Analysis of Oil Biodegradation Products

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Abstract:

Oil that has undergone biodegradation or photooxidation, contains oxygenated compounds. These compounds cannot be analysed by standard extraction and gas-chromatographic methods. Conventional methods do not analyse for polar compounds and would not count them in the analytical results. Studies have shown that highly oxidized oil, including that undergoing biodegradation and photooxidation, is not properly analyzed by conventional techniques. Conventional analytical techniques may miss as much as 75% of the oil mass. Similarly for biodegradation analysis, conventional techniques may overstate biodegradation by as much as four times.

Five possible analytical solutions to this problem are suggested and include; thin layer chromatography, derivitization and then GC analysis, FTIR, NMR and HPLC. The latter two techniques are typically applied to only the separated polar fractions of the oil. These techniques are in their infancy and much more work needs to be carried out.

The biodegradation pathways of oil compounds are largely unknown. Biodegradation steps are known only for a few of the thousands of compounds in oils. Analytical methods for many of the biodegradation products are also absent or need development. The situation is very complex and only extensive research over dozens of years will improve the knowledge. The first step will be a generalized analysis step that includes most oxygenated compounds.

Introduction

It has been noted for some time that oil biodegradation and photooxidation results in oxygenated products that are difficult to analyse using conventional techniques.[1-4] Since many of the products of biodegradation are oxygenated products, misapplication of analytical techniques show that as much as 75% of the product will be missed or that biodegradation would be overstated by as much as 4 times.[1] The situation for biodegradation is similar to residues from spills. These residues may be produced by photooxidation or by biodegradation, typically both. Thus the same errors may apply. Further, these errors cannot be overcome by the simple use of conservative biomarkers or other simple techniques. The biomarkers are resistant to oxidation and thus are conserved and measurable by GCMS techniques. This will mislead analysis when there is a significant amount of biodegradation.

Most oils degrade to oxygenated products involving a complex pathway.[5,6]. Some of the pathways proceed down to more basic products such as CO_2. Pathways for most oil compounds have not been determined. This can be envisioned as a staircase. The top of the staircase is sometimes known, the first step or two is known for only a few of the thousands of compounds in oil and the multiple steps down to the very bottom is only known for a very, very few and only simple compounds. The situation will only improve with extensive research over many years. The biodegradation pathways are different from the metabolic pathways because the oxidation agents are different. Many or most of these oxygenated products cannot be analyzed by conventional techniques. It has long been presumed by many that conventional chromatographic-mass spectroscopic techniques can analyse most oil compounds. In fact they can analyze only up to about ¼ of highly oxygenated oils.

Literature Review

Although there are hundreds of papers on biodegradation, there are only a handful that show the degradation pathways which are of relevance to this particular paper. Further several of these papers contain methods of analysing oils such that the oxygenated compounds are actually measured. Unfortunately, the bulk of the literature does not present such methods or pathways. This is largely because this is a very difficult field of science and studies will go on many years.

Fernandez-Varela et al. monitored the residues of photooxidation of Prestige oil using attenuated total reflectance - mid IR spectrometry.[3] The Prestige fuel was weathered under natural conditions and under infrared radiation to study its weathering over time. A correlation was established using the 1690-1700 cm^{-1} carbonyl peak, from which it was deduced that IR radiation weathered the product two times faster than natural exposure. The use of 10 weathering indexes was carried out to confirm the main patterns given by factor analysis and to determine which main functional groups and structures increased or decreased during weathering. It was found that the carbonyl and sulphoxide indexes varied greatly, as well as the total aromaticity and long chains compounds. The substitution-related indexes pointed out that highly substituted aromatic structures increased although the total amount of isolated CH groups in aromatic structures remained the same.

Aeppli et al. studied the effect of weathering on surface slicks, oil-soaked sands, and oil-covered rocks and boulders over an exposure period of 18 months.[1,2] With time, oxygen content increased in the hydrocarbon residues. Furthermore, a weathering-dependent increase of an oxygenated fraction relative to the saturated and aromatic fractions was observed. This oxygenated fraction made up >50% of the mass of weathered samples, and had an average carbon oxidation state of -1.0, and an average molecular formula of $(C_5H_7O)_n$. The oxygenated

hydrocarbon residues were determined to be from a fossil source. The incorporation of oxygen into the oil's hydrocarbons was confirmed from the detection of hydroxyl and carbonyl functional groups and the identification of long chain (C_{10}-C_{32}) carboxylic acids as well as alcohols. The authors conclude that biodegradation and photooxidation were responsible for the accumulation of oxygen in the oil residues. These results show that molecular-level transformations of petroleum hydrocarbons lead to increasing amounts of oxyhydrocarbons that dominate the solvent-extractable material from oiled samples. Furthermore, use of conventional extraction and GC methodology would only account for about 25% of the material.

McKenna et al. analyzed Macondo residual oil using Fourier Transform – Ion Cyclotron Resonance Mass Spectrometry (FT ICR MS).[2] The analytical window for different methods was discussed, noting that only a small percentage of compounds in oils, especially weathered oils, is accessible by standard GC MS techniques. They characterize more than 30,000 acidic, basic and nonpolar compounds for Macondo to create a baseline for future analysis.

Maki et al. studied the photo-oxidation and biodegradation of oil as well as the toxicity of the photo-oxidized products.[4] They used NMR to analyse the products and found that the oxygen content of the resulting oil has gone up extensively. They too, noted that this would have been missed by conventional extraction and analysis methods.

Garrett et al. studied the effect of ultraviolet illumination on crude oil using a variety of techniques including gas chromatography/mass spectroscopy and X-ray absorption spectroscopy.[5] It was found that the saturated compounds were resistant to oxidation, but the aromatic compounds were particularly sensitive to photooxidation. Greater size and increasing alkyl substitution increase the oxidation of aromatic compounds. The photooxidized products appear in the resin and polar fractions as determined by thin-layer chromatography. The effect of photooxidation was claimed to be different from that of biodegradation, where larger and more substituted compounds are more resistant to degradation. X-ray absorption spectroscopy indicates that the aliphatic sulfur compounds are more readily oxidized than the thiophenic compounds with the sulfur being oxidized to sulfoxides, sulfones, sulfonates, and sulfates in approximately equal amounts. Photooxidation was found to be a significant process in the degradation of crude oil spilled at sea. Most of the photooxidation products do not appear in the standard extraction and analysis methods.

Charrie-Duhaut et al. compared abiotic oxidation to other alteration processes such as biodegradation, evaporation and water washing.[6] Bulk analyses revealed that increasing exposure was accompanied by an increase in oxygen content. Gas chromatographic-mass spectrometric analyses of polar fractions showed the presence of oxygen-containing compounds (steroid ketones, benzothiophenic acids and sulfones) which result from oxidation of petroleum lipids. Separate analysis was carried out for ketones and acids. It was found that oxygen incorporation generally occurred without any diastereomeric discrimination indicating a different between biodegradation and photooxidation. It was also noted that analysis was required by several methods including NMR, IR, to measure the oxygenated compounds.

Rojo summarizes current knowledge on alkane biodegradation, focussing on the biochemical pathways.[7] The oxidative pathways involve alcohols, aldehydes and acids.

Lin et al. summarize experiments in which bacterial strains isolated from oil refining wastewater sludge (Fuzhou, China) were used to biodegrade naphthalene in cultured medium. Under optimal conditions and initial naphthalene concentration of 50 mg/L, more than 99.1% was removed within 96 h. Of those factors influencing the biodegradation of naphthalene, salinity and inoculum concentration were of greatest importance. Furthermore, the

biodegradation kinetics of naphthalene corresponded with the first-order rate model. Degradation metabolites identified using GC-MS, included o-phthalic acid and benzoic acid, suggesting possible metabolic pathways.

Johnson et al. studied the biodegradation of four aromatic alkanoic acid isomers thats differed in alkyl side chain branching: (4'-n-butylphenyl)-4-butanoic acid (n-BPBA, least branched); (4'-iso-butylphenyl)-4-butanoic acid (iso-BPBA); (4'-sec- butylphenyl)-4-butanoic acid (sec-BPBA) and (4'-tert-butylphenyl)-4- butanoic acid (tert-BPBA, most branched). n-BPBA was completely metabolized within 49 days.[9,10] Mass spectral analysis confirmed that the more branched isomers iso-, sec- and tert-BPBA were transformed to their butylphenylethanoic acid (BPEA) counterparts at 14 days. The BPEA metabolites were generally less toxic than BPBAs as determined by Microtox assay. n-BPEA was further transformed to a diacid, showing that carboxylation of the alkyl side chain occurred. In each case, biodegradation of the carboxyl side chain proceeded through beta-oxidation, which depended on the degree of alkyl side chain branching, and a BPBA degradation pathway is proposed. Comparison of 16S rRNA gene sequences at days 0 and 49 showed an increase and high abundance at day 49 of Pseudomonas (sec-BPBA), Burkholderia (–, iso-, tert-BPBA) and Sphingomonas (–, sec-BPBA).

Izmalkova and co-workers studied the biodegradation of naphthalene using a pseudomonas strain. The oxidative pathway was followed for 5 steps.[11]

Zhang et al. studied thermophilic naphthalene- and aliphatic hydrocarbon-degrading bacterium SH-1 isolated from a deep oil well and identified as Geobacillus sp. n-alkanes from C12 to C33 in crude oil and naphthalene were effectively degraded by strain SH-1, and this strain could readily utilize these compounds as its sole carbon and energy resources.[12] During the degradation of naphthalene, strain SH-1 initiated its attack on naphthalene by a monooxygenation at its C-1 to give 1-naphthol and further monooxygenation at C-2 to produce 1,2-dihydroxynaphthalene. The ring of 1,2-dihydroxynaphthalene was cleaved to form trans-o-hydroxybenzylidenepyruvate. Subsequently, trans-o-hydroxybenzylidenepyruvate was transformed to (2E)-3-(2-hydroxyphenyl)prop-2-enal by losing a carboxyl group. Additionally, benzoic acid was identified as an intermediate in the naphthalene degradation pathway of this Geobacillus strain.

Aitken et al. report data from anaerobic crude oil degrading microcosms which showed significant differences between the acid metabolite profiles of crude oil degraded under sulfate-reducing or methanogenic conditions.[13] Under sulfate-reducing conditions fumarate addition and the formation of alkylsuccinate metabolites was the principal mechanism for the anaerobic degradation of n-alkanes and branched chain alkanes. Other than alkyl succinates that represent indigenous metabolites in the sediment inoculum, alkyl succinate metabolites were never detected in sediment microcosms where methane generation was quantitatively linked to n-alkane degradation.

Yassine and co-workers aerobic studied the biodegradation kinetics of soybean biodiesel and petroleum diesel in batch experiments. The model was built on the assumptions that biodegradation takes place in the aqueous phase according to Monod kinetics, and that the substrate dissolution kinetics at the oil/water interface is intrinsically fast compared to biodegradation kinetics. Further, due to the very low aqueous solubility of these compounds, the change in the substrate aqueous-phase concentration over time was assumed to approache zero, and that substrate aqueous concentration remains close to the saturation level while the non-aqueous phase liquid (NAPL) is still significant. No former knowledge of the saturation substrate concentration (Ssat) and the Monod half-saturation constant (Ks) was required, as the term

Ssat/(Ks + Ssat) in the Monod equation remained constant during this phase. The n-alkanes C10-C24 of petroleum diesel were all utilized at a relatively constant actual specific utilization rate of 0.01-0.02 mg-alkane/mg-biomass-hr, while the fatty acid methyl esters (FAMEs) of biodiesel were utilized at actual specific rates significantly higher with increasing carbon chain length and lower with increasing number of double bonds.

In summary, most investigators have found that biodegradation proceeds via oxidative addition, typically of end members. This process also typically proceeds via several steps to more oxygenated products. In most cases studied above, the oxidation process appears to end with a highly oxygenated product and not usually carbon dioxide. This has major implications on the analysis of biodegraded (or otherwise oxygenated) matter.

The Biodegradation Process

Most biodegradation does not convert oil compounds to carbon dioxide and water. This largely only occurs with smaller chain alkanes. Most larger compounds will partially degrade leaving oxygenated compounds. Several researchers have studied this in the past. Table 1 summarizes some of the reactions observed. The reactions in Table 1 are sometimes serial, that is the first product may degrade to the second product and so on. Table 1 clearly shows that the reaction often results in oxygenated products are rarely in CO_2.

A note is needed here about the use of CO_2 methods to analyze biodegradation. Since the molarity of oil is not known, and consists of hundreds of different compounds, the resulting molarity of CO_2 that should be evolved, is also not known. CO_2 methods merely show that some biodegradation has taken place, but not how much of which compound.

The classes of compounds of compounds that are subject to the discussion in this paper are many and included; PAHs or PolyAromatic Hydrocarbons (of all sizes and types), polar PAHs, alkanes, alcohols, steranes and other degradable biomarkers. In fact, most compounds in crude oils are thought to be subject to this limited understanding with respect to their degradation.

There are other issues associated with biodegradation that are not discussed in this short paper, such as the fact that some biodegradation products are more toxic to aquatic life than are the original compounds.[4] Other factors not discussed are that some microbes and enzymes can produce chemicals such as radicals or peroxides that also oxidize oil constituents.

Table 1 Reactions, Products and Analysis

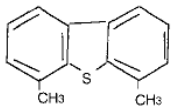
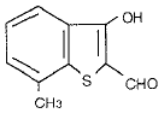
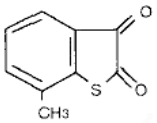
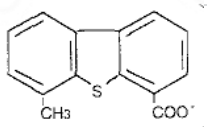
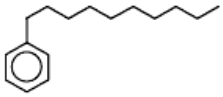
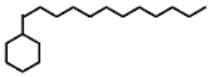
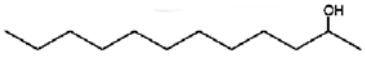
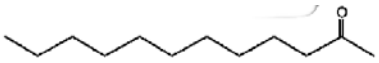
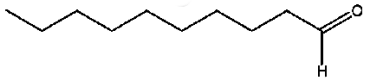
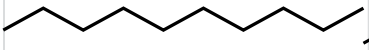
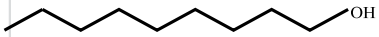
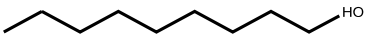
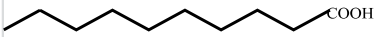
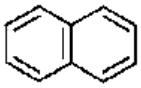
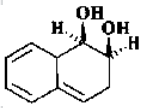
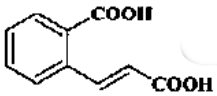
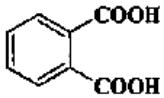
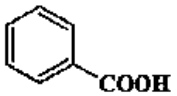
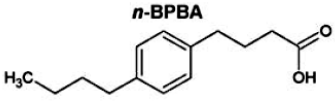
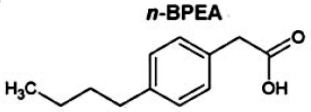
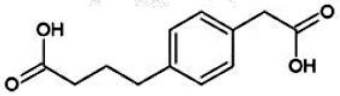
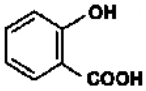
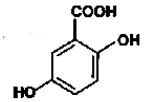
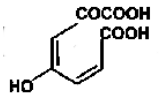
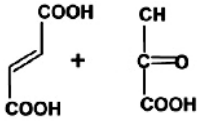
Reference	Reaction #	Starting Compound	Intermediate or Oxygenated Compound	CO_2 Measurement*	Product Standard GC Measurement**
4	1.1			0	nm
4	1.2			1 or 5 moles	nm
4	1.3			0	nm
5	2	Hexadecane	hexadecanoic acid	0	nm
5	3	 Phenyldecane	Phenyldecanoic acid	0	nm
5	4	 Pristane	Pristanoic acid	0	nm
5	5	 Dodecylcyclohexane	Cyclohexyldodecanoic acid	0	nm
6	6.1	 n-dodecane		0	maybe
6	6.2			0	nm
6	6.3			1 mole	maybe
6	6.4			0	nm
		* This column provides the amount of CO2 that would be produced by the specific reaction ** This column states if or not the product can be analysed by convention extraction GC methods, NM means not measured			

Table 1 *continued* Reactions, Products and Analysis

Reference	Reaction #	Starting Compound	Intermediate or Oxygenated Compound	CO_2 Measurement	Product Standard GC Measurement
7	7.1	 Nonane	 Nonanol	0	maybe
7	7.2			0	nm
7	7.3			0	nm
8	8.1	 Napthalene		0	maybe
8	8.2			1	nm
8	8.3			1	maybe
8	8.4		 Benzoic Acid	1	maybe
9	9.1			0	nm
9	9.2			0	nm
11	11.1			0	nm
11	11.2			0	nm
11	11.2			0	nm
		* This column provides the amount of CO2 that would be produced by the specific reaction ** This column states if or not the product can be analysed by convention extraction GC methods, NM means not measured			

Analysis of Oils and Oxygenated Products

The standard method for oil analysis is to use a gas chromatograph (GC). A small sample of the oil extract, often in hexane, and a carrier gas, usually helium or hydrogen, are passed through a capillary column.

The extraction of oil from the substrate and the cleanup procedures are important. The basic methods involve extracting the oil using dichloromethane (DCM), sometimes in combination with other solvents such as hexane. This procedure will leave the DCM insoluble material such as soil, wood, etc. and remove the DCM soluble material, which is largely petroleum oil. This typical procedure will typically take up oil-soluble materials but not highly polar materials. Compounds such as large-chain alcohols will be taken up but not short-chain alcohols. Most ketones, aldehydes and acids are not taken up by DCM/hexane extraction.

The second stage of the process is equally important, that of chromatographic conditions. If the chromatograph column is of non-polar phase, then most oxygenated products are not eluted. Thus most oxygenated biodegradation products are not analyzed by chromatography.

In summary, most biodegraded products are not extracted nor analyzed by typical chromatographic methods. Therefore the analysis of biodegradation experiments using typical chromatography is not quantitative and can miss up to 75% of the partially-degraded material, that is material that is merely oxygenated and not converted to carbon dioxide.[1]

Possible Analytical Methods

There may be some solutions to the problem of analysing oil biodegradation products. It is noted that several of the scientists in the references have carried out analysis of biodegradation by several methods. Standard methods (such as ASTM, EPA) have not evolved at this point in time.

Extraction Methods - One of the extraction methods that appears to remove a large portion of the oxygenated products is to use a DCM/MeOH mixture.

Analysis Methods - 1. Thin Layer Chromatography (e.g. Iatroscan) - this method employs a common laboratory instrument.[1] The thin-layer rods are analysed using a flame ionization detector. This method is functional but requires extensive calibration. Aeppli et al. used this technique and calibrated the single oxygen and double oxygen compounds separately.[1]

2. Derivitization - if a strong derivitization agent were used, the oxygenated compounds could be converted to compounds that will pass through a non-polar GC column. More work on this technique is required to ensure that a large percentage of the polar compounds are indeed analyzed. Calibration is more difficult than in standard GC analysis.[15]

3. FTIR - Fourier Transform Infrared has been used in the past to analyse oxygenated residues.[16,17] The biodegraded residue is typically separated into two fractions, one extracted by DCM/hexane for GC analysis and the remainder dissolved in a polar solvent such as DCM. The latter fraction is then analyzed by FTIR and the sum totalled to yield the total undegraded mass.

4. HPLC - High Pressure Liquid Chromatography also has been used to analyze the polar mass resulting from oxygenation or biodegradation.[18] The biodegraded residue is typically separated into two fractions, one for GC analysis as above, and the remainder dissolved in a polar solvent. The latter polar fraction is then analyzed by FTIR and the sum totalled to yield the total undegraded mass.

5. NMR - Nuclear Magnetic Resonance - NMR has been used to evaluate the amount of oxygenated compounds in a sample.[6] This method is involved and requires extensive calibration.

Conclusions

Biodegradation results in incomplete oxidation of many oil compounds. These compounds are typically oxygenated and may contain one, two or more oxygen molecules. These compounds are generally not amenable to standard GC analysis and thus biodegradation can be overstated by as much as 4 times. Studies have shown that highly oxygenated residues analysed by standard extraction and GC techniques, may only analyze 25% of the oxygenated oil mass because of the oxygen presence on various molecules.

Five possible analytical solutions to this problem are briefly noted and include; thin layer chromatography, derivitization and then GC analysis, FTIR, NMR and HPLC. The latter two techniques are typically applied to only the polar fractions of the oil.

Recommendations for Further Work

There are several approaches that should be followed:

1. There is an immediate need for analytical methods that are practical and can be used in many laboratories. Research for this should be supported and standards organizations need to develop standard methods. This will take several years before there are good initial methods.
2. Information on this problem needs to be transmitted to those performing biodegradation studies. The researcher needs to understand that simplistic analytical methods do not function to yield a reliable percentage of biodegradation. Temporary work-arounds might be suggested such as the use of thin-layer chromatography techniques. Currently only a few laboratories can apply this method.
3. The general transmission of information on this situation is important. This information should be widely disseminated to the oil spill community.
4. The pathways for biodegradation of more compounds need to be understood and measured. The factors relating to the various steps such as the kinetics of the steps and the limiting factors also need to be understood. Each study of this type could take years to complete. Thus, it will be many years before more data such as shown in Table 1, will be available.

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