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

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Geochemical and microbiological indicators of methane (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. Microbial and geochemical data indicate that there is biogenic CH_4 in the aquifer, but no methanogenic microbial activity. In addition, microbial community analysis showed that aerobic oxidation of CH_4 is occurring. 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; Zhang et al., 2016).

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In groundwater, CH_4 can originate from numerous sources (Barker and Fritz, 1981). The two main sources of CH_4 in shallow groundwater are 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 to 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 ($\delta^{13}C\text{-}DIC$) and dissolved organic carbon ($\delta^{13}C\text{-}DOC$) 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;

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74 Segarra et al., 2015; Sela-Adler et al., 2015; Currell et al., 2016). Other studies have
75 shown that the presence of active methanogenesis can be determined using isotopes of
76 hydrogen in the CH_4 (δ^2H-CH_4), and the surrounding formation water (δ^2H-H_2O)
77 (Schoell, 1980; Whiticar and Faber, 1986; Whiticar, 1999; Currell et al., 2016).
78 Additionally, recent studies have used clumped isotopes of CH_4 and their temperature
79 interpretations to ascribe a thermogenic versus biogenic source in groundwater (Stolper et
80 al., 2014). However, non-equilibrium (kinetic) processes may be responsible for an
81 overestimation of CH_4 formation temperatures (Wang et al., 2015). Therefore, combining
82 geochemistry and microbiology provides a robust method to assess CH_4 origin, as it
83 directly discriminates between microbiological communities involved in either production
84 or degradation processes.

85 Throughout the world the occurrence of freshwater aquifers adjacent to
86 unconventional gas production is common (Osborn et al., 2011; Moore, 2012; Roy and
87 Ryan, 2013; Vidic et al., 2013; Vengosh et al., 2014; Moritz et al., 2015). We have
88 previously shown that there may be local natural connectivity between the WCM and the
89 CRAA (Iverach et al., 2015). Here we show that a combination of geochemical data
90 ($[CH_4]$, $[SO_4^{2-}]$, $[NO_3^-]$, $[NO_2^-]$, $\delta^{13}C$-CH_4, $\delta^{13}C$-DIC, $\delta^{13}C$-DOC and δ^2H-H_2O), as well as
91 characterisation of microbiological communities present, can inform the discussion
92 surrounding the occurrence of CH_4, and its potential for upward migration in the
93 groundwater of the CRAA.
94

95 **1.1 Geochemical indicators of methanogenic processes**

96 Methanogenesis via acetate fermentation (Eq. 1) and carbonate reduction (Eq. 2) can be
97 restricted in groundwater with abundant dissolved SO_4^{2-} (> 19 mg/L) (Whiticar, 1999),

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because sulfate reducing bacteria (SRB) can outcompete methanogenic archaea for reducing equivalents (Lovley et al., 1985).

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

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

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

In addition, the $\delta^{13}C$-CH_4 of the underlying WCM in the study area has been characterised (Papendick et al., 2011; Hamilton et al., 2012; Hamilton et al., 2014). Thus the isotopic signature can be used to identify the potential source of the CH_4, however localised formation and oxidation processes that may occur either in the aquifer or during transport can confound the interpretation of mixing versus oxidation processes.

The isotopic composition of DIC and DOC are also useful indicators of CH_4 processes, as they can be used to determine the occurrence of methanogenesis (Kotelnikova, 2002; Wimmer et al., 2013). Kotelnikova (2002) found that ^{13}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

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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 monooxygenase (McDonald et al., 1995; Murrell et al., 2000). Recently, new groups of aerobic and anaerobic MOB distantly related to known methanotrophic groups have been discovered (Raghoebarsing et al., 2006; Stoecker et al., 2006; Op den Camp et al., 2009). Geochemically, aerobic 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; Knittel et al., 2005). Geochemical indicators can provide evidence for the occurrence of AOM, 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; Nordi and Thamdrup, 2014; Timmers et al., 2015).

2 Study Area

The CRAA is the primary aquifer in the Condamine Catchment (Figure 1). It is used primarily for irrigated agriculture, stock and domestic water supplies. There has been

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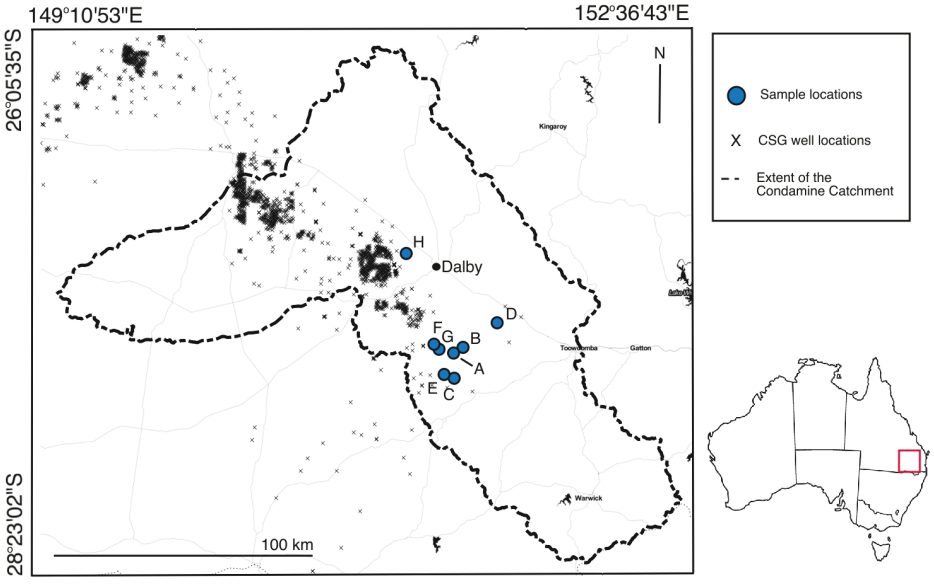
148 increased interest in the presence of CH_4 in the aquifer due to expanding CSG production
149 to the north-west of the study area (Figure 1). CSG production began in 2006 (Arrow
150 Energy, 2015) and has been expanding in the decade since then. This has raised concerns
151 regarding the quality and quantity of the groundwater in the CRAA.
152



153

154 **Figure 1.** Site map showing the extent of the study area and sample locations within the Condamine
155 Catchment, south-east Queensland, Australia. Map created in QGIS; data and imagery: Statem Toner, Open
156 Street Map and contributors, CC-BY-SA (QGIS, 2015). Modified with Corel Painter 2015 (Corel
157 Corporation, 2015).
158

159 **2.1 Hydrogeological setting**

160 The CRAA sits within the Surat Basin, which sits within the Great Artesian Basin (GAB)
161 in south-east Qld, Australia (Figure 1). Aquifers in the GAB vary between semi-confined
162 and confined (Kelly and Merrick, 2007; Dafny and Silburn, 2014).

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The environment of deposition for the Surat Basin was fluvio-lacustrine in the late Triassic-Jurassic and shallow marine and coastal in the Cretaceous (Hamilton et al., 2012). The middle-Jurassic WCM are a group of low-rank coal seams in the Surat Basin targeted for CSG production (Hamilton et al., 2012). The WCM are thicker (150 m to 350 m) along the western margin of the CRAA and thin to around 50 m in the east, where the unit outcrops (KCB, 2011), however, only around 10 % of this is coal. The unit consists of very fine- to medium-grained sandstone, siltstone, mudstone and coal, with minor calcareous sandstone, impure limestone and ironstone (KCB, 2011). The coal consists of numerous discontinuous thin lenses separated by sediments of low permeability (Hillier, 2010). The unit dips gently to the west (around 4^{o}), which is consistent with the general trend of the Surat Basin in this region.

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

The unconfined CRAA fills a paleovalley that was carved through the GAB (including the WCM). The valley-filling sediments are composed of gravels and fine- to course-grained channel sands interbedded with floodplain clays and, on the margins, colluvial deposits, which were deposited from the mid-Miocene to the present (Kelly and Merrick, 2007; Kelly et al., 2014). The valley-filling sediments have a maximum thickness of 134 m near Dalby (Dafny and Silburn, 2014). Along the eastern margin of the valley, the CRAA is bounded by the Main Range Volcanics. Estimations of the sources and quantity of recharge to the CRAA vary widely, however streambed recharge is generally considered to be the major source of freshwater to the aquifer (Dafny and Silburn, 2014).

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A low permeability layer (ranging from 8 x 10^{-6} to 1.5 x 10^{-1} m/d) has been reported between the CRAA and the underlying WCM (KCB, 2011; QWC, 2012). This has been referred to as the 'transition layer' (QWC, 2012) or a 'hydraulic basement' to the alluvium (KCB, 2011). However, the thickness of this layer varies between 30 m thick in some areas to completely absent in others. Thus, in some places the WCM immediately underlies the CRAA (Dafny and Silburn, 2014). This suggests that there is some level of connectivity between the CRAA and the WCM. Huxley (1982) and Hillier (2010) both suggest that the general decline in water quality downstream is due to some net flow of the more saline WCM water into the CRAA. Connectivity between the formations is not well understood; however, studies have been conducted to better understand the movement of both water and gas between the two aquifers. Duvert et al. (2015) and Owen and Cox (2015) both used hydrogeochemical analyses to show that there was limited movement of water between the two formations. However, Iverach et al. (2015) used the isotopic signature of CH_4 in the groundwater to show that there was localised movement of gas between the coal measures and the overlying aquifer. This research provides additional insight to inform the debate about the degree of connectivity between the WCM and the CRAA. The microbiological insights also inform the global research on biological CH_4 production and degradation in alluvial aquifers, in particular for zones distal to the river corridor.

3 Methods

From 22 January 2014 to 31 January 2014 we collected groundwater samples for geochemical analysis from 8 private irrigation boreholes in the Condamine Catchment. Iverach et al. (2015) outlines the complete methods for sample collection for [CH_4] and

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211 $\delta^{13}C\text{-}CH_4$ and subsequent analysis. The 8 samples collected from the unconfined CRAA
212 are representative of the aquifer, given their varied depths and locations.

213 Groundwater samples were collected by installing a sampling tube 2 m inside the
214 pump outlet of the borehole to avoid the air-water interface at the sampling point. Field
215 parameters (electrical conductivity (EC), oxidation-reduction potential (ORP), dissolved
216 oxygen (DO), temperature (T) and pH) were monitored in a flow cell to ensure
217 stabilisation before samples were collected. The boreholes had been pumping
218 continuously over the preceding month for irrigation and so stabilisation of the field
219 parameters was reached within minutes. Groundwater samples for major anions and
220 water-stable isotopes ($\delta^2H\text{-}H_2O$ and $\delta^{18}O\text{-}H_2O$) were collected after passing the water
221 through a 0.45 μm, high-volume groundwater filter, which was connected to the pump
222 outlet. Groundwater for anions and water stable-isotopes were stored in 125 mL high-
223 density polyethylene (HDPE) bottles and 30 mL HDPE bottles, respectively. Both had no
224 further treatment. The water for $\delta^{13}C$-DIC and $\delta^{13}C$-DOC was further filtered through a
225 0.22 μm filter and stored in 12 mL Exetainer vials and 60 mL HDPE bottles, respectively.
226 Samples to be analysed for DIC were refrigerated at 4 ^oC and samples to be analysed for
227 DOC were frozen within 12 hours of collection.

228 Groundwater samples for the microbiological analyses were collected between 8
229 December 2014 to 11 December 2014, and were collected from the same 8 private
230 irrigation boreholes used for the geochemical analyses. Groundwater samples for
231 microbiological analysis were collected in 2 L Duran Schott bottles and sealed (gas tight).
232 The groundwater was filtered through a 0.2 μm filter (Merck Millipore). We use aspects
233 of the geochemical data collected in the January campaign to inform our interpretation of
234 the microbial results from the December campaign.
235

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3.1 Geochemical analyses

The major ion chemistry in the groundwater samples was analysed at the Australian Nuclear Science and Technology Organisation (ANSTO) using Inductively Coupled Plasma - Ion Chromatography for anions. The samples for δ^2H-H_2O and δ^{18}O-H_2O were analysed at ANSTO and are reported as ‰ 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 method and δ^2H samples were run using an on-line combustion, dual-inlet IRMS method.

The isotopes of carbon in DIC were analysed at ANSTO using an established method on a Delta V Advantage mass spectrometer, and a GasBench II peripheral. The results are reported as a ‰ deviation from IAEA secondary standards that have been certified relative to V-PDB for carbon. The isotopes of carbon in DOC were analysed at UC-Davis Stable Isotope Facility and results are reported as ‰ and are corrected based on laboratory standards calibrated against NIST Standard Reference Materials with an analytical precision of ± 0.6‰. Samples were run using a total organic carbon (TOC) analyser connected to a PDZ Europa 20-20 IRMS using a GD-100 Gas Trap interface. The [SO_4^{2-}] were too low in 6 of the 8 samples for δ^{34}S and δ^{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 a Continuous Flow-Isotope Ratio Mass Spectrometry (CF-EA-IRMS) with an elemental analyser interfaced to a VG PRISM II mass spectrometer. The results are reported against V-CDT (Vienna Cañon Diablo Troilite). The oxygen isotope ratio was determined using a high temperature reactor coupled to an isotope ratio mass spectrometer in continuous flow mode.

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3.2 DNA extraction and Illumina sequencing

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

926F_Illum(5'-
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[AAACTYAAAKGAATTGRC
CG]-3'),

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

This universal primer pair targets the V6-V8 hyper-variable regions of the 16S ribosomal RNA gene and has been shown to capture the microbial diversity of Bacteria and Archaea in a single reaction (Wilkins et al., 2013). PCR products were purified using a magnetic bead capture kit from Agencourt AMPure XP beads (Beckman Coulter) and quantified using a fluorometric kit (RiboGreen, Qubit Assay Kit, Invitrogen). Purified amplicons

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were subjected to the Index PCR using the MiSeq platform (Ramaciotti Centre for Genomics, UNSW Australia) according to the manufacturer's specifications. Illumina sequences were checked for quality (FastQC, BaseSpace) and analysed using the BaseSpace cloud computing platform (Illumina, 2016) and MOTHUR (Schloss, 2009) with modified protocols (Schloss et al., 2009; Kozich et al., 2013). Taxonomy was assigned against the SILVA Database (Silva, 2016). To ensure even sampling depth for subsequent analyses, OTU abundance data were rarefied to the lowest number of sequences for a sample (8,300 sequences per sample).

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

Quantitative real-time PCR was used to determine abundances of bacterial and archaeal 16S rRNA gene targets and functional gene targets (*mcrA*, *pmoA*, *mmoX*, and *dsrA*), using the MJ Mini^{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). QPCRs were performed as described previously by Wilms et al. (2007). *PmoA* qPCR was performed using the *pmoA* primer-pair A189F (Holmes et al., 1999) and mb661R (Kolb et al., 2003) with a final total concentration of 0.8 µM. The qPCR programme for the amplification was performed as follows: 95ºC for 3 min followed by 40 cycles of 95ºC for 30 s, 64ºC for 45 s and 68ºC for 45 s. The *mmoX* gene fragment was quantified using the prime pairs mmoX-ms-945f

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and mmoXB-1401b at a final concentration of 0.8 μM. The qPCR conditions for the *mmoX* was as follows: 94ºC for 3 min followed by 45 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min. Bacterial and archaeal targets were measured in at least three different dilutions of DNA extracts (1:10, 1:100, 1:1000) and in triplicate. PCR products were checked by gel electrophoresis, using 2 % (w/v) agarose with TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM 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*^{T} (DSM 800), *Desulfovibrio vulgaris*^{T} (DSM 644), *Methylosinus sporium*^{T} (DSM 17706), and *Methylocella silvestris*^{T} (DSM 15510; DZMZ 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 8 private irrigation boreholes studied here (samples A-H) (Supplementary Table S3 online). These measurements were understood to have been mixing with regional background atmospheric CH_4 (1.774 ppm; -47‰) and therefore

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335 mixing plots were used to infer the isotopic source signature of the CH_4 off-gassing from
336 the groundwater. Iverach et al. (2015) found that samples E, G, and H plotted on a
337 regression line that had an isotopic source signature of -69.1‰ (90% CI, −73.2‰ to
338 −65.0‰), indicative of a biological source. However, samples A, B, C, D and F plotted on
339 a regression line that had an isotopic source signature of -55.9‰ (90% CI, −58.3‰ to
340 −53.4‰), suggesting either oxidation was occurring at the source or there was potential
341 upward migration of CH_4 from the underlying WCM.
342
343 **4.2 Limited geochemical and microbiological potential for methanogenesis in the**
344 **groundwater**
345 To further elucidate the source of the CH_4 reported in the groundwater (Iverach et al.,
346 2015), Illumina sequencing and quantitative real-time PCR (qPCR) was used to target
347 bacterial and archaeal 16S rRNA genes, as well as specific functional genes (*mcrA*, *pmoA*,
348 *mmoX* and *dsrA*) associated with CH_4 metabolism. Microbial abundances estimated by
349 SYBR Green I counts were between 10^3 and 10^5 cells/mL throughout all groundwater
350 samples (Figure 2). This was congruent with the qPCR data observed for bacterial and
351 archaeal cell concentrations.

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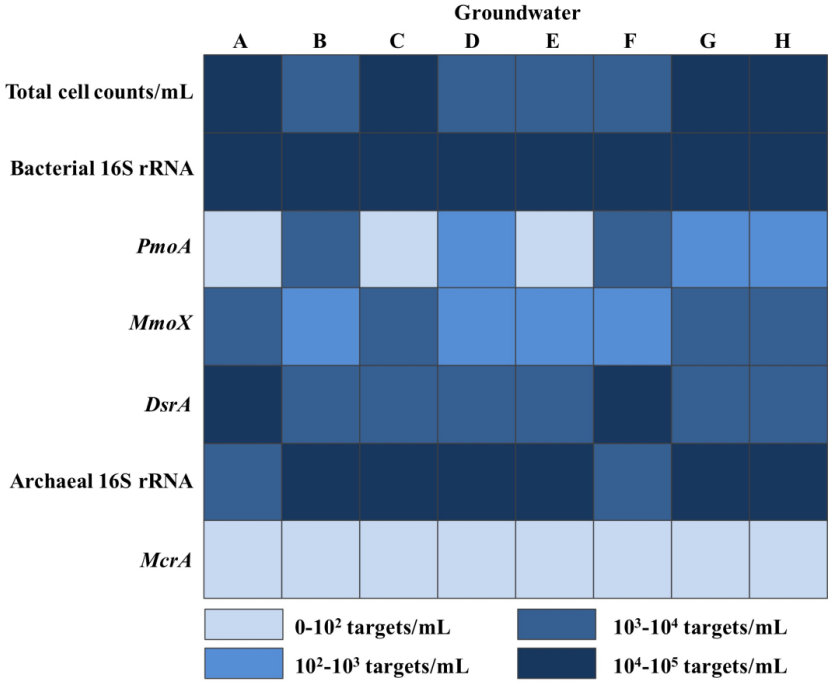

Figure 2. Total cell concentration and copy number abundances of bacterial and archaeal 16SrRNA genes and functional key genes for aerobic CH_4 oxidation (*pmoA* and *mmoX* genes), CH_4 production (*mcrA* gene) and sulfate reduction (*dsrA* gene) in the groundwater carried out by quantitative (q)PCR. Low abundances are highlighted in light blue. High abundances are highlighted in dark blue.

The groundwater community was primarily composed of bacteria (79 to 90 %), whilst archaea made up 10 to 21 % (Figure 3). The bacterial and archaeal community composition did not vary significantly between groundwater samples. Most of the bacterial sequences belonged to the phyla Proteobacteria (α-δ), Acidobacteria, Actinobacteria, Firmicutes and the Bacteroidetes/Chlorobi group (Figure 3). The phylum Thaumarchaeota dominated the archaeal communities with a relative abundance of 81 to 99 %, while Crenarchaeota made up 1 to 3 % of the archaeal community. Further sequences were related to other (if < 1 % relative abundance) and unclassified Bacteria and Archaea. No members of the Euryarchaeota, comprising the methanogenic archaea,

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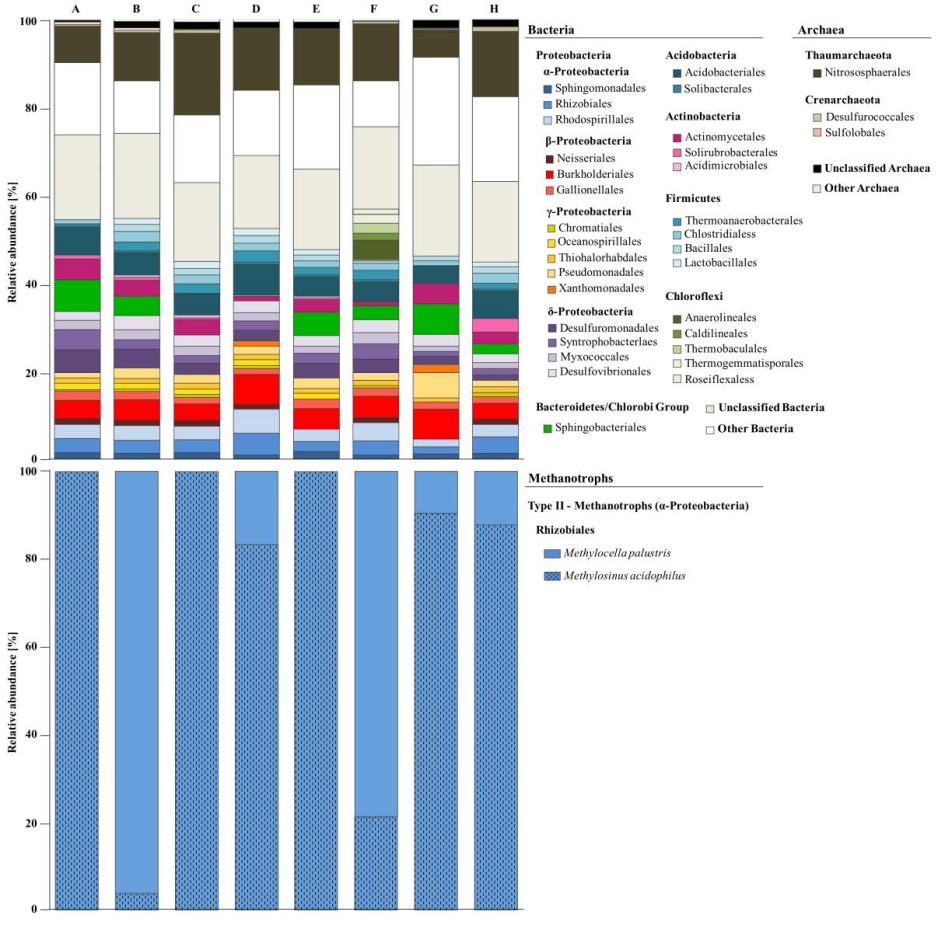
367 were observed. The archaeal *mcrA* gene, which encodes the methyl coenzyme M
368 reductase, was not detected in any of the groundwater samples (detection limit < 10
369 cells/mL; Figure 2). This was consistent with the Illumina sequencing results, and
370 suggests that the CH_4 observed off-gassing from the groundwater was not being produced
371 locally.



372

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

375

376 Our geochemical data also showed no evidence for the occurrence of
377 methanogenesis in the groundwater. As previously stated, a ^{13}C-enrichment in $\delta^{13}C$-DIC

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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 4a), 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 4b; Supplementary Table S1 online), it is evident that there is no noticeable δ^{2}H-enrichment that can be ascribed to methanogenesis in any of the groundwater samples (Cendón et al., 2015).

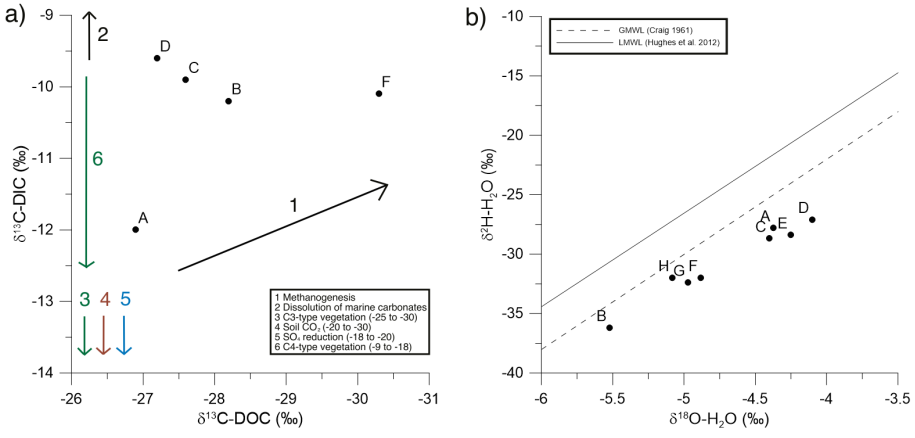

Figure 4. (a) A plot of δ^{13}C-DOC vs. δ^{13}C-DIC, highlighting the absence of correlation between these geochemical data, indicating that there is no methanogenic end member in our samples. Samples E, G and H are omitted because they were below the detection limit for δ^{13}C-DOC (Supplementary Table 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.

These geochemical analyses, along with the lack of classified methanogens, suggest that biogenic CH_4 production is not one of the major processes producing CH_4 in the CRAA. Therefore, the CH_4 reported in all samples in Iverach et al. (2015) must be

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398 coming from another source. We propose that the upward migration of CH_4 from the
399 WCM must be considered as the potential source. The isotopic signature of CH_4 from the
400 deeper coal measures has been characterised between -58.5‰ and -45.3‰, indicating
401 thermogenic CH_4 with a secondary biogenic component (Papendick et al., 2011; Hamilton
402 et al., 2012; Hamilton et al., 2014). Five of the 8 samples analysed in this study have an
403 isotopic source signature within this range, as reported in Iverach et al. (2015). This
404 implies that upward migration from the deeper WCM is the source of the CH_4 detected in
405 the groundwater.

406 However, the remaining 3 samples (samples E, G, and H) have a typically biogenic
407 isotopic source signature (-69.1‰). This could be the result of the replacement of
408 typically thermogenic gas in the shallow WCM by biogenic gas (Faiz and Hendry, 2006).
409 Thus, these three sites are potentially sourcing biogenic CH_4 from the shallow WCM,
410 resulting in a biological source signature despite the absence of methanogens in the
411 overlying aquifer.

412

413 **4.3 Sulfate reducers and aerobic methanotrophs potentially outcompete**
414 **methanogens**

415 Sulfate concentrations in most groundwater samples were low (3.2 mg/L to 11 mg/L)
416 (Supplementary Table S2 online). Groundwater samples D and H were higher with 55
417 mg/L and 29 mg/L, respectively (Supplementary Table S2 online). Sequence and
418 functional *dsrA* gene analysis (encoding the dissimilatory sulfite reductase of SRB)
419 revealed that SRB are present in all groundwater samples at relatively high abundances (5
420 - 10 % of the overall microbial community; Figures 2 and 3). These SRB are potentially
421 outcompeting methanogenic archaea for substrates such as acetate and H_2. Sulfate
422 concentrations higher than 3 mg/L, as detected in all groundwater samples (3.2 mg/L – 55

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mg/L), could potentially create a SO_4^{2-} -reducing environment with the predominance of SRB over methanogens. This would maintain the acetate at concentrations too low for methanogens to grow (Lovley et al., 1985). Deltaproteobacteria were dominant in all groundwater samples, and most of the sequences were closely related to acetate-oxidising, sulfate/sulfur-reducing bacteria (*Desulfovibrionales*, *Syntrophobacterales*, *Desulfuromonadales*; Figure 3). Additionally, *Methylocella* spp. are capable of using methanogenic substrates, such as acetate and methylamines, for their metabolism and therefore are not limited to growing on one-carbon compounds such as CH_4 (Dedysh et al., 2005). This could have major implications for the lack of methanogenic activity in the groundwater.

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

The functional gene for aerobic CH_4 oxidation (*pmoA*) was detected at relatively high concentrations (7.9×10^2 - 9.3×10^3 targets/mL) compared to the overall bacterial 16S rRNA concentration (2.5×10^4 - 5.1×10^4 targets/mL) (Figure 2). All groundwater samples were characterised with regard to the community structure of MOB. The samples harboured a low-diversity methanotrophic community associated with the order *Rhizobiales* (α-Proteobacteria), however MOB accounted for up to 7 % of the overall microbial community (Figure 3). All groundwater samples were dominated by two MOB, belonging to the type II methanotrophs (Figure 3). Five samples had both *Methylocella palustris* (family *Beijerinckiaceae*) and *Methylosinus acidophilus* (family *Methylocystaceae*) (samples B, D, F-H), whilst the remaining samples comprised *Methylosinus acidophilus* only (samples A, C and E) (Figure 3). These genera were characterised as aerobic CH_4 oxidisers, however aerobic MOB have been previously

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448 observed in micro-aerophilic and anaerobic environments (Bowman, 2000). This suggests
449 the existence of an alternative pathway for aerobic CH_4 oxidation in a suboxic/anaerobic
450 environment. Both species have previously been found and isolated from a variety of
451 freshwater habitats and *Methylosinus* spp. are known to be dominant methanotrophic
452 populations in groundwater (Bowman, 2000). *Methylocella* and *Methylosinus* spp. possess
453 a soluble CH_4 monooxygenase (*mmoX*) (McDonald et al., 1995; Murrell et al., 2000),
454 which is consistent with the high abundance of the *mmoX* gene targeted in all
455 groundwater samples (Figure 2). Interestingly, no *pmoA* gene, a biomarker for all MOBs,
456 has previously been detected in known *Methylosinus* spp. (Dedysh et al., 2005). This is
457 supported by our data, which shows the sole predominance of *mmoX* genes in 3 of the 8
458 groundwater samples that are exclusively dominated by *Methylosinus* sp. (samples A, C,
459 and E) (Figures 2 and 3).

460 In addition to low concentrations of CH_4 reported in Iverach et al. (2015), the
461 dissolved O_2 (DO) in our groundwater samples had a large range, from low to close to
462 saturation (0.91 mg/L to 8.6 mg/L). *Methylocella* spp. are not associated with the
463 previously known type II cluster of methanotrophs, but are closely related to a non-
464 methanotroph (Dedysh et al., 2005) suggesting different affinities to CH_4 and O_2,
465 compared to previously known type II methanotrophs (Amaral and Knowles, 1995).
466 There is no correlation between the methanotrophic community in each sample and the
467 CH_4 data reported in Iverach et al. (2015), nor is there any correlation between the
468 composition of methanotrophs and DO in the groundwater (Supplementary Table S2
469 online).

470 The sample with the most diverse bacterial community (Sample F; Figure 3) had the
471 most ^{13}C-enriched individual δ^{13}C-CH_4 relative to regional background (Iverach et al.,
472 2015) (Supplementary Table S3 online). A relatively high abundance (11 %) of relatives

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belonging to the Chloroflexi phylum was observed exclusively in this groundwater sample. This suggests that there are potential metabolic processes involved, such as the microbial conversion of denitrification products to nitrogen and oxygen, that are able to gain oxygen to facilitate the oxidation of CH_4 (Ettwig et al., 2010).

4.5 Absence of AOM

The lack of detection of the *mcrA* gene does not only indicate the absence of methanogens but also suggests the absence of anaerobic methanotrophs (Hallam et al., 2003). Details on the functional genomic link between methanogenic and methanotrophic archaea are discussed comprehensively in Hallam et al. (2003). Additionally, no sequences belonging to ANME-SRB clades were detected in the groundwater samples, indicating the absence of ANME activity. However, members of the phylum Thaumarchaeota dominated the archaeal community in the groundwater (Figure 3). Thaumarchaeota contains several clusters of environmental sequences representing microorganisms with unknown energy metabolism (Pester et al., 2011). Members of the Thaumarchaeota encode monooxygenase-like enzymes able to utilise CH_4, suggestive of a role in CH_4 oxidation.

Samples D and H had SO_4^{2-} concentrations of 55 mg/L and 29 mg/L, respectively. This suggests that the SO_4^{2-}concentration is high enough to support SO_4^{2-} -mediated AOM at these sites (Whiticar, 1999). The observed [SO_4^{2-}] was high enough in these 2 samples to be able to measure the stable isotopes in the SO_4^{2-}. This is useful because the isotopes yield a unique signature when SO_4^{2-} reduction is coupled to CH_4 oxidation in anaerobic conditions (Antler et al., 2015). However, because there are only two data points (Supplementary Table S2 online), determining a correlation between δ^{34}S-SO_4 and δ^{18}O-SO_4 is statistically invalid. The highest relative abundance of methanotrophs was found in

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497 samples D and H (Figure 3); however, these methanotrophs are not anaerobic oxidisers
498 and therefore the correlation may not imply causation.

499 The concentration of NO_3^- and NO_2^- in the groundwater was also very low, with
500 [NO_3^-] ranging from 1.2 mg/L to 2.3 mg/L and for all samples NO_2^- was below 0.05 mg/L
501 (Supplementary Table S2 online). Therefore, AOM coupled to denitrification is unlikely
502 to be occurring in the groundwater of the CRAA (Nordi and Thamdrup, 2014).

503 The δ^{13}C-DIC data indicates limited ^{13}C-depletion as a result of DIC formation
504 during AOM. Segarra et al. (2015) showed that maximum ^{13}C-depletion of DIC in the
505 zone of maximum AOM activity (0 – 3 cm) was highly dependent upon the isotopic
506 composition of the DIC before biological consumption. However, the difference between
507 maximum ^{13}C-depletion of DIC and ^{13}C-enrichment often exceeded 10‰. As our samples
508 are taken from deep in the aquifer (30 m or more below the ground surface), and the
509 difference between our most ^{13}C-depleted DIC value and the most ^{13}C-enriched was only
510 4‰ (Sample H; Supplementary Table SI online) it is unlikely that AOM is occurring in
511 the groundwater. Additionally, a previous study of the GAB geochemistry showed that
512 δ^{13}C-DIC values in this region are in the range -15‰ to -6‰ (Herczeg et al., 1991). All of
513 our samples fall within this regional range, and we see no obvious ^{13}C-depletion of DIC in
514 the groundwater that can be ascribed to AOM.

515 Therefore, any oxidation occurring in the groundwater would have been facilitated
516 by the two members of type II methanotrophs that we identified in the microbial
517 community analysis. Both of the species identified are classified aerobic CH_4 oxidisers,
518 agreeing with our geochemical data that no anaerobic oxidation was occurring. Despite
519 abundant SO_4^{2-} in 2 sample locations, the absence of anaerobic methanotrophic archaea
520 amongst other geochemical evidence (denitrification processes) suggests that it is unlikely
521 that AOM is occurring within the aquifer.

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522

523 **5 Conclusion**

524 We used geochemical and microbiological indicators to explain the occurrence of CH_4 in
525 the groundwater of an alluvial aquifer. Microbial community analysis and geochemical
526 data were consistent with respect to a lack of methanogenic archaea and methanogenic
527 activity in the aquifer. What is the original source of the CH_4 if not biologically produced
528 *in-situ*? One hypothesis to explain the presence of CH_4 despite there being no evidence of
529 methanogenesis is that there is localised upward migration of CH_4 from the WCM into the
530 CRAA via natural faults and fractures (Iverach et al., 2015).

531 Our geochemical data and microbiological community analysis both indicate that
532 AOM is not a major oxidation process occurring in the CRAA. However, the
533 microbiological data suggest the presence of aerobic CH_4 oxidisers. Due to the absence of
534 methanogenesis, the oxidation of CH_4 (facilitated by the aerobic methanotrophs present in
535 the groundwater) would require a secondary source of CH_4. The upwards migration of
536 CH_4 from the underlying WCM is the likely source.

537 Methane occurs naturally in groundwater, is produced via numerous biological
538 pathways, and can migrate through natural geological fractures. Therefore, determination
539 of the source of CH_4 using [CH_4] and δ^{13}C-CH_4 data alone doesn't discern all the
540 processes occurring. Our microbiological community analysis showed that there were no
541 methanogens present to produce the CH_4 measured in Iverach et al. (2015) and our
542 geochemical analyses supported the absence of methanogenesis in the alluvial aquifer.
543 Similarly, the geochemical and microbiological data revealed that oxidation may not have
544 as large an effect on the CH_4 due to the low abundance of aerobic oxidisers and the
545 absence of anaerobic archaea.

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Therefore, we suggest that the CH_4 detected in the CRAA in Iverach et al. (2015) is from the local upward migration of gas from the underlying WCM, through natural faults and fractures. A consideration of both geochemical and microbiological analyses is particularly important in this study area because of the immediate proximity of the underlying WCM and the proximity of the study area to CSG production. This research uses biogeochemical constraints on the origin of CH_4 in a freshwater aquifer to demonstrate the upward migration of CH_4 from an underlying coal seam.

Author Contributions

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

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Competing Interests

The authors declare that they have no conflict of interest.

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. 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.

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

Figure 4. (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.

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