Biogeochemical constraints on the origin of methane in an alluvial aquifer: evidence for the upward migration of methane from a coal seam.

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Geochemical and microbiological indicators of methane (CH₄) production, oxidation and migration processes in groundwater are important to understand when attributing sources of gas. The processes controlling the natural occurrence of CH₄ in groundwater must be understood, especially when considering the potential impacts of the global expansion of coal seam gas production on groundwater quality and quantity. We use geochemical and microbiological data, along with measurements of CH₄ isotopic composition (δ¹³C-CH₄), to determine the processes acting upon CH₄ in a freshwater alluvial aquifer that directly overlies coal measures targeted for coal seam gas production in Australia. Microbial and geochemical data indicate that there is biogenic CH₄ in the aquifer, but no methanogenic microbial activity. In addition, microbial community analysis showed that aerobic oxidation of CH₄ is occurring. The combination of microbiological and geochemical indicators suggests that the most likely source of CH₄, where it was present in the freshwater aquifer, is the upward migration of CH₄ from the underlying coal measures.

Keywords: Methane migration, groundwater, biogeochemistry, methanogenesis, methanotrophy, coal seam gas, aquifer connectivity

1 Introduction

Interest in methane (CH₄) production and degradation processes in groundwater is driven by the global expansion of unconventional gas production. There is concern regarding potential impacts of gas and fluid movement, as well as depressurisation, on groundwater quality and quantity in adjacent aquifers used to support other industries (Atkins et al., 2015; Heilweil et al., 2015; Iverach et al., 2015; Moritz et al., 2015; Zhang et al., 2016).
In groundwater, CH₄ can originate from numerous sources (Barker and Fritz, 1981). The two main sources of CH₄ in shallow groundwater are biological production (biogenic) and upward migration of CH₄ from deeper geological formations (thermogenic to mixed thermogenic to biogenic) (Barker and Fritz, 1981; Whiticar, 1999). This upward migration is via natural pathways such as geological faults and fracture networks (Ward and Kelly, 2007), however it can also be induced via poorly installed wells and faulty well casings (Barker and Fritz, 1981; Fontenot et al., 2013). The main focus of the debate about the occurrence of CH₄ in groundwater is whether it is naturally occurring or has been introduced by human activities. This research tests the hypothesis that a combination of geochemical indicators and microbiological data can inform production, degradation and migration processes of CH₄ in the Condamine River Alluvial Aquifer (CRAA) in Australia. This freshwater aquifer directly overlies the Walloon Coal Measures (WCM), the target coal measures for coal seam gas (CSG) production in the study area. Thus, our study has ramifications for global unconventional gas studies that investigate connectivity issues to freshwater aquifers.

Methane is subject to many production and degradation processes in groundwater (Whiticar, 1999). The carbon isotopic composition of CH₄ (δ¹³C-CH₄) gives insight into the source (Quay et al., 1999), but oxidation processes may enrich or deplete this signature (Yoshinaga et al., 2014). Therefore, it is very difficult to determine the potential source of CH₄ and processes occurring using CH₄ concentration and isotopic data alone.

Previous studies have used geochemical indicators, such as the concentration of sulfate [SO₄²⁻], nitrate [NO₃⁻] and nitrite [NO₂⁻], and the carbon isotopic composition of dissolved inorganic carbon (δ¹³C-DIC) and dissolved organic carbon (δ¹³C-DOC) to attribute the source of CH₄ in groundwater (Valentine and Reeburgh, 2000; Kotelnikova, 2002; Antler, 2014; Green-Saxena et al., 2014; Antler et al., 2015; Hu et al., 2015;
Segarra et al., 2015; Sela-Adler et al., 2015; Currell et al., 2016). Other studies have shown that the presence of active methanogenesis can be determined using isotopes of hydrogen in the CH$_4$ ($\delta^2$H-CH$_4$), and the surrounding formation water ($\delta^2$H-H$_2$O) (Schoell, 1980; Whiticar and Faber, 1986; Whiticar, 1999; Currell et al., 2016). Additionally, recent studies have used clumped isotopes of CH$_4$ and their temperature interpretations to ascribe a thermogenic versus biogenic source in groundwater (Stolper et al., 2014). However, non-equilibrium (kinetic) processes may be responsible for an overestimation of CH$_4$ formation temperatures (Wang et al., 2015). Therefore, combining geochemistry and microbiology provides a robust method to assess CH$_4$ origin, as it directly discriminates between microbiological communities involved in either production or degradation processes.

Throughout the world the occurrence of freshwater aquifers adjacent to unconventional gas production is common (Osborn et al., 2011; Moore, 2012; Roy and Ryan, 2013; Vidic et al., 2013; Vengosh et al., 2014; Moritz et al., 2015). We have previously shown that there may be local natural connectivity between the WCM and the CRAA (Iverach et al., 2015). Here we show that a combination of geochemical data ([CH$_4$], [SO$_4^{2-}$], [NO$_3^-$], [NO$_2^-$], $\delta^{13}$C-CH$_4$, $\delta^{13}$C-DIC, $\delta^{13}$C-DOC and $\delta^{2}$H-H$_2$O), as well as characterisation of microbiological communities present, can inform the discussion surrounding the occurrence of CH$_4$, and its potential for upward migration in the groundwater of the CRAA.

1.1 Geochemical indicators of methanogenic processes

Methanogenesis via acetate fermentation (Eq. 1) and carbonate reduction (Eq. 2) can be restricted in groundwater with abundant dissolved SO$_4^{2-}$ (> 19 mg/L) (Whiticar, 1999),
because sulfate reducing bacteria (SRB) can outcompete methanogenic archaea for reducing equivalents (Lovley et al., 1985).

\[ \text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \] (1)

\[ \text{CO}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \] (2)

Therefore, the presence or absence of [CH\(_4\)] and [SO\(_4^{2-}\)] are good preliminary indicators of the potential for methanogenesis.

In addition, the \(\delta^{13}\text{C-CH}_4\) of the underlying WCM in the study area has been characterised (Papendick et al., 2011; Hamilton et al., 2012; Hamilton et al., 2014). Thus the isotopic signature can be used to identify the potential source of the CH\(_4\), however localised formation and oxidation processes that may occur either in the aquifer or during transport can confound the interpretation of mixing versus oxidation processes.

The isotopic composition of DIC and DOC are also useful indicators of CH\(_4\) processes, as they can be used to determine the occurrence of methanogenesis (Kotelnikova, 2002; Wimmer et al., 2013). Kotelnikova (2002) found that \(^{13}\text{C}\)-depletion of \(\delta^{13}\text{C-DOC}\) in combination with a \(^{13}\text{C}\)-enrichment of \(\delta^{13}\text{C-DIC}\) was characteristic of methanogenesis in groundwater, consistent with the reduction of \(^{12}\text{CO}_2\) by autotrophic methanogens. Conversely, \(\delta^{13}\text{C-DIC}\) data are useful because DIC produced during CH\(_4\) oxidation was found to have a characteristically \(^{13}\text{C}\)-depleted signature (as depleted as -50‰) (Yoshinaga et al., 2014; Hu et al., 2015; Segarra et al., 2015).

**1.2 Methane oxidation in freshwater**

In groundwater, CH\(_4\) is oxidised by methane-oxidising bacteria (MOB; methanotrophs) that can utilise CH\(_4\) as their sole carbon and energy source. These methanotrophs are grouped within the Alpha- and Gamma-Proteobacteria (comprising type I and type II methanotrophs) and the Verrucomicrobia (Hanson and Hanson, 1996). The first step of
aerobic CH₄ oxidation is the conversion of CH₄ to methanol. This is catalysed by the particulate CH₄ monooxygenase (pMMO) encoded by the pmoA gene, which is highly conserved and used as a functional marker (Hakemian and Rosenzweig, 2007; McDonald et al., 2008). All known methanotrophs contain the pmoA gene, with members of Methylocella the exception (Dedysh et al., 2000; Dunfield et al., 2003). Type II methanotrophs and some type I members of the genus Methylococcus contain the mmoX gene, which encodes a soluble CH₄ monooxygenase (McDonald et al., 1995; Murrell et al., 2000). Recently, new groups of aerobic and anaerobic MOB distantly related to known methanotrophic groups have been discovered (Raghoebarsing et al., 2006; Stoecker et al., 2006; Op den Camp et al., 2009). Geochemically, aerobic CH₄ oxidation has been previously coupled to denitrification in groundwater (Zhu et al., 2016).

Besides methanotrophic bacteria, anaerobic CH₄ oxidising archaea (ANME) also play a significant role in the oxidation of CH₄ in both freshwater and saline water sources (Knittel and Boetius, 2009). These anaerobic methanotrophs are associated with the methanogenic Euryarchaeota within the clusters ANME-1, ANME-2, and ANME-3 and are closely related to the orders Methanosarcinales and Methanomicrobiales (Knittel et al., 2003; Knittel et al., 2005). Geochemical indicators can provide evidence for the occurrence of AOM, such as the prevalence of certain electron acceptors (SO₄²⁻, NO₃⁻, NO₂⁻ and Fe²⁺) (Valentine and Reeburgh, 2000; Ettwig et al., 2010; Sivan et al., 2011; Antler, 2014; Green-Saxena et al., 2014) and denitrification processes occurring in the groundwater (Ettwig et al., 2008; Nordi and Thamdrup, 2014; Timmers et al., 2015).

2 Study Area

The CRAA is the primary aquifer in the Condamine Catchment (Figure 1). It is used primarily for irrigated agriculture, stock and domestic water supplies. There has been
increased interest in the presence of CH$_4$ in the aquifer due to expanding CSG production to the north-west of the study area (Figure 1). CSG production began in 2006 (Arrow Energy, 2015) and has been expanding in the decade since then. This has raised concerns regarding the quality and quantity of the groundwater in the CRAA.

Figure 1. Site map showing the extent of the study area and sample locations within the Condamine Catchment, south-east Queensland, Australia. Map created in QGIS; data and imagery: Statem Toner, Open Street Map and contributors, CC-BY-SA (QGIS, 2015). Modified with Corel Painter 2015 (Corel Corporation, 2015).

2.1 Hydrogeological setting

The CRAA sits within the Surat Basin, which sits within the Great Artesian Basin (GAB) in south-east Qld, Australia (Figure 1). Aquifers in the GAB vary between semi-confined and confined (Kelly and Merrick, 2007; Dafny and Silburn, 2014).
The environment of deposition for the Surat Basin was fluvio-lacustrine in the late Triassic-Jurassic and shallow marine and coastal in the Cretaceous (Hamilton et al., 2012). The middle-Jurassic WCM are a group of low-rank coal seams in the Surat Basin targeted for CSG production (Hamilton et al., 2012). The WCM are thicker (150 m to 350 m) along the western margin of the CRAA and thin to around 50 m in the east, where the unit outcrops (KCB, 2011), however, only around 10% of this is coal. The unit consists of very fine- to medium-grained sandstone, siltstone, mudstone and coal, with minor calcareous sandstone, impure limestone and ironstone (KCB, 2011). The coal consists of numerous discontinuous thin lenses separated by sediments of low permeability (Hillier, 2010). The unit dips gently to the west (around 4°), which is consistent with the general trend of the Surat Basin in this region.

The WCM overlie the Eurombah Formation (consisting of conglomerate sandstone with minor siltstones and mudstone beds) and underlie the Kumbarilla Beds (mainly sandstone, with lesser mudstone, siltstones and conglomerates) (KCB, 2011).

The unconfined CRAA fills a paleovalley that was carved through the GAB (including the WCM). The valley-filling sediments are composed of gravels and fine- to course-grained channel sands interbedded with floodplain clays and, on the margins, colluvial deposits, which were deposited from the mid-Miocene to the present (Kelly and Merrick, 2007; Kelly et al., 2014). The valley-filling sediments have a maximum thickness of 134 m near Dalby (Dafny and Silburn, 2014). Along the eastern margin of the valley, the CRAA is bounded by the Main Range Volcanics. Estimations of the sources and quantity of recharge to the CRAA vary widely, however streambed recharge is generally considered to be the major source of freshwater to the aquifer (Dafny and Silburn, 2014).
A low permeability layer (ranging from $8 \times 10^{-6}$ to $1.5 \times 10^{-1}$ m/d) has been reported between the CRAA and the underlying WCM (KCB, 2011; QWC, 2012). This has been referred to as the ‘transition layer’ (QWC, 2012) or a ‘hydraulic basement’ to the alluvium (KCB, 2011). However, the thickness of this layer varies between 30 m thick in some areas to completely absent in others. Thus, in some places the WCM immediately underlies the CRAA (Dafny and Silburn, 2014). This suggests that there is some level of connectivity between the CRAA and the WCM. Huxley (1982) and Hillier (2010) both suggest that the general decline in water quality downstream is due to some net flow of the more saline WCM water into the CRAA. Connectivity between the formations is not well understood; however, studies have been conducted to better understand the movement of both water and gas between the two aquifers. Duvert et al. (2015) and Owen and Cox (2015) both used hydrogeochemical analyses to show that there was limited movement of water between the two formations. However, Iverach et al. (2015) used the isotopic signature of $\text{CH}_4$ in the groundwater to show that there was localised movement of gas between the coal measures and the overlying aquifer. This research provides additional insight to inform the debate about the degree of connectivity between the WCM and the CRAA. The microbiological insights also inform the global research on biological $\text{CH}_4$ production and degradation in alluvial aquifers, in particular for zones distal to the river corridor.

3 Methods

From 22 January 2014 to 31 January 2014 we collected groundwater samples for geochemical analysis from 8 private irrigation boreholes in the Condamine Catchment. Iverach et al. (2015) outlines the complete methods for sample collection for $[\text{CH}_4]$ and
δ^{13}C-CH_{4} and subsequent analysis. The 8 samples collected from the unconfined CRAA are representative of the aquifer, given their varied depths and locations.

Groundwater samples were collected by installing a sampling tube 2 m inside the pump outlet of the borehole to avoid the air-water interface at the sampling point. Field parameters (electrical conductivity (EC), oxidation-reduction potential (ORP), dissolved oxygen (DO), temperature (T) and pH) were monitored in a flow cell to ensure stabilisation before samples were collected. The boreholes had been pumping continuously over the preceding month for irrigation and so stabilisation of the field parameters was reached within minutes. Groundwater samples for major anions and water-stable isotopes (δ^{2}H-H_{2}O and δ^{18}O-H_{2}O) were collected after passing the water through a 0.45 μm, high-volume groundwater filter, which was connected to the pump outlet. Groundwater for anions and water stable-isotopes were stored in 125 mL high-density polyethylene (HDPE) bottles and 30 mL HDPE bottles, respectively. Both had no further treatment. The water for δ^{13}C-DIC and δ^{13}C-DOC was further filtered through a 0.22 μm filter and stored in 12 mL Exetainer vials and 60 mL HDPE bottles, respectively. Samples to be analysed for DIC were refrigerated at 4 °C and samples to be analysed for DOC were frozen within 12 hours of collection.

Groundwater samples for the microbiological analyses were collected between 8 December 2014 to 11 December 2014, and were collected from the same 8 private irrigation boreholes used for the geochemical analyses. Groundwater samples for microbiological analysis were collected in 2 L Duran Schott bottles and sealed (gas tight). The groundwater was filtered through a 0.2 μm filter (Merck Millipore). We use aspects of the geochemical data collected in the January campaign to inform our interpretation of the microbial results from the December campaign.
3.1 Geochemical analyses

The major ion chemistry in the groundwater samples was analysed at the Australian Nuclear Science and Technology Organisation (ANSTO) using Inductively Coupled Plasma - Ion Chromatography for anions. The samples for δ²H-H₂O and δ¹⁸O-H₂O were analysed at ANSTO and are reported as ‰ deviations from the international standard V-SMOW (Vienna Standard Mean Ocean Water). δ¹⁸O samples were run using an established equilibration, continuous flow IRMS method and δ²H samples were run using an on-line combustion, dual-inlet IRMS method.

The isotopes of carbon in DIC were analysed at ANSTO using an established method on a Delta V Advantage mass spectrometer, and a GasBench II peripheral. The results are reported as a ‰ deviation from IAEA secondary standards that have been certified relative to V-PDB for carbon. The isotopes of carbon in DOC were analysed at UC-Davis Stable Isotope Facility and results are reported as ‰ and are corrected based on laboratory standards calibrated against NIST Standard Reference Materials with an analytical precision of ± 0.6‰. Samples were run using a total organic carbon (TOC) analyser connected to a PDZ Europa 20-20 IRMS using a GD-100 Gas Trap interface.

The [SO₄²⁻] were too low in 6 of the 8 samples for δ³⁴S and δ¹⁸O analysis. The remaining 2 samples were analysed for their sulfur and oxygen isotope compositions at the University of Calgary Isotope Science Laboratory. Sulfur isotope ratios were analysed using a Continuous Flow-Isotope Ratio Mass Spectrometry (CF-EA-IRMS) with an elemental analyser interfaced to a VG PRISM II mass spectrometer. The results are reported against V-CDT (Vienna Cañon Diablo Troilite). The oxygen isotope ratio was determined using a high temperature reactor coupled to an isotope ratio mass spectrometer in continuous flow mode.
DNA extraction and Illumina sequencing

DNA was extracted from the biomass collected from filtering 2 L of groundwater. Briefly, DNA was extracted using a phenol-chloroform extraction method as described by Lueders et al. (2004). Subsequently, the DNA was precipitated using polyethylene glycol 6000 (Sigma Aldrich), and the DNA pellet was washed using 70 % (v/v) ethanol and resuspended in 50 μL nuclease free water (Qiagen). DNA concentration and purity were determined by standard agarose gel electrophoresis and fluorometrically using RiboGreen (Qubit Assay Kit, Invitrogen) according to the manufacturer’s instructions. The extracted DNA was used as a target for Illumina sequencing. Amplicon libraries were generated by following Illumina’s 16S Metagenomic Sequencing Library Preparation Protocol, using 12.5 ng of template DNA per reaction. The number of cycles for the initial PCR was reduced to 21 to avoid biases from over-amplification. The following universal primer pair was used for the initial amplification, consisting of an Illumina-specific overhang sequence and a locus-specific sequence:

926F_Illum(5’-TCGTCGCGAGCGATGTATAGAAGAC[AAACTYAAAAGGAATTGRC]-3’),

1392R_Illum(5’-GTCTCGTGGGCTCGAGATGTATAGAAAGAC[ACGGCGGTGTGRC]-3’).

This universal primer pair targets the V6-V8 hyper-variable regions of the 16S ribosomal RNA gene and has been shown to capture the microbial diversity of Bacteria and Archaea in a single reaction (Wilkins et al., 2013). PCR products were purified using a magnetic bead capture kit from Agencourt AMPure XP beads (Beckman Coulter) and quantified using a fluorometric kit (RiboGreen, Qubit Assay Kit, Invitrogen). Purified amplicons
were subjected to the Index PCR using the MiSeq platform (Ramaciotti Centre for Genomics, UNSW Australia) according to the manufacturer’s specifications. Illumina sequences were checked for quality (FastQC, BaseSpace) and analysed using the BaseSpace cloud computing platform (Illumina, 2016) and MOTHUR (Schloss, 2009) with modified protocols (Schloss et al., 2009; Kozich et al., 2013). Taxonomy was assigned against the SILVA Database (Silva, 2016). To ensure even sampling depth for subsequent analyses, OTU abundance data were rarefied to the lowest number of sequences for a sample (8,300 sequences per sample).

3.3 Quantification of bacterial and archaeal 16S rRNA and functional genes

Quantitative real-time PCR was used to determine abundances of bacterial and archaeal 16S rRNA gene targets and functional gene targets (mcrA, pmoA, mmoX, and dsrA), using the MJ Mini™ 96 Well Thermal Cycler (Bio-Rad, Hercules, CA). Each qPCR 25 μL reaction mixture contained 12.5 μL of premix solution from an iQ SYBRGreen qPCR Kit (Bio-Rad), 8 μL PCR-grade water, 1.5 μL of each primer (final concentration 0.2 – 0.5 μM), and 2 μL of template DNA (10 ng). Bacterial and archaeal 16S rRNA genes were amplified using the primer pairs 519F/907R (Lane 1991; Muyzer et al., 1995) and SDArch0025F/SDArch0344R (Vetriani et al., 1999). McrA and dsrA sequence fragments were amplified using the primer pairs ME1F/ME3R (Hales et al., 1996) and 1F/500R (Wagner et al., 1998; Dhillon et al., 2003). QPCRs were performed as described previously by Wilms et al. (2007). PmoA qPCR was performed using the pmoA primer-pair A189F (Holmes et al., 1999) and mb661R (Kolb et al., 2003) with a final total concentration of 0.8 μM. The qPCR programme for the amplification was performed as follows: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 64°C for 45 s and 68°C for 45 s. The mmoX gene fragment was quantified using the prime pairs mmoX-ms-945f.
and mmoXB-1401b at a final concentration of 0.8 µM. The qPCR conditions for the 
mmoX was as follows: 94°C for 3 min followed by 45 cycles of 94°C for 1 min, 50°C for 
1 min and 72°C for 1 min. Bacterial and archaeal targets were measured in at least three 
different dilutions of DNA extracts (1:10, 1:100, 1:1000) and in triplicate. PCR products 
were checked by gel electrophoresis, using 2 % (w/v) agarose with TBE buffer (90 mM 
Tris, 90 mM boric acid, 2 mM Na₂-EDTA; pH 8.0). The specificity of the reactions was 
confirmed by melting curve analysis and agarose gel electrophoresis to identify non-
specific PCR products. Amplification efficiencies for all reactions ranged from 96.3 % to 
110.5 % with an r² value of > 0.99 for standard curve regression. DNA calibration 
standards for qPCR were prepared as follows. The mcrA, dsrA, pmoA, and mmoX genes 
were amplified from pure cultures of Methanosarcina barkeriT (DSM 800), Desulfovibrio 
vulgarisT (DSM 644), Methylosinus sporiumT (DSM 17706), and Methylocella silvestrisT 
(DSM 15510; DZMZ Germany). The PCR amplicons were purified using the DNA Clean 
and Concentrator™-5 kit (Zymo Research, Irvine, CA), and eluted into 20 µL DNA 
elution buffer. DNA concentrations were quantified with 2 µL DNA aliquots using the 
Qubit® dsDNA BR Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA). Purified 
target gene PCR products were cloned into plasmids following the manufacturer’s 
instructions for the pGEM® – T Easy Vector System (Promega, Madison, WI).

4 Results and Discussion

4.1 Previous δ¹³C-CH₄ investigation

A previous study by Iverach et al. (2015) analysed the δ¹³C-CH₄ in the groundwater from 
an off-gassing port on the 8 private irrigation boreholes studied here (samples A-H) 
(Supplementary Table S3 online). These measurements were understood to have been 
mixing with regional background atmospheric CH₄ (1.774 ppm; ~47‰) and therefore
mixing plots were used to infer the isotopic source signature of the CH$_4$ off-gassing from the groundwater. Iverach et al. (2015) found that samples E, G, and H plotted on a regression line that had an isotopic source signature of -69.1‰ (90% CI, $-73.2‰$ to $-65.0‰$), indicative of a biological source. However, samples A, B, C, D and F plotted on a regression line that had an isotopic source signature of $-55.9‰$ (90% CI, $-58.3‰$ to $-53.4‰$), suggesting either oxidation was occurring at the source or there was potential upward migration of CH$_4$ from the underlying WCM.

4.2 Limited geochemical and microbiological potential for methanogenesis in the groundwater

To further elucidate the source of the CH$_4$ reported in the groundwater (Iverach et al., 2015), Illumina sequencing and quantitative real-time PCR (qPCR) was used to target bacterial and archaeal 16S rRNA genes, as well as specific functional genes (mcrA, pmoA, mmoX and dsrA) associated with CH$_4$ metabolism. Microbial abundances estimated by SYBR Green I counts were between $10^3$ and $10^5$ cells/mL throughout all groundwater samples (Figure 2). This was congruent with the qPCR data observed for bacterial and archaeal cell concentrations.
Figure 2. Total cell concentration and copy number abundances of bacterial and archaeal 16SrRNA genes and functional key genes for aerobic CH$_4$ oxidation (pmoA and mmoX genes), CH$_4$ production (mcrA gene) and sulfate reduction (dsrA gene) in the groundwater carried out by quantitative (q)PCR. Low abundances are highlighted in light blue. High abundances are highlighted in dark blue.

The groundwater community was primarily composed of bacteria (79 to 90 %), whilst archaea made up 10 to 21 % (Figure 3). The bacterial and archaeal community composition did not vary significantly between groundwater samples. Most of the bacterial sequences belonged to the phyla Proteobacteria ($\alpha$-,$\delta$), Acidobacteria, Actinobacteria, Firmicutes and the Bacteroidetes/Chlorobi group (Figure 3). The phylum Thaumarchaeota dominated the archaeal communities with a relative abundance of 81 to 99 %, while Crenarchaeota made up 1 to 3 % of the archaeal community. Further sequences were related to other (if < 1 % relative abundance) and unclassified Bacteria and Archaea. No members of the Euryarchaeota, comprising the methanogenic archaea,
were observed. The archaeal mcrA gene, which encodes the methyl coenzyme M reductase, was not detected in any of the groundwater samples (detection limit < 10 cells/mL; Figure 2). This was consistent with the Illumina sequencing results, and suggests that the CH₄ observed off-gassing from the groundwater was not being produced locally.

Figure 3. Bacterial, archaeal, and methanotrophic community profiles and relative abundances detected by Illumina sequencing.

Our geochemical data also showed no evidence for the occurrence of methanogenesis in the groundwater. As previously stated, a ¹³C-enrichment in δ¹³C-DIC
coupled with a $^{13}$C-depletion in the $\delta^{13}$C-DOC is characteristic of methanogenesis (Kotelnikova, 2002). Our groundwater data showed no correlation between $\delta^{13}$C-DOC and $\delta^{13}$C-DIC (Figure 4a), and the most $^{13}$C-enriched $\delta^{13}$C-DIC was also the second highest enriched $\delta^{13}$C-DOC value. Additionally, on a stable water isotope plot (Figure 4b; Supplementary Table S1 online), it is evident that there is no noticeable $\delta^2$H-enrichment that can be ascribed to methanogenesis in any of the groundwater samples (Cendón et al., 2015).

**Figure 4.** (a) A plot of $\delta^{13}$C-DOC vs. $\delta^{13}$C-DIC, highlighting the absence of correlation between these geochemical data, indicating that there is no methanogenic end member in our samples. Samples E, G and H are omitted because they were below the detection limit for $\delta^{13}$C-DOC (Supplementary Table S1.). Arrow 1 delineates the expected trend for methanogenesis and arrow 2 is the expected trend for the dissolution of marine carbonates (Currell et al., 2016). Arrows 3-6 highlight expected ranges for $\delta^{13}$C-DIC that are off the scale of the graph (Currell et al., 2016). (b) A plot of $\delta^{18}$O-H$_2$O vs. $\delta^2$H-H$_2$O showing that there is no $^2$H-enrichment in any of the groundwater samples. The GMWL (Craig, 1961) and LMWL (Hughes and Crawford, 2012) are also displayed.

These geochemical analyses, along with the lack of classified methanogens, suggest that biogenic CH$_4$ production is not one of the major processes producing CH$_4$ in the CRAA. Therefore, the CH$_4$ reported in all samples in Iverach et al. (2015) must be
coming from another source. We propose that the upward migration of CH$_4$ from the WCM must be considered as the potential source. The isotopic signature of CH$_4$ from the deeper coal measures has been characterised between -58.5‰ and -45.3‰, indicating thermogenic CH$_4$ with a secondary biogenic component (Papendick et al., 2011; Hamilton et al., 2012; Hamilton et al., 2014). Five of the 8 samples analysed in this study have an isotopic source signature within this range, as reported in Iverach et al. (2015). This implies that upward migration from the deeper WCM is the source of the CH$_4$ detected in the groundwater.

However, the remaining 3 samples (samples E, G, and H) have a typically biogenic isotopic source signature (-69.1‰). This could be the result of the replacement of typically thermogenic gas in the shallow WCM by biogenic gas (Faiz and Hendry, 2006). Thus, these three sites are potentially sourcing biogenic CH$_4$ from the shallow WCM, resulting in a biological source signature despite the absence of methanogens in the overlying aquifer.

4.3 Sulfate reducers and aerobic methanotrophs potentially outcompete methanogens

Sulfate concentrations in most groundwater samples were low (3.2 mg/L to 11 mg/L) (Supplementary Table S2 online). Groundwater samples D and H were higher with 55 mg/L and 29 mg/L, respectively (Supplementary Table S2 online). Sequence and functional $dsrA$ gene analysis (encoding the dissimilatory sulfite reductase of SRB) revealed that SRB are present in all groundwater samples at relatively high abundances (5 - 10 % of the overall microbial community; Figures 2 and 3). These SRB are potentially outcompeting methanogenic archaea for substrates such as acetate and H$_2$. Sulfate concentrations higher than 3 mg/L, as detected in all groundwater samples (3.2 mg/L – 55
mg/L), could potentially create a \( \text{SO}_4^{2-} \)-reducing environment with the predominance of SRB over methanogens. This would maintain the acetate at concentrations too low for methanogens to grow (Lovley et al., 1985). Deltaproteobacteria were dominant in all groundwater samples, and most of the sequences were closely related to acetate-oxidising, sulfate/sulfur-reducing bacteria (Desulfovibrionales, Syntrophobacterales, Desulfuromonadales; Figure 3). Additionally, Methylocella spp. are capable of using methanogenic substrates, such as acetate and methylamines, for their metabolism and therefore are not limited to growing on one-carbon compounds such as CH\(_4\) (Dedysh et al., 2005). This could have major implications for the lack of methanogenic activity in the groundwater.

### 4.4 Microbial methane oxidation in the groundwater catalyses upward migrating methane from the WCM

The functional gene for aerobic CH\(_4\) oxidation (\( pmoA \)) was detected at relatively high concentrations (7.9 x 10\(^3\) - 9.3 x 10\(^3\) targets/mL) compared to the overall bacterial 16S rRNA concentration (2.5 x 10\(^4\) - 5.1 x 10\(^4\) targets/mL) (Figure 2). All groundwater samples were characterised with regard to the community structure of MOB. The samples harboured a low-diversity methanotrophic community associated with the order Rhizobiales (\( \alpha \)-Proteobacteria), however MOB accounted for up to 7 % of the overall microbial community (Figure 3). All groundwater samples were dominated by two MOB, belonging to the type II methanotrophs (Figure 3). Five samples had both Methylocella palustris (family Beijerinckiaceae) and Methylosinus acidophilus (family Methylocystaceae) (samples B, D, F-H), whilst the remaining samples comprised Methylosinus acidophilus only (samples A, C and E) (Figure 3). These genera were characterised as aerobic CH\(_4\) oxiders, however aerobic MOB have been previously
observed in micro-aerophilic and anaerobic environments (Bowman, 2000). This suggests the existence of an alternative pathway for aerobic CH$_4$ oxidation in a suboxic/anaerobic environment. Both species have previously been found and isolated from a variety of freshwater habitats and *Methylosinus* spp. are known to be dominant methanotrophic populations in groundwater (Bowman, 2000). *Methylocella* and *Methylosinus* spp. possess a soluble CH$_4$ monooxygenase (*mmoX*) (McDonald et al., 1995; Murrell et al., 2000), which is consistent with the high abundance of the *mmoX* gene targeted in all groundwater samples (Figure 2). Interestingly, no *pmoA* gene, a biomarker for all MOBs, has previously been detected in known *Methylosinus* spp. (Dedysh et al., 2005). This is supported by our data, which shows the sole predominance of *mmoX* genes in 3 of the 8 groundwater samples that are exclusively dominated by *Methylosinus* sp. (samples A, C, and E) (Figures 2 and 3).

In addition to low concentrations of CH$_4$ reported in Iverach et al. (2015), the dissolved O$_2$ (DO) in our groundwater samples had a large range, from low to close to saturation (0.91 mg/L to 8.6 mg/L). *Methylocella* spp. are not associated with the previously known type II cluster of methanotrophs, but are closely related to a non-methanotroph (Dedysh et al., 2005) suggesting different affinities to CH$_4$ and O$_2$, compared to previously known type II methanotrophs (Amaral and Knowles, 1995). There is no correlation between the methanotrophic community in each sample and the CH$_4$ data reported in Iverach et al. (2015), nor is there any correlation between the composition of methanotrophs and DO in the groundwater (Supplementary Table S2 online).

The sample with the most diverse bacterial community (Sample F; Figure 3) had the most $^{13}$C-enriched individual $\delta^{13}$C-CH$_4$ relative to regional background (Iverach et al., 2015) (Supplementary Table S3 online). A relatively high abundance (11 %) of relatives
belonging to the Chloroflexi phylum was observed exclusively in this groundwater sample. This suggests that there are potential metabolic processes involved, such as the microbial conversion of denitrification products to nitrogen and oxygen, that are able to gain oxygen to facilitate the oxidation of CH$_4$ (Ettwig et al., 2010).

### 4.5 Absence of AOM

The lack of detection of the mcrA gene does not only indicate the absence of methanogens but also suggests the absence of anaerobic methanotrophs (Hallam et al., 2003). Details on the functional genomic link between methanogenic and methanotrophic archaea are discussed comprehensively in Hallam et al. (2003). Additionally, no sequences belonging to ANME-SRB clades were detected in the groundwater samples, indicating the absence of ANME activity. However, members of the phylum Thaumarchaeota dominated the archaeal community in the groundwater (Figure 3). Thaumarchaeota contains several clusters of environmental sequences representing microorganisms with unknown energy metabolism (Pester et al., 2011). Members of the Thaumarchaeota encode monooxygenase-like enzymes able to utilise CH$_4$, suggestive of a role in CH$_4$ oxidation.

Samples D and H had SO$_4^{2-}$ concentrations of 55 mg/L and 29 mg/L, respectively. This suggests that the SO$_4^{2-}$ concentration is high enough to support SO$_4^{2-}$-mediated AOM at these sites (Whiticar, 1999). The observed [SO$_4^{2-}$] was high enough in these 2 samples to be able to measure the stable isotopes in the SO$_4^{2-}$. This is useful because the isotopes yield a unique signature when SO$_4^{2-}$ reduction is coupled to CH$_4$ oxidation in anaerobic conditions (Antler et al., 2015). However, because there are only two data points (Supplementary Table S2 online), determining a correlation between δ$^{34}$S-SO$_4$ and δ$^{18}$O-SO$_4$ is statistically invalid. The highest relative abundance of methanotrophs was found in
samples D and H (Figure 3); however, these methanotrophs are not anaerobic oxidisers and therefore the correlation may not imply causation.

The concentration of NO$_3^-$ and NO$_2^-$ in the groundwater was also very low, with [NO$_3^-$] ranging from 1.2 mg/L to 2.3 mg/L and for all samples NO$_2^-$ was below 0.05 mg/L (Supplementary Table S2 online). Therefore, AOM coupled to denitrification is unlikely to be occurring in the groundwater of the CRAA (Nordi and Thamdrup, 2014).

The $\delta^{13}$C-DIC data indicates limited $^{13}$C-depletion as a result of DIC formation during AOM. Segarra et al. (2015) showed that maximum $^{13}$C-depletion of DIC in the zone of maximum AOM activity (0 – 3 cm) was highly dependent upon the isotopic composition of the DIC before biological consumption. However, the difference between maximum $^{13}$C-depletion of DIC and $^{13}$C-enrichment often exceeded 10‰. As our samples are taken from deep in the aquifer (30 m or more below the ground surface), and the difference between our most $^{13}$C-depleted DIC value and the most $^{13}$C-enriched was only 4‰ (Sample H; Supplementary Table SI online) it is unlikely that AOM is occurring in the groundwater. Additionally, a previous study of the GAB geochemistry showed that $\delta^{13}$C-DIC values in this region are in the range -15‰ to -6‰ (Herczeg et al., 1991). All of our samples fall within this regional range, and we see no obvious $^{13}$C-depletion of DIC in the groundwater that can be ascribed to AOM.

Therefore, any oxidation occurring in the groundwater would have been facilitated by the two members of type II methanotrophs that we identified in the microbial community analysis. Both of the species identified are classified aerobic CH$_4$ oxidisers, agreeing with our geochemical data that no anaerobic oxidation was occurring. Despite abundant SO$_4^{2-}$ in 2 sample locations, the absence of anaerobic methanotrophic archaea amongst other geochemical evidence (denitrification processes) suggests that it is unlikely that AOM is occurring within the aquifer.
Conclusion

We used geochemical and microbiological indicators to explain the occurrence of CH$_4$ in the groundwater of an alluvial aquifer. Microbial community analysis and geochemical data were consistent with respect to a lack of methanogenic archaea and methanogenic activity in the aquifer. What is the original source of the CH$_4$ if not biologically produced in-situ? One hypothesis to explain the presence of CH$_4$ despite there being no evidence of methanogenesis is that there is localised upward migration of CH$_4$ from the WCM into the CRAA via natural faults and fractures (Iverach et al., 2015).

Our geochemical data and microbiological community analysis both indicate that AOM is not a major oxidation process occurring in the CRAA. However, the microbiological data suggest the presence of aerobic CH$_4$ oxidisers. Due to the absence of methanogenesis, the oxidation of CH$_4$ (facilitated by the aerobic methanotrophs present in the groundwater) would require a secondary source of CH$_4$. The upwards migration of CH$_4$ from the underlying WCM is the likely source.

Methane occurs naturally in groundwater, is produced via numerous biological pathways, and can migrate through natural geological fractures. Therefore, determination of the source of CH$_4$ using [CH$_4$] and $\delta^{13}$C-CH$_4$ data alone doesn’t discern all the processes occurring. Our microbiological community analysis showed that there were no methanogens present to produce the CH$_4$ measured in Iverach et al. (2015) and our geochemical analyses supported the absence of methanogenesis in the alluvial aquifer. Similarly, the geochemical and microbiological data revealed that oxidation may not have as large an effect on the CH$_4$ due to the low abundance of aerobic oxidisers and the absence of anaerobic archaea.
Therefore, we suggest that the CH$_4$ detected in the CRAA in Iverach et al. (2015) is from the local upward migration of gas from the underlying WCM, through natural faults and fractures. A consideration of both geochemical and microbiological analyses is particularly important in this study area because of the immediate proximity of the underlying WCM and the proximity of the study area to CSG production. This research uses biogeochemical constraints on the origin of CH$_4$ in a freshwater aquifer to demonstrate the upward migration of CH$_4$ from an underlying coal seam.

**Author Contributions**

Experimental conceptualisation and design was carried out by D.I.C. & B.F.J.K. Fieldwork was conducted by C.P.I., S.B., D.I.C. & B.F.J.K. Geochemical analyses were conducted by D.I.C. Microbiological analyses were conducted by S.B., C.P.I. & M.M. The manuscript was written by C.P.I. and S.B. with input from all authors.

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**Competing Interests**

The authors declare that they have no conflict of interest.

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