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

Charlotte P. Iverach[1,2,*], Sabrina Beckmann[3], Dioni I. Cendón[1,2], Mike Manefield[3], Bryce F.J. Kelly[1]

[1]Connected Waters Initiative Research Centre, UNSW Australia, UNSW Sydney, NSW, 2052, Australia.

[2]Australian Nuclear Science and Technology Organisation, New Illawarra Rd, Lucas Heights, NSW, 2234, Australia.

[3]School of Biotechnology and Biomolecular Sciences, UNSW Australia, UNSW Sydney, NSW, 2052, Australia.

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 production on groundwater quality and quantity. We use geochemical and microbiological data, along with measurements of CH_4 isotopic composition ($\delta^{13}C$-CH_4), to determine the processes acting upon CH_4 in a freshwater alluvial aquifer that directly overlies coal measures targeted for coal seam gas 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 is occurring 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.

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

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

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 et al., 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 ($\delta^{13}C\text{-}CH_4$) 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) (Whiticar, 1999), because sulfate reducing bacteria (SRB) often outcompete methanogenic archaea for reducing equivalents (Lovley et al., 1985; Struchtemever et al., 2005).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1)$$

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

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 $\delta^{13}C\text{-}CH_4$ 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.

Table 1. Observed ranges of $\delta^{13}C_{CH4}$ and $\delta^{13}C_{DIC}$ for the WCM in previous studies.

Location of samples	$\delta^{13}C_{CH4}$ range	$\delta^{13}C_{CH4}$ median	$\delta^{13}C_{DIC}$	Source
WCM Surat Basin	-57.3‰ to -54.2‰	nd	nd	Draper & 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

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}C-depletion of $\delta^{13}C$-DOC in combination with a ^{13}C-enrichment of $\delta^{13}C$-DIC was characteristic of methanogenesis in groundwater, consistent with the reduction of $^{12}CO_2$ by autotrophic methanogens. Conversely, $\delta^{13}C$-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 *Gamma*-Proteobacteria (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

143 methanotrophs and some type I members of the genus *Methylococcus* contain the *mmoX*
144 gene, which encodes a soluble CH_4 monooxygenase (sMMO) (McDonald et al., 1995;
145 Murrell et al., 2000). Recently, new groups of aerobic and anaerobic MOB distantly
146 related to known methanotrophic groups have been discovered (Raghoebarsing et al.,
147 2006; Stoecker et al., 2006; Op den Camp et al., 2009). Geochemically, the expression of
148 the *pmoA and mmoX* is triggered by the amount of available Cu ions. In addition, aerobic
149 CH_4 oxidation has been previously coupled to denitrification in groundwater (Zhu et al.,
150 2016).

151 Besides methanotrophic bacteria, anaerobic CH_4-oxidising archaea (ANME) also
152 play a significant role in the oxidation of CH_4 in both freshwater and saline water sources
153 (Knittel and Boetius, 2009). These anaerobic methanotrophs are associated with the
154 methanogenic Euryarchaeota within the clusters ANME-1, ANME-2, and ANME-3 and
155 are closely related to the orders *Methanosarcinales* and *Methanomicrobiales* (Knittel et
156 al., 2003; Knittel et al., 2005). Geochemical indicators can provide evidence for the
157 occurrence of AOM, such as the prevalence of certain electron acceptors (SO_4^{2-}, NO_3^-,
158 NO_2^- and Fe^{2+}) (Valentine and Reeburgh, 2000; Ettwig et al., 2010; Sivan et al., 2011;
159 Antler, 2014; Green-Saxena et al., 2014) and denitrification processes occurring in the
160 groundwater (Ettwig et al., 2008; Nordi and Thamdrup, 2014; Timmers et al., 2015).
161

162 **2 Study Area**

163 The CRAA is the primary aquifer in the Condamine Catchment (Figure 1). It is used for
164 irrigated agriculture, stock and domestic water supplies. There has been increased interest
165 in the presence of CH_4 in the aquifer due to expanding CSG production to the north-west
166 of the study area (Figure 1). CSG production began in 2006 (Arrow Energy, 2015) and
167 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.

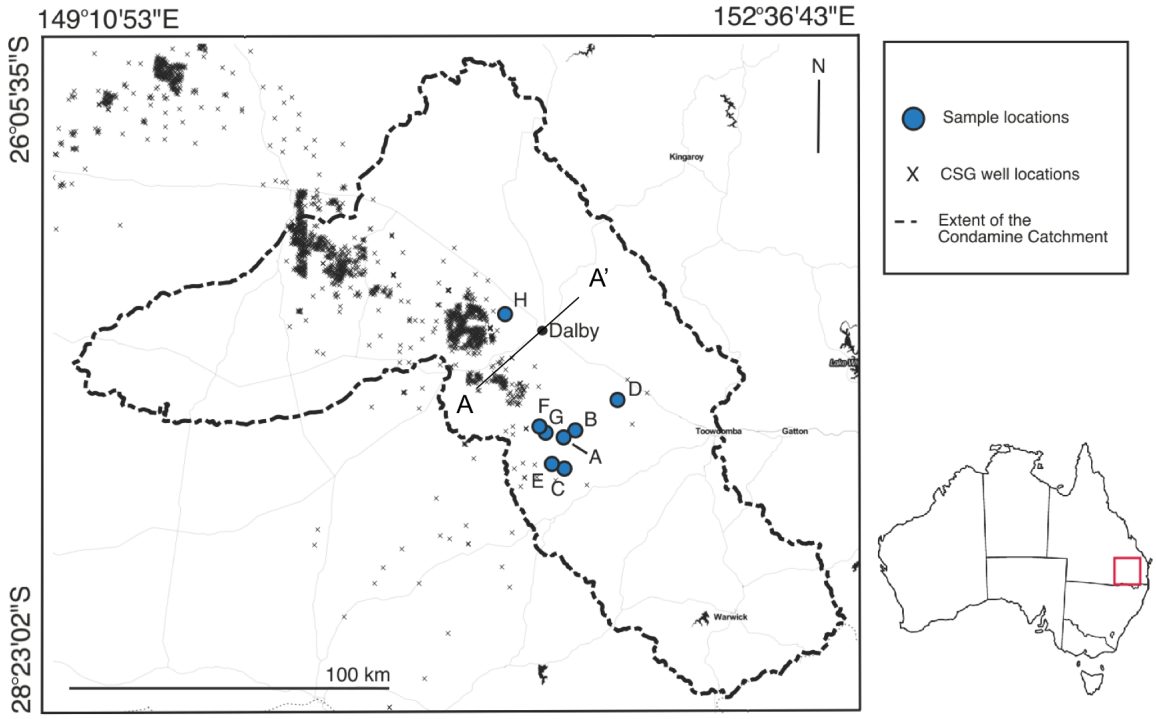


Figure 1. Site map showing the extent of the study area and sample locations within the Condamine Catchment, south-east Queensland, Australia. Map created in QGIS; data and imagery: Statem Toner, Open Street Map and contributors, CC-BY-SA (QGIS, 2015). Modified with Corel Painter 2015 (Corel Corporation, 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 (Radke et al. 2000; Ransley and Smerdon, 2012) (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

184 targeted for CSG production (Hamilton et al., 2012). The WCM are thicker (150 m to 350
185 m) along the western margin of the CRAA and thin to around 50 m in the east, where the
186 unit outcrops (KCB, 2011); however, only around 10% of this is coal. The unit consists of
187 very fine- to medium-grained sandstone, siltstone, mudstone and coal, with minor
188 calcareous sandstone, impure limestone and ironstone (KCB, 2011). The coal consists of
189 numerous discontinuous thin lenses separated by sediments of low permeability (Hillier,
190 2010). The unit dips gently to the west (around 4^{o}), which is consistent with the general
191 trend of the Surat Basin in this region.
192 The WCM overlie the Eurombah Formation (consisting of conglomerate sandstone
193 with minor siltstones and mudstone beds) and underlie the Kumbarilla Beds (mainly
194 sandstone, with lesser mudstone, siltstones and conglomerates) (KCB, 2011).
195 The unconfined CRAA fills a paleovalley that was carved through the GAB
196 (including the WCM). The valley-filling sediments are composed of gravels and fine- to
197 course-grained channel sands interbedded with floodplain clays and, on the margins,
198 colluvial deposits, which were deposited from the mid-Miocene to the present (Huxley,
199 1982; Kelly and Merrick, 2007; Dafny & Silburn, 2014). The valley-filling sediments
200 have a maximum thickness of 134 m near Dalby (Dafny and Silburn, 2014). Along the
201 eastern margin of the valley, the CRAA is bounded by the Main Range Volcanics.
202 Estimations of the sources and quantity of recharge to the CRAA vary widely; however,
203 streambed recharge is generally considered to be the major source of freshwater to the
204 aquifer (Dafny and Silburn, 2014).
205 A low permeability layer (ranging from 8×10^{-6} to 1.5×10^{-1} m/d) has been reported
206 between the CRAA and the underlying WCM (KCB, 2011; QWC, 2012). This has been
207 referred to as the 'transition layer' (QWC, 2012) or a 'hydraulic basement' to the
208 alluvium (KCB, 2011). 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 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.

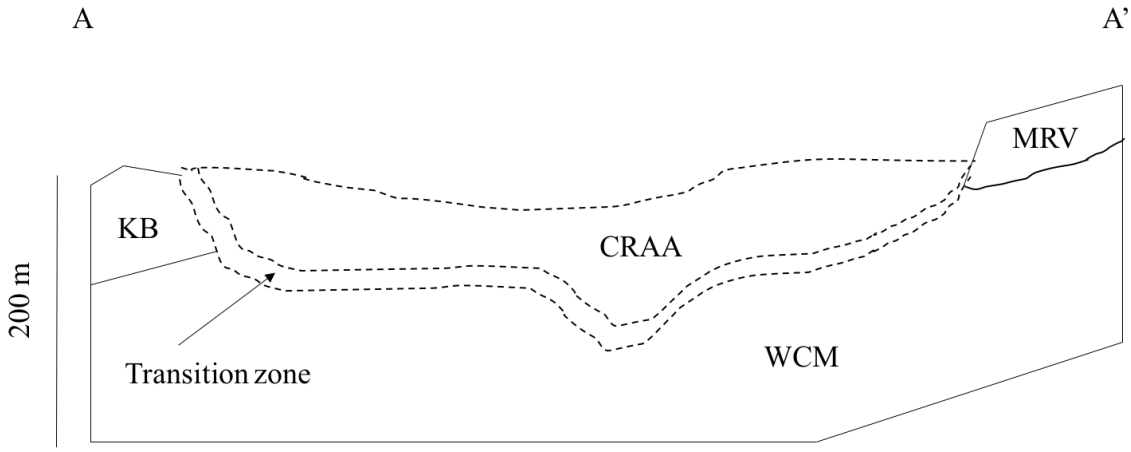


Figure 2. Geological cross section along A-A' in Figure 1 (adapted from Dafny & Silburn, 2014). KB-Kumbarilla Beds; MRV-Main Range Volcanics.

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 January 2014 to 31 January 2014 we collected groundwater samples for geochemical analysis from 8 private irrigation boreholes in the Condamine Catchment (locations shown in Figure 1). Iverach et al. (2015) outlines the complete methods for sample collection for $[CH_4]$ and $\delta^{13}C\text{-}CH_4$ and subsequent analysis. The 8 samples collected from the unconfined CRAA are representative of the aquifer, given their varied depths and locations (Table 2).

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

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

247 continuously over the preceding month for irrigation and so stabilisation of the field
248 parameters was reached within minutes. Groundwater samples for analysis of major
249 anions and water-stable isotopes (δ^2H-H_2O and δ^{18}O-H_2O) were collected after passing
250 the water through a 0.45 μm, high-volume groundwater filter, which was connected to the
251 pump outlet. Samples for analysis of anions and water stable-isotopes were stored in 125
252 mL high-density polyethylene (HDPE) bottles and 30 mL HDPE bottles, respectively.
253 Both had no further treatment. The water for δ^{13}C-DIC and δ^{13}C-DOC was further filtered
254 through a 0.22 μm filter and stored in 12 mL Exetainer vials and 60 mL HDPE bottles,
255 respectively. Samples to be analysed for DIC were refrigerated at 4 ^{o}C and analysed
256 within one month. Samples to be analysed for DOC were frozen within 12 hours of
257 collection.

258 Groundwater samples for the microbiological analyses were collected between 8
259 December 2014 and 11 December 2014 from the same 8 private irrigation boreholes used
260 for the geochemical analyses. Groundwater samples for microbiological analysis were
261 collected in 2 L Duran Schott bottles and sealed (gas tight). We used aspects of the
262 geochemical data collected in the January campaign to interpret the microbial results from
263 the December campaign.

264

265 **3.1 Geochemical analyses**

266 The major ion chemistry in the groundwater samples was analysed at the Australian
267 Nuclear Science and Technology Organisation (ANSTO) using inductively coupled
268 plasma atomic emission spectroscopy for cations and ion chromatography for anions. The
269 samples for δ^2H-H_2O and δ^{18}O-H_2O were analysed at ANSTO and are reported as ‰
270 deviations from the international standard V-SMOW (Vienna Standard Mean Ocean
271 Water). δ^{18}O samples were run using an established equilibration, continuous flow IRMS

method and δ^2H samples were run using an on-line 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 ‰ deviation from IAEA secondary standards that have been certified relative to V-PDB for carbon (Cendón et al., 2015). The isotopes of carbon in DOC were analysed at the UC-Davis Stable Isotope Facility; 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 (Meredith et al., 2016). The $[SO_4^{2-}]$ were too low in 6 of the 8 samples for $\delta^{34}S$ and $\delta^{18}O$ 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 Continuous Flow-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 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 (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

297 70% (v/v) ethanol and resuspended in 50 μL nuclease free water (Qiagen). DNA
298 concentration and purity were determined by standard agarose gel electrophoresis and
299 fluorometrically using RiboGreen (Qubit Assay Kit, Invitrogen) according to the
300 manufacturer's instructions. The extracted DNA was used as a target for Illumina
301 sequencing. Amplicon libraries were generated by following Illumina's 16S Metagenomic
302 Sequencing Library Preparation Protocol, using 12.5 ng of template DNA per reaction.
303 The number of cycles for the initial PCR was reduced to 21 to avoid biases from over-
304 amplification. The following universal primer pair was used for the initial amplification,
305 consisting of an Illumina-specific overhang sequence and a locus-specific sequence:
306 926F_Illum(5'-
307 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[AAACTYAAAKGAATTGRC
308 CG]-3'),

309 1392R_Illum(5'-
310 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[ACGGGCGGTGTGTRC]-3').

311 This universal primer pair targets the V6-V8 hyper-variable regions of the 16S ribosomal
312 RNA gene and has been shown to capture the microbial diversity of Bacteria and Archaea
313 in a single reaction (Wilkins et al., 2013). PCR products were purified using a magnetic
314 bead capture kit (Agencourt AMPure XP, (Beckman Coulter)) and quantified using a
315 fluorometric kit (RiboGreen, Qubit Assay Kit, Invitrogen). Purified amplicons were
316 subjected to the Index PCR using the MiSeq platform (Ramaciotti Centre for Genomics,
317 UNSW Australia) according to the manufacturer's specifications. Illumina sequences
318 were checked for quality (FastQC, BaseSpace) and analysed using the BaseSpace cloud
319 computing platform (Illumina, 2016) and MOTHUR (Schloss, 2009) with modified
320 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^{TM} 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). 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^{o}C for 3 min followed by 40 cycles of 95^{o}C for 30 s, 64^{o}C for 45 s and 68^{o}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* was as follows: 94^{o}C for 3 min followed by 45 cycles of 94^{o}C for 1 min, 50^{o}C for 1 min and 72^{o}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 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, Braunschweig, Germany). The PCR amplicons were purified using the DNA Clean and Concentrator^{TM}-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

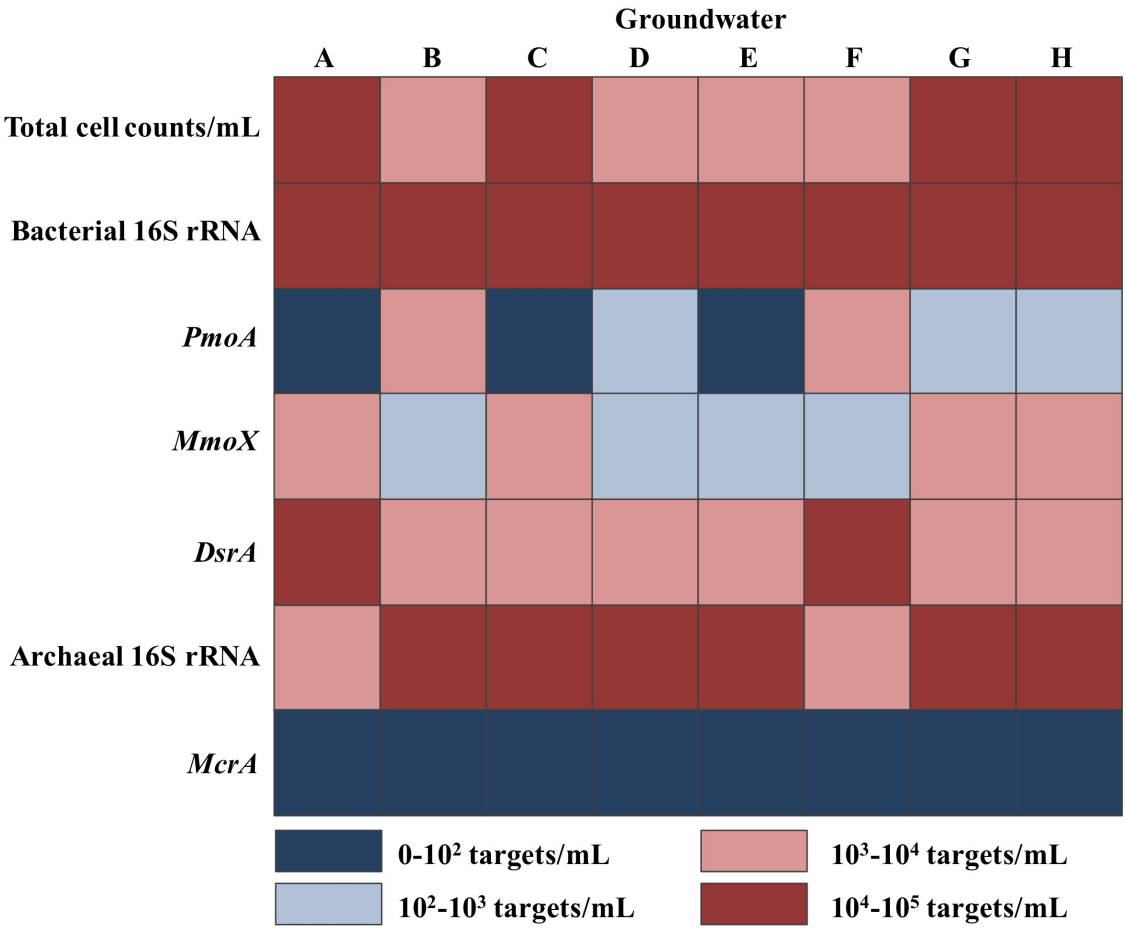
4.1 Previous δ^{13}C-CH_4 investigation

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) (Supplementary Table S3 online). 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 (qPCR) 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 SYBR Green I counts were between 10^3 and 10^5 cells/mL throughout all groundwater samples (Figure 3). This was congruent with the qPCR data observed for bacterial and archaeal cell concentrations.



387

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

393

394 The groundwater community was primarily composed of bacteria (79-90%), whilst
395 archaea made up 10-21% (Figure 4). The bacterial and archaeal community composition
396 did not vary significantly between groundwater samples. Most of the bacterial sequences
397 belonged to the phyla Proteobacteria *(α-δ)*, Acidobacteria, Actinobacteria, Firmicutes and
398 the Bacteroidetes/Chlorobi group (Figure 4). The phylum Thaumarchaeota dominated the
399 archaeal communities with a relative abundance of 81-99%, while Crenarchaeota made up

400 1-3%. Further sequences were related to other (if < 1 % relative abundance) and
401 unclassified Bacteria and Archaea. No members of the Euryarchaeota, comprising the
402 methanogenic archaea, were observed. The archaeal *mcrA* gene, which encodes the
403 methyl coenzyme M reductase, was not detected in any of the groundwater samples
404 (detection limit < 10 cells/mL; Figure 3). This was consistent with the Illumina
405 sequencing results, and suggests that the CH_4 observed off-gassing from the groundwater
406 was not being produced *in situ* within the CRAA.

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

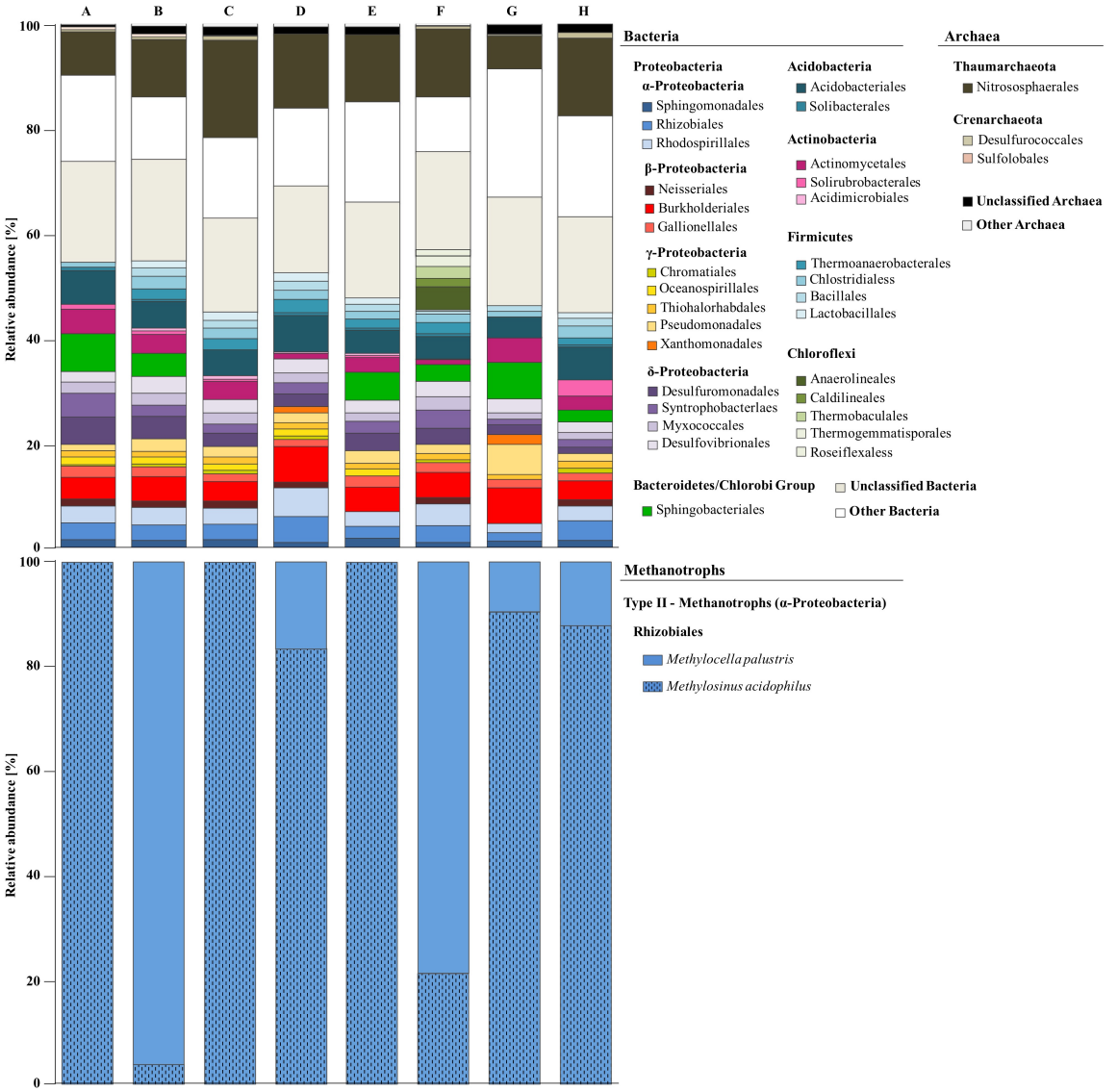
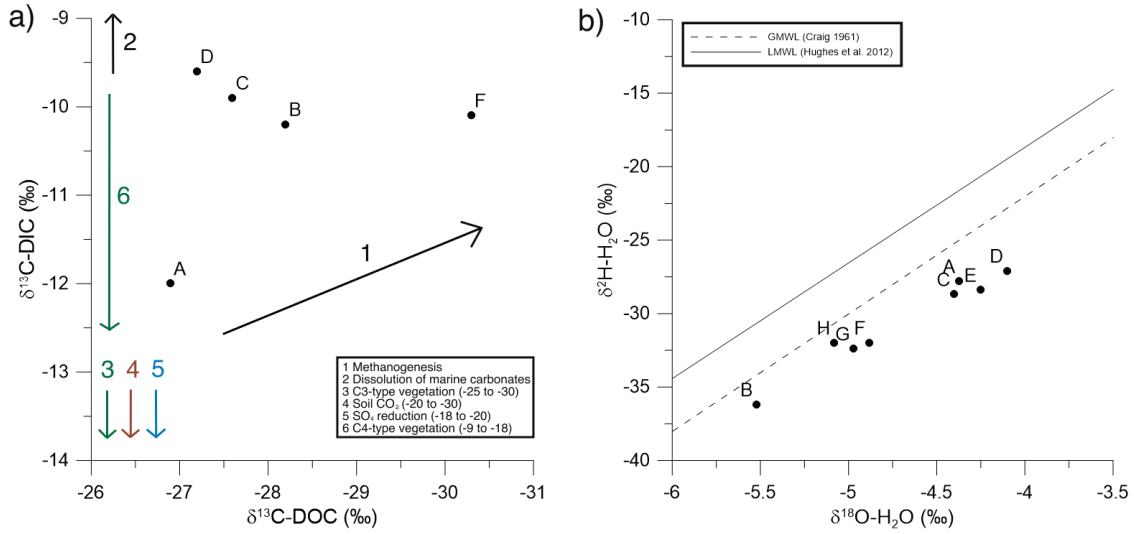


Figure 4. Bacterial, archaeal, and methanotrophic community profiles and relative abundances detected by Illumina sequencing.

Our isotopic geochemical data also showed no evidence for the occurrence of methanogenesis in the groundwater. As previously stated, a ^{13}C-enrichment in δ^{13}C-DIC coupled with a ^{13}C-depletion in the δ^{13}C-DOC is characteristic of methanogenesis (Kotelnikova, 2002). Our groundwater data showed no correlation between δ^{13}C-DOC and δ^{13}C-DIC (Figure 5a), and the most ^{13}C-enriched δ^{13}C-DIC was also the second highest enriched δ^{13}C-DOC value. Additionally, on a stable water isotope plot (Figure 5b; Supplementary Table S1 online), it is evident that there is no noticeable δ^{2}H-enrichment

425 that can be ascribed to methanogenesis in any of the groundwater samples (Cendón et al.,
426 2015).



427

428 **Figure 5.** (a) A plot of δ^{13}C-DOC vs. δ^{13}C-DIC. There is no correlation between these geochemical data,
429 indicating that there is no methanogenic end member in our samples. Samples E, G and H are omitted
430 because they were below the detection limit for δ^{13}C-DOC (Supplementary Table S1.). Arrow 1 delineates
431 the expected trend for methanogenesis and arrow 2 is the expected trend for the dissolution of marine
432 carbonates (Currell et al., 2016). Arrows 3-6 highlight expected ranges for δ^{13}C-DIC that are off the scale of
433 the graph (Currell et al., 2016). (b) A plot of $\delta^{18}O$-H_2O vs. $\delta^{2}H$-H_2O showing that there is no ^{2}H-enrichment
434 in any of the groundwater samples. The GMWL (Craig, 1961) and LMWL (Hughes and Crawford, 2012)
435 are also displayed.

436

437 These geochemical analyses, along with the lack of classified methanogens, suggest
438 that biogenic CH_4 production is not one of the major processes responsible for the
439 presence of CH_4 in the CRAA. Therefore, the CH_4 reported in all samples in Iverach et al.
440 (2015) must be derived from another source. We propose that the upward migration of
441 CH_4 from the WCM must be considered as the potential source. The isotopic signature of
442 CH_4 from the deeper coal measures has been characterised between -58.5‰ and -45.3‰,
443 indicating thermogenic CH_4 with a secondary biogenic component (Papendick et al.,

444 2011; Hamilton et al., 2012; Hamilton et al., 2014). Five of the eight samples analysed in
445 this study have an isotopic source signature within this range, as reported in Iverach et al.
446 (2015). This implies that upward migration from the deeper WCM is the source of the
447 CH_4 detected in the groundwater.

448 However, the remaining three samples (samples E, G, and H) have a typically
449 biogenic isotopic source signature (-69.1‰). Owen et al. (2016) recently characterised the
450 isotopic signature of both the WCM 'gas reservoir' and the 'shallow WCM' layer
451 between the 'gas reservoir' and the overlying alluvium (Table 1). The isotopic signature
452 for the shallow WCM samples was between -80‰ and -65‰. The three samples here,
453 which exhibit a source signature of -69.1‰, could potentially be sourcing CH_4 from the
454 shallow WCM. This would result in a biological source signature of the CH_4 in the
455 overlying aquifer despite the absence of methanogenic archaea.

456

457 **4.3 Sulfate reducers and aerobic methanotrophs potentially outcompete**
458 **methanogens**

459 Sulfate concentrations in most groundwater samples were low (3.2-11 mg/L)
460 (Supplementary Table S2 online). Groundwater samples D and H were higher with 55
461 mg/L and 29 mg/L, respectively (Supplementary Table S2 online). Sequence and
462 functional *dsrA* gene analysis (encoding the dissimilatory sulfite reductase of SRB)
463 revealed that SRB are present in all groundwater samples at relatively high abundances (5
464 - 10% of the overall microbial community; Figures 3 and 4). These SRB are potentially
465 outcompeting methanogenic archaea for substrates such as acetate and H_2. Sulfate
466 concentrations higher than 3 mg/L, as detected in all groundwater samples (3.2 – 55
467 mg/L), could potentially create a SO_4^{2-}-reducing environment with the predominance of
468 SRB over methanogens. This would potentially maintain the acetate at concentrations too

469 low for methanogens to grow (Lovley et al., 1985). Deltaproteobacteria were dominant in
470 all groundwater samples, and most of the sequences were closely related to acetate-
471 oxidising, sulfate/sulfur-reducing bacteria (*Desulfovibrionales*, *Syntrophobacterales,*
472 *Desulfuromonadales*; Figure 4). Additionally, *Methylocella* spp. are capable of using
473 methanogenic substrates, such as acetate and methylamines, for their metabolism and
474 therefore are not limited to growing on one-carbon compounds such as CH_4 (Dedysh et
475 al., 2005). This could have major implications for the lack of methanogenic activity in the
476 groundwater. In addition, the presence of SO_4^{2-} along with conditions favouring SRB is
477 further evidence that *in situ* methanogenesis is unlikely to be responsible for the presence
478 of CH_4 in the shallow aquifer.
479
480 **4.4 Microbial methane oxidation in the groundwater catalyses upward migrating**
481 **methane from the WCM**
482 The functional gene for aerobic CH_4 oxidation (*pmoA*) was detected at relatively high
483 concentrations (7.9×10^2-9.3×10^3 targets/mL) compared to the overall bacterial 16S
484 rRNA concentration (2.5×10^4-5.1×10^4 targets/mL) (Figure 3). All groundwater samples
485 were characterised with regard to the community structure of MOB. The samples
486 harboured a low-diversity methanotrophic community associated with the order
487 *Rhizobiales* (α-Proteobacteria), however MOB accounted for up to 7% of the overall
488 microbial community (Figure 4). All groundwater samples were dominated by two MOB,
489 belonging to the type II methanotrophs (Figure 4). Five samples had both *Methylocella*
490 *palustris* (family *Beijerinckiaceae*) and *Methylosinus acidophilus* (family
491 *Methylocystaceae*) (samples B, D, F-H), whilst the remaining samples comprised
492 *Methylosinus acidophilus* only (samples A, C and E) (Figure 4). These genera were
493 characterised as aerobic CH_4 oxidisers, however aerobic MOB have been previously

494 observed in micro-aerophilic and anaerobic environments (Bowman, 2000). This suggests
495 the existence of an alternative pathway for aerobic CH_4 oxidation in a suboxic/anaerobic
496 environment. Both species have previously been found and isolated from a variety of
497 freshwater habitats and *Methylosinus* spp. are known to be dominant methanotrophic
498 populations in groundwater (Bowman, 2000). *Methylocella* and *Methylosinus* spp. possess
499 a soluble CH_4 monooxygenase (*mmoX*) (McDonald et al., 1995; Murrell et al., 2000),
500 which is consistent with the high abundance of the *mmoX* gene targeted in all
501 groundwater samples (Figure 3). Interestingly, no *pmoA* gene, a biomarker for all MOBs,
502 has previously been detected in known *Methylosinus* spp. (Dedysh et al., 2005). This is
503 supported by our data, which show the sole predominance of *mmoX* genes in three of the
504 eight groundwater samples that are exclusively dominated by *Methylosinus* sp. (samples
505 A, C, and E) (Figures 2 and 3).

506 In addition to low concentrations of CH_4 reported in Iverach et al. (2015), the
507 dissolved O_2 (DO) in our groundwater samples had a large range, from low to close to
508 saturation (0.91 mg/L to 8.6 mg/L). The reported concentration of DO for the
509 groundwater was measured at the ground surface and is therefore not an accurate measure
510 of the *in situ* value. However, it could contribute to the absence of methanogenic archaea,
511 as well as the abundance of aerobic bacteria. In addition, the reduction of sulfate under
512 oxic conditions has been observed (Kieldsen et al. 2004; Fike et al., 2008), which would
513 explain the abundance of sulfate-reducing Deltaproteobacteria in most samples, despite
514 the high concentration of DO in the groundwater.

515 *Methylocella* spp. are not associated with the previously known type II cluster of
516 methanotrophs, but are closely related to a non-methanotroph (Dedysh et al., 2005)
517 suggesting different affinities to CH_4 and O_2, compared to previously known type II
518 methanotrophs (Amaral and Knowles, 1995). There is no correlation between the

519 methanotrophic community in each sample and the CH_4 data reported in Iverach et al.
520 (2015), nor is there any correlation between the composition of methanotrophs and DO in
521 the groundwater (Supplementary Table S2 online).

522 The sample with the most diverse bacterial community (Sample F, Figure 4) had the
523 most ^{13}C-enriched individual $\delta^{13}C$-CH_4 relative to regional background (Iverach et al.,
524 2015) (Supplementary Table S3 online). A relatively high abundance (11%) of relatives
525 belonging to the Chloroflexi phylum was observed exclusively in this groundwater
526 sample.

527

528 **4.5 Absence of anaerobic methane oxidation**

529 The lack of detection of the *mcrA* gene does not only indicate the absence of methanogens
530 but also suggests the absence of anaerobic methanotrophs (Hallam et al., 2003). Details
531 on the functional genomic link between methanogenic and methanotrophic archaea are
532 discussed comprehensively in Hallam et al. (2003). Additionally, no sequences belonging
533 to ANME-SRB clades were detected in the groundwater samples, indicating the absence
534 of ANME activity. However, members of the phylum Thaumarchaeota dominated the
535 archaeal community in the groundwater (Figure 4). Thaumarchaeota contains several
536 clusters of environmental sequences representing microorganisms with unknown energy
537 metabolism (Pester et al., 2011). Members of the Thaumarchaeota encode
538 monooxygenase-like enzymes able to utilise CH_4, suggestive of a role in CH_4 oxidation
539 (Pester et al., 2011).

540 Samples D and H had SO_4^{2-} concentrations of 55 mg/L and 29 mg/L, respectively.
541 This suggests that the SO_4^{2-}concentration is high enough to support SO_4^{2-}-mediated AOM
542 at these sites (Whiticar, 1999). The observed [SO_4^{2-}] was high enough in these 2 samples
543 to be able to measure the stable isotopes in the SO_4^{2-}. This is useful because the isotopes

544 yield a unique signature when SO_4^{2-} reduction is coupled to CH_4 oxidation in anaerobic
545 conditions (Antler et al., 2015). However, because there are only two data points
546 (Supplementary Table S2 online), determining a correlation between δ^{34}S-SO_4 and δ^{18}O-
547 SO_4 is statistically invalid. The highest relative abundance of methanotrophs was found in
548 samples D and H (Figure 4); however, these methanotrophs are not anaerobic oxidisers
549 and therefore the correlation may not imply causation.

550 The concentration of NO_3^- and NO_2^- in the groundwater was also very low relative
551 to groundwaters with the potential for AOM via denitrification (Nordi and Thamdrup,
552 2014). Our samples had [NO_3^-] ranging from 1.2 mg/L to 2.3 mg/L and [NO_2^-] below 0.05
553 mg/L (Supplementary Table S2 online). Therefore, AOM coupled to denitrification is
554 unlikely to be occurring in the groundwater of the CRAA (Nordi and Thamdrup, 2014).

555 The δ^{13}C-DIC data indicate limited ^{13}C-depletion as a result of DIC formation
556 during AOM. Segarra et al. (2015) showed that maximum ^{13}C-depletion of DIC in the
557 zone of maximum AOM activity (0–3 cm) was highly dependent upon the isotopic
558 composition of the DIC before biological consumption. However, the difference between
559 maximum ^{13}C-depletion of DIC and ^{13}C-enrichment often exceeded 10‰. As our samples
560 are taken from deep in the aquifer (30 m or more below the ground surface), and the
561 difference between our most ^{13}C-depleted DIC value and the most ^{13}C-enriched was only
562 4‰ (Sample H; Supplementary Table SI online), it is unlikely that AOM is occurring in
563 the groundwater. Additionally, a previous study of the GAB geochemistry showed that
564 δ^{13}C-DIC values in this region are in the range -15‰ to -6‰ (Herczeg et al., 1991). All of
565 our samples fall within this regional range, and we see no obvious ^{13}C-depletion of DIC in
566 the groundwater that can be ascribed to AOM.

567 Therefore, any oxidation occurring in the groundwater would have been facilitated
568 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.

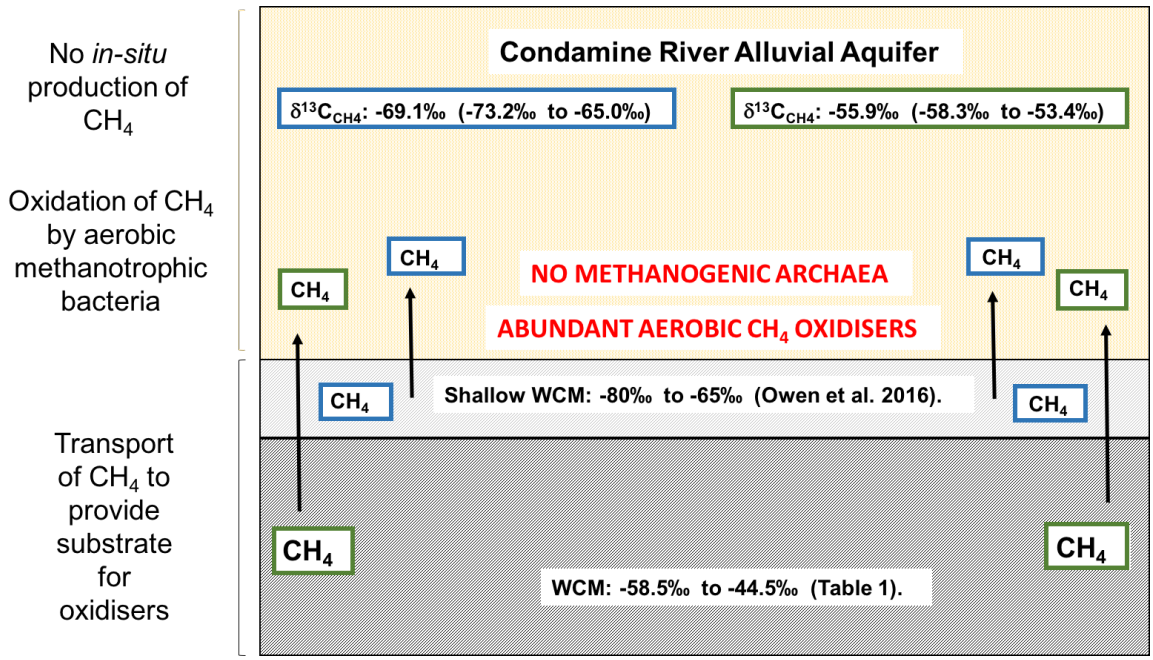


Figure 6. A conceptual schematic of the processes occurring between the WCM and the CRAA.

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*?

585 One hypothesis to explain the presence of CH_4 despite there being no evidence of
586 methanogenesis is that there is localised upward migration of CH_4 from the WCM into the
587 CRAA via natural faults and fractures (Iverach et al., 2015).
588 Our geochemical data and microbiological community analysis both indicate that AOM is
589 not a major oxidation process occurring in the CRAA. However, the microbiological data
590 suggest the presence of aerobic CH_4 oxidisers. Due to the absence of methanogenesis, the
591 oxidation of CH_4 (facilitated by the aerobic methanotrophs present in the groundwater)
592 would require a secondary source of CH_4. This, coupled with the isotopic signature of the
593 CH_4 and the concentration of SO_4^{2-} in the groundwater suggests that the upwards
594 migration of CH_4 from the underlying WCM is the likely source (Figure 6).
595 Methane occurs naturally in groundwater, is produced via numerous biological
596 pathways, and can migrate through natural geological fractures. Therefore, determination
597 of the source of CH_4 using [CH_4] and δ^{13}C-CH_4 data alone doesn't discern all the
598 processes occurring. Our microbiological community analysis showed that there were no
599 methanogens present to produce the CH_4 measured in Iverach et al. (2015), and our
600 geochemical analyses supported the absence of methanogenesis in the alluvial aquifer.
601 Similarly, the geochemical and microbiological data revealed that oxidation may not have
602 as large an effect on the CH_4 due to the low abundance of aerobic oxidisers and the
603 absence of anaerobic archaea.
604 Therefore, we suggest that the CH_4 detected in the CRAA in Iverach et al. (2015) is
605 from the local upward migration of gas from the underlying WCM, either through natural
606 faults and fractures, transport along poorly installed well casings, or direct leakage of gas
607 between the WCM and CRAA where the units are in direct contact. A consideration of
608 both geochemical and microbiological analyses is particularly important in this study area
609 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 were 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.

List of Figures

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).

Figure 2. Geological cross section along A-A' in Figure 1 (adapted from Dafny & Silburn, 2014). KB-Kumbarilla Beds; MRV-Main Range Volcanics.

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* 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.

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Figure 5. (a) A plot of δ^{13}C-DOC vs. δ^{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 δ^{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 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 (Craig, 1961) and LMWL (Hughes and Crawford, 2012) are also displayed.

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

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