FEMS Microbiology Ecology, 93, 2017, fix040

doi: 10.1093/femsec/fix040
Advance Access Publication Date: 22 March 2017
Research Article

RESEARCH ARTICLE

Elucidation of the methanogenic potential from coalbed microbial communities amended with volatile fatty acids

Christopher N. Lyles[1,*,†], Victoria A. Parisi[1,‡], William Howard Beasley[3], Joy D. Van Nostrand[1,2], Jizhong Zhou[1,2] and Joseph M. Suflita[1]

[1]Department of Microbiology and Plant Biology and the Institute for Energy and the Environment, University of Oklahoma, Norman, OK 73019-0390, USA, [2]Institute for Environmental Genomics, University of Oklahoma, Norman, OK 73019-0390, USA and [3]Howard Live Oak, LLC, Statistics, Norman, OK 72019, USA

*Corresponding author: Department of Biology, Microbiology, and Veterinary Technology, Northwestern State University, 135 Sam Sibley drive, Natchitoches, LA 71497, USA. Tel: +(318) 357–6514; E-mail: lylesc@nsula.edu
†Present Address: Northwestern State University, Department of Biology, Microbiology, and Veterinary Technology, Natchitoches, LA, USA
‡Present Address: ConocoPhillips Company, Applied Geosciences, Houston, TX.
One sentence summary: The potential for modern coalfield methanogenesis was assessed using formation water as inocula in nutrient-replete incubations amended with presumed intermediates of anaerobic coal biodegradation.
Editor: Tillmann Lueders

ABSTRACT

The potential for modern coalfield methanogenesis was assessed using formation water from the Illinois Basin, Powder River Basin and Cook Inlet gas field as inocula for nutrient-replete incubations amended with C_1–C_5 fatty acids as presumed intermediates formed during anaerobic coal biodegradation. Instead of the expected rapid mineralization of these substrates, methanogenesis was inordinately slow (~1 μmol day^{-1}), following long lag periods (>100 days), and methane yields typically did not reach stoichiometrically expected levels. However, a gene microarray confirmed the potential for a wide variety of microbiological functions, including methanogenesis, at all sites. The Cook Inlet incubations produced methane at a relatively rapid rate when amended with butyrate ($r = 0.98$; $p = 0.001$) or valerate ($r = 0.84$; $p = 0.04$), a result that significantly correlated with the number of positive *mcr* gene sequence probes from the functional gene microarray and was consistent with the *in situ* detection of C_4–C_5 alkanoic acids. This finding highlighted the role of syntrophy for the biodegradation of the softer lignite and subbituminous coal in this formation, but methanogenesis from the harder subbituminous and bituminous coals in the other fields was less apparent. We conclude that coal methanogenesis is probably not limited by the inherent lack of metabolic potential, the presence of alternate electron acceptors or the lack of available nutrients, but more likely restricted by the inherent recalcitrance of the coal itself.

Keywords: coalbed methane; coal basin; metabolite analysis; alkanoic acids; microbial communities; functional gene array

INTRODUCTION

Coal has been considered an unconventional natural gas reservoir where thermogenic or biogenic methane is stored predominantly in an adsorbed state (Suárez-ruiz 2012). Consistent reports in the literature suggest that a small fraction of methane from coalbeds is of modern origin (Thielemann 2004; Jones,

Received: 4 January 2017; **Accepted:** 16 March 2017

Voytek and Warwick 2008; Klein *et al.* 2008; Jones *et al.* 2010; Meslé, Dromart and Oger 2013). Biogenic coalbed methane is believed to contribute about 3%–4% of the total natural gas production in the United States (Strąpoć *et al.* 2011b). Considering the vast worldwide reserves of coal (Strąpoć *et al.* 2011b; Moore 2012), the ever-expanding need for energy across the globe and the environmental benefits that attend methane utilization as an energy source, there is considerable interest in stimulating the modern bioconversion of coal to methane (Green, Flanegan and Gilcrease 2008; Harris *et al.* 2008; Penner, Foght and Budwill 2010; Wawrik *et al.* 2011; Ünal *et al.* 2012, Ulrich and Bower 2008; Fallgren *et al.* 2013; Liu *et al.* 2013; Stephen *et al.* 2014).

Coal is an extremely heterogeneous organic material that is challenging to fully chemically characterize (Strąpoć *et al.* 2011b; Moore 2012). Given its inherent chemical complexity, coal is not an ideal substrate for methanogenic fermentation processes. However, it can reasonably be assumed that, like other forms of organic matter, the anaerobic mineralization of coal will require a nutritionally diverse assemblage of anaerobic bacteria that attack large molecular weight labile components and convert them to simpler chemical residues. The latter components ultimately get converted to a suite of volatile fatty acid (VFA) and alcohol intermediates (Leschine 1995; Faiz and Hendry 2006; Green, Flanegan and Gilcrease 2008; Strąpoć *et al.* 2008; Fry *et al.* 2009; Beckmann *et al.* 2011; Wawrik *et al.* 2011; Meslé, Dromart and Oger 2013; Orem *et al.* 2014). The anaerobic bioconversion of these critical intermediates has been the subject of many investigations. What seems clear is that the ultimate conversion of the small molecular weight intermediates to methane is often a rate-limiting process that requires a thermodynamically based microbial syntrophism. That is, substrate bioconversion by one microorganism occurs only when the resulting products, typically hydrogen, formate or acetate, are maintained at very low concentrations by a cooperating microbial partner. In electron-acceptor limited environments, these simple electron donors are maintained at the requisite low levels by acetoclastic, methylotrophic and hydrogenotrophic methanogens (Thauer, Jungermann and Decker 1977; Berry, Francis and Bollag 1987; Widdel and Rabus, 2001; McInerney *et al.* 2008; Meslé, Dromart and Oger 2013). Additionally, *Methermicoccus shengliensis* has been shown to directly utilize methoxylated aromatic compounds to produce methane from coal (Mayumi *et al.* 2016).

A considerable amount of research has been dedicated to identifying the requisite microorganisms associated with the bioconversion of coal to methane, and convincingly, a large body of work has been assembled identifying the presence of known fermenting and syntrophic bacteria as well as methanogenic archaea in coalfields worldwide (Thielemann 2004; Green, Flanegan and Gilcrease 2008; Harris, Smith and Barker 2008; Strąpoć *et al.* 2008, 2011a; Fry *et al.* 2009; Kimura *et al.* 2010; Midgley *et al.* 2010; Papendick, Downs and Vo 2011; Wawrik *et al.* 2011; Meslé, Dromart and Oger 2013; Wei *et al.* 2014). Based on these studies, it would seem that biogenic coalbed methane production is not likely to be hindered by a fundamental lack of metabolic potential (Wawrik *et al.* 2011). Of course, in addition to the presence of the requisite microorganisms, the environment should be suitable for microbial activity and growth. For efficient methanogenesis, this implies the lack of alternate electron acceptors, an appropriate and available supply of nutrients (C, N, P, S and trace elements), as well as pH and temperature conditions that are nominally compatible with the requirements of the indigenous microflora. Similarly, we presume that the major microbial activities in coalfields would be co-associated with the ready availability of moisture.

The maturity of coal can also substantially impact the production of methane. Previous studies have reported that lower-rank coal typically produced more biogenic methane than mature coals (Kotarba, 2001; Formolo, Martini and Petsch 2008; Harris, Smith and Barker 2008; Strąpoć *et al.* 2011b). Glombitza *et al.* (2009) and Vieth *et al.* (2008) reported that low-ranking coals had higher concentrations of extractable formate and acetate, thus potentially providing a carbon source for deep subsurface microbial communities. In contrast, Fallgren *et al.* (2013) reported a positive correlation between increasing coal rank and methane production. Other studies have indicated that coal maturity and methanogenesis are not highly correlated (Faiz and Hendry 2006; Formolo, Martini and Petsch 2008; Jones, Voytek and Warwick 2008; Wawrik *et al.* 2011), suggesting that rank alone is not a reliable indicator of the susceptibility of coal to methanogenesis.

For our study, we reasoned that if *in situ* coalbed methane was an active and ongoing process, the indigenous microflora from three different coalfields should be able to readily utilize low molecular weight fatty acids given their presumed importance in anaerobic coal biodegradation. In a previous study from the San Juan Basin, the microbial communities associated with 11 water samples produced little to no methane when inoculated in nutrient-replete, anaerobic incubations amended with either formate, acetate or propionate (Wawrik *et al.* 2011). To further investigate the ability of coalbed microbial communities to generate methane from potential fatty acid intermediates, we obtained formation water samples from multiple locations in the Illinois and Powder River coal basins as well as the Cook Inlet Alaska gas field (Fig. 1). The formation water samples were used as inocula for nutrient-sufficient anaerobic incubations that were amended with formate or acetate to assay for the methanogenic archaea. Similarly, methanogenesis was assessed in other incubations that were amended with the individual VFAs, propionate, butyrate or valerate to assay for the requisite syntrophic bacteria. The genetic potential for organic matter utilization was also evaluated at each site using a functional gene microarray (GeoChip 3.2 or 4.0). Lastly, the low molecular weight polar organic compounds in the formation waters, presumed metabolites associated with coal biodegradation processes, were also analyzed by mass spectrometry. Our findings suggest that the bioconversion of coal is not limited by the inherent lack of metabolic potential but more likely restricted by the recalcitrant nature of the coal itself.

MATERIALS AND METHODS

Sample collection and site description

A total of 17 water samples were obtained from multiple sites within three coalfields in the United States: the Illinois Basin, Cook Inlet gas field and Powder River Basin. Formation water was collected in 160 ml degassed (N_2), sterile serum bottles that contained 0.1 ml resazurin (1 g L^{-1}) and 1 ml of sodium sulfide (0.5 mM) and shipped overnight on ice to the University of Oklahoma. The preparation of the serum bottles was done at the University of Oklahoma and our collaborating industrial partners (ConocoPhillips Corporation, Houston, TX) collected the water samples in the field.

Coal maturity in the three coalfields differ markedly (Fig. 1; Calderwood and Fackler 1972; Pratt, Mavor and DeBruyn 1999; Walters 2002; Strąpoć *et al.* 2007, 2011b; Hartz *et al.* 2009; AOGCC 2011). The Illinois Basin encompasses the state of Illinois and parts of Indiana and Kentucky. The basin consists of high volatile bituminous coals (Strąpoć *et al.* 2007). Five samples were

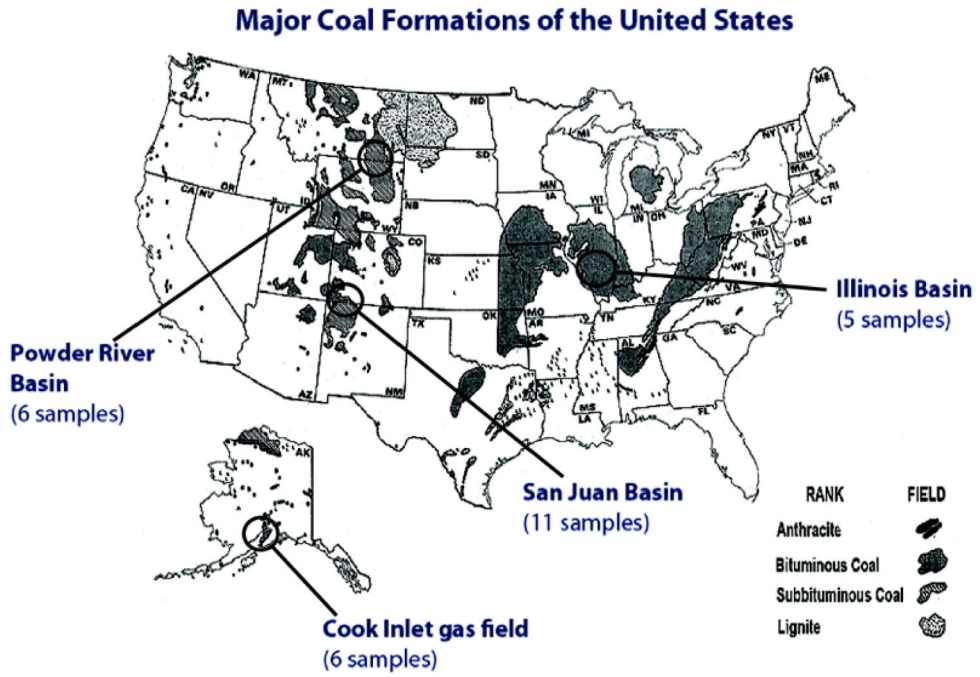

Figure 1. A map showing the major coal formations of the United States and the corresponding coal rank for each field (adapted from NETL, 2014). Seventeen water samples were collected from the Illinois Basin, Powder River Basin and the Cook Inlet gas field. Additionally, 11 sites from the San Juan Basin were investigated in a similar manner and previously published by Wawrik *et al.* (2011).

collected and designated 1–5. The Cook Inlet gas field is located along the western shoreline of Alaska (AOGCC 2011) and contains thin intercalated beds of claystone, sandstone, siltstone and lignitic to subbituminous coal (Calderwood and Fackler 1972). Six water samples were collected and designated 6–11. The Powder River Basin is located in Montana and northeast Wyoming and consists of subbituminous coals (Walters 2002). Six water samples were collected and designated 12–17.

Anaerobic incubations

A basal medium was prepared as previously described in McInerney, Bryant and Pfennig (1979), except rumen fluid and sulfate were omitted. Final concentrations of mineral components per liter were as follows: 57.4 mM K_2HPO_4, 32.5 mM $MgCl_2 \bullet 6H_2O$, 136.9 mM NaCl, 149.6 mM NH_4Cl and 9.01 mM $CaCl_2 \bullet 2H_2O$. The medium was also amended with a trace metal and vitamin solution described in Tanner (2002), a 0.001% solution of resazurin used as a redox indicator and 4.17 mM sodium bicarbonate ($NaHCO_3$). The medium (per liter) was reduced using 10 ml of a 2.5% cysteine sulfide solution. Strict anaerobic conditions were maintained as described by Widdel and Bak (1992). Nine-milliliter aliquots of the basal medium were dispensed into sterile 25 ml serum tubes in an anaerobic chamber (5% H_2 in N_2 gas phase). The serum tubes were closed with butyl rubber stoppers, crimped with aluminum seals and the headspace was exchanged to a N_2:CO_2 (80:20) atmosphere.

The basal medium was inoculated with 1 ml of a water sample from each of the 17 coalfield locations. Duplicate incubations were then amended with 5 mM of a specific low molecular weight VFA that included either formate, acetate, butyrate, propionate or valerate. Using the empirical equation derived by Buswell and Mueller (1952), theoretical yields of methane were predicted based on the initial concentration of VFA. The theoretical yields for formate, acetate, propionate, butyrate and valerate were 25, 50, 87.5, 125 and 162.5 μmol methane, respectively. Negative controls included sterile, VFA-unamended and uninoculated incubations. Incubations were monitored for methane production with time and values are corrected for the background level in the VFA-unamended controls. Rates of methanogenesis were calculated during periods of active methanogenesis and exclude the lag times for the incubations (Table S1, Supporting Information).

Analytical techniques

Methane production was measured using a gas chromatograph (Packard model 427, Downers Grove, IL, USA), equipped with a flame ionization detector and a Porapack Q column (Supleco, Inc., Bellefonte, Penn). The oven, detector and injector temperatures were set to 100°C, 100°C and 125°C, respectively. The concentration of chloride, nitrate and sulfate were measured using ion chromatography (Dionex model DX500, Sunnyvale, CA, USA) equipped with an IonPac AS4A column, ASRS 300 4 mm self-regenerating suppressor and a conductivity detector. The eluent consisted of 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate and was diluted 1:100 from a ready to use concentrate. The flow rate was 2.0 ml min^{-1} and the suppressor was set to 54 mA.

GeoChip functional gene array hybridization

The DNA extraction protocols were previously described (He *et al.* 2010; Lu *et al.* 2011). Approximately 100 ng of DNA was amplified using a Templiphi kit (GE Healthcare, Piscataway, NJ, USA), and labeled with Cy3 fluorescent dye (GE Healthcare) by random priming (Wu *et al.* 2006). The DNA was then purified with a QIAquick purification column (Qiagen, Valencia, CA) and dried in a SpeedVac (ThermoSavant, Milford, MA, USA) at 45°C for 45 min before hybridization. GeoChip analysis was performed

using either version 3.2 or 4.0. All hybridizations were carried out at 42°C with 40% formamide for 16 h on a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA). After hybridization, the arrays were scanned (NimbleGen MS200, Madison, WI, USA) at 100% laser power. The signal intensities were measured based on scanned images, and spots with signal-to-noise ratios lower than 2 for GeoChip 4.0 and 1.5 for GeoChip 3.2 were removed before statistical analysis as described previously (He *et al.* 2010; Lu *et al.* 2011).

GeoChip analysis

Approximately 800 unique functional genes were measured within each sample from the three coalfield basins. These genes were placed into 'gene categories' covering several fundamental microbial processes (He *et al.* 2010). Three changes were made to the organizational structure for GeoChip 3.2 and 4.0: the methyl coenzyme M reductase (*mcr*AB) genes were recategorized as methane production genes and the dissimilatory sulfite reductase (*dsr*AB) and adenosine-5′-phosphosulfate (*aps*AB) genes were recategorized as sulfate reduction genes, respectively. In order to reduce the complexity of the functional gene analysis, the nine most relevant categories are discussed within this paper. All data analyses were done using R (The R Foundation for statistical computing) and to facilitate reproducibility, the code and GeoChip raw data files are available at https://github.com/LiveOak/LylesCoalbedMethane2. For a complete description of the functional gene categories for GeoChip 3.2, see He *et al.* (2010) and for version 4.0 go to the Institute for Environmental Genomics (http://ieg.ou.edu/entrance.html).

Venn diagrams were used to display the genetic overlap of positive functional gene probes between the three coalfields. That is, if the probes were positive in any site within the coalfields, it was displayed as a shared functional gene. To further analyze the overlap, see Table S3 in the supplementary information. This table lists all the shared functional genes for the nine categories between the coalfields and how many sites per basin yielded a positive probe.

Correlation coefficients (r) were also used to indicate the strength and direction of the linear relationship between the total amount of methane produced from the various anaerobic incubations and the richness and abundance of *mcr* genes detected by the GeoChip. Strong correlation coefficients ($r > 0.79$) suggest a significant relationship (*t*-test, one-tail: $p < 0.05$) for the regression line between the two variables and indicate that the respective fatty acid amendment is likely a common metabolite throughout the assessed sample sites. Weak correlation coefficients ($r < 0.79$) are typically not significant (*t*-test, one-tail: $p > 0.05$) indicating that the two variables are independent and the fatty acid amendment is likely not a common metabolite throughout all the assessed sample sites. Additional statistical analysis of the GeoChip data is described in the supplemental information (Fig. S2, Supporting Information).

Metabolite profiling

Water samples from each of the coalfield sites were acidified with 50% HCl to a pH <2 and kept at 4°C until analyzed. One liter of acidified sample was extracted with ethyl acetate (10% vol/vol, four times) and dried over anhydrous Na_2SO_4. Extracted samples were concentrated by rotary evaporation and reduced further to approximately 100 μl under a flow of nitrogen gas (Duncan *et al.* 2009). The extract was derivatized using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, IL). Putative metabolites were identified using gas chromatography (Agilent model 6890, Santa Clara, CA) coupled with a mass spectrometer (Agilent model 5973, Santa Clara). Separations were performed using a HP-5 ms capillary column (30 m × 0.25 mm inner diameter × 0.25 μm film, J&W Scientific, Folsom, CA) with an initial oven temperature of 45°C for 5 min that was increased at 4°C min^{-1} to 270°C and held for 10 min (Aktas *et al.* 2010). All metabolite identifications were made by comparison of features with authentic standards.

Table 1. Chloride, nitrate and sulfate concentrations from 17 sample sites throughout three coalfield basins.

Sample Sites	Chloride (mM)	Nitrate (mM)	Sulfate (mM)
Illinois Basin			
1	93.3	BDL	0.038
2	58.7	0.058	0.075
3	48.7	0.050	0.143
4	2.92	BDL	0.090
5	1.20	BDL	0.625
Cook Inlet gas field			
6	3.23	BDL	0.066
7	4.23	0.018	0.072
8	9.42	BDL	0.037
9	35.4	BDL	0.063
10	0.58	BDL	0.035
11	0.59	BDL	0.034
Powder River Basin			
12	0.56	0.141	0.111
14	0.80	BDL	0.167
13	1.10	BDL	0.062
15	1.21	BDL	0.059
16	0.93	0.196	0.088
17	0.47	0.152	0.061

BDL = below detectable limit.

RESULTS

Water chemistry and metabolite analysis

Seventeen samples from the Illinois Basin, Cook Inlet gas field and Powder River Basin were analyzed for chloride, sulfate and nitrate anions (Table 1). The waters from the three coalfields were found to contain <0.5 mM of sulfate and nitrate, suggesting that alternate electron acceptors were unlikely to impact the endogenous rates of methanogenesis within the coalfields. The mean chloride concentrations for the Illinois Basin, Cook Inlet gas field and the Powder River Basin were 41.0 ± 39.2, 8.90 ± 13.4 and 0.843 ± 0.293 mM, respectively. Thus, the low chloride levels are also unlikely to be a substantive detriment to coalfield methanogenesis (Waldron *et al.* 2007).

Mass spectral analysis of water samples from the three coalfields was used to detect the presence of 19 alkanoic acids throughout the 17 sample sites (Fig. 2). The detection of C_1–C_5 alkanoic acids was particularly notable since we used the same compounds as substrates in the anaerobic biodegradation assays (below). Only one sample out of five in the Illinois Basin contained acetic, propionic and butanoic acids. Five out of six sites in the Cook Inlet gas field were positive for butanoic and pentanoic acids. Additionally, two sites contained acetic and propionic acid. Only one site within the Powder River Basin was positive for pentanoic acid. Longer chain fatty acids (octanoic through octadecanoic acids) were identified throughout

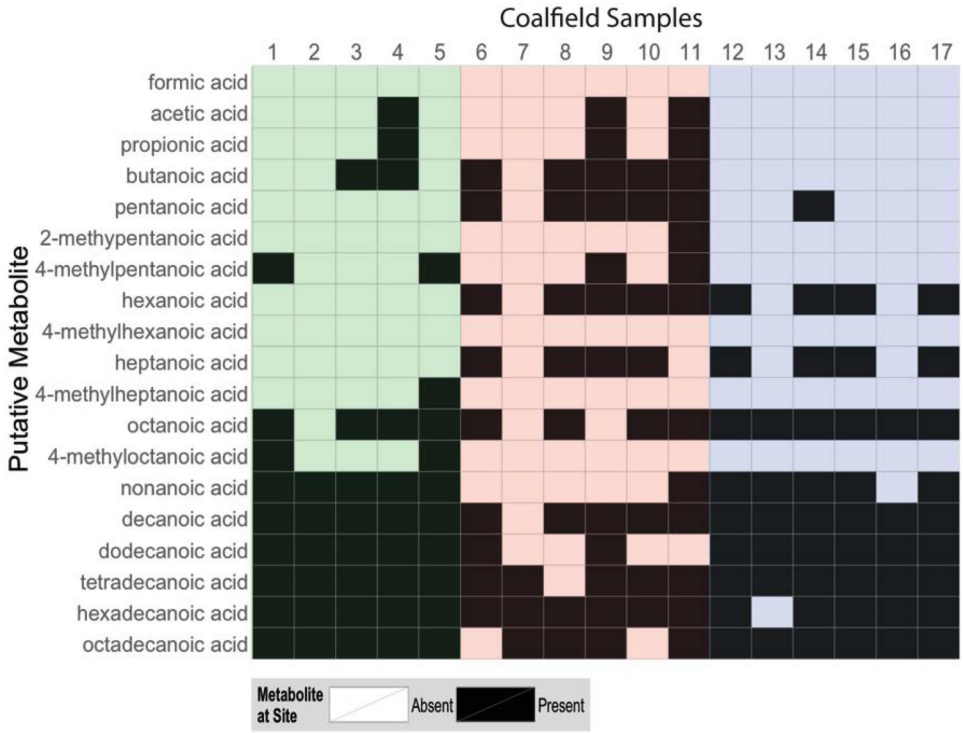

Figure 2. Alkanoic acids identified within the 17 sample sites from the Illinois Basin (Green), Cook Inlet gas field (Red) and Powder River Basin (Blue).

the samples from the three coalfields, though to a lesser extent within the Cook Inlet gas field.

Mass spectral analysis was also used to screen for an additional 71 polar organic compounds that could be putative intermediates associated coal biotransformation (Fig. S1, Supporting Information). The anaerobic metabolites for alkylbenzene degradation (benzylsuccinic acid through propylbenzylsuccinic acid) and polycyclic aromatic compounds (naphthylsuccinic through phenanthrene carboxylic acid) were only identified in a few samples throughout the Cook Inlet gas field. However, monoaromatic compounds (benzoate through 3-propyl phenol) were intermittently identified throughout the samples in the three coalfields. Additionally, alkylsuccinic acids (C_1 through C_{16}) were only identified in the Cook Inlet gas field samples. Overall, the Cook Inlet gas field had about twice the number of polar organic compounds than either the Illinois or Powder River Basins.

Anaerobic biodegradation assays

When laboratory incubations of coalfield water samples were amended with C_1–C_5 VFA compounds, slow rates of methanogenesis and long lag times were detected, and the stoichiometric expected amount of methane was not typically produced (Fig. 3). Prokaryotic biomass levels were estimated for each of the 17 samples (Table S2, Supporting Information). Cell numbers estimated based on the amount of DNA recovery ranged from $\sim 10^5$ to 10^7 cells ml^{-1} (Kubitschek and Freedman 1971; Button and Robertson 2001). There was no correlation between the estimated cell biomass levels and metabolic activity (Fig. 3; Table S2). The Illinois Basin (Fig. 3A) had two samples (1 and 2) out of five, where the inocula metabolized at least one of the C_1–C_5 VFAs to the theoretically expected amount of methane. Organisms within sample 1 incubations metabolized formate, acetate, butyrate and valerate to methane at a rate of 0.42 ± 0.10, 1.2 ± 0.8, 2.3 ± 0.88 and 0.48 ± 0.08 μmol CH_4 day^{-1}, respectively, following a 54- to 200-day lag period depending on the particular substrate. The microflora in sample 2 incubations amended with formate and acetate produced methane at a rate of 0.36 ± 0.04 and 0.52 ± 0.11 μmol CH_4 day^{-1} after a 50 and 25 day lag, respectively. Samples 3, 4 and 5 had negligible methanogenesis activity, with some lag times well in excess of 1 year. The Cook Inlet gas field (Fig. 3B) contained three samples (6, 7 and 8) out of six where the inoculum produced the theoretically expected amount of methane from the VFA amendments. The microbes in sample 6 incubations metabolized formate, acetate, propionate, butyrate and valerate to methane at a rate of 0.37 ± 0.08, 0.67 ± 0.06, 0.60 ± 0.003, 0.80 ± 0.10, 1.1 ± 0.14 μmol CH_4 day^{-1}, respectively. The lag time for these VFAs ranged from 35 to 150 days depending on the substrate. Sample 7 incubations metabolized formate, acetate and butyrate to methane at a rate of 0.03 ± 0.2, 0.34 ± 0.27 and 0.69 ± 0.02 CH_4 day^{-1}, respectively, after a 36- to 152-day lag period. Sample 8 incubations metabolized acetate and butyrate at a rate of 0.23 ± 0.3 and 0.98 ± 0.06 μmol CH_4 day^{-1}, respectively. The microflora in samples 9–11 did produce some methane from various VFA substrates, but none reached the theoretically expected amount after ~400 day incubation period. Only one sample (13) out of six from the Powder River Basin (Fig. 3C) produced the theoretically expected amount of methane from the VFA amendments. These incubations metabolized propionate at a rate of 0.73 ± 0.2 μmol CH_4 day^{-1} with a lag time of 149 days. Samples 12, 14–17 produced negligible methane after 450 days.

Functional gene analysis

A functional gene microarray (GeoChip 3.2 or 4.0) was used to assess the genetic potential in 14 of the 17 samples from the three

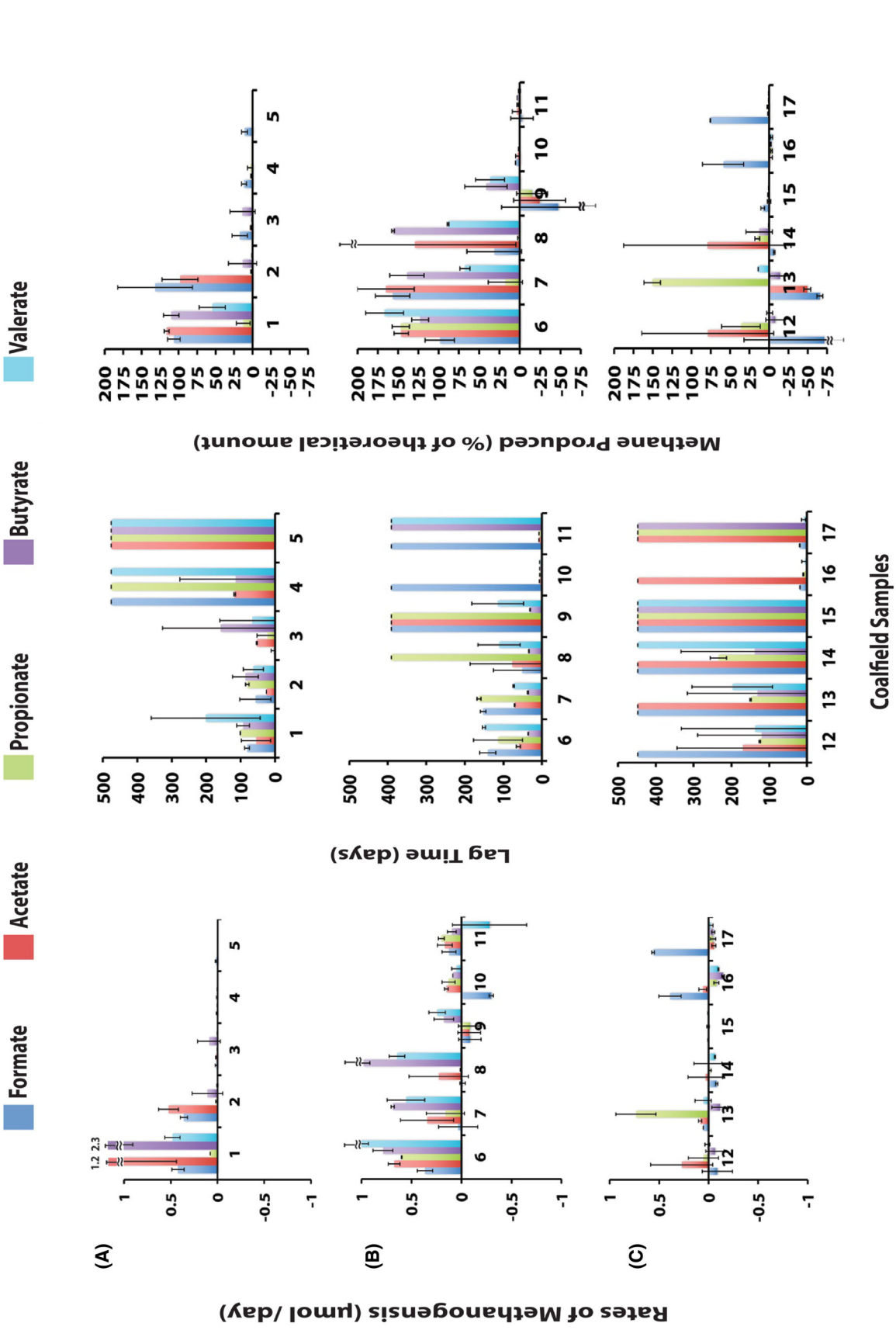

Figure 3. Rates of methanogenesis, lag time and the percentage of theoretical methane produced in incubations of the (A) Illinois Basin, (B) Cook Inlet gas field and (C) Powder River Basin. The error bars represent the difference between duplicate incubations.

coalfields. Samples 7, 16 and 17 were not included in this assay because the amount of DNA extracted was too low to amplify. We presume that an inhibitor interfered with either the extraction or amplification procedures (He, Van Nostrand and Zhou 2012). Venn diagrams were used to display the functional gene overlap between the three coalfields. Even though the two versions of the GeoChip differed in the number of functional gene probes, there were several shared genes amongst the nine categories (Fig. 4). Of particular interest are the two shared *mcr*A probes under the methane production category. These probes were originally derived from *Methanocorpusculum labreanum* type strain Z (Zhao *et al*. 1989) and an uncultured *Methanomicrobiales* archaeon (Banning *et al*. 2005); both methanogens are hydrogenotrophic and were present in the majority of sites assessed with the functional gene microarray (Table S3, Supporting Information). The detection of these probes suggests that the majority of sites from all three coalfields should readily be able to utilize formate as a methanogenic substrate. However, only about ∼50% of these sites produced methane from formate amendments (Fig. 3). Additional analysis of the functional gene microarray data indicated that the variability of the nine gene categories across the 14 samples was not significantly different (Bartlett test of homogeneity of variances: $K^2 = 4.6$, $p = 0.80$) and no significant difference was observed between the mean percentages for each of the nine gene categories ($F(8, 117) = 0.4$, $p = 0.9$). This result suggests that the variability and mean percentage of the functional gene categories are relatively the same throughout the three coalfields. This null finding is visually demonstrated in Fig. S2. Overall, the results suggest that there is no lack of functional genes associated with the coalbed microbial communities (Fig. 4 and Fig. S2).

Relationships between putative metabolites, produced methane and the methyl coenzyme M reductase (*mcr*) functional gene

Generally, the *in situ* presence of C_1–C_5 alkanoic acids (Fig. 2) in the coalfield samples was not associated with the ability of a particular inoculum to metabolize the same compounds *in vitro* (Fig. 3). However, exceptions were noted at the Cook Inlet site, in which butanoic and pentanoic acids were identified in the production waters from samples 6 and 8–10. Correlations between *mcr* richness or abundance and the total amount of methane produced from anaerobic incubations amended with the methanogenic substrates (i.e. formate, acetate) or the syntrophic substrate (i.e. propionate, butyrate, valerate) were generally quite low in most coalfield samples ($r \leq 0.65$; Figs 5 and 6). To investigate how the richness of *mcr* genes (α-diversity indices available in Table S4, Supporting Information) impacts methanogenesis within the coalfields, the number of positive *mcr* probes associated with the GeoChip analysis was totaled and compared to the total amount of methane measured within the various VFA amendments. The relationship between functional gene diversity and the structure of a microbial community has been previously demonstrated or discussed (He *et al*. 2012; Kang *et al*. 2013; Störmer, Wichels and Gerdts 2013). The strongest correlations were observed for Cook Inlet gas field samples that were amended with acetate ($r = 0.92$; $p = 0.01$), butyrate ($r = 0.98$; $p = 0.001$) and valerate ($r = 0.81$; $p = 0.04$; Fig. 5). Thus, as the number of positive *mcr* probes increased throughout the Cook Inlet gas field sites, more methane was produced from these fatty acid substrates. This suggests that acetate, butyrate and valerate could be important intermediates of biogenic coalbed methane production within the Cook Inlet gas field. Neither the Illinois nor Powder River Basins showed a significant relationship between the total amounts of methane produced in various incubations with increasing positive *mcr* probes for the particular sites assessed in this study.

However, it is not obvious why gene richness per se would correlate with microbial activity. That point notwithstanding, we also investigated whether the abundance of *mcr* genes, measured as signal intensity during the GeoChip analysis, correlated with methanogenesis. Previous work demonstrated a quantitative relationship between signal intensity and the concentration of DNA on the functional gene microarray (Wu *et al*. 2006). Additionally, *mcr*A gene copy number has been found to correlate with methanogen abundance (Nunoura *et al*. 2008) and methane production (Traversi *et al*. 2012) though more recently *mcr*A transcription was also highly correlated with metabolic activity (Wilkins *et al*. 2015). Thus, *mcr* gene signal intensities were totaled as a crude measure of methanogen abundance and compared to the total amount of methane produced from VFA-amended incubations. These relationships were generally weak ($r \leq 0.66$; Fig. 6), with the strongest correlations being observed in the Cook Inlet gas field when amended with acetate ($r = 0.61$; $p = 0.13$; Fig. 6) and butyrate ($r = 0.66$; $p = 0.11$; Fig. 6). While these correlation coefficients are not significant, they are still fairly high and help support the finding that acetate and butyrate are likely important metabolites throughout the Cook Inlet gas field sites. Again, neither the Illinois nor Powder River Basin showed a significant relationship between the total amount of methane produced and the abundance of *mcr* genes for the particular sites assessed in this study.

DISCUSSION

While it is clear that coalfield waters harbor a diverse indigenous microflora, the major ecological factors that influence the bioconversion of coal to methane remain unresolved. Jones, Voytek and Warwick (2008) and Jones *et al*. (2010) identified several possible limitations to biogenic coalbed methane, mainly *in situ* nutrient limitations and the bioavailability of the carbon within coal. Bates *et al*. (2011) suggested that carbon, nitrogen and phosphorous species are relatively low throughout groundwater in the Powder River Basin. Conceivably, the depletion of these nutrients could alone or in combination limit *in situ* methanogenesis. Nevertheless, multiple enrichment studies from this basin have reported the bioconversion of coal to methane *in vitro* when incubated with coal or potential intermediate compounds (i.e. acetate, ethanol, etc.). The same studies have also shown dramatic increases in methane production when incubations were amended with trace elements, acetate, yeast extract or slight changes in temperature or pH (Green, Flanegan and Gilcrease 2008; Harris, Smith and Barker 2008; Ulrich and Bower 2008; Ünal *et al*. 2012; Liu *et al*. 2013; Stephen *et al*. 2014). Similar results have been reported from enrichment studies in other coalfields such as the San Juan Basin (Wawrik *et al*. 2011) and coalbeds in Alberta, Canada (Penner, Foght and Budwill 2010). Taken together, this body of research suggests that indigineous microbial communities within coalfield waters could produce modern methane from coal-related organic matter. However, under laboratory conditions lag times for methane production from coal or coal-related intermediates can range from ∼120 to 450 days (Harris, Smith and Barker 2008; Krüger *et al*. 2008; Ulrich and Bower 2008; Wawrik *et al*. 2011), suggesting that even with adequate nutrient supply, methanogenesis from these substrates was still a relatively slow process.

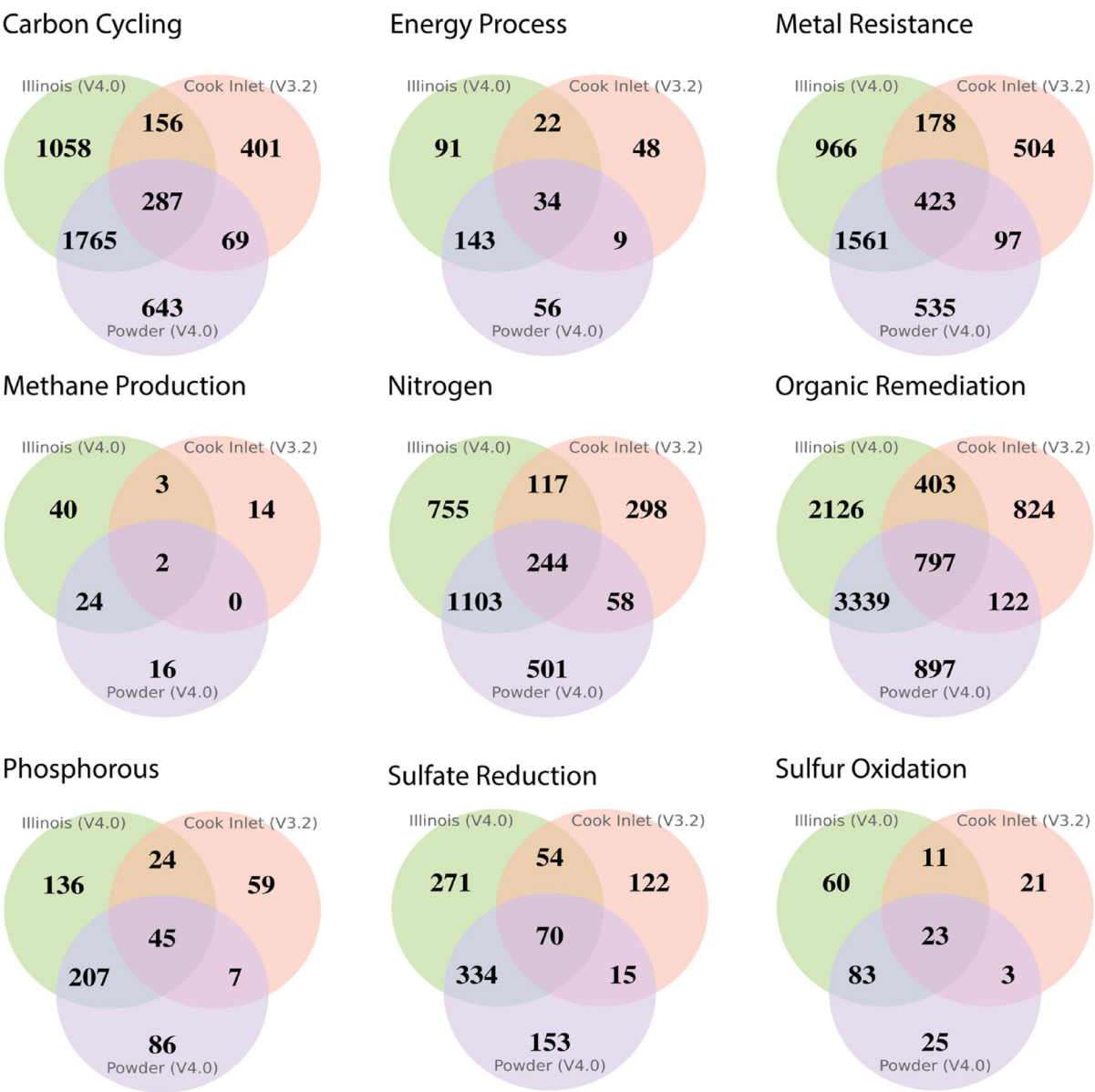

Figure 4. Venn diagrams showing the genetic overlap of functional genes associated with nine gene categories from three coalfields using the GeoChip functional gene microarray (3.2 and 4.0). The numeric value indicates the number of shared genes between the coalfields. A list of the shared genes between all three coalfields can be found in the supplemental information (Table S3).

In our study, we wanted to assess the methanogenic potential of coalbed microbial communities when amended with low molecular weight VFAs given their presumed importance in anaerobic coal biodegradation. To investigate this objective, production water samples were collected from three different coalfields and used as inocula for anaerobic incubations consisting of a basal medium and C_1–C_5 VFA substrates. The medium contained nitrogen and phosphorous sources as well as trace metals and vitamins in an attempt to overcome potential nutrient limitations that might restrict methanogenesis *in vitro*. The concentration of electron acceptors (Table 1; nitrate and sulfate) was low enough that methanogenesis would likely not be substantively inhibited in the 17 coalfield samples. However, our analysis did not comprehensively address all possible alternative electron acceptors that would potentially inhibit methanogenesis, most notably iron and other metals that could be introduced via the metal casing of the sampling wells. Additionally, the chloride levels (Table 1) fall within the range that is compatible with methanogenesis (Waldron *et al.* 2007). The fact that methane was produced from various C_1–C_5 VFA amendments in several incubations across the three coalfields suggests that the medium itself was sufficient and was therefore unlikely to restrict methanogenesis.

Low molecular weight VFAs were chosen as assay substrates because they have previously been identified as potential intermediates formed during the anaerobic bioconversion of coal to methane (Wawrik *et al.* 2011). We added formate and acetate as methanogenesis precursors and other VFAs as substrates requiring syntrophic interactions for ultimate mineralization. The C_1–C_5 VFAs are water soluble, and coal was specifically not added in the incubations to eliminate complications associated with substrate adsorption limiting bioavailability and

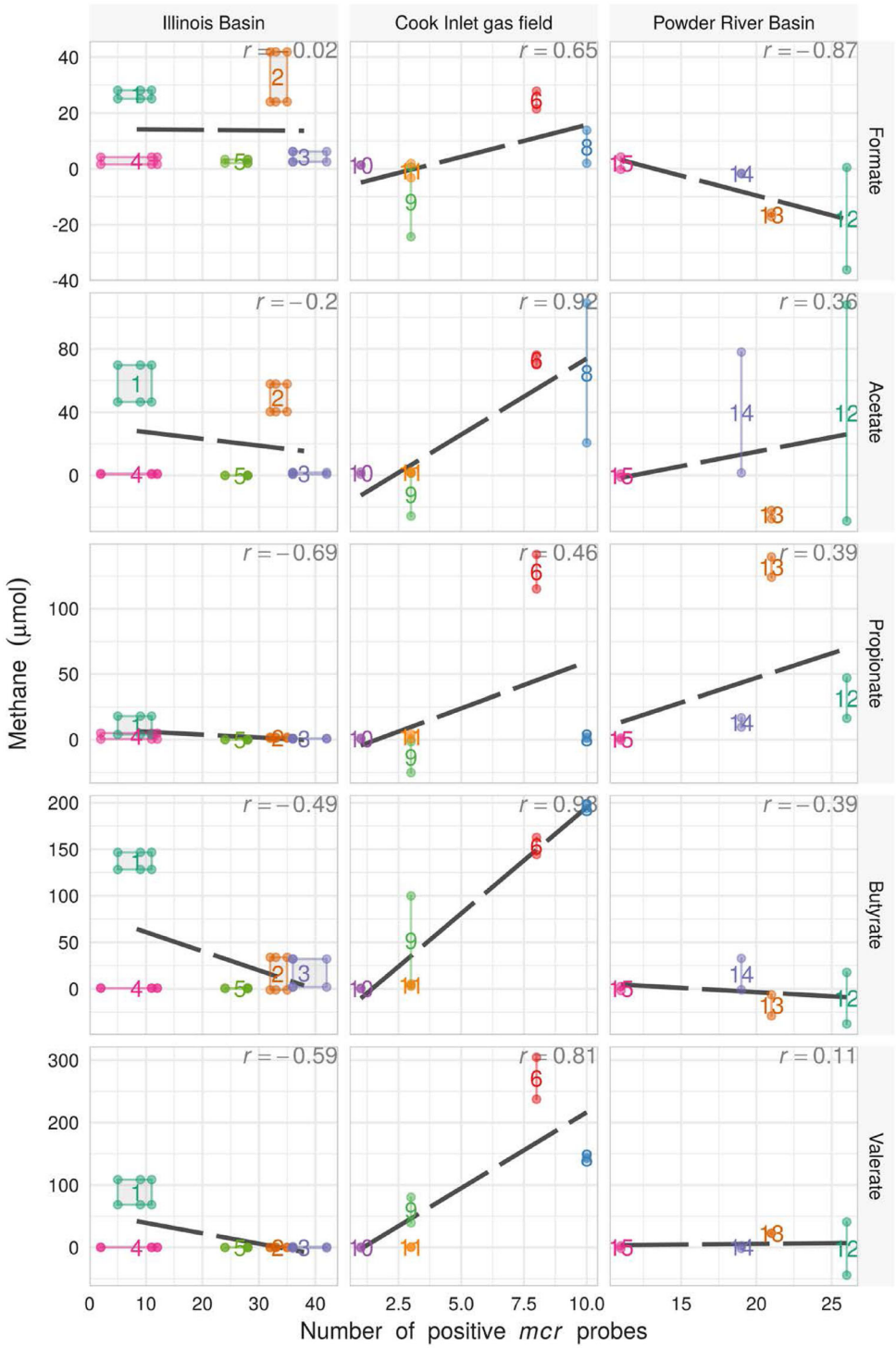

Figure 5. A scatterplot correlating the total amount of methane (μmol) to the number of positive *mcr* probes as measured by the GeoChip. Strong correlations (where $r \geq 0.79$; $p < 0.05$) indicate a significant relationship between the richness of methanogens and the total amount of methane produced. A weak correlation (where $r \leq 0.78$; $p > 0.05$) suggests that there is no relationship between the two variables. The boxes associated with the Illinois Basin data represent the range of error between three technical replicates of the GeoChip and the methane produced in duplicate incubations. The vertical bars for the Cook Inlet gas field and the Powder River Basin represent the error between the methane produced in duplicate incubations.

concomitant methanogenesis. We expected the coalfield inocula to easily biodegrade these low molecular weight VFAs and produce the stoichiometrically expected amount of methane. However, this was rarely the case. Anaerobic incubations using coalfield production waters as inocula exhibited long lag periods (~100 days) and methanogenesis rates that were rather slow (~1 μmol CH_4 day^{-1}). Moreover, these incubations typically did not reach the stoichiometrically expected amounts of methane, even after ~400 days of incubation (Fig. 3). Additionally, the slow rates of methanogenesis measured in the

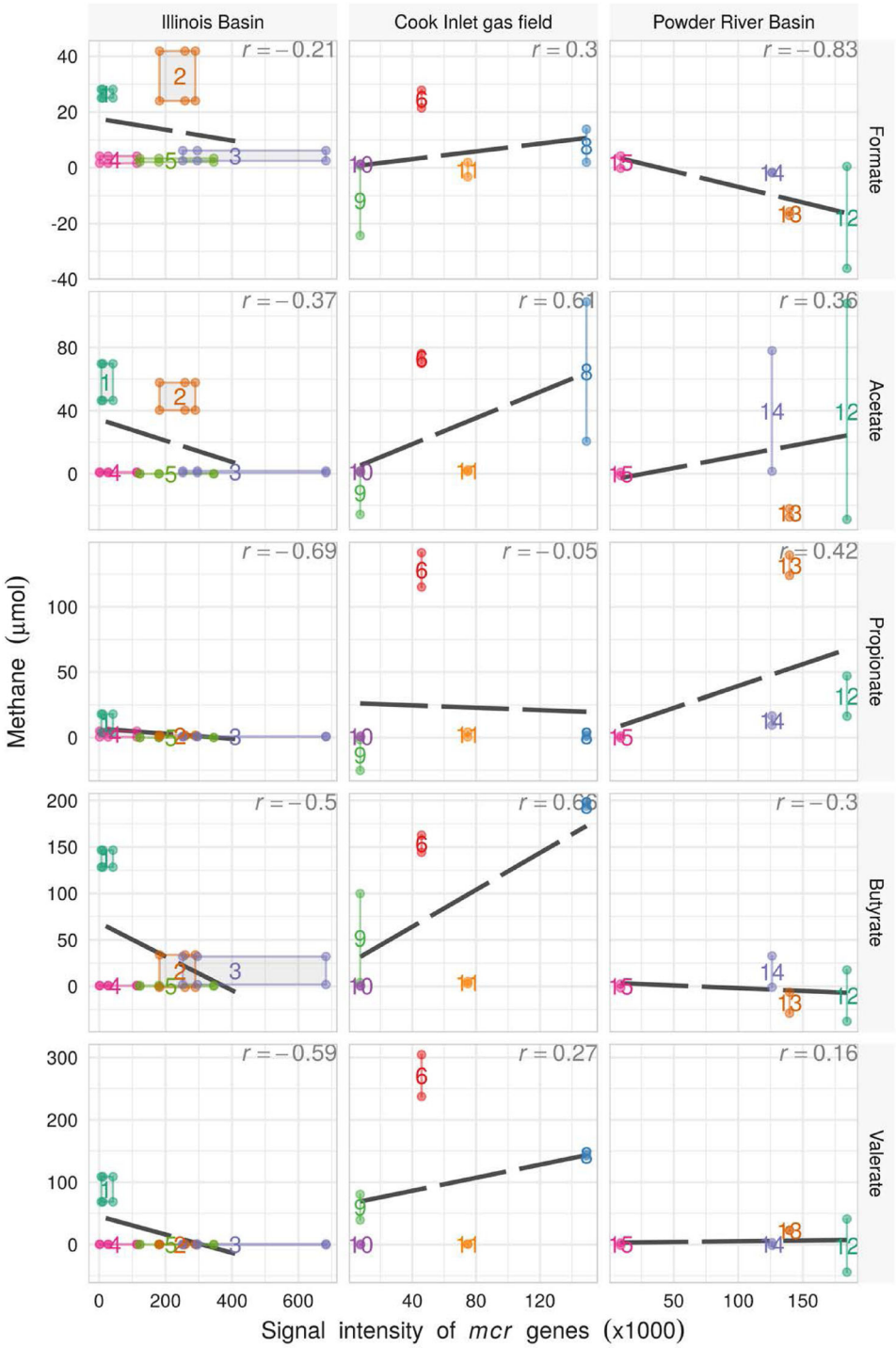

Figure 6. A scatterplot correlating the total amount of methane (μmol) to the signal intensities of *mcr* probes as measured by the GeoChip. Strong correlations (where $r \geq 0.79$; $p < 0.05$) indicate a significant relationship between methane production and the abundance of *mcr* genes. A weak correlation (where $r \leq 0.78$; $p > 0.05$) suggests that there is no relationship between the two variables. The boxes associated with the Illinois Basin data represent the range of error between three technical replicates of the GeoChip and the methane produced in duplicate incubations. The vertical bars for the Cook Inlet gas field and the Powder River Basin represent the error between methane produced in duplicate incubations.

VFA-amended incubations (Fig. 3) could not be explained by differences in total or methanogen biomass levels (Table S2, Supporting Information). Of the three coalfield basins, several samples from the Cook Inlet gas field were the only ones that harbored the inherent microbial community that completely mineralized the VFA amendments. The negative values of methane production in Fig. 3 indicate that several VFA amendments had a slight inhibitory impact on methanogenesis relative to substrate-unamended controls. In the few cases where it was observed, we presume that it was most likely due to the relatively high concentration of VFAs employed. Methane production exceeding 100% of the theoretical value was undoubtedly

due to endogenous electron donors already in the coalfield production waters as detected in the metabolite-profiling assay (Fig. 2 and Fig. S1).

The metabolic results from the anaerobic incubations were surprising, considering that the microbial communities from some of these coalfields, specifically the Powder River Basin, have been shown to oxidize coal in the laboratory (Green, Flanegan and Gilcrease 2008; Harris, Smith and Barker 2008; Ulrich and Bower 2008; Liu *et al.* 2013). However, there is much debate whether or not planktonic communities in production water reflect the sessile communities associated with coal. In most coal bed methane studies, production water is used as inocula due to the difficulty of obtaining actual coal samples from investigation sites (Strąpoć *et al.* 2011b). Klein *et al.* (2008) suggest that methanogens associated with coal per se are both phylogenetically and functionally different than those identified in coal production waters of the Powder River Basin. Additionally, Wei *et al.* (2014) also show that only three microbial genera are shared between the surface of the coal, mine water and enrichment samples. Thus, despite the potential differences in planktonic and sessile microbial communities throughout the three coalfields, the GeoChip analyses indicate that the genetic potential for complex carbon biodegradation and methanogenesis is present in all samples (Fig. 4), and that the mean relative abundance and variability between gene categories across the three coalfields was not significantly different (Fig. S2). Thus, methane production was not likely inhibited due to a lack of functional genes, and by extension, the absence of requisite microorganisms.

Compared to the methane production using the Illinois and Powder River Basin inocula, methanogenesis from C_1–C_5 VFA metabolism was substantially higher in comparable Cook Inlet incubations. That is, methane was produced when formate, acetate, butyrate or valerate was used as an amendment, though sometimes the mineralization of these substrates was incomplete (Fig. 3B). The differences between the Cook Inlet gas field and the Illinois or Powder River coalfield is particularly apparent when assessing the polar organic compound profiles. The abundance of putative metabolites in production waters from the Cook Inlet (Fig. S1) suggests that the metabolic fate of the softer lignitic to subbituminous coal within this formation was substantially different than the subbituminous to high volatile bituminous coals found within the Powder River or Illinois Basins. Harris, Smith and Barker (2008) previously reported that coals collected from Fort Yukon, Alaska, contained higher concentrations of organic-extractable carbon (maltene fraction) than the coals from the Powder River Basin. Therefore, it is likely that the coal from the Cook Inlet provides a higher concentration of labile carbon for bacteria to metabolize, thus producing more total polar organic molecules within production waters. The presence of butanoic and pentanoic acids (Fig. 2; samples 6, 8–10) in the Cook Inlet gas field corresponded with the ability of the inocula to metabolize butyrate and valerate amendments (Fig. 3B). A strong correlation was also observed between the diversity of *mcr* genes *in situ* and the total amount of methane when amended with acetate, butyrate and valerate *in vitro* (Fig. 5). The Cook Inlet gas field has previously been reported to have a high diversity of universal and obligate acetoclastic, methylotrophic and hydrogenotrophic methanogens (Strąpoć *et al.* 2011a). Thus, the strong correlations between the methane produced in butyrate- and valerate-amended incubations and the diversity of *mcr* genes suggest that the metabolic coupling between the syntrophic bacteria and methanogens is an important process potentially limiting anaerobic bioconversions in coal seams. However, the GeoChip assay does not contain functional genes that differentially target syntrophic bacteria, and we were unable to correlate the relationship between the quantity of syntrophs and methanogenesis. Regardless, the only possible fate for butyrate and valerate was the syntrophic bioconversion of these VFAs to the stoichiometrically predicted values of methane.

Collectively, these results suggest that the soft lignite to subbituminous coal in the Cook Inlet is easier to degrade than the subbituminous and bituminous coals of the Illinois and Powder River Basins. The degradation of the softer coal in turn leads to the formation of more polar organic compounds (i.e. butanoic, pentanoic acids) within production waters that are then syntrophically converted to methane. Thus, the major ecological difference between the Cook Inlet and the Illinois and Powder River Basins is the recalicitrant nature of the coal itself. Therefore, we propose that the inherent biodegradability of coal and the subsequent production of polar organic substrates that require syntrophic microbial metabolism is ultimately what governs the bioconversion of coal to methane. Of course, we do acknowledge that our analysis of a limited number of samples may not reflect the ecological conditions inherent throughout the entirety of the coalfields. Such coalfields are major deposits of organic carbon and it is certainly conceivable that other ecological factors could positively or negatively impact the ultimate bioconversion of coal to methane at individual locations. Additionally, Mayumi *et al.* (2016) reported that some methanogens can directly utilize methoxylated aromatic compounds in coal to produce methane. Our analysis does not differentiate these newly discovered methoxydotropic methanogens from acetoclastic, methylotrophic and hydrogenotrophic methanogens that use one- to two-carbon substrates as electron donors. Nevertheless, the general trends that we observe would indicate that modern coalbed methane production is slow at best and primarily limited by the chemistry of the coal itself.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGEMENTS

We would like to thank Darius Strąpoć and Courtney Turich for collecting the water samples used in this study. We would also like to thank Steve H. Harris and Boris Wawrik for reviewing this manuscript.

An earlier version of this manuscript was published as part of the Ph.D. thesis of Christopher N. Lyles accepted at the University of Oklahoma and available online at Shareok.org.

FUNDING

This work was funded by ConocoPhillips Corporation, Houston TX.

Conflict of interest. None declared.

REFERENCES

Aktas DF, Lee JS, Little BJ *et al.* Anaerobic metabolism of biodiesel and its impact on metal corrosion. *Energ Fuel* 2010;**24**: 2924–8.

Alaska Oil and Gas Conservation Commission (AOGCC). *Beluga River Unit, Beluga River Undefined Gas Pool*, 2011. http://doa.alaska.gov/ogc/annual/current/17_Gas_Pools/Beluga%20River%20-%20Gas/1_Gas_1.htm (22 April 2013, date last accessed).

Banning N, Brock F, Fry JC *et al.* Investigation of the methanogen population structure and activity in a brackish lake sediment. *Environ Microbiol* 2005;**7**:947–60.

Bates BL, McIntosh JC, Lohse KA *et al.* Influence of groundwater flowpaths, residence times, and nutrients on the extent of microbial methanogenesis in coal beds: Powder River Basin, USA. *Chem Geol* 2011;**284**:45–61.

Beckmann S, Krüger M, Engelen B *et al.* Role of bacteria, archaea and fungi involved in methane release in abandoned coal mines. *Geomicrobiol J* 2011;**28**:347–58.

Berry DF, Francis AJ, Bollag J-M. Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiol Rev* 1987;**51**:43–59.

Buswell AM, Mueller HF. Mechanism of methane fermentation. *J Ind Eng Chem* 1952;**44**:550–2.

Button DK, Robertson BR. Determination of DNA content of aquatic bacteria by flow cytometry. *Appl Environ Microb* 2001;**67**:1636–45.

Calderwood KW, Fackler WC. Proposed stratigraphic nomenclature for Kenai Group, Cook Inlet Basin, Alaska. *AAPG Bull* 1972;**56**:730–54.

Duncan KE, Gieg LM, Parisi VA *et al.* Biocorrosive thermophilic microbial communities in Alaskan North Slope oil facilities. *Environ Sci Technol* 2009;**43**:7977–84.

Faiz M, Hendry P. Significance of microbial activity in Australian coal bed methane reservoirs—a review. *B Can Petrol Geol* 2006;**54**:261–72.

Fallgren PH, Jin S, Zeng C *et al.* Comparison of coal rank for enhanced biogenic natural gas production. *Int J Coal Geol* 2013;**115**:92–6.

Formolo MJ, Martini AM, Petsch ST. Biodegration of sedimentary organic matter associated with coalbed methane in the Powder River and San Juan Basins, U.S.A. *Int J Coal Geol* 2008;**76**:86–97.

Fry JC, Horsfield B, Sykes R *et al.* Prokaryotic populations and activities in an interbedded coal deposit, including a previously deeply buried section (1.6–2.3 Km) above ~ 150 Ma basement rock. *Geomicrobiol J* 2009;**26**:163–78.

Glombitza C, Mangelsdorf K, Horsfield B. A novel procedure to detect low molecular weight compounds released by alkaline ester cleavage from low maturity coals to assess its feedstock potential for deep microbial life. *Org Geochem* 2009;**40**:175–83.

Green MS, Flanegan KC, Gilcrease PC. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, U.S.A. *Int J Coal Geol* 2008;**76**:34–45.

Harris SH, Smith RL, Barker CE. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. *Int J Coal Geol* 2008;**76**:46–51.

Hartz JD, Kremer MC, Krouskop DL *et al.* Preliminary engineering and geological evaluation of remaining Cook Inlet gas reserves. In: Decker PL (ed.). *Alaska Division of Oil and Gas Report*, 2009, p. 37, Available online at: http://www.dog.dnr.state.ak.us/oil/.

He Z, Deng Y, Van Nostrand JD *et al.* GeoChip 3.0 as a high-throughput tool for analyzing microbial community composition, structure and functional activity. *ISME J* 2010;**4**:1167–79.

He Z, Van Nostrand JD, Zhou J. Applications of functional gene microarrays for profiling microbial communities. *Curr Opin Biotechnol* 2012;**23**:460–6.

Jones EJP, Voytek MA, Corum MD *et al.* Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. *Appl Environ Microb* 2010;**76**:7013–22.

Jones EJP, Voytek MA, Warwick PD. Bioassay for estimating the biogenic methane-generating potential of coal samples. *Int J Coal Geol* 2008;**76**:138–50.

Kang S, Van Nostrand JD, Gough HL *et al.* Functional gene array-based analysis of microbial communities in heavy metals-contaminated lake sediments. *FEMS Microbiol Ecol* 2013;**86**:200–14.

Kimura H, Nashimoto H, Shimizu M *et al.* Microbial methane production in deep aquifer associated with the accretionary prism in Japan. *ISME J* 2010;**4**:531–41.

Klein DA, Flores RM, Venot C *et al.* Molecular sequences derived from Paleocene Fort Union Formation coals vs. associated produced waters: implications for CBM regeneration. *Int J Coal Geol* 2008;**76**:3–13.

Kotarba MJ. Composition and origin of coalbed gases in the upper Silesian and Lublin Basins, Poland. *Org Geochem* 2001;**32**:163–80.

Krüger M, Beckmann S, Engelen B *et al.* Microbial methane formation from hard coal and timber in an abandoned coal mine. *Geomicrobiol J* 2008;**25**:315–21.

Kubitschek HE, Freedman ML. Chromosome replication and the division cycle of Escherichia coli B/r. *J Bacteriol* 1971;**107**:95–9.

Leschine SB. Cellulose degradation in anaerobic environments. *Annu Rev Microbiol* 1995;**49**:399–426.

Liu Y, Urynowicz MA, Bagley DM. Ethanol conversion to methane by a coal microbial community. *Int J Coal Geol* 2013;**115**:85–91.

Lu Z, Deng Y, Van Nostrand JD *et al.* Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. *ISME J* 2011;**6**:451–60.

McInerney M, Bryant M, Pfennig N. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch Microbiol* 1979;**122**:129–35.

McInerney MJ, Struchtemeyer CG, Sieber J *et al.* Physiology, ecology, phylogney, and genomics of microorganisms capable of syntrophic metabolism. *Ann NY Acad Sci* 2008;**1125**:58–72.

Mayumi D, Mochimaru H, Tamaki H *et al.* Methane production from coal by a single methanogen. *Science* 2016;**354**:222–5.

Meslé M, Dromart G, Oger P. Microbial methanogenesis in subsurface oil and coal. *Res Microbiol* 2013;**164**:959–72.

Midgley DJ, Hendry P, Pinetown KL *et al.* Characterisation of a microbial community associated with a deep, coal seam methane reservoir in the Gippsland Basin, Australia. *Int J Coal Geol* 2010;**82**:232–9.

Moore TA. Coalbed methane: a review. *Int J Coal Geol* 2012;**101**:36–81.

National Energy Technology Laboratory (NETL). *United States Department of Energy*, 2014. http://www.netl.doe.gov/research/coal/energy-systems/gasification/gasifipedia/coal (20 March 2017, date last accessed).

Nunoura T, Oida H, Miyazaki J *et al.* Quantification of mcrA by fluorescent PCR in methanogenic and methanotrophic microbial communities. *FEMS Microbiol Ecol* 2008;**64**:240–7.

Orem W, Tatu C, Varonka M *et al.* Organic substances in produced and formation water from unconventional natural gas extraction in coal and shale. *Int J Coal Geol* 2014;**126**:20–31.

Penner TJ, Foght JM, Budwill K. Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. *Int J Coal Geol* 2010;**82**:81–93.

Papendick SL, Downs KR, Vo KD. Biogenic methane potential for Surat Basin, Queensland coal seams. *Int J Coal Geol* 2011;**88**:123–34.

Pratt T, Mavor M, DeBruyn R. Coal gas resource and production potential of subbituminous coal in the Powder River Basin. *In SPE Rocky Mountain Regional Meeting*, Gillette, Wyoming, 1999; 195–204.

Stephen A, Adebusuyi A, Baldygin A *et al.* Bioconversion of coal: new insights from a core flooding study. *RSC Adv* 2014;**4**:22779–91.

Störmer R, Wichels A, Gerdts G. Geo-Chip analysis reveals reduced functional diversity of the bacterial community at a dumping site for dredged Elbe sediment. *Mar Pollut Bull* 2013;**77**:113–22.

Strąpoć D, Ashby M, Wood L *et al.* Significant contribution of methyl/methanol-utilising methanogenic pathway in a subsurface biogas environment. In: Whitby C Skovhus TL (eds) Applied Microbiology and Molecular Biology in Oilfield Systems. Dordrecht, the Netherlands: Springer, 2011a, 211–6.

Strąpoć D, Mastalerz M, Dawson K *et al.* Biogeochemistry of microbial coal-bed methane. *Annu Rev Earth Pl Sc* 2011b;**39**:617–56.

Strąpoć D, Mastalerz M, Eble C *et al.* Characterization of the origin of coalbed gases in southeastern Illinois Basin by compound-specific carbon and hydrogen stable isotope ratios. *Org Geochem* 2007;**38**:267–87.

Strąpoć D, Picardal FW, Turich C *et al.* Methane-producing microbial community in a coal bed of the Illinois Basin. *Appl Microb Environ* 2008;**74**:2424–32.

Suárez-ruiz. Organic petrology: an overview. In: Ismail Al-Juboury A (ed) *Petrology - New Perspectives and Applications*. Intech, 2012. http://www.intechopen.com/books/petrology-new-perspectives-and-applications/organic-petrology-an-overview (20 March 2017, date last accessed).

Tanner RS. Cultivation of bacteria and fungi. In: Crawford RL Knudsen GR, McInerney MJ *et al.* (eds) Manual of Environmental Microbiology, 2nd edn. Washington, DC: ASM Press, 2002, 62–70.

Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. *Microbiol Mol Biol R* 1977;**41**:100–80.

Thielemann T. Coalbed methane in the Ruhr Basin, Germany: a renewable energy resource? *Org Geochem* 2004;**35**:1537–49.

Traversi D, Villa S, Lorenzi E *et al.* Application of a real-time qPCR method to measure the methanogen concentration during anaerobic digestion as an indicator of biogas production capacity. *J Environ Manage* 2012;**111**:173–7.

Ulrich G, Bower S. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. *Int J Coal Geol* 2008;**76**:25–33.

Ünal B, Perry VR, Sheth M *et al.* Trace elements affect methanogenic activity and diversity in enrichments from subsurface coal bed produced water. *Front Microbiol* 2012, doi: 10.3389/fmicb.2012.00175.

Vieth A, Mangelsdorf K, Sykes R *et al.* Water extraction of coals – potential for estimating low molecular weight organic acids as carbon feedstock for the deep terrestrial biosphere. *Org Geochem* 2008;**39**:985–91.

Waldron PJ, Petsch ST, Martini AM *et al.* Salinity constraints on subsurface archaeal diversity and methanogenesis in sedimentary rock rich in organic matter. *Appl Environ Microb* 2007;**73**:4171–9.

Walters BA. Coalbed gas systems, resources, and production and a review of contrasting cases from the San Juan and Powder River Basin. *AAPG Bull* 2002;**86**:1853–90.

Wawrik B, Mendivelso M, Parisi VA *et al.* Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. *FEMS Microbiol Ecol* 2011;**81**:26–42.

Wei M, Yu Z, Jiang Z *et al.* Microbial diversity and biogenic methane potential of a thermogenic-gas coal mine. *Int J Coal Geol* 2014;**134-135**:96–107.

Widdel F, Bak F. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A Truper HG, Dworkin M *et al.* (eds) The Prokaryotes, 2nd edn. New York, NY: Springer, 1992, 3352–78.

Widdel F, Rabus R. Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotech* 2001;**12**: 259–76.

Wilkins D, Lu X-Y, Shen Z *et al.* Pyrosequencing of mcrA and archaeal 16s rRNA genes reveals diversity and substrate preferences of methanogen communities in anaerobic digesters. *Appl Environ Microb* 2015;**81**:604–13.

Wu L, Liu X, Schadt CW *et al.* Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. *Appl Environ Microb* 2006;**72**:4931–41.

Zhao Y, Boone DR, Mah RA *et al.* Isolation and characterization of methanocorpusculum labreanum sp. nov. from the LaBrea Tar Pits. *Int J Syst Bacteriol* 1989;**39**:10–3.