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REPORT ON THE POTENTIAL FOR EXPLOITING METHANE OXIDISER GENES FOR MONITORING STRAY METHANE INTRUDING INTO AQUIFERS AND ASSESSMENT OF THE AREA THAT CAN BE MONITORED

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Public introduction

Subsurface Evaluation of CCS and Unconventional Risks (SECURe) is gathering unbiased, impartial scientific evidence for risk mitigation and monitoring for environmental protection to underpin subsurface geoenergy development. The main outputs of SECURe comprise recommendations for best practice for unconventional hydrocarbon production and geological CO2 storage. The project is funded from June 2018–May 2021.

The project is developing monitoring and mitigation strategies for the full geoenergy project lifecycle; by assessing plausible hazards and monitoring associated environmental risks. This is achieved through a program of experimental research and advanced technology development that includes demonstration at commercial and research facilities to formulate best practice. We will meet stakeholder needs; from the design of monitoring and mitigation strategies relevant to operators and regulators, to developing communication strategies to provide a greater level of understanding of the potential impacts.

The SECURe partnership comprises major research and commercial organisations from countries that host shale gas and CCS industries at different stages of operation (from permitted to closed). We are forming a durable international partnership with non-European groups; providing international access to study sites, creating links between projects and increasing our collective capability through exchange of scientific staff.

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Executive report summary

Deliverable 4.3 reports the work completed in subtask 4.3.2 among British Geological Survey, Geological Survey of Denmark and Greenland, and TOTAL S.A. The main objective has been to characterize microbial communities' response to gas leaks and identify whether the response of specific microbial groups can be used for monitoring of groundwater.

Historically, detectable changes of the microbial communities in soil due to methane, ethane, propane, and butane oxidizers or the relationships between them have all been used as indicators of hydrocarbon deposits in industrial prospecting. Industries have applied culture-based methods, but more recently, there have been attempts to apply DNA techniques to reduce culture-bias and improve accuracy and precision.

Applying these methods to groundwater is more novel. Therefore, the work presented in this deliverable consists of controlled laboratory experiments and field sites considering groundwater with high biogenic or thermogenic hydrocarbon content. Samples have been analysed by molecular and culture-based methods to identify potential indicator organisms or if the abundance of methylotrophs based on 16S sequencing can be linked to hydrocarbon concentrations. The main results are:

- The microbial composition of groundwater samples from both the Sleen and Vale of Pickering sites revealed that blanket application of 16S amplicon sequencing is insufficient to capture the microbial response to elevated hydrocarbon concentration.
- Culture-based studies identified certain Actinobacterial genera (*Corynebacterium*, *Nocardia*, *Mycobacterium*, *Rhodococcus* complex) as potential indicators of hydrocarbon leakage.
- Controlled microcosm experiments revealed an increase in the abundance of *Methylobacillus*, *Methylobacter Methylomonas*, *Methyloversatilis*, *Terrimicrobium*, and *Crenothrix*. Hence, these may be indicator candidates. However, the abundance of above-listed indicator candidates in groundwater samples did not correlate with dissolved methane concentrations.
- "*Candidatus* Methanoperedens" represented up to 30% of the entire community from Sleen or Vale of Pickering groundwater samples and could potentially be of interest as an indicator.

Improvement to the current work would include additional amplicon sequencing of the two relevant functional genes, *pmoA* and *mcrA,* that are directly related to methane oxidation. Further, the absolute quantification of specific bacteria by qPCR rather than the relative abundance needs to be evaluated as a better proxy for elevated hydrocarbon concentrations. This work is currently ongoing and if successful will be included in D.4.6.

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Contents

Public introduction ii

Executive report summary iii

Contents iv

1 Introduction 1

2 Field sites, Materials and Methods 2

2.1 Field Sites 2

2.2 Sample collection 5

2.3 Microcosm setup 6

2.4 Molecular techniques 6

2.5 Bioinformatics 7

2.6 Culture based methods 7

3 Results 10

3.1 Background data from fieldsites 10

3.2 Method development 11

3.3 Natural diversity 13

3.4 Microcosms 17

3.5 Selected microbial groups in groundwater samples 22

3.6 Automated image analysis 24

4 Discussion 25

4.1 Community Diversity 25

4.2 Indicator microorganisms 27

5 Relevance to future leak detection 29

Acknowledgements 30

Glossary 31

6 References 32

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FIGURES

Figure 1. Shallow stratigraphy shows the dominant lithology of each formation and the approximate location, depth, length, and designation of each wellbore and individual monitoring well (Schout et al. 2018). In red are marked wells that were sampled for DNA and culturable studies. 3

Figure 2. Geological cross-section of the upper layers of the Stenlille natural gas underground storage. Approximate location and depth of monitoring screens in K1 and K2. The red colour indicates the probable distribution of gas after the St14 leakage in August 1995 (modified from Laier, (2012). 4

Figure 3. Methane concentrations in the superficial aquifer (Ward et al 2020)...5

Figure 4. VoP Site 3, Feb20. The left image shows growth on methanol the right image is on propanol. Within each image the Petri dishes are as follows: top to bottom time from sample collection 1 day, 2 days, 5 days and 8 days. Left of image filtered in the field, right of image, filtered in the laboratory. 12

Figure 5. Filter membranes from Sleen 6.2 grown on methanol (left Petri dish in each image), propanol (right Petri dish in each image), showing difference between filtering in field (top) and laboratory (bottom).12

Figure 6. Principal component analysis (PCA) plot of the groundwater microbial community from Sleen (left) and Vale of Pickering (right). Circled coloured areas indicate if microbial communities cluster due to methane concentrations. 13

Figure 7. Comparison of diversity indices using manual cell counts for methane (x axis) and propane (y axis). 14

Figure 8. Comparison of diversity indices using manual cell counts for methanol (x axis) and propanol (y axis). Legend as in Figure 7. 14

Figure 9. Sleen. Total amounts in the system in mmoles per microcosm. A, B and D are aerobic treatments, including control (A), methane treatment (B), and methane and ethane treatment (D). C and E are anaerobic treatments, including methane treatment (C) and methane and ethane treatment (E). Note the difference in days for aerobic and anaerobic treatments. 18

Figure 10. Stenlille. Total amounts in the system in mmoles per microcosm. A, B and D are aerobic treatments, including control (A), methane treatment (B), and methane and ethane treatment (D). C and E are anaerobic treatments, including methane treatment (C) and methane and ethane treatment (E).19

Figure 11. Microbial community influenced the different microcosm treatments for Sleen (left figure) and Stenlille (right figure). No: no additional gas. 'No' gas control label from Sleen is underneath the Methane label. 20

Figure 12. Composition of the top 12 microbiological orders from microcosms from the Sleen site comparing Day 0 with oxygen, oxygen/methane and oxygen/methane/ethane headspace................................ 20

Figure 13. Composition of the top 12 orders from microcosms from the Stenlille site comparing Day 0 with oxygen, oxygen/methane and oxygen/methane/ethane headspace.. 21

Figure 14. Distribution of genera identified in Table 12 and Table 13 in the Sleen site. Wells are organized with an increase in methane concentration from left to right. Upper figure are bacteria and lower figure archaea.. 22

Figure 15. Distribution of genera identified in Table 12 and Table 13 from VoP samples. Sites are organized with an increase in C_1:C_2 ratio from left to right (Site 15 Feb20 to Site B42 Feb20). No ethane was detected in remaining sites (B43 Aug19 to B42 Aug19) so are organised by increasing methane concentration. Upper figure are bacteria and lower figure archaea 23

Figure 16. Example output from CellProfiler and self-organising map. Top row: absolute difference between grey and individual colour channels, left to right blue, green, and red. Middle row: object identification using individual colour channels, left to right blue, green, and red. Bottom left: combined objects, middle: original image with an overlay of combined objects, right: self-organising map. 24

Figure 17. Comparison of manual cell counts with automated presumed cell counts using CellProfiler and Self-Organising map. Comparisons in each of the diversity indies are shown. 25

TABLES

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Table 1. Overview of target genes for amplicon sequencing. 7

Table 2. Overview of target genes for qPCR. 7

Table 3. Summary of variables tested during method development 8

Table 4. PCR primers used for colony PCR 9

Table 5. PCR protocols for colony PCR. D. – denaturing, A. – annealing, E. – extension 9

Table 6. Sleen chemical data from water samples from the wells and screens... 10

Table 7. VoP chemical data from groundwater samples. 10

Table 8. Additional chemical data from the Sleen groundwater samples 11

Table 9. Refinement of sample parameters 11

Table 10. Use of ratio of species richness to predict C_1:C_2 ratio in groundwater 15

Table 11. Details for isolates where 16S identification could be made. Yes, PCR product of correct size, No – no PCR product, ? multiple PCR products or of incorrect size. Green samples high C_1/C_2, orange: medium C_1/C_2, red: low C_1/C_2. *mmoX* gene was also tested for however no isolates yielded a positive PCR product. MeOH- methanol, PrOH - propanol 15

Table 12. Inorganic parameters from groundwater well K1, Stenlille and 69.2, Sleen. 17

Table 13. Bacterial groups, where there was a significant difference between oxygen treatment and either of the two methane treatments for aerobic experiments and for anaerobic experiments comparing the control with the two methane treatments. For “*Candidatus* Methanoperedens” there was a significant decrease in abundance. 21

Table 14. Bacterial genera, where there was a significant difference between oxygen treatment and either of the two methane treatments for aerobic experiments and for anaerobic experiments comparing the control with the two methane treatments. 21

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1 Introduction

Within the Advanced Sensors for Challenging Monitoring Scenarios task of the SECURe project (task 4.3), a microbiology sub-task focussed on biological methods of sensing gas leaks from shale gas extraction. Various field and laboratory studies have previously shown that there are certain microbial groups present in the environment which respond to gas leaks (Farhan Ul Haque, Crombie et al. 2018, Kuloyo, Ruff et al. 2020). These studies raise the question of whether the microbial response to natural gas (methane plus other short-chain alkanes (SCA)) is distinct from the response to exposure to methane alone and, if that is the case, whether they can be used as a tool to identifying the presence of leaking gas and to help distinguish thermogenic methane sources from biological ones. This sub-task is focussed on characterising the response of microbial communities to gas leaks and identifying whether the response of specific microbial groups can be used for monitoring purposes.

Hydraulic fracturing for recovery of unconventional hydrocarbon reservoirs (or 'fracking') often attracts public concern on a number of grounds, ranging from water usage and disposal, excess traffic, pollution of groundwater, seismic activity and fugitive greenhouse gas emissions (Howarth, Ingraffea et al. 2011, Jaspal and Nerlich 2014, Bomberg 2017). Small but significant numbers of hydrocarbon wells have been observed to emit methane (Watson and Bachu 2009, Boothroyd, Almond et al. 2016, Kang, Christian et al. 2017), and within this context, suitable tools for monitoring leakage are critical. This is particularly needed if unconventional wells are at greater risk of suffering from issues, as suggested by Ingraffe *et al.*, (2014). However, this may be due to the introduction of stricter regulations and, therefore, an increase in violation reports (Jackson 2014). A better understanding of well integrity is needed, and a monitoring system for these wells should be in place (Davies, Almond et al. 2014, Jackson 2014). Understanding microbial response would aid in understanding well integrity and could be used in monitoring.

Gas emitted from hydrocarbon sources is predominantly methane but also contains smaller amounts of carbon dioxide and other SCA (Etiope 2015). This contrasts to methane that is biologically produced (via methanogenesis), which is almost entirely methane. While there are isolated examples of ethane and possibly propane being microbially produced in the subsurface (Oremland, Whiticar et al. 1988, Taylor, Sherwood Lollar et al. 2000, Hinrichs, Hayes et al. 2006, Sassen and Curiale 2006), it is not generally considered to be common.

Two methods are commonly applied to determine the source of methane. Firstly, gas wetness ratios can relate to the pathway and starting material; a wet gas is one with a higher proportion of SCA to methane (Golding, Boreham et al. 2013). Oil reservoirs tend to produce wetter gases than natural gas sources, which in turn are wetter than microbial sources. Secondly, isotopic methods rely upon the principle that biochemical processes favour the use of lighter isotopes, enabling the use of carbon ($^{13}C/^{12}C$) and hydrogen ($^{2}H/^{1}H$) isotopes of methane to characterise the source. Thermogenic sources of methane tend to have a greater proportion of heavier isotopes. It is possible that a seep can be composed of gases from more than one process due to mixing and the action of multiple production pathways make these data difficult to interpret (Golding, Boreham et al. 2013). For example, secondary methane production by methanogenesis occurring after uplift of hydrocarbon bearing strata, will result in gas originating from both sources, which will affect the results of both these methods (Martini, Walter et al. 1998, Strąpoć, Mastalerz et al. 2011). Alternatively, depletion of lighter methane isotopes through methanotrophy can lead to isotope signatures more typical of thermogenic sources (Golding, Boreham et al. 2013). For these reasons, techniques based on the changes in alkane oxidising microorganisms (or other associated microbial changes) would provide an additional method to identify gas source(s).

The use of microbial monitoring for the detection of gas seeps is not new, and techniques described as Microbial Prospecting for Oil and Gas (MPOG) or Microbial Oil Survey Techniques (MOST) have been used by the oil and gas industry for decades to survey soil microorganisms to prospect for underlying hydrocarbon sources (Brisbane and Ladd 1965, Tucker and Hitzman 1996, Wagner, Wagner et al. 2002). These methods rely upon detecting changes to soil microbial communities caused by gas seeping through microfractures. Atmospheric compositions above active gas fields have been observed to have an increased methane component with an increase in propane and butane, strongly suggesting the gas extraction process was the cause (Karion, Sweeney et al. 2013). This results in detectable changes to the microbial communities in the soil, particularly methane, ethane, propane and butane oxidisers, or the relationships between them. These have all been used as indicators of gases leaking from hydrocarbon deposits (Rasheed, Patil et al. 2013). Historically, industry relied on culture-based methods, but more recently, DNA techniques have been trialled

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to reduce culture-bias and improve accuracy and precision (e.g. (Miqueletto, Andreote et al. 2011, Zhang, He et al. 2014, Zhang, He et al. 2017)). These techniques claim to have success rates of up to 90% (Wagner, Wagner et al. 2002). Most of this work is based on soil microbiology, and few groundwater studies have been undertaken. Field-scale methane injection studies show that groundwater microbial communities remained in a disturbed state after 253 days (Cahill, Steelman et al. 2017), and laboratory microcosm studies show that methylotrophs may be suitable markers of methane contamination events, as they persisted for weeks after methane supply had stopped (Kuloyo, Ruff et al. 2020). Methylotrophs not known to oxidise methane has also been suggested as possible indicator species in sediments and seawater (Redmond, Valentine et al. 2010). The advantage of a microbial-based approach is that, in comparison to direct measurement of soil gas flux or concentration, microbial communities may be more stable over time and provide an indication of leakages in areas that are exposed to intermittent leakage for weeks or months (INC 2017).

Of the microorganisms known to respond to gas leaks, methanotrophs (organisms able to oxidise methane) are a subset of the methylotrophs that can oxidise single carbon molecules, such as methane and methanol (unless otherwise stated, the term methanotrophs is used to describe organisms capable of oxidising methane, while methylotrophs describe the broader group of organisms capable of oxidising single carbon molecules, including methane). Methanotrophs use methane monooxygenases in their soluble (sMMO) or particulate form (pMMO), to oxidise methane. Most methanotrophs express pMMO (McDonald, Bodrossy et al. 2008), the exception being the *Methylocella* and *Methyloferula* genera, which possess only the sMMO form (Dedysh, Liesack et al. 2000, Vorobev, Baani et al. 2011). A small group of other methanotrophs express both forms, with the sMMO enzyme expressed at low copper to biomass concentrations (Semrau, Jagadevan et al. 2013). The sMMO enzyme can oxidize C_2-C_8 alkanes in addition to methane (Colby, Stirling et al. 1977). *Methylocella* is a particularly interesting genus that is capable of growth on a variety of multi-carbon compounds such as ethanol and acetate (Dedysh, Knief et al. 2005) and has been observed to increase in relative abundance (compared to other methanotrophs) around hydrocarbon seeps (Dunfield and Dedysh 2014, Farhan Ul Haque, Crombie et al. 2018, Farhan Ul Haque, Crombie et al. 2019).

Microbial degradation of alkanes is possible by a broad range of genera (van Beilen and Funhoff 2007). These organisms might be particularly important to monitor, as methanogenesis in the subsurface could obscure a signal from thermogenic methane and detecting organisms capable of growth on SCAs may be useful to differentiate between sources. Non-methylotrophic organisms from many genera possess soluble di-iron monooxygenases (SDIMOs) and copper monooxygenases (CuMMO), the enzymes that enable oxidising a diverse range of substrates, including SCAs (Leahy, Batchelor et al. 2003, Coleman, Le et al. 2012). SDIMO has been used as a target in oil and gas prospecting (Miqueletto, Andreote et al. 2011). Microbial communities from gas seepage sites respond differently to non-seepage sites gas when exposed to butane, with Gammaproteobacteria and Betaproteobacteria showing increased abundance after exposure (Deng, Deng et al. 2018) with the SDIMO enzyme *bmoX* thought to be responsible for butane oxidation in key genera such as *Pseudomonas, Giesbergeria* and *Ramlibacte*r. Another study suggested that *Mycobacterium* and *Pseudomonas* could be indicators of hydrocarbon leakage (Deng, Yu et al. 2016).

Given the broad range of microorganisms that are potential indicators, this study uses both total bacterial community analysis based on the 16S rRNA gene and analysis of targeted functional genes, combined with culture-based methods to try to determine methods of microbial monitoring that could be used to detect gas leaks from shale gas extraction. The results from targeted functional genes will be described in deliverable D4.6 within SECURe.

2 Field sites, Materials and Methods

2.1 Field Sites

Three field sites have been used in this subtask. All three have known elevated methane levels and have access to groundwater via boreholes. Samples have been collected to compare inorganic parameters with microbiological parameters. At the Sleen site (The Netherlands), groundwater had elevated hydrocarbon concentrations (Table 6) due to an underground blowout on December 1, 1965. At Stenlille (Denmark), a leak from a gas storage facility in 1995 is still causing elevated methane and ethane levels in the aquifers above. The Vale of Pickering (VoP, UK) has been subject to independent baseline monitoring of water quality, along with other parameters, since 2015 in the vicinity of a then-proposed potential shale gas site.

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2.1.1 Sleen

On December 1, 1965, an underground blowout during an exploratory drill with a catastrophic outcome occurred near Sleen, The Netherlands. During approximately 2.5 months, near-continuous leakage of large amounts of natural gas was released into the subsurface. After the blowout, the local drinking water production company installed a network of groundwater monitoring wells to monitor for possible adverse effects on groundwater quality at the blowout site. Today, more than 50 years after the blowout, the groundwater is still impaired (Schout, Hartog et al. 2018).

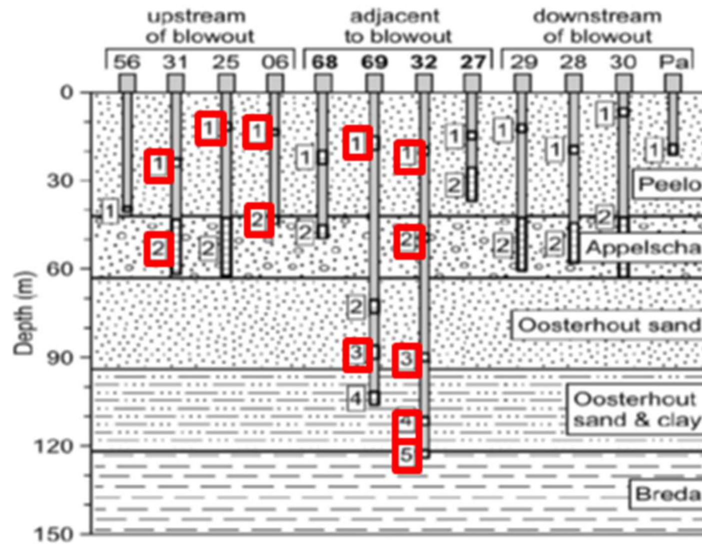

Figure 1. Shallow stratigraphy near the Sleen Site (The Netherlands) shows the dominant lithology of each formation and the approximate location, depth, length, and designation of each wellbore and individual monitoring well (Schout, Hartog et al. 2018). In red are marked wells that were sampled for DNA and culturable studies.

2.1.2 Stenlille

The Stenlille natural gas underground storage facility is located 70 km SW of Copenhagen and has been in operation since 1989. The storage operation was established to buffer the supply of gas from the North Sea. The storage facility has been re-developed over time to increase storage capacity. The two observation wells K1 and K2 have been established where the risk of leakage was considered highest. Observation well K1 allows water sampling from a melt-water sand aquifer at 36 m depth and 98 m in Palaeocene calcareous sand. Observation well K2 was established in 1993 with a screen in the sandy aquifer at 25–39 m depth (Figure 2).

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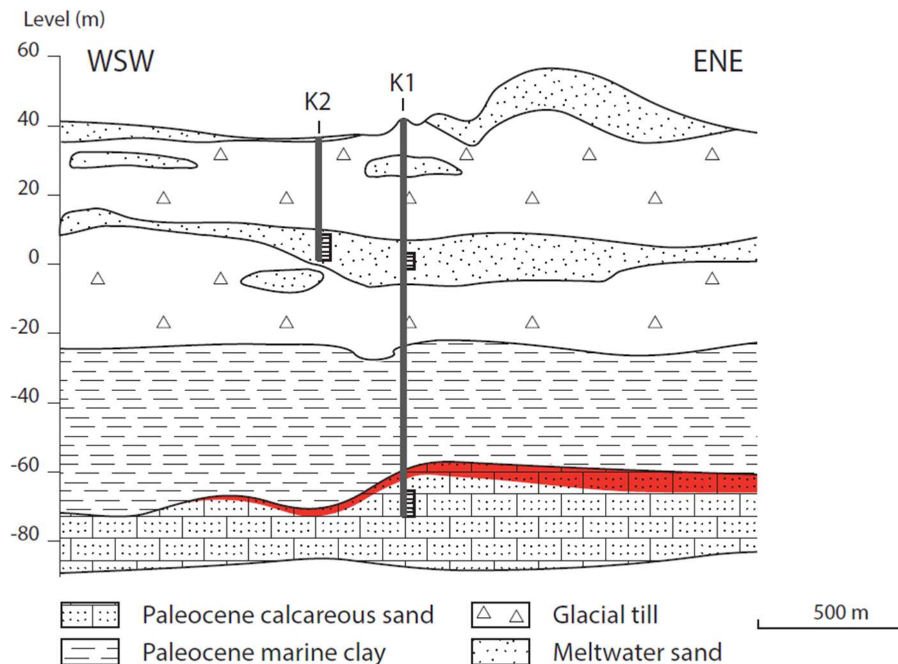

Figure 2. Geological cross-section of the upper layers of the Stenlille natural gas underground storage. Approximate location and depth of monitoring screens in K1 and K2. The probable distribution of gas after the St14 leakage in August 1995 is shown in red (modified from Laier,(2012)).

Before operational onset, the groundwater had biogenic methane with concentrations <0.5 mg/L (Laier and Øbro 2009). Hence, leaks from the gas storage are simple to monitor because of low methane background concentrations and no higher hydrocarbons. The injected gas consists of methane (89.5%), ethane (6.9%), and propane (2.6%) (Laier and Øbro 2009). Therefore, traces of ethane in groundwater would be a very sensitive indicator of gas leakage, considering the analytical detection limit of a few µg/L.

During the 30 years of operation, there has only been one known leakage. In September 1995, there was a leak due to technical problems during gas injection. Even though it was quickly stopped, an estimated 5000 m^3 leaked to geological formations above the reservoir cap rock. For further details, see Laier and Øbro, (2009). This leak has resulted in elevated methane and ethane concentrations in the deep K1 monitoring well.

2.1.3 Vale of Pickering (VoP)

Groundwater quality at VoP, North Yorkshire, UK has been monitored at one to three-month intervals since September 2015. Initially, this was to gather baseline data on water, soil and air quality ahead of proposed shale gas explorations, along with real-time monitoring of seismicity. Due to changes in the political environment in the UK, shale-gas exploration is not now expected to proceed. However, these data provide insights into effects of urban, industrial and natural processes on air and water quality (Ward, Smedley et al. 2020). It includes detailed time series of methane concentrations in a network of boreholes (Figure 3). The near surface geology of VoP consists of a series of Jurassic clays, sandstones and limestones, covered by superficial Quaternary sediments (Bearcock, Smedley et al. 2015). There are two main shallow aquifers, one encompassing the superficial sediments and sandy horizons within clay dominated sequences of the Jurassic Ampthill and Kimmeridge clay. The second is Corallian limestone which generally has lower methane concentrations (Ward, Smedley et al. 2019). Samples from the superficial aquifer (Kimmeridge and Quaternary) were used in this report.

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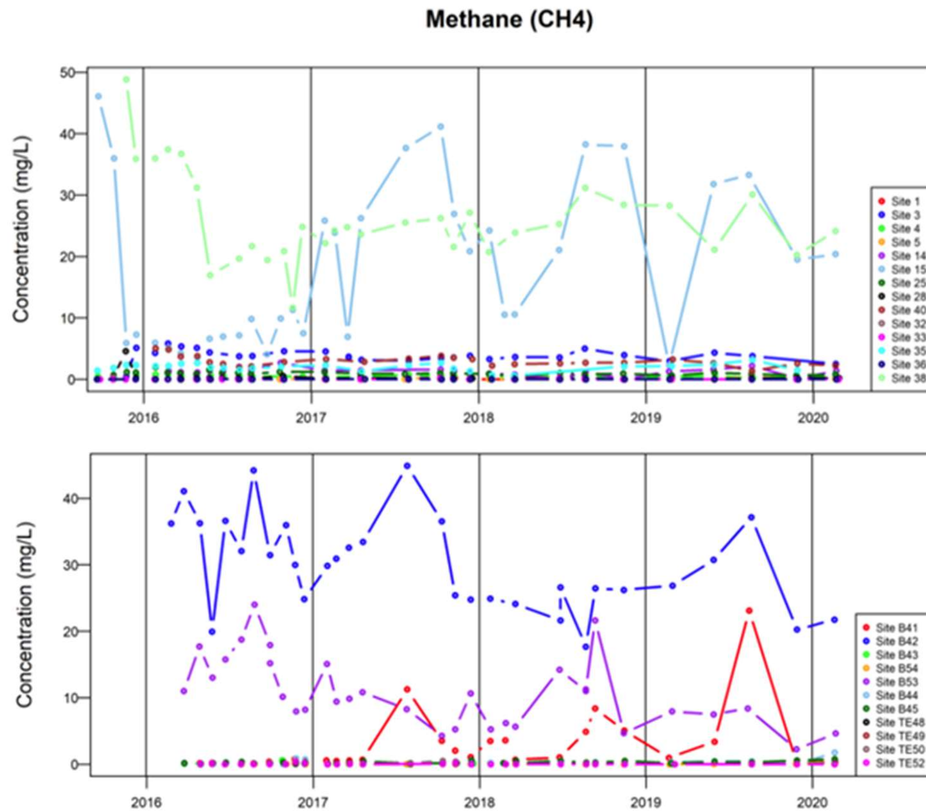

Figure 3. Methane concentrations in the superficial aquifer in the VoP (Ward, Smedley et al. 2020).

Negative $\delta^{13}C$ methane isotope data and high C_1/C_2 ratios indicate that the methane in the superficial groundwater is of biological origin, likely originating in the shallow parts of the Kimmeridge clay (Ward, Smedley et al. 2017). However, some of the isotope data and C_1/C_2 ratios lie just outside of the typical biological window (Ward, Smedley et al. 2017). The exact origin of these anomalies is unknown. The thermally mature Bowland Shale lies at ~2km below the study area. However, the lower-than-expected C_1/C_2 ratios could be due to unusually high generation of biological ethane (Taylor, Sherwood Lollar et al. 2000) and the less negative methane isotopic values can be generated by biological oxidation of methane (Golding, Boreham et al. 2013). Also, propane has not been detected in any of these groundwater samples.

2.2 SAMPLE COLLECTION

2.2.1 Field site sampling for DNA analysis

Water samples were taken when pH, O_2, and electrical conductivity (EC) electrode readings (WTW 3410 multimeter - Sleen) measured in a flow cell had stabilized. At the Sleen site, water was sampled from 14 different screens (Figure 1) in October 2019. At VoP, groundwater samples were collected for DNA analysis at nine sites in August 2019. Samples were collected from eight of these sites in February 2020.

At Sleen and VoP, 0.5–2.5 L of groundwater was filtered through a Sterivex cartridge (0.45 µm, Millipore SVHV010RS, Merck) for DNA analysis. After filtration, the inlet and outlet of the Sterivex cartridge were sealed and stored at -20 °C until further processing.

At Sleen only, samples for hydrocarbons were collected in 15 mL serum bottles with rubber septa and stored on ice until analysis within two days from sampling. Analysis of C_1-C_4 hydrocarbons was done by GC-FID (Shimadzu GC2010 equipped with a capillary column (GS-Gaspro, 60 m, 0.32 mm)) with a detection limit of 0.002 mg/L for both methane and larger hydrocarbons. Samples for major cations and alkalinity were filtered (0.2 µm) in the field into 20 mL polypropylene vials and stored cold. Samples for DOC, nitrate, ammonia, and sulphate were all filtered in the field (0.2 µm) and stored cold and frozen within 8 hours.

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2.2.2 Water sampling for culture studies

Groundwater samples from VoP were collected on three occasions, in May 2019, August 2019 and February 2020. The first two campaigns were aimed at method development; therefore, the main results section only contains data from the third VoP campaign, along with samples from Sleen. Groundwater samples were collected into sterilised 500 mL bottles. Bottles were washed out a minimum of three times in sample water before final collection. Water was filled to the top before closing. Due to the high gas content in some of the Sleen samples, degassing during sample transport created some headspace between sample collection and processing.

To test different sample processing and transport techniques, selected samples were filtered in the field. Water samples were filtered onto 47 mm diameter, 0.22 µm gamma sterile cellulose nitrate membranes (Sartorius, Gottingen, Germany). Filtering in the field was conducted using a vacuum filter housing and a handheld pump (Merck Millipore, Darmstadt, Germany), it was sterilised by autoclave before transportation to the field site and rinsed in sample water six times before use. A 50 mL sample was transferred to a vacuum filter using a 50 mL syringe. Filter membranes were handled at the edge of the membrane with tweezers sterilised using wipes, and transported in sterile 25 mL universals with top surface of membrane facing inwards. All samples and filter membranes were kept cool (on ice in the field, and fridge on return to BGS) and transferred to media within four days of collection, excluding longer term storage samples. Where possible, filtering was conducted upwind from the field team, and the filter housing was covered with a lid to minimise contamination.

2.3 MICROCOSM SETUP

In controlled microcosm experiments, methanotrophy under both aerobic and anaerobic conditions were tested on a microbial community from the deep K1 well at Stenlille and from screen 69.2 from Sleen. Both communities have adapted to elevated thermogenic methane concentrations. A total of 2400 L of groundwater was pumped and filtered over a PES filter with a pore size of 0.3 µm and 293mm in diameter (Sterlitech, Kent, WA, USA) using a submersible pump (Grundfos MP1, Grundfos, Bjerrringbro, Denmark) with a pump rate of 8–10 L/min. The filter was stored on ice after sampling, and microcosms were set up within 24 hours of collection. The filter was divided into 24 fragments, and each fragment was placed in a 583 mL flask containing 90 mL of K1 well groundwater and 10 mL salt solution. Stock solution (g/L) $MgSO_4 \cdot 7H_2O$ (1.53), KNO_3 (1.0), NH_4Cl (0.47), $NaHCO_3$ (0.065), NaCl (0.30), $CaCl_2 \cdot 2H_2O$ (0.76), $CaCO_3$ (5.0) and $Fe^{3+}OOH$ (0.44). Flasks were crimp sealed with butyl rubber stoppers and flushed with He before creating a controlled headspace comparing 1) untreated control, 2) CH_4, and 3) CH_4 and C_2H_6 under both aerobic and anaerobic conditions. Approximately 100 mL methane and 10 mL ethane were added to the headspace. Flasks were incubated in the dark at 10 °C on a rotational shaker at 75 rpm. The headspace gas concentration of CH_4, C_2H_6, O_2 and CO_2 was quantified by GC-FID (Shimadzu GC2010 equipped with a capillary column (GS-Gaspro, 60 m, 0.32 mm)) with a detection limit of 0.002 mg/L for both methane and larger hydrocarbons.

2.4 MOLECULAR TECHNIQUES

2.4.1 DNA extraction

Sterivex filters were separated from their casing before proceeding with DNA extraction. Manufacturers' protocols were followed and PowerWater DNA extraction kit (Qiagen N. V., Hilden, Germany) was used for Sleen and VoP samples. Aliquots of DNA extract from VoP were shipped on wet ice to GEUS, DK for amplicon library preparation and subsequent analysis. DNA from microcosm experiments were filtered through a 0.2 µm Supor MicroFunnel (Pall Corporation, New York, USA). Filters were stored at -20 °C before DNA extraction. DNA was extracted with DNeasy PowerWater (Qiagen).

2.4.2 Amplicon library preparation

A 2-step protocol prepared the 16S/*pmoA/mcrA* gene amplicon libraries according to details in Table 1. In the first PCR the PCR mastermix contained 10 µL Phusion buffer mix, 0.6 µL DMSO, 0.5 µL reverse and forward primers, 6.4 µL milli-Q water and 2 µL DNA template. The PCR program was set to 98 °C for 30 seconds initial denaturation step, followed by 30 cycles with 98 °C for 10 seconds, 55 °C (annealing temperature for 16S rRNA) for 30 seconds, and 72 °C for 30 seconds for denaturation, annealing, and elongation steps. A final elongation step at 72 °C for 10 minutes concluded the first PCR. For *pmoA* and *mcrA*, the annealing temperatures were 68 °C and 57.5 °C, respectively. Product purity and length was verified by agarose gel electrophoresis.

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The second step PCR was used to attach Illumina index barcodes. The PCR mix contained 10 µL Phusion buffer mix, 1 µL P5 and P7 primers, 11 µL milli-Q water, and 2 µL PCR product form the first reaction. The temperature program was set to 98 °C for 30 seconds followed by 7 cycles with 98 °C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A final elongation step of 72 °C for 10 minutes concluded the second PCR. Products were also verified on an agarose gel for purity and size. PCR products were cleaned up using a MagBio clean-up system. PCR products were quantified by Qubit fluorimeter and normalized before sequencing.

Table 1. Overview of target genes for amplicon sequencing.

Primer	Function	Target	Sequence (5'-3')	Amplicon size	Reference
515F **926R**	Generic bacteria	16S	GTGYCAGCMGCCGCGGTAA CCGYCAATTYMTTTRAGTTT	441	(Quince, Lanzen et al. 2011, Parada, Needham et al. 2016)
A189 **mb661**	Aerobic	*pmoA*	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	472	(McDonald, Bodrossy et al. 2008)
MLf **Mlr**	Anaerobic	*mcrA*	GGTGGTGTMGGATTCACACAR-TAYGCWACAGC TTCATTGCRTAGTTWGGRTAGTT	550	(Luton, Wayne et al. 2002)

2.4.3 qPCR

The abundance of the 16S rRNA in groundwater samples was analysed using quantitative real-time PCR in a CFX96 real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table 2. Overview of target genes for qPCR.

	Target	Sequence (5'-3')	Standard	Amplicon size	Reference
1369F **1492R** **TM1389F**	16S	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT CTTGTACACACCGCCCGTC	E. coli K12	123	(Suzuki, Taylor et al. 2000)

The 16S qPCR amplification was performed in 30 µL reactions containing 15 µL Lo-Rox Probe Mix (PCR Biosystems, London, UK), 1.2 µL of both primers (100 pmol/µL), 0.6 µL probe (Eurofins Genomics, Galten, Denmark), 11 µL water and 1 µL DNA template. The enzyme was activated for 3 min at 95°C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The reaction efficiencies were between 103 and 107%.

2.5 BIOINFORMATICS

The Sleen samples were performed in duplicates and 778 838 sequences were obtained. The VoP samples were conducted individually and 567 464 sequences were obtained. For the microcosm experiments, 1 359 415 sequences were obtained. The sequence read quality was controlled with FastQC (Andrews 2010), and the analysis was performed with the FROGS pipeline (Escudie, Auer et al. 2018).

The reads were merged with VSEARCH (Rognes, Flouri et al. 2016) and filtered according to the following criteria: expected amplicon size of 450 bp, minimal length of 350 bp, and maximal length of 450 bp, and no ambiguous nucleotides were allowed. The primer sequences and sequences where the two primers were not present were removed, and the sequences were dereplicated. The sequences were clustered in two steps using the swarm method (Mahe, Rognes et al. 2014). First, clustering is performed with aggregation distance equal to 1 for denoising and high clusters definition; then the second step with an aggregation distance equal to 3 on the seeds of first clustering. The chimera was removed using the VSEARCH tool with the UCHIME de novo method (Edgar, Haas et al. 2011, Rognes, Flouri et al. 2016) combined with a cross-sample validation. The taxonomic affiliation was performed using the SILVA database (Silva 132).

2.6 CULTURE BASED METHODS

Groundwater samples were vacuum filtered onto 0.22 µm gamma sterile membranes. For lab processed samples, filter housing was sterilised by autoclave between each sample and performed in laminar flow cabinet. Filters were handled at the edge of the membrane with 70% methanol sterilised and dried tweezers. All culture experiments were incubated at 25 °C in ammonium mineral salts 784 media: (per L) K_2HPO_4 (0.7 g), KH_2PO_4 (0.54 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), $CaCl_2 \cdot 2H_2O$ (0.2 g), NH_4Cl (0.5 g), $FeSO_4 \cdot 7H_2O$ (4 mg), agar

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(15 g) and trace element solution (final concentration per L): $FeSO_4 \cdot 7H_2O$ (4.0 mg), $ZnSO_4 \cdot 7H_2O$ (100 µg), $MnCl_2 \cdot 4H_2O$ (30.0 µg), H_3BO_3 (300 µg), $CoCl_2 \cdot 6H_2O$ (200 µg), $CuCl_2 \cdot 2H_2O$ (10.0 µg), $NiCl_2 \cdot 6H_2O$ (20.0 µg) and $Na_2MoO_4 \cdot 2H_2O$ (60.0 µg). Final pH 6.9. If required, filter sterilised methanol or prop-1-ol was added after autoclave to final concentration of 0.5%. For methane and propane incubations, Petri dishes were incubated in AnaeroGen bags (AN0010, Thermo Fisher Scientific Inc. Waltham, MA), modified with gas tight valves to allow for exchange of headspace gas. Samples were supplied with headspace of 10% methane or propane in air.

Table 3. Summary of variables tested during method development

Variable	Specifics
Volume filtered	10, 50 and 250 mL
Cell transfer	Impressions vs direct application
Time	1, 2, 3 and 4 weeks
Media delivery	Agar and liquid soaked pads
Carbon source	Methane / propane and methanol / propanol
Sample transport	Filtered in field vs lab
Time stored	1, 5 and 8 days

2.6.1 Colony description and population measurements

Photographs of plates were taken at two time points (between 2 and 4 weeks) and colonies counted after 2 weeks for Sleen and 3 weeks for VoP. Within each plate, colonies were primarily characterised on colour and size, and then counted. Due to their small size, often 1 mm or less, it was not possible to make consistent record of elevation, form and margin; however, these were noted if distinctive. The presence of fungal hyphae was noted but not included in diversity statistics.

Four measures of the microbial diversity were made for each of the plates, total number of colonies (N), species richness (S),

$$S = \frac{s}{\sqrt{N}} \qquad (1)$$

where S = number of colony types,
Shannon diversity index (H')

$$H' = -\sum_{i=1}^{s} p_i ln(p_i) \qquad (2)$$

where p = proportion of individual colonies (n/N), n = individual colonies of one type, and
Simpson's index (D)

$$D = \frac{1}{\sum_{i=1}^{s} {p_i}^2} \qquad (3)$$

2.6.2 Colony DNA analysis

Two samples from Sleen and two samples from VoP were selected for further analysis. Unique CFU grown with methanol and propanol were picked and streaked on separate plates to obtain single colonies. This was repeated until visual inspection indicated pure colonies. If colonies that matched the original description were obtained, these were picked with sterile 10 µL pipette tip and placed in 50 µL nuclease free water, then heated at 95 °C for 5 minutes forming crude DNA extracts. PCR was as follows 10 µL x2 iProof master mix, 0.2 pmol/µL of forward and reverse primer (Table 4), 2 µL crude DNA extract and made up to 20 µL with nuclease free water. Thermal cycler programme in Table 5. If no PCR product was amplified, PCR was repeated on same crude extract. A third attempt was made on different colony and crude extract. Purity was checked by gel electrophoresis. PCR was purified using Diffinity RapidTips (Merck Life Sciences, Darmstadt, Germany) before sequencing by Eurofins Genomics (Wolverhampton, UK) using 341F and 907R primers.

Where both forward and reverse sequences were successful, the reverse compliment of reverse sequences was obtained before alignment to forward sequence using Clustal Omega (Madeira, Park et al. 2019). The overlapping portion of aligned sequences was used for colony identification by BLASTn (Coordinators 2018), accesses 12th August 2020 for Sleen sequences, and 15th September 2020 for VoP42 sequences.

If a unique DNA sequence was returned, the crude DNA extracts were also tested for the presence of *pmoA*, *mmoX*, *Methylocella* specific *mmoX* and SDIMO functional genes (Table 4). Only one round of PCR was performed for these. PCR was as follows 5 µL x2 DreamTaq (Bio-Rad Laboratories, Hercules, CA), 0.2

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pmol/µL of forward and reverse primer (Table 4), 1 µL crude DNA extract and made up to 10 µL with nuclease free water. Thermal cycler programmes in Table 5.

Table 4. PCR primers used for colony PCR

Target	Primer name	Sequence	Reference
16S (full)	27F	AGAGTTTGATCCTGGCTCAG	
	1387R	GGGCGGWGTGTACAAGGC	
Methane mono-oxygenase (pmoA)	A189f	GGNGACTGGGACTTCTGG	*Costello and Lidstrom (1999)*
	mb661r	CCGGMGCAACGTCYTTACC	
Methane mono-oxygenase (mmoX)	mmoX206f	ATCGCBAARGAATAYGCSCG	*Hutchens et al. (2004)*
	mmoX886r	ACCCANGGCTCGACYTTGAA	
***Methylocella* specific methane mono-oxygenase (mmoX)**	mmoXLF	GAAGATTGG GGCGGCATCTG	*Rahman et al. (2011)*
	mmoXLFR	CCCAATCATCGCTGAAGGAGT	
SDIMO	NVC57	CAGTCNGAYGARKCSCGNCAYAT	*Coleman et al. (2006)*
	NVC66	CCANCCNGGRTAYTTRTTYTCRAACCA	
Sequencing primers	341F	CCTACGGGAGGCAGCAG	
	907R	CCGTCAATTCMTTTRAGTTT	

Table 5. PCR protocols for colony PCR. D. – denaturing, A. – annealing, E. – extension

Target	Hot start (°C / min)	D. (°C / s)	A. (°C / s)	E. (°C / s)	Cycles	D. (°C / s)	A. (°C / s)	E. (°C / s)	Cycles	Final E. (°C / min)
16s rRNA	95 / 2					95 / 30	66 / 30	72 / 120	35	72 / 5
SDIMO	95 / 2								35	72 / 5
pMMO (*pmoA*)	95 / 2	95 / 10	68 (-1) / 30	72 / 25	10	95 / 10	58 / 30	72 / 25	25	72 / 5
sMMO (*mmoX*)	95 / 2	95 / 10	72 (-1) / 30	72 / 45	10	95 / 10	62 / 30	72 / 45	25	72 / 5
Methylocella (*mmoX*)	95 / 2	95 / 10	68 (-0.5) / 30	72 / 30	10	95 / 10	65 / 30	72 / 45	25	72 / 5

2.6.3 Image analysis

Manual cell counts of colonies is both time consuming and open to user bias, therefore automated cell counting options were explored. Four mobile phone apps were tested for automated cell counts: Promega Colony counter (Promega), ADP Colony Counter App Lite (ADP Lab), Microbial Colony counter (RoboMX) @BactLAB (TCS Biosciences Ltd) and CountThings from Photos. Only BactLab has the ability to distinguish colonies of different morphologies, however this was linked to bespoke microbial analyses and did not have any flexibility in terms of classification by a range of size, shape or colour.

Images of plates were taken with phone camera (Sony Xperia Premium ZX) and were cropped manually using ImageJ (V 1.51) to contain the Petri dish only. CellProfiler (V 4.0.2) was used to identify the presence of colonies on a plate. Colony identification is primarily based on taking the absolute difference between each colour channel and the grayscale image of the combined channels, primarily to reduce the effects of the gridded background. Each channel is then used to identify objects (presumed colonies) before the objects are combined and data exported.

These parameters were passed onto a script that creates a Self-Organising Map, with each object forming a point on the map, written in R (V4.0.2) using Kohonen library. A Self-Organising Map is an unsupervised machine learning algorithm which seeks to cluster similar points together. In this case, objects with similar parameters are clustered together. The script automatically places similar objects into distinct clusters and automatically determines the number of object types present. By then presenting the user with plots of how objects are assigned, the number of objects can be adjusted if mis-classifications are visible. The script is used as a backend for an interactive dashboard where a user is able to upload their own image and output from CellProfiler and receive output plots and counts for each object type. The results are available as a .csv output.

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3 Results

3.1 BACKGROUND DATA FROM FIELDSITES

Table 6 and Table 7 gives an overview of hydrocarbon concentrations concurrently with field parameters in groundwater samples. Additional inorganic parameters and dissolved organic carbon for the Sleen site are presented in Table 8.

Table 6. Sleen chemical data from water samples from the wells and screens.

Well/screen	Depth	Formation	CH_4 mg/L	C_2H_6 mg/L	C_3H_8 mg/L	pH	Dissolved Oxygen %	Conductivity µS/cm
6.1	-12.7	Peelo	2.2	0.0	0.0	6.92	1.7	647
6.2	-41.2	Appelscha	5.2	0.0	0.0	6.82	1.6	392
25.1	-12.5	Peelo	0.2	0.0	0.0	7.0	2.0	448
25.2	-56.2	Appelscha	0.4	0.1	0.0	5.7	0.2	292
31.1	-27.0	Peelo	0.1	0.0	0.0	5.6	0.1	287
31.2	-55.0	Appelscha	0.1	0.0	0.0	6.4	0.1	330
32.1	-20.0	Peelo	6.0	0.2	0.0	7.0	3.7	1530
32.2	-49.0	Appelscha	9.5	0.3	0.0	7.0	0.0	350
32.3	-88.0	Oosterhout	16.3	1.1	0.0	7.4	1.4	357
32.4	-109.0	Oosterhout	48.6	3.1	0.1	7.6	2.1	542
32.5	-120.0	Breda	36.9	2.2	0.1	8.1	1.0	1698
69.1	-17.5	Peelo	8.1	0.5	0.0	6.9	2.0	1464
69.2	-72.5	Oosterhout	42.2	2.5	0.1	7.4	0.9	308
69.3	-87.5	Oosterhout	39.1	2.6	0.1	7.5	0.3	590

ND – not determined

Table 7. VoP chemical data from groundwater samples.

Site name	Date	CH_4 mg/L	C_2H_6 mg/L	pH	Dissolved Oxygen %	Conductivity µS/cm
Site 3	Aug-19	3.776	0.0013	8.96	0.008	1020
Site 15	Aug-19	33.284	0.481	8.21	0.002	2370
Site 25	Aug-19	0.8561	0	7.36	0	737
Site 38	Aug-19	30.099	0.168	8.44	0.004	1618
Site B42	Aug-19	37.141	0	7.4	0	2001
Site B43	Aug-19	0.0004	0	8.59	0.023	2561
Site B45	Aug-19	0.262	0	6.96	0.008	2139
Site B53	Aug-19	8.389	0	7.24	0.006	964
Site B55	Aug-19	17.492	0.0242	12.2	0.013	5400
Site 3	Feb-20	2.4879	0.005089	8.41	0.044	986
Site 15	Feb-20	20.37	0.3580	8.32	0.028	2229
Site 25	Feb-20	0.791	0	7.41	0.007	720
Site 38	Feb-20	24.14	0.1299	8.34	0.016	1587
Site B42	Feb-20	21.75	0.00375	7.37	0.013	1905
Site B43	Feb-20	0.2159	0	8.47	0.048	2508
Site B45	Feb-20	0.7595	0	6.94	0.062	2958
Site B53	Feb-20	4.630571	0	7.05	0.019	1093
Site 5	Feb-20	0.111571	0	6.91	0.047	737
Site 40	Feb-20	2.2162	0.018348	8.9	0	2276
Site 34	Feb-20	0.000286	0	7.22	1.012	454

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Site B54	Feb-20	0.0055	0	7.84	0.024	1078

Table 8. Additional chemical data from the Sleen groundwater samples

Well/screen	DOC mg/L	NH_4 µg/l	Alk (HCO_3) meqv/L	Na mg/L	Mg mg/L	Al mg/L	K mg/L	Ca mg/L	Fe mg/L	Mn mg/L
6.1	7.6	1004	3.14	42.13	6.56	0.35	3.10	74.15	31.63	0.95
6.2	6.6	112.0	2.00	32.54	3.26	0.23	1.15	35.83	21.81	0.58
25.1	1.4	1465	0.73	19.80	2.57	0.17	1.64	22.99	13.37	0.19
25.2	2.3	1345	0.82	19.20	4.82	0.11	2.11	49.09	14.92	0.25
31.1	2.8	165.9	0.48	31.73	0.54	0.07	4.47	2.88	29.28	0.11
31.2	1.0	188.2	0.34	25.09	2.83	0.05	1.18	20.52	24.78	0.22
32.1	11.3	651.5	10.50	157.5	13.20	0.04	2.26	166.3	64.57	0.72
32.2	2.1	615.6	1.10	25.78	5.02	0.04	2.07	32.25	8.72	0.46
32.3	0.7	261.9	3.65	12.90	6.17	0.05	2.42	59.71	2.86	0.06
32.4	0.8	198.7	6.73	83.93	4.81	0.04	4.09	34.23	1.14	0.10
32.5	4.1	686.6	4.27	296.4	11.56	0.04	13.72	32.32	0.84	0.09
69.1	11.5	807.5	6.84	149.5	11.91	0.02	2.94	129.1	54.40	0.42
69.2	2.1	86.6	2.54	28.80	3.66	0.02	1.47	33.89	2.29	0.03
69.3	1.2	201.3	4.11	52.85	7.71	0.03	3.86	68.60	3.56	0.04

3.2 METHOD DEVELOPMENT

Samples from two field campaigns in VoP (May19 and Aug19) were used to refine sampling strategy and microbial growth conditions. Table 9 shows the refinements tested during these two field campaigns and common outcomes applied to subsequent samples from Sleen and VoP Feb20. For the final two parameters (sample transport and time stored), additional samples were collected at Sleen and VoP Feb20.

Table 9. Refinement of sample parameters

Variable	Specifics	Observations	Outcome
Volume filtered	50 and 250 mL	Frequently colonies were too numerous at 250 mL	50 mL
Cell transfer	Impressions vs direct application of membrane to growth media	Limited growth by transferring cells using an impression, reasonable growth observed by direct application	Direct application
Incubation time	1, 2, 3 and 4 weeks	Colonies appear after 1 week, but generally too small to reliably count	Images at minimum of two time points, between 2 and 4 weeks, colonies counted at one of these timepoints
Media delivery	Agar and liquid soaked pads	Minimal growth on soaked pads	Agar
Carbon source	Methane / propane and methanol / propanol	Different colony numbers, morphologies and distribution with each carbon source	All
Sample transport	As filters or as water	Viable growth observed when filtered in field and laboratory, with potential difference in microbial diversity (Figure 4). More fungal growth was apparent with in field filtering	Use samples filter in laboratory, further investigations needed
Time stored	0, 1, 4 and 7 days	Growth was still viable after 7 days storage, with no clear trends (Figure 4). However prolonged storage of some Sleen samples allowed for precipitate to form (Figure 5), leading to changes in microbial growth	Test within 4 days of collection, further investigations needed with some sample types

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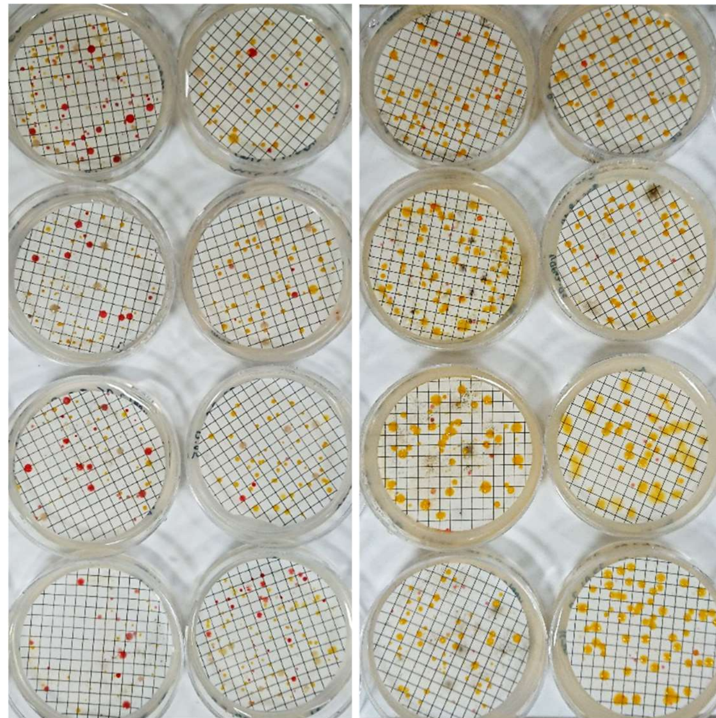

Figure 4. VoP Site 3, Feb20. The left image shows growth on methanol the right image is on propanol. Within each image the Petri dishes are as follows: top to bottom time from sample collection 1 day, 2 days, 5 days and 8 days. Within the image, the left column of Petri dishes is filtered in the field, right column of Petri dishes is filtered in the laboratory.

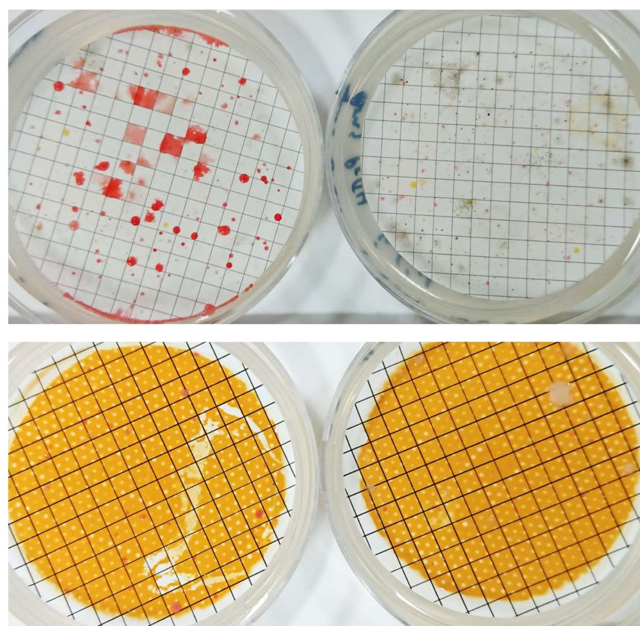

Figure 5. Filter membranes from Sleen 6.2 grown on methanol (left Petri dish in each image), propanol (right Petri dish in each image), showing difference between filtering in field (top) and laboratory (bottom).

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3.3 NATURAL DIVERSITY

3.3.1 DNA based

From the different wells at the Sleen site, the bacterial community seems to be influenced by methane concentrations ranging from 0.1 to 48.6 mg/L as listed in Table 6. As shown in Figure 6, low methane concentrations (<0.2 mg/L) cluster separately from the rest of the wells. Wells 6.1 and 6.2 are from the upstream wells (Figure 1) and cluster together.

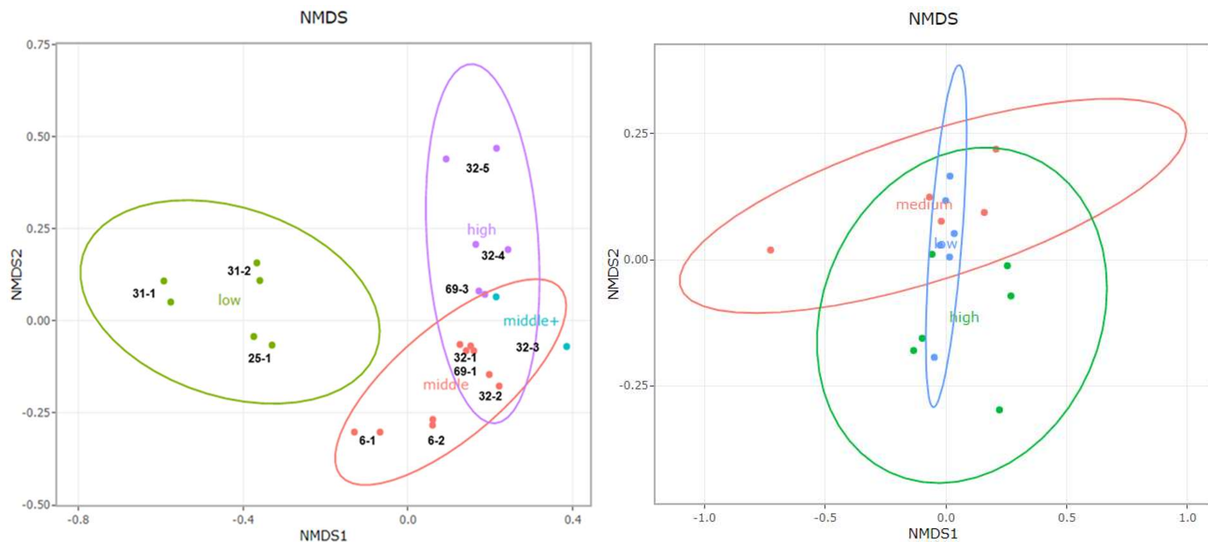

Figure 6. Principal component analysis (PCA) plot of the groundwater microbial community from Sleen (left) and Vale of Pickering (right). Circled coloured areas indicate if microbial communities cluster due to methane concentrations.

Samples from VoP cluster more closely and there are no distinct groups as influenced by the methane concentration. The sample on the right of the image shows a distinct community and has a much higher pH (12.2) than other samples (6.91 to 8.96). Many of the wells are affected by biogenic methane, which may be influenced by seasonal changes and hence methane concentrations.

3.3.2 Culture based

A total of 28 groundwater samples we collected from two sites. Selected samples were filtered in duplicate or triplicate, including filtering in the field giving a total of 38 analyses. Using the results from method development (Sections 3.2), 50mL fluid was filtered onto membranes before incubating with all four carbon sources (methane, propane, methanol and propanol). Diversity indices were calculated for each carbon source. Samples were classified by methane/ethane molar ratio: <100 – low, 100-1000 medium and >1000 high and growth with C_1 and C_3 sources was compared (Figure 7 and Figure 8).

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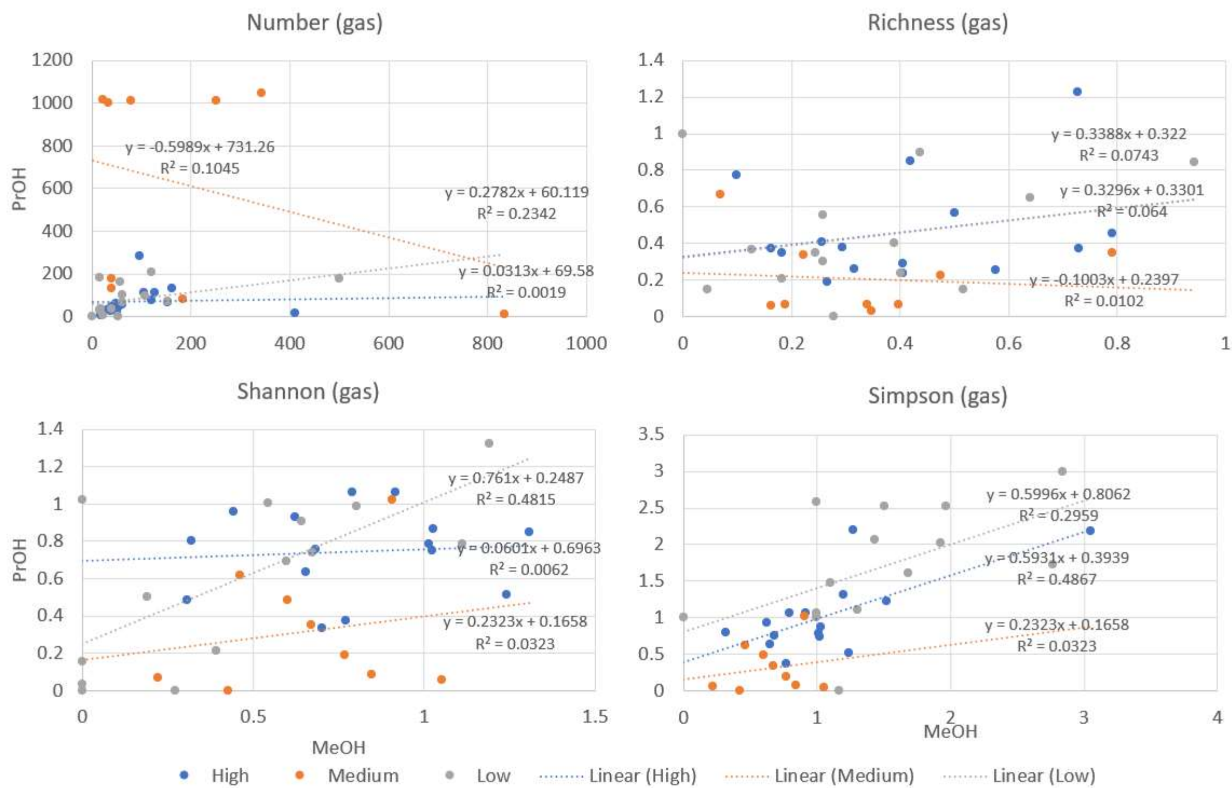

Figure 7. Comparison of diversity indices using manual cell counts for methane (x axis) and propane (y axis).

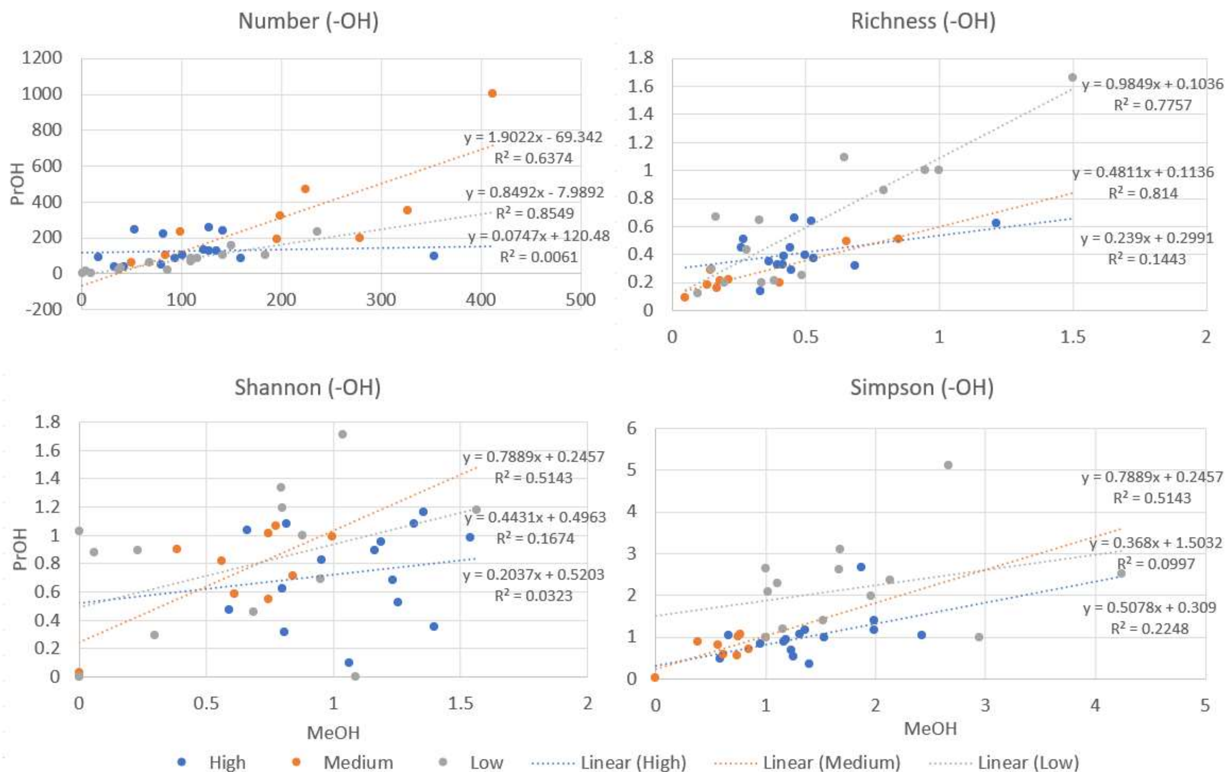

Figure 8. Comparison of diversity indices using manual cell counts for methanol (x axis) and propanol (y axis). Legend as in Figure 7.

If species diversity is related to C_1:C_2 ratio of the sample it would be expected that the slope of the high C_1:C_2 samples would be the shallowest, the low C_1:C_2 ratio the steepest and the mid intermediate to these two. This

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pattern was seen with richness when grown on methanol and propanol. Removal of outliers could reveal similar patterns with other indices. The ratio of richness from the methanol vs propanol was selected as predictor of C_1:C_2 ratio in original groundwater (Table 10).

Table 10. Use of ratio of species richness to predict C_1:C_2 ratio in groundwater

C_1:C_2 ratio in groundwater	Ratio of richness methanol / richness propanol	
	>1	<1
High	11	4
Medium	5	4
Low	3	11

Two samples from Sleen (plated in duplicate) and two samples from VoP Feb 20 (plated in triplicate) were selected for colony PCR. From these samples 43 isolates with unique 16S rRNA sequences were obtained (Table 11). The most common genus identified was *Methylobacterium* (21.6% of those identified to genus level or below), and was found in all samples and grows in presence of methanol and propanol. The following observations were made when more than one representative from the same genera or otherwise closely related species were observed. The following genera were observed growing on methanol only: *Hyphomicrobium*, *Methylophilus*, *Methylorubrum*, *Pedobacter* and *Sphingomonas*. For propanol only the genera were: *Rhodococcus* and *Xanthobacter*. *Ancylobacter* was observed growing with methanol and propanol.

Only four isolates were identified to genus level from samples with low C_1:C_2 ratio, eight from medium C_1:C_2 and 25 from high C_1:C_2 ratio meaning any observations on patterns in the samples can only be used as potential indicators. *Methylophilus, Methylorubrum* and *Pedobacter* were observed with high and medium C_1:C_2 ratios, the remaining methanol only isolates were all from high C_1:C_2 ratio samples (*Hyphomicrobium* and *Sphingomonas*). *Corynebacterium*, *Nocardia* and *Rhodococcus* form a clade of known propane oxidisers, of the four isolates identified from this clade, three are from low or medium C_1:C_2. In contrast to the previous complex, of the five *Xanthobacter* isolates, four were isolated from samples with high C_1:C_2 ratio.

Table 11. Details for isolates where 16S identification could be made. Yes, PCR product of correct size, No – no PCR product, ? – multiple PCR products or of incorrect size. Green samples high C_1:C_2, orange: medium C_1:C_2, red: low C_1:C_2. *mmoX* gene was also tested for however no isolates yielded a positive PCR product. MeOH- methanol, PrOH - propanol

Isolate	Sample	plate	number	colour	Top match	Identity (%)	SDIMO*	Methylocella*	pmoA*
SL01	06.2	MeOH	1	translucent	Uncultured bacterium clone	93.44	?	Yes	No
SL02	06.2	MeOH	1	cream	*Hyphomicrobium* sp.	98.00	Yes	Yes	No
SL03	06.2	MeOH	27	pink	*Methylorubrum extorquens*	100	No	No	No
SL04	06.2	MeOH	7	pale pink	*Methylobacterium* sp.	99.79	No	Yes	No
SL05	06.2	PrOH	7	orange/ cream	*Rhodococcus erythropolis*	99.80	Yes	Yes	No
SL06	06.2	PrOH	25	pink	*Methylobacterium* sp.	96.94	Yes	No	No
SL07	06.2	PrOH	4	white	*Curvibacter* sp.	99.01	No	Yes	No
SL16	06.2	MeOH	18	cream	*Hyphomicrobium* sp.	100	Yes	Yes	No
SL17	06.2	MeOH	33	dark pink	*Methylobacterium* sp.	100	No	Yes	No
SL21	06.2	PrOH	15	yellow	*Xanthobacter* sp.	97.88	Yes	Yes	No
SL49	32.4	MeOH	2	yellow-cream	Uncultured bacterium	99.50	No	Yes	No

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SL51	32.4	MeOH	67	pink	*Methylobacterium* sp.	100	Yes	Yes	No
SL52	32.4	PrOH	1	cream	*Corynebacterium pseudotuberculosis*	99.28	Yes	Yes	No
SL53	32.4	PrOH	35	yellow	*Chitinophaga* sp.	100	No	No	No
SL54	32.4	PrOH	7	yellow	*Xanthobacter* sp.	99.38	Yes	No	No
VP101	Site 15	PrOH	2	pink	Uncultured bacterium	100	Yes	Yes	No
VP102	Site 15	PrOH	4	pale pink	*Methylobacterium* sp.	99.80	No	No	No
VP103	Site 15	PrOH	105	yellow	*Pseudoxanthomonas* sp.	97.66	No	Yes	No
VP104	Site 15	PrOH	78	white/cream	*Nocardia* sp.	99.50	No	No	Yes
VP106	Site 15	PrOH	10	orange	*Rhodococcus* sp.	99.70	No	No	No
VP110	Site 15	PrOH	104	pink	Uncultured Bacteroidetes	95.32	No	No	No
VP111	Site 15	MeOH	49	pale pink	*Methylobacterium* sp.	100	No	Yes	?
VP115	Site 15	MeOH	56	pale pink	*Pedobacter* sp.	98.32	?	No	No
VP116	Site 15	MeOH	16	white/ translucent	Alpha proteobacterium	99.60	No	Yes	No
VP117	Site 15	MeOH	10	translucent/ cream	*Ancylobacter lacus*	98.72	No	No	No
VP118	Site 15	MeOH	2	dark yellow	*Methylophilus flavus*	99.60	No	Yes	Yes
VP119	Site 15	MeOH	71	pink	*Methylorubrum* sp.	100	No	Yes	No
VP120	Site B42	PrOH	?	cream	*Brevundimonas mediterranea*	99.90	Yes	No	No
VP123	Site B42	PrOH	1	cream	Uncultured bacterium	99.80	No	Yes	No
VP124	Site B42	PrOH	59	yellow	*Ancylobacter lacus*	99.11	Yes	Yes	No
VP125	Site B42	PrOH	2	orange	*Xanthobacter polyaromaticivorans*	99.80	No	No	No
VP126	Site B42	PrOH	60	yellow	*Xanthobacter* sp. strain	100	No	Yes	No
VP128	Site B42	PrOH	180	yellow	*Xanthobacter* sp.	99.21	No	Yes	No
VP130	Site B42	MeOH	72	pale pink	*Pedobacter ginsengisoli*	98.99	Yes	No	No
VP131	Site B42	MeOH	38	pink	*Methylobacterium* sp.	99.10	No	Yes	No
VP133	Site B42	MeOH	206	cream	*Methylopila oligotropha*	99.90	No	Yes	No
VP134	Site B42	MeOH	17	yellow	*Pedobacter* sp.	96.22	Yes	No	No
VP136	Site B42	MeOH	14	pink	*Sphingomonas* sp.	99.29	Yes	Yes	No
VP137	Site B42	MeOH	12	yellow	*Mesorhizobium* sp.	100	No	No	No
VP138	Site B42	MeOH	22	cream	*Sphingomonas* sp.	98.08	No	No	No
VP139	Site B42	MeOH	18	yellow	Uncultured *Methylophilus* sp.	99.61	?	No	No
VP140	Site B42	MeOH	63	pale pink	*Sphingomonas* sp.	98.97	No	No	No

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VP141	Site B42	MeOH	1	cream	*Methylobacterium* sp.	100	No	Yes	No

*Although isolates have been re-streaked, purity was not confirmed and therefore presence of these genes could be from contaminants

3.4 MICROCOSMS

3.4.1 Changes in gas composition

Filtered groundwater from the two sites differs in inorganic parameters as listed in Table 12. The estimated concentration of dissolved methane was 10 mg/L in the microcosm setup. Therefore, the Stenlille sample was exposed to a higher concentration than the 0.58 mg/L in the well. For the Sleen sample the opposite situation occurred, where the concentration in the well was 42.2 mg/L.

Table 12. Inorganic parameters from groundwater well K1, Stenlille and screen 69.2, Sleen.

Well	depth	CH_4	C_2H_6	C_3H_8	O_2	F	Cl	Br	NO_3	PO_4	SO_4
	m	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
K1	98-130	0.58	0.03	0	0	5,22	1399	8,74	<0,05	<0,05	62,0
69.2	80-90	42.2	2.5	0.1	0	0,06	16,82	0,51	<0,05	<0,05	0,06

From Stenlille and Sleen, oxidation of methane only occurred under aerobic conditions even though the added salt solution contained NO_3^-, SO_4^{2-} and Fe^{3+}, shown to work as electron acceptors during anaerobic methane oxidation (Ettwig, Zhu et al. 2016, Ren, Ma et al. 2018). Anaerobic methane oxidation is known to be carried out by a subset of methanogens who are able to form syntrophic relationships with sulphate, nitrate or iron reducing microorganisms in order to reverse the methanogenesis pathway to oxidise methane (including the *mcrA* gene) (Knittel and Boetius 2009). During the 206-day incubation study from Stenlille, the average methane oxidation rate was 35.97 µmol L^{-1} day^{-1} and 27.99 µmol L^{-1} day^{-1} in a headspace of methane or methane/ethane, respectively. The average ethane oxidation rate was 11.07 µmol L^{-1} day^{-1}. During the 63-day Sleen incubation study, the average oxidation rate was 414 µmol L^{-1} day^{-1} and 413 µmol L^{-1} day^{-1} in a headspace of methane or methane and ethane, respectively. The average ethane oxidation rate was 89 µmol L^{-1} day^{-1}. A faster oxidation rate from the Sleen samples can be explained by a bacterial community that is already adapted to higher methane concentrations.

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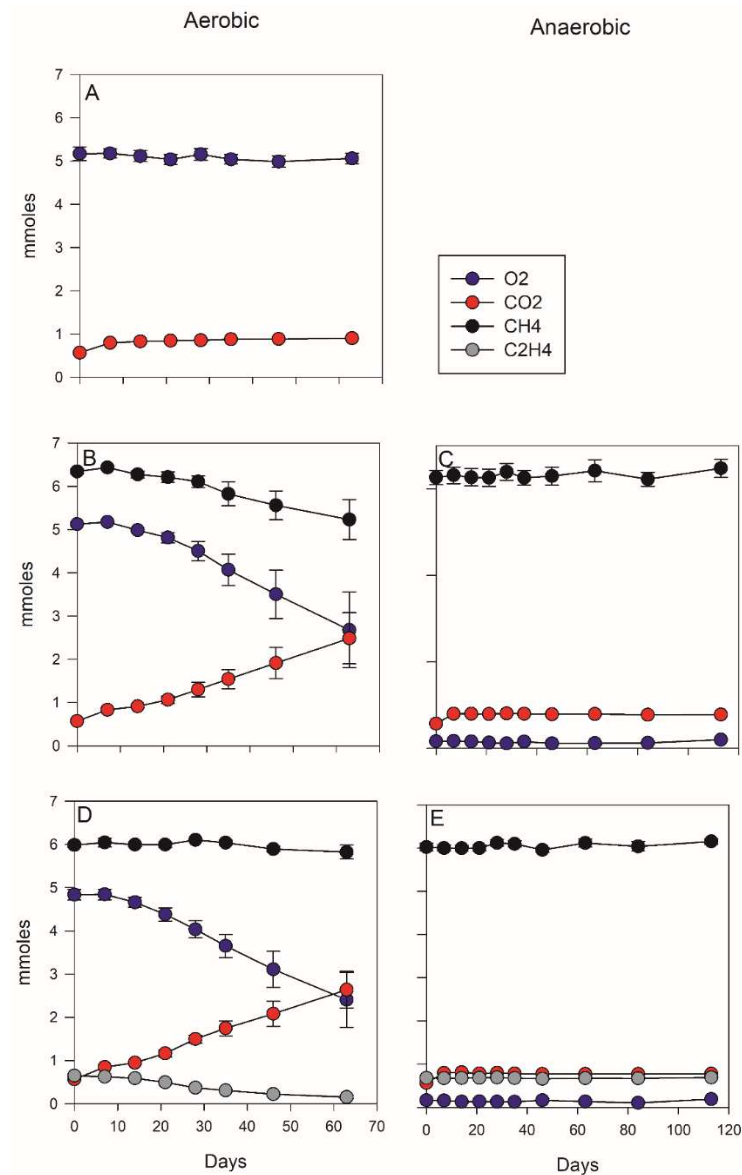

Figure 9. Sleen: total amounts in the system in mmoles per microcosm. A, B and D are aerobic treatments, including control (A), methane treatment (B), and methane and ethane treatment (D). C and E are anaerobic treatments, including methane treatment (C) and methane and ethane treatment (E). Note the difference in days for aerobic and anaerobic treatments.

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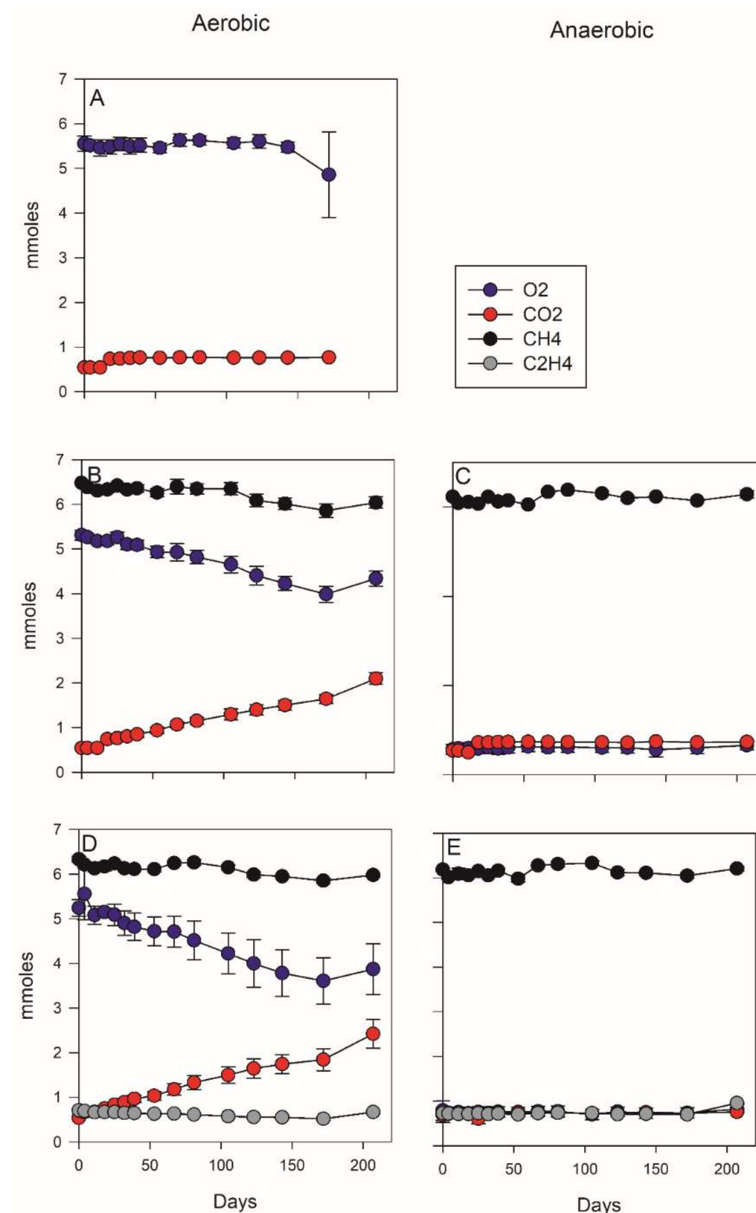

Figure 10. Stenlille: total amounts in the system in mmoles per microcosm. A, B and D are aerobic treatments, including control (A), methane treatment (B), and methane and ethane treatment (D). C and E are anaerobic treatments, including methane treatment (C) and methane and ethane treatment (E).

3.4.2 Natural microbial composition

The natural bacterial community from the Sleen site is dominated by Proteobacteria (Gammaproteobacteria), Firmicutes (Desulfotomacula), Verrucomicrobia (Omnitrophia), and Bacteroidetes (Bacteroides). In contrast the natural bacterial community from Stenlille was dominated by Desulfobacteria (Desulfovibrionia, Desulfobacteria), Caldatribacteriota (JS1), and Bacteroidetes (Bacteroides, Ignavibacteria). Desulfobacteria correlates with the higher concentration of sulphate at Stenlille (Table 12). Initial bacteria diversity of the sites is presented as Day 0 in Figure 12 and Figure 13.

PCA was applied to 16S analysis for each site to look for differences among the treatments (Figure 11). For both sites there is a difference between Day 0 (start of the experiment) and the end of the experiments. The Sleen samples are scattered where the two anaerobic treatments (methane and methane/ethane) cluster with No gas control. The three aerobic treatments are more separated. At Stenlille, the three aerobic treatments (oxygen, oxygen/methane and oxygen/methane/ethane) cluster as well as the three anaerobic treatments (No, methane and methane/ethane).

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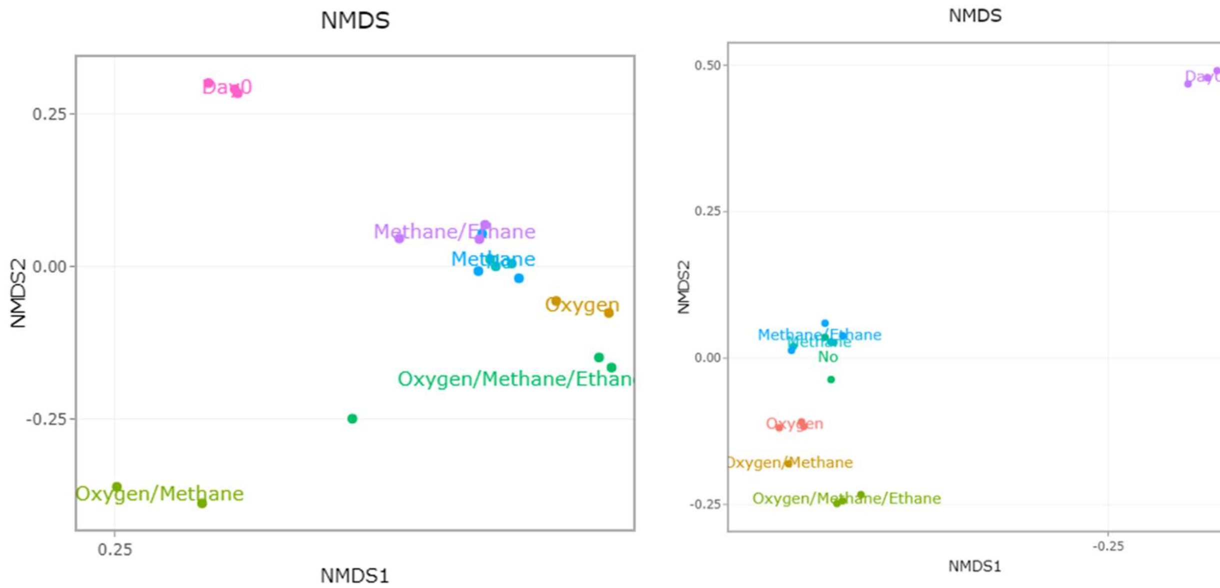

Figure 11. Microbial community influenced the different microcosm treatments for Sleen (left) and Stenlille (right). No: no additional gas. 'No' gas control label from Sleen is underneath the Methane label.

3.4.3 Changes in microbial composition

Figure 12 shows strong development of Gammaproteobacteria with oxygen and oxygen/methane/ethane at the Sleen site. In these microcosms the Burkholderiales is mainly *Hydrogenophaga*. Figure 13 shows strong development of Gammaproteobacteria (Burkholderiales) in oxygen, oxygen/methane and oxygen/methane/ethane in microcosms from Stenlille. Burkholderiales is dominated by *Acidovorax* and *Methyloversatilis* genera.

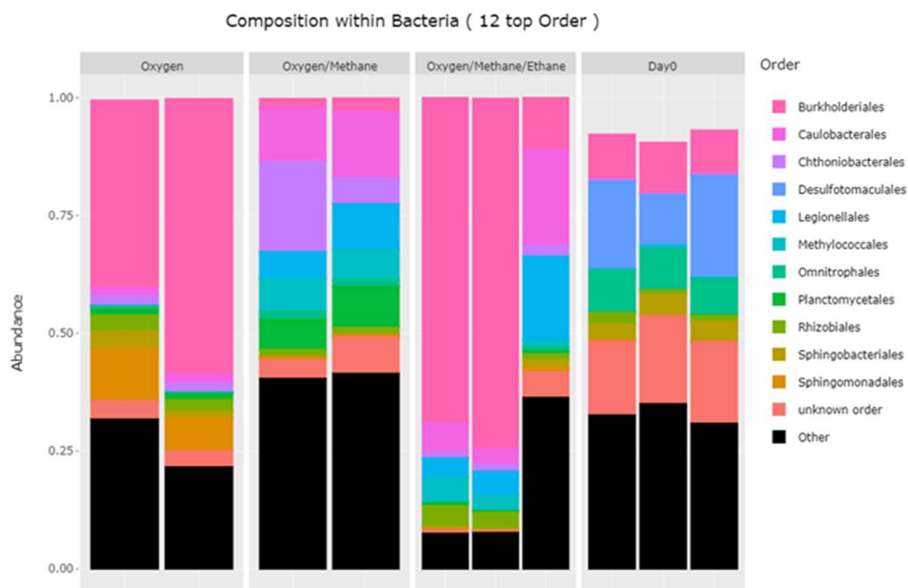

Figure 12. Composition of the top 12 microbiological orders from microcosms from the Sleen site comparing Day 0 with oxygen, oxygen/methane and oxygen/methane/ethane headspace.

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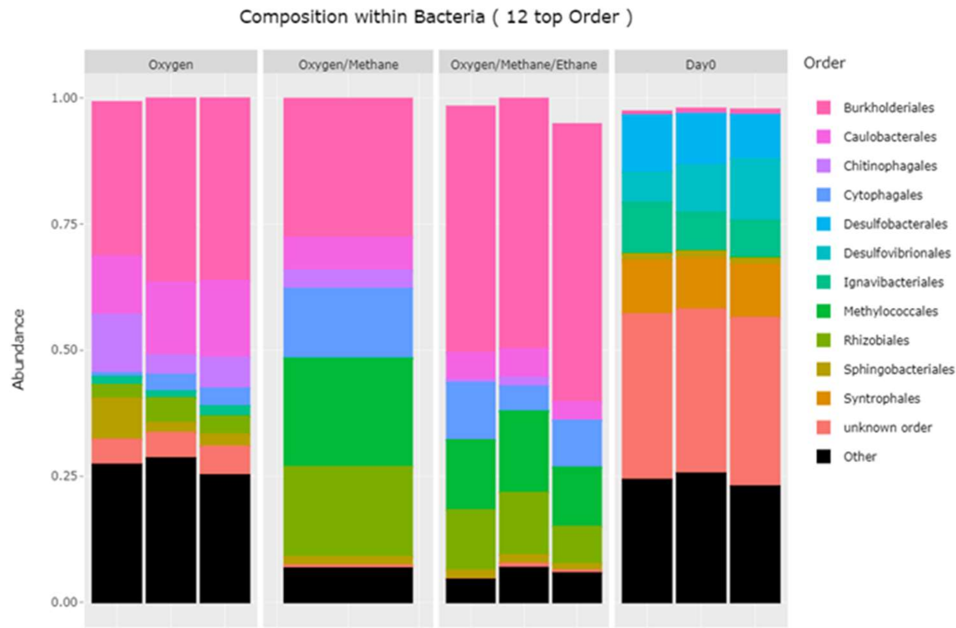

Figure 13. Composition of the top 12 orders from microcosms from the Stenlille site comparing Day 0 with oxygen, oxygen/methane and oxygen/methane/ethane headspace.

Table 13 and Table 14 provide an overview of the operational taxonomic units (OTUs) that changed significantly during the microcosm experiment, and are known to be related to methylotrophy. For the Sleen experiment amplicon reads were normalized to 2900 OTU. The strongest development of *Terrimicrobium* was seen under the aerobic methane treatment covering 25-30% of the total community. In the Stenlille experiment, there was a significant increase in *Methylomonas* and *Methyloversatilis* in the aerobic treatments and an increase in *Methylobacillus* and *Methylobacter* in the anaerobic treatment. Significant differences in the 16 S dataset were established when the p-value was less than 0.05 according to a student t-test.

Table 13. Bacterial groups, where there was a significant difference between oxygen treatment and either of the two methane treatments for aerobic experiments and for anaerobic experiments comparing the control with the two methane treatments. For "*Candidatus* Methanoperedens" there was a significant decrease in abundance.

Sleen	Day 0	O_2	O_2, CH_4	O_2, CH_4 C_2H_6	No	CH_4	CH_4, C_2H_6
Hyphomicrobium	4.3	2.0	24.5	29.7	1.7	1.7	1.3
Terrimicrobium	15.0	7.5	885.0	108.3	4.0	6.3	12.0
Methylomonas	0.0	0.0	43.5	12.0	0.0	0.7	0.0
Methylophilaceae	0.3	0.0	1.0	19.7	0.0	0.3	0.0
Crenothrix	19.7	0.5	392.5	228.7	0.0	1.7	0.7
"*Candidatus* Methanoperedens"	36.0	5.5	0.0	0.0	1.7	3.7	1.7
Methylocella*	49.7	34.0	15.0	56.7	16.7	23.7	44.0

*For *Methylocella* there was not a significant change due to treatment, but this has been included because of predicted importance and presence of *Methylocella* related *mmoX* genes from isolates from both sites

Table 14. Bacterial genera, where there was a significant difference between oxygen treatment and either of the two methane treatments for aerobic experiments and for anaerobic experiments comparing the control with the two methane treatments.

Stenlille	Day 0	Control O_2	O_2, CH_4	O_2, CH_4 C_2H_6	Control He	CH_4	CH_4 C_2H_6
Methylobacillus	1.3	2.7	0.0	0.0	7.3	141.7	107.0
Methylobacter	10.3	0.0	0.0	27.3	1.7	140.7	107.3

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Methylomonas	0.7	1.3	1527	923.7	0.0	3.7	1.3
Methyloversatilis	0.0	5.3	458	1333	6.3	5.0	9.7

3.5 SELECTED MICROBIAL GROUPS IN GROUNDWATER SAMPLES

The abundance of the selected microbial groups listed in Table 13 and Table 14 were used to interrogate the two datasets from Sleen and VoP groundwater samples. Figure 14 shows the abundance of these indicators per well from the Sleen site. Archaea and bacteria OTU were normalized to 2900 OTU per well from the Sleen site which means that “*Candidatus* Methanoperendens” is the most common methanotroph covering up to 10% of the total community. In general, bacterial OTU were much lower (<1%) than archaea. There is also no clear trend between the abundance of selected groups and the methane concentration of the wells.

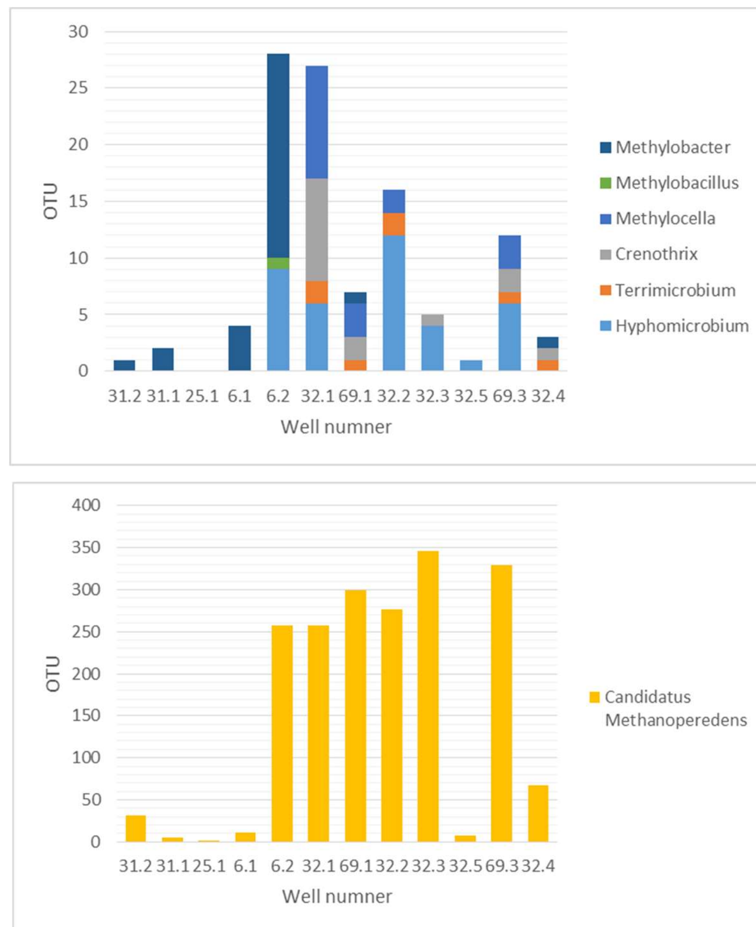

Figure 14. Distribution of genera identified in Table 13 and Table 14 in the Sleen site. Wells are organized with an increase in methane concentration from left to right. Upper figure are bacteria and lower figure archaea.

From the VoP groundwater samples total amplicon reads for archaea and bacteria OTU were normalized to 6900 OTU per well which means that “C*andidatus* Methanoperendens” is the most common methane oxidizing microorganism covering up to 25% of the total community. Again, for both sites there is no clear correlation between concentration of methane or ethane and the abundance of the selected indicator OTU.

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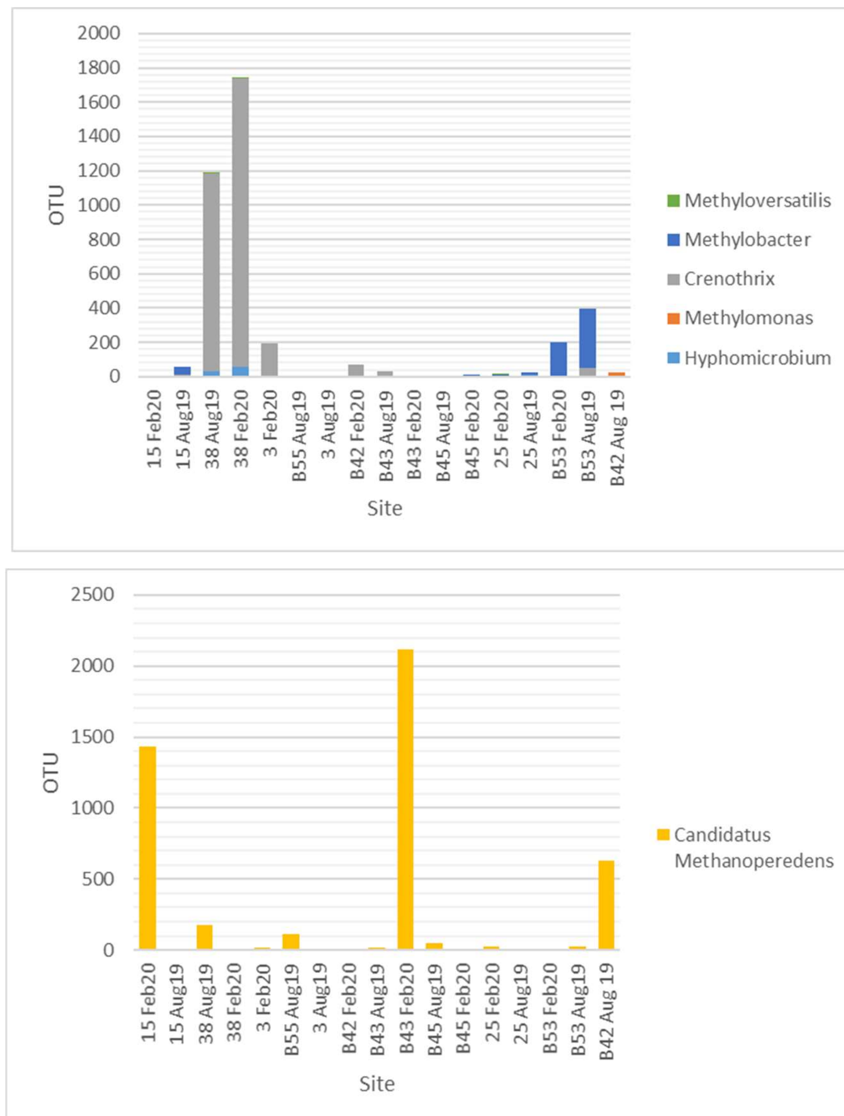

Figure 15. Distribution of genera identified in Table 13 and Table 14 from VoP samples. Sites are organized with an increase in C_1:C_2 ratio from left to right (Site 15 Feb20 to Site B42 Feb20). No ethane was detected in the remaining sites (B43 Aug19 to B42 Aug19), so they are organised by increasing methane concentration. Upper figure are bacteria and lower figure archaea

The genera and groups isolated and identified during cell culture (Table 11) were also used to interrogate the 16S dataset. *Hyphomicrobium* and *Sphingomonas* were only observed in samples with high C_1:C_2 ratio. *Sphingomonas* was only found in VoP samples, with a much higher prevalence of OTUs found in high C_1:C_2 ratio samples. *Hyphomicrobium* was identified in low, medium and high C_1:C_2 ratio samples and was also identified as sensitive to headspace gas in the microcosm experiments (Figure 14 and Figure 15).

Corynebacterium, *Nocardia* and *Rhodococcus* were concentrated in samples with low or medium C_1:C_2 ratio, whereas *Xanthobacter* dominated in samples with high C_1:C_2 ratio. *Corynebacterium* was identified in three low C_1:C_2 samples (up to 2 to 5 OTUs), Representatives from *Nocardia* only found in one sample (3 OTUs and high C_1:C_2) and *Rhodococcus* OTUs were found in multiple samples with low, medium and high C_1:C_2 ratios, in five, three and eight samples respectively with a maximum of 50 OTUs. *Mycobacterium* was not identified from any of the isolates; however, they form part of the same complex of propane oxidisers. Like *Rhodococcus* was no observable dependence on C_1:C_2 ratio and had a maximum of 24 OTUs. *Xanthobacter* OTUs were only identified in VoP samples, although OTUs associated with unidentified genera from the Xanthobacteraceae family were found in both Sleen samples with high C_1:C_2 ratio and in about half the samples with low C_1:C_2 ratios.

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3.6 AUTOMATED IMAGE ANALYSIS

Images were taken of culture-based studies described in Section 3.3.2 to investigate potential for automating cell counts, CellProfiler pipeline was used to identify Objects, presumed colonies (Figure 16). CellProfiler can be used to distinguish between Objects using ClassifyObjects function but this requires inputting the boundaries for the different Object classification that may equate to colony size, colour or other features. As these parameters are specific for the image, they would be required to be entered manually for each image and becomes time intensive and open to bias. CellProfiler outputs numeric parameters for each object which are derived from the object shape, size and colour. To automate the identification of colonies, the numeric outputs from CellProfiler were fitted to a Self-Organising Map to cluster similar objects and provide presumed colony counts for different colony types.

Figure 16. Example output from CellProfiler and self-organising map. Top row: absolute difference between grey and individual colour channels, left to right blue, green, and red. Middle row: object identification using individual colour channels, left to right blue, green, and red. Bottom left: combined objects, middle: original image with an overlay of combined objects, right: self-organising map.

Cell counts and number of colony types tend to be higher with the automated cell counts than manual cell counts (Figure 17), so were Shannon and Simpson's diversity indices. Richness was generally higher for manual counts. No clear relationship was observed between the automated and manual cell counts for any of the measures used. However, since no quality control measures were used, for either counting method, then spurious results are likely, especially for the automated counts.

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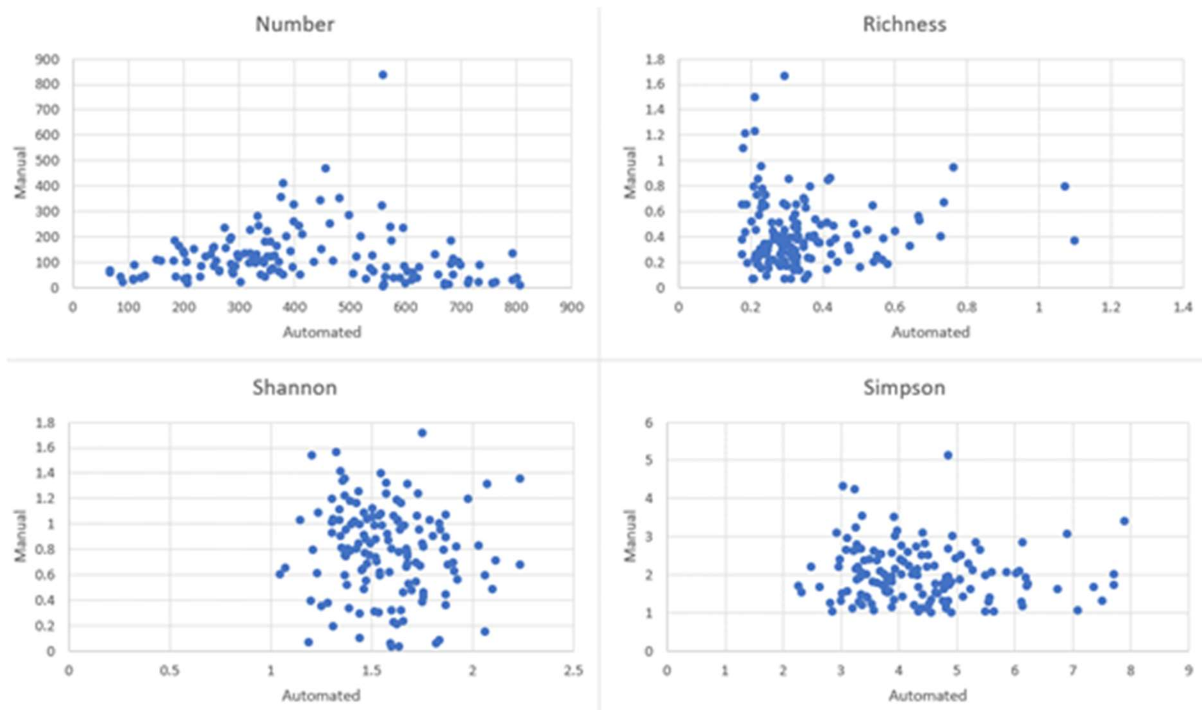

Figure 17. Comparison of manual cell counts with automated presumed cell counts using CellProfiler and Self-Organising map. Comparisons in each of the diversity indices are shown.

4 Discussion

Groundwater ecology is a relatively young discipline, compared to surface ecosystems, in its contribution to scientific theories. Many proposed ecological and evolutionary principles still need to be tested in groundwater ecosystems (Griebler, Malard et al. 2014). However, over the last two decades, groundwater ecology has embraced concepts and tools from multiple disciplines to enter a new era of research on the evolution, distribution, and functional role of biodiversity. First, the increased availability of large-scale environmental and species data has led community ecology in groundwater to shift from local to global perspectives (Cornu, Eme et al. 2013). Second, concepts and experiments in functional ecology have stimulated research on the role of key species, disturbance, functional redundancy, and trophic interactions within groundwater food webs in promoting important ecosystem services such as the cycling of organic carbon and nutrients (Mermillod-Blondin 2011).

Aquifers are complex ecosystems of critical importance for geochemical cycles (Griebler and Lueders 2009). Methane is a common trace constituent of groundwater (Zhang, Li et al. 1998) and can represent more than 20% of the total carbon in this environment (Barker and Fritz 1981). Nonetheless, these aquifers are sensitive to changes (Datry, Malard et al. 2004), and therefore, increases in methane and trace concentrations of other alkanes may alter the groundwater ecosystem. Historically, surveying soil microorganisms have been used to detect the presence of underlying hydrocarbon resources. However, groundwater less so. The details of microbial prospecting techniques are limited in the literature; yet, the idea that microbial response to C_1 and SCA carbon sources could indicate the origin of methane sources was explored as part of this report.

The main objective of this subtask was to test if microbial composition changes can be linked to thermogenic stray methane or ethane intruding into aquifers. This discussion is divided into the potential of looking at the diversity of a community as well as the presence of indicator organisms.

4.1 COMMUNITY DIVERSITY

All theoretical and conceptual models in community ecology can be explained by four processes: selection, dispersal, genetic drift, and speciation. Selection is the result of biotic and abiotic pressures causing variation in reproductive success across individuals and species. Genetic drift is the change in the frequency of gene

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variants and depends on population size and chance events. Dispersal governs the degree to which individuals move among communities, and speciation creates new species (Vellend 2010). These four processes have hardly been evaluated in groundwater ecosystems (Griebler, Malard et al. 2014).

Data from two geological formations revealed selection to dominate over genetic drift and dispersal in fine-grain sediments. Selection still exceeded genetic drift and dispersal in highly permeable sediments, while the latter increased in importance (Stegen, Lin et al. 2013). Therefore, the effect of highly variable methane and ethane concentrations at both the Sleen and VoP sites were expected to reveal a large impact on the microbial community.

4.1.1 Molecular techniques

The diversity of 16S rRNA gene provides information on the microbial classifications of a community. This approach was applied to DNA extracts from VoP and Sleen sites to test whether microbial diversity is linked to methane concentration or C_1:C_2 ratio. The microbial community from samples with a low methane concentration (0.2 mgL^{-1}) were distinct from sites with higher concentrations at Sleen. The three low methane samples arise from two different upstream boreholes, from two geological formations and cover a range of other chemical characteristics (Figure 1, Table 6 and Table 8). The VoP samples cluster together, with only one outlier, which had a higher pH than the other samples. There was no clear clustering based on the C_1:C_2 ratio from the different wells. This is possibly because SDIMO and hydrocarbon oxidising genes are distributed across multiple groups of organisms (Leahy, Batchelor et al. 2003, Coleman, Le et al. 2012).

Alternatively, there is no clear link between methane and ethane concentration and microbial composition because this work is based on microbes present in the groundwater. It has been shown from various low-energy aquifers that biogeochemical processes are primarily linked to the activity of microbes attached to the sediments and rocks rather than to bacteria suspended in groundwater. The cell abundance and activity of sediment microbial communities are typically one to four orders of magnitude higher than in groundwater (Baho, Peter et al. 2012). Sediment communities also display more diversity than the suspended communities, are more temporally stable, and more consistent across replicates and treatments (Kuloyo, Ruff et al. 2020).

Although some distinction between microbial communities using 16S rRNA genes was observed, the high number of other influences reduces the applicability of diversity of 16S rRNA gene as a sole indicator of methane, or methane ethane ratio in groundwater. The diversity of functional genes involved in microbial methane oxidation may provide further insight and is being investigated subsequently within the SECURe project.

4.1.2 Cell culture

Culturable microorganisms account for a small proportion of microbial diversity. However, conditions can be selected to encourage growth of certain groups and the response of a microbial community can be assessed under different conditions. The principal behind microbial prospecting for oil and gas relies on a different response of a microbial community in the presence of different carbon sources. If a groundwater microbial community is exposed to dissolved SCA, then it is expected that a community capable of oxidising SCA will develop. In a similar way, if a community is exposed to methane, a specific community of methylotrophs develops. Therefore, when a groundwater community is exposed to SCA as the sole carbon source a higher diversity is expected for groundwater samples containing SCA, and the opposite when the same sample is exposed to methane. On the contrary, if the community develops in the presence of methane and in the absence of SCA then a greater number and diversity of microbes could be expected when grown under C_1 compounds only. As many of the genes that are involved in C_2 metabolism are also capable of metabolising other SCA, the actual SCA present in environment or cell culture is unlikely to be important.

Along with being able to 'test' the same sample for different conditions, cell culture offers a low cost, low expertise option to detect methane leaks in groundwater, in particular when compared to molecular methods. To replicate the current setup the only required equipment is an autoclave, or pressure cooker to sterilise media, filtration equipment suitable for 47 mm filter membranes, an incubator capable of 25ºC and a sterile working area, the last of which can be achieved by working in the shadow of a Bunsen burner. The ability to use alcohol (methanol or propanol) instead of gas (methane or propane) as the source of carbon removes the requirement for pressurised gases, regulators and suitable gas tight containers, simplifying the system further.

Using relative species richness for the methanol and propanol ratios the success rate of predictions was 70%, although this is from a limited number of samples. After decades of development, the reported success rates for MPOG are 90% (Wagner, Wagner et al. 2002). Meaning with more time there is potential to improve the

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reliability of microbial diversity as a predictor of methane leaks and source. This could be achieved by replicate analyses and application of quality control measures and include the following.

- Replicate analyses – microbial communities can be highly variable, even when physical and chemical parameters have stabilised, there is still potential for microbial community changes in an aquifer. Groundwater samples could be taken at different timepoints in pumping to test natural diversity in community.
- No clear trends were observed during storage or with field vs laboratory filtration techniques, however, there were differences observed when samples were filtered in the field and in the laboratory. These could favour the survival of particular groups, hence influencing predictions of the nature of the methane source. Further investigation into the effect of storage time and transport method is required to identify trends and to develop robust protocols to minimise any inconsistencies.
- Quality control measures are required to reject outliers of unrepresentative samples. This could include high variability among replicates or refinement of volume filtered to give reliable cell counts for that sample. For automated analyses these could include image quality and confirmation of artefact removal.

4.2 INDICATOR MICROORGANISMS

Within the scope of this work several microorganisms have been identified to family level, or below, either because they were isolated during cell culture (Table 11) or an OTU that changed significantly during microcosm experiments (Table 13 and Table 14). Below are short descriptions of microbes of interest, including previous associations with methane, SCA, methanol and propanol. Previous research offers an idea of studies that these groups have been associated with and is not an exhaustive list.

If indicator organisms can be identified, bespoke assay development could allow for quicker and lower cost sample analyses (e.g. quantitative PCR assays).

Microbial families or orders of interest

The order Actinomycetales contains a complex of known propane oxidisers, using the propanol pathway (*Corynebacterium*, *Nocardia*, *Mycobacterium*, *Rhodococcus* complex).

Methylomonadaceae consists of Gram-negative, aerobic bacteria which utilize only one-carbon compounds as a source of carbon. It contains among others *Methylobacter, Methylomonas* and *Crenothrix*.

Methylophilaceae includes the genera *Methylophilus*, *Methylobacillus*, *Methylovorus*, and *Methylotenera*. They are Betaproteobacteria and contain species that are obligate and restricted facultative methylotrophs capable of utilizing methanol or methylamine as a sole source of carbon and energy. They do not use methane (Doronina et al., 2014).

Rhodocyclaceae members display many different modes of living including methylotrophy by the genus *Methyloversatilis*.

Methanoperedenaceae (formerly known as ANME-2d) were shown to be capable of anaerobic oxidation of methane coupled to nitrate and iron reduction. "*Candidatus* Methanoperedens" is anaerobic, methanotrophic, nitrate-reducing archaeon (Guerrero-Cruz et al., 2018).

Microbial genera of interest

Ancylobacter lacus is a facultative methylotroph that is also able to grow on ethanol along with additional carbon sources (Chemodurova et al 2000). Other members of this genus are able to grow weakly on propanol (van der Wijngaard et al 1992).

Brevundimonas mediterranea could not grow on any of the methylated compounds from Biolog substrate utilisation test (Fritz, Strompl et al. 2005). However other members of *Brevundimonas* genus have been associated with hydrocarbon degradation (Mansur, Adetutu et al. 2014).

Chitinophaga has a diverse 16S sequence similarity and typically grow on a range of carbohydrates (Kampfer, Young et al. 2006). Members of this genus have been associated with methanol sink in forest soils (Morawe, Hoeke et al. 2017).

Hyphomicrobium are facultative anaerobic methylotrophs, meaning they can switch between oxygen and nitrate as electron acceptors. *Hyphomicrobium* are metabolically variable. Methanotrophy is possible with low concentration of oxygen (Amaral and Knowles 1995).

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Mesorhizobium are generally known for symbiotic relationships with legumes, and some have been associated with hydrocarbon degradation (Yang, Wen et al. 2016). No evidence for growth on C_1 compounds (Sy, Giraud et al. 2001).

Methylocella are capable of growth on a variety of multi-carbon compounds such as ethanol and acetate (Dedysh, Knief et al. 2005) and have been observed to increase in relative abundance (compared to other methanotrophs) around hydrocarbon seeps (Dunfield and Dedysh 2014, Farhan Ul Haque, Crombie et al. 2018, Farhan Ul Haque, Crombie et al. 2019).

Methylobacterium is known to use methanol as well as SCA (Hanson and Hanson 1996).

Methylopila are a genus of aerobic facultatively methylotrophic species (Doronina, Kaparullina et al. 2014).

Methylorubrum are closely related to *Methylobacterium* (Green and Ardley 2018).

Pedobacter are soil associated aerobic bacteria that are generally reported to grow on organic carbon sources including heparin (Steyn, Segers et al. 1998). Reported facultative methylotrophic strains (Del Rocio Bustillos-Cristales, Corona-Gutierrez et al. 2017).

Pseudoxanthomonas are capable of using refractory organic carbon and has been observed in conjunction with “*Candidatus* Methanoperedens” during degradation of methylated compounds (Fu, Bai et al. 2019).

Sphingomonas are metabolically diverse (Balkwill, Fredrickson et al. 2006) and contain reported facultative methylotrophic strains (Del Rocio Bustillos-Cristales, Corona-Gutierrez et al. 2017).

Terrimicrobium is a member of the class Spartobacteria represent a dominant verrucomicrobial lineage in soil, freshwater and marine environments. Their metabolic capacities and ecological roles remain poorly understood because of few cultured representatives and sequenced genomes. One isolate within this genus has been sequenced, *Terrimicrobium sacchariphilum* NM-5T (JCM 17479T, CGMCC 1.5168T), and was isolated from an anoxic rice paddy field and was characterized as a mesophilic anaerobic bacterium utilizing simple carbohydrates for fermentative growth (Qiu, Wang et al. 2017).

Xanthobacter are able to grow in a variety of alcohols as the sole carbon source (Wiegel 2015), and earliest members were originally classified as *Corynebacterium* (Wiegel, Wilke et al. 1978).

4.2.1 Molecular techniques

The advantage of using molecular methods for potential methylotrophic indicator organisms is that methylotrophs can be hard to culture regardless of whether they are aerobic or anaerobic. When identifying potential candidates, it is important to consider characteristics of the investigated environment. As discussed in the above section, the microbial community is influenced by several interrelated parameters. Hence, parameters such as oxic/anoxic conditions, pH, and temperature will affect the abundance of specific indicator organisms and may camouflage any abundance changes related to hydrocarbons.

The microcosm experiment tested how two aquatic microbial communities responded to methane or ethane under aerobic and anaerobic conditions. Anaerobic treatments revealed a lack of methane and ethane oxidation. Lack of anaerobic methane oxidation has also been observed in other studies (Cahill, Steelman et al. 2017, Kuloyo, Ruff et al. 2020). Nonetheless, this result may be explained by laboratory conditions that differ from the groundwater aquifers' actual condition or a too-short incubation time. Despite no measurable anaerobic methane oxidation, a significant increase in the microbial abundance of *Methylobacillus* and *Methylobacter* from the Stenlille microcosms indicates a longer incubation time may have revealed changes in the headspace gas composition.

Few individual OTUs associated with methylotrophic genera showed significant increase in abundance with aerobic hydrocarbon treatments as listed in Table 13 and Table 14. The treatment effect was more apparent from Stenlille, which may be explained by an increase in dissolved methane and ethane compared to the original concentration in groundwater. In contrast, from the Sleen site, the natural methane concentration was higher than the 10 mg/L in the microcosm setup. Hence, when comparing the microbial composition change from both microbial aquatic communities, no consistent patterns pointed to niche differentiation among variants within genera. *Methylomonas* was the only genus found to increase in abundance from both Stenlille and Sleen under aerobic conditions.

The abundance of selected methylotrophic indicator organisms was linked to methane concentration from Sleen and VoP microbial communities (Figure 14 and Figure 15). Unfortunately, there was no observed correlation between selected microbial indicators and methane concentrations. 16S amplicon sequencing

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provides information on relative microbial abundance but does not provide information on actual concentrations. Therefore, the next step would be to quantify specific genes from the different groundwater samples and test if they correlate with methane concentrations.

When evaluating the relative abundance, it is seen that "*Candidatus* Methanoperedens" plays an essential part in methane oxidation covering 10% of the microbial community from Sleen samples and up to 30% in VoP samples. From the microcosm experiments, this archaeon significantly decreased in abundance, consistent with this group being hard to culture.

4.2.2 Cell culture

Although 37 isolates were identified to genus level or below, only four of these were identified from the ethane rich samples making inferences on specific indicator organisms from this dataset limited. Molecular analysis of the isolates shows no clear pattern linking species or presence of particular genes to C_1:C_2 ratio of original sample. However, using the genera identified as a basis for research revealed a few groups that could be targeted in further investigations.

Hyphomicrobium and *Sphingomonas* were both isolated from samples with high C_1:C_2 and in presence of methanol. *Hyphomicrobium* was also identified in microcosm experiments as its abundance increased in the presence of methane and methane/ethane headspace, but no difference was seen between methane and methane/ethane headspace. *Sphingomonas* OTUs were most abundant in samples with high C_1:C_2 ratio, but were only found in VoP samples. However, they have been isolated from groundwater elsewhere (Nielsen, Kot et al. 2015). *Hyphomicrobium* OTUs were found in the majority of samples, spanning both sites and C_1:C_2 ratios, however no trend was observed in numbers of OTUs with either methane concentration or C_1:C_2 ratio.

Corynebacterium, *Nocardia* and *Rhodococcus* are part of a complex of known propane oxidisers that can also use propanol (this complex typically also includes *Mycobacterium*). These genera were only found on propanol plates and were concentrated in the groundwater samples with low C_1:C_2 ratio. OTUs related to these genera were found in Sleen and VoP samples, however in low numbers <0.75% of OTUs. There was an increase in relative abundance of these groups during cell culture compared to the 16S amplicon data. This is explained by these groups being culturable and the selective pressure of the carbon source. Hence, this group was easier to detect and more common in samples with SCA with cell culture. Additional samples would be required to confirm whether this group are more readily cultured in groundwater sample with SCA.

Xanthobacter are capable of oxidizing both methanol and propanol. Nonetheless, they were only isolated from propanol treatments. This could be due to the limited number of identified isolates. Or, as these isolates were generally identified in samples with high C_1:C_2 ratio, the flexible metabolism of this species could explain why they dominate when shifting from C_1 to C_3 carbon source, but were outcompeted by other groups capable of metabolising propanol when C_1:C_2 ratio was low.

5 Relevance to future leak detection

Microbial biosensors are less sensitive to short-term fluctuations than gas concentrations, so they are an excellent option to identify areas where intermittent leakage may occur.

Molecular techniques and cell culture offer distinct advantages and disadvantages. Molecular techniques are quicker, however, they require more complex sample preparation and higher capital costs. These can be reduced if indicator microorganisms can be identified. Cell culture does not require specialist equipment or knowledge. The use of alcohol as a carbon source means analyses can be completed with basic filtering equipment and has the potential to be developed into a portable system. However, improvements are needed in the reliability of this method. A significant amount of time is required for manual cell counts and the potential for automating this process is demonstrated in this report. However further refinement is required to reliably identify colonies in an image, and a measurement in the confidence of the cell counts. Once achieved this opens up the possibility for repeat analyses on the same sample, contributing towards improved reliability.

Distinct microbial communities using 16S rRNA gene are not associated with methane or methane and SCA concentrations for the sites studied here. However, several indicator microorganisms or groups have been identified as further interest. In particular *Corynebacterium*, *Nocardia*, *Mycobacterium*, *Rhodococcus* complex and "*Candidatus* Methanoperedens". To develop these techniques for routine monitoring leakage from shale gas operations the culture and molecular methods investigated here will need optimisation as described in this

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report. Developments could include optimisation of imaging techniques, the targeting of the development functional genes involved in methane and SCA oxidation or genus/species specific quantitative assays such as qPCR to monitor changes in key organisms. Another approach could be to use artificial intelligence-based approaches which can detect differences in community via linear regression of random forest classification approaches. This would require access to sufficient representative environments to have a suitable database to develop the algorithm. These developments would allow microbial techniques to be used alongside established geochemical methods for a more accurate assessment of the source of leaking gas.

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Glossary

Candidatus: a prefix used in taxonomy when a microorganism cannot be grown in culture, and is inferred by DNA sequencing

CuMMO: copper monooxygenases

DOC: dissolved organic carbon

Facultative: metabolism that can use a certain substrate

Functional genes: genes that transcribe proteins that are involved in a specific metabolic pathway

Genomic classification in descending order: Kingdom, phyla, class, order, family, genus, species

mcrA: methyl coenzyme M reductase

Obligate: metabolism that required a certain substrate

OTU: operational taxonomic unit

PCR: polymerase chain reaction

pmoA: particulate methane monooxygenase

SCA: short chain alkanes

SDIMO: soluble di-iron monooxygenase

VoP: Vale of Pickering

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