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Deep biosphere in the Main Dolomite (Upper Permian) hydrocarbon reservoir

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As a Professor Michał Szulczewski's master's student, I would like to express my special thanks to my supervisor for his mentorship, knowledge and instilling in me a passion for geology. As a student, I wanted to link my professional career with oil exploration. The supervisor who offered interesting topics related to sedimentology and biostratigraphy based on microfauna was Professor Michał Szulczewski. He agreed to pass on one of his topics to me. Thanks to the help of my mentor, I managed to develop a very interesting research topic, gaining specialist knowledge on facies analysis and stratigraphy of Devonian carbonates. Also thanks to the Professor's recommendation, I managed to find a job at the Polish Oil and Gas Company, where I work to this day, fulfilling my student dream.

Paweł Zdanowski

ABSTRACT:

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This study focuses on evaluating microbial activity in Main Dolomite reservoir rocks (Zechstein, Wuchiapingian–Permian) at 100°C. Core samples from two wells, one potentially microbiologically active (C-1 well, 100°C) and the other considered inactive due to high temperature (L-1 well, 155.5°C), were analysed. The core from L-1, treated similarly to C-1, was used to control for contamination. Microbial experiments and molecular analyses were performed on both core samples to ensure accurate results unaffected by laboratory processing contamination. Microbial incubation tests were successfully employed to demonstrate microbial hydrocarbon degradation and methane formation from ^{13}C-labelled acetate. A new method for staining microorganisms from rock samples was introduced to estimate biomass. The results indicated that microorganisms from the C-1 well exhibited both hydrocarbon biodegradation and acetoclastic methanogenesis during a 3-month incubation at 100°C. Fluorescence-stained and countable microorganisms were only observed in the C-1 samples, while no biodegradation or methanogenesis occurred in reference samples from L-1 well. The study provides valuable insights into microbial activities in extreme conditions, emphasizing the importance of proper controls and techniques to ensure accurate interpretation of results.

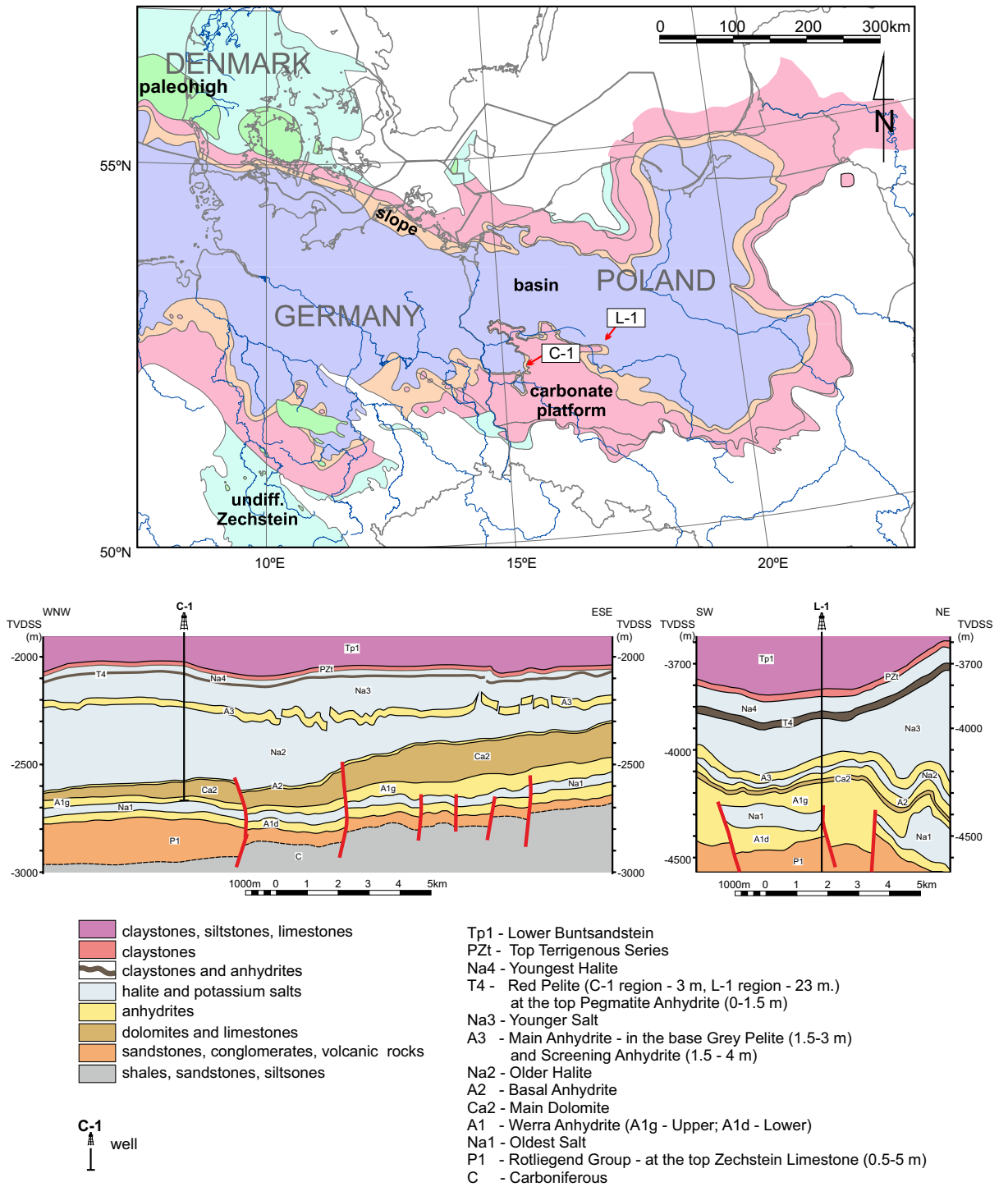
Key words: Deep biosphere; Main Dolomite; Microbial activity; Reservoir rock; Zechstein; Permian.

INTRODUCTION

It is known today that the microorganisms can exist within deep buried rocks where the temperature reaches the known life limit close to 100°C (Kashefi and Lovley 2003; Heuer *et al.* 2020). Numerous scientific papers have presented studies concerning deep drilling projects aimed at discovering microorganisms within various types of rock (Chapelle and Lovley 1990; Colwell *et al.* 1997; Onstott *et al.* 1998; Colman *et al.* 2017; Dai *et al.* 2021). All these attempts assumed that a deep biosphere can exist in rocks under thermodynamic constraints for many years in the geological time sense (Inagaki *et al.* 2015; Morono *et al.* 2020). It is not clear however, whether those microorganisms were introduced into the rock matrix due to the migration of formation water, or whether they have existed since the sedimentation processes and subsequent diagenesis. What is more, it is not clear whether those microorganisms can still actively transform organic matter (OM), or whether they exist in a dormant state (Heuer *et al.* 2019). It is now widely accepted that microbes can substantially change the composition of the formation water, petroleum and dry gas of such reservoirs (Ollivier and Magot 2005). Their ecological niches seem to be constrained primarily by toxicity and high temperature (Merino *et al.* 2019). Hydrogen sulphide, a common reservoir gas, affects cellular respiration and at high concentrations can be toxic even to its producers (Kushkevych *et al.* 2019). So, is it possible that the deep biosphere can truly affect the biotransformation of hydrocarbon compounds? Is it possible that the microorganisms in reservoir rocks can still produce methane or other gases like hydrogen sulphide on a scale that may affect the gas and oil production process? Such activity, depending on the reservoir conditions, should have a significant impact on the ratios between the individual components of the organic fraction. That, in turn, can strongly affect the geochemical interpretation and inference about sedimentation conditions.

It seems obvious that due to the specificity of the deep hot biosphere environment, the isolation and maintenance of pure cultures in the laboratory may only be possible in a few exceptional cases. Extremophiles extracted from hot hydrothermal vents have been cultivated in the laboratory at 122°C (Kashefi and Lovley 2003). Vegetative cells have been found in deep sediments at 120°C (Heuer *et al.* 2020; Beulig *et al.* 2022). However, for the purposes of studies related to the estimation of the biogenic component of a gas deposit, it is necessary to know whether the present microbial activity in reservoir rocks can be determined. Based on the course of exploitation of some gas deposits, it was found that the modern biogenic component may be as high as 20% (Cokar *et al.* 2010, 2013a, b). However, such estimations are usually based on a thermodynamic model of an already exploited gas field. There have been no attempts to estimate the biogenicity of deposits based on the actual microbiological activity associated with the generation of microbial methane. Another problem concerns the microbial community characterization in the rock samples. It is known that the rock samples can be problematic for genome DNA analyses. Some papers indicate that the isolation of DNA from core rock matrix is unjustified (Heuer *et al.* 2020). However, we tried to isolate DNA *via* Next Generation Sequencing (NGS) of the V3–V4 region of 16S rDNA and subjected the received data to criticism. We are convinced, that attempts like this should be made to achieve the best approach to such troublesome matrices. Secondly, comparative analyses of DNA isolated from drilling mud and from core samples can be treated as a contamination test. The drilling mud should contain many sequences that should not be found in the material extracted from the inner part of the core.

The geological formations where the C-1 and L-1 wells were drilled, were deposited within the European Southern Permian Basin during Zechstein time and represent one of the more interesting reservoir systems in the context of the deep biosphere. Today the Zechstein cyclic formations extend from the North Sea to Poland. Within the shallow, intracratonic, warm sea an evaporite-carbonate succession was deposited. The successive cycles left deposits with very diverse lithologyies, among which the Main Dolomite (Ca2; PZ2 cyclothem) is considered as a source and reservoir rock for oil and gas. On the PZ1 elevations built of the Lower Anhydrite (A1d) and Upper Anhydrite (A1g), the Main Dolomite was deposited in the facies of a carbonate platform, and its thickness varies from a few metres to a hundred metres. These deposits are strongly isolated and sealed by thick anhydrite and halite layers creating a closed hydrodynamic system containing the hydrocarbon reservoir rock (Kotarba and Wagner 2007; Krzywiec *et al* 2017; Bilkiewicz and Kowalski 2020). Hence, a theoretically ideal system for the development of a deep hot biosphere was created. The slow burying of the sediments caused gradual heat input. However, analysis shows that the temperature in the Main Dolomite from C-1 well did not reach life-critical values (120°C – the late oil window and the lower gas window) (Kotarba *et al.* 2020). Vitrinite reflectivity



Text-fig. 1. Location of the C-1 and L-1 wells in relation to the palaeogeographic map of the Main Dolomite basin. Map of the Southern Permian Basin in Ca2 time after Peryt et al. (Peryt *et al.* 2010).

measurements conducted on samples from this well indicate that the maximum temperatures reached were 105°C. Higher temperatures, which could lead to the paleopasteurization of the sediments, might also have occurred depending on the depth of burial and the local heat flux, as observed in the L-1 well.

The OM content may also have promoted microbial activity. It is therefore possible that microbial factors may have shaped both OM and gas composition within the Main Dolomite over millions of years. The latter is particularly interesting because within the Main Dolomite very large variations in gas composition are found. Dolomite rocks are found which have different methane contents (in the Polish part of SPB from 1,6% to 70%) and hydrogen sulphide concentrations (from 0 to 87%) (unpublished PGNiG data). In addition, nitrogen as high as 90% is often found. Large differences in the composition of gas and OM may occur in wells located very closely within one small carbonate platform. Although such differences can be explained by migration processes, a strong influence of contemporary microbiological activity can be also postulated. However, no attempts have been made to define such activity within the Main Dolomite deposits so far.

In our work, we attempted to determine the microbial activity in two wells, differing in temperature, which reached the Main Dolomite. Potentially microbially active C-1 well (2700–2741 m) which reached 100°C was chosen to test the working hypothesis about microbial activity in the Main Dolomite reservoir rocks (Text-fig. 1). Moreover, L-1 well (4313–4327 m) which reached 155°C was chosen as a reference well where the no microbial activity should be detected. Such an approach provides an excellent opportunity to test the hypothesis of microbiological activity in two wells reaching the Main Dolomite, but diametrically differentiated in temperature. That approach also provides the best method of controlling the results and determining the impact of possible contamination. The overriding goal was to determine whether it was possible to check how microbial activity could affect both gas production and hydrocarbon degradation. This problem is extremely important when assessing the biological productivity of gas deposits at the stage of exploration drilling. The assessment of microbial activity can both help to predict biodegradation processes still taking place in the deposit, but also facilitate the implementation of unconventional microbiological technologies to enhance gas and oil recovery.

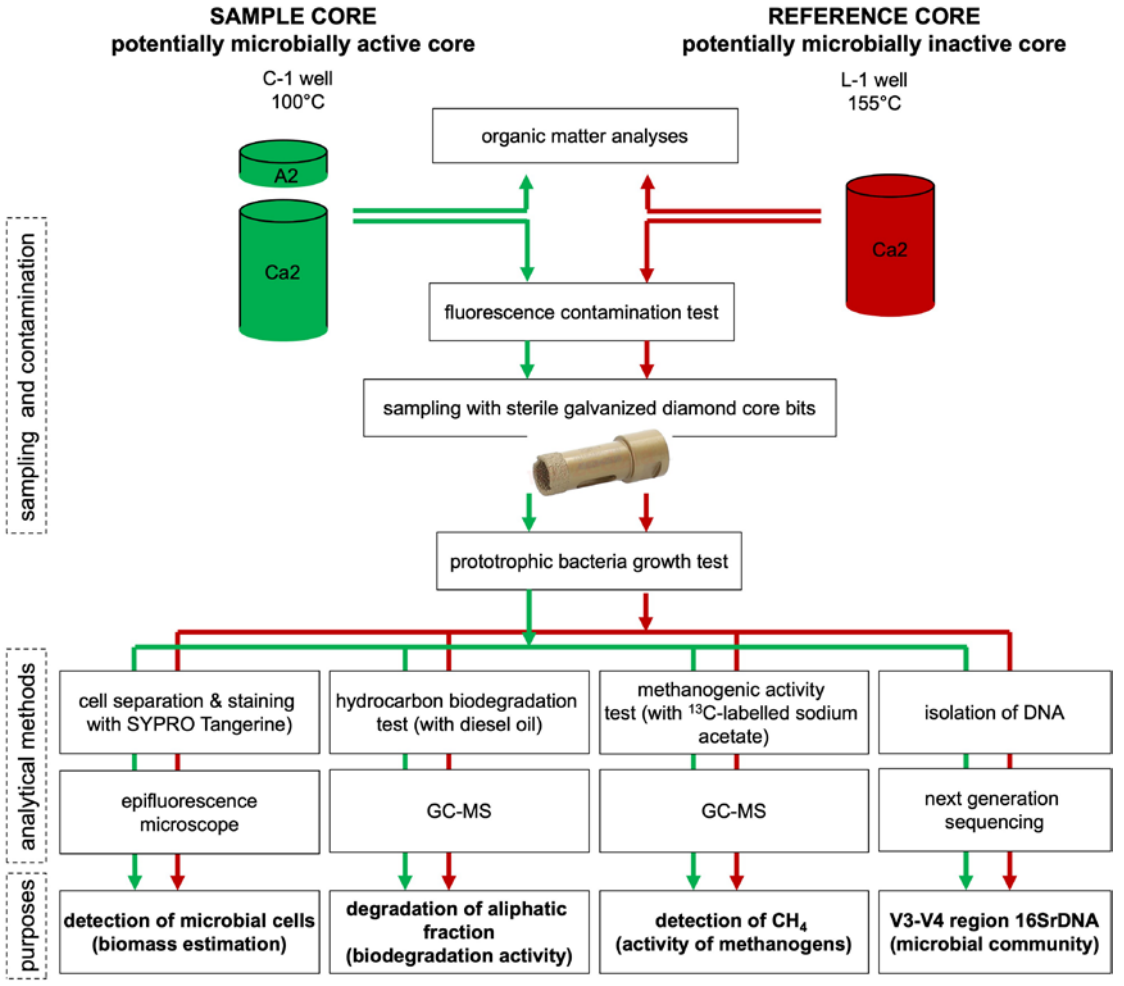
MATERIAL AND METHODS

Sampling

The samples were taken during a commercial exploration drilling project in 2021. Core samples (6.5 cm in diameter and ca. 10 cm in length) were collected on the rig site from the core barrel immediately after the lift operation using sterile stainless-steel tools (gas-flame sterilisation). Sixteen samples were taken from C-1 well (samples: Ch1–Ch16) and three samples from L-1 well (samples: Lis1–Lis3). Due to the quality of the samples from C-1, for the next experiments twelve samples were chosen. Water-based drilling mud from C-1 (used during the drilling process) was also taken (500 mL) for comparative molecular analyses. The samples were transported immediately to the laboratory under anaerobic conditions in tightly closed stainless-steel jars filled with helium. In the laboratory, the outermost part of the core, that was potentially contaminated by drilling mud, was removed using a sterile tool in a sterile laminar flow chamber. The core surface was sterilised by gas flame (for 5 s) and sterilised by UV radiation (235 nm, 8W for 30 min, working distance 15 cm). Then, the small cores (diameter 2 cm) were drilled using sterilised galvanized diamond core bits using a slow-speed drill (Makita Ltd, GA5040C). The core bits were sterilised as follows. Firstly, the bits were washed with an ultrasonic cleaner followed by washing with 70% ethanol with diethyl ether (Linegal, Poland) to remove mineral particles and organic impurities respectively. Then, the bits were fired in a flame and were washed in sodium hypochlorite (3%) to remove DNA residues. Next, the drill bits were washed again in alcohol to remove the rest of the hypochlorite and were then used for drilling. The small cores from the samples were collected with sterile aluminium foil and were used in the next experiments after grinding in a sterile mortar (sterilised with hypochlorite solution, alcohol, and flame sterilization). For the next experiments the parts of the drilled small cores were taken based on the results of contamination tests (Text-fig. 2).

Contamination protocol

The implemented contamination protocol assumes a multi-level control of possible core contamination: (i) the use of a reference core that was tested in an identical manner to the sample core; (ii) use of a fluorescent marker to indicate possible contamination during laboratory handling; (iii) using a microbiological test to exclude contamination of the collected samples with common bacteria; and (iv) using comparative molecular analyses of the drilling mud and the core. Due to the commercial nature of the drilling project, the application of a fluorescent microspheres marker directly during the drilling process was not possible. However, the use of such mark-



Text-fig. 2. Analytical flowchart.

ers is also problematic due to the high temperature of the reservoir (see discussion).

Reference core

Here, it was assumed that the best contamination test is the use of two cores from the Main Dolomite horizon, but significantly different at reservoir temperatures. The C-1 well reached a temperature of 100°C and is considered a potentially microbially active well. The L-1 well reached a temperature of 155.5°C and is considered a reference well, microbially inactive (Text-fig. 2). Core samples from both wells were treated in the same way during the tests. It was assumed that despite the application of rigorous laboratory work procedures when handling the samples (such as using a laminar chamber, sterilizing tools, sterilizing sampling drills, etc.), the adopted analytical procedures could still potentially be a source of contamination. If this were the case, then the microbiological analyses performed on the samples from L-1 would likely yield false positive results for microbial activity. If this happened, then the research should be considered unreliable. Otherwise, it can be considered that the samples were not contaminated.

Fluorescent penetration test

Fluorescent penetration tests were performed to determine the extent of possible contamination while handling the material in the laboratory. A core sample, carefully cleaned up from the drilling mud residues, was immersed in two fluorescent marker solutions: (i) distilled water with rhodamine B (Merck, 1.07599.0025) 25 mg 500 mL, and (ii) drilling mud with rhodamine B, 25 mg 500 mL. Samples were kept

under conditions close to the reservoir temperature for 4 h and at room temperature for 12 h. Then, the samples were drilled with clean stainless-steel bits at the distances: 0, 0.5, 1, 5, 10 and 20 mm from the core's edge. The obtained drill powder was put onto a 96-well microplate and microscope slide. The fluorescence level of rhodamine B was measured with a microplate fluorometer (Varioskan LUX, ThermoScientific, Life Technologies Holdings Pte Ltd, Singapore 739256) at excitation light at 553 nm and emission at 627 nm. Based on the calibration curve, the sensitivity of the fluorescence of rhodamine in the rock matrix was estimated at 0.1 μg g^{-1}. The microscope slide with a small amount of powder was analysed under an epifluorescence microscope at excitation wavelength 510–550 nm (DM670 filter).

Microbial growth test

A prototrophic bacteria growth test was performed to check whether the collected samples were contaminated with bacteria commonly present in the environment. Powdered core fragments (1 g) were dispersed in 10 mL sterile saline (0.9 % NaCl). Then, 0.1 mL of the obtained suspension was placed on a Petri dish with the nutrient agar medium (Biomaxima, Poland) and incubated at 30°C for 48 h. The test interpretation is founded on the assumption, that the extremophiles living deep underground cannot grow on a simple nutrient agar medium under aerobic conditions. Hence, any growth observed on the agar plate after inoculating with rock sample (even of one colony), would be considered as contamination of whole core sample.

Drilling mud

Additionally, drilling mud was collected for molecular analysis. The mud samples are treated as a material carrying molecular information that could potentially affect the results of the molecular investigations of the cores. This would be the case if the adopted procedures were insufficient, and the internal part of the cores was contaminated by the drilling mud. A comparative analysis of the molecular composition of the drilling mud and the C-1 core is described in section 3.5 (see molecular analyses).

Microscopic analysis

The implemented new procedure assumes three main steps: (i) cell separation from rock samples, (ii) cell attachment to the organic-free dispersed mineral matrix (nano-SiO_2) after modification of the net surface charge by ionic liquid and (iii) staining with SYPRO Tangerine (Invitrogen, Thermo Fisher) (see Supplementary material S1). The powdered rock sample (0.5 g) was dispersed in sterile deionised water (5 mL) with sodium pyrophosphate (10 mM). Then, the suspension was shaken for 30 min (horizontal shake, 300 rpm) and the suspension was centrifuged for 60 s at 4427 rcf (6000 rpm, MPW-260R). Then, the supernatant containing cells was gently mixed with sterile nano-silica (Sigma-Aldrich, 637238-50G) to create the mineral bed for adsorption of cells. The cell adsorption may not be sufficient due to negative net surface charge of the cell wall and low isoelectric point of silica. Hence, the solution of ionic liquid (1-decyl-3-methylimidazolium chloride, Sigma-Aldrich, 690597-5G) was added (0.2 mL of ionic liquid solution 0.1 mL 10 mL^{-1}) to modify the net surface charge of the particles and enhance of adsorption of the cells to the agglomerated silica particles. Finally, the obtained suspension was centrifuged again for 30 s at 4427 rcf (6000 rpm) and the supernatant was discarded. The nano-silica samples with adsorbed cells were suspended in 20–200 μL of SYPRO Tangerine solution (10 μL 10 mL^{-1}), dependently on the desired degree of concentration of isolated microorganisms on silica. The SYPRO Tangerine solution was prepared and filtered before using. Next, 10 μL of dispersed suspension was put on the Bürker chamber and the observation under fluorescence microscope was conducted with blue filter (DM500 with a bandpass 460–490 nm excitation filter). A minimum 10 fields (one field covered 0.003 mm^3 under magnification 400^x) were collected and visible cells were counted. The negative controls were: (i) the negative sample without rock matrix (pure water) and (ii) the rock samples without staining procedure to assess the impact of possible organic particles on fluorescence under the microscope.

Microbial activity

Two sets of incubation tests were prepared: (i) test for determination of the biodegradation of hydrocarbons and (ii) test for determination of methanogenic activity. The powdered rock sample (4 g) was placed in a clean sterile glass vial (20 mL) with a septum. An aliquot of 2.5 mL liquid mineral medium was added to the sterile glass vial and diesel oil (0.1 mL 50 mL^{-1}) or ^{13}C-labelled sodium acetate (1% sodium acetate-2-^{13}C, Sigma Aldrich 279315) was added to the medium. Next, the culture was purged with pure nitrogen for one minute. The glass vial was then tightly sealed with a screwcap featuring a septum. It

was subsequently placed in an incubator at a temperature of 99–100°C for a duration of three months. The control cultures ("abiotic samples") were prepared in the same manner. However, the rock samples were sterilised at 132°C for 30 min. Additionally the blank control sample (only reagents) was prepared without rock samples.

The mineral medium used here was prepared according to McInerney et al. (McInerney *et al.* 1979) with modifications (Cokar *et al.* 2013b). Basal medium: KH_2PO_4 2.5 g; $MgCl_2 \cdot 6H_2O$ 1.65 g; NaCl 2 g; NH_4Cl 2 g; $CaCl_2 \cdot 2H_2O$ 0.25 g; distilled water 250 mL. To 250 mL of basal medium, 0.2 mL of trace elements were added (H_3BO_4 570 mg; $MnCl_2 \cdot 4H_2O$ 360 mg; $ZnSO_4 \cdot 7H_2O$ 44.6 mg; $Na_2MoO_4 \cdot 2H_2O$ 78 mg; $CoCl_2$ 8 mg; $CuCl_2$ 8mg; distilled water 100 mL). Additionally, the medium was supplemented with cysteine·HCl (final concentration 0.05 g 250 mL^{-1}) and vitamin B12 (final concentration 10 mg 250 mL^{-1}). The basal medium and the trace elements solution were sterilised at 121°C for 30 min., the cysteine and vitamin solution were sterilised by filtration.

The microbial tests with ^{13}C labelled sodium acetate after 3-month incubation were analysed with Delta Plus Finnigan Mass Spectrometry coupled with Hewlett Packard 6890 gas chromatograph equipped with a Chrompack packed column (27.5 m × 0.32 mm). Helium (purity >99.999%) was used as the carrier gas, with a fixed flow rate of 2 mL/min. Split ratio was 1:3. The GC oven temperature increased from 27°C to 60°C at 5°C/min. A 100 µL sample of the headspace gas was directly loaded into the gas chromatograph. The hydrocarbons from the cultures with diesel oil after 3-month incubation were 24-h extracted with 5 mL of hexane. Then, the extracts were evaporated and 1 mL of hexane with internal standard (1-Chlorooctadecane, 8.6 mg 100 mL^{-1}) was added. The chromatographic analysis was conducted under the same conditions as for the OM analysis (see below).

Organic matter analysis

The samples were powdered with a sterile mortar and a sterile pestle in a sterile laminar flow chamber. The organic matter was extracted from the powdered samples (5 g/sample) using the 15 mL DCM:MeOH (2:1, vol./vol.) in an ultrasonic water bath for 20 min. The supernatant solutions containing maltenes were collected. Following this, the solvent was removed through evaporation. The vials were then weighed to obtain the Total Lipid Extract (TLE) content. Finally, the solution was dissolved in hexane with 1-Chlorooctadecane (internal standard, 8.6 mg 100 mL^{-1}). The distribution of the compounds present in the aliphatic fractions was determined with gas chromatography-mass spectrometry (GC-MS), using an Agilent 7890A equipped with a fused silica DB-5MS capillary column (60 m × 0.25 mm × 0.25 µm) and an autosampler. Helium (purity >99.999%) was used as the carrier gas, with a fixed flow rate of 0.5 mL/min. The GC and MS parameters were as follows: Aux-2 temperature 280°C, MS source temperature 230°C, inlet temperature 300 °C. The GC oven temperature increased from 70°C to 210°C at 10°C/min, was ramped up to 310°C at 3°C/min, and then held at 310°C for 26 min. The ionisation energy was 70 eV.

DNA extraction, sequencing, and decontamination *in silico*

DNA isolation and sequencing

The genomic DNA from the rock samples was isolated using an EURx kit for complex matrix (Soil DNA Purification Kit, no E3570, EURX Ltd. Poland) according to instructions. The protocol assumes the mechanical homogenisation of the samples to enable release of the cells from the rock matrix. The isolated genomic DNA was subjected to metagenomic analysis (metabarcoding). Sequencing of the hypervariable V3–V4 region of the 16S rRNA gene was commissioned to two independent laboratories (LAB_1 – Genomics Core Facility, Center of New Technologies (CeNT), University of Warsaw, and LAB_2 – Genomed S.A. in Warsaw). Specific primer (with adapter) sequences (Klindworth *et al.* 2013):

341F:5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNCCTACGGGNGGCWGCAG-3'

785R:5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNGACTACHVGGGTATCTAATCC-3' (LAB_1)

341F:5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'

785R:GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' (LAB_2)

were used to amplify the selected region and prepare the library. PCR was carried out using Q5 Hot Start High-Fidelity 2X Master Mix, reaction conditions according to the manufacturer's recommendations. Sequencing was performed on a MiSeq sequencer, using paired-end (PE) technology, 2x300nt, using Illumina's v3 kit.

Construction of a database with common contaminant sequences

The database of common contaminant sequences was built based on the sequences derived from NCBI nucleotide database (Sayers *et al.* 2022) (16S rRNA sequences) based on the literature (Salter *et al.* 2014). Moreover, microbiomes of throat (SRS042628), faeces (SRS042628), and saliva (SRS050640) from Human Microbiome Project (The Human Microbiome Project Consortium 2012a, b), and skin microbiome (NCBI SRP187334) (Bayal *et al.* 2019) have been also included. The obtained database (10.5281/zenodo.10208599) was used to *in silico* decontamination.

In silico decontamination and analysis of sequences

Prior to the *in silico* decontamination, fastq files were processed with fastp (0.23.2) (Chen *et al.* 2018) in order to enhance the quality of raw sequences (cutting adapters, filtering out bad quality reads, trimming, etc.). Then, the *in silico* decontamination method was applied. BBduk (39.01) (Bushnell *et al.* 2017) has been used with the constructed database. Based on the previous trials, the option k=100 was chosen. This option allows for setting up the sensitivity of the algorithm. Lower values of k caused too restrictive *in silico* decontamination, while k>100 did not change the results significantly. The sequences that did not align (not present in the database) were exported to a fastq file. The decontaminated sequences have been further analysed with Kraken2 according to the protocol of the authors (Wood *et al.* 2019). For the taxonomic analysis, Silva database (v.138) was used (Quast *et al.* 2013). Then Bracken (a part of KrakenTools), set at the *Family* level with threshold set at 5, has been applied on kraken2 reports (Lu *et al.* 2022).

Data analysis and statistics

Before hierarchical analyses, metagenomic results were imputed to solve the problem of zero-values and subsequently were transformed with centred log-ratio, due to its compositional nature using zCompositions package for R (Aitchison 1982; Quinn *et al.* 2019). Pairwise plots of correlation matrices were performed using Heatmapper free software (Babicki *et al.* 2016). Agglomerative hierarchical clustering of samples was performed using the average linkage algorithm and Manhattan distance. To show groupings among variables (taxa), the distance calculated from correlation coefficients based on symmetric pivot coordinates was employed (Kynčlová *et al.* 2017). The statistical analyses including nonparametric test for differences (U-Mann-Whitney test; U-M-W test) were conducted with Statistica 13 software (StatSoft Inc., Tulsa, OK, USA).

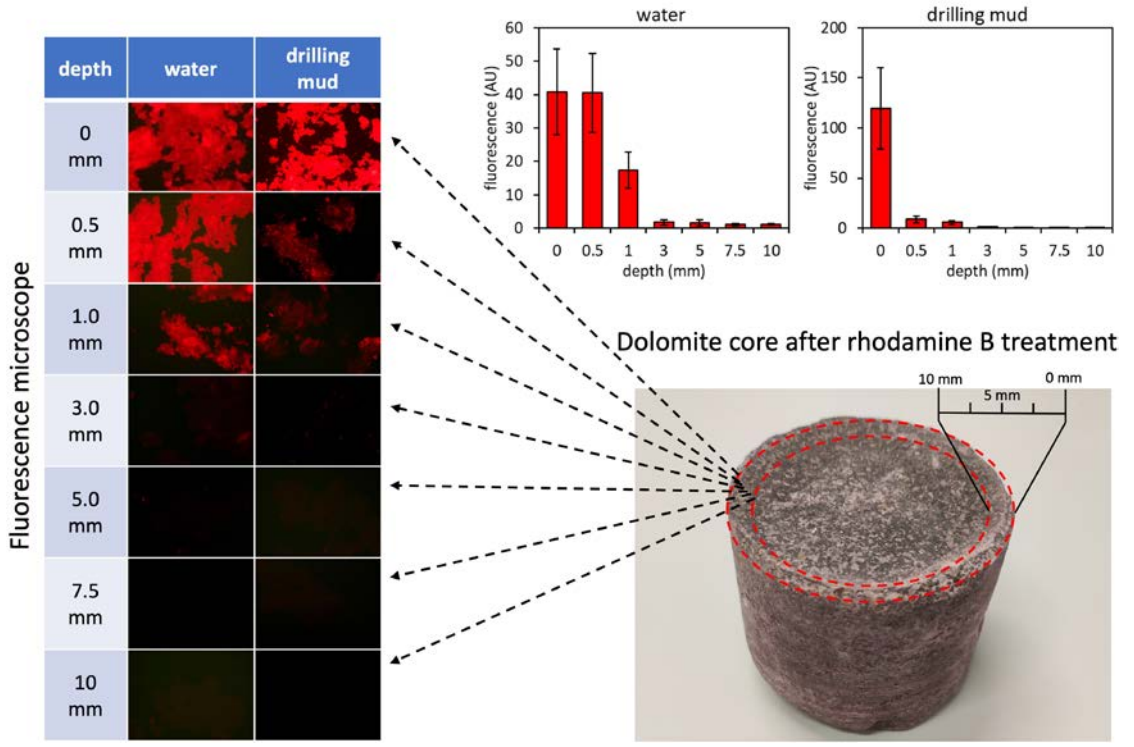
RESULTS

Fluorescent contamination tests

Two tests were conducted to check the possible contamination of the samples. The fluorescence level of the rhodamine marker that penetrated core after incubation indicated that the zone of possible contamination did not exceed 1–2 mm (Text-fig. 3). The differences in penetration range and fluorescence intensity in the two diagrams (water vs. drilling mud) indicate that the rhodamine-labelled water penetrated slightly deeper. This shows the theoretical maximum penetration range, unattainable for the much denser mud. The core incubation with rhodamine-labelled drilling mud demonstrated that under such conditions, penetration was less effective. Text-fig. 3 presents both the fluorescence measurement of powder (diagrams) and an image of drill cuttings from each depth under the fluorescence microscope. With knowledge of the possible contaminated zone, samples were taken, assuming a greater depth of contamination of about 20 mm. After preparation for main analyses, additional microbiological contamination tests were also conducted. These tests showed that the obtained samples from all investigated cores were not contaminated by microorganisms from the surroundings, which can easily grow on nutrient agar plates under laboratory conditions.

Organic matter

Generally, the Ca2 samples from C-1 well were not rich in OM. The total lipid extract (TLE) in all analysed samples did not exceed 1g $100g^{-1}$ of rock sample (Text-fig. 4). Maximum TLE content was found in Ch12 sample, the lowest TLE was noted in sample Ch1 (0.8 and below 0.2 g $100g^{-1}$ of rock sample respectively). The aliphatic fraction was the chief constituent of extractable organic matter. The highest concentration was noted in sample Ch4 (40 mg $100g^{-1}$ of rock sample), whereas in sample Ch16, a 0.05 mg $100g^{-1}$ of rock sample was found. The differences between investigated samples were clearly visible in the total ion chromatograms of the aliphatic fraction (see the chromatograms on the right in Text-fig. 4). The presented chromatograms are in the same scale. It should be noted that the distinct unresolved com-



Text-fig. 3. The fluorescence rhodamine penetration test. Diagrams show the fluorescence measurement of drill cuttings. Bars represent the mean value of fluorescence and error bars represent the standard deviation. The images present the drill cuttings from each depth under the fluorescence microscope.

plex mixture (UCM) can be seen at chromatograms from samples Ch4 and less visible in Ch10 and Ch12. Analogous analyses were conducted also in samples from L-1 well. However, the content of the OM was below the detection limit of the implemented procedure (the concentration of the aliphatic fraction was <0.01 mg $100g^{-1}$ of rock sample). The chromatograms show a clear baseline, and few peaks exceeded 5% of the baseline signal value.

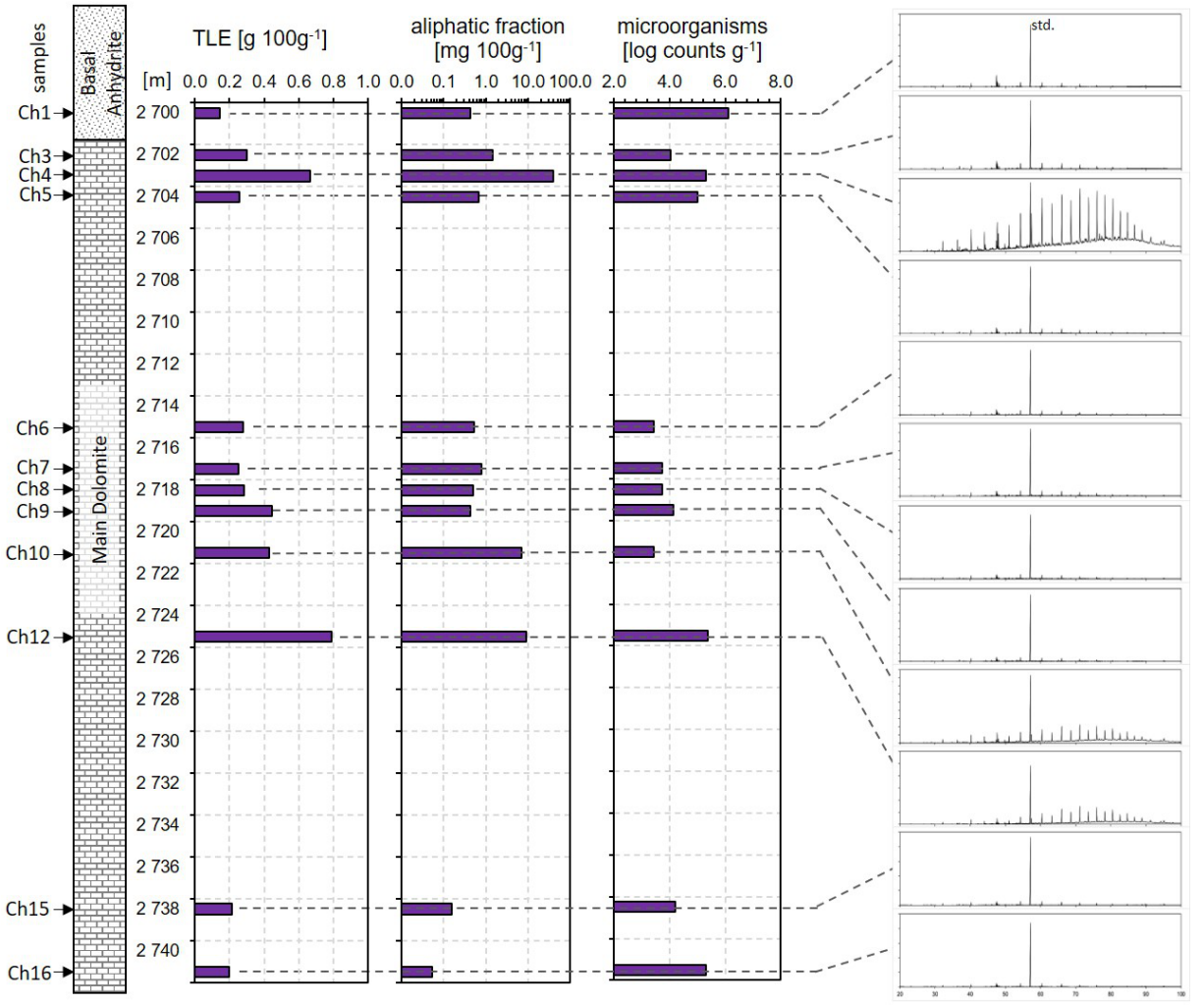
Biomass estimation

Text-fig. 4 shows the number of microorganisms in the C-1 samples. Bacteria-like particles were observed only in the samples from the C-1 well (Text-fig. 5), after staining with the new procedure for staining cells from the rock matrix. However, the count of these particles was very different, depending on the sample. For instance, the most abundance sample was Ch1 where ca. 10^6 bacteria-like particles g^{-1} were noted. On the contrary, in samples Ch6 and Ch10 the lowest value was found (10^4 particles g^{-1}). In the remaining samples, the number of bacterial-like particles ranged from 10^4–10^5 g^{-1}. In the reference samples from L-1 well, such particles were not found. Only a few organic-like particles were noticed, but their colour did not indicate microorganisms as assumed by the staining procedure. Such organic-like particles were rarely visible in both samples from the C-1 and L-1 wells (see Text-fig. 5, white arrows).

Microbial activity

Biodegradation of aliphatic fraction from diesel oil

The experiment conducted with the C-1 samples demonstrated the potential degradation of aliphatic hydrocarbons during incubation at a temperature of 100°C (Text-fig. 6A). The incubations were carried out in two variants: abiotic, with samples post-sterilization, and biotic, with native samples. The average degradation of hydrocarbons was approximately 60–70%. Interestingly, while the difference between the abiotic and biotic variants was not very large (thermal degradation may have occurred in both cases), it was significant in the C-1 samples when comparing all samples, both biotic and abiotic. In the pure control test (reagents with diesel oil without rock



Text-fig. 4. Total lipid extract (g 100 g^{-1} of rock sample), content of aliphatic fraction (mg 100 g^{-1} of rock sample) and number of microorganisms in samples (Ch1–Ch16) obtained from C-1 core. On the right, the TLE chromatograms were presented in the same scale for comparison (std. – internal standard).

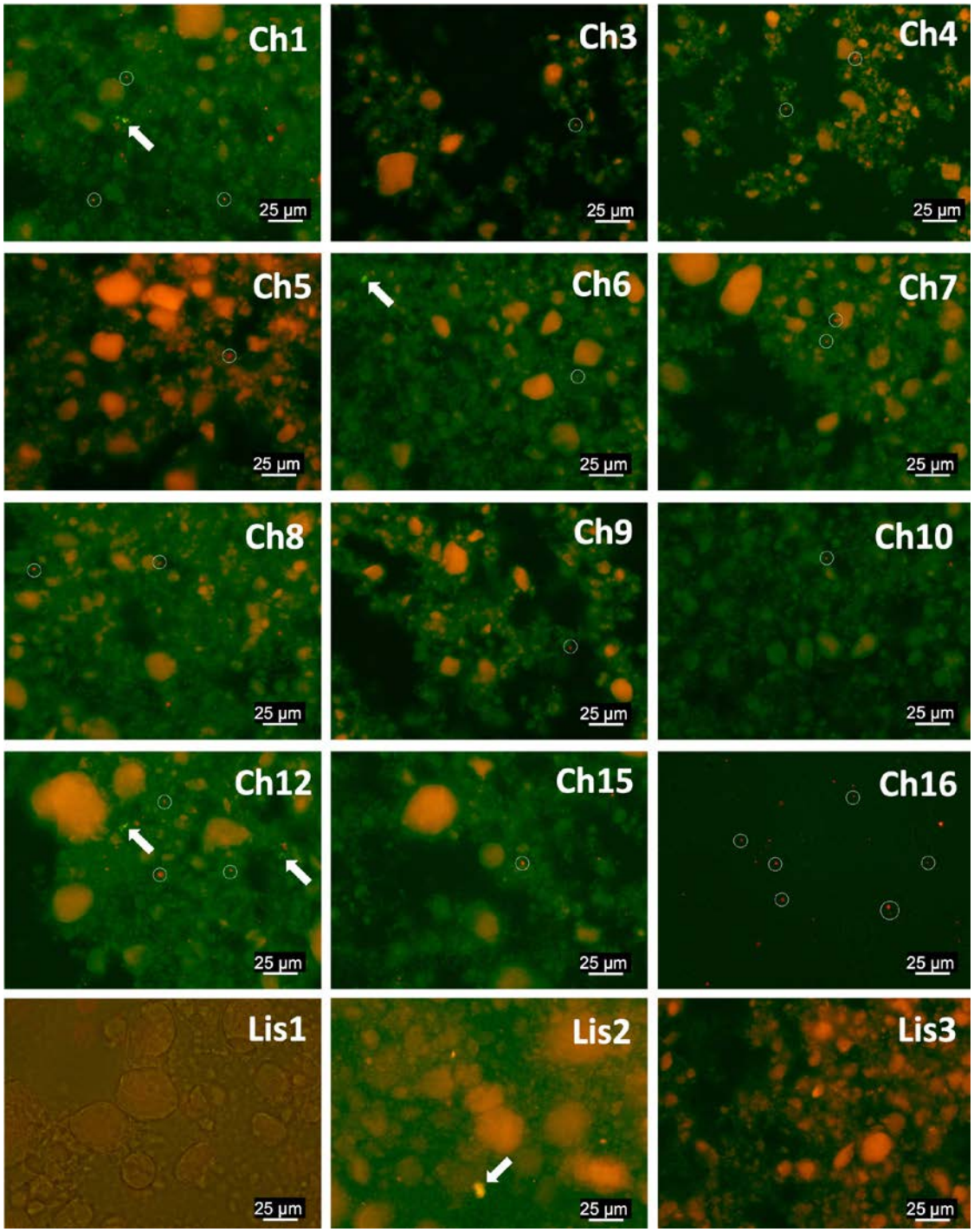
matrix), degradation reached 38%. This suggests a strong matrix effect in abiotic tests with rock samples. However, the samples did not exhibit a consistent level of degradation. Therefore, it may be more informative to compare the ratio of biotic to abiotic degradation, as illustrated in the diagrams below in Text-fig. 6A. A ratio above 1 indicates a higher probability that the sample underwent not only temperature degradation but also biodegradation. For the C-1 samples, usually the ratio is greater than 1, while for L-1 this value is around 1. Indeed, this approach may not be entirely convincing. Therefore, it is crucial to examine how selected parameters, which characterize the studied aliphatic fraction, changed after the degradation process in both biotic and abiotic samples (Text-fig. 6B and C). For example, parameters have been selected: Pristane-Phytane ratio (Pr/Ph), Pristane-C17 n-alkane ratio (Pr/C17), Phytane-C18 n-alkane ratio (Ph/C18), Average Chain Length (1) (Andersson *et al.* 2011),

$$ACL = \frac{\sum_{n=27}^{31}(c_n \cdot n)}{\sum_{n=27}^{31} c_n} \quad (1)$$

Carbon Preference Index (2),

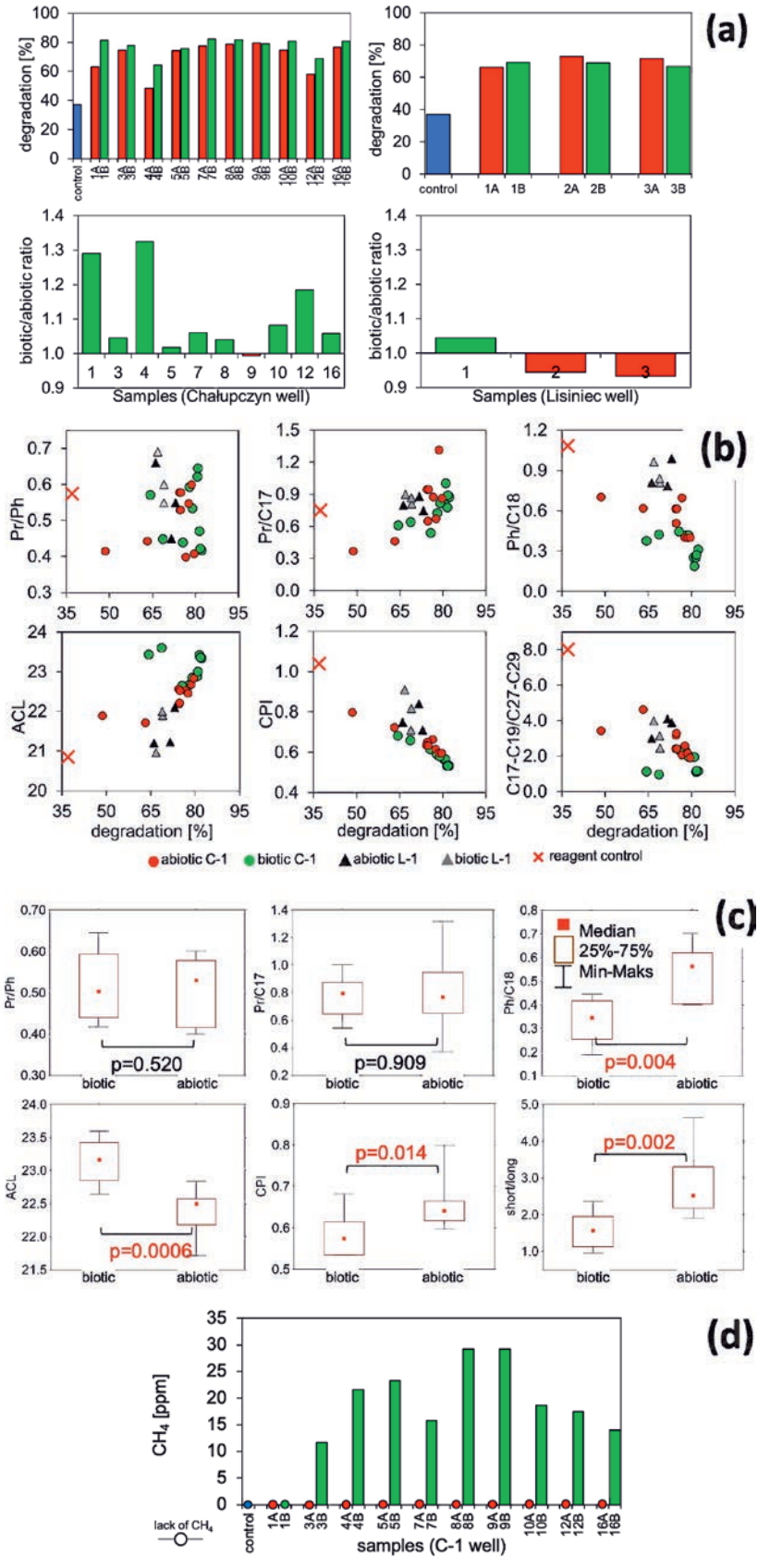
$$CPI = \frac{(c_{17}+c_{19}...+c_{27}+c_{29})+(c_{19}+c_{21}...+c_{29}+c_{31})}{2(c_{18}+c_{20}...+c_{28}+c_{30})} \quad (2)$$

and quite simple n-alkanes parameter C17–C19/C27–C29 showing medium chain to long chain n-alkanes



Text-fig. 5. Fluorescence microscope images of the samples from C-1 and L-1 wells after staining procedure. Distinct but very small red dots indicate possible microorganism cells (circles indicate exemplary bacteria-like particles), green or orangish particles are probably organic matter particles visible due to autofluorescence (for example indicated by white arrows).

ratio. Such an approach comes from the assumption that the biodegradation should result in changes of aliphatic fraction composition (alkanes and isoprenoids) in relation to aliphatic compounds that have undergone only temperature degradation. Besides the Pr/Ph and Pr/C17, the parameters differentiated the



Text-fig. 6. The degradation of the aliphatic fraction test and detection of methanogenesis. (a) – Degradation (%) in abiotic (suffix A) and biotic (suffix B) tests with samples from C-1 (left) and L-1 (right) at temperature 100°C. Lower diagrams show the biotic/abiotic ratios. (b) – The relationship between degradation (%) and the parameters characterizing the aliphatic fraction extracted from C-1 samples after the degradation test. (c) – Nonparametric test (U-M-W test) for differences between biotic and abiotic samples C-1 after the degradation test. The p-value marked in red indicates significant differences (at $p<0.05$). (d) – Methanogenic activity test. The concentration of methane after the 3-month incubation of the samples from C-1 core. Suffix A – abiotic samples (after sterilization); suffix B – biotic samples; control – reagent incubation without rock sample.

aliphatic fraction extracted after biotic and abiotic tests. What is more, it is clearly visible that samples from the L-1 well, both biotic and abiotic, were similar to the abiotic samples from the C-1 well. But are the differences statistically significant? Apart from the mentioned Pr / Ph and Pr / C17, the differences between the biotic and abiotic tests in the C-1 samples were significant (Text-fig. 6 C).

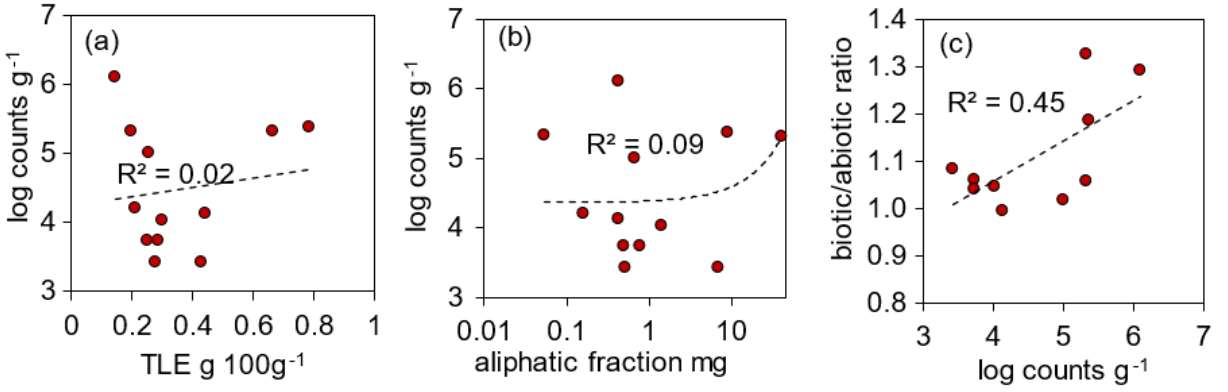
Despite the strong matrix effect mentioned earlier, which influenced the degree of degradation of the aliphatic fraction, significant differences were observed when comparing parameters characterizing saturated hydrocarbons after sample incubation. This was true for both biotic and abiotic samples from the C-1 well. Specifically, statistically significant differences were found in the case of Ph/C18, ACL, CPI, and short/long parameters. Moreover, the degradation *vs* parameters graph (Text-fig. 6 B) shows that the reagent control (marked by a red '×' in the graph) is positioned separately, almost as if it were extending a hypothetical trend line. However, it is important to note that determining a linear regression that interpolates all the results may not be appropriate, given that they come from different data populations (i.e., different wells). The control reagent sample, for instance, represents the pure saturated fraction incubated without the rock sample. This distinction is crucial in interpreting the results accurately.

Methanogenesis test

A methanogenesis test with ^{13}C-labeled acetate indicated that samples from well C-1 could contain methane-producing microorganisms via acetoclastic methanogenesis. Methane was detected in all biotic samples except the Ch1 sample which was derived from the anhydrite part of the core (Text-fig. 6D). The concentration of methane detected after the end of a 3-month incubation ranged from approximately 10 ppm (sample Ch3) to almost 30 ppm (in samples Ch8 and Ch9). Based on the isotopic composition, it was concluded that the generated methane came from the labeled acetate. The isotopic composition of the methane, expressed as the $^{13}C/^{12}C$ ratio, clearly indicated the presence of heavy carbon in it. Additionally, it should be noted here that the acetate used was labeled with only one type of carbon (sodium acetate-2-^{13}C), the one that is incorporated into methane in the process of acetoclastic methanogenesis. Methane was not detected in either the abiotic or control samples. Importantly, in the case of L-1, methane was not found in any of the samples after incubation.

Relationship between biomass and organic matter

A fairly important issue about the reservoir rock environment is the relationship between biomass and OM. Here, two parameters characterizing the OM content were distinguished: TLE, and the concentration of the saturated fraction. These parameters are related to the methodology of the biodegradation activity testing, where diesel fuel was used as a substrate for microorganisms. It can be assumed that the OM content should somehow correlate with the biomass content. However, no statistically significant relationships were noted (Text-fig. 7a, b) between those parameters. It is therefore possible that the biomass determination procedures used were not efficient enough. Although, considering the sample matrix, this should not come as a surprise. However, if the obtained values of the number of cells are a derivative of the actual biomass in the tested samples, then a correlation between the number of cells and the biodegradation of hydrocarbons can be expected. Indeed, such a correlation does exist (Text-fig. 7c). A weak correlation at 5% significance level was found



Text-fig. 7. Rectilinear correlation between number of microorganisms (log counts g^{-1}) and TLE content (a), aliphatic fraction content (b, logarithmic scale) and biotic/abiotic ratio (c). R^2 – coefficient of determination.

between the number of cells and the biotic/abiotic ratio (eliminating the matrix effect) from the biodegradation experiment ($r = 0.67$, $R^2 = 0.45$, p-val = 0.047; two-tailed permutation test of a Pearson correlation coefficient). These findings enhance the thesis about possible microbial activity in the C-1 samples.

Molecular analyses

The isolation of DNA was not always successful. Hence the construction of a genome library before sequencing was not always possible. In all the L-1 samples (reference well), probably due to lack of DNA, the sequencing revealed either no results, or contaminants only (in one sample, 4–415 reads, popular contaminants belonging to: Actinobacteria, e.g. *Corynebacterium*, *Micrococcus*; Alphaproteobacteria, *Paracoccus* only; Bacilli, e.g. *Staphylococcus*; Bacteroidia, e.g. Bacteroides; Campylobacteria, *Helicobacter* only; Clostridia, e.g. *Faecalibacterium*; Cyanobacteriia; Gammaproteobacteria, mainly *Escherichia* and *Pseudomonas*; Negativicutes and Vampirivibronia). The samples from C-1 well were also analysed (NGS) and the obtained results were slightly different (mainly in relation to reads) dependently on the laboratory (see Supplementary material S2). In Lab_1 ten samples and drilling mud were successfully sequenced, in Lab_2 seven samples and drilling mud were sequenced, and the data were obtained. Generally, the number of reads were in range 122,642–160,339 from Lab_1. In case of Lab_2, the number of reads were 89,510–129,752 except one sample (173 reads from Ch10 – probably due to lack of PCR products at the stage of library construction).

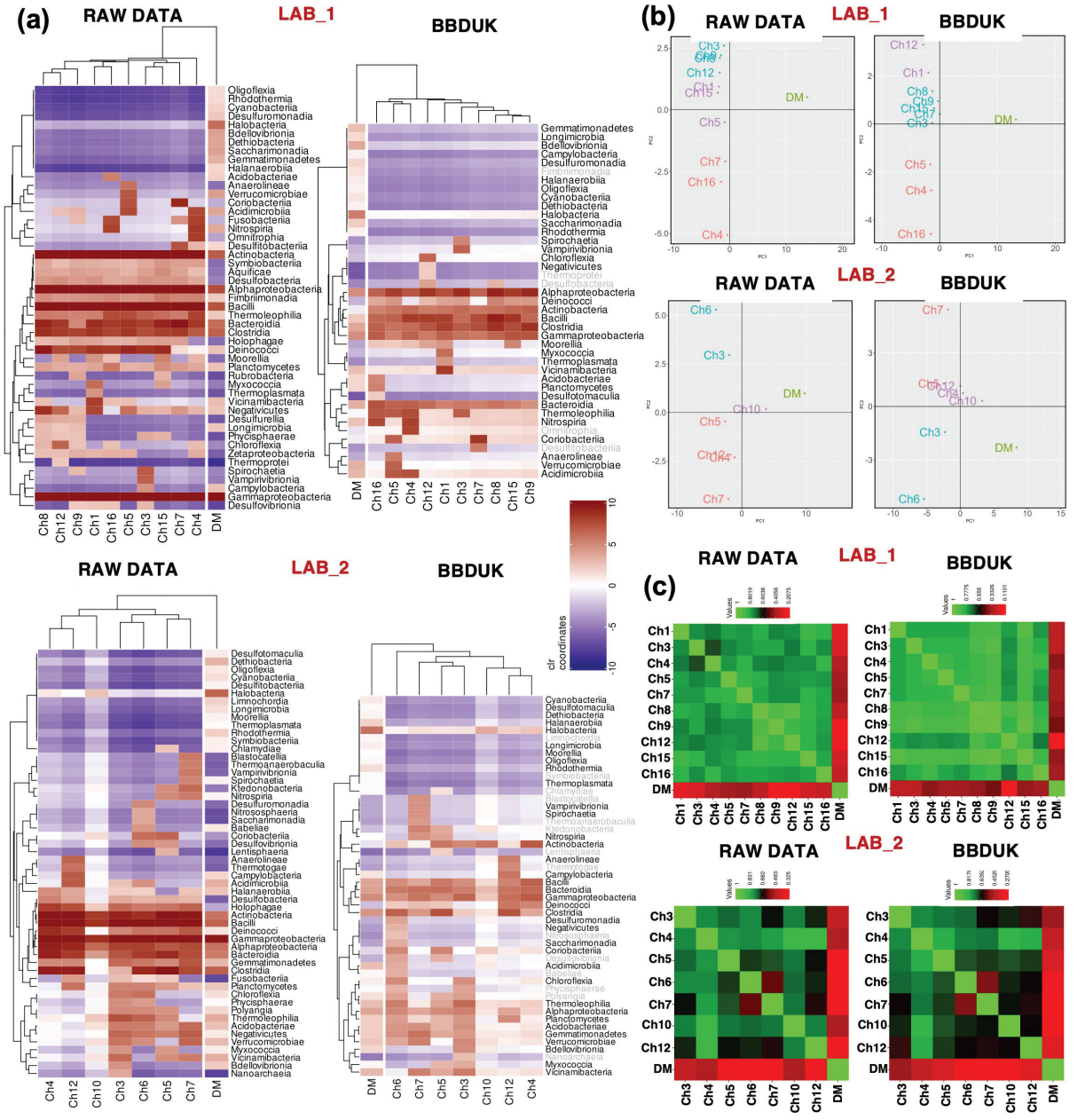
In the figure of metabarcoding analyses (Text-fig. 8), the data before *in silico* decontamination are presented as "RAW DATA"; while data after decontamination as "BBDUK". The overall pattern of classes is similar in both data from the laboratories, but some differences can be noted. On the one hand the main classes in both cases are Actinobacteria, Alphaproteobacteria, Bacilli and Gammaproteobacteria. On the other hand, the ratios between these classes are different. What is important is that in both cases the composition of the drilling mud (DM) is different from the composition of the core samples, this being well revealed by a hierarchical analysis (Text-fig. 8a). The decontamination step changed the composition of the data. Firstly, a reduction in the number of readings can be noted. However, it was observed that sequences present in the drilling mud were absent from the core samples. Moreover, this pattern is present in the data from both laboratories. This is particularly evident in the class of Halobacteria, the halophilic Archaea, which are entirely absent from samples from all cores (see data structure in Supplementary material S2; in Text-fig. 8a, the data were transformed due to its compositional nature). In the case of other Archaea, Nanoarchaeia and Nitrososphaeria were detected in the core samples. However, these groups were detected only in the LAB_2 analysis. Differences between LAB_1 and LAB_2 also concern bacterial groups, e.g. Desulfobacteria and Desulfitobacteriia were noted after sequencing in LAB_1. On the other hand, Desulfovibronia was detected in the LAB_2 data. This is probably the effect of using different primers characterized by different specificity. However, in general, the cluster analysis revealed a similar arrangement of possible groups of samples. There were some differences noted in the location of individual samples within clusters, but always the DM sample was located separately. Some of the samples before and after decontamination were clustered together, such as Ch4 and Ch14 samples from LAB_1 and Ch4, Ch10 and Ch12 samples from LAB_2. The abundance of the different classes of microorganisms before and after *in silico* decontamination was reflected in the Principal Components Analysis (Text-fig. 8b). The result of the analysis showed that the samples do group quite similarly, even considering the origin of the results from two different laboratories. Moreover, the DM sample always locates separately from the rest of the samples.

The correlation matrices showed that the drilling mud (DM) was distinct, within which individual grade readings were weakly correlated with readings from other core samples (Text-fig. 8c). Such a pattern suggests that most core-derived samples have a similar relationship between the number of readings and the microbial classes. *In silico* decontamination did not disturb this result. There is a clear lack of correlation (or weak correlation) between the drilling mud sample and the other samples. It can also be seen that no or weak correlation in relation to the other samples was found for samples Ch7, Ch16 and sample Ch10 from LAB_1 and LAB_2, respectively.

DISCUSSION

Contamination issue

The contamination tests that are commonly used in drilling should in principle be considered to be indirect and imperfect methods. The most import-



Text-fig. 8. Metabarcoding 16S rDNA analyses for raw data (RAW DATA) and after *in silico* decontamination (BBDUK) from two different laboratories (LAB_1 and LAB_2). (a) – Cluster analysis with heatmaps after imputation of zero values and transformation (clr). Gray fonts at the heatmaps (decontaminated datasets) indicate taxa positively identified by only one laboratory. (b) – Principal Component Analysis showing the location of samples in Euclidean space. (c) – Correlation matrices (pairwise plots) between samples. The higher the correlation coefficient of the readings for classes of microorganisms between samples, the greener colour. A higher correlation coefficient may indicate that the samples were more likely to originate from a similar niche. A red colour indicates no (or weak) correlation between readings of microbial classes within the samples being compared.

ant test is always to have reference samples taken as controls, as is usual for all microbiological testing. In the literature on deep biosphere studies, the problem of contamination of cores with drilling mud is raised (Morono and Inagaki 2016). It has been proposed to use microspheres as an additive to a drilling mud, but these markers are expensive and unstable at high temperature (Morono and Inagaki 2016; Friese *et al.* 2017). It has been shown that fluorescent dyes such as rhodamine and fluorescein can be used as markers

for contamination (Wandrey *et al.* 2010). Laboratory tests showed that increasing the pressure from ambient to 90 bar does not necessarily cause an increase in the depth of penetration of the drilling mud into the core and usually does not exceed a few millimetres (Pellizzari *et al.* 2013). It has been noted that during drilling, the drilling fluid penetration may be slightly higher, especially in sandstone rocks. However, even in these cases, it only reaches approximately 20 mm. (Pellizzari *et al.* 2013). Generally, the inner part of the core remains intact. The increased pressure of the drilling mud does not push out the brine from the core. The problem of pressure seems to be overestimated in many cases. The drilling mud does not penetrate deep into the core and into the walls of the wells, as it could be assumed in advance when thinking about the high pressures that may be generated there (which is also a mistake in assumption). During the drilling process, the safe mud window for drilling (according to Zoback 2007) – 'the difference between the minimum and maximum mud weight one should use when drilling at given depth' is intended for the wellbore stability. The minimum mud weight is usually taken to be a little higher than that of the pore pressure and collapse pressure so that a well does not flow while drilling and does not collapse. The upper boundary of the mud window cannot be higher than the pressure of hydraulic fracturing of the formation. Therefore, we can conclude that the bottom pressure exerted by the mud column during drilling is close to the initial pore pressure in the rock formation. The drilling process cannot significantly interfere with the pressure regime in the formation because mud invasion into the formation is minimized by creating mud cake.

If the samples were contaminated, then false positive results of microbiological activity in samples from the L-1 well should also be obtained. Both microscopic and methanogenic activity studies clearly show that this did not happen. Therefore, it can be concluded that the applied procedures are appropriate, and the results are reliable. What is more, the sample from the anhydrite part of the C-1 core also showed no methanogenic activity. This is an important result. It is known that the presence of sulphates can significantly inhibit the activity of the methanogenic Archaea (Lovley and Klug 1983; Karhadkar *et al.* 1987; Lovley and Phillips 1987) although there may be some exceptions where methane can be produced simultaneously with the reduction of sulphates (Oremland *et al.* 1982). One more point needs to be raised here. Paradoxically, testing "hot" wells for microbial activity is less prone to contamination interference than simply testing the microbial activity of various samples at room temperature. It is impossible to believe that common contaminators can grow at temperatures as extreme as 100°C. Things are different when it comes to molecular research. It is much easier to contaminate with the DNA molecules commonly found in the natural environment and human surroundings. This problem will be further considered in the discussion of molecular investigations. It should be emphasized, that molecular tests of drilling mud can be used as a very good contamination test. The mud should have a different molecular composition due to contact with non-sterile drill pipes and other rock layers. It can therefore be assumed that some molecular signals should not be found inside the tested core. This approach, assuming the analysis of the molecular composition of the drilling mud and the tested core, seems to make more sense than the use of artificial markers, especially when those markers may be instable at high temperatures.

Biomass in the reservoir rocks

All the issues mentioned above are strongly linked with the detection of the microbial activity that can be conducted under laboratory conditions. Firstly, to conclude on possible microbial activity, a method for determining the biomass of microorganisms which are present in rocks is needed. Many studies implement the microscopic methods using a fluorescence dye such as SYBR Gold (Middelboe *et al.* 2011; Beulig *et al.* 2022). However, that approach has the potential to generate some problems due to the presence of bacteria-like particles. Such particles, often "flecks" of organic matter, can exhibit their own fluorescence, causing false-positive results. On the other hand, there are methods based on e.g., the analysis of the fatty acid content. However, these methods can be extremely sensitive to lab-derived contamination (Grosjean and Logan 2007; Brocks *et al.* 2008). In fact, all research in this area is based on the counting of particles similar to bacteria that glow green under a fluorescence microscope. The issue lies in the fact that the procedure employed does not eliminate the rock matrix. This matrix is typically filled with particles that exhibit autofluorescence when excited with monochromatic light in the 400–450 nm range. As a result, an image of numerous green-glowing dots is obtained, which cannot be distinguished from microorganisms. In addition, these cells are expected to be very small, perhaps even extremely small due to the spatial constraints of the rock matrix. In the available literature, the presented photos usually even show a large cell size (Dai *et al.*

2021). Dai et al. (2021) also obtained surprisingly large numbers of cells in rocks, reaching 10^8 cells g^{-1} in metamorphic rocks(!). The question is whether some of these results are false positive due to the similar fluorescence of cells and organic particles. This fact and the above problems raise doubts as to the correctness of such data. In the analytical approach presented in our work, we have shown another way, though also imperfect. First, we assume that the cells from the rock matrix should be separated (as much as possible). Second, the cells should be concentrated as much as possible. We have shown that in this approach it is possible to use a modified artificial silica matrix to adsorb all cells from the suspension. For this purpose, an imidazolium ionic liquid changing the net surface charge was used (Borkowski *et al.* 2019). In this way, a material suitable for dying is obtained with cells dispersed on silica. The silica with adsorbed bacteria can be easily suspended in a defined volume in the microscopic counting chamber, and it is also very easy to concentrate at a low speed of the centrifuge. Our modification also concerned the dye used. Sypro®Tangerine stains cells from red to orange. It seems that after such staining it is easier to distinguish potential cells from material of other origin, usually showing green fluorescence when viewed under blue light. Particles of probable OM glowing green also happen, but there are considerably fewer of them. The procedure does not preclude the use of other fluorescent dyes. The use of an ionic liquid also does not interfere with the commonly used dyes for staining bacteria.

Microbial activity

The most important tests conducted during these studies concerned methanogenic activity. The obtained results seem unambiguous. Firstly, it was shown that the dolomite samples from the C-1 well revealed methanogenic activity at a temperature close to 100°C. Identically incubated samples from the L-1 well showed no such activity, as well as abiotic control samples from the C-1 well. Second, the anhydrite sample from the C-1 well showed no methanogenic activity. In the control reagent sample, the presence of methane was also not found. These data strongly support the fact that the C-1 well is microbiologically active. Moreover, it was possible to show this activity under laboratory conditions. The results also showed that there was no contamination of the samples. It should be noted that the addition of sodium acetate could simply have activated the microorganisms in the rock sample, but this does not contradict the claim that living cells exist in the deposit‘s conditions, even if we assume that they are in a dormant state. Hence, it is possible and justified to try to use the method of measuring methanogenic activity under laboratory conditions for the assessment of the contemporary methanogenic activity in reservoir formations, as it was suggested in previous works on the estimation of the contemporary biogenic gas component (Cokar *et al.* 2010, 2013b). It is not necessary to use only sodium acetate, as it also seems possible to use a ^{13}C-labelled H_2/CO_2 mixture for the assessment of the hydrogenotrophic activity. This aspect should be examined in further studies. However, the use of acetate is not an artificial treatment, as volatile fatty acid salts are commonly found in reservoir rocks (Li *et al.* 2021; Beulig *et al.* 2022). In addition, the handling of acetate solutions is usually easier than a gas mixture, where also the pressure would be of importance in setting up the incubation. A separate problem is the possibility of detecting changes in the number of microorganisms after the end of incubation tests. In the case of surface environments, which are usually not subject to such significant thermodynamic constraints, microbiological growth can be easily detected after sample incubation. However, in the case of rock samples, detecting an increase in the number of microorganisms can be very difficult. This is primarily due to the extremely long generation time of microorganisms inhabiting deep rocks (Jørgensen, 2011). It should be emphasized that the detected amounts of methane after 3 months of incubation were quite small. Therefore, it seems that although this time was sufficient to detect methanogenic activity, it would probably not be sufficient to detect an increase in biomass in the samples.

The degradation test with diesel oil is less convincing. The obtained results may be strongly affected by the matrix effect. This effect may manifest itself through different adsorption of hydrocarbons on the rock matrix, and thus affect the efficiency of the subsequent extraction after the end of the incubation. Secondly, hydrocarbons can be degraded abiotically at the incubation temperature, especially when rock is present in the sample. This is evident when compare the abiotic sample (with the sterilized rock matrix) to the reagent control sample. Hence, this test should be considered less useful in such studies. However, some careful conclusions can be drawn. It seems that if microbial degradation has occurred, some features characterizing the hydrocarbons should be different from those characterizing hydrocarbons after temperature degradation. So, such parameters as the average chain length or the ratios between differ-

ent aliphatic hydrocarbons should be different. Our studies showed that such differences exist. Moreover, they are statistically significant. Mostly, these results align with those from other tests. For instance, the anhydrite sample showed significant activity, as it is characterized by a significant number of visible cells in the microscopic test. However, in the methanogenesis test, it obviously showed no activity. This is quite a strong argument to demonstrate that the microbiological tests performed showed a reliable picture of the activity in the samples from the C-1 well.

Molecular studies

It is commonly known that environmental microbiological studies should be supported by molecular analyses. However, it should be emphasized that the rock samples, especially rock cores, are very difficult material due to: (i) low biomass abundance; (ii) inhibitors of PCR; (iii) problematic mineral matrix that makes difficulties during isolation of genomic DNA. However, the most important problem seems to be low biomass. That problem generates another one – the low abundance of DNA from the sample leads to an increase in the importance of contamination. That DNA contamination comes from the surroundings, mainly from human skin, air, laboratory equipment and from kits for DNA isolation. The latter is the so called "kitome" (Salter *et al.* 2014; Stinson *et al.* 2019). However, it can be assumed that a careful approach to isolation of DNA from the rock matrix can bring important information about microbial communities. This is acceptable, even though some works indicate that the DNA quantities were often insufficient to obtain reliable data concerning the structure of microbial communities (Heuer *et al.* 2020; Beulig *et al.* 2022). Indeed, if it is possible to incorporate a decontamination step (*in silico*) into DNA analysis after sequencing, then the resulting data may be more reliable. Of course, it is necessary to construct a good database for the decontamination step. We constructed such a database based on literature data indicating common contaminants and with access to contaminant DNA sequences (NCBI).

The data obtained are therefore devoid of common contaminants. Hence, it can be assumed that in some part they may reflect the actual microbiological composition of the samples studied. However, data derived from such specific matrices as rocks should always be approached with caution. If one assumes that the results obtained are reliable, it can be noted that: (i) in fact, the two laboratories independently revealed quite similar results; (ii) the drilling mud sample contained sequences that were absent in the rock samples; (iii) some of the sequences from the mud may indicate its contact with microbially colonized salt layers (Older Salt and Younger Salt above the Main Dolomite). These are very important observations and conclusions. First, the drilling mud has shown that it is most likely that the salt formations above the Ca2 may be microbially colonized. Second, it seems that some molecular sequences from the drilling mud can serve as an excellent marker of core contamination during drilling. It is a much better marker than artificially introduced mud components, which are usually limited in their use. In this regard, the results obtained seem very promising and justify attempts to isolate DNA from drill cores. However, due to the complex matrix and the scarce amount of DNA, the acquisition of molecular data should not be conducted by cloning. *In silico* decontamination has shown that, in light of the data obtained, the results obtained by cloning (e.g., in the papers of Zhang *et al.* (2006); Dai *et al.* (2021)) are basically exclusively or almost exclusively contaminants. Identifying the source of contamination can be challenging. It could originate during the genomic library creation, the sequencing process (potentially due to reagent contamination), or even during laboratory manipulations. Samples from the reference well (L-1) either turned out to be completely sterile in terms of the presence of DNA or revealed only the presence of contaminants.

The data obtained were generally consistent with the structure of microbial communities obtained from molecular analyses of various deep wells (Inagaki *et al.* 2015; Tsesmetzis *et al.* 2018; Sahu *et al.* 2022) or mine water (Keshri *et al.* 2015). Usually, the dominant classes of microorganisms observed in such analyses were Clostridia, Acidobacteriae, Actinobacteria, Bacilli, Bacteroidia and classes from Proteobacteria, mainly Alpha- and Gammaproteobacteria.

In that context, the Archaeal sequences revealed in the obtained data were pivotal. Archaea comprise the microorganisms able to grow under extreme conditions and are often involved in transformation of OM in reservoir systems. In the core samples, Nitrososphaeria and Nanoarchaeia were found. In the drilling mud, Halobacteria were detected. Some of the detected Archaea belong to Thaumarchaeota, which are generally thermophilic microorganisms involved in the nitrogen cycle and oxidation of methane (Sinninghe Damsté *et al.* 2002; de la Torre *et al.* 2008; Schouten *et al.* 2013). These findings are important. However, it is not clear why these microorganisms comprised only a very small group of the sequences found. Probably, either the PCR primers used multi-

plicated bacterial rather than archaeal DNA, or archaeal sequences were concealed during the NGS by more abundant bacterial sequences. Additionally, NGS primers may produce under- and over-representation of some Archaea and Eubacteria, respectively (Wear *et al.* 2018).

CONCLUSIONS

There is no doubt that the samples tested from the two different wells reaching the Main Dolomite had different microbiological characteristics. The data obtained suggest that the Main Dolomite at 100°C may be colonised by active microbial communities. It is plausible that some Permian petroleum systems may exhibit modern microbial gas generation and transformations. These processes could lead to hydrocarbon degradation and the production of methane, as well as carbon dioxide and potentially hydrogen sulphide. It is impossible to believe that the data obtained could have been the result of contamination. Indeed, given (i) the high incubation temperatures of the samples for methanogenesis and biodegradation tests, (ii) the molecular signals differentiating the C-1 samples from the L-1 reference samples and from the drilling mud, and (iii) the lack of methanogenesis in the anhydrite sample from the C-1 well, it is likely that the data obtained are indeed indicative of measurable modern microbial activity of the hot biosphere within the Main Dolomite. Hence, the biosphere can strongly affect the quality of gas in reservoir rocks and, equally importantly, modify the composition of hydrocarbons which should be considered in routine organic geochemistry studies. From a practical point of view, two conclusions from the study should be mentioned here. Firstly, it is possible to determine the modern biogenic component in gas reservoirs by measuring acetoclastic methanogenesis activity in well samples. Secondly, it appears that molecular comparative studies of the microbial composition of mud and core samples are a natural test of contamination without the introduction of artificial markers. It should also be noted here that the molecular composition of the mud may bear important microbiological information about the rocks through which the borehole was drilled.

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Data availability

The datasets generated during and/or analysed during the current study are available in the SRA repository, https://www.ncbi.nlm.nih.gov/sra/PRJNA1068119. The database for *in silico* decontamination can be found in Zenodo: https://zenodo.org/doi/10.5281/zenodo.10208598.

Author's contributions

Conceptualization & supervision: AB, PZ; methodology: AB; sample acquisition and handling: PZ; contamination tests: AB, PD; laboratory analysis (chemical and microbiological): AB, TK, PD; molecular analysis: PD; molecular data processing, statistical analysis, and data visualization: AB, PD, TS; writing (original draft): AB; writing (review & editing): PZ, PD, TS.

Competing interests

The contact author has declared that none of the authors has any competing interests.

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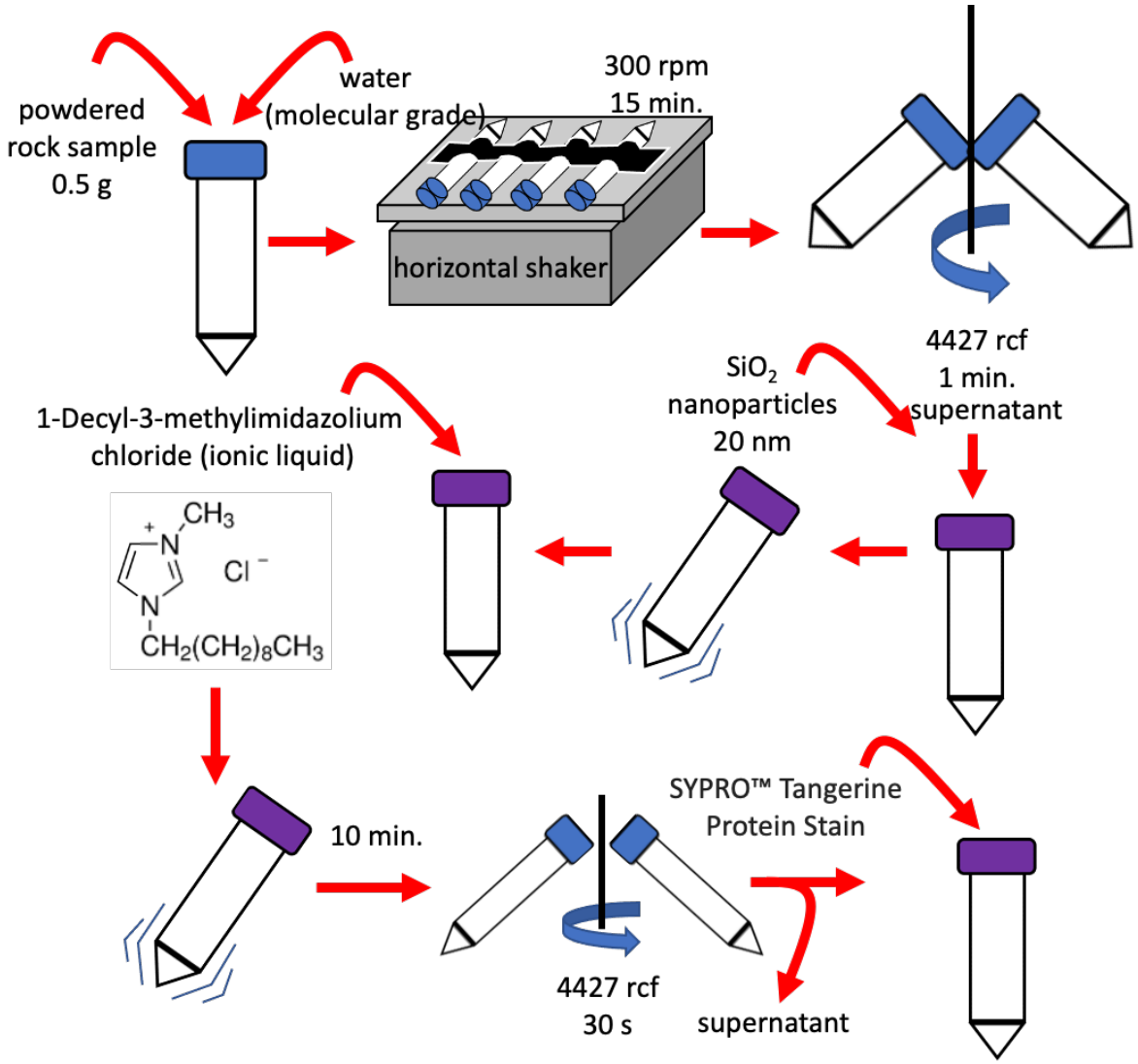
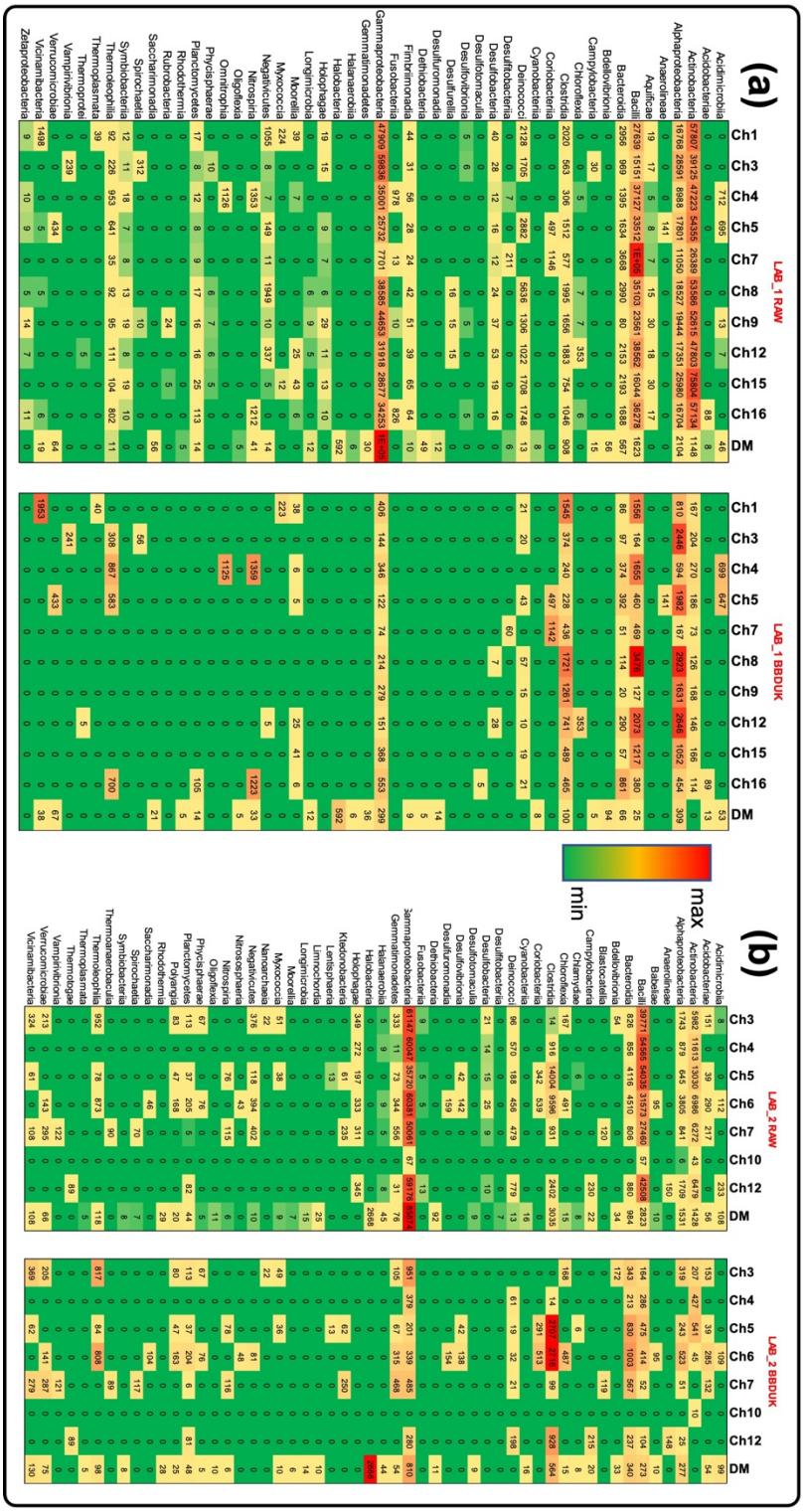
Supplementary materials



S1. Scheme of the microorganisms staining procedure implemented for dolomite and anhydrite rock samples.



S2. Data structure after metabarcoding analysis. Totals of readings for individual classes of microorganisms. (a) – Data from Lab_1 (raw data and after *in silico* decontamination). (b) – Data from Lab_2 (raw data and after *in silico* decontamination).