

METHANOTROPHIC ACTIVITY OF ROCKS SURROUNDING BADENIAN SALTS IN THE “WIELICZKA” SALT MINE

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Abstract: Microbial life in Earth's deep subsurface is currently known to reach depths of several kilometers within the continental crust. Methanotrophs are a subset of a physiological group of bacteria known as methyloolithotrophs. Phylogenetically, methanotrophs belong to α - or γ -*Proteobacteria*, but there are also some representatives affiliated as *Verrucomicrobia* and NC10 phylum. Halotolerant and halophilic methanotrophs have been isolated from seawater, coastal lagoons, and several soda lakes. The aim of this study was to assess the possibility of oxidation of methane by microorganisms inhabiting the rocks surrounding salt deposits in the “Wieliczka” Salt Mine. The research material was taken from rocks lying in the immediate vicinity of the “Wieliczka” Salt Mine deposits. The research material was diverse in terms of the depth, lithology, and surrounding salt deposit types. The determinations of the ability of the microbial community from the tested rocks to oxidize methane were performed by incubation of a crushed rock under methane atmosphere (10 % (v/v)). Gas composition was measured by the gas chromatography technique. Methanotrophic activity was determined based on the dynamics of methane consumption and expressed as micromole per grams of dry weight of sample per day. The results show the dynamics of CH₄, O₂, CO₂, and N₂ concentrations in the gaseous phase during incubation of the rocks. This revealed that methane uptake in the investigated samples is due to the activity of methanotrophs. In all incubated samples, utilization of CH₄ and O₂ was observed after 77 days, which was confirmed by the parallel drop in the concentration of those gases and simultaneous growth of the CO₂ ratio. The highest methanotrophic activity ($0.252 \pm 0.090 \mu\text{MCH}_4 \text{ g}_{\text{dw}}^{-1} \text{ day}^{-1}$) was found in W4 rock (siltstones with veins of fibrous salt and lenses of anhydrite), characterized by pH of cca. 7.43 ± 0.004 , Eh at the level of 374 ± 1.36 mV, and salinity of cca. 3.6 %.

Keywords: salt mine, methanotrophs, methanotrophic activity, methane emission, Badenian salts

1. INTRODUCTION

Microorganisms from the “Wieliczka” Salt Mine were first recognized in 1913 (Namysłowski, 1913). However, the research was limited only to brine investigations. Radax et al. (2001) reported on strain PW5. 4, originating from “Wieliczka” Salt Mine water. Later “Wieliczka” Salt Mine microfauna studies were concentrated on assemblages of foraminifers (Łuczkowska & Rolewicz, 1990) and were related to recognition of novel species of amoeba - *Selenaiion koniopes* (Park et al., 2012). In 1960, Reiser & Tasch (1960) isolated bacteria in brine (Permian salt mine, Kansas); later, Nehrkorn & Schwartz (1961) identified microorganisms from the Braunschweig mine. Furthermore, Vreeland &

Huval (1991), who investigated brines formed by dissolution of salt deposits by surface water, isolated many strains of haloversatile types (0 ± 17 % (w/v) NaCl), moderate halophiles (2 ± 20 % (w/v) NaCl), and extreme halophiles (12 ± 32 % (w/v) NaCl). In the Winsford salt mine (UK), researches were conducted by Norton et al. (1993), Norton & Grant (1988), and Rodriguez-Valera et al. (1980). Various kinds of moderately/extremely halophilic bacteria and Archaea were isolated from ancient salt deposits, in which several new taxa were identified (Dombrowski 1966, Grant et al. 1998, Norton & Grant 1988, Norton et al. 1993). Dombrowski (1963) and Tasch (1963) were the first who isolated microorganisms from rock salt. Reiser & Tasch (1960) succeeded in isolating diplococci, which they had observed in fluid

inclusions in Permian salt (Kansas, USA). Dombrowski (1963) performed similar work on Permian rocks in Europe and older rocks worldwide (Dombrowski, 1963). Enriched salt-saturated mediums, obtained from several sites, yielded to strains that resemble *Bacillus circulans* (Dombrowski 1963). Another isolate called *Pseudomonas halocrenaea* (Dombrowski 1963) was subsequently shown to be indistinguishable from *Pseudomonas aeruginosa* (DeLey et al., 1966), and therefore had to be considered as a contaminant. Although they were able to see fossil bacteria, Schwartz and colleagues failed to isolate microorganisms from 30 samples of rock salt (Bien & Schwartz 1965). Bibo et al. (1983) attempted to repeat Dombrowski's isolations with numerous controls and tests of surface sterilization. They isolated extreme halophiles described as Gram-positive cocci and rod-shaped spore formers from primary Permian Zechstein salt cores. Norton et al. (1993) isolated seven haloarchaeal strains from rock salt in the Winsford salt mine; three belonged to the genus *Halorubrum* and four to *Haloarcula*. The next microorganisms were isolated in the Permian Salado formation, New Mexico (Vreeland et al., 1998) and the Permian Zechstein deposit mined at Bad Ischl (Stan-Lotter et al., 2000); preliminary characterization places them in the genera *Halococcus*, *Haloarcula*, and *Halorubrum* (Stan-Lotter et al., 2000). The latter two genera were also found in rock salt from Winsford (Norton et al., 1993). In a recent microbial diversity study of the Yipinglang salt mine in southwest China, a moderately halophilic, facultatively alkaliphilic strain YIM Y15T *Salinicoccus kunmingensis* sp. nov. was isolated from a brine sample (Chen et al., 2007). Next, Yang et al. (2008) isolated *Thermobifida halotolerans* sp. nov. in a sample from the same mine. Akhtar et al. (2008) conducted research on biodiversity and phylogenetic analysis of culturable bacteria indigenous to Pakistan's Khewra salt mine. They found bacteria similar to those of the genus *Bacillus*, *Escherichia*, *Staphylococcus*. Moreover Xiao et al. (2012) isolated *Roseivivax sediminis* sp. nov., a moderately halophilic bacterium from the sediment from a salt mine in China. At the same time, *Fodinibius salinus* gen. nov., sp. nov., another moderately halophilic bacterium, was isolated in China by Wang et al. (2012). Chauhan & Garlapati (2013) reported on bacterial strain *Staphylococcus arlettae* JPBW-1 isolated from a rock salt mine in Darang, India. This strain produces lipase, which may have considerable potential for industrial application because of its tolerance towards industrial extreme conditions of pH, temperature, salt, and solvent. In 2012, Yildiz et

al. (2012) isolated and characterized 8 different isolates from a salt mine located in central Anatolia, Turkey. These are only a few examples of research on the identification of microorganisms from salt mines.

To our knowledge, no methanotrophs that can colonize such an ecological niche have been isolated yet. Methanotrophic bacteria are a subset of a physiological group of bacteria known as methylotrophs (Hanson & Hanson, 1996). Phylogenetically, methanotrophs belong to α - or γ -*Proteobacteria*, but there are also some representatives affiliated as *Verrucomicrobia* and NC10 phylum. These organisms are unique in their ability to utilize methane as a sole carbon and energy source and play an important role in the global biogeochemical cycles of carbon and nitrogen. The majority of known methanotrophic species are nonhalophilic neutrophiles (Hanson & Hanson, 1996). Halotolerant and halophilic methanotrophs have been isolated from seawater, coastal lagoons, and several soda lakes (Lidstrom et al., 1988; Lees et al., 1991; Khmelenina et al., 1997). Among halophilic methanotrophs, *Methylobacterium alcaliphilum*, *Mm. buryatense*, *Mm. kenyense*, *Methylobacter marinus*, *M. modestohalophilus* have been isolated (Kalyuzhnaya et al., 2001 and 2008, Khmelenina et al. 1999 and 2010; Trotsenko & Khmelenina, 2002). Moreover, Doronina et al. (1998 and 2003) isolated methanol and methylamine-utilizing bacteria from the genera *Methylophaga* and *Methylarcula*. Only a few strains of marine methanotrophs, growing at salinities of 0.15–4 % NaCl, have been isolated (Lidstrom, 1988; Lees et al., 1991). Furthermore, Heyer et al. (2005) isolated two strains of methanotrophic bacteria from hypersaline lakes in the Crimean Peninsula. Strains 10KiT and 4Kr are moderate halophiles that grow optimally at 5.8–8.7 % NaCl and tolerate a NaCl concentrations from 1.2 to 15 %.

The presence of methane in the "Wieliczka" Salt Mine was shown by exploitation of salt deposits in the Middle Ages, when a 'penitent' with torches was engaged in its removal. Methane is a gas released from rocks during exploitation of salt deposits. At a 4.5-15% CH₄ mixture with oxygen, it may be explosive. Since we have been dealing with the methanotrophic microorganisms for several years and studying their presence in coal mines (Stępniewska et al., 2013; Wolińska et al., 2013; Pytlak et al. 2014), we can formulate a hypothesis that the rocks associated with the Wieliczka salts can provide a favorable ecological niche for the growth of methanotrophs, for which methane is the only carbon and energy source.

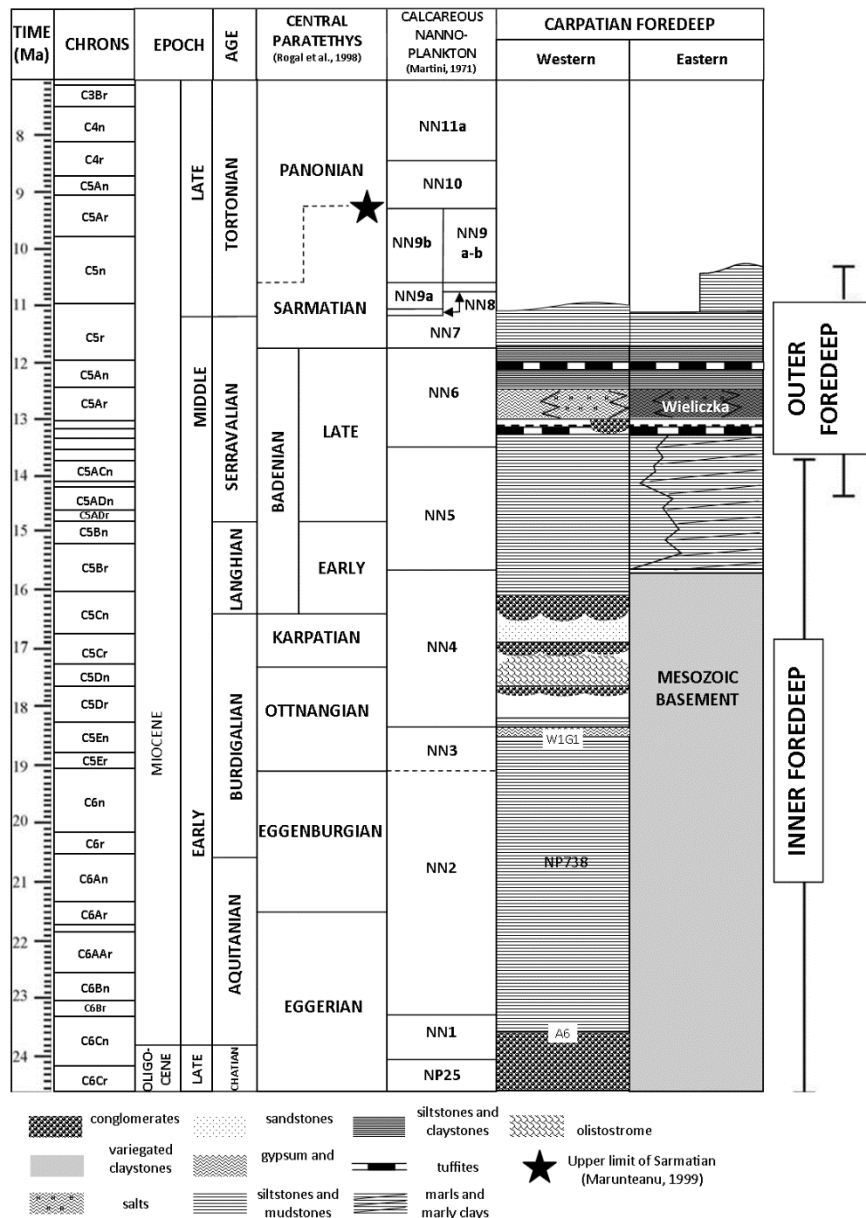


Figure 1. Lithostratigraphic table of the Miocene deposits from the Polish Carpathian foredeep basin (Oszczypko et al., 2005; Krzywiec & Vergés, 2007; modified)

2. MATERIAL AND METHODS

2.1. Geographical and geological setting

Rock salts occur in Poland in two evaporite formations: a Middle Miocene (Badenian Stage) one in Southern Poland (Carpathian Foredeep area) and an upper Permian (Zechstein) one in Northern and Central Poland area. The Carpathian orogenic belt consists of three main tectonostratigraphic domains: the older Inner Carpathians, the younger Outer Carpathians which are separated by the Pieniny Klippen Belt. Wieliczka salt deposit is in the eastern part of the Carpathian Foredeep. The latest lithostratigraphy data of the Miocene deposits allows

to classify the Wieliczka formation to late Badenian at the bottom NN6 level (Brudnik et al., 2010), (Fig. 1). The Badenian evaporate series is dated at 13.81 ± 0.08 Ma and is estimated to have lasted 200–600 k.y. (De Leeuw et al., 2010).

The Miocene period abounded in substantial transformations in the geological structure of the Earth's crust. Because of tectonic plates collisions, new mountain ranges were formed, among them the Carpathians. In the rift located in their foreground, known as the pre-Carpathian basin, a marine environment was setup. It constituted a northern branch of the Paratethys Ocean. Various types of rock sedimented in the basin and rock salt layers were formed. The Wieliczka deposit formed over a

period of approximately twenty thousand years (Bukowski, 1997). It owes its final shape to the orogeny, which resulted in accumulation of salt deposits causing a several-fold increase in their original thickness. The salt deposit was relatively shallow, thanks to which, millions of years later, exploitation of the deposits could be started easily (Pawlikowski, 1976; Bukowski, 1997).

The “Wieliczka” Salt Mine is in southern Poland (N49° 59' E020° 03') 13 km northeast of Krakow but the deposits extend over a length of ca. 10 km (Fig. 2). The Wieliczka salt deposits have been exploited for over 750 years in underground mines. After 1996 exploitation of salt has finished. “Wieliczka” Salt Mine become cultural heritage site, and in 1979 Wieliczka was included on the First International List of the World Cultural and Natural Heritage UNESCO.

2.2. Air sample collection

To determine the composition of the air around the rock material samples, especially the methane concentration, air samples were collected into vials (20 ml) using syringe and transported to the laboratory.

2.3. Rock sample collection

Four samples of rocks surrounding the salt deposit in the “Wieliczka” Salt Mine were collected. The sampling places were selected at depth from 50-130 metres below ground level (Fig.2). The rock samples were different in terms of lithology and the accompanying salt type: W1 – marly claystone - blocky deposits with green salt blocks; W2 – specific clayey salt (zubers) (Garlicki, 2008, Wachowiak et al., 2012) with veins of fibrous salt and lenses of gypsum and anhydrite – layered deposits of spiza salts; W3 – siltstones and sandstone – layered deposits of spiza salts; W4 – siltstones with veins of fibrous salt and lenses of anhydrite – layered deposits of green salt (Fig. 2). Spiza salts is the uppermost parts of the Stratified Salt Member and is composed of detrital salt layers showing occasionally graded bedding or cross-lamination and sporadic intercalations of marls and mudstones with planktic foraminifera (Kolasa & Ślaczka, 1985). The sampling in the mine was carried out after consultation and with assistance of the mine geologists. The pieces of rock samples were taken from the salt mine, immediately put into sterile polyethylene bags, and transported to the laboratory.

2.4. Analysis of air composition

The analysis of the air composition was performed with the gas chromatograph technique (SHIMADZU, GC 2010) with a TCD (Thermal Conductivity Detector), ECD (Electron Capture Detector), and FID (Flame Ionization Detector) after CH₄, CO₂, O₂, and N₂ calibration.

2.5. Sample preparation in the laboratory

Rock pieces (ca. 2 kg, approximately 20 × 10 × 10 cm or larger) were irradiated under a UV lamp on each side for 20 min. Next, each sample was immersed in ethanol and immediately ignited with a gas burner. Afterwards, the surface rock's layers were removed with a sterile knife, and holes (3-5 in each rock) were slowly drilled with a sterile hand-driven drilling machine (Pokorný, 2005), (Fig.3). The resulting rock powder was a material for further research.

2.6. Physicochemical properties

Physicochemical properties of the rock material were determined as follows: 1) The pH, Eh were measured in a suspension of rocks (rock: distilled water – 1:2.5 m/v) with the potentiometric method using a multifunctional pH-meter (Hach Lange or Radiometer Analytical S.A.) and suitable electrodes. The pH measurement was conducted using a combined glass electrode integrated with a chloride reference electrode and a temperature sensor, after calibration in buffers (pH 4, 7, 10). The redox potential was determined using a combined electrode with a platinum indicator electrode, a chloride reference electrode, and a temperature sensor. 2) Salinity was determined by conductometric measurements and expressed in mS·cm⁻¹. Next, the NaCl concentration (g·dm⁻³) was determined based on the previously prepared calibration curve. 3) The moisture content was determined by drying the sample to constant mass at 105±5 °C. 4) The total water capacity of each rock was determined gravimetrically in plastic cylinders by saturating ground rock material with deionized water up to a constant weight and after measurement of water absorption the density of the ground material was calculated. 5) The surface area and pore size analysis was performed in liquid nitrogen using an analyzer of specific surface area Sorptomat QUADRASORB SI, (Quantachrome Instruments, 2009) at the Department of Physical Chemistry of Porous Materials of The Bohdan Dobrzański Institute of Agrophysics, the Polish Academy of Sciences.

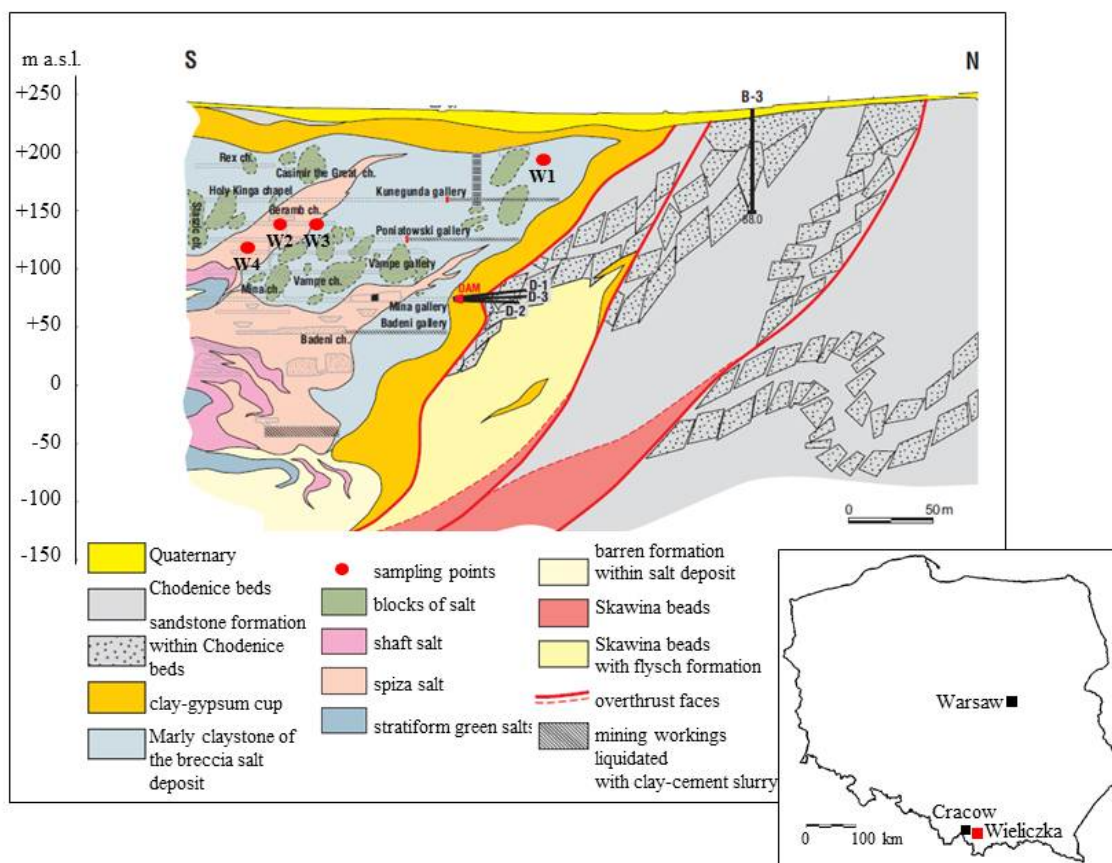


Figure 2. Geological section of the Wieliczka salt deposits and sampling places (Brudnik et al., 2010 and Garlicki, 1974; modified).



Figure 3. Preparation of rock samples

Volume and pore distribution was determined for the desorption curve and calculated with the BJH and DH methods. 6) The granulometric composition of incubated rocks was tested with the Casagrande areometric method modified by Proszynski, which consists in measuring the density of the rock suspension during progressive sedimentation of rock particles at a constant temperature. The density measurements were made with the Proszynski hydrometer, and the density of the rock suspension was read in the periods indicated in the tables developed by

Proszynski (Gliński et al., 2002). 7) The biogenic forms of N (N-NH_4^+ , N-NO_2^- , N-NO_3^-) and S (SO_4^{2-}) were measured using an AutoAnalyzer (Bran + Luebbe AAII/AAIII) after calibration. 8) The forms of carbon (TOC, TC, IC) were measured with the TOC-V_{CSH} SHIMADZU, TOC 5000 with SSM-5000A module. 9) The total Mg, Ca, K, Na, Fe, Cu, Zn contents in the rock samples were determined after mineralization of 0.25 g samples with a concentrated HNO_3 and HF mixture (30 min., temp. 200 °C) in an open microwave system (Ethos One, Milestone). After filtration, the extracts were diluted with distilled water to the volume of 100 ml. Level of participation of metals was measured with the AAS technique with flame atomization - FAAS (Z-8200, HITACHI), in three replications.

2.7. Determination of methanotrophic activity

The methanotrophic activity of bacteria inhabiting the rocks was determined in oxic conditions at a temperature of 10 and 20°C. Triplicates of each rock (15 g) were placed in dark bottles (60 cm³), filled with deionized water to obtain moisture adequate to 100 % of total water capacity, closed with rubber septa, capped with an

aluminium cap, and sealed with paraffin. For the experiments, an initial concentration of ca. 1 and 10% (v/v) CH₄ was obtained by replacing an appropriate volume of air with high purity (99.99 %) methane (Praxair) using a gastight syringe (5 ml, SGE). Simultaneously, control samples were prepared. Three times autoclaved rock samples were incubated in the same conditions. Methanotrophic activity was determined based on the dynamics of methane consumption. The gas concentration was determined by gas chromatography (SHIMADZU, GC 2010) equipped with a TCD, ECD, and FID detector. Methanotrophic activity of salt mine materials was calculated from the slope of the regression line of the measured CH₄ molar amounts vs. time and expressed in $\mu\text{MCH}_4 \text{ g}_{\text{dw}}^{-1} \text{ day}^{-1}$. Adjustments were made with $R^2 \geq 0.95$ with Sigma Plot 10.0 software.

2.8. Statistical analyses

Statistical analysis of the obtained data was performed using Statistica 12 (STATSOFT). The significance of differences between the rock material characteristics and methanotrophic activity were tested at the level of $p < 0.05$. Distributions were assessed using the Shapiro-Wilk test. Pearson or Spearman correlation coefficients (r_s) were calculated to evaluate the relationship between methanotrophic activity and the properties of the rock materials. Parametric data was further analysed with ANOVA, and non-parametric data with the Kruskal-Wallis test.

3. RESULTS

3.1. Analysis of air composition

Chromatographic analysis of the air samples indicates the presence of N₂, O₂, CO₂, and CH₄. The concentration of N₂, O₂, and CO₂ was similar to the composition of Earth's atmosphere. At a depth of 50 m.b.s (meters below surface), i.e. in the location of the W1 (marly claystone) sampling site, the CH₄ concentration was at the level 11.512 ± 1.354 ppm. The air composition around the W2 (zubers with veins of fibrous salt and lenses of gypsum and anhydrite) and W3 (siltstones and sandstone) samples at a depth of ca. 110 m.b.s contained 7.160 ± 0.797 ppm and 7.240 ± 0.653 ppm of methane respectively. The highest level of CH₄ (13.033 ± 1.204 ppm) was observed at a depth of 130 m.b.s, where the W4 (siltstones with veins of fibrous salt and lenses of anhydrite) sampling site was located (Fig. 4).

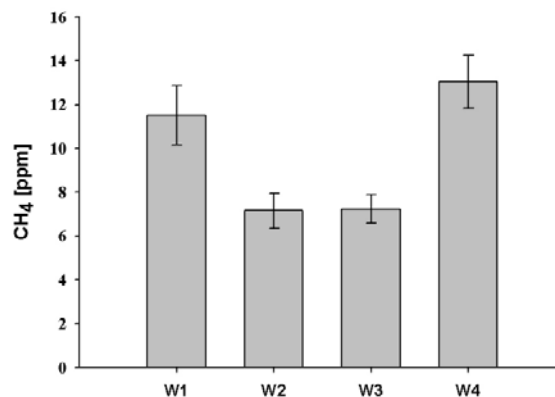


Figure 4. Methane concentration at a sampling site

3.2. Physicochemical properties

The collected rock samples differed in terms of physicochemical properties (Tab. 1). The pH was in the range of 7.43 ± 0.004 (W4) and 8.90 ± 0.06 (W1). The lowest value of Eh was noted in the W1 sample (294 ± 1.08 mV) and the highest in W2 (376 ± 0.20 mV). The W1 and W3 samples were similarly saline (1.232 ± 0.03 and 1.335 ± 0.03 % NaCl). The NaCl content in the W2 and W4 material was higher, i.e. 11.05 ± 3.46 and 3.607 ± 1.26 % NaCl, respectively. Moreover, different moisture contents were found ranging from 4.3 ± 0.02 % to 12.6 ± 0.10 % for the sample W2 and W1, respectively. Simultaneously, we determined water capacity, which was between 31.517 ± 1.223 % (W2) and 51.937 ± 3.336 % (W3). The surface area of the incubated rock material ranged from $14.753 \pm 0.021 \text{ m}^2 \cdot \text{g}^{-1}$ (W2) to $27.661 \pm 0.043 \text{ m}^2 \cdot \text{g}^{-1}$ (W1). The porosity of all rock samples was similar, i.e. approximately $0.054 \text{ cm}^3 \cdot \text{g}^{-1}$.

The total carbon concentration ranged from 1.3 ± 0.05 % in the W4 rock to 3.5 ± 0.10 % in W2. However, the W1 sample was characterized by similar contents of inorganic and organic carbon at the level of 1.463 ± 0.234 and 1.237 ± 0.234 %. The W3 and W4 samples contained greater amounts of inorganic carbon than organic carbon, which constituted 64 % and 63 % of total carbon. On the other hand, there was a reverse trend in the W2 rock and higher organic carbon content of 3.306 ± 0.060 % was observed.

The content of biogens: N, P as well as Cu, Mn, Cr, Ca, K, Zn, Mg, Fe, which could determine the methanotrophic activity, is presented in table 2. Particularly noteworthy is the presence of essential nutrients of methanotrophs: ammonium, nitrate, phosphorus, copper, iron (III), or magnesium. Ammonium was detected in all samples at the level from 5.15 ± 0.05 (W1) to $20.89 \pm 0.22 \text{ mg} \cdot \text{dm}^{-3}$ (W4). Nitrate was present only in the W4 sample at the level $0.12 \pm 0.005 \text{ mg} \cdot \text{dm}^{-3}$ (Table 2).

Table 1. Physicochemical properties of the rock material

rock	pH	Eh [mV]	EC [mS·cm ⁻¹]	NaCl [%]	Moisture content [%]	Water capacity [%]	Surface area [m ² ·g ⁻¹]	Porosity [cm ³ ·g ⁻¹]
W1	8.91 ±0.06	294 ±1.08	13.807 ±0.032	1.335 ±0.03	12.6 ±0.10	50.952 ±1.230	27.661 ±0.043	0.054 ±0.012
W2	7.34 ±0.003	376 ±0.20	114.267 ±3.581	11.05 ±3.46	4.3 ±0.02	31.517 ±1.223	14.753 ±0.021	0.033 ±0.011
W3	7.91 ±0.06	353 ±0.51	12.737 ±0.031	1.232 ±0.03	10.0 ±0.11	51.937 ±3.336	19.959 ±0.027	0.048 ±0.008
W4	7.43 ±0.004	374 ±1.36	37.300 ±1.300	3.607 ±1.26	7.4 ±0.13	42.027 ±1.358	20.312 ±0.063	0.045 ±0.009

Table 2. The content of biogens, C forms and metals

properties	W1	W2	W3	W4
NO ₃ ⁻ [mg·dm ⁻³]	0.00	0.00	0.00	0.12 ±0.005
NH ₄ ⁺ [mg·dm ⁻³]	5.15 ±0.05	12.21 ±0.03	11.32 ±0.04	20.89 ±0.22
PO ₄ ³⁻ [mg·dm ⁻³]	0.06 ±0.003	0.13 ±0.03	0.05 ±0.004	0.05 ±0.005
SO ₄ ²⁻ [g·dm ⁻³]	1.10 ±0.11	7.75 ±0.87	5.63 ±0.33	6.42 ±0.67
TC [%]	2.7 ±0.002	3.5 ±0.09	2.9 ±0.26	1.4 ±0.04
TOC [%]	1.237 ±0.234	3.406 ±0.060	1.031 ±0.439	0.756 ±0.045
Cu [g·kg ⁻¹]	0.32 ±0.14	0.13 ±0.05	0.26 ±0.06	0.32 ±0.02
Mn [g·kg ⁻¹]	0.36 ±0.07	0.43 ±0.04	2.49 ±0.10	0.83 ±0.11
Cr [g·kg ⁻¹]	0.11 ±0.17	0.06 ±0.07	0.75 ±0.17	0.99 ±0.22
Ca [g·kg ⁻¹]	14.85 ±11.43	97.50 ±42.09	145.25 ±16.74	230.16 ±23.95
K [g·kg ⁻¹]	95.58 ±144.11	13.84 ±4.87	168.46 ±20.33	254.59 ±28.95
Zn [g·kg ⁻¹]	0.03 ±0.04	0.00	2.91 ±1.46	0.98 ±0.19
Mg [g·kg ⁻¹]	2.71 ±2.39	4.77 ±0.85	0.00	5.76 ±0.50
Fe [g·kg ⁻¹]	10.80 ±1.28	10.21 ±1.02	106.05 ±36.89	103.27 ±52.36

The characteristics of the rock material were supplemented by analysis of its granulometric composition (Table 3), which is determined by rock types. The maximum grain diameter of the ground samples was 1 mm.

Moreover, we show a statistically significant correlation between the physicochemical properties of the rock material, which may indirectly affect the microbiological activity. One of the main factors determining microbial activity may be the salinity of the medium. The NaCl concentration exhibited a

positive correlation with SO₄²⁻ ($r_s = 0.685$), Eh ($r_s = 0.776$), and Mg ($r_s = 0.613$) and a negative correlation with pH ($r_s = -0.837$), moisture content ($r_s = 0.784$), water capacity ($r_s = -0.902$), porosity ($r_s = -0.777$), TC ($r_s = -0.588$), Cu ($r_s = -0.615$), K ($r_s = 0.622$), and Zn ($r_s = 0.653$).

Table 3. Percentage of rock material fractions.

rock	fractions [mm]					
	1.0- 0.1	0.1- 0.05	0.05- 0.02	0.02- 0.005	0.005- 0.002	<0.002
	[%]					
W1	8	1	8	25	19	39
W2	4	7	7	27	2	47
W3	14	4	13	18	47	4
W4	24	10	12	38	4	12

3.3. Methanotrophic activity

The incubation of all the rock samples were characterized by the dynamics of the gaseous phase. A steady decrease in the methane and oxygen concentration was shown, as well as accumulation of carbon dioxide (Fig. 5).

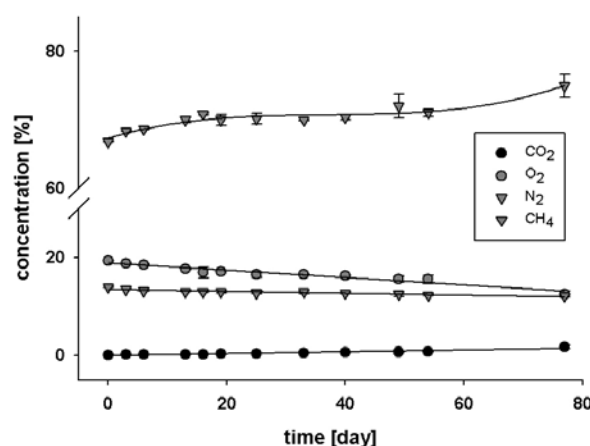


Figure 5. Dynamics of the tested gases during the incubation of rocks material.

The growth rate of the methanotrophic

microbial consortium was measured by methanotrophic activity. The dynamics of the CH₄, O₂, CO₂ and N₂ concentrations in the gaseous phase of the methanotrophic microbial consortium incubations revealed that the methane uptake in the investigated samples was due to the activity of methanotrophic bacteria. In almost all incubated samples, utilization of CH₄ and molecular oxygen was noted after 77 days, which was confirmed by the parallel drop in the concentration of those gases and simultaneous growth of the CO₂ ratio.

The statistical analysis of methanotrophic activity showed the relationship between MA and incubation conditions: temperature and CH₄ concentration ($p = 0.010$). Optimal conditions for the CH₄ oxidation by methanotrophs existing in “Wieliczka” Salt Mine is 20°C, 10% CH₄ and 100% water capacity. Thus, the highest methanotrophic activity ($0.220 \pm 0.066 \mu\text{MCH}_4 \text{ g}_{\text{dw}}^{-1} \text{ day}^{-1}$) was found in the W4 rock incubations. In the other conditions, the methanotrophic activity of W4 rock was lower and ranged from 0.015 ± 0.012 to $0.058 \pm 0.014 \mu\text{MCH}_4 \text{ g}_{\text{dw}}^{-1} \text{ day}^{-1}$. The methanotrophic activity of the other rocks ranged from 0.007 ± 0.002 under 10% CH₄ in 10°C to $0.073 \pm 0.019 \mu\text{MCH}_4 \text{ g}_{\text{dw}}^{-1} \text{ day}^{-1}$ under 10% CH₄ in 20°C (Fig. 6). Methanotrophic activity of rock samples material incubated under 1% of CH₄ at 10°C was not detected. The statistical analysis showed that the methanotrophic activity was positively correlated with the nutrient concentration: NH₄⁺ ($r_s = 0.698$), NO₃⁻ ($r_s =$

0.608) and the content of magnesium ($r_s = 0.704$), ($p < 0.05$). Moreover, what is important, MA was positively correlated with *in situ* CH₄ emission ($r_s = 0.67$).

4. DISCUSSION

Methanotrophic bacteria are ubiquitous and exist wherever there is a source of methane (Hanson & Hanson, 1996). High concentrations of H₂, CH₄ and higher hydrocarbon gas levels were detected in deep, saline fracture waters from mines in Canada, South Africa, and the Fennoscandian Shield (Lollar et al., 2008). We detected CH₄ concentration in “Wieliczka” Salt Mine air. The concentration of methane at the level described above is not hazardous. Methane hazard in Polish mines was estimated in the Polish Ordinance of the Minister of Economy of 28 June 2002 on occupational safety and health, operation, and special fire provisions in underground mines.

However, it should be noted that the methane concentration in the Earth’s atmosphere is lower and now in Poland is less than 2 ppm (Zimnoch et al., 2010; Stepniewska et al., 2014a). *In situ* methane emission is associated with the release of methane from porous formations, such as sandstones, where it was formed due to geological processes, as well as the activity of methanogens (biological sources of CH₄) and the mine ventilation system (Karacan et al., 2011).

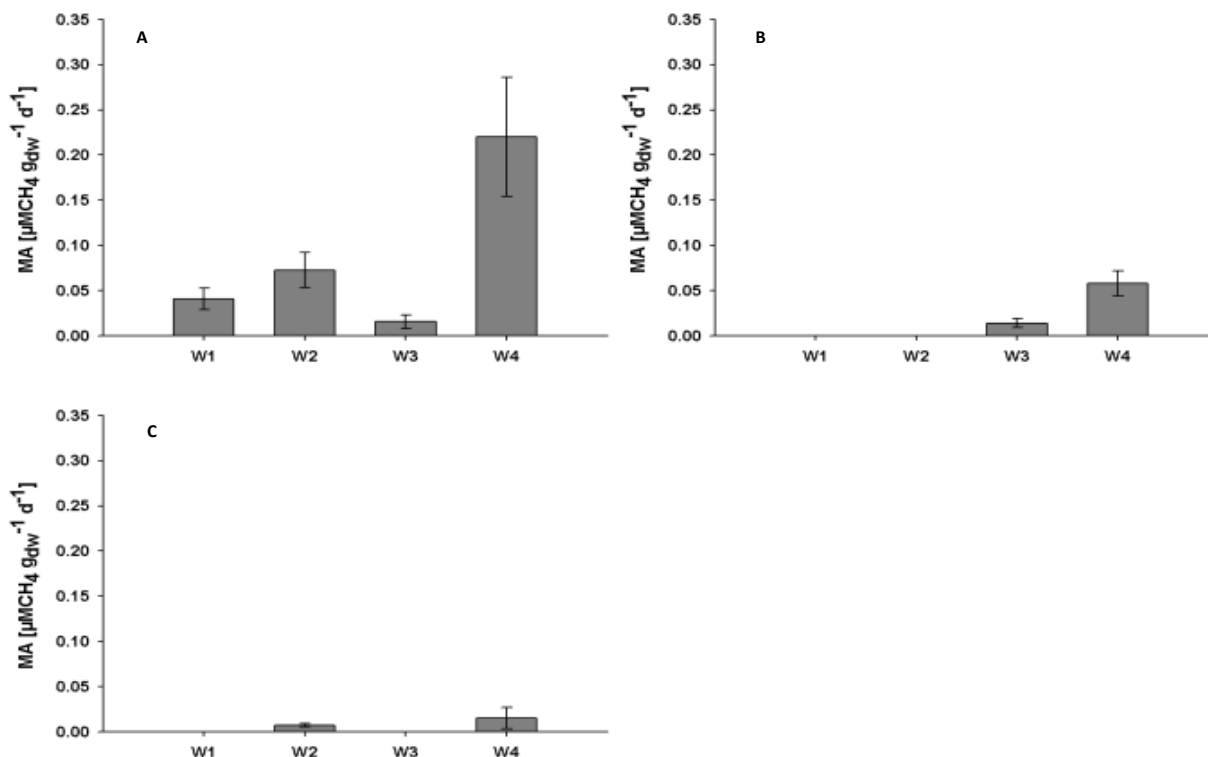


Figure 6. Methanotrophic activity of rock materials A – 10% CH₄ in 20°C; B – 1 % CH₄ in 20°C; C - 10% CH₄ in 10 °C.

It would seem that if the substrate to microbial CH_4 oxidation is present, the existence of methanotrophic microorganisms is possible. However, there are many factors limiting subsurface microbial life such as low oxygen levels. Only in the W1 sample, Eh dropped below 320 mV, which was suggested by (Stepniewska 1988) as a threshold value between oxygenated and reduced soil conditions in the transformation of iron. High redox potential suggests the availability of molecular oxygen as a terminal electron acceptor for methane oxidation (Stepniewska & Pytlak, 2008a). Moreover, one of the potential limitations for subsurface communities in the continental crust is low nutrient availability. With an increase in depth, photosynthetically derived organic carbon becomes limited and hardly accessible (Pedersen, 2001). Nitrogen sources are important factors stimulating methanotrophs (Bodelier et al., 2000), and the ability to fix atmospheric nitrogen is confined to some species (Auman et al., 2001; Dedysh et al., 2004). The highest content of ammonium and nitrate was observed in W4 material where the highest methanotrophic activity was observed. The concentration of organic carbon was detected in all rocks and range from 3.406 ± 0.060 to 0.756 ± 0.045 %. However, there was no relationship between MA and the presence of organic carbon.

The high content of certain elements is due to the lithology of rock materials. Calcium is an essential component of samples containing anhydrite (CaSO_4), (W2 and W4) or gypsum ($\text{CaSO}_4 \times 2\text{H}_2\text{O}$), (W2). Siltstones (W3) may also contain carbonates, in particular calcite (CaCO_3). Moreover, siltstones and sandstones (W3) contain a binder, for instance CaCO_3 . Clay minerals contained in claystones (W1), zuber (W2), and siltstones (W3 and W4) are responsible for the presence of iron and magnesium (Table 2). The presence of copper is also noteworthy. In most methane oxidizing bacteria (methanotrophs), the copper-containing membrane bound methane monooxygenase (pMMO) catalyzes the first step in methane oxidation (Murrell et al., 2000; Ho et al., 2013). Moreover, this rock laden with reduced electron donors for microbial growth such as SO_4^{2-} , Fe(II), and Mn(II) could be appropriate for the growth of anaerobic methanotrophs (Beal et al., 2009). Thus, rocks surrounding salt deposits in the “Wieliczka” Salt Mine can be sources and sinks of micronutrients to different geomicrobial processes. Besides, we show a statistically significant correlation between the physicochemical properties of rock material, which may indirectly affect the microbiological activity.

The granulometric composition (Tab. 3), results from the rock types. The W1 rock was classified as marly claystone, where clay minerals

account for 75-95 %. Claystone is a sedimentary rock made of more than 67 percent clay-size (<0.002 millimeter) particles (Soil Survey Division Staff, 1993). Zuber (W2) is a sedimentary rock made of NaCl and clay. Therefore, it is clear that the W1 and W2 samples were characterized by the largest fraction of <0.002 mm. The W3 and W4 samples were described as siltstones mixed with sandstone and siltstones with anhydrite. Siltstones are primarily composed (greater than 2/3 parts) of silt-sized particles, defined as grains $0.05 - 0.002$ mm (Soil Survey Division Staff, 1993). The silt fraction (coarse and fine) constitutes 78 % and 54 % of the W3 and W4 fractions (Table 3). The lithology of rock materials and granulometric composition may also determine the porosity and pore size of the research material, which is positively correlated with the percentage of sand and silt fractions ($r_s = 0.800$) and negatively correlated with the presence of the clay fraction ($r_s = -0.800$). Porosity and the size of pores control the distribution of microbial populations in different porous media, for example soil (Violante, 2002).

The information about methanotrophic microorganisms inhabiting salt-surrounding rocks is scarce; however, these studies agree with publications on the presence of aerobic methane-oxidizing bacteria in coal mine formations, especially in the Lublin Coal Basin (Stepniewska & Pytlak, 2008a; 2008b; Stepniewska et al., 2014b), which is characterized by very low methane content. Severe nutrient deprivation and low moisture content in the deep subsurface result in very slow cell growth, with cell doubling times that are estimated to reach hundreds to thousands of years (Chivian et al., 2008; Hoehler & Jorgensen, 2013). With long cell doubling times, microorganisms in the deep subsurface may tend to live in a semi-inactive state, waiting for conditions that are favorable for growth and reproduction (Chivian et al., 2008; Lomstein et al., 2012).

Occurrence of coal in the “Wieliczka” Salt Mine was confirmed. Coal material is present in the central part of Wieliczka salt deposit in sub-salt sandstone with claystones and layers of anhydrite (Bukowski, 1997). The numerous accumulations of coal are well above spiza salt (around W2, W3 samples) and in the complex marly claystones within boulder deposits (around W1 sample) (Wagner et al., 2008). Coal in profile of Wieliczka salt is visibly allochthonous. The petrographic composition indicates the presence of salinized moors of fen type, generally forest swamp and open marsh. It is probable that there were also raised bogs, evidently related to rheotrophic, riverine zone. Organic material was washed off and then transported in the organic soil

and deposited in the salted water reservoir or it was transported to the reservoir in the form of lithoclasts of peat and detritus. Peat soils are known as natural sources of methane (Szafraniek-Nakonieczna et al., 2010 and 2012 and 2015). In salt marsh methanogenic and methanotrophic bacteria, can also occur. Modern research about this type of peatlands indicates the presence of methanotrophic microorganisms with the genus *Methylococcus* (Irvine et. al., 2012). Spores of paleopeatland microorganisms can survive for extended periods of time and possess the ability to return to life in suitable conditions (water content, temperature, methane, nutrients), (Abel-Santos, 2012)

Our results indicate that subsurface environments should receive much more attention because their microbial inhabitants may play an important role in biogeochemical carbon cycling. These findings hold large implications for understanding the ultimate limits for life on Earth and the potential for life to exist in the subsurface of other planets, such as Mars (Simkus, 2013). Further investigations are necessary to evaluate the age, origin, and phylogeny of the microorganisms responsible for the observed methane oxidation as well as their influence on the methane levels in mining atmospheres.

4. CONCLUSIONS

- 1) The highest uptake of CH₄ was found in the W4 rock incubations exhibiting maximum water capacity (MA = 0.220±0.066 μMCH₄ g_{dw}⁻¹ day⁻¹)
- 2) MA of the other rocks ranged from 0.015±0.008 to 0.073±0.019 μMCH₄ g_{dw}⁻¹ day⁻¹
- 3) MA was positively correlated with the nutrient concentration: NH₄⁺ (Rs = 0.698) and NO₃⁻ (Rs = 0.608) and the content of magnesium (Rs = 0.704), (p<0.05).
- 4) MA was positively correlated with *in situ* emission (Rs = 0.67).
- 5) Further investigations are necessary to evaluate the age, origin, and phylogeny of the microorganisms responsible for the observed methane oxidation as well as their influence on the methane levels in mining atmospheres.

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