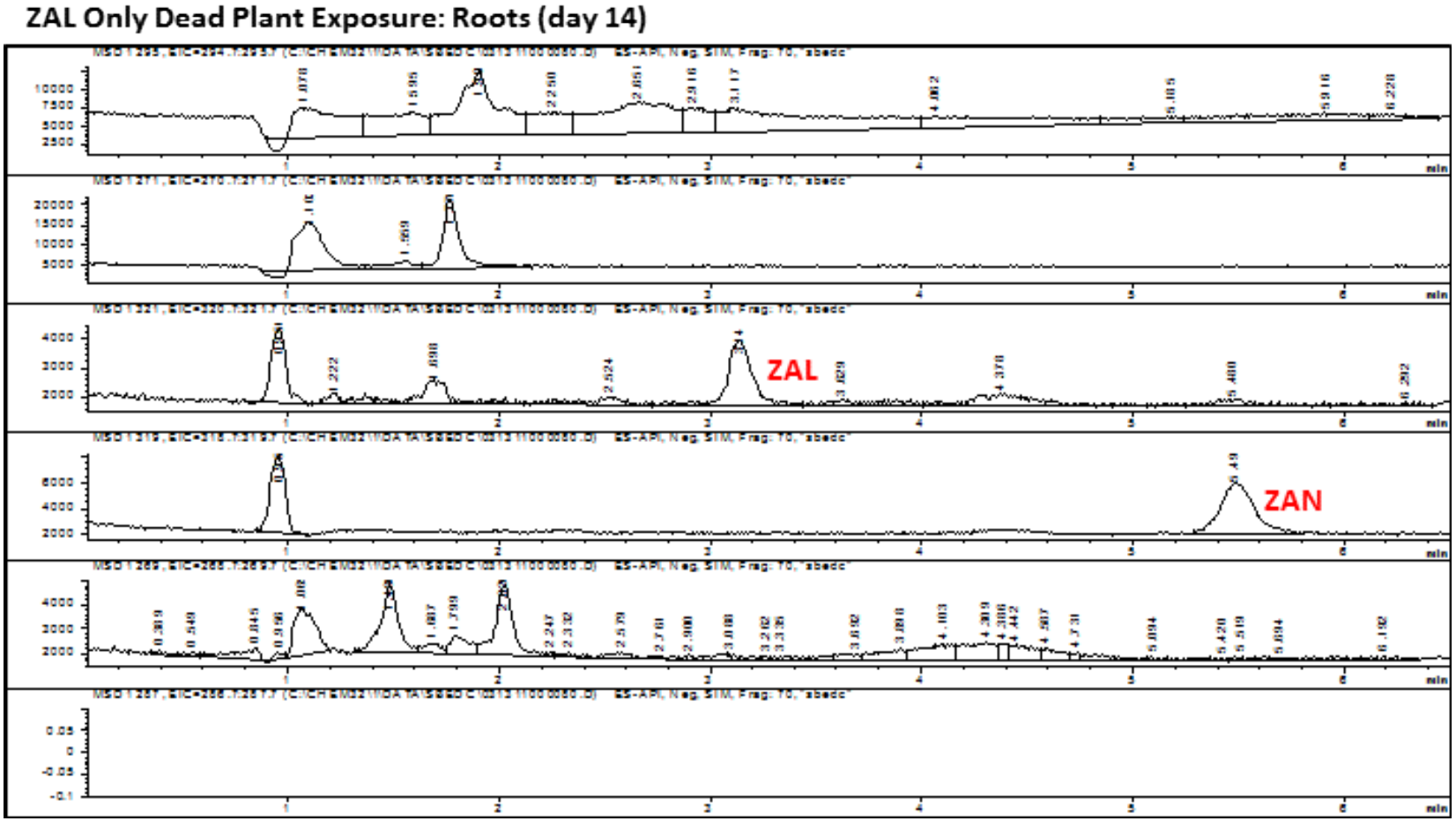

Figure A-22 Example chromatogram from the roots of the ZAL only dead plant exposure on day 14 of the softstem bulrush experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR THE HYBRID POPLAR EXPERIMENT

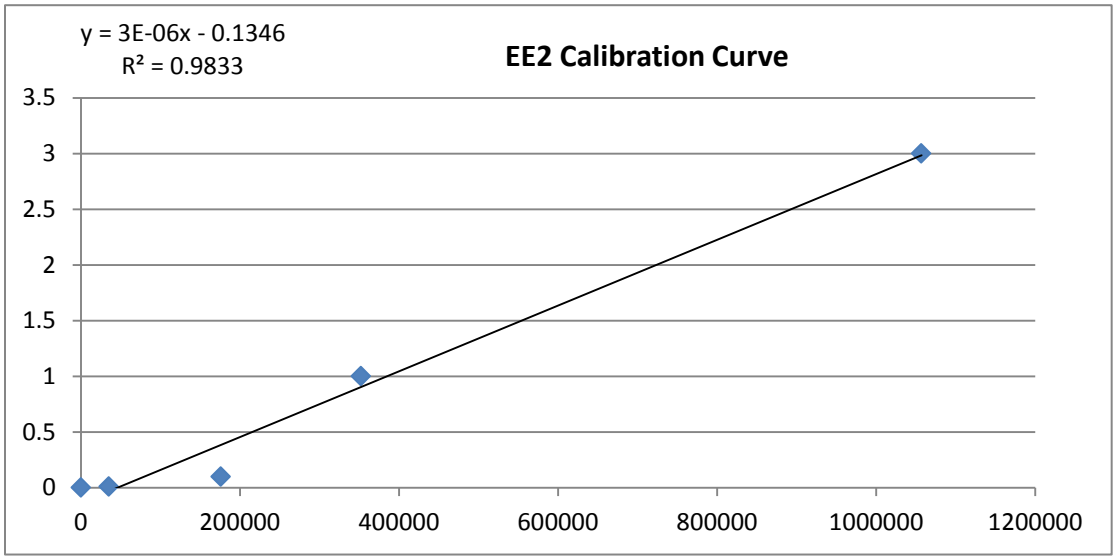

Figure B-1 Standard curve based on measured peak areas from LC/MS analysis for parent EE2 in the hybrid poplar experiment.

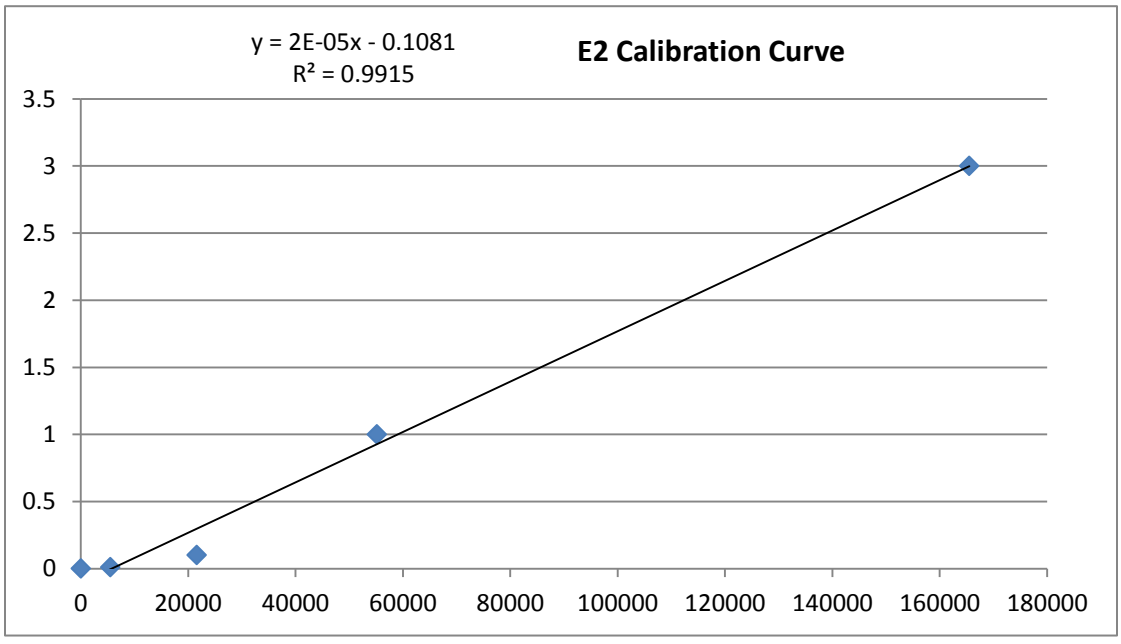

Figure B-2 Standard curve based on measured peak areas from LC/MS analysis for parent E2 in the hybrid poplar experiment.

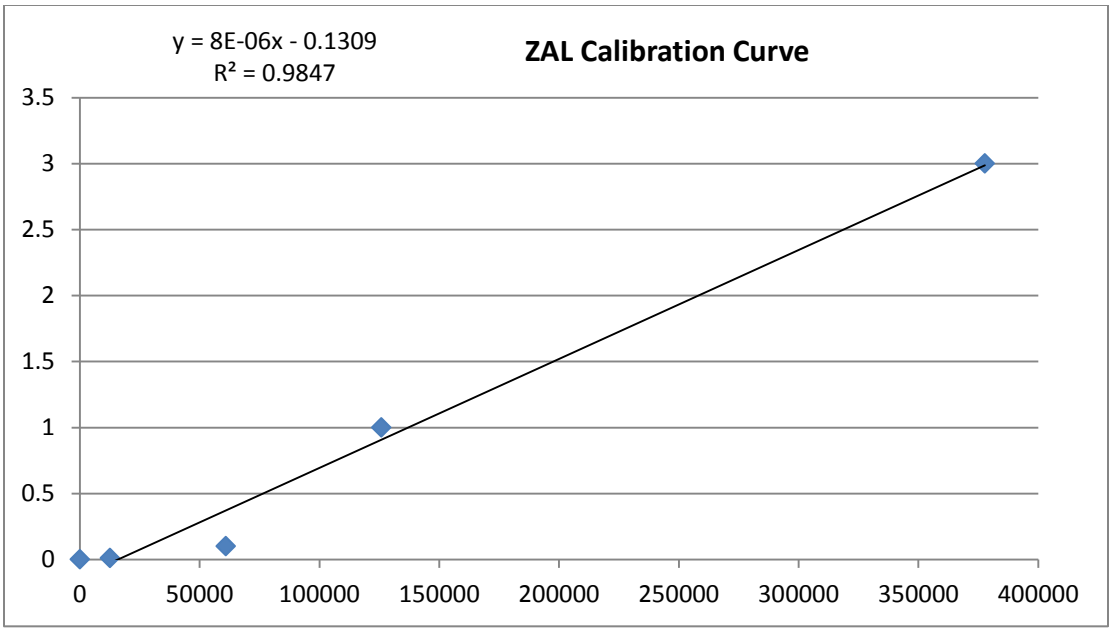

Figure B-3 Standard curve based on measured peak areas from LC/MS analysis for parent ZAL in the hybrid poplar experiment.

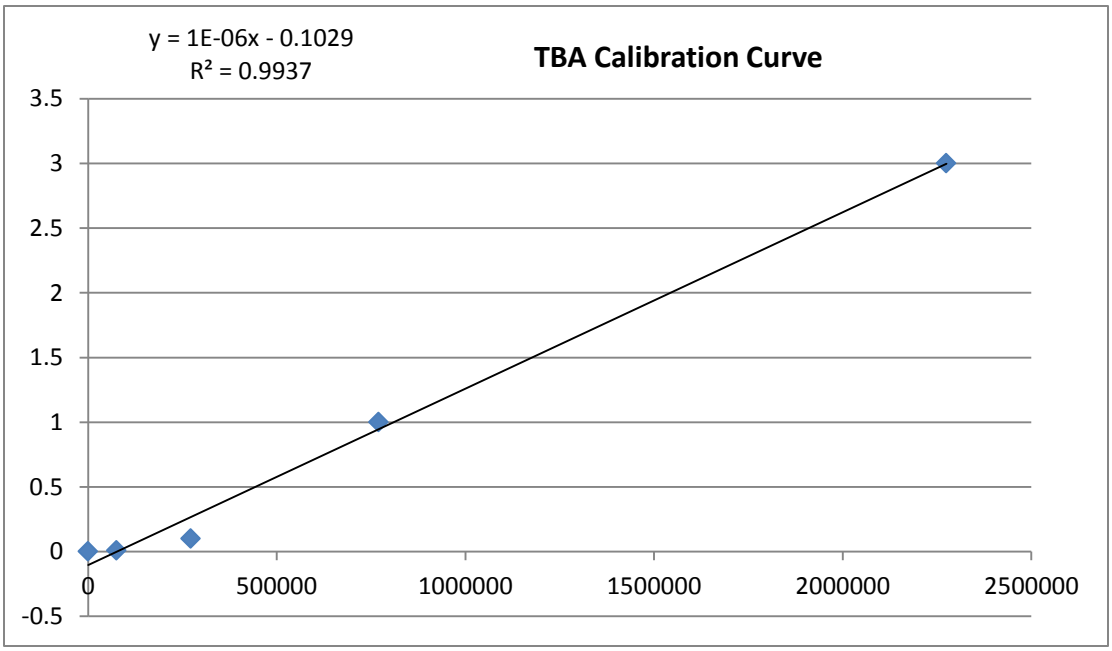

Figure B-4 Standard curve based on measured peak areas from LC/MS analysis for parent TBA in the hybrid poplar experiment.

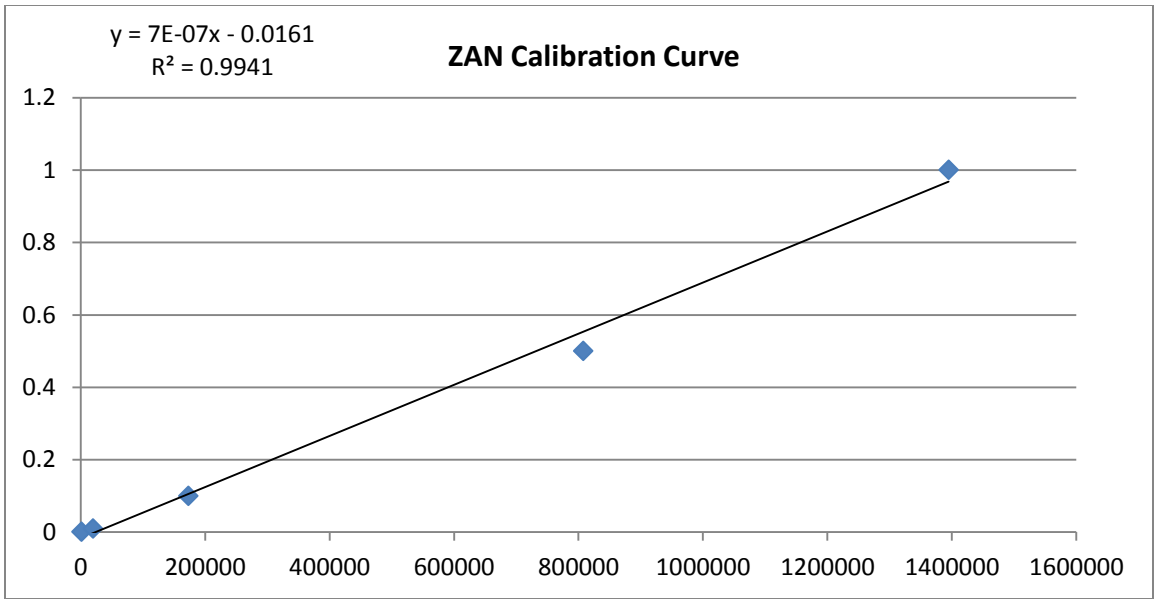

Figure B-5 Standard curve based on measured peak areas from LC/MS analysis for the metabolite ZAN in the hybrid poplar experiment.

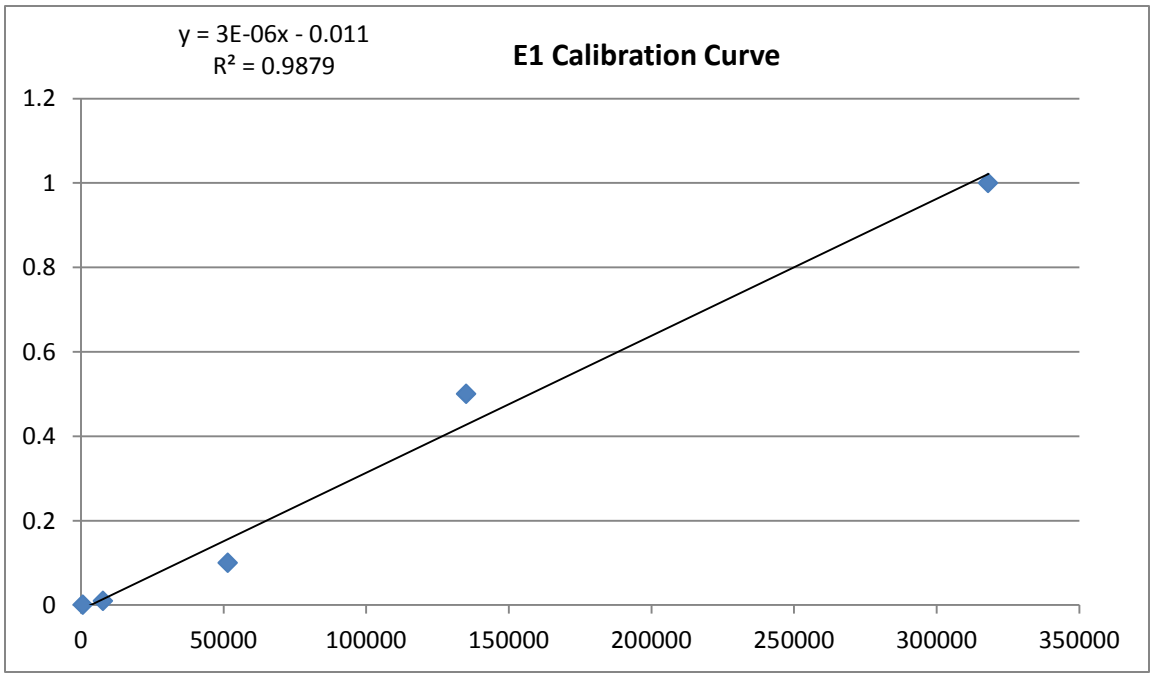

Figure B-6 Standard curve based on measured peak areas from LC/MS analysis for the metabolite E1 in the hybrid poplar experiment.

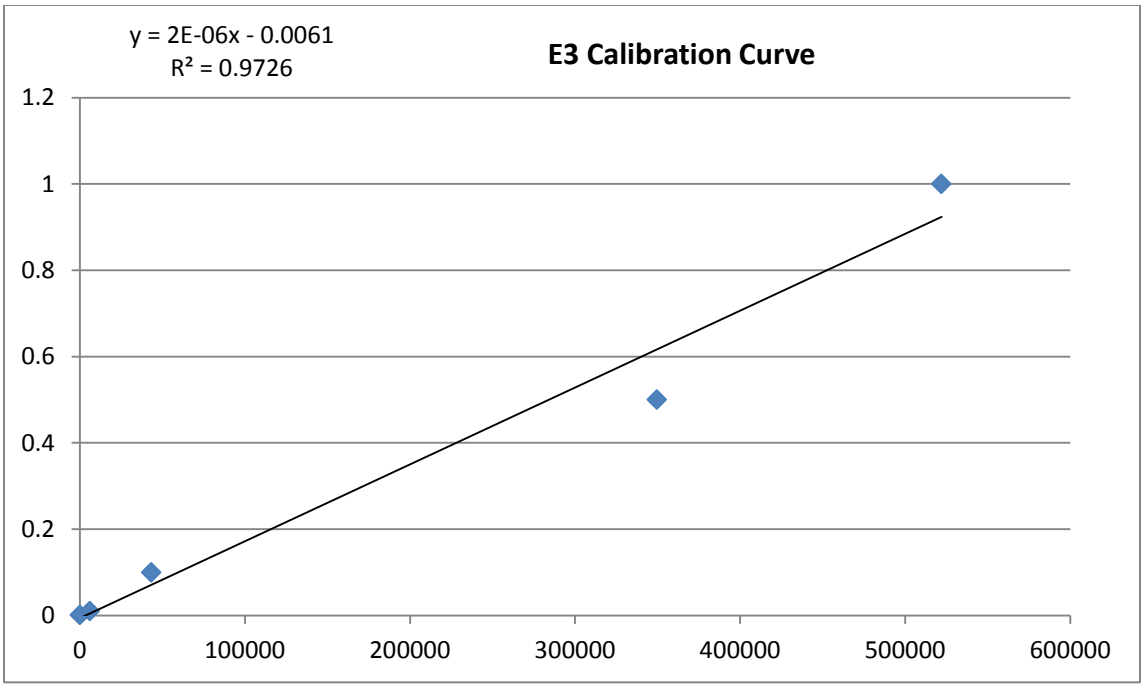

Figure B-7 Standard curve based on measured peak areas from LC/MS analysis for the metabolite E3 in the hybrid poplar experiment.

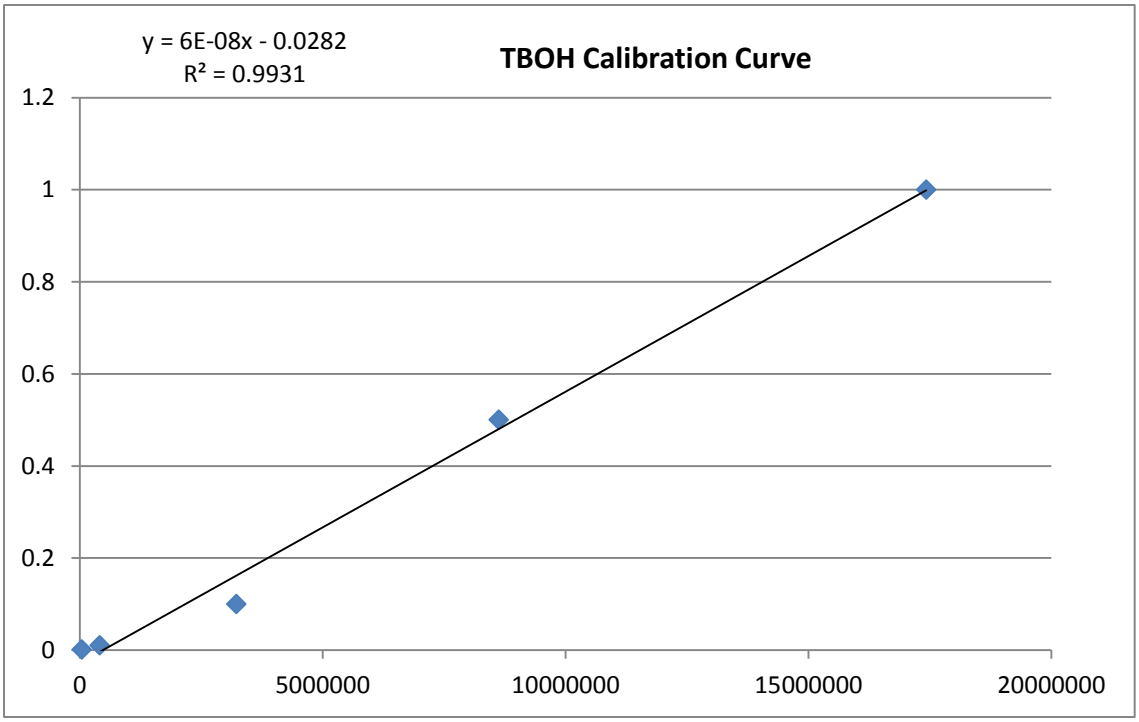

Figure B-8 Standard curve based on measured peak areas from LC/MS analysis for the metabolite TBOH in the hybrid poplar experiment.

Method detection limits (MDLs), found using the chromatogram peaks on the LC/MS analysis was calculated using the following equation:

$$MDL = \frac{\text{Lowest concentration of detected compound}}{\text{compound peak height}/3*\text{noise peak height}}$$

Table B-1 Method Level Detection Limits in the hybrid poplar experiment for each parent compound and their respected metabolites using the equation shown above.

Hormone Compound	EE2	E2	ZAL	TBA	E1	E3	ZAN	TBOH
Mass To Charge Ratio (*m/z*)	295	271	321	311	269	287	319	271
Retention Time (min)	3.9	3.1	2.9	4.1	4.4	1.4	5.1	2.4
MDL (µg/L)	6.41	7.94	0.042	0.378	0.617	0.362	0.286	0.185

Table B-2 Evapotranspiration rates for each sample in the excised plant, full plant, and dead plant exposures over the 10 day experiment.

Excised Evapotranspiration (ml/day)										
Sample #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	4									
2	4.5									
3	5									
4	4.9	6.2								
5	5.6	5.1								
6	6.7	7.4								
7	5.3	7.6	6.2	6.4	7.4					
8	4.8	5.2	6.1	7.4	6.5					
9	6	6.3	6.8	7.3	7.1					
10	6.8	7.2	6.6	8.4	8.1	7.1	6.1	7.2	8.1	7.8
11	5.9	6.6	6.7	6.2	6.2	6.4	5.9	6.6	7.3	7.5
12	5.5	6.5	8.1	7.2	6.3	6.6	6.4	8.2	7.9	6.4
								Average= 6.55		
Full Plant Evapotranspiration (ml/day)										
Sample #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	102									
2	112									
3	126									
4	104	109								
5	99	107								
6	123	142								
7	114	109	132	142	136					
8	108	116	120	108	121					
9	125	126	118	114	118					
10	129	132	115	109	106	112	124	120	132	108
11	98	107	110	108	111	109	112	115	99	107
12	105	135	158	119	108	110	118	124	120	117
								Average= 116.8		
Dead Plant Evapotranspiration (ml/day)										
Sample #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.1									
2	2									
3	2.1									
4	1.8	1.6								
5	1.2	1.1								
6	1.3	1.2								
7	1.4	1.2	1.4	1.5	1.4					
8	1.2	1.1	1.3	1.2	1.4					
9	1.1	1.3	1.3	1.1	1.3					
10	0.9	1.1	1	1.1	1.1	1.2	1.3	1.2	1.4	1.4
11	1.3	1.2	1.2	1.4	1.3	1.4	1.1	1.2	1.2	1.3
12	1.8	1.6	1.7	1.5	1.6	1.5	1.5	1.4	1.5	1.6
								Average = 1.34		

Figure B-9 Experimental set up for hydroponic exposure experiment with hybrid poplar in growth chamber.

Figure B-10 Picture of individual flasks for full plant, excised plant and dead plant exposures.

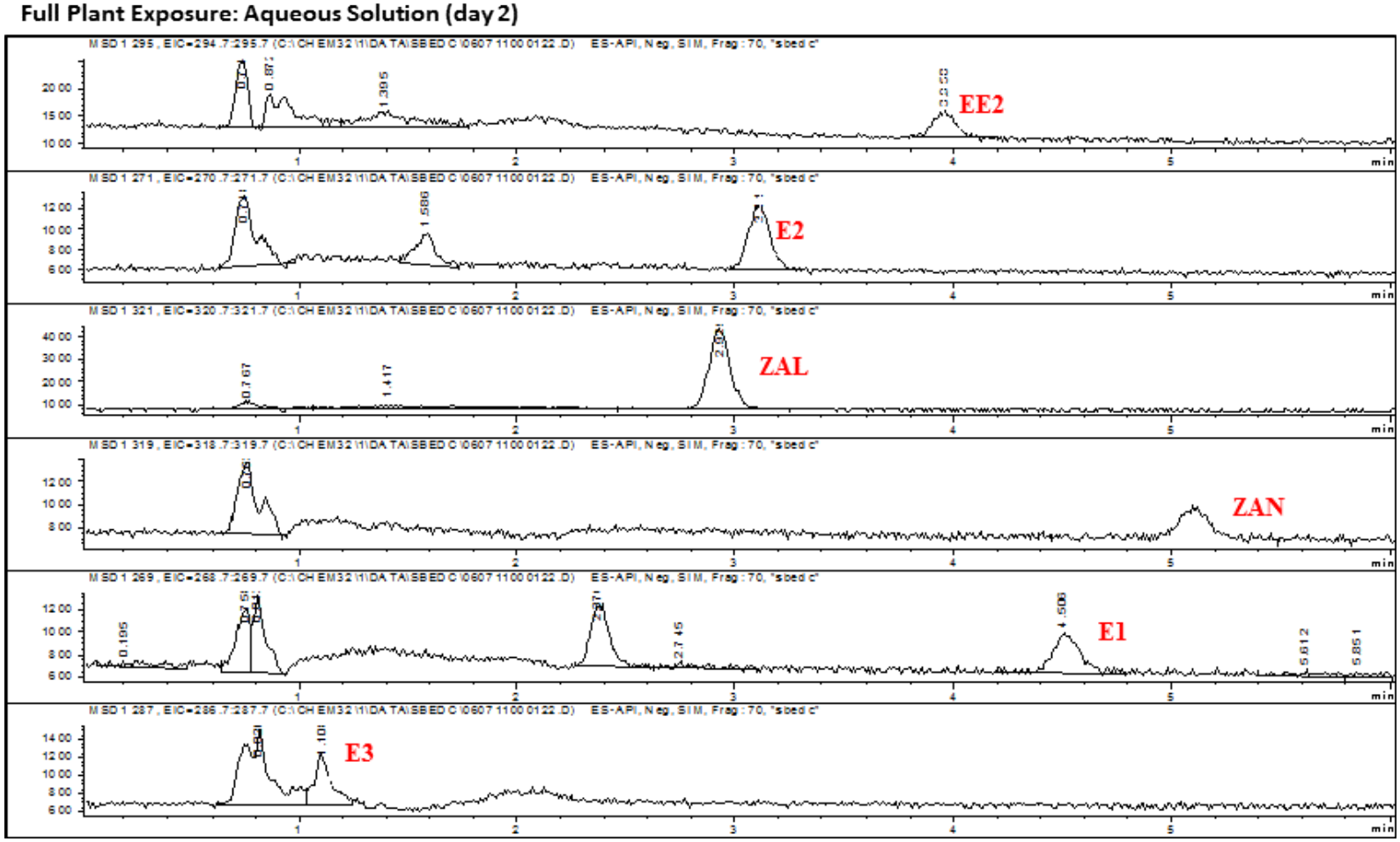

Figure B-11 Example chromatogram in negative ion mode from the aqueous solution of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

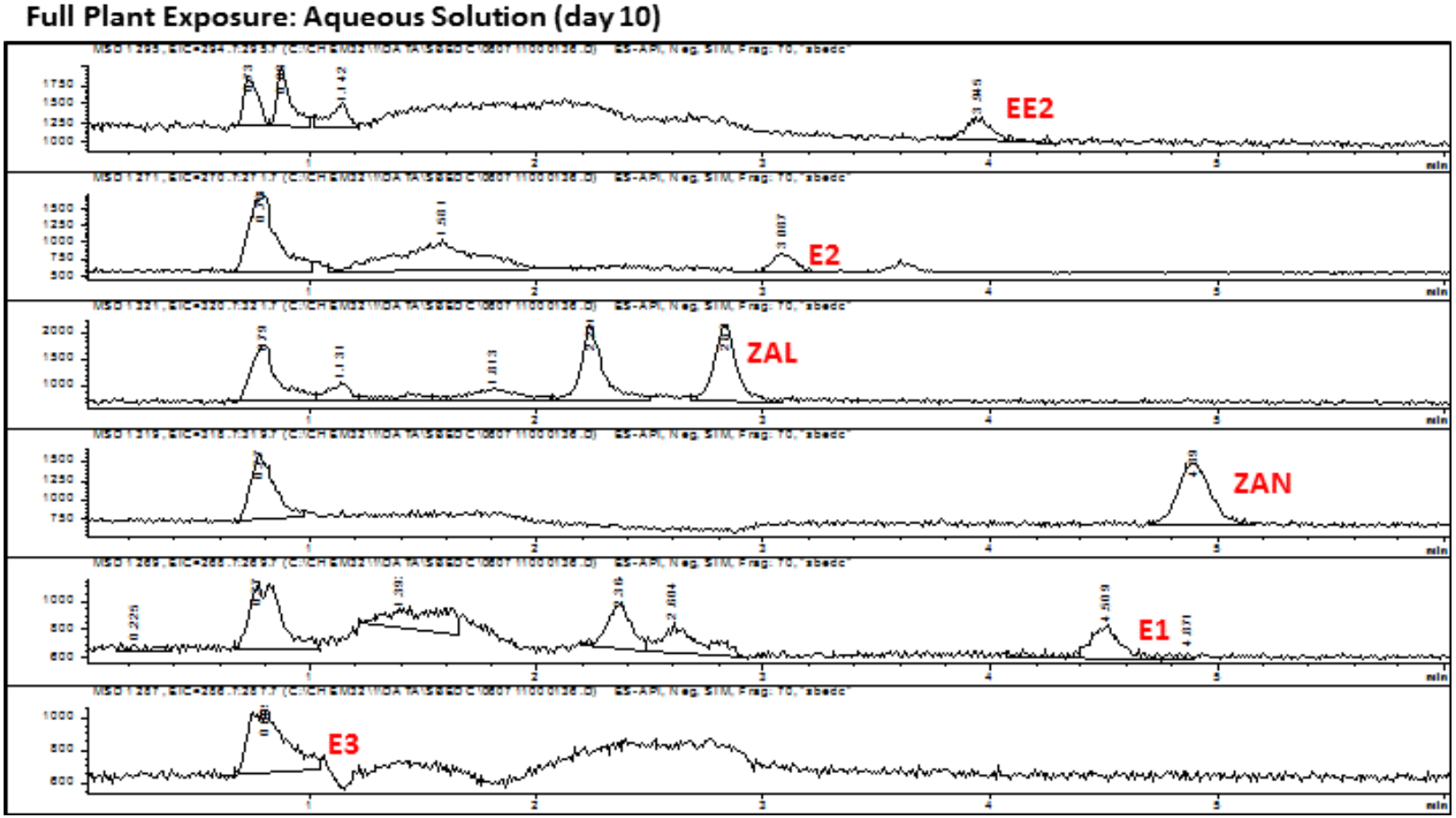

Figure B-12 Example chromatogram in negative ion mode from the aqueous solution of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red on the right side of the associated peaks.

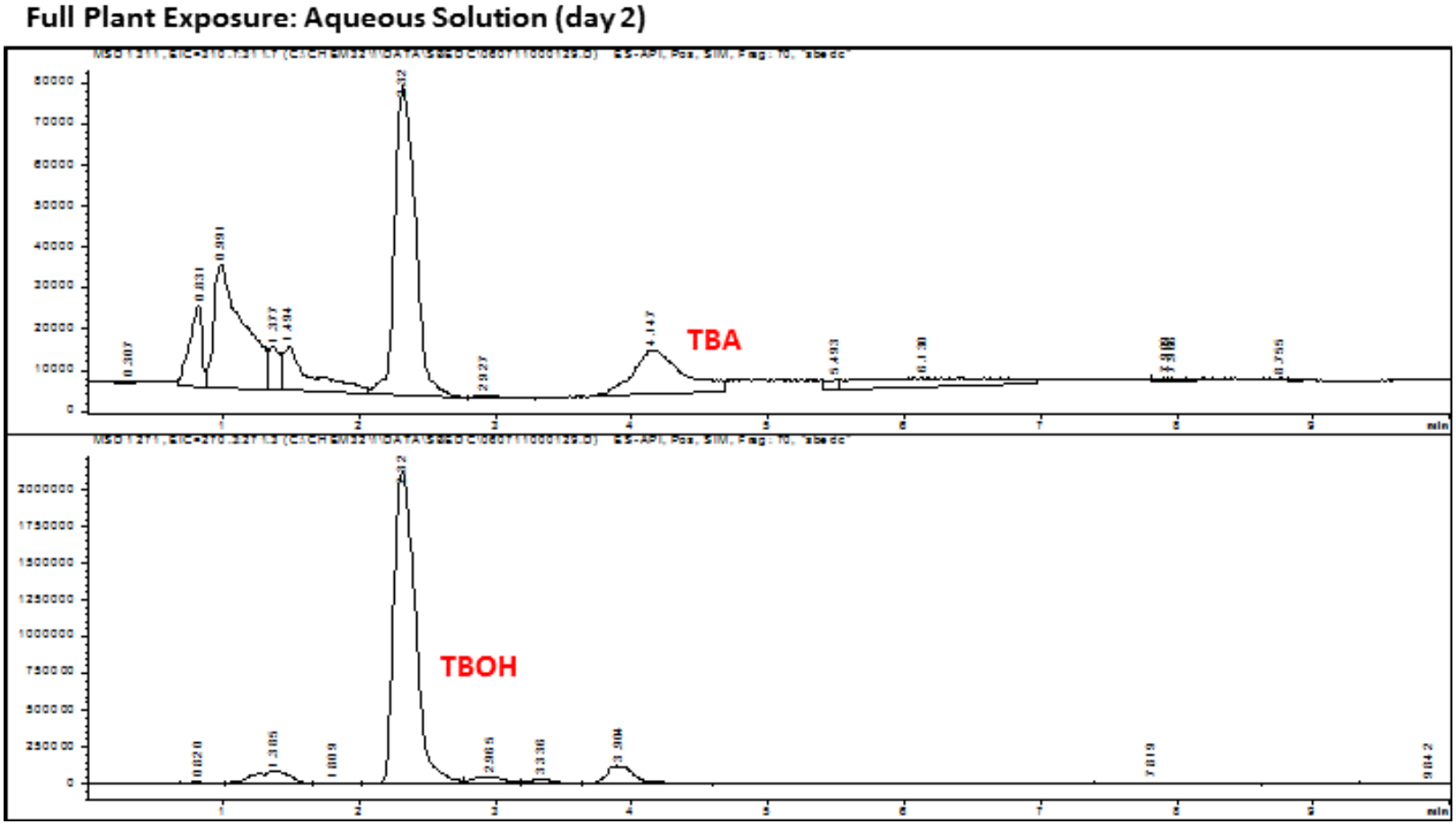

Figure B-13 Example chromatogram in positive ion mode from the aqueous solution of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

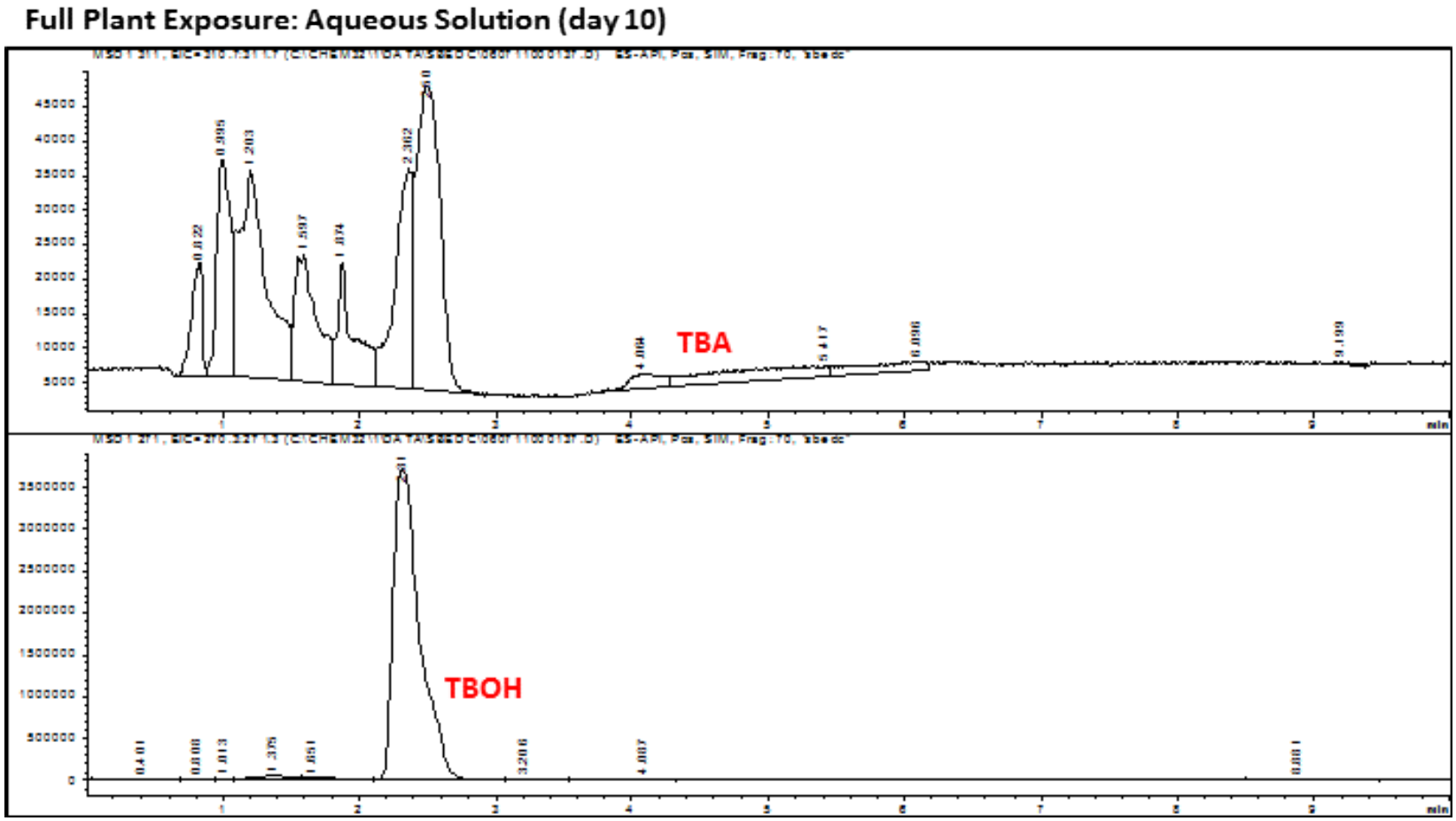

Figure B-14 Example chromatogram in positive ion mode from the aqueous solution of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

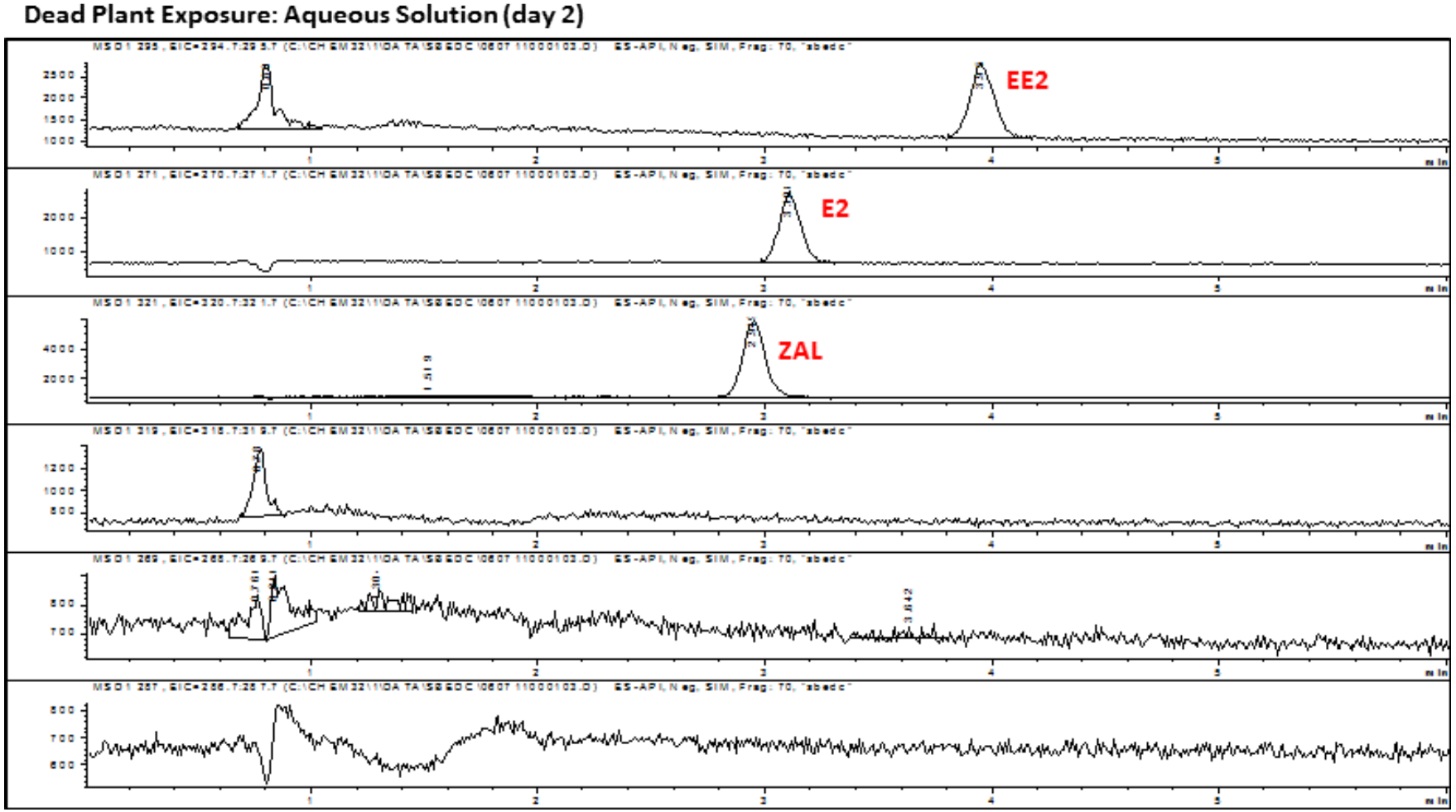

Figure B-15 Example chromatogram in negative ion mode from the aqueous solution of the dead plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

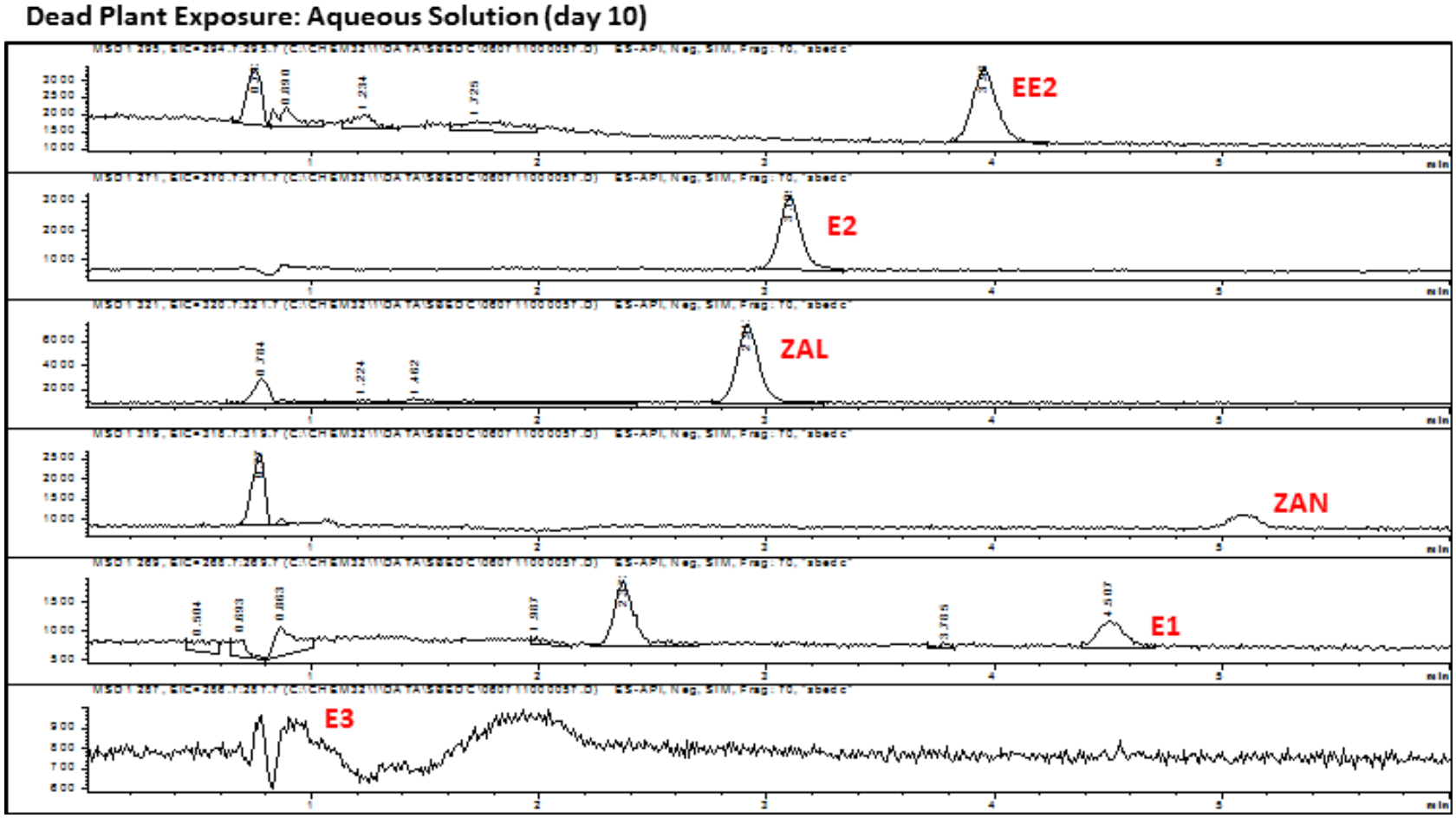

Figure B-16 Example chromatogram in negative ion mode from the aqueous solution of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

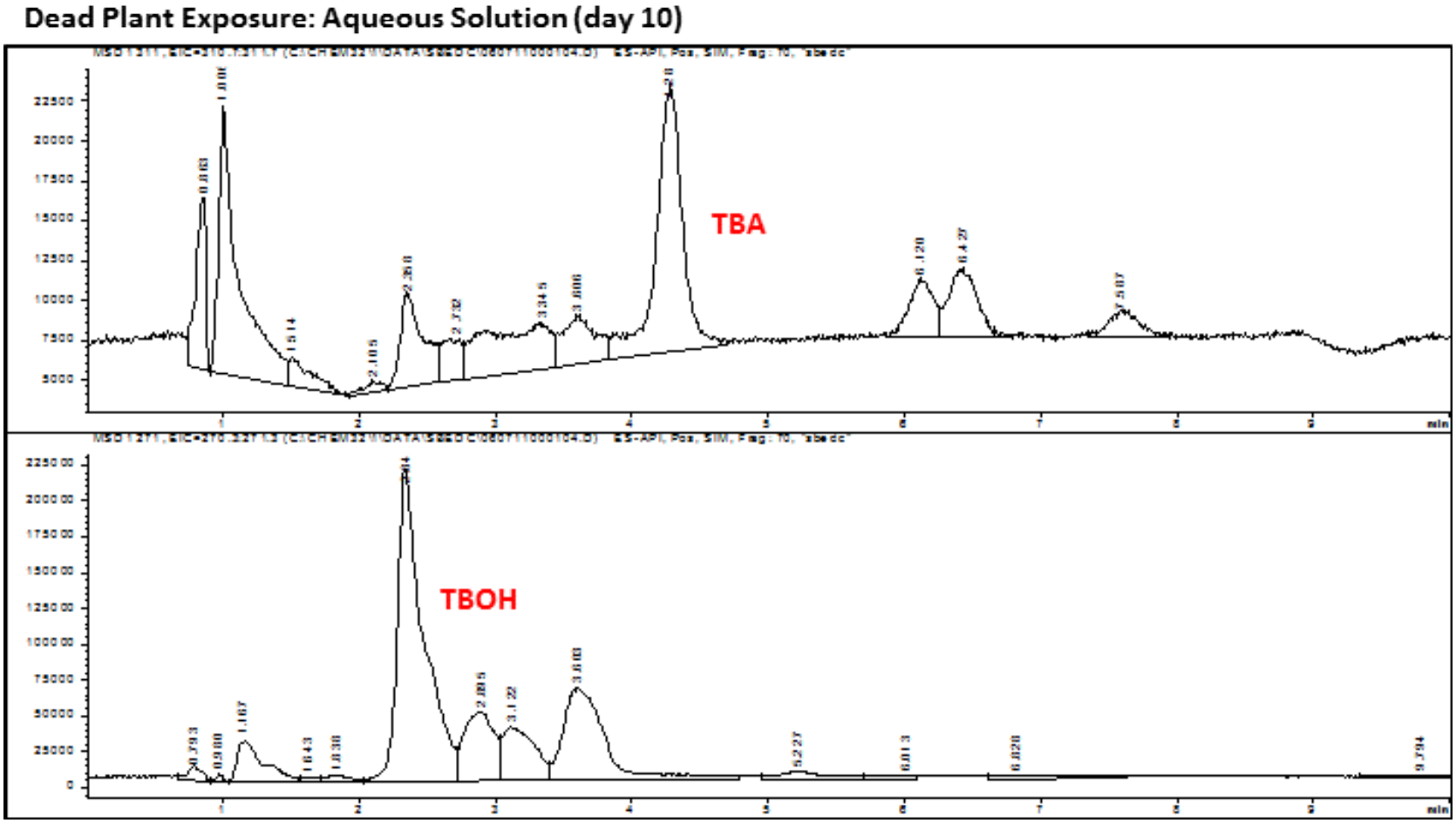

Figure B-17 Example chromatogram in positive ion mode from the aqueous solution of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

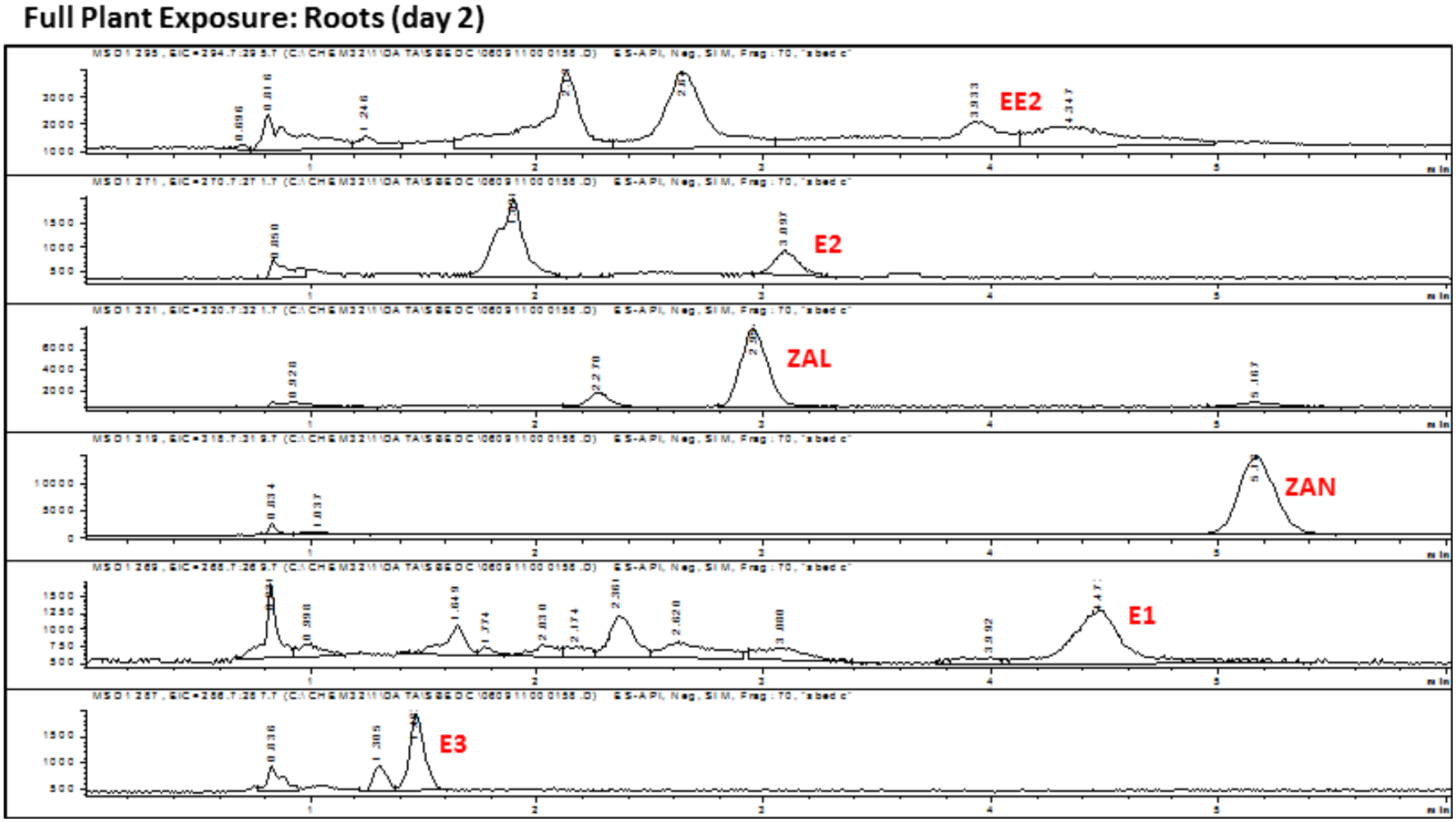

Figure B-18 Example chromatogram in negative ion mode from the roots of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

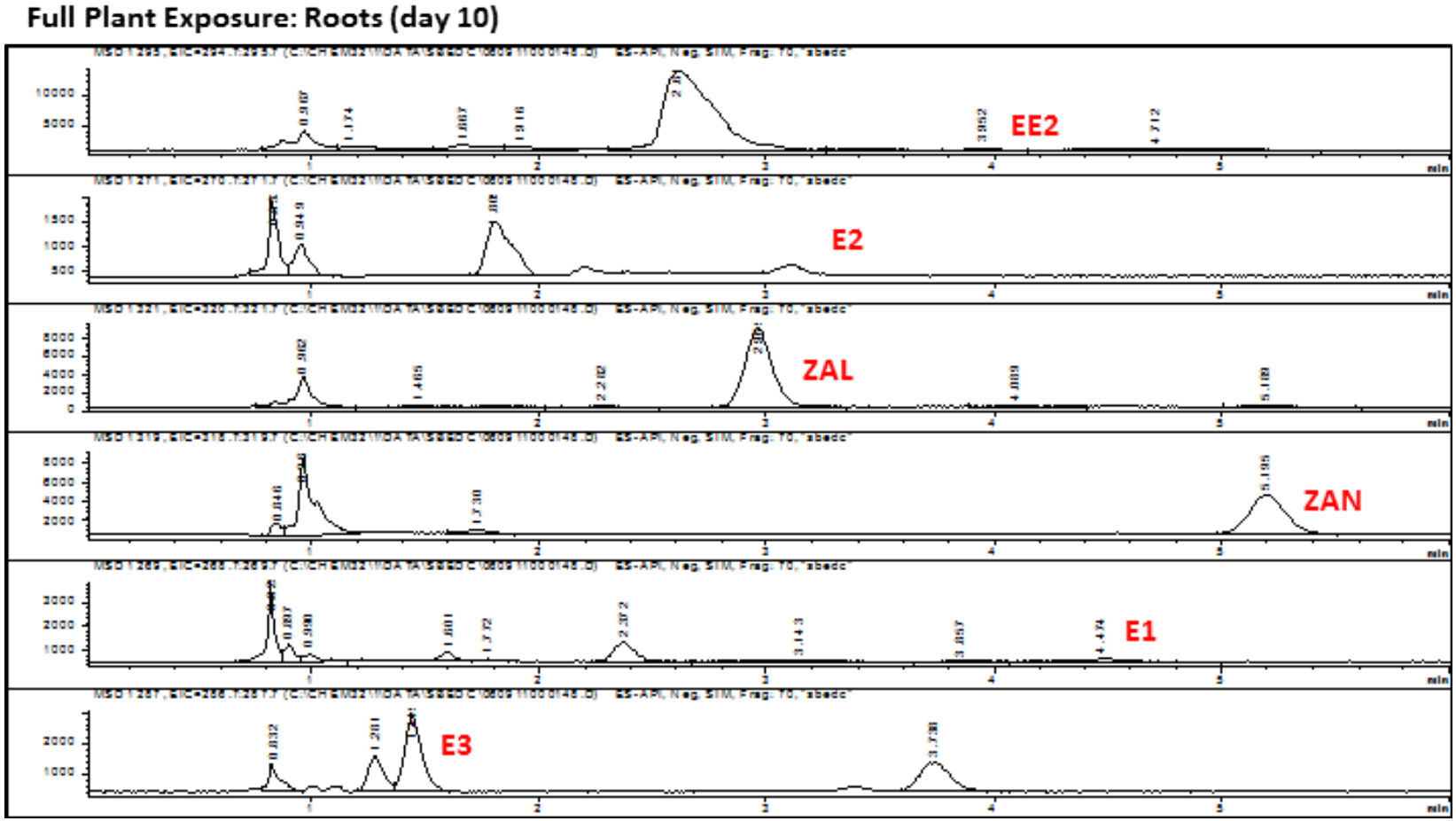

Figure B-19 Example chromatogram in negative ion mode from the roots of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

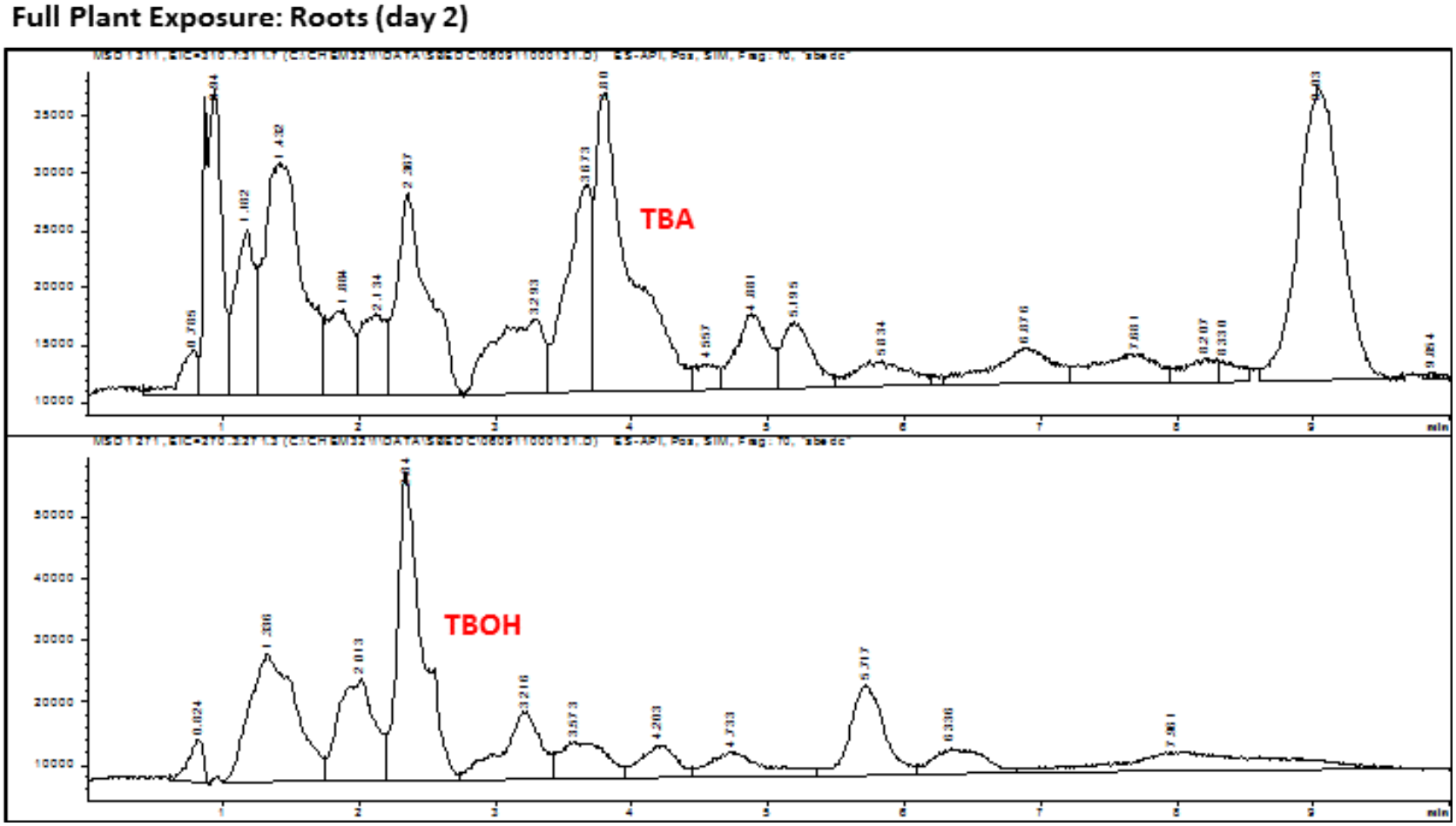

Figure B-20 Example chromatogram in positive ion mode from the roots of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

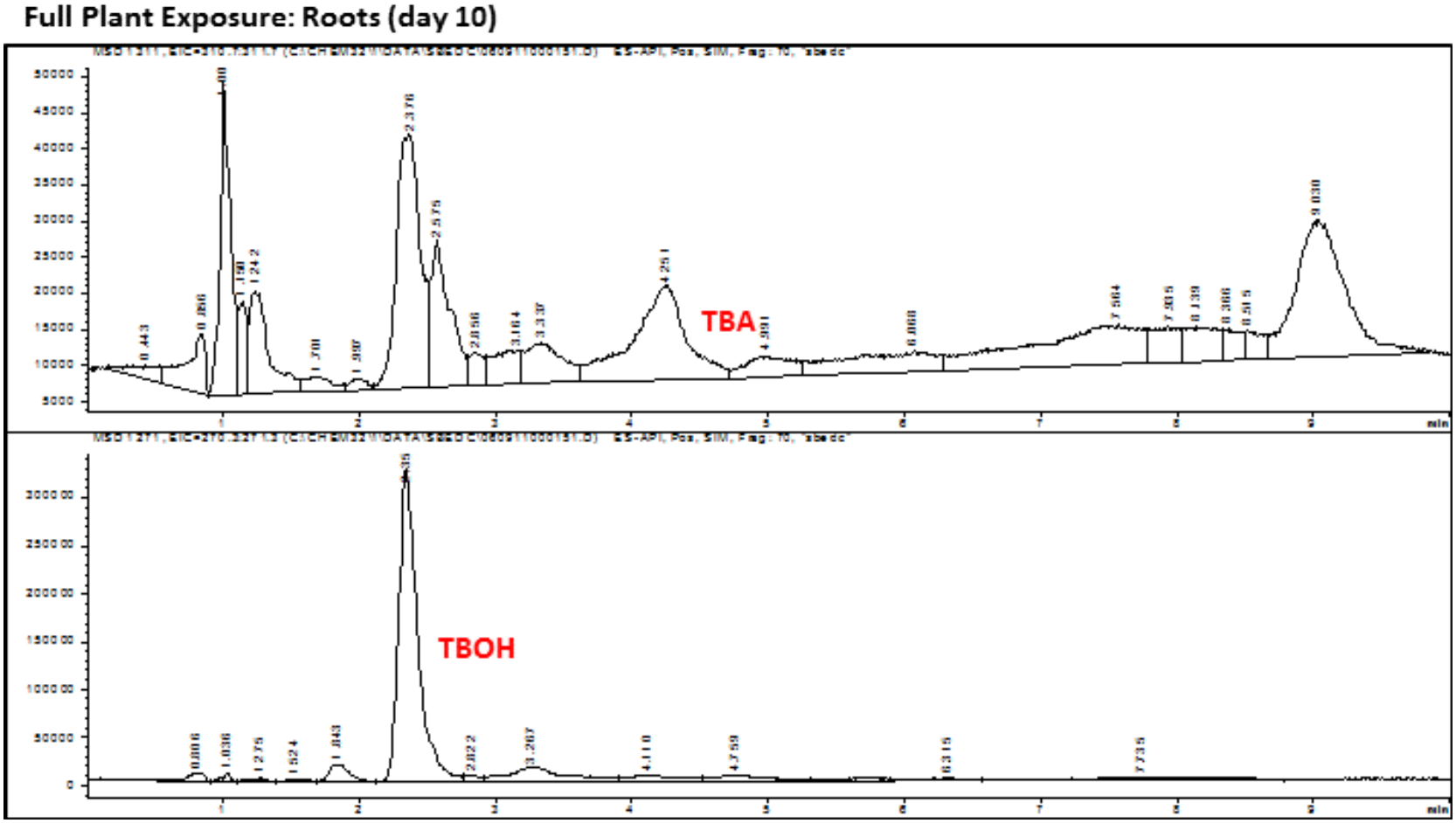

Figure B-21 Example chromatogram in positive ion mode from the roots of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

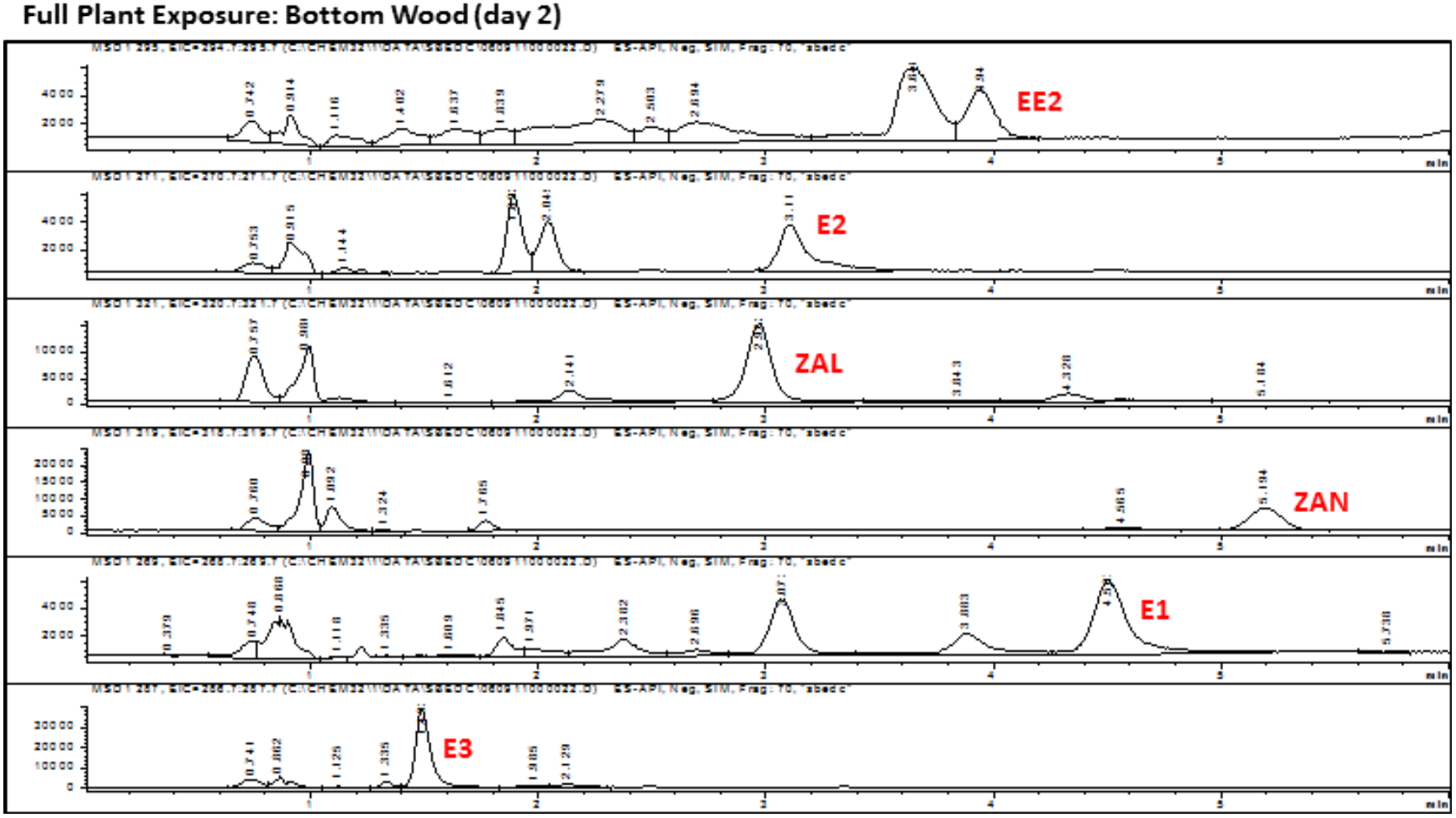

Figure B-22 Example chromatogram in negative ion mode from the bottom wood of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

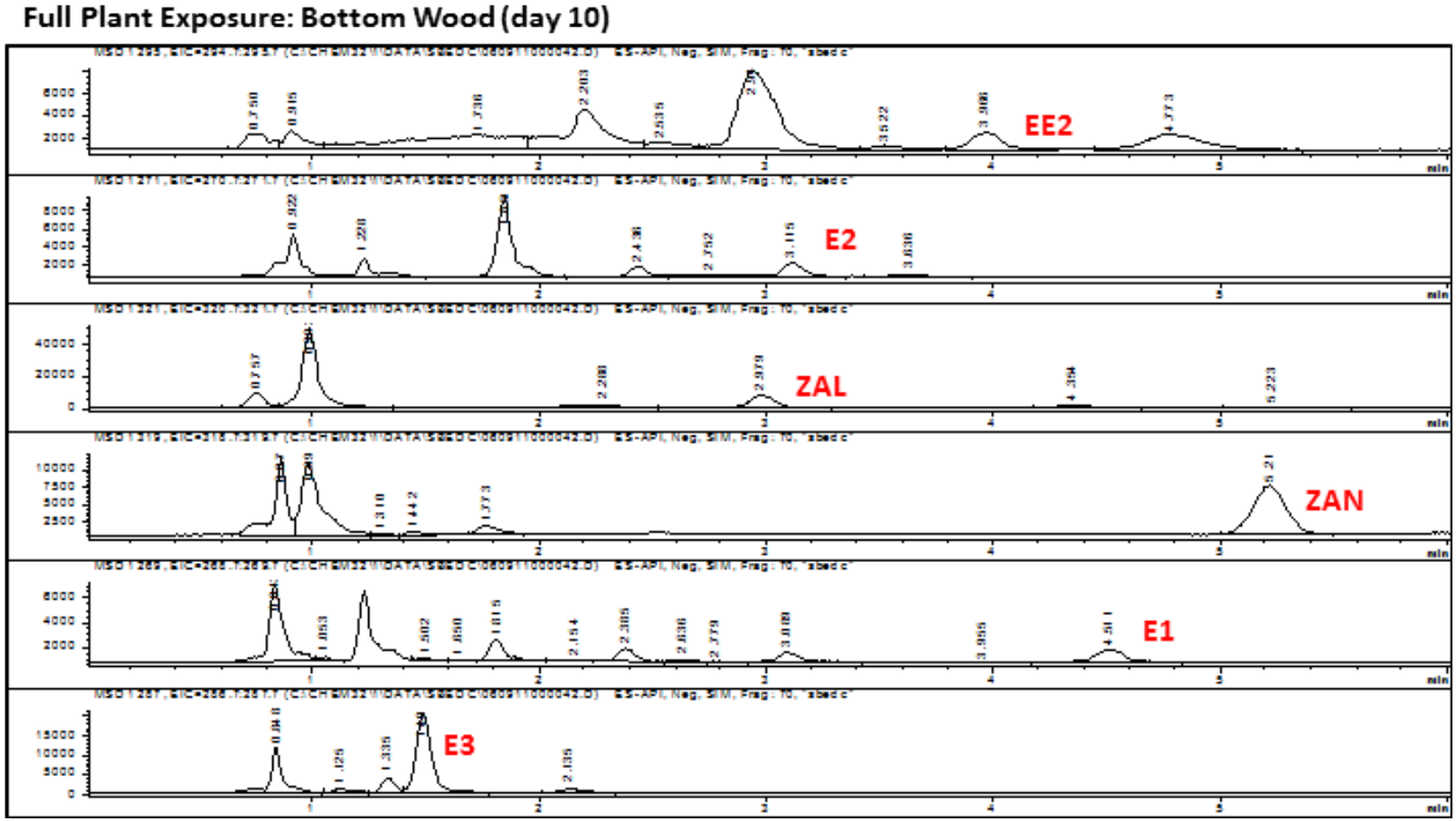

Figure B-23 Example chromatogram in negative ion mode from the bottom wood of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

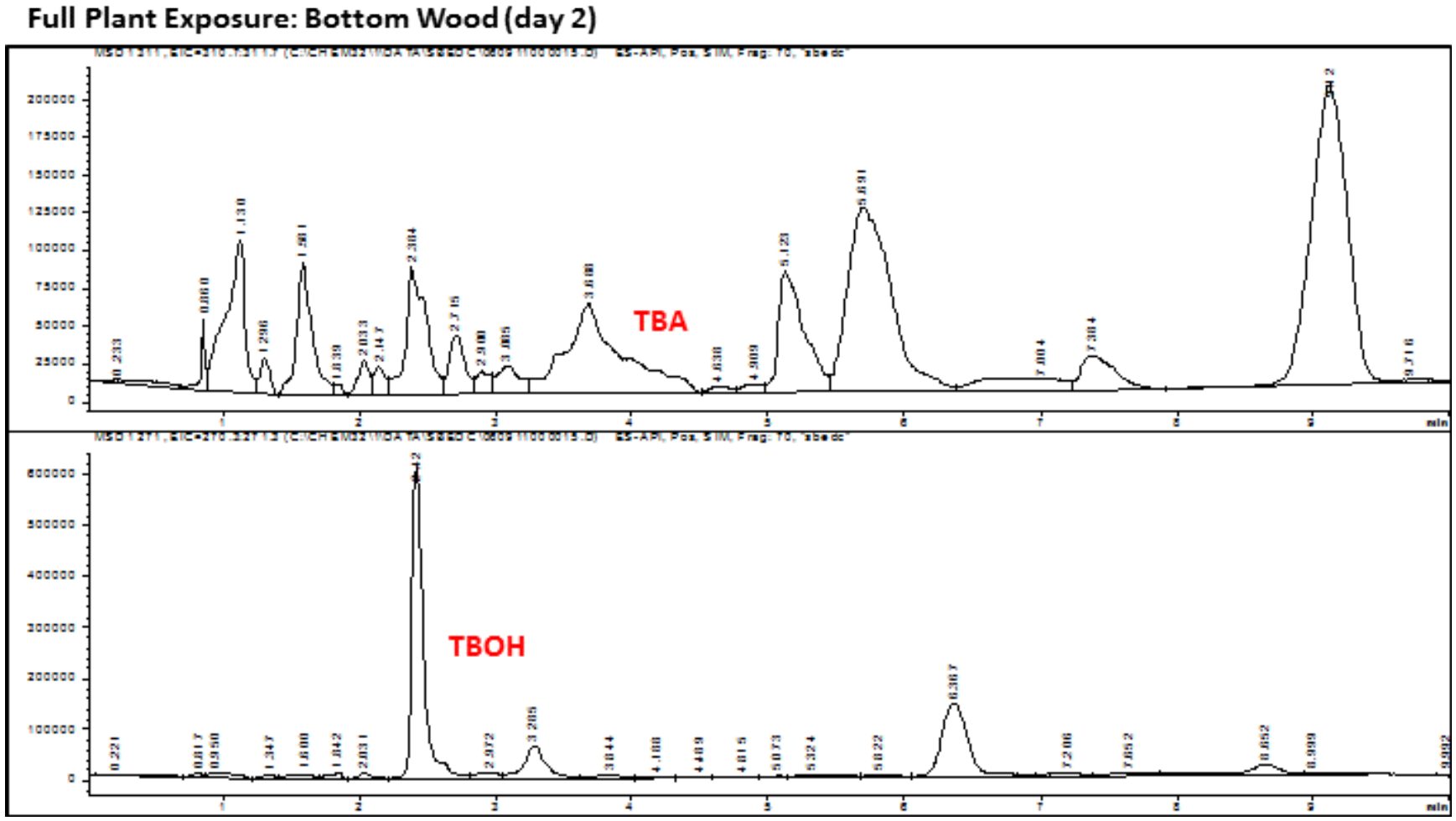

Figure B-24 Example chromatogram in positive ion mode from the bottom wood of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

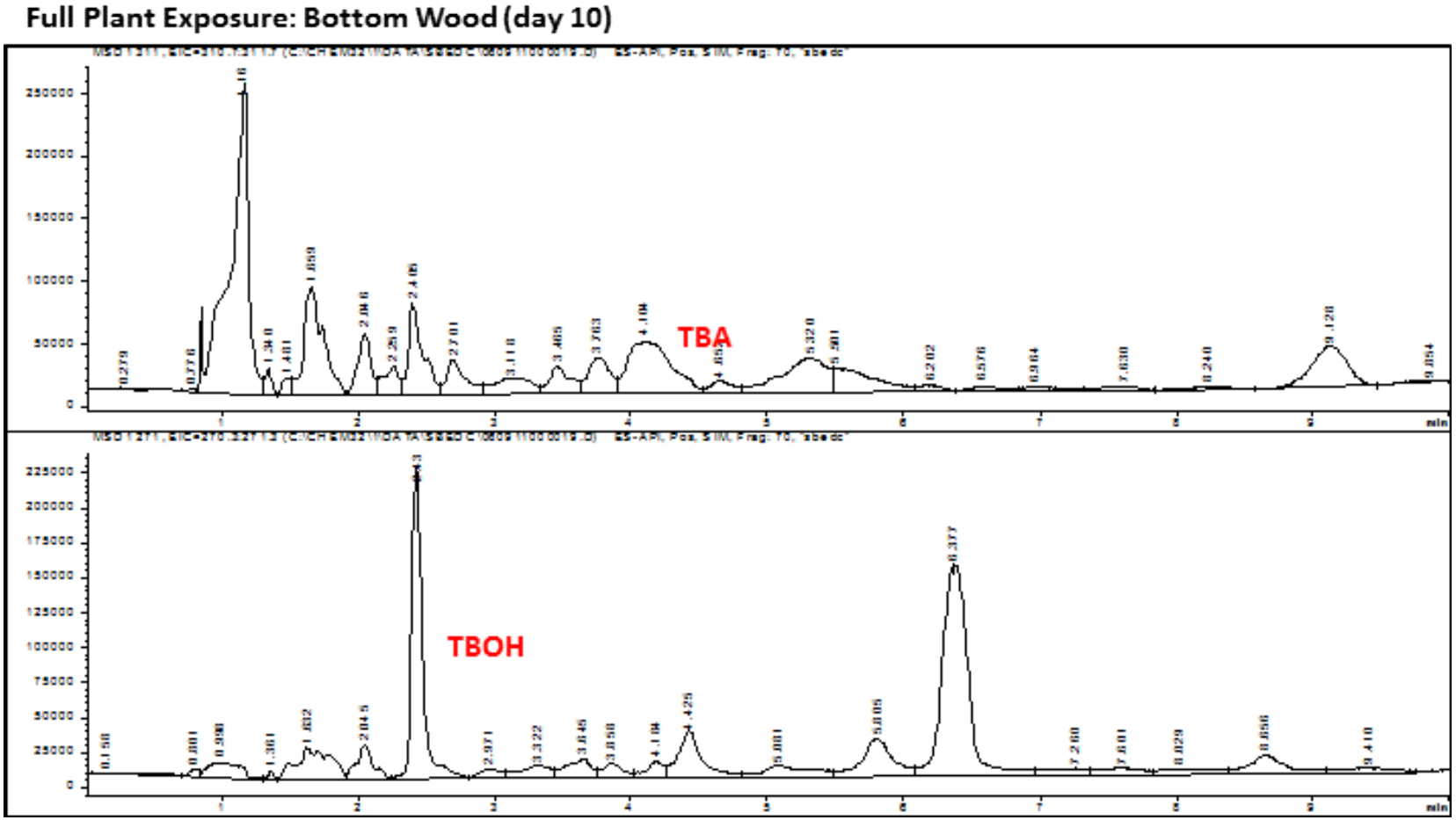

Figure B-25 Example chromatogram in positive ion mode from the bottom wood of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

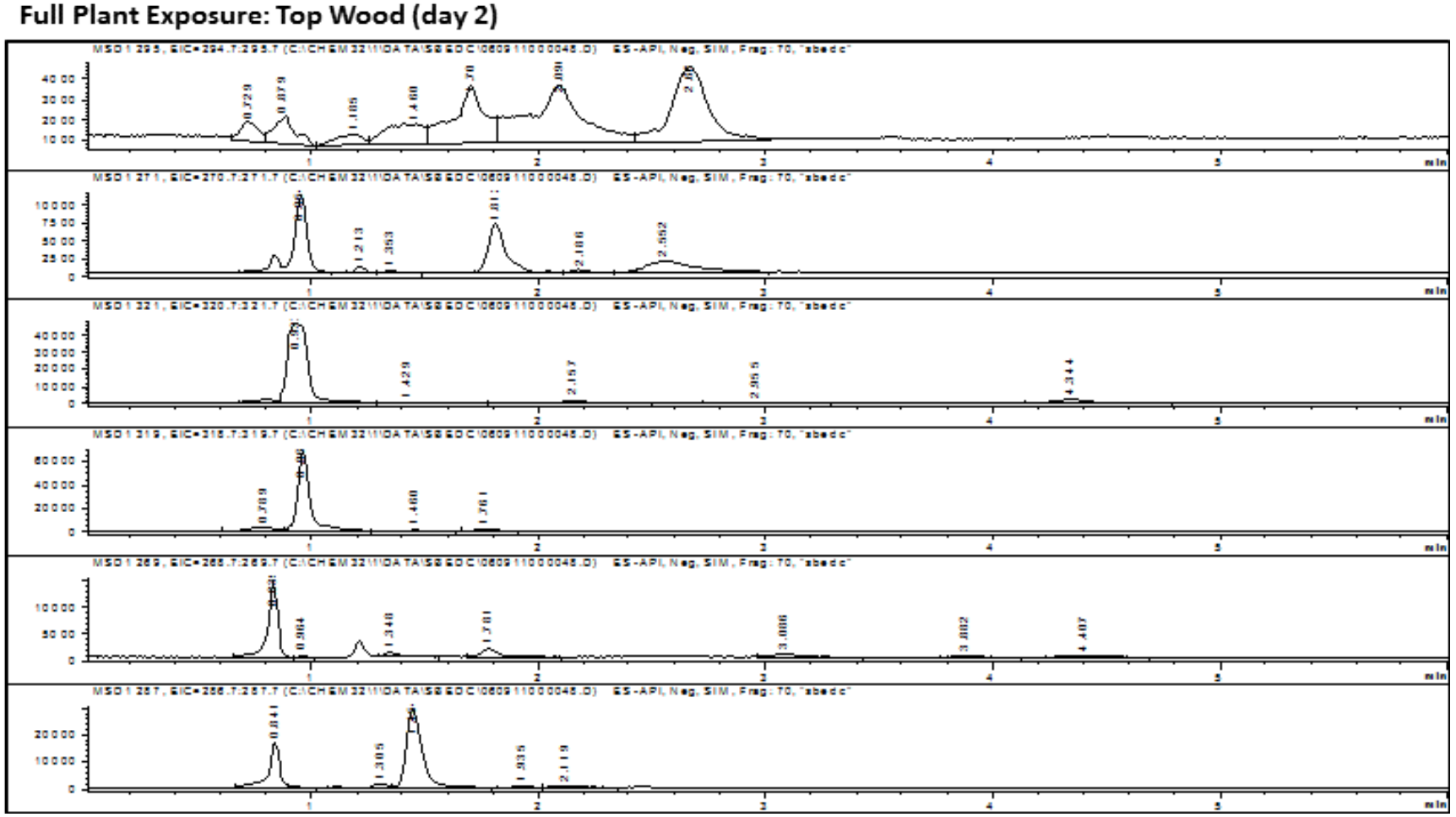

Figure B-26 Example chromatogram in negative ion mode from the top wood of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

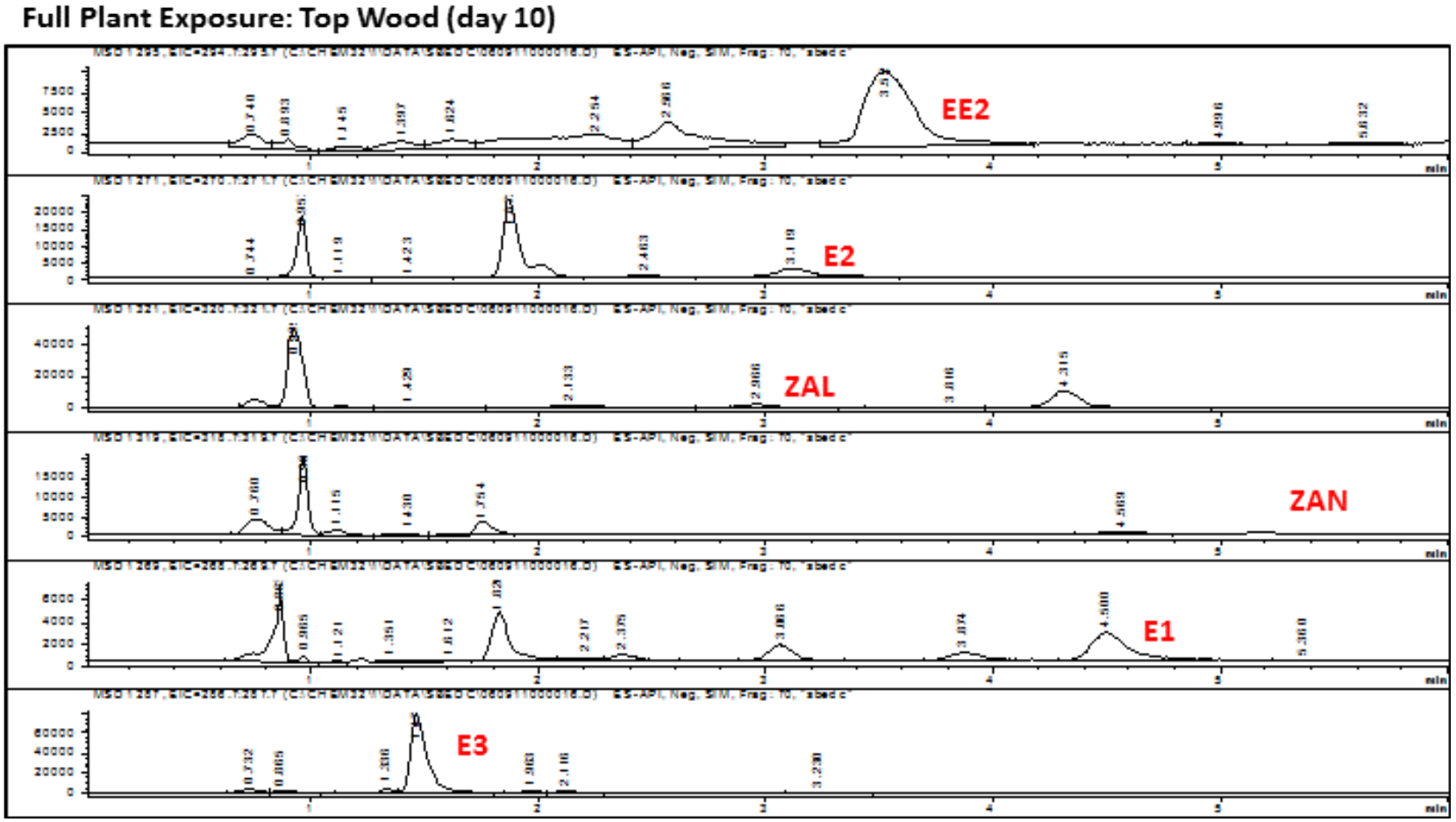

Figure B-27 Example chromatogram in negative ion mode from the top wood of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

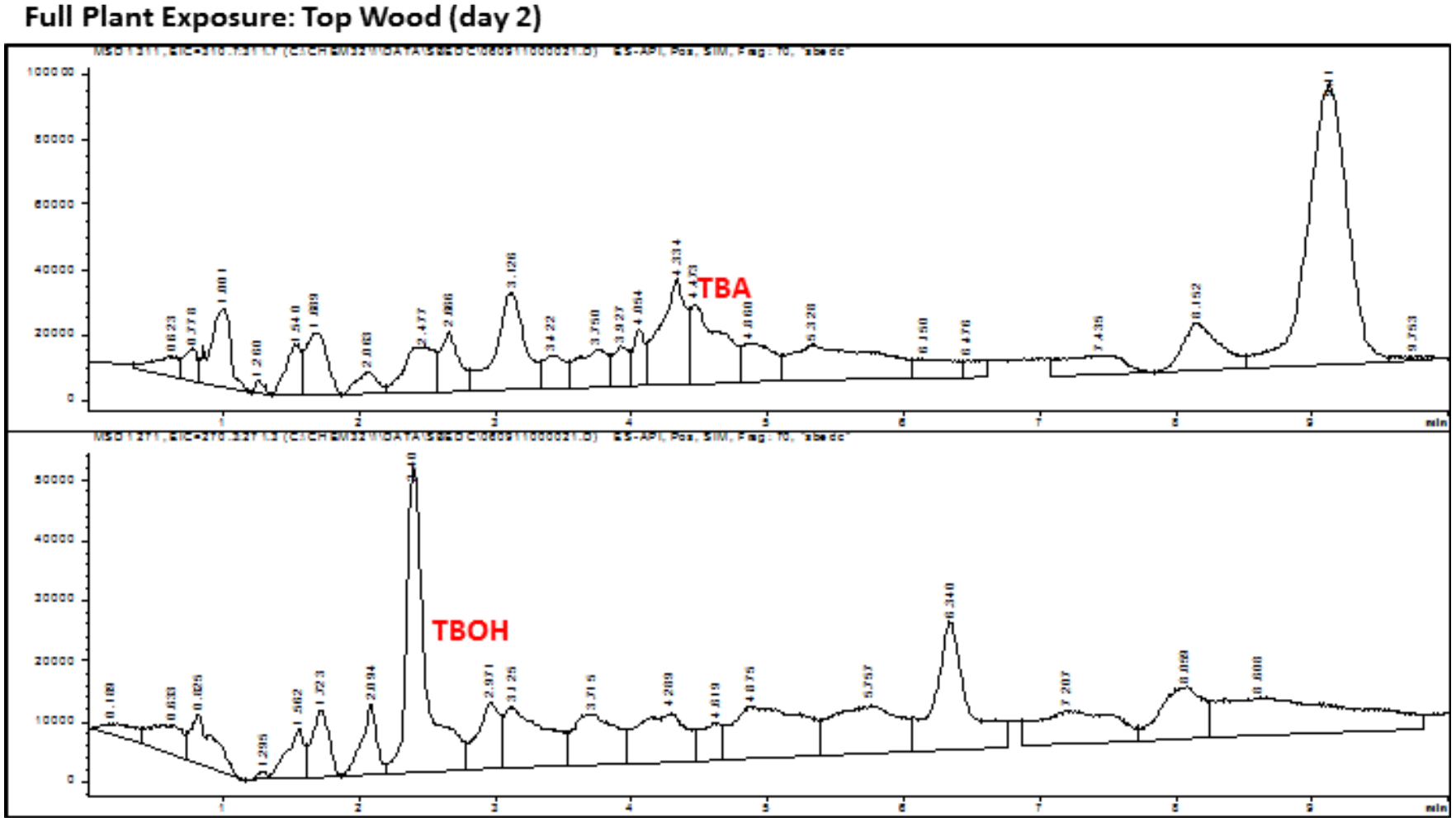

Figure B-28 Example chromatogram in positive ion mode from the top wood of the full plant exposure on day 2 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

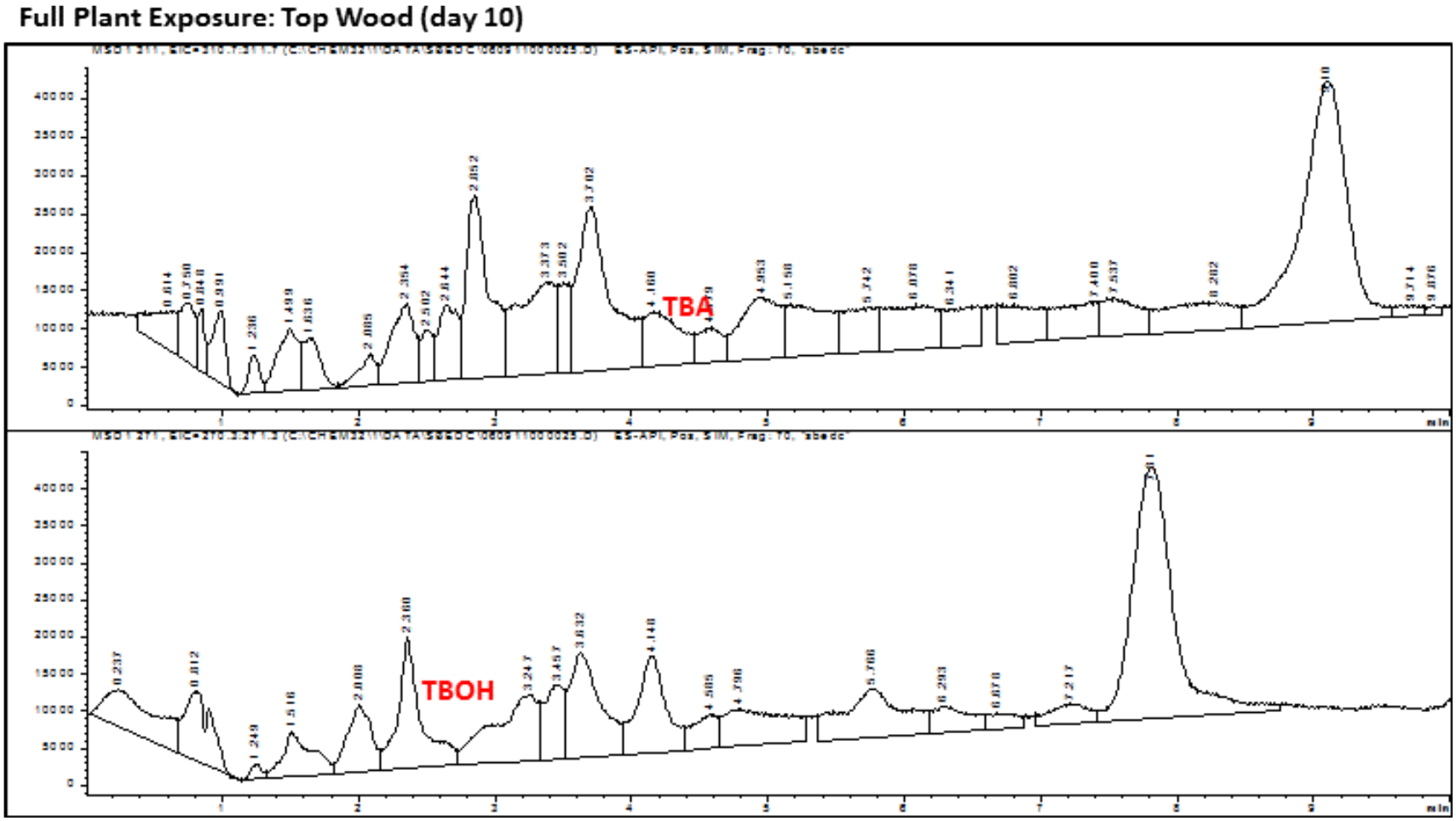

Figure B-29 Example chromatogram in positive ion mode from the top wood of the full plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

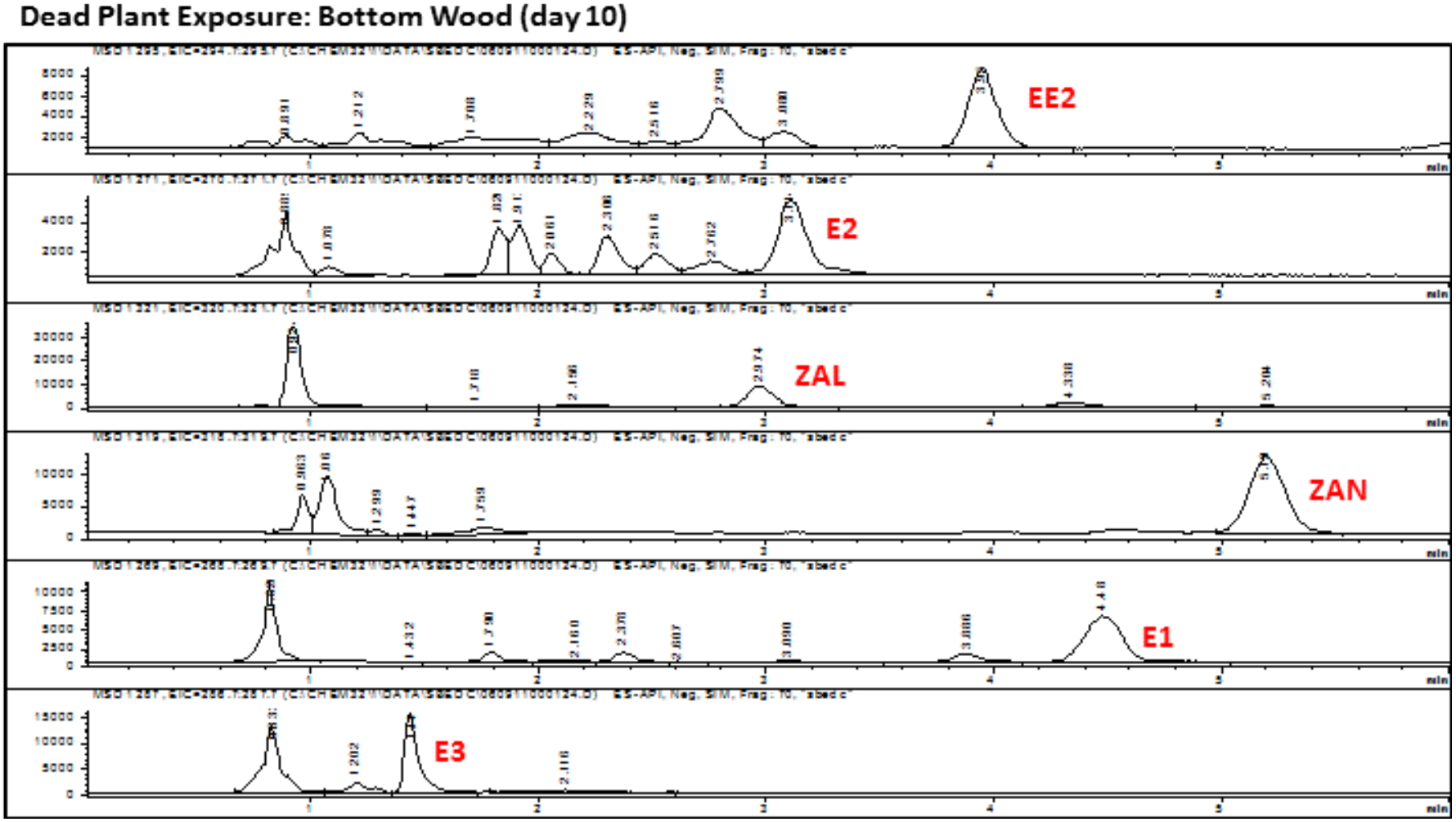

Figure B-30 Example chromatogram in negative ion mode from the bottom wood of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

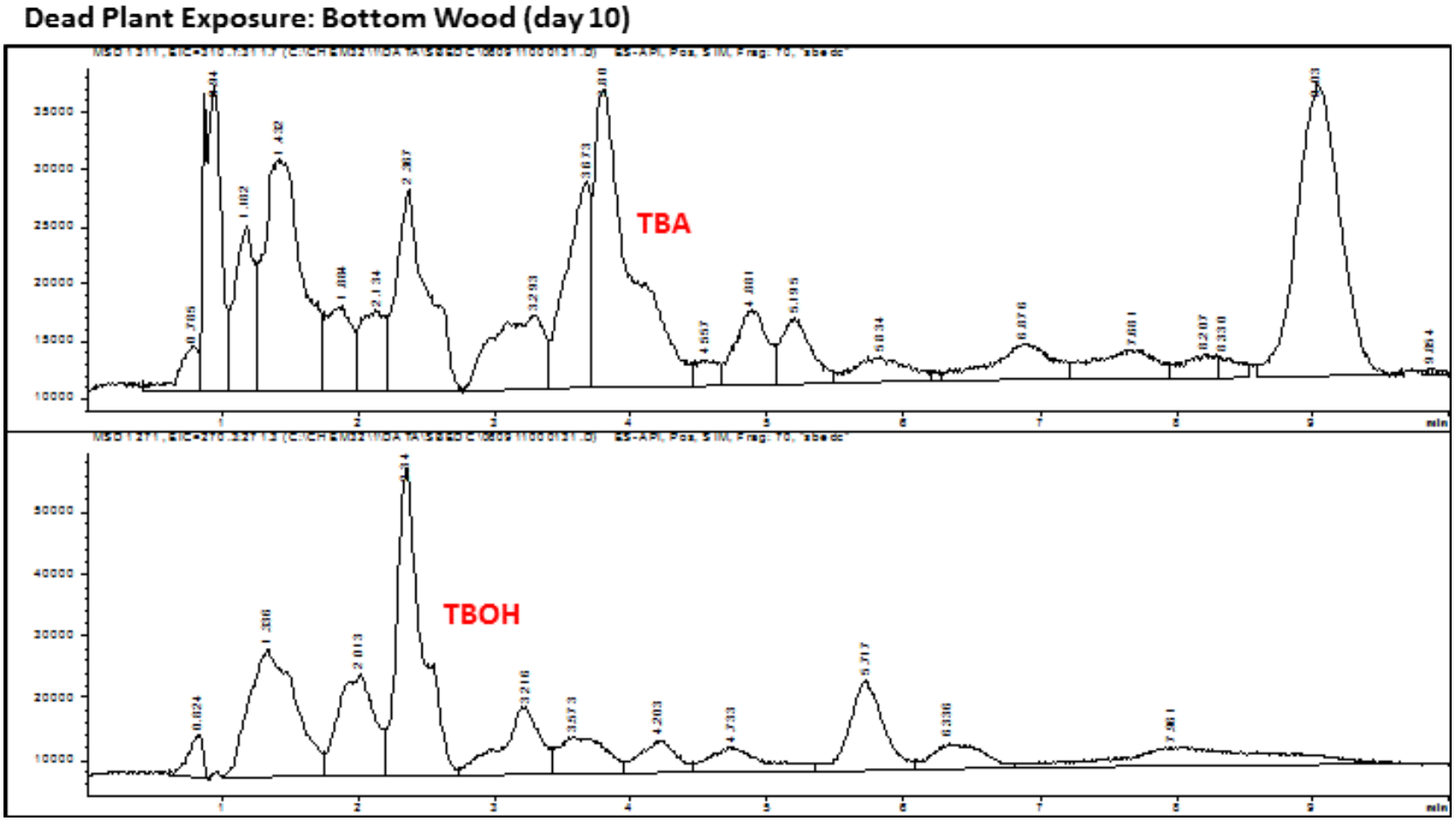

Figure B-31 Example chromatogram in positive ion mode from the bottom wood of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

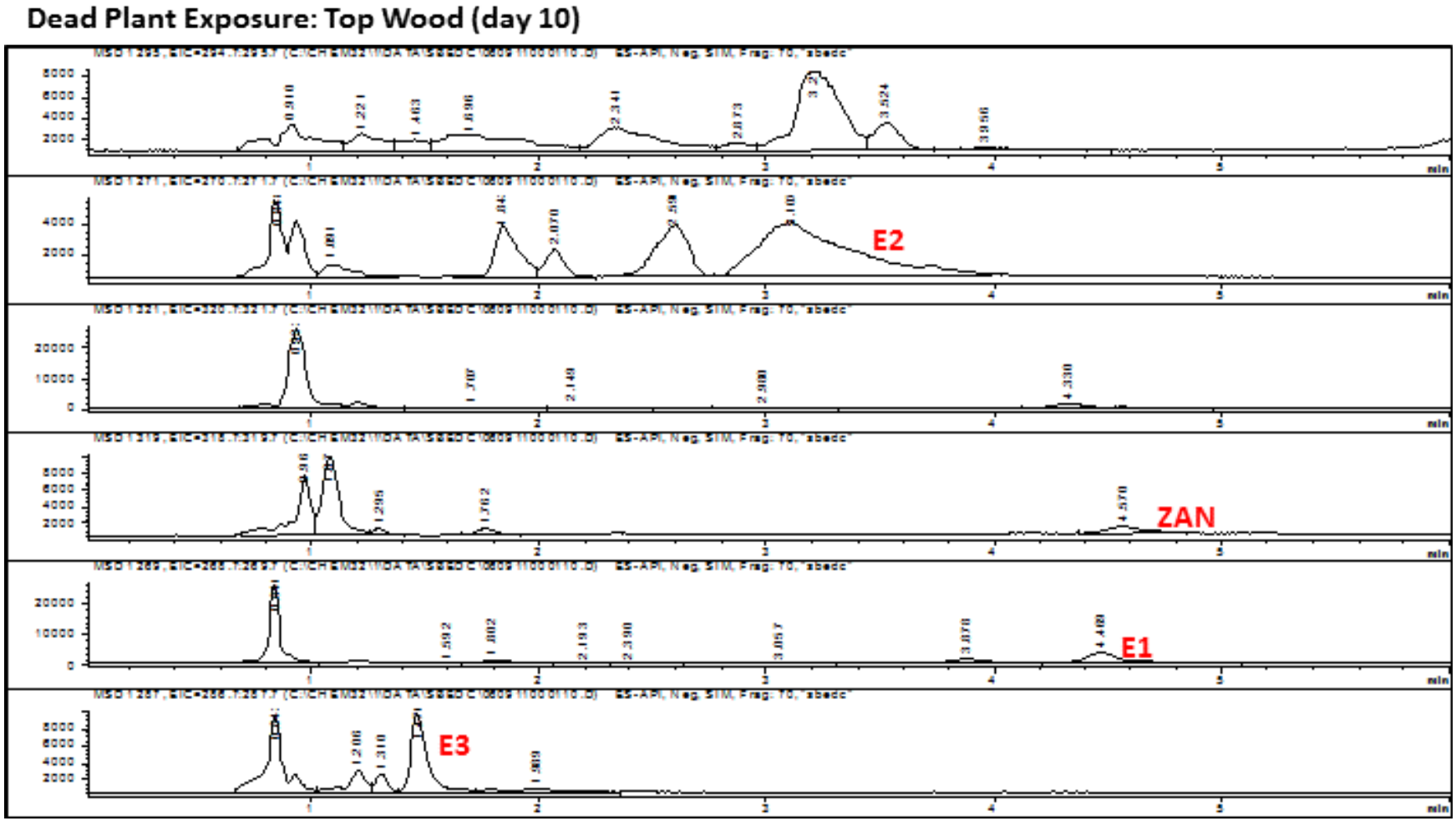

Figure B-32 Example chromatogram in negative ion mode from the top wood of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peaks.

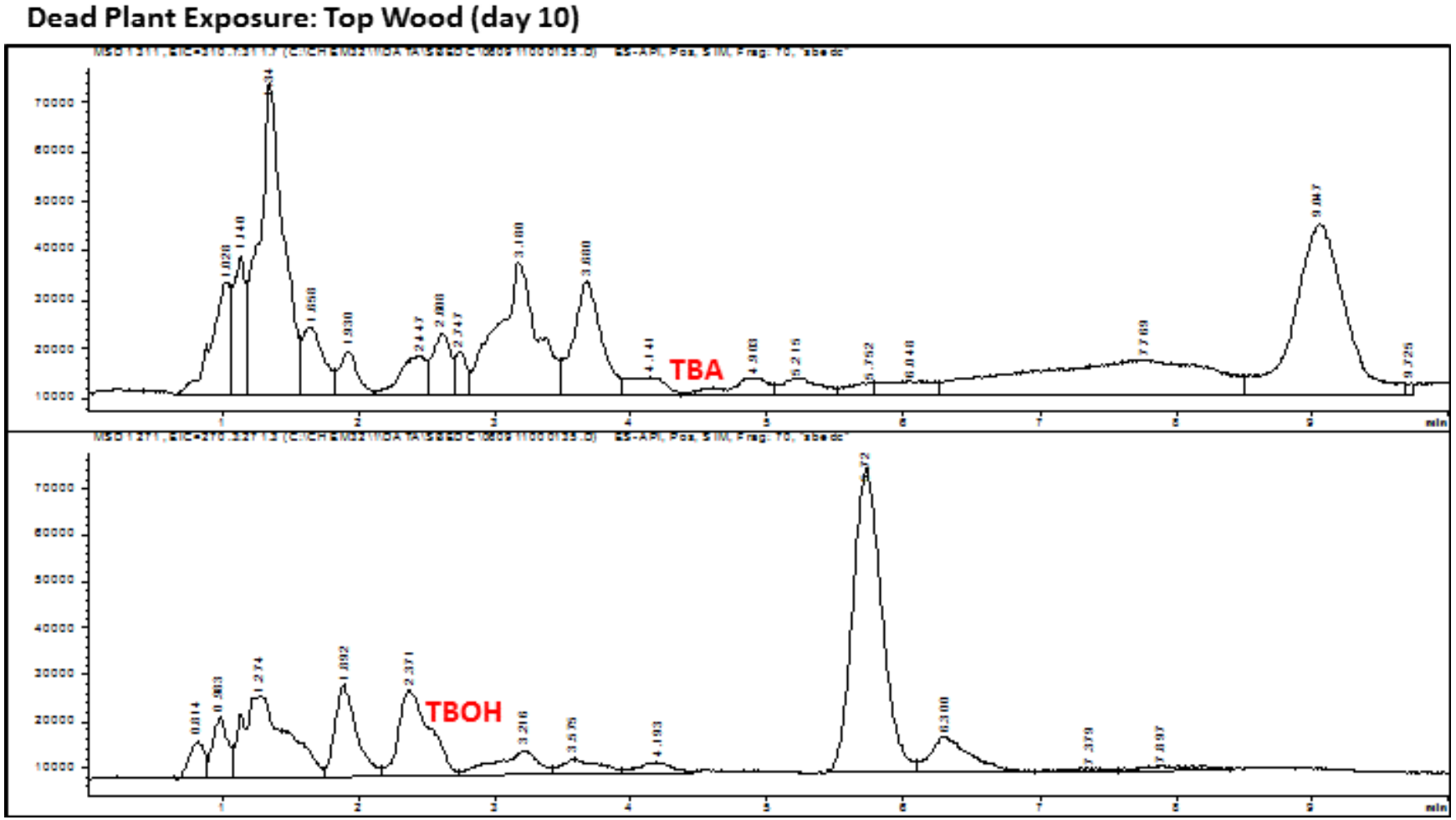

Figure B-33 Example chromatogram in positive ion mode from the top wood of the dead plant exposure on day 10 of the hybrid poplar experiment. The X-axis represents the retention time and the Y-axis represents the peak heights with the target compound names listed in red directly on the right side of the associated peak.