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Biogeochemical constraints on the origin of methane in an alluvial aquifer: evidence for the upward migration of methane from underlying coal measures

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Abstract. Geochemical and microbiological indicators of methane (CH_4) production, oxidation and migration processes in groundwater are important to understand when attributing sources of gas. The processes controlling the natural occurrence of CH_4 in groundwater must be understood, especially when considering the potential impacts of the global expansion of coal seam gas (CSG) production on groundwater quality and quantity. We use geochemical and microbiological data, along with measurements of CH_4 isotopic composition (δ^{13}C-CH_4), to determine the processes acting upon CH_4 in a freshwater alluvial aquifer that directly overlies coal measures targeted for CSG production in Australia. Measurements of CH_4 indicate that there is biogenic CH_4 in the aquifer; however, microbial data indicate that there are no methanogenic archaea in the groundwater. In addition, geochemical data, particularly the isotopes of dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC), as well as the concentration of SO_4^{2-}, indicate limited potential for methanogenesis in situ. Microbial community analysis also shows that aerobic oxidation of CH_4 occurs in the alluvial aquifer. The combination of microbiological and geochemical indicators suggests that the most likely source of CH_4, where it was present in the freshwater aquifer, is the upward migration of CH_4 from the underlying coal measures.

1 Introduction

Interest in methane (CH_4) production and degradation processes in groundwater is driven by the global expansion of unconventional-gas production. There is concern regarding the 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; Owen et al., 2016; Zhang and Soeder, 2016).

In groundwater, CH_4 can originate from numerous sources (Barker and Fritz, 1981). The two main sources of CH_4 in shallow groundwater are in situ biological production (biogenic) and upward migration of CH_4 from deeper geological formations (thermogenic to mixed thermo-biogenic 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 CH_4 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 CH_4 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 of freshwater aquifers.

Methane is subject to many production and degradation processes in groundwater (Whiticar, 1999). The carbon isotopic composition of CH_4 (δ^{13}C-CH_4) 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 CH_4 and processes occurring using CH_4 concentration and isotopic data alone.

Previous studies have used geochemical indicators, such as the concentration of sulfate [SO_4^{2-}], nitrate [NO_3^-] and nitrite [NO_2^-], and the carbon isotopic composition of dissolved inorganic carbon (δ^{13}C-DIC) and dissolved organic carbon (δ^{13}C-DOC) to attribute the source of CH_4 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 (δ^2H-CH_4) and the surrounding formation water (δ^2H-H_2O) (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). Microbiological indicators (in addition to geochemical data) may resolve some of the uncertainties associated with the determination of CH_4 origin, as they directly discriminate between microbiological communities involved in either production or degradation processes. There are no studies using combined geochemical and microbiological indicators to assess CH_4 production and degradation processes in a freshwater aquifer. We aim to fill this gap in the literature.

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^-], δ^{13}C-CH_4, δ^{13}C-DIC, δ^{13}C-DOC and δ^2H-H_2O), 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-} ($> 19\,mg\,L^{-1}$) (Whiticar, 1999) because sulfate-reducing bacteria (SRB) often outcompete methanogenic archaea for reducing equivalents (Lovley and Klug, 1985; Struchtemever et al., 2005).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(R1)}$$

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \quad \text{(R2)}$$

Therefore, the presence or absence of [CH_4] and [SO_4^{2-}] are good preliminary indicators of the potential for in situ methanogenesis.

In addition, the δ^{13}C-CH_4 of the underlying WCM in and around the study area has been characterised. Draper and Boreham (2006) characterised the isotopic signature of the WCM to be between −57.3 and −54.2 ‰. Hamilton et al. (2014) and Baublys et al. (2015) expanded this range to be from −58.5 to −45.3 and −57 to −44.5 ‰, respectively. Recently, Owen et al. (2016) have established a "shallow" WCM directly underlying the alluvium and a deeper "gas reservoir". The isotopic signatures of these range from −80 to −65 and −58 to −49 ‰, respectively. These values are summarised in Table 1, along with available ranges of $\delta^{13}C_{DIC}$ for the study area. 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 compositions 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}C depletion of δ^{13}C-DOC in combination with a ^{13}C enrichment of δ^{13}C-DIC was characteristic of methanogenesis in groundwater, consistent with the reduction of $^{12}CO_2$ by autotrophic methanogens. Conversely, δ^{13}C-DIC data are useful because DIC produced during CH_4 oxidation was found to have a characteristically ^{13}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 Gammaproteobacteria (comprising type-I and type-II methanotrophs) and the Verrucomicrobia (Hanson and Hanson, 1996). The first step of aerobic CH_4 oxidation is the conversion of CH_4 to methanol. This is catalysed by the particulate CH_4 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 CH_4

Table 1. Observed ranges of $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{DIC}$ for the WCM in previous studies.

Location of samples	$\delta^{13}C_{CH_4}$ range	$\delta^{13}C_{CH_4}$ median	$\delta^{13}C_{DIC}$	Source
WCM Surat Basin	−57.3 to −54.2 ‰	nd	nd	Draper and Boreham (2006)
WCM – upper and lower measures	−58.5 to −45.3 ‰	−51.8 ‰	nd	Hamilton et al. (2014)
WCM – upper and lower measures	−57.0 to −44.5 ‰	−52.1 ‰	14.2 ‰ (median)	Baublys et al. (2015)
WCM gas reservoir	−58.0 to −49.0 ‰	−51.6 ‰	9.0 to 23.0 ‰	Owen et al. (2016)
WCM shallow	−80.0 to −65.0 ‰	−75.0 ‰	−15.9 to −3.5 ‰	Owen et al. (2016)

monooxygenase (sMMO) (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, the expression of the *pmoA* and *mmoX* is triggered by the amount of available Cu ions. In addition, aerobic CH_4 oxidation has been previously coupled to denitrification in groundwater (Zhu et al., 2016).

Besides methanotrophic bacteria, anaerobic CH_4-oxidising archaea (ANME) also play a significant role in the oxidation of CH_4 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, 2005). Geochemical indicators can provide evidence for the occurrence of AOM (anaerobic oxidation of methane), such as the prevalence of certain electron acceptors (SO_4^{2-}, NO_3^-, NO_2^- and Fe^{2+}) (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; á Norði and Thamdrup, 2014; Timmers et al., 2015).

2 Study area

The CRAA is the primary aquifer in the Condamine Catchment (Fig. 1). It is used 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 (Fig. 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 (especially with respect to CH_4 concentrations) and quantity of the groundwater in the CRAA.

Hydrogeological setting

The CRAA sits within the Surat Basin, which sits within the Great Artesian Basin (GAB) in south-east Qld, Australia (Radke et al., 2000; Ransley and Smerdon, 2012) (Fig. 2). 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 fluviolacustrine 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 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 (Huxley, 1982; Kelly and Merrick, 2007; Dafny and Silburn, 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 8×10^{-6} to 1.5×10^{-1} m day^{-1}) 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). The thickness of this layer varies between 30 m in some areas to completely absent in others. Thus, in some places the WCM

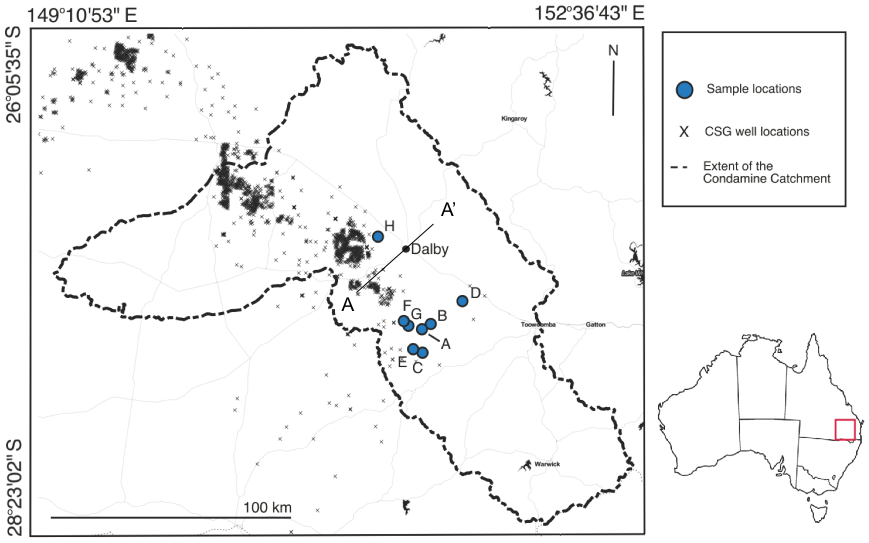

Figure 1. Site map showing the extent of the study area and sample locations within the Condamine Catchment, south-east Queensland, Australia. Map created in QGIS; data and imagery: Statem Toner, Open Street Map and contributors, CC-BY-SA (QGIS 2.8.2 Wien, 2015). Modified with Corel Painter 2015 (Corel Corporation, 2015).

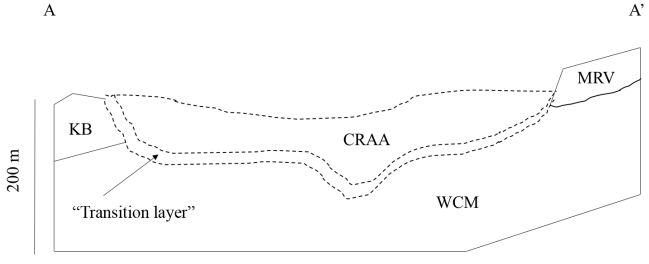

Figure 2. Geological cross section along A–A′ in Fig. 1 (adapted from Dafny and Silburn, 2014). KB – Kumbarilla Beds; MRV – Main Range Volcanics.

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 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. By contrast, Iverach et al. (2015) used the isotopic signature of CH_4 in the groundwater to show that there was localised movement of gas between the coal measures and the overlying aquifer.

More recently, a report prepared by the Office of Groundwater Impact Assessment, Department of Natural Resources and Mines, found that a low-permeability transition layer exists between the CRAA and the zones of the WCM that could contain commercially viable CSG. The report concluded that, overall, the level of hydraulic connectivity between the CRAA and the WCM is low (OGIA, 2016). This research provides additional insight to inform the debate about the degree of connectivity for both water and gas between the WCM and the CRAA. The microbiological insights also inform the global research on biological CH_4 production and degradation in alluvial aquifers, in particular for zones distal to the river corridor.

3 Methods

From 22 to 31 January 2014 we collected groundwater samples for geochemical analysis from eight private irrigation boreholes in the Condamine Catchment (locations shown in Fig. 1). Iverach et al. (2015) outlines the complete methods for sample collection for $[CH_4]$ and δ^{13}C-CH_4 and subsequent analysis. The eight samples collected from the unconfined CRAA are representative of the aquifer, given their varied depths and locations (Table 2).

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 the analysis of major anions and water-stable isotopes (δ^2H-H_2O and δ^{18}O-H_2O) were

Table 2. Slotted depth intervals for the 8 samples.

Sample	Depth interval (m)
A	46.6–60.3
B	64.9–69.5
C	33.9–41.8
D	19.5–35.7
E	23.6–42.5
F	28.6–40.8
G	31.7–35.4
H	25.3–50.3

collected after passing the water through a 0.45 µm, high-volume groundwater filter, which was connected to the pump outlet. Samples for the analysis of 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 analysed within 1 month. Samples to be analysed for DOC were frozen within 12 h of collection.

Groundwater samples for the microbiological analyses were collected between 8 and 11 December 2014 from the same eight 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). We used aspects of the geochemical data collected in the January campaign to interpret 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 atomic emission spectroscopy for cations and ion chromatography for anions. The samples for δ^2H-H_2O and δ^{18}O-H_2O were analysed at ANSTO and are reported as per mill deviations from the international standard V-SMOW (Vienna Standard Mean Ocean Water). δ^{18}O samples were run using an established equilibration, continuous-flow IRMS (isotopic ratio mass spectrometry) method and δ^2H samples were run using an online combustion, dual-inlet IRMS method (Cendón et al., 2015).

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 per mill deviation from IAEA secondary standards that have been certified relative to V-PDB (Vienna Pee Dee Belemnite) for carbon (Cendón et al., 2015). The isotopes of carbon in DOC were analysed at the UC-Davis (University of California, Davis) Stable Isotope Facility; results are reported as per mill 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 (Meredith et al., 2016). The [SO_4^{2-}] were too low in six of the eight samples for δ^{34}S and δ^{18}O analysis. The remaining two samples were analysed for their sulfur and oxygen isotope compositions at the University of Calgary Isotope Science Laboratory. Sulfur isotope ratios were analysed using continuous-flow elemental analysis isotope ratio mass spectrometry (CF-EA-IRMS) with an elemental analyser interfaced to a VG PRISM II mass spectrometer (Cendón et al., 2015). The results are reported against V-CDT (Vienna Canyon Diablo Troilite). The oxygen isotope ratio was determined using a high temperature reactor coupled to an isotope ratio mass spectrometer in continuous-flow mode (Cendón et al., 2015).

3.2 DNA extraction and Illumina sequencing

DNA was extracted from the biomass collected from filtering 2 L of groundwater using a 0.2 µm filter (Merck Millipore). Briefly, DNA was isolated using a phenol-chloroform extraction method as described by Lueders et al. (2004). The DNA was then 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′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[AAACTYAAAKGAATTGRCCG]-3′),

1392R_Illum(5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[ACGGGCGGTGTGTRC]-3′).

This universal primer pair targets the V6–V8 hypervariable 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 (Agencourt AMPure XP (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 specifica-

tions. 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 according to the SILVA Database (SILVA, 2016). To ensure even sampling depth for subsequent analyses, OTU (operational taxonomic unit) abundance data were rarefied to the lowest number of sequences for a sample (8300 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 (quantitative real-time PCR) 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). qPCR was 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 primer concentration of 0.8 µM. The qPCR programme for the amplification was 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 primer pairs mmoX-ms-945f and mmoXB-1401b at a final total concentration of 0.8 µM. The qPCR conditions for the *mmoX* were 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. To maintain inter-assay reliability, standards ranging from 10^8 to 10^2 copies µL^{-1} were included on each assay plate to account for slight variations between runs. A no-template control (NTC) of molecular-biology-grade H_2O was also included on each plate to detect PCR contamination. PCR products were checked by gel electrophoresis using 2 % (w/v) agarose with TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM Na_2-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 r^2 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 barkeri* (DSM 800), *Desulfovibrio vulgaris* (DSM 644), *Methylosinus sporium* (DSM 17706) and *Methylocella silvestris* (DSM 15510; DSMZ, Brunswick, 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 δ^{13}C-CH_4 investigation

A previous study by Iverach et al. (2015) analysed the δ^{13}C-CH_4 in the groundwater from an off-gassing port on the eight private irrigation boreholes studied here (samples A–H) (Table S3 in the Supplement). These measurements were understood to have been mixing with regional background atmospheric CH_4 (1.774 ppm; −47 ‰); 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 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 were 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 SYBRGreen I counts were between 10^3 and 10^5 cells mL^{-1} throughout all groundwater samples (Fig. 3). This was congruent with the qPCR data observed for bacterial and archaeal cell concentrations.

The groundwater community was primarily composed of bacteria (79–90 %), whilst archaea made up 10–21 % (Fig. 4). 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 (Fig. 4). The

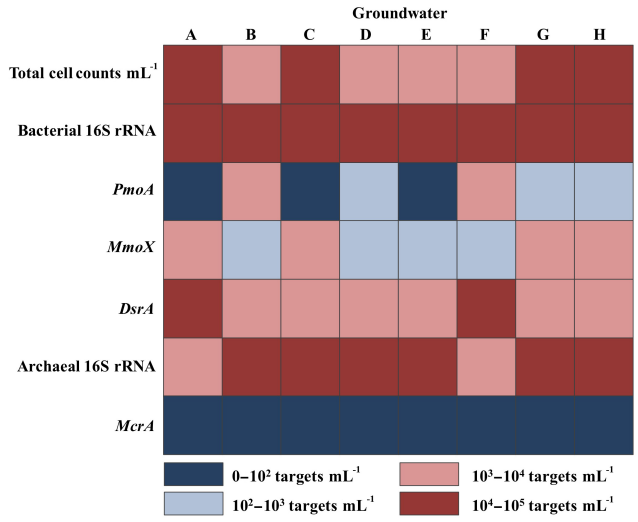

Figure 3. Total cell concentration and copy number abundances of bacterial and archaeal 16SrRNA genes and functional key genes for aerobic CH_4 oxidation (*pmoA* and *mmoX*), CH_4 production (*mcrA*) and sulfate reduction (*dsrA*) in the groundwater carried out by quantitative PCR. Low abundances are highlighted in dark blue. High abundances are highlighted in dark red. The calculated standard deviations for replicate quantifications of one sample were consistently between 10–20 %.

phylum Thaumarchaeota dominated the archaeal communities with a relative abundance of 81–99 %, while Crenarchaeota made up 1–3 %. 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^{-1}; Fig. 3). This was consistent with the Illumina sequencing results and suggests that the CH_4 observed off-gassing from the groundwater was not being produced in situ within the CRAA.

The microbial community in the groundwater was assumed to reflect that of the geological formations because when we sample the groundwater, we are also sampling fine particles with biomass attached. Additionally, Maamar et al. (2015) found that the microbial community composition of groundwater was controlled by groundwater residence times and flow paths, independent of the geology. Further, the intense purging of the production wells in the Condamine alluvium ensure that we are sampling groundwater that is representative of the sampled formations.

Our isotopic geochemical data also showed no evidence for the occurrence of methanogenesis in the groundwater. As previously stated, a ^{13}C enrichment in $\delta^{13}C$-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 (Fig. 5a), 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 (Fig. 5b; Table S1 in the Supplement), it is evident that there is no noticeable δ^2H enrichment that can be ascribed to methanogenesis in any of the groundwater samples (Cendón et al., 2015).

These geochemical analyses, along with the lack of classified methanogens, suggest that biogenic CH_4 production is not one of the major processes responsible for the presence of CH_4 in the CRAA. Therefore, the CH_4 reported in all samples in Iverach et al. (2015) must be derived 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, 2014). Five of the eight 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 three samples (samples E, G and H) have a typically biogenic isotopic source signature (−69.1 ‰). Owen et al. (2016) recently characterised the isotopic signature of both the WCM gas reservoir and the shallow WCM layer between the gas reservoir and the overlying alluvium (Table 1). The isotopic signature for the shallow WCM samples was between −80 and −65 ‰. The three samples here, which exhibit a source signature of −69.1 ‰, could potentially be sourcing CH_4 from the shallow WCM. This would result in a biological source signature of the CH_4 in the overlying aquifer despite the absence of methanogenic archaea.

4.3 Sulfate reducers and aerobic methanotrophs potentially outcompete methanogens

Sulfate concentrations in most groundwater samples were low (3.2–11 mg L^{-1}) (Table S2 in the Supplement). Groundwater samples D and H were higher with 55 and 29 mg L^{-1}, respectively (Table S2 in the Supplement). 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; Figs. 3 and 4). These SRB are potentially outcompeting methanogenic archaea for substrates such as acetate and H_2. Sulfate concentrations higher than 3 mg L^{-1}, as detected in all groundwater samples (3.2–55 mg L^{-1}), could potentially create a SO_4^{2-}-reducing environment with the predominance of SRB over methanogens. This would potentially maintain the acetate at concentrations too low for methanogens to grow (Lovley and Klug, 1985). Deltaproteobacteria were dominant in all groundwater samples, and most of the sequences were

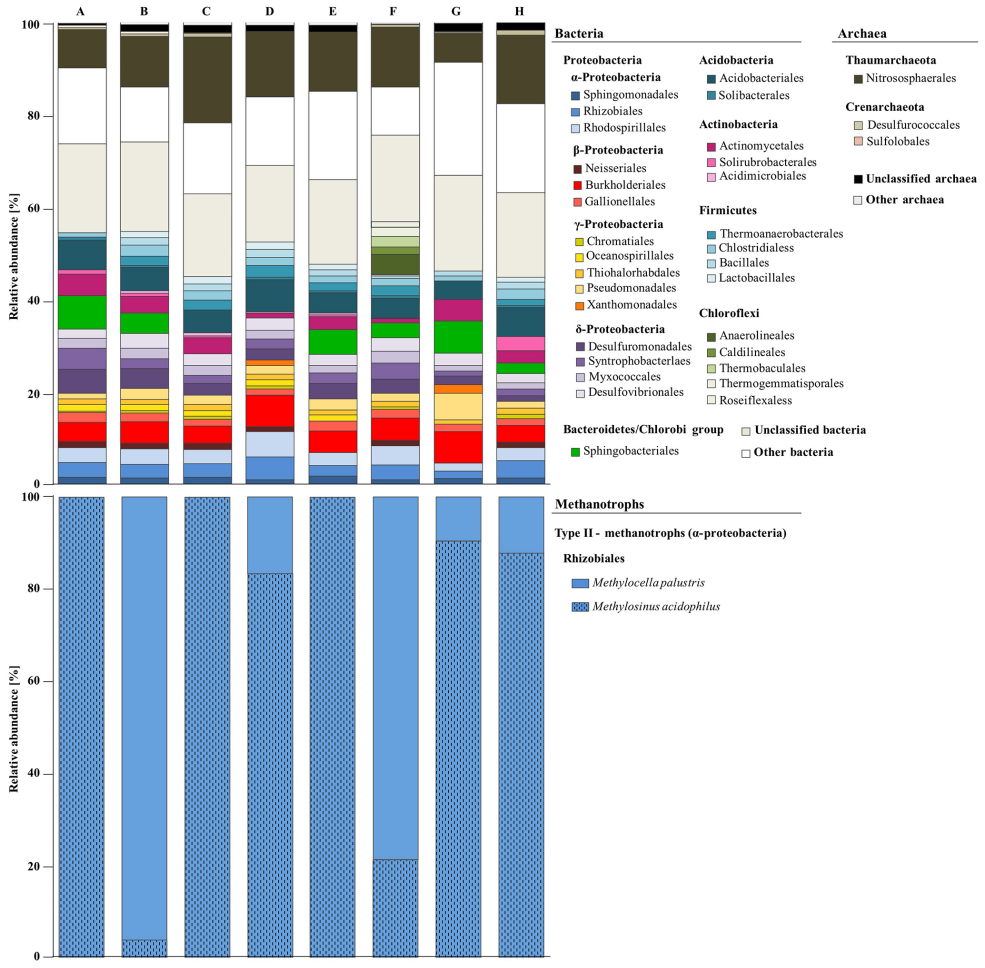

Figure 4. Bacterial, archaeal and methanotrophic community profiles and relative abundances detected by Illumina sequencing.

closely related to acetate-oxidising, sulfate/sulfur-reducing bacteria (*Desulfovibrionales*, *Syntrophobacterales, Desulfuromonadales*; Fig. 4). 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. In addition, the presence of SO_4^{2-} along with conditions favouring SRB is further evidence that in situ methanogenesis is unlikely to be responsible for the presence of CH_4 in the shallow aquifer.

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 (7.9×10^2–9.3×10^3 targets mL^{-1}) compared to the overall bacterial 16S rRNA concentration (2.5×10^4–5.1×10^4 targets mL^{-1}) (Fig. 3). 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* (Alphaproteobacteria); however, MOB accounted for up to 7 % of the overall microbial community (Fig. 4). All groundwater samples were dominated by two MOB, belonging to the type-II methanotrophs (Fig. 4). 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) (Fig. 4). These genera were characterised as aerobic CH_4 oxidisers; however, aerobic MOB have been previously observed in microaerophilic 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,

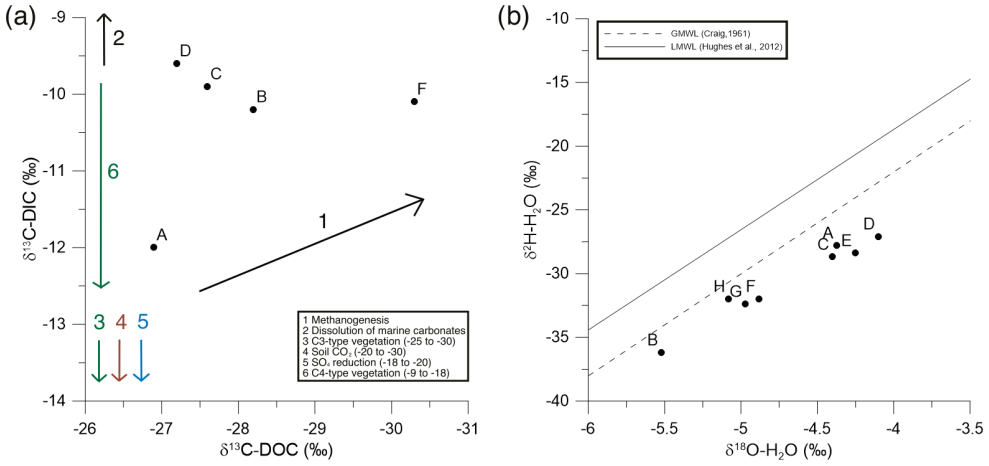

Figure 5. (a) A plot of δ^{13}C-DOC vs. δ^{13}C-DIC. There is no correlation between these geochemical data, indicating that there is no methanogenic end member in our samples. Samples E, G and H are omitted because they were below the detection limit for δ^{13}C-DOC (Table S1 in the Supplement). Arrow 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 δ^{13}C-DIC that are off the scale of the graph (Currell et al., 2016). **(b)** A plot of δ^{18}O-H_2O vs. δ^{2}H-H_2O showing that there is no ^{2}H enrichment in any of the groundwater samples. The GMWL (global meteoric water line; Craig, 1961) and LMWL (local meteoric water line; Hughes and Crawford, 2012) are also displayed.

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 (Fig. 3). 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 show the sole predominance of *mmoX* genes in three of the eight groundwater samples that are exclusively dominated by *Methylosinus* sp. (samples A, C and E) (Figs. 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 to 8.6 mg L^{-1}). The reported concentration of DO for the groundwater was measured at the ground surface and is therefore not an accurate measure of the in situ value. However, it could contribute to the absence of methanogenic archaea, as well as the abundance of aerobic bacteria. In addition, the reduction of sulfate under oxic conditions has been observed (Kieldsen et al., 2004; Fike et al., 2008), which would explain the abundance of sulfate-reducing Deltaproteobacteria in most samples, despite the high concentration of DO in the groundwater.

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 (Table S2 in the Supplement).

The sample with the most diverse bacterial community (Sample F, Fig. 4) had the most ^{13}C-enriched individual δ^{13}C-CH_4 relative to regional background (Iverach et al., 2015) (Table S3 in the Supplement). A relatively high abundance (11 %) of relatives belonging to the Chloroflexi phylum was observed exclusively in this groundwater sample.

4.5 Absence of anaerobic methane oxidation

The lack of detection of the *mcrA* gene not only indicates 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 (Fig. 4). Thaumarchaeota contains several clusters of environmental sequences representing microorganisms with an 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 (Pester et al., 2011).

Samples D and H had SO_4^{2-} concentrations of 55 and 29 mg L^{-1}, 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 two 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 (Table S2 in the Supplement), 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 (Fig. 4); 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 relative to groundwaters with the potential for AOM via denitrification (á Norði and Thamdrup, 2014). Our samples had [NO_3^-] ranging from 1.2 to 2.3 mg L^{-1} and [NO_2^-] below 0.05 mg L^{-1} (Table S2 in the Supplement). Therefore, AOM coupled to denitrification is unlikely to be occurring in the groundwater of the CRAA (á Norði and Thamdrup, 2014).

The δ^{13}C-DIC data indicate 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; Table S1 in the Supplement), it is unlikely that AOM is occurring in the groundwater. Additionally, a previous study of the GAB geochemistry showed that δ^{13}C-DIC values in this region are in the range of −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 as aerobic CH_4 oxidisers, agreeing with our geochemical data that no anaerobic oxidation was occurring. Despite abundant SO_4^{2-} in two 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.

The above geochemical and microbiological data place constraints on the active process, gas origin and pathways of migration. Figure 6 presents a conceptual schematic of the processes occurring between the WCM and the CRAA.

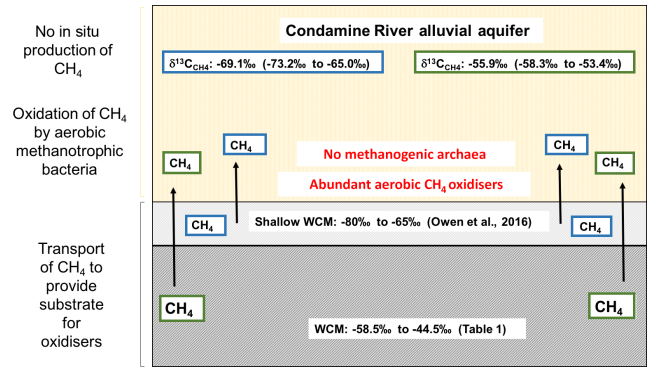

Figure 6. A conceptual schematic of the processes occurring between the WCM and the CRAA.

5 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 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. This, coupled with the isotopic signature of the CH_4 and the concentration of SO_4^{2-} in the groundwater, suggests that the upwards migration of CH_4 from the underlying WCM is the likely source (Fig. 6).

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 δ^{13}C-CH_4 data alone does not 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 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, either through natural

faults and fractures, transport along poorly installed well casings, or direct leakage of gas between the WCM and CRAA where the units are in direct contact. 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.

6 Data availability

We have provided our data set in the Supplement. All of our findings in this article can be reproduced by the data that we provided, either in the text itself or the Supplement.

In addition, data from Iverach et al. (2015) have been used.

The Supplement related to this article is available online at doi:10.5194/bg-14-215-2017-supplement.

Author contributions. Experimental conceptualisation and design were carried out by Dioni I. Cendón and Bryce F. J. Kelly. Fieldwork was conducted by Charlotte P. Iverach, Sabrina Beckmann, Dioni I. Cendón and Bryce F. J. Kelly. Geochemical analyses were conducted by Dioni I. Cendón. Microbiological analyses were conducted by Sabrina Beckmann, Charlotte P. Iverach and Mike Manefield. The paper was written by Charlotte P. Iverach and Sabrina Beckmann with input from all authors.

Competing interests. The authors declare that they have no conflict of interest.

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