SHORTENED IN VIVO BIOCONCENTRATION FACTOR

TESTING IN *Cyprinus carpio*

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Bioconcentration factor testing serves as the most valuable surrogate for the assessment of bioaccumulation. The assessment of potentially harmful chemicals is crucial to not only the health of aquatic environments, but to humans as well. Chemicals that possess the ability to persist in the environment or that have the potential to bioaccumulate, pose a greater risk to organisms that are exposed to these chemicals. The Organization for Economic Cooperation and Development Guideline 305 outlines specific protocols to run an accurate and reliable aquatic flow-through test. However, since its adoption in 1996, very few changes have been made to accommodate the endeavor to lowering the amount of test species to run one of these said tests. Running an aquatic flow-through test, according to 305, takes much time and money as well as numerous amounts of fish. Such burdens can be eliminated through simple modifications to the standard protocols. In this study, we propose an abbreviated study design for aquatic bioconcentration testing which effectively alleviates the burdens of running a flow-through test. Four chemicals were used individually to evaluate the usefulness of the proposed shortened design; 4-Nonyphenol, Chlorpyrifos, Musk Xylene, and DDT. The study consisted of exposing *Cyprinus carpio* for 7 days followed by 7 days of depuration, for a total of a 14-day study. Our results for each of the four compounds are consistent with literature values, thus, demonstrating that BCF<sub>k</sub> can be accurately predicted in an abbreviated *in vivo* test.
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CHAPTER 1
INTRODUCTION

1.1 Background

1.1.1 Fish Bioconcentration Factor Testing

Fish bioconcentration factor (BCF) testing serves as the most valuable source for determining a chemical’s ability to bioaccumulate in the environment. Guidelines have been set to standardize and validate testing protocols to increase repeatability and reliability among results. To obtain a reliable and valid assessment of a compound’s bioaccumulative property, four key criteria must be met: 1) clear specification of the test compound and fish being tested, 2) chemical analysis in the fish as well as analysis of the exposure medium, 3) no toxicological effects from the chemical should be observed in the test, and 4) steady-state conditions must be met (Parkerton et al., 2008). Information from BCF studies can be obtained from both environmental and laboratory settings, however most testing procedures take place within laboratory settings due to lack of protocol guidelines for field studies and increased variability factors among testing results. Additionally, BCF values can be used to formulate quantitative structure-activity relationships (QSARs), which can be used to help predict certain compounds’ potential to bioaccumulate within the environment (Arnot et al., 2009).

Regulation of chemicals in the environment is crucial to help reduce exposure to not only wildlife but humans as well. Chemicals that persist in the environment have the potential to accumulate to higher-tropic level organisms, most notably humans. Environmental organizations look at three characteristics to assess if a chemical should or should not be placed under certain restrictions to eliminate potential over-exposure within the environment and
potentially to humans. Agencies assess the compounds persistence (P), bioaccumulative potential (B), and environmental toxicity (T) to determine if a compound should be subjected to tighter regulations as well as determining if a chemical is environmentally safe and should be placed on the market for commercial use (Weisbrod et al., 2008).

Chemicals that have the ability to persist in the environment for longer periods of time, will pose a greater risk for organisms that are indigenous to the area of exposure. In other words, the longer the chemical can persist in a marine environment; the greater the chances of an organism to come into contact with that chemical. When evaluating the persistence of a substance, actual half-life data under desired marine conditions should be used to determine the substances persistence. Substances with a half-life of >60 days in marine water or >40 days in freshwater will fulfill the persistence criterion. Also, a substance with a half-life that is >180 days in marine sediment or >120 days in freshwater sediment would be considered persistent. In more extreme cases, some substances have the potential to be very persistent. Substances that have a half-life >60 days in both marine and freshwater or >180 days in both marine and freshwater sediment would be considered very persistent (vP) (European Chemicals Bureau 2003).

Aquatic organisms have the potential to become exposed to substance directly from the water (bioconcentration), or uptake through the foodchain (biomagnification). Actual measured data on bioconcentration in aquatic species should be used to assess a substances bioaccumulative property. When measured data is not available, log \( K_{ow} \) or BCF based on mathematical models can be used to predict bioaccumulative potential. Substances with a BCF value exceeding of 2,000 will fulfill the B criterion, and any substance that exceeds a value of
5,000 would be considered very bioaccumulative (vB). When actual measured data is not available, a log $K_{ow}$ value exceeding 4.5 will fulfill this criterion (European Chemicals Bureau 2003). Requirement to fulfill this criterion varies from country to country. For example, the United States, Japan, Canada, and Australia use criterion that follows the Stockholm Convention that states a chemical may be considered bioaccumulative if its BCF value is greater than 5,000.

Toxicity data should ideally assess long-term effects to fulfill the toxic criterion. However, data available for most chemicals are acute effects and used to drive initial selection. A substance would meet this criterion if chronic long-term no observed effect concentration (NOEC) for marine or freshwater organisms is $< 0.01 \text{ ml/l}$, or when a substance has been classified as carcinogenic, mutagenic or toxic for reproduction. Also, if there is evidence of long-term toxicity, such as endocrine disruption, a substance would be considered to fulfill this criterion (European Chemicals Bureau 2003).

Regulatory agencies look at PBT characteristics in a chemical to determine if tighter regulations should be put in place. For example, the European Registration, Evaluation, Authorization, and Restriction of Chemical substances (REACH) program places greater responsibility on industries to provide information on chemicals for safer and smarter handling of the distribution of substances. Regulation of chemicals has been a milestone in reducing the amount of potentially harmful chemicals to the environment. Not only has regulation been pivotal for protecting our environment, but it has reduced exposure to humans as well. Although some protocols for environmental assessment are in need to be updated, newer and cheaper methods for environmental assessment have proven to be effective at predicting bioaccumulation potential of a compound.
1.1.2 OECD 305: Bioconcentration Assessment: Flow-Through Fish Test

The Organization for Economic Cooperation and Development Guideline 305 outlines the protocols used in the United States that are needed for an accurate and reliable aquatic flow-through test to be administered (OECD, 1996). The testing consists of two phases: exposure (uptake) and post-exposure (depuration) phases. The protocol calls for a 28 day uptake phase, however if steady-state is not reached (BCF$_{ss}$) by day 28 then the uptake phase should be lengthened until BCF$_{ss}$ is met or up to 60 days. BCF$_{ss}$ can be derived from taking the concentration in the fish (C$_f$) and dividing that by the concentration in the water (C$_w$) (OECD, 1996).

\[
(BCF = C_f/C_w)
\]  

Along with the length of the test, two different concentrations should be used to conduct each study. Sampling of the fish should take place at least five different time points during the uptake phase and at least four different time points for the depuration phase. Water should be sampled prior to the addition of the fish being studied to determine if nominal concentrations have been reached. Guideline 305 calls for at least four fish per concentration at each of the time points to be sampled for accurate analysis of BCF.

Standard fish BCF analysis can be derived from two first order kinetic processes that can accurately describe uptake and depuration (OECD, 1996).

Rate of uptake = $k_1 \times C_w$  

Overall loss rate = $(k_2 + k_e + k_m) \times C_f$
By combining the rate of uptake and overall loss rate equations we can derive kinetic BCF ($BCF_k$).

\[
(BCF_k = \frac{k_1}{k_2})
\]  \hspace{1cm} (4)

$BCF_k$ is a ratio between $k_1$ and $k_2$ and should be equal to steady-state BCF ($BCF_{ss}$) that is obtained from the concentration in fish to the concentration in water. Once $BCF_{ss}$ has been reached, or 60 days have passed, whichever comes first, the exposure phase has come to an end and the aquatic organisms should be moved to a clean tank to begin the depuration phase. With this procedure an experiment could run as long as three months and fish used for the experiment could range from 70 to 90 fish, or more depending on when $BCF_{ss}$ is reached.

The length of the test, the required amount of fish to be tested, and the amount of chemical needed for that length of time in a flow-through system can be very taxing on any budget for labs performing these tests. Development for a shortened and applicable BCF testing protocol would alleviate many monetary concerns that are often a burden for laboratories. A reduction in the amount of time would also reduce the amount of fish and chemical needed for a BCF test. Alleviating these burdens will open opportunities for more chemical screening as many chemicals have already been screened but there are still thousands more that can be found in the environment that need to be tested.

The organism often used for BCF assessments is the common carp. The use of this organism is efficient in that it develops and grows very slowly, allowing for a more accurate assessment for the potential to bioaccumulate. Using an organism that develops rapidly could skew the results as the organism’s ability to metabolize and excise chemicals could increase.
1.2 Environmental Relevance

1.2.1 Chemical Interaction

Chemicals that are found in the environment are not found alone, but in a complex of chemicals that aquatic organisms can potentially be exposed to. Many BCF tests have focused on one particular chemical at a time and how an organism will uptake and depurate that specific chemical. As stated, chemicals are found as a complex; therefore it has been proposed that mixture studies are needed to validate many of these findings. Interactions between these chemicals within the environment or the organism can alter the BCF values that we see during the test. For example, it has been shown that musk xylene induces CYP2B enzymes in mice; however activity of this enzyme was not observed (Chou and Dietrich, 1999; Hawkins et al., 2002; Lehman-McKeeman et al., 1995). These findings suggest that musk xylene, while inducing certain enzymes; will inhibit metabolic proteins as well. CYP2B is a major liver enzyme that is required to break down many chemicals within the liver and inhibition of this enzyme could very well prevent the metabolism of other chemicals present within the organism.

1.2.2 Future Testing Procedures

Certain steps are being made to better screen chemicals in order to reduce the amount of testing and to filter out any chemicals that could potentially be a higher risk to humans and the environment. Quantitative structure-activity relationships (QSARs) are mathematical models that can potentially predict complex relationships between complex chemical reactions and biological systems they may occupy. The predictability of these QSARs is limited and based on homogeneity of the compound throughout the investigation of the chemical. Limits can be
widened if the biological activity is unspecific or narrowed if the biological endpoints involve specific mechanisms of action (Suter-Eichenberger et al., 1998).

Metabolism studies with S9 or microsomal fractions have increased in popularity in regards to relating these data to BCF work. In vitro metabolism assays can accurately show how well an organism may or may not break a chemical down (Cowan-Ellsberry et al., 2008). In addition to showing relevant metabolism data, in vitro tests can be performed much faster and cheaper. Mixture studies can also be performed to determine if there are any reactions taking place that may skew the results of how well an organism can break down multiple compounds.

1.2.3 Xenobiotic Biotransformation

1.2.3.1 Liver Metabolism

Metabolism of compounds that are foreign to an internal environment has been well studied and documented for many years. Studies that look at different modifications of xenobiotic compounds range from pharmaceuticals that are found in waste water treatment to pesticides that leach into different ecosystems. Although many different cell types possess the appropriate enzymes to metabolize xenobiotics, the liver is the main site for most biotransformation. Biotransformation of foreign substances can be subdivided into two phases: modification and conjugation. The objective for phase I (modification) reactions are to introduce reactive and polar groups to substrates that have been introduced into a system (Guengerich, 2001). The most common reactions are oxidation, reduction, and hydrolysis (Goeptar et al., 1995). Phase I reactions, more often than not, involve the enzyme cytochrome P450, which is a large superfamily of enzymes that catalyze reactions (Guengerich, 2008). If the newly formed metabolites have become sufficiently polar, they can be excreted out of the
system. However, metabolites that have not acquired the polarity needed for excretion, the metabolites will undergo Phase II reactions, conjugation. In Phase II reactions, metabolites are conjugated with different species such as glutathione, sulfate, glycine, or glucuronic acid (Ritter, 2000; Buckley and Klaassen, 2007). Conjugation can occur on carboxyl, hydroxyl, amino, or sulfhydryl groups of the metabolite, depending on the chemical structure of the metabolite (Parkinson and Oglivie, 2007). The end product of conjugation is a more polar metabolite; therefore, it cannot diffuse across membranes and can be actively transported.

Phase I and II reactions are vital for the detoxification of potentially poisonous foreign substances. However, this is not always the case. Some metabolites that are formed during biotransformation are more detrimental than their parent compound. The biotransformation of chlorpyrifos is a good example of how a metabolite can be more toxic than the parent molecule itself. Chloropyrifos-oxon, a metabolite of chlorpyrifos, has been well studied and has shown to inhibit acetylcholinesterase activity in neurons. Although biotransformation is essential for detoxifying xenobiotics, there are a few instances where biotransformation can be toxic in itself.

1.2.3.2 Subcellular Fractions

In vitro work has increased in popularity over the past two decades, due to the constraints of cost and time. Approximately 70-80% of the liver is composed of hepatocyte cells, where many different biological processes occur. Homogenized liver tissue can be centrifuged for a certain amount of time to isolate cytosol and microsomes. Cytosol contains the necessary transferases for phase II metabolism, while microsomes contain cytochrome P450 isoforms necessary for phase I metabolism. Both cytosol and microsomes form what is
called S9 fractions, where the ‘S’ stands for supernatant and the ‘9’ represents a 9,000g 20
minute centrifuge spin to isolate the fraction from cellular debris. In vitro metabolism data,
using S9 fractions, can accurately assess and determine xenobiotic biotransformation from
multiple species. One strong point of in vitro work is the ability to show biotransformation
differences among different species. Along with variation among species, in vitro has also
helped reduce the number of animals needed for experimentation.

1.3 Experimental Design

1.3.1 Test Materials

The proposed testing materials include nonylphenol, dichlorodiphenyltrichloroethane,
chlorpyrifos, and musk xylene.

1.3.1.1 Chlorpyrifos

\[ \begin{align*}
\text{OC}_2\text{H}_5 & \quad \text{S} \\
\text{Cl} & \quad \text{Cl}
\end{align*} \]

Figure 1.1. Molecular structure of chlorpyrifos (Cole et al., 2012).

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) was first
synthesized by Dow Chemical Company in 1965 as an organophosphate (OP) insecticide (Fig.
1.1), under the trade names include Dursban and Lorsban. The OP works at the neurological
level by blocking acetylcholinesterase, which is an enzyme that is vital for acetylcholine (ACh) to
be broken down and enables the pre-synaptic cleft to re-uptake the chemical (Cole et al., 2012).
Blockage of acetylcholinesterase leads to an increase in ACh levels in neuronal tissues and can
cause a wide range of effects, depending on the level of exposure. Severe intoxications can
eventually result in death, usually from respiratory failure. Metabolism of chlorpyrifos is dependent on P450 enzymes found in hepatic cell lines. CYP3A4/5 has showed the highest activity for metabolism but due to lack of specificity CYP2B6 and CYP2C19 have shown high activity as well (Robert et al., 2007). Specifically, CYP2B6 enzyme is responsible for desulfuration, CYP2C19 for dearylation, and CYP3A4/5 for both pathways (Smith et al., 2011). P450 enzymes may either detoxify the chemical (dearylation), or it may activate oxon metabolites (desulfuration) (Cole et al., 2012). During desulfuration, the sulfur group is removed and a double bonded oxygen molecule is put in its place (CPS-oxon). CPS-oxon metabolite demonstrates a much higher affinity for the ß and ß subunits esterases that causes unwanted side effects (Bicker et al., 2005). Oxon binds irreversibly to the ß-esterase and reversibly to the ß-esterase through hydrolyzing the oxon metabolite. As mentioned earlier, inactivation of these results in an increase in ACh and overstimulation of cholinergic nerves.

Elimination of chlorpyrifos and its metabolites is mainly achieved via the kidneys (Nolan et al., 1984; Griffin et al., 1999; Timchalk et al., 2002). Urinary excretion data in acute chlorpyrifos exposures in humans is available for DETP, DEP, and TCPγ, all primary metabolites for chlorpyrifos. Elimination, however, is highly dependent on the mode of absorption. Dermal absorption of chlorpyrifos has an apparent half-life that is twice as long as an oral dose of chlorpyrifos, this is also true for the elimination phase of chlorpyrifos (Griffin et al., 1999). The processes of absorption, distribution, metabolism and excretion are highly dependent on route and vehicle of administration. These processes can vary greatly and this variety can cause discrepancies among various amounts of exposure (Smith et al., 2009). Specifically for CPS-oxon, metabolism can occur by hepatic and extrahepatic esterases, such as paraoxonase (PON-
1) (Pond et al., 1998), and tissue β-esterases to form two intermediates TCPγ and diethylphosphate (DEP) (Chanda et al., 1997). Excretion of chlorpyrifos is facilitated through passage of urine as DEP, DETP, TCPγ, or a glucuronide, sulfate, or other conjugates of TCPγ (Bakke et al., 1976; Nolan et al., 1987).

Figure 1.2. Biotransformation pathway of chlorpyrifos as adapted from Smith et al., 2011.

Table 1.1. Chlorpyrifos properties. (NIH 2012)

<table>
<thead>
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<th>Property</th>
<th>Value</th>
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<tr>
<td>Chemical Name</td>
<td>O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>1.4 mg L (25 °C)</td>
</tr>
<tr>
<td>Kow</td>
<td>4.7</td>
</tr>
<tr>
<td>BCF</td>
<td>100-4,667</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>350.59 g/mol</td>
</tr>
<tr>
<td>Formula</td>
<td>C9H11Cl3NO3PS</td>
</tr>
</tbody>
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1.3.1.2  Nonylphenol

Figure 1.3. Molecular structure of nonylphenol (Eriksson et al., 2003).

Nonylphenol (4-(2,4-dimethylheptan-3-yl)phenole) is another compound that is commonly found in the environment, Fig. 1.3. Microbial organisms are known to breakdown alkylphenol polyethoxylates (APEs) in wastewater treatment, as seen in Fig 1.4. One product that is created during the shortening of APEs is nonylphenol.

Figure 1.4. Degredation of nonylphenol polyethoxylates in wastewater treatment microorganisms as adapted from Giger et al., 1984 and Di Corcia et al., 1998.

Industrial and agricultural use of nonylphenol is also apparent as it is used as a surfactant in many different processes that include detergents, emulsifiers, and wetting agents (Eriksson et al., 2003; Arukwe et al., 2003). The USEPA considers nonylphenol toxic to aquatic organisms due to its estrogenic mimicry effects that are shown in laboratory testing. In rainbow
trout, it has also been suggested that nonylphenol directly induces the expression of the estrogen receptor and vitellogenin gene (Arukwe et al., 2003; Lalah, et al., 2003). That being said, nonylphenol is considered to be an endocrine-disrupting chemical (EDC) as it feminizes male fish and increases vitellogenin expression, a gene that is only expressed in female fish (Ren and Lech, 1997). In regards to human health, nonylphenol has been shown to proliferate MCF-7 breast tumor cells (Lee and Lech 1998). Jensen et al. presented a correlation in the increased number of cases involving testicular cancer, decreased in semen quality and hypospadias.

In rainbow trout, studies have shown that the highest concentrations of residues were seen in bile and intestinal contents/feces (Coldham et al., 1998; Kleinow et al., 1992; Cravedi et al., 1985). This finding leads to the indication that the primary route of excretion is biliary or fecal (Thibaut et al., 1998). In rats, biotransformation of nonylphenol is achieved by UDP-glucuronosyltransferase (UGT) 2B1 (Daidoji et al., 2003). Likewise, in trout, treatment of hepatocytes with β-glucuronidase demonstrated that conjugation with glucuronic acid is the primary biotransformation pathway (Cravedi et al., 2001). However, some studies have indicated that a few phase I enzymes are responsible for biotransformation in rats (Lee et al., 1998). Although phase II liver enzymes effectively metabolize nonylphenol, the more polar glucuronide conjugate can still persist for long periods of time (Daidoji et al., 2003). As in trout studies, orally administered nonylphenol in rats is absorbed by the gastrointestinal tract and persist in intestinal tissues for long periods of time (Daidoji et al., 2005).
Figure 1.5. Proposed metabolic pathway of nonylphenol as adapted from Thibaut et al., 1998.

Table 1.2. Nonylphenol properties. (NIH 2012)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Nonylphenol</td>
<td>4-(2,4-dimethylheptan-3-yl)phenole</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>6 mg L⁻¹ (20°C)</td>
</tr>
<tr>
<td>K&lt;sub&gt;ow&lt;/sub&gt;</td>
<td>5.71</td>
</tr>
<tr>
<td>BCF</td>
<td>2-350</td>
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<tr>
<td>Molecular Weight</td>
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<tr>
<td>Formula</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;O</td>
</tr>
</tbody>
</table>

1.3.1.3 DDT

Dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) is different than most organochlorine insecticides in that it works by opening voltage-gated Na+ channels causing them to open spontaneously leading to repetitive discharging of axonal action potentials. It was first synthesized in the late 1800’s but was not used as an insecticide until the
latter part of World War II to control malaria and typhus. It wasn’t until 1962 when Rachel Carson published Silent Springs that the dangers of DDT were made apparent to the public. The book outlined the dangers of indiscriminately spraying DDT without knowledge of the impact it could have on the environment or to human health. DDT is a class II organochlorine that is no longer in use in the United States. Low acute exposure to DDT can lead to symptoms like headaches, nausea, vomiting, confusion, and can even produce tremors and lower coordination. At higher doses exposure can produce convulsions, or spasms, which are caused by Na\(^+\) channels spontaneously opening in neuronal tissue causing action potentials to fire abruptly and can eventually lead to death. The metabolism of DDT causes the formation of its two metabolites DDD and DDE, seen in Fig. 1.6.

![Biotransformation of DDT](image)

**Figure 1.6.** Biotransformation of DDT to form DDD (by reductive dechlorination, right) and DDE (by elimination of HCl, left).

Since legislation passed banning DDT from commercial and agricultural use, concentrations of the compound have decreased significantly within the environment. However, even with this decrease in concentration there still persist some levels of DDT in certain organisms and environments. Even still, the discontinued use of the organophosphate was a major contributor to the bald eagle population making a turn around. DDT, and its
metabolites, is highly lipophilic which gives the compounds the ability to accumulate in sediment, soils, and in adipose tissue of animals. Because of its lipophilic nature, DDT has a very high potential to bioaccumulate and biomagnify up the food chain, which is the cause for the decline of the birds of prey population.

Figure 1.7. Proposed metabolic pathway of DDT as adapted from van den Berg et al., 2003.

Increased levels of CYP3A4 mRNA levels suggest that this enzyme plays an important role in the xenobiotic metabolism of DDT (Litvak et al., 1968).
Table 1.3. DDT properties. (NIH 2012)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT</td>
<td>1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>5.50x10^-3 mg/L @ 25°C</td>
</tr>
<tr>
<td>Kow</td>
<td>6.91</td>
</tr>
<tr>
<td>BCF</td>
<td>600-84,500</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>354.49 g/mol</td>
</tr>
<tr>
<td>Formula</td>
<td>C_{14}H_{9}Cl_{5}</td>
</tr>
</tbody>
</table>

1.3.1.4 Musk Xylene

Figure 1.8. Molecular structure of Musk Xylene (Sari and Itoh, 2012).

Musk xylene (1-tert-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene) is a nitro musk that is very persistent and very bioaccumulative within the environment (Fig. 1.8). Since the mid-1980s, its use has declined greatly due to safety concerns regarding human and environmental health. Commercially, musk xylene was used as a synthetic fragrance to mimic natural musks (Della Tore et al., 2011). Such applications included perfumes and some cosmetics. Musk xylene is an analogue of trinitrotoluene (TNT), and for all intents and purposes nitro musks were first discovered and studied to produce new high explosives. The nitro musks decline in use was, in part, due to the residues obtained in the environment. Under the REACH regulation program the European Chemicals Agency labeled musk xylene as a substance of very high concern (SVHC), saying that the chemical has very persistent and very bioaccumulative (vPvB) properties; however the musk does meet the criteria to be considered toxic to humans or for the environment. Musk xylene is not only persistent in aquatic environments but in human
adipose and breast tissue as well. In rats and mice, musk xylene has been shown to increase transcriptional mRNA levels of CYP2B enzymes in hepatic fractions. However, the increase of expression is not followed by an increase in activity (Hawkins et al., 2002; Lehman-McKeeman et al., 1995; Lehman-McKeeman et al., 1999). This suggests that inhibition of the CYP2B enzyme may be caused by musk xylene or its metabolites (Lehman-McKeeman et al., 1995).

In human volunteer studies, Riedel and Dekant (1998) demonstrated the toxicokinetics of musk xylene via oral and dermal administration. What they found was that musk xylene was rapidly absorbed in the gastrointestinal tract and penetration through the skin was very slow, stating that the concentration of musk xylene was 40-fold lower than that of the orally administered volunteers (Riedel and Dekant, 1998). The previously described study also showed that plasma concentrations demonstrate a rapid initial decrease of musk xylene from blood to a second compartment (body fat), with a half-life of elimination of 70 days.

Figure 1.9. Biotransformation of musk xylene in humans as adapted from Riedel and Dekant 1998.

Lehman-McKeeman et al. (1999) showed hepatic effects of musk xylene in rats, increasing total liver weight as well as increasing total microsomal P450 content in a dose-
dependent manner. Interestingly, the increase in P450 activity was coupled with a decrease, or inhibition of specific activity. Specifically, CYP2B mRNA expression increases while the compound and its metabolites inhibit the proteins activity. CYP1A and CYP3A showed increased mRNA expression as well, however, this is not followed by a decrease in enzymatic activity, as seen with CYP2B.

Table 1.4. Musk Xylene properties. (NIH 2012)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Musk Xylene</td>
<td>1-tert-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>0.472 mg/L @ 25°C</td>
</tr>
<tr>
<td>Kow</td>
<td>4.369</td>
</tr>
<tr>
<td>BCF</td>
<td>1,400-6,740</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>297.264 g/mol</td>
</tr>
<tr>
<td>Formula</td>
<td>C_{12}H_{15}N_{5}O_{6}</td>
</tr>
</tbody>
</table>

1.3.2 Test Organism

The organism commonly used for BCF assessments is the common carp. The use of this organism is efficient in that it develops and grows very slowly, allowing for a more accurate assessment for the potential to bioaccumulate. As an organism grows and develops its ability to metabolize and excise chemicals increases, as the enzymes become more refined. Since common carp develop very slowly, this reduces any variability while running a test with the organism. Along with the advantage of slow growth, carp are very resilient and viable in many different environments. Resilience is especially important when the organism is placed under conditions that could increase the stress levels of the organism. Tolerant of many different environmental conditions, carp can live in water with a pH ranging from 6.5-9.0 and temperatures ranging from 3 to 35°C. These characteristics of the common carp make it an ideal test organism for flow-through BCF studies.
1.4 Specific Aims

In order to assess the potential of a shortened BCF protocol to accurately predict 28-day BCF values specific days during the study were selected for sampling. Days 1, 3, and 7 will be used as testing time points to accurately assess the uptake of the chemical. To accurately assess the depuration of a chemical, Day 7, 10, and 14 were used as sampling time-points. Shortening the exposure time and consequently the amount of fish used for BCF assessments could significantly reduce the cost for laboratories to perform these tests. This could ultimately lead to a large reduction in cost that would allow more chemicals being tested in the future.

1.5 Objectives and Hypothesis

Objective 1: Determine the difference in $BCF_k$ values between whole body and tissue specific samples.

- Hypothesis 1: $H_0$: Tissue specific BCF values will not be different than the whole body BCF values

Objective 2: Determine if the proposed shortened BCF protocol is sufficient to screen for bioconcentration potential.

- Hypothesis 2: $H_0$: 14-day BCF value will not be different than 28-day BCF values.

Objective 3: Determine if there is difference in the in vivo BCF of individual chemicals vs. BCF values when fish are exposed to a mixture of all chemicals.

- Hypothesis 3: $H_0$: Individual BCF values will not be different than mixture BCF values.

Objective 4: Determine if there is difference in the in vitro metabolic activity of individual chemicals vs. in vitro metabolic activity with a mixture of three different chemicals.

- Hypothesis 4: $H_0$: Individual in vitro values will not be different than mixture in vitro values.
1.6 Chapter References


Scott H. Jackson, Christina E. Cowan-Ellsberry, and Gareth Thomas. Use of Quantitative Structural Analysis To Predict Fish Bioconcentration Factors for Pesticides. *Journal of Agricultural and Food Chemistry* 2009 57 (3), 958-967


CHAPTER 2

DEVELOPMENT OF AN ABBREVIATED IN VIVO BIOCONCENTRATION FACTOR TEST

IN COMMON CARP

2.1 Introduction

Bioconcentration factor testing serves as the most valuable surrogate for the assessment of bioaccumulation (Parkerton et al., 2008). The assessment of potentially harmful chemicals is crucial to not only the health of aquatic environments, but to humans as well. Chemicals that possess the ability to persist in the environment or that have the potential to bioaccumulate, pose a greater risk to organisms that are exposed to these chemicals (European Commissions Bureau, 2003). The Organization for Economic Cooperation and Development Guideline 305 outlines specific protocols to run an accurate and reliable aquatic flow-through test (OECD, 1996). However, since its adoption in 1996, very few changes have been made to accommodate the endeavor to lowering the amount of test species to run one of these said tests. Running an aquatic flow-through test, according to OECD guideline 305, takes much time and money as well as numerous amounts of fish. Such burdens can be eliminated through simple modifications to the standard protocols.

When performing environmental assessments, regulatory agencies look at different criteria in a compound to determine if they pose any danger to human or environmental health. In 2007, the REACH program (the European Union regulation on the Registration, Evaluation and Authorization of Chemicals) was set in force. Under this program, chemicals that are imported into the EU at quantities of one tonne or more per year are required to register with European Chemicals Agency. The REACH program was a novel stepping-stone for the
regulation of chemicals and for the companies that are making these chemicals to be held responsible for making sure the compound is safe for human and environmental health.

Regulatory agencies look at three criteria: persistence (P), bioaccumulation (B), and toxicity (T) (Weisbrod et al., 2008). BCF tests are the most common studies performed to assess bioaccumulation of a compound. OECD guideline 305 outlines the protocol to accurately and reliably run a BCF test for aquatic organisms. Along with the few changes that have been made since 1996, the guideline only outlines testing procedures for one compound at a time. However, in the environment compounds are not found individually, rather in a cocktail of multiple compounds. This could pose a potential problem for the normal absorption, distribution, metabolism and excretion (ADME) characteristics of a compound in an organism. Different compounds have different effects within a biological system that could alter the ADME properties of a compound and, in turn, alter $BCF_k$ values that are generated in a single compound BCF study. Thus, it is important to study the effects that chemicals have within a biological system with mixture studies to determine if there are increased risks for chemicals to bioaccumulate.

2.2 Materials and Methods

2.2.1 Chemicals

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphate, CAS# 2921-88-2), methyl-chlorpyrifos (CAS# 5598-13-0), 4-Nonylphenol (4-(2,4-dimethylheptan-3-yl)phenol, CAS# 104-40-5), D4-Nonylphenol (CAS# 1173019-62-9), DDT (1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane, CAS# 50-29-3), DMF (N,N-dimethylformamide CAS# 68-12-2) and DCBP (4,4'-Bis(9-carbazolyl)1-1'-biphenyl, CAS# 583828-31-7), were all obtained from Sigma Aldrich.
Corp (St. Louis, MO). Musk Xylene (1-tert-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene CAS# 81-15-2) was obtained from City Chemical LLC (West Haven, CT). HPLC grade methanol, dichloromethane, ethyl acetate, and n-hexane were obtained from Fisher Scientific (Houston, TX). Milli-Q water was obtained from in house Milli-Q water system (Millipore, Billerica, MA).

2.2.2 Animals and Housing

Animals were stored in appropriate conditions for common carp, 18°C in a living stream and fed every other day. Carp were obtained from Osage Catfisheries (MO) and transported to the University of North Texas. Housing took place in a separate room from where the fish were exposed to prevent any contamination of fish stocks. Fish were allowed to acclimate for a week before conducting BCF tests, which were conducted according to OECD 305 guideline (OECD 1996). Fluorescent lights were kept at a constant light and dark cycle of 16 h light/ 8 h dark. Water quality was checked every 3 days and measurements of (mean ± SD) temperature, pH, DO, alkalinity, and water hardness for the 14 d BCF were 25 ± 2°C, 8.0 ± 2.2, 7.8 ± 0.6 mg/L, 90 ± 16 mg/L, respectively. Carp were fed ad libitum twice a day during the experiment.

2.2.3 Fish Exposure

For the 14 d BCF test, carp (n=60) were randomly chosen and distributed into two solvent control tanks and two exposure tanks, with all aquaria having a 20 L capacity. The exposure tanks received a continuous flow of 2.5 μg/L test compound solution using dimethylformamide (DMF) as a carrier solvent (<0.01 %). Carp were exposed for 7 d and then moved into clean tanks containing only dilution water for the remaining 7 d depuration period.

2.2.3.1 Chemical Stocks

Once chemicals arrived, a 10,000 mg/L master stock of each of the chemicals was made
with the appropriate solvent. The master stock was made by measuring out 10 mg of the compound and then adding 1 mL of solvent. For DDT, musk xylene, and DCBP stocks were made up in DCM, and for rest of the compounds stocks were made up in methanol. From the 10,000 mg/L master stocks, diluted stocks were made from 10,000 down to 1 mg/L. Dilutions went as follows: from 10,000 mg/L to 1,000 mg/L (1 in 10 dilution) 100 μL aliquot of 10,000 mg/L into 900 μL of solvent, the 1,000 mg/L stock was vortexed and another 100 μL aliquot was taken out of the 1,000 mg/L solution into 900 μL of solvent producing a 100 mg/L stock solution (another 1 in 10 dilution). This was repeated down to 1 mg/L stock solution.

For the syringe pumps a 60 mg/L solution needed to be prepared. From the 10,000 mg/L master stock 60 μL was aliquoted out into a 100 mL volumetric. The carrier solvent (DMF) was then added to the volumetric to fill up to the 100 mL line.

2.2.3.2 Flow-Through System

A flow-through system was set up to deliver solvent (DMF) and compound at a steady rate. This was to ensure a constant uptake of chemical was being delivered during the initial phase of the experiment. The peristaltic pump (Masterflex® L/S®, Cole Palmer®, Veron Hills, IL) pumped water at a constant rate of 120 ml/min into a 5 L mixing chamber that would deliver the water and chemical to the aquaria. The 5 L mixing chamber was placed on top of a stir plate with a stir bar in the mixing chamber to distribute the compound throughout the water. A syringe pump was used to transfer the compound from 30 mL polycarbonate syringes (Becton Dickinson, Franklin Lakes, NJ) to the mixing chambers at 5 μm/min, both flows were checked once every other day during the experiment. Plastic tubing transported the mixed water-compound/solvent into the 20 L aquaria that housed the testing species. The 20 L aquaria were
placed into a large tub to allow the out-flowing water to drain into a single outlet. At the end of
the outlet, carbon traps were set in place to absorb any chemical from leaching out into any
water systems.

The 30 mL polycarbonate syringes were loaded with the compound and DMF with a
concentration of 60 mg/L. The compound was slowly pumped into the 5 L mixing chambers and
then finally drained into 20 L aquaria, making the final concentration that the fish were exposed
to 2.5 μg/L. From the syringes to the 20 L aquaria results in a 26,000-dilution factor that results
in a 2.5 μg/L final concentration from 60 mg/L. Water concentrations were checked before
placing the fish in the exposure tanks to determine nominal concentrations had been met.

2.2.3.3 Test Organism and Sample Prep

*Cyprinus carpio* (obtained from Osage Catfisheries) was used in the experiment to test
bioaccumulative properties of each of the testing compounds. For each of the four test
chemicals, fish were exposed to a nominal concentration of 2.5 μg/L. A solvent control
delivering only DMF to the aquaria was included to account for any possible side effects. Water
concentrations were verified on each of the fish sampling days and 10 mL of water was taken
from both the dosed tanks and solvent control tanks. The samples were then dried down by
lightly applying nitrogen gas. Once dried down the test materials were transferred to 2 mL
amber vials using the same solvent and then analyzed.

Fish were anesthetized with up to 100 mg/L MS222 prior to taking muscle and liver
samples as well as whole body analysis. Samples were placed into vials with solvent (1:1 hexane
ethyl-acetate) and spiked with the appropriate internal standard then homogenized. After
homogenization, samples were transferred into centrifuge glass vials. 0.5 mL of MQ and 0.5 mL
of solvent were added to the vials for better separation between solvent and tissue debris. Vials containing samples were mixed with a vortex and then centrifuged for 20 minutes at 2000 rpm. Once separation was achieved, the solvent layer was then extracted (i.e. top layer for 1:1 hexane ethyl-acetate and bottom layer for DCM). Using pasteur pipettes, the appropriate solvent layer was extracted into a scintillation vial, this was repeated once more to collect a total amount of solvent and lipid of about 1 mL. The solvent was lightly dried down with nitrogen gas and the final lipid weight was taken to use for lipid normalization. With 1 mL of 1:1 hexane ethyl-acetate lipid material was transferred to 2 mL amber vials. The sample was dried down using a zephyr of nitrogen gas. 100 µL aliquots of acetonitrile was used to wash down the sides of the dried sample and transferred to a glass insert and frozen in a -20°C freezer for approximately 1 hour to allow any lipid debris to settle. After an hour, 60 µL of the sample was transferred to a new amber vial for further processing (i.e. 4-NP derivatization) or reconstitution with appropriate solvent for analysis with ESI+ LCMSMS or GC-MS.

2.2.4 Analytical Chemistry

2.2.4.1 4-Nonylphenol Analysis

Derivatizing the hydroxyl group of the phenol ring with dansyl chloride was necessary to analyze 4-nonylphenol using ESI+ LC-MSMS. The process involved nitrogen dry-down of the 60 µL aliquots taken after the freezing step of the processed sample (see Sample Prep). Once dry, 50 µL of 10 M sodium bicarbonate (pH 10) and 50 µL of 1 g/L dansyl chloride (in acetone) were added to sample vials and derivatized in an oven at 60°C for 3 minutes. Once the samples were derivatized they were liquid/liquid back-extracted into 500 µL of ethyl acetate (2x). The pooled layers of ethyl acetate (1000 µL total volume) were gently dried under nitrogen and residue
reconstituted to glass spring inserts with a final volume of 50 µL methanol (containing 0.1% formic acid) to fix the final concentration. Deuterated (D4) 4-nonylphenol was used as the analytical internal standard (at 250ppb final concentration). Analytes were analyzed using multiple reaction monitoring (MRM) with parent to daughter mass transitions of 453.9>171.0 for 4-nonylphenol and 457.9>170.6 for D4-4-nonylphenol monitored.

2.2.4.2 Chlorpyrifos Analysis

Chlorpyrifos did not require derivatization for accurate analysis by the ESI+ LC-MSMS. The 50 µL aliquot taken after freezing the reconstituted samples (see Sample Prep) were gently dried down with nitrogen and re-suspended in an equivalent volume (50 µL) of methanol (containing 0.1% formic acid). As with 4-nonylphenol, samples were placed in a glass spring-loaded insert due to the small volume. Parent to daughter mass transition (MRM) of 350>198 were analyzed for chlorpyrifos. Methyl-chlorpyrifos was used as the internal standard (at 250ppb final concentration).

2.2.4.3 DDT and Musk Xylene Analysis

DDT and musk xylene were analyzed by GC-MS using GC 6890N (Agilent, Palo Alto, CA) fitted with a 30 m x 0.25 mm x 0.25 µm EC™-5 capillary column (Alltech, Deerfield, IL). Ultrapure Helium (Air Liquide, Houston, TX) was used as the carrier gas and the instrument was operated in constant pressure (9.5 psi) mode with an initial flow of 1.2 mL/min and an average velocity of 40 cm/sec. Samples were auto-injected (2.0 µL) in pulsed splitless mode at 260°C and the separation was achieved using the following temperature program for the GC oven: initial temperature at 40°C for 3 minutes, increased to 200°C at 10°C/min with no hold time followed by the final ramp to 300°C at 15°C/min and held at this temperature for 5 minutes for
baking the column in preparation for the next injection. After the separation in the GC column, DDT and musk xylene were identified from the mass spectra obtained by electronic impact ionization (70eV; positive ion polarity) in a Mass Selective Detector, MSD 5973 (Agilent) that was operated under Selection Ion Monitoring (SIM) with dwell time of 50 ms and at transfer line temp of 280°C.

2.2.5 Data Analysis

Data analysis was prepared using GraphPad Prism® (version 5.02, La Jolla, CA) and SAS® (version 9.1, SAS institute Inc, Cary, NC). K₁ and K₂ values were obtained from SAS® statistical analysis program, where BCFₖ estimates were calculated. Standard error of the mean (SEM) was calculated in Graphpad for each of the BCF tests that were run.

2.3 Results

2.3.1 Single Compound Study

For the single exposure studies, our BCFₖ values show to be in range of known literature values (Tables 2.1 & 2.2). Reported ranges for all chemicals seem to coincide with the results shown here. According to the MITI (Ministry of International Trade and Industry) database reported BCF values for 4-nonylphenol range from 90-330. For chlorpyrifos, reported BCF values range from 49-2880 and both of our studies fall within this range of values. DDT and musk xylene also fall within the reported range with DDT ranging from 5100-25900 and musk xylene ranging from 1440-6740. Using the benchmark tool, in this case musk xylene seems to be helpful in analysis data points in regards to BCF calculations. The benchmark values can be determined by dividing the calculated BCFₖ value by the calculated BCFₖ value of the benchmark compound. Musk xylene is a useful compound to use as a benchmark because of its known
bioaccumulation properties in aquatic environments. Muscle data shown in tables 5 & 6 appear to be good representation of whole body homogenate data, while increases liver values suggests this is a major site for accumulation.

Even with the shortened exposure time, steady-state was met in the muscle tissue during the single compound exposures. For DDT, not surprisingly steady-state was never met; however DDT has been notorious for its ability to not reach steady-state. In the whole body homogenate we saw a different trend in regards to steady-state being met. BCF<sub>ss</sub> can be defined as sampling tissue on two different days without the test compound increasing in concentration within the organism. Data in Appendix A shows that steady-state criteria was met, thus indicating that a shortened approach to BCF tests can be effective and accurate.

Table 2.1: Table of BCF<sub>k</sub> values from single exposure study in carp muscle tissue.

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>Uptake (K&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>Depuration (K&lt;sub&gt;2&lt;/sub&gt;)</th>
<th>BCF&lt;sub&gt;k&lt;/sub&gt;</th>
<th>MX Benchmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Compound exposure</td>
<td>4-Nonylphenol</td>
<td>107.70</td>
<td>0.48</td>
<td>220.93</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Chlorpyrifos</td>
<td>62.57</td>
<td>0.13</td>
<td>999.68</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Musk Xylene</td>
<td>1155.10</td>
<td>0.64</td>
<td>1814.36</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DDT</td>
<td>765.55</td>
<td>0.04</td>
<td>20077.70</td>
<td>11.07</td>
</tr>
</tbody>
</table>

Table 2.2: Table of BCF<sub>k</sub> values from single exposure study in carp liver tissue.

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>Uptake (K&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>Depuration (K&lt;sub&gt;2&lt;/sub&gt;)</th>
<th>BCF&lt;sub&gt;k&lt;/sub&gt;</th>
<th>MX Benchmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Compound exposure</td>
<td>4-Nonylphenol</td>
<td>372.65</td>
<td>0.39</td>
<td>808.93</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Chlorpyrifos</td>
<td>180.40</td>
<td>0.29</td>
<td>829.18</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Musk Xylene</td>
<td>2340.00</td>
<td>0.43</td>
<td>5396.52</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DDT</td>
<td>7296.30</td>
<td>0.16</td>
<td>124369.57</td>
<td>23.05</td>
</tr>
</tbody>
</table>

With the exception of chlorpyrifos, increased BCF<sub>k</sub> values can be seen in the liver indicating that this may be the primary site for metabolism for each of these compounds. Also
due to the increased lipid content within the liver, as compared to the muscle, may explain why there is such a large increase in $BCF_k$ values.

2.3.2 Mixture Study Results

Results from this study show that mixture studies could prove to be valuable tools in environmental data analysis. The mixture study we performed came out with different and surprising results, however. While $BCF_k$ results for muscle tissue remained equivalent to the single exposure study results, in the liver we saw a dramatic increase in $BCF_k$ of nonylphenol and musk xylene. These findings indicate that in the presence of certain compounds, metabolism and/or accumulation can be affected in certain organisms. As stated earlier, musk xylene is a known inhibitor of CYP enzymes in rat/mice livers; this may be the cause of why we see such a dramatic increase of $BCF_k$ in carp liver samples. Tables 7 & 8 show the results from the mixture exposure experiment. These findings also indicate the importance for mixture BCF tests to represent real world bioaccumulation.

When running an aquatic BCF flow-through study, agencies and laboratories only look at one compound at a time; the results of the mixture study indicate the importance of running mixture studies on a regular basis to represent real-world bioaccumulation. Certain legislations were set in place to not only protect environmental exposures, but human exposure as well so it is important to assess chemicals in conditions that can represent environmental conditions, as results can vary in laboratories while running only one compound.
Table 2.3: Table of BCF_k values from mixture study exposure in carp muscle tissue.

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>Uptake (K1)</th>
<th>Depuration (K2)</th>
<th>BCF_k</th>
<th>MX Benchmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture Compound exposure</td>
<td>4-Nonylphenol</td>
<td>85.58</td>
<td>0.28</td>
<td>306.55</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Chlorpyrifos</td>
<td>30.34</td>
<td>0.11</td>
<td>286.21</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Musk Xylene</td>
<td>828.20</td>
<td>0.10</td>
<td>8457.69</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DDT</td>
<td>1239.60</td>
<td>0.03</td>
<td>46495.83</td>
<td>10.99</td>
</tr>
</tbody>
</table>

Table 2.4: Table of BCF_k values from mixture study exposure in carp liver tissue.

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>Uptake (K1)</th>
<th>Depuration (K2)</th>
<th>BCF_k</th>
<th>MX Benchmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture Compound exposure</td>
<td>4-Nonylphenol</td>
<td>68.58</td>
<td>0.005</td>
<td>13864.91</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>Chlorpyrifos</td>
<td>59.53</td>
<td>0.05</td>
<td>1119.54</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Musk Xylene</td>
<td>2411.10</td>
<td>0.20</td>
<td>11826.60</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DDT</td>
<td>4721.00</td>
<td>0.21</td>
<td>22964.88</td>
<td>1.94</td>
</tr>
</tbody>
</table>

2.3.3 Whole-Body Homogenate Results

In a separate experiment, we ran both single and mixture exposures in carp, however, instead of just taking specifically muscle and/or liver tissue we sampled whole body homogenate and compared them to our tissue-specific sample analysis. Especially in a species like carp, where liver tissue is disperse and hard to gather, taking whole body homogenate tissue could help regulatory agencies improve the efficiency of BCF tests as this whole body homogenate approach could further streamline the process of data analysis.

Muscle data would indicate that our muscle data more closely represents single compound whole body BCF_k values than the liver does. By extracting out specific tissues we can pinpoint exact sites for bioaccumulation within an organism, however, this does not completely represent whole body bioaccumulation as the whole body homogenate does. In the mixture study, criterion for BCF_{50} was met for nonylphenol and chlorpyrifos; however, these criteria
were not met for DDT and musk xylene during the time allotted for the experiment. Such would be expect for DDT due to its extreme lipophilic nature and increased bioaccumulation results even during an extended BCF flow-through test.

Table 2.5: Summary of BCF<sub>k</sub> values calculated from whole body homogenate in carp from single and mixture studies.

<table>
<thead>
<tr>
<th>Whole Body Homogenate</th>
<th>Single Compound</th>
<th>Uptake (K&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>Depuration (K&lt;sub&gt;2&lt;/sub&gt;)</th>
<th>BCF&lt;sub&gt;k&lt;/sub&gt;</th>
<th>MX Benchmark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Musk Xylene</td>
<td>764.70</td>
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<td>Exposure</td>
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<td>Chlorpyrifos</td>
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<td></td>
<td></td>
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<tr>
<td>Whole Body Homogenate</td>
<td>Mixture</td>
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<tr>
<td>Exposure</td>
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<td>0.03</td>
<td>213689.82</td>
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2.4 Discussion

In this study, we propose an abbreviated study design for aquatic bioconcentration testing which effectively alleviates the burdens of running a flow-through test. Four chemicals were used individually to evaluate the usefulness of the proposed shortened design; 4-Nonylphenol, Chlorpyrifos, Musk Xylene, and DDT. The study consisted of exposing *Cyprinus carpio* for 7 days followed by 7 days of depuration, for a total of a 14-day study. Our results for each of the four compounds are consistent with literature values, thus, demonstrating that BCF<sub>k</sub> can be accurately predicted in an abbreviated *in vivo* test. A shortened approach could increase productivity for both regulatory agencies and industries. Alleviating monetary and time burdens would help move environmental assessment forward a little faster and allow for more chemicals to be screened. Only a small percentage of chemicals that are being produced
in high quantities and that are found in the environment have published BCF or BAF values, and
even fewer with published BMF values. BCF$_{ss}$ was met in the short amount of time the
organisms were exposed to the chemicals, thus, demonstrating that a shortened approach
could be accurate and reliable for further aquatic BCF testing. Chemical exposure to aquatic
organisms to test bioaccumulation has been used for many years, but ever since, little has been
done to refine, reduce, and replace animal testing in the laboratory. To further the expansion of
a growing line of work, efficiency and improvement must be taken into account. Using this
method for bioaccumulation will aid regulatory agencies and private labs to further streamline
the process of chemical BCF testing, which will allow for more chemicals in question to be
tested.

Also, it is important to note the BCF values that we found with the mixture study. BCF$_k$
for 4-nonylphenol and DDT increased tremendously in the mixture exposure indicating that
there are chemical interactions that may be taking place within the organism that are affecting
the ADME characteristics of these chemicals. It is interesting to note that the most dramatic
changes were seen in the liver tissue, a site where metabolism of xenobiotic chemicals occur
the most. Most BCF tests are ran with only one compound being tested at a time. However, we
show here that in the presence of multiple chemicals, BCF values have the potential to be
dramatically altered.

There can be many reasons for skewed BCF results; one possibility is that one of the
compounds, musk xylene, is a known CYP2B inhibitor in rat and mouse hepatocyte cell lines.
This inhibition, in turn, could affect the metabolism of the other compounds where CYP2B is
important for metabolism. Further experiments would need to be done to verify the results we see here, and to better determine why exactly we saw these results.

Our whole body homogenate results indicate that it may be possible to use this approach when running an aquatic BCF flow-through test. However, since our results in the mixture differ so greatly, this approach may only be prudent in single exposure studies. This variation in results could be due to adsorption in the presence of high lipid content, or in the case of nonylphenol adsorption to vessels used to extract it out for chemical analysis. The whole body homogenate results indicated that it could be used as a replacement for muscle-specific BCF\(_k\) data. However, in the mixture studies the results varied, meaning, more testing would need to be completed to verify the data shown here.

2.5 Chapter References


Scott H. Jackson, Christina E. Cowan-Ellsberry, and Gareth Thomas. Use of Quantitative Structural Analysis To Predict Fish Bioconcentration Factors for Pesticides. *Journal of Agricultural and Food Chemistry* 2009 57 (3), 958-967


CHAPTER 3

IN VITRO METABOLISM OF FOUR COMPOUNDS AND THEIR EFFECTS THEY HAVE ON EACH OTHERS METABOLISM

3.1 Introduction

Sub-cellular in vitro metabolism assays have increased in popularity and usage in the past decade and have been extremely helpful in screening for metabolic properties of xenobiotics (Plant, 2004). Not only have in vitro assays been crucial for drug companies in their decision-making process of which chemicals to discard and which to progress further for development, but they have also been novel in helping environmental regulatory agencies and laboratories around the world screen for potentially harmful chemicals in the environment (Lipscomb and Poet 2008; Cowan-Ellsberry et al., 2007). With respect to environmental impact assessment, sub-cellular in vitro metabolism assays offer a quick and cost-effective way to determine metabolism potential of certain compounds (Cowan-Ellsberry et al., 2007).

Humans and different environments are constantly exposed to xenobiotic chemicals and to assess if there are any harmful impacts, regulatory agencies look at the persistence (P), bioaccumulation (B), and toxicity (T) characteristics of thousands of chemicals European Commission, 2003). However, with as many chemicals that have been screen, it has been estimated that reliable BCF data only exists for <4% of commercial organic compounds (Arnot and Gobas, 2006). Fish BCF can be measured using the OECD guideline 305 (OECD, 1996). Guideline 305 can become a very long and expensive test, thus lengthening to time it takes to acquire BCF data. Computer models, such as quantitative structure-activity relationships (QSARs), have also been novel in assisting with BCF prediction of certain compounds. However,
these models can provide inaccurate estimates of bioaccumulation potential (Dimitrov et al., 2003; Weisbrod et al., 2007). Model inaccuracies can be attributed to the lack of reliable and accepted methods for estimating biotransformation in fish (Cowan-Ellsberry et al., 2007).

Furthermore, chemicals are found in the environment as a complex of chemicals not just a single compound. Along with BCF tests, in vitro assays are normally conducted with one chemical at a time. Due to the complex nature of chemical interactions, inside or out of an organism, it is important to note the metabolic changes a compound exerts in an organism in the presence of other chemicals. To clarify, musk xylene is a known inhibitor of the CYP2B enzyme in rat and mouse liver fractions (Hawkins et al., 2002; Lehman-McKeeman et al., 1995; Lehman-McKeeman et al., 1999). If present, musk xylene could potentially inhibit the biotransformation of a compound where CYP2B is the primary pathway taken by that compound. Chemical interaction should be considered important not only for biotransformation data, but for BCF testing data as well.

In a BCF flow-through experiment, a mixture of chemicals was used at once to see if there were any differences in BCFk values. Because of the dramatic increase of values in the mixture study, as compared to the single compound study, it is necessary to find out exactly why this increase was seen in the aquatic organisms. The previous chapter of this work outlines data found in both experiments, and as the liver showed the largest increase in BCF values it would thought that this difference could be explained by running in vitro S9 assays to assess any inhibitory effects chemicals may have on the liver. Because the liver is the primary site for most compounds to be metabolized, it seems beneficial to run these experiments to assess any correlation between liver metabolism and BCF results we saw in the previous chapter.
3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Chlorpyrifos (\(O,O\)-diethyl 3,5,6-trichloropyridin-2-yl phosphate, CAS# 2921-88-2) methyl-chlorpyrifos (CAS# 5998-13-0), 4-Nonylphenol (CAS# 104-40-5), D4-Nonylphenol (CAS# 1173019-62-9), DDT (1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane, CAS# 50-29-3), DCBP (4,4’-Bis(9-carbazolyl)1-1’-biphenyl, CAS# 583828-31-7), B-Nicotinamide adenine dinucleotide phosphate (NADPH, CAS# 100929-71-3), isocitric dehydrogenase (NADP, CAS# 9028-48-2) and DL-isocitric acid trisodium salt hydrate (ISO, CAS# 1637-73-6) were all obtained from Sigma Aldrich Corp (St. Louis, MO). Musk Xylene (1-\(\text{tert}\)-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene, CAS# 75-05-8) was obtained from City Chemical LLC (West Haven, CT). HPLC grade methanol, DCM, ethyl acetate, and n-hexane were obtained from Fisher Scientific (Houston, TX). Milli-Q water was obtained from in house Milli-Q water system (Millipore, Billerica, MA). Resuspension buffer (0.02M KH\(_2\)PO\(_4\), 0.08M K\(_2\)HPO\(_4\), 0.1M KCl, 1mM EDTA, and glycerol) materials were obtained from Fisher Scientific (Houston, TX).

3.2.2 Fish Culture

Both male and female trout (\textit{Oncorhynchus mykiss}) weighing approximately 1 kg were used in the study. Fish were obtained from Pond King (Gainesville, TX) and transported to the University of North Texas. The fish were kept in a living stream at approximately 15°C, fed daily with commercial trout chow, and placed under fluorescent lights with a 16 h light/ 8 h dark photoperiod.

3.2.3 Liver S9 Preparation

Fish were anesthetized with up to 100 mg/L MS222 until immobile prior to sacrificing.
The liver was then perfused with a saline solution in order to remove hemoglobin from the organ. Livers were pooled from 3 different trout, weighed and then homogenized on ice in PBS buffer solution in a 2:1 ratio of buffer to liver. The PBS buffer (pH 7.4) was composed of 50mM potassium phosphate, 0.15 M potassium chloride, and 0.2 M sucrose. The homogenate was placed into chilled centrifuge tubes and centrifuged at 9,000g for 20 min at 4°C. The S9 (supernatant) was pulled off from the tissue debris with careful pipetting and 1 mL was transferred to chilled 1.5 mL microfuge tube then stored in a -80°C freezer for later use. 300 μL of S9 was set aside to analyze CYP activity using spectrophotometer as described in (Paper David found) and total protein content was measured by conducting a Bradford assay.

3.2.4 Microsomal Preparations

For specific phase I metabolism analysis, microsomal fractions were prepared from trout S9 fractions. S9 was placed in chilled ultracentrifuge tubes and centrifuged at 100,000g for 1 h at 4°C. The supernatant was removed from the tubes and 500 μL resuspension buffer (0.02 M KH2PO4; 0.08 M K2HPO4; 0.1 M KCl; 1 mM EDTA; 1mM DTT; 20% glycerol or 20 g in 100 mls) was added to remove the bottom pellet. Using a thin brush, the microsomes were gently removed from the bottom of the tube and mixed in with the resuspension buffer. The resulting solution was then placed in a -80°C freezer for storage, and as with the S9 300 μL was set aside for protein and CYP analysis.

3.2.5 In Vitro Assay

Trout S9 and microsomes were diluted to 1 mg/mL protein content in PBS (pH7.4) containing 50mM potassium phosphate and 0.15 M potassium chloride. A NADPH regeneration system was used and composed of isocitric (7 mM), isocitric dehydrogenase (0.5 units of
activity ml$^{-1}$) and β-NADPH (600 mM) (Sigma-Aldrich, St. Louis, MO). All reagents were made in PBS (pH7.4) on the day of the assay. The regeneration system was aliquoted into 20 mL vials that contained the diluted S9 or microsomal fractions, and the reaction mix was placed in a 25°C shaking water bath and allowed to equilibrate for several minutes before adding the testing compound. To initiate biotransformation, an ethanol (<1% solvent) stock containing the compound (10 μM) was added to each of the reaction mixes. Over a period of 1 h, 0.5 mL aliquots of the solution were removed and placed in 4 mL vial containing 0.5 mL 1:1 hexane-ethyl acetate solution to stop the reaction. Time points to assess biotransformation were 0 min, 15 min, 30 min, 45 min and 60 min. The vial was then vortexed and centrifuged to remove the supernatant for analysis on either a LC-MS/MS or a GS-MS.

3.2.6 Control Groups

Three different controls were used while running each of the S9 or microsomal assays: a buffer control, which contained only buffer and compound (10 μM); a no NADPH control, PBS (pH7.4) was added into the reaction mix to fill the missing volume; and a heat inactivated control, diluted protein was placed in an oven at 100°C until protein was visibly coagulated and clumped. The controls were only taken at time points 0 min and 60 min to assess any inaccuracies that may have arisen. Buffer controls are used to assess loss of parent due to volatility or adsorption onto surfaces, no NADPH controls are used to assess whether or not the reaction is phase I as CYP enzymes require the donated hydrogen from the NADPH, and the heat inactivated controls denatures the proteins and can be used to assess the loss of parent due to adsorption to the reaction vessel, volatilization, or abiotic degradation.
3.2.7 CYP-Specific Inhibition

To show that the presence of certain compounds can alter the metabolism profile of other compounds, musk xylene was added and run in separate assays. A solution of musk xylene (10 μM) was added to the standard NADPH regeneration solution with the diluted protein in two different ways: 1) at the same time that the test compound was added, and 2) 30 min before the test compound solution was added. By doing adding musk xylene at different times we can assess whether or not the parent compound is responsible for the inhibition of metabolism, or its metabolites.

3.2.8 Data Analysis

Data analysis was prepared using GraphPad Prism® (version 5.02, La Jolla, CA). A One-way ANOVA was performed to determine statistical significance in all experiments, a $P$ value less than 0.05 was considered significant. Within the reaction mix vessels, levels of all compounds were expressed as Means ± SEM.

3.3 Results

The results of the following assays were representative of literature findings in regards to nonylphenol (NP) metabolism in trout. Two assays were used to assess metabolism: S9 and microsomal fractions. The results show that with phase I assays (microsomes) NP is not readily metabolized or degraded (data not shown); however, with S9 fractions we see a 50% loss in parent compound (Fig. 3.1). Also, in the S9 assay there is an almost equivalent loss of parent in the buffer control. In a separate assay, we ran a reaction mix with a buffer control and at time point 60 min we washed the sides of the reaction vessel with hexane ethyl-acetate and transferred residue to a 4 mL vial. By doing this we were able to recover 100% of NP in the
buffer controls (data not shown). In the no NADPH control with the S9 assay the loss of parent was almost equivalent to the loss in the reaction mix, this alongside with the microsomal results indicates that NP is metabolized mainly by phase II enzymes.

Figure 3.1: Biotransformation of 4-NP in trout S9 fractions over a 60 min period.

Chloropyrifos (CPS) results differed than that of the nonylphenol results. We saw no significant loss in parent for the CPS assay, indicating that trout may not be able to metabolize

Figure 3.2: Biotransformation of NP in trout S9 fractions. Data here showing different reaction vessels with MX added.
CPS as well as mammals can. Surprisingly, the results from the musk xylene (MX) treated and MX + CPS assays were somewhat counterintuitive. In the reaction vessel that was treated with 10 µM MX 30 mins prior to adding CPS, we see a significant drop in parent compound by time point 60 min. In the reaction vessel where CPS was added simultaneously with MX (10 µM each) we see a steady decline in the parent compound as the reaction takes place, indicating that perhaps a metabolite of MX (rather than MX itself) is responsible for the increased loss of parent. In multiple rat and mice studies, results have shown that while inducing CYP2B expression, MX simultaneously inhibits CYP2B activity (Hawkins et al., 2002; Lehman-McKeeman et al., 1995; Lehman-McKeeman et al., 1999). Being that a CYP2B enzyme is important in mammalian metabolism of CPS, it would seem that MX would inhibit the metabolism of CPS however, this is not the case.

Figure 3.3: Biotransformation of CPS in trout S9 fractions.
3.4 Discussion

The results of this study show that in the presence of musk xylene some type of interaction is taking place within the reaction mix to alter the metabolic characteristics of trout S9 fractions. Musk xylene is a known CYP2B inhibitor in rats and mice but little is known about its effects in trout. Chemical inhibition is especially important for regulatory agencies that run BCF tests according to protocol OECD 305. In the presence of multiple chemicals, interactions may be altering certain ADME characteristics of an organism, either by certain interactions that are affecting metabolic enzymes of the organism or if there is some sort of chemical-to-chemical interaction taking place. We show here that in the presence of musk xylene, results of loss-of-parent metabolism assays are altered. These results, however, differ between chlorpyrifos and 4-nonylphenol.

For 4-nonylphenol, in the presence of musk xylene, metabolism seems to be inhibited to a certain point, preventing the S9 fraction from breaking the parent molecule down. Being that the duality of musk xylene is that it not only induces transcription of certain CYP enzymes in rat livers but it also inhibits the activity of these enzymes, therefore, the results of the 4-nonylphenol assays are not surprising. Two reaction mixes were ran with musk xylene and 4-nonylphenol; 1) a reaction mix with the S9 and all co-factors treated with musk xylene for 30 mins before aliquoting in 4-nonylphenol, and 2) a reaction mix where musk xylene and 4-nonylphenol were aliquoted in the reaction mix vial simultaneously. In the reaction mix that was treated with musk xylene, no metabolism was seen in 4-nonylphenol over a period of 1 hr. Indicating that musk xylene had successfully inhibited or prevented 4-nonylphenol from being broken down. In the second reaction mix, when 4-nonylphenol was added simultaneously with
musk xylene, we observed a gradual decrease in metabolic activity over a period of 1 hr. Within 30 mins we observed a 30% loss of parent in 4-nonylphenol, however, over the next 30 mins we observed >5% loss of parent in the parent compound. These data suggests that a metabolite of musk xylene may be more responsible for inhibiting metabolic activity rather than musk xylene itself.

For chlorpyrifos, the results were somewhat counterintuitive as compared to our 4-nonylphenol results. For the chlorpyrifos assay we ran the same reaction mixes with one reaction mix treated with musk xylene 30 mins before adding chlorpyrifos and another where we added them simultaneously. What we observed was the exact opposite of what we saw in 4-nonylphenol. For the 30 mins treated reaction mix we saw the most metabolism after 1 hr, and for the second reaction mix we observed an increase in loss-of-parent over the period of 1 hr. In the second reaction mix, we saw little loss of parent in the first 30 mins and over the next 30 mins we see that loss almost double in scale. These data suggests that musk xylene may have had the opposite effect as originally hypothesized. We also ran a reaction mix that was included only S9, co-factors, and chlorpyrifos, where we saw little to no significant loss of parent in the results. Along with these results and the results from the previously mentioned reaction mixes, it would seem that musk xylene (or one of its metabolites) actually increases the rate or ability for trout S9 to metabolize chlorpyrifos.

The reactions that we observed taking place are not well understood, however, what is apparent is that in the presence of musk xylene other compounds metabolic properties change – and not in the same way. The results of this experiment show that it is important for us to try and understand these interactions better, as they may affect how certain regulatory agencies
handle chemical impact assessment. Most notably, for aquatic BCF tests results may vary in the presence of multiple chemicals. Environmental impact assessment protocols were set in place to help protect not only the environment but humans as well, as it is possible for us to be exposed to these chemicals. Realistic environmental conditions do not comprise of only one chemical but a mixture of chemicals and it is important for us to understand these interactions as they may alter BCF test results, which would not represent real-world interaction within the environment.

3.5 Chapter References


CHAPTER 4

CONCLUSION

4.1 Introduction

Environmental impact assessment has grown and developed since Rachel Carson wrote her eye-opening book ‘Silent springs’, however, over the past decade little has been done to improve how we go about testing compounds that are of concern. One goal for researchers is to apply the 3Rs whenever possible; refine, replace and reduce. Referring specifically to the number of animals required to run a BCF test, we can apply these ‘3Rs’ to aquatic BCF testing. Current protocols (OECD 305) outline specific guidelines to run a reliable and reproducible aquatic flow-through test, however there are some limitations that hinder the process from testing more chemicals in a timely manner. Being that there is very little reliable data for the amount of chemicals that are present in the environment, it is becoming increasingly important to alleviate certain constraints that are present in current testing protocols.

4.2 BCF Testing

OECD guideline 305 outlines how to run an aquatic flow-through test, however the protocol can be a burden on a laboratory. The time the protocol requires to run an aquatic flow-through and the amount of fish needed can become costly. If steady-state has not been reached within 28 days, the protocol states that one should keep running the test until steady-state has been met, or up to 60 days. After the uptake phase in completed, the depuration phase initiates. The length of these two phases combined with the number of sampling days demonstrates how much of a burden running one of these tests can be. Therefore, in these
experiments we demonstrate a shortened testing design that could alleviate not only a monetary burden, but time as well.

The results of the 14-day BCF tests show that accurate and reliable \( \text{BCF}_k \) can be calculated in only 14 days, rather than a 42-day study as proposed by OECD 305. Our results here are comparable to literature BCF results and accurately represent compound \( \text{BCF}_k \) in juvenile common carp. By shortening the time it takes to run an aquatic BCF test, we could effectively alleviate the burdens of current protocols, which would ultimately allow for more compounds to be screened. As previously stated, there are many chemicals present in the environment that still need to be assessed, and BCF results of many chemicals that have been tested may not be as reliable as others. By using the proposed shortened procedure here, more chemicals could be pushed through evaluation.

We also evaluated the differences between using tissue-specific samples and whole-body homogenate samples for the BCF tests. What we found was that there are little differences between using tissue-specific sampling and whole-body homogenate. While we observed differences in \( \text{BCF}_k \) between specific tissues (e.g. liver and muscle), the \( \text{BCF}_k \) values of the whole-body homogenate fell in between the values for muscle and liver (in the single compound studies). Especially with species such as juvenile carp, it can become very time consuming to extract out specific tissues such as liver or muscle, therefore using whole-body homogenate can shorten some of the time for sample preparations. Also, being that the \( \text{BCF}_k \) results between muscle and liver differ so greatly, using whole-body homogenate can provide a more accurate \( \text{BCF}_k \) that is present throughout the whole fish.
Along with shortening current protocols, we ran shortened flow-through tests with only one compound as well as a mixture of compounds. While most aquatic BCF tests are ran with only one test chemical in question, it is important to note that within the environment certain chemical interactions could take place that will affect the BCF in certain organisms. Our data suggests the importance of real-world environmental exposures in aquatic organisms as certain properties of chemicals may change in the presence of multiple chemicals. Our mixture exposure included the compounds 4-nonylphenol, chlorpyrifos, DDT, and musk xylene. In our results for the mixture studies we observed a dramatic increase in BCF for 4-nonylphenol and musk xylene. Musk xylene is a known CYP2B inhibitor in mammalian livers, however, little is known about its affect in the common carp. And while musk xylene is only one example of this alteration, there are many compounds present in the environment that could skew BCF results.

4.3 S9 In Vitro Assay

Due to the results of our mixture studies, we found it prudent to run in vitro S9 assays to see if there were any differences in metabolism in the presence of multiple chemicals. Using trout S9 fractions we were able to detect any affects that may be occurring within the organism that would alter its ability to excise chemicals. What we found was quite surprising in that the results varied between specific compounds. In the presence of musk xylene, the trout S9 was unable to reduce the parent compound, as it normally should without musk xylene present. We also observed a decrease in the loss of parent of time when musk xylene and 4-nonylphenol were simultaneously added to the reaction mix, indicating that a metabolite of musk xylene may be responsible for this inhibition rather than musk xylene itself. The exact opposite was observed in the chlorpyrifos assay. The trout S9 was unable to metabolize the parent
compound without musk xylene present in the reaction mix. However, when musk xylene was added 30 mins prior to adding chlorpyrifos, we observed a significant loss in parent chlorpyrifos. Along with these results, when musk xylene was added with chlorpyrifos simultaneously we observed a gradual increase in loss-of-parent over time, indicating again that a metabolite of musk xylene may be responsible to this induction of metabolism rather than musk xylene itself. The results observed in the in vitro assays correlate with the results that we see in our single and mixture BCF tests. BCF$_k$ for 4-nonylphenol dramatically increased in the mixture study, indicating that the organism was unable to excise the compound properly as it did in the single study exposure. BCF$_k$ for chlorpyrifos remained unchanged in the mixture study design when compared to the single compound study. Results from the in vitro assays could help explain why our BCF results dramatically differed between our single and mixture studies. However, more testing should be done to clarify the differences in BCF results.

4.4 Conclusions

In any case, the results from both the BCF tests and in vitro assays indicate that further studies should be done to better understand how chemicals behave within certain organisms. The work done for this thesis showed the following:

- 14-day BCF tests can accurately and reliably represent BCF results from a 42-day study.
- Whole-body homogenate sampling for BCF tests can be used to represent BCF$_k$ of the entire fish and not just specific tissues.
- Mixture BCF studies should become regular in practice to represent real-world interactions in the environment.
- In the presence of certain chemicals, in vitro clearance can change, further indication the importance of mixture studies.
Environmental impact assessment is absolutely essential to the health of our environment and to humans; so it should be equally important to continually question and update current protocols that are set in place for assessment. There is much work that could be done to help move environmental assessment forward, and the work done here shows that certain steps could be taken to help move impact assessment forward to allow for more chemicals to be tested and to help obtain better and more accurate ‘real-world’ BCF values.

4.5 Chapter References


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APPENDIX A

BCF GRAPHS FROM SINGLE EXPOSURE
Figure A.1: Concentration of DDT in muscle tissue over 14 days with a nominal concentration of 2.5 ppb.

Figure A.2: Concentration of DDT in liver tissue over 14 days with a nominal concentration of 2.5 ppb.
Figure A.3: Concentration of 4-NP in muscle tissue over 14 days with a nominal concentration of 2.5 ppb.

Figure A.4: Concentration of 4-NP in liver tissue over 14 days with a nominal concentration of 2.5 ppb.
Figure A.5: Concentration of MX in muscle tissue over 14 days with a nominal concentration of 3.2 ppb.

Figure A.6: Concentration of MX in liver tissue over 14 days with a nominal concentration of 3.2 ppb.
Figure A.7: Concentration of CPS in muscle tissue over 14 days with a nominal concentration of 2.5 ppb.

Figure A.8: Concentration of CPS in liver tissue over 14 days with a nominal concentration of 2.5 ppb.
APPENDIX B

BCF GRAPHS FROM MIXTURE EXPOSURE: CONCENTRATION OF MULTIPLE COMPOUNDS IN WHOLE BODY HOMOGENATE OVER A 14-DAY PERIOD
Whole Body BCF Values for Mixtures Study

Whole Body Concentration (ng/gram) vs. Time (Days)

- Chlorpyrifos
- Musk Xylene
- 4-NP
- DDT

Uptake and Depuration phases are indicated.

Data points and error bars shown for each chemical over the time period of 1 to 14 days.