All line numbers refer to the marked-up pdf manuscript at the end of both reviewer comments.

We thank the reviewer for their time and constructive comments on our manuscript. We have addressed all concerns raised below.

M. Currell (Referee)

Iverach et al. present a novel approach to the determination of methane sources in shallow groundwater in the Condamine Alluvium aquifer, Australia. I think the study is of high scientific significance, for two main reasons:

1. The use of combined geochemical and microbiological indicators to study the origins of methane in groundwater is novel. Studies of this kind are relatively rare in the literature, and the microbiological analysis provide insight about the methane sources and degradation processes that couldn't otherwise be gained from the isotopic analyses alone
2. The topic and research question(s) are of high importance, given the current debate about environmental impacts of coal seam gas (and other unconventional gas), both in this particular area of Australia, and worldwide.

There are some minor issues and corrections needed, and some areas where additional information could be included to make the paper more solid. However, overall I think this is a high quality manuscript.

Specific comments

Abstract Line 33-34: Which data? I like to see some actual data values or description of the particular aspects of the data set of greatest significance (and supporting the conclusions described) included in the abstract. If more space is needed in order to do this, I suggest removing the second sentence of the abstract, as this is background information that can be included in the introduction.
Lines 34-39: A description of the particular data that provide the greatest significance (no methanogenesis *in-situ*) has been included in the abstract. We mention the isotopes of DIC and DOC and the concentration of SO_4^{2-} as being the pertinent geochemical data, and the absence of methanogenic archaea being the important microbial data presented to support the conclusions in the manuscript.

Introduction Line 50: I suggest adding the term 'in situ' when discussing biological production of methane in the shallow groundwater. This makes it clear that you are distinguishing two different potential gas sources- one produced in the shallow aquifer itself, and another whereby gas from another unit has migrated to the aquifer.
Line 61: We have added 'in situ' when discussing biological production of methane in the shallow groundwater.

Line 81: 'Therefore' is not really the best word here. It does not follow logically from the preceding discussion that combining geochemistry/microbiology can discriminate the relevant processes; rather you could say that microbiological indicators have the potential to resolve some of the uncertainties just mentioned (e.g. methanogenesis and methane degradation processes), that can't be otherwise determined on the basis of geochemical data alone. Here you could also note the general absence of published studies which have

combined geochemical and microbiological indicators to look at methane sources and degradation in an applied setting (an important point to make in your introduction).
Lines 93-95 and 97-99: 'Therefore' has been removed and sentence has been rewritten following the suggestion above. We have also mentioned that there are no studies using geochemical and microbiological indicators to assess CH_4 production and degradation processes in a freshwater aquifer and that this study aims to fill this gap in the literature.

Line 103: See previous comment; this could be clarified by adding 'in situ methanogenesis' to distinguish from gas migration from another unit.
Line 122: As above, the term 'in situ' has now been added.

Line 104-108: I think you should expand this paragraph and include some of the actual data, e.g. the observed ranges and mean/median values of d13CCH4 and d13CDIC found in the WCM from other published studies. This can be included in the text (e.g. ranges, mean values etc), as well as in a table. This would help to strengthen your isotopic lines of evidence to support the hypothesised migration mechanism later in the manuscript. Note that Baublys et al 2015 (Int. J. Coal Geol v.147-8, pp85-104) have also reported extensive data on isotopic composition of gases and water in the WCM, which should be included along with other recent published studies.
Lines 124-130: This paragraph has been expanded to include some actual data reported for the WCM. Data ranges have been provided in text as well as in a table. Isotopes for DIC weren't available for all of the studies, but included where possible. Baublys et al. 2015 has been added to the references here.

Study area Line 146-47: Try to avoid repetition (primary/primarily)
Line 178: Primarily has been removed from the second sentence.

Line 151: Suggest adding 'including methane concentrations' at the end of this sentence, to highlight the significance of what you are looking at (mostly the methane in groundwater).
Line 185: We have added 'especially with respect to CH_4 concentrations' after groundwater quality to highlight that it is the methane in groundwater that we are concerned with.

2.1 Hydrogeological setting. Could you include a cross section or at least a stratigraphic column to go with your description of the geological units?
Line 244: We have included a cross section to go with the description and provided a reference to the literature.

Line 160: 'The CRAA sits within the Surat Basin, which is a major sub-province of the Great Artesian Basin'. Perhaps refer to one of the Geoscience Australia and/or CSIRO hydrogeology reports on the GAB (e.g. Ransley and Smerdon, 2012).
Line 195: We have now referenced the abovementioned report, as well as the extensive work by Radke et al. 2000 on the hydrodynamics and hydrochemistry of the GAB (Radke et al. Hydrochemistry and implied hydrodynamics of the Cadna-owie Hooray Aquifer Great Artesian Basin, 2000).

Line 188: The recent studies by the Office of Groundwater Impact Assessment (OGIA) may have more detail about the connectivity between the CRAA and the WCM and the extent of the aquitard(s), e.g. the Surat Underground water impact report (OGIA, 2016).
Lines 248-254: This reference has been included in the connectivity section of the hydrogeology, with a sentence explaining their more recent findings on the connectivity

between the WCM and the CRAA.

Line 203: Connectivity for gas? water? both?
Line 255-256: This has been clarified in the manuscript. It is connectivity for both gas and water.

Method Line 212: Here you should refer to a figure and/or table which includes your sample depths and locations
Line 263 and 266: Figure 1 has been referred to in the methods for the locations of the samples and a small table has now been included to show the slotted interval depth of each bore that was sampled.

Line 233-34: Were the physico-chemical parameters (EC, pH, DO) monitored during the second round of sampling? If so, you could report these and use as evidence that the water composition between the two sampling events did not change substantially (if this is true).
Unfortunately, the physico-chemical parameters were not monitored during the second round of sampling. However, thirty years of studies have shown that the groundwater chemistry has remained fairly consistent (Huxley 1982).

Line 238-239: What about cations?
Line 295: Our groundwater samples were analysed for cations, however we don't use any cation data in this manuscript. For completeness, we have now added the analysis method that the groundwater underwent for cations.

Line 242-243: Can you refer to a published paper where the same method was used? Same for the DIC isotopes (line 245).
Line 317-333: Published papers have now been referred to for all of the analytical techniques used for the geochemical analyses.

Results & Discussion Line 371: Suggest writing 'in situ within the CRAA' instead of 'locally' to be clearer.
Line 491: This has been changed.

Line 377: Do you mean the major ion data? Which particular aspects (e.g. sulfate and nitrate concentration data)?
Line 508: At this point the discussion is just on the DIC and DOC isotopic data. The beginning of the sentence has been changed to "Our isotopic geochemical data" to make it clearer.

Line 396: Suggest changing to: 'major processes resulting in CH4 in the CRAA' rather than 'producing CH4 in the CRAA' (or you could say 'responsible for the presence of CH4').
Line 532-533: This has been changed.

Line 398: Suggest changing 'coming from' to 'derived from'.
Line 534: This has been changed.

Line 406-411: This paragraph is a bit confusing and needs re-writing. Is the gas in the WCM really 'typically thermogenic'? All of the isotopic data for 13CCH4 I have seen for gases and water in the WCM indicates a bacterial source of methane (e.g. 13CCH4 values around -50permil) rather than thermogenic (which should have values higher than -40permil). Is there

anything else distinctive about the samples with more depleted 13CCH4, such as a much lower CH4 concentrations or differences in the major ions that could explain the isotopic difference?
Lines 547-553: Stating that the gas from the WCM was thermogenic was a large oversight and this sentence has now actually been completely removed in the re-write of the paragraph. A new reference that was published after this manuscript was originally submitted has been added (Owen et al., 2016). This paper describes an isotopic signature for a 'shallow WCM' – a unit between the WCM 'gas reservoir' and the overlying alluvium. This signature is between -80permil and -65permil. Therefore, the -69.1permil that these three samples exhibit (despite no methanogens) could be a result of CH_4 from this 'shallow WCM', rather than the deeper 'gas reservoir'. This is discussed in text now.

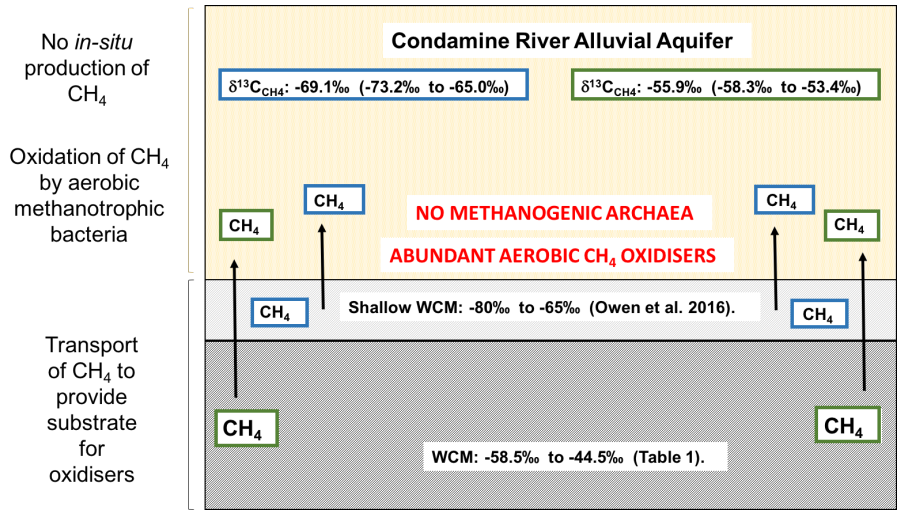
Line 431-432: Yes, and further, the evidence about the presence of sulfate and conditions favouring SRB is a further line of evidence that in situ methanogenesis is unlikely to be responsible for the CH4 in the shallow aquifer
Line 588-590: This further line of evidence has been included to strengthen the manuscript.

Line 434 - 476: The section on methane oxidation is insightful; good use of the microbiological methods to combine with the isotopic data and yield some new insights.
Thank you.

Line 478: Use the full name for AOM in the title.
Line 654: The full name for AOM is now used in the title

Line 499-500: Relative to what? Other water in the CRAA?
Lines 687-689: It was relative to groundwaters that have the potential for AOM to occur via denitrification. This has been clarified in text with the appropriate reference.

General comment I think including a figure showing your isotopic compositions (13CCH4) and concentrations of methane, (using the data from Iverach 2015) and comparing with other published data on isotopic characteristics of WCM gases would be helpful, to strengthen the evidence for the proposed hypothesis (together with the microbiological indicators).
Line 721: A conceptual figure has been included that highlights that there is no *in situ* CH_4 production in the aquifer, there is the presence of CH_4 in the aquifer and there are abundant CH_4 oxidisers in the aquifer. Hence, there is CH_4 migrating upwards to provide the substrate for those oxidisers. Isotopic signatures from the literature provided for the WCM, as well as the signature for the more depleted shallow WCM and measured isotopic signatures for the CRAA (from Iverach et al. 2015) have been included.



Conclusions Line 536: You could also note your other lines of evidence here (e.g., that this is supported by the co-existence of CH4 with sulfate in the groundwater, and the isotopic composition of the methane).
Lines 740-741: The isotopic signature of CH_4 and the concentration of SO_4 have been added as further evidence (on top of the microbial data) that methane is being oxidised (hence needs a source to oxidise) and is not being produced *in-situ*.

Line 547-548: Your study does not really provide information about the precise pathway(s) by which methane migrates from the WCM to the CRAA, only strong evidence that such migration occurs. Hence, the statement about 'through natural faults and fractures' is really just speculation. Unless you can support it with some geological evidence, other mechanisms may also be responsible (such as transport along wells that are not fully sealed, direct leakage of gas between the units where the aquitard is absent). I suggest either talking about all possible path ways (including these), or simply leaving out the discussion of the pathway altogether and sticking to what your data shows.
Lines 754-755: We have included all pathways that the gas could be taking to migrate upwards.

We thank the reviewer for their time and constructive comments on our manuscript. We have addressed all concerns raised below.

General comments

Generally, the manuscript address scientific questions within the scope of BG; proving the source of methane in shallow aquifer is a relevant and important issue. The author's present data which indicate that methane detected in an alluvial aquifer is not produced in the aquifer itself but is produced in the underlying coal seam and subsequently migrates upwards to the aquifer. This finding would be of fundamental interest for the risk assessment regarding the occurrence of methane in shallow aquifers. However, three of the authors (including the first and last author) published already in 2015 a paper in which basically the same conclusion has been drawn (Iverach et al., 2015); moreover, essential data – the carbon isotope signatures of methane – shown in the present manuscript have been already published by Iverach et al. (2015). This reduces the originality and novelty of this paper.

The microbiological data presented in this paper are unique and vastly improve our understanding of this aquifer system. A small portion of the geochemical data from the previous manuscript was reproduced here for ease of reading the paper.

The overall presentation is well structured and clear, including an accurate title, a proper abstract and introduction into the topic, and adequate citations of related work.

The applied methods and assumptions are valid; some of the used scientific methods are not clearly described and cannot be reproduced (see specific comments). Generally, the results are sufficient to support the main conclusion that the source of the methane detected in the alluvial aquifer was the underlying coal seam. Some interpretations based on the geochemical and microbiological data are certainly speculative (see specific comments) and need to be supported by literature/experimental data; if not possible, these parts should be condensed or deleted.

We have added citations to all mentioned methods, and we have addressed the specific speculative comments below.

On the other hand, one important result of this study, the oxygen concentrations of the investigated groundwater samples, is not seriously presented and discussed in the main manuscript (the data are somewhat hidden in the supplemental information). The oxygen data indicate that the studied aquifer zones are predominantly aerobic, a fact that could explain the absence of strictly anaerobic methanogens in the groundwater samples. Due to the presence of methanotrophs and availability of oxygen in the aquifer, the question arises to which extent methane is oxidized and whether aerobic oxidation of methane is trackable in the aquifer by compound specific stable isotope analysis, as this reaction is characterized by strong carbon and hydrogen isotope fractionation (Feisthauer et al., 2011). Unfortunately, this aspect is not discussed in the manuscript.

The dissolved oxygen data in the groundwater were measured using a YSI probe on the surface that was also measuring the pH, EC, TDS, temp. As such, it is not a completely accurate representation of the DO conditions in the aquifer, as the degassing caused by pumping and the effect of the barometric pressure needs to be considered. However, we have mentioned the high DO concentration (line 541), addressing the comments above, as well as

the DO concerns raised below. Unfortunately, tracking methane oxidation was outside the scope of this study, which aimed at characterising for the first time the microbial community in this freshwater aquifer and seeing if it was possible to use microbes to help elucidate the source of CH_4 detected in the aquifer. It would be a very useful future study, but we have not mentioned it in the text because it is outside the scope of this investigation.

Specific comments

Lines 96-103: This statement is too strict. It's true that sulfate reducers generally outcompete methanogens but not always, see Struchtemeyer et al. (2005).

Lines 117-118: This statement has been softened: "…because SRB often outcompete methanogenic archaea…" and the suggested reference has been included.

Lines 119-133: I suggest mentioning that the expression of the particulate and soluble methane monooxygenase is triggered by the amount of available copper ions.

Lines 162-163: This has been mentioned at the suggested location in the text.

Lines 208-212: For clarity, I suggest indicating the depth at which each well was sampled. I do not understand why the eight samples are representative of the aquifer, please explain in detail.

Line 266: A table indicating the slotted interval for each sample has been included in the methods now. We understand that eight samples are a small dataset, however they are at varying depths and locations throughout the aquifer. Physico-chemical parameters and the spread of geochemical data indicate that the samples are representative of the spread of the conditions of the aquifer as a whole.

Line 226: How long were the DIC samples stored before measurement? Please indicate.

Lines 282-283: The DIC samples were analysed within one month and this information has now been included in the manuscript. They were also filtered through a 0.22 µm filter in the field, which is the best way to maintain the sample (provided refrigeration and proper storage) (Doctor et al. 2008). In addition, DIC samples from another field site were analysed 1 week after collection, and then re-analysed 6 months later and were found to have no difference in measurement.

Lines 228-230: I wonder why samples for geochemical and microbiological analyses were not sampled at the same time, which would have strengthened the main conclusions of this paper.

Insights from the original hydrogeochemical survey indicated that microbiological data would refine our understanding of the processes. Therefore we returned and collected microbiological data (at a limited number of sites due to budget constraints). In December of the same year (when the aquifer is under the same stress as in January), additional funding was granted and we were able to sample for the microbiology.

Lines 232: Probably, any nanobacteria (prokaryotes smaller than 0.2 µm) were lost during this procedure?

A 0.2 µm filter is standard for filtering microbial communities. The filtrate was also screened using SYBRGREEN I staining and microscopy and there was no detection of cells.

Lines 241-259: Give references for the methods of d2H-H2O, d18O-H2O, d13C-DIC, d13C-DOC, d18O-SO4, d34S-SO4 analysis or describe the methods in detail that they can be reproduced.

Lines 317-333: References for the methods of analysis for all geochemical data have been provided in this section.

Lines 262 ff. A critical question is whether the microbial community of a groundwater sample will truly reflect the microbial community of the subsurface from which the groundwater was extracted from. This aspect should be briefly discussed (probably in the Results & Discussion section).

We do believe that the microbial community of the groundwater is reflecting the microbial community of the subsurface. Maamar et al. (2015) found that the microbial community composition of groundwater was controlled by groundwater residence times and the location of samples along the groundwater flow path, independent of the geology, stating that "hydrogeologic circulation exercises a major control on microbial communities". They also state: "…Thus, geochemical conditions, and in particular the availability of electron donors and acceptors, are a major driver of microbial community composition and diversity in groundwater and the geological substratum".

Additionally, when we sample the groundwater, we are also sampling fine particles with biomass attached. Further, the Condamine production wells are drawing water that is representative of the sampled formations and the intense purging ensures that this is the case. The ^{14}C and ^{3}H activities suggest that we are not drawing a modern/old mixed groundwater component, therefore whatever water is sampled is representative of the formation, and we presume the microbial communities within it.

Lines 492-498: A small paragraph explaining the above has been included in the discussion.

Figure 2: In the Figure, five ranges are shown (indicated by 5 different colors) whereas only four ranges are given in the legend. I recommend using different colors for each order of magnitude for higher resolution. A general drawback of Figure 2 is the lack of any statistics, what are the standard deviations of the data?

Lines 458-462: Figure 2 has been changed - 4 different colours have been used for 4 different ranges. Standard deviations have been added to the figure legend and qPCR specific validations are in the methods.

Line 420 ff. See comment above. It's true that sulfate reducers generally outcompete methanogens but not always, see Struchtemeyer et al. (2005). I recommend discussing with more caution.

Lines 562-581: We have clarified the language above, however in the text at this location we do already say "These SRB are potentially outcompeting methanogenic archaea…", implying that this may not be the case. We then proceed with additional evidence as to why the lack of methanogenic archaea could be a result of this competition.

Lines 425-428: It is very speculative to conclude that the detected phylotypes affiliated to sulfate or sulfur reducers will oxidize acetate (or outcompete methanogens). I suggest discussing with more caution. Deducing specific metabolic activities from partial 16S rDNA sequences is questionable.

Lines 566-581: We have clarified our discussion. Because most of the Deltaproteobacteria sequences detected in the groundwater were closely related to acetate-oxidising sulfate/sulfur reducing bacteria (*Desulfovibrionales, Syntrophobacterales, Desulfuromonadales*), it is reasonable to assume that the lack of methanogenic archaea could potentially be a result of competition from sulfate reducers taking the acetate, which is the methanogenic substrate required.

Lines 428-432: I do not understand this argumentation. Methylocella are aerobic organisms, whether methanogens are strictly anaerobic. They probably do not exist in the same ecological niche.

Aerobic and anaerobic microorganisms can exist in the same environment. They are not strictly separated; e.g. anaerobic methanogens can occur in anoxic or suboxic microniches in mainly aerobic environments (Kato et al., 2007; Dimikić et al., 2011).

Lines 448-450: What could be an alternative pathway for aerobic methane oxidation in an anaerobic environment? The initial methane oxidation reactions will always depend on molecular oxygen, hence aerobic methane oxidation cannot take place in the absence of oxygen. Why not discussing the detected (high) oxygen concentrations of the groundwater samples in this context?

Lines 630-636: As previously mentioned, the detected high concentrations of dissolved oxygen in the groundwater have been discussed now and it has been stated that these are most likely the reason for abundant aerobic methanotrophs in the groundwater. Therefore, an alternative pathway for aerobic methanotrophs, potentially using other electron acceptors, has not been discussed.

Lines 460-462: I wonder why the oxygen data are not shown in more detail. Some wells seem to be fully aerobic, a result which does not correspond to the observation of the dominance of sulfate or sulfur reducing deltaproteobacteria in most of the samples. On the other hand, the presence of oxygen explains well the presence of methanotrophs and other aerobes in the groundwater samples. Probably, the discrepancy might be explained by the sampling artifacts; the pumped groundwater may contain strictly anaerobic organisms originally attached to the aquifer solids in which anoxic microenvironments exist.

As mentioned previously, the DO data are not a completely accurate representation of DO concentration within the aquifer - this is why they were included in the supplementary material but not highlighted in the text. If the discrepancy between DO and deltaproteobacteria is to be explained by sampling artifacts, it would probably be this, not microbial sampling methods.

Aerobic and anaerobic microorganisms can live alongside each other in many habitats in microniches. Sulfate reduction under oxic conditions has been observed and previously published; e.g. in cyanobacterial mats or periodically in activated sludge (Kjeldsen et al. 2004; Fike et al. 2008).

We have now explicitly referred to the role that the high concentration of DO is potentially playing in the absence of methanogenic archaea and abundance of aerobic bacteria (lines 630-636). In addition, we have explained why the deltaproteobacteria are dominant in most samples despite the presence of O2.

Lines 470-476: This hypothesis is very, very speculative. Are there any indications for the presence of nitrate in the groundwater? Why Chloroflexi should convert denitrification products to oxygen? The hypothesis needs more arguments (support by literature or own experimental data); if no other arguments are available, I suggest deleting this passage.

We have removed this hypothesis.

Lines 487-488: Give references for this statement.

Line 665: A reference has been given for this statement (Pester et al. 2011).

Lines 490-491: I doubt that the methane concentrations were high enough to allow sulfate-dependent AOM. Please discuss.

We agree that methane concentrations were most likely not high enough to allow sulfate-dependent AOM in this groundwater. However, at this location in the manuscript we are going step-wise through our data providing evidence either for or against potential processes affecting the occurrence of CH4 in this groundwater – at this particular point, it is the possible occurrence of AOM in the groundwater. Hence, we state that the sulfate concentrations are potentially high enough to mediate AOM at 2 locations, however, we go on to state that further geochemical evidence (including lack of detected ANME's) indicate that this process is not occurring.

Cited literature:

Feisthauer S, Vogt C, Modrzynski J, Szlenkier M, Krüger M, Siegert M, Richnow HH (2011) Different types of methane monooxygenases produce similar carbon and hydrogen isotope fractionation patterns during methane oxidation. Geochim. Cosmochim. Acta 75: 1173-1184

Iverach CP, Cendón DI, Hankin SI, Lowry D, Fisher RE, France JL, Baker A, Kelly BFJ (2015) Assessing connectivity between an overlying aquifer and a coals seam gas resource using methane isotopes, dissolved organic carbon and tritium. Sci. Rep. 5: 1-11

Struchtemeyer CG, Elshahed MS, Duncan KE, McInerney MJ (2005) Evidence for aceticlastic methanogenesis in the presence of sulfate in a gas condensate-contaminated aquifer. Appl. Environ. Microbiol. 71: 5348-5353

Technical comments
Line 322: DSMZ, Braunschweig, Germany

This has been corrected.

References:

Dimikić, M., Pušić, M., Majkić-Dursun, B. & Obradović, V. Certain implications of oxic conditions in alluvial groundwater. *Water Res. Manage.* **1(2)**, 27-43, (2011).

Fike, D.A., Gammon, C.L., Ziebis, W. & Orphan, V.J. Micron-scale mapping of sulfur cycling across the oxycline of a cyanobacterial mat: a paired nanoSIMS and CARD-FISH approach. *ISME J.* **2**, 749-759, (2008).

Kato, M.T., Field, J.A. & Lettinga, G. Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. *Braz. J. Chem. Eng.* **14(4)**, (1997).

Kjeldsen, K.U., Joulian, C. & Ingvorsen, K. Oxygen tolerance of sulfate-reducing bacteria in activated sludge. *Environ. Sci. Technol.* **38(7)**, 2038-2043, (2004).

Maamar, S.B., Aquilina, L., Quaiser, A., Pauwels, H., Michon-Coudouel, S., Vergnaud-Ayraud, V., Labasque, T., Roques, C., Abbott, B.W. & Dufresne, A. Groundwater Isolation Governs Chemistry and Microbial Community Structure along Hydrologic Flowpaths. *Fron. Microbiol.* **6**: 1457, (2015).

Pester, M., Schleper, C., Wagner, M. (2011) The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. Current Opinion in Microbiology, 14: 300-306.

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

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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.

Deleted: Microbial and geochemical data indicate that there is biogenic CH_4 in the aquifer, but no methanogenic microbial activity.

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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.

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Methane is subject to many production and degradation processes in groundwater (Whiticar, 1999). The carbon isotopic composition of CH_4 ($\delta^{13}C$-CH_4) gives insight into the source (Quay et al., 1999), but oxidation processes may enrich or deplete this signature (Yoshinaga et al., 2014). Therefore, it is very difficult to determine the potential source of CH_4 and processes occurring using CH_4 concentration and isotopic data alone.

Previous studies have used geochemical indicators, such as the concentration of sulfate [SO_4^{2-}], nitrate [NO_3^-] and nitrite [NO_2^-], and the carbon isotopic composition of dissolved inorganic carbon (δ^{13}C-DIC) and dissolved organic carbon (δ^{13}C-DOC) to attribute the source of CH_4 in groundwater (Valentine and Reeburgh, 2000; Kotelnikova, 2002; Antler, 2014; Green-Saxena et al., 2014; Antler et al., 2015; Hu et al., 2015; Segarra et al., 2015; Sela-Adler et al., 2015; Currell et al., 2016). Other studies have shown that the presence of active methanogenesis can be determined using isotopes of hydrogen in the CH_4 (δ^2H-CH_4), and the surrounding formation water (δ^2H-H_2O) (Schoell, 1980; Whiticar and Faber, 1986; Whiticar, 1999; Currell et al., 2016). Additionally, recent studies have used clumped isotopes of CH_4 and their temperature interpretations to ascribe a thermogenic versus biogenic source in groundwater (Stolper et al., 2014). However, non-equilibrium (kinetic) processes may be responsible for an overestimation of CH_4 formation temperatures (Wang et al., 2015). Microbiological indicators (in addition to geochemical data) may resolve some of the uncertainties associated with the determination of CH_4 origin, as they directly discriminate between microbiological communities involved in either production or degradation processes. There are no studies using combined geochemical and microbiological indicators to assess CH_4 production and degradation processes in a freshwater aquifer. We aim to fill this gap in the literature.

Throughout the world the occurrence of freshwater aquifers adjacent to unconventional gas production is common (Osborn et al., 2011; Moore, 2012; Roy and Ryan, 2013; Vidic et al., 2013; Vengosh et al., 2014; Moritz et al., 2015). We have previously shown that there may be local natural connectivity between the WCM and the CRAA (Iverach et al., 2015). Here we show that a combination of geochemical data ([CH_4], [SO_4^{2-}], [NO_3^-], [NO_2^-], δ^{13}C-CH_4, δ^{13}C-DIC, δ^{13}C-DOC and δ^2H-H_2O), as well as

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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.

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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

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

Besides methanotrophic bacteria, anaerobic CH_4-oxidising archaea (ANME) also play a significant role in the oxidation of CH_4 in both freshwater and saline water sources (Knittel and Boetius, 2009). These anaerobic methanotrophs are associated with the methanogenic Euryarchaeota within the clusters ANME-1, ANME-2, and ANME-3 and are closely related to the orders *Methanosarcinales* and *Methanomicrobiales* (Knittel et al., 2003; 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 for irrigated agriculture, stock and domestic water supplies. There has been increased interest in the presence of CH_4 in the aquifer due to expanding CSG production to the north-west of the study area (Figure 1). CSG production began in 2006 (Arrow Energy, 2015) and has been expanding in the decade since then. This has raised concerns regarding the

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quality (especially with respect to CH_4 concentrations) and quantity of the groundwater in the CRAA.

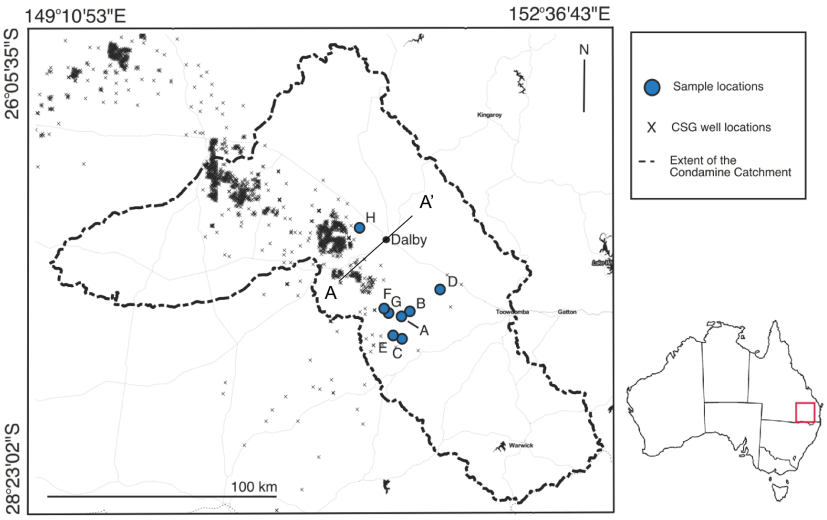

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Figure 1. Site map showing the extent of the study area and sample locations within the Condamine Catchment, south-east Queensland, Australia. Map created in QGIS; data and imagery: Statem Toner, Open Street Map and contributors, CC-BY-SA (QGIS, 2015).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

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 (Huxley, 1982; Kelly and Merrick, 2007; Dafny & Silburn, 2014). The valley-filling sediments have a maximum thickness of 134 m near Dalby (Dafny and Silburn, 2014). Along the eastern margin of the valley, the CRAA is bounded by the Main Range Volcanics. Estimations of the sources and quantity of recharge to the CRAA vary widely; however, streambed recharge is generally considered to be the major source of freshwater to the aquifer (Dafny and Silburn, 2014).

A low permeability layer (ranging from 8×10^{-6} to 1.5×10^{-1} m/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). The thickness of this layer varies between 30 m thick in some

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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.

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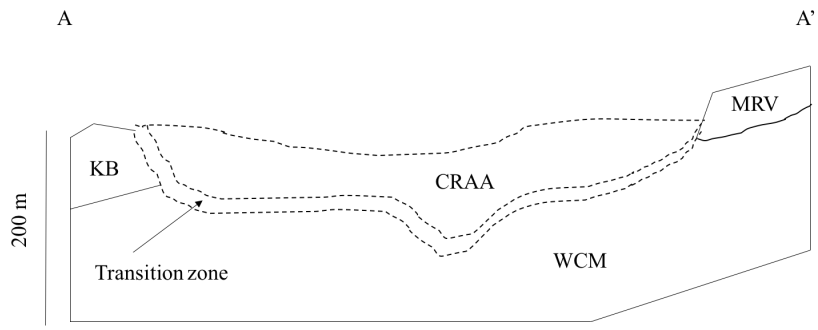

Figure 2. Geological cross section along A-A' in Figure 1 (adapted from Dafny & Silburn, 2014). KB-Kumbarilla Beds; MRV-Main Range Volcanics.

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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

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

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

3.1 Geochemical analyses

The major ion chemistry in the groundwater samples was analysed at the Australian Nuclear Science and Technology Organisation (ANSTO) using inductively coupled plasma atomic emission spectroscopy for cations and ion chromatography for anions. The samples for δ^2H-H_2O and δ^{18}O-H_2O were analysed at ANSTO and are reported as ‰ 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

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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

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

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

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

This universal primer pair targets the V6-V8 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 (Agencourt AMPure XP, (Beckman Coulter)) and quantified using a fluorometric kit (RiboGreen, Qubit Assay Kit, Invitrogen). Purified amplicons were subjected to the Index PCR using the MiSeq platform (Ramaciotti Centre for Genomics, UNSW Australia) according to the manufacturer's 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

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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

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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™-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).

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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

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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.

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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.

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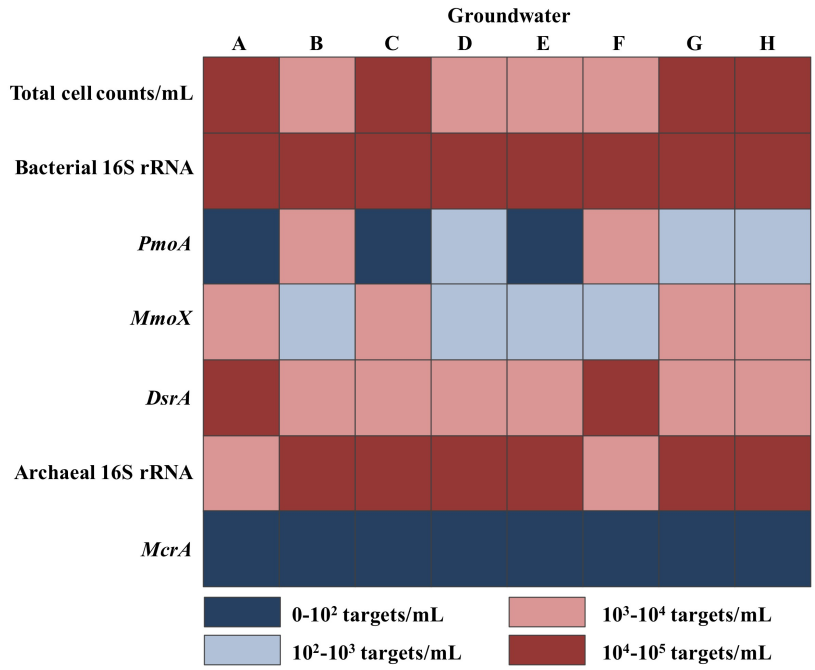
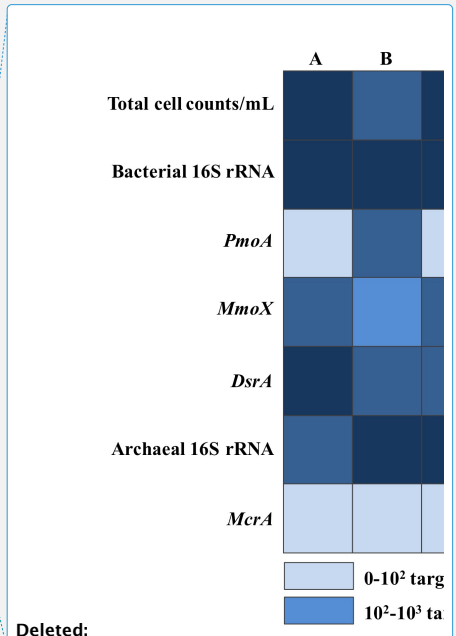


Figure 3. Total cell concentration and copy number abundances of bacterial and archaeal 16SrRNA genes and functional key genes for aerobic CH_4 oxidation (*pmoA* and *mmoX*), CH_4 production (*mcrA*) and sulfate reduction (*dsrA*) in the groundwater carried out by quantitative (q)PCR. Low abundances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

The groundwater community was primarily composed of bacteria (79-90%), whilst archaea made up 10-21% (Figure 4). 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 4). The phylum Thaumarchaeota dominated the archaeal communities with a relative abundance of 81-99%, while Crenarchaeota made up



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

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

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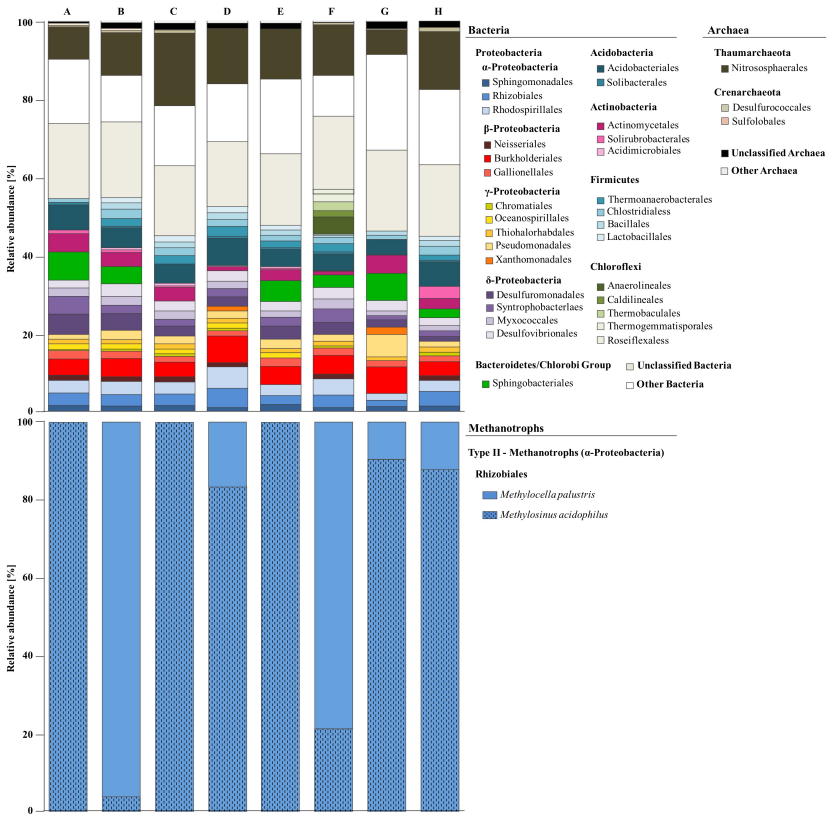

Figure 4. Bacterial, archaeal, and methanotrophic community profiles and relative abundances detected by Illumina sequencing.

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

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519 that can be ascribed to methanogenesis in any of the groundwater samples (Cendón et al.,
520 2015).

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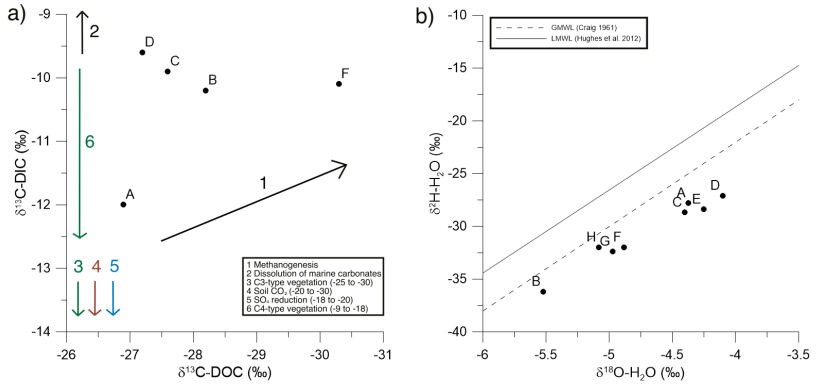

521

522 **Figure 5.** (a) A plot of δ^{13}C-DOC vs. δ^{13}C-DIC. There is no correlation between these geochemical data,
523 indicating that there is no methanogenic end member in our samples. Samples E, G and H are omitted
524 because they were below the detection limit for δ^{13}C-DOC (Supplementary Table S1.). Arrow 1 delineates
525 the expected trend for methanogenesis and arrow 2 is the expected trend for the dissolution of marine
526 carbonates (Currell et al., 2016). Arrows 3-6 highlight expected ranges for δ^{13}C-DIC that are off the scale of
527 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
528 in any of the groundwater samples. The GMWL (Craig, 1961) and LMWL (Hughes and Crawford, 2012)
529 are also displayed.

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531 These geochemical analyses, along with the lack of classified methanogens, suggest
532 that biogenic CH_4 production is not one of the major processes responsible for the
533 presence of CH_4 in the CRAA. Therefore, the CH_4 reported in all samples in Iverach et al.
534 (2015) must be derived from another source. We propose that the upward migration of
535 CH_4 from the WCM must be considered as the potential source. The isotopic signature of
536 CH_4 from the deeper coal measures has been characterised between -58.5‰ and -45.3‰,
537 indicating thermogenic CH_4 with a secondary biogenic component (Papendick et al.,

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

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

4.3 Sulfate reducers and aerobic methanotrophs potentially outcompete methanogens

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

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Deleted: This could be the result of the replacement of typically thermogenic gas in the shallow WCM by biogenic gas (Faiz and Hendry, 2006). Thus, these three sites are potentially sourcing biogenic CH_4 from the shallow WCM, resulting in a biological source signature despite the absence of methanogens in the overlying aquifer.

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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 4). Additionally, *Methylocella* spp. are capable of using methanogenic substrates, such as acetate and methylamines, for their metabolism and therefore are not limited to growing on one-carbon compounds such as CH_4 (Dedysh et al., 2005). This could have major implications for the lack of methanogenic activity in the groundwater. In addition, the presence of SO_4^{2-} along with conditions favouring SRB is further evidence that *in situ* methanogenesis is unlikely to be responsible for the presence of CH_4 in the shallow aquifer.

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

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

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

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

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

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

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

4.5 Absence of anaerobic methane oxidation

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 4). 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 (Pester et al., 2011).

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

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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 $\delta^{34}S$-SO_4 and $\delta^{18}O$-SO_4 is statistically invalid. The highest relative abundance of methanotrophs was found in samples D and H (Figure 4); however, these methanotrophs are not anaerobic oxidisers and therefore the correlation may not imply causation.

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

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

Therefore, any oxidation occurring in the groundwater would have been facilitated by the two members of type II methanotrophs that we identified in the microbial

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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.

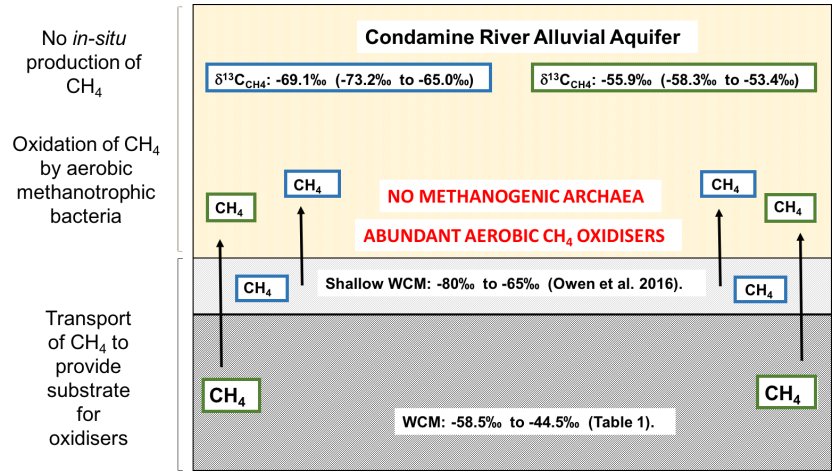

Figure 6. A conceptual schematic of the processes occurring between the WCM and the CRAA.

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*?

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One hypothesis to explain the presence of CH_4 despite there being no evidence of methanogenesis is that there is localised upward migration of CH_4 from the WCM into the CRAA via natural faults and fractures (Iverach et al., 2015).

Our geochemical data and microbiological community analysis both indicate that AOM is not a major oxidation process occurring in the CRAA. However, the microbiological data suggest the presence of aerobic CH_4 oxidisers. Due to the absence of methanogenesis, the oxidation of CH_4 (facilitated by the aerobic methanotrophs present in the groundwater) would require a secondary source of CH_4. This, coupled with the isotopic signature of the CH_4 and the concentration of SO_4^{2-} in the groundwater suggests that the upwards migration of CH_4 from the underlying WCM is the likely source (Figure 6).

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

Therefore, we suggest that the CH_4 detected in the CRAA in Iverach et al. (2015) is from the local upward migration of gas from the underlying WCM, either through natural faults and fractures, transport along poorly installed well casings, or direct leakage of gas between the WCM and CRAA where the units are in direct contact. A consideration of both geochemical and microbiological analyses is particularly important in this study area because of the immediate proximity of the underlying WCM and the proximity of the

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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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Acknowledgements

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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.

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

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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