Petroleum Metabolites

Literature Review and Assessment Framework

Technical Resource Document
San Francisco Bay Regional Water Quality Control Board

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Executive Summary

The evaluation of the adverse effects on human health and the environment from exposure to petroleum contamination, from crude oil as well as refined products, is difficult to quantify because such releases are usually mixtures composed of hundreds to thousands of individual chemicals. Petroleum mixtures present challenges at multiple levels, from the determination of the exact concentration of each chemical to the evaluation of the toxic effects on different receptors individually and in combination. Weathering further complicates these issues because it creates numerous additional compounds, which often behave differently than the parent petroleum hydrocarbons.

Fresh petroleum releases contain primarily hydrocarbons in the strict sense, that is, molecules consisting only of carbon and hydrogen. Although most hydrocarbons have low solubility in water, water samples from sites with large, aged petroleum releases commonly contain significant concentrations of dissolved petroleum-derived chemicals. These chemicals are typically intermediate degradation products derived from complex, multi-step oxidation processes catalyzed by microbes that break down the parent petroleum hydrocarbon molecules. The extent and rate of biodegradation depends on an adequate supply of redox cofactors and the presence of the right combination of microorganisms. Photo-oxidation also contributes to the breakdown of petroleum released at the surface to soil and surface water. While petroleum hydrocarbon-derived partial degradation or breakdown products are known by many terms such as *polar compounds* (in reference to the increased solubility in water that is the result of oxygen in the molecules), *petroleum metabolites* (in reference to biodegradation), or simply *degradates* (a more general term), this document mainly refers to them as ***petroleum metabolites***.

Regional Water Board staff regularly receives technical reports for petroleum release sites with risk evaluations and site management recommendations that ignore petroleum metabolites and focus only on the parent hydrocarbons. In practice, this involves using silica gel cleanup (SGC) to remove the metabolites from samples in which they would normally be quantified as part of common laboratory analyses for total petroleum hydrocarbons. Often dischargers cite Chapter 13 of the State Water Board's 2012 Leaking Underground Fuel Tank Guidance Manual to justify the use of SGC because the document includes a statement that metabolites have a low potential toxicity to humans. The main basis for this statement appears to be a sole conference abstract for a unique and proposed relative toxicity ranking system as the technical reference. However, the few readily available examples of well-studied individual petroleum metabolites with toxicity criteria are not consistent with this conclusion. This led us to conduct a thorough review of the available literature, which is described in this document. In addition, we provide brief explanations of those aspects important for understanding of the behavior of petroleum releases in the environment such as analytical laboratory methods (Chapter 2) and an overview of how weathering affects fate and transport (Chapter 3).

The review of the available literature on the toxicity of petroleum metabolites is presented in Chapter 4 "Human Health Risk" and Chapter 5 "Ecological Risk," which include an overview of research trends, specific publications, and a discussion of their relevance to the work of the Regional Water Boards. Published research documenting adverse effects of metabolites on

both human and ecological receptors confirms the overall concerns of Regional Water Board staff and supports the conclusion that petroleum metabolites clearly pose risks to human and environmental receptors. Thus, we conclude the polar breakdown products should be considered as part of site-specific, petroleum-related risk assessments and also when evaluating cumulative risk. As a result of the review and analysis described in this document, Regional Water Board staff will continue requiring evaluation of petroleum metabolites at sites where: 1) significant petroleum contamination is detected; and 2) human or ecological receptors are present. Regarding SGC of samples prior to analysis of total petroleum hydrocarbons (TPH), Regional Water Board staff will accept it only for the purpose of comparison of duplicate samples processed with and without SGC to assess the relative degree of biodegradation. Routine samples used for determining the full extent of petroleum contamination and evaluating potential risks should be analyzed without SGC.

The Regional Water Board staff's overall approach to petroleum site cleanup remains the same: adequate investigation and delineation; source control to the extent practicable; groundwater plume remediation (natural attenuation where appropriate); sufficient monitoring to demonstrate plume stability; and institutional controls (e.g., deed restrictions, risk management plans, etc.) when necessary. Consideration of additional action is mainly driven by heightened concerns at a site, such as the presence of nearby receptors (e.g., supply wells, aquatic receptors) that could be affected by the groundwater plume (composed of hydrocarbons and/or petroleum metabolites).

Lastly, during our literature review, in addition to the significant body of research available in the area of petroleum metabolites, Regional Water Board staff discovered that novel technologies are being adapted for evaluating a range of toxic effects of multiple contaminant mixtures, including petroleum-related releases, in the environment. As new publications become available on improved laboratory methods, the toxicity of petroleum hydrocarbons and their breakdown products, and related topics, Regional Water Board staff will evaluate and incorporate the information into guidance and/or technical resource documents, as appropriate.

1. Introduction

Spills and leaks of petroleum hydrocarbons such as gasoline, diesel, motor oil, petroleum solvents, bunker fuels and crude oils have caused widespread contamination in the environment (USEPA 1999). The majority of petroleum releases to the environment consist of complex mixtures of many chemicals. In fresh releases most of these chemicals belong to a class of compounds referred to as **hydrocarbons** (molecules containing only carbon and hydrogen atoms). **Crude oils** also contain significant amounts of polar organic molecules that contain heteroatoms (nitrogen, sulfur, or oxygen atoms or metals), refined fuels and products may contain additives, and weathered releases contain partially oxidized compounds, all of which are more polar than the parent hydrocarbons due to larger differences in electronegativity between the constituent atoms. Crude oils are refined by various techniques, which also remove many of the polar compounds intrinsic to crude oil, to produce different petroleum fuels and products based on boiling point range (e.g., gasoline, diesel fuel, motor oil, bunker fuel). However, refined petroleum fuels and products are still complex mixtures consisting of many types of hydrocarbons, including the nonpolar aliphatic and somewhat polar aromatic molecules.

After release to the environment, these petroleum mixtures undergo **weathering**,[1] a stepwise process that produces countless oxygen-containing intermediates. A number of other terms have been used for these petroleum breakdown products, including *polar compounds* in reference to their increased solubility, *petroleum metabolites* in the case of biodegradation, or *petroleum degradates* (a more general term that encompasses both biodegradation as well as photo-oxidation).[2] In this document, we refer to them as **petroleum metabolites** since biodegradation is ubiquitous at soil and groundwater petroleum release sites.

In addition to petroleum-derived polar compounds, there are naturally occurring organic compounds that resemble hydrocarbons with polar functional groups, which we refer to as **biogenic organic compounds (BOCs)**.

Over the past several decades, various arguments have promoted the use of adsorbents to remove the polar compounds from the sample prior to analysis, such as: 1) the polar compounds are naturally occurring compounds (Zemo and Synowiec 1995); 2) the metabolites are no longer hydrocarbons in the strict sense and therefore should not be considered (Zemo and Foote 2003); and 3) the metabolites have such low potential toxicity as compared to the parent hydrocarbon, so they can be ignored (Zemo et al. 2013a).

The argument that petroleum metabolites have low potential toxicity lacks plausibility because numerous examples in both the pharmacological and the toxicological literature demonstrate

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[1] Weathering is a general term for a variety of processes that change the composition of chemicals and mixtures released to the environment. The processes pertinent to the subsurface include: evaporation or volatilization; dissolution or leaching; adsorption to soil particles; and microbial biodegradation. For surface releases, an additional process is photo-oxidation.

[2] The literature refers to the petroleum breakdown products by various terms: organic acids, naphthenic acids, non-volatile dissolved organic carbon, polar compounds, petroleum metabolites, polar hydrocarbons, polar nonhydrocarbons, and oxyhydrocarbons.

that intermediate metabolites are often more toxic than parent compounds. For example, it is well known that the carcinogenic risk associated with vinyl chloride generated by the breakdown of tetrachloroethene (PCE) in the environment is much higher than the toxicity of the parent compound. Similarly, the petroleum hydrocarbon metabolite 2-hexanone is about 200 times more toxic than n-hexane based on comparisons of their USEPA-derived oral reference doses (RfDs) (USEPA IRIS 2016 for 2-hexanone and USEPA 2009 for n-hexane). Moreover, there is evidence that microbial fluoranthene and pyrene metabolites released into the environment are more toxic than the parent compounds (Zielinska-Park 2004). In fact, it is typical that it is the contaminant metabolites mediate the biological effects. This apparent conflict led us to conduct a thorough review of the available literature that is described in this technical resource document, which contains brief descriptions of:

- Laboratory analytical methods and limitations for detecting and quantifying petroleum metabolites;
- The nature, fate, and transport of petroleum metabolites;
- Human health risks: background on our current approach to evaluating threats to drinking water, literature review regarding potential adverse health risks, and discussion;
- Ecological risks: background on how we regulate surface water discharges from permitted facilities, aquatic toxicity testing at petroleum release sites in the region, and our current approach to evaluating threats to aquatic receptors posed by contaminated groundwater discharges; literature review regarding potential adverse effects to aquatic species, and discussion; and
- Conclusions and a summary of the Regional Water Board staff's overall recommendations for the site-specific evaluation of metabolites at petroleum release sites.

Much of the currently available information on the nature and toxicity of petroleum degradation products comes from the study of marine oil spills or the oil sands process waters associated with the Athabasca Oil Sands in Alberta, Canada. These studies have focused on chemical characterization with increasingly sophisticated detection methods and a level of toxicity testing (primarily aquatic species), including:

- Toxicity to Pacific herring embryos from the Cosco Busan oil spill (e.g., Incardona et al. 2012; Lemkau et al. 2014);
- Weathering of the oil released from the Deep Water Horizon spill (e.g., Aeppli et al. 2012; McKenna et al. 2013; Wickliffe et al. 2014);
- Ecological toxicity of the polar polycyclic aromatic hydrocarbons (PAHs) that are also known as oxygenated PAHs (e.g., Lundstedt et al. 2007; Carney et al. 2008; USGS 2011; and Wincent et al. 2015);

- Chemistry of the petroleum/metabolite groundwater plume at the National Crude Oil Spill Research Site in Bemidji, Minnesota (Baedecker et al. 1993; Bennett et al. 1993; Eganhouse et al. 1993; Cozzarelli et al.1994; Thorn and Aiken 1998; Cozzarelli et al. 2001; Amos et al. 2012; Ng et al. 2014; Cozzarelli et al. 2015; Bekins et al. 2016);[3]
- Chemistry of metabolite plumes at California sites (Mohler et al. 2013; Zemo et al. 2013a; O'Reilly et al. 2015);
- Chemistry and toxicity of the naphthenic acids associated with the process waters from mining of the Athabasca oil sands (Rogers et al. 2002; Clemente and Fedorak 2005; Scott et al. 2005, Grewer et al. 2010; Scarlett et al. 2012; Toor et al. 2013; McKee et al. 2014).

During our literature review, we discovered a significant body of research in the area of petroleum metabolites. Likewise, novel technologies are being adapted for evaluating a range of toxic effects of contaminant mixtures (Schroeder et al. 2016; Jahnke et al. 2016), including specifically petroleum-related releases in the environment (Kassotis et al. 2016). As new laboratory analytical methods or information becomes available on the toxicity of petroleum hydrocarbons and their breakdown products, Regional Water Board staff will evaluate and incorporate them into guidance or technical resource documents, as appropriate.

2. Laboratory Methods and Limitations

Petroleum mixtures contain many large hydrocarbons. The more carbon atoms a hydrocarbon has, the more isomers of that molecule can exist. Isomers differ in their three-dimensional structure, which can have important implications not only for their biological activities but also for the investigation of such mixtures by common laboratory methods. For instance, during separation of environmental samples by gas chromatography many of these compounds co-elute because they have nearly the same boiling point, which complicates analysis particularly for those compounds with more than eight carbons (C_8). These unresolved compounds manifest as a hump in chromatograms of conventional analytical methods, referred to as the **unresolved complex mixture (UCM)** (TPHCWG 1998a). UCMs are especially pronounced in biodegraded petroleum and certain refined mixtures such as lubricating oils (Gough and Rowland 1990). The number of compounds detected in crude oil UCMs is at least 60,000 (Marshall and Rodgers 2008), although as many as 250,000 compounds may be present (Sutton et al. 2005). UCMs have been demonstrated to persist in the environment (Thomas et al. 1995; Watson et al. 2002; Scott et al. 2005; Booth et al. 2007; Melbye et al. 2009). Available structural data of these UCM compounds indicates many are highly branched or have different types of complex structures. The degree of branching and the presence of cyclic moieties correlate with increased resistance to biodegradation (Gough and Rowland 1990; Watson et al. 2002; Scott et al. 2005; Booth et al. 2007).

Partial biodegradation or photo-oxidation further increases the complexity of the mixture present at weathered petroleum release sites. Conventional gas-chromatograph methods (GC-FID and

[3] USGS Bemidji Webpage: http://mn.water.usgs.gov/projects/bemidji/

GC-MS) have limited ability to separate, identify, and quantify compounds in complex mixtures (Frysinger et al. 2002; Booth et al. 2007; Melbye et al. 2009; McKenna et al. 2013; Mohler et al. 2013). For example, Aeppli et al. (2012) studied the oxygenated residues that resulted from the weathering of the Deep Water Horizon spill, and estimated the compounds detected by these conventional methods account for only about 25% of a crude oil mass. Even many of the parent hydrocarbons remain unidentified.

A common analysis method used to evaluate the extent of petroleum contamination in environmental media measures bulk hydrocarbons as TPH. The TPH analysis method has been used for decades to gain insights into the full extent of petroleum releases in recognition that it is neither possible nor practicable to detect and quantify each and every hydrocarbon compound. TPH analysis for diesel and motor oil range compounds includes extraction with a nonpolar solvent, molecular separation by gas chromatography (i.e., based on boiling point), and flame ionization detection (FID). Because the method was not designed for individual compound separation/detection, all hydrocarbons eluting within a specified boiling point range (for example, TPH-gasoline, TPH,-diesel, etc.) are lumped and quantified together. The method does not reveal any specific information about detected compounds, such as their identity or structure. While optimized for detection of nonpolar hydrocarbons (i.e., non-degraded hydrocarbons) between about C_5 and C_{36}, this method can detect and quantify petroleum metabolites, albeit with less efficiency. In other words, some fraction of the metabolites will typically be detected and quantified as TPH, which helps with the estimation of the remaining petroleum-related mass as well as risks associated with the release.

This section presents information regarding the TPH analysis method by GC-FID (description of the method, potential interferences, and fractionation techniques); analysis of petroleum metabolites; and resolving interferences from BOCs.

a. Total Petroleum Hydrocarbon Analysis Method

i. Description

The key steps of TPH analysis are sample preparation/extraction, separation, and detection/quantification. Each of these steps has the potential for unequal recovery of sample constituents. Following extraction from an environmental sample with a nonpolar solvent (e.g., hexane or methylene chloride also known as dichloromethane), the sample is introduced into the GC where it is vaporized without decomposition and separated as the compounds travel through a single separation column. The column separates the compounds based on boiling point, with the more volatile compounds exiting the separation column first, followed by the less volatile compounds. After exiting, the compounds are analyzed in the attached detector. An FID ionizes (burns) the carbon-containing molecules thus generating a signal proportional to the mass present in the sample. The results are plotted on a graph (chromatogram) with either boiling point or carbon number on the x axis and intensity of the response on the y axis. The concentration is determined by comparing the area under the sample peak/curve within a selected carbon range against the response from an unweathered fuel standard (e.g., USEPA 1996f).

The preparation methods differ for the more volatile mixtures (e.g. gasoline) and less volatile mixtures (e.g., diesel and motor oil):

- TPH-gasoline (aka gasoline range organics or GRO) – For gasoline-range compounds, TPH-gasoline is analyzed after a preparation step (e.g., purge and trap, which is: USEPA Method 5030B for water samples, USEPA 1996d; and 5035 for soil samples; USEPA 1996e).
- TPH-diesel and TPH-motor oil (aka diesel range organics or DRO and motor oil range organics or MORO, respectively)[4] – For diesel- and motor oil-range compounds, the TPH-diesel and TPH-motor oil analyses are usually performed after liquid-liquid extraction using dichloromethane, and then the extract is evaporated to concentrate the extract before analysis (USEPA Method 3510C, USEPA 1996a).

ii. Interferences

Simple solvent extraction of samples will also to some extent extract similar but unrelated chemicals that may be present at the site, which would bias detections high (interfering compounds) (TPHCWG 1998a). For example, BOCs can be extracted and then detected in the TPH-diesel and TPH-motor oil analyses (Zemo and Synowiec 1995; Zemo and Foote 2003; Lundegard and Sweeney 2004; Lang et al. 2009; and Zemo et al. 2013b). Clearly, the BOCs interfere with the extractable TPH method because the objective is to measure the compounds associated with releases of petroleum mixtures.

iii. Fractionation Methods

The techniques employed to extract the analytes of interest can frequently extract interfering compounds (TPHCWG 1998a). Solvent extracts may be cleaned up or further fractionated in order to accomplish one or more of the following:

- Remove non-petroleum compounds;
- Isolate a particular petroleum fraction; or
- Concentrate the analytes of interest.

Several limitations to various cleanup steps for petroleum hydrocarbons (TPHCWG 1998a) exist:

- Sample loading may exceed the capacity of cleanup columns and cartridges.
- Nonpetroleum compounds like BOCs may have chemical structures similar to petroleum compounds and may behave like a petroleum compound. Such compounds may not be removed during the cleanup.
- The cleanup may not have been properly performed.
- Some analytes of interest may be removed.
- For some samples, no single cleanup technique can remove all interferences.

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[4] In this document, we frequently use the term extractable TPH to refer to both TPH-diesel and TPH-motor oil.

Examples of reagents that can be used to cleanup extracts include alumina and silica gel.

a. Alumina Cleanup

Alumina cleanup (USEPA Method 3611; USEPA 1996b) uses neutral pH alumina in combination with different solvents to separate petroleum mixtures into up to three fractions: aliphatic fraction using hexane; aromatic fraction using methylene chloride, and the polar fraction using methanol.

b. Silica Gel Cleanup (SGC)

Silica gel is a non-specific reagent that has been used for more than a century for the separation of compounds that differ in polarity for preparative and analytical purposes. The fraction of a compound that is bound to and eluted from silica gel depends not only on its polarity but also, to a great extent, on the exact experimental conditions and the solvents chosen. Because silica gel is so non-specific, it allows only for recovery or removal of a fraction of the compounds of interest, and is subject to substantial variability unless experimental conditions are carefully controlled. SGC in accordance with USEPA Method 3630 (USEPA 1996b) can be used for the separation of compounds of differing chemical polarity. SGC, which is an additional cost to the extractable TPH analysis, removes both BOCs and petroleum metabolites, and does not distinguish between the two.

It is not possible to separate the gasoline-range petroleum hydrocarbons (non-polar) and petroleum metabolites. This is because the silica gel separation process would not work with water, which is polar, and the process could volatilize the hydrocarbons.

b. Analysis of Petroleum Metabolites

The (polar) petroleum metabolites can be detected and quantified in bulk by the extractable TPH analysis to some extent, but not if SGC is used.

i. Use of the Extractable TPH Method

To some extent, the (polar) petroleum metabolites can be detected and quantified in bulk by the extractable TPH analysis. Typically, the petroleum metabolites detected in groundwater samples subjected to a standard extractable TPH analysis manifest as an UCM. Although some authors have reported that the UCM tends to occur in the C_{15} through C_{28} range (Lundegard and Sweeney 2004; Lang et al. 2009), Regional Water Board staff have also observed the polar UCM to extend into the early portion of the TPH-motor oil range (C_{24}-C_{36} or C_{40}) in some sample chromatograms. Review of the scaled chromatograms from samples with detections, standards, and blanks can be helpful for interpretation of results.

Nevertheless, the TPH analysis method has shortcomings for detecting polar petroleum metabolites. **In a study of two crude oil spill sites, the USGS found that the extracted DRO compounds represent one-third to one-half of the total concentration of petroleum oxidation products found in groundwater (Bekins et al. 2016).** The USGS determined the main reasons for the difference resulted from three aspects of the DRO analysis method:

1) Polar compounds do not extract well in nonpolar organic solvents like methylene chloride or hexane (TPHCWG 1998a), the solvents typically used for EPA Method 3510C (USEPA 1996a). Alternative techniques to isolate and recover the full spectrum of polar compounds, including high concentrations of high concentrations of ketone, quinone, carboxylic acid and alcohol groups are resin adsorption techniques and electrodialysis/reverse osmosis procedures (Aiken et al. 1992; Green et al. 2015).
2) Many polar compounds do not readily pass through the GC column without derivatization (TPHCWG 1998a), thus, important compounds of interest may be retained in the column.
3) The narrow boiling point range of the DRO method (e.g., C_{10} to C_{24}) means that some metabolites will not be measured. Some metabolites of diesel fuel and crude oils are above or below this range. As noted above, polar UCMs often extend beyond C_{24}.

Consequently, the extractable TPH analysis underestimates petroleum metabolite concentrations and better methods are needed.

ii. Alternative Methods

Several studies attempting to resolve or identify the individual polar compounds within the UCM report that thousands of co-eluting compounds are present, and few can be matched to mass spectral libraries (Melbye et al. 2009; Mohler et al. 2013). Most compounds within UCMs, both nonpolar and polar, remain unidentified and there are few laboratory standards for these compounds (Melbye et al. 2009, Toor et al. 2013).

There are alternative, research-level methods for studying individual compounds, including petroleum metabolites, but all have limitations. For example, Mohler et al. (2013) used comprehensive, two dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC-ToF-MS) and were able to match 750 compounds to known standards but this represented only 23 percent (%) of the total ion chromatogram. Further, accurate quantitation was not possible due to a lack of standards. Some researchers have synthesized small numbers of compounds to develop laboratory standards for accurate identification and quantitation (Smith et al. 2001; Rowland et al. 2011). The drawbacks to the research-level techniques are that: 1) no single preparation/analysis method can detect all the compounds; 2) the methods are expensive; 3) laboratory standards for quantification are available for limited numbers of compounds; and 4) the methods are not standardized. Detection and quantitation of these compounds in field samples is a developing field. Furthermore, toxicity evaluations for each individual petroleum metabolite are in the early stages of development. Therefore, **at this time, a compound-specific approach for the petroleum metabolites is not practicable.**

Another option for an inexpensive laboratory analysis to quantify more of the metabolites than the extractable TPH method is a non-volatile, dissolved, organic carbon laboratory analysis

(NVDOC)[5] which the USGS uses at the Bemidji, Minnesota site. Similar to the extractable TPH analysis, its use would require upgradient testing to ascertain potential BOC contributions (see next section).

c. Resolving Interferences to the Extractable TPH Analysis from Biogenic Organic Compounds (BOCs)

There are several terms used for naturally occurring organic compounds that could potentially introduce errors during the evaluation of TPH contamination including biogenic organic compounds (BOCs), natural organic matter (NOM) and dissolved organic matter (DOM). In this document, we use the general term BOCs for addressing the naturally occurring compounds in soil and water. NOM includes both particulate and colloidal matter, whereas DOM represents the truly dissolved matter.

The term BOCs has been used for certain aliphatic compounds synthesized by living plants that resemble hydrocarbons found in petroleum mixtures as well as biological molecules from decaying matter that might resemble the polar breakdown products of petroleum hydrocarbons. Some of these compounds can be co-extracted from samples undergoing the extractable TPH analysis (Wang et al. 2012). At heavily vegetated sites (e.g., marshes) with organic-rich soils, the co-extraction and analysis of these compounds could result in overestimation of petroleum contamination.

Near petroleum release areas (i.e., source areas), the concentrations of the petroleum metabolites typically far exceed the concentrations of BOCs (Zemo and Foote 2003; Zemo et al. 2013a), and the potential for significant interference from BOCs is negligible. However, during an initial investigation, when interpreting whether extractable TPH concentrations reflect a petroleum release, BOCs, or both, estimating the relative contribution of BOCs can be important. **Potential interferences to the extractable TPH method from BOCs are most readily assessed by testing both with SGC (hydrocarbons only) and without SGC (hydrocarbons, metabolites, and BOCs) in an area of the site with no known release and with a similar hydrologic and vegetation setting as indicated in Table 1.** This can be accomplished by testing upgradient areas or downgradient locations. However, at San Francisco Bay (Bay) margin release sites, testing at a cross-gradient area (the same distance from the Bay margin) and outside the petroleum-affected area, may be more representative than testing in an upgradient upland area. Consultation with the overseeing regulatory agency is recommended before implementing a background testing program.

The BOCs can manifest in the chromatogram as single peaks that do not resemble a polar UCM, and so chromatogram review can, in some cases, be a useful tool. However, the approach outlined in Table 1 is more robust.

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[5] The U.S. Geological Survey methodology for NVDOC analysis includes filtering the samples through 0.20-µm Supor® or Nuclepore filters into baked glass bottles that are preserved with hydrochloric acid to a pH of <2. The samples are analyzed by a high temperature combustion technique using a Shimadzu TOC Vcsn analyzer (Shimadzu Corporation, Kyoto, Japan) (Bekins et al. 2016).

Table 1 – Recommended Approach for Distinguishing Between Hydrocarbons, Metabolites, and BOCs	
Laboratory Analysis/Evaluation	**Result is a Measurement of:**
Step 1 (Measurement of Hydrocarbons, Metabolites, and Background BOCs) – Samples collected near source area and downgradient.	
1a – Extractable TPH without SGC	HCs + Metabolites + BOCs
1b – Extractable TPH with SGC	HCs
Difference (1a minus 1b)	Metabolites + BOCs (typically negligible, if any)
Step 2 (Assessment of Background BOCs) – Samples collected from upgradient or at known unimpacted location with similar setting as release area. [Optional]	
2a – Extractable TPH without SGC	BOCs
2b – Extractable TPH with SGC	HCs (expected to be non-detect if an unimpacted location was selected)
Difference (2a minus 2b)	BOCs
Notes and Abbreviations: Step 2 is optional but is recommended at heavily vegetated sites Extractable TPH – TPH diesel (diesel range organics) and TPH motor oil (motor oil range organics). Note that this analysis likely underestimates all polar compound concentrations because these compounds are not readily extracted with hexane or methylene chloride. BOCs – Polar biogenic organic compounds unrelated to petroleum Metabolites – Intermediate breakdown products of petroleum hydrocarbons from biodegradation (metabolites) or photo-oxidation. These are polar compounds and are a more soluble class of compounds than the parent hydrocarbons. HCs – Hydrocarbons SGC – Silica gel cleanup	

More sophisticated methods for differentiating between petroleum hydrocarbons and petroleum metabolites and BOCs include:

- Lang et al. (2009) recommended the use of a petroleum-specific marker compound like 1-adamantol to distinguish diesel-derived polar compounds. 1-Adamantol is a polar metabolite of adamantanes ($C_{10}H_{16}$ is the simplest adamantane), which are diamondoids. Diamondoids result from crude oil formation at high temperature and pressures and have no other known natural precursors (i.e., they are unique to petroleum).
- Thorn and Aiken (1998) used carbon dating and elemental sulfur content to distinguish between BOCs and crude oil-derived polar compounds in groundwater.
- Lang (2011) developed a reportedly simple and inexpensive defunctionalization technique that can differentiate petroleum hydrocarbons and polar compounds in TPH extracts of groundwater samples based on their carbon skeletons. Defunctionalization consists of a series of reactions that essentially converts the complex polar compounds into readily-analyzable hydrocarbons by removing the polar functional groups. After defunctionalization and analysis by conventional methods, the origin of the UCM compounds can be determined. For instance, the presence of diamondoid carbon skeletons would be indicative of petroleum because these are ubiquitous in petroleum and do not occur elsewhere in nature.

For soil, Wang et al. (2012) indicated that the presence of petroleum-characteristic, alkylated PAH homologues and biomarkers can be used to distinguish between petroleum-contaminated soils and BOCs. The analysis of the alkanes and use of diagnostic ratios (e.g., carbon preference index) can be used to apportion contributions from petroleum versus BOCs.

d. Discussion – Laboratory Methods and Limitations

The TPH analysis remains the primary tool to detect and quantify the bulk components of petroleum releases, including petroleum metabolites. Extractable TPH analysis with SGC quantifies the petroleum hydrocarbons whereas extractable TPH analysis without SGC provides an estimate of the amount of both the petroleum hydrocarbons and petroleum metabolites. The difference is used to estimate the petroleum metabolite concentration while keeping in mind that extraction and other analysis steps likely have been biased (i.e., not all the petroleum metabolites are extracted and thus concentrations likely are underestimated). Although interferences from BOCs are typically expected to be minimal near petroleum source areas for a given site, BOCs can be evaluated by testing appropriate locations or, if necessary, other techniques (Table 1). Table 2 illustrates the overall TPH analysis approach for petroleum releases to estimate both hydrocarbons and metabolites.

Table 2 – TPH Analysis Approach for Petroleum Releases

Carbon Range of Petroleum Release	Recommended TPH Analysis and Target Compounds		
	TPH gasoline	TPH diesel	TPH motor oil
Low (C_5-C_{12})	Yes (HC)	Yes (M)[1]	--
Medium (C_{10}-C_{24})	--	Yes (HC + M)	Yes (M)
High (C_{24}-$_{36}$)			Yes (HC)

Abbreviations:

HC – Petroleum hydrocarbons

M – Petroleum metabolites. The breakdown products typically boil at higher temperatures. For instance, breakdown products of low carbon range releases typically boil in the diesel range, and breakdown products of medium carbon range releases boil in both the later diesel and early motor oil range.

3. Nature and Fate and Transport of Petroleum Metabolites

a. Nature

The petroleum metabolites include: 1) the transformation products from the microbial biodegradation of the parent petroleum hydrocarbons; 2) transformation products from the photo-oxidation of surface releases; and 3) polar compounds in unrefined crude oil that contain nitrogen, sulfur or oxygen (NSO) atoms. Although the degradation of petroleum hydrocarbons has been studied, the factors affecting the relative persistence of these intermediate

transformation products (i.e., the petroleum metabolites) at a given site are not fully understood because the microorganism, mechanisms and pathways of degradation depend on site-specific conditions. However, we do understand the following:

- Thousands of individual polar compounds resulting from the degradation of petroleum hydrocarbons are possible (Mohler et al. 2013). Petroleum metabolite molecules contain oxygen in addition to carbon and hydrogen atoms. For example, at the crude oil release site near Bemidji, Minnesota, studied by the USGS, a representative molecular formula of $C_{19}H_{24}O_6$ was determined for the non-volatile dissolved organic acids (i.e., polar compounds) (Thorn and Aiken 1998; Ng et al. 2014). At some sites polar compound molecules with sulfur or nitrogen atoms may also be present, especially at crude oil release sites.
- Unrefined crude oil contains polar compounds (e.g., resins, asphaltenes, and compounds containing NSO atoms; Aeppli et al. 2012). These compounds are removed during the refining process for gasoline and partially removed for mid-boiling fuels like jet fuel and diesel (Zemo and Foote 2003). These compounds are not removed from residual fuels[6] (e.g., bunker fuels).
- Due to the presence of strongly electronegative atoms (e.g., oxygen, sulfur, nitrogen), polar compounds are more water-soluble than petroleum hydrocarbons.
- The water-soluble constituents of undegraded petroleum hydrocarbon mixtures are largely limited to aromatic hydrocarbon molecules with 14 or fewer carbon atoms (C_{14} and smaller), and smaller aliphatic hydrocarbon molecules with 6 or few carbon items (C_6 and smaller) (Zemo and Synowiec 1995).
- In contrast, petroleum metabolites with greater molecular masses and aliphatic structures remain soluble within limits. Progressively larger molecular weights and chain lengths limit the solubility of polar compounds. Overall greater solubility due to weathering is apparent from the results of extractable TPH analyses from groundwater samples collected near petroleum source areas with polar-compound-only concentrations ranging as high as 10,000 to 100,000 µg/L (Lundegard and Knott 2001; Zemo and Foote 2003; Lundegard and Sweeney 2004; Lang et al. 2009; and Zemo et al. 2013a), which are one to two orders of magnitude greater than hydrocarbon-only concentrations.
- Given their greater solubility, it is not a surprise that groundwater plumes from weathered petroleum releases are dominated by petroleum metabolites rather than petroleum hydrocarbons.

b. Fate and Transport

Intermediate breakdown products of petroleum hydrocarbons alter the fate and transport properties of the release such as solubility or volatility. Although hydrocarbons are chemically very inert (Labinger and Bercaw, 2002), many petroleum releases will at least partially degrade in the environment. Petroleum hydrocarbon breakdown is a complicated, multi-step process that requires a large number of catalytic activities. In addition, complete oxidation of large

[6] Residual fuels are residues from crude oil that remaining after distillation or cracking. They are heavier than diesel fuel/middle distillates.

hydrocarbon molecules, which represent the fully reduced state of carbon, depends on a large supply of terminal electron acceptors such as oxygen. For instance, complete mineralization of a single molecule of octadecane ($C_{18}H_{38}$) requires 55 oxygen atoms. Thus, for petroleum release sites with larger residual petroleum mass in soil it is not uncommon to observe different stages of biodegradation with different terminal electron acceptors besides oxygen. When electron acceptors are limited, petroleum metabolites can be continually generated over many years, which is likely why petroleum-generated groundwater plumes can persist for decades.

At sites where biodegradation is occurring the catalytic activities are provided by a large collection of different microorganisms sometimes called a microbial consortium. The rate and extent to which hydrocarbons degrade depends also on site-specific factors and on the size and structural features of individual hydrocarbon constituents. For example, hydrocarbons can have elements that are aliphatic, aromatic, cyclic (alicyclic), polycyclic, unsaturated or branched. All of these elements require different types of microorganisms for breakdown, and some are fairly resistant to biodegradation, such as branched groups (Schaeffer et al. 1979). Site-specific controlling factors include the availability of oxygen or alternate electron acceptors, geochemistry, moisture, temperature, and the presence of other contaminants which alter conditions, for example, by using up oxygen. Only if all of the necessary factors and all required microorganisms (catalytic activities) are present in adequate quantities and proportions, and in the proper sequence, can hydrocarbon releases be mineralized, that is, all carbon atoms converted to carbon dioxide. Hydrocarbons can also be degraded under anaerobic conditions, albeit much less efficiently. Since for each type of hydrocarbon in the release the requisite microorganism may only catalyze a few steps under given site conditions, intermediate metabolites can accumulate. Examples of (partial) breakdown products can be found in reviews by Kiyohara and Nagao (1978), Foght (2008) and Rojo (2009). Lack of oxygen or other redox cofactors or production of intermediate products toxic to subsequent microbes may limit the extent of breakdown. Some anaerobic conditions, that is, combinations of certain redox factors and microorganisms, lead to methane production. Mineralization is most likely to occur at the edges of the plume under aerobic conditions.

Few published studies have focused specifically on the fate and transport of petroleum metabolites. In addition, few of the published studies of metabolites at petroleum-contaminated sites provide complete conceptual site models (CSMs). As a result, little is known about groundwater plume size or length, the relative proportions of petroleum hydrocarbons to petroleum metabolites, molecular composition of polar UCMs, or other transport-related properties. However, several studies, summarized below, have yielded interesting and relevant information.

i. Crude Oil-Contaminated Groundwater Studied for Over 30 Years

The 1979 light crude oil release from a ruptured surface pipeline near Bemidji, Minnesota, is probably the best studied petroleum-contaminated groundwater site with a highly-developed CSM. The following is a summary of key elements of the CSM synthesized from Bennett et al. 1993, Eganhouse et al. 1993, Cozzarelli et al. 1994, Thorn and Aiken 1998, Amos et al. 2012, and Ng et al. 2014:

- Source Composition – The original crude oil composition was 58 to 61% alkanes (dominated by n-alkanes with some branched, and negligible cyclic alkanes), 33-36% aromatics (BTEX, alkylbenzenes, and PAHs like naphthalene and phenanthrene), 4-6% resins, and 1-2% asphaltenes (Eganhouse et al. 1993).
- Residual Source – The original spill was estimated to be 10,500 barrels (about 441,000 gallons). Emergency response efforts removed an estimated amount of about 75% of that volume (330,000 gallons). Therefore, the residual comprises about 110,000 gallons (Delin and Herkelrath 2014).
- Geochemical Conditions – Anaerobic conditions were established within a decade following the release. Dissolution of the oil from the source area has resulted in a contaminant plume with a progression of biodegradation reactions, from the source area to the downgradient edge of the plume, including methanogenesis (near the source area) with a dissolved methane plume, iron reduction, manganese reduction, and aerobic oxidation along the plume edges (Baedecker et al. 1993; Bennett et al. 1993).
- Environmental Fate – The weathering and transport differs depending on the nature of the hydrocarbons, as follows:
    - The alkanes (aliphatics) degrade in or near the source area under methanogenic conditions with a local buildup of aliphatic and alicyclic organic acids (i.e., petroleum degradates). After 30 years of weathering, the n-alkanes and the alkyl side chains of the alkylcyclohexanes have significantly degraded, but the branched alkanes are largely unaffected (Ng et al. 2014).
    - Although toluene and ortho-xylene degraded near the source area, other aromatics particularly benzene and ethylbenzene generally dissolve then migrate before being biodegraded in the iron-reducing zone (Cozzarelli et al. 2001).
    - Over time, a persistent non-volatile dissolved organic carbon (NVDOC; aka non-volatile dissolved organic acids)[7] plume developed, which extends about 400 feet downgradient from the source area, as of 2010.
- NVDOC Plume – The composition and concentration of the NVDOC plume changes over time and space as degradation processes shift (Cozzarelli et al. 1994).
    - Plume Extent and Concentrations – In 1993, the concentrations of NVDOC in the different zones of the plume were: 3,000 µg/L (Zone I, upgradient/background); 16,000 µg/L (Zone II, pipeline spray zone); 45,000 µg/L (Zone III, source area); 21,000 µg/L (Zone IV, suboxic zone where plume mingles with oxygenated groundwater); and background concentrations (Zone V, downgradient native

[7] NVDOC is measured by a combustion method and has not yet been correlated with the extractable TPH analysis at the Bemidji site.

groundwater). The NVDOC plume has continued to expand and as of 2010 concentrations exceed 40,000 µg/L at a distance greater than 300 feet downgradient of the source area.

- Plume Composition – Thorn and Aiken (1998) concluded that the NVDOC compounds are the intermediate transformation products of C_{18} or larger alkylaromatic, naphthenoaromatic, and sulfur-containing constituents of the crude oil, including possibly the resins and asphaltenes. In other words, these are the polar biodegradation metabolites of crude oil. They determined a representative molecular formula of $C_{19}H_{24}O_6$. The oxygen-containing functional groups include primary and secondary alcohols, phenolic hydroxyls, esters, lactones, ketones, aldehydes, quinones, carboxylic acids and α-keto carboxylic acids. Low-molecular-weight (LMW) organic acids are part of the NVDOC plume. Near the source area they are present at concentrations of 3,100 µg/L constituting 3% of the NVDOC plume (Eganhouse et al. 1993). The low-molecular-weight organic acids area complex mixture of aliphatic, alicyclic, and aromatic acids (Cozzarelli et al.1994). The LMW aliphatic and alicyclic acids are degraded near the source, but the aromatic acids migrate in the anoxic portion of the plume. These aromatic acids consist of alkyl-substituted benzoic acids, and are primarily C_3 and C_4 molecular structures. Based on mass balance calculations, Cozzarelli et al. (1994) estimated that the LMW organic acids are produced not only by biodegradation of the parent hydrocarbons but also by degradation of the more complex acids. Thus, although the plume persists, the data on the LMW organic acids indicates the molecular compositions within the plume are in flux and vary through space. Similarly, data presented in Bekins et al. (2016) showed that the percentages of NVDOC in three resin-extracted categories change with distance.

- Secondary Plumes – The NVDOC plume, which is a significant electron donor source, in turn gives rise to secondary plumes including: depleted dissolved oxygen; large concentrations of metals; other elevated major ion concentrations (e.g., Fe^{2+}); large concentrations of dissolved methane (CH_4); and changes in alkalinity and pH (Ng et al. 2014). As the Fe(III) in the aquifer sediment is reduced by the NVDOC plume, Fe(II) and naturally occurring arsenic are mobilized, and then redeposited on the sediments in the anoxic zone (Cozzarelli et al. 2015). Benzene migrates to where fresh Fe(III) is present on the aquifer sediments before biodegrading.

These studies provide detailed insights into the fate and transport of the crude oil and soluble components through space and time. The analyses document the geochemical conditions and degradation processes that allow the advance and persistence of a plume dominated by petroleum degradates with the secondary effect of mobilizing metals such as arsenic due to locally-altered geochemical conditions.

ii. Diesel-Contaminated Soil Leaching Tests over Time

Mao et al. (2009) studied the biodegradation of diesel-range hydrocarbons in two soil microcosms to characterize changes in chemistry and toxicity of the soil and leachate over 20 weeks. One microcosm consisted of a sandy soil spiked with commercially-available unweathered diesel fuel (A soil), and the other consisted of a soil sample containing weathered diesel-like oil from a site in Old Antwerp Harbor, Belgium (B soil).

a. Testing Methods

The testing program for both microcosms included:

- Chemistry – The chemistry was evaluated using a unique silver-modified high-pressure liquid chromatography (HPLC) column as a prefractionation step before GCxGC-FID and GCxGC-ToF-MS. Deconvolution software was used to resolve co-eluting compounds and provide tentative identifications and quantitation estimates.
- Soil Ecological Toxicity – For the residual TPH in soil, the terrestrial acute ecological toxicity was evaluated using a seedling emergence and growth test with cress (*Lepidium sativum*) (Organisation for Economic Co-operation and Development or OECD Test No. 208).
- Aquatic Toxicity – For the degradation intermediates (i.e., petroleum metabolites) in leachate, the acute aquatic toxicity was evaluated using the Microtox toxicity assay in accordance with ISO 11348-3, which measures the inhibition of light emission from the marine bacterium *Vibrio fischeri*.

b. Results

Because our primary focus is aged (weathered) petroleum releases, the remainder of the summary is focused on the B soil.

- Chemistry – TPH concentrations decreased from 12,000 milligrams per kilogram (mg/kg) over the course of the 20-week test to about 4,000 mg/kg.
- Soil Ecological Toxicity – Toxicity to the seedlings based on germination and biomass production remained about the same throughout the study at about 50% inhibition. The authors indicate that toxic intermediates (i.e., petroleum metabolites) may have formed during biodegradation.
- Aquatic Toxicity – Samples of the leachate were chemically analyzed and tested for aquatic toxicity at time zero (T0), 8 weeks (T8), 12 weeks (T12) and 20 weeks (T20). The results are summarized below:
    - Time Zero (T0) – The compound types detected using GCxGC-ToF-MS included: 1) aromatic hydrocarbons at low concentrations; 2) oxygenated hydrocarbons (petroleum metabolites) at much larger concentrations and described as aromatic ketones, diones, and lactones; 3) significant concentrations of sulfur-containing heterocyclics attributed to the diesel fuel itself including benzothiophenes, dibenzothiophenes, and alkylated homologues; and 4) other complex compounds that were tentatively identified as monoaromatic and naphthenic monoaromatic ketones or aldehydes as well as some alcohols/phenols. Based on the Microtox bacteria test, the toxicity of the leachate was significant.
    - Eight Weeks (T8) – Concentrations remained high, but the type of compounds differed significantly from those detected at T0. Most of the compounds detected at T0 were no longer detected. Newly detected compounds were complex structures that were both heavier and more polar and severely co-eluted (i.e., individual compounds could not be identified). Based on the presence of carboxylic functional groups, these were tentatively identified as mononaphthenic and monoaromatic carboxylic acids and esters, which have demonstrated toxicity to micro-organisms according to the authors. There were also mono- and bicyclic-ketones and alcohols

as well as complex structures with more than one oxygen atom that the authors designated as “polyoxygenated hydrocarbons.” One of these compounds was tentatively characterized as a cyclic compound with four oxygen atoms, thus explaining its high polarity and water solubility. The toxicity of the leachate was similar to the T0 leachate.

- Twelve Weeks (T12) – The molecular composition differed from T8. Instead of the carboxylic acids and esters, another group of compounds formed that are heavier and have greater polarity. These appeared to be complex spatial structures with heteroatoms, including nitrogen and sulfur, and the authors called these “spatial heteroatom oxygenated hydrocarbons.” The leachate did not show toxicity to the Microtox bacteria, although the soil retained toxicity as previously discussed.
- Twenty Weeks (T20) – The spatial heteroatom oxygenated hydrocarbons were still present at T20, but at lower concentrations indicating that these compounds were not persistent and continue to degrade. Like T12, these compounds did not show toxicity to the Microtox bacteria, although the soil retained toxicity.

This study provided insights into the chemical and toxicological changes of the diesel mixture in soil over time and with continued aerobic biodegradation. Ever more polar and larger intermediate compounds were detected in the leachate over time. The leachate intermediates at T0 and T8 were similarly toxic, but toxicity eventually decreased based on the Microtox bacteria test at T12 and T20. The toxicity of the soil remained stable during the study. While this laboratory leaching study was useful, the results may not be readily transferable to actual field conditions where toxic effects may persist for decades due to large source areas depleted at a very slow rate and where the metabolites can persist or are continually generated for decades.

iii. Five Gasoline- and Diesel-Contaminated Groundwater Sites in California

Zemo et al. (2013a) and Mohler et al. (2013) studied the petroleum metabolites in groundwater at five anonymous sites in California: four upland sites and one site adjacent to a marine water body. Limited CSM information was provided, but includes:

- Source Composition – The sites had known historical releases of both gasoline and diesel fuels. Although the ages of the releases were not presented, in a recent presentation the authors indicated that the plumes were in a highly biodegraded condition (Zemo 2015).
- Source Control/Residual Mass – No information was presented regarding remediation or volume of contaminant mass remaining in soil.
- Geochemical Conditions – Elevated methane was present in groundwater in the source areas. Overall dissolved oxygen concentrations were low.

- Contaminant Plume – The plume configurations at the sites were known (i.e., lateral and vertical extents were defined).
    - Source Area Concentrations and Composition – Concentrations from the TPH-diesel analysis quantified from C_{10} through C_{28} without SGC ranged from 1,000 to 8,100 µg/L in source area wells. The number of detected petroleum metabolites ranged from 80 to 737. The authors reported more complex molecular compositions (e.g., bicyclic and polycyclic aromatic structures) in the source area.
    - Downgradient Concentrations and Composition – Concentrations from the TPH-diesel analysis quantified from C_{10} through C_{28} without SGC ranged from 98 to 1,700 µg/L in downgradient wells. The number of detected petroleum metabolites ranged from 28 to 40. The molecular structures were less complex than in the source area samples.

The authors noted that these results confirm the continued biodegradation of the petroleum metabolites and ultimately natural attenuation with increasing distance from the source area.

In addition, as part of this study, the authors compared the results from the analyses of groundwater samples collected from the five sites using GC-MS versus using GCxGC-ToF-MS. For the GC-MS analysis, laboratory standards were obtained for 57 potential petroleum metabolites, which became the primary analytes. Only one of these analytes, dodecanoic acid ($C_{12}H_{24}O_2$), a carboxylic acid, was detected in one sample, at 11 µg/L. The maximum number of tentatively identified compounds for the GC-MS analysis was 27 (Site 5). Samples from these same wells were analyzed by Mohler et al. (2013) using GCxGC-ToF-MS. The maximum number of tentatively identified compounds for the GCxGC-ToF-MS analysis was 772 (Site 5). The authors further highlighted the shortcomings of conventional methods like GC-MS in identifying, detecting, and quantifying individual petroleum metabolites.

This study provided a comparison of the petroleum metabolite concentrations (using the TPH-diesel analysis) and molecular compositions between the source and downgradient areas of well-defined and highly biodegraded plumes produced from gasoline and diesel releases. Specifically, source areas have more complex molecular compositions than downgradient areas. These results are generally consistent with those from the Bemidji site, although it remains to be determined whether such results would be consistent under different CSM scenarios (i.e., when the plumes represent a less biodegraded state).

iv. Naphthenic Acids Associated with the Athabasca Oil Sands

The naphthenic acids are a well-known and studied example of petroleum metabolites. Naphthenic acids[8] are a complex mixture of monocyclic and polycyclic alkanes containing a carboxylic group, and many of the cyclic compounds have a methyl group (Toor et al. 2013). The naphthenic acids are naturally-occurring components of crude oil due to either incomplete catagenesis or biodegradation (Tissot and Welte 1984). In a laboratory study, Watson et al. (2002) demonstrated that carboxylic acids are formed from the biodegradation of crude oil. In

[8] Naphthenes are another term for cycloalkanes that have one or more rings of carbon atoms in the molecular structure. They are not related to naphthalene.

the crude oil refining process, the naphthenic acids are removed to prevent corrosion of refinery equipment, and the acids are separately refined for some commercial applications.

The Athabasca Oil Sands in Alberta, Canada contain reserves of bitumen recovered via surface mining. Bitumen is a significantly biodegraded petroleum that is a viscous, tar-like material. An alkaline, hot water process is used to extract the bitumen. The tailings, consisting of a slurry of sand, silt, clay, and residual bitumen, are contained in ponds for settling. Due to the toxicity of naphthenic acids to aquatic species, the industry operates under a zero discharge policy as required in their permits (Clemente and Fedorak 2005). The oil sands process waters are recycled in the extraction process, but there is a growing inventory of water held in the tailings ponds (about 800,000 acre-feet as of 2010; Toor et al. 2013). Concentrations of naphthenic acids in the tailings pond water range between 20,000 and 120,000 µg/L (Clemente and Fedorak 2005). Naphthenic acids have been detected in groundwater near the ponds at concentrations between 400 and 51,000 µg/L, and in Athabasca River water at concentrations as high as 900 µg/L.

Due to their complexity, the naphthenic acids pose a challenge similar to other petroleum metabolites for conventional analytical methods (Scott et al. 2005; Grewer et al. 2010). The naphthenic acids are known to present acute and chronic toxicity to aquatic species and are the primary toxicant in tailings pond water. The compounds are soluble and have low volatility, are relatively stable, and sorption to organic matter is limited by the polarity of the compounds. The naphthenic acids smaller than C_{17} are subject to biodegradation, while the larger compounds are more recalcitrant (Scott et al. 2005). Watson et al. (2002) similarly found during a biodegradation experiment that C_{20} or larger branched and cyclic carboxylic acids appeared as a UCM, which was resistant to further biodegradation during the experiment. Initially, significant concentrations of medium molecular weight (C_{10} to C_{20}) carboxylic acids were generated, but as biodegradation proceeded, the concentrations of heavier molecular weight (C_{20} or larger) carboxylic acids increased.

c. Discussion – Fate and Transport

We draw the following conclusions from these studies:

- Significant residual mass can sustain persistent petroleum metabolite plumes by continually generating or supplying metabolites. The Bemidji crude oil release site represents an example of a significant petroleum metabolite groundwater plume being sustained due to the large residual mass remaining in the source area. This plume persists long after the initial release and continues to expand more than 30 years later. It now extends to approximately 400 feet in length. Such large residual sources can alter geochemical conditions locally and may additively contribute to toxicity by mobilizing naturally occurring metals (e.g., arsenic) or transforming them into a more toxic state (e.g., methylmercury).
- The polar UCM compounds resist degradation due to their branched and cyclic nature, which explains their long-term persistence (i.e., decades) at some sites.
- The molecular composition of the groundwater polar UCM compounds varies spatially (proximity to source versus downgradient) and with the degree of weathering.

- Combining toxicity testing with extractable TPH analysis is important because toxicity appears to vary with molecular composition and the degree of weathering.
- The petroleum metabolites are an important component of the CSM of petroleum release sites. In lieu of any other means, they should be quantified using the extractable TPH analysis (both with and without SGC) in groundwater samples to understand the full extent of the effects of the petroleum release.

4. Human Health Risk

As discussed, groundwater plumes typically persist for decades where a significant mass of contaminated soil is present (Essaid et al. 2009; Wisconsin Department of Natural Resources 2009; and Bekins et al. 2016). At a weathered (biodegraded) petroleum release site, petroleum-related alcohols, ketones, acids, esters, aldehydes, phenols and other intermediate breakdown products typically constitute the majority of the contaminant mass in groundwater, due to their overall greater solubility (Thorn and Aiken 1998; Lundegard and Knott 2001; Zemo and Foote 2003; Lundegard et al. 2004; Lang et al. 2009; Mohler et al. 2013; Zemo et al. 2013). Based on one study that used advanced analytical methods to detect these compounds, there were hundreds to thousands of these breakdown products present in groundwater samples at weathered release sites (Mohler et al. 2013). These compounds are more polar than the parent hydrocarbons and can be separated from the hydrocarbons through use of SGC at the laboratory. Few studies have addressed the risk posed by these petroleum metabolite mixtures, which is essential for making defensible decisions regarding the need for active remediation and/or longer term management of contamination at a site. This is particularly important for sites in the proximity of critical receptors such as drinking water wells or aquatic habitats.

However, the derivation of quantitative descriptions of the human health risk posed by fresh petroleum hydrocarbon mixtures has been challenging. Generally, toxicological studies suitable for obtain quantitative criteria either: 1) test the toxicity of the whole petroleum hydrocarbon mixture or 2) test the toxicity of each compound present in the mixture individually. Toxicity data are only available for a small proportion of the individual petroleum compounds.

A well-known approach created to estimate the risk posed by petroleum mixtures (TPH) or to derive risk-based screening levels for petroleum-contaminated media is the fraction-based approach, of which there are several variations (ATSDR 1999; TPHCWG 1997a, 1997b, 1998a, 1998b, and 1999; MADEP 2003; USEPA 2009; Hawaii DOH 2011; and Regional Water Board 2013b and 2016b). In general, the fraction-based approaches define petroleum hydrocarbon fractions on the basis of fate and transport properties and analytical methods that are commonly used to identify and quantify petroleum hydrocarbon environmental contamination (USEPA 2009). Surrogate chemicals or mixtures for which toxicity values can be obtained are selected to represent the toxicity of these fractions following an approach consistent with USEPA mixture risk assessment methods where dose-addition or response-addition is assumed (USEPA 1986, 2000).

Accounting for petroleum metabolites in the toxicological evaluation complicates things further because even less is known about the toxicity and abundance of these compounds. The composition of contaminant mixtures at petroleum release sites depends on site-specific

conditions that affect biodegradation (e.g., microbial population present, temperature, soil and groundwater chemistry, etc.), and the distance from the source area and the stage of weathering (roughly time). While useful for understanding the relative toxicity of these mixtures in general, recently published papers on the toxicity of petroleum metabolites (e.g. Zemo et al. 2013, O'Reilly et al. 2015) lack adequate detail to quantitatively assess toxicity to the degree required for decision-making as part of an environmental investigation (Brewer and Hellmann-Blumberg 2014; Hellmann-Blumberg et al. 2016) The scarcity of detailed information about the toxicity of petroleum metabolites is not surprising given the challenges in detecting and quantifying these compounds with conventional laboratory analytical technologies, especially GC-FID and GC-MS (see Chapter 2).

This section presents the Regional Water Board staff's current screening approach for evaluating threats to drinking water, a literature review to support this approach covering the potential adverse effects on human health from petroleum metabolites, and a discussion of the implications of these studies for the screening approach presented below.

a. Approach to Evaluating Threats to Drinking Water from Petroleum and Metabolites

In order to screen groundwater sample data for sites where the beneficial uses include municipal and domestic supply (i.e., existing or potential drinking water sources), contaminant concentrations are compared to the lowest of federal or California maximum contaminant levels (MCLs), health-risk-based criteria (e.g., tapwater ESLs), nuisance criteria (odor or taste), or gross contamination criteria (i.e., non-aqueous phase liquid or NAPL; aka free product). Promulgated standards for individual compounds are presented as Water Quality Objectives (WQOs) in the Basin Plan (Regional Water Board 2013a). However, for the soluble or fractionally soluble petroleum mixtures typically reported as TPH including TPH-gasoline and TPH-diesel, neither an MCL nor a numeric WQO have been developed. Assessment of TPH data for groundwater is therefore typically carried out through comparison to risk-based tapwater ESLs for TPH developed using a fraction-based approach (Regional Water Board 2016b).

As a default, Regional Water Board staff considers that the toxicity of petroleum metabolite mixtures is similar to the parent hydrocarbon mixtures. Effective December 2013, the ESL User's Guide specifically recommends comparing data from extractable TPH groundwater analyses (e.g., TPH-diesel in the absence of SGC) to approximate the risk of the sum total of both nonpolar, parent hydrocarbons and polar metabolites. The results are to be compared against the corresponding risk-based extractable TPH-diesel ESL. This method had been used at the majority of sites overseen by Regional Water Board staff in the past. Although better laboratory methods are still needed, the inclusion of the petroleum metabolites in the extractable TPH analysis provides a measure of protection by treating the toxicity and risk posed by the metabolites identically to those posed by the parent hydrocarbons.

b. Literature Review: Human Health Risks Posed by Petroleum Metabolites

The potential for chemicals to cause adverse effects in humans is typically extrapolated based on *in vitro* (in an artificial environment) or *in vivo* (on an animal, such as the rat, mouse, rabbit, guinea pig, hamster, dog, or monkey) experiments (USEPA 1989). *In vitro* assays using biological target molecules or cultured cells (including genetically engineered cell lines) can be used as screening tests that produce mostly qualitative and mechanistic information about a chemical's toxicity. In addition, computational tools are often employed for helping predict a chemical's toxicity using known toxicity data for similar compounds (e.g., quantitative structural activity relationships or QSARs). Such *in vitro* and computational studies can then be used to prioritize more time-consuming and costly animal studies. While QSAR-based tools are often employed for obtaining an approximate toxicity estimate for individual chemicals or groups of compounds that have not been tested in the laboratory, quantitative dose response experiments on mammals are the preferred source for derivation of quantitative toxicity values for cancer endpoints (e.g., oral slope factor or SFo, inhalation unit risk or IUR) or noncancer endpoints (e.g., oral reference dose or RfD, inhalation reference concentration or RfC) that are used for risk assessments. Summaries of several studies of differing significance are presented below; starting with those that used the most desirable *in vivo* toxicity studies (i), to those using qualitative computational toxicity studies (ii), and finishing with those using undocumented toxicity data (iii) and (iv).

i. Dose Response Testing of Naphthenic Acid Mixtures

Rogers et al. (2002) studied the acute and subchronic toxicity to rats from a mixture of C_{14} to C_{18} naphthenic acids isolated from Athabasca Oil Sands process-affected waters (OSPW) (see Chapter 3). Naphthenic acids are complex mixtures of monocyclic and polycyclic alkanes containing a carboxylic functional group (COOH), and many of the cyclic compounds have an alkyl group (Toor et al. 2013). Naphthenic acids are naturally-occurring components of crude oil formed from either incomplete catagenesis or biodegradation (Tissot and Welte 1984). There are likely to be structural overlaps between naphthenic acids and organic acids and related metabolites from other types of petroleum releases.

To date, most toxicity testing of the naphthenic acids involve aquatic species. Aquatic toxicity is discussed in more detail in Chapter 5. Several mechanisms by which naphthenic acids cause adverse biological effects have been identified. Naphthenic acids interact with multiple biological targets and cause disruptions that may lead to disease or cause adverse effects.

Rogers et al. (2002) suggest the liver is a target organ for exposure to naphthenic acids. In the subchronic study, for female rats, oral exposure to 60 mg/kg-day of naphthenic acids resulted in significant increases in liver and brain weights, and in seizures after 40 days of dosing. Based on these studies, 6 mg/kg/day is the no observed effect level (NOEL) and 60 mg/kg/day is the lowest observed effect level (LOEL).

Although McKee et al. (2014) studied a slightly different mix of C_{10} to C_{16} naphthenic acids refined for use in commercial applications, the findings similarly suggested the liver was a target organ. The overall no observed adverse effects level (NOAEL) for all target organ and

developmental effects was 100 mg/kg-day. McKee et al. (2014) attributed the differences between their results and those of Rogers et al. (2002) to variations in the mixture compositions and in the absorbed doses. For instance, Rogers et al. (2002) delivered the doses as aqueous solutions, whereas McKee et al. (2014) used corn oil as the delivery vehicle.

While both the McKee et al. (2014) and Rogers et al. (2002) studies are relevant, the widely cited Rogers et al. (2002) study is of particular interest for the evaluation of the cumulative effects of chemicals from weathered petroleum releases because the experiment tested a mixture of naphthenic acids from OSPW rather than fresh commercially-available mixtures. **For discussion purposes, by treating the LOEL from the Rogers et al. (2002) as the low observed adverse effect level (LOAEL) an RfD of 6.0E-02 mg/kg-day can be extrapolated using standard USEPA uncertainty factors totaling 1,000. This does is about the same as the RfD for naphthalene (2.0E-02 mg/kg-day; IRIS 2015) and the medium carbon range aromatic TPH fraction (3.0E-02 mg/kg-day; USEPA 2009), which represent the soluble components of diesel fuels and crude oil.**

ii. Screening of Naphthenic Acids for Multiple Human and Environmental Toxicity Endpoints

Scarlett et al. (2012) evaluated the potential toxic effects of 54 chemically characterized OSPW naphthenic acids. The authors used ADMET predictor™ software, a compilation of QSAR models developed for pharmacological applications, to predict the likelihood of any of the 54 naphthenic acids causing adverse effects when a range of environmental and human endpoints were considered.

As described in Chapter 3, the OSPW naphthenic acids consist of a complex mixture of cyclic and noncyclic carboxylic acids. The naphthenic acid fraction of the OSPW is considered largely responsible for the observed toxicity of these waters (Clemente and Fedorak 2005). Scarlett et al. (2012) emphasized that naphthenic acids also are used extensively in many industries and are likely to be widespread, non-monitored environmental contaminants. Only small quantities of individual naphthenic acids are available for toxicity testing because they first have to be synthesized. For this reason, besides the time and cost associated with animal studies, the authors elected to employ computer models first to help identify the more harmful compounds or groups of compounds for later focused testing.

Scarlett et al. (2012) divided the 54 naphthenic acids into 8 structural classes: 6 aliphatic n-acids; 6 aliphatic branched acids; 6 aliphatic cyclic (cyclohexyl-containing) acids; 6 aliphatic bicyclic acids; 6 aliphatic tricyclic acids; 6 aliphatic tetra- and pentacyclic acids; 12 monoaromatic acids; and 6 polycyclic aromatic acids. The use of 6 or more compounds from each structural class allows for insights about inter-compound variability within the structure class.

The ADMET predictor™ software uses chemical structures and experimental data to create QSAR models which are in turn used to predict properties of individual molecules for a range of physico-chemical outputs. For their initial investigation Scarlett et al. (2012) selected toxic effects they considered likely to occur as the result of exposure to at least some of the naphthenic acids. For example, liver toxicity was selected because some naphthenic acids have

structural similarity to pharmaceuticals known to cause liver toxicity as a side effect. Whenever available, the authors included multiple endpoints for each area of toxicity. Thus, they selected a module that predicted the effect of the naphthenic acids on five liver enzymes commonly used to test whether the liver is functioning normally or whether it has been impacted by injury or disease. In addition, possible interactions with enzymes that are involved in the processing of toxic chemicals and pharmaceuticals, the cytochrome P450 enzymes (CYPs), were investigated.

Of particular interest are endpoints related to the potential of a chemical to increase the probability of cancer developing. Chemicals considered genotoxic and mutagenic act in the early stages of carcinogenesis whereas other carcinogens play a role in later stages. Scarlett et al. (2012) modeled the activity of the naphthenic acids with respect to several cancer-related endpoints.

Chemicals with structural similarity to naphthenic acids have also been found to interfere with the proper function of certain hormones. In other words, they act as endocrine disruptors. Some types of endocrine disruptors alter the effect of the normal endogenous levels of estrogens or androgens produced by a living organism. A variety of adverse effects can occur when the estrogen balance in a female or the androgen balance in a male or both are disrupted. Often this leads to reproductive problems and developmental defects in early life stages.

The results from Scarlett et al. (2012) are summarized in Table 3.

The carcinogenic rat model predicted that the most toxic naphthenic acid structural classes were, in decreasing order: 1) the polycyclic aromatic naphthenic acids; 2) monoaromatic naphthenic acids and the aliphatic n-acids; 3) aliphatic branched acids, aliphatic cyclic acids, aliphatic bicyclic acids, and aliphatic tetracyclic/pentacyclic acids; and 4) the aliphatic tricyclic acids. All of the classes were predicted to result in an elevated abundance of at least one liver enzyme, but none were predicted to be mutagenic or affect the estrogen receptor, with the exception of the polycyclic aromatic acids. The classes predicted to affect the androgen receptor were the aliphatic tetracyclic/pentacyclic acids and the polycyclic aromatic acids. The classes predicted to affect reproduction were the aliphatic n-acids (one compound), aliphatic tricyclic acids (two compounds), and the polycyclic aromatic acids. All of the classes were predicted to be a substrate for at least one CYP enzyme.

These predictions indicate that the naphthenic acids as a group are probably toxic. The authors concluded that the classes potentially most harmful to human health are the polycyclic monoaromatic acids and the aliphatic pentacyclic acids, and recommended further testing of those classes. They did not recommend eliminating any classes from further consideration, however.

It is important to note that the selected models test only a small number of endpoints. Therefore, a negative result for a single endpoint does not necessarily mean that a chemical does not have a certain type of adverse effect. For example, in order to ascertain that a chemical is unlikely to increase the risk of cancer, several genotoxic and, if those are all negative, non-genotoxic endpoints should be evaluated, whether the evaluation involves experimental testing, modeling or both.

Table 3 – Summary of Toxicity Prediction for Naphthenic Acids (from Scarlett et al. 2012)							
Structural Class	Liver[1]	CYP[2]	Carcino-genicity (TD50)[3]	Mutagenic	Estrogen Receptor	Androgen Receptor	Repro-duction
	Number of compounds with predicted effect / number of compounds tested						
Ali n-acids	**6/6**	**6/6**	**High-Medium**	0/6	0/6	0/6	1/6
Ali branched	**6/6**	**6/6**	**High-Low**	0/6	0/6	0/6	0/6
Ali cyclic	**6/6**	**6/6**	**Medium-Low**	0/6	0/6	0/6	0/6
Ali bicyclic	**6/6**	**6/6**	Low	0/6	0/6	0/6	0/6
Ali tricyclic	**6/6**	**6/6**	Low	0/6	0/6	0/6	2/6
Ali tetra/ pentacyclic	**6/6**	**6/6**	**High-Medium**	0/6	0/6	**3/6**	0/6
Mono-aromatic	**12/12**	**12/12**	**High**	0/12	0/12	0/12	0/12
Polycyclic	**6/6**	**6/6**	**High**	0/6	2/6	**4/6**	**3/6**

Notes:

Bold font is used to highlight the more significant predicted responses.

1 – Indicates that one or more of the five liver enzymes showed an increase in relative abundance.

2 – Cytochrome P450 enzymes. Indicates that compounds in the class were predicted to be a substrate for one or more of the five CYP enzymes.

3 – Carcinogenicity TD50 (rat model) – TD50 corresponds to the daily dose-rate (in mg/kg/body weight/day) for life, to induce tumors in half of test animals that would have remained tumor-free at zero dose. Presented by relative toxicity as follows: (a) high (<50 mg/kg-day); (b) medium (50 to 100 mg/kg-day) and low (>100 mg/kg-day).

iii. Toxicity of Ester Metabolites Detected at Fuel Release Sites

Recently, 197 likely ester metabolites resulting from petroleum degradation were identified in groundwater samples from fuel release sites in California (O'Reilly et al. 2015). While the authors express the belief that esters in general have low toxicity, this is not consistent with numerous examples of ester toxicity described in the literature. Moreover, some of the esters identified have been shown to have a variety of biological effects that may adversely impact human health, for example several of the phthalate esters, or esters of 1,2-benzenedicarboxylic acid, as they are referred to in the O'Reilly et al. 2015 paper.

Computational as well as experimental toxicity information is readily available for some of the esters identified, in particular mono(2-ethylhexyl) phthalate (MEHP), which is an active and in this case toxic metabolite of di(2-ethylhexyl)phthalate (DEHP; also known as bis(2-ethylhexyl)phthalate or BEHP), and monobutyl phthalate (MBP), which is a metabolite of either dibutyl phthalate (DBP) or benzylbutyl phthalate (BBP) (Singh and Li, 2011). Singh and Li (2011) used modeled interactions between these compounds and proteins produced from

genes in the Comparative Toxicogenomics Database, to identify likely phthalate toxicity categories, which included heart disease, liver disease and kidney disease in addition to the well-known endocrine disrupting potential of a number of phthalates. The database search complements numerous experimental studies involving cell lines, rodents, and a few correlative studies in humans that identified additional endpoints and mechanisms for toxicity.

Mono(2-ethylhexyl) phthalate has been shown to induce oxidative stress (Yang et al. 2012; Tetz et al. 2013), which in turn can cause multiple adverse cancer and non-cancer health effects. Carcinogenic potential has specifically been documented in the case of DEHP (Caldwell 2012; Ruslyn and Corton 2012). Several phthalates, including DEHP and DBP, are listed in the USEPA Regional Screening Levels (RSLs). Endocrine-disrupting effects influencing metabolic (Venkata et al. 2006; Ellero-Simatos et al. 2011) or reproductive (Hauser et al. 2006; Jurewicz et al. 2013, Wang et al. 2015) processes are among the most frequently identified effects for MEHP and are particularly noteworthy because they can occur at very low concentrations (Zoeller and Vandenberg 2015). Phthalates have also been shown to adversely affect aquatic life (Zhai et al. 2014, Ye et al. 2015). In addition, other esters identified by O'Reilly et al. (2015) appear structurally related to naphthenic acids and therefore may have similar toxicities. In this context it should be noted that microorganism are expected to synthesize, utilize and excrete esters rather than free acids but that esters are subject to hydrolysis by enzymes or acidic or basic pH. **Esters and acids (and combinations of metabolites belonging to either of these groups) may affect human and ecological receptors at very low individual concentrations in water resources that are well below the "no effect" levels determined by traditional testing.**

iv. Proposed Relative Toxicity Ranking Approach for Petroleum Metabolites

A unique approach for estimating the relative oral toxicity of the petroleum metabolites based on the RfDs of compounds with particular polar functional groups (surrogates) was developed by Tiwary et al. (2013), as referenced in an abstract for the Society of Toxicology conference in March 2013. This proposed relative toxicity ranking approach was subsequently summarized in Zemo et al. (2013a), and applied to a study of petroleum metabolites in groundwater at five California bulk terminal-refinery sites published in a companion paper presenting the chemical characterization methods and findings (Mohler et al. 2013). However, a companion paper presenting a comprehensive explanation of the toxicological basis for this relative ranking system has not been published. Nevertheless, the authors (Zemo & Associates 2014) have proceeded to make unsubstantiated statements such as "Zemo et al (2013) showed that the metabolites present in groundwater are primarily compounds with RfDs of 0.1 or higher (or at least 3 times less toxic than the aromatics). At highly biodegraded sites where the 'TPH' plume is exclusively metabolites dominated by organic acids, the mixture RfD would likely be closer to 1.0 (or 30 times less toxic than the aromatics)."

Based on our review of the proposed relative toxicity ranking approach presented in Tiwary et al. (2013) and Zemo et al. (2013a), we have identified a number of concerns (see also Brewer and Hellmann-Blumberg 2014; and Hellmann-Blumberg et al. 2016). Available data are not adequate to broadly conclude that petroleum metabolites individually and as part of mixtures present a negligible health risk to humans (aquatic toxicity is addressed in Chapter 5).

Data from related, peer-reviewed literature supports a conclusion that petroleum metabolites can pose potential adverse effects to humans, even when concentrations of relatively nonpolar, parent compounds in water are well below risk-based screening levels. The data generated thus far does not support dismissing petroleum metabolites from further testing and evaluation, as has been proposed both in the above-referenced papers and in other publications by the same authors (e.g., Zemo and Foote 2003; Zemo et al. 2013b). Below is a summary of the Tiwary et al. (2013) approach, followed by a discussion of specific concerns. Although it is our understanding that the ranking approach was developed by Tiwary et al. (2013), in the text below we also refer to specific elements presented in Zemo et al. (2013a). Thus, we will cite Zemo et al. (2013a) as the main reference for the ranking approach.

a. Description

For the development of the proposed relative toxicity ranking approach Tiwary et al. (2013) reviewed the literature to identify polar chemicals with oxygen-containing functional groups expected to occur in petroleum metabolites mixtures. As described in Zemo et al. (2013a), five families (alcohols, acids/esters, aldehydes, ketones, and phenols) were selected based on the oxygen-containing polar groups. Each family was then subdivided based on the hydrocarbon skeleton into a total of 22 structural classes.

A search for existing toxicology information was also carried out for specific petroleum metabolites within the identified functional groups considered to be potential surrogates for petroleum metabolites. The primary sources for toxicology information were USEPA and other databases from regulatory agencies and, where necessary to ensure that a functional group class was represented, the scientific literature. However, a comprehensive list of information sources or specific references is not provided in Zemo et al. (2013a). These publications indicate only that the surrogate compounds include those with RfDs from the USEPA 2012 RSLs. The initial list of 83 petroleum metabolites was later reduced to 57 compounds due to laboratory analytical considerations. Thus, 57 surrogate compounds are listed in Zemo et al. (2013a).

A relative toxicity ranking approach (Low, Low to Moderate, Moderate) was developed by converting a LOAEL of 1.0E+02 mg/kg-day to an RfD-equivalent criterion of 1.0E-01 mg/kg-day using a total uncertainty factor of 1,000 (a breakdown of the total uncertainty factor was not provided). This was defined to be the more toxic end of the “Low” toxicity category. The surrogate compounds were placed into the following categories based on the estimated RfDs: low toxicity (RfDs greater than 1.0E-01 mg/kg-day); low to moderate toxicity (RfDs between 1.0E-01 mg/kg-day and 1.0E-02 mg/kg-day); and moderate toxicity (RfDs between 1.0E-02 mg/kg-day and 1.0E-03 mg/kg-day). The authors state that these summary rankings are consistent with both USEPA and United Nations ranking programs. Their ranking approach is illustrated in Figure 1 of Zemo et al. (2013a). The RfDs for 51 of the 57 surrogate petroleum metabolites in the three narrative toxicity categories are as follows: low toxicity (25 compounds); low-moderate toxicity (21 compounds), and moderate toxicity (5 compounds). It is unclear why the RfDs for 6 of the 57 surrogate compounds are not plotted. The authors’ state that the majority of the petroleum metabolites detected at the downgradient extent of the groundwater

plumes at the five sites studied (see Chapter 3) fall into the proposed structural classes believed to be low toxicity. Moreover, Tiwary et al. (2013) states:

> "The ranking approach was applied to groundwater samples collected from biodegrading petroleum sites and the results show that the vast majority of the polar biodegradation products are in structural classes that may present 'Low' hazard to humans. Overall, the polar mixtures are unlikely to present a significant risk to human health if consumed as drinking water."

b. Concerns

While the proposed relative toxicity ranking approach by Tiwary et al. (2013) adds to the discussion regarding the petroleum metabolites, several aspects of this work are in conflict with the peer-reviewed literature discussed earlier. This in particular includes the conclusion that the petroleum metabolites are unlikely to present a significant risk to human health if consumed in drinking water. The lack of a listing of the toxicity studies reviewed during development of the toxicity ranking approach and the lack of a comprehensive technical explanation for the proposed relative toxicity ranking system in a peer-reviewed journal or an even less formal white paper presents some review challenges. Despite this, the following key concerns and observations have been identified thus far:

1. Representativeness of the Surrogate Petroleum Metabolites

The representativeness of the selected surrogates for petroleum metabolites is questionable. Petroleum-contaminated groundwater can include thousands of potential petroleum metabolites, as acknowledged in the Tiwary et al. (2013) and Zemo et al. (2013a) papers. Given the significant limitations for detecting and characterizing petroleum metabolites using commercially available analytical methods, there is substantial uncertainty whether the surrogates for the petroleum metabolites (individual compounds) described in the papers can be reliably applied to represent the toxicity of complex mixtures of petroleum metabolites at petroleum release sites in general. The number and type of compounds employed in the ranking system are likely insufficient to make meaningful predictions about the large and unknown number of possible metabolites at a specific release site.

A full listing of the chemicals with the reference citations of the source along with the details indicating how the RfDs were derived (identification of principal study, critical effect, basis for the uncertainty factor, and confidence level in the study) is required before the selected surrogates can be fully reviewed, but to date this critical information has not been presented. We note that the RfDs for only 6 of the 57 compounds are from regulatory toxicity value sources consistent with the USEPA toxicity value hierarchy (USEPA 2003, 2013, 2016), and the toxicity value hierarchy employed in our ESLs (Regional Water Board 2016).

In addition, there should be a discussion regarding uncertainties since the ranking system has not been validated. This information is needed to further evaluate the ranking approach and the authors' findings and conclusions.

2. Three-Dimensional Molecular Structure Not Considered

The proposed relative toxicity ranking approach is an unorthodox approach compared to traditional toxicological studies. The approach does not take into account the three-dimensional shape of the molecule and how it might interact with biological molecules, for example receptors, to produce a biological effect, which in some cases means toxicity. Seemingly small differences in structure can lead to a profoundly different biological effect of a molecule having the same functional groups. Examples of isomer pairs that differ dramatically in their biological effects include limonene (the three dimensional configuration dictates whether the smell is that of oranges versus lemons); ibuprofen (one structure results in an anti-inflammatory effect while the other structure is biologically inactive; Smith 2009); the RfDs (toxicity values) for petroleum metabolite isomers 2,4-dimethylphenol (2.0E-02 mg/kg-day) and 2,6-dimethylphenol (6.0E-04 mg/kg-day) differ by a factor of 33 (see Zemo et al. 2013a Table 1); and thalidomide (one structural orientation results in sedative effects while the other is teratogenic; Smith 2009). The three-dimensional shape of the molecule and position of the functional group can make a significant difference in the biological response (toxicity). A ranking approach based on a single compound with an RfD in a particular structural class likely has limited predictive power. For instance, in the Scarlett et al. (2012) study described earlier, 6 to 12 compounds were considered for each structural class and differential toxicity was observed.

3. Appropriateness for Categorizing the Toxicity of Large Groups of Compounds

The ranking approach consists of narrative categories with a range of RfDs. This approach, according to the authors, is similar to USEPA and United Nations' approaches which prioritize chemicals for further evaluation in commerce or for safe handling of chemicals to facilitate trade. They are not intended for use in quantitative environmental risk assessments, nor were they intended to exempt large classes of compounds, for example alcohols, from further consideration based on the presence or absence of a functional group.

4. Lack of Consideration of Exposure

As discussed in Chapter 3, the petroleum metabolites, due to their greater polarity, can achieve dissolved concentrations one to two orders of magnitude greater than the parent hydrocarbons. Thus, even if the metabolites were 3 to 30 times less toxic than the parent hydrocarbons (Zemo & Associates 2014), then the resulting risk/hazard could be equivalent to or even higher than that posed by the parent hydrocarbons, given the greater metabolite concentration in groundwater (Regional Water Board 2014; Brewer and Hellmann-Blumberg 2014). Focusing solely on suspected toxicity values to the exclusion of exposure (e.g., concentration) is not appropriate from a public health perspective.

5. Lack of Consideration of Cumulative Risk/Hazard

As a result of the transformation processes that happen to petroleum mixtures in the environment, petroleum metabolites are a natural consequence of petroleum releases. Zemo et al. (2013a) recommend that the metabolites should always be removed using SGC prior to analysis (and therefore not measured). This reduces the apparent magnitude of the contamination by removing petroleum metabolites and in term lessens the apparent risk posed by a release. The literature presented in this review suggests that risks posed by the metabolites are not negligible. The risk is at least comparable to that of the parent hydrocarbons, particularly when the greater solubility and concentrations are considered. Therefore, the full extent of petroleum releases, both hydrocarbon and metabolites, should be characterized and risks evaluated. The cumulative risk and hazard estimate should include all chemicals found in all media at a petroleum release site and that includes the metabolites.

Moreover, the available literature indicates that petroleum metabolites preferentially target certain biological pathways. Disruption of a pathway in different places and/or by multiple agents can lead to more severe disruption than what would be expected based on simple additive effects. Recent research suggests that the joint action of chemically-distinct groups of compounds derived from petroleum (e.g., resins or polars and saturates or aliphatics) can induce synergistic effects (Jonker et al. 2015).

Lastly, we are unaware of any published toxicity testing by this group or other groups that would support or validate the proposed relative toxicity ranking approach. Nor are we aware of any concurrence with the proposed relative toxicity ranking approach by regulatory agencies that typically review toxicity studies and develop regulatory toxicity values (e.g., USEPA, ATSDR, and OEHHA).

c. Discussion – Human Health Risk

Very little is known about the composition of petroleum breakdown products at a given release, which depends on site-specific conditions (geochemistry, microbial communities) and changes during weathering. While the studies summarized above are welcome, they are merely the start of a developing research area. Making risk-based decisions for weathered petroleum release sites using data from groundwater samples subjected to SGC that removes metabolites is not supportable by existing information and is not prudent from a public health perspective for the following reasons:

1) There is evidence that individual metabolites as well as mixtures are toxic. For example, the Rogers et al. (2002) and McKee et al. (2014) studies indicate the petroleum metabolite mixtures are toxic to test animals and therefore, potentially to humans. The Scarlett et al. (2012) study on predictive toxicity to human endpoints is consistent with this conclusion.
2) Available evidence does not support the notion that partial degradation causes a substantial reduction in risk associated with petroleum releases.

The rationale outlined by Zemo et al. (2013a) is insufficient to defensibly conclude that polar, petroleum-derived compounds are significantly less toxic than the parent petroleum hydrocarbons, as stated by Tiwary et al. (2013). The O'Reilly et al. (2015) paper provides examples of petroleum metabolites which have shown several toxic effects in a number of

different experimental settings. This highlights the fact that the chemistry of complex, metabolite mixtures in the environment is not fully understood. The toxicity of these mixtures is even less well understood.

The studies described in sections 4b(i), 4b(ii), and 4b(iii) above further support the need to measure and assess petroleum breakdown compounds in conjunction with remaining, parent petroleum hydrocarbons as part of site-specific evaluations. This is required to understand the full, cumulative effects of petroleum releases on human health and the environment.

5. Ecological Risk

Once a compound has been identified and chemically characterized, its toxicity can be evaluated in different ways. Qualitative and mechanistic information can be gained from *in vitro* tests whereas quantitative information, such as dose response, can be used for the derivation of toxicity criteria that is usually obtained from animal experiments.

The ecological risk from petroleum hydrocarbon mixtures has been studied by performing toxicity tests *in vivo* on a variety of marine and freshwater aquatic species and endpoints (e.g., mortality, reduction in growth, reproductive impairment, changes in numbers of species, bioaccumulation of residues in non-target organisms, and disruption of community and ecosystem-level functions). Much of this testing has been focused on developing dose-response relationships for specific compounds, but there has been some testing of whole mixtures. There is a significant body of literature regarding the aquatic toxicity posed by the direct releases of petroleum hydrocarbon mixtures from oil/fuel spills in aquatic environments.

This section presents background information regarding regulation of discharges to surface water from permitted facilities in the San Francisco Bay region, previous aquatic toxicity testing at petroleum sites in the region, and our existing approach to evaluating petroleum-contaminated groundwater discharges to surface water. The section also summarizes published studies regarding the ecological toxicity of weathered petroleum hydrocarbon mixtures.

a. Background

i. Regulation of Discharges to Surface Water in the San Francisco Bay Region

Surface waters in the region consist of inland surface water (freshwater lakes, rivers, and streams), estuaries, enclosed bays, and ocean waters. Historical and ongoing wasteloads to surface water bodies in the region come from upstream discharges carried into the region via Delta outflows, direct input in the forms of point and nonpoint sources, and indirect input via groundwater seepage (Regional Water Board 2013a). A point source usually refers to waste emanating from a single, identifiable location, while a nonpoint source usually refers to waste emanating from diffuse locations. The Regional Water Board may require control of either type of discharge.

Point source discharges to surface waters are generally controlled through waste discharge requirements issued under the federal National Pollutant Discharge Elimination System (NPDES) permits. Although the NPDES program was established by the federal Clean Water

Act, the permits are prepared and enforced by the State and Regional Water Boards per California's delegated authority for the act.

The Clean Water Act has two broad goals: 1) elimination of pollutants; and 2) protection of water quality. This leads to two different types of effluent limits for permitted facilities: 1) technology-based limits for treatment facility performance, such as biological oxygen demand and oil and grease; and 2) water quality-based limits based on receiving water health, which generally involves on-going acute and chronic toxicity testing.

Discharges are classified as either deep water or shallow water discharges. This classification influences the allocation of dilution ratios. In order to be classified as a deep water discharge, waste must be discharged through an outfall with a diffuser and must receive a minimum initial dilution of 10:1, with generally much greater dilution. All other dischargers are classified as shallow water discharges.

Shallow water discharges have less potential for dilution and a higher chance for toxicity as the water flows through sediments where benthic organisms that form the base of the food chain can be exposed to the full brunt of a contaminated discharge. Shallow water dischargers are subject to a discharge prohibition, which is intended to protect beneficial uses in areas that receive very limited, if any, dilution. When an exception to the prohibition is granted, it is generally not appropriate to allocate dilution credits for purposes of calculating effluent limitations, because these shallow aquatic environments are often biologically sensitive or critical habitats. However, dilution credit may be granted on a discharger-by-discharger and pollutant-by-pollutant basis if the discharger demonstrates an aggressive pretreatment and source control program is in place.

Refineries may be a significant source of petroleum and breakdown products. Refineries in our region treat their waste, which is then discharged to surface waters. The refinery discharge locations in our region are all deep water discharges with dilution, which further diminishes toxicity. Even so, petroleum-derived naphthenic acids have been identified at refineries as contributing toxicity in some toxicity identification evaluations (TIEs). For example, as part of one TIE, naphthenic acids were found to decrease survival of Threespine Stickleback fish (*Gasterosteus aculeatus*) in a 96-hour, flow-through bioassay (van Compernolle et al. 1985) at a concentration of 2,500 µg/L.

ii. Aquatic Toxicity Testing at Petroleum Release Sites in the San Francisco Bay Region

Aquatic toxicity testing performed on samples collected from two Bay margin contaminated sites deemed sufficiently robust to be used for developing site-specific screening criteria include the Naval Station Treasure Island and the Presidio of San Francisco. The studies are summarized below:

- Naval Station Treasure Island (Treasure Island) – Aquatic species chronic toxicity testing was performed using contaminated soil collected from four petroleum release sites on the base (PRC Environmental Management, Inc. 1997). The petroleum fuels released at the sites included gasoline, diesel, Bunker C fuel, and unknown fuels. Elutriates[9] were prepared from soil samples with filtered seawater and commercial sea salts and used to simulate water-column effects in the toxicity tests. The maximum detected concentrations in the elutriates were 24,000 µg/L TPH-gasoline, 300,000 µg/L TPH-diesel, and 31,000 µg/L TPH-motor oil. It was reported that the diesel-range detections were due to aged diesel. The toxicity tests performed included: 1) an echinoderm abnormal development bioassay using *Strongylcentrotus purpuratus* (purple sea urchin) and 2) a bivalve shell development bioassay using *Mytilus edulis* (blue mussel). Confounding factors (e.g., metals, ammonia, sulfide, etc.) were evaluated, and confounded results were excluded. The EC50[10] values were determined to range from total TPH (sum of TPH-gasoline, TPH-diesel, and TPH-motor oil) concentrations between 607 µg/L (greater toxicity) and 169,000 µg/L (little or no toxicity). The test results and report were used in the development of a basewide screening level for protection of ecological receptors considering groundwater discharge to the Bay of 1,400 µg/L total TPH (Tetra Tech EMI, Inc. 2001); the criterion was applied at the edge of the Bay.
- Presidio of San Francisco (Presidio) – Aquatic species chronic toxicity testing was performed to develop surface water point of compliance (POC) concentrations for future wetland aquatic receptors within the Presidio's saltwater ecological protection zone (IT Corporation 1997) and to develop surface water and sediment POC concentrations for a future freshwater stream (Montgomery Watson 1999). The results of these two studies are summarized below:
    - Saltwater Species Testing – Elutriates were prepared with soil samples collected from two petroleum release areas (one contaminated with aged gasoline and the other by aged fuel oil) and laboratory grade seawater. The elutriates were used in toxicity tests with *Americamysis bahia* (crustacean) and *Mytilus sp.* (mussel) for development of surface water POC concentrations. Confounding factors (e.g., metals, ammonia) were evaluated, and confounded results were excluded. The

[9] Elutriates (also known as eluates) are used to predict the release of contaminants to surface water columns resulting from open water disposal of dredged materials (US Army Corps of Engineers and USEPA 1998). They are prepared by combining sediment with dilution water in a ratio of 1:4 and then vigorously mixed for 30 minutes. The supernatant is siphoned off for use in testing. For fine-grained sediments that do not readily settle, centrifuging may be used to remove suspended sediment.

[10] EC is the effects concentration. EC50 is the concentration at which 50% of the organisms exhibit an effect(s).

IC25[11] results for *Americamysis bahia* and the EC25 results for *Mytilus sp* were used to develop saltwater POC concentrations of 1,200 µg/L TPH-gasoline and 2,200 µg/L TPH-fuel oil (applicable to diesel and fuel oil).

Whole sediment toxicity tests using *Eohaustorius estuarius* (amphipod) were conducted to develop POC concentrations for surface sediment (top three feet of the sediment surface) in the future wetlands. Aliquots of site soil were mixed with reference soils and then overlain with seawater to simulate wetland sediment. Reference site toxicity and confounding factors (e.g., metals, ammonia) were evaluated, and confounded results were excluded. The LC25[12] results were used to develop sediment POC concentrations of 11.6 mg/kg TPH-gasoline and 144 mg/kg TPH-fuel oil (applicable to diesel and fuel oil).

- Freshwater Species Testing – As part of the reuse plan for the Presidio, a riparian corridor was proposed that would include a freshwater stream. Two former gasoline station sites were inside or adjacent to the corridor. Groundwater was collected from two monitoring wells at one of the sites for use in toxicity testing of species considered likely to inhabit the freshwater stream: three brood *Ceriodaphnia dubia* (water flea) test; 7-day larval *Pimpephales promelas* (fathead minnow) test; and 4-day algal growth test with *Selenastrum capricornutum* (green algae). The results showed a dose-response relationship only for *Ceriodaphnia dubia*, and those results were used for derivation of a freshwater POC concentration based on the EC25 value, which was 443 µg/L TPH-gasoline. Assuming equilibrium partitioning from the surface water POC concentration, a stream sediment POC concentration of 140 mg/kg TPH-gasoline was derived.

None of these tests were performed with SGC, so it is unknown whether petroleum metabolites contributed to the detected concentrations. However, due to their solubility relative to the hydrocarbons, lack of separate-phase hydrocarbons, and the age (weathering) of these releases, petroleum metabolites were most likely present in significant quantities and constituted the majority of the dissolved-phase constituents detected in the TPH-diesel and TPH-motor oil analyses at these sites.

iii. Approach to Evaluating Discharges of Petroleum-Contaminated Groundwater to Surface Water

Aquatic toxicity testing at petroleum-contaminated groundwater sites in our region clearly demonstrates that petroleum metabolites can be toxic. Therefore, when petroleum and petroleum metabolite concentrations in groundwater discharges to surface water exceed aquatic habitat screening levels, consistent with our NPDES program, our approach to evaluating the impact to aquatic receptors includes several options, one of which is aquatic toxicity testing.

The concerning scenario regarding petroleum metabolites is a site with a large residual petroleum source in soil that, through biodegradation and solubilization, sustains a persistent groundwater plume containing petroleum hydrocarbons and/or petroleum metabolites in contact

[11] IC is the inhibition concentration. IC25 is the concentration at which organisms exhibit 25 percent reduction in a biological measurement like reproduction or growth.

[12] LC is lethal concentration. LC25 is the lethal concentration required to kill 25% of the population.

with or discharging directly to surface water, thereby potentially impacting benthic organisms and habitats.

Although there are processes in surface water that may disperse or dilute discharges depending on the location, time, and hydrologic conditions, the use of surface waters for the treatment of petroleum releases without source removal and/or control and without a permit, is inconsistent with the Basin Plan (Regional Water Board 2013a; see Section 4.25.2 – Requirements for Site Investigation, Cleanup, and Site Closure – Policies and Procedures for Investigation and Cleanup and Abatement of Discharges). Further, benthic organisms can be subjected to the full effects of a discharge and they constitute the base of the food chain.

Furthermore, there is no vested right or existing Regional or State Water Board permit to discharge petroleum metabolites to the waters of the State (this applies to both groundwater and surface water discharges). The Porter Cologne Water Quality Control Act (Water Code) Section 13260 prohibits the discharge of any substance to waters of the State without first filing a report of waste discharge to the Regional Water Board. Pursuant to Water Code Section 13263, the Regional Water Board may prescribe requirements for a waste discharge.

Our current approach for evaluating discharges of contaminated groundwater to surface water is to initially compare groundwater sample concentrations to the groundwater ESL for the protection of aquatic receptors, which is a surface water criterion. For TPH-diesel and TPH-motor oil, the marine criterion of 640 µg/L (without SGC) is based on chronic toxicity testing of unweathered Jet A fuel using the *Americamysis bahia* (mysid shrimp)[13] where the receiving water was the Bay at the San Francisco International Airport (Burns and McDonnell 1999; Regional Water Board 1999b). For sites proximal to the Bay and where groundwater concentrations exceed this criterion, further evaluation typically includes the following options:

- Fate and transport modeling (e.g., development of dilution-attenuation factors and a validation study);
- Near-shoreline hydrogeologic investigations of groundwater discharge (e.g., identification of groundwater discharge zones and sampling of groundwater/sediment porewater in those zones and estimation of mass discharge; Chadwick and Hawkins 2008, Washington Department of Ecology 2009); and/or,
- Site-specific aquatic toxicity testing using appropriate species, lethal and sublethal endpoints, and contaminated site groundwater (representing the actual hydrocarbon mixture that was released and the plume at that stage of weathering). Similarly to the National Pollutant Discharge Elimination System (NPDES) permits for our jurisdiction, it is important to include a sensitive species (e.g., *Americamysis bahia*) in the testing program. In addition, consideration should be given to testing of the early life stage as well as the full life-cycle.

The point of compliance for groundwater is the area outside the influence of the surface water body (i.e., where the chemistry reflects contaminated groundwater rather than surface water). In general, a multiple lines-of-evidence approach becomes necessary the closer the site is to a

[13] *Americamysis bahia* was previously named *Mysidopsis bahia*.

surface water body and therefore likely becomes increasingly subject to surface water influence (e.g., tidal mixing zone, hyporheic zone).

The hyporheic zone, where present, is the saturated sediment zone beneath and adjacent to a surface water body where the surface water readily exchanges. It's an important area of biogeochemical cycling of nutrients, and provides habitat and refugia for a range of organisms. Landmeyer et al. (2010) studied the attenuation (concentration reduction) of oxygenates in four different hyporheic zones and found that attenuation was primarily the result of physical processes such as dilution and dispersion, with a small contribution to attenuation by biodegradation. While some contaminant degradation may be enhanced within this zone, there is also potential for contaminant accumulation due to organic-rich sediments, resulting in toxicity to benthic organisms, which form the base of the food chain. The hyporheic zone can be damaged by the construction of shoreline improvements (e.g., seawalls) or filling (Environment Agency 2009). It is not appropriate to assume contaminant degradation will occur to a sufficient degree within the hyporheic zone to avoid benthic organism toxicity without site-specific evidence.

b. Literature Review: Aquatic Toxicity Testing of Fresh and Weathered Petroleum Mixtures or Compounds

Toxic effects are commonly observed in aquatic species as a result of exposure to dissolved petroleum hydrocarbon contamination, including weathered petroleum. The mechanism of action of this toxicity is widely accepted to be the result of narcosis, which is a non-specific toxic effect. Conventional thought has been that the monoaromatic hydrocarbons (MAHs), PAHs, and phenols pose the greatest toxicity threat to aquatic species. However, for some time now, researchers have been recognizing the aquatic toxicity exhibited by petroleum releases is not entirely driven by these constituents. Studies have shown the petroleum metabolites are significant contributors to overall toxicity (Barron et al. 1999; Neff et al. 2000; Melbye et al. 2009). A selection of these studies is summarized below, in chronological order. Key points are in bold and italic font.

i. Toxicity Enhancement of an Aliphatic Petrogenic Unresolved Complex Mixture (UCM) by Chemical Oxidation

Thomas et al. (1995) investigated the toxicological importance of the chemical oxidation of an aliphatic UCM because aliphatic UCMs are widespread in the environment and the aliphatics themselves are considered to have little direct toxicological significance. They evaluated the toxicological effect of chemically oxidizing an aliphatic UCM from lubricating oil on the feeding rate of *Mytilus edulis* (mussel).

The aliphatic UCM was isolated from the lubricating oil and then water-washed to remove any soluble constituents. The UCM was chemically oxidized, and then toxicant solutions of the UCM and UCM oxidation products were prepared using filtered seawater and acetone/toxicant. Two experiments were conducted. Experiment 1 employed a 1,000 µg/L toxicant solution in a 24-hour exposure to 16 mussels fed with an algal culture in a single container. After 24 hours, the mussels were transferred by pairs into new containers with fresh toxicant and algae and the

clearance rates were measured. The authors indicated that the experiment 1 results showed a statistically significant difference (5% significance level) between the experimental and control at about 7%. Experiment 2 employed a 2,000 µg/L toxicant solution in an otherwise similar test. The authors reported the results as a highly significant difference at about 46%. **The authors postulated that the increase in toxicity following oxidation was due to the fact that the oxidation byproducts, carboxylic acids, ketones, and lactones, are still hydrophobic but have a greater solubility than UCM hydrocarbons.** They also pointed out that QSAR studies predict such compounds should be narcotic toxicants and that polar products of oxidation, such as carboxylic acids, have measureable half lives in marine environments.

ii. Toxicity of Polar and Non-Polar Fractions in Sediments Oiled by Crude Oil

Wolfe et al. (1995) studied sediments at two sites impacted by the 1989 Exxon Valdez crude oil spill. Although considerable weathering had occurred and overall toxicity was low, ***the findings from ecological toxicity tests performed on sediment samples demonstrated that the polar compound fraction had about the same toxicity as the aromatic hydrocarbon fraction.***

iii. Toxicity of Soluble Fractions of Three Weathered Middle Distillate Oils to Marine Species

Barron et al. (1999) tested the hypothesis that the toxicity of the water-soluble fraction (WSF)[14] of three weathered middle distillate oils from a coastal California oilfield was attributable to the aromatic hydrocarbons (i.e., monoaromatics or MAHs and PAHs). The three oils were compositionally similar to diesel fuel with a large UCM, few resolved peaks in the chromatograms, and low concentrations of MAHs and PAHs (only the two- and three-ring PAHs were detected). Toxicity testing was performed using the *Americamysis bahia* (mysid crustacean or shrimp) using survival and growth tests (sublethal). LC50s ranged from 900 µg/L to 1,500 µg/L extractable TPH. EC20s ranged from 130 µg/L to 1,100 µg/L extractable TPH. ***Toxicity of the WSF was not well correlated with the specific constituents detected (MAHs and PAHs), but was correlated with the TPH concentrations. The authors concluded that aromatic hydrocarbon content was not the major determinant of toxicity in the WSF of these weathered oils. Rather, based on analysis of an extended range of non-conventional analytes, the authors concluded the toxicity was due to more polar heterocyclic aromatic compounds***.

[14] WSFs are usually prepared by layering the oil (or petroleum hydrocarbon mixture) over water and gently mixing to enhance dissolution while avoiding emulsification or other entrainment of separate-phase hydrocarbon in the water. A typical oil-water loading ratio is 1:10. Then the water is carefully sampled to avoid separate-phase hydrocarbons. WSFs are also referred to as water-accommodated fractions (WAFs).

iv. Toxicity of Soluble Fractions of Artificially-Weathered Crude Oils and Diesel Fuel to Temperate and Tropical Marine Species

Neff et al. (2000) performed an extensive study of the chemical composition and acute toxicity to marine animals of the WSFs of three crude oils (two light and one medium) and a diesel fuel used in machinery on the platforms for future spill planning purposes. This testing was performed on the unweathered oils as well as artificially-weathered oils. The WSFs were artificially weathered by evaporation to simulate a spill in tropical marine waters off of northwestern Australia. Although the unweathered oils were analyzed for total hydrocarbons, alkanes, MAHs, PAHs, and C_0 to C_9 phenols, the unweathered and weathered WSFs only were analyzed for MAHs, PAHs, and phenols because the conventional thought is that these pose the greatest toxicity threat to marine organisms.

The acute toxicity of the WSFs was tested at three stages of weathering. Three species each of tropical/subtropical and temperate marine animals were used including fish, crustaceans, and larvae. The temperate species (*Menidia beryllina*, *Americamysis bahia*, and larvae from *Strongylocentrotus purpuratus* or *Dendraster excentricus*) are consistent with those used in USEPA methods (e.g., *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*; USEPA 2002). The toxicity of the unweathered oil WSFs varied with the two light crude oils showing the greatest toxicity followed closely by the diesel and then the medium crude oil, which the authors reported as almost non-toxic. The weathering effects on the toxicity of the crude oil WSFs varied. For the two light crude oils, the toxicity of the WSFs decreased with increased weathering for the fish species and one larva. However, the toxicity remained constant for the mysid, shrimp (tropical), and the other larvae. For the medium crude oil, the toxicity of the WSFs was low to non-toxic for all species except the mysids. For the diesel fuel, there was little change in the toxicity of the increasingly weathered WSFs. This observed toxicity did not correlate with the hazard indices calculated for the analyzed components (individual MAHs, PAHs, and phenols), which were well below one. ***The authors concluded that the unanalyzed constituents, polar compounds produced by oil degradation, contributed to or were the major cause of toxic effects observed.***

v. Bioassays of Contaminated Groundwater at an Oil Field

At an oil field in California, preparative HPLC was used to remove polar compounds from solvent extracts of groundwater samples to determine the contribution of petroleum metabolites to groundwater samples (Lundegard and Knott 2001). Crude oil distillate had been released and the groundwater plumes were old and stable. Polar compound concentrations were as high as10,000 µg/L near source areas and comprised as much as 83% of the dissolved components in the samples. ***Bioassays performed with frog embryos and other receptors indicated toxicity, and that the toxicity decreased in the direction of groundwater flow (downgradient)***.

vi. Developmental Toxicity of Four PAH Metabolites to Medaka Embryos

Carney et al. (2008) evaluated the toxicity of four PAH metabolites that were structural isomers of hydroxynaphthoic acid ($C_{11}H_8O_3$) using *Oryzias latipes* (medaka fish, a euryhaline species) embryos and eleutheroembryos. The four compounds are bacterial metabolites of

phenanthrene, anthracene, and 2-methylnaphthalene: 1-hydroxy-2-naphthoic acid (1H2NA), 2-hydroxy-1-naphthoic acid (2H1NA), 2-hydroxy-3-naphthoic acid (2H3NA), and 6-hydroxy-2-naphthoic acid (6H2NA). Multiple endpoints were evaluated including: mortality; embryonic development; abnormal heart index; frequency of selected teratogenic effects; behavioral abnormalities; heartbeats per minute; and craniofacial index. Based on the effects data for mortality, development, and heart defects, ***the four isomers differed significantly in their toxicity to tested species***. 1H2NA was the most toxic, about twice that of 2H1NA and 2H3NA, which were similar, and lastly 6H2NA, which was essentially non-toxic. The authors noted previous research indicated 1H2NA demonstrated less sorption in organic estuarine sediments than its parent compound phenanthrene as well as greater toxicity. ***The authors concluded that risk assessments conducted using information on parent compounds only will not reflect real-world scenarios where the parent compounds are metabolized*.**

vii. Toxicity of Water Leached from Diesel-Contaminated Soil over Time

Mao et al. (2009) studied the acute ecological toxicity of water leached through soil microcosms over a 20-week period (see Section 3b for greater detail) using the Microtox toxicity assay in accordance with ISO 11348-3, which measures the inhibition of light emission from the marine bacterium *Vibrio fischeri*. This study provided some indication of the molecular-level changes during the course of aerobic biodegradation of the B soil (weathered diesel-like oil) with ever more polar and larger intermediate compounds being detected in the leachate. ***The leachate at time zero (T0) and at 8 weeks (T8) was similarly toxic, but the toxicity decreased based on the Microtox bacteria test at weeks 12 (T12) and 20 (T20).***

viii. Effect-Directed Identification of Naphthenic Acids as Important Environmental Estrogen Agonists and Androgen Antagonists

Thomas et al. (2009) studied the potential estrogenic and anti-androgenic effects of petrogenic naphthenic acids in water from North Sea offshore produced water discharges. Produced water from offshore oil production platforms is a complex mixture of heavy metals, hydrocarbons, phenols, organic acids, and oil production chemicals (Roe 1999). Some of the produced water components are known to be *in vitro* estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) agonists and androgen receptor (AR) antagonists. A frequently observed effect of AR antagonism is the demasculinization of male fish through interference with natural androgen signaling which can lead to feminization when it occurs during sensitive periods of development. This may act in concert with the feminization due to ER agonists. Short-chain petrogenic alkylphenols previously have been identified as responsible for about 35% of the ER agonist activity measured *in vitro*, but the compounds responsible for AR antagonism are unknown. Both alkylphenols and PAHs have been identified as environmental AR antagonists.

The main objective of the study was to identify the unidentified ER agonists and AR antagonists present in offshore produced water discharges. This goal was accomplished through a combined compound class fractionation using HPLC, determination of endocrine disrupting potential by yeast estrogen and androgen screens, and compound identification by gas chromatography-time-of-flight, high-resolution mass spectrometry (GC-ToF HRMS). Samples from two oil platforms were collected at the last sample point before discharge to the sea after

following treatment to remove oil and clean the process water. Both samples had comparable AR antagonist potency, but one sample extract was non-detect for ER agonist potency from the employed assay. The sample extract with detectable ER agonist activity was further characterized to identify additional estrogenic chemicals. As mentioned above, previous testing had linked about 35% of the ER agonist activity to alkylphenols. Fractionation resulted in 31 fractions. Although only 19% of the total, pre-fractionation ER activity was recovered following fractionation, 5 of the 31 fractions tested positive for the presence of ER agonists. Of the recovered ER activity, approximately 90% was in Fraction 31, which represented the heaviest compounds that were recovered during final flushing of the fractionation column. Since that final column flush had not been previously performed, the authors concluded Fraction 31 comprises previously uncharacterized ER agonists. Twenty of the 31 fractions tested positive for AR antagonists, and the total AR activity recovered was comparable to the pre-fractionation total.

Chemical characterization of Fraction 31 indicated it was a complex mixture of naphthenic acids. **The study demonstrated that the naphthenic acids behave as environmental ER agonists and AR antagonists along with the alkylphenols.**

ix. Toxicity of a Soluble Fraction of Artificially-Weathered Crude Oil to Liver Cells from Rainbow Trout

Melbye et al. (2009) studied the UCMs in a WSF of an artificially-weathered Norwegian Sea crude oil by chemical analysis and toxicity testing. The WSF of the crude oil was separated into 14 increasingly polar fractions by preparative HPLC. After the chemical characterization and toxicological analysis of the individual fractions, three fractions were selected for in-depth studies:

- F1 (least polar, dominated by $C_{0\ to\ 3}$ naphthalenes, 3-ring PAHs, $C_{0\ to\ 3}$ alkylated PAHs);
- F6 (medium polar, containing 3- to 5-ring alkylated PAHs, alkylphenols, and benzothiophenes); and
- F11 (highly polar, UCM-dominated and accounting for 71.1% of the WSF at 1,500 µg/L).

The GCxGC-ToF-MS analysis of the UCM-dominated F11 indicated the presence of more than 3,000 compounds, but only a few could be spectrally matched in the National Institute of Standards and Technology mass spectral library.

The *in vitro* toxicity of the fractions was characterized in a primary culture of liver cells (hepatocytes) from rainbow trout (*Oncorhynchus mykiss*). The endpoints included cytotoxicity (metabolic inhibition), 7-ethoxyresorufin O-deethylase (EROD) activity, and the estrogenic biomarker vitellogenin. The F11 fraction was the main contributor to toxicity. However, after normalization of *in vitro* responses to the mass in each fraction, the authors found that toxicity in the individual fractions was proportional to the amount of material present (i.e., concentration). ***Both polar and non-polar compounds contributed additively to crude oil toxicity. Nevertheless, due to the large concentrations, the polar UCM fraction still dominated the toxicity after the results were normalized. The authors concluded that the polar compounds in the UCM may be the most toxicologically important portion of the WSF.***

c. Discussion – Ecological Risk

Often, in aquatic toxicity studies of the water-soluble constituents from petroleum mixtures, the toxicological responses are frequently attributed only to those compounds that are readily resolved and identified by conventional GC-FID or GC-MS such as PAHs and phenols (Barron et al. 1999; Melbye et al. 2009). However, the studies summarized herein indicate that both unweathered and weathered petroleum demonstrate toxicity to aquatic species; this includes the hydrocarbons, the polar compounds in crude oils, and the degradates from refined fuels (e.g., diesel) as well as unrefined petroleum products (i.e., crude oil). Some studies indicate these intermediate breakdown products can be just as toxic as the aromatic hydrocarbons or are the major cause of toxic effects. Furthermore, the oxidation that takes place during weathering can actually enhance toxicity as well as solubility. The increased solubility makes these compounds potentially more bioavailable (USEPA 2012). Clearly, these compounds should be included as part of risk assessments for petroleum releases.

6. Conclusion and Recommendation for a Site-Specific Evaluation Approach

Our overall conclusion is that petroleum metabolites clearly pose risks to humans and ecological receptors. The polar breakdown products should be considered as part of site-specific, petroleum-related risk assessment and also when evaluating cumulative risk. In addition, they provide information about the stage or degree of weathering. Therefore, metabolites should be evaluated as part of the CSM for specific petroleum release sites.

As a result of the review and analysis described in this document, Regional Water Board staff will continue requiring evaluation of petroleum metabolites at sites where: 1) significant petroleum contamination is detected; and 2) human or ecological receptors are present.

- **Sites of heightened concern** are those with significant remaining contaminant mass in soil, proximity to receptors (insufficient distance for adequate biodegradation/ attenuation) such as groundwater supply wells or aquatic habitats, and uncertainty in the CSM regarding the nature and extent and fate and transport of the petroleum release in question being evaluated.

- **Routine extractable TPH analyses (e.g., TPH diesel and TPH motor oil) for delineation of extent, comparison to ESLs, or risk assessment purposes should be performed without SGC**. The data for TPH-diesel and TPH-motor oil should be added and compared against the TPH-diesel criterion. This is because: 1) motor oil range hydrocarbons are virtually insoluble and are unlikely to add significantly to the water sample result; and 2) at weathered petroleum sites, sample chromatograms show a polar UCM that occurs mostly in the diesel range (C_{10} to C_{24}) with a portion extending into the motor oil range (C_{24} to C_{36}). It is preferable to have the laboratory integrate and quantitate the entire UCM against a single standard (e.g., diesel) rather than against two standards (diesel and motor oil). However, the TPH-diesel and TPH-motor oil results for each sample can be added after the laboratory analysis as an approximation.

- **While SGC should not be used on samples for determining the extent of a plume or for comparison of data against risk-based screening levels, SGC can be useful in assessing the degree of degradation or contribution of BOCs.** Although we recommend the analysis of extractable TPH without SGC for delineation of extent, comparison to ESLs, or risk assessment purposes, we encourage the analysis of samples both with and without SGC once or twice during the course of a project to get a sense of the relative proportions of the hydrocarbons versus metabolites and see if those proportions significantly change.

This approach provides a more complete evaluation of the full, cumulative impacts associated with petroleum releases. While this document illustrates why the petroleum metabolites should be measured and evaluated as part of site-specific evaluations, Regional Water Board staff's overall approach to petroleum site cleanups remains the same: adequate investigation and delineation; source control to the extent practicable; groundwater plume remediation (including natural attenuation where appropriate); sufficient monitoring to demonstrate plume stability; and institutional controls (e.g., deed restrictions, risk management plans, etc.) when necessary. Consideration of additional action is mainly driven by heightened concerns at a site, such as the presence of nearby receptors (e.g., supply wells, aquatic receptors) that could be affected by the groundwater plume (composed of hydrocarbons and/or petroleum metabolites).

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Acronyms and Abbreviations

AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ATSDR	Agency for Toxic Substances and Disease Registry
BBP	Benzylbutyl phthalate
BEHP	Bis-2-ethylhexylphthalate; aka di(2-ethylhexyl)phthalate (DEHP)
BOC	Biogenic organic compound
CSM	Conceptual Site Model
CYP	Cytochrome P450 enzyme
DBP	Dibutyl phthalate
DEHP	Di(2-ethylhexyl)phthalate; aka bis-2-ethylhexylphthalate (BEHP)
DOM	Dissolved organic matter
DRO	Diesel-range organics; aka TPH-diesel
EC	Effects concentration. EC50 is the concentration at which 50% of the organisms exhibit an effect(s).
ER	Estrogen receptor
ESL	Regional Water Board Environmental Screening Level
FID	Flame ionization detector
GC	Gas chromatograph
GCxGC-ToF-MS	Comprehensive, two dimensional gas chromatography with time-of-flight mass spectrometry
GC-MS	Gas-chromatograph with mass spectrometry detector
HPLC	High-performance liquid chromatography
IC	Inhibition concentration. IC25 is the concentration at which organisms exhibit 25 percent reduction in a biological measurement like reproduction or growth.
ISO	International Standards Organization
IUR	Inhalation unit risk
LC	Lethal concentration. LC25 is the lethal concentration required to kill 25% of the population.
LOEL	Low-observed effects level
MAH	Monoaromatic hydrocarbons
MBP	Monobutyl phthalate
MCL	Maximum contaminant level
MEHP	Mono(2-ethylhexyl) phthalate
MORO	Motor-oil range organics; aka TPH-motor oil

MS	Mass spectrometry or mass spectrometer
NOAEL	No observed adverse effects level
NOEL	No observed effects level
NOM	Natural organic matter
NPDES	National Pollutant Discharge Elimination System
NSO	Nitrogen, sulfur, oxygen
NVDOC	Non-volatile dissolved organic carbon
OECD	Organisation for Economic Co-operation and Development
OEHHA	Office of Environmental Health Hazard Assessment
OSPW	Oil sands process waters
POC	Point of concentration
QSAR	Quantitative structural activity relationship
Regional Water Board	San Francisco Bay Regional Water Quality Control Board
RfD	Oral reference dose (non-carcinogens)
RfC	Inhalation reference concentration
RSL	USEPA Regional Screening Level
SFo	Oral cancer slope factor
SGC	Silica gel cleanup
TIE	Toxicity identification evaluation
TPH	Total petroleum hydrocarbons
USEPA	U.S. Environmental Protection Agency
USGS	United States Geological Survey
WAF	Water-accommodated fraction
WSF	Water-soluble fraction
mg/kg	Milligram per kilogram
µg/L	Micrograms per liter