Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D2.7

New chemical fingerprinting method for detailed monitoring of changes in >40 groups of alkanes and alkylated PACs

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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 an automated data analysis approach to reach to the assessment of the efficiency of the applied cleanup and remediation technologies. The approach is an integrated multivariate oil hydrocarbon fingerprinting approach using the CHEMSIC method. In this method, GC-MS chromatograms are treated as pixels in images in an automated procedure. First, chromatographic and detection artifacts such as retention time shifts and changes in sensitivity are removed. Data are then scaled and normalized prior to multivariate analysis (e.g., principal component analysis) of the processed chromatograms.

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.

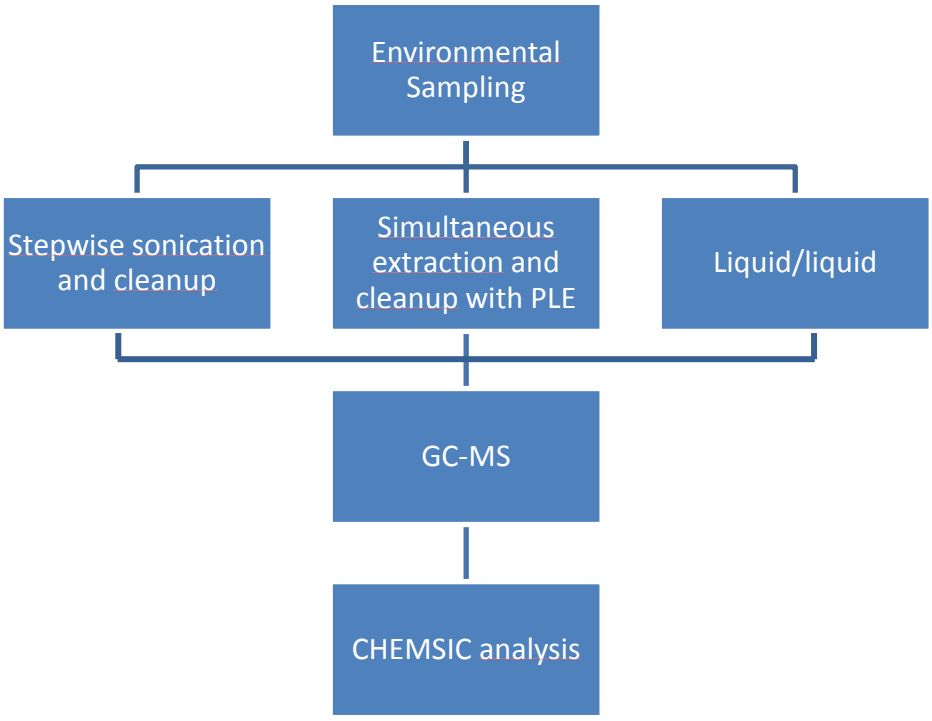

Figure 1. Diagram showing the content of this protocol, covering sample preparation, chemical analysis and data treatment.

2 Introduction

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

Efficient extraction, preconcentration and cleanup are indispensable prior to the determination of PAHs in water, soil and sediments. There exists wide varieties 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;Sporring 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. CHEMSIC is based on image analysis of selected chromatograms relative to the original unaffected oil, and therefore internal standards are not needed. However, the CHEMSIC approach can be utilized for samples containing internal and recovery standards. See deliverable 2.6 in the Kill-Spill products for descriptions of standards.

3 Extraction from sediment

3.1 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 particular 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. dryweight and PAC extraction= 3x(3x5 g+5 g)=60 g.

3.1.1 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.

3.2 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.

3.2.1 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 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

3.2.2 Chromatographic column cleanup of 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 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.

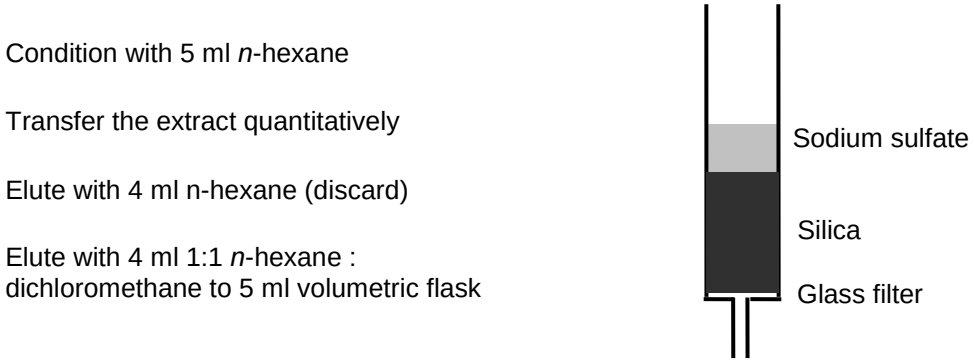

Figure 2. Illustration of the preparation of a small chromatographic column, packed from its base with a glass filter, activated silica and anhydrous sodium sulfate. Also a brief description of the sample cleanup procedure is shown.

3.3 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).

3.3.1 Preparations of extraction components

1) PLE extraction cells

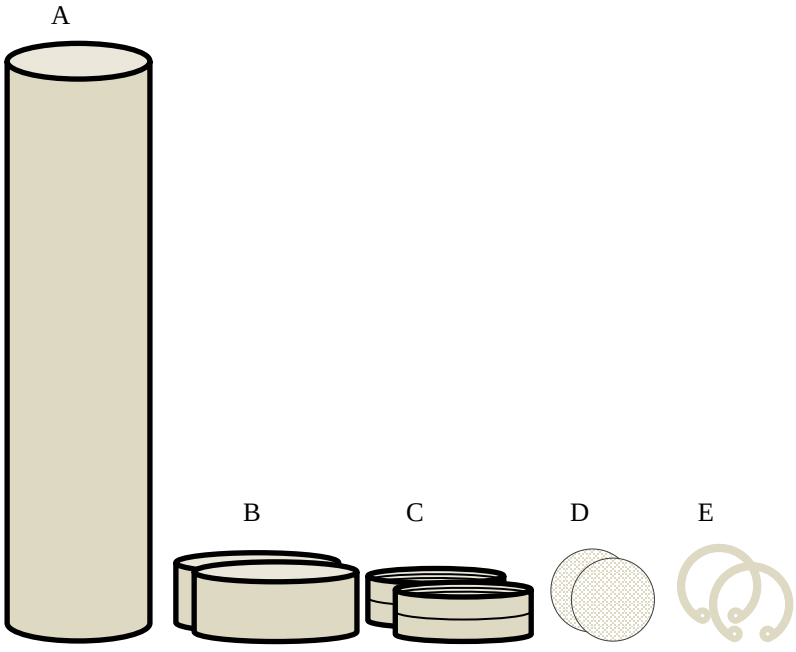

Figure 3. PLE extraction cell components.

a) Separate the extraction cell completely and remove old sample material form 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).

3.3.2 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
      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) Optional 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.

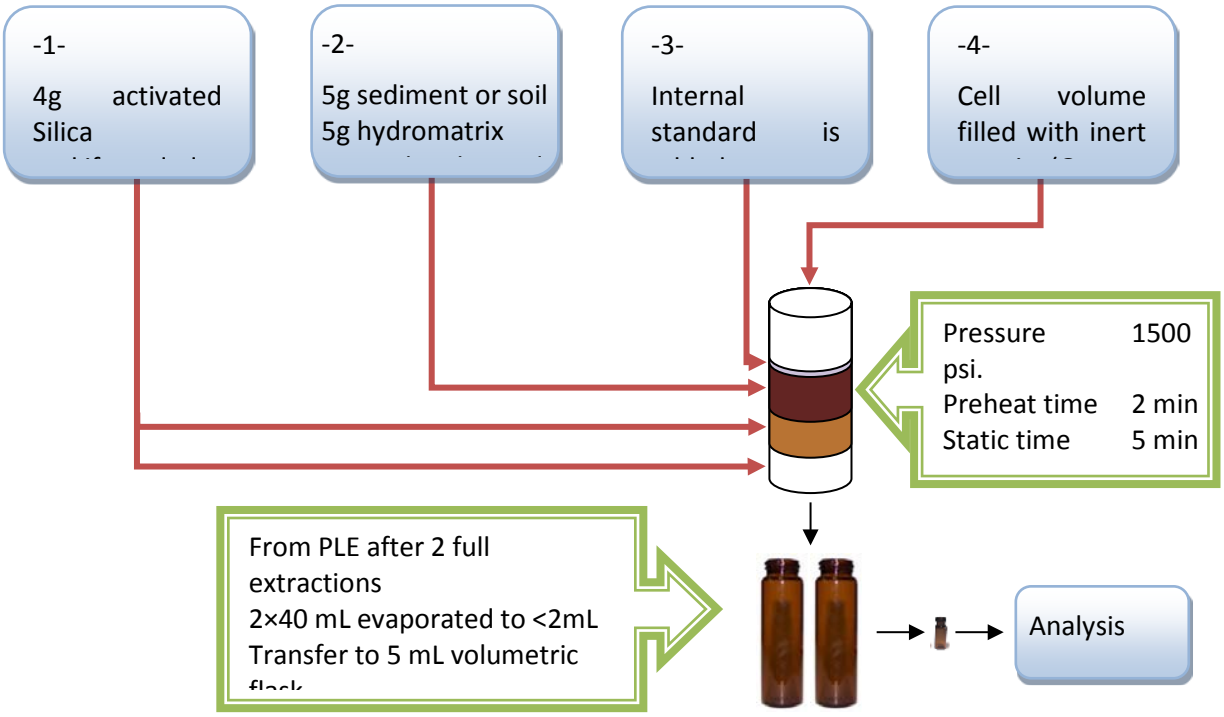

Figure 4. Scheme of the extraction protocol

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

3.4.1 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. Optional 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.

3.4.1.1 Chromatographic column cleanup of water extracts for GC analysis

1. A small glass column (1.4 id × 7 cm) is prepared per extract (See Figure 4). 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. Optional 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.

3.4.2 Alternative water extractions

3.4.2.1 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.

3.4.2.2 Extraction with solid phase extraction

Oil in water can be extracted using solid phase extraction. We have successfully used Waters HLB columns 6cc, 500mg 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 <5ml under a gentle stream of nitrogen. The extract is added recovery standard (in methanol) if needed and adjusted to 5ml.

4 GC-MS method for Petroleum hydrocarbons

4.1 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 **Fehler! Verweisquelle konnte nicht gefunden werden.**) as well as for diagnostic purposes using semi-quantitative approaches.

The CHEMSIC (Chemometric analysis of sections of selected ion chromatograms) data treatment methodology is based on the publication by Christensen, Hansen et al. (2005b) and Gallotta and Christensen (2012), run on an Agilent 6890N/5975 GC-MS.

4.2 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).

4.3 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).

4.3.1.1 Tuning

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

Table 1 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**
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**

4.3.1.2 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%. **Fehler! Verweisquelle konnte nicht gefunden werden.** 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 2 shows the key ions of compounds that are used for monitoring the chromatographic conditions.

a) **b)**

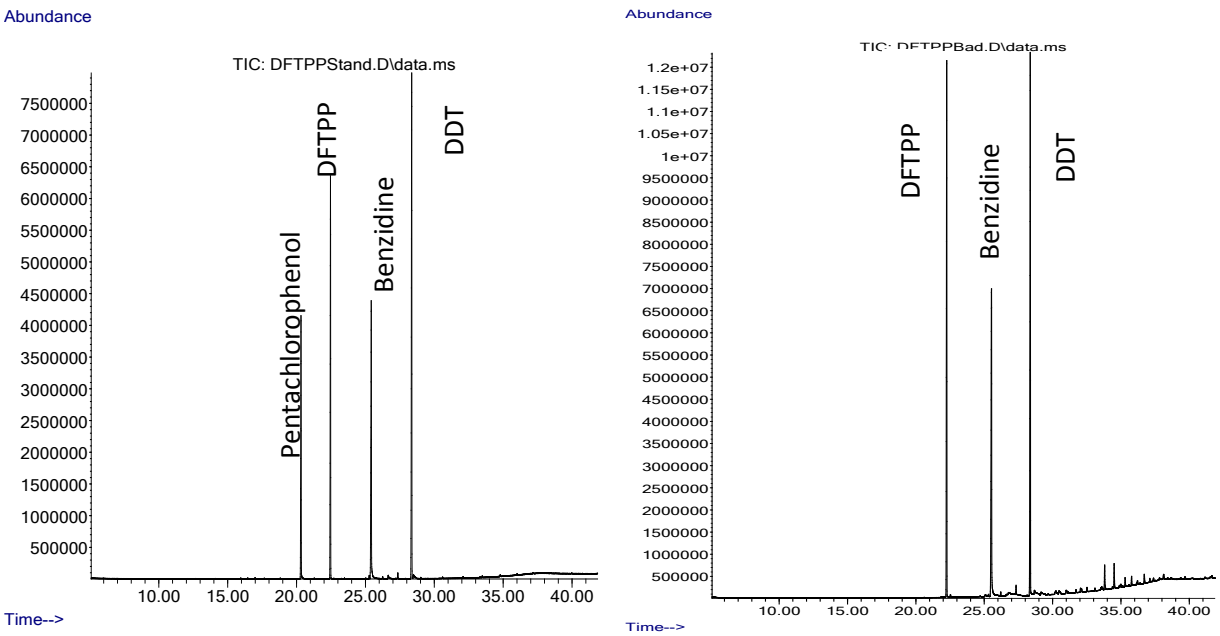

Figure 5. Chromatograms of injections of a mix of DFTPP, Pentaclorophenol, Benzidine and DDT from a GC-MS operating in ideal conditions (a) and a GC-MS in bad conditions (b).

Table 2 Ions of the key compounds responsible for the control of chromatographic conditions.

Compounds	Primary ion	Secondary ions
Benzidine	184	92, 185
Pentaclorophenol	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.

4.3.1.3 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).

4.3.1.4 Inlet

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

4.3.1.5 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.

4.3.1.6 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 0 for ion and group details.

Table 3 List of compounds, SICs and corresponding groups of GC-MS/SIM as described by (Gallotta and Christensen 2012)

	Group number												
	1	2	3	4	5	6	7	8	9	10	11	12	
nCx	0	13	14	15	16	18	19	21	23	24	27	32	n-alkane (RT of n-alkane to be used for group division)
SIC													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

	Group number												
	1	2	3	4	5	6	7	8	9	10	11	12	
nCx	0	13	14	15	16	18	19	21	23	24	27	32	n-alkane (RT of n-alkane to be used for group division)
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
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, Benzonaphtothiophene
240								x	x	x			C_4-Dibenzothiophenes, d12-Benzo(a)anthracene, d12-Chrysene
242										x	x		C_1-Chrysenes

	Group number												
	1	2	3	4	5	6	7	8	9	10	11	12	
nCx	0	13	14	15	16	18	19	21	23	24	27	32	n-alkane (RT of n-alkane to be used for group division)
244								x					d14-p-Terphenyl
248										x	x		C_1-Benzonaphtothiophenes
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**	

4.3.1.7 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. The extended sequence is needed in order to conduct automated chemometric data analysis (CHEMSIC).

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.

5 GC-MS data analysis

5.1 Chemometric analysis of selected ion chromatograms using the CHEMSIC approach

The CHEMSIC approach is based on automated comparison (based on principal component analysis, PCA) of changes in signal at a given retention time across all selected samples (at each selected m/z). It is a requirement that non-sample variation is removed prior to the data analysis. Non-sample variation includes retention time shifts, changes in response (due to e.g. suppression or enhancements in the sample loading or ionization) and baseline changes. In Figure 6, the progress of the data preprocessing is shown on a section of a chromatogram (nC17 to phytane of m/z 85). Especially the effect of retention time alignment and response normalization is visible.

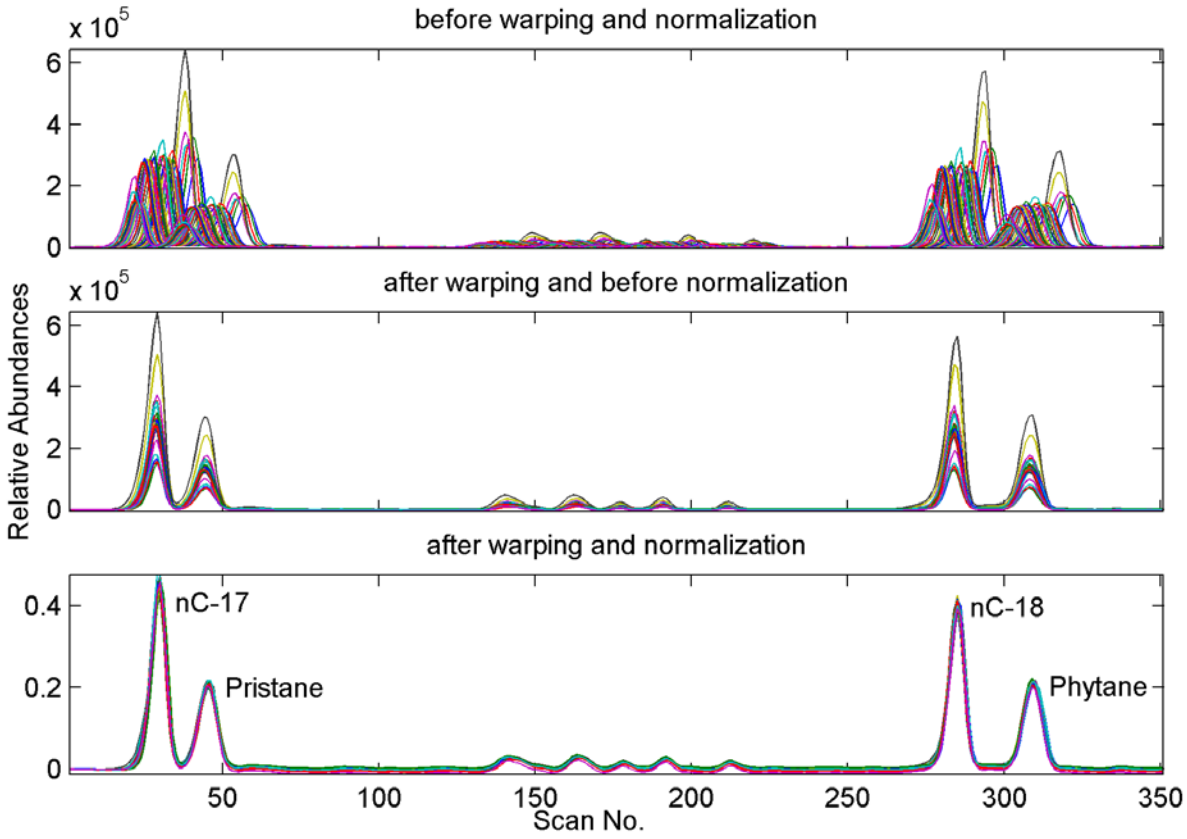

Figure 6. Retention time shifts, warping and normalization of 39 replicate analyses of an oil sample, showing the retention time window from nC17 to phytane of m/z 85 as unprocessed data (top), warped data (middle) and warped and normalised data (bottom).

Briefly, the baseline is removed by calculating the first derivatives of the chromatographic data (point-by-point subtraction). The retention time alignment is performed in two steps: (i) applying rigid shifts (i.e. without compression or expansion) on the chromatograms, and (ii) employing the COW algorithm (Nielsen, Carstensen et al. 1998, Tomasi, van den Berg et al. 2004). The COW algorithm aligns a sample chromatogram towards a target chromatogram by stretching or compressing sample segments along the retention time axis using linear interpolation. The Selected Ion Chromatograms (SICs) for each m/z are aligned separately to the SICs of a target sample. The target for the alignment should be a reference samples (e.g. a mix of all samples in the sample set).

Although baseline removal and retention time alignment are essential steps to prepare the data, the methods involved are not important for the further interpretation of the model. However, data normalisation affects the interpretation of the results and focus of the subsequent data analysis. The aims of the normalization step are to remove variations unrelated to the chemical information such as time related changes in sensitivity; and to focus the subsequent chemometric data analyses on different types of aspects (viz. compound concentrations, differences between groups of compounds (SICs) or differences in relative concentrations within SICs). Three types of normalization can be applied: 1. Normalization to an internal (e.g. deuterated) standard, leading to data analysis where concentration is kept as a variable. 2. Normalization to Euclidian norm (sum of all data points across the chromatogram), leading to data analysis where concentration is taken out as a variable and changes in content of individual isomers and homologue groups are related to each other across samples. 3. In the third normalization method, the second procedure is applied, however here data is normalized to the Euclidian norm within each SIC, and therefore, the PCA will focus solely on chemical variations within each SIC such as differences within homologue isomer patterns and biomarker fingerprints.

5.2 Example using the CHEMSIC approach on oil biodegradation data

5.2.1 Introduction and data description

An example of the entire process is visualized in data from a microbial degradation experiment carried out as a slurry experiment with sediment from a non-contaminated site (1 g), heavy fuel oil (0.1 g) and autoclaved seawater (200 mL). The experiment ran for 70 days, and triplicate samples (separate flasks) were taken at day 0, 3, 7, 14, 21, 35, 42, 49, 63 and 70, together with one negative control sample at each sampling time (autoclaved sediment was used at negative controls).

Sediment and water (as a collected pool) was extracted following the stepwise ultrasonication extraction method, described in section 3.2, and the extracts were run on GC-MS.

The raw data were extracted into Matlab and the signals from trace 85, 216, 230 and 242 (comprising *n*-alkanes, methylpyrenes, dimethylpyrenes and methylchrysenes respectively) of up to 6957 data points were further reduced, to only comprise the areas with peaks of interest corresponding to respectively 319, 232, 246 and 145 data points. The data sets were merged leading to a data set of 40 samples × 942 data points. Since negative control-samples after 63 and 70 days showed effect of biodegradation they were not included in the subsequent PCA, and the data set was reduced to contain 38 samples. For the normalization, the second approach was used, i.e. normalization to the Euclidian norm of the entire merged chromatogram for each sample. The resulting PCA-model (consisting of PC1 and PC2) with the above-mentioned specifications explained in total 96.6% of the variance in the data set.

5.2.2 Results and discussion

The results are visualized as a loading plot (Figure 7) and a score plot (Figure 8). With the chromatograms as loadings, we can interpret the differences in the scores as contributions of each peak with high positive or negative loadings. In other terms, samples with high positive PC1 scores have relative to the average sample, high contents of what is positive in the loading plot, and relatively low contents of what is negative in the loading plot. The opposite is applicable for negative scores. It is important to remember that all loadings are relative to the average chromatogram. An increase of a compound therefore translates into a higher content of this relative to the average sample, and vice versa.

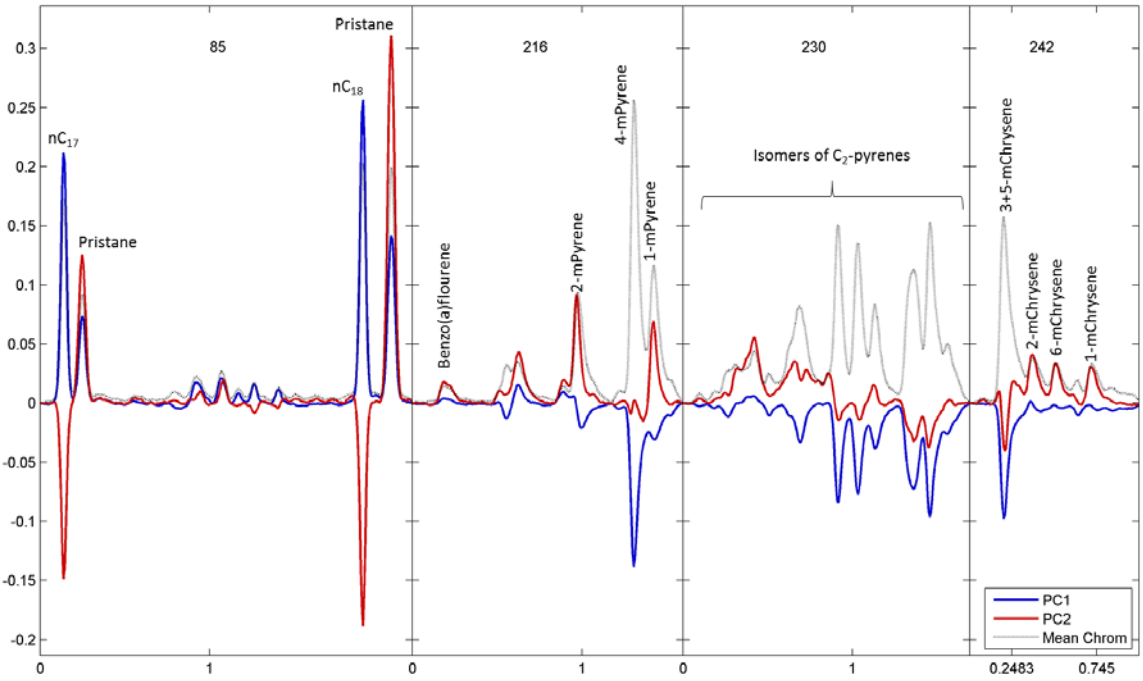

Figure 7. Loading plot. PC1 (blue line), PC2 (red line) and mean chromatogram (dashed black line). The four selected m/z chromatograms are 85: n-alkanes and isoprenoids (narrowed into comprising nC17, pristane, nC18 and phytane), 216: methylpyrenes, 230: dimethyl- and ethylpyrenes, 242: methylchrysenes.

For the example, the long term exposed samples have negative PC1 and small negative PC2 scores. Negative PC1 and PC2 scores are interpreted (via the loading plot) to: Low contents of nC17, pristane, nC18 and phytane, low content of the methylpyrenes, except the 4-methylpyrene isomer, high contents of the C2 pyrenes, high content of the 3/5-methylchrysene isomer. This indicates a situation where substantial microbial degradation has occurred to the samples, thereby removed n-alkanes, isoprenoids and several isomers of mono- and di- methylated pyrenes and chrysenes.

Positive PC2 scores and close-to-zero PC1 scores, translates into low content of n-alkanes, but high pristane and phytane contents, high contents of methylpyrene and methyl chrysene isomers. This describes a situation of mild biodegradation, where only the easiest degradable compounds (n-alkanes) are degraded.

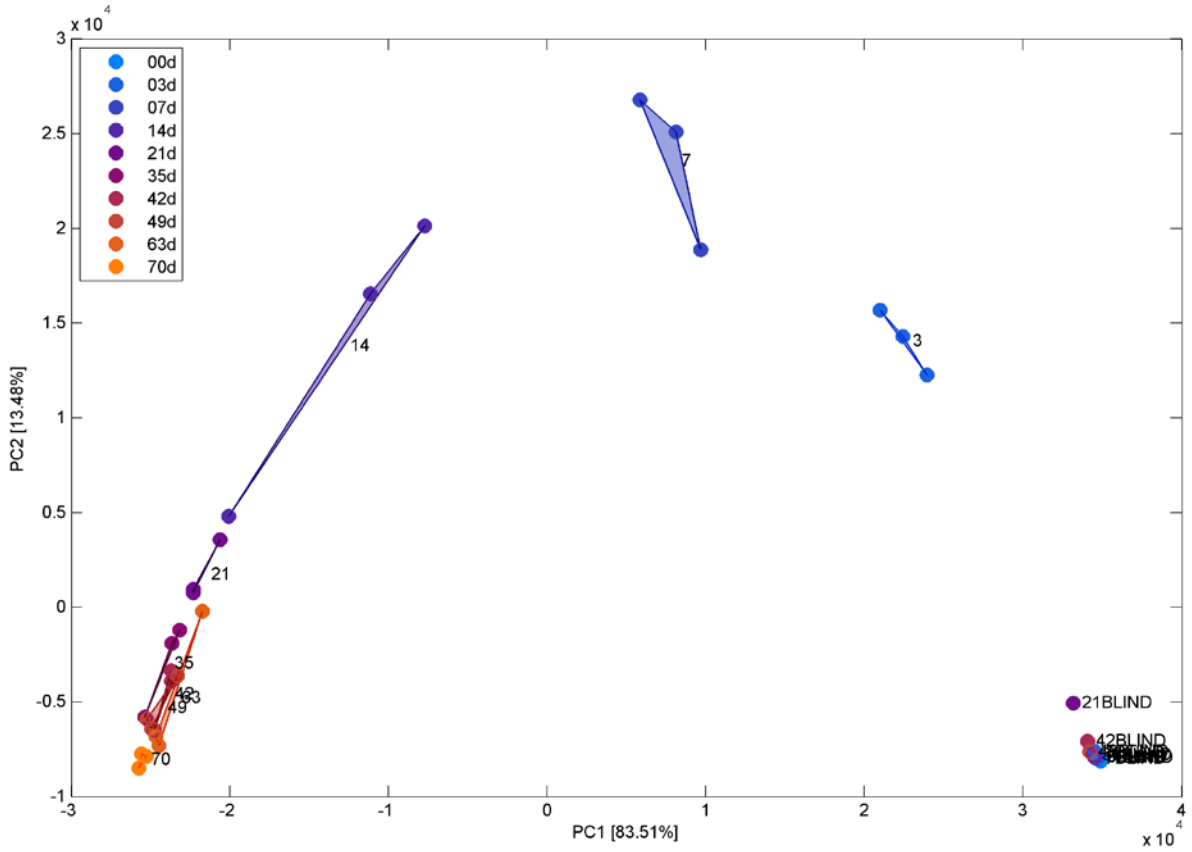

Figure 8. Score plot of the effects of microbial degradation on selected parts of ion chromatograms for m/z 85, 216, 230 and 242. The numbers refer to incubation time in days, replicate samples are connected with lines, and the area is filled.

The data shows that some peaks in the chromatograms are removed during degradation, whereas others are not, even within the same homologue group of compounds. This is termed isomer specific degradation, and previous studies shows that isomer specific degradation of alkylated PAHs does occur. The strength of the CHEMSIC approach is that it aids to find associations in the data that was not looked for, and not least, that it shows results that can be interpreted biologically. For other examples of CHEMSIC and comparable methods to extract information automatically, see: Christensen, Hansen et al. (2005a), Christensen, Hansen et al. (2005b), Malmquist, Olsen et al. (2007), Christensen, Tomasi et al. (2010), Gallotta and Christensen (2012), Soleimani, Farhoudi et al. (2013).

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