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

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1 **Comprehensive two-dimensional gas**
2 **chromatography-mass spectrometry of complex**
3 **mixtures of anaerobic bacterial metabolites of**
4 **petroleum hydrocarbons**

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27 **Highlights**

- 28
- Two year incubations of crude oil with sediment bacteria under anaerobic

29 sulfate-reducing conditions.

 - Complex mixtures of acid and diacid metabolites studied by GCxGC-MS as

30 methyl esters.

 - Range of known metabolites extended from those of benzene through to

31 those of methylphenanthrenes.

 - Compounds identified by mass spectra and co-chromatography with synthetic

32 acids (methyl esters).

 - Sequential order of degradation of aromatic hydrocarbons established and

33 compared with those reported for reservoired petroleum.

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39 ABSTRACT

40 Anaerobic biotransformation of petroleum hydrocarbons is an important alteration
41 mechanism, both subsurface in geological reservoirs, in aquifers and in anoxic deep
42 sea environments. Here we report the resolution and identification, by
43 comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-
44 MS), of complex mixtures of aromatic acid and diacid metabolites of the anaerobic
45 biodegradation of many crude oil hydrocarbons. An extended range of metabolites,
46 including alkylbenzyl, alkylindanyl, alkyltetralinyl, alkyl-naphthyl succinic acids and
47 alkyltetralin, alkyl-naphthoic and phenanthrene carboxylic acids, is reported in
48 samples from experiments conducted under sulfate-reducing conditions in a
49 microcosm over two years. The range of metabolites identified shows that the
50 fumarate addition mechanism applies to the alteration of hydrocarbons with up to C₈
51 alkylation in monoaromatics and that functionalisation of up to three ring aromatic
52 hydrocarbons with at least C₁ alkylation occurs. The GC×GC-MS method might now
53 be applied to the identification of complex mixtures of metabolites in samples from
54 real environmental oil spills.

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

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64 1. Introduction

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

74

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

83

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

88 [12,17], but the importance of anaerobic mechanisms has been realised increasingly
89 (e.g. reviewed in [17-24] and references therein).

90

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

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

103

104 Wilkes et al. [37] studied the biotransformation of several crude oils for 60 days when
105 incubated with two strains of sulfate-reducing bacteria. They observed
106 transformation of C₁₋₅ alkylbenzenes with production of C₁₋₂ alkylated benzoic acids
107 and traces of the corresponding succinates. Townsend et al. [30] studied the
108 biodegradation of two crude oils by microorganisms from an anoxic aquifer
109 previously contaminated by natural gas condensate, under methanogenic and
110 sulfate-reducing conditions. They showed that whilst n-alkanes were relatively labile,
111 bicyclic aromatic hydrocarbons were more recalcitrant and the biodegradation of

112 these substrates appeared to be sulfate-dependent and homologue-specific.

113 However, the metabolites of the hydrocarbons were not studied.

114

115 Aitken et al., [22] conducted a two-year anaerobic incubation of a North Sea crude oil

116 under sulfate-reducing and methanogenic conditions and studied both the

117 degradation of the hydrocarbons and production of aliphatic acid metabolites.

118 Comparison of hydrocarbon compositional information after ~ 700 days of anaerobic

119 biodegradation revealed that, under sulfate-reducing conditions, changes were

120 observed. However, the complex nature of the acid profiles when examined by GC-

121 MS of the methyl esters restricted identifications of the aromatic metabolites, even

122 though the distributions of many of the aromatic hydrocarbons had changed.

123

124 Jarling et al. [21] also studied the metabolites produced by anaerobic degradation of

125 crude oil and binary mixtures of hydrocarbons, by 11 individual bacterial strains. By

126 GC-MS they identified, not only co-metabolically formed alkyl and arylalkylsuccinates

127 from fumarate addition, but also products of anaerobic hydroxylation of

128 alkylbenzenes, by sulfate reducers.

129

130 In the present study, we examined by GC×GC-MS, the aromatic acid metabolites

131 produced during two year incubations of a crude oil under sulfate-reducing

132 conditions. The study focuses almost entirely on the analytics; identification of the

133 products helped to reveal the extent and further indicated evidence, of multiple

134 pathways of transformation. Up to three ring aromatic hydrocarbons were

135 biotransformed, extending the range of those observed in studies of pure

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

138

139 **2. Experimental**

140 *2.1 Incubations*

141 The conditions and methods used for the two year anaerobic incubation of a North
142 Sea crude oil have been described fully elsewhere [22]. Briefly, microcosms were
143 prepared in sterile glass serum bottles (120 mL) in an anaerobic cabinet (Coy
144 Laboratory Products Inc., MI, USA) fitted with an oxygen sensor and with a regulated
145 atmosphere of nitrogen (99.5%) and hydrogen (0.5%) (BOC Special Gases Ltd). All
146 microcosms were incubated in the cabinet in the dark to ensure anaerobic conditions
147 were maintained. Each microcosm comprised a carbonate buffered nutrient medium
148 containing sources of nitrogen and phosphorous, vitamins, and trace minerals,
149 prepared in deionised water for brackish conditions and 10 mL of sediment slurry (10
150 % v/v) from the River Tyne. The total volume of liquid in each microcosm was 100
151 mL with 20 mL of headspace. All microcosms were prepared in triplicate. Sulfate
152 reducing conditions were established in microcosms by the addition of Na₂SO₄ (final
153 concentration 20 mM) to the medium and Na₂S (0.5 mM) was added as a reducing
154 agent. Approximately 300 mg of North Sea oil was added as a carbon and energy
155 source to all the microcosms. Control microcosms with sodium molybdate (20 mM)
156 added to inhibit sulfate reduction were also prepared. Additional controls included
157 microcosms Pasteurised by heating at 95°C for 2 hours, and microcosms containing
158 no added oil (to assess sulfate reduction in the absence of oil). Microcosms were
159 incubated at room temperature (ca. 20°C) and for each treatment 6 sets of
160 microcosms were prepared for sacrificial sampling over a two year period to allow

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

164

165 Sediment samples used as inoculum were taken from the River Tyne near
166 Scotswood Bridge, Newcastle, UK (54.96 °N, 1.68 °W). Sulfate concentrations from
167 16 cm long sediment cores taken at low tide ranged from 2.91 mM (surface) to 7.04
168 mM (8cm depth) with a value of 6.25 mM at 16cm, the average depth of sediment
169 samples taken for inoculum in microcosms. The acids of killed controls (molybdate or
170 Pasteurised), undegraded oil and sediment inoculum, were all studied as methyl
171 esters, in addition to samples of oil degraded for 22, 176 and 686 (three replicates)
172 days under sulfate-reducing conditions. (Whilst samples were also studied at 302
173 days in a previous study of non-aromatic metabolites [22], only analyses of the
174 aforementioned samples were made herein). Sulfate reduction was monitored by
175 hydrocarbon depletion and methane (non-) generation [22].

176 *2.2 Metabolite identification and measurement by GCxGC-MS*

177 GCxGC-MS analyses of acid fractions derivatised by refluxing with BF₃-methanol
178 were conducted essentially as described previously for other mixtures [12]. Briefly,
179 analyses were conducted using a model 7890A gas chromatograph (Agilent
180 Technologies, Wilmington, DE, USA) fitted with a ZX2 GCxGC cryogenic modulator
181 (Zoex, Houston, TX, USA) interfaced with a BenchTOFdx™ time-of-flight mass
182 spectrometer (Almsco International, Lantrisant, UK) operated in positive electron
183 ionisation mode and calibrated with perfluorotributylamine. The scan speed was 50
184 Hz. The first-dimension column was a 95% dimethyl polysiloxane 5% diphenyl
185 polysiloxane fused-silica capillary HP-5ms (30m × 0.25 mm × 0.25 μm; Agilent

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

199

200 *2.3 Authentic acids*

201 1- and 2-naphthoic acids and phenanthrene-3- and 4-carboxylic acids were
202 purchased from Sigma-Aldrich (Poole, UK). 1- and 2-tetralin carboxylic acids and the
203 corresponding decalin acids were synthesised by partial or complete hydrogenation
204 of the aromatic analogues [12]. Phenanthrene-2- and phenanthrene-9-carboxylic
205 acid were synthesised by refluxing a mixture of either 2-acetylphenanthrene or 9-
206 acetylphenanthrene (Sigma-Aldrich, Poole, U.K.) with a 5% solution of sodium
207 hypochlorite for 24 h according to the procedure of Dixon and Neiswender [38].

208

209 3. Results and discussion

210 Aitken et al., [22] have described the overall changes in incubated crude oil
211 hydrocarbon composition during the 686 days of the sulfate-reducing microcosm in
212 some detail. Briefly, the microcosms initially all contained the same undegraded
213 North Sea crude oil, nutrients and sediment-associated micro-organisms. Sulfate
214 was added to induce sulfate-reducing conditions, which were monitored by the
215 depletion of alkanes and the (non-) production of methane [22]. Analysis of River
216 Tyne sediments sampled at the same location and depth as the inoculum (the
217 source of the microbes) confirmed the presence of only low concentrations of sulfate
218 in the sediments initially [22]. Cumulative methane generation was less than 100
219 μmoles by 300 days and in microcosms containing inhibitors, cumulative headspace
220 methane was $<250 \mu\text{moles}$ over 686 days. Thus sulfate reduction, not
221 methanogenesis, was the major anaerobic mechanism. Total bacterial numbers
222 changed little over the incubation period, but specific anaerobes implicated in
223 hydrocarbon degradation were enriched by 3 orders of magnitude to 10^8 cells/g
224 sediment [22]. As a general indication of the progress of the overall removal of oil,
225 alkane concentrations (n-C₇₋₃₄) decreased from about 2000 μmoles carbon to about
226 250 μmoles carbon over the 686 days. Approximately two thirds of the n-C₁₂₋₂₆
227 alkanes were consumed by 302 days, after which their rate of removal decreased,
228 such that at 686 days only about 10% of the initial n-alkanes remained [22].

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

232

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

241

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

258 applicable to the production of kinetic data for such environments. Rather, the
259 variations (Table 1) indicate the sequence of biotransformation events and
260 progressive metabolism of the substrate hydrocarbons and intermediate acids
261 (Figures 6-9). Whilst aromatic and non-aromatic hydrocarbons can be separated
262 routinely by open column chromatography techniques before analysis by GC-MS
263 (even though many of these are then still revealed only as UCMs), separation of the
264 corresponding aromatic acids and di-acids (e.g. succinates) from the non-aromatic
265 acids (as acids or esters) is less trivial. The use of GCxGC-MS is particularly
266 advantageous in resolving the aromatic analytes of the total esterified acid mixtures,
267 particularly in the second dimension (Figure 1) and even the non-aromatic analytes
268 were better resolved by GCxGC-MS from co-eluting interferents, than by GC-MS (cf
269 [22]).

270 Fumarate addition is perhaps the best known mechanism for anaerobic
271 transformation of aromatic hydrocarbons (reviewed in [17], [21]). The proposed
272 mechanism produces 'signature' succinate metabolites, usually by addition to the
273 benzylic position in alkylaromatics with C₁₋₃ alkyl groups, or by addition to the omega
274 minus two or omega minus three positions in aromatics with longer side chains (e.g.
275 Figure 2; [21]). These were revealed by GCxGC-MS herein (Figures 1, S1).
276 Alternatively, anaerobic hydroxylation of aromatic hydrocarbons can occur, with
277 further transformations resulting in aromatic carboxylic acids (Figure 2; [21]).
278 However, such simple acids are less specific to anaerobic processes than the
279 characteristic succinates, since these acids can result from both processes by further
280 transformation (Figure 2; [12, 21]). Nonetheless, these could also be identified by
281 GCxGC-MS (Figures 1, S1).

282

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 benzy succinates (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 methylbenzy succinates (Figure 2). It was thus not surprising that benzy succinate
291 and methylbenzy succinates 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 benzy succinate and
297 methylbenzy succinates were also present in the 176 and 686 day samples (e.g.
298 Table 1; Figure S3). No benzy succinates or E-phenylitaconates or associated
299 methylbenzy 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),

308 alkyltoluenes (AT; Figure 2) and alkylxylenes (AX). These are common in crude oils
309 and oil-polluted environmental samples (e.g. [40, 41]). Consistent with this, the
310 samples incubated herein for 176 days contained alkylbenzylsuccinates (Figure 2),
311 identified from the mass spectra herein (Figure 3), as possessing C₀₋₈ alkyl groups.
312 The spectra were typified by low abundance molecular ions, ions due to loss of
313 methoxy (M-31), ions due to loss of methyl formate (M-60) and base peak ions (B⁺)
314 due to benzylic fragmentation (Figure 3). (Similar fragmentations occurred for the
315 higher benzologues (e.g. Figures 4-6)).

316 A series of benzylsuccinates with carbon chain lengths from 5 to 10 was detected in
317 deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill
318 [19], but no spectra were published. Some of the C₁ analogues of the latter have
319 been shown to derive from anaerobic transformation of xylenes (e.g. [39, 42]) and it
320 is a reasonable assumption that the succinates with longer chain alkyl substituents in
321 the environment were derived from the corresponding AB from the Macondo oil, as
322 supported by the identifications in the present laboratory study.

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

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

337 Aerobic biodegradation of a crude oil previously showed production of C₀₋₃
338 phenylalkanoic acids within 5 days incubation, with their complete removal within 80
339 days [44], so accumulation of these acids might be a useful indication of the
340 operation of dominant anaerobic processes, as suggested previously [37].

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

357

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

359 Biotransformation of C₀₋₂ 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 C₂ and C₃
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 C₂₋₃
376 alkylnaphthalenes, in addition to the methylnaphthalenes, were observed, extending
377 the known range of metabolites and consistent with the removal of up to C₃
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 GCxGC-MS (Table 1) and

383 trends in the respective metabolites could be followed (Table 1; Figure 6). The data
384 showed that up to 176 days the incubation proceeded with generation of both 1- and
385 2-naphthoic acid, with the latter predominating (Figure 6A). This is consistent with
386 the known faster depletion of 2-methylnaphthalene, though it is unlikely that even the
387 2 year laboratory microcosms reproduce the kinetic effects observed in oil reservoirs.
388 After 176 days, both isomers were degraded further in the 'live' samples (Figure 6A).
389 Only trace quantities were present in the original oil and the sediment inoculum,
390 again with the 2-naphthoic acid isomer predominating (Figure 6A). No production
391 was observed in the 176 days Pasteurised sample.

392

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

406 markers of such processes. These can be monitored by GCxGC-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

431 The relative concentrations of 2-MP between days 0 and 686 reduced by ~3%. Over
432 the same period, changes in the relative distributions and amounts of phenanthrene-
433 2-carboxylic acid, identified by GCxGC-MS versus the synthesised compound, were
434 monitored (Figure 9). Changes in the other phenanthrene carboxylic acids were
435 minimal (Figure 9; Figure S7). These changes exceeded any influence produced by
436 the small amounts of the acids present in the sediment inoculum and the
437 undegraded crude oil (Figure 9). No succinates of the methyl phenanthrenes were
438 detected, possibly due rapid further transformation of these to the carboxylic acids.

439 No changes were observed in the dimethyl- and trimethylphenanthrenes, suggesting
440 the extent of biodegradation in this slow anaerobic sulfate-reducing process was only
441 sufficient to effect up to the MP isomers. A small amount of methylphenanthryl
442 carboxylic acid was tentatively identified herein by GCxGC-MS, suggesting incipient
443 degradation of C₂-phenanthrenes by day 686. These results indicate the greater
444 sensitivity of monitoring incipient biotransformation via production of the acid
445 metabolites, compared to monitoring changes in the hydrocarbons.

446

447 Some changes were also observed in the sulfur-containing aromatics. Minor
448 amounts of a benzothiophene and a C₁ benzothiophene carboxylic acid were
449 detected in the sediment inoculum, by comparison of the mass spectra with those of
450 authentic samples; these were not detected in the undegraded oil. Traces of these
451 compounds were present in the 22 and 176 day incubated samples but these were
452 absent by 686 days, suggesting further metabolism. Three dibenzothiophene
453 carboxylic acids were detected in the sediment inoculum, by comparison of the mass
454 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 C₈ alkylation in monoaromatics and
467 that functionalisation of up to three ring aromatic hydrocarbons with at least C₁
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.

479 In addition, the increased exploitation and processing of heavier and non-
480 conventional crude oils and other hydrocarbons (e.g. [7]), many of which have been
481 at least partially biodegraded and which contain higher than normal proportions of
482 so-called 'polar' NSO compounds (such as the diacids (O₄) and heteroacids (e.g.
483 SO₂) identified herein) is likely to lead to increased proportions of these polar
484 compounds, including some of the acids studied herein, in process and production
485 waters. An important example is the large proportions of acids in the process-
486 affected waters resulting from the oil sands industries [reviewed in 16]. Studies such
487 as those herein will lead to an increased understanding of the origins and fate of
488 such pollutants.

489

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

495

496

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509

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664

665

666 **Table and Figure Legends**

667 **Table 1**

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

671 Key: = not detected

672 = present in increasing relative amounts within compound class

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

675 **Figure 1.**

676 Extracted ion current A. (*m/z* 278) mass chromatogram and B-F, mass spectra of tentatively
677 assigned C₃-benzylsuccinate (dimethyl ester) and C_{4,5,6,7}-benzylsuccinates (dimethyl esters),
678 in acidic extract of crude oil incubated for 176 days with Tyne sediment inoculum under
679 sulfate reducing conditions.

680 **Figure 2.**

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

685 **Figure 3.**

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

689 **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 GCxGC/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 GCxGC/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.**

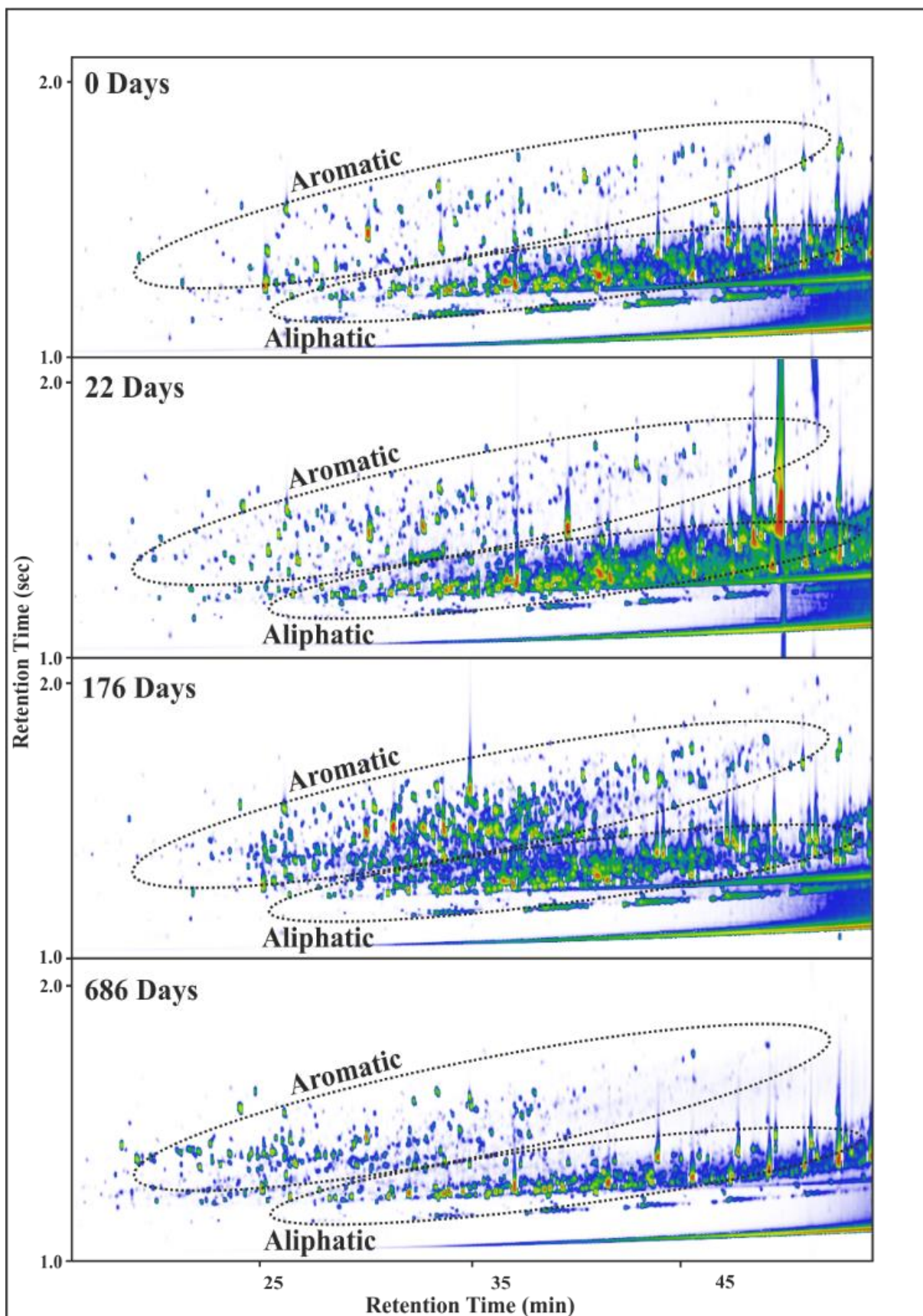
702 Concentrations of 2-, 3- and 4-biphenyl carboxylic acid (versus internal standard) measured
703 as methyl esters by GCxGC/MS in acidic extracts of crude oil and in crude oil incubated for
704 0-686 days with Tyne sediment under sulfate reducing conditions. Past = Pasteurised
705 control; MOLY = molybdate killed control. Data for 686 day are shown for mean of n=3
706 replicates.

707 **Figure 7.**

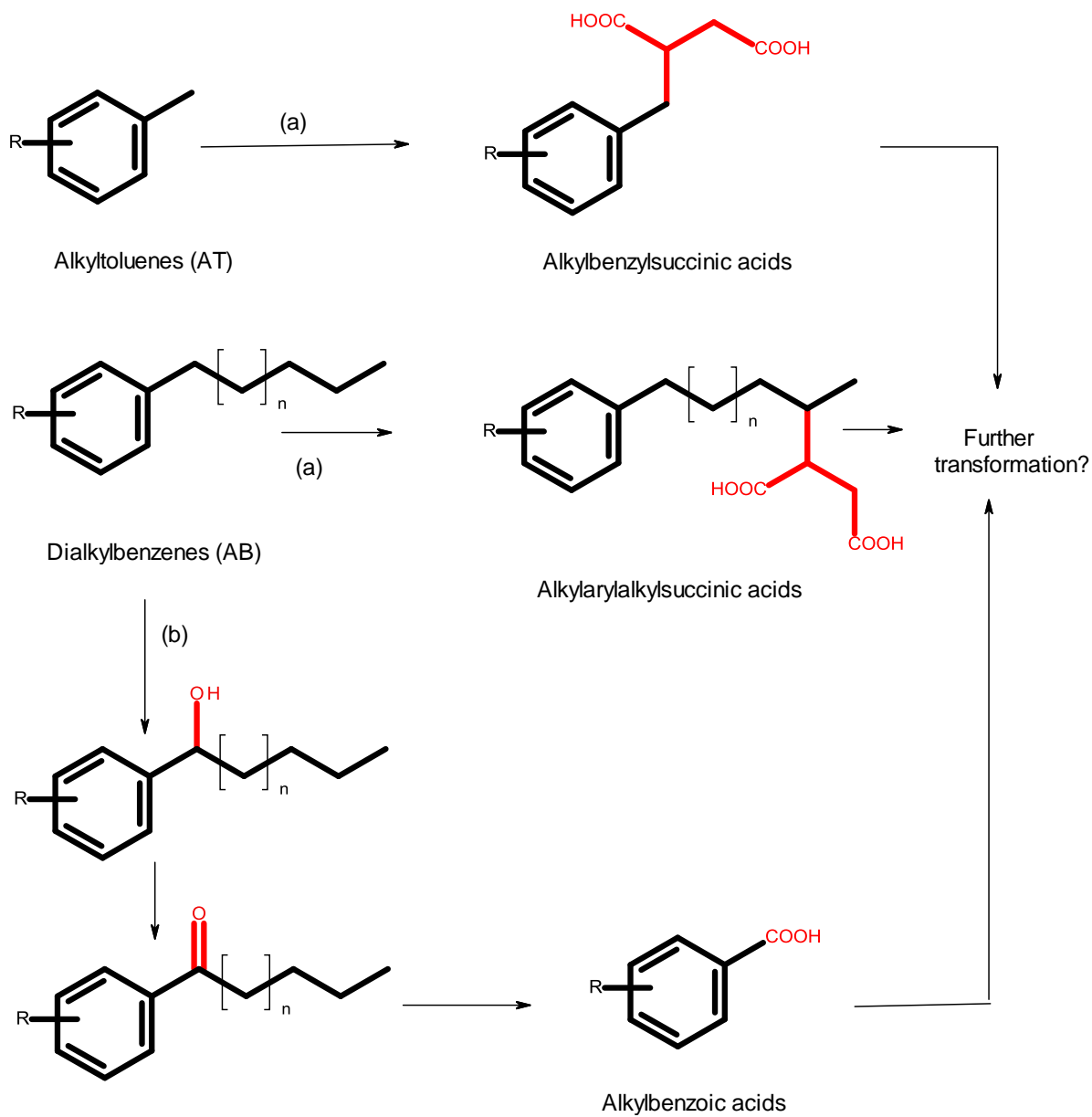
708 (A) Concentrations of 1-, 2-, 3- and 9- (4-absent) phenanthrene carboxylic acids (versus
709 internal standard) measured as methyl esters by GCxGC/MS in acidic extracts of crude oil
710 and in crude oil incubated for 0-686 days with Tyne sediment under sulfate reducing
711 conditions. Past = Pasteurised control; MOLY = molybdate 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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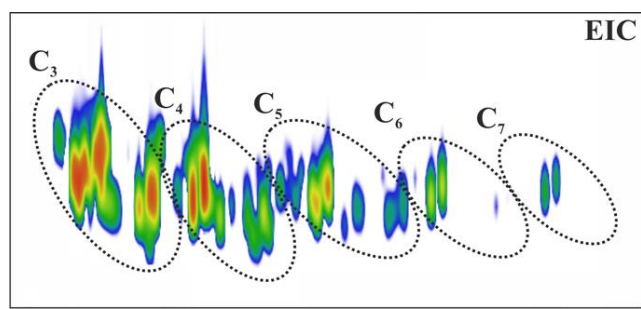


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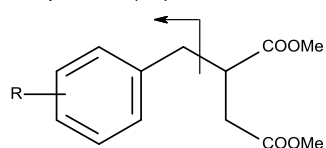
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(A)

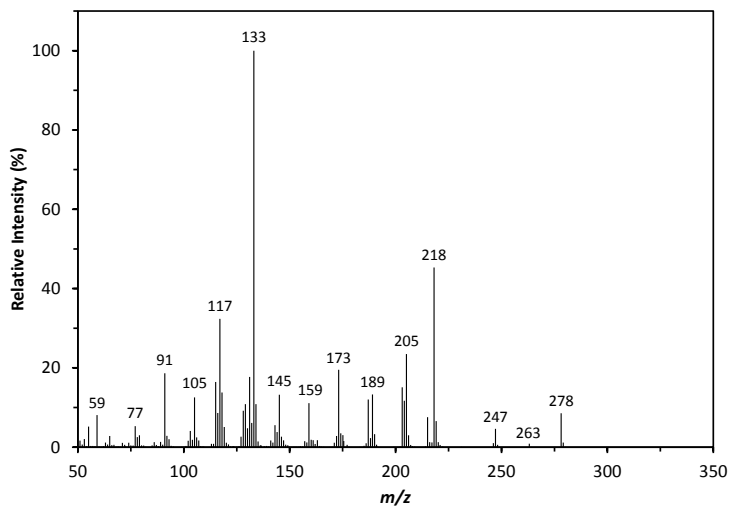


Base peak ion (B+) 133,147,161,175,189

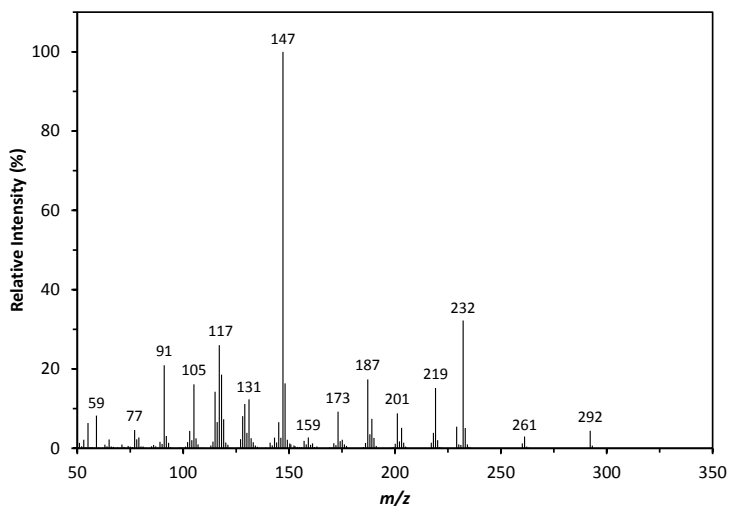
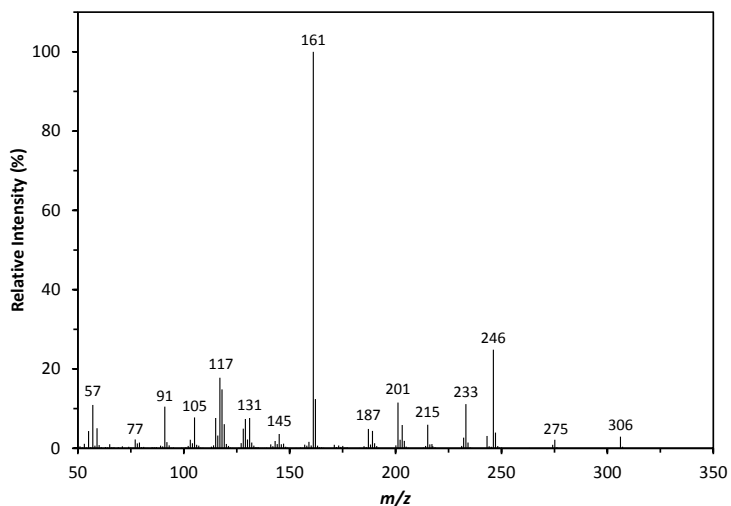


R=C3-7 (C)

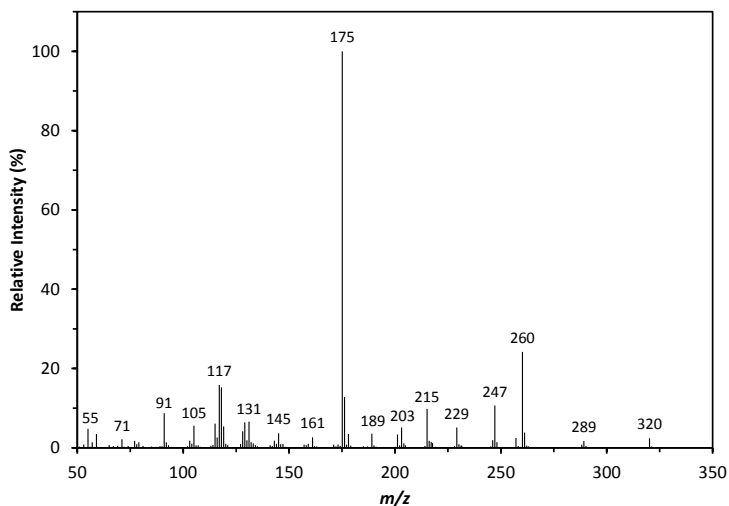
(B)



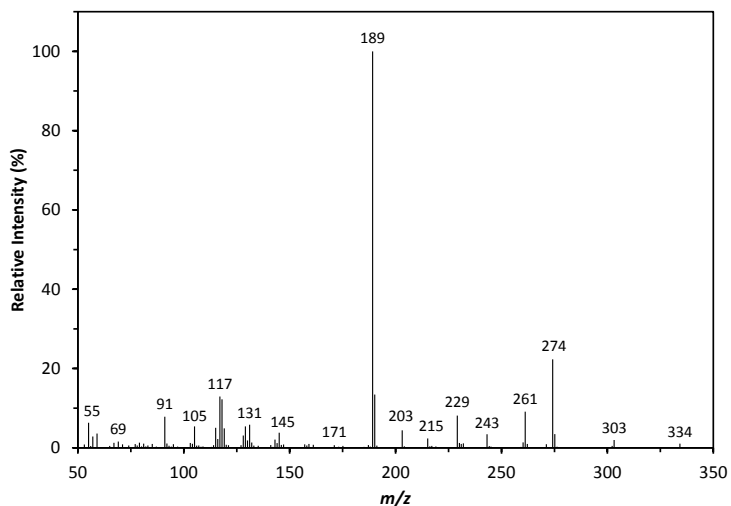
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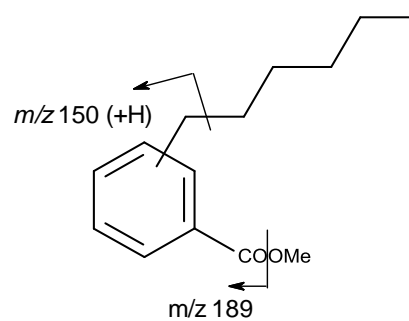
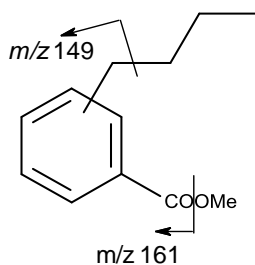
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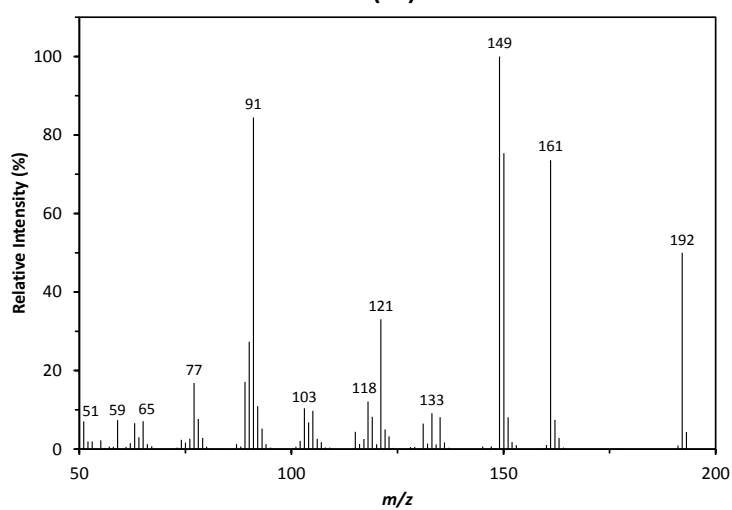
(F)



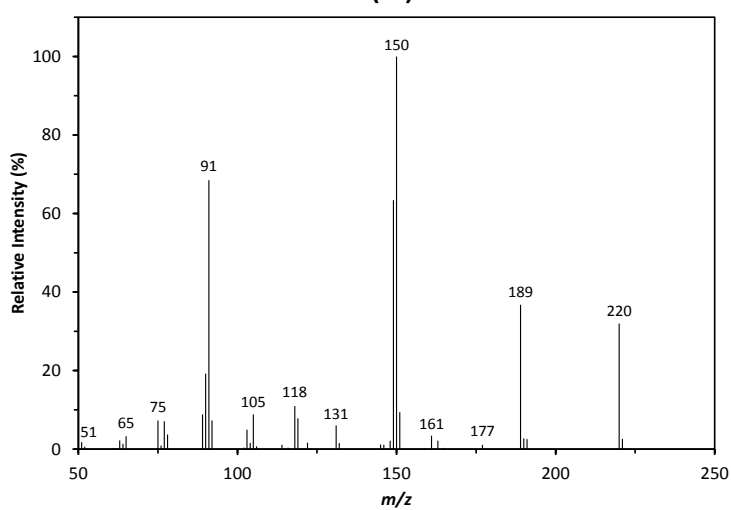
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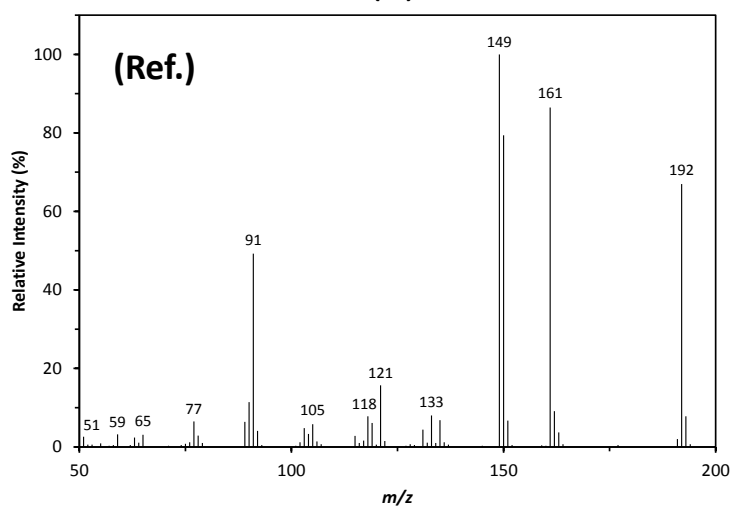
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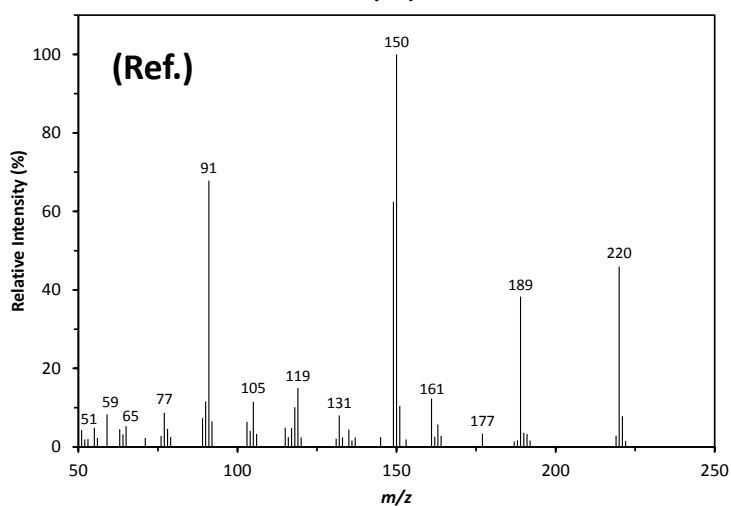
(B)



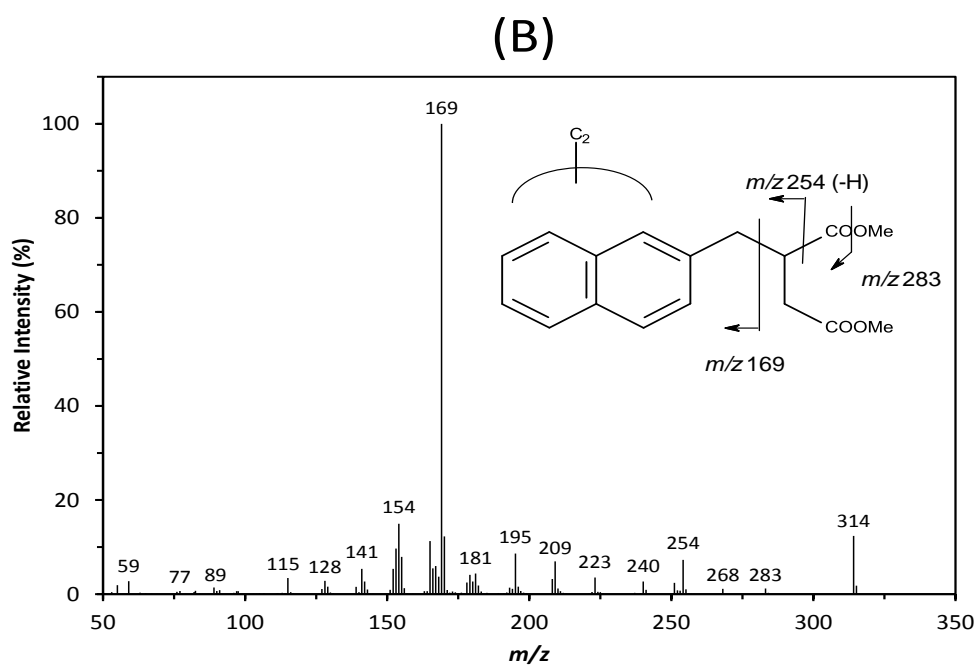
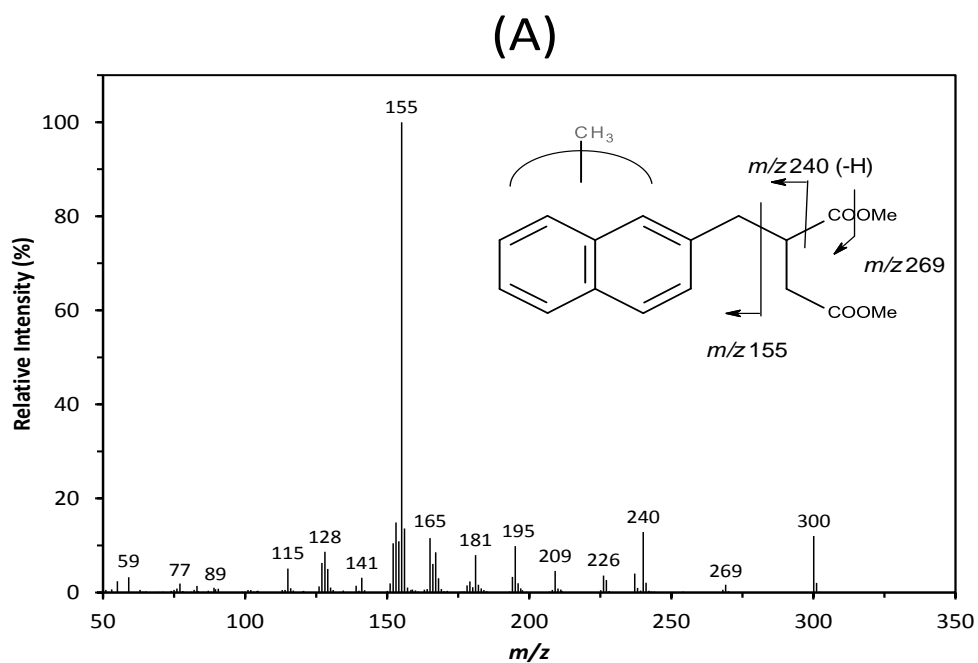
(C)



(D)

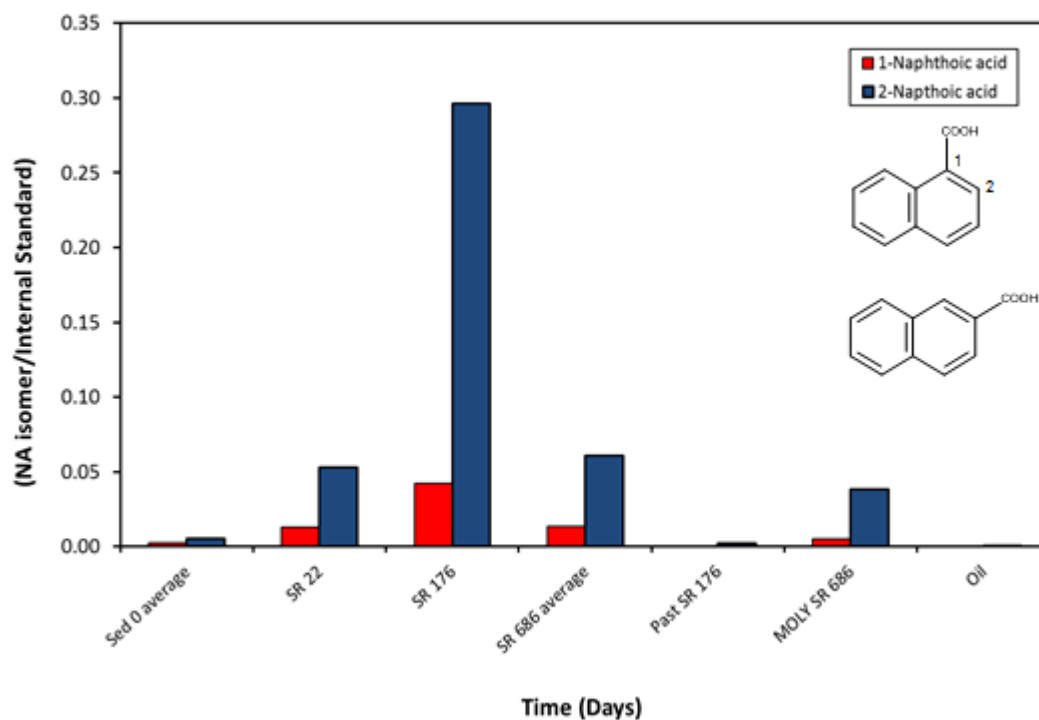


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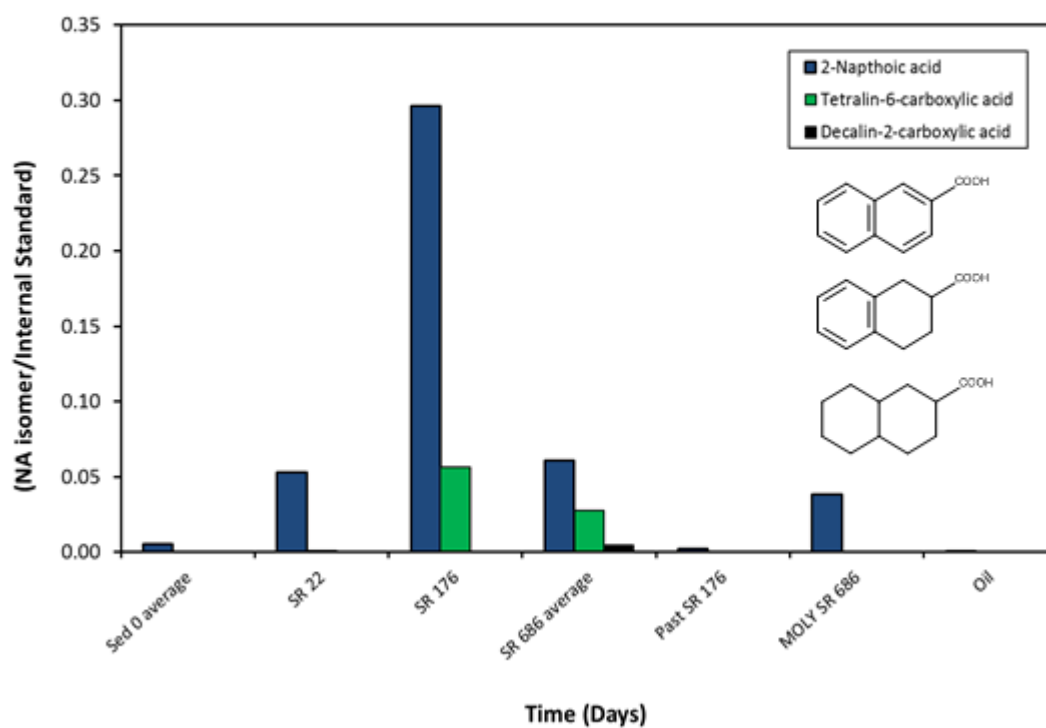


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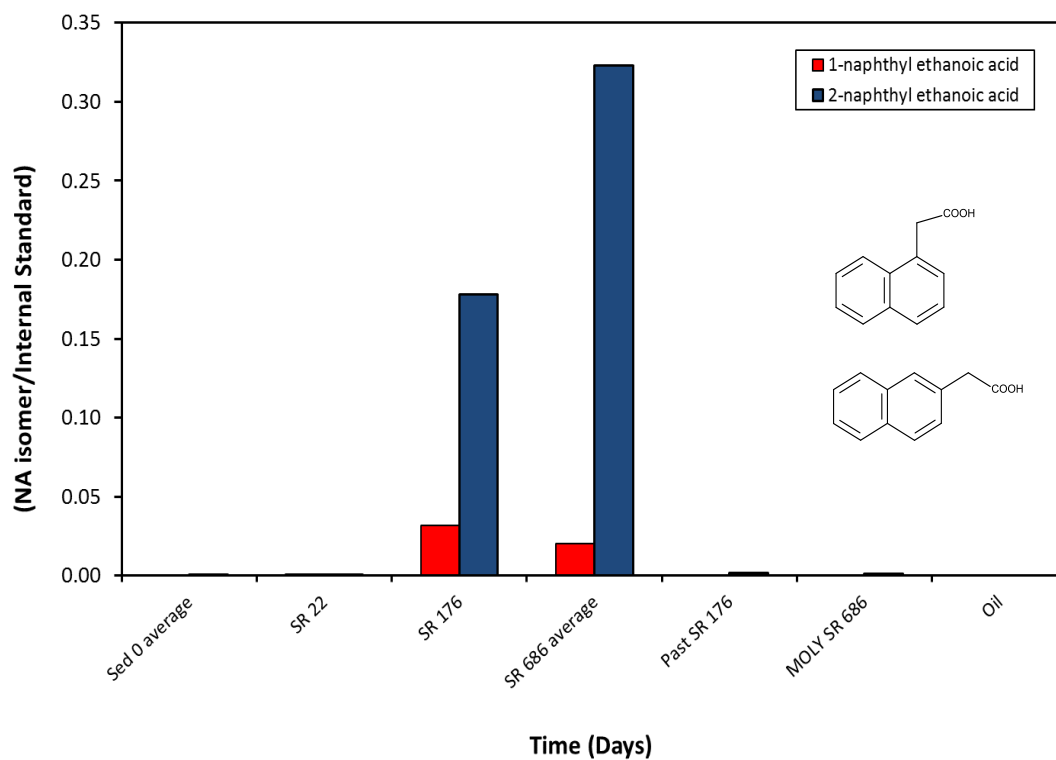
(A)



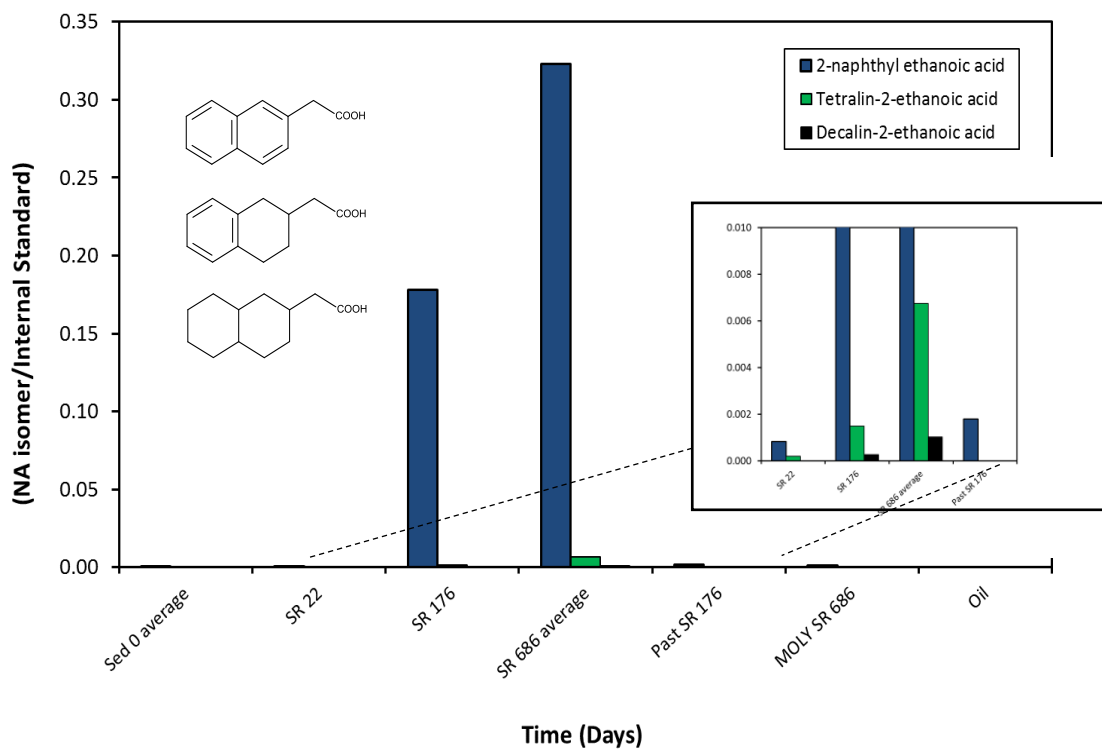
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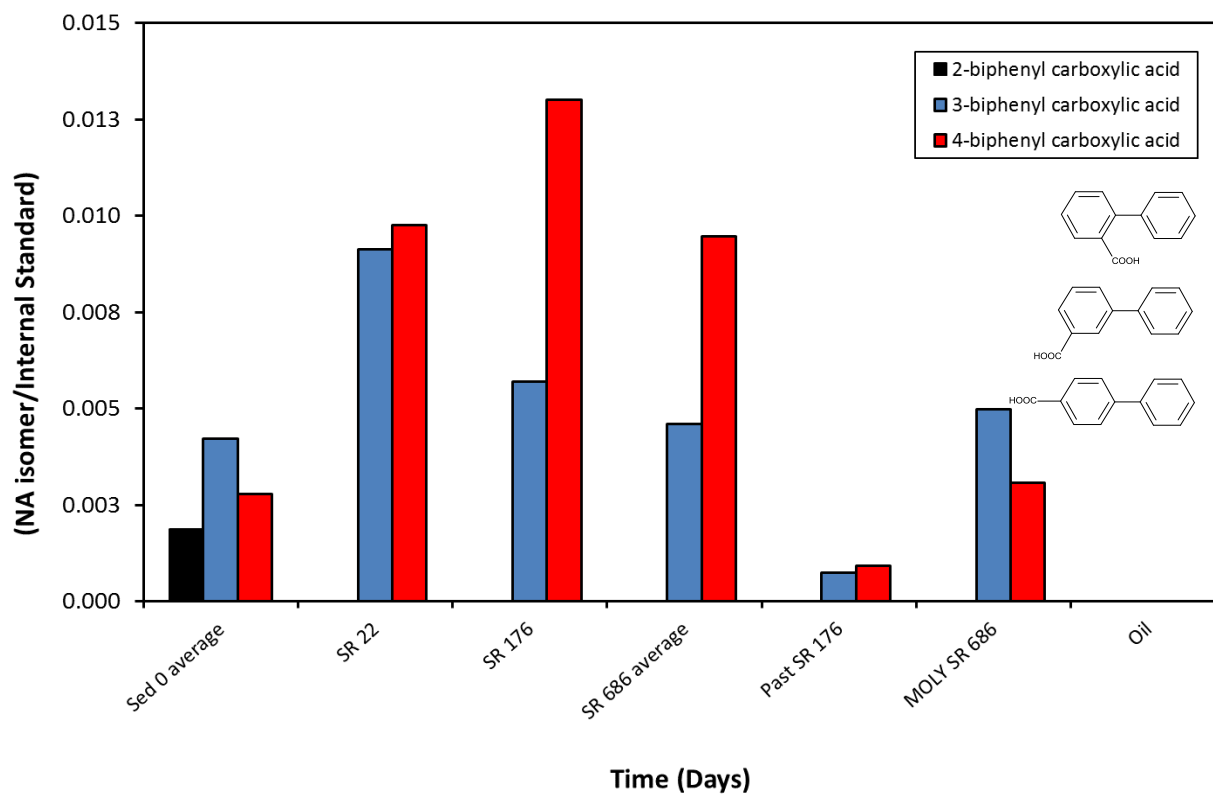
(A)



(B)

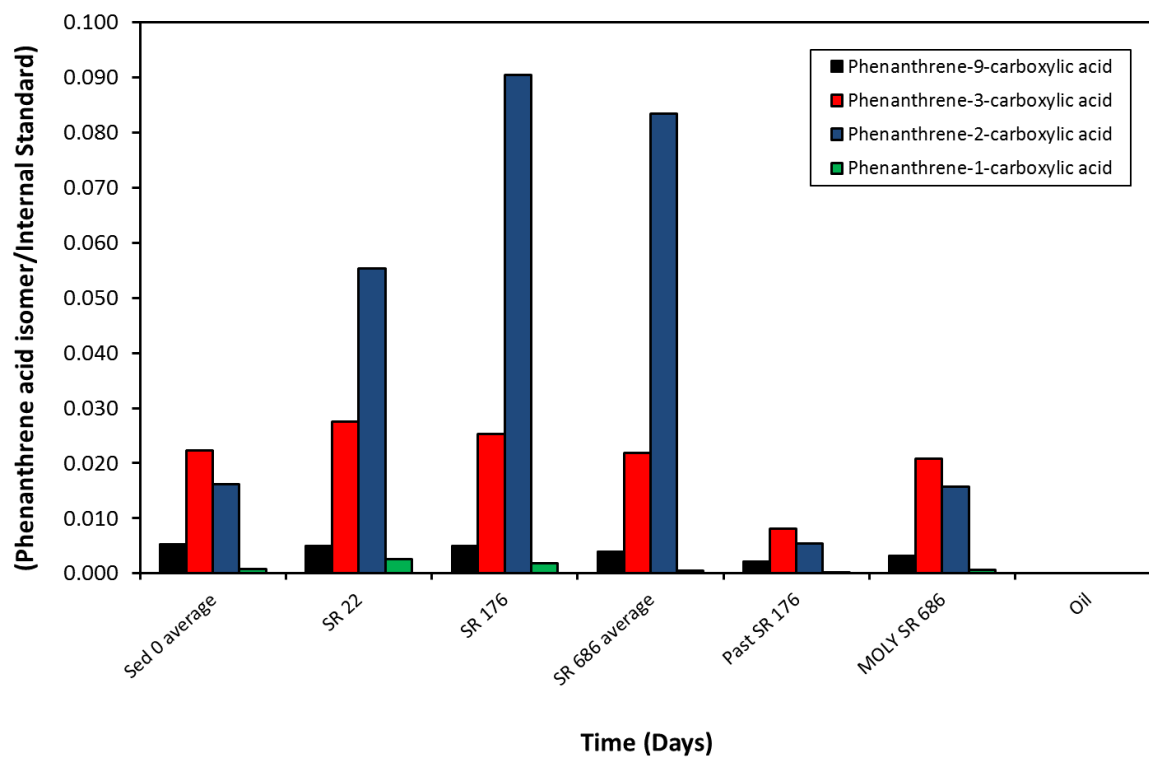


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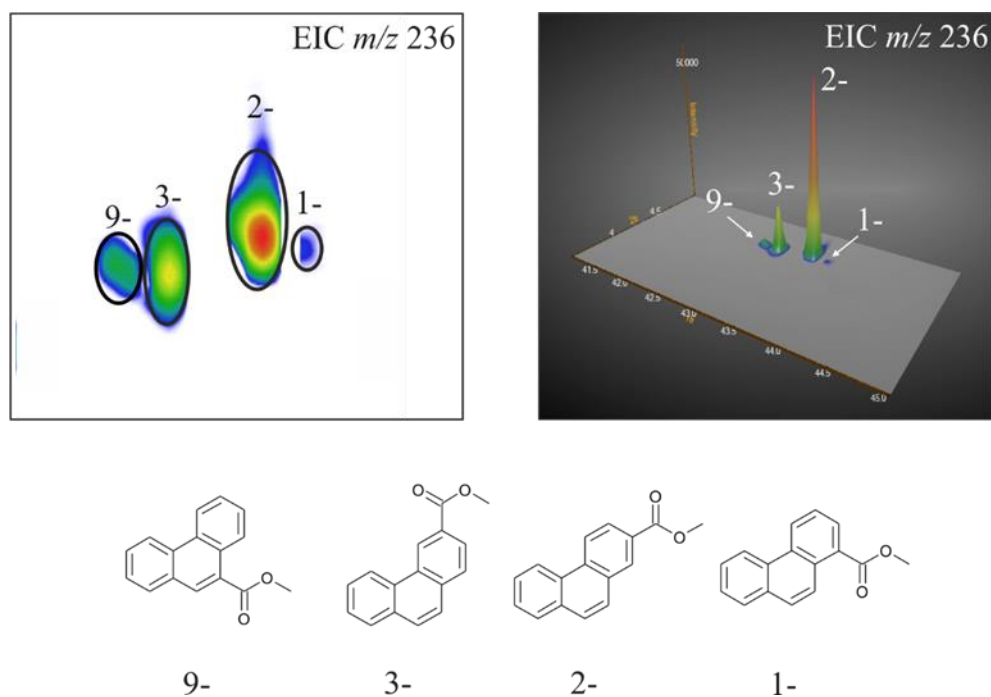


726

(A)



(B)



Supplementary Information

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728

729

Comprehensive two-dimensional gas

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chromatography-mass spectrometry of complex

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mixtures of anaerobic bacterial metabolites of

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petroleum hydrocarbons

733

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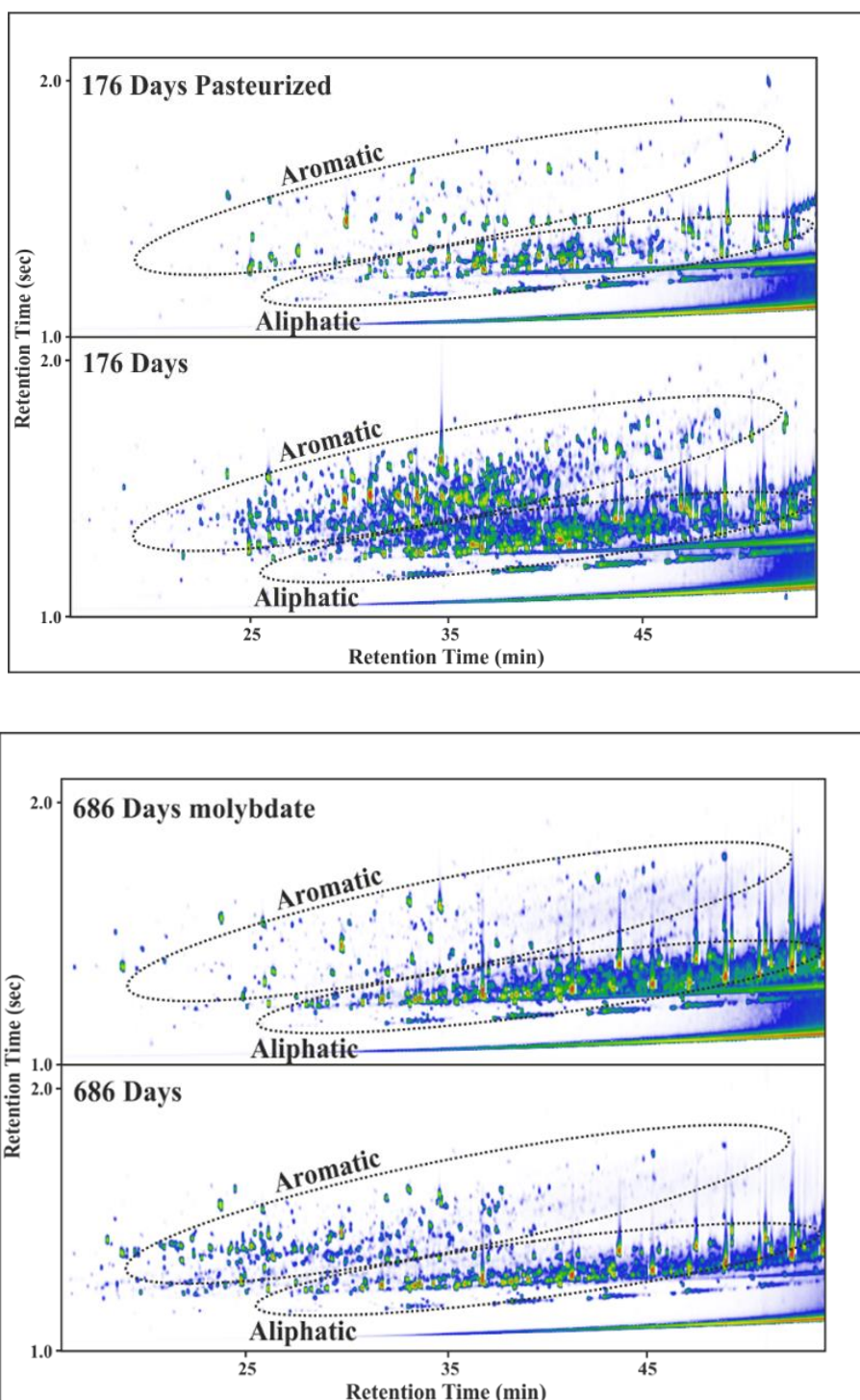
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750

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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756
757

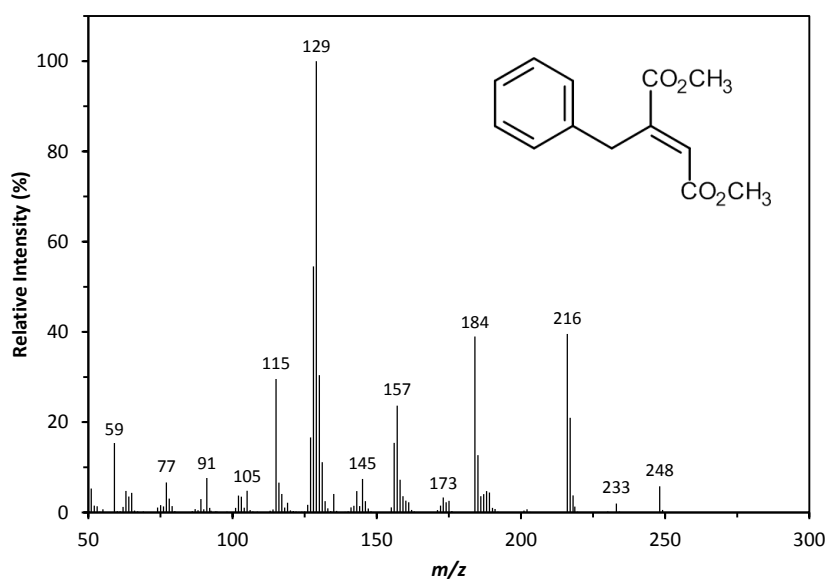


758 **Figure S2.**

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.

762

763



764 **Figure S3.**

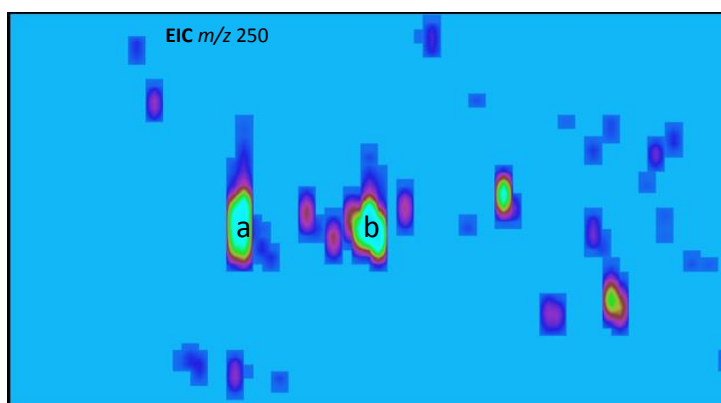
765 Extracted ion current (m/z 250) mass chromatogram and mass spectra of tentatively assigned
766 methylbenzylsuccinates (dimethyl esters; a, b) in acidic extract of crude oil inoculated for 176
767 days with Tyne sediment inoculum under sulfate reducing conditions.

768

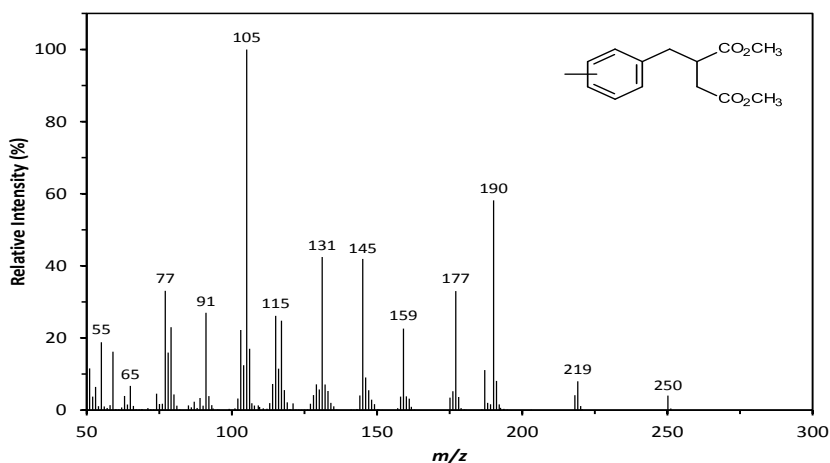
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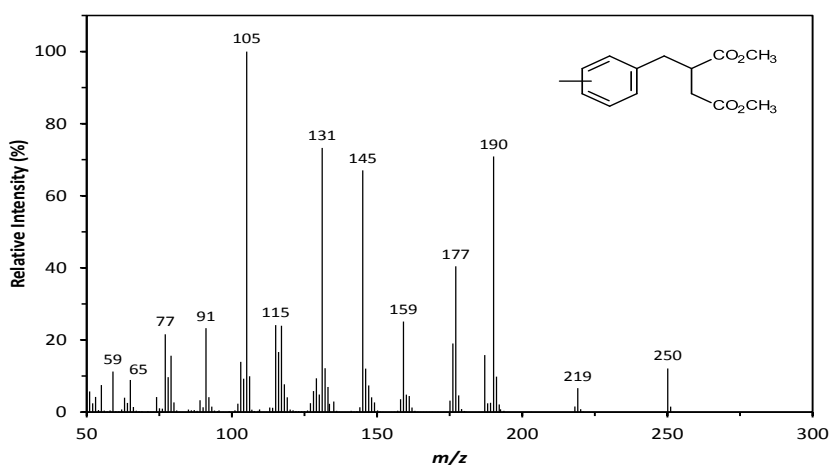
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a

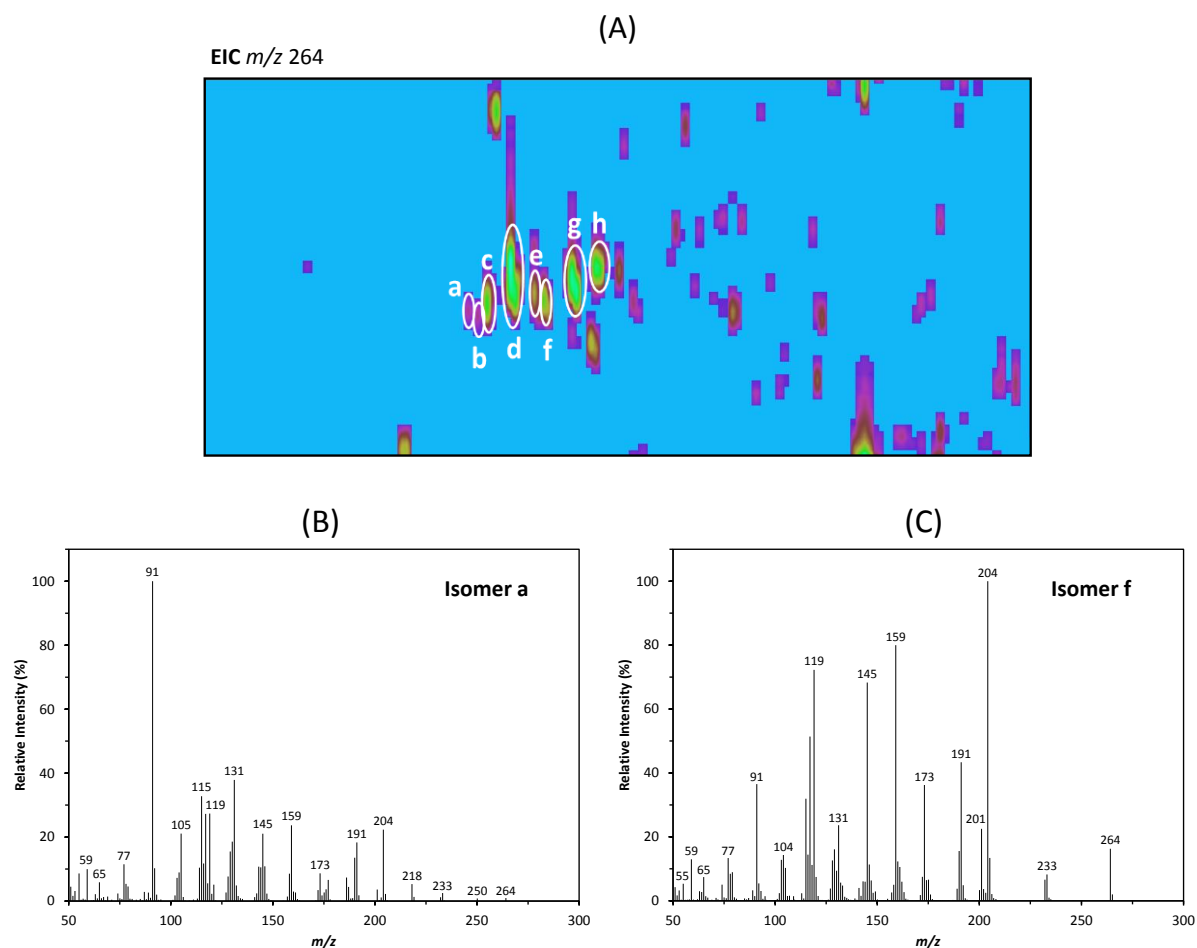


b



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.

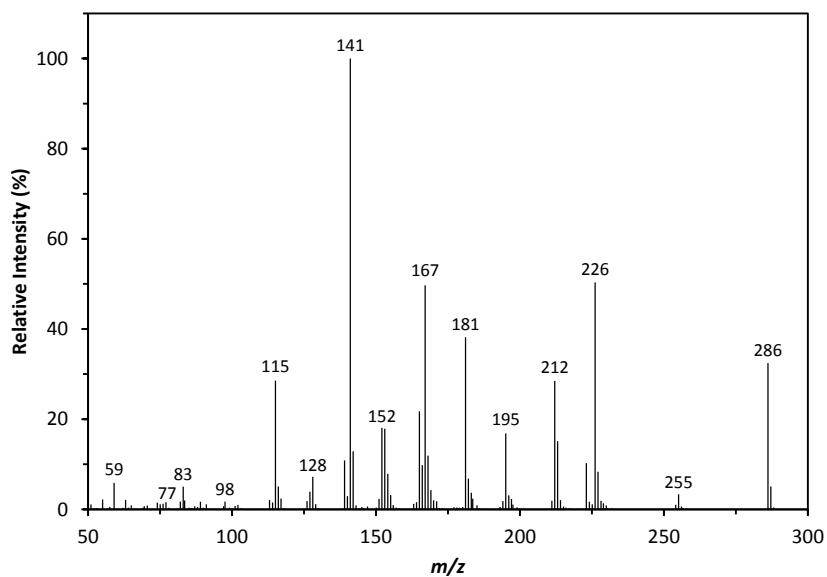


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778 **Figure S5.**

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.



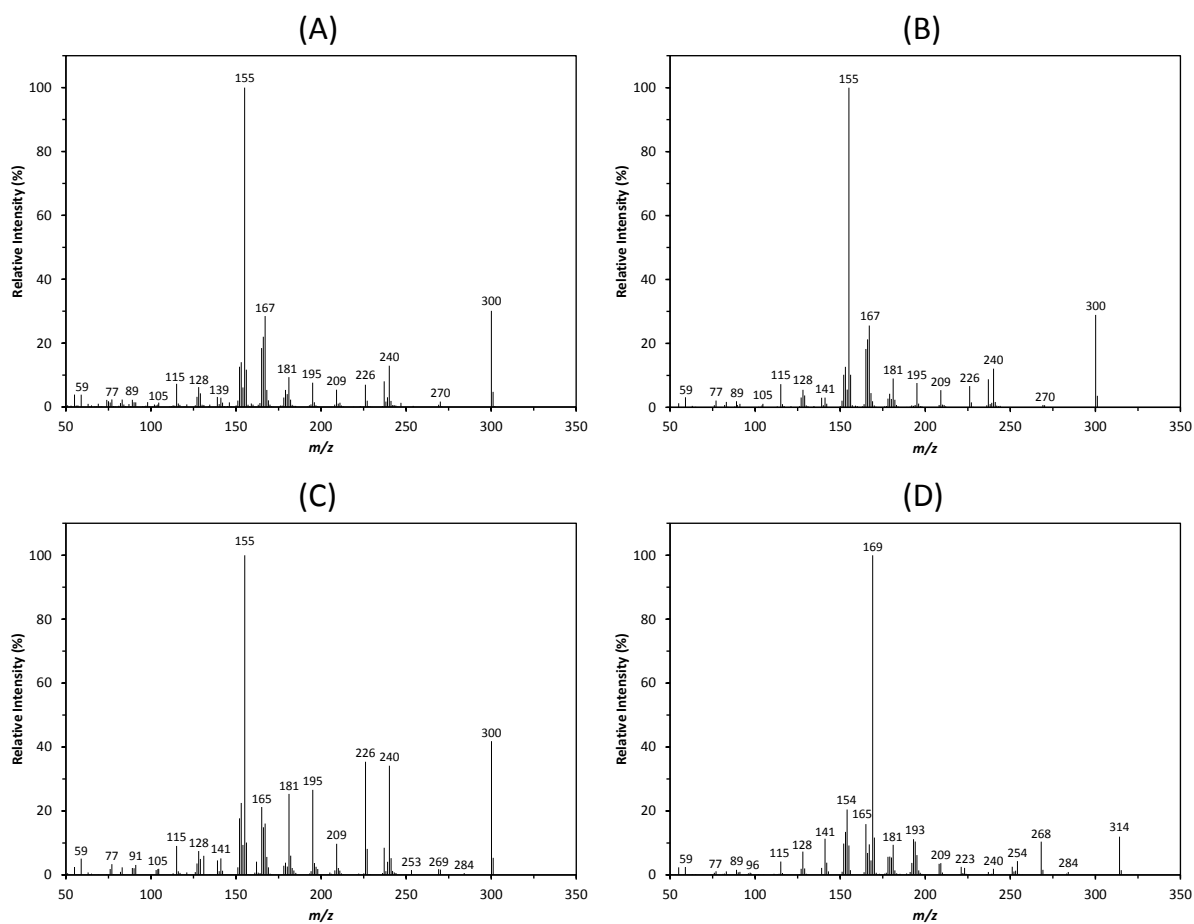
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783

784 **Figure S6.**

785 Mass spectra of C₁ and C₂ 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



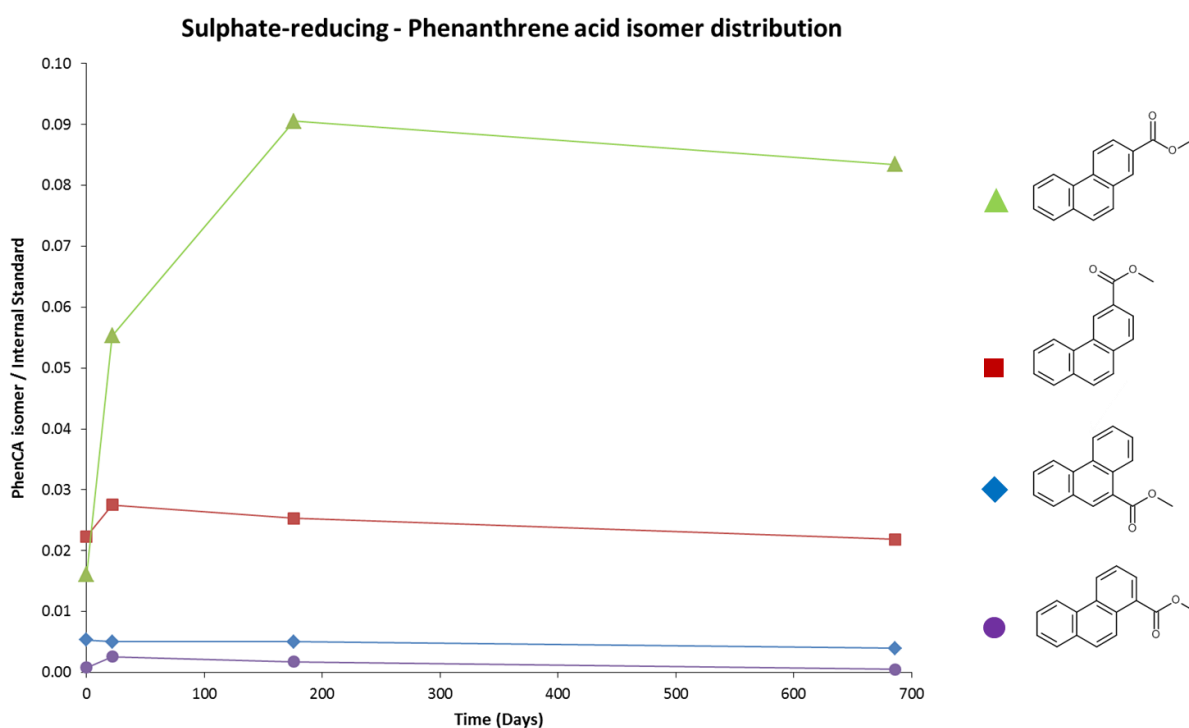
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790

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

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