

**KILL
SPILL**



Kill•Spill

Integrated Biotechnological
Solutions for Combating
Marine Oil Spills

Deliverable D2.6

New isomer-based
methods for monitoring of
bioremediation



This project is supported by the European Union under the Food, Agriculture and Fisheries and Biotechnology theme of the 7th Framework Programme for Research and Technological Development under GA no. 312139

Work package	WP2 Research and technological development
Deliverable no.	D2.6
Deliverable title	New isomer-based methods for monitoring of bioremediation
Due date:	2016-01-31 (Month 36)
Actual submission date:	2016-04-22 (Month 40)
Start date of project:	2013-01-01
Deliverable Lead Beneficiary (Organisation name)	UCPH
Participant(s) (Partner short names)	UCPH ¹ , GEUS ² .
Author(s) in alphabetic order:	J. Aamand ² , J. Christensen ¹ , A. Johnsen ² , L. Malmquist ¹
Contact for queries:	Linus Malmquist, UCPH Thorvaldsensvej 40, 1871 Frb C (Denmark) T: +45 35320486 E: linus@plen.ku.dk
Dissemination Level: (PUBlic, Restricted to other Programmes Participants, REstricted to a group specified by the consortium, COntidential only for members of the consortium)	PU
Deliverable Status:	final



Table of Contents

1	About this deliverable	1
2	Introduction.....	2
2.1	Internal Standards used in sample preparation.....	2
2.1.1	Internal standards for biomarker quantification (GC-MS)	2
2.1.2	Internal standards for semi-quantitative diagnostic isomer ratio calculations	2
2.1.3	Internal standards for GC-MS analysis for PAC quantification.....	2
2.2	Extraction from sediment.....	3
2.2.1	Sampling	3
2.2.2	Extraction model 1: Stepwise ultrasonication extraction and silica gel cleanup	4
2.2.3	Extraction model 2: Simultaneous extraction and cleanup using pressurized liquid extraction (PLE)	5
2.2.4	Extraction and cleanup of oil from water and oil/water emulsions	7
3	GC-MS method for PAC and biomarker analysis.....	9
3.1	Introduction.....	9
3.2	Sample preparation	9
3.3	Standards.....	9
3.3.1	GC-MS analysis for PAC quantification and diagnostic ratio calculations.....	9
3.3.2	Biomarker quantification (GC-MS)	10
3.4	Instrument settings and equipment.....	10
3.5	GC-MS data analysis	15
3.5.1	Peak area quantification.....	15
4	References.....	21



List of Figures

Figure 1.	Diagram showing the content of this protocol, covering sample preparation, chemical analysis and data treatment. Extraction and cleanup of petroleum hydrocarbons for GC analysis.....	1
Figure 2.	Illustration of the preparation of a small chromatographic column, packed from its base with a glass filter, activated silica and anhydrous sodium sulfate. Also a brief description of the sample cleanup procedure is shown.....	5
Figure 3.	PLE extraction cell components.....	5
Figure 4.	Scheme of the PLE extraction cell packing and extraction parameters.	7
Figure 5.	Chromatograms of injections of a mix of DFTPP, Pentaclorophenol, Benzidine and DDT from a GC-MS operating in ideal conditions (a) and a GC-MS in bad conditions (b).....	11
Figure 6.	PW-plot. Example of relative changes in composition of n-alkanes in soil extracts compared to original oil after 1 year of weathering.	16
Figure 7.	Example of relative changes in composition of mono-, di- and tri cyclic aromatic compounds in soil extracts compared to original oil after 1 year of weathering.	17
Figure 8.	Example of relative changes in composition of sulfur containing di- and tricyclic aromatics in soil extracts compared to original oil after 1 year of weathering.	18
Figure 9.	Employment of diagnostic ratios to show the degree of weathering of an oil after one year.	19

List of Tables

Table 1.	List of the most appropriate internal and recovery standards for the quantification of 19 selected PAHs.....	3
Table 2.	List of the most appropriate internal and recovery standards for the 19 PAHs.....	10
Table 3.	DFTPP key ions and ion abundance criteria. (USEPA 1998) See USEPA method 8270D, t3 for DFTPP acceptance criteria.	10
Table 4.	Ions of the key compounds responsible for the control of chromatographic conditions.	11
Table 5.	List of compounds, SICs and corresponding groups of GC-MS/SIM as described by (Gallotta and Christensen 2012).....	13
Table 6.	Biodegradation diagnostic ratios (DRs) of selected petroleum hydrocarbons.....	20



1 **About this deliverable**

This protocol standardizes the extraction, sample preparation and analysis of Total Petroleum Hydrocarbons (TPH), Polycyclic Aromatic Compounds (PACs) and biomarkers from sediment and soil samples. With this protocol, samples evaluating remediation technologies of oil spills are standardized, allowing comparisons and summation across different remediation approaches. The protocol is based on current governmental standard methods (USEPA, NIST, CEN Bonn-OSInet, etc.) and published scientific data analysis approaches developed to take full advantage of state-of-the-art instrumentations for detailed analysis of the progress and extent of employed remediation technologies.

The protocol first describes two methods for extraction and cleanup of petroleum hydrocarbons from sediment and one for extraction of petroleum hydrocarbons from water. Furthermore, the protocol includes an analytical approach, GC-MS, and three data analysis approaches to reach to the assessment of the efficiency of the applied cleanup and remediation technologies. These are:

- I. Calculation of the total weathering degree. Data are obtained from quantifications of $C_{30-\alpha,\beta}$ -hopane in the oil.
- II. Chemical fingerprints of C_0 - C_4 alkyl-substituted polycyclic aromatic compounds (PACs).
- III. Calculation of process-specific diagnostic ratios to assess the contribution of different weathering and degradation processes to the total removal.

The protocol lists quantification standards, certified reference materials and quality control procedures that would be beneficial to employ in standard analyses, when assessing cleanup and remediation technologies applied to oil spills. See Figure 1 for an overview of the deliverable.

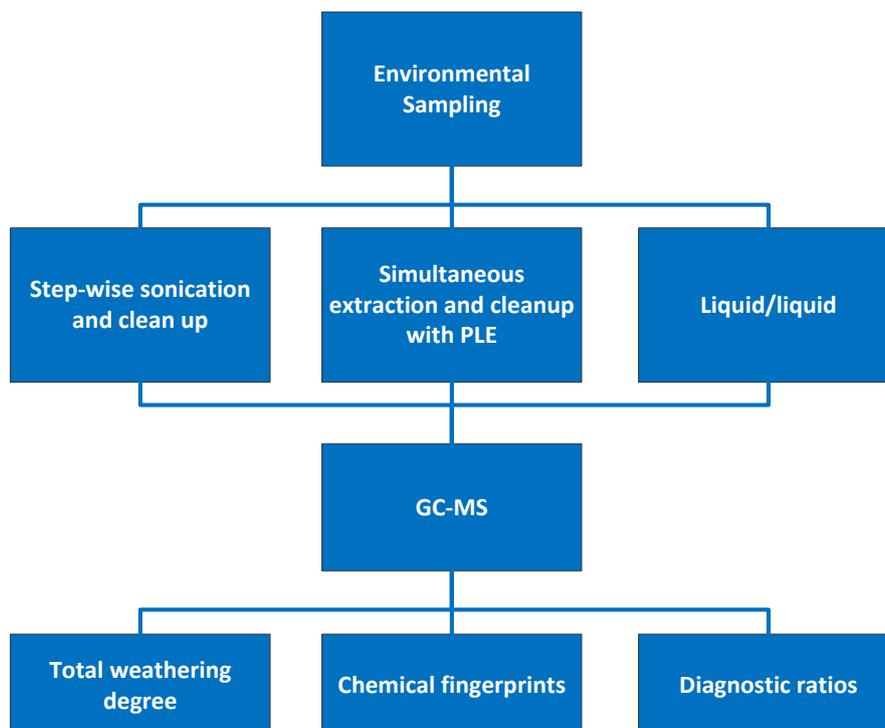


Figure 1. Diagram showing the content of this protocol, covering sample preparation, chemical analysis and data treatment. Extraction and cleanup of petroleum hydrocarbons for GC analysis



2 Introduction

This deliverable standardizes the extraction of petroleum hydrocarbons from sediment and water samples to be used for the assessment of applied remediation technologies.

Efficient extraction, preconcentration and cleanup are indispensable prior to the determination of PAHs in water, soil and sediments. There exists a wide variety of solvent extraction techniques commonly used for extracting hydrocarbons from soil and sediments. Soxhlet extraction, which has high extraction efficiency, has been the standard method for preparing a solvent extract of solid matrices containing PAHs. Other traditional extraction procedures include ultrasonication (Luque-Garcia and de Castro, 2003; Banjoo and Nelson, 2005), mechanical shaking (Berset et al., 1999), reflux with methanolic KOH and steam distillation. Modern instrumental techniques include supercritical fluid extraction (SFE) (Barnabas et al., 1995; Benner, 1998), pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) (Sporring and Bjorklund, 2004; Sporrying et al., 2005) and microwave assisted extraction (MAE) (Ericsson and Colmsjo, 2000). Each technique has its own merits and the choice of extraction depends on several factors including capital cost, operating cost, simplicity of operation, amount of organic solvent required, sample throughput and availability of appropriate equipment and a standardized method. For soil and sediment extraction, we display a simple technique with stepwise extraction and cleanup viz. ultrasonication, and a more sophisticated approach with simultaneous extraction and cleanup using pressurized liquid extraction (PLE). For oil in water, we describe the simple liquid/liquid extraction.

2.1 **Internal Standards used in sample preparation**

The employed analytical platform and the purpose of the analysis defines the type of standard (internal and external) that should be used. This protocol distinguishes between GC-FID analysis for TPH quantification, GC-MS analysis for PAC and biomarker quantification and GC-MS analysis for weathering specific semi-quantitative diagnostic ratio calculations.

2.1.1 *Internal standards for biomarker quantification (GC-MS)*

Since weathering of oil only rarely affect the content of C_{30} - α,β -hopane, this compound is often used as an internal preserved reference compound for the assessment of specific oil compound removal within the oil. If exact quantifications of C_{30} - α,β -hopane and other biomarkers is needed (ex. for total weathering degree calculation), we suggest to use the thermally unstable (and therefore rarely seen in oil) isomer C_{30} - α,α -hopane as an internal standard, referring to the suggestion given by Nytoft and Bojesen-Koefoed (2001).

2.1.2 *Internal standards for semi-quantitative diagnostic isomer ratio calculations*

Since this method is based on changes in selected C_0 - C_4 -alkylated PACs relative to internal preserved reference compounds (such as C_{30} - α,β -hopane or highly alkylated PACs) and the original undegraded oil, internal standards as such are not needed in the sample preparation. However, if a need for quantification of PACs exists, we provide a list of recommended standards and concentrations below. It is recommended to always use internal and recovery standards, if a need for quantification should arise, or if a need for evaluation of extraction efficiency, evaporative losses during sample prep or discrimination within the analytical instrument.

2.1.3 *Internal standards for GC-MS analysis for PAC quantification*

Internal standards: Naphthalene-d8, acenaphthene-d10, fluorene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo(k)fluoranthene-d12 and benzo(g,h,i)perylene-



d12 (8µg/mL in isooctane giving 0.32µg/mL in final sample). Label the solution (**RED 3**). See Table 1 for an overview of all standards.

Recovery standards: acenaphthylene-d8, anthracene-d10, fluoranthene-d10, benz(a)anthracene-d12, benzo(a)pyrene-d12 and indeno(1,2,3-c,d)pyrene-d12 (8µg/mL in isooctane giving 0.32µg/mL in final sample). Label the solution (**RED 4**). See Table 1 for an overview of all standards.

The solutions are prepared in pre-cleaned 5 mL volumetric flasks, transferred to 8 mL amber glass vials, and stored in the freezer at -20 °C until use.

Table 1. List of the most appropriate internal and recovery standards for the quantification of 19 selected PAHs.

Internal standard	PAHs	Recovery standard
Naphthalene-d8	Naphthalene, Acenaphthylene	Acenaphthylene-d8
Acenaphthene-d10	Acenaphthene	Anthracene-d10
Fluorene-d10	Fluorene	Anthracene-d10
Dibenzothiophene-d8	Dibenzothiophene	Anthracene-d10
Phenanthrene-d10	Anthracene, phenanthrene	Anthracene-d10
Pyrene-d10	Pyrene, fluoranthene	Fluoranthene-d10
Chrysene-d12	Chrysene, benzo(a)anthracene	Benzo(a)anthracene-d12
Benzo(k)fluoranthene-d12	Benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene	Benzo(a)pyrene-d12
Benzo(g,h,i)perylene-d12	Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene, indeno(1,2,3-c,d)pyrene	Indeno(1,2,3-c,d)pyrene-d12

2.2 Extraction from sediment

2.2.1 Sampling

The importance of obtaining a representative sample for analysis cannot be overemphasized. Without it, results may be meaningless or even grossly misleading. Sampling is particularly crucial where a heterogeneous material is to be analyzed. It is vital that the aims of the analysis are understood and an appropriate sampling procedure adopted. In some situations, a sampling plan or strategy may need to be devised to optimize the value of the analytical samples such as sediment samples. It is strongly recommended to examine relevant literature for standardized protocols for the specific compartment and analytes. For shipment of samples, at least three times the amount needed for an analysis should be sampled, i.e. dry weight and PAC extraction= $3 \times (3 \times 5 \text{ g} + 5 \text{ g}) = 60 \text{ g}$.

2.2.1.1 Determination of dry weight and organic matter content:

1. Representative sampled sediment samples are homogenized thoroughly and large fragments are removed.
2. 3×5 gram of material is weighed into ceramic crucibles (the exact weight is noted) and placed in an oven at 105 °C overnight. The cooled samples are weighed and the exact weight noted.



3. Samples are put in muffle oven at 550 °C for at least 6 hours, and the cooled samples are weighed.

2.2.2 Extraction model 1: Stepwise ultrasonication extraction and silica gel cleanup

Ultrasonication procedures have been compared with the Soxhlet method for extraction of PACs in samples, with short time ultrasonic extractions producing equivalent or better recoveries than 6-8 hours of Soxhlet extraction (Lundstedt et al., 2000). Since the equipment for ultrasonication is simple, easy to operate and relatively fast, ultrasonication is often a preferred extraction method. To separate the analytes from the sample matrix that could interfere with the analysis, a column cleanup procedure (with silica and sodium sulfate) is applied.

2.2.2.1 Sample extraction and cleanup

Extractions are performed with 2 blank extractions (procedure blanks), 1 extraction of an appropriate reference sample (e.g. NIST SRM 1941b - Organics in sediment) used for proficiency testing and sample extractions where 1 of these are extracted in duplicate.

1. Accurately weighed known amounts (≈ 5 gram to reach a final concentration of about 2000 μg oil/mL solvent) of wet sediment/soil is ground thoroughly with 5 gram of hydromatrix.
2. Each sample is subsequently transferred quantitatively to a 100 mL Erlenmeyer flask and internal standard solution is added on to the top of the sample. The sample is left to stand for sorption of the added standards, capped for at least 20 min.
3. 30 mL solvent (*n*-hexane and acetone, 1:1, v/v) is added to the Erlenmeyer flask and the suspension is ultrasonicated at room temperature for 10 min, after which the solution from the settled mixture is decanted. The remaining suspension is washed with approximately 2 mL solvent and withdrawn using a Pasteur pipette.
4. The extraction process is subsequently repeated twice (a total of three extractions per sample) and the extracts obtained from the ultrasonication are filtered (in-between each extraction) to remove sediment particles by use of a glass funnel with filter paper directly into a 250 mL round bottom flasks.
5. 1 mL of isoctane is added to the combined extract as keeper and the combined extracts are evaporated slowly (maximum 5 mL/min) to approximately 1 mL on a rotary evaporator

2.2.2.2 Chromatographic column cleanup of extracts for GC analysis

1. A small glass column (1.4 id \times 7 cm) is prepared per extract (Figure 2). Each column is dry packed from its base with a glass filter, 1 gram of activated silica (180°C for 20 hours) and 0.2 cm of anhydrous sodium sulfate. The column is covered with alumina foil until use.
2. The column is conditioned with 5 mL of *n*-hexane.
3. Before the column runs dry, the extract is transferred quantitatively to the top of the column using a Pasteur pipette. Then $\frac{1}{2}$ mL of *n*-hexane is transferred to the Erlenmeyer flask using a clean pipette and the first pipette is used to wash the flask and transfer the wash to the top of the column. The wash procedure is repeated three times to obtain a quantitative transfer of the extract.
4. The column is washed with 4 mL of *n*-hexane, which is discarded.
5. The column is subsequently eluted with 4 mL of a 1:1 mixture of *n*-hexane:dichloromethane into a 5 mL volumetric flask.
6. If needed, recovery standard solution is added.
7. The volume of the flask is adjusted to 5 mL with *n*-hexane:dichloromethane (1:1), and stored in amber glass vials at -20 °C until analysis.

Condition with 5 mL *n*-hexane
 Transfer the extract quantitatively
 Elute with 4 mL *n*-hexane (discard)
 Elute with 4 mL 1:1 *n*-hexane :
 dichloromethane to 5 mL volumetric flask

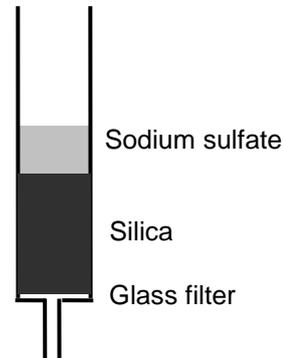


Figure 2. Illustration of the preparation of a small chromatographic column, packed from its base with a glass filter, activated silica and anhydrous sodium sulfate. Also a brief description of the sample cleanup procedure is shown.

2.2.3 Extraction model 2: Simultaneous extraction and cleanup using pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) is a fast and efficient alternative to traditional extraction methods for many types of contaminants. The technique, which is based on extraction at elevated pressure and temperature, can be used with the same solvents as those used for Soxhlet extraction. The method used in this protocol is based on the development by Lundstedt, Haglund et al. (2006), and Boll, Christensen et al. (2008).

2.2.3.1 Preparations of extraction components

1) PLE extraction cells (Figure 3)

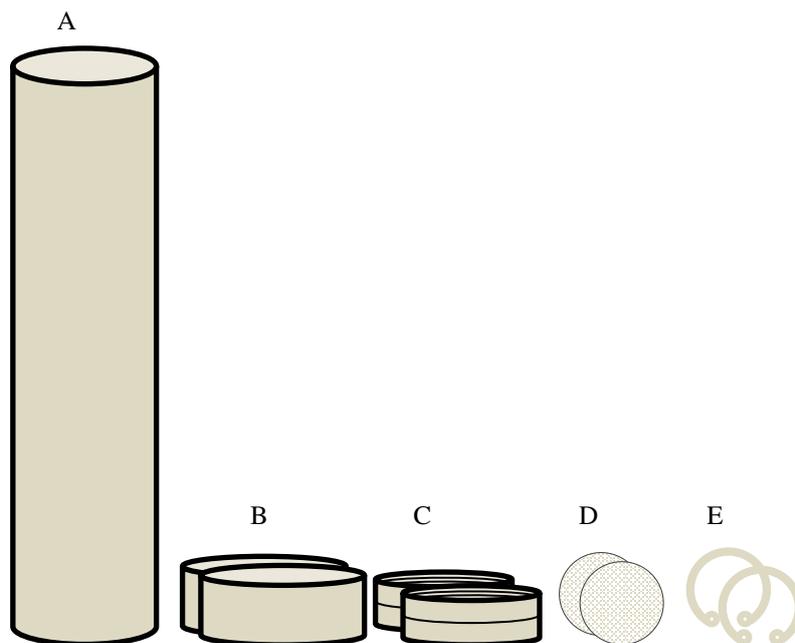


Figure 3. PLE extraction cell components.

a) Separate the extraction cell completely and remove old sample material from the cylinder (A)



- b) Clean the cylinder inside with a tissue
 - c) Wash all parts (A, B, C, D and E) in detergent
 - d) Rinse all parts (A, B, C, D and E) in tap water
 - e) Rinse all parts (A, B, C, D and E) in deionized water
 - f) Sonicate small parts (D and E) for 5 minutes in methanol, the larger parts (A, B and C) are rinsed with methanol
 - g) Sonicate small parts (D and E) for 5 minutes in pentane:acetone (1:1), the larger parts (A, B and C) are rinsed with pentane:acetone (1:1)
 - h) Let all parts dry on alumina foil
- 2) Cellulose filters
 - a) Sonicate cellulose filters two times 5 minutes with dichloromethane, and leave to dry on alumina foil
 - 3) Hydromatrix:
 - a) Hydromatrix (e.g. Agilent Technologies) is placed in a thin layer (max 1 cm) on alumina foil and burned in a muffle oven at 400°C overnight. Cooled hydromatrix is stored in red cap bottles in a dry environment (e.g. desiccator).
 - b) Sodium sulfate (pro analysis, Merck) is dried in an oven at 85°C overnight. Dry sodium sulfate is stored in red cap bottles in a dry environment (e.g. desiccator).
 - 4) Silica gel:
 - a) Silica gel 60 (0.063-0.200 mm, Merck) is activated at 180°C overnight. Activated silica gel is stored in red cap bottles in a dry environment (e.g. desiccator).
 - 5) Copper powder (used for removal of elemental sulfur in sediments)
 - a) Fine copper powder (Fluka, 99% pro analysis) is activated with concentrated hydrochloric acid immediately before use and rinsed with milliQ water followed by acetone and pentane to remove the hydrochloric acid. Residual hydrochloric acid will lead to degradation of PAHs.
 - 6) Ottawa sand:
 - a) Ottawa sand is placed on alumina foil, and burned in a muffle oven at 550°C overnight. The layer of Ottawa sand on alumina foil should be no more than 2 cm in thickness. The Ottawa sand is cooled with alumina cover and when cold, stored in red cap bottles in a dry environment (e.g. desiccator).

2.2.3.2 Sediment and soil extraction procedures with PLE

Extractions are performed batch-wise: A batch of samples consists of 15 extractions: 2 blank extractions (procedure blanks), 1 extraction of an appropriate reference sample (e.g. NIST SRM 1941b - Organics in sediment) used for proficiency testing, 11 sample extractions where 1 of these are extracted in duplicate.

- 1) Integrated extraction and cleanup is performed by pressurized liquid extraction (PLE) in this case with a Dionex ASE 200 accelerated solvent extractor.
 - a) Packing of PLE cells (see Figure 4)
 - i) A 33 mL stainless steel extraction cell is packed from the bottom with two cellulose filters, 4 gram of activated silica gel and 4 gram of wet activated (acid treated) copper (Note: activated copper is added in order to remove elemental sulfur – and is only necessary for anoxic sediment samples. Thus, activated copper is not added in the analysis of soil samples).
 - ii) Accurately weighed known amounts (≈5 gram) of wet sediment/soil is ground thoroughly with 5 gram of hydromatrix. The homogenized and dried sample is transferred quantitatively to the extraction cell.
 - iii) Internal standard is added directly on top of the sample.

- iv) The remaining cell volume is filled with an inert matrix (burned Ottawa sand) and the extraction cells are closed and inserted in the PLE-autosampler.
- v) The PLE-program elaborated in Figure 4 is used for the extractions.
- vi) Each PLE cell is extracted two times (into two separate glass vials)
- vii) The two extracts for each sample are concentrated to 15 mL under a gentle stream of nitrogen and heat (40°C) and the second extract is transferred quantitatively to the first extract. This extract (added 2 mL isooctane as keeper) is then concentrated to 2 mL under a gentle stream of nitrogen and transferred quantitatively to a 5 mL volumetric flask.
- viii) QS with n-pentane:dichloromethane (90:10, v/v) and store in amber glass vials at -20°C until analysis.

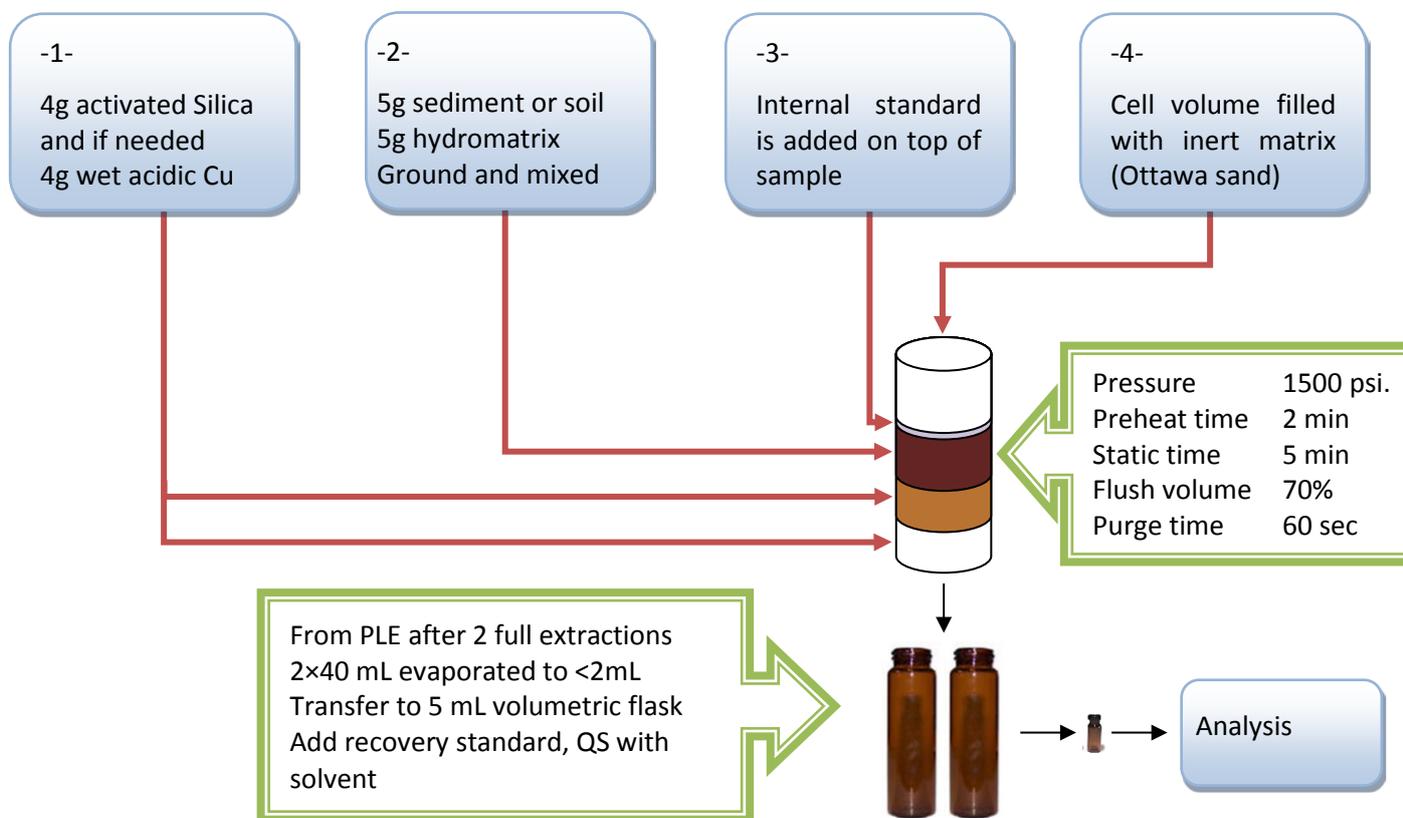


Figure 4. Scheme of the PLE extraction cell packing and extraction parameters.

2.2.4 Extraction and cleanup of oil from water and oil/water emulsions

2.2.4.1 USEPA method 3510C/8270D

Referring to USEPA method 3510C/8270D, organic compounds can be extracted in a simple manner by three successive liquid/liquid extractions in dichloromethane. For TPH analysis measures should be taken to reach oil concentrations of 2-2000 mg/L in the final dichloromethane extract. Adding 10 mL dichloromethane to 1 liter of water is appropriate. Internal standard is added to the water, before adding dichloromethane.

The extracts obtained from the liquid/liquid extraction are filtered to remove particles by use of a glass funnel with filter paper directly into a 250 mL round bottom flasks. 1 mL of isooctane is added to the combined extract as keeper and the combined extracts are evaporated slowly (maximum 5 mL/min) to approximately 1 mL on a rotary evaporator.



Chromatographic column cleanup of water extracts for GC analysis

1. A small glass column (1.4 id × 7 cm) is prepared per extract (Figure 2). Each column is dry packed from its base with a glass filter, 1 gram of activated silica (180°C for 20 hours) and 0.2 cm of anhydrous sodium sulfate. The column is covered with alumina foil until use.
2. The column is conditioned with 5 mL of *n*-hexane.
3. Before the column runs dry, the extract is transferred quantitatively to the top of the column using a Pasteur pipette. Then ½ mL of *n*-hexane is transferred to the flask using a clean pipette and the first pipette is used to wash the flask and transfer the wash to the top of the column. The wash procedure is repeated three times to obtain a quantitative transfer of the extract.
4. The column is washed with 4 mL of *n*-hexane, which is discarded.
5. The column is subsequently eluted with 4 mL of a 1:1 mixture of *n*-hexane:dichloromethane into a 5 mL volumetric flask.
6. Recovery standard is added.
7. The volume of the flask is adjusted to 5 mL with *n*-hexane:dichloromethane (1:1), and stored in amber glass vials at -20 °C until analysis.

2.2.4.2 Alternative water extractions

Pentane as organic phase and increasing ionic strength of water-phase

Oil in water is extracted as per the USEPA method 3510C/8270D, however, salt is added to the water. This increases the ionic strength of the water and thereby more strongly force analytes into the organic phase. Adding pentane instead of dichloromethane gives an organic phase in the top instead of in the bottom of the two-layer system. The procedure is otherwise the same as the above described.

Extraction with solid phase extraction

Oil in water can be extracted using solid phase extraction. We have successfully used Waters HLB columns 6cc, 500 mg sorbent for extracting PAHs from samples up to 500 mL. The SPE cartridges are preconditioned by washing 3 times with 3 mL methanol and flushing 3 times with 5mL deionized water. Water (added internal standard in methanol) is extracted with a flow of approximately 10 mL per minute. After extraction, PAHs are eluted 3 times with 3mL of methanol. The combined eluate is then concentrated to < 5 mL under a gentle stream of nitrogen. The extract is added recovery standard (in methanol) and adjusted to 5 mL.



3 GC-MS method for PAC and biomarker analysis

3.1 Introduction

GC-MS analysis is performed to assess the relative quantity of PACs and biomarkers in oil, water and sediment samples. The method is sensitive and can be used for full quantification of single PACs in the concentration range from 2-2,500 µg/kg (by addition of appropriate internal and recovery standards, see 0) as well as for diagnostic purposes using semi-quantitative approaches.

3.2 Sample preparation

Samples should be prepared according to sample preparation protocol, and preferably be in n-pentane:dichloromethane (90:10, v/v), alternatively in another water-free low boiling point solvent (e.g dichloromethane, pentane or hexane).

3.3 Standards

3.3.1 GC-MS analysis for PAC quantification and diagnostic ratio calculations

- 1) Quantification of 19 parent PAHs including the 16 US-EPA PAHs
 - a) Exhaustive list of PAHs with authentic standards: Naphthalene, acenaphthene, acenaphthylene, dibenzothiophene, anthracene, phenanthrene, fluorene, fluoranthene, pyrene, chrysene, benzo(a)anthracene, benzo(a)pyrene, perylene, benzo(g,h,i)perylene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, benzo(e)pyrene, benzo(b)fluoranthene and indeno(1,2,3-c,d)pyrene.
 - b) Single PAH concentration range: 2.5 – 2000 µg/kg sample
- 2) 11 point standard curves are made (see excel sheet): 3-6-12-25-50-100-300-500-1000-1500-2000 ng/mL; Internal standards (5 – 200 ng/mL); and Recovery standards (150 ng/mL). **Naphthalene-d8, acenaphthene-d10, fluorene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo(k)fluoranthene-d12 and benzo(g,h,i)perylene-d12** are used as internal standards, while **acenaphthylene-d8, anthracene-d10, fluoranthene-d10, benz(a)anthracene-d12, benzo(a)pyrene-d12 and indeno(1,2,3-c,d)pyrene-d12** are used as recovery standards.

3.3.1.1 Preparation of standards

- 1) Prepare Quantification stock solution (here labelled **RED1**): consisting of 19 PAHs. The stock solution contain approximately 4 µg/mL of each individual PAH (see excel preparation sheet)
- 2) Prepare a 10 time dilution of the Quantification stock solution - Label **RED2**: The solution contain approximately 0.4 µg/mL of each individual PAH (see excel preparation sheet)
- 3) Prepare internal stock solution: Naphthalene-d8, acenaphthene-d10, fluorene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo(k)fluoranthene-d12 and benzo(g,h,i)perylene-d12: Label **RED3**: 8 µg/mL (see excel preparation sheet)
- 4) Prepare recovery stock solution: acenaphthylene-d8, anthracene-d10, fluoranthene-d10, benz(a)anthracene-d12, benzo(a)pyrene-d12 and indeno(1,2,3-c,d)pyrene-d12: Label **RED4**: Approx. 8 µg/mL (see excel preparation sheet)
- 5) Prepare the 11 standard solutions from the four **Red** solutions: **RED1, RED2, RED3** and **RED4** using the "Preparation of Quant std" excel sheet (see excel preparation sheet). The solutions are prepared in pre-cleaned 5 mL volumetric flasks, transferred to 8 mL amber glass vials, and stored in the freezer at -20 °C until use. See Table 2 for an overview of all standards.



Table 2. List of the most appropriate internal and recovery standards for the 19 PAHs

Internal standard	PAHs	Recovery standard
Naphthalene-d8	Naphthalene, Acenaphthylene	Acenaphthylene-d8
Acenaphthene-d10	Acenaphthene	Anthracene-d10
Fluorene-d10	Fluorene	Anthracene-d10
Dibenzothiophene-d8	Dibenzothiophene	Anthracene-d10
Phenanthrene-d10	Anthracene, phenanthrene	Anthracene-d10
Pyrene-d10	Pyrene, fluoranthene	Fluoranthene-d10
Chrysene-d12	Chrysene, benzo(a)anthracene	Benzo(a)anthracene-d12
Benzo(k)fluoranthene-d12	Benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene	Benzo(a)pyrene-d12
Benzo(g,h,i)perylene-d12	Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene, indeno(1,2,3-c,d)pyrene	Indeno(1,2,3-c,d)pyrene-d12

3.3.2 Biomarker quantification (GC-MS)

C₃₀- α,β -hopane is quantified in oil samples where C₃₀- α,α -hopane is used as an internal standard.

3.3.2.1 Preparation of standards

Five-point calibration curve in desired concentration levels is constructed with varying concentrations of C₃₀- α,β -hopane and constant concentrations of the internal standard C₃₀- α,α -hopane.

3.4 Instrument settings and equipment

Tuning and performance of the GC-MS system is monitored through daily analyses of a standard tuning sample (following instructions in EPA method 8270D). The tuning sample comprise 50 ng of each of DFTPP, DDT, benzidine and pentachlorophenol in DCM. Tuning and performance samples can be purchased at e.g. <http://www.accustandard.com> (M-TS-625 or M-TS-625-20X).

3.4.1.1 Tuning

The mass spectrometer should be able to produce the mass spectrum for the compound DFTPP that meets the criteria of Table 3 where 1 μ l of the standard GC-MS tuning sample is injected.

Table 3. DFTPP key ions and ion abundance criteria. (USEPA 1998) See USEPA method 8270D, t3 for DFTPP acceptance criteria.

Mass	Ion abundance criteria
51	10-80% of base peak
68	<2% of mass 69
70	<2% of mass 69
127	10-80% of base peak
197	<1% of mass 198
198	base peak, or >50% of mass 442
199	5-9% of mass 198



Mass	Ion abundance criteria
275	10-60% of base peak
365	>1% of mass 198
441	Present but <24% of mass 443
442	base peak, or >50% of mass 198
443	15-24% of mass 442

3.4.1.2 Performance

Inlet liner and column performance is monitored with DDT, benzidine and pentachlorophenol. Benzidine and pentachlorophenol should be present at their normal responses (based on the experience of the laboratory) and should not show peak tailing (peak deterioration will reveal the presence of active sites in the liner). The degradation of DDT to DDE and DDD should not be greater than 20%. Figure 5 illustrates a chromatogram injected to a GC-MS operating in ideal conditions (a) and a chromatogram of a sample run with GC-MS in bad conditions (b). Table 4 shows the key ions of compounds that are used for monitoring the chromatographic conditions.

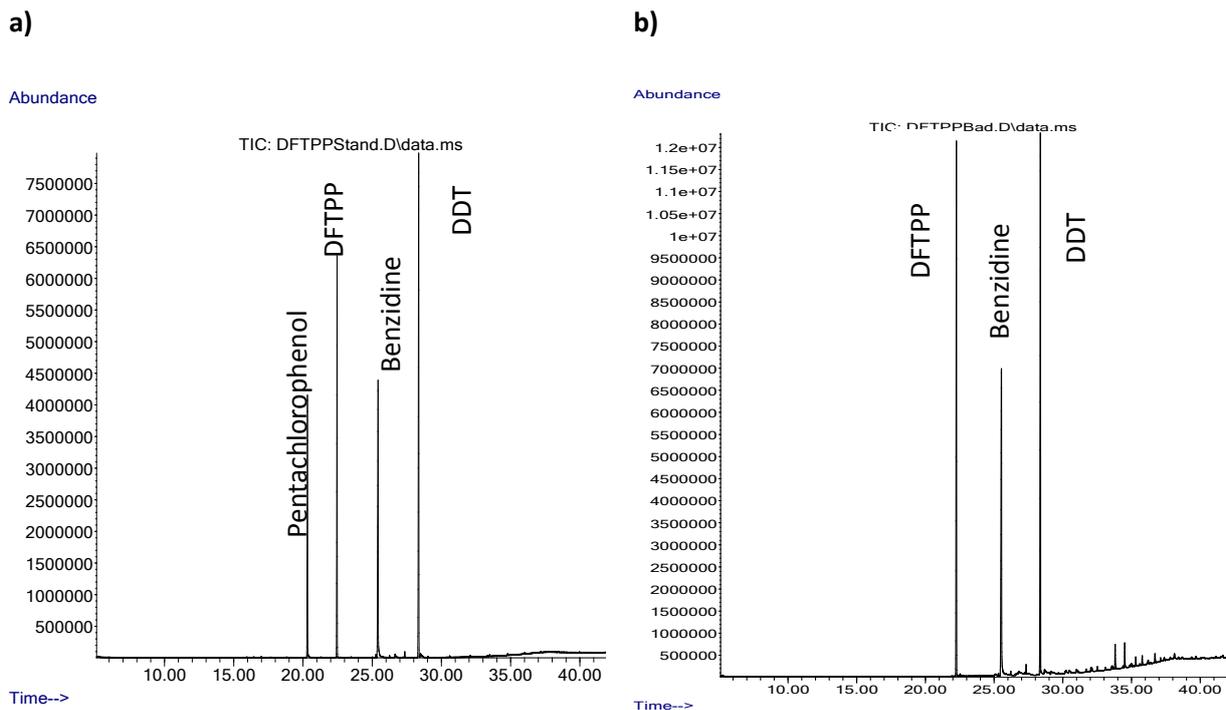


Figure 5. Chromatograms of injections of a mix of DFTPP, Pentachlorophenol, Benzidine and DDT from a GC-MS operating in ideal conditions (a) and a GC-MS in bad conditions (b).

Table 4. Ions of the key compounds responsible for the control of chromatographic conditions.

Compounds	Primary ion	Secondary ions
Benzidine	184	92, 185
Pentachlorophenol	266	264, 268
4,4'-DDT	235	237, 165
4,4'-DDE	246	148, 176
4,4'-DDD	235	237, 165



After injection of many samples, the peaks of Benzidine and Pentachlorophenol may show tailing or disappear completely. The Benzidine is a reactive compound and its peak will tail if the liner is not inert. The DTT will decompose in the injector, if this contains any reactive sites (e.g. silanol interactions).

If these symptoms occur, replace the injector liner and cut about 15-30 cm of the start of the column. At the same time, the injector septum can be changed. While the injector is reheated to the original temperature, set the temperature to 10°C below maximum temperature of the column and hold it for 5 minutes in order to remove residual contaminants.

3.4.1.3 Reference materials and standards

Quantification of analytes should meet recommendations associated with standard reference materials such as NIST SRM 1582 (Crude oil) and NIST SRM 1941 (Organics in sediment).

3.4.1.4 Inlet

1µL injected in pulsed splitless mode, injector temperature: 315°C

3.4.1.5 Column

60 m ZB-5 (0.25 mm ID, 0.25 µm film thickness) capillary column. Helium should be used as carrier gas at 1.1 mL/s. Initial temperature: 40°C hold for 2 min, 25°C/min to 100°C, and 5°C/min to 315 (hold for 13.4 min), total analysis time 60.8 min. Transfer line should be 315°C.

3.4.1.6 MS

Ion source and quadropole temperatures should be 230°C and 150°C respectively. The ion source should be operating in electron ionization mode. For PAC and diagnostic ratio analysis, twelve groups of ions with 13 ions in each should be monitored in SIM mode, with a dwell time of 25 ms, giving 2.81 scans/s. Number of ions should be consistent between groups, to avoid differences in scanning frequency, see Table 5 for ion and group details.



Table 5. List of compounds, SICs and corresponding groups of GC-MS/SIM as described by (Gallotta and Christensen 2012)

nCx	Group number												n-alkane (RT of n-alkane to be used for group division)
	1	2	3	4	5	6	7	8	9	10	11	12	
SIC	0	13	14	15	16	18	19	21	23	24	27	32	Compound name
83	x	x	x	x	x	x	x	x	x	x	x	x	n-Alkyl cyclo hexanes
85	x	x	x	x	x	x	x	x	x	x	x	x	Alkanes
105	x	x	x	x	x	x	x	x	x	x	x	x	Alkyl toluenes
123	x	x	x	x	x	x							Sesquiterpanes
128	x												Naphthalene
134	x												Benzo(b)thiophene
136	x												d8-Naphthalene
138	x												Decalin
142		x											C ₁ -Naphthalenes
148	x	x											C ₁ -Benzo(b)thiophenes
152	x	x	x										C ₁ -Decalins, Acenaphthylene
154		x	x	x									Acenaphthene
156			x										C ₂ -Naphthalenes
160			x										d8-Acenaphthylene
162		x	x										C ₂ -Benzo(b)thiophenes
164			x	x									d10-Acenaphthene
166	x	x			x								C ₂ -Decalins, Fluorene
168		x	x	x									Dibenzofuran
170				x	x								C ₃ -Naphthalenes
176				x	x								C ₃ -Benzo(b)thiophenes, d10-Fluorene
178						x							Phenanthrene, Anthracene
180	x	x	x		x								C ₃ -Decalins, C ₁ -Fluorenes
182				x	x	x							C ₁ -Dibenzofurans
184				x	x	x							C ₄ -Naphthalenes, Dibenzothiophene
188						x							d10-Phenanthrene, d10- Anthracene
190				x	x								C ₄ -Benzo(b)thiophenes
191								x	x	x	x		Tricyclic terpanes, Hopanes
192					x	x	x						C ₁ -Phenanthrenes/anthracenes d8-Dibenzothiophene
194	x	x	x	x		x	x						C ₄ -Decalins, C ₂ -fluorenes
196					x	x	x						C ₂ -Dibenzofurans
198						x	x						C ₁ -Dibenzothiophenes



nCx	Group number												n-alkane (RT of n-alkane to be used for group division)
	1	2	3	4	5	6	7	8	9	10	11	12	
	0	13	14	15	16	18	19	21	23	24	27	32	
202							x	x	x				Fluoranthene, Pyrene
206							x	x	x				C ₂ -Phenanthrenes/anthracenes
208						x	x						C ₃ -Fluorenes
212							x	x					C ₂ -Dibenzothiophenes, d10-Fluoranthene, d10-Pyrene
216								x	x				C ₁ -Fluoranthenes/pyrenes
217								x	x	x	x	x	Steranes
218										x	x	x	Steranes
220							x	x	x				C ₃ -Phenanthrenes/anthracenes
226							x	x	x				C ₃ -Dibenzothiophenes
228										x			Benzo(a)anthracene, Chrysene
230									x	x			C ₂ -Fluoranthenes/pyrenes
231										x	x	x	Triaromatic steranes
234								x	x	x			C ₄ -Phenanthrenes/anthracenes Retene, Benzonaphthothiophene
240								x	x	x			C ₄ -Dibenzothiophenes, d12-Benzo(a)anthracene, d12-Chrysene
242										x	x		C ₁ -Chrysenes
244								x					d14-p-Terphenyl
248										x	x		C ₁ -Benzonaphthothiophenes
252											x	x	5 Rings PAHs
256											x		C ₂ -Chrysenes
264											x	x	d12-Benzo(k)fluoranthene, d12-Benzo(a)pyrene, d12-Perylene
270											x	x	C ₃ -Chrysenes
276												x	6 Rings PAHs
278												x	6 Rings PAHs
288												x	d12-Indeno(1,2,3-cd)pyrene, d12-Benzo(g,h,i)perylene
Total	13	13	13	13	13	13	13	13	13	13	13	13	



3.4.1.7

3.4.1.8 GC-MS sequence

This section describes the samples that are included in the GC-MS analysis of one extraction batch. The samples include those from the PLE extraction as well as quantification standards and quality control samples to control instrumental, procedural and other variations.

- 2) The sequence for analysis of each extraction batch (See extraction protocol) can include
 - a) Instrument quality control sample (DFTPP mix). Analyzed as first and last sample in the sequence.
 - b) Two procedure blank samples (Quality control, limit of detection (LOD) and limit of quantification (LOQ))
 - c) Reference samples
 - d) Sample extracts
 - e) Duplicate of one of the sample extracts
 - f) Quantification standards (1-11). Standard6 (0.1 µg/mL) is analyzed in-between every five extracts from the sample extraction batch
 - g) Total petroleum hydrocarbon mixture (TPH) (500 ng/mL for each individual compound) is analyzed in-between every five extracts from the sample extraction batch. The mixture is used for quality control during the analysis in order to detect discrimination in the inlet liner (the relative ratios between alkanes should remain constant in the RT window relevant for the analytes of interest)
 - h) Solvents. These samples are analyzed in-between every five extracts from the sample extraction batch and used for quality control (system contamination)
 - i) An example of a sequence: 1: DFTPP, 2: DCM, 3: Standard6, 4: TPH, 5: Extract2, 6: Blank1, 7: Extract8, 8: Extract3, 9: Standard1, 10: DCM, 11: Standard6, 12: TPH, 13: Standard6, 14: ReferenceMaterial, 15: Extract2, 16: Standard8, 17: Standard11, 18: DCM, 19: Standard6, 20: TPH, etc., etc., etc., final: DFTPP.

3.5 GC-MS data analysis

3.5.1 Peak area quantification

1. The relevant peaks are integrated in ChemStation, standard curves and quantifications are made in Microsoft Excel, see Excel-Template.
2. The concentrations of PAHs in extracts are calculated batch-wise (one batch at a time) using the internal standard approach and the replicate analysis of one specific standard solution (standard6)
3. The internal standard method is used and the deuterated PAHs added directly to the sample are used as internal standards. A list of the most appropriate internal standards for quantification of the 19 PAHs are listed in table 1.

3.5.1.1 Total weathering degree data analysis

The total weathering degree is calculated as the ratio of biomarker concentration in the source oil relative to that of the weathered oil.

$$\text{Equation 1. } P(\%) = \left(1 - \frac{C_{\text{Hopane in source oil}}}{C_{\text{Hopane in weathered oil}}}\right) \cdot 100\%$$



3.5.1.2 Chemical fingerprints

The relevant peaks are integrated in ChemStation, and the results are exported to excel. To aid for the identification of peaks and groups of peaks, please refer to the CEN guideline (CEN/TR 2012).

Alkane fingerprints

Semiquantitative results of alkanes are reported as areas (A) of alkanes normalized to the area of a sufficiently high-recovered high boiling point compound that is robust to weathering. In some cases linear alkanes, such as nC₂₃ will be sufficient, but in cases of severe weathering, compounds proven robust to weathering such as C₃₀-α,β-hopane should be used. All n-alkanes-areas are normalized to the areas of the selected normalization compound, and the result can hereafter be normalized to the same ratio in the original oil, whereby percent removal from the original oil is calculated (see Equation 2).

$$\text{Equation 2. } \frac{A_{nC_x(\text{weathered sample})} / A_{nC_{23}(\text{weathered sample})}}{A_{nC_x(\text{unweathered sample})} / A_{nC_{23}(\text{unweathered sample})}} \cdot 100\% =$$

% left related to unweathered oil

Equation 2 will, when calculated for all alkanes in the sample give rise to the PW-plot, as seen in Figure 6.

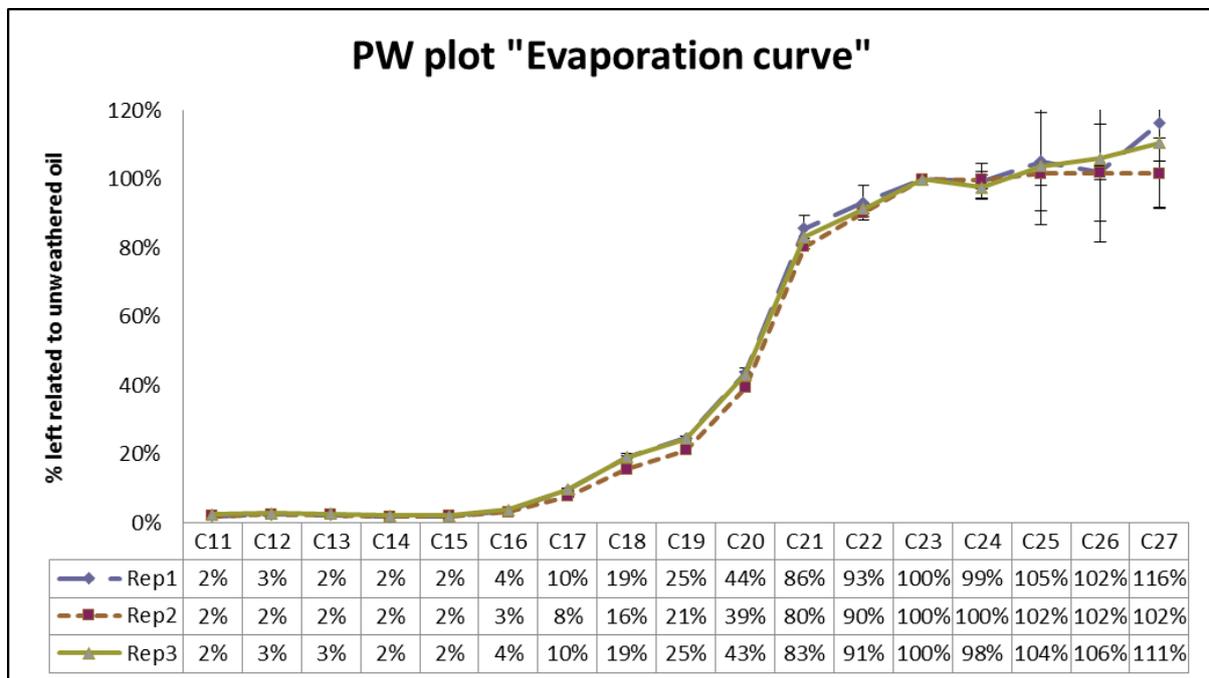


Figure 6. PW-plot. Example of relative changes in composition of n-alkanes in soil extracts compared to original oil after 1 year of weathering.

The PW-plot is sometimes referred to as an “evaporation curve”, however this calculation cannot distinguish between evaporation and biodegradation, since these processes will have the same effect on the alkanes (the shorter the alkane, the faster the removal). Therefore, the PW plot shows the combined effects of evaporation and biodegradation.



PAC fingerprints

Corresponding to the PW-plot, PAC removal can be assessed by choosing appropriate normalization compounds. As an example, biodegradation efficiency of a crude oil can be assessed by normalizing content of individual PACs to the sum of C₃- and C₄-dibenzothiophenes (DBT, m/z 226 and 240). Due to the high molecular weight and high complexity of C₃- and C₄-DBTs have high boiling point and are relatively recalcitrant to biodegradation. They can therefore serve as internal markers for PAC removal as a combined function of biodegradation and short-term physical processes, such as evaporation and dissolution. Likewise, other HMW PACs, such as C₃- and C₄-chrysenes can be used as internal standards. Example plots of PAC fingerprinting ratios are seen in Figure 7 (mono- di and tri-aromatic PAHs) and Figure 8 (di- and tricyclic sulfur containing PACs).

$$\text{Equation 3. } \frac{A_{PAC(\text{weathered sample})} / \sum(A_{C_3DBT} + A_{C_4DBT})_{\text{weathered sample}}}{A_{PAC(\text{unweathered sample})} / \sum(A_{C_3DBT} + A_{C_4DBT})_{\text{unweathered sample}}} \cdot 100\% =$$

% left related to unweathered oil

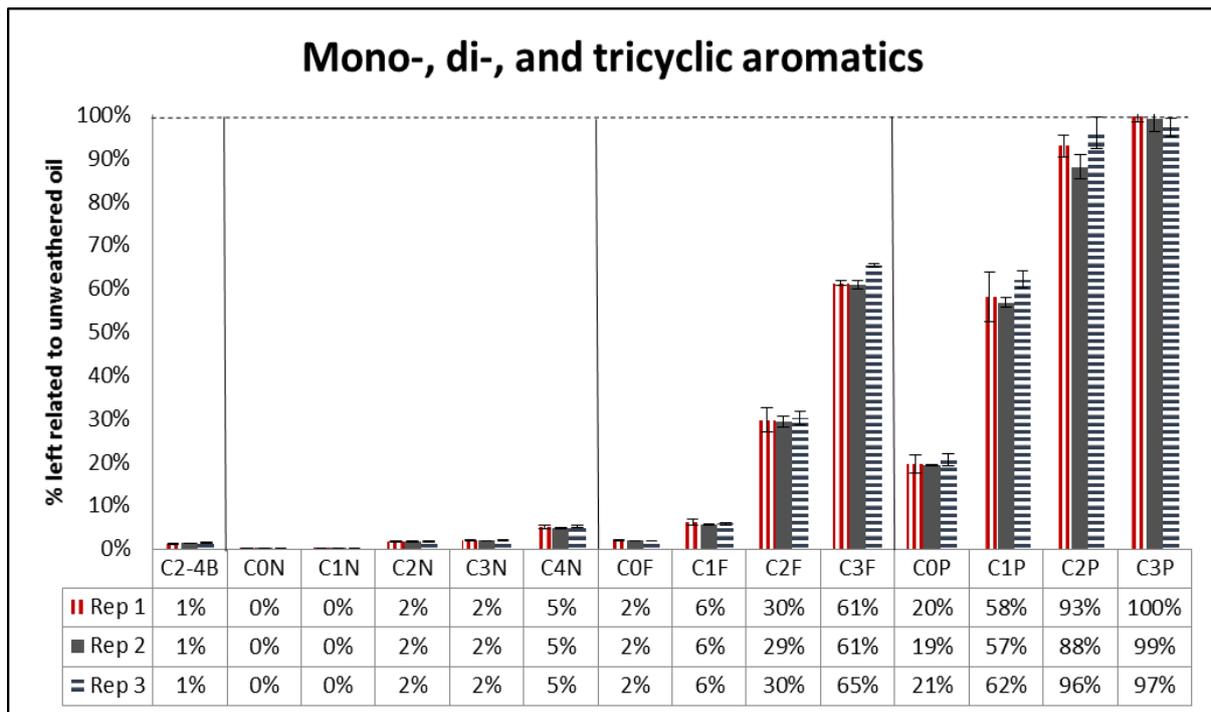


Figure 7. Example of relative changes in composition of mono-, di- and tri cyclic aromatic compounds in soil extracts compared to original oil after 1 year of weathering.

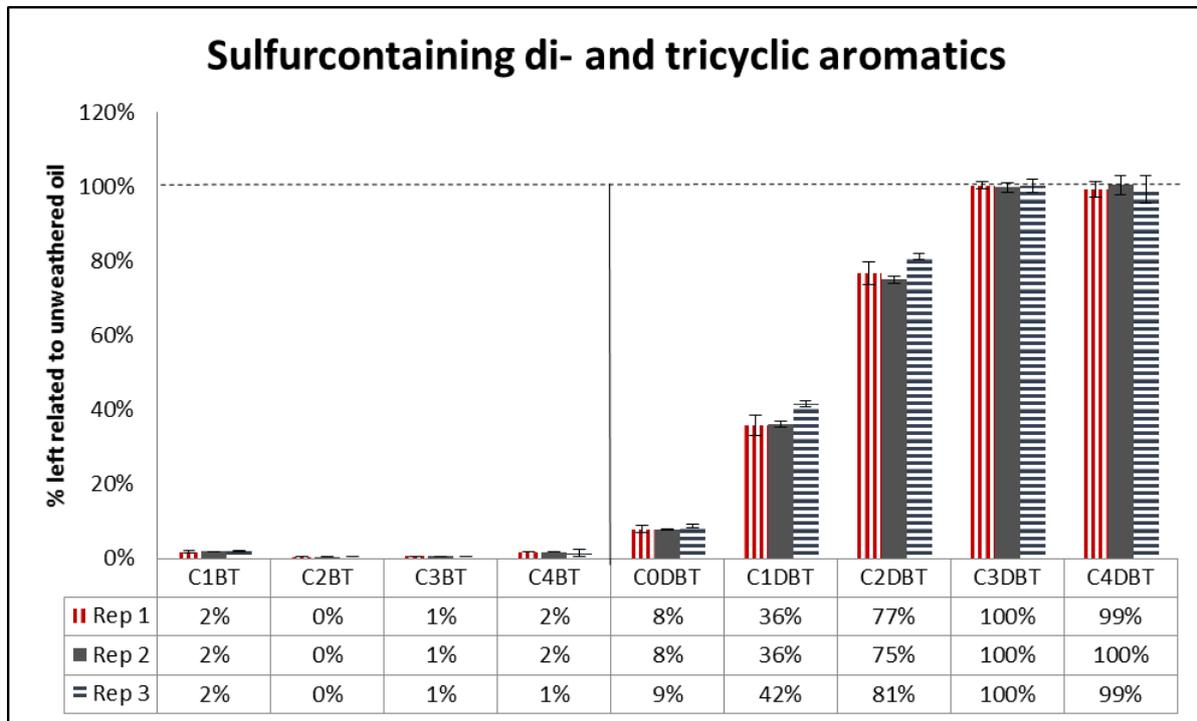


Figure 8. Example of relative changes in composition of sulfur containing di- and tricyclic aromatics in soil extracts compared to original oil after 1 year of weathering.

Diagnostic ratios

Certain weathering processes involve highly selective chemical reactions, and therefore large differences in degradation of closely related compounds can appear. This is apparent for photo oxidation and bacterial degradation of certain alkylated PAHs. Based on empirical evaluations and understanding of the reactions, several relationships have been constructed to distinguish between short-term physical weathering processes (evaporation and dissolution), photo oxidation and bacterial degradation. The relationships result in semiquantitative diagnostic ratios (DRs) that can indicate the progress of the individual weathering processes. Table 6 lists DRs for different processes, again with references to the CEN guideline for identification help (CEN/TR 2012), and Figure 9 shows a plot of selected DRs for the description of weathering of oil in a contaminated soil.

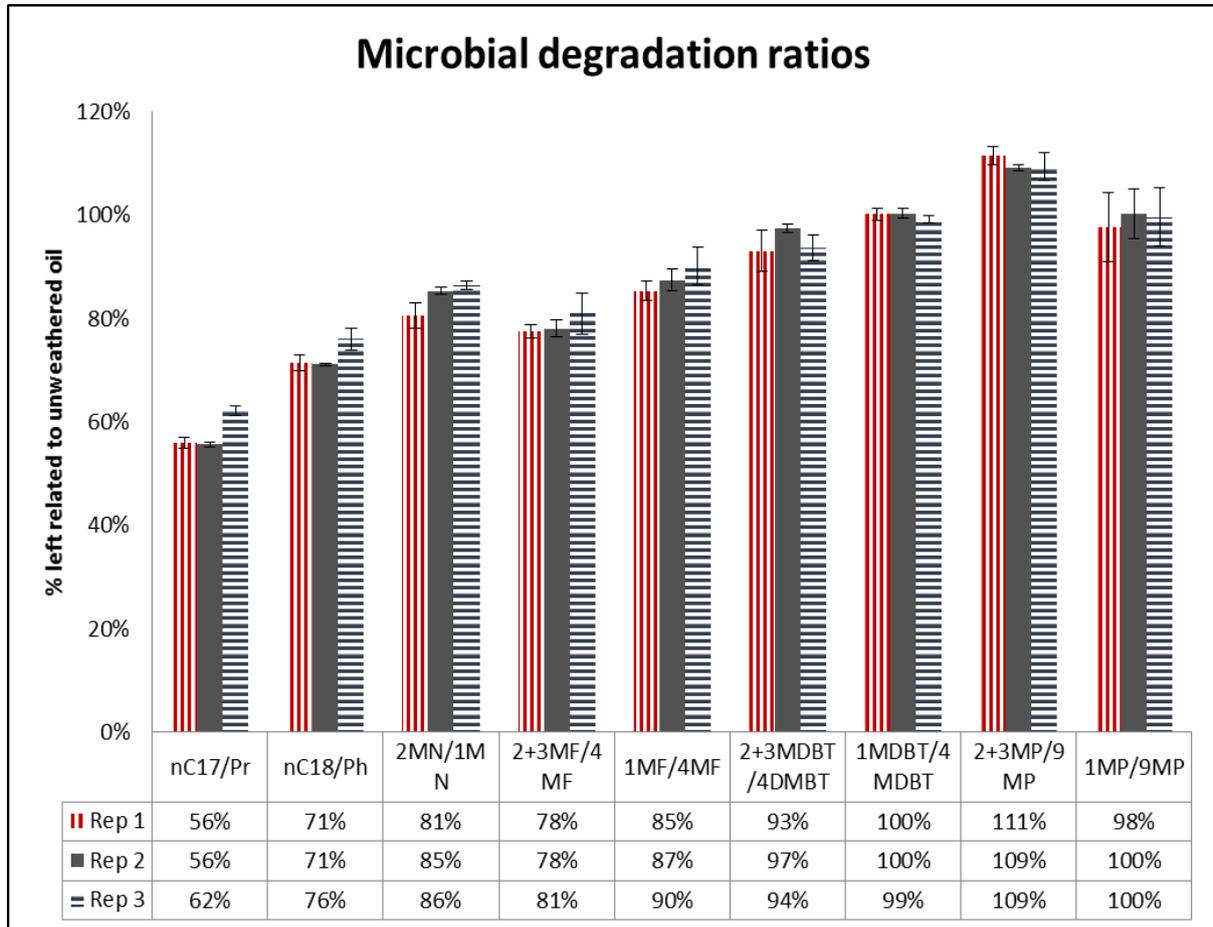


Figure 9. Employment of diagnostic ratios to show the degree of weathering of an oil after one year.



Table 6. Biodegradation diagnostic ratios (DRs) of selected petroleum hydrocarbons.

Diagnostic ratio	Process	Degradation severity	Reference
nC ₁₆ /norpristane	Biodegradation	Initial	
nC ₁₇ /pristane	Biodegradation	Initial	
nC ₁₈ /phytane	Biodegradation	Initial	
Pristane/phytane	Biodegradation	Initial	
2mN/1mN	Biodegradation	Initial	
1,3+1,6dmN/C ₂ N	Biodegradation	Initial	(Wang, Fingas et al. 1998)
2,6+2,7dmN/C ₂ N	Biodegradation	Initial	(Bayona, Albaiges et al. 1986)
2+3mF/4mF	Biodegradation	Initial	(Christensen, Hansen et al. 2005a)
2+3mF/1+2+3+4mF	Biodegradation	Initial	(Christensen, Hansen et al. 2005a)
1mPy/2mPy	Photooxidation	Initial	(CEN/TR 2012)
2+3mDBT/4mDBT	Biodegradation	Moderate	(Wang, Fingas et al. 1998)
1mDBT/4mDBT	Biodegradation	Moderate	(Lamberts, Johnsen et al. 2008)
1mPhe/9+4mPhe	Biodegradation	Moderate	(Wang, Fingas et al. 1998)
2+3mPhe/1+9+4mPhe	Biodegradation	Moderate	(Wang, Fingas et al. 1998)
2,4+2,6+3,7+1,3C ₂ DBT/4,6+3,6+1,4+1,6+1,8C ₂ DBT	Biodegradation	Moderate	(Douglas, Bence et al. 1996)
C ₂ DBT/C ₂ DBT+C ₂ Phe	Biodegradation	Moderate	(Douglas, Bence et al. 1996)
C ₃ DBT/C ₃ DBT+C ₃ Phe	Biodegradation	Moderate	(Douglas, Bence et al. 1996)
C ₂ DBT/C ₂ DBT+C ₂ Chry	Biodegradation	Moderate	(Douglas, Bence et al. 1996)
C ₃ DBT/C ₃ DBT+C ₃ Chry	Biodegradation	Moderate	(Douglas, Bence et al. 1996)
C ₂ Chry/C ₂ Chry+C ₂ Py	Biodegradation	High	(Douglas, Bence et al. 1996)
C ₃ Chry/C ₃ Chry+C ₃ Py	Biodegradation	High	(Douglas, Bence et al. 1996)
2mChry/1mChry	Biodegradation	High	(Bayona, Albaiges et al. 1986, Diez, Sabate et al. 2005)
2mPy/1mPy+4mPy	Biodegradation	High	(Bayona, Albaiges et al. 1986, Diez, Sabate et al. 2005)



4 **References**

- Bayona, J. M., J. Albaiges, A. M. Solanas, R. Pares, P. Garrigues and M. Ewald (1986). "Selective Aerobic Degradation of Methyl-Substituted Polycyclic Aromatic-Hydrocarbons in Petroleum by Pure Microbial Cultures." International Journal of Environmental Analytical Chemistry **23**(4): 289-303.
- Boll, E. S., J. H. Christensen and P. E. Holm (2008). "Quantification and source identification of polycyclic aromatic hydrocarbons in sediment, soil, and water spinach from Hanoi, Vietnam." Journal of Environmental Monitoring **10**(2): 261-269.
- CEN/TR (2012). CEN/TR 15522-2:2012 Oil spill identification - Waterborne petroleum and petroleum products - Part 2: Analytical methodology and interpretation of results based on GC-FID and GC-MS low resolution analyses, European Committee for Standardization.
- Christensen, J. H., A. B. Hansen, U. Karlson, J. Mortensen and O. Andersen (2005a). "Multivariate statistical methods for evaluating biodegradation of mineral oil." Journal of Chromatography A **1090**(1-2): 133-145.
- Christensen, J. H., A. B. Hansen, J. Mortensen and O. Andersen (2005b). "Characterization and matching of oil samples using fluorescence spectroscopy and parallel factor analysis." Analytical Chemistry **77**(7): 2210-2217.
- Christensen, J. H., G. Tomasi, A. D. Scofield and M. D. G. Meniconi (2010). "A novel approach for characterization of polycyclic aromatic hydrocarbon (PAH) pollution patterns in sediments from Guanabara Bay, Rio de Janeiro, Brazil." Environmental Pollution **158**(10): 3290-3297.
- Diez, S., J. Sabate, M. Vinas, J. M. Bayona, A. M. Solanas and J. Albaiges (2005). "The Prestige oil spill. I. Biodegradation of a heavy fuel oil under simulated conditions." Environmental Toxicology and Chemistry **24**(9): 2203-2217.
- Douglas, G. S., A. E. Bence, R. C. Prince, S. J. McMillen and E. L. Butler (1996). "Environmental Stability of Selected Petroleum Hydrocarbon Source and Weathering Ratios." Environmental Science & Technology **30**(7): 2332-2339.
- Gallotta, F. D. and J. H. Christensen (2012). "Source identification of petroleum hydrocarbons in soil and sediments from Iguacu River Watershed, Paraná, Brazil using the CHEMSIC method (CHEMometric analysis of Selected Ion Chromatograms)." Journal of Chromatography A **1235**: 149-158.
- Lamberts, R. F., A. R. Johnsen, O. Andersen and J. H. Christensen (2008). "Univariate and multivariate characterization of heavy fuel oil weathering and biodegradation in soil." Environmental Pollution **156**(2): 297-305.
- Lundstedt, S., P. Haglund and L. Oberg (2006). "Simultaneous extraction and fractionation of polycyclic aromatic hydrocarbons and their oxygenated derivatives in soil using selective pressurized liquid extraction." Analytical Chemistry **78**(9): 2993-3000.
- Malmquist, L. M. V., R. R. Olsen, A. B. Hansen, O. Andersen and J. H. Christensen (2007). "Assessment of oil weathering by as chromatography-mass spectrometry, time warping and principal component analysis." Journal of Chromatography A **1164**(1-2): 262-270.
- Nielsen, N.-P. V., J. M. Carstensen and J. Smedsgaard (1998). "Aligning of single and multiple wavelength chromatographic profiles for chemometric data analysis using correlation optimised warping." Journal of Chromatography A **805**(1-2): 17-35.
- Nytoft, H. P. and J. A. Bojesen-Koefoed (2001). "17 α ,21 α (H)-hopanes: natural and synthetic." Organic Geochemistry **32**(6): 841-856.
- Soleimani, M., M. Farhodi and J. H. Christensen (2013). "Chemometric assessment of enhanced bioremediation of oil contaminated soils." Journal of Hazardous Materials **254**: 372-381.



Grant Agreement no. 312939

Deliverable D2.6

New isomer-based methods for monitoring of bioremediation

- Tomasi, G., F. van den Berg and C. Andersson (2004). "Correlation optimized warping and dynamic time warping as preprocessing methods for chromatographic data." Journal of Chemometrics **18**(5): 231-241.
- USEPA (1998). METHOD 8270D: SEMIVOLATILE ORGANIC COMPOUNDS BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) SW-846 Ch 4.3.2 Update IVA, United States Environmental Protection Agency.
- Wang, Z. D., M. Fingas, S. Blenkinsopp, G. Sergy, M. Landriault, L. Sigouin, J. Foght, K. Semple and D. W. S. Westlake (1998). "Comparison of oil composition changes due to biodegradation and physical weathering in different oils." Journal of Chromatography A **809**(1-2): 89-107.