

Comprehensive two-dimensional gas chromatography-mass spectrometry of complex mixtures of anaerobic bacterial metabolites of petroleum hydrocarbons

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Highlights

- Two year incubations of crude oil with sediment bacteria under anaerobic sulfate-reducing conditions.
- Complex mixtures of acid and diacid metabolites studied by GCxGC-MS as methyl esters.
- Range of known metabolites extended from those of benzene through to those of methylphenanthrenes.
- Compounds identified by mass spectra and co-chromatography with synthetic acids (methyl esters).
- Sequential order of degradation of aromatic hydrocarbons established and compared with those reported for reservoired petroleum.

ABSTRACT

Anaerobic biotransformation of petroleum hydrocarbons is an important alteration mechanism, both subsurface in geological reservoirs, in aquifers and in anoxic deep sea environments. Here we report the resolution and identification, by comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS), of complex mixtures of aromatic acid and diacid metabolites of the anaerobic biodegradation of many crude oil hydrocarbons. An extended range of metabolites, including alkylbenzyl, alkylindanyl, alkyltetralinyl, alkylnaphthyl succinic acids and alkyltetralin, alkylnaphthoic and phenanthrene carboxylic acids, is reported in samples from experiments conducted under sulfate-reducing conditions in a microcosm over two years. The range of metabolites identified shows that the fumarate addition mechanism applies to the alteration of hydrocarbons with up to C_8 alkylation in monoaromatics and that functionalisation of up to three ring aromatic hydrocarbons with at least C_1 alkylation occurs. The GC×GC-MS method might now be applied to the identification of complex mixtures of metabolites in samples from real environmental oil spills.

Keywords: anaerobic biodegradation/ petroleum acids/sulfate reduction/ GC×GC-MS

1. Introduction

Analysis of complex mixtures of organic compounds is often facilitated by the application of combined chromatography-mass spectrometry techniques [1, 2]. Amongst these, gas chromatography-mass spectrometry (GC-MS) is a well-accepted, powerful method of choice [2]. Indeed, application of GC-MS to some of the most complex mixtures known, such as those of biodegraded crude oils, has resulted in major advances in understanding of the mechanisms of petroleum hydrocarbon biotransformation (e.g. [3-8]). However, the vast complexity of biodegraded oils often means that even use of GC-MS leaves a fraction of such oils unresolved and unidentified [8,9].

Such unresolved complex mixtures (UCMs) or “humps” of hydrocarbons [9], can be resolved further by comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS; [10, reviewed in 11]). Few studies have used this method to study the metabolites of biodegradation until recently, when GC×GC-MS was shown to be well suited to the analysis of acid metabolites in reservoired and refined crude oils and oils sands process waters (e.g. [12-16]). GC×GC-MS has yet to be used to study the metabolites resulting from laboratory studies of crude oil transformations under anaerobic conditions, to our knowledge.

Microbial transformation of crude oil hydrocarbons is an important mechanism, which alters the composition of petroleum, both subsurface (e.g. in deep geological reservoirs; reviewed in [7]), aquifers (reviewed in [17]) and after environmental oil spills (reviewed in [18,19]). Both aerobic and anaerobic processes may occur

[12,17], but the importance of anaerobic mechanisms has been realised increasingly (e.g. reviewed in [17-24] and references therein).

Evidence for anaerobic mechanisms has been produced mainly by either laboratory studies of the removal of single substrate hydrocarbons and production of metabolites (e.g. [3, 25-28]); by detection of signature metabolites of the hydrocarbons in extracts from underground aquifers; by incubations of crude oil with microbes from such reservoirs (e.g. [4, 5, 29, 30]); or by hydrocarbon and metabolite profiling of deep subsurface oil reservoirs (e.g. [6, 23, 31]). Many anaerobic processes are possible, including sulfate reduction, (reviewed in [22, 32-36]).

One way to approach a study of the anaerobic degradation of crude oil is to conduct controlled, long term laboratory incubations of anaerobes with petroleum. Such studies of whole crude oils, in addition to those of pure compounds, are important, since petroleum hydrocarbons in pollution scenarios (and in-reservoir), exist as these complex mixtures [3].

Wilkes et al. [37] studied the biotransformation of several crude oils for 60 days when incubated with two strains of sulfate-reducing bacteria. They observed transformation of C_{1-5} alkylbenzenes with production of C_{1-2} alkylated benzoic acids and traces of the corresponding succinates. Townsend et al. [30] studied the biodegradation of two crude oils by microorganisms from an anoxic aquifer previously contaminated by natural gas condensate, under methanogenic and sulfate-reducing conditions. They showed that whilst n-alkanes were relatively labile, bicyclic aromatic hydrocarbons were more recalcitrant and the biodegradation of

these substrates appeared to be sulfate-dependent and homologue-specific. However, the metabolites of the hydrocarbons were not studied.

Aitken et al., [22] conducted a two-year anaerobic incubation of a North Sea crude oil under sulfate-reducing and methanogenic conditions and studied both the degradation of the hydrocarbons and production of aliphatic acid metabolites. Comparison of hydrocarbon compositional information after ~ 700 days of anaerobic biodegradation revealed that, under sulfate-reducing conditions, changes were observed. However, the complex nature of the acid profiles when examined by GC-MS of the methyl esters restricted identifications of the aromatic metabolites, even though the distributions of many of the aromatic hydrocarbons had changed.

Jarling et al. [21] also studied the metabolites produced by anaerobic degradation of crude oil and binary mixtures of hydrocarbons, by 11 individual bacterial strains. By GC-MS they identified, not only co-metabolically formed alkyl and arylalkylsuccinates from fumarate addition, but also products of anaerobic hydroxylation of alkylbenzenes, by sulfate reducers.

In the present study, we examined by GC×GC-MS, the aromatic acid metabolites produced during two year incubations of a crude oil under sulfate-reducing conditions. The study focuses almost entirely on the analytics; identification of the products helped to reveal the extent and further indicated evidence, of multiple pathways of transformation. Up to three ring aromatic hydrocarbons were biotransformed, extending the range of those observed in studies of pure

hydrocarbons or oils previously. The major aim of the present paper was to illustrate the applicability of GCxGC-MS to such studies.

2. Experimental

2.1 Incubations

The conditions and methods used for the two year anaerobic incubation of a North Sea crude oil have been described fully elsewhere [22]. Briefly, microcosms were prepared in sterile glass serum bottles (120 mL) in an anaerobic cabinet (Coy Laboratory Products Inc., MI, USA) fitted with an oxygen sensor and with a regulated atmosphere of nitrogen (99.5%) and hydrogen (0.5%) (BOC Special Gases Ltd). All microcosms were incubated in the cabinet in the dark to ensure anaerobic conditions were maintained. Each microcosm comprised a carbonate buffered nutrient medium containing sources of nitrogen and phosphorous, vitamins, and trace minerals, prepared in deionised water for brackish conditions and 10 mL of sediment slurry (10 % v/v) from the River Tyne. The total volume of liquid in each microcosm was 100 mL with 20 mL of headspace. All microcosms were prepared in triplicate. Sulfate reducing conditions were established in microcosms by the addition of Na_2SO_4 (final concentration 20 mM) to the medium and Na_2S (0.5 mM) was added as a reducing agent. Approximately 300 mg of North Sea oil was added as a carbon and energy source to all the microcosms. Control microcosms with sodium molybdate (20 mM) added to inhibit sulfate reduction were also prepared. Additional controls included microcosms Pasteurised by heating at 95°C for 2 hours, and microcosms containing no added oil (to assess sulfate reduction in the absence of oil). Microcosms were incubated at room temperature (ca. 20°C) and for each treatment 6 sets of microcosms were prepared for sacrificial sampling over a two year period to allow

analysis of crude oil composition over time. To monitor the contribution of any hydrocarbons initially present in the inoculum sediment added to microcosms, triplicate sediment samples were extracted and analysed.

Sediment samples used as inoculum were taken from the River Tyne near Scotswood Bridge, Newcastle, UK (54.96 ^{o}N, 1.68 ^{o}W). Sulfate concentrations from 16 cm long sediment cores taken at low tide ranged from 2.91 mM (surface) to 7.04 mM (8cm depth) with a value of 6.25 mM at 16cm, the average depth of sediment samples taken for inoculum in microcosms. The acids of killed controls (molybdate or Pasteurised), undegraded oil and sediment inoculum, were all studied as methyl esters, in addition to samples of oil degraded for 22, 176 and 686 (three replicates) days under sulfate-reducing conditions. (Whilst samples were also studied at 302 days in a previous study of non-aromatic metabolites [22], only analyses of the aforementioned samples were made herein). Sulfate reduction was monitored by hydrocarbon depletion and methane (non-) generation [22].

2.2 Metabolite identification and measurement by GCxGC-MS

GC×GC-MS analyses of acid fractions derivatised by refluxing with BF_3-methanol were conducted essentially as described previously for other mixtures [12]. Briefly, analyses were conducted using a model 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a ZX2 GC×GC cryogenic modulator (Zoex, Houston, TX, USA) interfaced with a BenchTOFdx™ time-of-flight mass spectrometer (Almsco International, Lantrisant, UK) operated in positive electron ionisation mode and calibrated with perfluorotributylamine. The scan speed was 50 Hz. The first-dimension column was a 95% dimethyl polysiloxane 5% diphenyl polysiloxane fused-silica capillary HP-5ms (30m × 0.25 mm × 0.25 μm; Agilent

Technologies J & W, Wilmington, DE, USA) and the second-dimension column was a 50% phenyl polysilphenylene-siloxane BPX50 (3m × 0.1 mm × 0.1 μm; SGE, Melbourne, Australia). Samples (1 μL) were injected at 280 °C splitless. The oven was programmed from 40 °C (held for 1 min), heated to 300 °C at 5 °C min^{-1} and then at 10 °C min^{-1} to 320 °C (held for 10 min). The modulation period was 5 sec. The mass spectrometer transfer line temperature was 280 °C and the ion source temperature 300 °C. Data were collected in ProtoTof (Markes International, Llantrisant, Wales, U.K.) and processed with ChromSpace (Markes International Limited, Llantrisant, Wales, UK) or GC Image v2.3 (Zoex, 328 Houston, TX). Acid metabolites were quantified as the methyl esters using integration of the volumes of the GC×GC-MS base peak ions or molecular ion of the analytes, relative to that of the base peak ion of the internal standard 1-phenyl-1-cyclohexane carboxylic acid (methyl ester), assuming a response factor of unity, as previously [22].

2.3 Authentic acids

1- and 2-naphthoic acids and phenanthrene-3- and 4-carboxylic acids were purchased from Sigma-Aldrich (Poole, UK). 1- and 2-tetralin carboxylic acids and the corresponding decalin acids were synthesised by partial or complete hydrogenation of the aromatic analogues [12]. Phenanthrene-2- and phenanthrene-9-carboxylic acid were synthesised by refluxing a mixture of either 2-acetylphenanthrene or 9-acetylphenanthrene (Sigma-Aldrich, Poole, U.K.) with a 5% solution of sodium hypochlorite for 24 h according to the procedure of Dixon and Neiswender [38].

3. Results and discussion

Aitken et al., [22] have described the overall changes in incubated crude oil hydrocarbon composition during the 686 days of the sulfate-reducing microcosm in some detail. Briefly, the microcosms initially all contained the same undegraded North Sea crude oil, nutrients and sediment-associated micro-organisms. Sulfate was added to induce sulfate-reducing conditions, which were monitored by the depletion of alkanes and the (non-) production of methane [22]. Analysis of River Tyne sediments sampled at the same location and depth as the inoculum (the source of the microbes) confirmed the presence of only low concentrations of sulfate in the sediments initially [22]. Cumulative methane generation was less than 100 µmoles by 300 days and in microcosms containing inhibitors, cumulative headspace methane was <250 µmoles over 686 days. Thus sulfate reduction, not methanogenesis, was the major anaerobic mechanism. Total bacterial numbers changed little over the incubation period, but specific anaerobes implicated in hydrocarbon degradation were enriched by 3 orders of magnitude to 10^8 cells/g sediment [22]. As a general indication of the progress of the overall removal of oil, alkane concentrations (n-C_{7-34}) decreased from about 2000 µmoles carbon to about 250 µmoles carbon over the 686 days. Approximately two thirds of the n-C_{12-26} alkanes were consumed by 302 days, after which their rate of removal decreased, such that at 686 days only about 10% of the initial n-alkanes remained [22].

The latter authors reported the production of succinate metabolites from the alkanes, but the complexity of the mixtures hindered analysis of the metabolites of the aromatic hydrocarbons.

Previous studies have shown the complexity of the acid metabolite mixtures produced by the biotransformation of crude oils in the laboratory [8]. These acids are typically observed as unresolved complex mixtures (UCMs) when converted to the methyl esters and examined by GC-MS and have thus proved virtually impossible to identify previously [8]. The so-called 'naphthenic acid' biodegradation products of petroleum, most dramatically revealed by the processing of oil sands deposits, are also unresolved by GC-MS of the esters and also represented similar analytical challenges until the application of GCxGC methods [reviewed in 16].

In the present study, use of GCxGC-MS helped overcome the difficulty of identifying many of the components of the complex acid mixtures. Thus, when methylated acidic extracts of the microcosm sediments incubated for 22-686 days under sulfate-reducing conditions were examined, the expanded peak capacity of GCxGC-MS, over conventional GC-MS [reviewed in [11], produced good resolution of the complex mixtures of aromatic metabolites from the non-aromatic (aliphatic) acids, due to the high phenyl content of the second dimension GC column (Figures 1,S1). This then allowed electron ionisation mass spectra, relatively free of ions from co-eluting interferents, to be obtained (e.g. Figures 3-5,S2-6), which was not possible previously [22] and identifications to be made by comparison of mass spectra with those of spectral libraries and/or those of synthesised reference compounds (e.g. Figure 4). It was then possible to monitor variations in the relative proportions of a wide range of metabolites throughout the duration of the two year experiment (Table 1). We report only variations in the relative proportions of metabolites (Table 1; Figures 6-9) since the conditions in the microcosm probably do not reflect those in any given oil reservoir or aquifer. Thus, the absolute concentrations are probably not

applicable to the production of kinetic data for such environments. Rather, the variations (Table 1) indicate the sequence of biotransformation events and progressive metabolism of the substrate hydrocarbons and intermediate acids (Figures 6-9). Whilst aromatic and non-aromatic hydrocarbons can be separated routinely by open column chromatography techniques before analysis by GC-MS (even though many of these are then still revealed only as UCMs), separation of the corresponding aromatic acids and di-acids (e.g. succinates) from the non-aromatic acids (as acids or esters) is less trivial. The use of GCxGC-MS is particularly advantageous in resolving the aromatic analytes of the total esterified acid mixtures, particularly in the second dimension (Figure 1) and even the non-aromatic analytes were better resolved by GCxGC-MS from co-eluting interferents, than by GC-MS (cf [22]).

Fumarate addition is perhaps the best known mechanism for anaerobic transformation of aromatic hydrocarbons (reviewed in [17], [21]). The proposed mechanism produces 'signature' succinate metabolites, usually by addition to the benzylic position in alkylaromatics with C_{1-3} alkyl groups, or by addition to the omega minus two or omega minus three positions in aromatics with longer side chains (e.g. Figure 2; [21]). These were revealed by GCxGC-MS herein (Figures 1, S1). Alternatively, anaerobic hydroxylation of aromatic hydrocarbons can occur, with further transformations resulting in aromatic carboxylic acids (Figure 2; [21]). However, such simple acids are less specific to anaerobic processes than the characteristic succinates, since these acids can result from both processes by further transformation (Figure 2; [12, 21]). Nonetheless, these could also be identified by GCxGC-MS (Figures 1, S1).

283

284 *3.1 Identification of metabolites of monoaromatic hydrocarbons by GCxGC-MS*

285 The route of transformation of toluene by sulfate reducing bacteria has been shown
286 in several studies in which the pure hydrocarbon has been incubated, to proceed via
287 formation of benzylsuccinates (e.g. Figure 2; [39]). These same 'signature'
288 metabolites and sometimes the associated *bssA* genes, have also been detected in
289 numerous field studies of hydrocarbon-polluted aquifers, as have the associated
290 methylbenzylsuccinates (Figure 2). It was thus not surprising that benzylsuccinate
291 and methylbenzylsuccinates were identified in the day 22 incubated samples in the
292 present study, by comparison of mass spectra with those of synthesised compounds
293 (Table 1; cf [39]). The corresponding E-phenylitaconates and
294 methylphenylitaconates, which have been proposed as further transformation
295 products [21], were also identified in the day 22 sample by comparison of mass
296 spectra with those already published (Figure S2). The benzylsuccinate and
297 methylbenzylsuccinates were also present in the 176 and 686 day samples (e.g.
298 Table 1; Figure S3). No benzylsuccinates or E-phenylitaconates or associated
299 methylbenzyl compounds were detected in the sediment inoculum or the crude oil
300 used in the incubations (Table 1). This is good evidence that transformation of
301 toluene and xylenes occurred via the known fumarate addition pathway in the
302 present incubations (Figure 2; [17], [21]). These 'signature' metabolites of sulfate-
303 reducing anaerobic transformation of toluene and xylenes were not detected in the
304 molybdate-treated day 686 samples.

305

306 In addition to benzene, toluene and xylenes, the incubated North Sea crude oil, like
307 many crude oils, contained homologous series of alkylbenzenes (AB; Figure 2),

alkyltoluenes (AT; Figure 2) and alkylxylenes (AX). These are common in crude oils and oil-polluted environmental samples (e.g. [40, 41]). Consistent with this, the samples incubated herein for 176 days contained alkylbenzylsuccinates (Figure 2), identified from the mass spectra herein (Figure 3), as possessing C_{0-8} alkyl groups. The spectra were typified by low abundance molecular ions, ions due to loss of methoxy (M-31), ions due to loss of methyl formate (M-60) and base peak ions (B^+) due to benzylic fragmentation (Figure 3). (Similar fragmentations occurred for the higher benzologues (e.g. Figures 4-6)).

A series of benzylsuccinates with carbon chain lengths from 5 to 10 was detected in deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill [19], but no spectra were published. Some of the C_1 analogues of the latter have been shown to derive from anaerobic transformation of xylenes (e.g. [39, 42]) and it is a reasonable assumption that the succinates with longer chain alkyl substituents in the environment were derived from the corresponding AB from the Macondo oil, as supported by the identifications in the present laboratory study.

Further metabolism of the benzylsuccinates might conceivably lead to production of alkylbenzoic and methylalkylbenzoic acids [43]. In the present anaerobic experiments (Table 1), data were obtained for the relative proportions of phenylalkanoic and alkylbenzoic acids as possible metabolites of AB and AT (e.g. Figure 2). By day 176, both classes of acids were present and their abundance increased still further by day 686 (Table 1). No alkylbenzoic or related acids, and no phenylalkanoic acids, were detected by mass chromatography in the sediment inoculum, in the undegraded North Sea oil, or in the degraded oil up to day 22. Alkyl substituents with 3 to at least 6 carbon atoms were identified in the alkylbenzoic acids (e.g. Figure 4), which were identified by comparison of mass spectra with

those of library or of synthesised acids. The number of carbon atoms in the alkanoate chains of the phenylalkanoates ranged from 2 to at least 6. Some spectra for reference compounds were available for comparison (Figure 4). The abundance of the acids had increased still further by day 686.

Aerobic biodegradation of a crude oil previously showed production of C_{0-3} phenylalkanoic acids within 5 days incubation, with their complete removal within 80 days [44], so accumulation of these acids might be a useful indication of the operation of dominant anaerobic processes, as suggested previously [37].

The above observations of succinates, itaconates and carboxylic acids suggest that products of numerous steps in the fumarate addition mechanism are present in these complex mixtures (Figure 2). The incubation of complex mixtures of hydrocarbons herein is realistic of the natural environment, such as oil reservoirs or oil spills. However, unlike experiments in which single substrates are incubated and where the product metabolite mixtures are relatively simple, it is more difficult to deconvolute exactly which hydrocarbon is transformed by which mechanism in such incubations. For example, it is not known whether the carboxylic acid products come from the same metabolic mechanism as the succinate products, by further transformation (Figure 2), or if they are derived from a different mechanisms, such as benzylic hydroxylation, followed by further transformation (Figure 2). Neither can it be easily deduced whether the observed chemicals are "end products" of metabolism or intermediate transition products. However, by monitoring the relative proportions of the products of these transformation routes it is possible to speculate on the relationships between the observed metabolites. This proved easier with the diaromatic metabolites, as discussed below.

358 *3.2 Identification of metabolites of diaromatic hydrocarbons by GCxGC-MS*

359 Biotransformation of C_{0-2} naphthalenes under sulfate-reducing conditions is known in
360 experiments in which pure or binary mixtures of hydrocarbons were incubated. This
361 also proceeds by fumarate addition with, (for naphthalene and 2-
362 methylnaphthalene), formation of naphthyl-2-methylsuccinic acid and naphthyl-2-
363 methylenesuccinic acid and the central intermediate, 2-naphthoic acid [23]. Detection
364 of the succinate signature metabolites in polluted aquifer water has been taken as
365 evidence of anaerobic biodegradation of alkylnaphthalenes (e.g. [3, 5]).

366

367 In the present study, naphthyl-2-methylsuccinic acid was identified in the day 22 to
368 day 686 samples by comparison of mass spectra with published spectra (Figure S5);
369 but was not present in the crude oil or the sediment inoculum (Table 1). A series of
370 alkylnaphthyl-2-methylsuccinic acids was also identified in the incubated samples by
371 interpretation of the mass spectra: these are metabolites of the C_2 and C_3
372 alkylnaphthalenes (Figure 5). Only one such compound has been reported
373 previously [3], whereas numerous isomers were identified herein, with spectra
374 showing the expected benzyl fragmentations (base peak ion B^+ *m/z* 155; e.g. Figure
375 5). Acids consistent with transformation of the multiple isomeric C_{2-3}
376 alkylnaphthalenes, in addition to the methylnaphthalenes, were observed, extending
377 the known range of metabolites and consistent with the removal of up to C_3
378 naphthalenes.

379

380 Also identified in the incubated samples were the expected downstream metabolites
381 of these succinates. Thus, individual naphthoic, (and tetralin and decalin) acids, as
382 well as numerous higher homologues, were identified by GC×GC-MS (Table 1) and

trends in the respective metabolites could be followed (Table 1; Figure 6). The data showed that up to 176 days the incubation proceeded with generation of both 1- and 2-naphthoic acid, with the latter predominating (Figure 6A). This is consistent with the known faster depletion of 2-methylnaphthalene, though it is unlikely that even the 2 year laboratory microcosms reproduce the kinetic effects observed in oil reservoirs. After 176 days, both isomers were degraded further in the 'live' samples (Figure 6A). Only trace quantities were present in the original oil and the sediment inoculum, again with the 2-naphthoic acid isomer predominating (Figure 6A). No production was observed in the 176 days Pasteurised sample.

Aitken et al. [6] proposed that increased proportions of the downstream metabolism of 2-naphthoic acid accounted for the observation of the corresponding tetralin and decalin acids in reservoired crude oils, providing evidence for an anaerobic biodegradation mechanism. This is supported by the increasing occurrence of these acids in the present incubated samples, with observation of the tetralin acid at 176 days and additionally of the decalin acid by day 686 (Figure 6B). The relative proportions of these acids depend on the relative rates of production and on further metabolism. Such trends in production of the corresponding naphthyl ethanoic acids and the corresponding tetralin and decalin acids (Figure 7), which were identified by comparison of mass spectra with those of the known compounds, were also observed herein, lending further support to this hypothesis. The high relative concentrations of the naphthyl-2-ethanoic acid, even after 2 years incubation under sulfate-reducing conditions (Figure 7), suggests such metabolites may be recalcitrant

406 markers of such processes. These can be monitored by GC×GC-MS using the
407 methods reported herein.
408 Examination of the metabolites of transformation of the three isomers of
409 methylbiphenyls herein, showed no evidence of succinates, but production was
410 observed of both biphenyl-3- and 4-carboxylic acids by day 22, above the relative
411 amounts in the sediment inoculum (and absence in the undegraded oil; Figure 8). By
412 176 days, the amount of the 3-isomer had reduced, followed by reduction in the 4-
413 isomer by day 686. These results suggest that both 3- and 4-methylbiphenyl
414 degraded to the corresponding acids, which were then degraded further (to unknown
415 products). Detection of the corresponding metabolite of 4-methylbiphenyl (i.e. the
416 acid) seems to be a sensitive method for monitoring incipient biotransformation. The
417 corresponding succinates of these acids were not identified, likely because they had
418 been metabolised further to the acids in the intervening period.
419
420 *3.3 GCxGC-MS identification of metabolites of triaromatic hydrocarbons*
421 The effects of aerobic biodegradation of phenanthrene and particularly of
422 methylphenanthrenes (MP) have been studied in laboratory experiments of crude oil
423 hydrocarbons and in numerous field studies [45-47]. These have often shown an
424 isomer-specific degradation, usually of the beta-substituted 2- and 3-MP. In addition,
425 laboratory studies of MP hydrocarbon degradation under anaerobic conditions have
426 been reported [47], whilst Gieg et al. [23] showed that pure MP substrates were
427 degraded with the production of phenanthrene carboxylic acids. However, production
428 of MP acid metabolites under anaerobic conditions in a crude oil mixture does not
429 appear to have been shown in the laboratory, so far as we are aware.
430

The relative concentrations of 2-MP between days 0 and 686 reduced by ~3%. Over the same period, changes in the relative distributions and amounts of phenanthrene-2-carboxylic acid, identified by GC×GC-MS versus the synthesised compound, were monitored (Figure 9). Changes in the other phenanthrene carboxylic acids were minimal (Figure 9; Figure S7). These changes exceeded any influence produced by the small amounts of the acids present in the sediment inoculum and the undegraded crude oil (Figure 9). No succinates of the methyl phenanthrenes were detected, possibly due rapid further transformation of these to the carboxylic acids. No changes were observed in the dimethyl- and trimethylphenanthrenes, suggesting the extent of biodegradation in this slow anaerobic sulfate-reducing process was only sufficient to effect up to the MP isomers. A small amount of methylphenanthryl carboxylic acid was tentatively identified herein by GC×GC-MS, suggesting incipient degradation of C_2-phenanthrenes by day 686. These results indicate the greater sensitivity of monitoring incipient biotransformation via production of the acid metabolites, compared to monitoring changes in the hydrocarbons.

Some changes were also observed in the sulfur-containing aromatics. Minor amounts of a benzothiophene and a C_1 benzothiophene carboxylic acid were detected in the sediment inoculum, by comparison of the mass spectra with those of authentic samples; these were not detected in the undegraded oil. Traces of these compounds were present in the 22 and 176 day incubated samples but these were absent by 686 days, suggesting further metabolism. Three dibenzothiophene carboxylic acids were detected in the sediment inoculum, by comparison of the mass spectrum with that of an authentic sample, not detected in the undegraded oil and

455 the traces present in the 22 and 176 day incubated samples were again absent by
456 686 days.
457
458 No changes in the well-known mono- or triaromatic tetracyclic steroids present in
459 most crude oils, including the oil used herein, were observed and as expected, the
460 acid products of these were not observed.

461 **4. Conclusions**

462 A wide range of extended chain metabolites of aromatic hydrocarbons, including
463 those not reported previously, were formed during two year laboratory
464 biotransformation of crude oil hydrocarbons under sulfate-reducing conditions. The
465 range of metabolites identified shows that the fumarate addition mechanism applies
466 to alteration of aromatic hydrocarbons with up to C_8 alkylation in monoaromatics and
467 that functionalisation of up to three ring aromatic hydrocarbons with at least C_1
468 alkylation occurs, even in these very complex petroleum mixtures within 2 years. The
469 spectral data presented herein may be important for the future identification of a
470 wider range of degraded crude oil pollutants and studies of their fate and toxicity. For
471 example, whilst the occurrence of suspected succinate metabolites of alkylbenzenes
472 thought to be derived from spilled Macondo oil in the Gulf of Mexico required only
473 GC-MS analysis [19], the application of GCxGC-MS techniques may extend the
474 inventory of metabolites still further. In the sediments in such regions, sulfate
475 reduction is likely to occur. The alkylbenzoic acids may be useful dead-end
476 metabolites [37]. They thus may be preserved indicators of such processes, as may
477 naphthyl ethanoic acid, whereas the succinates and other acids (e.g. naphthoic and
478 phenanthrene acids) may be degraded further.

In addition, the increased exploitation and processing of heavier and non-conventional crude oils and other hydrocarbons (e.g. [7]), many of which have been at least partially biodegraded and which contain higher than normal proportions of so-called 'polar' NSO compounds (such as the diacids (O_4) and heteroacids (e.g. SO_2) identified herein) is likely to lead to increased proportions of these polar compounds, including some of the acids studied herein, in process and production waters. An important example is the large proportions of acids in the process-affected waters resulting from the oil sands industries [reviewed in 16]. Studies such as those herein will lead to an increased understanding of the origins and fate of such pollutants.

Despite the quite extended nature of the present laboratory experiments (i.e. 2 years incubation) it is clear that a use of GC×GC-MS techniques to study naturally degraded crudes, which may have proceeded beyond the transformation of the alkylphenanthrenes studied herein, will be advantageous and will add further to the database of known anaerobic (and aerobic) metabolites of crude oil degradation.

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Table and Figure Legends

Table 1

Acid metabolites detected by GC×GC-MS during laboratory biotransformation of a North Sea crude oil for 686 days under sulfate-reducing conditions. Data for Pasteurised or molybdate treated samples are given in parentheses.

Key: = not detected

= present in increasing relative amounts within compound class

a. 1= published mass spectrum; 2=synthetic compound (GC×GC & MS); 3= MS interpretation.

Figure 1.

Extracted ion current A. (*m/z* 278) mass chromatogram and B-F, mass spectra of tentatively assigned C_3-benzylsuccinate (dimethyl ester) and $C_{4,5,6,7}$-benzylsuccinates (dimethyl esters), in acidic extract of crude oil incubated for 176 days with Tyne sediment inoculum under sulfate reducing conditions.

Figure 2.

Electron ionisation mass spectra of tentatively assigned (A) *n*-butyl and (B) *n*-hexylbenzoic acids (methyl esters) in acidic extract of crude oil incubated for 686 days with Tyne sediment inoculum under sulfate reducing conditions and NIST reference mass spectra of authentic compounds (C & D).

Figure 3.

Electron ionisation mass spectra of tentatively assigned (A) methyl and (B) dimethyl (or ethyl)naphthyl-2-methyl succinates (dimethyl esters) in acidic extract of crude oil incubated for 176 days with Tyne sediment inoculum under sulfate reducing conditions.

Figure 4.

690 (A) Concentrations of 1- and 2-naphthoic acid (versus internal standard) and (B)
691 concentrations of 2-naphthoic, tetralin-6 and decalin-2-carboxylic acids measured as methyl
692 esters by GC×GC/MS in acidic extracts of crude oil and in crude oil incubated for 0-686 days
693 with Tyne sediment under sulfate reducing conditions. Past = Pasteurised control; MOLY =
694 molybdate killed control. Data for 686 day are shown for mean of n=3 replicates.
695 **Figure 5.**
696 (A) Concentrations of 1- and 2-naphthyl ethanoic acid (versus internal standard) and (B)
697 concentrations of 2-naphthoic ethanoic, tetralin-6 and decalin-2-ethanoic acids measured as
698 methyl esters by GC×GC/MS in acidic extracts of crude oil and in crude oil incubated for 0-
699 686 days with Tyne sediment under sulfate reducing conditions. Past = Pasteurised control;
700 MOLY = molybdate killed control. Data for 686 day are shown for mean of n=3 replicates.
701 **Figure 6.**
702 Concentrations of 2-, 3- and 4-biphenyl carboxylic acid (versus internal standard) measured
703 as methyl esters by GC×GC/MS in acidic extracts of crude oil and in crude oil incubated for
704 0-686 days with Tyne sediment under sulfate reducing conditions. Past = Pasteurised
705 control; MOLY = molybdate killed control. Data for 686 day are shown for mean of n=3
706 replicates.
707 **Figure 7.**
708 (A) Concentrations of 1-, 2-, 3- and 9- (4-absent) phenanthrene carboxylic acids (versus
709 internal standard) measured as methyl esters by GC×GC/MS in acidic extracts of crude oil
710 and in crude oil incubated for 0-686 days with Tyne sediment under sulfate reducing
711 conditions. Past = Pasteurised control; MOLY = molydate killed control. Data for 686 day are
712 shown for mean of n=3 replicates. (B) Extracted ion mass chromatograms (*m/z* 236)
713 showing distributions of phenanthrene carboxylic acid isomers (as methyl esters) in day 686
714 sample (replicate 3).

Acid metabolite (as methyl, or dimethyl esters)	**Crude oil**	**Sediment inoculum Day 0**	**Sediment inoculum Day 22**	**Sediment inoculum Day 176 (Pasteurised)**	**Sediment inoculum Day 686 (Molybdate)**	**Identification[a]**
(Alkyl)benzyl succinic	x	x	●	● (•)	● (•)	1
Phenylitaconic	x	x	•	x (x)	x (x)	1
(Alkyl)benzoic	x	x	x	● (x)	● (x)	1,2
(Alkyl)indanyl succinic	x	x	x	● (x)	x (x)	3
(Alkyl)indanoic	x	x	x	● (x)	● (x)	1-3
(Alkyl)naphthyl methyl succinic	x	x	•	● (x)	● (x)	1
(Alkyl)naphthoic	x	•	●	● (x)	● (•)	1-3
(Alkyl)tetralin methyl succinic	x	x	x	● (x)	x (x)	3
(Alkyl)tetralin	x	x	•	● (x)	● (•)	1-3

Benzothiophene methyl succinic	x	x	x	• (•)	x (x)	1
Dibenzothiophene carboxylic	x	•	•	● (x)	x (x)	1, 2
Phenanthrene carboxylic	x	•	●	● (•)	● (•)	2,3

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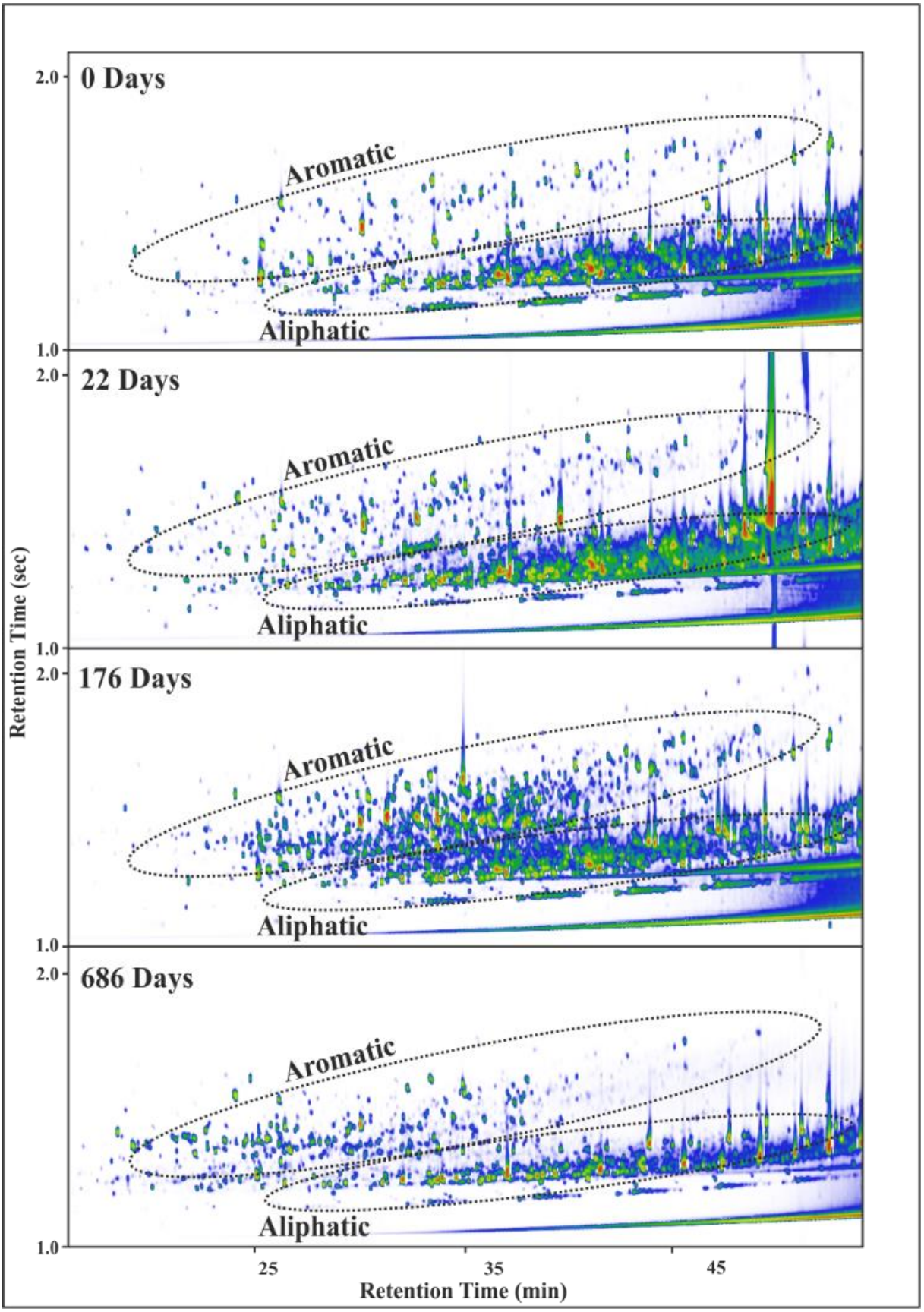

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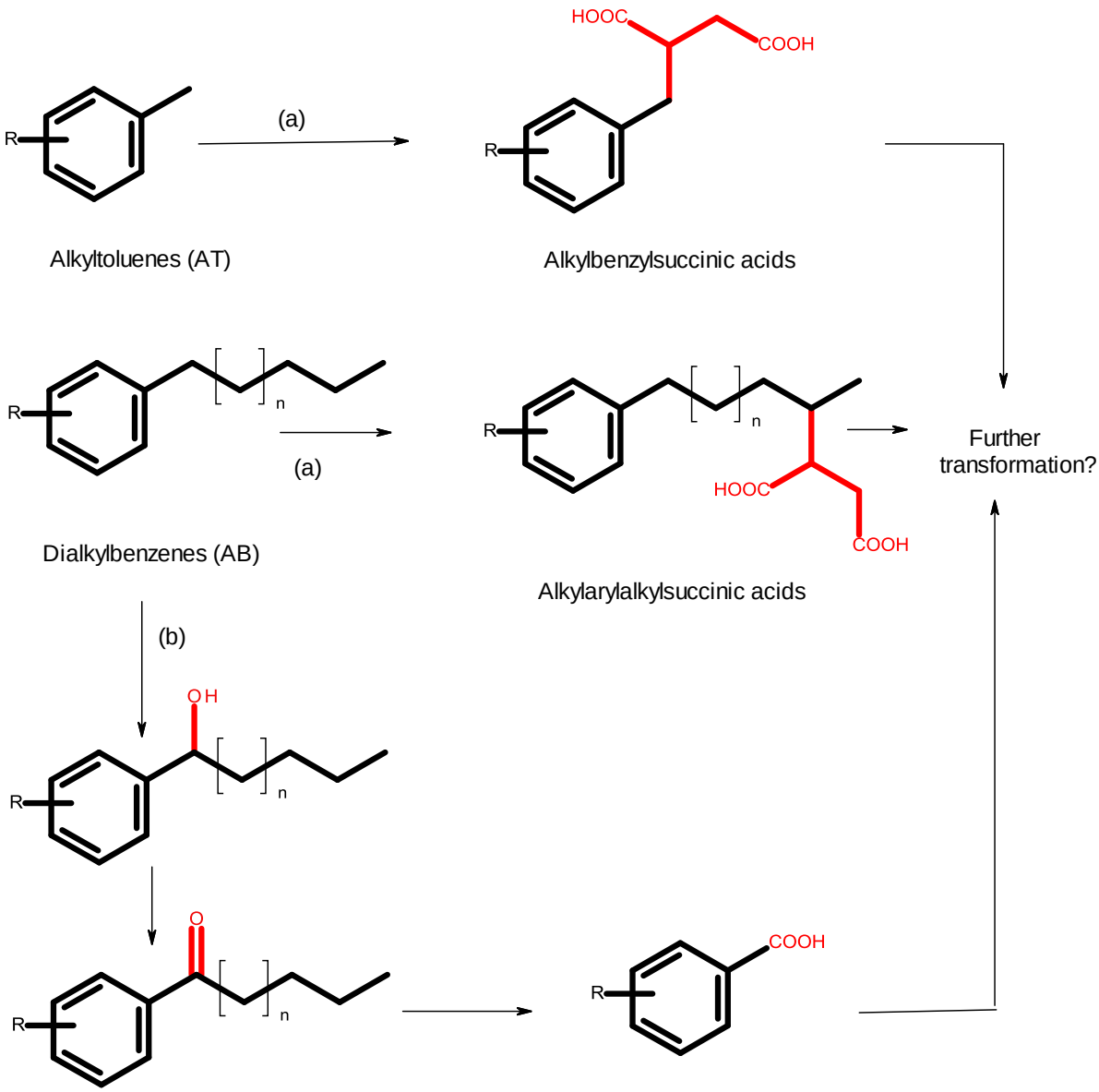

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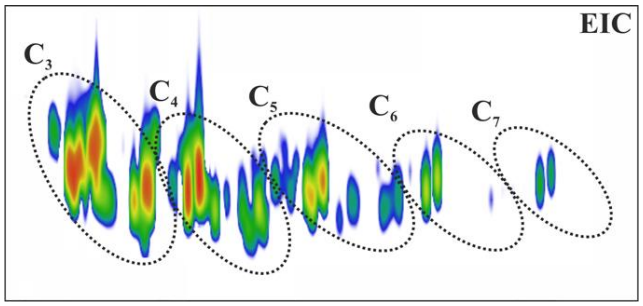
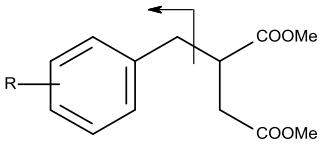


Base peak ion (B+) 133,147,161,175,189



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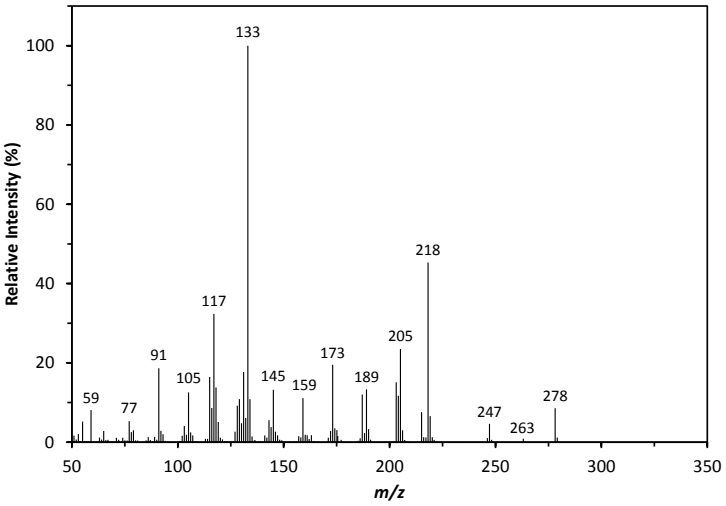

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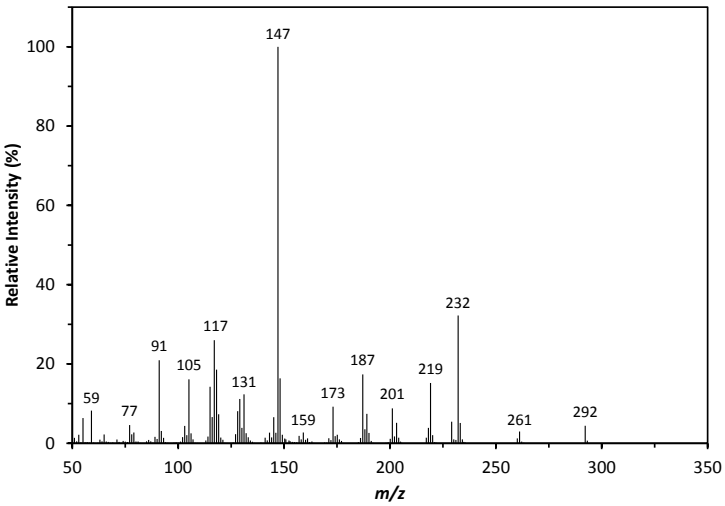

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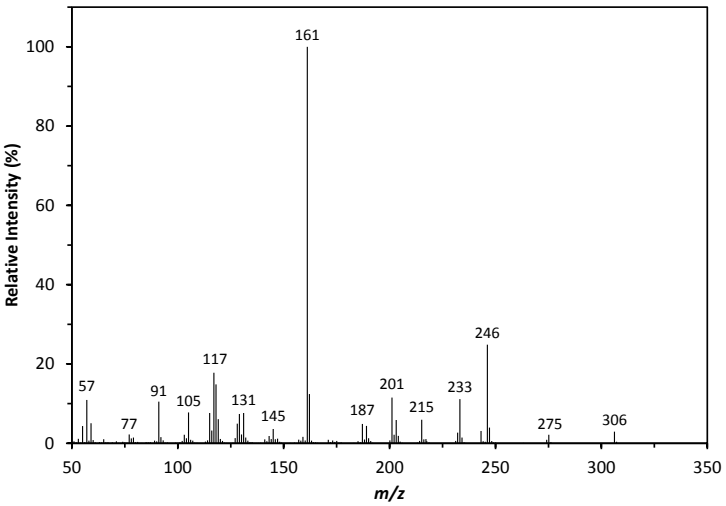

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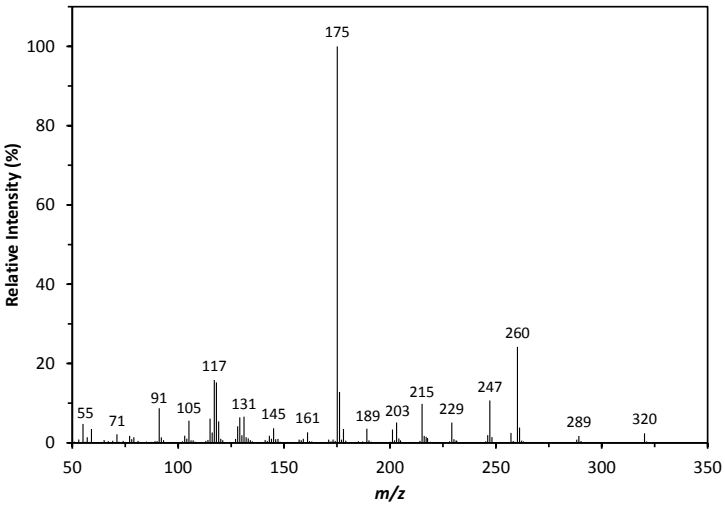

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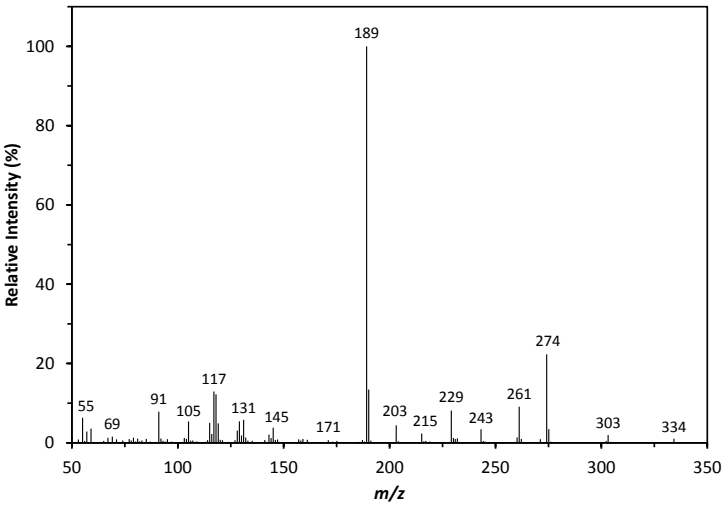

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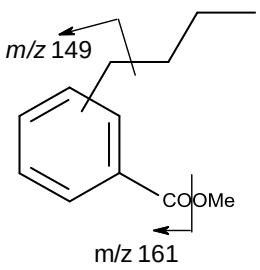

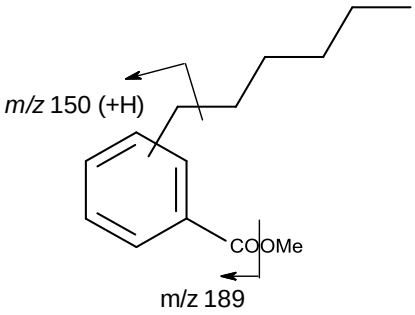

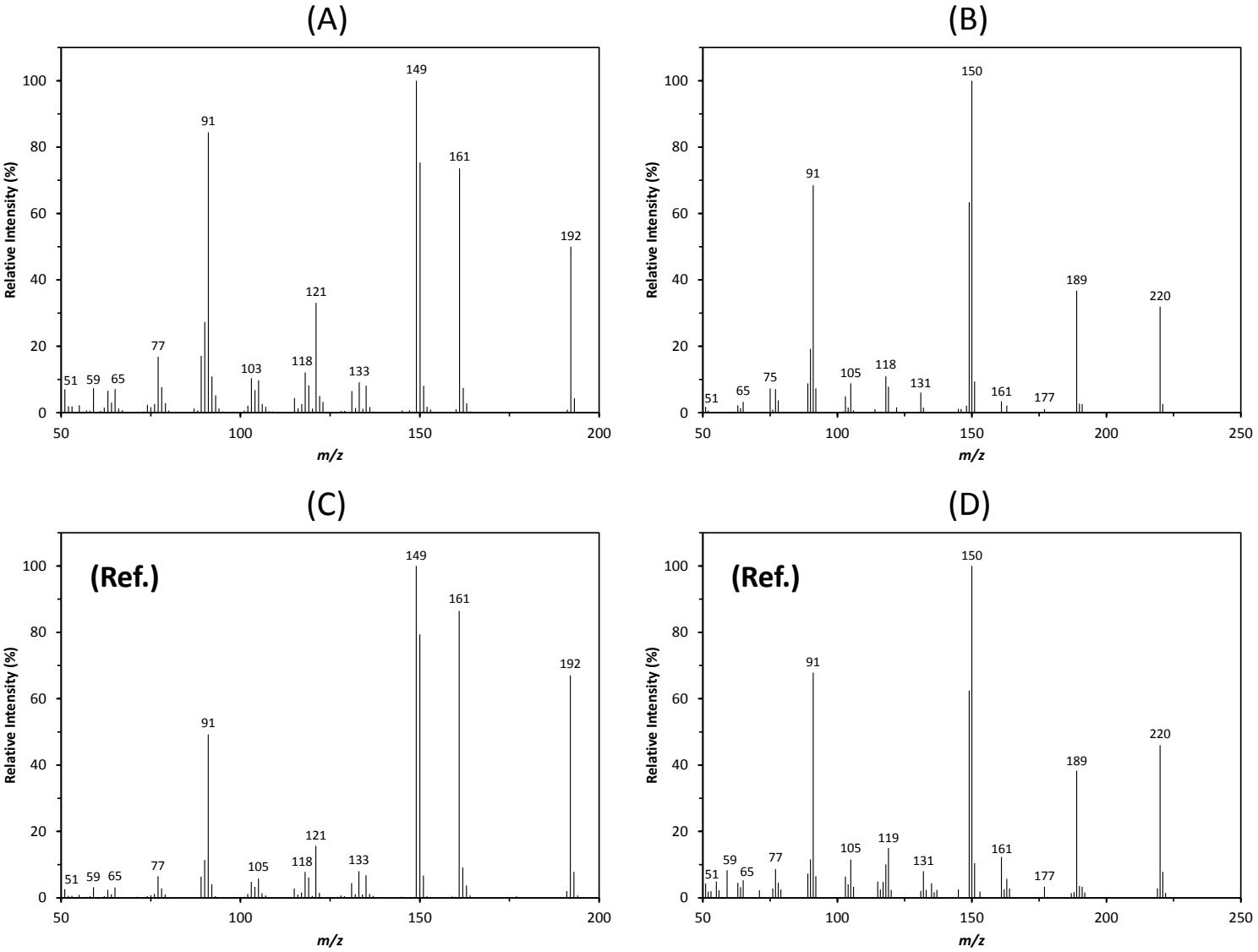

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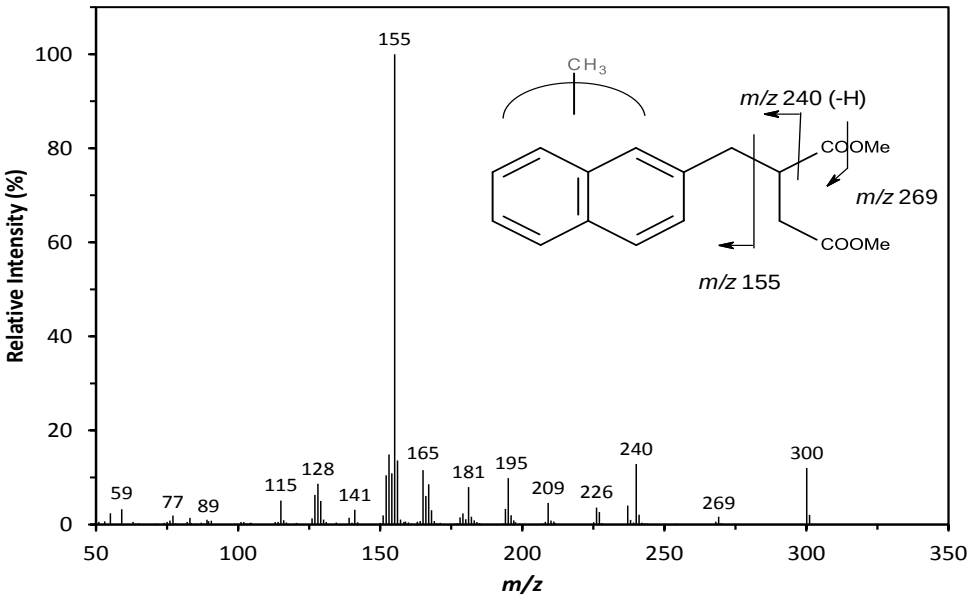

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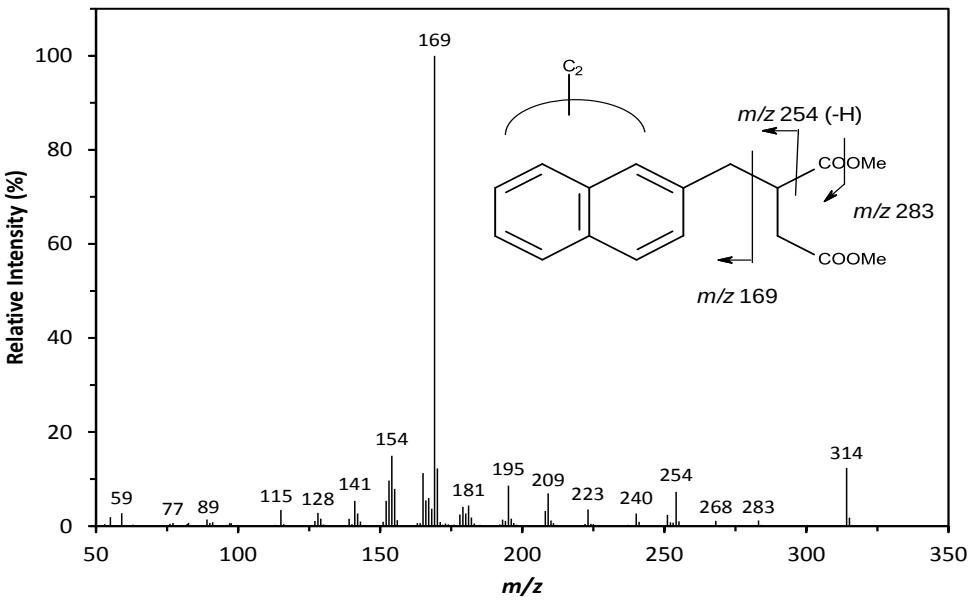

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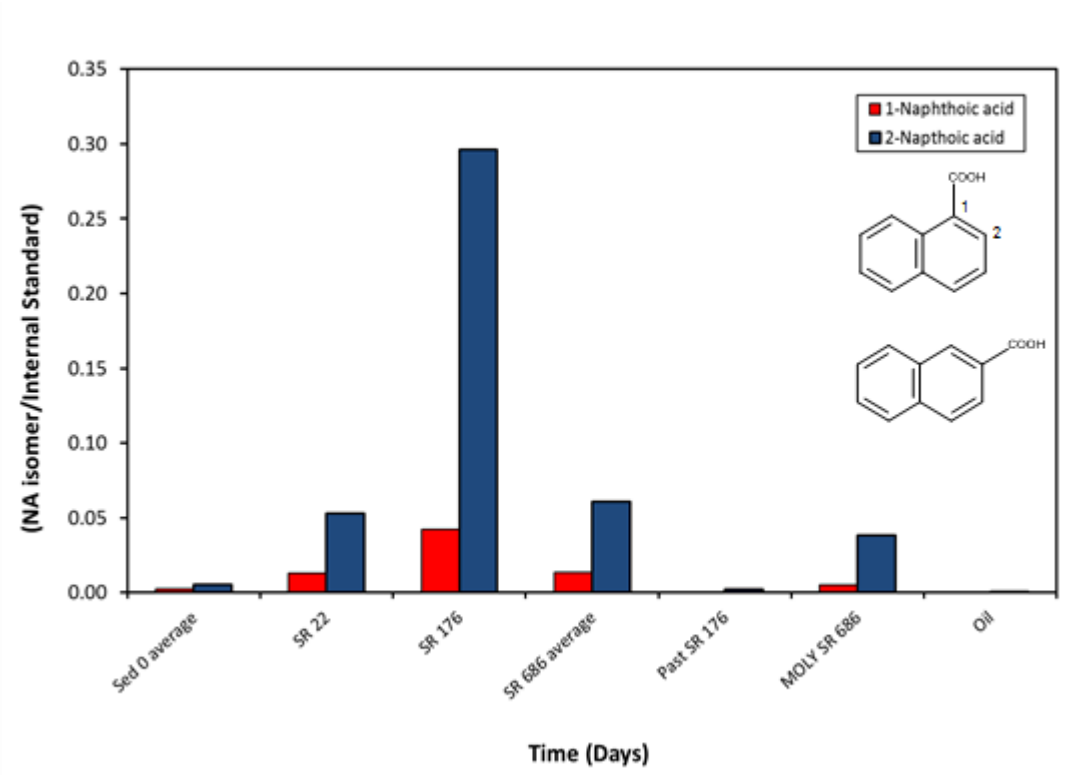

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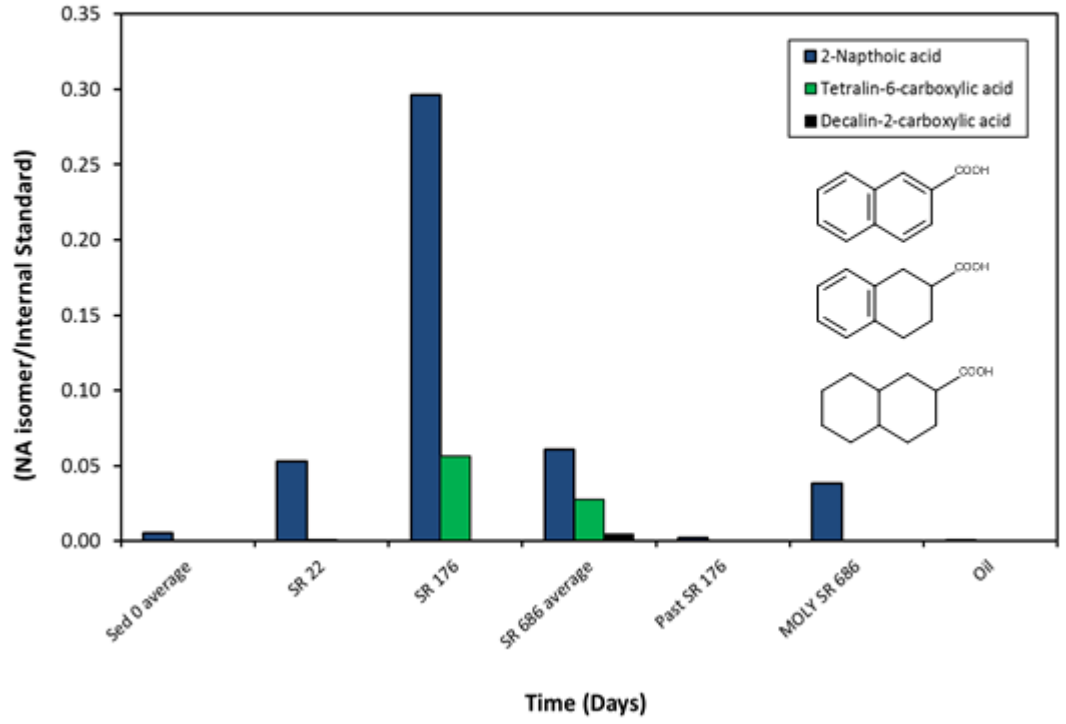

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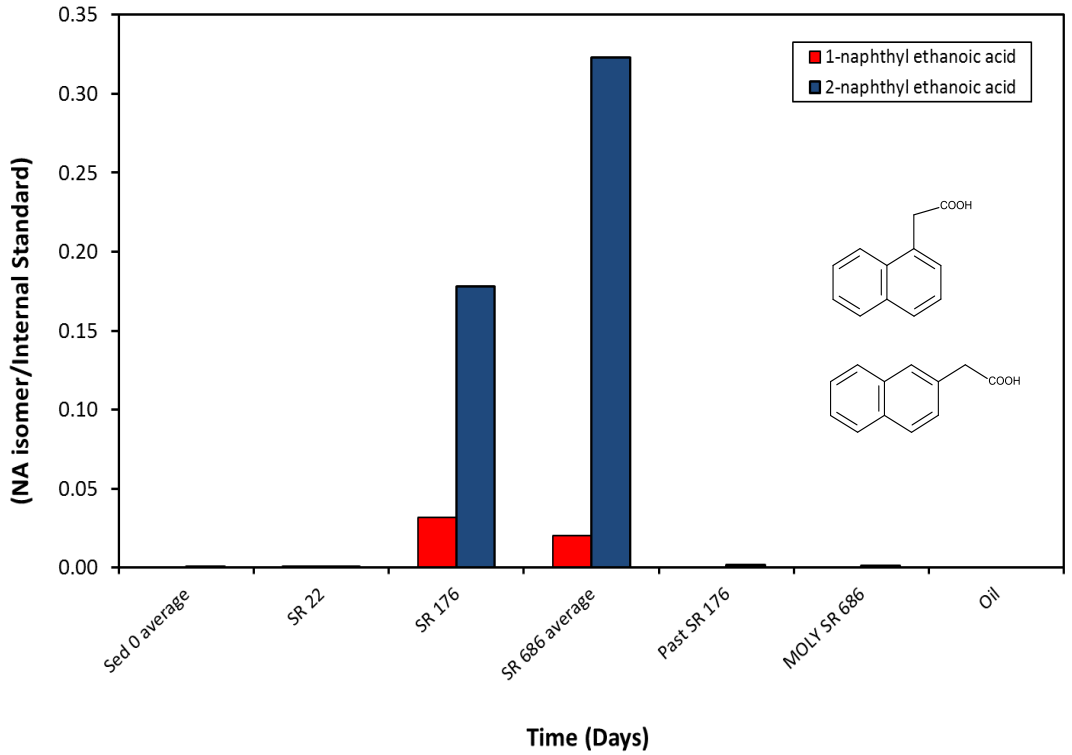

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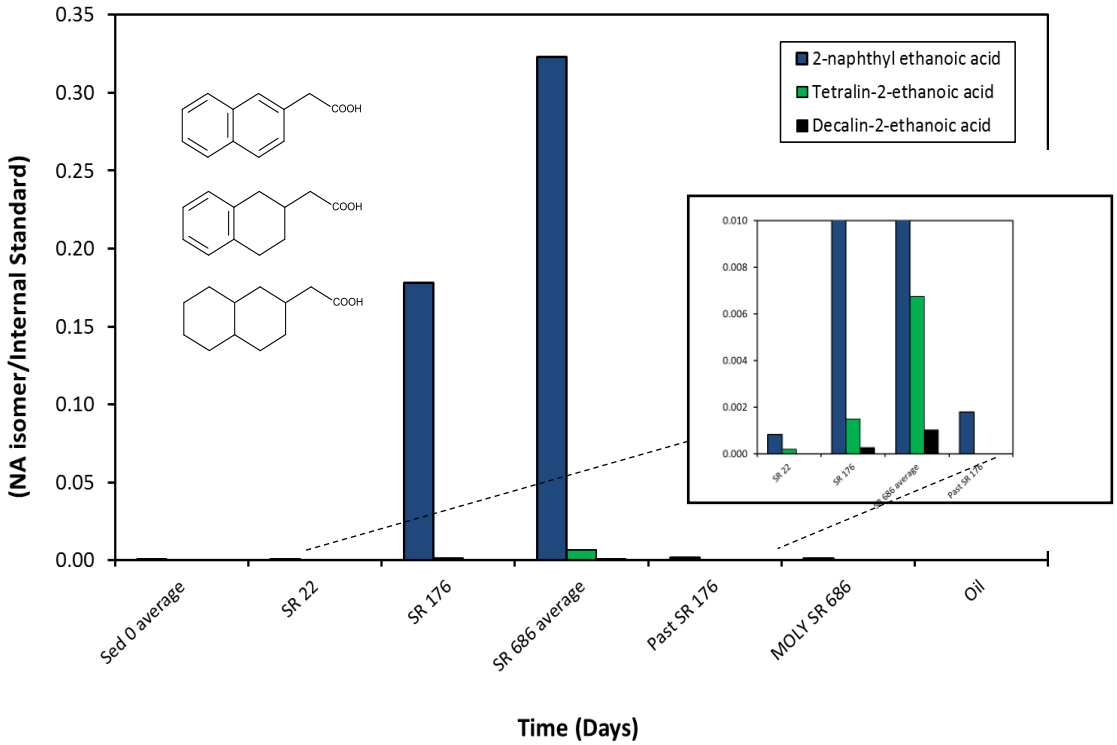

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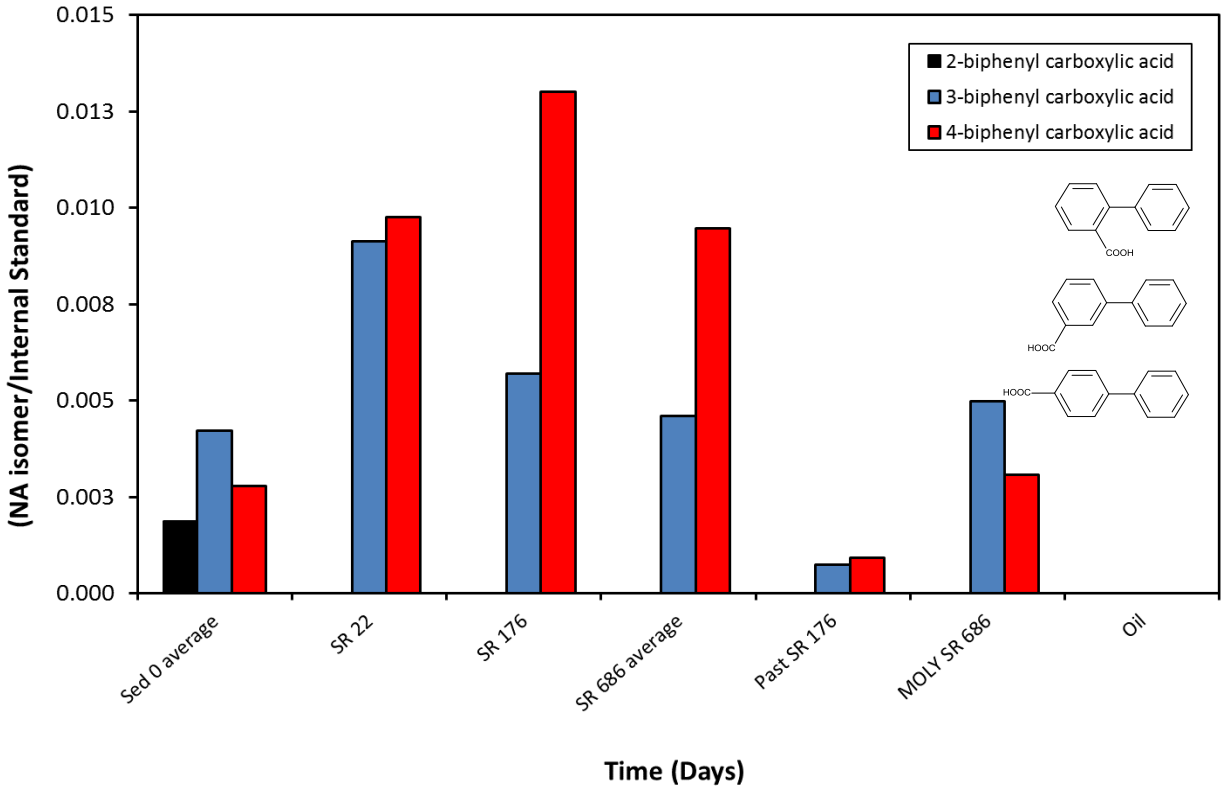

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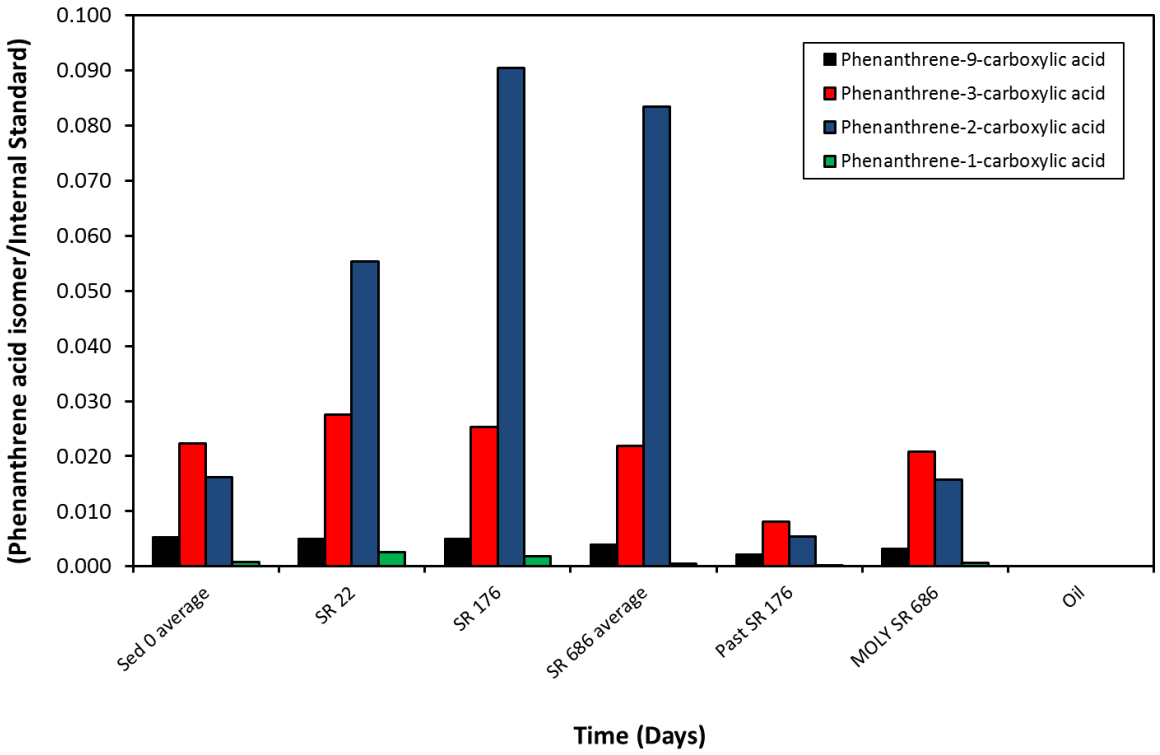

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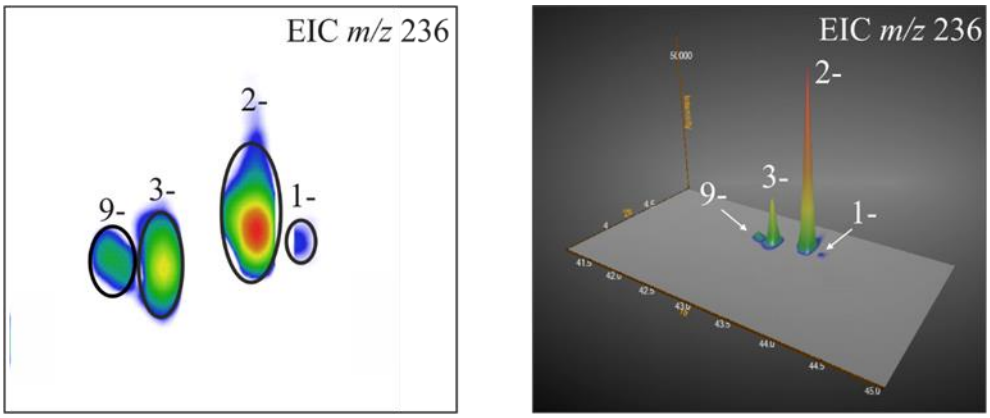

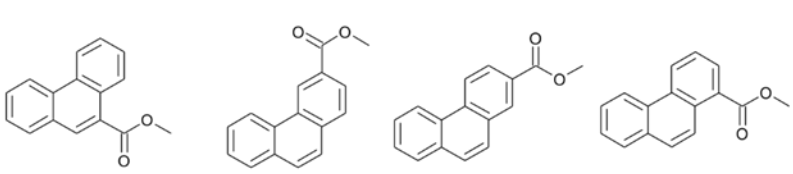

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Supplementary Information

Comprehensive two-dimensional gas chromatography-mass spectrometry of complex mixtures of anaerobic bacterial metabolites of petroleum hydrocarbons

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751 **Figure S1**

752 GCxGC-MS Total ion current chromatograms illustrating differences in acid metabolite (methyl
753 ester) profiles under Pasteurised and killed control (molybdate-treated) conditions at 176 and
754 686 days.

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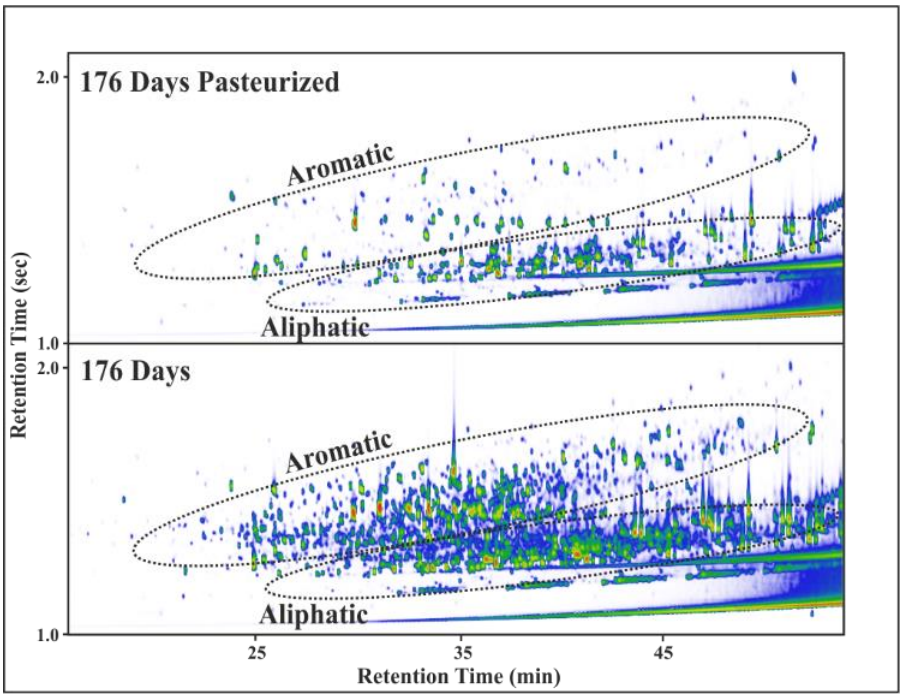

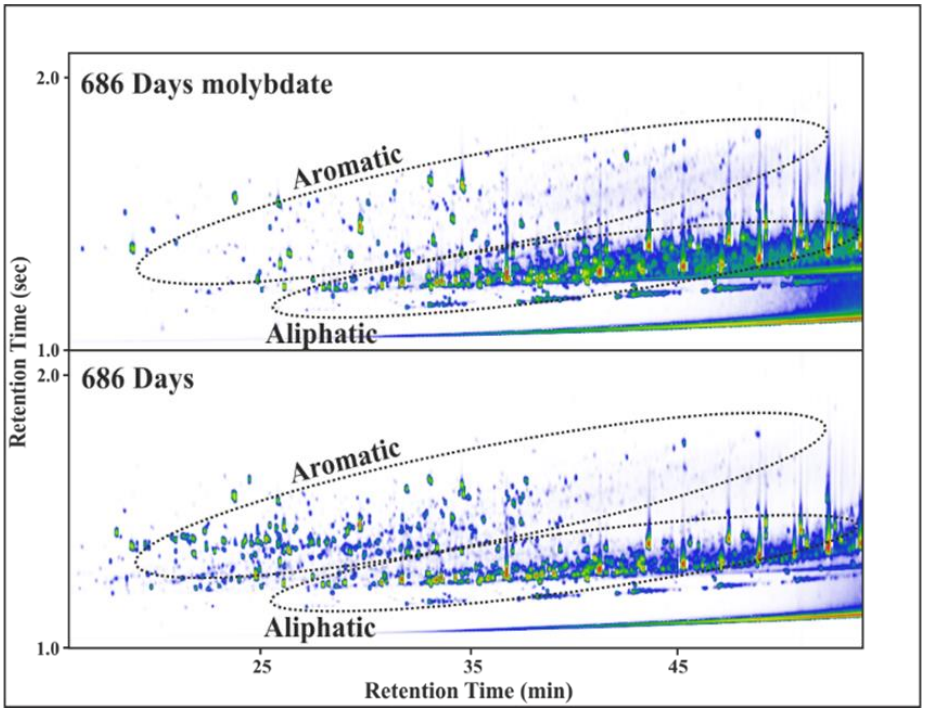

758 **Figure S2.**

759 Electron ionisation mass spectrum of one of three tentatively assigned methylphenylitaconates
760 (dimethyl esters) acidic extracts of crude oil inoculated for 22 days with Tyne sediment under
761 sulphate reducing conditions.

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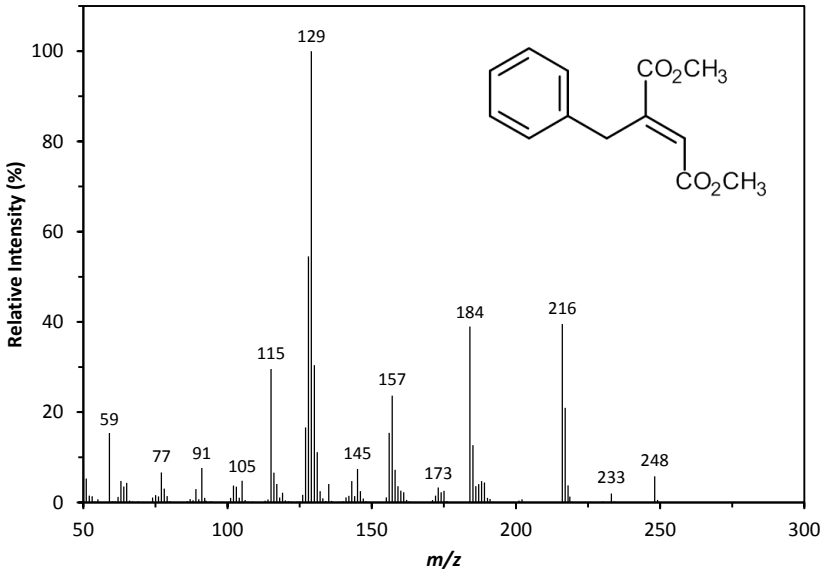

764 **Figure S3.**

765 Extracted ion current (m/z 250) mass chromatogram and mass spectra of tentatively assigned
766 methylbenzylsuccinates (dimethyl esters; a, b) in acidic extract of crude oil inoculated for 176
767 days with Tyne sediment inoculum under sulfate reducing conditions.

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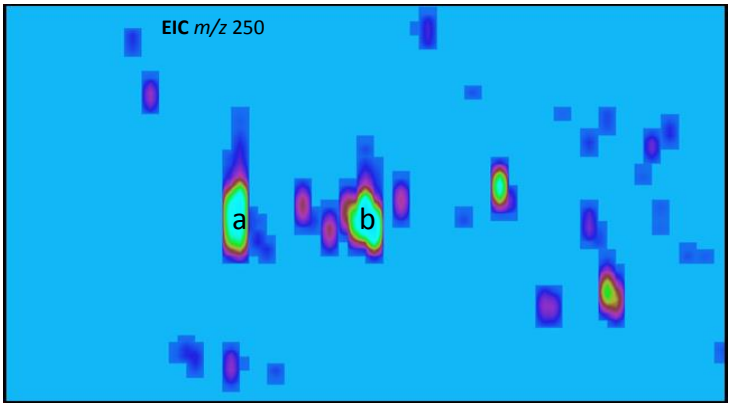
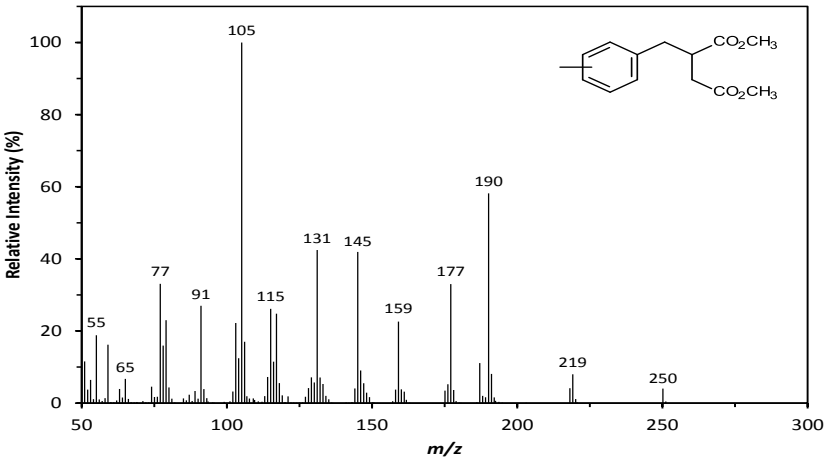
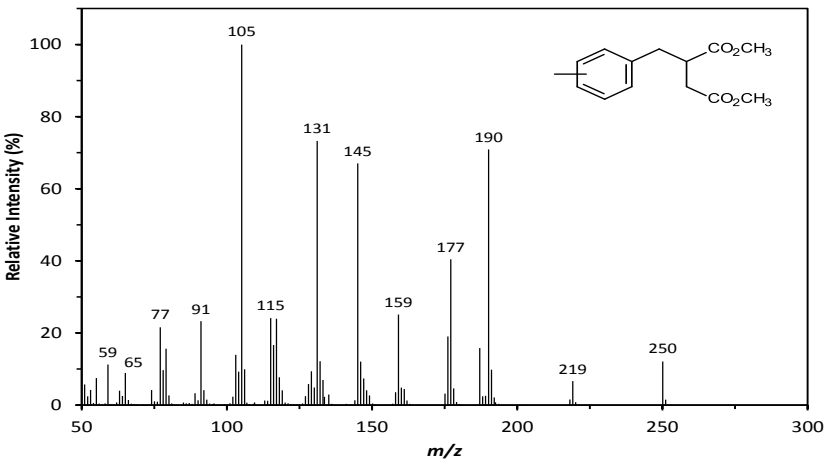


a

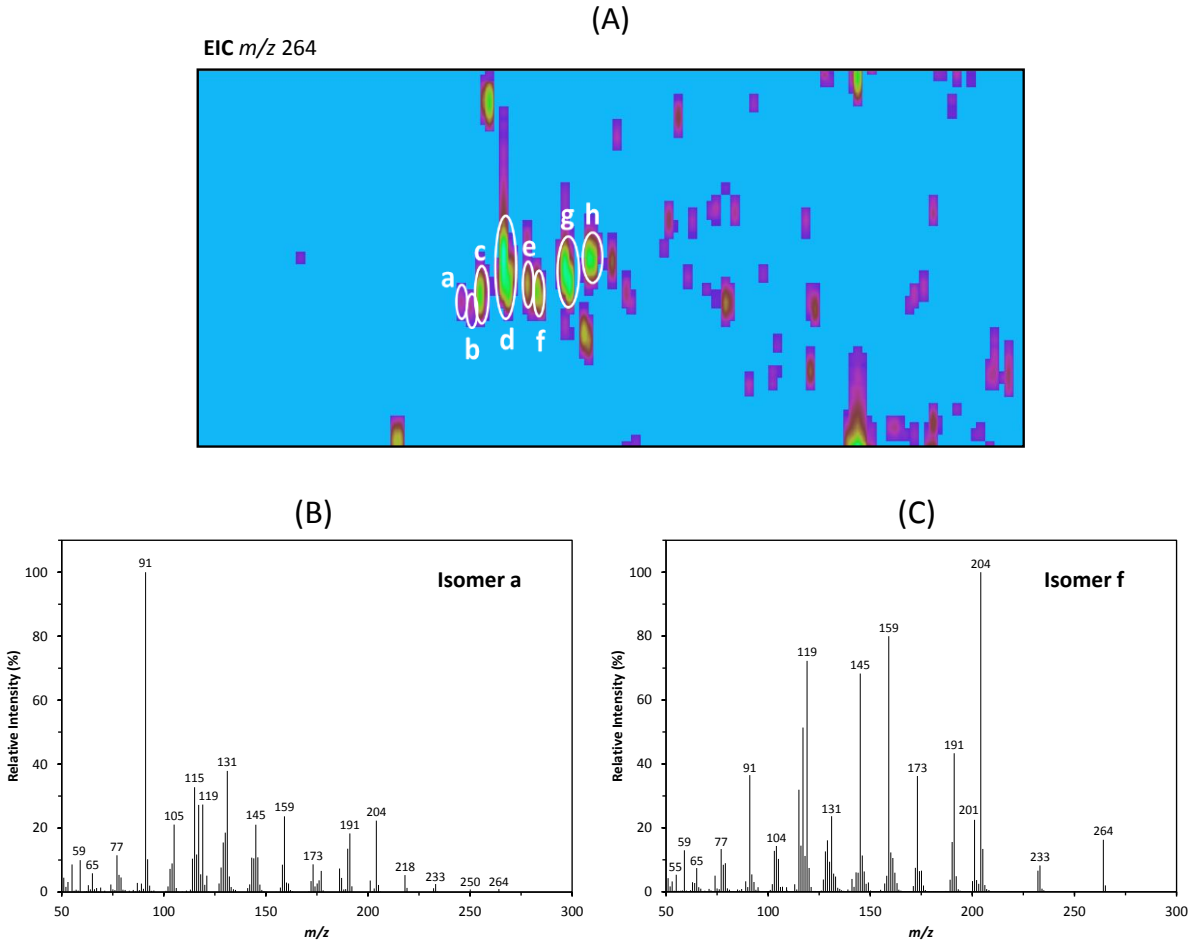


b



772 **Figure S4.**

773 Extracted ion current (m/z 264) mass chromatogram (A) and mass spectra (B,C) of two of eight
774 tentatively assigned dimethylbenzylsuccinates (dimethyl esters; a-e) in acidic extract of crude
775 oil inoculated for 176 days with Tyne sediment inoculum under sulfate reducing conditions.



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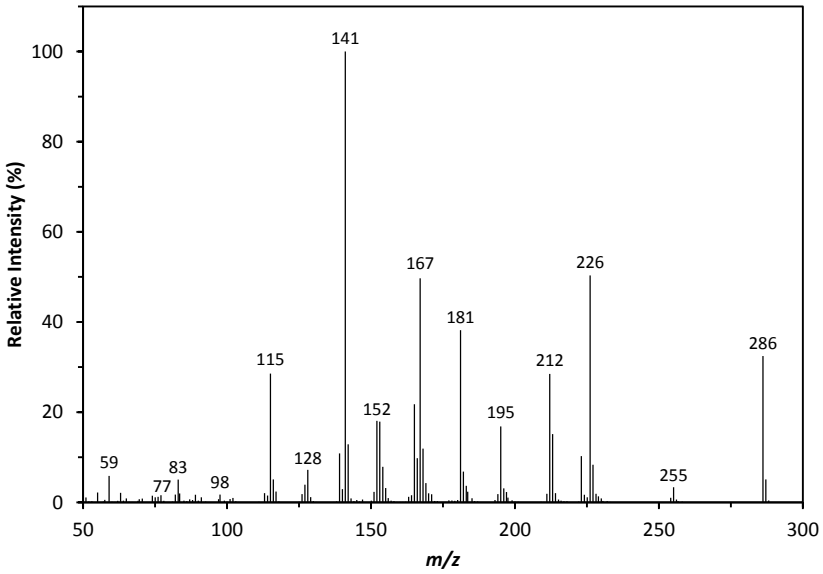
778 **Figure S5.**

779 Electron ionisation mass spectrum of naphthyl-2-methylsuccinate (dimethyl ester) in acidic
780 extract of crude oil inoculated for 176 days with Tyne sediment under sulphate reducing
781 conditions.



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783

784 **Figure S6.**

785 Mass spectra of C_1 and C_2 naphthyl-2-methylsuccinates (dimethyl esters; a-d) in acidic extract of
786 crude oil inoculated for 176 days with Tyne sediment inoculum under sulfate reducing
787 conditions.

788

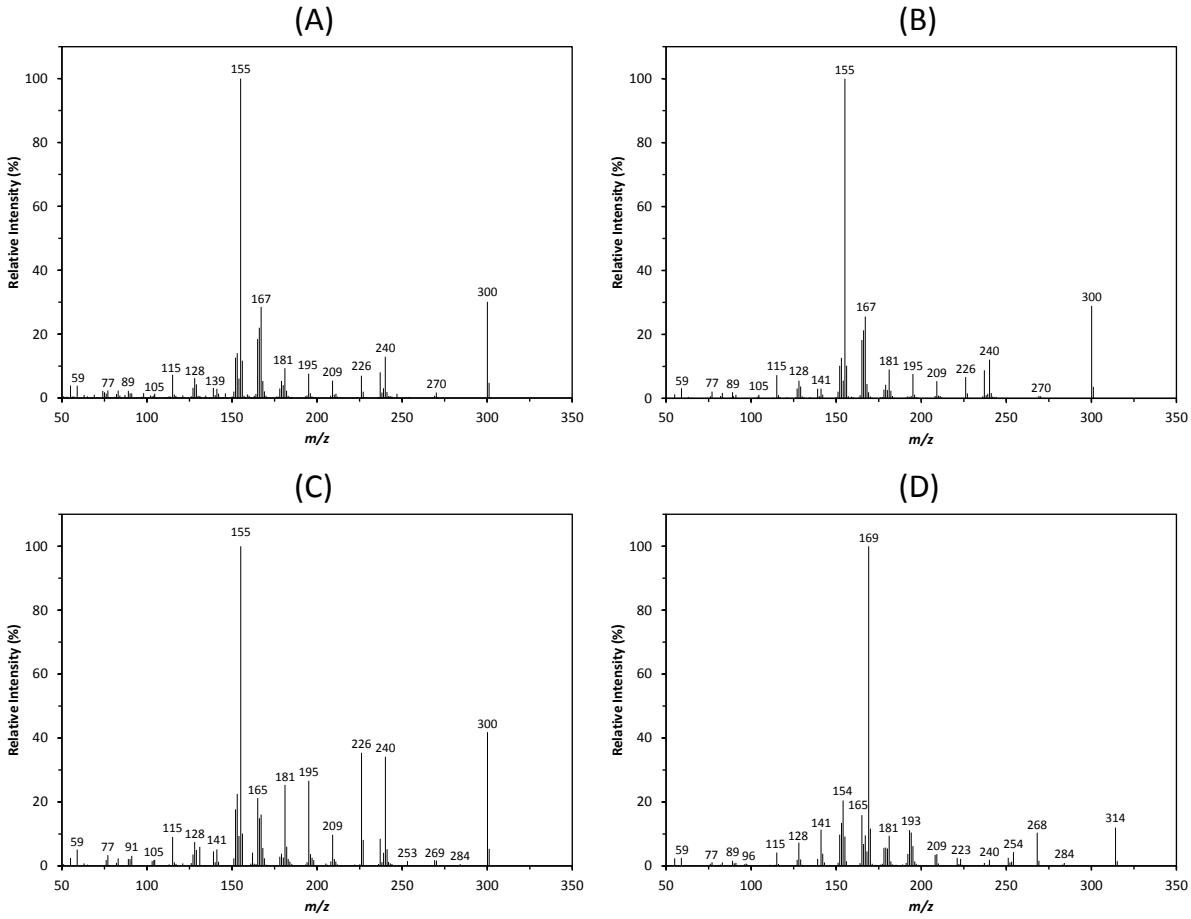

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Figure S7. Concentrations of 1- Purple), 2- (green) ,3- (red) and 9- (blue) phenanthrene carboxylic acids (versus internal standard) measured as methyl esters by GC×GC-MS in acidic extracts of crude oil and in crude oil inoculated for 0-686 days with Tyne sediment inoculum under sulfate reducing conditions. Data for 686 day incubated sample is mean shown for n=3 replicates.