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Long-term anaerobic biodegradation of Miocene lignite by indigenous microbial communities: insights into carbon and nitrogen cycling

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To better understand processes in coal environment Miocene detritic lignite from the opencast mine was incubated under anaerobic conditions in the dark (headspace atmosphere 85% N_2, 10% CO_2, 5% H_2) for 3 years. The natural microbial community of detritic lignite was stimulated for growth through the addition of trace elements, vitamins, and carbon-bearing compounds such as yeast extract, nutrient broth, methanol, and sodium acetate. A visual indicator of microbial activity was observed as the color of the fermentation solutions changed over time: from colourless to light yellow (after 3 months), dark brown (after 6 months), and finally black (after 1 year). This progression suggests the dissolution of fulvic and humic acids. At the end of the cultivation, the total nitrogen and total inorganic nitrogen (TIN) contents in the solutions were significantly reduced whereas in incubations with sodium acetate, dissolved organic nitrogen (DON) content significantly increased compared to the initial levels. In most cases, total carbon content significantly increased due to biodegradation, except for the incubations where methanol was added. A GC-MS analysis of the total extracts from lignite revealed that the main macromolecule decomposed by microorganisms was lignin, along with its diagenetic derivatives. The biogas released during the process contained CO_2 and trace amounts of CH_4 (up to 50 ppm). Isotopic data indicated the occurrence of possible anaerobic CH_4 oxidation. Notably, 16S rRNA gene sequencing identified taxa affiliated with lineages that include known diazotrophs in all investigated samples, members of the order *Rhizobiales* (families *Beijerinckiaceae, Rhizobiaceae*).

KEYWORDS
anaerobic biodegradation, lignite, microbial community from coal, molecular composition, N2-fixation, near-surface organic matter cycling, rhizobiales

1 Introduction

Organic-rich sediments, including coals, are habitats for microorganisms such as Bacteria, Archaea, and Fungi. Coal decomposition processes under aerobic and anaerobic conditions are widely studied to better understand the formation of microbial gases rich in CH_4 and H_2 (Vinson et al., 2017). Stimulation of natural microbial communities from organic-rich habitats can result in the formation of coal bed methane (CBM), which is a biotechnological method alternative to traditional industrial processes of methane production (Fallgren et al., 2013; Pytlak et al., 2021; Ritter et al., 2015; Singh et al., 2012; Strąpoć et al., 2011; Szubert et al., 2020). Bacteria isolated from lignite deposits can also degrade some aliphatic and aromatic hydrocarbons under fully aerobic conditions (Supel et al., 2026), which may be applicable for bioremediation of contaminated sites. For these reasons, carbon cycling in organic-rich environments is studied in detail, while nitrogen cycling in coal-bearing strata is a relatively rarely undertaken research topic (Flores and Moore, 2024). The positive exception is the monitoring of NOx emissions from the combustion of lignite or hard coal (Scheffknecht et al., 2011). The lack of proper studies of nitrogen cycling in coal environments is mainly caused by very low nitrogen content in the coal matrix (which is usually lower than 2%) (Nelson et al., 1992). However, nitrogen cycling is the second most important element after carbon for controlling the natural diversity, dynamics, and functioning of marine, freshwater, and terrestrial ecosystems (Stankiewicz and Van Bergen, 1998).

The main pool of nitrogen on Earth is the atmosphere, which contains 99.96% of total nitrogen (TN) as gaseous N_2. The remaining 0.04% is the nitrogen bound in organic (57%) and inorganic form (43%) (Stankiewicz and Van Bergen, 1998). Organic nitrogen occurs in living biomass of terrestrial and aquatic origin and products of its decomposition as sedimentary organic matter (OM). The nitrogen cycle is complex, and identification of transformations of nitrogen compounds in OM is very difficult and requires sophisticated analytical methods, e.g., stable isotope studies (Deb et al., 2024; Müller et al., 2014). However, many questions remain to be answered, especially when considering N_2O and N_2 fluxes from anthropogenic sources or natural habitats (e.g., peatlands, wetlands) (Yu et al., 2020). Recently, it was found that heterotrophic nitrification, a process where nitrites and N_2O are produced during the decomposition of soil OM, may play an important or even a dominant role in N_2O emissions (Lewicka-Szczebak et al., 2021; Zhang et al., 2015), however this process has been often ignored in nitrogen balance. Hence, the combined studies of OM decomposition and nitrogen transformations may open new insights into soil nitrogen cycling. Processes such as N_2-fixiation, denitrification, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), and anaerobic ammonium oxidation (ANAMMOX) occur commonly in many environments (Müller and Clough, 2014).

The main forms of nitrogen in sedimentary OM are biomolecular structures proteins (e.g., collagen, keratins, myosin), amino acids (as DNA, RNA), amino sugar polymers (e.g., chitin) or porphyrins (Ding et al., 2018; Liu et al., 2024). Proteins and amino acids are easily degraded by microorganisms, and they are relatively unstable during deposition and later after diagenesis. Moreover, chitin and porphyrins, much more resistant materials, are minor contributors to the nitrogen in the bio- and geosphere. Nitrogen is often linked with aromatic structures of non-labile fractions of OM (Haynes, 2000). Lignin may contain nitrogen in its structure, e.g., as–NCH_3 groups (Whitehead and Quicke, 1960). In summary, in many organic-rich sediments, the total amount of nitrogen is very low. For example, Miocene lignites from Konin and Bełchatów (Poland) contain 0.2%–0.6% nitrogen (Bucha et al., 2020; Kwiatos et al., 2018). Moreover, TN content is up to two or three times higher in detritic lignites than in xylites, due to a higher proportion of lignin to cellulose.

The decomposition of any OM is slowed if the C:N ratio is > 30 (Mortier et al., 2016). Thus, the bioavailability of nitrogen in coal habitats is a limiting factor for the growth of microorganisms like in many oligotrophic environments (Strąpoć et al., 2011). Leschine et al. (1988) discovered the bacteria able for lignocellulose degradation and anaerobic N_2-fixation. Dey et al. (2021) showed that N_2-fixing bacteria are active under anaerobic conditions and use humic substances as extracellular electron mediators. This discovery indicates that bacteria can fix nitrogen not only in an oxygen-rich atmosphere. It seems that microorganisms adapt to conditions and sources of biophilic elements. In soils N_2-fixing bacteria called rhizobia can form a symbiotic relationship with legumes. The result of this symbiosis is forming the nodules on the plant root, within which the bacteria can convert atmospheric nitrogen into ammonia that can be used by the plant. Recent studies revealed microorganisms able for nitrogen processing in coal-rich sediments. Guo et al. (2015) detected the microbial taxa related to nitrogen metabolism in Chinese coals, including N_2-fixing taxa and denitrifying taxa. Shi et al. (2021) found that microbial nitrogen metabolism affected OM decomposition in coals such as the decomposition of cellulose and carbohydrate. Therefore, it is very important to better understand the role of nitrogen cycling (especially N_2-fixing), and lignocellulose degradation under anaerobic conditions.

In this work, we show preliminary premises of possible anaerobic activity of N_2-fixing microorganisms that naturally occurred in Miocene detritic coals from Poland. The basic microbiological studies are supplemented with geochemical data, including biogas composition (concentrations of CH_4, CO_2 and its stable carbon isotopes values), elemental content of fermentation liquids (total carbon and different forms of nitrogen) as well as GC-MS studies of degraded coals (molecular composition of biodegradation products). The results of 16 S rRNA amplicon sequencing of microbiological samples from the experimental coals and coals without any cultivation were compared to preliminary observation of N_2-fixing bacteria activity under anaerobic conditions during coal degradation. The novelty of this work is the simulation of natural OM decomposition by maintaining anaerobic conditions of lignite cultivation for a few years (3 years) and the stimulation of microorganisms naturally occurring in raw lignite samples. Geochemical and microbiological methods were used to provide a very preliminary indication anaerobic N_2-fixation in lignite deposits and initially identify the biodegradation processes of detritic lignite by autochthonous microorganisms.

TABLE 1 Experimental setup of the lignite cultivation and controls (bold font—variants selected for microbiome structure profiling; in brackets symbols referring to the key characteristics of each treatment).

Name	Silica gel (SG) [g]	Lignite [g]	Sterile redestilled water (SRW) [mL]	M9 minimal media (M9) [mL]	Trace solution (TS) [mL]	Yeast extract (Y) [mg/L]	Nutrient broth (N) [mg/L]	Sodium acetate (AC) [mg/L]	Methanol (ME) [mg/L]	Repetitions
M9		10		150						2
M9_Y		10		150		100				2
M9_N		10		150			100			2
M9_Y_N		10		150		100	100			2
M9_AC		10		150		100	100	2500		2
M9_ME		10		150		100	100		2100	2
M9_TS		10		150	1					2
M9_TS_Y		10		150	1	100				2
M9_TS_N		10		150	1		100			2
M9_TS_Y_N		10		150	1	100	100			2
M9_TS_AC		10		150	1	100	100	2500		2
M9_TS_ME		10		150	1	100	100		2100	2
Controls										
SRW		10	150							2
SG	10			150						2
SG_Y	10			150		100				2
SG_N	10			150			100			2
SG_Y_N	10			150		100	100			2
SG_AC	10			150		100	100	2500		2
SG_ME	10			150		100	100		2100	2
SG_TS	10			150	1					2
SG_TS_Y	10			150	1	100				2
SG_TS_N	10			150	1		100			2
SG_TS_Y_N	10			150	1	100	100			2
SG_TS_AC	10			150	1	100	100	2500		2
SG_TS_ME	10			150	1	100	100		2100	2

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2 Materials and methods

2.1 Sample collection and separation

Miocene detritic lignite was collected in 2019 from the Jóźwin IIB open pit (52°24.781′N, 18°10.150′E) of the "PAK Kopalnia Węgla Brunatnego Konin" mine in central Poland. This immature lignite is dominated by the detritic lithotype with xylite fragments and represents an early stage of microbial transformation and diagenesis (Fabiańska, 2007; Fabiańska and Kurkiewicz, 2013). Detailed geological and geochemical characteristics of the deposit have been reported previously (Bechtel et al., 2019; Bechtel et al., 2020; Marynowski et al., 2021; Widera, 2016a; Widera, 2016b; Widera et al., 2017, Widera et al., 2021; Zieliński and Widera, 2020).

Freshly exposed lignite blocks were collected from the exploited mine wall, transported in N_2-flushed glass jars, and transferred on the same day to an anaerobic chamber (N_2 85%, CO_2 10%, H_2 5%). The material was subsequently crushed into ~1 × 1 cm fragments under anaerobic conditions, and only the inner part of the lignite was used for cultivation experiments.

2.2 Experimental setup

Approximately 10 g of lignite (macroscopically identified as detritic lignite) was placed into each 250 mL serum bottle. The samples were neither dried nor homogenized before incubation to avoid disturbance of the native microbial community. Consequently, slight variability in lignite properties between bottles is a limitation of the experimental design; however, lignite weight deviations did not exceed ±0.2 g.

Each bottle contained 150 mL of sterilized modified M9 mineral medium (Miller, 1972), in which $MgSO_4$ was replaced by $MgCl_2$ (190 mg/L). The medium (pH 7.0) contained no glucose. Selected cultivations were supplemented with yeast extract and nutrient broth (100 mg/L each; BTL Sp. Z o. o., Department of Enzymes and Peptydes, Łodź, Poland) as sources of vitamins and growth factors (Zinder, 1993). Half of the cultivations also received trace mineral solution (modified Wolin's mineral solution; DSMZ Medium 826) - M9_TS; M9_TS_Y; M9_TS_N; M9_TS_Y_N; M9_TS_AC; M9_TS_ME. Methanol, sodium acetate, or their mixtures were added to selected bottles to stimulate indigenous microbial activity. No external inoculum was introduced.

Abiotic controls (SRW) consisted of lignite and sterile redistilled water (EC < 3 μS/cm), whereas medium-only controls contained silica gel instead of lignite. Sterile (killed) controls were not included because thermal sterilization substantially alters lignite organic matter (thermal-pressure treatment of OM). This limitation should be considered when interpreting the results. The scheme of the repetitions, carbon-bearing additives used for cultivation and controls are presented in Table 1.

Batch cultures were incubated in the dark at 20 °C for 3 years. To minimize gas leakage, bottles were stored upside down and remained unopened throughout the experiment. Biogas was sampled after first from four bottles and after 3rd from all bottles. This approach was designed to maintain stable, undisturbed anoxic conditions.

2.3 Analyses of biogas

Gas composition was analyzed using a Shimadzu Nexis GC-2030 gas chromatograph equipped with a barrier ionization discharge detector (BID) and a thermal conductivity detector (TCD). Headspace samples (1 mL) were manually injected and separated on RT-Msieve 5 A and Carboxen 1010 PLOT capillary columns. CH_4 and N_2O were measured using the BID detector, whereas O_2 was measured using TCD. Oxygen and N_2O analyses were performed primarily to verify the absence of atmospheric contamination. Carbon dioxide concentrations were determined during separate analyses using a modified detector configuration, with final measurements performed by TCD. Detailed chromatographic conditions are provided elsewhere (Bucha et al., 2025).

Detection limits were 0.1% for O_2 and CO_2 (TCD), 1.6 ppm for CH_4, and 300 ppb for N_2O (BID). Carbon isotopic compositions of CH_4 and CO_2 were determined using a Picarro G2201-i cavity ring-down spectroscopy (CRDS) analyzer. No isotopically labeled tracers were added, and only natural $\delta^{13}C$ abundances were measured.

At the end of the three-year incubation, 5 mL biogas samples were collected from duplicate cultivations and diluted in 2 L Tedlar bags filled with N_2 to reduce CO_2 concentrations and obtain sufficient CH_4 for isotopic analysis. Samples from duplicate bottles were combined because CH_4 concentrations were too low for reliable individual measurements. This approach was necessary due to the low CH_4, high CO_2 concentrations, and the limited headspace volume (100 mL) of the incubations.

The instrumental drift was +13.9‰ for $\delta^{13}C(CH_4)$ and 0.0‰ for $\delta^{13}C(CO_2)$, relative to the laboratory atmospheric-air standard. Measurement precision was better than ±0.55‰ for $\delta^{13}C(CH_4)$ and ±0.16‰ for $\delta^{13}C(CO_2)$, consistent with the manufacturer's specifications.

2.4 Analyses of fermentation liquids

Fermentation liquids were analyzed using a Multi N/C 2100 analyzer. Samples (500 μL) were combusted at 800 °C in O_2-atmosphere, and total carbon (TC) and total nitrogen (TN) concentrations were determined using nondispersive infrared (NDIR) and electrochemical detectors, respectively. The TC and TN values were quantified using calibration curves based on sucrose and potassium nitrate standards. The analytical precision was ±1.66 mg/L for TC and ±0.11 mg/L for TN.

Nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) concentrations were determined using a SLANDI LF300 photometer and dedicated kits Slandi, cat. No. ZW 20342, cat. No. ZW 20352, cat. No. ZW 20212, respectively. Due to the dark color of the media, samples were diluted 20-fold before analysis. Absorbance was measured at 520 nm (NO_3^-), 560 nm (NO_2^-), and 610 nm (NH_4^+). Detection limits were 0.1–50.0 mg/L for NO_3^-, 0.02–1.0 mg/L for NO_2^-, and 0.01–5.0 mg/L for NH_4^+.

Total inorganic nitrogen (TIN) was calculated as the sum of nitrate and ammonium nitrogen, as nitrites were not detected. Dissolved organic nitrogen (DON) was calculated as the difference between TN and TIN.

The pH and electrical conductivity (EC) values were measured using WTW and Mettler Toledo Seven Compact S230 instruments,

respectively. Measurements were performed at the beginning and end of the incubation period to minimize disturbance of the long-term cultivation.

2.5 GC-MS analyses of the lignite extracts

Lignite samples recovered after incubation were separated from the liquid phase, frozen at −20 °C, and freeze-dried. Raw lignite from the mine was air-dried at room temperature. Approximately 1 g of each sample was extracted using a dichloromethane/methanol mixture (1:1, v/v) in a Dionex ASE 350 accelerated solvent extractor. Total extracts were derivatized to trimethylsilyl (TMS) derivatives using BSTFA with 1% trimethylchlorosilane and pyridine (3 h, 70 °C). Internal standards (ethyl vanillin, ribonic acid, or trans-cinnamic acid) were added prior to derivatization. After evaporation of excess reagent under N_2, samples were dissolved in *n*-hexane. A procedural blank (baked silica gel) was processed and analyzed using the same protocol.

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out with an Agilent Technologies 7890 A gas chromatograph and Agilent 5975C Network mass spectrometer with Triple-Axis Detector (MSD). Helium (6.0 Grade) was used as a carrier gas at a constant flow of 2.6 mL/min. Separation was obtained on a fused silica capillary column (J&W HP5-MS, 60 m × 0.25 mm i. d., 0.25 μm film thickness) coated with a chemically bonded phase (5% phenyl, 95% methylsiloxane), for which the GC oven temperature was programmed from 45 °C (1 min) to 100 °C at 20 °C/min, then to 300 °C at 3 °C/min (hold 40 min), with a solvent delay of 10 min.

The GC column outlet was connected directly to the ion source of the MSD. The GC-MS interface was set at 280 °C, while the ion source and the quadrupole analyzer were set at 230 °C and 150 °C, respectively. Mass spectra were recorded from m/z 45–550 (0–40 min) and m/z 50–700 (>40 min). The MS was operated in the electron impact mode, with an ionization energy of 70 eV. Analysis was performed in the Institute of Earth Sciences, Faculty of Natural Sciences, University of Silesia in Katowice. Quantification of organic compounds was performed using MSD ChemStation E.02.02.143, Openchrome Lablicate version 1.5.0,and the NIST mass spectral database.

2.6 Total DNA isolation, 16S rRNA profiling, and functional predictions

Total DNA from raw lignite was extracted using a modified protocol based on previously published methods (Alawi et al., 2014; Töwe et al., 2011; Bag et al., 2016; Narayan et al., 2016; Schulze-Makuch et al., 2018). Microbial cells were separated from lignite by washing, filtration, and subsequent chemical and mechanical lysis. DNA was purified by phenol–chloroform extraction and polyethylene glycol precipitation. A parallel blank extraction was processed to verify the absence of laboratory contamination.

Due to the expected low DNA yield, DNA concentration and amplifiability were assessed by qPCR targeting the 16S rRNA gene using primers MF341 and MR907 (Markowicz et al., 2021). Quantification was performed on a LightCycler 96 instrument (Roche, Switzerland) using a plasmid standard containing a 16 S rRNA gene fragment. DNA from microbial communities developed during incubation was extracted from lignite–liquid mixtures using the PowerSoil Pro DNA Isolation Kit with minor modifications (Detman et al., 2018).

Amplification, library preparation, and sequencing were performed by Genomed S.A. (Warsaw, Poland). The V3–V4 region of the 16S rRNA gene was amplified using primers 341 F and 785R, followed by paired-end sequencing (2 × 300 bp) on an Illumina MiSeq platform. The resulting reads were quality filtered using FIGARO (Weinstein et al., 2019) and Cutadapt (Martin, 2011).

Sequence processing was performed in QIIME2 (Bolyen et al., 2019). Denoising, sequence merging, dereplication, and chimera removal were conducted with DADA2 (Callahan et al., 2016). Taxonomic classification was performed using the QIIME2 feature classifier (Bokulich et al., 2018) trained on the Greengenes2 database (McDonald et al., 2024). All raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA1190450 (BioSample–SAMN45045376, SAMN45045377, SAMN45045378, SAMN45045379, SAMN45045380, SAMN45045381, SAMN45045382).

Functional prediction of microbial communities was carried out using PICRUSt2 (Douglas et al., 2020), incorporating phylogenetic placement with EPA-NG (Barbera et al., 2019) and gappa (Czech et al., 2020), hidden-state prediction with castor (Louca and Doebeli, 2018), and pathway inference with MinPath (Ye and Doak, 2009). Predicted genes and metabolic pathways were classified using the MetaCyc database (Caspi et al., 2020; Kanehisa and Goto, 2000). Detailed version of the protocol can be found in Supplementary Data.

3 Results

3.1 Visual observations

The first evidence of the activity of the autochthonic/native microorganisms in the first month of the cultivation was the growth of the biofilm (Figure 1). At the beginning of the cultivation, the fermentation solution was colourless. The colour started to change after 3 months of cultivation. The solutions became light yellow, which could be an effect of the dissolving of fulvic acids. After 6 months of cultivation, these microcosms contained a dark brown solution (Figure 1), which changed to almost black after 12 months. These observations indirectly suggest the dissolving of humic and fulvic acids and/or as biodegradation of OM by microbial communities/microorganisms from lignite-beds. These acids were not dissolved in the control cultivations–solutions in all control cultivations were colourless.

3.2 Biogas analyses

For the first testing (after 1 year) 4 samples were checked using GC methods and CH_4 as well CO_2 were not present in the headspace gas. The main component of the headspace at that time was N_2, the role of H_2 is unknown because we did not have the possibility of checking its concentration during cultivation. Headspace gas analysis after 3 years incubation revealed that the main gaseous product of lignite's decomposition was CO_2 (Figure 2;

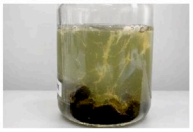
START OF INCUBATION	ANAEROBIC INCUBATION IN THE DARK AT THE TEMPERATURE 25 °C				END OF INCUBATION
Field sampling	1 YEAR			2 YEARS	3 YEARS
October 2019	3 months	6 months	12 months		October 2022
Collection of raw detritic lignite samples from the opencast mine in Konin (Poland) Beginning of the experiment under O_2-free atmosphere				NO ACTIONS / NO SHAKING ONLY STORAGE UPSIDE DOWN IN THE DARK WITHOUT INTERFERING THE INCUBATION	
OBSERVATIONS / TASKS	Biofilm growth in the fermentation liquid	Dissolving of fulvic acids and humic substance	Organic matter biodegradation **NO BIOGAS!**	—	Fermentation liquids are black **CO_2 PRODUCTION !**
• *DNA extractions* • *GC-MS studies of OM* • *GC: CH_4 and CO_2* • *TC and TN from media* • *NO_3^{2-} and NH_4^+* • *pH and EC*			• GC: CH_4 and CO_2 (only 4 samples)		• *DNA extractions* • *GC-MS studies of OM* • *GC: CH_4 and CO_2* • *TC and TN from media* • *NO_3^{2-} and NH_4^+* • *pH and EC* • *$\delta^{13}C$ of CH_4 and CO_2*

FIGURE 1
Time diagram of the research tasks and changes in the colour of the fermentation liquid during anaerobic cultivation.

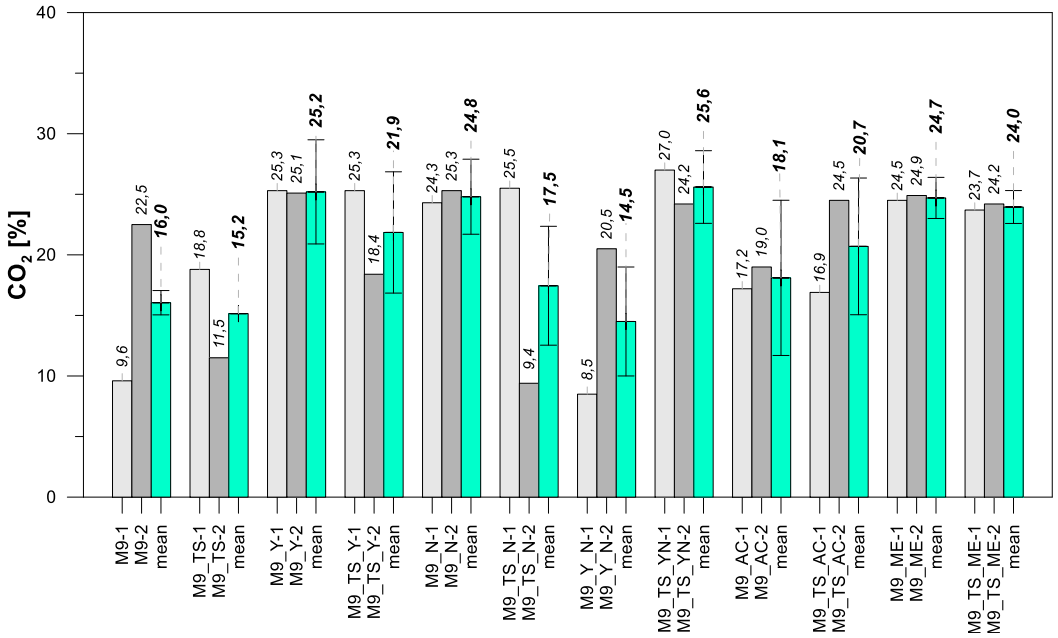


FIGURE 2
The concentration of CO_2 after 3-year anaerobic lignite cultivation (first two bars - repetitions; green bar - mean value with SD).

Supplementary Table S1). Its concentration ranged from 14.5% to 25.6% (mean concentration 20.7%). The trace component of the biogas was CH_4 whose concentration ranged from 11 to 49 ppm (mean concentration 37 ppm) (Figure 3; Supplementary Table S1). Biogas production was not observed in control cultivations.

The lowest concentrations of CH_4 were determined for incubations with the additive of methanol (M9_ME and M9_TS_ME). The high concentrations of CH_4 were characterised by cultivations with nutrient broth and yeast extract – 48.8 ppm for M9C and 44.4 ppm for M9_TS_Y_N. However, an unexpectedly high CH_4 concentration, 47.2 ppm, was observed in the experiment containing M9 medium and trace solution (M9_TS). All concentrations were much higher than atmospheric concentrations (1.8 ppm), which indicates the biological origin of CH_4. The lowest concentrations of CO_2 were detected in cultivation with M9 medium (M9 and M9_TS). Relatively high CO_2 concentrations were characterized for cultivations with methanol and equalled 24.7% for M9_ME and 24.0% for M9_TS_ME. However, CO_2 concentrations in almost half of the cultivations indicate a high variability expressed by the SD index above 5. The

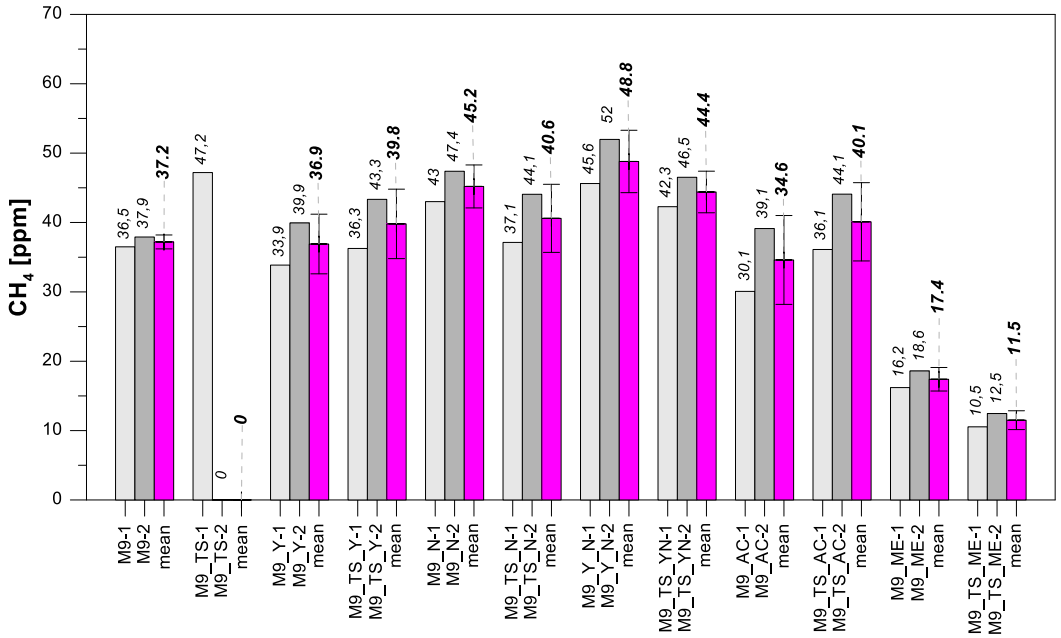


FIGURE 3
The concentration of CH_4 after 3-year anaerobic lignite cultivation (first two bars - repetitions; violet bar - mean value with SD).

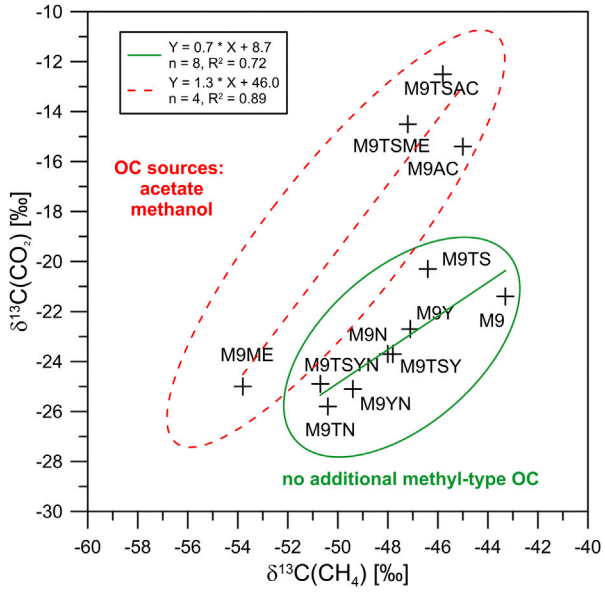


FIGURE 4
The $\delta^{13}C$ in CH_4 and CO_2 in headspace gas after 3-year anaerobic lignite cultivation ("_" in sample names is removed for better visibility).

concentration of O_2 and N_2O was checked to detect potential leakage or contamination with atmospheric air. In all analysed headspace gas samples, no O_2 was detected (always below the LOD <0.1% measured by TCD). N_2O was not detected as well (below detection limit of 300 ppb measured by BID).

The $\delta^{13}C(CH_4)$ values were in range from −53.8 to −43.3‰. The $\delta^{13}C(CO_2)$ values ranged from −25.1 to −12.5‰ (Figure 4; Supplementary Table S1). The headspace gas samples can be based on that divided for two groups–with addition of methyl-type organic carbon (from acetate and methanol) and group without addition of methyl-type organic carbon. The isotope fractionation factor $\alpha^{13}C_{CO2\text{-}CH4}$ ranged from 1.023 to 1.0267 in most cultivations containing pure M9 media, or additives of nutrient broth and yeast extract. Higher values above 1.03 were characterized for the cultivations containing sodium acetate (M9_AC and M9_TS_AC) and methanol (M9_ME and M9_TS_ME).

3.3 Fermentation liquids analyses

The total carbon (TC) concentrations in the fermentation liquids at the beginning of the cultivation ranged from 0 to 1128.4 mg/L (Figure 5). After 3-year cultivation, these concentrations were in the range from 481.7 to 1686.6 mg/L (detailed data showed in Supplementary Table S2). The highest total carbon concentrations were observed in the cultivations including yeast extract, nutrient broth, and sodium acetate (M9_AC and M9_TS_AC). The highest decrease of TC was observed in cultivations containing only M9 medium (M9 and M9_TS).

The pH of the liquids decreased slightly during the cultivation from 7.5 to 6.6 (or near 6.9–7.0 in cases of cultivation with sodium acetate). The EC increased during the cultivation which resulted most probably from dissolving of HCO_3^- ions (balance processes of CO_2 in the solutions) and dissolving of the compounds from the lignite (like inorganic mineral matter, e.g., sulphates or iron hydroxides). It is also worthy to note that M9 minimal medium used in this work has EC value equalled 1250 µS/cm. A similar value of EC (1290 and 1295 µS/cm) was measured for the M9_ME and M9_TS_ME cultivations with the addition of methanol (Supplementary Table S2).

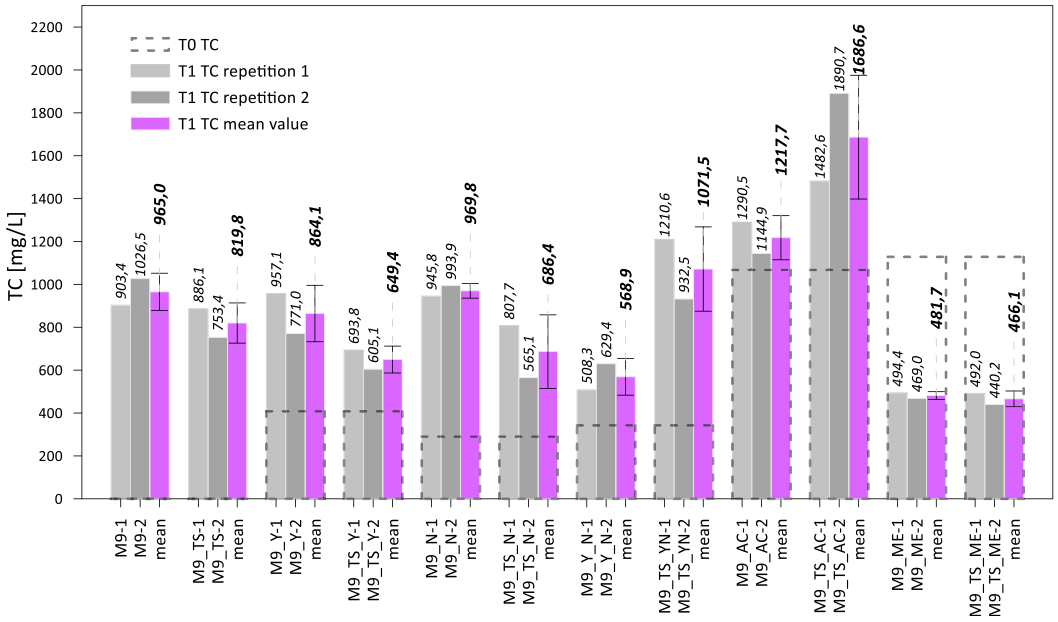


FIGURE 5
The TC values of fermentation liquids at the beginning (T0) and after 3-year anaerobic lignite cultivation (T1).

The total nitrogen (TN) content in the fermentation liquid ranged from 261.9 to 324.0 mg/L at the beginning of the cultivation and from 125.0 to 218.2 mg/L at the end of the cultivation (after 3 years) (Figure 6; Supplementary Table S3). The highest TN concentrations were observed in the cultivation with yeast extract, nutrient broth, and methanol (M9_ME and M9_TS_ME). Total inorganic nitrogen (TIN) content decreased in all the samples at the end of cultivation. The lowest TIN values were determined in cultivation with sodium acetate (M9_AC and M9_TS_AC) in the range from 68.6 to 70.2 mg/L. The highest TIN values were in cultivation with methanol (M9_ME and M9_TS_ME) in the range from 129.3 to 164.7 mg/L. The photometric measurements revealed that NO_2^- were not detected, while NO_3^- varied in range from 0 to 13.3 mg/L (mean value 3.9 mg/L) and NH_4^+ in range from 54.6 to 190.7 mg/L (mean value 108.9 mg/L). In all cultivations the concentrations of NH_4^+ decreased at the end of incubation (Supplementary Table S2). The compound responsible for the high values of TN in the fermentation liquid was NH_4^+, originally present in the minimal media M9 (NH_4Cl concentration 1 g/L) (Supplementary Table S2). The dissolved organic nitrogen (DON) in samples at the beginning of the experiments originated from the additives–yeast extract and nutrient broth and ranged from 43.5 to 61.9 mg/L. In the samples at the end of cultivation, DON values were in the range from 43.0 to 11.5 mg/L and differed between the cultivations without any correlation. The cultivation with minimal media M9 named M9 and M9_TS indicates an increase of DON values from 0 to 54.3 and 43.0 mg/L respectively (Figure 6).

3.4 GC-MS analyses

The total extract of OM from detritic lignite indicates significant changes in their molecular composition (Figure 7). These changes include mainly decrease in compound concentrations and the appearance of new compounds that are products of the decomposition of lignite and/or biomass.

The compounds which were degraded are exo-borneol; benzoic acid; 3-hydroxybenzaldehyde; 4-hydroxybenzaldehyde; trans-calamene; vanillin; 4-hydroxybenzoic acid; cadalene; vanillic acid; 18-norabietane; 2,3-dehydroferruginol; ferruginol; communic acid; dehydroabietic acid; sugiol; oleanolic acid; ursolic acid. Changes of their concentrations are presented in Table 2. The labile compounds present in lignite, which were easily available to microorganisms, were levoglucosan; α-glucose; β-glucose, and sucrose. These anhydro-, mono-, and disaccharides were almost fully degraded (Figure 8). The products of biodegradation were glycerol, succinic and azelaic acid. As a result of lignite biodegradation, the relative abundances [%] of carboxylic acids and alkanols increased in all analysed samples (Supplementary Table S4).

3.5 Microbiological analyses

Microbiome structure analysis based on 16 S rDNA sequencing showed a decrease in diversity in all cultivated samples where autochthonous microflora was stimulated, compared to the raw lignite (Supplementary Table S6; Supplementary Figure S2). Pronounced changes in the microbiome composition were observed between samples (Supplementary Figures S2-S3; Figure 9). There was decrease in detectable family's number from 123 for lignite sample to 60 in M9_TS and 30, 26 and 25 for M9_TS_Y_N, M9_TS_AC and M9_TS_ME respectively (Supplementary Figure S2). In the lignite 74 unique families were detected, while M9_TS sample had 24 unique families. Only 11 families were present in each sample type and less than 10 families were detected in different samples or were unique for rest of the culture conditions (Supplementary Figure S2). However, in

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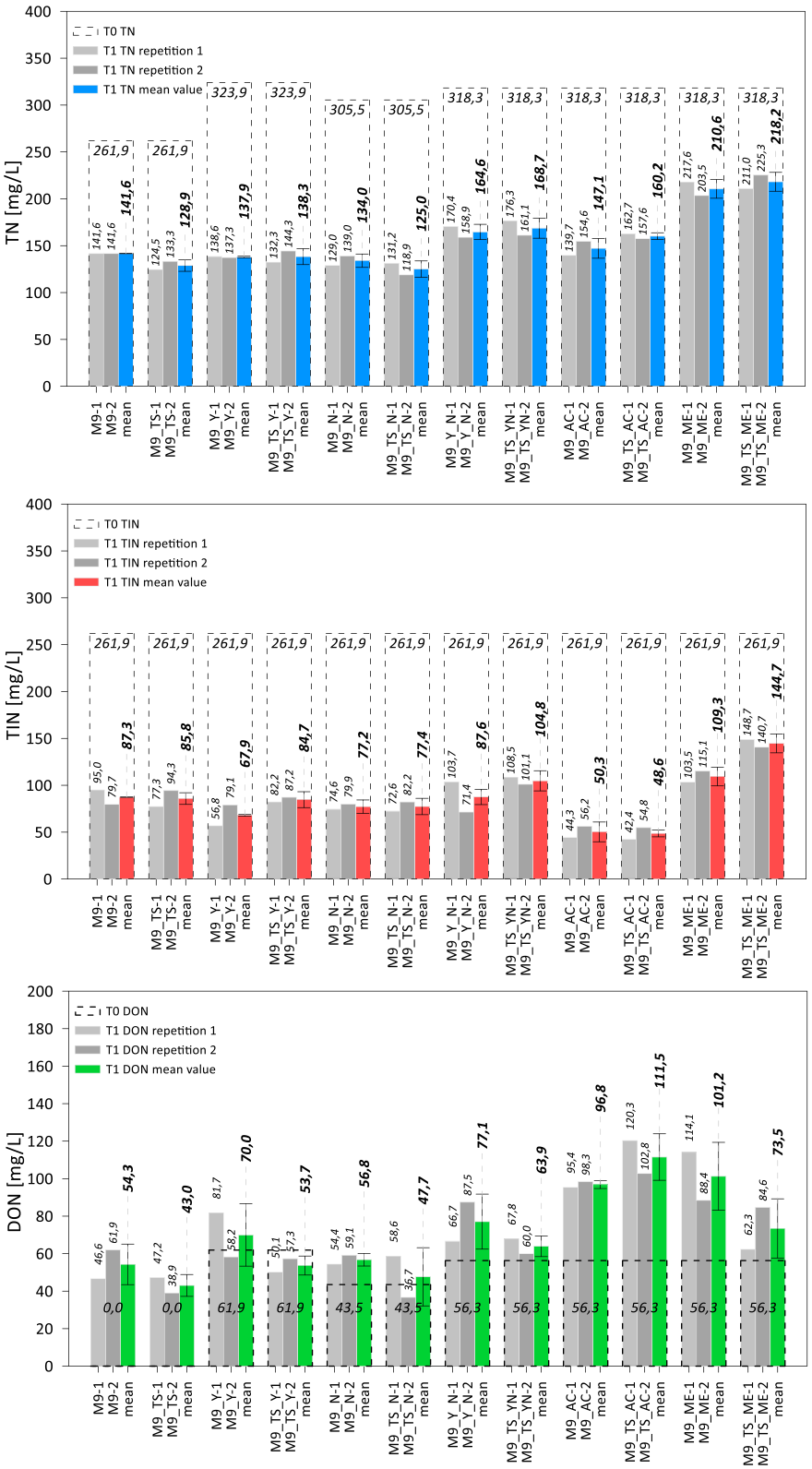


FIGURE 6
The concentrations of different nitrogen forms (TN, TIN, DON) in fermentation liquids at the beginning (T0) and after 3-year anaerobic lignite cultivation (T1).

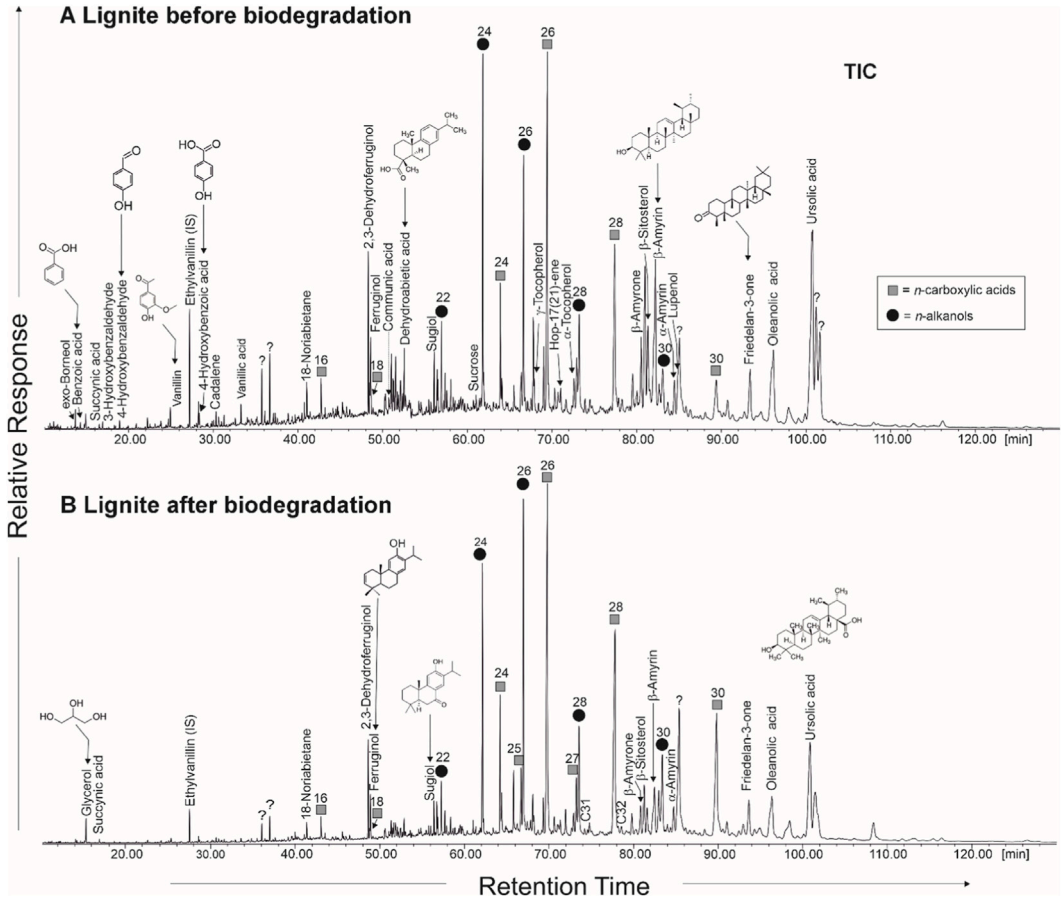


FIGURE 7
Total ion chromatogram of the detritic lignite at the beginning **(A)** and after 3-year anaerobic cultivation experiment **(B)**.

culture samples were detected families that was not detected in lignite (Supplementary Figure S3).

In all cultures abundance of *Micrococaceae* was increased in comparison to lignite, and in case of samples M9_TS and M9_TS_Y_N this was the most enriched family relative to lignite, with 14.59% and 38.24% increase respectively (Figure 9). For M9_TS_AC members of family *Dermathophilaceae* increased the most (24.11%), and for M9_TS_ME *Methylophilacea_502230* family increased the most (27.11%), while other *Methylophilaceae* decreased in abundance in all sample for average of 5.42%. The most abundant family in lignite samples, *Gallionellaceae* completely diminished from the culture samples (Figure 9). The second highest decrease occurred for collective group of less dominant taxa ('other'on Figure 9) except sample M9_TS_ME where it was for *Burkholderiaceae_A_59252*.

The microbial communities in the cultured samples (M9_TS, M9_TS_Y_N, M9_TS_AC, M9_TS_ME) exhibit enrichment in nitrogen-cycling bacteria compared to the original lignite sample, with a clear increase of nitrogen cycling bacteria in the cultured samples compared to the lignite sample. In particular, *Rhizobiales* (involved in N_2-fixation) (Deb et al., 2024) and *Burkholderiales* (involved in both N_2-fixation and denitrification) (Berkelmann et al., 2020) are significantly enriched in M9_TS and M9_TS_AC, while denitrifying bacteria *Sphingomonadaceae* (Dai et al., 2022) and *Pseudomonadaceae* are more abundant in M9_TS_Y_N and M9_TS_ME.

Based on the microbiome structure functional predictions have been performed. Data for the relative abundance of pathways involved in the degradation of OM, nitrogen and sulphur metabolism based on the MetaCyc classification are presented on Supplementary Figure S3. Overall, 399 pathways have been predicted to be present in the sample. Among pathways that were predicted to increase in culture where these connected with OM degradation, including degradation of aromatic compounds as well as with fermentation. Predicted abundance of almost all pathways for carbon assimilation were reduced in M9_TS_Y_N samples while reductive TCA cycles were predicted to be increased in M9_TS_AC and M9_TS_ME samples. From oxidative pathways, methanol oxidation to CO_2 was predicted to be increased in all culture samples (Figure 10).

For nitrogen cycling the denitrification pathway (DENITRIFICATION-PWY) was predicted to be more prominent in M9_TS_Y_N and M9_TS_ME samples (Figure 9). In contrast urea cycle (PWY-4984), leading to conversion of ammonia to urea, was predicted to be more prevalent in M9_TS and M9_TS_AC samples, while assimilatory nitrate reduction pathway (PWY-490–3) was predicted to be increased relative to lignite in all cultures, although with different degree (Figure 10; Supplementary Figure S3).

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TABLE 2 Concentrations of biodegraded compounds in detritic lignite after 3-year anaerobic cultivation experiment.

	Concentration [µg/g of sample]				
Compound	lignite	M9_TS	M9_TS_Y_N	M9_TS_AC	M9_TS_ME
exo-Borneol	1.30	0.76	2.39	1.55	0.20
Benzoic acid	0.42	0.37	0.99	0.55	0.22
3-Hydroxybenzaldehyde	0.63	0.25	0.46	0.05	0.19
4-Hydroxybenzaldehyde	0.57	0.30	0.50	0.39	0.11
trans-Calamene	0.92	0.58	1.86	1.09	0.28
Vanillin	1.55	0.58	0.95	0.62	0.24
4-Hydroxybenzoic acid	1.09	0.66	2.02	1.30	0.42
Cadalene	1.28	0.00	1.18	0.87	0.00
Vanillic acid	1.66	0.00	3.44	0.00	0.00
18-Norabietane	3.94	5.43	10.41	4.91	1.55
2,3-Dehydroferruginol	14.20	34.82	35.08	26.00	8.85
Ferruginol	2.61	3.55	3.98	4.80	1.25
Communic acid	2.58	2.60	6.16	4.17	0.33
Dehydroabietic acid	8.71	7.43	19.06	10.71	3.96
Sugiol	5.50	11.95	15.18	1.21	3.81
Oleanolic acid	22.32	8.22	43.42	19.36	20.17
Ursolic acid	99.47	171.87	141.44	122.62	66.33

4 Discussion

Anaerobic biodegradation of immature sedimentary organic matter (OM) has been extensively studied because of its relevance to microbial CH_4 production and its potential industrial applications. Most previous studies have relied on enriched microbial consortia or pretreated coal materials to enhance CH_4 yields. While these approaches increase process efficiency, they do not accurately represent natural conditions. In this study, we investigated the long-term activity of indigenous microorganisms naturally present in Miocene lignite during a three-year incubation experiment. As expected, cultivation reduced microbial diversity and promoted the enrichment of specific taxa depending on medium composition. Despite taxonomic differences among treatments, functional predictions indicated similar metabolic capabilities, particularly those related to lignite-derived OM degradation, which was consistent with geochemical evidence. Although these predictions should be validated by future transcriptomic or gene-expression analyses, they suggest that key processes, including aromatic compound degradation, nutrient cycling, greenhouse gas production, and biomass growth, were maintained across different cultivation conditions.

Nitrogen availability is a major factor controlling lignite biodegradation (Stankiewicz and Van Bergen, 1998) because lignites typically exhibit very high C:N ratios (120–150 in the Konin deposits) (Bucha et al., 2020), far above the threshold considered optimal for microbial decomposition. In our experiments, NH_4Cl supplied the main readily available nitrogen source, while additional nitrogen originated from organic supplements and N_2 present in the headspace. The observed decrease in TN concentration most likely reflected microbial biomass formation and adsorption onto coal particles rather than nitrogen loss from the system.

Gradual color changes of the cultivation liquids provided visual evidence of ongoing biodegradation. Slight yellowing observed during the first months was attributed to dissolution of fulvic acids (Czechowski and Jezierski, 1997), whereas brown to black coloration after 6 months to 1 year indicated increasing mobilization and transformation of humic substances and lignite-derived organic matter (Bucha et al., 2020; Detman et al., 2018; Killops and Killops, 2005). These observations, together with geochemical analyses, confirm progressive degradation of lignocellulosic and lignin-derived structures. Methanol and acetate additions likely stimulated microorganisms capable of utilizing methylated substrates, including methanogens. Future experiments using ^{13}C-labelled compounds could help directly trace these metabolic pathways and quantify their contribution to CH_4 formation.

Natural leaching of organic matter from lignite may contribute to changes in solution composition and colour; however, its significance in the Miocene lignite used in this study is considered negligible. This conclusion is supported by abiotic controls containing lignite and sterile redistilled water (SRW), as well as previous experiments demonstrating that incubation with water alone does not alter the molecular composition of lignite organic matter (Bucha et al., 2018). Szubert et al. (2020) reported that anaerobic biodegradation of lignite resulted by increased

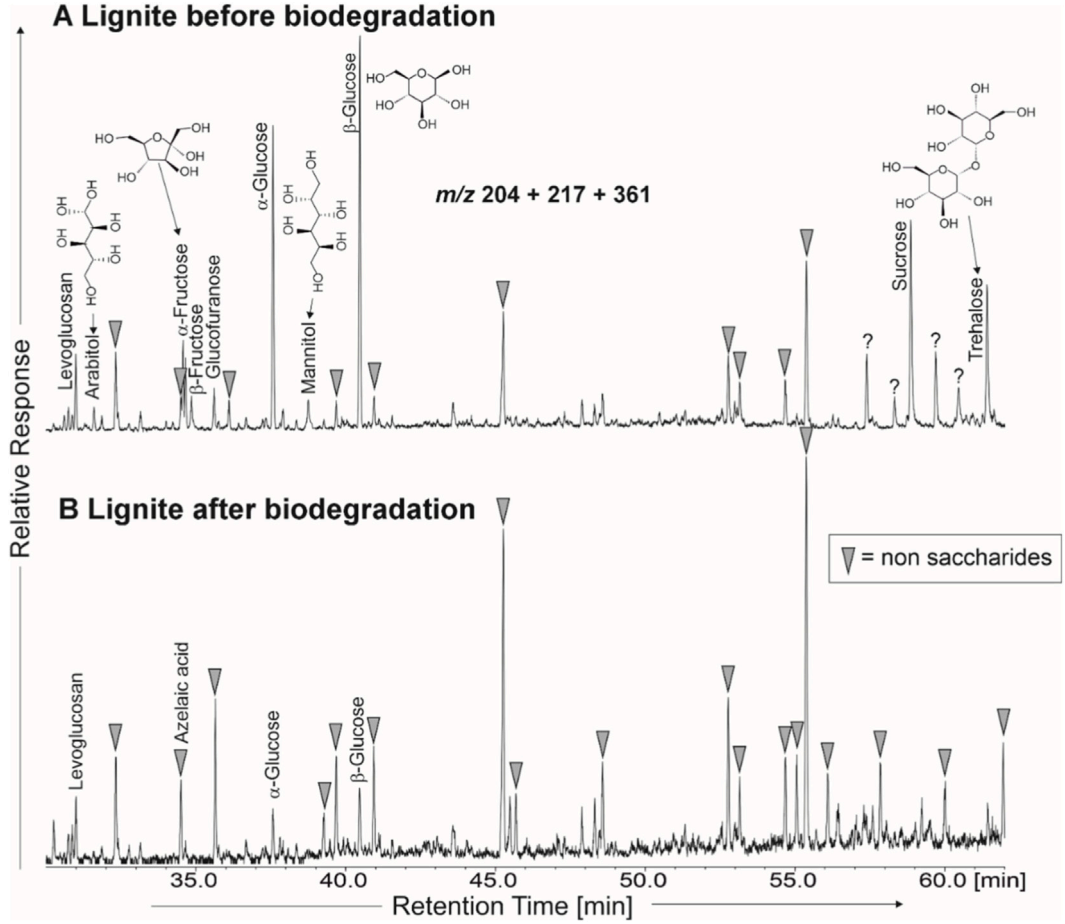


FIGURE 8
Distribution of saccharides in lignite at the beginning **(A)** and after 3-year anaerobic cultivation experiment **(B)**.

content of humic acids from 1.60% to 2.74% (measured after chemical treatment). Dissolution and extraction of humic acids require the use of chemical reagents, and water alone can only cause negligible dissolution of fulvic acids (whose content in lignite does not exceed 0.5%). Furthermore, lignite extracts are characterized by the absence of easily mobilized low-molecular-weight hydrocarbons (Fabiańska, 2007), and the amount of humic substances released by water is typically very low (Marynowski et al., 2023).

The other limitation of our study was natural heterogeneity of lignite. Although mechanical grinding could average molecular composition of OM, such treatment may disturb indigenous microbial communities and alter the original rock structure. Therefore, intact lignite material was used to better preserve natural conditions. The sterile M9 medium and its supplements contained insufficient amounts of organic carbon to explain the observed colour changes or gas production in the absence of microbial activity. Similar control experiments conducted previously with sterile mineral media and organic-free substrates produced no comparable effects (Bucha et al., 2018).

The next challenge limiting the interpretation of the data from this study is sterilization of lignite (or any other immature OM-sediments) without altering its physicochemical properties. Conventional autoclaving promotes dissolution of organic compounds, whereas alternative approaches such as UV or gamma irradiation are either ineffective or impractical. Nevertheless, the observed changes in solution colour and chemistry are most consistently explained by microbial activity and biodegradation processes, in agreement with previous studies on Polish lignites and hard coals (Pytlak et al., 2020).

The OM decomposition during the first year of incubation produced only minor amounts of biogas. After 3 years, the headspace gas was dominated by CO_2 (16%–25%), whereas CH_4 was detected only at trace concentrations (~50 ppm). Elevated CO_2 concentrations, together with increased EC resulting from HCO_3^- ions formation, indicate that CO_2 was the principal end-product of OM degradation. Stable isotope data suggest that anaerobic CH_4 oxidation represented the major CH_4 sink and an important source of CO_2 formation (Figure 11). The detected gases were most likely generated during biodegradation rather than released from residual gas trapped within lignite pores, as the sampled lignite had been exposed to atmospheric conditions for an extended period prior to collection, allowing earlier degassing processes to occur.

However, to prove anaerobic CH_4 oxidation maintaining strictly anaerobic conditions was essential for the experiment. No oxygen contamination was detected throughout the three-year incubation

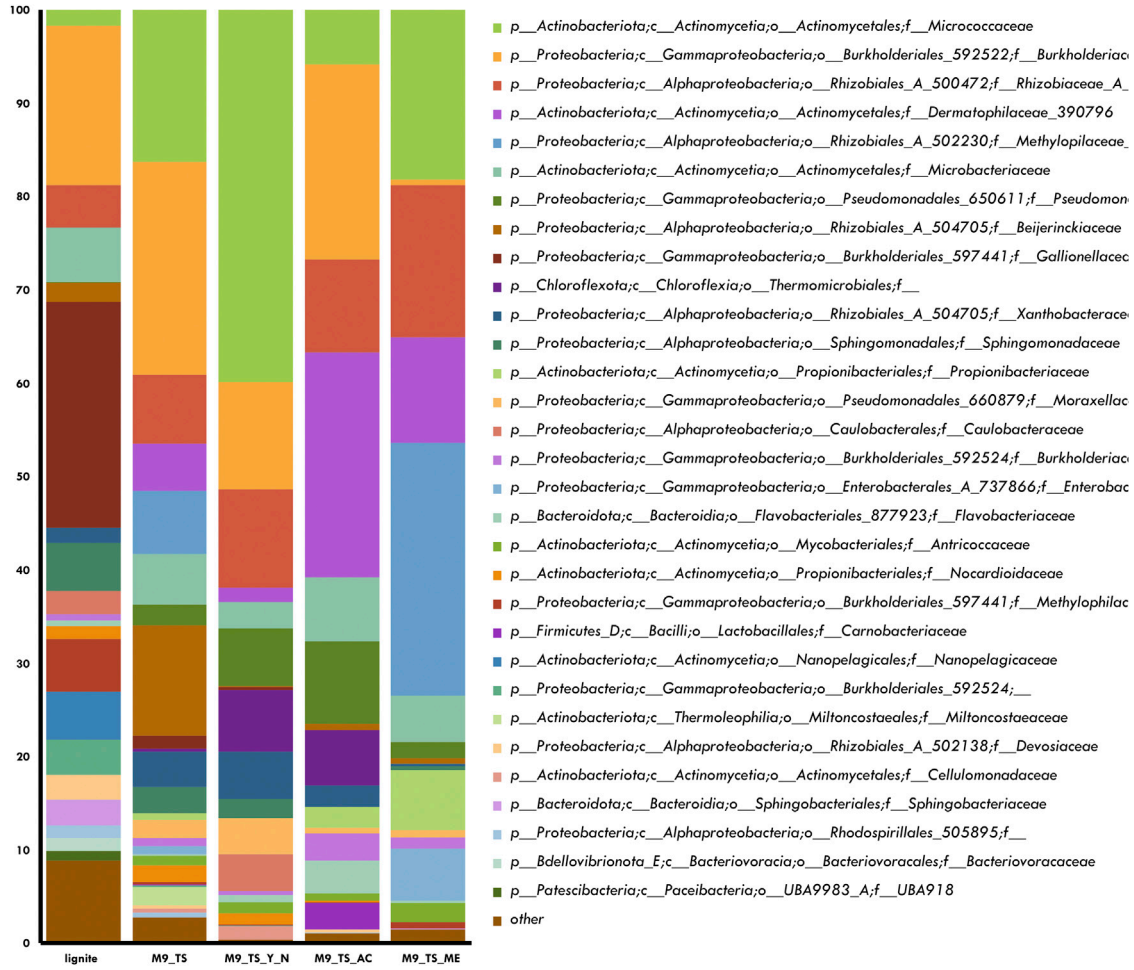


FIGURE 9
Microbial communities' structure in lignite and culture samples at the family level. Families that had abundance below 1% across all the samples were merged into a single group 'other'. For the lignite sample, the mean value of three repeats is shown.

period, and the cultivation bottles remained sealed, minimizing the risk of atmospheric intrusion. Under such conditions, CH_4 oxidation may have been coupled to alternative electron acceptors, including phosphate (Nijman et al., 2022) present in the culture medium or naturally occurring metal species within the lignite matrix (e.g., Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn), although the latter hypothesis requires further geochemical verification. The extremely low CH_4 concentrations (but significantly higher than in ambient air) and the absence of detectable methanogenic taxa indicate that methanogenesis was either limited or that produced CH_4 was rapidly consumed by CH_4-oxidizing microorganisms. Nevertheless, isotopic evidence supports the occurrence of CH_4 oxidation at trace levels, confirming the persistence of anaerobic carbon cycling processes throughout the experiment.

GC–MS analysis of lignite OM revealed characteristic biodegradation products, providing direct evidence of lignocellulose-degrading microbial activity. Lignite decomposition was reflected by increased abundances of long-chain alkanols (C_{24}–C_{28}) and carboxylic acids (C_{24}–C_{30}) (Supplementary Table S4), consistent with the degradation of aromatic compounds, organic acids, and carbohydrates reported previously (Fabiańska, 2007; Bucha et al., 2018; Detman et al., 2018). Sugars are naturally present in lignite, particularly within fossil wood fragments commonly dispersed in detritic lignite (Marynowski et al., 2018). Consequently, sucrose likely represented a readily available supplementary carbon source for microbial communities in our cultivations. Carbon metabolism during incubation was primarily associated with CO_2 production, CH_4 oxidation, and the transformation of humic and fulvic substances. Coal degradation also generated low-molecular-weight organic acids, including succinic and azelaic acids, which coincided with a decrease in pH from approximately 7.5 to 6.5. However, the relationships between carbon transformations, temporal changes in CH_4 and CO_2 concentrations, and TC dynamics require further investigation.

Functional prediction analyses complemented the geochemical data and identified metabolic pathways related to lignite-derived organic compounds. Predicted functions included pyruvate fermentation to isobutanol and propionate, homolactic fermentation, protocatechuate degradation, carbohydrate utilization (glucose and xylose degradation), and urea cycling. These pathways are consistent with the composition of lignite degradation products detected by GC–MS and indicate active



FIGURE 10
Prediction of pathways' abundance in the culture samples relative to the lignite samples. Bars indicate the increase (green) or decrease (red) in pathway abundance in the culture samples compared to lignite (relative in sample >0.1%).

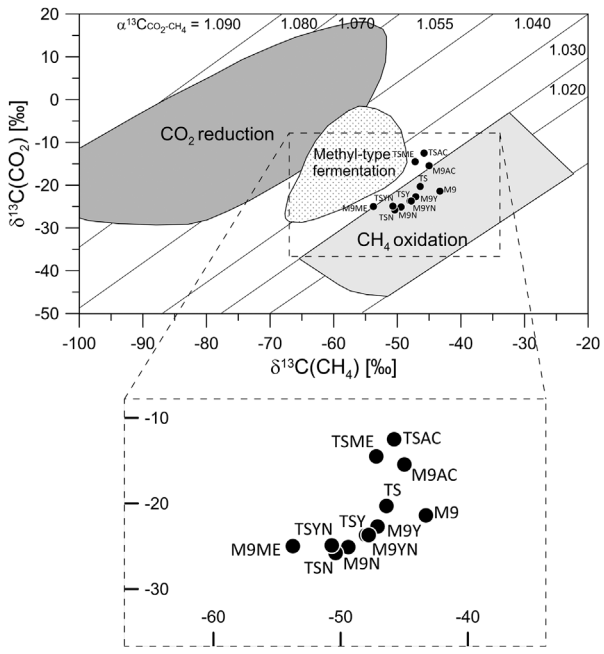


FIGURE 11
The isotopic composition of biogas (CH_4 and CO_2) at the end of 3-year cultivation of detritic lignite from Konin ("_" in sample names is removed for better visibility).

microbial processing of both carbon and nitrogen substrates. Nitrogen cycling also appeared to play a significant role during incubation. Geochemical analyses revealed changes in TN, TIN, and DON values, with NH_4^+ representing the principal contributor to TN variability (R^2 = 0.50; Supplementary Figure S4). Ammonium availability is likely critical because lignite OM contains relatively low TN concentrations, typically ranging from 0.2 to 0.6 wt% in the Konin deposits (Bucha et al., 2020). Given the ongoing diagenetic transformation and microbial decomposition of these low-rank coals (Fabiańska and Kurkiewicz, 2013), nitrogen availability may progressively decline during their long-term geological evolution. The deposits are outcropped, so the rainfall or weathering conditions (as oxidizing of sulphides) occurs naturally (Chang and Berner, 1999; Li et al., 2023; Fabiańska et al., 2024). This can transfer some of the N_2-fixing microorganisms into the layers of coal as well as mineral nutrients together with rainfall water (Pytlak et al., 2020). It is also worth noting that the deposit has contact with ambient air, which is the largest pool of nitrogen (as atmospheric N_2) on Earth (Müller and Clough, 2014; Lewicka-Szczebak et al., 2021; Deb et al., 2024).

The predominance of inorganic over organic nitrogen in fermentation liquids suggests that nitrogen was actively assimilated by microorganisms. Since most microbial biomass was likely attached to lignite particles, decreases in dissolved TN and TIN should not be interpreted as net nitrogen losses from the closed system. Instead, nitrogen may have been incorporated into biomass or associated with solid organic fractions, which is supported by increasing DON concentrations in some cultivations. Additional processes, including adsorption to mineral surfaces or humic substances, may also have contributed to nitrogen retention. Nevertheless, interpretations remain partly constrained by analytical uncertainty, limited replication, sample heterogeneity, and the inability of GC–MS to detect high-molecular-weight humic substances. Although the combined geochemical and microbiological evidence supports ongoing lignite biodegradation, several proposed mechanisms remain unexplained and require direct experimental verification. Future studies employing improved molecular and analytical

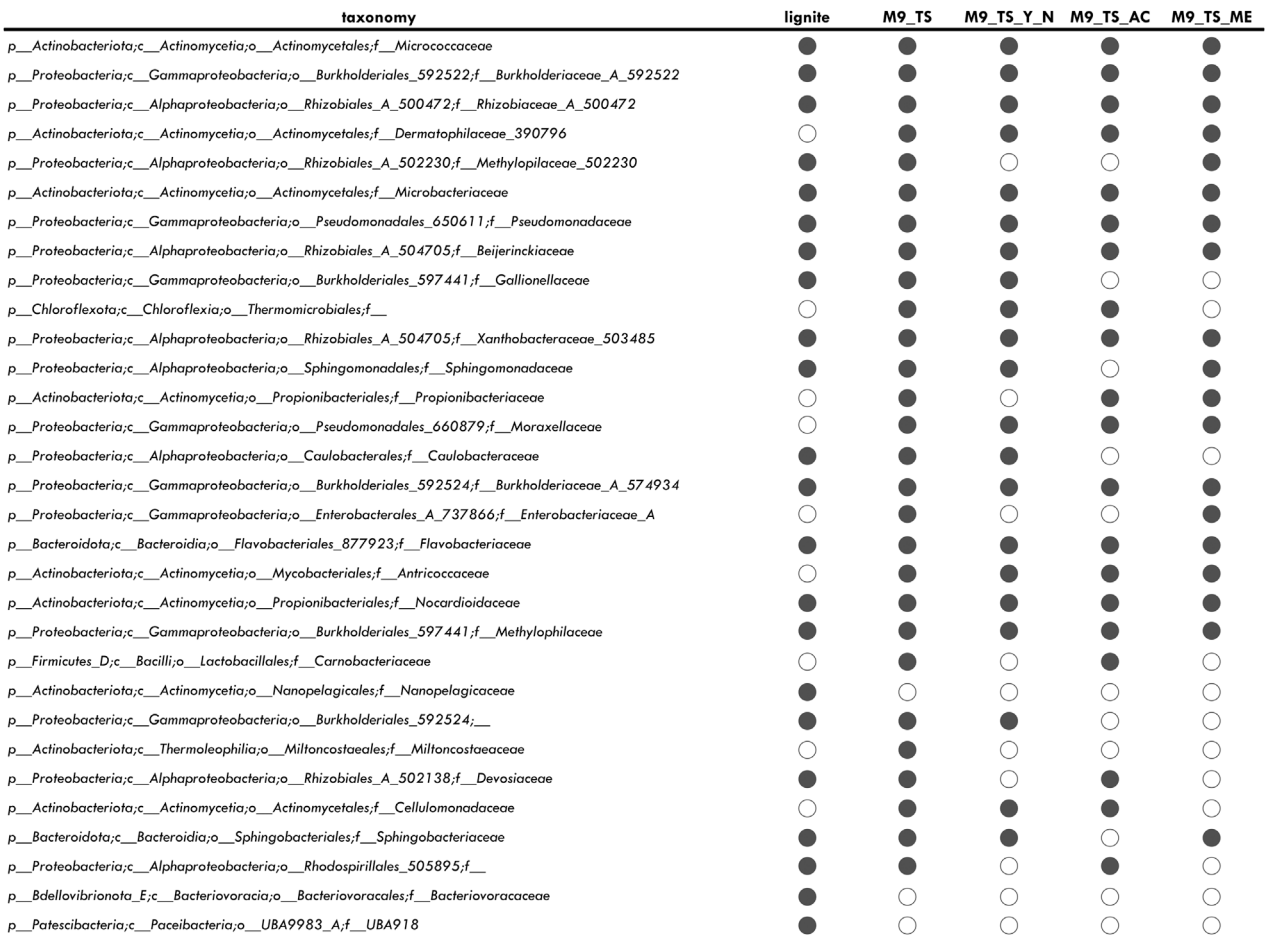


FIGURE 12
Dot plot showing the detection of specific families in particular samples. Black dots indicate the presence of the family, white dots indicate no detection of the family. Families which at least in one sample showed relative abundance >1% are included.

approaches will be essential for confirming the pathways responsible for carbon and nitrogen transformations.

The decomposition of lignite OM may also have been linked to biological N_2-fixation under anaerobic conditions. Under anaerobic conditions, NH_4^+ can be directly converted to organic nitrogen by many organisms and plants. Nitrogen assimilation relates to anaerobic CH_4 oxidation, but this process is not yet sufficiently known (Larmoia et al., 2014). Recent studies have demonstrated the ecological importance of anaerobic N_2-fixation in organic-rich, anoxic environments (Minamisawa et al., 2016; Hara et al., 2022), although analogous processes in lignite systems remain poorly understood. Notably, taxa affiliated with lineages containing known diazotrophs became enriched during cultivation relative to the original lignite samples. Many of these microorganisms are also associated with the degradation of complex organic compounds, suggesting a potential coupling between lignite decomposition and N_2-fixation. While direct evidence for N_2-fixation was not obtained, the observed microbial community shifts are consistent with this hypothesis and warrant further investigation.

Three *Rhizobiales* families predominated in the studied communities: *Beijerinckiaceae*, *Rhizobiaceae*, and *Methylopilaceae*. Members of *Beijerinckiaceae* include chemoorganoheterotrophs, facultative methylotrophs, facultative methanotrophs, and obligate methanotrophs (Rosenberg et al., 2014). Their metabolic versatility enables the utilization of a broad spectrum of multicarbon compounds, although the substrate range varies among ecological groups (Tamas et al., 2010; Yurimoto et al., 2021). These bacteria tolerate a wide pH range, including acidic conditions, and frequently utilize sugars as carbon sources. Species affiliated with the genus *Beijerinckia* are commonly associated with acidic soils, rhizosphere, and phyllosphere environments and are capable of degrading polycyclic aromatic hydrocarbons, including anthracene, biphenyl, dibenzofuran, and phenanthrene (Kiyohara et al., 1983; Tamas et al., 2010). Members of *Rhizobiaceae* utilize diverse carbohydrates and organic acids as carbon sources and ammonium, nitrate, and amino acids as nitrogen sources. *Methylopilaceae* comprise methylotrophic bacteria capable of growth on methanol; notably, *Methylopila oligotropha* has been reported from saline environments and exhibits tolerance to salt stress (Dedysh et al., 2005; Beck et al., 2015).

Taxa involved in nitrogen cycling were detected in all samples (Figure 12), although their abundance increased markedly in cultivated communities, likely in response to the N_2-enriched atmosphere used during incubation. The most prominent groups

included *Rhizobiales*, *Burkholderiales*, *Pseudomonadaceae*, and *Sphingomonadaceae*, all of which contain representatives associated with N_2-fixation and/or denitrification. Their enrichment suggests that experimental conditions favored microorganisms capable of active nitrogen transformation. The increased abundance of *Rhizobiales*, particularly *Rhizobiaceae*, may indicate the presence of trace amounts of oxygen below the analytical detection limit. This interpretation is supported by the predicted occurrence of aerobic respiration pathways, including aerobic oxidation I (cytochrome c), which remained detectable across all treatments. Although the relative abundance of these pathways decreased in cultivated samples compared to raw lignite, their persistence suggests reduced rather than eliminated oxygen availability.

In contrast, pathways directly associated with N_2-fixation were not predicted, despite the enrichment of taxa known to contain diazotrophic representatives. This apparent discrepancy likely reflects the limitations of functional prediction approaches, which infer metabolic capabilities from taxonomic composition rather than direct gene detection. Predictions may therefore be less reliable for poorly characterized microbial lineages or taxa with uncertain taxonomic assignments. Consequently, comprehensive metagenomic and metatranscriptomic investigations will be required to verify the metabolic potential and activity of these communities. In addition, ^{15}N stable-isotope labelling experiments would provide direct evidence for biological N_2-fixation.

The microbial communities differed considerably among cultivation variants. In summary, M9_TS and M9_TS_AC were characterized by the enrichment of *Rhizobiales* and *Burkholderiales*, whereas M9_TS_Y_N and M9_TS_ME showed increased abundances of *Pseudomonadaceae* and *Sphingomonadaceae*, taxa frequently associated with denitrification processes. These shifts demonstrate the strong influence of cultivation conditions on the structure and ecological functions of lignite-associated microbial communities and highlight the potential importance of nitrogen cycling in these systems.

Previous investigations of detritic coals also reported the presence of *Rhizobiales* within indigenous microbial communities (Bucha et al., 2020). Together with the present results, this finding suggests that diazotrophic microorganisms may represent an important functional component of coal and lignite microbiomes. Nitrogen may have been assimilated from the mineral medium during biomass production, while additional organic nitrogen sources could originate from lignin degradation products (Fioretto et al., 2005). Moreover, the N_2-rich headspace atmosphere provided a potentially accessible nitrogen reservoir for diazotrophic microorganisms, including those active under anaerobic conditions (Dey et al., 2021).

Although N_2- fixation and denitrification are likely to be important processes in lignite-associated environments, direct experimental evidence remains limited. Some researchers (Finzi et al., 2007; Van Groenigen et al., 2015; Zak et al., 2003) revealed unknown mechanisms of N_2-fixation at high levels of atmospheric CO_2. Additional microbiological and geochemical experiments are needed to unequivocally confirm the N_2-fixation processes. Future studies integrating microbiological, molecular, isotopic, and geochemical approaches are needed to constrain nitrogen sources, transformation pathways, and their coupling with organic matter decomposition. Particular attention should be given to changes in OM composition, C:N ratios, and stable-isotope signatures ($\delta^{13}C$ and $\delta^{15}N$), as these parameters can improve quantification of carbon and nitrogen fluxes during diagenesis and provide valuable insight into greenhouse-gas generation, global biogeochemical cycling, and potential mitigation strategies (Zaman et al., 2021).

The importance of this study is related to fundamental research on coal-rich sediments. It provides new insights into how indigenous microbial communities can convert recalcitrant OM under anaerobic conditions, which may have several future industrial and biotechnological applications. From an industrial perspective, the study supports the development of microbial enhanced coal bed methane (MECBM) technologies (Ritter et al., 2015; Wang et al., 2023; Niu et al., 2024). The results indicate that nitrogen availability may be a key factor controlling microbial activity in coal deposits, alongside carbon availability. By stimulating indigenous microorganisms and optimizing nutrient conditions, it may become possible to enhance the biological conversion of low-rank coals into CH_4, creating an alternative route for energy recovery from coal resources that are uneconomical or environmentally undesirable to exploit through conventional mining and combustion. The findings are also relevant for the management of post-mining environments and lignite waste deposits. Understanding how microbial communities degrade lignite and recycle nitrogen can improve predictions of long-term carbon metabolism, greenhouse-gas production, and biogeochemical transformations occurring in reclaimed mining areas. Such knowledge could contribute to more sustainable mine closure and land-reclamation strategies (Wu et al., 2024).

5 Conclusion

Our study showed that under anaerobic conditions the natural microbial communities present in lignite from open pit are active and cause significant changes to OM molecular composition. The degradation of the lignite polymer was manifested by changes in liquid colour to the dark brown or black solution within 1 year. Microbial activity was supported by the production of CO_2 and its release to the headspace. Interestingly, no significant amounts of methane were detected in the headspace. Its concentrations were most often below 50 ppm. The results of stable isotope analyses of CH_4 and CO_2 indicate possible CH_4 oxidation processes. The dominant degradation process was lignin decay. Geochemical analyses revealed that the concentration of several aromatic compounds, organic acids and saccharides decreased due to OM decomposition. At the end of incubation experiments (after 3 years) carboxylic acids (C_{24}, C_{28}, C_{30}) and alkanols (C_{24}, C_{26}, C_{28}, C_{30}) were the dominant compounds in extracts of detritic lignite. Analysis of bacterial structure showed that *Rhizobiales* were one of the most abundant orders in the communities from the analysed cultures. The role of nitrogen cycling in lignite deposits remains still not explained, but our results are in accordance with previous studies and confirm the increasing amount of *Rhizobiales* in all analysed samples during lignite incubation. Explanation of nitrogen cycling in coal habitats requires further studies (e.g., gene expression studies, cultivation for specific conditions). Prediction of microbial community metabolic activity is in accordance with other results showing lignite degradation. The important processes during lignite decomposition were also determined and confirmed by geochemical investigation. These are pyruvate fermentation to

propionate, homolactic fermentation, protocatechuate degradation, glucose and xylose degradation, and urea cycle. The majority of predicted fermentation pathways present in the communities also increased in abundance in culture conditions. This prediction correlates with observed lignite biotransformation products and supports the degradation of OM from lignites. More research is needed to be certain which pathways are, in fact active in the communities during lignite biotransformation. This important topic should be further investigated in the future. Based on our observations we suggest that ^{15}N-stable isotope labelling can help to directly prove N_2-fixation processes during lignite biodegradation. Our study suggests that anaerobic N_2-fixation may be a previously overlooked factor controlling lignite biodegradation and CH_4 generation. By revealing this link, it opens new opportunities for microbial coal conversion, lignocellulosic biomass bioprocessing, advanced biogas technologies, and sustainable management of carbon-rich geological resources.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, Bioproject - PRJNA1190450.

Author contributions

MB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. PS: Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft. AD-I: Formal Analysis, Investigation, Methodology, Writing – review and editing. SD: Formal Analysis, Investigation, Methodology, Writing – review and editing. AB: Formal Analysis, Investigation, Methodology, Writing – review and editing. DK: Formal Analysis, Investigation, Methodology, Writing – review and editing. AC: Formal Analysis, Investigation, Methodology, Writing – review and editing. DKu: Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing. DL-S: Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing. AS: Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing, Supervision. LM: Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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