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Methanogenesis and metal leaching on anaerobic decomposition of graptolite argillite

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ABSTRACT

Estonian sedimentary deposits (e.g., graptolite argillite) with relatively high content of organic matter and metals (V, Mo, U and Zn) are habitat for microorganisms. Metals exist in argillite as sulfides, in the composition of organometallic compounds or in silicate minerals. While the role of pyrite oxidizing microorganisms in bioleaching of metals is well described, less is known about the processes related to transformation of organometallic complexes. The current study examines impact of microorganisms on argillite organic matter decomposition as well as on metals leaching and/or precipitation. The microbial diversity of methane producing, and possible metal leaching consortium is described, the role of betaine in biodegradation of argillite organic matter is explained and a tentative mechanism for organic matter degradation with simultaneous methanogenesis is proposed.

1. Introduction

Deposits of black shale ores worldwide contain considerable amounts of base, heavy, rare and precious metals (Pasava, 1993; Sawlowicz, 1993; Stribrny et al., 2000; Orberger et al., 2003; Vera et al., 2013).

Several black-shale hosted metal enrichments offering significant economic interest are among the world largest base metal reserves like the Ni–Cu bearing black-shales in Talvivaara, Finland (Luokola-Ruskeeniemi and Heino, 1996). Most of the elements of interest in black shales are typically in metal sulfides or silicate minerals, but also in organometallic compounds including the metallo-porphyrins. For example, the Late Permian black shales widely distributed over Central Europe and known as Kupferschiefer contain 5%–14% organic matter of marine origin (type II kerogen) (Gouin et al.,

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Abbreviations

δ^{13}C (V-PDB)	Carbon isotope composition relative to the V-PDB (Vienna *Belemnitella Americana*, Peedee Formation, Cretaceous Period, South Carolina) standard
CELMS	Collection of non-medical environmental and laboratory microbial strains at the Institute of Molecular and Cell Biology, University of Tartu
EEUTARGCON5	Methane generating microbial community isolated from graptolite argillite
GA	Graptolite argillite
NC	Negative control
NCBI SRA	National Centre of Biotechnology Information Sequence Read Archive
PCR	Polymerase chain reaction
R2A	Reasoner medium, suitable for isolation of microorganisms from the environment
SRB	Sulfate reducing bacteria
SULFAMMOX	Sulfate reducing ammonium oxidation.

2007; Kamradt et al., 2012), and are rich in Ni, Pb, Co, Cu, Mg, Zn, V, Al, Cr as well as Sn, Te, W, Pt and Zr that form a range of metallo-porphyrins and metallo-porphyrin-derivatives (Orberger et al., 2003). Similarly, the Ordovician black shale (called as graptolite argillite — GA) in Estonia and NW Russia containing 10%–20% of organic matter (kerogen) in feldspar, quartz and clay mineral matrix (Maremäe, 1988) is significantly enriched with U, Mo and V, and occasionally Zn (Voolma et al., 2013). These high concentrations of certain metals may be potentially useful and hazardous at the same time. During the Soviet Occupation of Baltic countries, the GA was mined in Estonia for uranium production. Between 1964 and 1991, approximately 73 million tons of graptolite argillite was mined and piled into waste heaps from a covering layer of phosphorite ore at Maardu, near Tallinn (Soesoo et al., 2020).

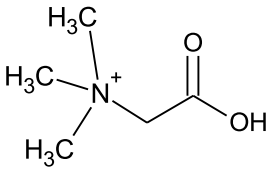
Commonly different pyrometallurgical processes are used to extract useful components from black shales. In last decades, however, hydrometallurgical processes have been advocated as less environmentally harmful and economic methods (Kaksonen et al., 2018; Vind and Tamm, 2021). Metals can be leached from organic rich shales with acids, oxidation and hydrogenation; (Lippmaa et al., 2009, 2011).

Alternatively, the microbially induced (bio-)leaching has been successfully used to retrieve the metals of interest (Mishra and Rhee, 2010; Watling, 2015; Kaksonen et al., 2020). Microbial technology offers an economic substitute for the mining industry, at a time when high grade mineral resources are being exhausted (Poulin and Lawrence, 1996). While the role of pyrite oxidizing microorganisms in bioleaching of metals is well described (Cockell et al., 2011; Esnault et al., 2013; Vera et al., 2013; Li et al., 2014; Dumett and Keener, 2014; Crundwell, 2014), much less is known about the microbial decomposition of organometallic complexes.

Black shales are known as habitat for diverse microorganisms (Esnault et al., 2013; Mesle et al., 2013), and number of studies have detailed the diversity of culturable species in mineral material (Watling, 2015). Two strains of heterotrophic bacteria, *Bacillus cereus* and *Bacillus amyloliqueficiens*, were isolated and used to extract metals (at pH 7) from the organometallic component of Kupferschiefer black shale ore (Farbiszewska-Kiczma et al., 2004). Isolates of heterotrophic bacteria degrading organometallic components included *Pseudomonas, Acinetobacter, Aeromonas, Brevibacillus, Microbacterium* and *Bacillus species* (Brombacher et al., 1998; Farbiszewska-Kiczma and Farbiszewska, 2005; Matlakowska et al., 2007; Matlakowska and Sklodowska, 2009). Based on metagenome and metaproteome studies, these microbial processes have now been described (Wlodarczyk et al., 2018).

Although biodegradation of geopolymers has been reported in several studies (Widdel and Rabus, 2001; Strapoc et al., 2011; Schlegel et al., 2013; Kutschke et al., 2015; Ritter et al., 2015; Rabus et al., 2016) the mechanisms of biotransformation of such polymers, incl. geo-porphyrins to natural gas were not fully understood. Recent studies by (Stasiuk and Matlakowska, 2021a) have shed light to these processes presenting the key metabolic pathways. In line with other substrates for methanogens (Jones et al., 2008; Harris et al., 2008; Strapoc et al., 2011; Urios et al., 2012; Mesle et al., 2013; Schlegel et al., 2013; Urios et al., 2013), methanol and trimethylamine (Pfeiffer and Ulrich, 2010; Wuchter et al., 2013) as methylotrophic substrates have been used to stimulate methanogenesis. However, to our knowledge, no reports on the use of betaine for this task could be found. Methanogens, consuming betaine directly have nevertheless been described (Watkins et al., 2014; Ticak et al., 2015).

The degradation of N-containing porphyrin geopolymers can be compared with similar processes in anaerobic treatment of high-level nitrogen and sulfur containing wastewaters. Favorable coexistence of sulfate reducing bacteria (SRB) and methanogens has been observed, explained by the metabolism of trimethylamine — a degradation product of betaine (**Formula** (1)) (Koplimaa et al., 2010). Thus, the observation of (Fdz-Polanco et al., 2001) confirms sulfate reducing ammonium oxidation (SULFAMMOX) and simultaneous methanogenesis in a reactor containing betaine (Eq. (1)).



Formula (1). Chemical structure of betaine (N, N, N-trimethyl glycine).

$$2\,NH_4^+ + SO_4^{2-} \rightarrow N_2 + S_0 + 4\,H_2O,\ \Delta G_0 = -46\ \text{kJ reaction}^{-1} \quad (1)$$

In the presence of sulfate more NH_4^+ is consumed than the stoichiometric ratio would predict (Rikmann et al., 2012; Kong et al., 2021). Similar processes have been observed in sediments (King, 1983; Schrum et al., 2009). Microorganisms responsible for SULFAMMOX (sulfate reduction dependent ammonium oxidation) were not typical anammox organisms – *Planctomycetes* – but rather other phyla, e.g. *Verrucomicrobia* (Rikmann et al., 2014). Betaine is degraded stepwise (Eq. (2)) (Naumann et al., 1983; Zumbusch et al., 1994; Moune et al., 1999), and in the final steps methylated amines are consumed by methylotrophic archaeans (methanogens) resulting in production of ammonium and gaseous methane (Eq. (3)) (Tschobanoglous et al., 2003). Thus, methanogens gain advantage over sulfate reducing microorganisms, avoiding generation of H_2S.

$$\text{betaine} + 1.32\ \text{serine} + H_2O \rightarrow (CH_3)_3N + 2\,CH_3COOH + 1.32\,CO_2 + 1.32\,NH_3 \quad (2)$$

$$4\,(CH_3)_3N + 12\,H_2O \rightarrow 9\,CH_4 + 3\,CO_2 + 6\,H_2O + 4\,NH_3 \quad (3)$$

The aim of this study is to describe the microbial diversity of methane producing and metal leaching community enriched from Estonian graptolite argillite. An attempt was also made to obtain implications on the mechanism of these processes.

2. Materials and methods

2.1. Substrates and media

Graptolite argillite samples from a drill core provided by Ashtree Ltd, Estonia (Laagri, Harju County, Estonia, 59°20′57″N, 24°35′32″E, depth of 32,5 m) were used as substrate in cultivation experiments. The outer weathered layer of the drill core (approx. 3–4 cm thick) was removed and about 500 g of argillite taken from the drill core was crushed (pieces with dimensions of 0.5 to 1 cm). The crushed argillite samples were kept in mini grip bags at +4 °C. The resulting argillite samples served both as an inoculum (source of facultative and obligatory anaerobic bacteria and methanogenic archaeans) as well as a substrate (source of nutrients). The organic matter content of the sample was 11.4% and sulfur content 2.3%. R2A, a suitable medium for isolation of microorganisms from the environment was used as the main medium (Reasoner and Geldreich, 1985). Various carbon sources (glucose, ethanol), substrates favoring methanogenesis (Na-acetate, methanol, betaine, vinasse), buffer components ($NaHCO_3$) and reducing agents (cysteine, Na_2S) were added to the main medium of R2A (Fig. 1, **Appendix A, MMC S1**).

2.2. Inoculum

In enrichment experiments, no specific inoculum was used, all the microbial consortia originated from the GA samples themselves and were used in the experiments without prior sterilization, autoclaving or any other thermal treatment. In cultivation experiments of the microbial community (named as EEUTARGCON5) with graptolite argillite (native or gamma sterilized at 50 kGy), glycerol stock cultures stored at −80 °C were used, with inoculation into 15 ml of liquid medium R2A, growing anaerobically for 2–4 days under argon and then adding to the experiment with the ratio 1:20 at an optical density of 0.05.

2.3. Cultivation experiments

2.3.1. Serum bottles

For enrichment a methane-producing microbial community from argillite, 6 parallel experiments at 36 °C and 2 parallel experiments at 25 °C in serum bottles were started with glucose, ethanol, butyric acid, propionic acid and whey as additional carbonaceous substrates, and acetate, methanol, betaine and vinasse as methanogenic substrates in addition to the main medium R2A (Fig. 1). 55 ml of medium was measured into each serum bottle and 7 g argillite was added. Before closing, the serum bottles were flushed with the mixture of CO_2: N_2 (20:80) for 15 s. The production of biogas was monitored. For liquid sampling, one serum bottle at 36 °C was opened every two weeks and one serum bottle at 25 °C every 4 weeks. Immediately after sampling, the cell cultures were filtered using 0.2 μm Whatman syringe filter. DNA was extracted using PowerSoil® DNA Isolation Kit. Additionally, negative controls (NC) were used to verify integrity of extraction method. Extracted DNA concentrations were measured with Appliskan in plate format or with Qubit in tube format. This was followed by PCR analysis with primers specific for acetogens (primer pair Fhs1-FTHFSr) or methanogens (primer pairs MLf-MLr, MCRf-MCRr, ME1-ME2) and preparation of amplicons with a pyrosequencing label by PCR (with primer pairs BSF8-BSR357 and Arch349F-A934b) and pyrosequencing.

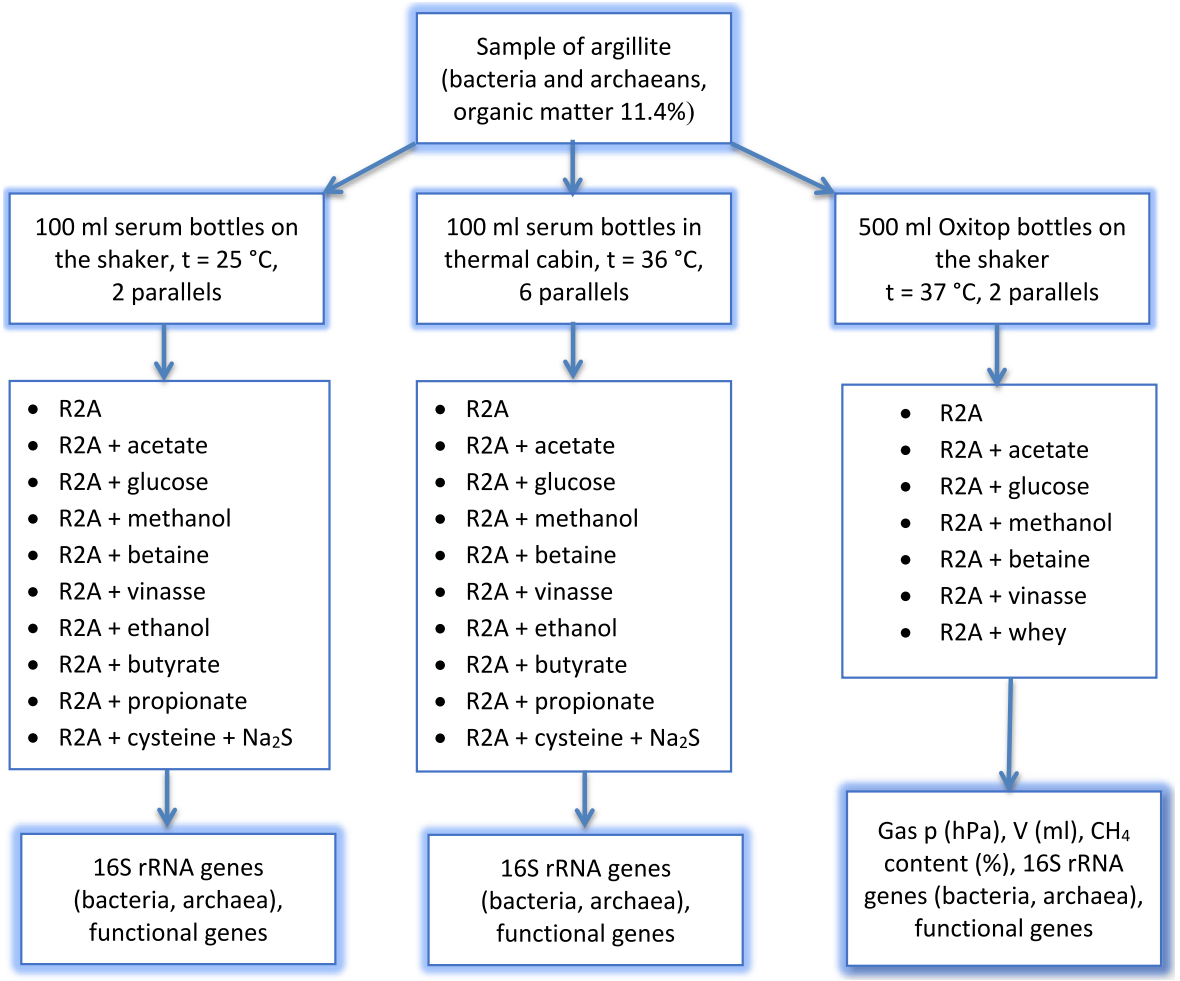


Fig. 1. Scheme for preparation of media for various experiments and analysis performed; $NaHCO_3$ buffer was added to all media variants.

2.3.2. OxiTop® system

Media containing additional carbon sources (glucose, whey) as well as media containing additional methanogenic substrates (acetate, methanol, betaine, vinasse) were used; the experiments with the use of ethanol, propionate and butyrate were omitted (Fig. 1). All the experiments were performed with two replicas. Experiments with the main medium R2A and with supplements methanol and vinasse were performed twice. Cultivation experiments were performed with the OxiTop® system (WTW/ Xylem Analytics) in hermetically sealed 250 ml, 500 ml or 1000 ml volume reactors with manometric sensors, data acquisition device and Achat OC 3.2.0.0. software. The 250 ml and 1000 ml bottles have two lateral connection hubs enabling liquid and gas sampling. The internal pressure in bottle and the composition of gas mixture were analyzed. Biogas released by anaerobic degradation increases the pressure in the hermetical and calibrated OxiTop® system, based on which the growth curve of microorganisms can be generated. The pressure is measured in hectopascals (1 hPa = 102.1 Pa = 1 mbar). From pressure change, the amount of gas released can be calculated from Eq. (4) (Pereira et al., 2012; Menert, 2015):

$$pV = nRT, \text{ where} \tag{4}$$

p — the pressure of the gas, N/m^2 or hPa;
V — the volume of the gas, m^3;
R — the universal gas constant, J/mol*K; $R = 8.314$ J/mol*K;
T — the absolute temperature of the gas, K;
n — the amount of the substance of gas (number of moles) [mol]; the number of moles $n = 1$ corresponds to 22.4 l gas under normal conditions.

Reactor bottles with the main medium (R2A) were autoclaved, labile media components ($NaHCO_3$, betaine, cysteine, Na_2S) were added by filter sterilization (0.2 μm pore size Whatman syringe filter). Subsequently the samples of argillite

and inoculum (in all the experiments, except the enrichment ones) were added and the OxiTop® reactors were assembled with pressure sensors and sampling ports. As argillite indigenous microbial community supposedly contains obligatory anaerobes, air in the headspace of reactor bottles was replaced by argon gas with flushing for 4 min before the beginning of the experiments. The completed reactor bottles were placed into a thermostated shaker at t = 37 °C. The DNA extraction, PCR analysis and pyrosequencing were performed as in the case of serum bottle experiments.

2.4. Analytical methods

2.4.1. Determination of the amount of gas

From the raw file data obtained from the OxiTop® data acquisition device, the cumulative pressure (hPa) in the reactor was first calculated:

$$P_{sum,t} = P_{sum,\,t-1} + P_t - P_{t-1}, \tag{5}$$

where $P_{sum,\,t-1}$ — the total pressure in the previous test point, hPa;
P_t — the current value of pressure at given test point, hPa;
P_{t-1} — the current value of pressure at the previous test point, hPa.
It was then possible to calculate the cumulative amount of biogas in millimoles:

$$n_{biogas} = \frac{P_{biogas} \cdot V_{biogas}}{R \cdot T}, \tag{6}$$

where P_{biogas} — the pressure of biogas in the reactor, hPa;
V_{biogas} — the volume of biogas in the reactor, m^3;
R — the universal gas constant, m^3 hPa/ (K·mol);
T — the temperature, K.
Before starting the experiments with the inoculum EEUTARGCON5, the cells were not washed, so the inoculum also contained a small amount of nutrients that could be degraded into biogas. The amount of biogas (n_{inoc}) originated from the degradation of the nutrients derived from the inoculum was not determined. Instead, to find out the gas pressure value (p_{subs}) for experiments, the gas pressure value in EEUTARCON5 culture grown in the absence of argillite (p_{inoc}) was subtracted from the total gas pressure value (p_{biogas}). The cumulative amount of biogas released only by substrate decomposition in millimoles is calculated analogously:

$$n_{subs} = n_{biogas} - n_{inoc} \cdot \frac{m_{inoc\,in\,mixture}}{m_{inoc}}, \tag{7}$$

where n_{biogas} — the amount of biogas generated with EEUTARCON5 culture during the decomposition of the substrate (argillite) and cultivation medium, mmol;
n_{inoc} — the amount of biogas released during the cultivation of EEUTARCON culture in the absence of argillite, mmol;
$m_{inoc\,in\,mixture}$ — the mass of the inoculum added to the substrate, g;
m_{inoc} — the mass of inoculum in the blank experiment, g.
Using volume fractions of gas components, the cumulative amount of methane (or any other gas component) in biogas can be calculated (mmol):

$$n_{i,\,t} = n_{i,\,t-1} + (n_{biogas,\,t} - n_{biogas,\,t-1}) \cdot v_{i,\,\%} \Big/ \sum (v_{i,\,\%}), \tag{8}$$

where $n_{i,\,t}$ — the cumulative amount of gas component i at given test point, mmol;
$n_{i,\,t-1}$ — the cumulative amount of gas component i in the previous test point, mmol;
$n_{biogas,\,t}$ — the cumulative amount of biogas at given test point, mmol;
$n_{biogas,\,t-1}$ — the cumulative amount of biogas at previous test point, mmol;
$v_{i,\%} / \sum (v_{i,\%})$ — the volume fraction of gas component i at the given test point.
Cumulative amount of biomethane released by substrate degradation per mass of graptolite argillite (m^3/t GA):

$$V_{\mathrm{CH_4},A} = \frac{n_{\mathrm{CH_4},subs} \cdot 22.4 \cdot 1000}{m_{sample,GA}}, \tag{9}$$

where $n_{CH4,\,subs}$ — the cumulative amount of biomethane released by the substrate degradation, mol;
22.4 – the volume of one mole of gas under normal conditions, l;
$m_{sample,\,GA}$ — mass of graptolite argillite, g;
1000 — for converting the result to tons (without the conversion the unit is l/g or m^3/kg). (Menert, 2015; Appendix A, MMC S1).

2.4.2. Chromatographic analyses

The composition of the released gas mixture in all cultivation experiments was measured with the gas chromatograph Shimadzu GC-2014 (methane determination range 10 ppb — 30%) and with a gas chromatograph Varian Inc. Model CP-4900 (methane determination range 1%–100%). (Luna-delRisco et al., 2011).

Table 1
Results of gas pressure, gas composition and analysis of acetogenic and methanogenic activity by functional genes analysis in the OxiTop enrichment experiment at t = 37 °C[a].

No	Culture medium	Day	pH	Gas pressure, hPa	CO_2, %	CH_4, %	N_2O, ppb	DNA conc, ng/µl	PCR Fhs1-FTHFSr	PCR MLf-MLr	PCR MCRf-MCRr	PCR ME1-ME2
1	R2A + GA, 1st experiment[b]	24	n.d.	465	6.83	0.00060	355	n.d.	n.d.	n.d.	n.d.	n.d.
2	R2A + GA, 2nd experiment	31	6.9	454	n.d.	n.d.	n.d.	0.9	+	–	–	–
3	R2A + Na-acetate + GA, 1st experiment	43	6.7	416	n.d.	n.d.	n.d.	2.2	+	–	–	–
4	R2A + Na-acetate + GA, 1st experiment	43	6.9	363	n.d.	n.d.	n.d.	0.8	+	–	–	–
5	R2A + glucose + GA, 1st experiment	43	6.2	794	n.d.	n.d.	n.d.	1.1	–	–	–	–
6	R2A + glucose + GA, 1st experiment	43	5.3	434	n.d.	n.d.	n.d.	3.3	+	–	–	–
7	R2A + methanol + GA, 1st experiment	81	n.d.	561	6.21	0.0051	79	n.d.	n.d.	n.d.	n.d.	n.d.
8	R2A + methanol + GA, 2nd experiment	24	n.d.	633 ± 21	10.94	0.00199	1908	n.d.	n.d.	n.d.	n.d.	n.d.
9	R2A + methanol + GA, 2nd experiment	31	7.7	625 ± 6.0	n.d.	n.d.	n.d.	0.1	+	–	–	–
10	R2A + methanol + GA, 2nd experiment	47	n.d.	639 ± 27	0.06	0.00034	326	n.d.	n.d.	n.d.	n.d.	n.d.
11	R2A + vinasse + GA, 1st experiment	43	6.7	377 ± 95	n.d.	n.d.	n.d.	6.4	+	–	–	–
12	R2A + vinasse + GA, 2nd experiment	24	n.d.	705 ± 15	7.61	0.00077	3226	n.d.	n.d.	n.d.	n.d.	n.d.
13	R2A + vinasse + GA, 2nd experiment	31	6.7	701 ± 11	n.d.	n.d.	n.d.	2.2	+	+	–	–
14	R2A + vinasse + GA, 2nd experiment	47	n.d.	708 ± 17	7.34	0.00026	4393	n.d.	n.d.	n.d.	n.d.	n.d.
15	R2A + GA + betaine[c], 1st experiment	56	n.d.	800	7.33	15.58	1443	n.d.	n.d.	n.d.	n.d.	n.d.
16	R2A + GA + betaine, 1st experiment	63	n.d.	1090	7.00	28.06	1010	n.d.	n.d.	n.d.	n.d.	n.d.
17	R2A + GA + betaine, 1st experiment	77	7.9	1202	6.26	27.18	413	6.8	+	+	+	+
18	R2A + GA + betaine, 1st experiment	91	n.d.	1601	5.11	18.96	252	n.d.	n.d.	n.d.	n.d.	n.d.
19	R2A + GA + betaine, 1st experiment	102	n.d.	1957	5.25	33.00	181	n.d.	n.d.	n.d.	n.d.	n.d.
20	R2A + GA + betaine, 1st experiment	112	n.d.	2275	12.60	27.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21	R2A + GA + betaine, 1st experiment	124	n.d.	2574	11.78	37.45	8.79	n.d.	n.d.	n.d.	n.d.	n.d.

[a]$NaHCO_3$ buffer added to all combinations of media, additionally reducing agents Na_2S (0.225 g/l) and cysteine (0.45 g/l) were added in the 2nd series of experiments.
[b]Both experiments with R2A + GA were performed with one replica.
[c]Only the data for the methane producing replica are presented.

3. Results and discussion

3.1. Obtaining enrichment cultures in serum bottle experiments

Provided several metals in argillite could be combined with organic matter, a hypothesis was posed consisting in initiating methanogenesis and releasing metals by disruption of porphyrin cycle rather than in simple stimulating it with methanogenic substrates. The mechanism for degradation of organic component of argillite and the most efficient combination of microbes conducting this process could thus be identified.

The effect of supplements at 36 °C was quite modest. More gas was released at higher pH values in the experiments with vinasse, methanol, ethanol and acetate. Primer pairs binding to the methanogenesis marker gene *mcrA*, encoding the methyl coenzyme M reductase (MCR) subunit A, were used to detect methanogenic archaeans. Primer pair binding to the acetogenesis marker gene *fhs* encoding formyltetrahydrofolate synthetase (FTHFS), was used to detect acetogenic bacteria. Since vinasse was used without sterilization, growth of acetogens was also observed in control samples with vinasse. Positive for gene specific to acetogens were culture media R2A, R2A + methanol, R2A + ethanol, R2A + vinasse and R2A + reducing agents (Na_2S and cysteine). Media supporting the growth of methanogens were R2A and R2A + reducing agents. Thus, all the supplements, except organic acids propionate and butyrate promoted the growth of acetogens and/or methanogens. For the first time, detectable amounts of both CO_2 and methane were observed with all the supplements since day 56. The higher concentrations of methane were obtained with vinasse (44 ppm) and ethanol (53 ppm).

The changes in pH and gas generation in serum bottle experiments at 25 °C were similar. The following media were tested positive for specific to acetogens: R2A, R2A + acetate, R2A + methanol, R2A + ethanol, R2A + betaine, R2A + vinasse, R2A + butyrate. Only R2A + ethanol was tested positive for methanogens. Detectable amounts of biogas components were observed only since day 56. All the data concerned are presented in **Appendix A, MMC S1**.

3.2. Obtaining enrichment cultures with the OxiTop system

Only the experimental conditions with positive results on acetogen and/or methanogen marker gene detection in serum bottle enrichment experiments were selected for gas generation monitoring with the OxiTop® system (Fig. 2). In both experiments, gas generation results with the media R2A and with R2A + methanol were rather similar. Vinasse gave some increase in gas pressure, however the methane content in gas phase remained insignificant (77 ppm, Table 1). Acetate addition had no effect on gas production as compared to R2A as main medium. Slightly higher gas generation

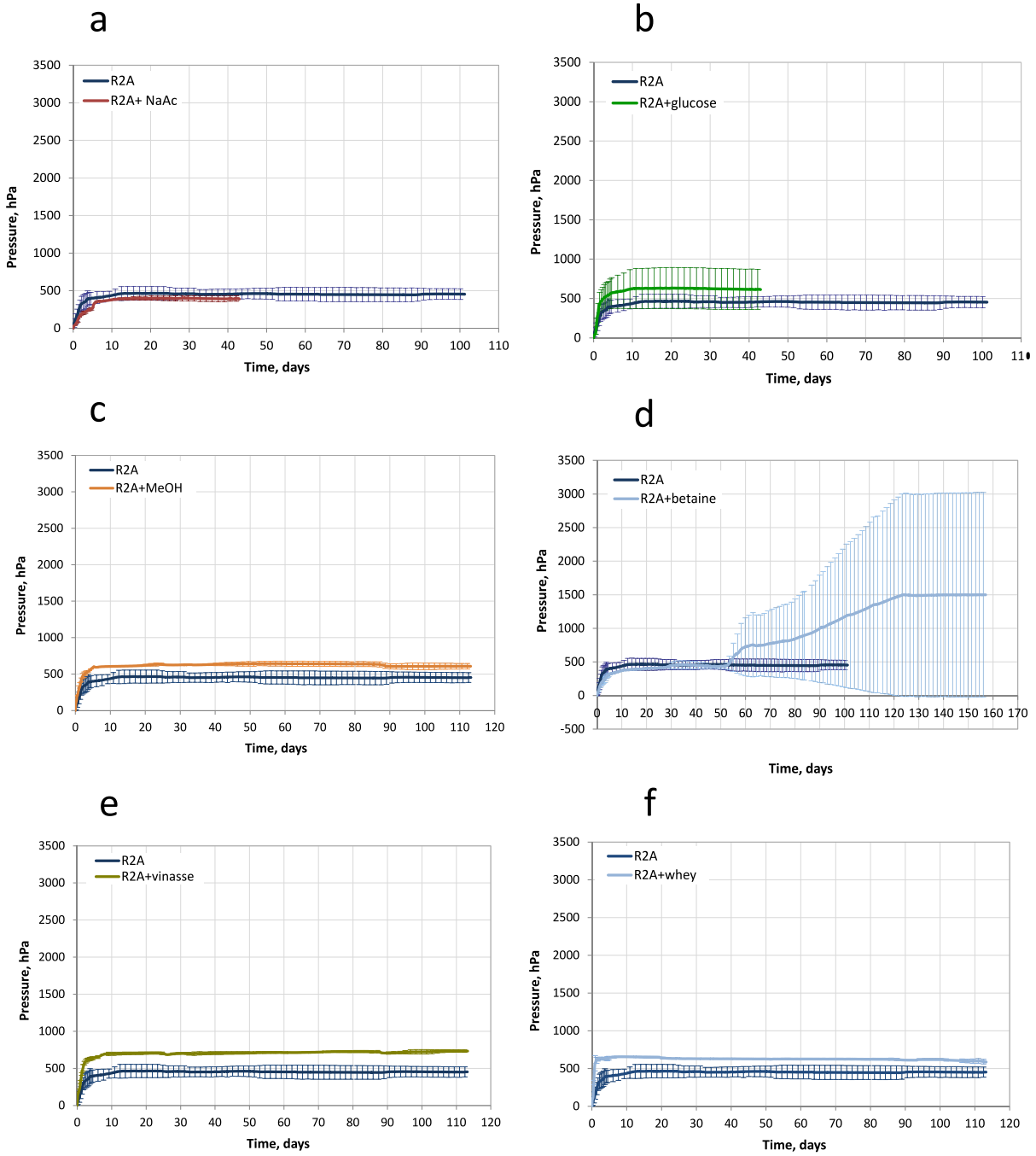


Fig. 2. Pressure changes in microcosm OxiTop experiments of argillite samples (8.3% suspension) with various supplements added to R2A base medium as compared to medium R2A without supplements, t = 37 °C. In the experiments with the media R2A, R2A + methanol, R2A + vinasse and R2A + whey, also reducing agents Na_2S and cysteine were added to culture medium. a. — Na-acetate; b. — glucose; c. — methanol; d. — betaine; e. — vinasse; f. — whey.

was achieved with glucose, methanol, vinasse and whey (Fig. 2c, e, f). The best supplement to sustain higher pH and thus promote methanogenesis was again vinasse, and additionally betaine (Table 1). In the experiments with the media R2A, R2A + methanol, R2A + vinasse and R2A + whey, also reducing agents Na_2S and cysteine were added to culture medium, as this combination had been previously shown to promote acetogenesis and methanogenesis in t = 36 °C serum bottle experiments.

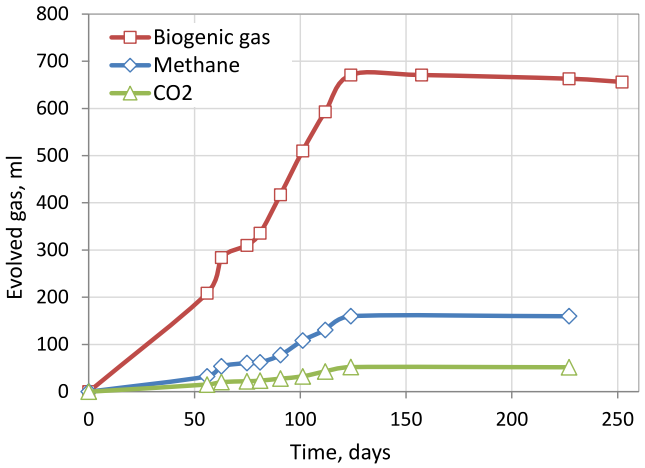


Fig. 3. The amounts of total biogenic gas and its components methane and CO_2 released from argillite with an indigenous microbiological consortium and with medium R2A + $NaHCO_3$ + betaine, calculated by cumulative gas pressure and volume fractions of gas components.

With the medium R2A + betaine, the presence of methanogenesis pathway gene *mcrA* was in good correlation with the actual appearance of methane into the gas phase (16% on day 56, Table 1). The taxonomic composition of the microbial community based on the 16S ribosomal RNA gene sequence is described in Sections 3.3 and 3.4. When cultivating methanogens in a mixed culture, the buffering capacity of the medium is of utmost importance because metabolites from fermentative microorganisms acidify the environment rapidly while methanogens prefer solely alkaline region (pH 6.8–7.5). Availability of microelements and vitamins is important; supplement of metabolic intermediates and methanogenic substrates also facilitates the growth of methanogens. All these conditions were fulfilled solely in one of the replicas with the cultivation medium R2A + betaine, obviously promoting gas generation at the expense of degradation of argillite organic matter (Fig. 2d).

Based on the measurements with the OxiTop device, the changes in cumulative gas pressure (hPa) in the reactor were first calculated (Eq. (5)). Further the cumulative amount of biogas in millimoles was calculated from Eqs. (6), (7) and finally, using the volume fractions of gas components, the cumulative amounts of methane and other gas components (mmol) in biogas were found by Eq. (8). The volume of biomethane evolved can be calculated and expressed also as per mass of argillite (Eq. (9)). Using 25 g of crushed argillite as a substrate (with particle dimensions of 0.5–1 cm) with 300 ml medium R2A + $NaHCO_3$ + betaine, within 90 days 417 ml of biogenic gas with methane content from 15 to 28%, with a yield of 3.1 L of methane per 1 kg of argillite was obtained (Fig. 3). 671 ml of biogenic gas with methane content of up to 37.5% was evolved as a maximum within 124 days, which means a yield of 6.4 liters methane per 1 kg of mineral (argillite) (**Appendix A, MMC S1**).

3.3. Taxa identification of microbial communities from enrichments

From the samples of microbial communities enriched from argillite in OxiTop experiments with various cultivation media (Fig. 1), microbial DNA was extracted, amplicons with a pyrosequencing label were prepared by PCR and sequenced. According to the results of pyrosequencing, the medium stimulating methane generation – R2A + betaine was dominated by the class *Bacilli* – the genus *Ureibacillus;* by 16S rRNA gene-specific primers Arch349F-A934b the genus *Bacillus* was present (consortium EEUTARGCON5, Fig. 4a, b). The Ni-enzyme urease containing genus *Ureibacillus* accounted for 85.41% of total reads (**Appendix A, MMC S2**).

The detailed list of all taxa identified in indigenous communities from the enrichment experiments of graptolite argillite in various growth media in both serum bottle and OxiTop experiments with primer pair BSF8-BSR357 are presented in **Appendix A, MMC S2**.

The genus *Methanosarcina* belonging to domain *Archaea*, accounted for 10.46% of total taxa of EEUTARGCON5 (Fig. 4b, **Appendix A. MMC S3**). These archaeans contain Co- and Ni-enzymes and are capable for methane generation by three different metabolic pathways — hydrogenotrophic, aceticlastic and methylotrophic ones (Glass and Orphan, 2012; Vinson et al., 2017). The overall diversity of microorganisms of this microbial community was higher than that of with base medium R2A. Taxa identified from this community can be functionally divided into organic matter degraders (genus *Bacillus*), sulfur metabolism-related taxa (class *Desulfotomaculia*), and methanogenesis-related archaeans (genus *Methanosarcina*). By contrast, the cultivation media R2A and R2A + methanol, were dominated by the representatives of class *Desulfotomaculia* (formerly *Clostridia* Parks et al., 2018; Chakraborty et al., 2022), mainly genus *Desulfotomaculum* related to sulfur metabolism, which accounted correspondingly for 49 and 75% of all taxa assigned (Fig. 4a). Similar response of dormant microbial communities to organic substrate amendments has been observed in marine sediments (Chakraborty

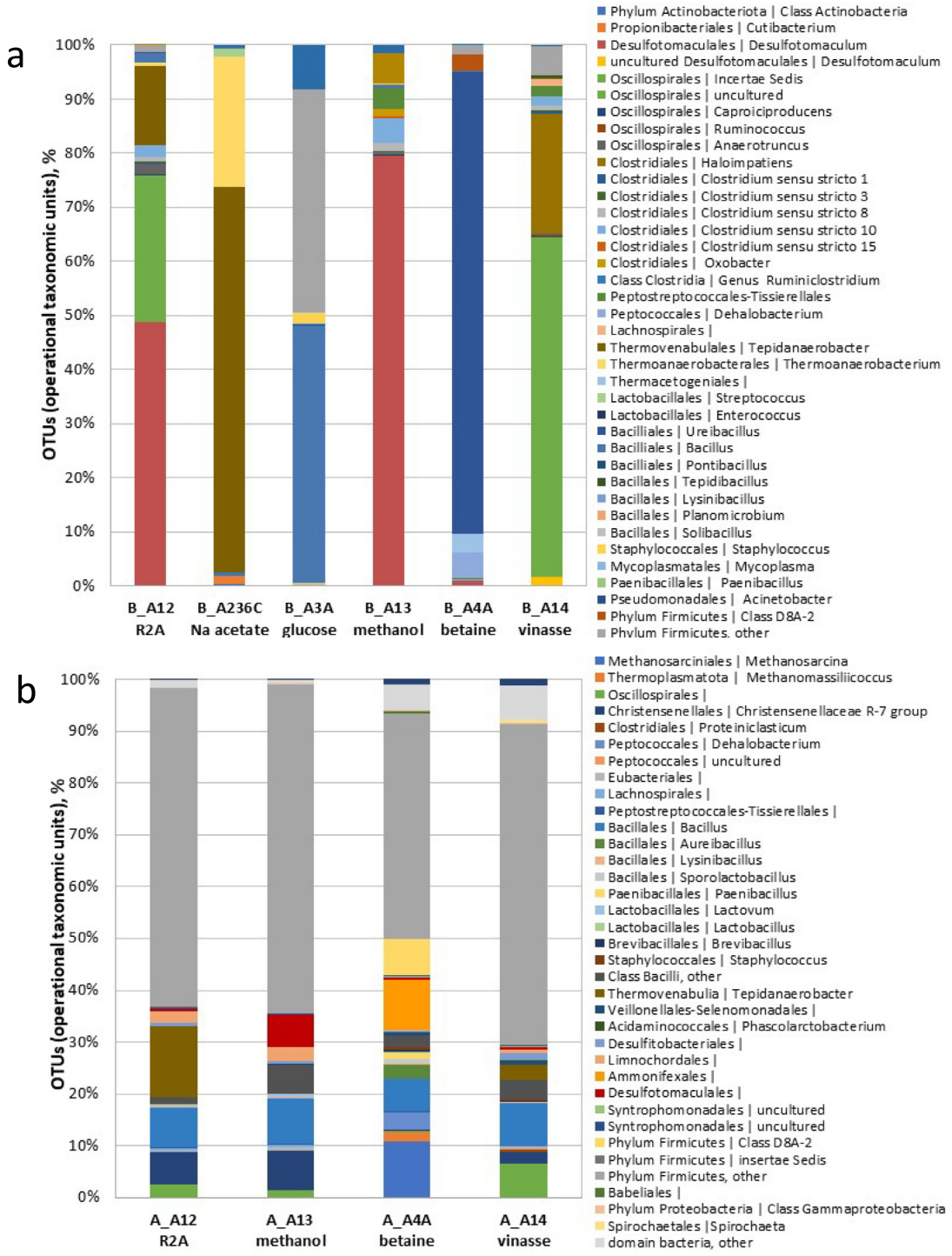


Fig. 4. The main taxa identified in the indigenous communities from the enrichment experiments of graptolite argillite with OxiTop system in various growth media. a. — with primer pair BSF8-BSR357, suitable for the bacterial 16S ribosomal RNA V2 gene region (McKenna et al., 2008; Fabrice and Didier, 2009) (Table 2, **Appendix A. MMC S2**); b. — with archaeal 16S ribosomal RNA V3-V5 genes region using primers Arch349F-A934b (Grosskopf et al., 1998; Takai and Horikoshi, 2000); GTDB taxonomy (Parks et al., 2018) (Table 2, **Appendix A. MMC S3**).

Table 2
NCBI SRA Accession numbers for the indigenous communities from the enrichment experiments of graptolite argillite.

Medium	Sample code	Primers	NCBI SRA Accession number	Sample code	Primers	NCBI SRA Accession number
R2A	B_A12	BSF8-BSR357	SRR23179999	A_A12	Arch349F-A934b	SRR23498591
R2A + Na – acetate	B_A236C	BSF8-BSR357	SRR23179998	n.d.	n.d.	n.d.
R2A + glucose	B_A3A	BSF8-BSR357	SRR23179994	n.d.	n.d.	n.d.
R2A + methanol	B_A13	BSF8-BSR357	SRR23179993	A_A13	Arch349F-A934b	SRR23498590
R2A + betaine	B_A4A	BSF8-BSR357	SRR23179992	A_A4A	Arch349F-A934b	SRR23498589
R2A + vinasse	B_A14	BSF8-BSR357	SRR23179991	A_A14	Arch349F-A934b	SRR23498588

et al., 2022). Strict anaerobes from the class *Clostridia* (order *Clostridiales*) dominated in serum bottle experiments with medium R2A + ethanol (64 and 83%). In both, serum bottle and OxiTop experiments with medium R2A + vinasse, the most abundant was also class *Clostridia* but order *Oscillospirales* (65 and 79%). A higher proportion of facultative anaerobes from class *Bacilli* and lower proportion of strict anaerobes from class *Clostridia* (order *Lachnospirales)* was observed in serum bottle experiments with media R2A, R2A + methanol and R2A + reducing agents. The list of all taxa identified in indigenous communities from the enrichment experiments of graptolite argillite in both serum bottle and OxiTop experiments with primer pair Arch349F-A934b is presented in **Appendix A, MMC S3**. In line with methanogens also bacterial taxa, e.g., genus *Tepidanaerobacter*, genus *Bacillus,* order *Desulfotomaculales*, order *Ammonifexales* were identified (Fig. 4b). As primer pair Arch349F-A934b has been designed for detection of archaeans, incl. methanogenic archaeans, only 29%–47% of bacterial taxa could be identified on order/ genus level. In Oxitop experiments, methanogenic archaeans were detected neither with other supplements than betaine, nor they were detected in serum bottle experiments.

3.4. Changes in community structure during cultivation of methane generating consortium on graptolite argillite

3.4.1. Changes in the concentrations of gaseous metabolites

It is probable that during the first weeks with modest biogas generation, only the first two phases of anaerobic decomposition of organic matter — hydrolysis and fermentation take place, and the pressurizing gases produced are CO_2, H_2, NH_3 and water vapor (Angelidaki et al., 2011). This assumption was tested by gas chromatographic analysis (Fig. 5a). Using freshly ground argillite and growth medium R2A plus supplements, a new experiment was launched with the inoculum EEUTARGCON5 in a 250 mL OxiTop® system with 150 ml culture medium at a temperature of 37 °C and at pH 7.5. Using the volume fractions of gas components, the cumulative amount of methane and other gas components (μmol) in biogas was found by Eq. (8) and the amount of methane evolved was calculated and expressed as per mass argillite (Eq. (9)).

Indeed, considerable amounts of methane were produced only since day fifteen, indicating the start of later phases of anaerobic decomposition — acetogenesis and methanogenesis. Considerable amounts of N_2 indicate on the possibility of heterotrophic denitrification (Dominika et al., 2021) and minor amounts of O_2 on the possible decomposition of reactive oxygen species (ROS) (Sabumon, 2007).

3.4.2. Changes in the microbial community in cultivation experiments

In addition to gas composition measurements, we sampled three time points for microbial community determination. The data on microbial community composition correspond to the same cultivation experiment as in Fig. 5a. Depending on the time of the sampling, on day 0, 16 or 44, the composition of consortium was very different. Analogously to the enriched cultures (Fig. 4a, b), taxa, identified from the communities can be functionally divided into organic matter degraders (genus *Bacillus*), sulfur metabolism-related taxa (genus *Desulfotomaculum*), and methanogenesis-related archaeans (genus *Methanosarcina*) (Fig. 5b, **Appendix A, MMC S4**). The genus *Methanosarcina* was detectable in all three sampling points, being most numerous in the middle sampling point, in accordance with methane generation. Strict anaerobes dominated in all sampling points, order *Clostridiales* in the inoculum and in the middle sampling point and order *Thermoanaerobacteriales* in the end sampling point. Diversity was the highest in the middle sampling point (**Appendix A, MMC S4**).

3.5. Determination of the origin of the methane by isotopic analysis

The origin of methane was tested by $\delta^{13}C$ isotopic analysis in a separate experiment using a sample from the first enrichment experiment with betaine on day 129 (8.3% GA suspension) with two different betaine concentrations (1.35 g/l and 0.675 g/l) and with and without argillite. The average values for $\delta^{13}C$ (‰V-PDB) for methane from the samples containing graptolite argillite and medium and from the samples without argillite, containing only medium (blank samples) were -51.99 ± 4.60 ‰ and -72.86 ± 5.35 ‰, respectively (Table 3). The typical $\delta^{13}C$ value for methane originating from kerogen matter generated by aceticlastic pathway is known to be -50‰ (Whiticar, 1999; Ashley et al., 2021). In the samples with adapted consortium solely in the medium (R2A plus betaine, 1.35 g/L or 0.675 g/L) the $\delta^{13}C$ (V-PDB) values are much more negative ($\delta^{13}C$ (V-PDB) $= -72.86 \pm 5.35$‰), indicating methane originating from

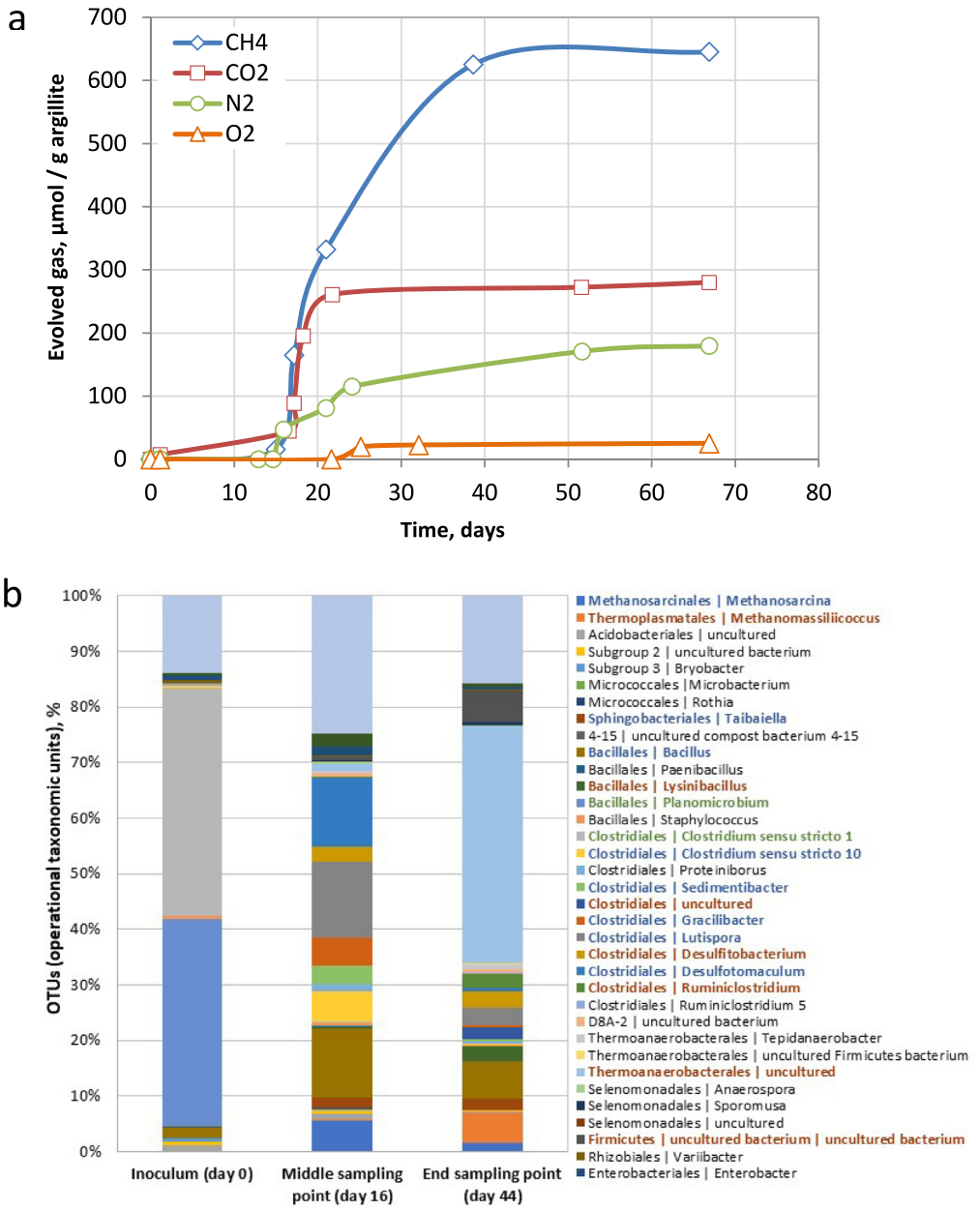


Fig. 5. Changes in community structure during cultivation of methane generating consortium EEUTARGCON5 on graptolite argillite (1% suspension) with OxiTop system and medium R2A + $NaHCO_3$ + betaine + Na_2S + cysteine. a. — changes in the concentrations of gaseous metabolites; b. — taxa identified in various growth phases with primer pair 515F-926R targeting 16S ribosomal RNA v4-v5 genes regions (Parada et al., 2016). Green — the main taxa in the inoculum (day 0), NCBI SRA Accession number SRR23193069; blue — the main taxa in the middle sampling point (day 16), NCBI SRA Accession number SRR23193068; red — the main taxa in the end sampling point (day 44), NCBI SRA Accession number SRR23193067; NCBI taxonomy (Parks et al., 2018) (**Appendix A. MMC S4**). . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrogenotrophic methanogenesis. Thus, betaine was not used for methane generation directly in either case, but methane originated more likely from the organic part of graptolite argillite. There is also a noticeable positive trend between methane isotopic composition and the proportion of methane in gas phase. The $\delta^{13}C$ (V-PDB) in the solid samples of GA and growth medium were also measured (-31.87 ± 0.31 and -28.87 ± 0.05, respectively) but showed no significant difference ($p = 0.64$). On the other hand, the average values of $\delta^{13}C$ (V-PDB) in methane gas from the samples with GA and medium and from the samples without GA had a significant difference ($p = 0.0001$) (see Table 3).

Table 3
Determination of the origin of the methane by isotopic analysis ($\delta^{13}C$ method).

No.	Sample	Content of growth medium	$\delta^{13}C$ (VPDB), ‰	CH_4 in gas phase, %
1	Adapted consortium	argillite, R2A (3.0 g/L) plus betaine (1.35 g/L)	−49.40 ± 2.43	40.70 ± 4.62
		argillite, R2A (1.5 g/L) plus betaine (0.675 g/L)	−53.93 ± 5.17	15.78 ± 5.10
		Average	−51.99 ± 4.60	26.45 ± 14.04
2	Adapted consortium	R2A (3.0 g/L) plus betaine (1.35 g/L)	−71.47 ± 3.30	13.16 ± 9.04
		R2A (1.5 g/L) plus betaine (0.675 g/L)	−74.95 ± 8.84	5.73 ± 1.34
		Average	−72.86 ± 5.35	10.19 ± 7.61

3.6. Leaching of metals from graptolite argillite

Leakages are a major problem with black shales deposits and mines (Parviainen and Loukola-Ruskeeniemi, 2019). The analysis of the supernatant from the enrichment experiment samples showed solubilization of several metal ions (Ni, Co). With the growth medium R2A (3.0 g/L) plus betaine (1.35 g/L) without any microbial inoculum added, 23.1% cobalt and 14.4% nickel of the maximum concentration of these metals in argillite was leached into the growth medium under anaerobic conditions in argon atmosphere (Fig. 6a). Both elements are necessary as cofactors of bacterial and archaeal enzymes (Glass and Orphan, 2012). Betaine is known to promote the synthesis of vitamin B12 (cobalamin), which is required for the growth of microorganisms. The structures of cobalamin (the central atom is cobalt) and porphyrin in argillite (central atoms are different metals) are similar. It is possible that microorganisms naturally present in argillite can take the microelements (Ni, Co) required for the synthesis of vitamin B12 from argillite and thereby cleave the porphyrin structure to some extent (Wlodarczyk et al., 2018; Stasiuk et al., 2021). As the pH was in the neutral region where most of the metal sulfides of interest (Zn, Cu, Mo, Ni, Co, Fe) are insoluble, they were precipitated. Metal extraction efficiency/release was affected by the addition of betaine to the growth medium. Leaching of Co was smaller in the case of both betaine (promoter of methanogenesis in the present study) and methanol (known methanogenic substrate) but was not at all affected in the case of R2A and glucose (Fig. 6a). In cultivation experiments with graptolite argillite and EEUTARGCON5, the concentrations of Co and Ni, however, decreased during the experiment (Fig. 6b), as opposed to the increase of their concentrations in the experiment without the inoculum added (Fig. 6c). Addition of microbes also affects metal leaching when comparing different time points with and without inoculum for Mo and U, as well as for Co, Ni and As (day 13). It is possible that Ni and Co were precipitated as some metal sulfides under neutral conditions of the experiment, as sulfidogenic species (genus *Desulfotomaculum*) were present.

In the current study, the effect of betaine in this decomposition process is noticeable only starting from a certain concentration (1.35 g/L). As methane-producing microorganisms prefer a neutral or slightly alkaline growth medium (pH 6.8–7.5), the success of methane production lies in maintaining a neutral pH for as long as possible. Betaine allows the pH to be maintained by occasionally releasing some NH_4^+ ions, thereby buffering the environment. NH_4^+ is also a source of nitrogen for microorganisms and the products of anaerobic digestion of betaine – acetate and methylamines – are substrates for methanogenesis, which accelerate methanogenesis in argillite giving methanogens a growth advantage over sulfate-reducing bacteria (Eqs. (2), (3), (10), (11)) (Moune et al., 1999; Kotelnikova, 2002; Tschobanoglous et al., 2003).

$$2.5\,\text{betaine} + 4.04\,H_2 \rightarrow \text{2-propanol} + 2.5(CH_3)_3N + 0.95\,CH_3COOH + 0.1\,CO_2 + 1.9\,H_2 \quad (10)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (11)$$

This indicates that the role of betaine might be propagation of methanogenesis through providing substrate for methylotrophic organisms and facilitating the synthesis of enzyme cofactors (B12) (Asakawa et al., 1998; Ticak et al., 2015). Ni is a central atom in coenzyme F430 (Holliday et al., 2007). Cobalt and nickel needed for the synthesis of the cofactors and coenzymes could be taken from argillite after the porphyrin structure has been cleaved (Stasiuk and Matlakowska, 2021b). The average content of cobalt in Estonian argillite is 15 mg/kg (Petesell, 2014) and the content of nickel varies between 85–185 mg/kg (Petersell, 1997). Methanogenesis is one of the most metal-rich enzymatic pathways in biology (Gartner et al., 1993; Thauer, 1998; Zerkle et al., 2005; Hagemeier et al., 2006; Glass and Orphan, 2012).

Recent studies by Stasiuk et al. (2021) have shown that bacteria degrade or cleave aliphatic and aromatic substituents of nickel and vanadyl geoporphyrins, also the tetrapyrrole ring. Shale rock bacteria may use geoporphyrins as a carbon or nitrogen source even though simpler compounds are present. The authors also propose that bacteria originating from black shale may use other sources of heme. Based on metagenomic and metaproteomic analyses the authors further suggest the possibility of utilizing fossil primary or modified sedimentary porphyrins, as well as their bacterial degradation products, as precursors of tetrapyrrole cofactors.

As the analysis of the communities in anaerobic culture of microorganisms on graptolite argillite showed a large proportion of taxa involved in sulfur metabolism, we hypothesized that the removal and recovery of metals from the growth medium can proceed by sulfidogenesis, i.e., by oxidation of simple organic compounds or H_2 under anaerobic conditions where sulfur compounds are converted to H_2S or hydrogen sulfide and form sulfides with metals released (Florentino et al., 2016). The presence of sulfate reducing bacteria in black shale Kupferschiefer has been documented (Wlodarczyk et al.,

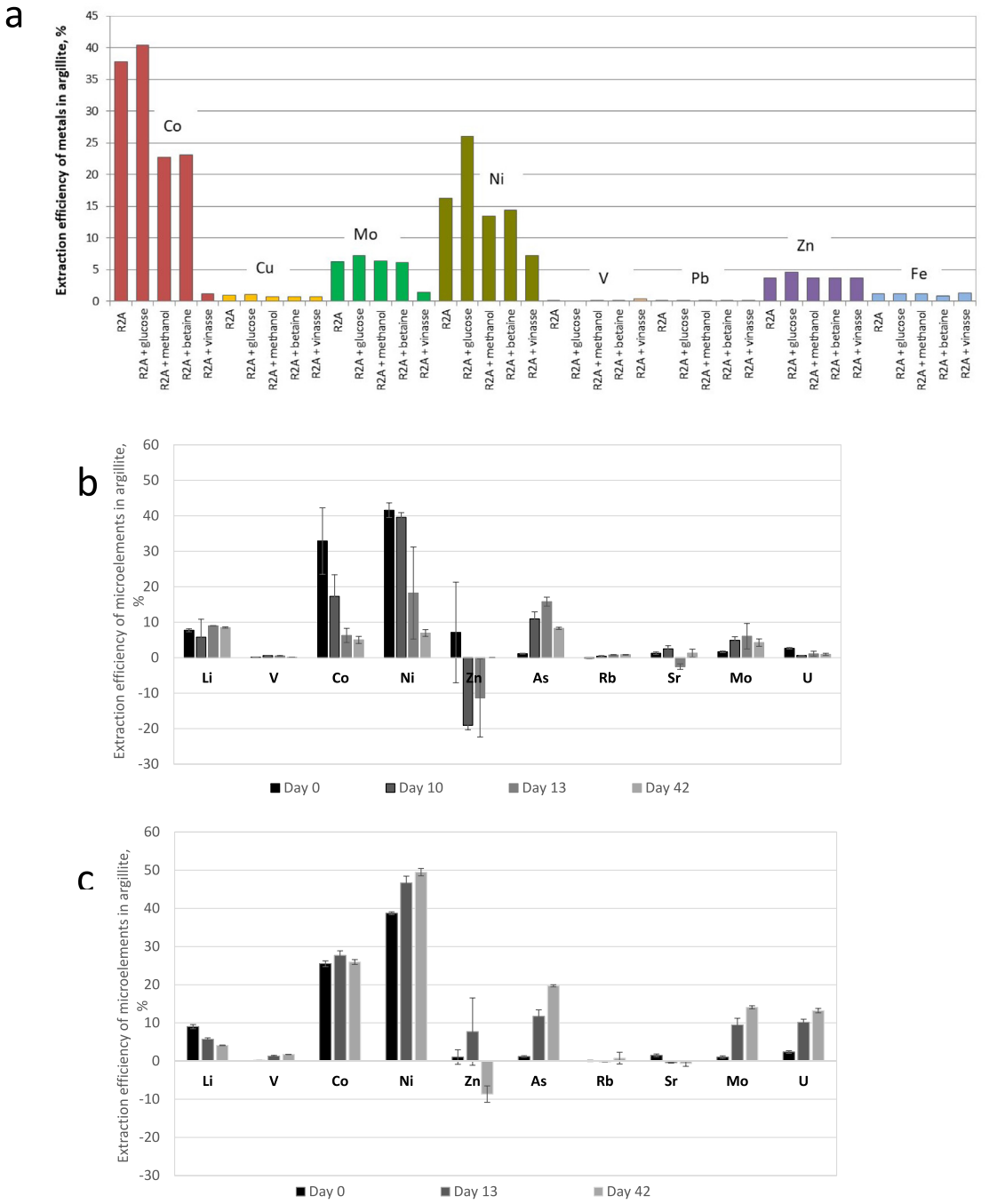


Fig. 6. Dissolution of metals from graptolite argillite. *Y*-axis represents the cumulative extraction efficiency of metals (%) relative to the metal concentration in argillite. a. — native GA (8.3%) with various growth media (without microbial inoculum addition); b. — sterilized GA (1%) + culture medium with R2A + inoculum EEUTARGCON5, c. — sterilized GA (1%) + culture medium with R2A (**Appendix A. MMC S5**).

2018). Metals can be dissolved from sulfides afterwards by acidophilic microorganisms, e.g., *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*.

In argillite, the organic matter for methane production is in a finely dispersed state, unevenly distributed and bound to the trace elements by organometallic compounds. Therefore, metals (Mo, Ni, Re, U, V) forming porphyrin rings, or in silica minerals or primary sulfides are not easy to leach (Breit and Wanty, 1991; Voolma et al., 2013). Recent studies have shown that the situation might be even more complex, e.g. (Lu et al., 2021) have demonstrated for V, that authigenic V in ancient black shales is hosted mainly by clay-organic nanocomposites.

4. Conclusions

For enrichment of a microbial consortium decomposing organometallic complexes in graptolite argillite, a liquid cultivation medium suitable to use is R2A, supplemented with betaine, and cultivation in anaerobic batch reactor (under argon atmosphere) at a temperature of 37 °C. The pH of culture medium 7.0–7.5 should be maintained until the end of cultivation. Detection of the methanogenesis marker gene *mcrA* encoding the subunit A of methyl coenzyme M reductase (MCR) in the studied microbial consortium indicated the presence of methanogenic archaeans. Media R2A and R2A + reducing agents (Na_2S and cysteine) had also a positive result in PCR-analysis with a primer pair specific for methanogens, MLf-MLr.

The results of the DNA sequencing analysis were confirmed by data from gas chromatography. The release of methane was significantly above the atmospheric baseline with media components that gave a positive result with specific primers to acetogens and/ or methanogens. As argillite organic material is a recalcitrant carbon source, its decomposition takes longer than, for example, biomethane production from traditional substrates (biomass). Cultivation of the adapted consortium EEUTARGCON5 in the presence of 1% graptolite argillite suspension in R2A plus $NaHCO_3$ plus betaine plus Na_2S plus cysteine medium enabled to generate 14.5 L of methane per kg argillite (645 μmol/g) in 38 days.

Despite modest metal leaching, the joint action of methanogens and sulfidogens contributes to precipitation of metals decreasing their concentration and adverse effect to microorganisms in the liquid phase, thereby facilitating the growth of all microorganisms, incl. the methanogens as pointed out earlier by (Paulo et al., 2017). Thus, using betaine as a substrate for methanogenic archaeans allows them to thrive within sulfate reduction zone. The solid residue from this anaerobic process can be used as starting material for traditional, acidophilic bioleaching, e.g., with *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*.

As it is not yet clear into which part of mineral (organic or silica) the metals in graptolite argillite are bound, the decomposition of organic material was obviously not sufficient to facilitate leaching of all the metals of interest. Furthermore, for most of the metal ions the pH was out of the range of solubility. However, for the first time, the two goals for using black shale (graptolite argillite) as a raw material were combined — first as a source for methane generation and second as an ore for several metals. Further optimization of post treatment of the residue of this process is needed.

CRediT authorship contribution statement

Anne Menert: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Triin Korb:** Investigation. **Kaja Orupõld:** Investigation. **Alar Teemusk:** Investigation. **Holar Sepp:** Investigation. **Ülo Mander:** Resources, Funding acquisition, Writing – review & editing. **Tanel Ilmjärv:** Formal analysis, Data curation. **Jaak Truu:** Formal analysis, Data curation, Funding acquisition, Writing – review & editing. **Päärn Paiste:** Investigation. **Kalle Kirsimäe:** Investigation, Resources, Writing – review & editing, Funding acquisition. **Terje Menert:** Methodology. **Inna Kamenev:** Methodology. **Eeva Heinaru:** Methodology. **Ain Heinaru:** Conceptualization. **Sirli Sipp Kulli:** Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition. **Maia Kivisaar:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared my data as attached files

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2023.103139.

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