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LOYOLA UNIVERSITY CHICAGO

DEVELOPMENT OF METHODS FOR THE REMOVAL OF SELECTED POLLUTANTS FROM SEVERAL MATRICES AND IDENTIFICATION OF UNKNOWN POLLUTANTS ADSORBED ONTO PLASTICS COLLECTED FROM FRESHWATER

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY
KATHRYN M. RENYER
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Figure 112. Diagram of a triple quadrupole mass spectrometer.\textsuperscript{48} (Reprinted with permission from Skoog, D. A.; et.al. \textit{Principals of Instrumental Analysis}; Thomson Brooks/Cole: Belmont, California, 2007; pg. 525)
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS SAN</td>
<td>Acetonitrile Butadiene Styrene</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>AHC</td>
<td>Aliphatic Hydrocarbon</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol A</td>
</tr>
<tr>
<td>CaSO₄·2H₂O</td>
<td>Calcium Sulfate Dihydrate</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical Ionization</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-Induced Dissociation</td>
</tr>
<tr>
<td>CNL</td>
<td>Constant Neutral Loss</td>
</tr>
<tr>
<td>DC</td>
<td>Direct Current</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved Organic Matter</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron Capture Detector</td>
</tr>
<tr>
<td>EI</td>
<td>Electron Impact</td>
</tr>
<tr>
<td>EIC</td>
<td>Extracted Ion Chromatogram</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
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</table>
ESS    Endosulfan Sulfate
FMG    Fluorescent Green Microspheres
FTICR  Fourier Transform Ion Cyclotron Resonance
IPW    International Pellet Watch
IUPAC  International Union of Pure and Applied Chemistry
GC     Gas Chromatography
GC-ECD Gas chromatography Electron Capture Detection
GC-ECD/FID Gas Chromatography Electron Capture Detection/Flame Ionization Detection
GC/MS  Gas Chromatography Mass Spectrometry
GC/MS/MS Gas Chromatography Tandem Mass Spectrometry
GC-TOF/MS Gas Chromatography Time-of-Flight Mass Spectrometry
HCH    Hexachlorohexane
HCl    Hydrochloric Acid
HDPE   High Density Polyethylene
HOC    Hydrophobic Organic Chemical
KCl    Potassium Chloride
LC_{50} Lethal Concentration to Kill Half a Population
LC     Liquid Chromatography
LC/MS  Liquid Chromatography Mass Spectrometry
LC/MS/MS Liquid Chromatography Tandem Mass Spectrometry
LD_{50} Lethal Dose for Half a Population
LDPE   Low Density Polyethylene
LLE    Liquid-Liquid Extraction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
</tr>
<tr>
<td>LUREC</td>
<td>Loyola University Ecology and Retreat Campus</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium Sulfate</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl Tert-Butyl Ether</td>
</tr>
<tr>
<td>MSW</td>
<td>Municipal Solid Waste</td>
</tr>
<tr>
<td>M/Z</td>
<td>Mass to Charge Ratio</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium Hypochlorite</td>
</tr>
<tr>
<td>NCI</td>
<td>Negative Chemical Ionization</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared Region</td>
</tr>
<tr>
<td>NP</td>
<td>Nonylphenol</td>
</tr>
<tr>
<td>OCP</td>
<td>Organochlorine Pesticide</td>
</tr>
<tr>
<td>OPFR</td>
<td>Organophosphorus Flame Retardant</td>
</tr>
<tr>
<td>PA</td>
<td>Polyamide</td>
</tr>
<tr>
<td>PAH</td>
<td>Polyaromatic Hydrocarbon</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybromodiphenyl Ether</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated Biphenyl</td>
</tr>
<tr>
<td>PCB 126</td>
<td>3,3’,4,4’,5-Pentachlorobiphenyl</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PCB 153</td>
<td>2,2',4,4',5,5'-Hexachlorobiphenyl</td>
</tr>
<tr>
<td>PCB 189</td>
<td>2,3,3',4,4',5,5'-Heptachlorobiphenyl</td>
</tr>
<tr>
<td>PCDD</td>
<td>Polychlorinated Dibenzodioxin</td>
</tr>
<tr>
<td>PCDF</td>
<td>Polychlorinated Dibenzofuran</td>
</tr>
<tr>
<td>PCN</td>
<td>Polychlorinated Naphthalene</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene Terephthalate</td>
</tr>
<tr>
<td>PI</td>
<td>Product Ion</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly (methylmethacrylate)</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>POP</td>
<td>Persistent Organic Pollutant</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>PPB</td>
<td>Parts per Billion</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per Million</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PS-E</td>
<td>Expanded Polystyrene</td>
</tr>
<tr>
<td>PUR</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride</td>
</tr>
<tr>
<td>Pyr-GC/MS</td>
<td>Pyrolysis Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>QqQ</td>
<td>Triple Quadrupole Mass Spectrometer</td>
</tr>
<tr>
<td>qTOF</td>
<td>Quadrupole Time-of-Flight</td>
</tr>
<tr>
<td>ReTOF</td>
<td>Reflectron Time-of-Flight Mass Spectrometer</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per Minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
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<tr>
<td>SRM</td>
<td>Selected Reaction Monitoring Scan</td>
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<tr>
<td>TED-GC/MS</td>
<td>Thermal Desorption Gas Chromatography Mass Spectrometry</td>
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<td>TGA</td>
<td>Thermogravimetric Analysis</td>
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<td>TOF</td>
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<td>TOF-NCI</td>
<td>Time-of-Flight Negative Chemical Ionization</td>
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<td>U.S.</td>
<td>United States</td>
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ABSTRACT

Plastic pollution represents one of the greatest anthropogenic threats to the environment. Five to ten billion tons of plastic are manufactured every year. Currently, Earth’s ecosystem is contaminated with billions of tons of plastic debris, much of which cannot be recycled. Over time, this plastic debris decomposes into small particles. Small plastic particles are known to adsorb toxic compounds in marine environments. My research is concerned with creating novel methods for the detection and quantification of selected persistent organic pollutants from several media. Specifically, I developed methods for the detection and quantification of endosulfan sulfate (ESS) from *Lumbricus terrestris* tissue and excrement as well as soil used in the organism exposure for bioaccumulation studies. I also developed methods for detecting and quantifying 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) from *Daphnia magna* tissue and polyethylene (PE) polymer beads related to bioaccumulation studies. I also defined the limits of quantification for a modified method to quantify PCB 126 in water samples from the same bioaccumulation studies. My research is also concerned with understanding what types of compounds attach to the surfaces of plastic particles in freshwater environments. Plastics collected from the Chicago River were extracted and analyzed for potentially toxic compounds. Triclosan, a commercially used antimicrobial agent, and the bacteria metabolite methyl triclosan were identified on plastic deliberately incubated in the river. This is a novel study of compound adsorption on plastics in a freshwater environment.
CHAPTER ONE

A SUMMARY OF PROBLEMSPOSED BY PLASTIC POLLUTION

What is Plastic and How Do We Use It?

The International Union of Pure and Applied Chemistry (IUAPC) defines polymers as substances composed of molecules with high relative molecular mass whose structures are composed of repeating units derived from molecules of low relative molecular mass. Synthetic polymers are materials with a broad range of physical properties commonly referred to as plastics. They are used in construction of numerous modern-day items for commercial and private use. Plastics have drastically improved the daily life for most of the world’s human population yet there are significant drawbacks to their widespread creation and use.

A Brief History of Plastics Manufacturing and Recycling

The first fully synthetic plastic polymer was developed by Baekeland in the early 1900s. Chain polymerization, a technique used to create modern plastics, was developed during the 1930s-1950s. Synthesis of polyamides (PAs) and polyesters were awarded a Nobel Prize in 1953 and the development of catalysts to aid in the synthesis of polyethylene (PE) and polypropylene (PP) won in 1963. Manufacturing of plastic increased dramatically following the end of World War 2. It has been constantly expanding since then, with the most recent increase going from 299 to 348 million tons from 2013-2017. There are 12 frequently produced plastics that dominate the market. PlasticsEurope has tracked the demand of these for several years, with the most recent data available for 2017 (Table 1).
The increase in manufacturing parallels an increase in environmental plastic debris.\textsuperscript{4-6} Discarded plastic increased approximately fourfold from 1980-2008.\textsuperscript{4} Recycling was first tracked by the U.S. Environmental Protection Agency (EPA) in 1980, when 20 thousand U.S. tons were recycled. The data steadily increased until 2015, where the number dropped slightly, with 8.37\% of US produced plastic recycled in 2017.\textsuperscript{7} Overall recycling percentages in Europe have steadily increased from 1995 to 2017.\textsuperscript{5,8}

Most common plastics are not biodegradable. They are discarded as municipal solid waste (MSW) when not recycled. One report suggests that between 25-50\% of the plastic produced per year is discarded as MSW rather than recycled.\textsuperscript{3} MSW is either incinerated for energy recovery or dumped in a landfill.\textsuperscript{9-11} Plastics designed to biodegrade often do so sluggishly resulting in environmental accumulation.\textsuperscript{9,10} Modern trash-burn facilities have recycling sorting on the front end to maximize recycling efforts, which works for only easily identifiable and easy to grab plastics.\textsuperscript{11} No efficient method exists for large scale identification of plastics of all sizes, thus recycling processes are underutilized.\textsuperscript{10,11} Plastic contaminated with biological materials cannot be recycled.\textsuperscript{9} Biodegradable plastic increases the difficulty of sorting bulk plastics for recycling as it needs to be identified and removed from non-biodegradable plastic.\textsuperscript{9,10} The result is greater up-front costs for recycling centers and incineration facilities. Currently, a significant amount of plastic is mistakenly or carelessly discarded as waste each year. Environmental plastic debris will cause several negative impacts on humans and wildlife from habitat contamination and ingestion, among other issues. This is despite the increased recycling efforts of several countries.
Table 1. Identity of Commonly Produced Plastics. The 12 most produced plastics, their chemical structures, and the approximate amount produced according to PlasticsEurope for 2017.

<table>
<thead>
<tr>
<th>Plastic Type</th>
<th>Chemical Formula</th>
<th>Chemical Structure</th>
<th>Approximate Production (million tons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypropylene (PP)</td>
<td>$(C_3H_6)_n$</td>
<td><img src="image" alt="Polypropylene Chemical Structure" /></td>
<td>9.7</td>
</tr>
<tr>
<td>Low Density Polyethylene (LDPE)</td>
<td>$(C_2H_4)_n$</td>
<td><img src="image" alt="Low Density Polyethylene Chemical Structure" /></td>
<td>8.8</td>
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<tr>
<td>High Density Polyethylene (HDPE)</td>
<td>$(C_2H_4)_n$</td>
<td><img src="image" alt="High Density Polyethylene Chemical Structure" /></td>
<td>6.1</td>
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<tr>
<td>Polyvinyl Chloride (PVC)</td>
<td>$(C_2H_3Cl)_n$</td>
<td><img src="image" alt="Polyvinyl Chloride Chemical Structure" /></td>
<td>5.0</td>
</tr>
<tr>
<td>Polyurethane (PUR)</td>
<td>$(C_{17}H_{16}N_2O_4)_n$</td>
<td><img src="image" alt="Polyurethane Chemical Structure" /></td>
<td>3.7</td>
</tr>
<tr>
<td>Polyethylene Terephthalate (PET)</td>
<td>$(C_{10}H_{8}O_4)_n$</td>
<td><img src="image" alt="Polyethylene Terephthalate Chemical Structure" /></td>
<td>3.7</td>
</tr>
<tr>
<td>Polystyrene (PS)</td>
<td>$(C_8H_8)_n$</td>
<td><img src="image" alt="Polystyrene Chemical Structure" /></td>
<td>1.9</td>
</tr>
<tr>
<td>Expanded Polystyrene (PS-E)</td>
<td>$(C_8H_8)_n$</td>
<td><img src="image" alt="Expanded Polystyrene Chemical Structure" /></td>
<td>1.5</td>
</tr>
<tr>
<td>Acetonitrile Butadiene Styrene (ABS SAN)</td>
<td>$(C_{15}H_{17}N)_n$</td>
<td><img src="image" alt="Acetonitrile Butadiene Styrene Chemical Structure" /></td>
<td>1</td>
</tr>
<tr>
<td>Polyamide (PA)</td>
<td>Must be macromolecules linked by amide bonds</td>
<td><img src="image" alt="Polyamide Chemical Structure" /></td>
<td>0.9</td>
</tr>
<tr>
<td>Polycarbonate (PC)</td>
<td>Must contain carbonate groups</td>
<td><img src="image" alt="Polycarbonate Chemical Structure" /></td>
<td>0.7</td>
</tr>
<tr>
<td>Poly(methyl methacrylate) (PMMA)</td>
<td>$(C_3O_2H_5)_n$</td>
<td><img src="image" alt="Poly(methyl methacrylate) Chemical Structure" /></td>
<td>0.4</td>
</tr>
</tbody>
</table>
Classification of Plastic Debris by Size

Plastic debris was difficult to describe when it was first being collected, counted, and analyzed for classification and identification of adsorbed pollutants. There was no regulatory text, so descriptive terms had to be developed. There are currently several terms used with varying size definitions. Megaplastics are plastics larger than 100 mm or 1000 mm in size which include plastic bags, straws, packaging, and other items crafted for consumer use.\textsuperscript{12-14} Plastic bags have been targeted by numerous countries for bans or taxes for their single use and well documented interference with wildlife. Debris from plastic bags has been found in digestive organs of multiple marine organisms including fish, turtles, seals, and whales.\textsuperscript{15-20} Countries including France and Botswana have banned plastic bags in an attempt to limit further damage to marine life.\textsuperscript{21,22} The United States has not enacted a federal ban. Individual states must decide how they are going to tackle the single-use plastic problem. Two solutions enacted by counties and cities are banning plastic bags (Bethel, Alaska and Chatham, Massachusetts) or taxing their usage in large retail stores (Chicago, Illinois).\textsuperscript{23-25} These are beneficial in aiding the prevention of new megaplastics from entering the environment. However, they do nothing to alter the level of plastic debris already present in the environment.

Macro- and mesoplastics are smaller than megaplastics. Both terms have been used in literature to describe plastics of similar sizes. They have been defined numerous ways. Some researchers do not use the term megaplastics, but rather use macroplastics to describe any plastics larger than 20 mm.\textsuperscript{26} The ranges of 25 cm – 1 m, 1 – 5 mm, and 20 – 100 mm have also been used to classify macroplastics.\textsuperscript{12-14} Mesoplastics have been defined in the ranges of 5-20 mm.\textsuperscript{12,26} Smaller classifications of plastic debris are micro- and nanoplastics. Microplastics are
the most common type of plastic debris studied. They have been defined as plastics with the upper limit 5 mm in size and the lower limit being either 330 µm or 1 µm.26-30 The current smallest plastic debris classification is nanoplastics which are defined as smaller than 1 µm.27

Meso- and microplastic pollution may be formed through two means. The first is the direct deposition of meso- and microplastic particles from consumer products. Primary meso- and microplastic particles are manufactured for numerous consumer purposes including industrial scrubbers, plastic powders, and those designed for use in personal care products (facial scrubs, shower gels, etc.).31,32 Microbeads are a common form of microplastics that are often used as abrasives. They became common in consumer products during the 1990s.33,34 A 2015 Business Insider report listed over 90 commercial products from several brands which contained microbeads, the majority of which are marketed as facial scrubbers or cleansers.35 Microbeads also have industrial applications. They have been used as abrasive media for cleaning ships and garments. Microbeads have also been used in automotive molding and commonly used anti-slip products.36 Countries, including the United States (2015) and Canada (2016), have banned microbeads.37,38 The second method of forming meso- and microplastic pollution is through the breakdown of larger plastics. This can occur through mechanical tearing and ripping of large plastics into continuously smaller fragments. Larger plastics can also be broken down by photo-oxidation weakening the bonds of the molecule, resulting in fragmentation.39 The large-scale use of plastic and it’s inability to degrade naturally classifies plastic debris as a significant environmental concern.
Decomposition of Plastics in the Environment

Traditional plastics are exceptionally stable under environmental conditions and do not readily biodegrade.\textsuperscript{40,41} Their stability at temperatures up to 200\degree C may come from their high molecular weights (4000-28000 Daltons) and possible hydrophobicity, which would impede breakdown through hydrolysis.\textsuperscript{40,42} Additional chemicals such as antioxidants and stabilizers are often added to plastics to improve various qualities throughout the manufacturing process. These additives increase plastic resistance to environmental degradation through the limitation of biodegradation pathways.\textsuperscript{40} Common forms of degradation of plastics are photo- and thermo-oxidation.\textsuperscript{41-43} Both cause the plastics to lose tensile strength, making them brittle through the formation carbonyl functional groups. Brittle plastics crumble into progressively smaller fragments.\textsuperscript{40,44}

Two main classes of plastics are thermoplastics and thermoset plastics. Thermoplastics are long, sole carbon chain polymers produced by breaking a double bond of an olefin or via condensation between a carboxylic acid and an alcohol or amine.\textsuperscript{40} Thermoplastics may be reheated and cooled several cycles without breaking the carbon backbone. The backbone is what renders them difficult to degrade. Thermoplastics include PE, polyvinyl chloride (PVC), PP, polystyrene (PS), acetonitrile butadiene styrene (ABS SAN), PA, and poly (methylmethacrylate) (PMMA).\textsuperscript{40,45} Thermoset plastics have cross-linked backbones creating a polymer network during the curing process.\textsuperscript{46} These are formed by curing a liquid prepolymer with heat, radiation, pressure, or use of a catalyst. Molds are used to shape products created from thermoset plastics as they cannot be reformed once cured. Thermoset plastics include polyethylene terephthalate
(PET), polyurethane (PUR), unsaturated polyester and vinyl esters.\textsuperscript{40,45,46} Polycarbonate (PC) can be classified as either thermoplastic or thermoset dependent upon how is produced.\textsuperscript{47}

The smaller the plastic fragments, the easier it becomes for microbes to biodegrade either class of plastic. Studies have observed various microbes and their degradation of plastics.\textsuperscript{43,44} They are quick to point out that photo- or thermo-oxidation must often occur before microbes can begin biodegradation of the plastics. Biofilms form on the surface area of the fragments. These biofilm surfaces are often hydrophobic, facilitating potential adsorption of hydrophobic pollutants.\textsuperscript{44} This breakdown of plastics is measurable by monitoring loss of mass and measuring tensile strength alongside comparison of spectroscopic data.\textsuperscript{43}

**Determination of Polymer Identification Constituting Plastic Debris**

Plastic debris needs to be identified by polymer type once collected. There are numerous different techniques that may be utilized. Plastic debris needs to be isolated from the environmental matrix before it can be identified. This can be accomplished using filtration, density separation, or sieving. Any of these methods are followed by visual separation using a confocal microscope before polymer identification analysis.

Two vibrational spectroscopic techniques used for polymer identification are Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) and Raman spectroscopy. Both are frequently used to identify the polymer compositions because they are nondestructive methods. ATR-FTIR, as the name implies, generates spectra using infrared radiation. It is absorbed by molecules during a net change in a dipole moment when vibrating.\textsuperscript{48,49} Net changes in dipoles are most often between polar bonds in molecules. The radiation frequency must match a natural vibrational frequency of the molecule for absorption to
occur. This changes the amplitude of the vibration, generating a peak in the spectrum. Raman spectroscopy uses visible and near-infra red light to measure the scattered radiation at a 90° angle to the sample. The sample is polarized by the incoming radiation. Polarization takes place in molecules that have bonds undergoing symmetric stretching, such as some non-polar bonds.48,49

The two techniques are complementary of each other. Additionally, some molecules are both FTIR and Raman active. Both techniques have been used in studies of collected plastic debris.50-53 They are more effective at identification of plastic than by visual inspection alone.49,52 Reviews have studied the use of both Raman and ATR-FTIR on polymer identification in plastic debris. Debris may be composed of multiple polymers, so it is best to utilize both techniques in order to garner a complete identification of the polymer(s) that compose it.49,54

A microscopy technique has recently been pioneered to analyze the chemical composition of plastic debris. Scanning electron microscopy (SEM) analyzed for inorganic material that composed plastic debris. A focused beam of electrons is swept across the surface. This generates several types of signals, including X-ray fluorescence photons.48 Characteristic line spectra and an X-ray continuum are generated. The characteristic lines are used to identify metals that are often used for pigmentation or used in conjunction with organic materials for plasticizers.55

Two destructive gas chromatography techniques have been developed for polymer identification. Pyrolysis gas chromatography mass spectrometry (Pyr-GC/MS) analyzes a small mass (0.1-0.5 mg) of plastic debris. A platinum coil is heated upwards of 750°C. The intense heat breaks the polymer down into smaller molecules that are transferred to the GC/MS for analysis and identification.46,55-58 The second technique combines thermogravimetric analysis (TGA) on solid-phase absorbers with analysis via thermal desorption gas chromatography mass
spectrometry (TED-GC/MS). TGA is performed by heating a sample under a nitrogen atmosphere to around 600°C. The analytes are collected on a piece of polydimethylsiloxane for GC/MS analysis.\textsuperscript{59}

Spectral differentiation needs to be achievable between low-density polyethylene (LDPE) and high-density polyethylene (HDPE) for separation and recycling purposes. It is currently difficult to use TED-GC/MS to differentiate between the spectra. There is only one peak that differentiates the two.\textsuperscript{59} This peak could be amplified in an environmental sample by an additive or other adsorbed pollutant. The two PE types can been seen in the near infrared region (NIR) using ATR-FTIR but resolution between the two is difficult due to their nearly overlapping wavenumbers.\textsuperscript{60} The most accurate method for currently differentiating between LDPE and HDPE is Raman spectroscopy. Raman spectroscopy can differentiate between the two due a difference in peak ratios at 1060 and 1130 cm\textsuperscript{-1}, which are unique to either HDPE or LDPE.\textsuperscript{61}

All the above techniques may also facilitate additive identification in various plastics. Additive identification is useful for identifying the source(s) of the plastic debris. Raman spectroscopy may be used to identify metal additives that are used in plastic manufacturing which are not observed ATR-FTIR.\textsuperscript{49,54} ATR-FTIR can be used to identify organic additives on plastics.\textsuperscript{62} Spectrum subtraction may be necessary to resolve the potential overlap of the plastic and additive spectra. Pyr-GC/MS is frequently used for additive identification because it can be performed concurrently with polymer identification using a small amount of sample.\textsuperscript{46,55-58,63,64} It does not require extraction of the additives from the surface of plastics. All three of these techniques allow for the concurrent identification of polymers and additives.
Additives may be extracted from the surface of the plastics for identification. The extraction process may or may not be destructive, depending on the solvents used and the polymer. It is in best practice to use a non-destructive technique to identify the polymer before extracting additives to avoid loss of sample. Extracts can be analyzed using a variety of mass spectrometry techniques dependent upon which additives are targeted for analysis. Liquid chromatography mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS) are two techniques that can be used to search for molecules across a wide range of molecular weights as well as those with acid-base functionality.65-67

A second mass spectrometry method for additive analysis is matrix assisted laser desorption/ionization (MALDI) mass spectrometry. MALDI is used to obtain high resolution masses of biomolecules and large organic molecules including polymers up to thousands of Daltons in weight.68 The matrix that contains the analyte must absorb strongly at the wavelength of the laser.48 The matrix may be the polymer itself, depending on the laser source.68 A third mass spectrometry technique used for additive identification is Pyr-GC/MS. This technique has been used for polymer identification of the plastic sample.55,57,58,69 Analysis of the plastic sample without purification facilitates the identification of additives concurrent with bulk polymer identification. Pyr-GC/MS has been used for the identification of several additive types including flame retardants and lubricants.63,64

**Environmental Microplastic Debris**

Microplastic pollution has been documented in aqueous environments in the world’s oceans, seas, lakes, rivers as well as on their respective shorelines.31,70-72 Microplastic pollution is a complex mixture of different fibers and particles in both size and chemical structure.
Microplastics are ingested by organisms across trophic levels. Filter feeders and herbivores mistake them for food, and they may accumulate in the organisms until death. Microplastics then re-enter the environment or are ingested by a predator. Ingestion is not the only threat these plastics present. There is concern about toxic pollutants adsorbed on the surfaces of the debris. Pollutants may desorb in the organism after plastic ingestion occurs, then accumulate in tissues.\textsuperscript{73,74} The concentration of toxic pollutants over the lifetime of an organism may lead to long-term negative health effects for the organism or its predators.\textsuperscript{74-76}

The compounds adsorbed onto plastic debris can be classified in three categories: persistent organic pollutants (POPs), additives, and residual monomers.\textsuperscript{9,31,75,77} Adsorbed compounds on debris are partially determined by the degree of debris hydrophobicity and what chemicals the plastics are exposed to in the manufacturing process. POPs are chemicals that have been found to be resistant to biodegradation, facilitating their environmental accumulation.\textsuperscript{78} They have been used for several applications historically including pesticides, solvents, and flame retardants among others.\textsuperscript{79} Chemical additives are used in the making of plastics for various reasons including enhancing the functionality and improving flame resistance. Additives are commonly found on secondary microplastics.\textsuperscript{31,32,80,81} The last class of compounds are residual monomers. Polymers can break down and leave monomers and oligomers adsorbed to the debris.\textsuperscript{77} All three classes are hydrophobic. Their hydrophobicity propels them to find other hydrophobic molecules on surfaces to create micelles.\textsuperscript{82} The surfaces of most plastic debris (regardless of size) are hydrophobic. These surfaces create an opportune environment for hydrophobic molecules to adsorb. POPs and small molecule plastic additives are major toxicity concerns for organisms who ingest plastic debris.
Marine Microplastic Pollution

Commonly identified marine plastic pollutants include PE, PP, PS, PA, polyesters, and polyoxymethylene. The most prevalent environmental samples are from aqueous marine environments and the surrounding beaches. Marine waters have an average density of 1.027 g/mL and the common marine plastic pollutants have relatively low densities, ranging from 0.917-1.61 g/mL. Their similarity is why most plastic debris floats on or near water surfaces.

The conventional sampling method for aqueous microplastic identification is to tow neuston nets, a mesh net that tapers to a point designed to sample the top 10 cm of a body of water, with a small pore size behind a boat across a section of a body of water. Researchers may also stand in shallow waters and submerge much of the net below water level. A majority of heavier density polymer microplastics and small plastic debris that has settled out into the sedimentation at the bottom of the waterbeds are missed. This skews the available data towards those polymers of lighter densities. Particles collected from beaches are often visually sorted from the sand and separated using tweezers.

A conservative estimate put the number of small plastic particles floating in the ocean at 5.25 trillion, weighing approximately 268,000 tons. This estimate does not consider particles with larger densities that accumulate below the commonly sampled water depth or deposited into the sedimentation. A 2013 study explored the presence of microplastic pollution in deep sea sediment. The sampling range spanned across the Atlantic Ocean and Mediterranean Sea with a maximum sampling depth of approximately 5000 meters. It reported microplastic pollution in 75% of the sampled locations. The measurements reflected the lower limit of total plastic pollution in the sediment due to the limited depth of core samples. Further studies of numerous
core depths are necessary to obtain a complete scope of the microplastic pollution within deep sea sediment. Studies are also being performed on sediment samples collected from remote Arctic ice for microplastic pollution characterization. One recent study performed in 2017 by Bergmann and collaborators found a range of 42-6595 microplastic particles/kg of sediment with an average of 4,356 microplastic particles/kg of Arctic sediment. All current research supports the claim that plastic pollution is ubiquitous in any environment.

The majority of the adsorbed plastic pollutant identification studies have been conducted on those collected from marine environments. Hydrophobic organic chemicals (HOCs) are a class of POPs known to bind to particulate, sediment, and plastics that are often observed as adsorbed pollutants to marine plastic debris. The plastic, predominantly microplastics, may have a greater surface area than that of the surrounding sediment. Mato et.al. hypothesized that microplastics in marine environments can adsorb pollutants at concentrations up to 100 times greater than the surrounding sediment. Targeted analysis studies were conducted to identified adsorbed pollutants. They involved searching for specific compounds, usually several within a compound class, in each extracted sample. Compounds may also be quantified and reported as the overall average concentration of the compound class. Studies have found numerous adsorbed compounds, suggested by their chemical structures to have adverse health effects, adsorbed to the plastic debris (Table 2). Antibiotics have not been observed to have been adsorbed to collected marine plastics in detectable quantities.
Table 2. An overview of marine plastic debris studies for identified adsorbed compounds.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sampling Location</th>
<th>Identified POPs</th>
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</thead>
<tbody>
<tr>
<td>28</td>
<td>Water</td>
<td>BPA NPs</td>
</tr>
<tr>
<td>72</td>
<td>Water</td>
<td>AHCs OCPs PAHs</td>
</tr>
<tr>
<td>75</td>
<td>Beach</td>
<td>DDTs NPs</td>
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<tr>
<td>90</td>
<td>Beach, Water</td>
<td>DDTs PAHs PBDEs</td>
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<td>Beach</td>
<td>DDTs HCHs</td>
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<tr>
<td>92</td>
<td>Beach</td>
<td>DDTs OCPs OPFRs PAHs PBDEs PCBs</td>
</tr>
<tr>
<td>93</td>
<td>Beach</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>Beach, Water</td>
<td>AHCs DDTs PAHs PCBs</td>
</tr>
<tr>
<td>97</td>
<td>Beach</td>
<td>DDTs HCHs</td>
</tr>
<tr>
<td>98</td>
<td>Beach</td>
<td>DDTs HCHs</td>
</tr>
<tr>
<td>99</td>
<td>Beach</td>
<td>DDTs HCHs OCPs PAHs PCBs</td>
</tr>
<tr>
<td>101</td>
<td>Beach, Water</td>
<td>DDTs HCHs</td>
</tr>
<tr>
<td>102</td>
<td>Beach</td>
<td>DDTs PAHs</td>
</tr>
<tr>
<td>103</td>
<td>Beach</td>
<td>OCPs PAHs</td>
</tr>
</tbody>
</table>

* Aliphatic Hydrocarbons (AHCs), Bisphenol A (BPA), dichlorodiphenyltrichloroethane and metabolites (DDTs), hexachlorohexanes (HCHs), nonylphenols (NP), organochlorine pesticides (OCPs), organophosphorus flame retardants (OPFRs) polyaromatic hydrocarbons (PAHs), polybrominated diphenylethers (PBDEs), and polychlorinated biphenyls (PCBs)
Freshwater Microplastic Pollution

The pollution of freshwaters with plastic debris is understudied. Bletter et.al in 2018 found that only 13% of plastic pollution studies examined freshwaters rather than marine waters. Large plastic debris was not documented as a freshwater pollutant until 1996 with microplastics not being reported before 2011. This is despite the recent estimate that between 1.15 – 2.41 million tonnes of plastic enters the oceans from freshwater sources each year. A significant part of current research is concerned with identifying which freshwaters are contaminated with plastic debris. Some studies may go a step further in identifying which types of plastic have contaminated freshwaters. Strungaru et.al. reviewed the literature in 2019 and found that the top 12 frequently produced plastics were found as freshwater plastic debris.

Two studies have examined the adsorbed pollutants from plastics collected in freshwaters. Faure et.al. performed a targeted analysis study in 2015 on plastic debris collected from Swiss waters. The search looked for numerous known POPs, including polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), and nonylphenols (NPs). All 50+ compounds were detected above the established limits of detection (LODs) and limits of quantification (LOQs). Ravit et.al. performed a non-targeted analysis study in 2017 on plastic debris from New Jersey freshwaters. A non-targeted analysis study differs from a targeted study by not having a set list of compounds to search for within the sample. Unknown peaks are matched to spectral libraries for preliminary identification. Analysis of a standard may be carried out under the same chromatographic conditions for further proof of identification. They identified 2-butanone, 4-(2,6-trimethyl-1-cyclohexen-1-yl) among other
compounds as adsorbed pollutants through matching the unknown mass spectra to spectral libraries.\textsuperscript{110}

Freshwater studies are heavily concerned with the threat that POPs pose once the plastic debris has been ingested by the organism. Pollutants may desorb from the plastic surface and interact with the organism’s tissues. Current research seeks to understand what characteristics determine if a pollutant desorbs into the gut and tissues of an organism or stays adsorbed to the plastic surface. Coffin et.al. found that endocrine-disrupting compounds significantly desorbed from commercial plastic items under mimics of seabird and fish gut conditions.\textsuperscript{111} Studies like those conducted by Bakir et.al. suggest that plastic debris does not quantitatively increase the uptake of adsorbed pollutant by aquatic organisms.\textsuperscript{112} It has been suggested that plastic additives pose a greater threat to desorb and accumulate in organisms than adsorbed pollutants.\textsuperscript{111,112}

A literature search revealed two current approaches to determine the impact of specific pollutants. The first is to elect a plastic, preferably one commonly identified as plastic debris, a pollutant, and an organism. Model organisms are often filter feeders or others in low trophic levels that are most likely to ingest a significant amount of small plastic debris in their natural environments. The plastic and pollutant are mixed and allowed to equilibrate in the constructed matrix before organism exposure. Observations are made during the exposure period regarding organism behavior. They could exhibit symptoms of toxicity including a decreased body size, activity, mobility, as well as a higher mortality rate than the control. Organisms are exposed for a specific time duration. Results are compared to control organisms exposed to the same concentration of pollutant without the plastic present.\textsuperscript{113-115} These observations may be done over a range of concentrations of the chosen pollutant.
The second approach centers around collecting organisms from their natural habitats. Blood and tissue samples are collected from the organisms to be tested for known pollutants. Stomach contents are often examined closely to quantify ingested plastic debris at time of death. The amount of ingested plastics is compared to the sum of the concentrations of known pollutants to identify any correlation. Current research uses this method to focus on a commonly identified adsorbed pollutants—pollutants. The results of this approach influences chosen pollutants used in the first targeted pollutant approach described previously with the most abundant pollutants targeted.

**Proposed Research**

The research proposed herein has two main components. The first is to develop and refine novel extraction techniques for hydrophobic pollutants from organism tissues, the matrices that they live in, and small polymer beads they are exposed to. We examined these different media specifically for three chlorinated pollutants: 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) and 2,3,3’,4,4’,5,5’-heptachlorobiphenyl (PCB 189) with respect to PE beads and the freshwater organism daphnia, and endosulfan sulfate (ESS) with respect to PMMA beads and the terrestrial organism earthworms (Figure 1). The second component is to perform a non-targeted analysis of plastic extracts collected from Salt Creek, a tributary of the Chicago River, to identify novel, adsorbed pollutants from plastics exposed to freshwater.

![Chemical structures of the poly-chlorinated pollutants](image)

**Figure 1.** Chemical structures of the poly-chlorinated pollutants that are being studied in the proposed research: A) PCB 189, B) PCB 126 and C) ESS.
CHAPTER TWO

EXPERIMENTAL DESIGNS, METHODOLOGIES, AND INSTRUMENTATION

Introduction

This chapter will discuss information key to all three projects (extraction of polychlorinated pollutants from daphnia, earthworms, and the deliberate exposure of polyethylene, PE, in freshwater). First, I will discuss in detail the materials needed and methods used to extract PCBs 126 (3,3’,4,4’,5-pentachlorobiphenyl) and 189 (2,3,3’,4,4’,5,5’-heptachlorobiphenyl) from the collected daphnia, beads, and water samples. Next, I will discuss the materials needed and the development of extraction methods for the removal of endosulfan sulfate (6,9-Methano-2,4,3-benzodioxathiepin, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-, 3,3-dioxide, ESS) from worm tissue, worm excrement, beads, and soil. Part three of this chapter will discuss what materials were used to deliberately expose PE in a local Chicago creek and the methods used to analyze the collected plastic for numerous potential pollutants. The last part of this chapter will discuss the various instrumentation used to analyze samples from all three projects.

Extraction of PCBs 126 and 189 from D. magna Tissue, Water, and PE Beads

Materials

Both PCB 126 and PCB 189 were acquired from AccuStandard (New Haven, CT, USA) and dissolved in octane (Reagent grade, Thermo Fischer Scientific, Waltham, MA, USA). Calibration solutions were prepared in hexane (GC-ECD/FID grade, SupraSolv, Darmstadt, Germany). Daphnia magna were obtained from the Hoang group (Institute of Environmental
Studies, Loyola University Chicago, IL, USA) exposure studies and culture. Fluorescent green PE polymer beads size 63-75 µm in diameter and density 1.005 g/cc were acquired from Cospheric LLC (Santa Barbara, CA, USA).

PCBs 126 and 189 Extraction Procedures

**D. magna Tissue Extraction Method One.** Samples provided from the Hoang group exposure studies were brought to room temperature for extraction and analysis. Organisms were transferred to test tubes from storage vials. Vials were rinsed with 1 mL aliquots of dichloromethane (DCM, CH₂Cl₂, ACS grade, Merck) twice to ensure full transfer of PCBs. Samples were concentrated using a Savant Speed Vac Sc110 (Thermo Fisher Scientific) set on low heat (25°C) for 10 minutes. Vials were rinsed twice more with 1 mL aliquots of DCM and concentrated. Samples were stored at 4°C and brought to constant weight. They were then reconstituted in a 1 mL aliquot of DCM (GC-ECD/FID grade, SupraSolv) and vortexed. Extraction was performed by sonication in a benchtop sonicator for 25 minutes. Samples were then concentrated to dryness using the Savant Speed Vac set on low heat. Samples were reconstituted for analysis in 500 µL hexane (GC-ECD/FID Grade, Suprasolv). Adult daphnia and neonates were extracted with this method.

**D. magna Tissue Extraction Method Two.** Modifications were made for daphnia tissue. Organisms were dried over desiccant then brought to constant weight. Screw-top glass culture tubes were used in place of small test tubes for solvent evaporation prevention during the sonication process. Samples were sonicated using a benchtop sonicator for 25 minutes and were left uncovered overnight for the solvent evaporation. They were capped and stored pending
analysis once dry. Neonates were unable to be transferred to culture tubes used to extract the adults due to their extreme light weight after drying under the desiccant. They were extracted in the storage containers via sonication for 10 minutes. The solvent was transferred to a glass screw-top culture tube and evaporated to dryness under nitrogen. Samples were reconstituted in 500 µL hexane (GC-ECD/FID Grade, Suprasolv) for analysis.

**PE Isolation from Water.** All water samples collected after daphnia removal from the Hoang group exposure studies were filtered through P2 fine porosity filter paper (Thermo Fischer Scientific) using a water-aspirator. Care was taken with all samples containing PE to collect all PE that gathered on the bottom of the glass apparatus with the filter paper for quantitative transfer. Samples from the first exposure were covered until analysis. Second and third exposure samples were dried in an oven set at 60°C and brought to constant weight. The filter paper used to collect the sample had been pre-rinsed with the same type of moderately hard water used in the daphnia exposures and dried in the oven before use for the third exposure.

**Water Extraction Method One.** A liquid-liquid extraction (LLE) method was used for PCB extraction. A volume of water (50 mL) was added to a separatory funnel followed by a 15 mL aliquot of DCM (GC-ECD grade, SupraSolv). Funnels were shaken vigorously, vented, and then let sit for layer separation. The bottom DCM layer was transferred to a glass screw-cap culture tube and evaporated to dryness under nitrogen. Water samples were extracted three times into the same culture tube for PCB concentration maximization. Samples were reconstituted in 400 µL of hexane (GC-ECD/FID grade, SuprasSolv) for analysis.
**Water Extraction Method Two.** The LLE method and a solid phase extraction method (SPE) were tested for maximum recovery at low concentrations of PCB 126 prior to extraction of the samples from the second organism exposure. Results determined that the SPE method should be used for sample workup. Samples were acidified to contain 0.25% formic acid. A volume of 200 mL was passed through an Oasis HLP SPE cartridge (Waters Corp, Milford, MA, USA) which has been previously conditioned with DCM (ACS grade, Merck) followed by distilled water containing 0.25% formic acid. The cartridges were dried for 2 minutes via water aspiration. Cartridges were eluted using three aliquots of DCM (3 mL). The eluate was evaporated to dryness under nitrogen and reconstituted in 400 µL of hexane (GC-ECD/FID grade, SupraSolv) for analysis.\textsuperscript{119}

**Polyethylene Extraction Method One.** Filter paper bearing the PE beads collected from the Hoang group exposure studies was placed in a glass jar with 10 mL of methanol (ACS grade, Merck). Samples were sonicated for 20 minutes in a bench top sonicator. The methanol was transferred to a glass screw-top culture tube and centrifuged to pellet out any transferred PE. The supernatant was transferred to a new culture tube and evaporated to dryness under nitrogen. The beads in the jar and first culture tube were sonicated in methanol (15 mL) for 20 minutes a total of five times. The supernatant was transferred to the same secondary culture tube for evaporation under nitrogen. Samples were reconstituted in 400 µL of hexane (GC-ECD/FID grade, SupraSolv) for analysis.

**Polyethylene Extraction Method Two.** Samples were scraped off filter paper into a pre-weighed glass 4-dram vial. The four replicates for each day were combined to maximize signal.
The extraction solvent was switched to iso-octane for increased PCB 126 solubility. PE beads were sonicated for 10 minutes in a benchtop sonicator in iso-octane (2 mL) (ACS grade, Thermo Fischer Scientific). The solvent was transferred to a glass autosampler vial and centrifuged. The supernatant was drawn off into a new glass autosampler vial for storage pending analysis. A sample known to contain PCB 126 was extracted a total of 5 times and each aliquot was analyzed separately to determine the amount of PCB 126 removed with each extraction.

**Polyethylene Bead Loading and Extraction Method Three.** Green PE beads (1 mg) were transferred to a small glass vial. PCB 126 (0.500 mg) dissolved in octane was added to each replicate. Additional octane (Reagent grade, Thermo Fischer Scientific) was added to each vial to reach a final volume of 1 mL. Samples were vortexed then concentrated for 20 minutes using the Savant Speed Vac at low temperature. Further 1 mL aliquots of octane were added and removed a total of three times for maximum concentration of PCB 126 on the PE surface. Nanopure water (1 mL) was added to each replicate, vortexed, and concentrated off at low temperature. A small amount (10 mg) was removed from each replicate three times and placed into glass autosampler vials for extraction. Samples were sonicated for 20 minutes in hexane (GC-FID/ECD) SupraSolv, 1mL) using a benchtop sonicator. The hexane was drawn off and stored for analysis.

**Extraction of ESS from *L. terrestris* Tissue, Excrement, Soil, and PMMA Beads**

**Materials**

Endosulfan sulfate (6,9-Methano-2,4,3-benzodioxathiepin, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-, 3,3-dioxide) was acquired from Sigma Aldrich and used without
further purification. External calibration curve solutions were prepared in hexane (GC-ECD/FID grade, SupraSolv), and methanol (ACS grade, Merck, Boston, USA). White poly (methyl methacrylate) (PMMA) microplastic beads of diameter 30 µm were acquired from Polysciences, Inc. (Warrington, PA, USA).

**ESS Extraction Procedures**

*L. terrestris* Tissue Extraction Method One

Worms collected from the Hoang group exposure studies were thawed and separated before being weighed and cut into fine pieces with dissection scissors. They were transferred to glass beakers containing 15 mL of DCM (ACS grade, Merck) per gram of worm tissue. Samples were micro-sonicated for cell lysing using a micro-tip sonicator (Model Q125, QSonica LLC, Newtown, CT, USA). It was set at 60% amplitude, pulse 10 seconds on followed by 5 seconds off, for a run time of 6 minutes per gram of worm tissue. Benchtop sonication followed for 5 minutes to ensure homogeneity. A portion of the sample (15 mL) was drawn off into a plastic centrifuge tube for analysis. Activated carbon (250 mg, Thermo Fischer Scientific) was added as a decolorizing agent. Samples were centrifuged for 20 minutes at 4000 RPM in a large tabletop centrifuge. The supernatant was transferred to conical glass centrifuge tubes for evaporation to dryness under nitrogen. Samples were reconstituted in 400 µL hexane for analysis (GC-ECD/FID grade, SupraSolv).

A significant experimental design error was identified following analysis of the first exposure results. Soil, excrement, and PMMA that remained inside the worms were transferred along with the resulting worm tissue pieces into glass beakers for analysis. It was observed during the sample workup that an unknown amount of PMMA would precipitate when dried
samples were reconstituted. We hypothesized that some of the ESS quantified in the PMMA exposed samples could be retained or otherwise trapped on the precipitated PMMA. The measured ESS concentrations of worm tissue for those samples would be lower than expected. The procedure was adapted to avoid this significant error in the second exposure.

**L. terrestris** Tissue Extraction Method Two. The processing of the worm tissue was modified. Each worm from the replicate was sliced down the middle, length wise. Once the entire worm was split open, the resulting dirt was rinsed out with distilled water. Care was taken to not remove the organs from the head. Rinsing continued until no visible dirt remained within the worm (Figures 2, 3). The body was then patted dry with a paper towel and weighed before being cut into fine pieces using a razor blade. This was the only modification made to the extraction procedure from the first exposure.

**L. terrestris** Excrement Extraction Method One. Excrement samples collected from the second Hoang exposure study were homogenized once thawed. An aliquot was collected on pre-weighed P2 filter paper using water aspirated vacuum filtration. Samples were dried in an oven at 100°C for a minimum of 12 hours to remove all moisture then brought to constant weight. Samples and filter paper were placed in a glass jar containing 10 mL of methanol (ACS grade, Merck). They were sonicated in a benchtop sonicator for 20 minutes. The methanol was drawn off to a glass screw top culture tube and centrifuged. The supernatant was transferred to a new culture tube and evaporated to dryness under nitrogen. A further 10 mL of methanol was added to the aliquot jar and methanol (5 mL) was also added to the first culture tube that contained the pelleted excrement. Each aliquot was extracted a total of five times and evaporated in the same
inal culture tube. Samples were reconstituted in 400 µL of hexane (GC-ECD/FID grade, SupraSolv) for analysis.

**Soil Extraction Method One.** Soil from the initial Hoang exposure study were tested following the conclusion of the exposure to determine their ESS concentration. The soils would be reused for the second exposure dependent upon the results. Samples were brought to room temperature from -2°C and homogenized. An aliquot was transferred to a plastic centrifuge tube for analysis. Four grams of magnesium sulfate (MgSO₄, Thermo Fischer Scientific), 1.7 grams of sodium acetate (NaC₂H₃O₂, Sigma-Aldrich, St. Louis, MO, USA) 0.05 grams of activated carbon (Thermo Fischer Scientific), and 10 mL of a 1% acetic acid in acetonitrile solution were added to each centrifuge tube. Samples were vortexed and centrifuged for 5 minutes at 4000 RPM in a large tabletop centrifuge. Supernatants were transferred to conical glass centrifuge tubes and evaporated to dryness using nitrogen. Samples were reconstituted in 400 µL of distilled water containing 0.25% formic acid for analysis.

**Soil Extraction Method Two.** Soil ESS concentrations following the Hoang group’s second exposure were analyzed. Half a gram of each of soil type was transferred into glass screw-cap culture tubes with methanol (10 mL) (ACS grade, Merck) then vortexed. The samples sat covered overnight at room temperature. Samples were vortexed after 24 hours then centrifuged for 5 minutes using a tabletop benchtop centrifuge. Supernatants were transferred to new culture tubes and concentrated off using the Savant Speed Vac on low heat (Thermo Fisher Scientific). Samples were reconstituted in 400 µL of hexane (ACS grade, Merck) for analysis.
Figure 2. Uncut worm from the second ESS exposure study.

Figure 3. Cut and rinsed worm from the second ESS exposure study
**PMMA Bead Loading and Extraction Method One.** White PMMA beads (100 mg) were transferred to a small glass vial. ESS (1 mg) dissolved in methanol added to each replicate. Additional methanol (ACS Grade, VWR) was added to each vial to reach a final volume of 1.475 mL. Samples were vortexed then concentrated for 20 minutes using the Savant Speed Vac at low temperature. Further 1 mL aliquots of methanol were added and removed a total of three times for maximum concentration of ESS on the surface of the beads. Nanopure water (1 mL) was added to each replicate, vortexed, and concentrated off at low temperature. A small amount (10 mg) was removed from each replicate three times and placed into glass autosampler vials for extraction. Samples were sonicated for 20 minutes in hexane (GC-FID/ECD) SupraSolv, 1mL) using a benchtop sonicator. The hexane was drawn off and stored for analysis.

**Exposure of Plastic Debris in Chicago Area Freshwaters**

**Materials**

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) used for compound identification was purchased from Sigma Aldrich and used without further purification. PE plastic tarp (product no 626234) used in controlled litter exposure experiments was obtained from Berry Plastics Corporation (Evansville, IN, USA). Tryptic soil broth used to grow microbes for methyl triclosan experiments was obtained from Bacto (Sigma Aldrich). Each liter of broth made was a soybean-casein digest medium composed of 17 grams of Bacto tryptone, 3 g of Bacto soytone, 2.5 g of Bacto dextrose, 5 g of sodium chloride (NaCl), and 2.5 g of dipotassium phosphate (K₂HPO₄). The broth powder was heated slightly to ensure all particles completely dissolved.

Methyl triclosan (4-chloro-1-(2,4-dichlorophenoxy)-2-methoxy-benzene) used for compound identification was prepared according to a procedure adopted from Knapp.² Briefly,
triclosan (25 mg) was dissolved in 50 mL of dry acetone (ACS Grade, VWR) and combined with a large molar excess (6x) of powdered potassium carbonate (K₂CO₃, Thermo Fischer Scientific) after grinding. A large molar excess (10x) of methyl iodide (CH₃I, Sigma Aldrich) was added dropwise. It was stirred for 48 hours in a stoppered round bottom flask at room temperature. Distilled water (100 mL) was added and the methyl triclosan was extracted with DCM (10 mL) (ACS Grade, VWR) three times then evaporated to dryness under nitrogen. The residue was reconstituted in hexane (2 mL) over dry sodium sulfate (Na₂SO₄, VWR) until analysis.

Study Designs

Environmental Exposure of Virgin PE Litter Study Design. PE tarp pieces were exposed in the Salt Creek tributary of the Chicago River for 142 days using the ‘litter bag’ method common in stream ecology. A blue 2.8’ x 3.7’ (thickness approximately 0.15 mm) PE plastic sheet (i.e., a drop cloth for painting was purchased from Berry Plastics Corporation and cut into triangular plastic shapes. Thirteen pieces of PE were placed into a mesh bag (mesh size = 3.3 mm, made of polypropylene by Cady Bag Company, Pearson, GA) and arranged with 2 bags attached together by plastic cable ties in the stream.¹²¹ They were placed in unobstructed water flow and secured to the streambed using rebar. Samples were removed from Salt Creek on 5 collection dates over autumn, winter, and spring of 2016-2017. Samples were cut into 4 cm² for chemical analysis and stored at 4°C in borosilicate glass vials pending extraction.

Methyl Triclosan Transformation Study Design. Tryptic soil broth (22.5 g) was dissolved in a water sample (1500 mL) from the North Branch of the Chicago River which was obtained the previous day and stored at 4°C. The water was split into three replicates. Optical density of all replicates was measured. Samples were shaken overnight at 125 RPM and room
temperature. Optical density was measured again to track microbial growth. PE tarp was rinsed with distilled water and methanol (ACS grade, Merck) before triclosan (1.21 mg) was added. Three PE squares were added to each tryptic soy broth replicate once the methanol evaporated. The experiment ran for 18 days at room temperature with constant stirring. PE squares were sampled weekly from each replicate and stored at 4°C in glass vials pending extraction. Two weeks into the exposure, 10 mL of the liquid was drawn off each replicate and extracted the same day.

**Analyte Extraction**

**Plastic Extraction.** Samples were brought to room temperature and 1 mL of hexane (GC-ECD/FID grade, SupraSolv) was added. They were sonicated for 20 minutes using a benchtop sonicator. Extracts were transferred to a glass autosampler vial and stored over dry sodium sulfate (VWR) until analysis. A select few PE tarp samples were sonicated for up to 60 minutes in aim to differentiate additives that were embedded in the PE during the manufacturing process from adsorbed environmental pollutants. Unexposed PE tarp was extracted as a control.

**PE Tryptic Soy Broth Media Extraction.** An LLE was performed on the liquid media to extract any triclosan and potential methyl triclosan. Samples (10.00 mL) were drawn off into a small beaker. Concentrated sulfuric acid (200 μL) (H₂SO₄, Sigma Aldrich) and saturated sodium chloride solution (10.00 mL) were then added. Copper sulfate (CuSO₄, Sigma Aldrich) in 50 mg amounts was added to each extraction to aid in distinguishing between the two layers. The solution was added to a 125 mL separatory funnel followed by 3 mL of methyl tert-butyl ether (MTBE, ACS Grade, Sigma Aldrich). Funnels were shaken vigorously, vented, and then let sit for the layers to separate. The bottom layer of tryptic soy broth media was drained into a waste
beaker. The MTBE layer was transferred via Pasteur pipettes to glass autosampler vials containing dry sodium sulfate (VWR) and stored until analysis. A 400 µL aliquot was drawn off for analysis.

**Instrumentation**

**Chromatography Separation Methods**

Several methods of chromatography may be used to separate out analytes from various matrices. Two main classifications of chromatography are gas and liquid chromatography. Gas chromatography can be performed as gas-liquid or gas-solid chromatography. Liquid chromatography (LC) has numerous methods that may be used depending upon the analyte and matrix. Partition, adsorption, ion exchange, size exclusion, and affinity chromatography are all forms of liquid chromatography. What differentiates these chromatographic techniques from each other is the stationary phases of the columns and the types of equilibrium achieved on them.

**Gas Chromatography.** Gas-liquid chromatography, commonly referred to as gas chromatography (GC), separates volatile compounds through the interaction of the vaporized sample with the stationary phase of the column.\(^{48,122}\) The stationary phase of the column is a liquid that has been immobilized on the walls of capillary tubing. An inert gas serves as the mobile phase (carrier gas) in the system. The pressure of the carrier gas moves the sample through the system. The sample is injected onto the column and vaporized. The inlet is held at a temperature at minimum 50°C higher than the boiling point of the least volatile analyte. Samples may then be split before they reach the column. Use of a split largely depends on the analyte concentrations. Split samples have only a small volume reach the column with the majority flowing directly to waste. The remaining sample (or whole sample if splitless injection is used) is
purged onto the column.\textsuperscript{48,122} Separation on the capillary column occurs through manipulation of column oven temperatures. The lower the temperatures with respect to the analytes’ boiling point, the better the resolution and longer retention time.

\textbf{Partition Chromatography.} Partition chromatography is used to separate molecules with a wide range of molecular weights and solubility in both organic solvents and water.\textsuperscript{48,122} Two solvents of different polarities are used in conjunction with a high-pressure system to force the analytes into the column. Small guard columns may be placed before the column to remove particulates and contaminants from the mixture. Modern columns are liquid-bonded-phase columns where the solid phase is chemically bonded. This renders it insoluble to the mobile phase. Pressure forces the mixture through the column where the analyte interacts with the column interior. The packing of the column depends on the type of partition chromatography used.\textsuperscript{48} Normal phase chromatography has a polar stationary phase and uses non-polar solvents as eluents. The compounds with least polarity elute first in normal phase chromatography. Reverse phase chromatography has a non-polar stationary phase used with polar solvents. Compounds with high polarity elute first in reverse phase chromatography. In both types of partition chromatography, the solvent composition is varied to remove compounds of different polarities from the column. The analytes exit the column in the liquid phase.\textsuperscript{48,122}

\textbf{Mass Analyzer Ion Sources}

The eluate flows from the column into the ionization chamber of mass analyzer. Mass analyzers separate ions generated by the loss or gain of electrons, referred to as the molecular ions. Molecular ions are produced by ionization sources and have specific mass-to-charge (m/z) ratios. Sources are classified as either hard or soft. Hard ionization methods transfer enough
energy to analyte molecules to leave them in a highly excited energy state. The molecules relax, rupturing bonds to produce fragment ions. Fragment ions have smaller m/z ratios than the molecular ion. Examples of hard ion sources include chemical ionization (CI) and electron impact (EI). Soft ionization sources cause little to no fragmentation. Soft ion source examples include electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).48,122

**Electron Impact.** EI is the most common source used in gas chromatography mass spectrometry (GC/MS). Electrons are generated by a heated filament. An anode is placed opposite the filament to attract the electrons in a perpendicular path to the column eluate vapor stream. Electrons bombard the vapor stream, causing fragmentation of the analyte and ion formation. Generated fragment ions pass through charged focusing plates forming an ion beam that travels to the mass analyzer (Figure 4).48,122

**Electrospray Ionization.** ESI is often used in conjunction with liquid chromatography mass spectrometry (LC/MS). The liquid column eluate must be vaporized before it enters the mass analyzer. It is pumped through a charged steel capillary needle. Charged eluate spray is passed through another capillary where the solvent is evaporated, imparting a charge. Charge density grows on the molecules until the Raleigh limit is reached and Coulombic explosions occur. Droplets are continuously torn apart until no solvent remains. Ions are channeled into the mass analyzer using a focusing skimmer and a difference in pressures (Figure 5).48,122
Figure 4. EI source schematic.⁴⁸ (Adapted from Skoog, D. A.; et.al. *Principals of Instrumental Analysis*; Thomson Brooks/Cole: Belmont, California, 2007; pg. 504)
Figure 5. Schematic of an ESI source. (Adapted from Skoog, D. A.; et.al. *Principals of Instrumental Analysis*; Thomson Brooks/Cole: Belmont, California, 2007; pg. 512)
Mass Analyzers

Mass analyzers are used to further separate, identify, and quantify analytes. They manipulate magnetic and/or electric fields to control the detection of specific m/z values. Mass analyzers include Fourier-Transform ion cyclotron resonance (FTICR), ion traps, magnetic-sectors, Orbitraps, single quadrupoles, triple quadrupoles (QqQ), and time-of flight (TOF).48,122

Single Quadrupole. Single quadrupole mass analyzers filter ions using a combination of a time-independent (direct current, DC) and time-dependent (alternating current, AC) potential through a set of four parallel rods (Figure 6). These rods produce nearly hyperbolic electric fields (Equation 1). The trajectories of ions travelling through the rods are manipulated by both the AC and DC potentials. For each AC waveform and in the absence of DC potential, the electrodes along the x-axis spend half the cycle at a positive potential and half at a negative potential. A beam of ions is focused on the onto the center axis in between the rods along the z plane when the waveform is at a positive potential. A negative potential sends the beam toward negatively charged electrodes and forces it off the center axis. The DC potential applied along this same x-z axis is positive which focuses the heavy ions onto the center beam along the z-axis. Ions light enough to experience a large acceleration crash into electrode and doesn’t reach the detector. Together, this forms the high pass mass filter.

The low pass mass filter controls ions in the y-z plane. Potentials applied to the electrodes along the y-axis are opposite in charge but equal in magnitude to that applied to those on the x-axis. AC potentials in along the x-z plane are out of phase with potentials applied along the y-z plane. DC potentials applied along the x-z plane and those applied along the y-z plane are of opposite charges. The negative DC potential eliminates heavy ions that experience a large
acceleration and crash into electrodes. The AC potential corrects the trajectory of the light ions and keeps them on the center z-axis.

Ions traveling to the detector must remain stable along both the x-z and y-z planes. The forces exerted by the electric fields generated by the electrodes along the axes are determined by the magnitude of the electric field times the charge of the ions (Equations 1-6). Trajectory of any ion in terms of its initial condition can be found by using Newton’s law of force (Equations 7-9). Further manipulation of these equations produces Mathieu’s differential equation (Equation 10). Solutions classify the trajectory of an ion as either stable or unstable dependent upon if the solution is bound or unbound respectively. Stability of the trajectory is based on parameters a and q which are related by a ratio, identified as the mass resolution of the quadrupole.

\[
E_x = -\frac{d\Phi}{dx} = -[U + VCos(\omega t)] \times \frac{x}{r_0^2}
\]

Equation 1. Magnitude of the electric field produced by electrodes in a quadrupole along the x axis.

\[
F_x = -[U + VCos(\omega t)] \times \frac{ex}{r_0^2}
\]

Equation 2. Force of the electric field produced by electrodes in a quadrupole along the x axis.

\[
E_y = -\frac{d\Phi}{dy} = -[U + VCos(\omega t)] \times \frac{y}{r_0^2}
\]

Equation 3. Magnitude of the electric field produced by electrodes in a quadrupole along the y axis.

\[
F_y = -[U + VCos(\omega t)] \times \frac{ey}{r_0^2}
\]

Equation 4. The force of the electric field produced by electrodes in a quadrupole along the y axis.
\[ E_z = -\frac{d\Phi}{dz} = 0 \]

Equation 5. Magnitude of the electric field produced by electrodes in a quadrupole along the z axis.

\[ F_z = 0 \]

Equation 6. Force of the electric field produced by electrodes in a quadrupole along the z axis.

\[
\frac{d^2x}{dt^2} + \frac{ex}{mr_0^2} [U + VCos(\omega t)] = 0
\]

Equation 7. Differential equation describing the trajectory of an ion in the x-plane based on its initial conditions.

\[
\frac{d^2y}{dt^2} - \frac{ex}{mr_0^2} [U + VCos(\omega t)] = 0
\]

Equation 8. Differential equation describing the trajectory of an ion in the y-plane based on its initial conditions.

\[ \frac{d^2z}{dt^2} = 0 \]

Equation 9. Differential equation describing the trajectory of an ion in the z-plane based on its initial conditions.

\[
\frac{d^2u}{d\xi^2} + [a_u + 2q_uCos2\xi]u = 0
\]

Equation 10. Canonical form of Mathieu’s differential equations. Solutions to this equation are classified as bound (stable trajectories) or unbound (unstable trajectories).

Ions that successfully pass through the quadrupole exit to the ion transducer detector.

Dwell time is how long the quadrupole is set at a chosen ratio frequency. This dictates the number of ions at the allowed m/z ratio successfully reach the detector. Potentials can be swept from a set minimum to maximum to scan a wide range of m/z values. The maximum m/z range
for a quadrupole is 3000. This is caused by the lack of momentum ions have when they strike the detector, resulting in an unmeasurable signal.48,122

**Triple Quadrupole.** A QqQ is composed of three quadrupoles. Ions are accelerated out of the ionization source to the first quadrupole (Q). It manipulates ions trajectories through the rods as a single quadrupole does. Selected ions of a specific m/z range pass through into the second quadrupole (q). It is operated in radio frequency (RF) mode so all ions can pass through or it can also be filled with an inert gas such as nitrogen to facilitate collision-induced dissociation (CID). Ions are accelerated into the third quadrupole (Q). It also functions as the first, filtering ions based on selected m/z values before they reach the detector (Figure 7).48,122 Triple quadrupoles face the same m/z limits as a single quadrupole.

**Time-of-Flight.** TOF mass analyzers separate ions by m/z ratios as do other mass analyzers. They achieve this by giving them all the same kinetic energies and using the differences in velocities based on weight to separate when ions reach the detector. The column eluate is pulsed in front of a pulsed bombardment of electrons, secondary ions, or photons. The pulse has a lifetime of 0.25 ms.48,122 There are several variations of TOF instruments that manipulate the ion path to increase resolution. The variations include linear TOFs and reflectron TOFs (reTOF).122 Linear TOFs are the basic model. A pulse of the same frequency lagging behind the ionization pulse accelerates the ions into a meter-long, field free drift tube. Ions travel through it based on their relative velocities (Figure 8). Ions of lighter mass will reach the detector first.48,122,123 Linear TOFs have no upper limit for detectable m/z values.

ReTOFs use an ion mirror generated by an electric field to increase resolution. Pulsed ions enter the field and slow until they have no kinetic energy. They are then re-accelerated
toward the detector. Lengths of ion flight paths differ based on their penetration depth as they exit the electric field (Figure 9). Differences in flight paths account for minor differences in kinetic energies of ions with the same m/z value, ensuring they arrive at the detector concurrently. A reTOF has a lower upper mass limit than a linear TOF. Ions that penetrate too deep into the ion mirror are neutralized when they strike the back plate and are undetected.

TOF instruments have better mass resolution than traditional quadrupoles. Resolution is limited the most by the initial spatial and velocity spreads of ions in the z dimension. Ions with opposing initial velocities than the rest of the pulsed pack must first get turned around by the extraction pulse, falling and staying behind other ions. This can be corrected by a stronger electric field but is limited by the correctional ability of the electrostatic mirror. A longer TOF also increases mass resolution, but there are practical and cost-prohibitive limits to consider.

**Quadrupole Time-of-Flight (qTOF).** The fragmentation ability of a quadrupole may be combined with the increased sensitivity of a time-of-flight mass analyzer. This mass analyzer combines two quadrupoles followed by the collision cell of a triple quadrupole and then connects with a reTOF. The first quadrupole (Q0) is often an RF quadrupole to increase dampening of ion trajectories. Ions travel into the second quadrupole (Q1) which is used either to continue the transmission of all ions or to select a molecular ion for fragmentation. Fragmentation occurs in third quadrupole (q2) if the collision cell is turned on, otherwise it transmits all ions from the previous quadrupoles to the reTOF analyzer (Figure 10). The use of a TOF analyzer provides concurrent recording of all ions rather than having to sweep across a mass range like a quadrupole, providing increased the increase in sensitivity of all ions.
Figure 6. Schematic of a single quadrupole mass analyzer.48 (Adapted from Skoog, D. A.; et.al. Principals of Instrumental Analysis; Thomson Brooks/Cole: Belmont, California, 2007; pg. 259)
Figure 7. Diagram of a triple quadrupole mass spectrometer.48 (Adapted from Skoog, D. A.; et.al. Principals of Instrumental Analysis; Thomson Brooks/Cole: Belmont, California, 2007; pg. 525)
Figure 8. Schematic of a linear TOF.\textsuperscript{122} (Adapted from Throck Watson, J.; David Sparkman, O. \textit{Introduction to Mass Spectrometry: Instrumentation, Applications, and Strategies for Data Interpretation}; John Wiley & Sons, Ltd: West Sussex, England, 2007; pg. 63)
Figure 9. Schematic of the electric field of a reTOF.122 (Adapted from Throck Watson, J.; David Sparkman, O. Introduction to Mass Spectrometry: Instrumentation, Applications, and Strategies for Data Interpretation; John Wiley & Sons, Ltd: West Sussex, England, 2007; pg. 69)
Figure 10. Schematic of a QTOF that has the additional Q0 to aid in ion focusing.\textsuperscript{123} (Adapted from Chernushevich, I. V. et.al. An introduction to quadrupole–time-of-flight mass spectrometry. \textit{J.Mass Spectrom.} \textbf{2001}, \textit{36}, 850)
Mass Analyzer Scans

Mass analyzers may isolate ions through different techniques called scans. All mass analyzers may perform full scans and selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) scans. These look at either a large group or a small select set of ions. Some mass analyzers that can cause further fragmentation after formation of molecular ions can perform product ion (PI) and constant neutral loss (CNL) scans. These scans isolate the molecular ion, fragment it, and looks at specific m/z ranges for the fragments.

**Full Scan.** A scan of the complete possible range of m/z values is a full scan. It should be conducted regularly on standards and analyte samples. A chromatogram will be generated showing peaks or bands for all eluting ions within the time gradient. The length of time it takes for ions to interact with and travel through the column before reaching the ion detector at the end of the mass analyzer is the retention time of the compound. Chromatographic peaks are composed of several mass spectra taken at a constant rate. An average of the individual mass spectra spanning the chromatographic peak may be generated. This mass spectrum spans the entire scanned range of m/z values that eluted within the chosen range.

**Selected Reaction Monitoring and Multiple Reaction Monitoring.** A small number of m/z values are monitored rather than a large range in both SRM and MRM scans. Monitoring a small select number of m/z values increases the dwell time on each. The standard is to monitor 3 m/z values for each analyte. This is typically the molecular ion and two ions that are of high intensity and unique to the molecule. SRM and MRM scans are best utilized once the retention time and mass spectrum of the analyte are determined. The matching retention times and presence of monitored ions, and matching isotopic distribution are used to for analyte detection.
Detectors

Multiple ion detection systems have been engineered to pair with mass analyzers and chromatographic methods. A choice typically is made between accuracy and response time depending upon the type of ion analysis being conducted. Common detectors for coupling to a mass analyzer include electron multipliers, multichannel plates, and photomultiplier tubes. Other notable detectors include gas chromatography electron capture detectors (GC-ECD).

**Electron Multiplier Transducers.** Electron multiplier transducers are rugged detectors in a mass spectrometer. They can be either discrete-dynode or continuous-dynode. Discrete-dynodes traditionally had copper-beryllium dynode plates with each held at a continually higher voltage. An ion stream exits the mass analyzer and hits the first dynode. The ion strike causes a burst of electrons to leave the dynode, attracted towards the next. This pattern continues, generating a greater burst of electrons with each dynode plate hit until the electrons reach the amplifier\(^4\).\(^{122}\) (Figure 11A). Discrete-dynodes are not currently in widespread use, but those that are used are made of lead-doped glass.\(^1\)\(^{122}\)

Continuous-dynodes are cornucopia shaped glass doped with lead. A voltage is applied across the length. A gradient spanning the dynode is generated. Ions from the mass analyzer strike near the mouth of the cornucopia. The ion strikes generate bursts of electrons. These skip along the surface, generating further bursts of electrons down the voltage gradient to the amplifier (Figure 11B). A continuous-dynode provides a current gain of \(10^5\).\(^4\)\(^,\)\(^{122}\)

**Multi-channel Plates.** Multi-channel plates (MCP) function similarly to electron multiplier transducers. They are composed of a honeycomb arrangement of channels, each of a small diameter (10 µm), and separated by 12 µm gaps (Figure 12).\(^{122}\) An electrical gradient is
applied across the detector by a potential difference at opposite ends of the channel. Ions exit the mass analyzer and enter the channels slightly off axis ensuring they strike the walls of the channel. Their impact generates secondary electrons which continue to ricochet down the channel. This amplifies the original signal from the ion.

**Photomultiplier Tube.** Photomultiplier tubes (PMT) measure signals from radiation in a similar fashion to electron multiplier transducers. They are highly sensitive to light and damage to the inner surfaces may occur if PMTs are exposed to high incidence light. The quartz tube contains a cathode and additional dynodes for signal amplification. Photons strike the cathode, emitting electrons. They are propelled toward the first dynode which is held at a higher positive voltage (Figure 13). A chain of electron emissions at each additional dynode occurs. The cascade of electrons is collected at the anode and measured.

**Electron Capture Detector.** An ECD is a selective detector that will only give a response for molecules with high electronegativity. Compounds such as those containing halogens, nitrile groups, and nitro groups are able to be detected by an ECD. The eluate from the column is passed over a β-particle emitter, typically nickel-63. The radioactive metal emits a constant stream of electrons to ionize the carrier gas, producing a burst of ions. A constant current between two electrodes is generated when no electron withdrawing groups are present (Figure 14). If a halogen or other electronegative group is present, the current decreases as the electrons are captured by the functional groups. The decrease in current is what is transformed and reported by the data analysis program.
Figure 11. Schematics of A) discrete-dynode and B) continuous-dynode electron multipliers.48 (Adapted from Skoog, D. A.; et.al. Principals of Instrumental Analysis; Thomson Brooks/Cole: Belmont, California, 2007; pg. 256)
Figure 12. Design of a multi-channel plate.\textsuperscript{48} (Adapted from Skoog, D. A.; et.al. Principals of Instrumental Analysis; Thomson Brooks/Cole: Belmont, California, 2007; pg. 258)

Figure 13. Cross section schematic of a PMT.\textsuperscript{48} (Adapted from Skoog, D. A.; et.al. Principals of Instrumental Analysis; Thomson Brooks/Cole: Belmont, California, 2007; pg. 178)
Figure 14. Schematic of an ECD.48 (Adapted from Skoog, D. A.; et.al. *Principals of Instrumental Analysis*; Thomson Brooks/Cole: Belmont, California, 2007; pg. 727)
Instrumentation Used to Conduct Sample Analysis

**GC/MS.** Analysis of samples from the PE bead loading study, samples related to the Hoang’s group second daphnia exposure, soil samples related to the Hoang’s group second earthworm exposure, and triclosan transformation products from attempted microbial growth related to the Salt Creek plastic extracts was completed using a 6890N Agilent GC coupled to an Agilent G2614A autosampler and an Agilent 5973 single quadrupole mass spectrometer (Agilent Technologies). A DB-5MS column, 30 m, 0.250 mm internal diameter column was used to carry out separations. The carrier gas used was helium at a flow rate of 1.5 mL/minute. Splitless injection was used for analysis and one microliter of sample injected for each analysis.

**Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS).** Soil samples related to the Hoang group’s first worm exposure were analyzed on an Agilent G6460 Triple Quadrupole Tandem Mass Spectrometer interfaced to an Infinity 1290 LC system (Agilent Technologies). It was operated under a flow rate of 0.2 mL/min utilizing gradient conditions using an XTerra C18 column (100 x 2.1 mm, 5 micron) with a C18 guard column (10 x 2.1 mm, 5-micron particle size, Waters Corp.) and a water/methanol gradient containing 0.25% formic acid. All samples were analyzed using electrospray ionization in negative ion mode. One microliter was injected for each analysis.

**QTOF.** The environmental plastic sample hexane extracts collected from Salt Creek were analyzed by GC-TOF/MS using an Agilent 7200 GC-qTOF/MS (Agilent Technologies, Santa Clara, CA, USA) in positive ion (electron impact, EI) mode. A 30-meter Agilent J&W DB-5MS UI column was used for analysis. A GC gradient with holds before and after was used (start hold at 32°C , 32° to 300°C at 15°C/min, followed by hold at 300°C for 3 and up to 30 minutes for
clearing soluble debris from the plastic itself coming through the column) for the separation. The source temperature was maintained at 280°C. One microliter was injected for each analysis.

**GC-ECD.** The complete analysis samples related to the Hoang group’s first daphnia and earthworm exposures, the PMMA loading study, and a significant portion samples related to the Hoang group’s second exposure of earthworms was completed using a Shimazdu (GC-ECD) GC-2010 Plus connected to a Shimazdu AOC-20i autosampler (Shimazdu Scientific Instruments, Columbia, MD, USA). A Restek (Bellfonte, PA, USA) RTx-5MS column, 60 m, 0.25 mm internal diameter column was used to carry out separations. The carrier gas was helium at a flow rate of 1.5 mL/minute. Nitrogen was used as the make-up gas at a flow rate of 30 mL/minute. Splitless injection was used for analysis, with one microliter of sample injected for each analysis.
CHAPTER THREE

NOVEL METHOD DEVELOPMENT FOR DETECTION AND QUANTIFICATION OF ENDOSULFAN SULFATE FROM ORGANISM TISSUE, EXCREMENT, AND SOIL

RESULTS

Introduction

We have analyzed the terrestrial organism *Lumbricus terrestris* (earthworms) following exposure to a potentially toxic insecticide metabolite endosulfan sulfate (6,9-Methano-2,4,3-benzodioxathiepin, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-, 3,3-dioxide, ESS) in soil with and without poly (methylmethacrylate) (PMMA) beads present to determine the concentration of ESS in various media. The original hypothesis put forth by the Hoang group was worms exposed to ESS in soil in the presence of PMMA beads would absorb more of the ESS than worms exposed to ESS in soil alone. My research goal was to develop novel extraction methods for the media related to the exposures of the worms. Dr. Hoang’s group selected worms as the model organism for these exposures for several reasons. Several aspects of earthworm biology including burrowing, foraging, mating and oviposition, mucus excretion and casting of feces are well known. *L. terrestris* are a deep burrowing species that build vertical burrows. A cocoon is excreted for up to 12 months following mating and hatchlings emerge between 2-5 months. Castings (natural casings of excrement) are created as the worms excrete digested soil and litter. They have an ingestion rate of 3.5 mg cast/g worm/day. It takes approximately
two days for the worms to completely empty their guts once starved. Changes to these behaviors and aspects based upon alterations in controlled environments are well documented in laboratory studies.

Earthworms of several species including *L. terrestris* are often used as bio-indicators for soil health as they are one organism that links the top layer of soil to the layers underneath, recycling nutrients and increasing water infiltration. Their bodies are covered in a secondary water film which acts as a lubricant as they move through the burrows. This mucus coating facilitates direct contact with soil contaminants. Direct exposure to harmful substances in the soil has potential for higher bioaccumulation of the contaminants in the worms and their predators. *L. terrestris* have been used as indicators of heavy metal contamination. The impact of various pesticides on *L. terrestris* have been investigated as well. Doses as high as 1.4 g/kg soil of isoproturon had no lethal effect on earthworms after 60 days of exposure.

PMMA was chosen as the model plastic for the Hoang group exposures. It has been utilized as a substitute for glass in illuminated signs and is commonly known as Plexiglass. PMMA has been used in different aspects of human medicine including bone screws and cement, contact lenses, and filler for cavities. Other common uses of PMMA include acrylic paint, modern furniture, DVDs and optical fibers. Numerous uses generate numerous pathways for PMMA to appear in terrestrial environments through waste disposal. The crushing, burning, and other manipulation of trash will fragment larger pieces of PMMA to smaller sizes. Thirty-micron diameter PMMA spheres were selected for this study because it is within the size of particles the worms are capable of ingesting. It is within the size of soil particles that the worms ingest.
*L. terrestris* has been observed to interact with microplastics and microfibers. Prendergast-Miller et al. report polyester-derived microfibers were ingested by worms while burrowing over the 35-day exposure. High concentrations of microfibers lowered cast production. PE was used in both microplastic studies, one using beads of four diameters ranging from 710-2800 µm and the other used beads smaller than 150 µm in diameter. Huerta Lwanga et al. found the worms were transporting particles smaller than 50 µm in diameter in their casts while particles up to the maximum 150 µm were incorporated into the burrows. Growth rates and weight decreased over the 60 day exposure. Rilling et al. reported that presence of microplastic particles increased through 3 soil layers (3.5 cm deep each) in the presence of worms. All studies support the conclusion that earthworms transport microplastics of several sizes in soils through burrow creation and excretion of casts.

ESS was the model compound utilized for the Hoang exposures. It is the aerobic oxidation degradation product of the pesticide endosulfan and known to collect on the surface of plastic litter in marine environments. Endosulfan was first marketed by the German company Fabwerke Hoechst in 1954 as a pesticide suitable to treat cereals, vegetables, fruits, and tobacco. The Stockholm Convention named it as one of the persistent organic pollutants (POPs) to be eliminated from use, though there are some exceptions for the treatment of cotton. Technical endosulfan is comprised of the two isomers, *α*-endosulfan (endosulfan 1) and *β*-endosulfan (endosulfan 2) which have half-lives of 37-65 days and 104-265 days in soil respectively. Both endosulfan isomers oxidize to form several products including endosulfan sulfate and endosulfan diol. ESS is a toxic, durable oxidation degradation product of endosulfan.
Multiple studies have suggested that ESS is toxically equivalent to technical endosulfan. This toxicity coupled with a half-life of several years classifies ESS a POP. ESS has been identified as both an aquatic and terrestrial pollutant. Endosulfan is oxidized to ESS when bound to soil and sediment. Runoff from crop fields transports the ESS to freshwaters. It is not currently monitored nor regulated. ESS was selected as the model compound for this exposure study because of its toxicity, long half-life, and identification both on plastic debris and as a terrestrial pollutant.

Herein I will discuss the results of the analysis of the organism tissue, organism excrement, and soil used to conduct the two exposures by the Hoang group. Both studies exposed worms to two different concentrations of ESS, 3 mg ESS/kg soil and 45 mg ESS/kg soil with and without PMMA present. The results of the first exposure led to our conducting of experiments to determine if residual PMMA was negatively impacting the results of the organism tissue analysis. These experimental results were followed by the development of a modified extraction procedure used for samples from the second exposure. The Hoang group provided samples from all exposures for the development and refinement of analytical methods for the detection and quantification of ESS in the various matrices. The quantification of ESS in these matrices led to development of a new hypothesis based on the surface chemistry interactions between the polymer beads and the ESS molecules. We propose that the interaction of these two regulates the transfer of compounds in the organism tissue and thus the excrement. We propose that the hydrophobic plastic particles bind strongly to hydrophobic molecules, decreasing the amount transferred into the tissue and excrement. Plastics with hydrophilic surfaces of the same particle size would transfer a greater amount of the compound into the
tissue when compared to the hydrophobic plastic. I end the chapter with proposing an experiment to test the new hypothesis.

**Analysis of Worm Tissue and Soil to Determine the Concentration of ESS Following the Hoang Group’s First Exposure**

A preliminary calibration curve, solvent blanks, and method blanks were analyzed before analysis of the exposed worm samples following the conclusion of the first exposure. Method and solvent blanks were analyzed to determine if there were any co-eluting peaks that would cause interference in the exposed samples (Figure 16). Solvent blanks were GC-ECD grade hexane and method blanks were generated by extracting unexposed worms that frozen at the beginning of the exposure. There was no co-elution in the solvent blank (Figure 16A). The method blanks did not generate signals above the limit of quantification (Figure 16B). The lower limit of quantification was established at 100 ng/L (Figure 16C). We determined that this co-eluting compound from the worm tissue would not significantly interfere with detection of ESS in the exposed worm samples and proceeded to analyze them.
Figure 15. The proposed mechanism for the aerobic oxidation of α-endosulfan through a monooxygenase enzyme to form endosulfan sulfate.β-Endosulfan must undergo isomerization to form α-endosulfan before it can be oxidized. (Adapted from Mudhoo, A.; et.al. Endosulfan removal through bioremediation, photocatalytic degradation, adsorption and membrane separation process: A review. Chem. Eng. 2019, 360, pg. 917)
Figure 16. Gas chromatography electron capture detection (GC-ECD) partial chromatograms of A) a solvent blank, B) an exposed worm method blank and C) an ESS standard at the concentration of 100 ng/L all around retention times of 35.45 minutes.
**Determination of ESS Concentration in Worm Tissue**

We were given samples from the Hoang group pertaining to a bioaccumulation exposure study. There were two large sample sets presented for analysis. Each large sample set contained two subsets. The two subsets were related by the concentration of ESS present in the exposure and differed in the presence of a polymer. Samples exposed in the presence of ESS and the polymer versus only ESS were expected to have two different concentrations. Our developed extraction methods allowed for analysis and quantitation of ESS present in all samples. The quantitation of the two samples sets is summarized in Figures 17 and 18. The subset for each sample set exposed to only ESS had a higher concentration than the subset exposed to ESS in the presence of the polymer.

It was observed in some cases while processing the subsets exposed to ESS and PMMA that a sticky, opaque, plastic-like film would form when the individual samples were reconstituted in hexane for GC-ECD analysis. We theorized that an unknown amount of ESS in the subset samples exposed to both ESS and PMMA could have been retained or otherwise trapped by the polymer during the extraction and sample workup. ESS concentrations measured in these subsets would be reported as falsely lower than they should have been if this is true. As a result, we performed an experiment to test whether samples exposed to these conditions were retaining the PMMA (described below). If so, the tissue extraction procedure for all samples would need to be modified to correct for this error and maintain consistency in the sample workup across sample types. Recovery studies based on the original method of tissue extraction were not performed based on the above concern and the results of the following experiments.
Figure 17. Bar graph comparing the average concentration of ESS/g worm for the sample set that had the lower initial concentration of ESS with and without the polymer present.
Figure 18. Bar graph comparing the average concentration of ESS/g worm for the sample set that had the higher initial concentration of ESS with and without the polymer present.
Determination of ESS Retention by PMMA Following Sample Extraction

Our hypothesis concerning the incorporation of ESS into the PMA during the extraction process during the first exposure was dependent on PMMA being retained in the worms’ gut after ingestion and not yet excreted upon analysis. Laboratory notes stated that worms across all samples contained varying amounts of soil when their bodies were opened during the extraction process. Worms exposed to soils containing PMMA would, in theory, have retained PMMA as well though this was not visible to the naked eye. The following experiments were designed to test this.

Beakers were labelled and weighed before a set volume (15.00 mL) of homogenized worm tissue in dichloromethane (DCM) was added. Six samples, two each from days 7, 14, and 21 with and without PMMA were used. The beakers were left uncovered in the lab hood at room temperature until all DCM had evaporated. Samples were further dried overnight in an oven at 120°C to ensure any remaining moisture was driven off before being brought room temperature and re-weighed. Residues for worms in soil containing PMMA were consistently heavier than the residues produced from worms exposed to soil not containing PMMA (Table 3).

Table 3. Summary of the worm residue experiment to determine if exposed worms retained significant, measurable amounts of PMMA in their gut.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Difference in Residue Weights (mg PMMA/g worm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days exp.</td>
<td>7.0</td>
</tr>
<tr>
<td>14 days exp.</td>
<td>6.3</td>
</tr>
<tr>
<td>21 days exp.</td>
<td>10.0</td>
</tr>
</tbody>
</table>

These results of this experiment suggested that PMMA beads were being ingested and retained by the worms during the exposure period and thus significant amounts were retained by
the worms upon death. We proposed that ESS was being trapped or otherwise retained in this PMMA when samples were reconstituted. Our next experiment tested this theory.

Aliquots of DCM (15.00 mL) were spiked with ESS (60 ng) both with and without PMMA (10 mg) present and taken through the extraction process. The decision was made to analyze the samples using liquid chromatography tandem mass spectrometry (LC/MS/MS) as the GC-ECD was unavailable for several months. A standard of ESS was purchased and analyzed in full scan mode. It showed all detectable ions present. Fragmentation was attempted using a product ion scan with different collision energies with no success. The three most abundant ions from the full scan were selected for selected ion monitoring (SRM) analysis. The switch was made from full scan to MRM for sample analysis to increase the limits of detection through monitoring of 3 known ions of ESS rather than scanning a large range. The ions chosen for SIM analysis: m/z 422.90 (molecular ion), m/z 420.90 and m/z 418.90.

The three ions are molecular ions vary in the numbers of two chlorine isotopes, \(^{35}\text{Cl}\) and \(^{37}\text{Cl}\) as the six chlorines on the molecule. The ion at m/z 418.90 has six \(^{35}\text{Cl}\) atoms, the ion at m/z 420.90 has five \(^{35}\text{Cl}\) and one \(^{37}\text{Cl}\) atom, and the ion at m/z 422.90 has \(^{35}\text{Cl}\) and two \(^{37}\text{Cl}\) atoms. SIM scans were completed of standards before analysis of samples for calibration determination of the limits of quantification (Figure 19). The limit of quantification was found to be 350 ppm.

Samples were reconstituted in distilled water for analysis on the LC-MS/MS rather than GC-ECD hexane used for previous GC-ECD analysis. Solubility of ESS in distilled water has been reported as 0.48 ppm, and our samples had a maximum concentration of 0.20 ppm upon reconstitution if no ESS was bound or otherwise trapped by the PMMA.\(^{153}\) Analysis of the samples following the extraction process showed no ESS in the samples that contained PMMA.
Samples without PMMA gave detectable signal for ESS (Figure 20). This supports that once the ESS is bound or trapped to the PMMA during sample workup, it does not desorb from the surface of the plastic into the solvent used for sample analysis. Remaining soil and PMMA present in the worms’ gut at time of death need to be removed for quantitation of ESS in the worm tissue to be accurate. We modified the ESS extraction procedure for worm tissue in the second exposure to remove the PMMA.

**Modification of Isolation and Extraction of ESS from Worm Tissue**

Individual worms from same days of exposure (21 days) and replicate (replicate 1) were used to test the developed modification discussed in Chapter 2 for removing all ingested dirt and PMMA from the organisms. The first worm was extracted using the proposed modification for tissue isolation and the second extracted using the initial study procedure as a control. The worm extracted using the modified procedure had a concentration of 85.49 ppm while the worm extracted using the initial procedure had a concentration of 0 ppm. The modified tissue isolation procedure gave significantly increased signal, providing evidence for modification of the original procedure to include this technique prior to completion of the second exposure.

**Determination of ESS Concentration in Soil**

We checked the concentration of ESS in the soil following the conclusion of the first exposure by the Hoang group. If the concentrations were deemed acceptable, it would be reused for the second exposure. Original concentrations of ESS were 45.4 mg/kg soil for high concentration and 3.11 mg/kg soil for the low concentration soils. Access to the GC-ECD was unavailable and would be for some time so these samples were also analyzed using LC/MS/MS.
Figure 19. ESI triple quadrupole extracted ion chromatograms (EICs) of A) m/z 422.900, B) m/z 420.900, and C) m/z 418.900 for a 350 ppb ESS standard, the established limit of quantification. 1 µL was injected for analysis.
Figure 20. ESI triple quadrupole EICs of extracted DCM samples spiked with 60 ng of ESS A) without PMMA present and B) with 10 mg of PMMA present.
Soil samples were analyzed after the generation of a preliminary calibration curve. The control and low spike with PMMA present soils fell below the limit of quantification of 350 ppb. The low spike soil without PMMA present gave a signal that fell below the lower limit of detection of 200 ppb. Both the high spike soil with PMMA and high spike soil without PMMA present gave quantifiable signal. A decrease in soil ESS concentration was expected, but not to this degree.

We analyzed two control soil samples with a known amount of ESS added to determine if potential faults were present in the extraction procedure. One had had a stated concentration of 45 mg/kg and the second a stated concentration of 3 mg ESS/kg soil to reflect the concentrations used in the first exposure. The approximate 45 mg ESS/kg soil sample could not be analyzed. The solution became found thick and viscous. Attempts were made to remove particulates through centrifugation and were unsuccessful. Analysis of the approximate 3 mg ESS/kg soil sample was completed and quantified (Figure 21). ESS may successfully be extracted from soil samples using the method discussed in Chapter 2. Quantifiable signal from an externally spiked sample supports there being no quantifiable signal in the low spike soils with and without PMMA.
Figure 21. ESI triple quadrupole EICs of A) m/z 422.900, B) m/z 420.900, and C) m/z 418.900 for the external low spike soil sample.
The two high spike soil samples were quantified using an external calibration curve. The high spike soil sample without PMMA gave a concentration of 0.357 mg ESS/kg soil. The high spike soil with PMMA gave a concentration of 0.0130 mg ESS/kg soil. Both concentrations are far from the target concentration of 45.4 mg ESS/kg soil. These concentrations coupled with the unmeasurable signal of the low spike soil samples support the conclusion that the soil could not be reused for the second exposure study. The ESS could have been retained on the sides on the exposure containers or underwent hydrolysis to form endosulfan diol as it was over six months between the end of the exposure and the re-testing of the soil.

**PMMA Adsorption of ESS**

The goal of this study was to determine if measurable quantities of ESS would adsorb to the surface of PMMA beads and if it were possible to remove the majority of the bound ESS. Small quantities (10 mg) of PMMA beads exposed to a chosen quantity of ESS were extracted and analyzed. The quantity of ESS (1 mg) was chosen because it was close to the low spike concentration of ESS (3 mg/kg) and should have provided significant signal for detection. Five sequential extracts were analyzed. The first extracts showed the greatest concentrations of ESS. Quantifiable amounts of ESS were present in all five extracts in decreasing amounts (Figure 22). The first three rounds of sonication removed an average of 93.3% of all measured ESS. The results of this study show that it is possible to remove the bulk of bound ESS from PMMA within three rounds of sonication. This study laid the groundwork for procedure used to analysis soil and excrement samples from the second exposure. The developed procedure streamlined the sample workup and reduced the materials needed for extraction.
Figure 22. Histogram displaying the percentage of ESS removed from PMMA beads following each round of sonication in hexane.
Analysis of Worm Tissue, Excrement, and Soil to Determine the Concentration of ESS Following the Hoang Group’s Second Exposure

Determination of ESS Concentration in Worm Tissue

We were given organism (earthworm) samples from the Hoang group pertaining to a second bioaccumulation exposure study. Again, there were two large organism sample sets presented for analysis and each contained two subsets. They were related by the concentration of ESS present in the exposure and differed in the presence of a polymer. Organism samples exposed in the presence of ESS and the polymer versus only ESS were expected to have two different concentrations. The modified organism tissue extraction method allowed for analysis and quantitation of ESS present in all samples. Quantitation of the two sample sets is summarized in Figure 23. The sample set pertaining to the initial lower ESS concentration showed that the subset exposed to only ESS had a higher relative concentration than the subset exposed to ESS in the presence of the polymer. The sample set pertaining to the initial higher ESS concentration showed that the subset exposed to ESS in the presence of a polymer had a higher relative concentration than the subset exposed to only ESS. For the subset exposed to ESS in the presence of a polymer, the last data set within the subset should not be considered when evaluating the data for trends as it represents only one replicate due to excessive worm death during the Hoang exposure. The use of the modified organism tissue extraction method resulted in organism tissue concentrations approximately 1000x less than those calculated using the original organism tissue extraction method. This is most likely due to the removal of the excess dirt and ESS from the organisms before extraction.
Recovery studies were performed using unexposed worms given to us by Dr. Hoang’s group to aid in method development. Excess soil and polymer were removed the gut of the organisms. A glass syringe was used to add ESS (320 ng total) to various parts of the organism tissue. The samples in this recovery set were then processed using the modified extraction method. Analysis of the samples was performed under the GC-ECD conditions used to analyze both sample sets from the Hoang exposure study. The average percent recovery was calculated to be 116%. The values for each sample set from the Hoang exposure were adjusted accordingly.

Statistical analysis was performed on all subset data that had a minimum of 2 replicates to determine if the differences observed in the data could be considered statistically different. An f-test was performed on each small data set within the subsets to check for equal variances. A t-test for equal or unequal variances was performed on the same data set based on the f-test results. Data is considered statistically significantly different if the p-value is equal to or less than 0.05. The sample set related to the lower initial ESS concentration had one set of subset data points with a p-value \( \leq 0.05 \). The sample set related to the higher initial ESS concentration had two sets of subset data points with p-values \( \leq 0.05 \).
Figure 23. Bar graph comparing the average concentration of µg ESS/gram of worm tissue in both the initial low and initial higher sample sets from the second Hoang exposure.
Determination of ESS Concentration in Worm Excrement

We were given samples from the Hoang group pertaining to a second bioaccumulation exposure study. The excrement samples consisted of were two large sample sets that each contained two subsets presented for analysis. The subsets were related by the concentration of ESS present in the exposure and differed in the presence of a polymer. The two sample sets were expected to have differing concentrations. Our developed excrement extraction method allowed for the analysis and quantitation of ESS present in all samples. The quantitation of the two excrement sample sets is shown in Figure 24. A trend was identified in both sample sets. The sample set corresponding to the initial lower ESS concentration showed that the subset exposed to ESS in the presence of the polymer had a relatively higher ESS concentration than the subset exposed to only ESS. The sample set corresponding to the initial higher ESS concentration showed that the subset exposed to only ESS had a relatively higher ESS concentration than the subset exposed to ESS in the presence of a polymer. This trend is reversed for the data point within the subset (Figure 24).

Recovery studies were performed on excrement collected from worms in the Hoang exposures that did not contain ESS. A known amount (2 µg) of ESS was added to each recovery sample and extracted using the established procedure outlined in Chapter 2. Analysis of the samples was performed under the GC-ECD conditions used to analyze both sample sets from the Hoang exposure study. The average percent recovery was calculated to be 80.65%. The values for each sample set from the Hoang exposure were adjusted accordingly.
Figure 24. Bar graph comparing the average concentration of mg ESS/gram of worm excrement in both the initial low and initial higher sample sets from the second Hoang exposure.
Determination of ESS Concentration in Soil

Soil ESS concentrations for all subsets within both sample sets were determined following the conclusion of the Hoang exposure study. We sought to ascertain if a difference in soils for the subsets that were supposed to have the same concentration of ESS with and without the polymer present. The results are summarized in Figure 25. The subsets pertaining to the initial lower concentration of ESS were found to have the same concentration, 0.898 mg ESS/kg soil. The subsets pertaining to the higher concentration of ESS were found to have different concentrations. The subset containing ESS and the polymer had a concentration of 5.45 mg ESS/kg soil and the subset containing only ESS had a concentration of 6.74 mg ESS/kg soil. Statistical analysis was performed on the subsets pertaining to the initial higher concentration of ESS to determine if they were statistically significantly different. An f-test showed unequal variances and the subsequent appropriate t-test had a p-value less than 0.05, meaning the data points are statistically significantly different.

Recovery studies were performed using samples that were unexposed to ESS. A known amount of ESS (11.5 µg) was added to each sample. Samples were then extracted using the procedure outlined in Chapter 2. Analysis of the samples was performed under the GC/MS same conditions as the samples from the Hoang exposure study. The average recovery was calculated to be 74.72%. The values from the Hoang exposure were adjusted according. The recovery study verified the developed extraction method for the soil. The developed procedure is not responsible for the statistical difference seen within the sample set for the higher initial ESS concentration. There is an unknown reason as to why the concentrations are statistically significantly different.
Figure 25. Bar graph comparing the average concentration of ESS in excrement from the first and last sampling day of the exposure study to the post-exposure concentrations of the bulk soils. Inset are the low spike soil results to an appropriate scale.
Conclusions

Methods for the extraction of ESS from *L. terrestris* tissue, *L. terrestris* excrement, and soil samples, some containing plastic particles were developed. There is no available reference tissue for *L. terrestris* tissue or excrement. The tissue extraction method was found to have a limit of quantification (LOQ) of 100 ppb and a linear range of 0.100 ppm to 20 ppm. Recovery studies were performed using unexposed organisms and a known amount of ESS to validate the efficiency of the method and were found to be 116%. Recovery amounts may be over 100% due to an unseen, consistent error or bias in the sample preparation. The excrement extraction method was found to have an LOQ of 1 ppb and a linear range 1 ppb to 500 ppb. Recovery studies using unexposed excrement and a known amount of ESS found the average recovery to be 80.65%. The soil extraction method has an LOQ of 10 ppb and a linear range of 10 ppb to 10 ppm. Many soil types, differing in the presence of PMMA and concentration of ESS were analyzed using the developed method. Accurate quantification of the amount of ESS in all soil types, the recovery method used was to take control soils with and without the plastic present and add a known amount of ESS to them. The average recovery using this method was calculated to be 74.72%.

ESS in worm tissue has been studied in *Eisenia fetida*, commonly known as the tiger worm by Park et.al. in 2012.154 The extraction method added phosphate buffer to the tissue and homogenized the sample followed by filtration and centrifugation. The pellet was what was resuspended in buffer for analysis.154 Our method differs using DCM rather than phosphate buffer as the extraction solvent and the removal of gut contents is done prior to extraction of the organism. Our first tissue extraction method did not remove the gut contents prior to extraction and interference in the tissue ESS concentrations was observed. The GC-ECD methods used for
detection and quantitation differed in the use of 30-m column by Park et.al. and a 60-m column by us. The use of a longer column allows for better separation of ESS from potential interferences due to the increase of the number of theoretical plates in the column as compared to a traditional 30-m column. A longer column may also result in a longer analysis time than if a traditional 30-m column was used.

ESS have been studied in reference soil regarding potential identification in landfill leachate. The developed method by Manier et.al. in 2012 was used to look for a wide variety of compounds including heavy metals and polyaromatic hydrocarbons (PAHs). ESS was one of many compounds that they attempted to quantify in the reference soil. The concentration was less than 0.001 mg/kg Ms, the established LOQ. The details of their extraction methods of the soil and leachate is unclear, as the study was focused on the impact of the highest quantified compounds in the leachate on two different organisms. Zhang et.al. extracted sediment collected from a marine environment to determine several HOC concentrations. They established a method limit of detection (MDL) to be 1.4 pg/g dry weight. Both our method and the Zhang method used sonication to extract the pollutants. A key difference in the Zhang method and our developed method using ultrasonic sonication are the solvents used. The Zhang method used a 1:1 composite of hexane and DCM for extraction whereas our method used only hexane. DCM was attempted to be used as an extraction solvent in our method but was deemed unsuitable due to the presence of the plastic. PMMA exposed to DCM was found to dissolve after several hours. The PMMA formed a sticky film coating when the samples were reconstituted in hexane for analysis. The warping of the plastic would interfere with the ability to accurately quantify the ESS in the soil. A similar interference was observed in the worm tissue using our first developed
extraction method and proven with subsequent experiments discussed above. The Zhang et.al. method did not have to be concerned with the impact of DCM on the structure of PMMA whereas our method did have to take that into account. Park et.al. analyzed soil containing endosulfan that *E. fetida* were exposed to for several endosulfan oxidation products including ESS. The soils were extracted in acetone by mechanical shaking for 9 hours total.

Our novel extraction method differs from both the Manier et.al. and Zhang et.al. methods in that our method was tailored for maximum extraction and detection of only one pollutant, ESS, rather than several of different compound classes. Our method also differs through the use of GC/MS for detection and quantification of ESS rather than GC-ESI-MS/MS as used by Zhang et.al. One key difference between our developed method and the one used by Park et.al. is the total time it takes to extract a sample. Each soil sample extracted using our method took 20 minutes of sonication in a benchtop sonicator versus a total of 9 hours for the Park method.

Park et.al. also extracted excrement produced by *E. fetida* during their experiments. The samples were extracted using acetone with a homogenizer followed by centrifugation and evaporation. Our method differed in the use of hexane as compared to acetone. Hexane was used as it was known not to interfere with the determined concentration of ESS through warping of the plastic structure. Acetone would need to be verified to not interfere before it could used on excrement containing PMMA.

These methods were used to analyze data from the Hoang earthworm exposures and quantitate the amount of ESS present in all related media once they were validated through the recovery methods. Analysis of the data using these methods inspired the Chiarelli group to examine possible surface chemistry explanations for what was observed. Data collected from
low concentration exposures suggests that ESS has a greater affinity for the hydrophobic PMMA surface than the hydrated worm tissue. Data collected from high concentration exposures suggests that after a specific, unknown concentration is reached, the interaction between the PMMA surface and ESS molecules weakens considerably. This would facilitate easier removal of the ESS molecules from the polymer beads.

Saturation of PMMA particle surfaces with ESS could lead to a significant leaching of ESS into the surrounding media. The ESS would congregate and bind to the hydrophobic PMMA. The outer layers of ESS would be less tightly bound to the PMMA. These lightly bound layers would require less energy to desorb from the PMMA than the inner layers. Further bead adsorption studies should be conducted to further prove or disprove this hypothesis.

Studies using both hydrophilic and hydrophobic compounds should be performed to aid in the determination if PMMA could act as a sink for specific compound classes rather than exposure organisms to potentially toxic compounds. Compounds with a range of polarities and halogens mirroring those found in landfill sites and other areas of congregated plastic debris should be tested under controlled conditions. Studies could also be performed using a range of hydrophilic and hydrophobic polymers with a chosen set of pollutants. Each set of proposed studies would expand our knowledge on the surface interaction between polymers and pollutants that have varying degrees of hydrophilicity and hydrophobicity.
CHAPTER FOUR
NOVEL METHOD DEVELOPMENT FOR DETECTION AND QUANTIFICATION OF PCB 126 FROM ORGANISM TISSUE, WATER, AND POLYETHYLENE POLYMER BEADS

RESULTS

Introduction

We have analyzed the aquatic organism Daphnia magna following the Hoang group’s exposure to two dioxin-like PCBs in aqueous solutions with and without polyethylene (PE) beads present to determine the PCB concentrations in the organism tissue, water, and PE beads. The original hypothesis as proposed by the Hoang group was that D. magna exposed to 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) and 2,3,3’,4,4’,5,5’-heptachlorobiphenyl (PCB 189) in moderately hard water in the presence of PE beads would absorb a greater amount of the PCBs than daphnia exposed to the PCBs in only moderately hard water. My research goal was to develop novel methods for the analysis of samples related to the exposures of the daphnia. D. magna were chosen as the model organisms by Dr. Tham Hoang for this exposure for several reasons. They are rapidly producing filter feeders that will ingest plastic particles as they seek natural food sources and have been documented to uptake small plastic litter.157 Daphnia are well studied as a standard test organism in ecotoxicological studies as well.158 The large amount of toxicological data available on D. magna and their documented ability to ingest small plastic litter made them a model organism for our study of the potential transfer of toxic adsorbed pollutants in freshwater. PE was selected for the model plastic used in these exposures. It is one
of the most produced plastics and one of the frequently identified types of plastic debris in marine water.\textsuperscript{5} PE is most often used in packaging such as plastic bags, films, and bottles. All these items are frequently discarded as waste, generating multiple potential pathways for PE to enter freshwater. Ripping and tearing along with photo-oxidative processes facilitate the breakdown of PE into microplastic fragments. The diameter of the PE beads used in our exposures was 50-75 µm which are small enough for daphnia to ingest.\textsuperscript{157,159}

The removal of PCBs 18, 40, 128, and 209 from daphnia in the presence of fluorescent green microspheres, FMG, a proprietary polymer of Cospheric LLC, at a diameter of 1-5 µm was studied in daphnia.\textsuperscript{160} Neonates were exposed to a mixture of the four PCBs for four days followed by a PCB free diet with FMG present. The removal of the PCBs from the neonate tissue was four times faster in daphnia exposed to FMG microspheres than in aqueous solution alone. This suggests that plastic debris may serve as a sink for hydrophobic, relatively high molecular weight compounds.\textsuperscript{160} None of the five PCBs in these studies are dioxin-like.\textsuperscript{160,161} The use of highly chlorinated compounds for our exposures would provide low limits of detection for GC-ECD analysis, allowing the study of more environmentally relevant concentrations. PCBs 126 and 189 were chosen as model compounds for this study due to their dioxin-like toxicity, differences in solubilities, amenity to GC-ECD analysis, and lack of specific study of them in daphnia.

Herein I will discuss the results of the analysis of the organisms, PE beads, and water used to conduct the three exposures by the Hoang group. The first study exposed daphnia to 1 µg/L of PCBs 126 and 189 each. The results of the first exposure led to the decision to proceed with only one analyte, PCB 126, for the second and third exposures. The second exposure used a
concentration of 1 µg/L. The third exposure used an increased concentration of 5 µg/L. The concentration was increased to above the solubility limit to ensure the PCB would adsorb on the PE during the time of exposure. The Hoang group provided samples from all exposures for the development and refinement of analytical methods for the detection and quantification of PCBs in the various matrices. The quantification of PCB 126 in these numerous matrices led to the development of a new hypothesis based on the surface chemistry interactions. We propose that the interaction between the hydrophobicity of the plastic surface and the chosen compounds regulates the transfer of the compounds to the tissues. The hydrophobic plastic surface should bind strongly to hydrophobic compounds, decreasing the amount that is transferred to the tissues. A plastic with a hydrophilic surface of the same particle size would transfer a larger amount of the compound to the tissue compared to the hydrophobic plastic. I end the chapter with proposing an experiment to test this new hypothesis.

**Determination of PCBs 126 and 189 Concentrations in *D. magna* Tissue, Water, and PE Beads Following the Hoang Group’s First Exposure**

Standards of PCBs 126 and 189 were analyzed to determine retention times (Figures 26, 27). Limits of detection for both analytes were found to be 1 ng/µL. Control samples were then analyzed as method blanks to ensure no interferences were present at quantifiable levels (Figure 28). Samples derived from the exposures were analyzed next for preliminary analysis of pollutant uptake identification within the organisms. No recovery studies were performed for any sample types for this exposure as it was an exploratory study to determine in what matrices the PCBs were detected. No quantification was performed.
**Determination of PCBs 126 and 189 Concentrations in D. magna Tissue**

We were given samples from the Hoang group pertaining to a bioaccumulation exposure study. There were two sample types: controls and trials. The trial sets differed by the presence of PCBs 126 and 189 in addition to the presence of the polymer. Our goal for these samples was to determine if PCB 126 and 189 were detectable. Both trial samples contained quantifiable levels of PCB 189 and no detectable levels of PCB 126 (Figure 29).

**Determination of PCBs 126 and 189 Concentrations in Water**

Water samples were obtained from the Hoang group pertaining to a bioaccumulation exposure study. There were two sample types, controls and trials. The control samples were prepared by the Hoang group with the salt and food mixture described in Appendix A. The organism and polymer were not present in these samples. Post-exposure water samples were collected from the Hoang group once the organisms were removed and filtered once to collect the polymer. All samples from both sample types were analyzed. The trial samples showed detectable quantities of PCB 126 and no detectable levels of PCB 189 (Figure 30).
Figure 26. GC-ECD chromatogram of a 1 ng/µL PCB 126 standard. 1 µL was injected for analysis.
Figure 27. GC-ECD chromatogram of a 1 ng/µL PCB 189 standard. 1 µL was injected for analysis.
Figure 28. GC-ECD chromatogram of daphnia exposed to moderately hard water (method blank). No signals are observed at retention times corresponding to PCBs 126 or 189.
Figure 29. GC-ECD chromatogram of daphnia tissue extract displaying a positive signal for PCB 189 and no detectable signal for PCB 126. Daphnia were exposed to 1 µg/L of PCB 126 and 1 µg/L of PCB 189 in the presence of PE.
Figure 30. GC-ECD chromatogram of a daphnia water sample extract displaying a positive signal for PCB 126 and no detectable signal for PCB 189. Daphnia were exposed to 1 µg/L of PCB 126 and 1 µg/L of PCB 189 in the presence of PE.
Figure 31. GC-ECD chromatogram of PE bead extract displaying a positive signal for PCB 189 and no detectable signal for PCB 126. Daphnia were exposed to 1 µg/L of PCB 126 and 1 µg/L of PCB 189 in the presence of PE.
Determination of PCBs 126 and 189 Concentrations on PE Beads

Polymer beads collected from the water samples provided by the Hoang group pertaining to a bioaccumulation study. These beads were only from the trial samples of the study. The samples showed detectable quantities of PCB 189 and no detectable levels of PCB 126 (Figure 31).

Determination of PCB 126 Concentration in D. magna Tissue, Water, and PE Beads

Following the Hoang Group’s Second Exposure

PCB 126 was selected as the sole analyte for the second exposure for several reasons. It is 10 times more soluble than PCB 189. PCB 126 is over 300 times more toxic than PCB 189 based on their TEF values. This increased toxicity has led to PCB 126 being studied in greater detail with regards to toxicity effects on a variety of species. It was based on these reasons that we decided to proceed with the second exposure using PCB 126 as the only analyte.

Determination of PCB 126 Concentration in D. magna Tissue

Organism samples were obtained from the Hoang group which pertained to a bioaccumulation exposure study. There were two trial sample sets analyzed. They were related by the concentration of PCB 126 and differed in the presence of a polymer. The two sets were expected to have differing concentrations. Our developed extraction methods allowed for both analysis and quantitation of PCB 126 in all organism samples. The quantitation of the trial sample sets is summarized in Figure 32. The trial sample set exposed to PCB 126 in the presence of the polymer had a higher concentration than organisms exposed to only PCB 126.

Neonates (young organisms) were also presented by the Hoang group for analysis. Quantifiable signal was obtained from both trial sets of neonates. An accurate weight was not
obtained for the samples as there were too few to register on the analytical balance used. It had a minimum weight limit of 0.1 mg. Neonates from the trial sample set exposed to PCB 126 in the presence of polymer contained 0.1 ng PCB 126, with a concentration of 1 ppb based on a weight of 0.1 mg. Neonates from the trial sample set exposed to only PCB 126 contained 10.4 ng PCB 126, with a concentration of 104 ppb based on a weight of 0.1 mg. The extraction method was proven to work on both adults and neonate daphnia.

It was observed prior to analysis of the trial samples exposed in the presence of a polymer still contained the polymer. Bright green polymer particles were clearly visible in samples before extraction and remained in the sample tubes post extraction (Figure 33). The polymer may have contributed to the total PCB concentration found in the trial sample set. Removal of the polymer from the sample set prior to extraction was attempted for future samples but was unsuccessful.

**Determination of PCB 126 Concentration in Water**

The Hoang group provided water samples related to a bioaccumulation study. There were two sample sets for analysis. The sample sets differed by the presence of organisms prior to extraction and analysis. Each sample set contained two subsets that were related by PCB 126 concentration and differing by the presence of the polymer. The two sample sets were expected to have differing concentrations. The quantitation of the two sample sets is summarized in Figure 34. The sample set that was unexposed to organisms had a higher relative concentration than the sample set exposed to organisms. Samples within the set without the organism present were expected to have the same concentrations of PCB 126. It was not observed in the bulk of the sample. Statistical analysis would need to be performed on the sample values within each subset to determine if they are significantly statistically different with a p-value $=\ll 0.05$.  


Recovery studies were performed using prepared hard water provided from the Hoang group with a known amount of PCB 126 (1 µg) added in and homogenized. Samples were extracted using the solid phase extraction (SPE) cartridge method described in Chapter 2. Sample analysis was performed under the sample GC-ECD conditions used to analyze water samples from the Hoang exposures. The average percent recovery was determined to be 76.25%. The values for the water concentrations from the Hoang exposure were adjusted accordingly.

**Determination of PCB 126 Concentration on PE Beads**

Beads were collected from water samples obtained the Hoang group pertaining to a bioaccumulation study. There was only one sample set for analysis. Several aliquots for each sample within the set was analyzed. No detectable level of PCB 126 in any of the samples. Recovery studies were not performed because of the lack of signal within the sample set.

**Discussion**

Analysis of sample sets using the developed extraction techniques yielded quantifiable levels of PCB 126 in the organism tissue and water. No detectable levels of PCB 126 were found on the polymer beads and the developed method was not verified via a recovery study due to the lack of signal. Based on the initial amount of PCB 126 present, 1 µg/L per the Hoang group, 55% of the available PCB 126 was found in the *D. magna* tissues, 12.9% in the water media, 0% on the PE beads and 31.6% unaccounted for within the 3 examined media. We hypothesized that the lack of quantifiable PCB 126 on the exposed PE in part due to the initial concentration of PCB 126 (1 µg/L) but wanted to confirm that the PCB would adsorb to the bead surface. We designed the adsorption study discussed below to determine if PCB 126 would adsorb in quantifiable amounts.
Figure 32. Bar graph displaying the *D. magna* organism tissue concentrations of the two trial sample sets following the second Hoang exposure.
Figure 33. Daphnia from a sample subset exposed to PCB 126 in the presence of the polymer. The gut is filled with highly visible green PE microspheres. Picture provided by an undergraduate in Dr. Tham Hoang’s lab.
Figure 34. Bar graph displaying the PCB 126 concentrations determined for the water sample sets from the second Hoang exposure. Samples for day 7 T1 (+PE) and day 21 T2 (no PE) were not analyzed due to errors that occurred during the sample workup.
PE Adsorption of PCB 126 Study

Small quantities (10 mg) of PE polymer beads exposed to high concentrations of PCB 126 were extracted and analyzed. A large quantity of PCB 126 (0.5 mg) was used compared to that in the exposures (0.25 ug) as the goal was to determine if measurable quantities of the analyte would adsorb to the surface of the beads. Three sequential extracts were analyzed. The first extracts showed the largest concentration of PCB 126, with a small quantity being present in the second extracts and no quantifiable PCB 126 was present in the third extracts (Figure 35). The results show that a single extraction of PE beads should yield an average 95% of PCB 126 present below 0.5 mg of exposure, with the remaining 5% removed by a second round of extraction. The data supports the observation made for the second exposure that PCB 126 was not present at detectable levels in the polymer bead sample set. A subsequent exposure study should be performed at a higher concentration of PCB 126 (greater than 1 µg/L) to determine if the PE beads are playing a role in the uptake of PCB 126 by the daphnia as proposed by the Hoang group in their study objectives. It would also allow for verification of our developed polymer bead extraction method.
Figure 35. Histogram displaying the percentage of PCB 126 removed from PE beads following each round of sonication in hexane.
Determination of PCB 126 Concentration in *D. magna* Tissue, Water, and PE Beads

Following the Hoang Group’s Third Exposure

**Determination of PCB 126 Concentration in *D. magna* Tissue**

Organism samples were obtained from the Hoang group which pertained to a bioaccumulation exposure study. Samples from this study were exposed to a higher concentration of PCB 126 (5 µg/L) than previous exposure studies conducted by the Hoang group (1 µg/L). There were two trial sample sets analyzed. They were related by the concentration of PCB 126 and differed in the presence of a polymer. The two sets were expected to have differing concentrations. Our developed extraction method facilitated analysis and quantitation of PCB 126 in all organism samples. The quantitation of the trial sample sets is summarized in Figure 36. The trial sample set exposed to only PCB 126 had a higher concentration than the trial sample set exposed to PCB 126 in the presence of the polymer. Neonates were counted using a confocal microscope by the Hoang group prior to extraction. Neonates from the trial sample set exposed to only PCB 126 had a concentration of 17 pg PCB 126/neonate. Neonates from the trial sample set exposed to PCB 126 in the presence of the polymer had a concentration of 38 pg PCB 126/neonate. Recovery studies for method verification have not been performed yet but should be performed on both adult and neonate daphnia that have not been exposed to PCB 126.

**Determination of PCB 126 Concentration in Water**

The Hoang group provided water samples for analysis that were related to a bioaccumulation study. The two sample sets presented for analysis differed by the presence of organisms prior to extraction and analysis. The sample set exposed to organisms contained two
subsets that were related by PCB 126 concentration and differed by the presence of a polymer. The two large sample sets were expected to have differing concentrations. The quantitation of the two sets is summarized in Figure 37. The sample set unexposed to organisms had a higher relative concentration than the sample set exposed to organisms. The two subsets exposed to organisms appear to have similar concentrations. Statistical analysis would need to be performed on the sample values of the two subsets to determine if they statistically different with a p-value \( \leq 0.05 \). Recovery studies were not performed as they were extracted using the developed and verified SPE method discussed within the previous Hoang exposure study.

**Determination of PCB 126 Concentration on PE Beads**

Bead samples were collected from water samples provided by the Hoang group related to a bioaccumulation study. Two sample sets were analyzed, a control and a trial. The control sample set gave no detectable signal for PCB 126. Figure 38 summarizes the quantitative results for the trial sample set. There is slight variation in the samples and statistical analysis would need to be performed to determine if they are statistically different with a p-value \( \leq 0.05 \).

Recovery studies were performed using virgin PE polymer beads of the same size and color used in the Hoang exposures. A known amount (500 µg) of PCB 126 was added to 0.0998 g PE beads and homogenized. Several 10 mg aliquots were removed and extracted using the established procedure outlined in Chapter 2. Analysis was performed under the same GC/MS conditions used to analyze the bead samples from Hoang exposures. The average recovery was calculated to be 76.3% for one 20-minute sonication. The values from the Hoang exposure were adjusted accordingly.
Figure 36. Bar graph displaying the *D. magna* organism tissue concentrations of the two trial sample sets following the third Hoang exposure.
Figure 37. Bar graph displaying the PCB 126 concentrations determined for the water sample sets from the third Hoang exposure.
Figure 38. Bar graph displaying the PCB 126 concentrations determined for the trial polymer sample set from the third Hoang exposure.
Conclusions

Novel methods for the extraction of PCB 126 from organism tissue, and PE polymer beads were developed. An established method for PCB extraction from water was modified and used for all water samples. The daphnia tissue extraction method was found to have a limit of quantification (LOQ) of 50 ppb and a linear range of 50 ppb to 900 ppb. Recovery studies have not yet been performed using unexposed adults and neonates with a known amount of PCB 126 added to the tissue prior to extraction. This should be performed for validation of the method. The PE polymer bead extraction method was found to have an LOQ of 5 ppb and a linear range of 5 ppb to 50 ppb. Recovery studies using virgin PE beads and a known amount of PCB 126 found the average recovery to be 76.3%. The water extraction method was modified from the method Sethi et al. published in 2017. The conditioning solvent was switched from methanol to DCM to remove any potential interferences already present on the cartridge before the sample was loaded onto it. This modified method was found to have an LOQ of 5 ppb and linear ranges of 5 ppb to 100 ppb and 400 ppb to 800 ppb. Recovery studies were performed using prepared hard water from the Hoang group with a known amount of PCB 126. The average recovery was found to be 76.25%.

Daphnia organisms were extracted by Ek et al. in 2016. They exposed daphnia to four PCBs and extracted them through sonication in hexane followed by the addition of sulfuric acid before evaporation for analysis. Our developed method differs in the extraction solvent used. We used DCM as an extraction solvent. DCM was selected over hexane as PCBs have a higher solubility in DCM. This use of a solvent with greater overall analyte solubility would increase the LOQ of the method.
A Soxhlet extraction is often used to remove pollutants from the surfaces of environmental plastics. This method requires several hours and the sample may need further purification using different types of chromatography to remove specific compound classes. Ogata et al. used a soaking method for quantification of several compound classes on environmental pollutants in 2009. Their method soaked several pellets in hexane for 72 hours twice then separated using silica gel chromatography prior to analysis. Frias et al. used an accelerated solvent extraction method in 2010. This method used a hexane acetone mixture (1:1) and high pressure (1500 psi) to extract the compounds from the surface of the plastics. It is then followed by further chromatography, similar to the Ogata method. The method developed by Zhang et al. in 2013 used ultrasonic sonication and hexane to extract collected pellets followed by further chromatography purification. These methods all differ from our developed method in that they were designed and optimized for the extraction and quantification of several classes of pollutants. This requires not only the first extraction method, but purification using further chromatography methods. The Soxhlet extraction and soaking in solvent methods require several hours to extract a single sample. The Frias et al. method requires specialized equipment that was not available during our experiments.

The Zhang method is very similar to our developed method. Both use sonication to remove the pollutants from the polymers. We reduced the required sonication time from 30 minutes to 20 minutes. We also used iso-octane as an extraction solvent rather than hexane. Our chosen pollutant, PCB 126, has greater solubility in iso-octane than hexane. The use of a solvent that the analyte has a greater solubility in would increase the LOQ. The Zhang method had to use
hexane as it is a suitable solvent for several of the commonly identified compound classes on plastics collected from a marine environment.98

These methods were used to analyze data from the Hoang D. magna exposures and quantitate the amount of PCB 126 present in all related media. Analysis of the data using these methods in addition to the results from the developed methods for ESS extraction in earthworm related media led the Chiarelli group to examine possible surface chemistry explanations for the interaction of hydrophobic molecules and the surface of the polymer beads. We propose these hydrophilic plastics will interact differently with hydrophobic compounds such as PCB 126 when compared to its interaction with hydrophobic plastics including PE.

We propose a second bead adsorption study utilizing two different types of plastic. Hydrophilic plastic such as acetyl cellulose or hydroxylated PE will be used in addition to PE used in all previous exposures (Figure 41). Our initial hypothesis for this adsorption study is that the hydrophilic plastics would have a lesser affinity for PCB 126 than the hydrophobic plastic when exposed to the same concentration under the same conditions. This would be due to the weaker surface bonding between PCB 126 and the hydrophilic plastic. The chosen hydrophilic plastic would be shredded to fit within the 50-75 µm particle size used in the previous adsorption study. Trials using different amounts of PCB 126 (0.5 mg, 1 mg, 2 mg, etc) should be conducted with each plastic. The total amount removed across several rounds of sonication should be compared between different plastics at each concentration to prove or disprove our hypothesis.

Figure 39. Chemical structure for acetyl cellulose.
CHAPTER FIVE
NONTARGETED ANALYSIS FOR ADSORBED POLLUTANTS OF PLASTICS EXPOSED IN SALT CREEK

Introduction

Synthetic polymers are a class of materials with a broad range of physical properties which are commonly referred to as plastics. Plastic production rates have accelerated since the 1950s.\(^5\) Up to one half of plastic produced each year is discarded, translating to up to 87 million tons thrown away in 2017. Accordingly, plastic debris accumulation has been documented in ecosystems worldwide.\(^{30,90,107,163-165}\) Plastic litter is exposed to a wide array of chemical pollutants that can adsorb to the surface at significantly higher concentrations than that of the surrounding water due to their shared hydrophobicity.\(^{40,99}\) These saturated plastic particles may have important implications for aquatic ecosystems.\(^{73}\) Identification of pollutants adsorbed to the surfaces of plastic debris in aqueous environments has been an important goal of recent research. Plastic additives used during the manufacturing process may pose a potential threat. Plastic litter consumed by organisms may desorb the toxic pollutants and additives directly into their digestive systems. Therefore, it is possible that small plastic litter may concentrate adsorbed chemicals and serve as a transport vector for potentially toxic pollutants to aquatic organisms, some of which are consumed by humans.
The majority of research conducted on adsorbed pollutants from plastic debris has been performed on plastics isolated from marine environments and has reported detection of wide variety of chemical compounds.\textsuperscript{87,92,94-98,166} The International Pellet Watch (IPW) collected plastic pellets and debris and suggested that they may serve as passive samplers to monitor targeted pollutant levels.\textsuperscript{92,96} Compounds that have been found adsorbed onto plastic debris thus far are primarily persistent organic pollutants (POPs) of limited water solubility. These include polychlorobiphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and its degradation products, hexachlorocyclohexane (HCHs) isomers, polyaromatic hydrocarbons (PAHs), polybromodiphenyl ethers (PBDEs) and the plasticizer bisphenol A (BPA).\textsuperscript{73,87,92,94-98} Pesticides have been found adsorbed to plastic litter as well. These include heptachlor, aldrin, endrin, endosulfan and the degradation product endosulfan sulfate.\textsuperscript{95,98}

Freshwater environments are an important and potentially overlooked site to examine the dynamics between plastic and common pollutants that may adsorb to plastic. Urbanization increases plastic pollution in freshwater ecosystems, and urban waterways are known to contain POPs.\textsuperscript{107} The determination of adsorbed chemicals on plastics in urban, freshwater environments may provide insight into the identities of unknown pollutants and mechanisms of transfer in these aquatic environments.

To our knowledge, only two studies of compounds adsorbed on plastics collected from a freshwater environment have been published. Faure et.al. (2015) performed a targeted analysis study on plastics collected from Swiss surface waters.\textsuperscript{51} The plastics were analyzed for over 50 known POPs including PCBs, PAHs, organochlorine pesticides (OCPs) and nonylphenols (NPs). Individual pieces of plastic were collected from surface waters of lakes and the surrounding
beaches. Samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography tandem mass spectrometry (GC-MS/MS). Compounds were identified through high resolution mass and retention time matching. The study demonstrated that the amounts of the targeted pollutant (ng pollutant class/g microplastic) could vary greatly between groups of plastic analyzed even when they are collected in close proximity. Plastics collected from the Raritan River watershed in New Jersey, USA were also analyzed for adsorbed compounds in a non-targeted study. Analysis was performed using gas chromatography coupled with low resolution, ion trap mass analysis. Compound identification was performed by spectral library matching. Compounds identified included 2-butanone, 4-(2,6-trimethyl-1-cyclohexen-1-yl) among others as adsorbed pollutants.

Here we describe the non-targeted analysis of POPs adsorbed on polyethylene (PE) sheets deliberately incubated in a tributary of the Chicago River for 142 days. Plastics were analyzed for adsorbed POPs using accurate mass gas chromatography time-of-flight mass spectrometry (GC-TOF/MS). POPs were identified through use of an algorithm in Mass Hunter software that identifies compounds based on their empirical formulae and monoisotopic mass. We describe the detection of the anti-microbial compound triclosan and its metabolite methyl triclosan for the first time in any plastic debris analysis recovered from aquatic environments.

**Plastics Exposed in Salt Creek Results**

**Compound Class Search**

We sought to determine if frequently targeted classes of compounds identified as adsorbed pollutants on plastics samples from both marine and freshwaters could be identified on the plastic incubated in Salt Creek. A review of these studies was performed to generate a list of
commonly targeted compound classes.\textsuperscript{51,72,73,87,88,92,95-101} Agilent Mass Hunter Qualitative Analysis Version B.07.00 software was used to perform a custom library search based on the monoisotopic mass of common compounds within each class. The find by formula feature was used to search for matching compounds within 10 ppm. The charge state was changed between common organic molecules and common organic molecules (no halogens) depending on the classes of compounds. Any suspected compound that fell below an overall score of 80 was discarded automatically.

Potential positive ion identification was limited to -electron and +H. Identification score was set to > 70%. Match tolerance for masses was +/- 10 ppm and for retention time was +/- 0.350 min. Maximum number of peaks in an extracted ion chromatogram (EIC) was limited to 5 largest in height. Spectra included average scans > 10% of peak height. Peak spacing tolerance was 0.0025 m/z plus 7 ppm for isotope grouping. Isotope model used either common organic molecules or common organic molecules (no halogens).

A non-targeted study was completed on 13 PE samples that had been incubated in Salt Creek for 142 days. All samples were analyzed with the GC-TOF/MS to generate high resolution data. Data analysis determined the potential identities of compounds adsorbed onto the PE during the incubation period. We searched the mass spectra for 62 empirical formulae representing 10 compound classes. The chosen compound classes were selected due to their presence in targeted studies looking for adsorbed pollutants on plastics sampled from either marine or fresh waters. The non-targeted search led to the detection and identification of both triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) and the transformation product methyl triclosan (4-chloro-1-(2,4-
dichlorophenoxy)-2-methoxy-benzene). This is the first observation of these two pollutants in any study searching for adsorbed plastic pollutants.

Specifically, the 10 compound classes searched for were: BPA, DDTs, HCHs, NPs, OCPs, OPFRs (organophosphate flame retardants), PAHs, PBDEs, PCBs, and phthalates. One empirical formula each for BPA, HCHs, and NPs was searched for. The remaining seven compound classes had several formulae searched for. Three empirical formulae for DDTs, four for OCPs, 24 for OPFRs, eight for PAHs, five for PDBEs, 10 for PCBs, and five empirical formulae for phthalates were searched for on all incubated plastic. Positive tentative identifications based on the formulae search are summarized in Table 4. Five classes of compounds were identified on at least one of the PE extracts analyzed in addition to triclosan and methyl triclosan: DDTs, NPs, PAHs, PBDEs, and PCBs. Identification attempts for specific compounds within the 5 identified compound classes has not been performed at this time.
Table 4. Compound classes detected on PE plastic debris exposed in Salt Creek. Thirteen total data files were searched.

<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Number of Empirical Formulae Searched For</th>
<th>Empirical Formula</th>
<th>Frequency of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlorodiphenyltrichloroethane (DDTs)</td>
<td>3</td>
<td>C14H8Cl4</td>
<td>3</td>
</tr>
<tr>
<td>Nonylphenols (NPs)</td>
<td>1</td>
<td>C15H24O</td>
<td>12</td>
</tr>
<tr>
<td>Polyaromatic Hydrocarbons (PAHs)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polybrominated Diphenyl Ethers (PBDEs)</td>
<td>5</td>
<td>C12H4Br6O</td>
<td>2</td>
</tr>
<tr>
<td>Polychlorinated Biphenyls (PCBs)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-chloro-2-(2,4-dichlorophenoxy)phenol</td>
<td>1</td>
<td>C12H7Cl3O2</td>
<td>12</td>
</tr>
<tr>
<td>(Triclosan)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-chloro-1-(2,4-dichlorophenoxy)-2-methoxy-benzene</td>
<td>1</td>
<td>C13H9Cl3O2</td>
<td>12</td>
</tr>
</tbody>
</table>
**Identification of Triclosan and Methyl Triclosan on Deliberately Exposed Plastic**

The antimicrobial compound triclosan was observed in the hexane extracts for 12 of the 13 analyzed samples. Triclosan has been observed in numerous wastewaters, freshwaters, and the related sediment.\textsuperscript{167-172} Figure 40 shows an EIC from the hexane extract from sample 13 and the mass spectrum of the compound eluting at 18.25 minutes. Figure 41 is composed of an EIC of a triclosan standard and the corresponding mass spectrum for the peak eluting at 18.27 minutes. The retention time of the compound found in the hexane extracts is consistent with that of the triclosan standard. The mass spectra of the triclosan standard and extract display the same isotope distribution pattern for compounds containing 3 chlorine atoms with the abundance ratio of 100:96:31 for the molecular ion (A), molecular ion +2 (A+2), and molecular ion +4 (A+4). Two fragment ions were compared in the extract and standard. M/z 251.9 is formed by the loss of hydrochloric acid (HCl) from triclosan and m/z 215.9 is formed by further loss of HCl. Both fragment ions were observed in the standard and PE extracts. The combination of the consistent retention time, identical isotope distribution, presence of the same fragment ions, and similar masses of the molecular ions support the conclusion that the environmental adsorbed pollutant can be identified as triclosan.
Figure 40. GC-TOF/MS **A**) Extracted ion chromatogram (EIC) of m/z 287.95 and **B**) mass spectrum of the compound eluting at 18.25 minutes in sample 13. The theoretical masses for the three most abundant ions are 287.9512 (A), 289.9482 (A+2), and 291.9453 (A+4). The ions have a difference of 4.18 ppm, 3.81 ppm, and 3.43 ppm respectively.
Figure 41. GC-TOF/MS A) EIC of m/z 287.95 and B) mass spectrum of a triclosan standard. The theoretical masses for the three most abundant ions are 287.9512 (A), 289.9482 (A+2), and 291.9453 (A+4). The ions have a difference of 1.39 ppm, 1.04 ppm, and 0.69 ppm respectively. 
Triclosan has not been observed as an adsorbed plastic pollutant in previous studies. It has been found to adsorb to polyethylene debris under controlled laboratory conditions. Wu et.al. studied the absorbance of triclosan to PE debris with other pharmaceuticals and personal care products under a variety of conditions. Salinity and dissolved organic matter (DOM) content were altered to determine the impact on the adsorption of each compound to the debris. Triclosan was found to bind more strongly under increased salinity conditions. It bonded weaker to PE under increased DOM content.

Triclosan has been used as an antimicrobial agent in a plethora of consumer products including plastic packaging. It has also been added to plastics for odor prevention, to lessen discoloration, and to inhibit degradation of the plastic. Reports suggest triclosan has been added to plastics in range from 0.6 to 10%. Triclosan will leach from plastics in nanogram quantities. Unexposed PE tarp was extracted and analyzed to detect triclosan that was potentially added to the plastic during the manufacturing process. No detectable quantities of triclosan or the transformation product methyl triclosan were found in the control samples. Methyl triclosan has not been used as an additive in plastics to date.

The compound methyl triclosan (2,4-dichloro-1-(4-chloro-2-methoxyphenoxy) benzene) was observed in 12 of the 13 samples. It has been observed in waste-water effluent in Germany, Portugal, and the United States. Figure 42 shows an EIC for m/z 301.9 from Sample 1 and the corresponding mass spectrum of the compound which elutes at 18.14 minutes. Figure 43 is the EIC for m/z 301.9 of a synthesized methyl triclosan standard and the corresponding mass spectrum for the peak eluting at 18.14 minutes. The retention times are consistent between the two. Analysis of the mass spectra of the unknown and standard reveal the same isotope
distribution pattern in abundance ratios for a compound containing 3 chlorine atoms. Experimentally, the masses of these ions in the methyl triclosan standard are 301.9677, 303.9648, and 305.9613. The ions for the compound in the hexane abstract are 301.9663, 303.9632, and 305.9607. Both the standard and extract compound differ from the theoretical masses of methyl triclosan by less than 3 ppm. Fragmentation ions derived by the loss of chlorine (m/z 267.0) and further loss of CH₃Cl (m/z 251.9) were observed in both the standard and PE extracts. The combination of consistent retention times, identical isotope distribution, presence of the same fragment ions, and similar masses of the molecular ions support the conclusion that the second environmental adsorbed pollutant can be identified as methyl triclosan.

Triclosan has been shown to undergo methylation by the fungus *P. cinnabarinus*. Researchers have also discovered that triclosan will derivatize to form methyl triclosan in activated sludge used in wastewater treatment plants under aerobic conditions. We hypothesize that triclosan may be derivatized by bacteria that collected as a biofilm formed on plastic litter. The mechanism for the derivatization of triclosan into methyl triclosan should be explored further.
Figure 42. GC-TOFMS A) EIC of m/z 301.900 and B) mass spectra of the compound eluting at 18.14 minutes in Sample 1. The theoretical masses for the three most abundant ions are 301.9668, 303.9639, and 305.9609 respectively. The ions have a difference of 0.00 ppm, 0.33 ppm, and 0.98 ppm respectively.
Figure 43. GC-TOF/MS EI A) EIC of m/z 301.90 and B) mass spectrum of a methyl triclosan standard. The theoretical masses for the three most abundant ions are 301.9668, 303.9639, and 305.9609 respectively. The ions have a difference of 0.00 ppm, 0.33 ppm, and 0.98 ppm respectively.
Methyl Triclosan Transformation Study Analysis

The purpose of next experiment was to determine if the conversion of triclosan into methyl triclosan was faster adsorbed onto PE. Virgin PE tarp was incubated in water collected from the North Branch of the Chicago River with added triclosan (1 mg/mL). Our hypothesis is that the microbes present in the natural waters are responsible for the conversion of triclosan into methyl triclosan. We attempted to increase the number of microbes in the water believing that if the hypothesis was correct, the conversion of triclosan to methyl triclosan would be accelerated. We performed an exposure after increasing microbial growth using tryptic soy broth (22.5 g) as the growth media in a North Branch water sample (1500 mL). The sample was split into three replicates. The optical densities were measured of each at 480 nm. Samples were agitated for 24 hours on a shaker at room temperature then the optical densities were remeasured. Triclosan and methyl triclosan were added directly onto the surface of the PE and the solvent evaporated off before placed into the three chambers with the grown microbes. The optical density had to show a tenfold increase in concentration before the PE was added (Table 5).

Samples collected over 18 days showed a decrease in triclosan concentration but no detectable methyl triclosan (Figure 44). Water aliquots (10.00 mL) were drawn off the microbe exposure chambers after 16 days and extracted. They contained no detectable methyl triclosan. A limit of quantification was established at 0.1 mg/mL of methyl triclosan (Figure 45). The source of methyl triclosan in Salt Creek should be further investigated.

Table 5. Measured optical densities of waters collected from Salt Creek containing added tryptic soy broth over 72 hours.
Figure 44. GC/MS EICs for PE extract following 18 days of grown microbial exposure of A) m/z 288.00 and B) m/z 218.00 corresponding to triclosan then C) m/z 252.00 and D) m/z 352.00 corresponding to methyl triclosan.
Figure 45. GC/MS EICs of A) m/z 302.00 and B) m/z 252.00 for a 0.01 mg/mL methyl triclosan standard, limit of quantification. 1 µL was injected for analysis.
Discussion

**Comparison of Data to Published Literature.** There are several variables that must be taken into consideration when comparing our results to those from the two published freshwater studies. The first of those is surface area as this plays a key role in the quantity of adsorbed pollutants present on the plastic when sampled. Unfortunately, we cannot compare the surface areas of the plastics sampled in the three studies. The study of plastics collected from New Jersey waters looked at particles smaller than 5 mm. Less than 1 mg of microplastics was analyzed for chemical pollutants in each run.\textsuperscript{110} The shape and size of the plastics collected are not given, so surface area cannot be determined. The Swiss study examined micro-and macroplastics. The Swiss study examined 100 mg of 25 subsamples for adsorbed pollutants.\textsuperscript{51} Plastic surface area should be considered when attempting to identify and quantify pollutants. The larger the surface area, the greater the quantity of adsorbed pollutants.

Consideration should also be given to the local populations adjacent to the sampling sites. All three studies have analyzed plastics exposed in polluted urban waters. Each study had a highly populated city adjacent to the sampling location. The Swiss study sampled close to Zurich, the New Jersey study close to New Brunswick, and our study sampled near Chicago.\textsuperscript{51,110} These cities have widely different populations but a very similar population density (Table 6). The population sizes partially dictate the amount and classes of pollutants into freshwater systems. Differences in population sizes or density may account for differences in observed compound classes. Larger populations and densities would facilitate larger concentrations of pollutant compound classes in the surrounding environment. This could cause an increase in
adsorbed pollutant concentration for plastics exposed for the same duration in the freshwater systems.

Table 6. Comparison of the population and population densities of the three largest cities closest to the sampling sites in the three freshwater adsorbed pollutant studies. Data taken from the 2010 US Census and the Federal Statistical Office of Switzerland.

<table>
<thead>
<tr>
<th>City</th>
<th>Population (2010)</th>
<th>Population Density (people/km²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zurich (Switzerland)</td>
<td>415215</td>
<td>4700</td>
</tr>
<tr>
<td>Chicago (USA)</td>
<td>2695598</td>
<td>4572</td>
</tr>
<tr>
<td>New Bruinswick (USA)</td>
<td>55181</td>
<td>4075</td>
</tr>
</tbody>
</table>

Exposure duration must be taken into consideration when comparing the three studies. Our plastics were deliberately incubated for 142 days or 4.5 months. We do not know how long the plastics from the Swiss and New Jersey studies were exposed before collection. The plastics could have been present in the waterways for some time before being sampled. They would also undergo weathering, which could increase the likelihood of hydrophobic pollutants favorably adsorbing to the surfaces.

**Conclusion**

Virgin PE tarp was exposed in local Chicago freshwaters for 142 days then screened for commonly identified classes of adsorbed pollutants as well as novel freshwater pollutants using GC-TOF/MS. Five of the 10 compound classes screened for had positive identifications based on library matching. The antimicrobial compound triclosan and the transformation product methyl triclosan were identified as adsorbed pollutants on plastics collected from aquatic ecosystems for the first time.
APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER TWO
The laboratory of Dr. Tham Hoang in the Institute of the Environmental Sustainability (IES) at Loyola University Chicago provided the original hypotheses for both organism exposure studies and carried out the design and organism care. Samples were retrieved from Dr. Hoang’s group following the conclusion of the organism exposures for method development and pollutant quantification in all media. Two exposures were conducted for the earthworm exposure to endosulfan sulfate (6,9-Methano-2,4,3-benzodioxathiepin, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-, 3,3-dioxide, ESS) study and three exposures were conducted for the daphnia exposure to two polychlorobiphenyls (PCBs), specifically 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) and 2,2’,3,3’,4,4’,5-heptachlorobiphenyl (PCB 189). The necessary materials and the overall design of each exposure is described below.

**Daphnia Materials**

Both PCB 126 and PCB 189 were acquired from AccuStandard and dissolved in octane (Reagent grade, Thermo Fischer Scientific). The final concentrations of PCBs 126 and 189 in the exposure chambers were 1 µg/L each for the initial exposure. The second exposure utilized only PCB 126 at a final concentration of 1 µg/L. PCB 126 was dissolved in dimethyl sulfoxide (DMSO, VWR International, Radnor, Pennsylvania, USA) at a concentration of 5 mg/mL for use in the third exposure. A dilution was made to a concentration of 0.1 mg/mL for dosing the exposure samples to a final concentration of 5 µg/L. Moderately hard water was prepared by dissolving salts (CaSO\textsubscript{4}×2H\textsubscript{2}O, MgSO\textsubscript{4}, NaHCO\textsubscript{3}, and KCl) to achieve final concentrations of 96 g Na\textsuperscript{+}/mL, 60 g Ca\textsuperscript{2+}/mL, 60 g Mg\textsuperscript{2+}/mL, 4.0 g K\textsuperscript{+}/mL in distilled water.\textsuperscript{1} *Daphnia magna* were from the Hoang group (IES, Loyola University Chicago, IL, USA) culture. Fluorescent green PE polymer beads size 63-75 µm in diameter and density 1.005 g/cc were acquired from Cospheric
LLC. Daphnia testing was conducted under standard laboratory conditions: 23 ± 2°C, light:dark = 16hr:8hr, and a feeding regime of 0.1 mL of both algae (*Selenastrum capricornutum*) and yeast-trout chow-cerophyl (YTC) per organism per day.

**Daphnia Exposure Study Designs**

**First Exposure.** Five test chambers were used to conduct the initial exposure. There were three control chambers and two trial chambers, each filled with 250 mL of prepared hard water. PE beads (12.5 mg) were added to each chamber for a final concentration of 50 mg/L. Ten adult daphnia were placed in each chamber. PCBs 126 and 189 were added to the trial chambers at a final concentration of 1 µg/L with methanol (250 µL) added to aid in PCB solubility. Organisms were exposed for three weeks. Neonates were collected daily to ensure population control. Adult daphnia and PE beads were collected at the end of the exposure period. Water and PE in all chambers had to be replaced every two days due to algae growth. Fresh waters were prepared to the same conditions and equilibrated for 24 hours before replacement. Water samples were collected at the end of the three weeks for PE isolation and analysis of PCB 126 concentration in both water and PE. Daphnia were stored at -2°C, water samples were stored at 4°C, and PE samples were stored at room temperature prior to analysis.

**Second Exposure.** Twenty test chambers were used in total for five exposure types with four replicates each. The exposure types were: negative control (hard water), positive control 1 (hard water and DMSO), positive control 2 (hard water, DMSO, and PE), trial 1 (hard water, DMSO, PE, PCB 126), and trial 2 (hard water, DMSO, and PCB 126). PCB 126 was the only analyte studied in the exposure (target final concentration 1 µg/L). DMSO rather than methanol was used to aid in solubility. Previous studies exposing daphnia to haloperidol and PCBs utilized
DMSO as their analyte solvent. DMSO never composed more than 0.1% of the total volume of the test water. No adverse effects to daphnia were observed in the control studies of either experiment.\textsuperscript{2,3} The PE concentration (12.5 mg/250 mL), number of adult daphnia (10), and exposure duration (three weeks) were held constant from the first exposure. Neonates were collected daily to ensure population control. Adult organisms were exposed for three weeks. Replicate chamber water and PE were replaced every two days due to algae growth. Fresh water was prepared to the same conditions and equilibrated for 24 hours before replacement. Water samples were collected from Days 0, 7, 14, and 21 for PE isolation and analysis for the concentration of PCB 126 for both water and beads. Daphnia were stored at -2\textdegree C, water samples were stored at 4\textdegree C, and bead samples were stored at room temperature prior to analysis.

Third Exposure. Twenty test chambers were used in total for 5 exposure types with 4 replicates each. The exposure types were: negative control (hard water), positive control 1 (hard water and DMSO), positive control 2 (hard water, DMSO, and PE), trial 1 (hard water, DMSO, PCB 126), and trial 2 (hard water, DMSO, PCB 126, and PE). PCB 126 was the only analyte studied (target final concentration 5 µg/L). The PE concentration (12.5 mg/250 mL), number of daphnia (10), and exposure duration (three weeks) were the same as the previous studies. Neonates were collected daily to ensure population control. Adult daphnia were collected following the conclusion of the exposure period. Replicate chamber water and PE were replaced every two days due to algae growth. Fresh water was prepared and equilibrated for 24 hours before replacement. Water samples were collected from Days 0, 7, 14, and 21 for PE isolation and analysis of PCB 126 concentration in both the water and PE. Daphnia were stored at -2\textdegree C,
water samples were stored at 4°C, and bead samples were stored at room temperature prior to analysis.

**Earthworm Materials**

Endosulfan sulfate (6,9-Methano-2,4,3-benzodioxathiepin, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-, 3,3-dioxide) was acquired from Sigma Aldrich and used without further purification. ESS was added to the soil at final concentrations of 3.11 mg/kg for the low spike and 45.4 mg/kg for the high spike soils. Worms were acquired from Carolina Biological Supplies (Burlington, NC, USA). Soil was acquired from the Loyola University Ecology and Retreat Campus (LUREC) in Woodstock, Illinois, USA.

**Earthworm Exposure Study Designs**

**Initial Exposure.** Five soil chambers were set up for the various soil types: a control soil, a low concentration of ESS, a low concentration of ESS with PMMA, a high concentration of ESS, and a high concentration of ESS with PMMA in the soil. Soils had final target concentrations of 3.11 mg ESS/kg soil for low spike and 45.4 mg ESS/kg soil for high spike soils. All soils were equilibrated for several weeks then divided into four smaller replicates for 20 testing chambers total. Organism sampling was conducted over 21 days. At the start of the exposure a total of 15 worms were placed in each testing chamber. Three organisms were present to account for any mortality that occurred over the 21 days. In total, 300 worms were sampled during the experiment. Unexposed worms were analyzed as method blanks. Organisms were placed in a -80°C freezer for storage until analysis.

**Second Exposure.** Soil was prepared with ESS and PMMA concentrations held constant from the first exposure study. There were three additional modifications to the sampling set up.
Day three sampling was eliminated. This changed the number of worms placed into each replicate to 12, with three worms present again to account for any mortality that occurred. The second modification was the addition of a control soil (no ESS) with PMMA present, raising the total soil types to six. The first two modifications resulted in a total of 288 worms sampled over 21 days. The third modification occurred post-sampling of the organisms. Worms from each replicate were placed in a separate jar overnight to collect all excretion. They were then transferred to a new container. Worms and excrement were stored at -80°C until analysis.
APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER FIVE
Triclosan and methyl triclosan were searched for as possible adsorbed pollutants in each of the 10 samples. A search for potential nonylphenols and polychlorobiphenyls (PCBs) was conducted. The nonylphenol molecular ion of m/z 220 was searched for as well as the common fragment ions m/z: 107, 121, 135, and 149. Lacking the molecular ion did not eliminate the sample as a potential hit. The molecular ion of each class of PCB containing between 1-10 chlorine atoms were searched for. Common molecular ions for BPA, DDTs, HCHs, OCPs, OPFRs, PAHs, and PBDEs were searched for based on those identified in other freshwater and marine adsorbed pollutant studies.

**Sample 1**

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PBDEs

Figure 46.  
A1) Extracted ion chromatogram (EIC) of m/z values 287.95, 289.95, 291.94 and A2) an electron impact (EI) mass spectrum of triclosan identified in Sample 1.
Figure 47. **B1**) EIC of m/z values 301.96, 303.96, 305.96 and **B2**) an EI mass spectrum for methyl triclosan identified in Sample 1.

Figure 48. **C1**) EIC ranging from 15.06-15.68 mins for m/z values 107, 121, 135, 149 and **C2**) an EIC ranging from 15.06-15.68 mins for m/z 220. EI mass spectra of possible nonylphenols at retention times **C3**) 15.110 mins, **C4**) 15.181 mins, **C5**) 15.232 mins, **C6**) 15.290 mins, **C7**) 15.330 mins, **C8**) 15.401 mins, **C9**) 15.476 mins, **C10**) 15.527 mins, and **C11**) 15.601 mins in Sample 1.
Figure 49. **D1)** EIC of m/z values 315.93, 316.94, 317.93 and **D2)** an EI mass spectrum for a DDE isomer identified in Sample 1.

Figure 50. **E1)** EIC of m/z values 202.0777, 203.0855, and **E2)** an EI mass spectrum for fluoranthene identified in Sample 1. **E3)** EIC of m/z values 228.0934, 229.1012, and **E4)** an EI mass spectrum for chrysene identified in Sample 1. **E5)** EIC of m/z values 166.0777, 167.0855, and **E6)** an EI mass spectrum for fluorene identified in Sample 1.
No PCBs or PDBEs were found in Sample 1.

**Sample 2**

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PDBEs

Figure 51. **A1)** EIC of m/z values 287.95, 289.95, 291.94, and **A2)** an EI mass spectrum of triclosan identified in Sample 2.

Figure 52. **B1)** EIC of m/z values 301.96, 303.96, 305.96 and **B2)** an EI mass spectrum for methyl triclosan identified in Sample 2.
Figure 53. **C1)** EIC ranging from 15.06-15.68 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 15.06-15.68 mins for m/z 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.111 mins, **C4)** 15.179 mins, **C5)** 15.223 mins, **C6)** 15.287 mins, **C7)** 15.331 mins, **C8)** 15.399 mins, **C9)** 15.429 mins, **C10)** 15.473 mins, **C11)** 15.524 mins, and **C12)** 15.602 mins in Sample 2.

No DDTs were observed in Sample 2.
Figure 54. **E1)** EIC of m/z values 228.0934, 229.1012, and **E2)** an EI mass spectrum for chrysene identified in Sample 2. **E3)** EIC of m/z values 202.0777, 203.0855 and **E4)** an EI mass spectrum for fluoranthene identified in Sample 2.

No PCBs or PBDEs were observed in Sample 2.
Sample 3

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 55. A1) EIC of m/z values 287.95, 288.95, 291.94 and A2) an EI mass spectrum for triclosan identified in Sample 3.

Figure 56. B1) EIC of m/z values 301.96, 303.96, 305.96 and B2) an EI mass spectrum for methyl triclosan identified in Sample 3.
Figure 57: **C1)** EIC ranging from 15.06-15.70 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 15.06-15.70 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.112 mins, **C4)** 15.183 mins, **C5)** 15.234 mins, **C6)** 15.288 mins, **C7)** 15.332 mins, **C8)** 15.403 mins, **C9)** 15.474 mins, **C10)** 15.525 mins, and **C11)** 15.603 mins in Sample 3.

Figure S58. **D1)** EIC of m/z values 315.93, 316.94, 317.93 and **D2)** an EI mass spectrum for a DDE isomer identified in Sample 3.
No PCBs or PDBEs were observed in Sample 3.
Sample 4

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBS
G) PDBEs

Figure 60. **A1)** EIC of m/z values 287.95, 289.95, 291.95 and **A2)** an EI mass spectrum for triclosan identified in Sample 4.

Figure 61. **B1)** EIC of m/z values 301.96, 303.96, 305.96 and **B2)** an EI mass spectrum for methyl triclosan identified in Sample 4.
Figure 62. **C1**) EIC ranging from 15.00-15.80 mins for m/z values 107, 121, 135, 149 and **C2**) an EIC ranging from 15.00-15.80 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3**) 15.111 mins, **C4**) 15.179 mins, **C5**) 15.233 mins, **C6**) 15.287 mins, **C7**) 15.331 mins, **C8**) 15.406 mins, **C9**) 15.433 mins, **C10**) 15.474 mins, **C11**) 15.525 mins, **C12**) 15.562 mins, and **C13**) 15.599 mins in Sample 4.
Figure 63. **D1)** EIC of m/z values 315.93, 316.94, 317.93 and **D2)** an EI mass spectrum for a DDE isomer identified in Sample 4.

Figure 64. **E1)** EIC of m/z values 228.0934, 229.1012, and **E2)** an EI mass spectrum for chrysene identified in Sample 4. **E3)** EIC of m/z values 202.0777, 203.0855 and **E4)** an EI mass spectrum for fluoranthene identified in Sample 4.

No PCBs or PDBEs were found in Sample 4.
Sample 5

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PDBEs

Figure 65. A1) EIC of m/z values 287.95, 288.95, 291.95 and an A2) EI mass spectrum for triclosan identified in Sample 5.

Figure 66. B1) EIC of m/z values 301.96, 303.96, 305.96 and B2) an EI mass spectrum for methyl triclosan identified in Sample 5.
Figure 67. C1) EIC ranging from 15.00-15.70 mins for m/z values 107, 121, 135, 149 and C2) an EIC ranging from 15.00-15.70 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times C3) 15.113 mins, C4) 15.180 mins, C5) 15.234 mins, C6) 15.289 mins, C7) 15.329 mins, C8) 15.400 mins, C9) 15.475 mins, C10) 15.526 mins, and C11) 15.600 mins in Sample 5.

No DDTs were found in Sample 5.
Figure 68. **E1)** EIC of m/z values 228.0934, 229.1012, and **E2)** an EI mass spectrum for chrysene identified in Sample 5. **E3)** EIC of m/z values 202.0777, 203.0855 and **E4)** an EI mass spectrum for fluoranthene identified in Sample 5.
Figure 69. **F1)** EIC for tetrachlorobiphenyls at m/z values 289.9, 290.9, 291.9, and 292.9. EI mass spectra of potential tetrachlorobiphenyls at retention times **F2)** 18.665 mins and **F3)** 19.728 mins. **F4)** EIC for pentachlorobiphenyls at m/z values 323.8, 324.8, 325.8, and 326.8. EI mass spectrum of a potential pentachlorobiphenyl at retention time **F5)** 19.722 mins in Sample 5.

No PDBEs were found in Sample 5.
Sample 6

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 70. A1) EIC of m/z values 287.95, 289.95, 290.95 and A2) an EI mass spectrum for triclosan identified in Sample 6.

Figure 71. EIC of m/z values 301.96, 303.46, and 305.46 showing that methyl triclosan is not present in Sample 6.
Figure 72. C1) EIC ranging from 15.06-15.68 mins for m/z values 107, 121, 135, 149 and C2) an EIC ranging from 15.06-15.68 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times C3) 15.110 mins, C4) 15.181 mins, C5) 15.232 mins, C6) 15.286 mins, C7) 15.330 mins, C8) 15.401 mins, C9) 15.401 mins, C10) 15.472 mins, C11) 15.523 mins, and C12) 15.560 mins in Sample 6.

No DDTs were observed in Sample 6.
Figure 73. E1) EIC of m/z values 228.0934, 229.1012, and E2) an EI mass spectrum for chrysene identified in Sample 6. E3) EIC of m/z values 202.0777, 203.0855 and E4) an EI mass spectrum for fluoranthene identified in Sample 6.

Figure 74. F1) EIC for pentachlorobiphenyls at m/z values 323.8, 324.8, 325.8, and 326.8. EI mass spectrum of a potential pentachlorobiphenyl at retention time F2) 19.722 mins in Sample 6. No PDBEs were observed in Sample 6.
Sample 7

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PDBEs

Figure 75. A1) EIC of m/z values 287.95, 289.95, 290.95 and A2) an EI mass spectrum for triclosan identified in Sample 7.

Figure 76. B1) EIC of m/z values 301.96, 302.97, 303.96, 304.97, and B2) EI mass spectrum for methyl triclosan identified in Sample 7.
Figure 77. **C1**) EIC ranging from 15.05-15.72 mins for m/z values 107, 121, 135, 149 and **C2**) an EIC ranging from 15.05-15.72 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3**) 15.111 mins, **C4**) 15.182 mins, **C5**) 15.233 mins, **C6**) 15.287 mins, **C7**) 15.331 mins, **C8**) 15.402 mins, **C9**) 15.447 mins, **C10**) 15.524 mins, and **C11**) 15.602 mins in Sample 7.

No DDTs were found in Sample 7.
Figure 78. **E1)** EIC of m/z values 228.0934, 229.1012, and **E2)** an EI mass spectrum for chrysene identified in Sample 7. **E3)** EIC of m/z values 202.0777, 203.0855 and **E4)** an EI mass spectrum for fluoranthene identified in Sample 7. **E5)** EIC of m/z values 166.0777, 167.0855, and **E6)** an EI mass spectrum for fluorene identified in Sample 7.
Figure 79. **F1)** EIC for tetrachlorobiphenyls at m/z values 289.92, 290.92, 291.91, and 292.92. EI mass spectra of potential tetrachlorobiphenyls at retention times **F2)** 18.660 mins and **F3)** 19.717 mins. **F4)** EIC for pentachlorobiphenyls at m/z values 323.88, 324.89, 325.87, and 326.88. EI mass spectrum of a potential pentachlorobiphenyl at retention time **F5)** 19.720 mins in Sample 7. No PDBEs were found in Sample 7.
Sample 8
A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 80. A1) EIC of m/z values 287.95, 289.95, 290.95 and A2) an EI mass spectrum for triclosan identified in Sample 8.

Figure 81. B1) EIC of m/z values 301.96, 303.96, 305.96, and B2) EI mass spectrum for methyl triclosan identified in Sample 8.
Figure 82. **C1)** EIC ranging from 15.08-15.65 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 15.08-15.65 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.111 mins, **C4)** 15.182 mins, **C5)** 15.233 mins, **C6)** 15.287 mins, **C7)** 15.331 mins, **C8)** 15.402 mins, **C9)** 15.473 mins, **C10)** 15.524 mins, and **C11)** 15.602 mins in Sample 8.

No DDTs were found in Sample 8.
Figure 83. **E1)** EIC of m/z values 228.0934, 229.1012, and **E2)** an EI mass spectrum for chrysene identified in Sample 8.

Figure 84. **F1)** EIC for pentachlorobiphenyl m/z values: 323.88, 324.89, 325.87, and 326.88. EI mass spectrum for a potential pentachlorobiphenyl at retention time **F2)** 19.723 mins in Sample 8.

No PDBEs were found in Sample 8.
Sample 9

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 85. **A1)** EIC of m/z values 287.95, 289.95, 290.95 and **A2)** an EI mass spectrum for triclosan identified in Sample 9.

Figure 86. **B1)** EIC of m/z values 301.96, 303.96, 305.96, and **B2)** EI mass spectrum for methyl triclosan identified in Sample 9.
Figure 87. **C1)** EIC ranging from 15.07-15.66 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 15.07-15.66 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.110 mins, **C4)** 15.181 mins, **C5)** 15.232 mins, **C6)** 15.286 mins, **C7)** 15.330 mins, **C8)** 15.401 mins, **C9)** 15.476 mins, **C10)** 15.523 mins, and **C11)** 15.601 mins in Sample 9.

No DDTs were found in Sample 9.
Figure 88. E1) EIC of m/z values 228.0934, 229.1012, and E2) an EI mass spectrum for chrysene identified in Sample 9. E3) EIC of m/z values 202.0777, 203.0855, and E4) an EI mass spectrum for fluoranthene in Sample 9. E5) EIC of m/z values 166.0777, 167.0855 and E6) an EI mass spectrum for fluorene in Sample 9. E7) EIC of m/z values 179.0852, 180.0901, 181.0892, and E8) an EI mass spectrum for phenanthrene.
Figure 89. **F1)** EIC for tetrachlorobiphenyls at m/z values 289.92, 290.92, 291.91, and 292.92. EI mass spectra of potential tetrachlorobiphenyls at retention times **F2)** 18.663 mins and **F3)** 19.729 mins. **F4)** EIC for pentachlorobiphenyls at m/z values 323.88, 324.89, 325.87, and 326.88. EI mass spectrum of a potential pentachlorobiphenyl at retention time **F5)** 19.719 mins in Sample 9.

No PDBEs were found in Sample 9.
**Sample 10**

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PDBEs

Figure 90. **A1)** EIC of m/z values 287.95, 289.95, 290.95 showing that triclosan is not present in Sample 10.

Figure 91. **B1)** EIC of m/z values 301.96, 303.96, 305.96, and **B2)** EI mass spectrum for methyl triclosan identified in Sample 10.
No DDTs, PAHs, PCBs, or PDBEs were found in Sample 10.

Figure 92. **C1)** EIC ranging from 15.07-15.66 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 15.07-15.66 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.112 mins, **C4)** 15.179 mins, **C5)** 15.230 mins, **C6)** 15.288 mins, **C7)** 15.332 mins, **C8)** 15.403 mins, **C9)** 15.433 mins, **C10)** 15.471 mins, **C11)** 15.525 mins, **C12)** 15.562 mins, and **C13)** 15.603 mins found in Sample 10.
Sample 11

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 93. A1) EIC of m/z values 287.95, 289.95, 290.95 and A2) an EI mass spectrum for triclosan identified in Sample 11.

Figure 94. B1) EIC of m/z values 301.96, 303.96, 305.96, and B2) EI mass spectrum for methyl triclosan identified in Sample 11.
Figure 95. **C1)** EIC ranging from 15.23-15.85 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 5.23-15.85 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.257 mins, **C4)** 15.342 mins, **C5)** 15.400 mins, **C6)** 15.450 mins, **C7)** 15.491 mins, **C8)** 15.559 mins, **C9)** 15.582 mins, **C10)** 15.633 mins, **C11)** 15.698 mins, **C12)** 15.731 mins, and **C13)** 15.762 mins found in Sample 11.

No DDTs were found in Sample 11.
No PCBS or PDBEs were found in Sample 11.

Figure 96. **E1)** EIC of m/z values 252.0934, 253.1012 and EI mass spectra of benzo(a)pyrene at **E2)** 23.49 and **E3)** 22.78 minutes. **E4)** EIC of m/z values 228.0934, 229.1012, and EI mass spectrum of chrysene at **E5)** 20.59 minutes. **E6)** EIC of m/z values EIC of m/z values 202.0777, 203.0855, and EI mass spectrum for fluoranthene at **E7)** 18.156 minutes. **E8)** EIC of m/z values m/z values 166.0777, 167.0855 and EI mass spectrum for fluorene **E9)** at 20.960 minutes in Sample 11.

No PCBS or PDBEs were found in Sample 11.
Sample 12

A) Triclosan  
B) Methyl Triclosan  
C) Nonylphenols  
D) DDTs  
E) PAHs  
F) PCBs  
G) PDBEs

Figure 97. **A1)** EIC of m/z values 287.95, 289.95, 290.95 and **A2)** an EI mass spectrum for triclosan identified in Sample 12.

Figure 98. **B1)** EIC of m/z values 301.96, 303.96, 305.96, and **B2)** EI mass spectrum for methyl triclosan identified in Sample 12.
Figure 99. **C1)** EIC ranging from 15.23-15.83 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 5.23-15.83 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.258 mins, **C4)** 15.339 mins, **C5)** 15.397 mins, **C6)** 15.451 mins, **C7)** 15.492 mins, **C8)** 15.559 mins, **C9)** 15.583 mins, **C10)** 15.634 mins, **C11)** 15.695 mins, **C12)** 15.732 mins, and **C13)** 15.762 mins found in Sample 12.

No DDTs were found in Sample 12.
No PCBs were found in Sample 12.

Figure 100. **E1)** EIC of m/z values 252.0934, 253.1012 and **E2)** EI mass spectra of benzo(a)pyrene at 22.78 minutes. **E3)** EIC of m/z values 228.0934, 229.1012, and EI mass spectrum of chrysene at **E4)** 20.59 minutes. **E5)** EIC of m/z values EIC of m/z values 202.0777, 203.0855, and EI mass spectrum for fluoranthene at **E6)** 18.156 minutes. **E7)** EIC of m/z values m/z values 166.0777, 167.0855 and EI mass spectrum for fluorene **E8)** at 20.960 minutes in Sample 12.

Figure 101. **G1)** EIC of m/z values 483.7126, 484.7205, 485.7106 and EI mass spectrum of 1,2-dibromo-4-(2,4-dibromophenoxy)benzene at retention time **G2)** 20.771 minutes in Sample 12.
Sample 13

A) Triclosan
B) Methyl Triclosan
C) Nonylphenols
D) DDTs
E) PAHs
F) PCBs
G) PDBEs

Figure 102. **A1)** EIC of m/z values 287.95, 289.95, 290.95 and **A2)** an EI mass spectrum for triclosan identified in Sample 13.

Figure 103. **B1)** EIC of m/z values 301.96, 303.96, 305.96, and **B2)** EI mass spectrum for methyl triclosan identified in Sample 13.
Figure 104. **C1)** EIC ranging from 15.21-15.81 mins for m/z values 107, 121, 135, 149 and **C2)** an EIC ranging from 5.21-15.81 mins for m/z value 220. EI mass spectra of possible nonylphenols at retention times **C3)** 15.258 mins, **C4)** 15.343 mins, **C5)** 15.399 mins, **C6)** 15.451 mins, **C7)** 15.492 mins, **C8)** 15.559 mins, **C9)** 15.583 mins, **C10)** 15.634 mins, **C11)** 15.695 mins, **C12)** 15.732 mins, and **C13)** 15.762 mins found in Sample 13.

No DDTs were found in Sample 13.
Figure 105. **E1)** EIC of m/z values 252.0934, 253.1012 and EI mass spectra of benzo(a)pyrene at E2) 23.49, E3) 22.84, and E4) 22.78 minutes. **E5)** EIC of m/z values 228.0934, 229.1012, and EI mass spectrum of chrysene at E6) 20.59 minutes. **E7)** EIC of m/z values EIC of m/z values 202.0777, 203.0855, and EI mass spectrum for fluoranthene at E8) 18.16 minutes. **E9)** EIC of m/z values m/z values 166.0777, 167.0855 and EI mass spectrum for fluorene E10) at 20.95 minutes in Sample 13.

No PCBs or PDBEs were found in Sample 13.
APPENDIX C

A LITERATURE REVIEW OF DIOXIN RESEARCH: UPDATES IN SAMPLE PREPARATION, BIOASSAYS, ANALYSIS TECHNIQUES, AND THE NOVEL DEVELOPMENTS MADE TOWARDS IDENTIFYING BROMINATED AND MIXED DIOXINS
I. Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are types of persistent organic pollutants (POPs) that have been detected all over the world. Together, these compounds are often referred as “dioxins” (Hites 2011). Dioxins are a class of compounds that share similar chemical properties and a similar planar tricyclic structure (Wikoff et al. 2012). PCDDs/Fs are planar triple-ring compounds consisting of two chlorine substituted benzene rings and an oxygenated ring with either one oxygen atom (furan) shown in Figure 106A or two oxygen atoms (dioxin) shown in in Figure 106B. The most toxic and most heavily studied of these compounds include those with chlorines in the 2,3,7,8 positions (2,3,7,8-tetrachlorodibenzo-p-dioxin) often referred to as TCDD. Another class of POPs that can contain dioxin-like (DL) properties include polychlorinated biphenyls (PCBs); a group of dioxin-like compounds (DLCs). PCBs contain a chlorine substituted biphenyl group (two benzene rings connected by a single carbon-carbon bond) as shown in Figure 106C (National Research Council 2006).

![Figure 106. General structures of A) furans, B) dioxins, and C) PCBs.](image-url)
Due to their lipophilic properties, these chemicals bioaccumulate in fatty tissues causing their transport into the food chain. This causes these chemicals to intensify at higher tropic levels, including humans (Faroon & Ruiz 2016, Olsen 2012, Wikoff et al. 2012). Consequently, human exposure to dioxins and DLCs primarily occurs through ingestion, especially fish, red meats, and dairy products (National Research Council 2006, Wikoff et al. 2012). Other forms of human exposure are due to occupational and environmental exposures (Faroon & Ruiz 2016, Wikoff et al. 2012). Their half-lives in the body can range anywhere from years to decades depending on a number of factors such as age, body fat, and smoking habits (Milbrath et al. 2009).

Dioxins and DLCs have been linked to have adverse effects on human health and the environment. Due to a few unfortunate industrial accidents and occupational exposures, the extent of toxicity of these compounds to humans have been assessed and have shown to cause both chronic and acute effects (National Research Council 2006). The most common effect seen with high exposures of dioxins and DLCs is chloracne, a skin condition described as persistent acne-like lesions (Kogevinas 2001, National Research Council 2006, White 2009, Wikoff et al. 2012). Other non-cancer adverse effects seen in humans include endometriosis, reduced testosterone, developmental effects, thyroid function effects, cardiovascular disease, and diabetes (Kogevinas 2001, White & Birnbaum 2009).

It has been highly debated whether to classify these compounds as “carcinogenic to humans’ or “likely to be carcinogenic to humans.” While epidemiological data does not suggest an apparent increase in a single type of cancer due to dioxin exposure, there is a positive correlation between relatively high concentrations of TCDD in the body and increased deaths
from all cancers. Overall, epidemiological data and the additional support from animal studies data is enough to classify TCDD as cancerous to humans (National Research Council 2006, Wikoff et al. 2012).

These chemical classes have numerous congeners with varying numbers and positions of chlorine atoms that dictate their dioxin-like properties. Depending on where the chlorine atoms bind, there can be 210 chemically different PCDDs/Fs (Hites 2011). Out of the 210 PCDDs/Fs congeners, 17 of them are identified as toxic (Van den Berg et al. 2006a). DL-toxicity of PCB congeners specifically increases with a larger number of chlorines in the lateral positions coupled with one or no chlorines in the ortho position. This configuration allows the molecule to rotate into a coplanar or flat configuration (National Research Council 2006). There are 209 possible PCB congeners with 12 of them considered dioxin-like by the World Health Organization (Van den Berg et al. 2006a). Dibenzodioxins, dibenzofurans, and dioxin-like PCBs will be referred to as dioxins and DLCs for the purpose of this review.

A. Dioxin and DLC Toxicity

Dioxins and DLCs share a common mode of mechanism by binding to the aryl hydrocarbon receptor (AhR) (Kafafi et al. 1993, National Research Council 2006, Reiner 2010, Van den Berg et al. 1998). The AhR is a cytosolic receptor protein that is present in most vertebrate tissues. It is expressed in several organs including the lungs, liver, and kidneys (Esser & Rannug 2015, Sorg 2014). The numbers and positions of the chlorine atoms directly impact the toxicity of PCDDs/PCDFs and DLCs to the AhR because they must be able to rotate into the proper configuration to bind to the AhR. The ability to bind to the AhR is generally considered the first necessary step for the expression of dioxin-like activity (Olsen 2012). Dioxins and DLCs
have a high affinity for the AhR (Kafafi et al. 1993, Kulkarni et al. 2008, Watanabe et al. 1999). The AhR complex binds to the transcription factor AhR nuclear translocator protein (Arnt) after releasing chaperone proteins. The newly formed complex enters the nucleus and binds to xenobiotic-response elements (XRE), promotor regions on several genes (Esser & Rannug 2015, Luecke et al. 2010, Sorg 2014) (Figure 107).

![Figure 107](image)


Their common mechanism of action allows for toxicity measurements of dioxins and DLCs to be presented in relation to TCDD (Dopico & Gómez 2015, Van den Berg et al. 1998, van den Berg et al. 2013). The ratio of toxicity is presented as the toxic equivalency factor (TEF) for each compound (Dopico & Gómez 2015, Kulkarni et al. 2008, Van den Berg et al. 1998). TEFs are determined by examining all available *in vivo* and *in vitro* relative effect potency (REP) data. REP data for each dioxin and DLC is composed of any study on the effect of the compound
on various organisms and cell lines, including human cell lines (Trnovec et al. 2013). The concept of TEFs was first developed in the 1980s (Van den Berg et al. 2006a).

The World Health Organization (WHO) has defined TEFs for dioxins and DLCs internationally since the 1990s. The first was conducted in 1993, the second in 1997, and the most recent in 2005 (Ahlborg et al. 1994, Van den Berg et al. 1998, Van den Berg et al. 2006b). The most recent meeting defined the criteria that must be met for a compound to be included on the TEF list. They are that the compound must show a structural relationship to PCDDs and PCDFs, bind to the AhR, elicit an AhR-mediated biochemical and toxic response, and be persistent and accumulate in the food chain. All REP data for compounds meeting the 4 criteria was evaluated and the TEF values updated (Van den Berg et al. 2006a).

TCDD has an assigned TEF of 1.0 with the other TEFs depicting their toxicity in orders of magnitude comparatively (Table 7) (Dopico & Gómez 2015, Kulkarni et al. 2008, Van den Berg et al. 2006b). TEFs are used in combination with chemical residue data to calculate toxic equivalencies (TEQs) in various environmental matrices (Ahlborg et al. 1994, Reiner 2010, Van den Berg et al. 2006b). They are a figure of merit describing the toxicity of each dioxin, furan, and dl-PCB found in the given sample in relation to their concentrations. TEQs are calculated by summing the total of each TEF multiplied to the concentration of observed PCDDs/Fs, PCBs, and other DLCs (Ericson Jogsten et al. 2010) (Equation 11).

\[
TEQ = \sum_{i} [PCDD_i \times TEF_i] + \sum_{i} [PCDF_i \times TEF_i] + \sum_{i} [PCB_i \times TEF_i] \sum_{i} [DLC_i \times TEF_i]
\]

Equation 11. Calculation for TEQ of a sample.
Table 7. Compound names, structures, and TEF values for all dioxins, furans, and DL-PCBs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Structure</th>
<th>2005 WHO TEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD)</td>
<td><img src="image1" alt="Chemical Structure" /></td>
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<tr>
<td>1,2,3,7,8-Pentachlorodibenzo-p-dioxin (1,2,3,7,8-PeCDD)</td>
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<td>Compound</td>
<td>Chemical Structure</td>
<td>2005 WHO TEF</td>
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<td>--------------</td>
</tr>
<tr>
<td>2,3,3',4,4',5,5'-Heptachlorobiphenyl (PCB 189)</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>0.0003</td>
</tr>
</tbody>
</table>

B. Dioxin and DLC Formation

Dioxins and DLCs are produced mainly from anthropogenic sources, especially from those processes related to combustion. Dioxins and their products are unwanted byproducts of these combustion processes. PCDD/Fs formation is primarily driven by the simultaneous oxidation and chlorination of organic aromatic compounds at temperatures between 200-600°C. These newly formed compounds then undergo coupling reactions to generate planar PCDD/Fs. (Altarawneh et al. 2009, Lin et al. 2015). Unlike dioxins, PCBs were made intentionally and were widely used beginning in the early 20th century (White &Birnbaum 2009, Wikoff et al. 2012). Due to certain properties such as their chemical stability and non-flammability, these chemicals were used as lubricants, plasticizers and cooling fluids in electrical transformers. Eventually, the sale and use of PCBs was banned in the United States in 1979 and in 2001 the Stockholm Convention on POPs required that the production of PCBs be banned internationally (Erickson 2001, Szabo &Loccisano 2012, Wikoff et al. 2012). However, PCBs are still released into the environment from industrial incinerators and as leakage from ill maintained hazardous waste sites((ATSDR) 2014).

Numerous industrial and nonindustrial human activities facilitate the formation of dioxins and DLCs (Altarawneh et al. 2009, Dopico &Gómez 2015, Kulkarni et al. 2008). Significant release of dioxins into the environment began during the 1920s with the rise of the chemical
industry (Dopico & Gómez 2015). Dioxins and furans were formed as unwanted byproducts in
the reactions to produce various chlorophenols used for several purposes including pesticides,
fungicides, leather tanning, and wood treatment (Dobbs & Grant 1981, Hites 2011). Ignorance of
their harmful impact allowed for continued concentration increases in all media until the 1970s
when the exposure of veterans to Agent Orange was discovered alongside contamination events
in Missouri, USA and Seveso, Italy (Hites 2011). Legislation was introduced to regulate the
emissions from industrial sources following these incidents (Davy 2004, Watanabe et al. 1999).
Nonindustrial sources are near impossible to regulate (Dopico & Gómez 2015, Kulkarni et al.
2008).

Current industrial sources of dioxins include waste combustion, metal production, paper
mills, coal powered power plants, wastewater treatment facilities, and crematory incinerators.
Municipal solid waste incinerators (MSWI) are employed to burn common trash for energy
recovery and are the largest waste combustion source of dioxins (Altarawneh et al. 2009, Davy
2004, Dopico & Gómez 2015, Kulkarni et al. 2008). MSWIs are still used by several countries,
including several within Europe, Asia, and North and South America. Australia and Africa have
seen their first plants built in recent years. Iron and steel industries are the key metal production
source of dioxins (Davy 2004, Dopico & Gómez 2015, Kulkarni et al. 2008). Regulation and
monitoring of all these industrial sources will continually manage and lower dioxins emissions.
Nonindustrial sources may include accidental fires, diesel engines, illegal incineration of
household wastes, and the release of dioxins from pentachlorophenol-treated wood products
2018). Their lack of regulation results in a large variability of emission levels (Lee et al. 2004).
The effective regulation of industrial dioxin emissions has resulted in the shifting of focus to developing methodologies to accurately document and quantify emissions from nonindustrial sources.

Dioxins occurrence, concentration, and possible transformations in varying environments have been well documented (Dopico & Gómez 2015). Studies beginning in the 1990s focused on overall global dioxin concentrations (Alcock & Jones 1996, Brzuzy & Hites 1996, Jones & de Voogt 1999, Kirchner et al. 2019). Further studies focused on identifying the unique fingerprints from industrial sources and quantitation of their individual components (Charnley & Doull 2005, Everaert & Baeyens 2002, Liu et al. 2013, Ryu et al. 2006, Suarez et al. 2005). Current research is examining potential human exposure to dangerous levels of dioxins through consumption of numerous foods has been examined (Charnley & Doull 2005, Diletti et al. 2018, Hoogenboom et al. 2015, Pemberthy et al. 2016). Yet a need remains for the quantitation of dioxins from nonindustrial sources and the growing importance concerning environmental dioxin concentrations (Costopoloulou et al. 2010). The release of dioxins and DLCs from fires is of great concern due to the increased number of wildfires across the globe in addition to the recent expansion of burning technological waste (e-waste) (Carratt et al. 2017, Salian et al. 2019, Suzuki et al. 2016, Tue et al. 2016, Zeng et al. 2017).

II. Analytical Approach

A. Established Methods

It is important to have optimal analytical methods that can accurately monitor them whether biologically or environmentally considering the damaging effects that dioxins and DLCs may have. Therefore, many of these analytical methods have been standardized. These methods

B. Sample Preparation

Sample preparation methods of dioxins and DLCs often require multiple steps and can be complex. A preferred extraction method is chosen based on the sample matrix (Table 8). It is common that interfering compounds are present at much higher concentrations and therefore many of these steps cannot be avoided. Generally, the sample preparation steps are as follows: 1. Extraction 2. Clean-up 3. Isolation.

Table 8. Sample matrices and their appropriate extraction methods.

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Matrix</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soxhlet</td>
<td>Tissue</td>
<td>(EPA 1994, 2010)</td>
</tr>
<tr>
<td></td>
<td>Soil, Sediment, Fly ash, Paper pulp</td>
<td>(EPA 2007a)</td>
</tr>
<tr>
<td></td>
<td>Ambient air</td>
<td>(EPA 1999)</td>
</tr>
<tr>
<td>Dean Stark</td>
<td>Chemical waste</td>
<td>(EPA 2007b)</td>
</tr>
<tr>
<td></td>
<td>Fuel oil, Aqueous sludge</td>
<td>(EPA 2007a)</td>
</tr>
<tr>
<td>SDS, PLE, MAE</td>
<td>All solid samples (wet or dry)</td>
<td>(EPA 1994, 2007c, d, b, a, 2010)</td>
</tr>
<tr>
<td>LLE, SPE</td>
<td>Aqueous samples</td>
<td>(EPA 1994, 2007b, a, 2010)</td>
</tr>
</tbody>
</table>

The traditional Soxhlet is a common extraction method that has been used for over a century (Luque de Castro & Priego-Capote 2010). This technique extracts the sample with a
condensed solvent from a distillation flask repeatably until the process is complete (Luque de Castro & Priego-Capote 2010). In fact, Soxhlet is recommended by the most commonly used regulatory methods referred to as EPA 1613 and EPA 1668 for the analysis of PCDD/Fs and DLCs. Under these methods, the use of the Soxhlet extraction is solely used on tissue samples and the Soxhlet/Dean Stark (SDS) extractor is used on solid and multiphase samples (such as wet sediment). The SDS device, a combination of Soxhlet and Dean Stark, allows for the removal and measurement of water while simultaneously extracting the sample by Soxhlet. This is key of the SDS device as solid samples extracted by Soxhlet alone must be dried before extraction; a process that can take up to a day compared to SDS that requires no drying of the sample (Lamparski & Nestrick 1989).

In summary, a designated amount of homogenized sample (grinding or blending) is spiked before extraction with stable isotopically labeled ($^{13}$C$_{12}$) standard analogs of 2,3,7,8 substituted PCDD/Fs and PCBs used for internal standards. If working with fish or tissue, it is recommended that the sample should be dried for 18-24 hours after spiking and then extracted for 12-24 hours using Soxhlet and concentrated. Samples that are not tissue are then subjected to SDS using toluene for 16-24 hours and concentrated. The most common concentration method is by rotary evaporation (EPA 1994, 2010).

Various recent studies investigating dioxins and DLCs in different sources such as fish, breast milk (freeze dried), air (emissions from waste incinerators), sediment, and eggs have all taken advantage of the Soxhlet or SDS extractor (Conesa et al. 2016b, Ortuño 2015, Shen et al. 2012, Shin et al. 2016, Squadrone et al. 2015, Thacker et al. 2013, Wan et al. 2010, Zhang et al. 2010, Zhang et al. 2011). Extraction times ranged anywhere from 12 to 24 hours with solvent
volumes being as high as 400 ml. Commonly used solvents included hexane and dichloromethane (Ortuño 2015, Shen et al. 2012, Wan et al. 2010, Zhang et al. 2010, Zhang et al. 2011). Evidently, this method requires long extraction times and large amounts of solvent but is fairly simple to operate.

Newer methods such as the pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) by Dionex, and microwave assisted extraction (MAE) can also be used on solid samples such as soils, clays, sediments, sludges, and solid wastes (EPA 2007c, d, Pico 2017). These methods increase the diffusion and desorption rate of analytes from the sample matrix to the solvent and takes advantage of the potential use of elevated temperatures and/or pressure (Kettle 2013, Xu et al. 2013). These techniques require less solvent, sample, and time compared to traditional extraction methods such as Soxhlet extraction and therefore are sometimes referred to as green extraction techniques (Llompart et al. 2017, Pico 2017). ASE and MAE have also shown to have similar extraction results for the extraction of dioxins and DLCs (73% average surrogate recovery) compared to Soxhlet (75% average surrogate recovery); although it can require expensive equipment (Pico 2017, Richter et al. 1997, Wang et al. 2010b). PLE has continuously become a more common technique for the analysis of PCDDs/Fs and DLCs that has been applied to samples such as street dust, soil, umbilical cord serum, and infant formulae (Klees et al. 2015, Klees et al. 2013, Pandelova et al. 2010, Wu et al. 2018, Xu et al. 2020, Yu et al. 2019).

Aqueous samples are subjected to the traditional liquid-liquid extraction (LLE) or solid-phase extraction (SPE). In summary, the labeled analogs described before are spiked into a 1 L sample and subjected to either of these two techniques. Samples that have visible particles are
first filtered. If the sample is subjected to SPE or filtered, it is recommended that the filter and
disk be also extracted. (EPA 1994, 2010). LLE has been applied successfully to various recent
studies investigating PCDDs/Fs and DLCs in matrices such as eggs, breast milk, and dairy
products (Chovancová et al. 2011, Pizarro-Aránguiz et al. 2015, Shen et al. 2017, Zhou et al.
2017). LLE and SPE are also commonly used to determine the concentration of PCDDs/Fs and
DLCs in humans by extracting plasma or serum although recently LLE is not used as frequently
because it is considered more labor intensive (Patterson et al. 2011).

Several effective clean-up and fractionation steps are required after extraction depending
on how clean the sample is, and the instrumentation being used for analysis. This procedure can
also minimize the possibility of any co-elution problems during analysis. Gel permeation
chromatography (GPC) or size exclusion chromatography (SEC) may be used as a primary step
on samples that may have large amounts of high molecular weight interferences from samples
due to the lipophilic properties of dioxins and DLCs. This is important as these high molecular
weight interferences can cause the harm to the GC column (EPA 1994). Chromatography on
multi-layer adsorption columns with different combinations of acidic, neutral and basic silica,
Alumina, and Florisil are also commonly used in addition to GPC. Multilayer silica column can
retain compounds such as polycyclic aromatic hydrocarbons and lipids. Alumina and Florisil are
used to separate PCDD/Fs from ortho-substituted PCBs. A carbon column can be used to
separate PCDD/Fs from non-ortho-substituted PCBs or planar from non-planar compounds
a. Current Advancements in Sample Preparation

There has been a considerable amount of valuable effort to develop a cost-effective sample preparation method that is reliable, less time consuming, and less labor intensive for the analysis of dioxins and DLCs (Kedikoglou et al. 2018, Li et al. 2016, Lin et al. 2016, Rossetti et al. 2012, Roszko et al. 2013).

A study was published in 2012 that incorporated all the necessary column clean up techniques with an extraction technique. It was the first example that combined silica gel, florisil, celite, carbopak and alumina within a single automated PLE technique for the analysis of PCDD/Fs and dl-PCBs in fish tissue. This method eliminated the need for a GPC post extraction step because of the adsorbents ability to retain high molecular weight compounds. According to this study, sample preparation time was reduced by 95% and solvent volume was reduced by 65% in comparison to EPA 1613 (Subedi & Usenko 2012). This type of extraction is commonly referred to as selective pressurized liquid extraction (SPLE) and it incorporates interference retainers (clean-up adsorbents) upon PLE (Do et al. 2013, Ghosh et al. 2011, Kettle 2013, Subedi et al. 2015). In short, it combines the extraction and clean-up steps into one.

Cloutier et al. modified a Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS) extraction and clean-up for the analysis of PCDD/Fs and other POPs in biological samples (salmon and mussels) (Cloutier et al. 2017, Kalachova et al. 2011). This method used GPC and silica SPE and compared the results with the traditional Soxhlet extraction using NRC CARP-2 certified reference material. Average recoveries for surrogate PCBs and PCDD/S were 70% and 69% respectively with no statistical differences seen between the two compared methods.
Although, no significant statistical difference was seen, this *QuEChERS* method reduced solvent consumption and analytical time (Cloutier et al. 2017).

These are only a few ways that sample preparation methods have been improved, although studies continue to use traditional methods such as the Soxhlet extraction and a multi-step clean-up method that remains unchanged (Abalos et al. 2016). A significant amount of solvent waste is generated by each extraction. A single solid sample extraction can generate 0.50 L of solvent waste. Much of this waste is recovered and recycled when possible. (Labs 2020). Extraction and column clean-up methods are time consuming and can take up to a week for a set of 10-20 samples and can cost around $450 per sample.

**B. Single Column vs Dual Column Gas Chromatography**

Gas chromatography is often used to supply the analytes to a mass analyzer for identification and quantification. Isomeric compounds must be separated prior to mass analyzer analysis to provide the most accurate analysis of the composition and potential threat of the analyzed sample. The most common method for separate dioxins and furans is achieved using a 60-meter column, typically a DB-5ms (60 m x 0.25 mm i.d. x 0.25 µm) or a similar model (Liu et al. 2013). The DB-5ms is a nonpolar stationary phase composed of (5%-phenyl) methylpolysiloxane. Helium is the typical carrier gas used at flow rate of 1.2 mL/min (Stultz & Dorman 2020, Zhang et al. 2012). The temperature program established by the Environmental Protection Agency (EPA) begins at 200°C for 2 minutes, ramped to 220°C at 5°C/minute and held for 16 minutes, ramped to 235°C at 5°C/minute and held for 7 minutes, and lastly a final ramp to 330°C at 5°C/minute (EPA 1994, 2007b). Each injection has an analysis time of 60 minutes not including column temperature equilibrium between injections. This column and
gradient combination provide the best separation of isomers, though unfortunately no one column has been developed that can separate all 17 2,3,7,8-PCDD/F isomers in a single instrument run (Do et al. 2014).

Issues occur with separating 2,3,7,8-TCDD from 1,2,3,7-TCDD and 1,2,6,8-TCDD and with separating 2,3,7,8-TCDF from 1,2,4,9-, 1,2,7,9-, 2,3,4,6-, 2,3,4,7-, and 2,3,4,8-TCDF (EPA 2007b). 2,3,7,8-TCDD and 2,3,7,8-TCDF separation is key as the isomers they co-elute with are non-toxic to human health whereas 2,3,7,8-TCDD and 2,3,7,8-TCDF are the most toxic congeners of dioxins and furans. Lack of separation of 2,3,7,8-TCDD and 2,3,7,8-TCDF from their co-eluting isomers may result in a falsely high TEQ reported for the sample, The use of an additional 30-m DB-225 column can aid in the separation of 2,3,7,8-TCDF many of the isomers but it will still elute with 2,3,4,7-TCDF. Manipulations of the mass spectrometer conditions described in EPA Method 8290A in combination with the DB-225 column allows for quantification of 2,3,7,8-TCDF (EPA 2007a).

Dioxin-like PCBs may be separated using a 30-meter SPB-octyl film column such as a Supelco 2-4218 or equivalent (EPA 2010). This column can separate 10 of the 12 DL-PCBs. Congeners 156 and 157 co-elute using the SPB-octyl film column but may be separated using a DB-1 or equivalent column (EPA 2010). They do not have to be separated for TEQ analysis as they have the same TEF. Multiple injections are required for complete analysis of dioxins, furans, and DLCs using a single GC instrument.

Current research in chromatography for separating dioxins has been conducted in the alternative method of two-dimensional gas chromatography (GC x GC). Two columns, usually one non-polar such as a DB-5ms or equivalent, and one polar, often contains cyanopropyl
groups, are connected by a modulator to increase the separation power of the system before sending the analyte to the mass analyzer (Do et al. 2014, Muscalu & Górski 2018, Patterson Jr. et al. 2011, Xia et al. 2016). Columns used for dioxin separation may range in size from 60m x 0.32mm x 0.25 µm to 2m x 0.1mm x 0.1 µm.

The main modulator type used for GC x GC coupled to a mass analyzer for sample analysis is a cryogenic modulator. There are several designs of cryogenic modulators, with one of the most common being a two-jet cryo. These modulators have two mounted jets perpendicular to each other, one hot (up to 475°C) and one cold (as low as -189°C) (Figure 108). Carbon dioxide and nitrogen have both been used in liquid or gas states for composition of the hot and cold gas (Ramos 2009). The temperature of the cold jet is dependent upon if liquid nitrogen is used to cool the stream. The modulation tube loops twice in the paths of both jets for efficient focusing of bands to propel onto the second column. The sample enters the loop modulator from the first column and enters the path of the cold jet. The cold jet is fired, immobilizing and group the small portion of sample. This period lasts only a few seconds. The firing of the hot jet remobilizes the band of sample until it encounters the second portion of the loop exposed to the cold jet. The sample band is concentrated again, before being pushed into the second column by the firing of the hot jet (Hanari et al. 2013b, Patterson et al. 2011, Ramos 2009). Minimal sample loss occurs using a cryogenic modulator. Single or multiple detectors may be used.
Figure 108. A side-view of a loop modulator provided by Zoex Corporation. The cold jet is directed downward while the hot jet fires horizontally. The modulator tube containing the effluent is diagonal.

Use of two columns facilitates the separation of isomers for dioxin-like compounds that are difficult to separate with one column in complex samples (Dorman et al. 2014, Hanari et al. 2013a, Organtini et al. 2014, Xia et al. 2016). Orthogonal separation achieved using this technique increases the resolving power of the system (Patterson Jr. et al. 2011). Complete separation of the monitored dioxins and DLCs has been achieved using dual chromatography systems (de Vos et al. 2011b, Focant et al. 2005). GC x GC can be coupled with different mass analyzers and detectors so long as they are capable of fast scan rates and full scan capabilities (Muscalu & Górski 2018). A single injection method has been developed for separation of all monitored PCDDs, PCDFs, and DL-PCBs using a GCxGC method though multiple injections may be used to lessen the burden of data processing (Focant et al. 2005). Detection limits for 2,3,7,8-TCDD have been measured using a variety of GC techniques with the use of a cryogenic modulator increasing sensitivity from 20 fg to 313 ag (Patterson Jr. et al. 2011).
C. Mass Spectrometers

The most common mass analyzers used for dioxin and DLC analysis are high-resolution magnetic sector instruments and time-of-flight (TOF) instruments. Low resolution triple quadrupoles have been used in some methods for dioxin screening when a high-resolution mass analyzer is not readily available. Calibration above the limits of detection should be conducted with a minimum of 5 to 8 points for linearity determination no matter the mass spectrometer used (EC 2017, EPA 1994, 2007b, a). Analysis of a single sample consists of a solvent blank, a method blank, the standard for the lower limit of quantification, and the sample itself. Different blanks must be analyzed every 12 hours to ensure the instrument is operating within acceptable parameters (Labs 2020). GC-HRMS sample analysis currently costs between $900 - $1000 per sample, dependent upon the required extraction (Dindal et al. 2011, Labs 2020, Organization 2020). Both GC x GC-TOF/MS and GC x GC-MS/MS provide a cheaper option than GC-HRMS for dioxin and DLC analysis (de Vos et al. 2013, Xia et al. 2016).

a. Traditional Ionization Sources

The most common ionization source used for dioxin and DLC analysis is electron impact (EI) because dioxins and DLCs have no acid/base functional groups and therefore are not amenable to chemical ionization (CI) methods or electrospray ionization (ESI). Chlorine’s non-bonding electrons have the lowest ionization energy in the molecules. They are the most likely to be lost during the formation of radical cations during the ionization process. Heavy fragmentation typically occurs during EI and can detract from overall sensitivity because the fragment ions may not have unique structures i.e. they are produced from several different dioxins and DL-PCB congeners. The energy of the electron beam could be decreased but would
result in a decrease in overall sensitivity. The limits of detection for an instrument equipped with an EI source increases directly with the number of chlorines on the congener.

Negative chemical ionization (NCI) has been used for dioxin and DLC studies. NCI is a sensitive method for analysis of compounds containing electron withdrawing groups that capture electrons for ion formation. (Hass et al. 1978). Methane gas used for thermalization lowers the energy of 100 eV electrons to make them amenable for absorption. The 39 monitored dioxins/furans and DL-PCBs have at minimum four chlorines available for ionization all bonded to aromatic rings, making them strong electron withdrawing groups. NCI would be best used for analysis of the highly chlorinated PCBs and PCDD/Fs, which are the most toxic congeners (Patterson Jr. et al. 1989). It may provide better detection limits for these compounds, with picogram limits of detection achieved in foods (Korucu et al. 2014). Sensitivity decreases parallel to the decrease in chlorines present in the molecules by up to a factor of 8. NCI should be used as an ionization method for heavily chlorinated congeners for optimum results (Patterson Jr. et al. 1989).

b. Current Experimental Ionization Sources

Atmospheric pressure chemical ionization (APCI) is one of two ionization techniques that have been adapted for dioxin analysis (Bruce-Vanderpuije et al. 2019, Fernando et al. 2016, Portoles et al. 2016, Rivera-Austrui et al. 2017, van Bavel et al. 2015). A soft ionization source, it provides an alternative for less fragmentation than EI with enhanced sensitivity due to the abundance of molecular ions formed (Fernando et al. 2016, Portoles et al. 2016, van Bavel et al. 2015). The sample is carried by the mobile gas into the ion source and is vaporized with the nebulizer gas such as nitrogen. The gas mixture is heated before interacting with the corona
discharge electrode or a beta particle emitter electron stream. A heated drying gas is mixed with the sample before it enters the mass analyzer (Figure 109).

Figure 109. Schematic of an APCI source provided by Shimazdu Corporation.

Analysis of PCBs has been performed in positive ion mode and negative ion mode. Positive ionization mode forms ions through proton transfer to generate radical cations, $M^+$, for dioxin analysis. First, the solvent interacts with the discharged electrons to generate radical cation solvent ions (Equation 12). These charged solvent ions then collide with the analyte to form the radical cation (Equation 13). Negative ion mode generates phenoxide ions through the loss of a chlorine and addition of oxygen (Equations 14,15) (Fernando et al. 2016). APCI paired with a tandem mass spectrometer has given dioxin analysis results comparable to HRGC-HRMS
Detection limits for APCI-MS/MS analysis of serum were established at 1.2 pg/mL (Stubleski et al. 2018). Quantification limits for APCI-MS/MS analysis of emission samples was found to be between 0.5-20 pg/sample, comparable to quantification limits achieved analyzing the same samples using HRGC/HRMS equipped with an EI source (Rivera-Austrui et al. 2017).

It has been used to analyzed dioxins and DLCs in complex samples including hazardous, incineration, and municipal solid wastes, blood, fish oils, and animal fats with detection limits of picograms/g sample (Bruce-Vanderpuije et al. 2019, Rivera-Austrui et al. 2017, van Bavel et al. 2015). APCI analysis offers a less expensive and more flexible option for analysis than traditional high-resolution mass spectrometry analysis for dioxins (Fernando et al. 2016, Rivera-Austrui et al. 2017).

\[
S + e^- \rightarrow S^{++} + 2e^-
\]

Equation 12. Ionization of the solvent molecules in an APCI source to a generated radical cation solvent ion.

\[
S^{++} + M \rightarrow M^{++} + S
\]

Equation 13. Ionization of the analyte molecules in an APCI source to generate a radical cation analyte ion.

\[
O_2 + e^- \rightarrow O_2^-
\]


\[
O_2^- + M \rightarrow [M - Cl + O]^-\]

Equation 15. Ionization of the analyte molecules to generate a radical anion analyte phenoxide ion.
Atmospheric pressure photoionization (APPI) is the second ionization method which is beginning used to develop novel methods for dioxin analysis (McCulloch et al. 2017, Riddell et al. 2015). The sample is introduced to the vaporizer before exposed to the UV lamp as the photon source. The sample interacts with the make-up gas used to aid in the ionization of the analyte. The composition of the make-up gas determines the velocity of the molecules through the ionization region (Kersten et al. 2016). An investigation of suitable photo-ionizable solvents that would facilitate the ionization of all investigated PCDD/Fs found that toluene or methanol doped with 5% fluorobenzene provided the best results (Kersten et al. 2016, McCulloch et al. 2017, Riddell et al. 2015). Dioxide analysis has been performed in negative ion mode, which monitors the phenoxide ions. The source of the electrons differs from the APCI source. Electrons from an APPI source are generated by the solvent interacting directly with the energy from the light source to produce electrons (Equation 16). The electrons then interact with any oxygen in the source to form the phenoxide ions (McCulloch et al. 2017) (Equations S4, S5). One study that examined the detection limits for tetra- through octo-chloro congeners established them in the range of 0.11-4.61 pg/µL in atmospheric samples McCulloch et al. 2017).

\[ S + hv \rightarrow S^+ + e^- \]

Equation 16. Ionization of a solvent molecule using an APPI source to generate electrons.

Advantages of using APPI over traditional ionization sources are its increased sensitivity and selectivity, reduced matrix effects, and a larger linear dynamic range (McCulloch et al. 2017). Researchers hope that the source will decrease the amount of sample volume used and decrease analysis time (Riddell et al. 2015). Methods using both APCI and APPI ionization sources for dioxin analysis should be further developed and refined.
c. High Resolution Magnetic Sector and Time of Flight Mass Analyzers

Detection limits for dioxins were set at 1 ppt by the American Association of Science Team during 1970 (Reiner 2010). The only available instrumentation capable of measuring at such low concentrations was gas chromatography coupled with high resolution magnetic sector mass spectrometry (GC/HRMS) (Croes et al. 2013, Fromme et al. 2016, Liu et al. 2013, Muzembo et al. 2019). This instrumentation is still the gold standard and method of choice for both regulatory and litigation purposes regarding dioxins and DLCs (Bruce-Vanderpuije et al. 2019, McCulloch et al. 2017). The majority of current instruments in use at laboratories for dioxin testing are magnetic sector instruments from suppliers including ThermoFischer Scientific and Jeol as shown in Figure 110 (ALSGlobal 2020, Eurofins 2020, Labs 2020). Detection limits of pg/g have been achieved in several media including sewage sludge, blood, lipids, milk, fish, and ash (Bjurlid et al. 2018, Chirollo et al. 2018, Croes et al. 2013, de Vos et al. 2013, Fromme et al. 2016, Garcia-Bermejo et al. 2015, Hoang et al. 2014, Kedikoglou et al. 2018, Muzembo et al. 2019, Zhang et al. 2017).

![Schematic for a magnetic sector mass analyzer](image)

Figure 110. Schematic for a magnetic sector mass analyzer. (Reprinted with permission from Skoog, D. A.; et.al. *Principals of Instrumental Analysis*; Thomson Brooks/Cole: Belmont, California, 2007; pg. 517)
Researchers have also used use a time-of-flight (TOF/MS) as seen in Figure 111 to achieve the necessary limits of detection (Hashimoto et al. 2015, Stultz et al. 2018, Xia et al. 2016). These may be used in analyses in which isomers do not have to be resolved if they have the same TEF and response factor, so long as they can be separated from all other dioxins and DLCs (EC 2014, 2017, EPA 1994, 2007a, 2010). Both TOF and magnetic sector instruments must be able to monitor 12 unique m/z’s minimum at a minimum of 10,000 resolution (EC 2014, 2017). These high-resolution instruments allow for low limits of detection, typically femtograms and lower, and quantification to be achieved (EC 2017). Detection limits for several media analyzed using time of flight mass spectrometers have been documented at of pg/g in meats and soils and ng/g in soil among others (de Vos et al. 2013, de Vos et al. 2011a, Fernando et al. 2014, Xia et al. 2016).

Figure 111. Schematic of the electric field of a reTOF. (Reprinted with permission from Throck Watson, J.; David Sparkman, O. Introduction to Mass Spectrometry: Instrumentation, Applications, and Strategies for Data Interpretation; John Wiley & Sons, Ltd: West Sussex, England, 2007; pg. 69)
d. Low Resolution Triple and Single Quadrupole Mass Analyzers

Current research for mass analyzers has focused on further development and refinement of methods for using low resolution mass spectrometers when GC/HRMS is not readily available for compound identification and quantification (Abalos et al. 2016, Reiner 2010, Sun et al. 2017). Gas chromatography triple quadrupole mass spectrometry methods (GC-MS/MS) have been certified for some dioxin testing and screening (Figure 112) (Abalos et al. 2016, EC 2014, 2017, EPA 2007b). Fragmentation for ion screening is facilitated through an appropriate ion source such as EI or a collision cell. Multiple reaction monitoring (MRM) mode is used to obtain higher sensitivity and selectivity on a GC-MS/MS system. Two precursor ions and one corresponding transition product for each is monitored in the MRM (Abalos et al. 2016, EC 2014, 2017). The relative ion intensities for the transition product ions must be +/- 15% when compared to the calculated or measured values from standards under identical MS/MS conditions (EC 2017) Transition products may be either product ions such as the loss of [COCl]⁻ and 2[Cl]⁻ or ions generated from constant neutral loss (CNL) scans of ³⁵Cl, ³⁷Cl, ⁷⁹Br, and ⁸¹Br (Hashimoto et al. 2015, Sun et al. 2017). The instrument must be able to achieve unit mass resolution for minimization of interferences (EC 2014). Separation of isomers must be less than 25% peak to peak between 1,2,3,4,7,8-hexachlorodibenzofuran and 1,2,3,6,7,8-hexachlorodibenzofuran. Additionally, the LOQ must be one fifth of the maximum level and at least 1 ppt (EC 2017). Detection limits for samples analyzed using triple quadrupoles are reported as ng/g for fish, pg/g for sewage sludge, milk powder, fly ash, and feed samples, pg/sample for stack gas emissions (Cao et al. 2018, Garcia-Bermejo et al. 2015, Portoles et al. 2016, Rivera-Austrui et al. 2017, Sun et al. 2017)
III. Bioassays for Dioxin Analysis

The development of bioassays for the analysis of dioxins and DLCa are driven by the high costs (as discussed above) associated with the HRGC/MS methods and the need in many instances to analyze many samples. The goal of screening large number of samples quickly is to reduce the number of samples that are submitted for confirmation by mass spectrometry analysis. Examples of high-profile, large scale contamination events requiring the ongoing analysis of numerous samples include soil contamination in Vietnam, Elbe river sediment in Germany, animal food products in Belgium, and contamination caused by the explosion of a chemical plant in northern Italy.

Many publications state that dioxin analysis using cell culture methods are much less costly than those associated with mass spectrometry analysis, but no actual estimates are given. A comparison of CALUX cell culture analysis with HRGCMS provided by a commercial contractor (Hiyoshi Corporation) states that 16 cell culture samples can be analyzed in the time it
takes to do one HRGCMS analysis (approximately one hour). Sample sizes required for CALUX analysis are smaller (2-10 grams) than HRGCMS (50 grams). Turn around times (the time from submission of samples to the reporting of results) are shorter for CALUX as well compared to HRGCMS (3.5 days vs 6 weeks). Sample purification is based on the two column approach recommended by EPA method 1613b (EPA 1994).

Most bioassays designed to assess sample toxicity are based on cell cultures with sample extracts. The dioxin and dioxin-like components in the sample extract interact with the same AhR receptor proteins that bind to DLCs promote toxic effects. Ahr receptor proteins will bind both halogenated and non-halogenated planar PAHs and transport them into the nucleus of the cell. Dioxin-like Polychlorinated Biphenyls containing phenyl groups with no chlorine atoms in ortho positions are free to rotate about the biphenyl C-C bond and are capable of adopting a planar conformation all them to bind to the AhR receptor protein. The AhR protein-DLC complex then binds to an Aryl hydrocarbon receptor nuclear translocator protein (ARNT) in the nucleus of the cell. The ARNT:AHR heterodimer binds to the DNA sequence 5'-TGCGTG-3' within the dioxin response element (DRE) of the target gene promoter leading to the formation of enzymes whose reaction with a particular substrate generates a luminescent response.

The measurement of dioxin and other DLCs using cell culture techniques is attractive because several samples can be analyzed in a short period. Typically, samples can be cultured in 96 well plates. Measurements are made shortly after the substrate (when needed) is added. Several of the 96 well plates may be analyzed when the luminescence instrumentation is interfaced to an autosampler. Cell culture times may take up to 72 hours depending upon the sample, the cell type, and temperature. Therefore, when rapid on-site screenings are deemed
necessary, methods based on Immunoassays are preferred. The two cell culture methods used most frequently to screen dioxin samples (EROD and CALUX) are discussed below.

A. Limits of Detection associated with Cell-Culture Bioassays

As has been noted previously (Eichbaum, STOTEN), the LODs and LOQs associated with in vitro quantification assays are not always reported. This is due in part to the fact that the LODs and LOQs will vary based on several experimental conditions that might vary between laboratories. Factors such as the cell line chosen, the preparation method, culture times, the amount of carbon dioxide used in the incubation, and temperature will affect the LODs and LOQs. The limits of detection (when reported) associated with bioassays are normally expressed in equivalents of 2,3,7,8-TCDD rather than individual congener concentrations because the potency of entire extract is evaluated at once and referenced to a specific concentration of 2,3,7,8 TCDD. Most studies undertaken to improve detection limits of in vitro bioassays have focused on modifying new cell lines by inserting dioxin responsive elements (DRE) into the cellular DNA that will promote luminescence when cultured with dioxins and dlcs. Sub-picomolar detection limits have been demonstrated using cell lines derived from Japanese Rice Fish and Zebrafish for 2,3,7,8 TCDD equivalents in seawater (0.32 ng/L = 1 WHO-TEQ pg/gram). Cells derived from protoplastic cells were found to be sensitive to dioxin levels that are 10 times lower than the Japanese environmental standard for dioxins in soil (1000 pg/g). A new recombinant mouse hepatoma cell line, CBG2.8D, was shown to detect dioxin from ambient air samples at 0.1 pM levels. These most recent studies suggest that the LODs achieved with cell culture methods approach those achieved for high resolution mass spectrometry. The accuracy of newly developed cell culture bioassays is often evaluated by comparing the BEQs to the TEQs obtained
by mass spectrometry for the same samples. The closer the correlation coefficient ($R^2$) is to one, the more accurate the assay. The following discussion of dioxin bioassays will focus on such studies.

**B. EROD Bioassays**

The EROD (7-ethoxyresorufin-O-deethylase) assay for planar aromatic compounds is based on the induction of CYP1A gene to produce cytochrome P450 enzymes that oxygenate PAHs as part of the body’s natural detoxification system. In the EROD assay, the cytochrome P450 enzymes convert the substrate 7-ethoxyresorufin to resorufin.

Fluorescence spectroscopy is used to quantify the amount of resorufin produced by the enzyme. The amount of resorufin detected is proportional to the amount of dioxin and DLCs present in the extract in the cell culture. EROD assays may be carried out with two cell lines. One cell line is derived from rainbow trout liver (RTL-W1). The rat hepatoma cell line H4IIE is most often used in the EROD assay (sometimes called micro-EROD). In this particular bioassay, the reactivity of the deethylase with the 7-ethoxyresorufin can be compromised by the PCBs and PAHs in the sample, particularly when the concentrations of DLCs is high giving a result that may underestimate the true concentration of the DLCs being analyzed.

In an EROD bioassay, the toxicity equivalents that equate to TEQs derived from mass spectrometry measurements of DLCs are referred to as BEQs or EROD-TEQs and are calculated referencing the effective concentration of the extract to generate a fluorescence signal relative to 2,3,7,8-TCDD (Equation 17). Here the EC refers to the effective concentration needed to induce a certain percentage ($x$) of the maximum fluorescence response of 2,3,7,8-TCDD in the specific cell line used for culturing the sample.
\[
BEQ = \frac{TCDD \frac{pg}{mL}}{Extract \ EC_x \ \frac{g}{mL}}
\]

Equation 17. Calculation of a sample’s BEQ

The most recent examples of comparative EROD and HR mass spectrometry analysis have been carried out with soil and sediment samples. The levels of DLCs in European River sediment samples were studied by EROD and micro-EROD and two other mammalian cell lines to determine the LODs and LOQs (described in terms of BEQs) associated with these cell lines as well as the reproducibility of the assays when performed by different laboratories (In vitro..2018 giesy). The micro-EROD assay was found have lower detection and quantification limits and better reproducibility compared to the EROD method (0.5 and 0.7 pM TCDD vs. 0.94 and 1.74 pM). The EROD assay conducted with the RTL-W1 cell was faster (in terms of time required for the culturing of cells) and thus deemed more appropriate to screen large number of samples for prioritization regarding mass spectrometry analysis. The total TEQs estimated (determined using a magnetic sector mass spectrometer) were on average 25% of the BEQ values. For all four samples studied, the TEQs were larger than the BEQs for the PCDD/Fs in sediment while the analysis of dl-PCBs gave much larger BEQs than TEQs.

One study of eleven soil samples extracted at three different depths found that the concentration of DLCs decreased as the sampling depth increased when analyzed by EROD and mass spectrometry. However, the combined TEQs for both PCDD/F and dl-PCB measured by mass spectrometry were generally greater than the BEQs generated by micro=EROD analysis (13.9 to 60.5 pg/g and 8.8 to 34.1 pg/g, respectively) when samples were extracted and separated with the same method based on EPA 8290 using a two column method. The authors suggest that high concentrations of PCB 77 and PCB 126 were responsible for the reduction in EROD
activity relative to the HRGCMS analysis in several samples analyzed. When soil extracts were analyzed without the sulfuric acid/silica column cleanup, the BEQs detected by EROD increased by a factor of twenty on average, suggesting that soil samples contain other bioactive compounds that could skew the results of the EROD assay when analyzing for DLCs.

C. CALUX Bioassays

The most common screening method used to analyze DLC-contaminated samples is referred to Chemical Activated Luciferase gene eXpression (CALUX). CAFLUX (Chemical Activated FLUorescent protein eXpression) is an AhR receptor-based bioassay like CALUX except the DRE inserted into the cell chromosome generates an enhanced green fluorescent protein (EGFP) from jellyfish (*Aequorea victoria*). Bioluminescence measurements require a luminometer that measure the light produced by the chemical reaction using a photomultiplier tube detector. The luminometer analyzing light emitted from individual wells on a plate so many samples can be analyzed rapidly. Detection limits for the ProMega luminometer are stated as 3 zeptomoles of Luciferase. The CAFLUX assay is based on the fluorescence of the green jellyfish protein generated by the DRE and thus does not require the addition of a substrate. The cost of fluorescence instrumentation for the CAFLUX assay is generally lower as well. The CAFLUX assay is more susceptible to temperature variations relative to the CALUX assay. The detection limits associated with CAFLUX are larger than CALUX because the measurements are more susceptible to noise. CALUX assays are used to analyze a wide variety of planar PAH samples in variety of matrices in addition to dioxins and DLCs.

The efficacy of CALUX-based methods for the analysis of DLCs is tested by comparing the results of CALUX assays to the HR GC/MS methods for a particular set of samples. In such
studies CALUX TEQs (or BEQs) are calculated in a similar way to TEQs derived from HRGCMS analyses. CALUX TEQs are calculated by multiplying the CALUX REPs (relative potencies, similar to TEFs) by relative light units (RLUs) derived from the luminescence measurements of the cultured samples. CALUX REPs are determined for a particular DLC first by assigning 2378-TCDD an REP value of one and then comparing the dose-luminescence response curves of various DLCs to that of 2378-TCDD obtained when cells are cultured with a particular analyte. The values of the REPs will depend upon the cell line used in the CALUX methods. Two different CALUX methods that have been used for DLC analysis based on two different cell lines. One is XDS-CALUX (xenobiotic detection system) which employs a mouse hepatoma cell line and the other is DR-CALUX (Dioxin-responsive) that uses a rat hepatoma H4IIE cell line. The cell specified in the July 2014 revision of EPA method 4435 is the mouse hepatoma cell line modified with DREs necessary to generate the luciferase enzyme to produce recombinant cell line H1L6.1c3. The genes in both cell lines are modified to include a dioxin responsive element that generates the green fluorescent protein. In most comparative studies of CALUX and HRGCMS, the CALUX TEQs measured are greater than the WHO-TEQs determined for the same samples. This is because HRGCMS methods are designed to measure the concentrations of 17 PCDD/Fs and 12 DL-PCBs only. The CALUX assay will respond to any AhR agonist present in the sample. Over 3500 planar aromatic compounds may react with the AhR receptor. It is believed that the additive effects of these many low-concentration AhR binding agonists contribute to the elevated response of the CALUX assay compared to HRGCMS. For this reason, some researchers have postulated that in some cases the CALUX assay may present a better estimate of a particular sample’s toxicity compared to HRGCMS.
Ultimately, the goal of any CALUX study is to develop a firm correlation with HRGCMS results so that CALUX assays may be used in lieu of HRGCMS to analyze samples of the same matrix (food, soil, fly ash, etc.). Comparative analyses of HRGCMS and CALUX methods use the same extraction and separation techniques, which are based on EPA method 1613. CALUX methods used to determine the level of DLC contamination in soil surrounding Municipal Waste Incinerators was validated using five standard soil samples containing known quantities of DLCs typically analyzed by HRGCMS. CALUX analysis of the five soil samples of high concentration gave a CALUX TEQ range of 20.8 to 7795 pg TEQ/gram while the HRGCMS analysis gave a lower TEQ range, 9.7 to 6760 pg WHO-TEQ/gram, with a correlation coefficient ($R^2$) of 0.996. When soil samples ($n = 24$) acquired around four different waste incinerators were analyzed and compared the correlation was not as strong ($R^2 = 0.764$) given the lower concentrations of DLCs present (1.72 to 31.4 pg/g WHO-TEQ vs 1.71-44.2 CALUX-TEQ). The WHO-TEQ levels in 14 of the 24 samples fell below the 5 pg/g TEQ action level for remediation recommended by EU guidelines. The higher TEQ levels in the CALUX samples were attributed to low concentrations of other AhR agonists, particularly brominated and mixed brominated/chlorinated dioxins, furans, and PAHs. The REP values associated with brominated dioxins and furans are similar to the chlorinated analogues, suggesting that they may contribute significantly to the overall CALUX-TEQ measured.

Suzuki et al. carried out the most comprehensive comparative study of CALUX and HRGCMS methods for the analysis of DLCs. Twenty-three individual samples including soil, sediment, wastewater, indoor air samples, and three types of ash samples were analyzed for the standard mixture of 29 chlorinated DLCs along with twelve PBDD and PBDF congeners. DR-
CALUX and XDS-CALUX TEQs were plotted against WHO-TEQs with correlation coefficients of 0.969 and 0.985, respectively. The correlations are linear over at least 6 orders of magnitude down to 0.1 pg/g WHO-TEQ. The DR and XDS CALUX assays provide very similar responses when analyzing the same samples. Different types of samples were extracted and separated in the same fashion. Soil, sediment, and dust samples were extracted using an accelerated solvent extraction system that required almost a liter of combined solvents (acetone, hexane, and toluene). After the samples were purified on a 55% sulfuric acid-silica gel column, the chlorinated and brominated DLC fractions were separated on a 10% silver nitrate silica gel column. Almost the entire WHO-TEQs found in several house dust and surface soil (taken from an area of North Vietnam where electronic waste is burned openly) were due to brominated DLCs. The total PBDD and PBDF contributions to the total WHO-TEQs. Roughly 20% of the WHO-TEQs in several river sediment and fly ash samples were due to brominated DLCs. The twelve brominated PCDD/Fs were assigned the same TEF values as their corresponding chlorinated congeners. The average DR-CALUX-TEQ and XDS-CALUX/WHO-TEQ ratios were found to be 4.5 ± 3.6 and 5.0 ± 3.2 for all samples studied. The authors attribute the higher CALUX-TEQs values to differences in WHO-TEFs and CALUX_REPS when brominated DLCs were detected at elevated concentrations as well as the presence of low concentrations of unknown AhR agonists in the sample. This study clearly demonstrates the efficacy of CALUX-based methods for the analysis of DLCs in a variety of field samples.

The one exception to the observation that CALUX-TEQs are normally larger than WHO-TEQs can be found in the analysis of bird’s (Pelegrine Falcon) eggs. Bird’s eggs are known to accumulate chlorinated organic compounds and thus are regarded as appropriate indicator of
wildlife exposure to these compounds. When TEQs for HRGCMS derived from the standard 29 analytes were compared to DR-CALUX TEQs for 59 different eggs, it was determined that 67% of the total WHO-TEQs could be attributed to PCB-126. The CALUX REP value for PCB 126 using the DR cell line was found to .075, while the WHO-TEF is 0.1. The average CALUX-WHO TEQ ratio in this study was found to be 0.75, even though the CALUX method responded to 93 of the 177 analytes tested for in the eggs. The primary reason cited for reduced CALUX TEQs relative to WHO TEQs is the CALUX REP for PCB 126 determined for this cell line is 0.075 while the corresponding WHO TEF is 0.1. Poultry eggs are a primary source of human nutrition in a variety of cultures and recent studies of hen’s eggs suggest that they are primary route of human exposure to DLCs.

IV. Current Research on PBDD/Fs, PXDD/Fs

A. Formation of PBDD/Fs and PXDD/Fs

Recently, polybrominated dibenzo-p-dioxins (PBDDs), polybrominated dibenzofurans (PBDFs), and mixed bromo-chloro dibenzo-p-dioxins/furans (PXDDs/Fs) have been a rising concern (Duan et al. 2011, Song et al. 2019, van den Berg et al. 2013). They have not been assigned WHO-TEFs due to a lack of REP data, but it has been recommended that they be added as quickly as possible (van den Berg et al. 2013). Use of brominated flame retardants (BFRs) including polybrominated diphenyl ethers (PBDEs) have led to an increased chance of PBDD/F formation (Conesa et al. 2016a, Venkatesan & Halden 2014, Wang et al. 2010a, Yang et al. 2020). They are created through waste incineration, uncontrolled fires, and other thermal processes like PCDD/Fs (Conesa et al. 2016a, Song et al. 2019, Wang et al. 2010a). PBDDs/Fs have reached detectable levels in the environment and have been shown to have similar toxicities
as PCDDs/Fs (Conesa et al. 2016a, Song et al. 2019, van den Berg et al. 2013, Yang et al. 2020). They are more lipophilic than their chlorinated analogs and more susceptible to UV degradation (DeVito & Birnbaum 1994, Hagberg 2009). A study has shown that the photolysis of decabromodiphenyl ether (BDE-209) on the surface of car seats yielded PBDD/Fs as photoproducts (Khaled et al. 2018).

Mixed PXDDs/Fs (X = Cl or Br) are made as unintentional by-products from combustion and other chemical processes (Bruce-Vanderpuije et al. 2019). They have been detected in various biological and environmental samples. There is evidence that the thermal degradation and transformation of polybrominated diphenyl ethers (PBDEs) also produce PXDDs/Fs (Fernando et al. 2016, Zhang et al. 2016). PXDDs/Fs have been measured in greater concentrations than their purely chlorinated counterparts (Du et al. 2010). PXDDs/Fs, specifically those containing halogens in the 2,3,7, and 8 positions, can be just as toxic or exceed the toxicity of their chlorine and bromine analogs (Bruce-Vanderpuije et al. 2019, Fernandes et al. 2011, Fernando et al. 2016).

**B. Sample Prep Analysis of PBDD/Fs and PXDD/Fs**

Sample preparation steps are similar as those performed on PCDD/Fs in that require multiple intensive clean-up and fractionation steps depending on the analyte(s) of interest and available instrumentation (Chen et al. 2017, Conesa et al. 2016a, Duan et al. 2011, Minh Tue et al. 2016, Wang et al. 2010a). One example of these methods is a screening method for the separation of brominated and chlorinated dioxins in waste and environmental samples which has been validated with the CALUX assay (Suzuki et al. 2017). Clean-up was performed with a silica gel column impregnated with 55% sulfuric acid and another silica gel column impregnated with 10%
silver nitrate was used for separation with recovery rates ranging from 66-102%. No
debromination was observed during separation with average recoveries rates of PBDD/Fs found
to be 85-96% and 68-96% respectively. Separation of brominated and chlorinated dioxins
occurred due to a difference in bonding strength. Brominated dioxins bound more tightly to the
silver ions of the column. CALUX-TEQ ratios were generally comparable to WHO-TEQ ratios
for the chlorinated and brominated dioxin fragments. Some CALUX-TEQs obtained were
different than the WHO-TEQs obtained possibly due to unidentified compounds such as non-
2,3,7,8-substituted PCDD/Fs, non-2,3,7,8-substituted PBDFs, and PXDDs/Fs which may have
contributed to activity. This screening method can be useful in large scale investigations for field
samples contaminated with chlorinated and brominated dioxins (Suzuki et al. 2017).

C. Mass Spectrometry Analysis of PBDD/Fs and PXDD/Fs

Standard analysis of PBDDs/Fs and PXDDs/Fs is performed using HRGC/HRMS (Conesa et
al. 2016a, DeVito & Birnbaum 1994, Du et al. 2010, Duan et al. 2011, Fernandes et al. 2011,
Pajurek et al. 2019, Wang et al. 2010a). Challenges may arise from analysis of these compounds
due to lack of available reference standards, interferences found in complex matrices, thermal
degradation of analytes during gas chromatography, and the required selectivity and sensitivity
2013). Detection limits of pg/g have been documented for PBDD/Fs in eggs and fats and ng/Nm$^3$
or pg/Nm$^3$ for flue-gas samples (Bjurlid et al. 2018, Conesa et al. 2016a, Pajurek et al. 2019,
Wang et al. 2010a).

Mass spectrometry methods using a triple quadrupole and a quadrupole time-of-flight
(QTOF) have been established as alternatives to HRGC/HRMS analysis similar those used for
PCDD/Fs analysis. An GC-EI-MS/MS method established detection limits of PXDD/Fs in soil in the ng/g range. It should be noted that co-elution did occur between two analytes (Myers et al. 2012). Detection limits as low as 0.001 pg/g lipid weight in blood and 0.15-1.4 pg/g in fire debris have been established using GC-APCI-MS/MS for PBDD/Fs and PXDD/Fs (Bruce-Vanderpuije et al. 2019, Organtini et al. 2015). A Q-TOF method established detection limits of 100-600 femtograms in soil samples for PXDD/Fs (Fernando et al. 2016).

D. Separation and Analysis of PBDD/Fs, PCDD/Fs, PXDD/Fs, PCBs, and PBDEs

Numerous studies have been conducted where samples are extracted and analyzed for a combination of PBDD/Fs, PCDD/Fs, PXDD/Fs, PCBs, and PBDEs simultaneously, often using an established method for the determination of PCDD/Fs and PCBs (Bruce-Vanderpuije et al. 2019, Pajurek et al. 2019, Zacs et al. 2013). Clean-up consists of fractionation that separates the PCBs and PBDEs from PBDD/Fs, PCDD/Fs, and PXDD/Fs (Chen et al. 2017, Pajurek et al. 2019, Zacs et al. 2013). Analysis of samples, no matter the matrix, gave detectable and quantifiable signal of at least one PBDD/F in each study. Bruce-Vanderpuije and coauthors studied the background levels of PBDD/Fs, PCDD/Fs, PXDD/Fs, and dlPCBs in sera collected from pregnant woman in Ghana where e-waste is openly burned, exposing the population to higher levels of these compounds. While the average toxic equivalent concentration was found to be 5.3 pgTEQ/g lw, lower than the global average, PBDD/Fs and PXDD/Fs were found to contribute approximately 20% of the total toxicity (Bruce-Vanderpuije et al. 2019).
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VITA

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