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

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

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Abstract

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

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

1. Introduction

Oil sands operations in Canada produce more than 200 million barrels of crude oil per year (Del Rio et al., 2006). During oil-sand refining, vast quantities of wastewaters known as tailings are generated that have to be stored indefinitely in settling basins or ponds until strategies for reclamation are devised and approved. Tailings are composed of solids (e.g. sand, silt) and oil sands process-affected water (OSPW) that contains complex mixtures of toxic carboxylic acids known as naphthenic acids (NAs). It has been estimated that in Canada alone > 840 million m^3 of OSPW has accumulated and is being stored in tailings ponds (Siddique et al., 2011). There have been very few studies on anaerobic NA biodegradation despite the fact that, as most tailings ponds mature, they become anoxic (Whitby, 2010); and in sulfate-depleted ponds, methane is produced in very large quantities (Clemente & Fedorak, 2005; Fedorak et al., 2002; Holowenko et al., 2000). For example, methane released from Syncrude's largest tailings pond, the Mildred Lake Settling Basin, has been estimated to be up to 43000 m^3 day^{-1} (Holowenko et al., 2000, Siddique et al., 2006). It is not understood whether NAs present in tailings ponds can act as substrates in methane biogenesis *in situ*. Holowenko et al. (2001) demonstrated that methanogenesis was stimulated by the model NA compounds 3-cyclohexylpropanoic acid and 4-cyclohexanepentanoic acid in microcosms containing tailings pond water (Holowenko et al., 2001). Furthermore, Siddique et al. (2006; 2007; 2011) have shown that short chain alkanes, BTEX compounds (benzene, toluene, ethylbenzene and xylenes) and longer chain alkanes (up to C_{18}) are degraded under methanogenic conditions in OSPW (Siddique et al., 2011). Additionally, to date, only a limited number of hydrocarbons have been shown definitively to biodegrade anaerobically in other environments (reviewed by Widdel et al. (2010). This has been shown using

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

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

2. Methods

2.1 Environmental sample

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

2.2 PAH and NA compounds

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

2.3 Biodegradation experiment

The basal medium for all anaerobic cultures contained per litre of anaerobic water: K_2HPO_4, 0.652 g; $NaH_2PO_4.H_2O$, 0.173 g; NH_4HCO_3, 0.443 g; $NaHCO_3$, 3.73 g and 1 mL Resazurin solution (0.5 g L^{-1}). The basal medium for sulfate reducers was made as above with the addition of $NaSO_4$ at 4 g L^{-1}. Basal medium (92.5 mL) was dispensed into serum bottles which were closed with butyl rubber stoppers and crimp sealed. The gas phase was replaced with H_2/CO_2 (80%/20%) (in order to initiate anaerobic growth) to a pressure of 1.5 bar, and autoclaved. Thereafter, 2.5 mL each, of three filter sterilized solutions (A, B and C), were added by syringe to 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: Na_2S, 2.402 g. Solution C contained L^{-1} of anaerobic water:
129 $CaCl_2.2H_2O$, 4.410 g; $MgCl_2.6H_2O$, 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^{o}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 N_2/CO_2
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).
141
142 **2.4 Solvent extraction, GC-MS and analysis**
143 To remove hydrocarbon contamination all glassware was soaked overnight in
144 Decon^{90} (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

Scientific) using a separating funnel. Each HMW-PAH was extracted as above except samples were extracted with HPLC-grade acetone (Fisher). Solvent extracts were pooled, dried with 5–10 g anhydrous Na_2SO_4 (Fisher Scientific), and the organic acids concentrated by rotary evaporation (Buchi) at 40^{o}C. Samples were transferred to a gas chromatography vial (Chromacol) and stored at – 20^{o}C. Prior to analysis, all samples were reduced to dryness under a gentle stream of nitrogen at 40^{o}C and reacted with N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA, USA) at 60^{o}C for 20 min to form trimethylsilyl derivatives. Derivatized samples were resuspended in 1mL dichloromethane (HPLC, Acros Organics). Samples were separated by gas chromatography–mass spectrometry (GC-MS) using an Agilent 7890 GC interfaced with an Agilent 5975C MS. Samples were injected with a 1 µl splitless injection (injector temperature 250^{o}C) onto a 30 m x 250 µm x 0.25 µm Rtx – 1MS column using helium as the carrier gas at a constant flow of 1 mL min^{-1}. Oven temperatures were programmed with an initial increase from 40^{o}C to 250^{o}C at 10^{o}C min^{-1} and a final hold at 250^{o}C for 10 minutes. For PAHs, oven temperatures were programmed with an initial increase from 40^{o}C to 300^{o}C at 10^{o}C min $^{-1}$ and a final hold at 300^{o}C for 10 minutes. The transfer line was held at 230^{o}C onto a source for the MS which was in full scan mode (scan range 50-550 Da). Data was analysed and integrated with Agilent GC Chemstation. Agilent GC Chemstation was used to tentatively identify metabolites through mass spectral analysis.

2.5 Methane measurements

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

with the FID maintained at 150^{o}C. Gases were separated on a 1.5m stacked stainless steel column using N_2 as the carrier gas at a flow rate of 1 mL min^{-1}.

2.6 Sulfide measurements

Sulfide measurements were performed according to the method of Cord-Ruwisch (1985). A 50 µl sample of culture was mixed with 950 µl of 5 mM $CuSO_4$ and 50 mM HCl and the optical density measured at 480 nm on a spectrophotometer (Cecil CE2021).

2.7 Bacterial community analysis.

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

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

2.9 Statistical analysis

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

3. Results

3.1 Anaerobic degradation of 2-MN

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

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

cultures incubated with 2-MN under sulfate-reducing conditions contained 1.6 mM ± 0.389 sulfide , and by day 260 sulfide production had significantly increased to 2.68 mM ± 0.312 (p<0.05) (Fig. 1D).

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

3.2 Bacterial community analysis

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

5. Conclusions

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

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Titles to Figures and Tables

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

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

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

Table 1. Blast*n* analysis of the 16S rRNA genes from excised DGGE bands.

Supplementary information

Supplementary Fig. S1. Mass spectrum of metabolite 1, tentatively identified as 2-naphthoic acid (siliylated) 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.

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

644 **Composition of anaerobic media**

645 The basal medium for all anaerobic cultures contained L^{-1} of anaerobic water:
646 K_2HPO_4, 0.652 g; NaH_2PO_4. H_2O, 0.173 g; NH_4HCO_3, 0.443g; $NaHCO_3$, 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 $NaSO_4$ 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; $FeCl_2$. $4H_2O$, 2000mg; $CoCl_2$ x 6 H_2O, 190
651 mg; $ZnCl_2$, 70 mg; $CuCl_2$, 2 mg; H_3BO_3, 6 mg; Na_2MoO_4, 36 mg; $NiCl_2$ .6 H_2O, 24
652 mg. 50 mL portions were filter sterilised and stored in N_2 flushed, crimp sealed
653 serum bottles, with gas phase filled with N_2 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 N_2 flushed, crimp sealed serum bottles,
657 with gas phase filled with N_2 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; nicotinamide137.5 mg. Aliquots (50 mL) were filter sterilised and stored in N_2
662 flushed, crimp sealed serum bottles, with gas phase filled with N_2 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 N_2 flushed, crimp sealed serum
666 bottles, with gas phase filled with N_2 to 1.5 bar. The Na_2S solution for all anaerobic
667 cultures contained 100 mL^{-1} anaerobic water: Na_2S, 24.02 g. Aliquots (50 mL) were
668 filter sterilised and stored in N_2 flushed, crimp sealed serum bottles, with gas phase

669 filled with N_2 to 1.5 bar. The Ca and Mg solution contained L^{-1} of anaerobic water:
670 $CaCl_2.2H_2O$, 4.410 g; $MgCl_2.6H_2O$, 4.066 g. 50 mL portions were filter sterilised and
671 stored N_2 flushed, in crimp sealed serum bottles, with gas phase filled with N_2 to 1.5
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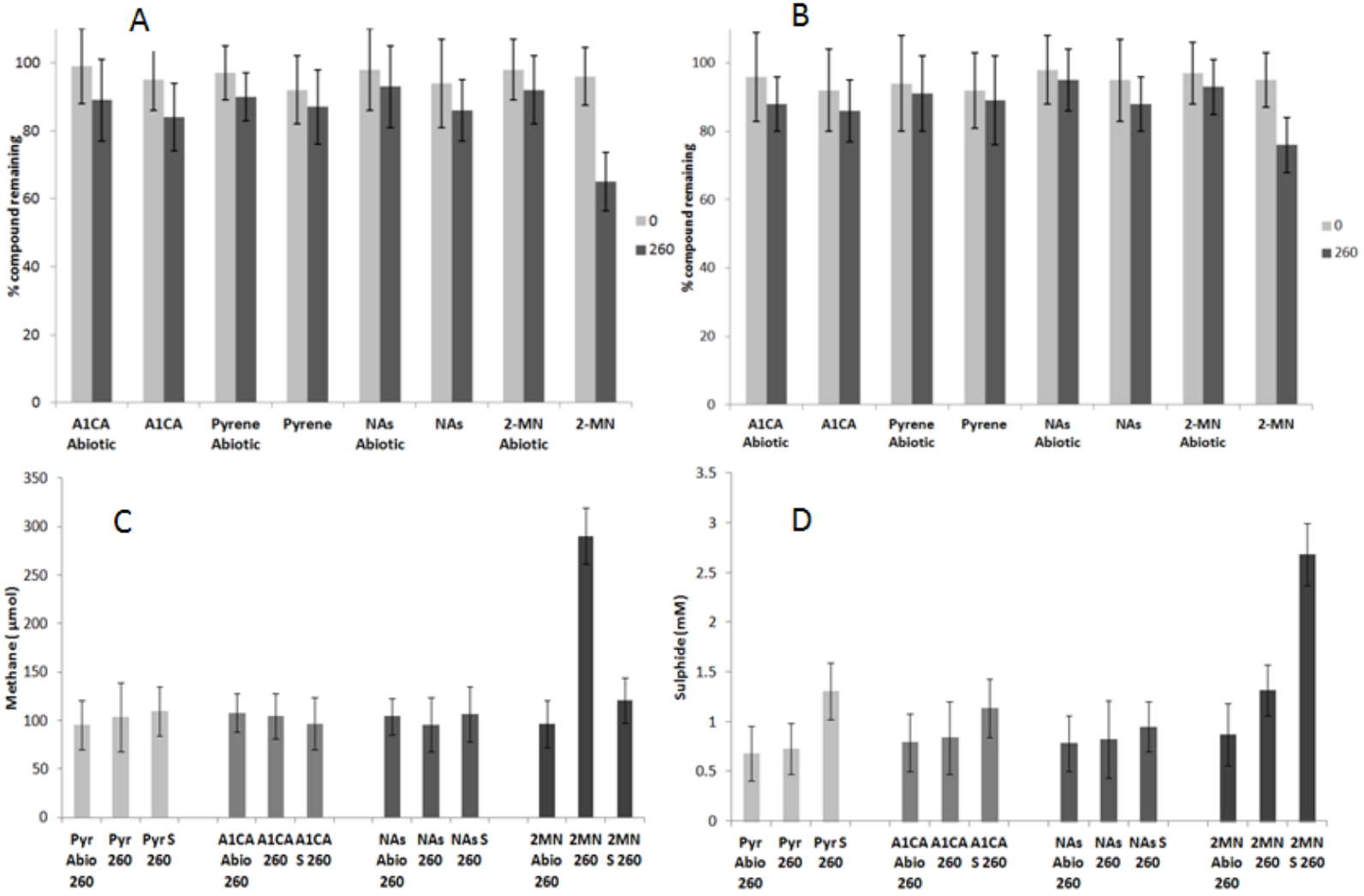
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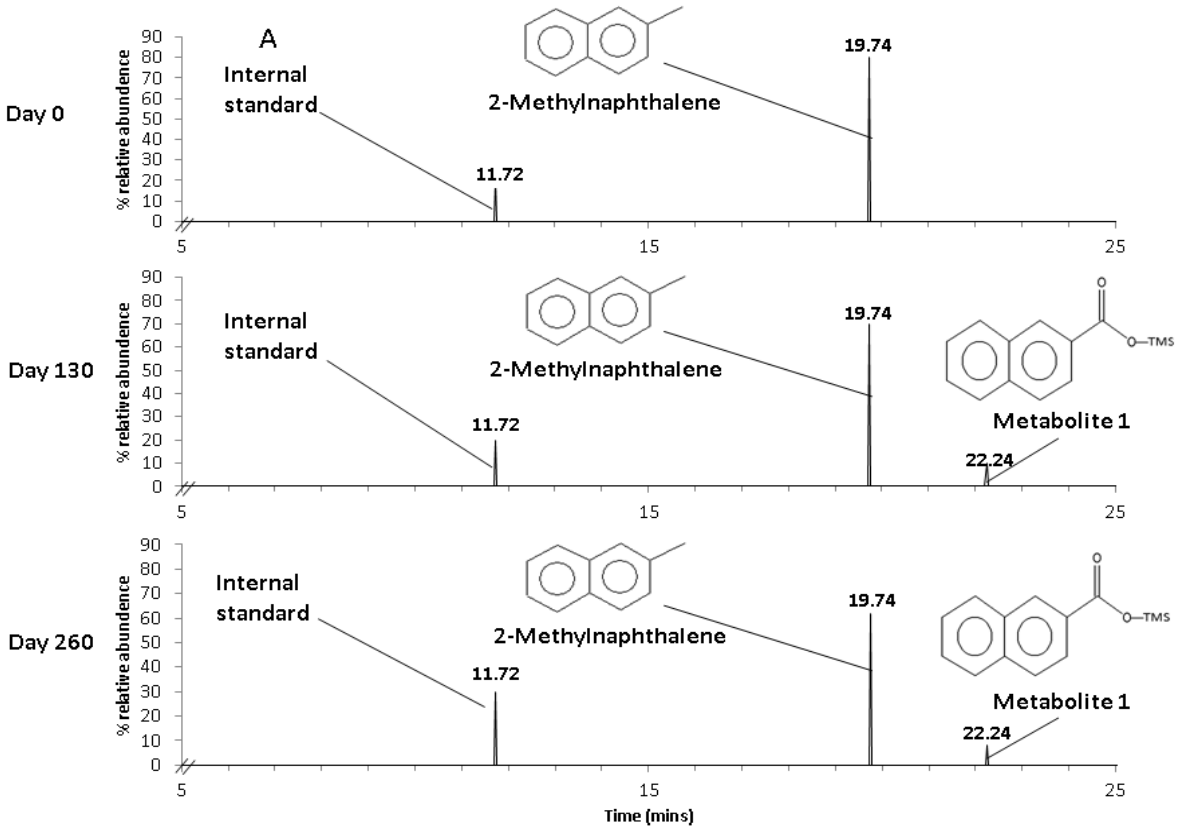
Fig. 1.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Metabolite 1
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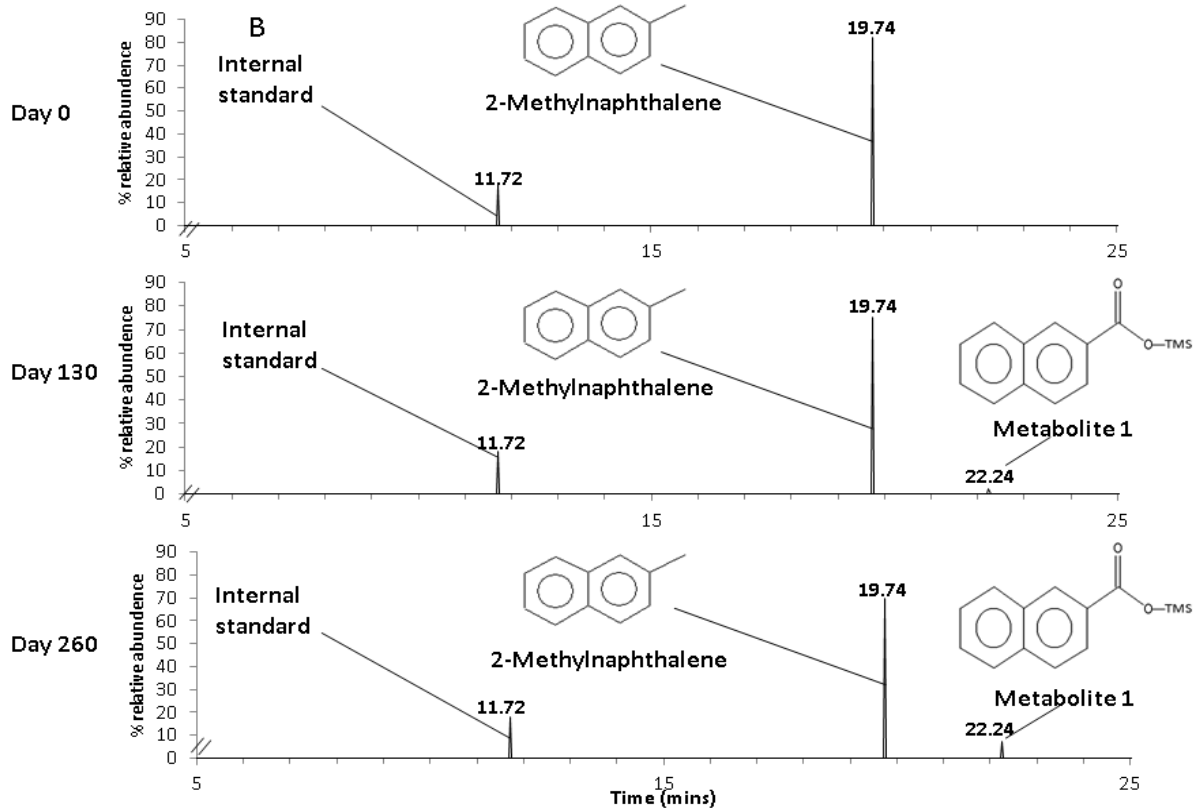
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2-Methylnaphthalene
Metabolite 1
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TMS
Time (mins)

Fig. 2.

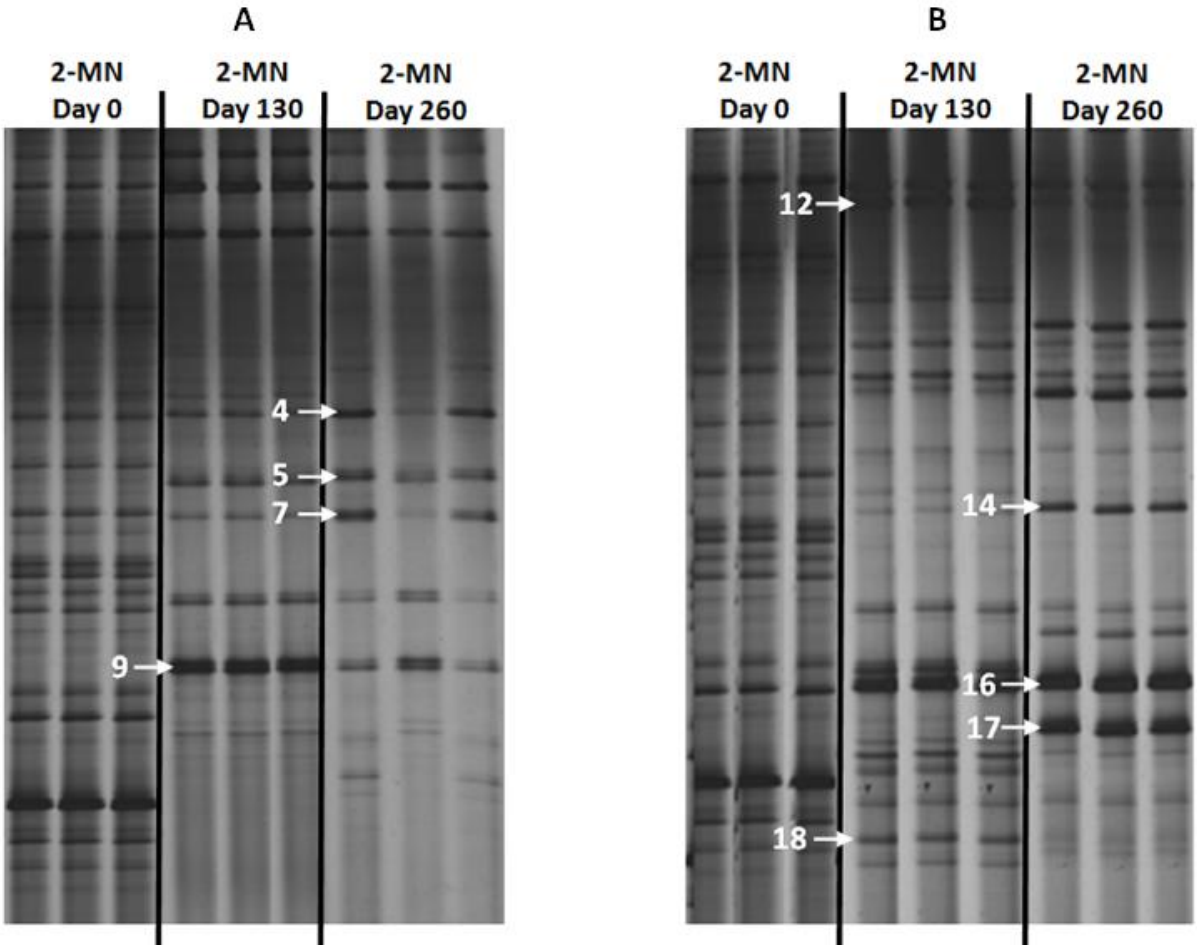
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Fig. 3.

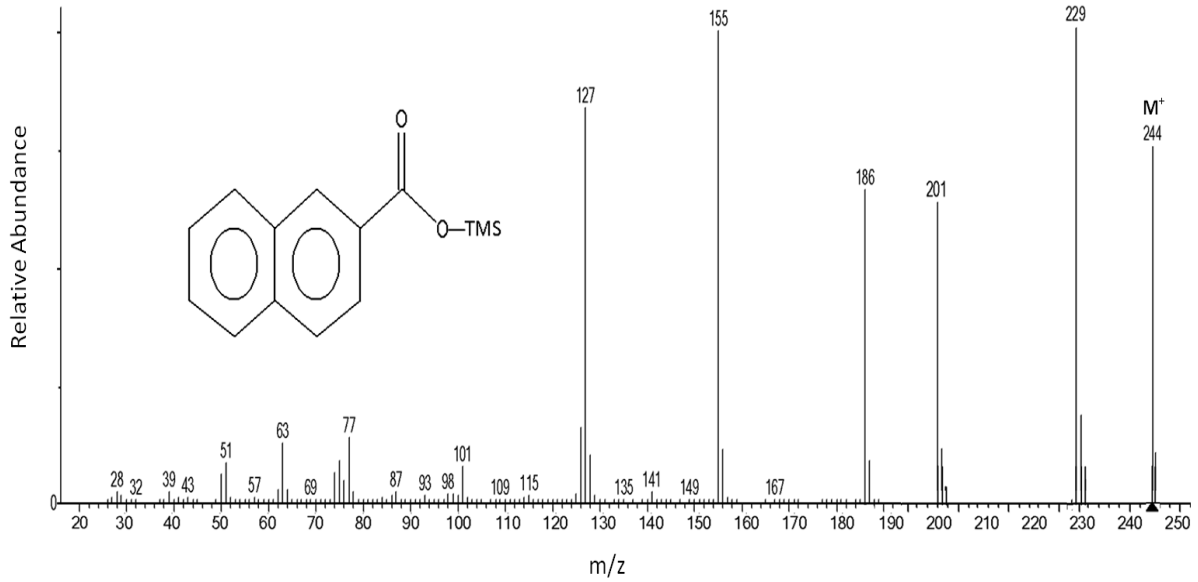
Table 1 Blast*n* analysis of the 16S rRNA genes from excised DGGE bands.

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	*Desulfobacterium autotrophicum*	193	96	Marine	NR_074942.1
5	*Desulfonatronum thiodismutans*	194	97	Freshwater	NR_025163.1
7	*Variovorax dokdonensis*	184	98	Soil	NR_043615.1
9	*Thaurea aromatica*	193	97	Activated sludge	NR_026153.1
12	*Fusibacter paucivorans*	192	95	Oil producing well	NR_024886.1
14	*Alkaliphilus* spp.	191	95	Methanogenic	NR_041892
16	*Sphingobium astaxanthinifaciens*	176	94	Freshwater	NR_041535.1
17	*Xanthobacter* spp.	205	97	Soil	NR_026353
18	*Hydrogenophaga caeni*	196	98	Activated sludge	NR_043769.1

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

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

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



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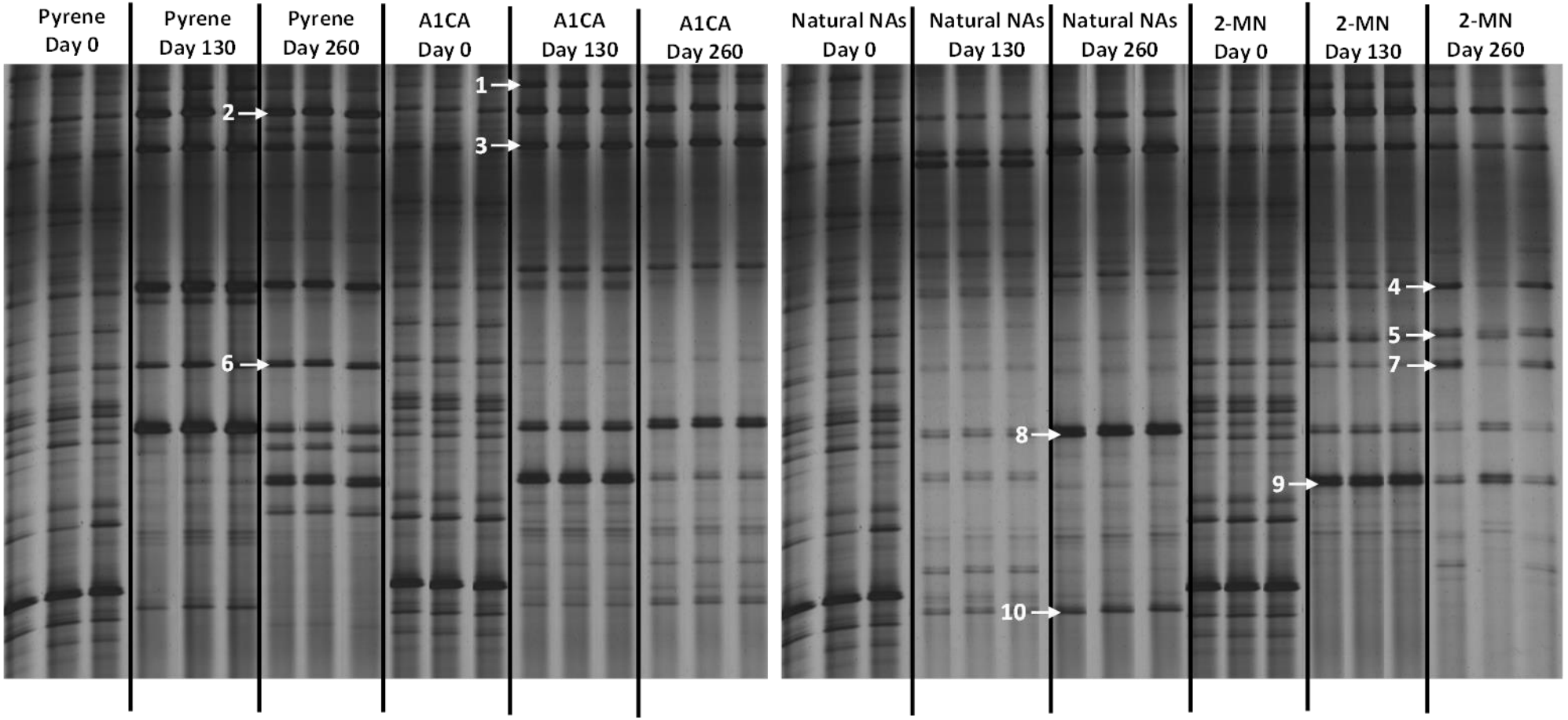

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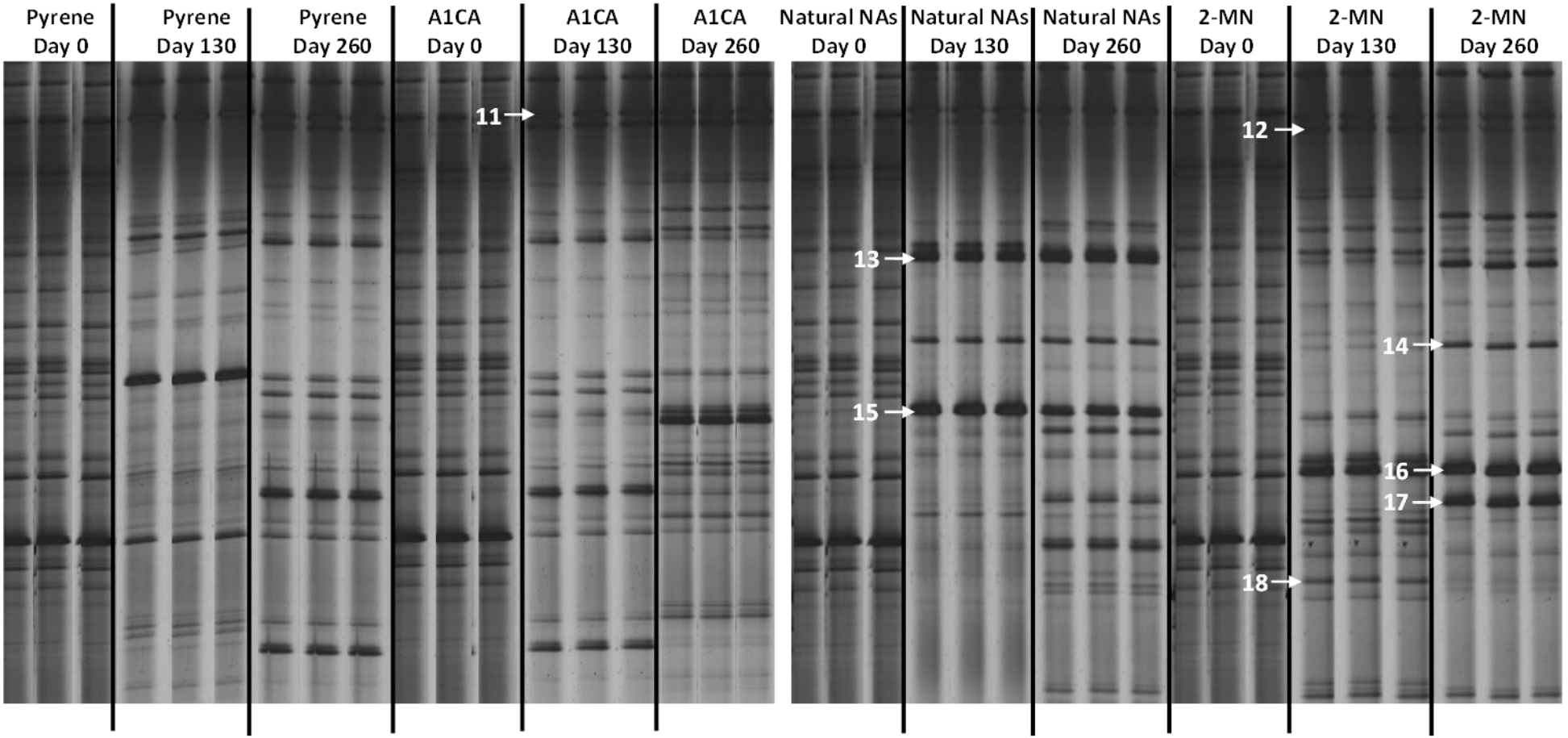

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