

1 **Exploring the capacity for anaerobic biodegradation of polycyclic aromatic**
2 **hydrocarbons and naphthenic acids by microbes from oil-sands process**
3 **affected waters.**

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8 Running title: Anaerobic biodegradation of PAHs and NAs by microbes from OSPW

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26 **Abstract**

27 Both polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs)
28 are natural components of fossil fuels but are also widespread, toxic and
29 environmentally persistent pollutants. They are the major cause of environmental
30 toxicity in oil sands process waters (OSPW). This study aimed to investigate the
31 anaerobic biodegradation of the PAHs: pyrene and 2-methylnaphthalene, and the
32 NAs, adamantane-1-carboxylic acid, and a 'natural' NA mixture (i.e. acid-extractable
33 NAs from OSPW) under sulfate-reducing and methanogenic conditions by a
34 microbial community derived from an oil sands tailings pond. Using gas-
35 chromatography mass spectrometry (GC-MS), the rate of biodegradation was
36 measured in relation to changes in bacterial community composition. Only 2-
37 methylnaphthalene was significantly degraded after 260 days, with significantly more
38 degradation under sulfate-reducing (40%) than methanogenic conditions (25%).
39 During 2-methylnaphthalene biodegradation, a major metabolite was produced and
40 tentatively identified as 2-naphthoic acid. Denaturing gradient gel electrophoresis
41 (DGGE) demonstrated an increase in intensity of bands, during the anaerobic
42 biodegradation of 2-methylnaphthalene, which derived from species of the genera
43 *Fusibacter*, *Alkaliphilus*, *Desulfobacterium*, *Variovorax*, *Thaurea* and
44 *Hydrogenophaga*. Despite the biodegradation of 2-methylnaphthalene, this study
45 demonstrates that, under anaerobic conditions, NAs and high molecular weight
46 PAHs are predominantly likely to persist in OSPW. Therefore alternative remediation
47 strategies are required.

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49 **Keywords** polycyclic aromatic hydrocarbon, naphthenic acids, oil sands process
50 waters, anaerobic biodegradation

51 **1. Introduction**

52 Oil sands operations in Canada produce more than 200 million barrels of
53 crude oil per year (Del Rio et al., 2006). During oil-sand refining, vast quantities of
54 wastewaters known as tailings are generated that have to be stored indefinitely in
55 settling basins or ponds until strategies for reclamation are devised and approved.
56 Tailings are composed of solids (e.g. sand, silt) and oil sands process-affected water
57 (OSPW) that contains complex mixtures of toxic carboxylic acids known as
58 naphthenic acids (NAs). It has been estimated that in Canada alone > 840 million m³
59 of OSPW has accumulated and is being stored in tailings ponds (Siddique et al.,
60 2011). There have been very few studies on anaerobic NA biodegradation despite
61 the fact that, as most tailings ponds mature, they become anoxic (Whitby, 2010); and
62 in sulfate-depleted ponds, methane is produced in very large quantities (Clemente &
63 Fedorak, 2005; Fedorak et al., 2002; Holowenko et al., 2000). For example, methane
64 released from Syncrude's largest tailings pond, the Mildred Lake Settling Basin, has
65 been estimated to be up to 43000 m³ day⁻¹ (Holowenko et al., 2000, Siddique et al.,
66 2006). It is not understood whether NAs present in tailings ponds can act as
67 substrates in methane biogenesis *in situ*. Holowenko et al. (2001) demonstrated that
68 methanogenesis was stimulated by the model NA compounds 3-cyclohexylpropanoic
69 acid and 4-cyclohexanepentanoic acid in microcosms containing tailings pond water
70 (Holowenko et al., 2001). Furthermore, Siddique et al. (2006; 2007; 2011) have
71 shown that short chain alkanes, BTEX compounds (benzene, toluene, ethylbenzene
72 and xylenes) and longer chain alkanes (up to C₁₈) are degraded under methanogenic
73 conditions in OSPW (Siddique et al., 2011). Additionally, to date, only a limited
74 number of hydrocarbons have been shown definitively to biodegrade anaerobically in
75 other environments (reviewed by Widdel et al. (2010). This has been shown using

76 either pure cultures or in microcosms (containing soil, river sediment, aquifer
77 material or marine sediment) under methanogenic conditions or using nitrate, iron III,
78 manganese IV, sulfate or carbon dioxide as terminal electron acceptors (Widdel et
79 al., 2010). In addition to the environmental problems caused by NAs, recalcitrant
80 polycyclic aromatic hydrocarbons (PAHs) have been found in environments
81 surrounding the oil sands developments in Alberta, Canada (Timoney & Lee 2009;
82 Kelly et al., 2009). Detailed information on anaerobic degradation of high molecular
83 weight PAHs (HMW-PAHs) is scarce, and there is debate whether PAHs having
84 three or more rings can support growth under anoxic conditions or whether they are
85 only partially oxidised through co-metabolism with growth substrates such as lower
86 molecular weight hydrocarbons (Ambrosoli et al., 2005; Meckenstock et al., 2004).

87 Addition of sulfate to tailings inhibits methanogenesis by stimulating bacterial
88 competition (Holowenko et al., 2000). Therefore, if NAs or other hydrocarbons in
89 OSPW, such as HMW-PAHs, can be degraded under sulfate-reducing conditions, it
90 will not only contribute to the bioremediation of these toxic, recalcitrant pollutants, but
91 it may also reduce the amount of methane produced by OSPW stored in tailings
92 ponds. The aim of the present study was to investigate the anaerobic biodegradation
93 of HMW-PAH, pyrene (Pyr), adamantane-1-carboxylic acid (A1CA, a NA), a 'natural'
94 NA mixture, (i.e. acid-extractable NAs from OSPW) compared to the more readily
95 degradable low molecular weight PAH (LMW-PAH) 2-methylnaphthalene (2-MN),
96 under sulfate-reducing and methanogenic conditions. The rate of biodegradation was
97 measured in relation to changes in bacterial community composition.

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101 **2. Methods**

102 **2.1 Environmental sample**

103 The tailings pond water sample used in this study, (designated 20m) was
104 supplied by L. Gieg, (University of Calgary) and was collected in summer 2010 at a
105 depth of 20 m from a Suncor tailings pond, in Alberta Canada. The sample was
106 maintained at 4°C prior to use.

107

108 **2.2 PAH and NA compounds**

109 Pyrene (Pyr), 2-methylnaphthalene (2-MN) and adamantane-1-carboxylic acid
110 (A1CA) were obtained from Sigma-Aldrich, Gillingham UK at > 98% purity. The
111 'natural' NA mixture was the acid-extractable fraction from a Suncor OSPW sample
112 and was supplied by L. Gieg (University of Calgary). The OSPW samples were
113 acidified to pH 2 and NAs were extracted using dichloromethane according to the
114 method of Holowenko et al. (2002).

115

116 **2.3 Biodegradation experiment**

117 The basal medium for all anaerobic cultures contained per litre of anaerobic
118 water: K₂HPO₄, 0.652 g; NaH₂PO₄·H₂O, 0.173 g; NH₄HCO₃, 0.443 g; NaHCO₃, 3.73
119 g and 1 mL Resazurin solution (0.5 g L⁻¹). The basal medium for sulfate reducers
120 was made as above with the addition of NaSO₄ at 4 g L⁻¹. Basal medium (92.5 mL)
121 was dispensed into serum bottles which were closed with butyl rubber stoppers and
122 crimp sealed. The gas phase was replaced with H₂/CO₂ (80%/20%) (in order to
123 initiate anaerobic growth) to a pressure of 1.5 bar, and autoclaved. Thereafter, 2.5
124 mL each, of three filter sterilized solutions (A, B and C), were added by syringe to
125 each bottle. Solution A contained: 1mL each of 4 vitamin solutions; 1 mL of trace

126 elements solution, and 1 mL of amino acid solution added to 20 mL sterile anaerobic
127 water (see supplementary information for details). Solution B contained L⁻¹
128 anaerobic water: Na₂S, 2.402 g. Solution C contained L⁻¹ of anaerobic water:
129 CaCl₂·2H₂O, 4.410 g; MgCl₂·6H₂O, 4.066 g. Individual cultures were amended with
130 either Pyr, A1CA, 'natural' NAs or 2-MN (5 mg L⁻¹ final concentration) as the sole
131 carbon and energy source as described in Johnson et al. (2011). Bottles were
132 inoculated with a 2% (v/v) of 20m tailing pond water sample. Abiotic controls
133 containing either Pyr, A1CA, 'natural' NAs or 2-MN (5 mg L⁻¹ final concentration) and
134 anaerobic medium were also prepared. Procedural blanks containing the 20m
135 inoculum and anaerobic media only were also prepared. All bottles were incubated
136 statically in the dark at 20°C for 260 days. Sampling of triplicate bottles was carried
137 out at 0, 130 and 260 days. After 130 days the gas phase was changed to N₂/CO₂
138 (80%/20%). At 0, 130 and 260 days a 30 mL sub-sample was removed, centrifuged
139 at 3435 x g for 10 min and acidified to pH 2 with HCl for ethyl acetate extraction
140 according to the method of Johnson et al. (2011).

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142 **2.4 Solvent extraction, GC-MS and analysis**

143 To remove hydrocarbon contamination all glassware was soaked overnight in
144 Decon⁹⁰ (Decon), rinsed three times with distilled water, baked until dry, and rinsed
145 three times with acetone (Fisher) (Johnson et al., 2011). The internal standard 4-
146 phenylbutanoic acid (Acros Organics) (final concentration 2 mg L⁻¹) was used for all
147 NA samples and 2-MN, whilst the internal standard 2-MN (Acros Organics) (final
148 concentration 2 mg L⁻¹) was used for all pyrene amended samples. Each NA from
149 the supernatants was extracted by acidifying to pH 2 (using 2 drops of concentrated
150 HCl) and extracted three times with 15 mL of HPLC-grade ethyl acetate (Fisher

151 Scientific) using a separating funnel. Each HMW-PAH was extracted as above
152 except samples were extracted with HPLC-grade acetone (Fisher). Solvent extracts
153 were pooled, dried with 5–10 g anhydrous Na₂SO₄ (Fisher Scientific), and the
154 organic acids concentrated by rotary evaporation (Buchi) at 40°C. Samples were
155 transferred to a gas chromatography vial (Chromacol) and stored at – 20°C. Prior to
156 analysis, all samples were reduced to dryness under a gentle stream of nitrogen at
157 40°C and reacted with N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte,
158 PA, USA) at 60°C for 20 min to form trimethylsilyl derivatives. Derivatized samples
159 were resuspended in 1mL dichloromethane (HPLC, Acros Organics). Samples were
160 separated by gas chromatography–mass spectrometry (GC-MS) using an Agilent
161 7890 GC interfaced with an Agilent 5975C MS. Samples were injected with a 1 µl
162 splitless injection (injector temperature 250°C) onto a 30 m x 250 µm x 0.25 µm Rtx
163 – 1MS column using helium as the carrier gas at a constant flow of 1 mL min⁻¹. Oven
164 temperatures were programmed with an initial increase from 40°C to 250°C at 10°C
165 min⁻¹ and a final hold at 250°C for 10 minutes. For PAHs, oven temperatures were
166 programmed with an initial increase from 40°C to 300°C at 10°C min⁻¹ and a final
167 hold at 300°C for 10 minutes. The transfer line was held at 230°C onto a source for
168 the MS which was in full scan mode (scan range 50-550 Da). Data was analysed
169 and integrated with Agilent GC Chemstation. Agilent GC Chemstation was used to
170 tentatively identify metabolites through mass spectral analysis.

171

172 **2.5 Methane measurements**

173 Headspace samples (50 µl) were taken to measure methane and analysed by
174 gas chromatography with flame ionization detection (GC-FID) (PU 4500
175 Chromatograph, Philips). The oven temperature of the GC-FID was set to 100°C,

176 with the FID maintained at 150°C. Gases were separated on a 1.5m stacked
177 stainless steel column using N₂ as the carrier gas at a flow rate of 1 mL min⁻¹.

178 **2.6 Sulfide measurements**

179 Sulfide measurements were performed according to the method of Cord-
180 Ruwisch (1985). A 50 µl sample of culture was mixed with 950 µl of 5 mM CuSO₄
181 and 50 mM HCl and the optical density measured at 480 nm on a spectrophotometer
182 (Cecil CE2021).

183

184 **2.7 Bacterial community analysis.**

185 Total nucleic acids were extracted from cell pellets obtained from centrifugation of 30
186 mL sub samples at each time point. Cells were suspended in lysis buffer and were
187 lysed with lysozyme (2 mg/ml), sodium dodecyl sulfate (1%), and lauryl-sarkosyl
188 (1%). Proteinase K (1 mg/ml) was added and the tubes were incubated at 37°C for 1
189 h. Lysates were first extracted with 1 volume of phenol– chloroform–isoamyl alcohol
190 (25:24:1, pH 8.0) and then with 1 volume of chloroform–isoamyl alcohol (24:1). The
191 nucleic acids in the aqueous phase were precipitated with 5 M NaCl (0.1 vol) and
192 100% ethanol (2 vol) at -20°C, and then washed with 70% ethanol. DNA pellets
193 were dried and suspended in 100 µl of water (Grabowski et al., 2005). PCR
194 amplifications were performed using the primers F341 GC
195 (CCTACGGGAGGCAGCAG) and R534 (CCAGCAGCCGCGGTAAT) and
196 thermocycling consisted of 95°C for 5 min followed by 35 cycles of 95°C for 30 s,
197 57°C for 30 s, 72°C for 1.5 min, 72°C for 10 min. as described by Muyzer et al.
198 (1993) using a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems).
199 Each 50 µl PCR reaction mixture contained approximately 50-100 ng of DNA
200 template, primers (0.4 µM), dNTPs (0.1 mM), Taq polymerase (1.25 U, Qiagen), and

201 1x PCR buffer (Qiagen). PCR products were analysed by 1% (w/v) 1 x TAE agarose
202 gel electrophoresis stained with ethidium bromide (10 mg mL⁻¹) and viewed under
203 UV transillumination (Alpha Innotech). DGGE analysis of 16S rRNA gene fragments
204 was performed as previously described (McKew et al., 2007) using the Bio-Rad D
205 Code system on 8% polyacrylamide gels and a denaturing gradient from 40% to
206 60% and silver stained using the modified protocol of Acuña Alvarez et al. (2009).
207 Selected DGGE bands were excised, re-amplified and sequenced by Source
208 Bioscience (Nottingham, UK).

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210 **2.9 Statistical analysis**

211 Statistical analysis was performed using SPSS PASW statistics version 18.0
212 and Primer E.

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226 3. Results

227 3.1 Anaerobic degradation of 2-MN

228 At day 260, the relative percentage of Pyr, A1CA and 'natural' NAs remaining
229 was not significantly different from that at day 0 under either sulfate-reducing (Fig.
230 1A) or methanogenic conditions (Fig. 1B) ($p=0.1$). After 260 days, the cultures grown
231 under sulfate-reducing conditions had degraded $40\% \pm 7.89$ of the 2-MN (Fig. 1A)
232 and under methanogenic conditions had degraded $25\% \pm 8.21$ of the 2-MN (Fig. 1B),
233 demonstrating significant degradation of 2-MN compared to the abiotic control
234 ($p<0.05$). Furthermore, the sulfate-reducing cultures degraded significantly more 2-
235 MN than the methanogenic cultures ($p<0.05$) by day 260. All killed controls showed
236 no significant abiotic losses.

237 After 130 days and 260 days there was no significant production of methane
238 in the cultures incubated with Pyr, A1CA or 'natural' NAs compared to abiotic
239 controls (Fig. 1C). After 130 days, 2-MN cultures under methanogenic conditions
240 demonstrated a significant increase in methane concentration compared to both the
241 2-MN abiotic controls and the 2-MN cultures under sulfate-reducing conditions
242 ($p<0.05$). At day 130 the cultures containing 2-MN grown under methanogenic
243 conditions contained 110 ± 19.2 μmol methane and 290 ± 28.9 μmol by day 260, a
244 significant increase compared to abiotic controls ($p<0.05$) (Fig 1C). In the sulfate-
245 amended enrichments, by day 130 and 260 there was no significant production of
246 sulfide in the cultures incubated with Pyr, A1CA or environmental NA cultures
247 compared to abiotic controls ($p=0.1$) (Fig. 1D). However, with cultures incubated with
248 2-MN under sulfate-reducing conditions, there was a significant increase in dissolved
249 sulfide concentration by day 130 and day 260 compared to the 2-MN abiotic controls
250 and the 2-MN cultures under methanogenic conditions ($p<0.05$). At day 130 the

251 cultures incubated with 2-MN under sulfate-reducing conditions contained $1.6 \text{ mM} \pm$
252 0.389 sulfide , and by day 260 sulfide production had significantly increased to 2.68
253 $\text{mM} \pm 0.312$ ($p < 0.05$) (Fig. 1D).

254 Degradation of 2-MN by cultures grown under sulfate-reducing and
255 methanogenic conditions resulted in the production of a major metabolite, the mass
256 spectrum of which is presented in Fig 2. The metabolite (denoted Metabolite 1) had a
257 molecular mass of 244 and was tentatively identified as the trimethylsilylated ester of
258 2-naphthoic acid (Supplementary information Fig.1). No metabolites were detected in
259 either the abiotic or killed controls.

260

261 **3.2 Bacterial community analysis**

262 PCR-DGGE analysis of the 16S rRNA gene was performed to determine
263 changes in the bacterial community composition, and the DGGE fingerprints for the
264 2-MN communities under both sulfate-reducing and methanogenic conditions are
265 shown in Fig. 3A and B. DGGE fingerprints from the other hydrocarbon enrichments
266 were also obtained, as shown in Supplementary Fig. S2 (sulfate-reducing
267 conditions), Supplementary Fig. S3 (methanogenic conditions) and Supplementary
268 Table S1. Bacterial community changes in cultures incubated with Pyr, A1CA and
269 environmental NAs were observed, however analysis of the DGGE banding patterns
270 demonstrated that 2-MN amended cultures were more distinct. Comparisons of the
271 bacterial community composition during the biodegradation of 2-MN under both
272 sulfate-reducing and methanogenic conditions show that changes are apparent by
273 day 130 and day 260 compared to day 0, with numerous bands becoming more
274 prominent. Reproducibility among triplicates remained at a high level throughout.
275 Bands were excised, sequenced, and the closest BLAST n match is presented in

276 Table 1. From the sulfate-reducing community, DGGE bands 5, 7 and 9 were
277 specific to the day 130 and day 260 2-MN communities and had a high 16S rRNA
278 gene sequence similarity to *Desulfonatronum thiodismutans* (97%), *Variovorax*
279 *dokdonensis* (98%) and *Thaurea aromatica* (97%), respectively. DGGE band 4 had a
280 16S rRNA gene sequence similarity (96%) to *Desulfobacterium autotrophicum* and
281 was present in enrichments containing 2-MN at days 0, 130 and 260.

282 Under methanogenic conditions, DGGE bands 12, 14, 17 and 18 were
283 uniquely present in the 2-MN enrichments at day 130 and day 260 and had a 16S
284 rRNA gene sequence similarity to *Fusibacter paucivorans* (95%), *Alkaliphilus* spp.
285 (95%), *Xanthobacter* spp. (99%) and *Hydrogenophaga caeni* (98%), respectively.
286 DGGE band 17 had 16S rRNA gene sequence similarity (94%) to *Sphingobium*
287 *astaxanthinifaciens* and was present in enrichments containing 2-MN at days 0, 130
288 and 260.

289

290 **4. Discussion**

291 To date there have been very few studies on anaerobic NA biodegradation,
292 despite the fact that as most tailings ponds mature they become anoxic (Whitby,
293 2010). In this study, the low molecular weight polycyclic aromatic hydrocarbon
294 (LMW-PAH), 2-methylnaphthalene (2-MN) was degraded under both sulfate-reducing
295 and methanogenic conditions to produce 2-naphthoic acid as an intermediate
296 metabolite. Previous studies have also shown the production of 2-naphthoic acid
297 during the degradation of 2-MN under both sulfate-reducing (Annweiler et al., 2000;
298 2002; Musat et al., 2009) and methanogenic conditions (Bergio-Clavijo et al., 2012).
299 2-MN metabolism is known to have a mechanism similar to that seen in anaerobic
300 toluene degradation (Annweiler et al., 2000; Meckenstock et al., 2004). In order to

301 form 2-naphthoic acid, a fumarate is added at the methyl group, resulting in the
302 intermediate naphthyl-2-methyl-succinic acid. Naphthyl-2-methyl-succinic acid is
303 then oxidized to naphthyl-2-methylene-succinic acid (Annweiler et al., 2000). Further
304 metabolism of 2-naphthoic acid proceeds in the same manner as described for
305 naphthalene (Zhang et al., 2000). However, in this study no further metabolites were
306 detected under either sulfate-reducing conditions or methanogenic conditions. The
307 inability to detect other metabolites in the present study may be due to the amount of
308 substrate added, as fumarate addition products were detected by Annweiler et al.
309 (2000), at a 2-MN concentration of 100 mg L⁻¹ whereas in this study 5 mg L⁻¹ of 2-
310 MN was used. However, Bergio-Clavijo et al. (2012) were unable to detect any
311 fumarate addition products at a 2-MN concentration of 130 mg L⁻¹ after 125 days
312 incubation after previously detecting them at this concentration during toluene
313 degradation over a period of 50 days (Fowler et al., 2012).

314 However, in the present study Pyr, A1CA and the 'natural' NAs were not
315 degraded anaerobically by day 260 under sulfate-reducing or methanogenic
316 conditions. The anaerobic biodegradation of HMW-PAHs has been tentatively
317 demonstrated under sulfate-reducing (Rothermich et al., 2002), nitrate-reducing
318 (Ambrolosi et al., 2005) and methanogenic (Trably et al., 2003; Chang et al., 2006;
319 Chang et al., 2003) conditions. In a comparison of all three conditions, Chang et al.
320 (2007) demonstrated biodegradation of a range of PAHs, including Pyr under the
321 various anaerobic conditions with communities isolated from river sediment.
322 Comparison of the PAH degradation rates under three different reducing conditions
323 produced the following order: sulfate-reducing > methanogenic > nitrate reducing
324 conditions (Chang et al., 2007). This supports the results of the present study in
325 which, by day 260, 40% of 2-MN was degraded under sulfate-reducing conditions

326 and 25% was degraded under methanogenic conditions. However, in the studies of
327 Chang et al. (2003), Chang et al. (2006) and Chang et al. (2007), there was no
328 detection of mineralisation, cell growth or characteristic metabolites, so the
329 measured decrease in PAH concentration could be ascribed to partial and co-
330 metabolic oxidation (Foght, 2008; Meckenstock et al., 2004; Safinowski &
331 Meckenstock., 2006).

332 Based on the appearance of DGGE bands during incubation with 2-MN at day
333 130 and day 260, (which were absent at day 0), the bacteria most likely to be
334 contributing to the biodegradation of the 2-MN under sulfate-reducing conditions,
335 were the Deltaproteobacterium *Desulfonatronum* (band 5), the Betaproteobacteria,
336 *Variovorax* (band 7) and *Thaurea* (band 9). *Thaurea* species have previously been
337 shown to degrade toluene under nitrate-reducing conditions (Mechichi et al., 2002)
338 and phenol in anoxic conditions (Breinig et al., 2000). *Variovorax* spp. have
339 previously been associated with anaerobic 2-MN degradation in bacterial
340 communities at 7°C (Eriksson et al., 2003) and benzene degradation in petroleum
341 contaminated aquifers (Rooney-Varga et al., 1999). However, neither
342 *Desulfonatronum* nor *Thaurea* have previously been associated with the degradation
343 of 2-MN under sulfate reducing conditions. A *Desulfobacterium* spp. (band 4) was
344 also detected, however it was not enriched during biodegradation of 2-MN, so it is
345 unlikely that it contributed directly to 2-MN degradation in this case. However,
346 *Desulfobacterium* spp. are known to degrade aromatic compounds such as *m*-cresol
347 (Müller et al., 1999), catechol (Gorny & Schink 1994) and phenol (Bak & Widdel
348 1986).

349 Under methanogenic conditions, the bacteria most likely contributing to the
350 biodegradation of the 2-MN were the Firmicutes, *Fusibacter* (band 12) and

351 *Alkaliphilus* (band 14), the Alphaproteobacterium *Xanthobacter* (band 17) and the
352 Betaproteobacterium *Hydrogenophaga* (band 18). *Hydrogenophaga caeni* have
353 demonstrated anaerobic growth on R2A agar (Chung et al., 2007) and
354 *Hydrogenophaga* sequences have been detected in anaerobic biofilms from OSPW
355 samples (Golby et al., 2012). *Fusibacter* species have previously been isolated from
356 oil-producing wells, which has led some to infer an ability to degrade hydrocarbons
357 anaerobically (Ravot et al., 1999), while *Alkaliphilus* species have been shown to
358 degrade crotonate, a short chain carboxylic acid, under methanogenic conditions
359 (Cao et al., 2003). Bacterial species closely related to the *Clostridiaceae* have
360 previously been detected in 2-MN methanogenic enrichment cultures, suggesting
361 that they may play an important role in the methanogenic metabolism of 2-MN
362 (Berdugo-Clavijo et al., 2012). An increasing number of studies are showing the
363 importance of organisms closely affiliated with members of the phylum Firmicutes
364 and class Clostridia in the anaerobic biodegradation of hydrocarbons. This was
365 summarised by Gray et al. (2010) who clustered microbial community information
366 from 26 different studies related to hydrocarbon-associated environments and found
367 that the Firmicutes were frequently detected in diverse habitats and represented
368 31% of the surveyed bacterial sequences (Gray 2010). It must be noted that the
369 change of gas phase at day 130 may be responsible for some of the community
370 changes and biodegradation observed, however no significant degradation of the 2-
371 MN was observed in the day 130 samples. In addition, the production of methane
372 during the biodegradation of 2-MN under methanogenic conditions suggests that
373 syntrophic metabolism between the hydrocarbon utilizing bacteria and hydrogen /
374 acetate utilizing archaeal methanogens was occurring. This was implied by Berdugo-
375 Clavijo et al. (2012) whose 2-MN degrading communities were shown to be

376 dominated by two archaeal members *Methanosaeta* and *Methanoculleus*. In the
377 present study, the focus was on the bacterial communities, as they were more likely
378 to be the primary hydrocarbon degraders in such a syntrophic relationship.

379 Previous studies into the anaerobic communities in OSPW have
380 demonstrated unexpectedly diverse communities to be present (Penner & Foght
381 2010). Bacterial clone libraries from mature fine tailings were shown to be composed
382 of *Proteobacteria* including presumptive nitrate, iron, or sulfate-reducing,
383 hydrocarbon-degrading species belonging to genera such as *Thauera*, *Rhodoferax*
384 and *Desulfatibacillum* as well as a number of Firmicutes within the order *Clostridiales*
385 as in the current study (Penner & Foght 2010). Ramos-Pedron et al. (2010)
386 characterized microbial communities by 16S rRNA gene pyrosequencing from
387 OSPW treated with gypsum. Deep anaerobic layers were dominated by syntrophs
388 (*Pelotomaculum*, *Syntrophus* and *Smithella* spp.), sulfate- and sulfur-reducing
389 bacteria (*Desulfocapsa* and *Desulfurivibrio* spp.), as well as other anaerobes that
390 have been previously implicated in hydrocarbon utilization such as *Thauera*, which
391 were found in the 2-MN degrading communities in the current study. However, the
392 nature of the substrates used by the identified organisms is not known (Ramos-
393 Pedron et al., 2010). Furthermore, Golby et al. (2012) cultivated mixed-species
394 biofilms from OSPW under anaerobic conditions. Pyrosequencing of the resulting
395 communities revealed they were composed of *Hydrogenophaga* (19.5%),
396 *Rhodoferax* (9.9%), *Methyloversatilis* (9.9%), *Magnetospirillum* (6.5%),
397 and *Acidovorax* (4.0%). Species from some of these genera have been shown to be
398 associated with NA biodegradation (Golby et al., 2012), and *Hydrogenophaga* were
399 found in methanogenic 2-MN communities in the present study.

400

401 **5. Conclusions**

402 The present study demonstrated that a microbial community from OSPW was
403 capable of the anaerobic biodegradation of significantly more 2-MN under sulfate-
404 reducing conditions than under methanogenic conditions and provided an insight into
405 the bacteria required to achieve this. However, in the same time period there was no
406 significant anaerobic degradation of the NAs or the HMW-PAH, pyrene. Therefore, if
407 a similar lack of biodegradation is found in anoxic OSPW, then natural attenuation by
408 anaerobic biodegradation is likely not to be a feasible means of removing both NA
409 and PAH contamination from OSPW. Possible solutions to this are: aeration, as both
410 NAs (Whitby 2010) and PAHs (Kanaly & Harayama 2010) are known to degrade
411 much more rapidly under oxic conditions, or enhancing biodegradation through co-
412 metabolism with lower molecular weight compounds (Meckenstock et al., 2004).
413 Nevertheless, it is conceivable that with sufficient time for communities to adapt,
414 anaerobic degradation of some of these compounds will occur. These results have
415 implications for future remediation strategies of tailings ponds because if microbial
416 communities cannot be influenced to anaerobically degrade NAs and other toxic
417 components of OSPW then, unless alternative remediation options are explored,
418 their long-term persistence will cause significant environmental issues.

419

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594 **Titles to Figures and Tables**

595 **Fig. 1.** Degradation of Pyr, A1CA, environmental NAs (NAs) and 2-MN after 260
596 days incubation compared to abiotic controls (at day 260) under both sulfate-
597 reducing (A) and methanogenic conditions (B). Relative percentage remaining after
598 0 days (light bars) and 260 days (dark bars). (C): Methane production in
599 methanogenic and sulfate-reducing cultures at day 260. (D): sulfide production in
600 methanogenic and sulfate-reducing cultures at day 260. S = cultures under sulfate-
601 reducing conditions. Error bars represent standard error of the mean ($n=3$).

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603 **Fig. 2.** Example gas chromatograms showing degradation of 2-MN over time under
604 sulfate-reducing (A) and methanogenic conditions (B). The internal standard was 4-
605 phenylbutyric acid.

606

607 **Fig. 3.** DGGE fingerprint of the bacterial communities grown on 2-MN under sulfate-
608 reducing (A) and methanogenic conditions (B). Numbers indicate the bands excised
609 and sequenced. Band numbers correspond to those in Table 1.

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611 **Table 1.** Blastn analysis of the 16S rRNA genes from excised DGGE bands.

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619 **Supplementary information**

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621 **Supplementary Fig. S1.** Mass spectrum of metabolite 1, tentatively identified as 2-
622 naphthoic acid (silylated) detected in 2-MN cultures under both sulfate-reducing and
623 methanogenic conditions.

624

625 **Supplementary Fig. S2.** Composite image of DGGE gel (using F341 and R534
626 primers) from the sulfate-reducing cultures demonstrating bacterial community
627 structure in the presence of Pyr, A1CA, Natural NAs and 2-MN at day 130 and day
628 260. The bands indicated (1-10) were excised and sequenced. Band numbers
629 correspond to those in Supplementary Table S1.

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631 **Supplementary Fig. S3.** Composite image of DGGE gel (using F341 and R534
632 primers) from the methanogenic cultures demonstrating bacterial community
633 structure in the presence of Pyr, A1CA, Natural NAs and 2-MN at day 130 and day
634 260. The bands indicated (11-18) were excised and sequenced. Band numbers
635 correspond to those in Supplementary Table S1.

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637 **Supplementary Table S1.** Blastn analysis of the 16S rRNA genes from excised
638 DGGE bands from the methanogenic and sulfate-reducing cultures.

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644 **Composition of anaerobic media**

645 The basal medium for all anaerobic cultures contained L⁻¹ of anaerobic water:
646 K₂HPO₄, 0.652 g; NaH₂PO₄·H₂O, 0.173 g; NH₄HCO₃, 0.443g; NaHCO₃, 3.73 g and
647 Resazurin solution (0.5 g L⁻¹) 1mL. The medium for sulfate reducers was made as
648 above with the addition of NaSO₄ at 4 g L⁻¹.

649 The trace elements solution for all anaerobic cultures contained L⁻¹ of
650 anaerobic water: concentrated HCl, 1 mL; FeCl₂·4H₂O, 2000mg; CoCl₂·6 H₂O, 190
651 mg; ZnCl₂, 70 mg; CuCl₂, 2 mg; H₃BO₃, 6 mg; Na₂MoO₄, 36 mg; NiCl₂·6 H₂O, 24
652 mg. 50 mL portions were filter sterilised and stored in N₂ flushed, crimp sealed
653 serum bottles, with gas phase filled with N₂ to 1.5 bar.

654 The amino acids solution for all anaerobic cultures contained 100 mL⁻¹
655 anaerobic water: Arginine, 871 mg; Histidine, 775.8 mg; Threonine, 595.6 mg. 50 mL
656 portions were filter sterilised and stored in N₂ flushed, crimp sealed serum bottles,
657 with gas phase filled with N₂ to 1.5 bar.

658 Vitamin solution A for all anaerobic cultures contained 250 mL⁻¹ anaerobic
659 water: Biotin, 12.5 mg; p-aminobenzoate (Na-salt), 55 mg; pantothenate (Ca-salt),
660 12.5 mg; folic acid (dihydrate), 5 mg; lipoic acid (thiocitic acid), 12.5 mg; pyridoxine,
661 25 mg; nicotinamide 137.5 mg. Aliquots (50 mL) were filter sterilised and stored in N₂
662 flushed, crimp sealed serum bottles, with gas phase filled with N₂ to 1.5 bar. Vitamin
663 solutions B, C and D for all anaerobic cultures contained 100 mL⁻¹ anaerobic water:
664 thiamine HCl, 10mg; riboflavine, 5 mg and cyanocobalamin, 25 mg respectively. 50
665 mL portions were filter sterilised and stored in N₂ flushed, crimp sealed serum
666 bottles, with gas phase filled with N₂ to 1.5 bar. The Na₂S solution for all anaerobic
667 cultures contained 100 mL⁻¹ anaerobic water: Na₂S, 24.02 g. Aliquots (50 mL) were
668 filter sterilised and stored in N₂ flushed, crimp sealed serum bottles, with gas phase

669 filled with N₂ to 1.5 bar. The Ca and Mg solution contained L⁻¹ of anaerobic water:
670 CaCl₂.2H₂O, 4.410 g; MgCl₂.6H₂O, 4.066 g. 50 mL portions were filter sterilised and
671 stored N₂ flushed, in crimp sealed serum bottles, with gas phase filled with N₂ to 1.5
672 bar.

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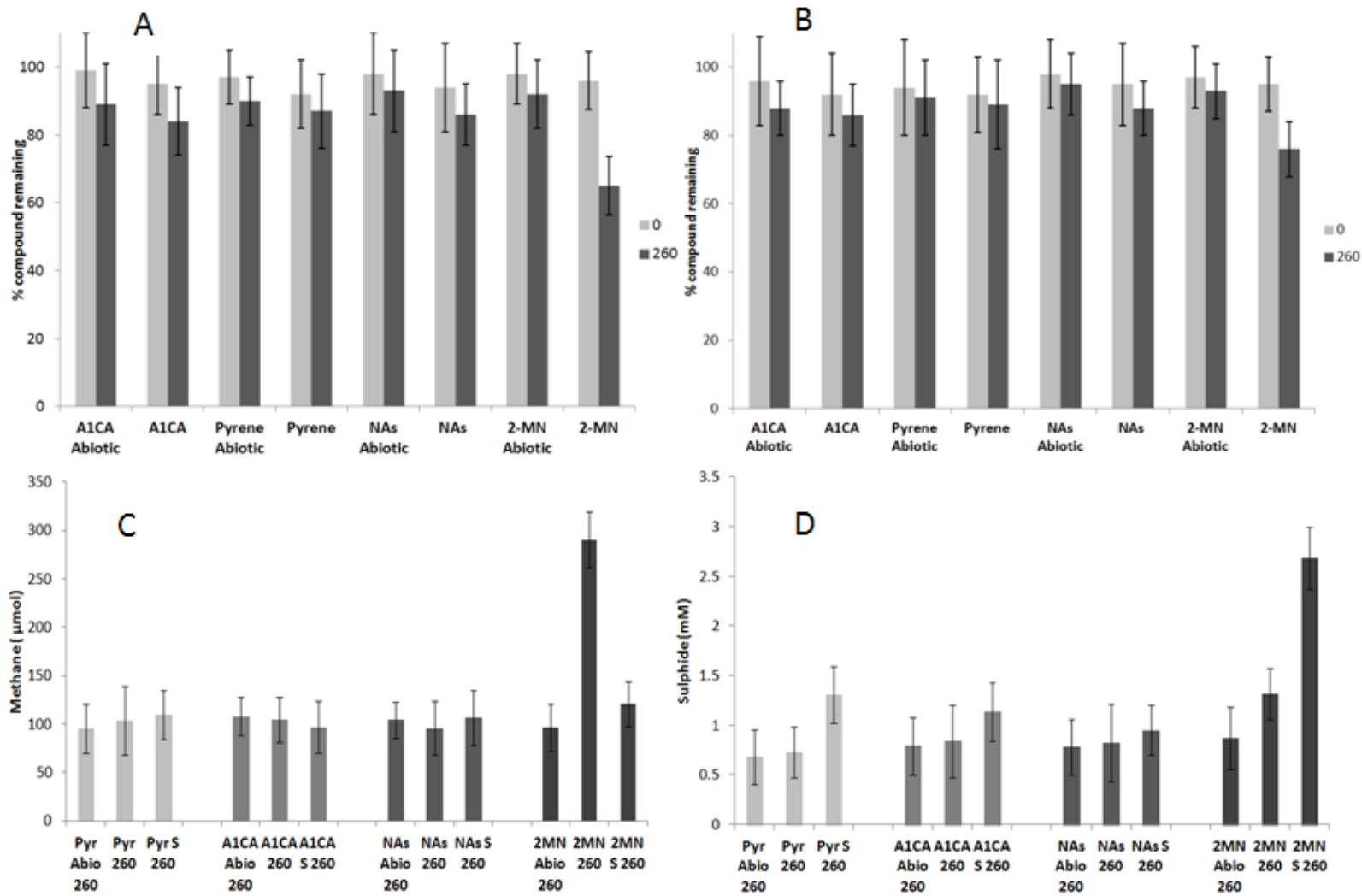


Fig. 1.

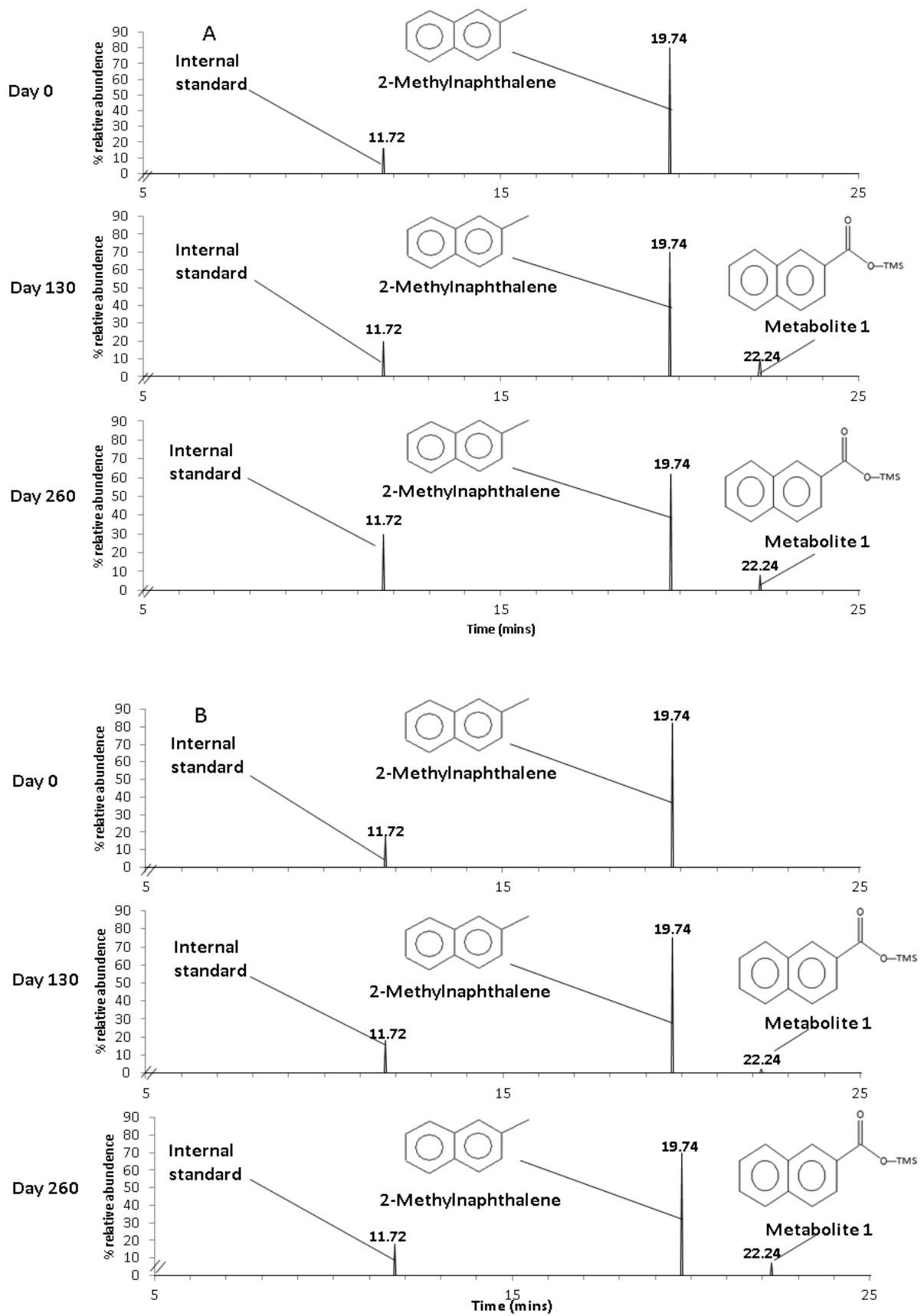


Fig. 2.

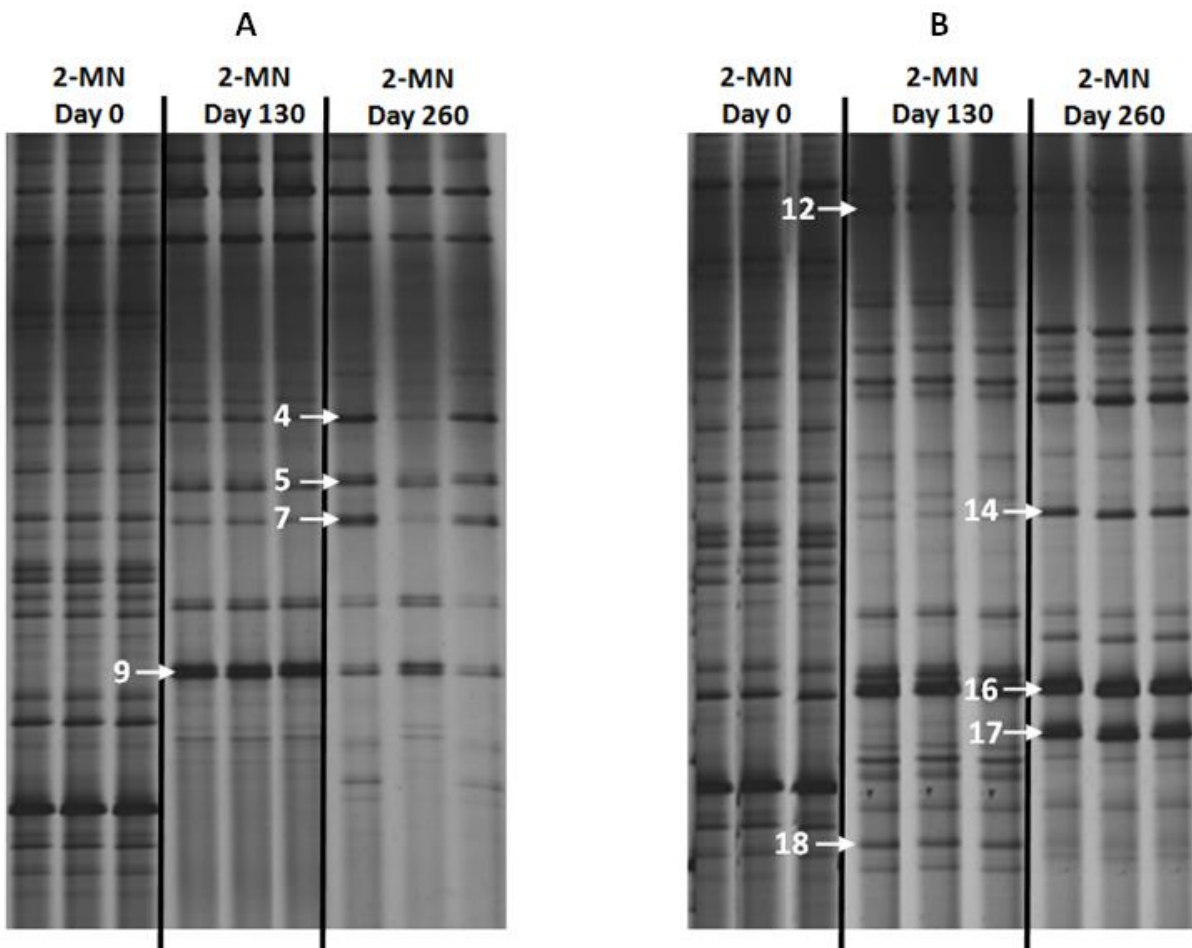


Fig. 3.

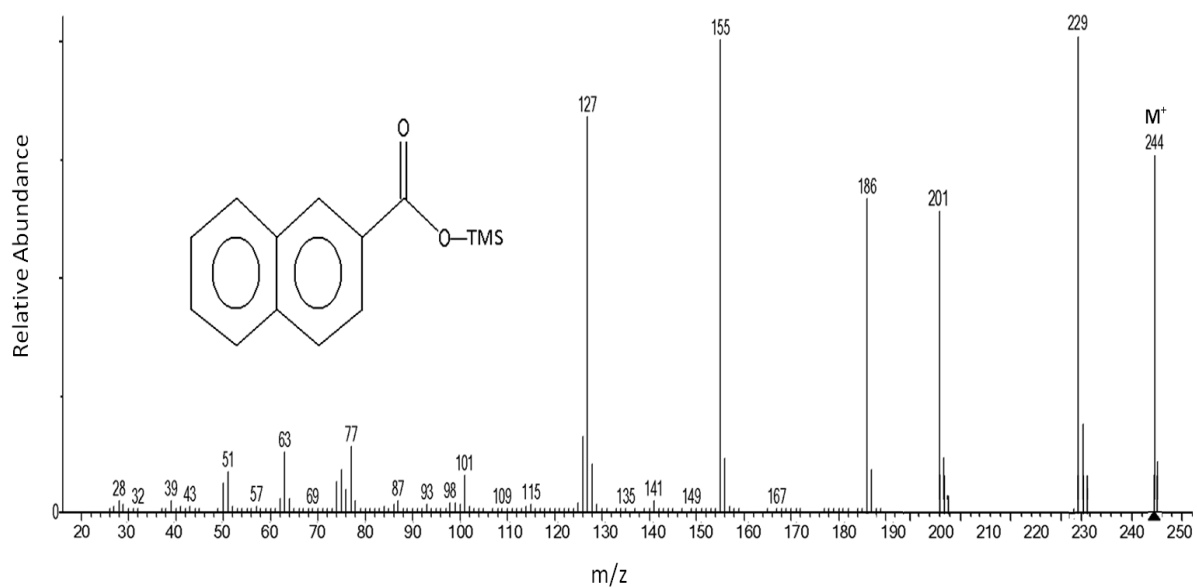
Table 1 Blastn analysis of the 16S rRNA genes from excised DGGE bands.

DGGE band	Closest match	Sequence length(bp)	% 16S rRNA gene sequence similarity	Environment from which closest match was derived	Genbank accession number of most closely related sequence
4	<i>Desulfobacterium autotrophicum</i>	193	96	Marine	NR_074942.1
5	<i>Desulfonatronum thiodismutans</i>	194	97	Freshwater	NR_025163.1
7	<i>Variovorax dokdonensis</i>	184	98	Soil	NR_043615.1
9	<i>Thaurea aromatica</i>	193	97	Activated sludge	NR_026153.1
12	<i>Fusibacter paucivorans</i>	192	95	Oil producing well	NR_024886.1
14	<i>Alkaliphilus</i> spp.	191	95	Methanogenic	NR_041892
16	<i>Sphingobium astaxanthinifaciens</i>	176	94	Freshwater	NR_041535.1
17	<i>Xanthobacter</i> spp.	205	97	Soil	NR_026353
18	<i>Hydrogenophaga caeni</i>	196	98	Activated sludge	NR_043769.1

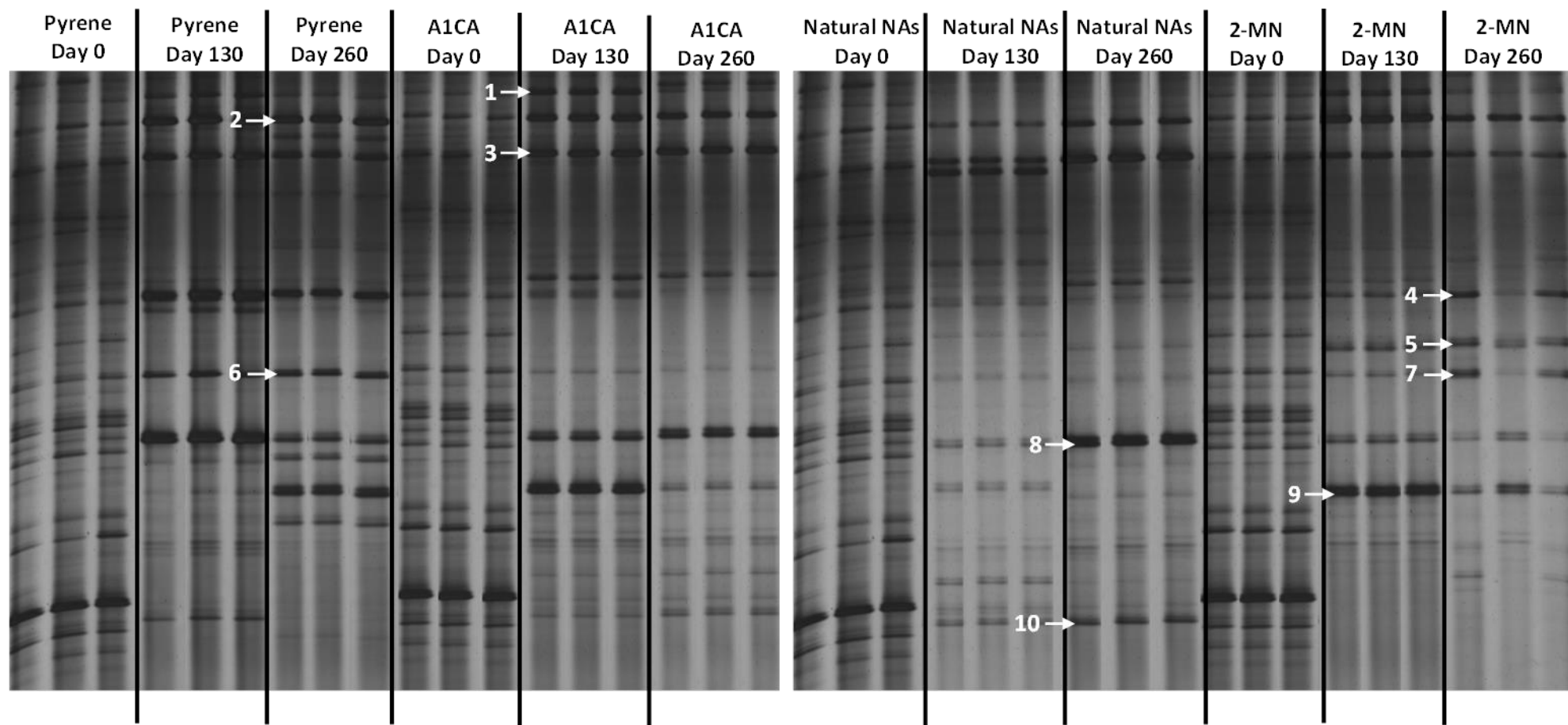
Supplementary Table S1. Blastn analysis of the 16S rRNA genes from excised DGGE bands from the methanogenic and sulfate-reducing cultures.

DGGE band	Closest match from Blastn	Sequence length (bp)	% 16S rRNA gene sequence identity	Environment from which closest match was derived	Genbank accession number of most closely related sequence
1	<i>Alkaliphilus</i> spp.	199	98	Methanogenic	NR_041892.1
2	<i>Fusibacter</i> spp.	217	95	Oil producing well	NR_024886.1
3	<i>Clostridium acetobutylicum</i>	210	99	Soil	AE_001437
4	<i>Desulfobacterium autotrophicum</i>	193	96	Marine	NR_074942.1
5	<i>Desulfonatronum thiodismutans</i>	223	97	Freshwater	NR_025163.1
6	<i>Sanguibacter</i> spp.	179	96	Marine sediment	NR_042311
7	<i>Variovorax dokdonensis</i>	184	98	Soil	NR_043615.1
8	<i>Acidovorax defluvii</i>	201	95	Activated sludge	NR_026506.1
9	<i>Thaurea aromatica</i>	193	97		NR_041011.1
10	<i>Perlucidibaca piscinae</i>	192	94	Freshwater	NR_043919.1

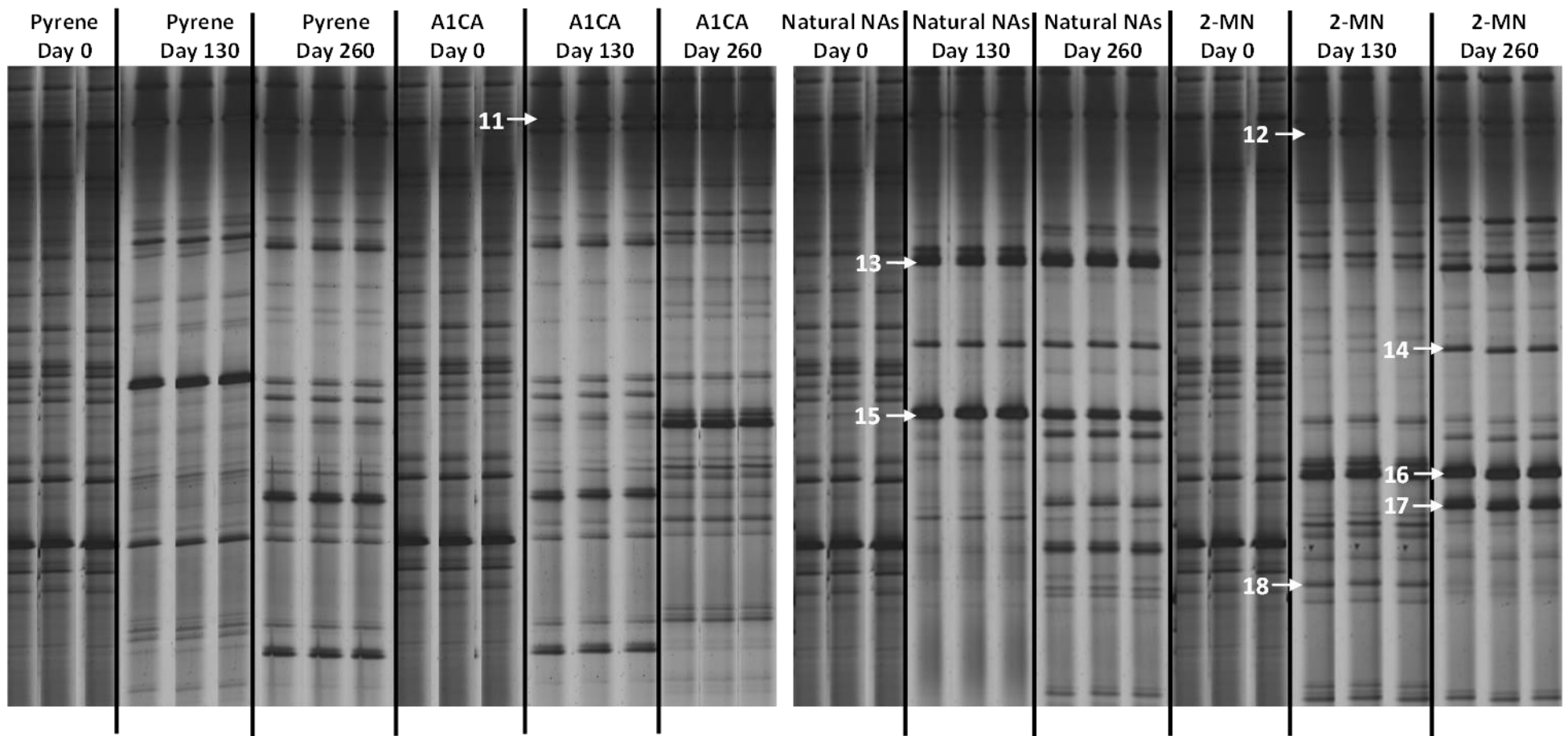
11	<i>Proteocatella sphenisci</i>	204	98	Antarctic soil	NR_041885.1
12	<i>Fusibacter paucivorans</i>	192	95	Oil producing well	NR_024886.1
13	<i>Anoxynatronum sibiricum</i>	218	94	Freshwater	NR_025256
14	<i>Alkaliphilus</i> sp.	221	95	Methanogenic	NR_041892
15	<i>Corynebacterium</i> spp.	187	98	Soil	NR_102500
16	<i>Sphingobium astaxanthinifaciens</i>	176	94	Freshwater	NR_041535.1
17	<i>Xanthobacter</i> sp.	205	97	Soil	NR_026353
18	<i>Hydrogenophaga caeni</i>	196	98	Activated sludge	NR_043769.1



Supplementary Fig. S1. Mass spectrum of metabolite 1, tentatively identified as 2-naphthoic acid (silylated) detected in 2-MN cultures under both sulfate-reducing and methanogenic conditions.



Supplementary Fig. S2. Composite image of DGGE gel (using F341 and R534 primers) from the sulfate-reducing cultures demonstrating bacterial community structure in the presence of Pyr, A1CA, Natural NAs and 2-MN at day 130 and day 260. The bands indicated (1-10) were excised and sequenced. Band numbers correspond to those in Supplementary Table S1.



Supplementary Fig. S3. Composite image of DGGE gel (using F341 and R534 primers) from the methanogenic cultures demonstrating bacterial community structure in the presence of Pyr, A1CA, Natural NAs and 2-MN at day 130 and day 260. The bands indicated (11-18) were excised and sequenced. Band numbers correspond to those in Supplementary Table S1.