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Microwave radiation and reactor design influence microbial communities during methane fermentation

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Abstract The effect of reactor design and method of heating on the efficiency of methane fermentation and composition of microbial communities, especially methanogenic *Archaea*, were determined. The research was carried out using submerge- and trickling-bed reactors fed with wastewater and the heat supply into the reactors included a convection heating method and microwave radiation. The polymerase chain reaction-denaturing gradient gel electrophoresis and relative real-time PCR were used in order to assess the biofilm communities. The best fermentation results and the highest abundance of methanogenic *Archaea* in biomass were observed in microwave heated trickling-bed reactors. The research proved that in reactors of identical design, the application of microwaves enabled a higher fermentation efficiency to be obtained and simultaneously increased the diversity of methanogenic *Archaea* communities that favors process stability. All the identified sequences of *Archaea* belonged to *Methanosarcina* sp., suggesting that species from this genera are susceptible to non-thermal effects of microwaves. There were no effects from microwaves on the bacterial communities in both types of reactors, however, the bacterial species composition varied in the reactors of different design.

Keywords Microwave radiation · Methanogenic *Archaea* diversity · *Methanosarcina* sp.

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Introduction

Methane fermentation is a process of anaerobic stabilization of organic matter that enables management of diversified industrial wastewater, as well as energy production in the form of biogas [16]. It is believed that during fermentation, microorganisms form an ecosystem of different trophic levels in the food chain. Excess products of one trophic level disturb the balance of the entire system, resulting in an accumulation of intermediates, pH changes, and reduced efficiency of the process. In the last phase, the products of the previous phases are fermented to methane by methanogenic *Archaea* [12, 14].

Fermentation is often carried out in reactors with immobilized biomass. The bedding for a biofilm development in the reactor is natural material or plastic. Selection of the reactor is one of the factors that determines the efficiency of methane fermentation. The efficiency of fermentation is also affected by temperature; that is why a significant proportion of the produced biogas is used for reactor heating. A reduction of energy consumption can be achieved by conducting the process at a lower temperature [18] or by application of microwave radiation that permits rapid heating of the reactor volume, the elimination of pathogens, easier control, and lower operational costs [1]. It has been also shown that microwaves enhance the solubilization of substrates, improve sludge dewaterability, and disrupt the network of exopolysaccharides accelerating the fermentation process [10, 32, 36].

The use of microwave heating in a reactor with a biofilm can cause two types of effects: thermal and non-thermal. Thermal effects are associated with a process in which heat is generated due to absorption of microwave energy by water or organic complexes. Polar water molecules in an external electric field are set in such a way that their dipole moment is consistent with the direction of the field. When the electric field is periodically changed, the molecules try to keep up with the changes and collide with neighboring molecules. During the collision, a portion of the electromagnetic energy generates heat, which increases the temperature of the heated substance [23]. Non-thermal effects are unrelated with increased temperature and can affect the structure and functioning of biofilms [37]. The different biological effects of the microwaves depend on field strength, frequency, and waveform, as well as the length of exposure [4]. The non-thermal effects of microwaves can also change enzymatic activity, modify the sensitivity to antibiotics, and inhibit or stimulate protein, DNA, and RNA synthesis in the cells of microorganisms [21].

The stability of the anaerobic processes in the reactor is proportional to the diversity of microorganisms inhabiting the biomass [25]. Changes in species composition during fermentation can lead to disturbances of the process. Species diversity is assessed not only by determining the total number of species involved in the analyzed transformations but also by determining the relative abundance of microorganisms, as components of diversity.

Despite the advantages of microwave use for the anaerobic treatment of wastewater, knowledge on their influence on microorganisms involved in the fermentation process is still very limited. Therefore, in the research we investigated the impact of microwave heating on methane fermentation efficiency and microbial communities composition in biofilm in submerge-bed and trickling-bed reactors fed with identical wastewater. The polymerase chain reaction-denaturing gradient gel electrophoresis was used in order to assess the biofilm communities. Phylogenetic affiliation of *Archaea* in the experimental reactors was also investigated. In order to compare the number of methanogenic *Archaea* in the biomass, relative real-time PCR was used. To our knowledge, this is the first study showing the non-thermal effects of microwaves on the methanogenic *Archaea* diversity in biofilm.

Materials and methods

Reactor setup and organization

The experiment was carried out in eight laboratory-scale submerge- and trickling-bed reactors with a diameter of 3 cm and a working volume of 0.5 l. The bedding of the reactors was polyethylene granulate transparent to microwaves (bedding porosity of $\varepsilon = 0.38$). The radiation absorption proceeded only as a result of biomass interaction with radiation.

The fermentation was carried out at 35 ± 1.5 °C. Two reactors of each type were placed in a chamber, in which reactors were heated by hot air and the required temperature was achieved with a set of heaters (controls). The other set of reactors was heated by microwave radiation (Fig. 1). Microwave chamber and the control chamber were thermostated at 20 ± 1.5 °C. The source of microwave radiation was magnetrons. There was a possibility of smooth adjustment of radiation power in order to obtain the desired temperature level. The magnetrons emitted the radiation in a continuous manner. The microwave radiation was supplied to the trickling-bed reactors in an amount of approximately 5 W/l, while in submerge-bed reactors an amount of approximately 12.8 W/l ensured a constant temperature inside the reactor. The microwave frequency was 2.45 GHz, so the microwave length was $\lambda = 12.24$ cm. At given reactor geometry, it can be assumed that the penetration depth of microwave irradiation used in experiment was unlimited.

In the trickling-bed reactors, wastewater from retention tanks located below the reactor was pumped to the surface of the bed (down-flow). In the submerge-bed reactors, wastewater from the reservoir was pumped from the bottom of the bed and returned to the reservoir (up flow) (Fig. 1). The hydraulic retention time (HRT) was 9 h. Once in 24 h the fermented substrate was carried away and physico-chemical analyses were then performed. The coefficient of biomass production was assessed on the basis of weight analysis of biofilm in the reactors at the beginning and at the end of the experimental series and the load of suspended solids (SS) in the effluent. The summary weight of the increased biofilm was referred to the removed load of COD. Analytical measurements were performed according to APHA [3] and pH was measured in filtered samples. The amount of biogas was analyzed in continuous mode using the mass flow analyzer (Aalborg, New York, USA), biogas composition was determined with an LMSxi/G4.18 analyzer (Gas Data Ltd, Whitley, UK).

In the study, a synthetic wastewater with composition given in Table 1 was used. Influent was made on the basis of water deoxygenated with nitrogen. Anaerobic conditions in the reactors were provided by their caulk and passing with nitrogen gas. The organic loading rate of both types of reactors was set at 2 g COD l/day. This meant that COD load introduced each day into the reservoir averaged approximately 1,000 mg COD/day. As an inoculum for the reactors, a digester sludge from a municipal wastewater treatment plant in Olsztyn (Poland) was used. The sludge with a concentration of 14.6 g TSS/l was pumped into the

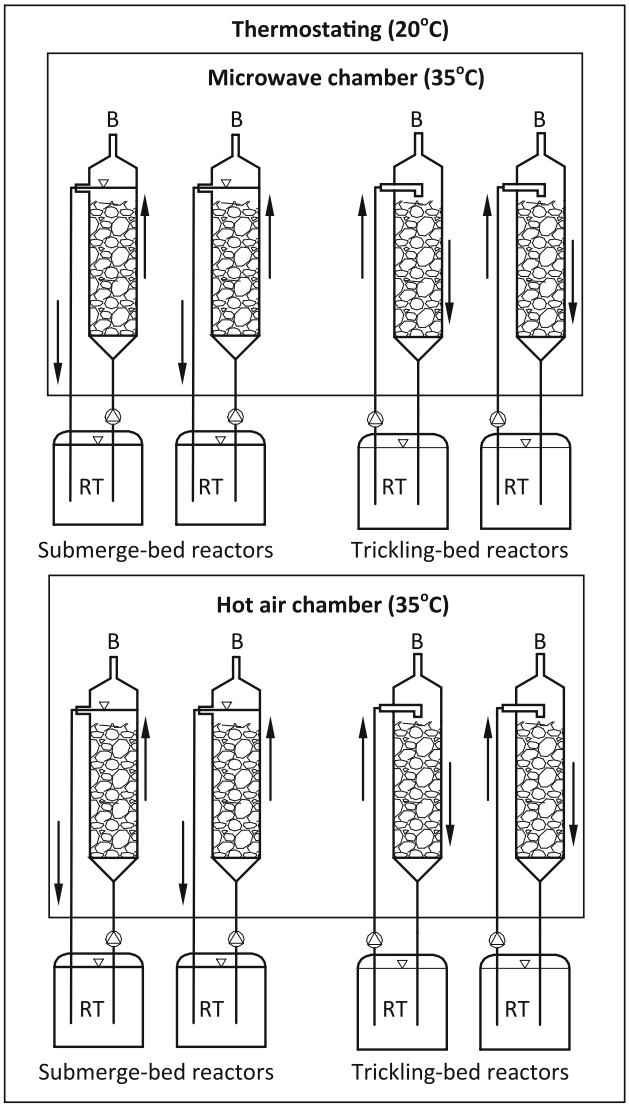

Fig. 1 The experimental stand; *RT* retention tank, *B* biogas

reactor for a period of 5 days with a flow rate of 416 ml/h; then the reactors were emptied and began operation. After a 30-day period of adaptation, the experiment was carried out for 28 days, and was completed with an intake of the probes of biomass for molecular analyses. Biomass samples were scratched from the bed fulfilling the reactors.

Biomass from the two repetitions of the each technological variant was subjected to molecular analysis. In studies, the following descriptions of samples of biomass were adopt:

MT—biofilm from trickling-bed reactor heated by microwaves,

MS—biofilm from submerge-bed reactor heated by microwaves,

CT—biofilm from trickling-bed reactor heated by convection (control),

Table 1 Composition of synthetic wastewaters [7]

Component	Concentration (mg/l)
CH_3COONa	160.0
NH_4Cl	76.1
$Na_2HPO_4 \cdot 12H_2O$	46.2
NaCl	10.1
KCl	4.7
$CaCl_2 \cdot 2H_2O$	4.7
$MgSO_4 \cdot 7H_2O$	16.7
$NaHCO_3$	243.3
Na_2CO_3	162.2
($FeCl_3 \cdot 6H_2O$, $ZnSO_4$, $MnSO_4 \cdot H_2O$, $CuSO_4$)	<0.2

CS—biofilm from submerge-bed reactor heated by convection (control).

PCR-DGGE

DNA was extracted from approximately 400 mg of centrifuged sample using a FastDNA® SPIN for Soil Kit (Q-BIOgene, Montreal, Canada). Concentration of the DNA was measured spectrophotometrically using a BioPhotometer (Eppendorf, Hamburg, Germany).

PCRs were performed in an Eppendorf Mastercycler Gradient (Eppendorf) using two primer sets: 341F/515R targeting V3 region within the bacterial 16S rDNA and 0691r/0357f targeting 16S rDNA of methanogenic *Archaea*. Information about primers and annealing temperatures are shown in Table 2. The PCR mixture contained 1.7 ng/µl of extracted DNA, 0.5 µM of each primer, 100 µM of deoxynucleoside triphosphate mixture (Promega, Madison, USA), 1.5 U of GoTaq DNA Polymerase (Promega), 6 µl of 10× reaction buffer supplied with polymerase, 1.5 mM $MgCl_2$ and sterile water to a final volume of 30 µl. The PCR amplifications were carried out using the following program: 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing for 45 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. The presence of PCR products was confirmed by analyzing 5 µl of the product on a 0.8 % agarose gel stained with ethidium bromide. DGGE procedure was performed and the gels were documented as described elsewhere [8].

Representatives of 16S rDNA amplicons of methanogenic *Archaea* that were clear and had a high intensity were excised from the DGGE gel, reamplified and sequenced in the Institute of Biochemistry and Biophysic, Polish Academy of Science (http://www.oligo.ibb.waw.pl). The determined nucleotide sequences were compared with sequences in the GenBank using the BLASTN program [2] and deposited in the GenBank under accession no.

Table 2 Primers used in the experiment

Primer set	Target organism(s)	Annealing temp (°C)	Amplicon/PCR product length (bp)	Reference
341F/515R	*Bacteria*	63 °C	16S rDNA/~230	[19]
0691r/0357f	Methanogenic *Archaea*	52 °C	16S rDNA/~300	[33]

JN828692-JN828703. The sequences determined in this study were aligned and genetic relationships were determined (the Maximum Likelihood method) using MEGA5 software [30].

Relative real-time PCR

To compare methanogenic *Archaea* abundance in the biomass, relative real-time PCR was performed. A reaction mixture contained 3 ng/µl of the template DNA, 12.5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA), 50 nM of each primer (0691r/0357f primer set, primer 0357f without GC clamp) and water to a final volume of 20 µl. The protocol for quantification was as follows: 2 min in 50 °C 10 min in 95 °C and 40 cycles consisting of 30 s in 95 °C, 45 s in 54 °C and 45 s in 60 °C. Each sample was amplified in triplicate in the presence of negative and positive controls. The amplification was followed by a denaturation step to confirm the melting temperature of the PCR products, the products were also electrophoresed in the presence of a molecular marker. Reactions were carried out in a 7,500 Real-Time PCR System (Applied Biosystems). The fluorescence signal was normalized by dividing the SYBR dye emission by the reference dye (ROX) signal intensity. Data were analyzed with sequence-detection software, version 1.3 (Applied Biosystems). Methanogenic *Archaea* relative abundance in the reactors was compared using a modification of the $2^{-\Delta\Delta Ct}$ method [17]. Reactions were normalized by adding the same amount of DNA for each reaction tube. The abundance of 16S rDNA gene was presented as $2^{-\Delta Ct}$ where ΔC_t (C_t sample x$-C_t$ sample MS) and sample MS is a calibration sample (1× abundance of methanogenic *Archaea*).

Calculation methods

The normality of the distribution was confirmed by Shapiro–Wilk test, whereas the hypothesis of the homogeneity of variances across the groups was verified on the basis of Levene's test. The differences between the mean values derived from particular groups were examined by RIR Tukey's test or by a two-sided paired Student's *t* test. All analyses were performed at the probability level of 95 %. On the basis of the DGGE patterns, distance matrix analyses were performed according to the method of Nei Li [20], using the DGGEstat 1.0 software (Eric van Hannen, The Netherlands Institute for Ecological Research, The Netherlands). The samples were clustered using the unweighted pair group method of arithmetic averages (UPGMA), bootstrapping was conducted with 1,000 replicates. The structural diversity of the microbial community was expressed by the Shannon-Wiener index of general diversity H2′ [28, 34] and calculated on the basis of the bands on the DGGE gel tracks, using the densitometric curves.

Results and discussion

The present study was carried out using two types of fermentation reactors namely submerge- and trickling-bed reactors heated by convection (controls) or by microwaves. The efficiency of the fermentation process was expressed through the rate of chemical oxygen demand (COD) removal, biogas production, and the methane content in biogas. In both types of reactors heated by microwaves, significantly better results were achieved in comparison to the control reactors.

The removal efficiency of organic compounds was associated with the design of the reactor and the type of heating method (Table 3). The highest efficiency was observed in the trickling-bed reactors that used microwave heating (680.3 ± 10.2 mg COD/day). The amount of COD/day removed in the microwave-heated reactors was on average about 20 mg higher than that of the control reactors that comprised statistically significant difference (RIR Tukey's test, $p = 0.001$ and RIR Tukey's test, $p = 0.009$ for submerge-bed and trickling-bed reactors, respectively).

Banik et al. [4] demonstrated that exposing the inoculum to microwaves with a frequency of 13–40 GHz for 2 h resulted in a shortening of the lag-phase and an earlier start of biogas production in the reactor. The biogas formed in microwave-heated reactors was characterized by a higher methane content than the biogas from the control reactors (Table 3). Eskicioglu et al. [9] reported a higher production of biogas at temperatures of 50, 75, and 96 °C in the biomass pre-exposed to microwave radiation at a frequency of 24.5 GHz. In our study, a much lower frequency of microwaves (2.45 GHz) was used and the biomass was not exposed to microwaves in the inoculum stage but steadily throughout the study period. Under such conditions, in both types of reactors we observed beneficial effects of the microwave radiation on the efficiency of biogas production.

Table 3 Results of technological research

	Removed COD (mg COD/day)	Biogas production (ml/day)	Methane conc. (%)	Biomass production (mg/mg COD)	COD in the effluent (mg COD/l)	TSS in the effluent (mg/l)
CT reactor	660.60 ± 12.20[a]	207.90 ± 3.27[a]	69	0.12	339.4 ± 13.1	25.4 ± 6.7
MT reactor	680.30 ± 10.20	219.70 ± 3.39	71	0.09	319.7 ± 9.8	19.2 ± 3.7
MS reactor	630.60 ± 7.20[a]	176.60 ± 2.05[a]	66	0.12	369.4 ± 6.4	29.7 ± 7.9
CS reactor	610.30 ± 17.80	164.80 ± 4.88	65	0.13	389.7 ± 15.7	33.3 ± 5.6

TSS total suspended solids, after ± standard deviation is given

[a] The differences between microwave and convection heated reactors of identical design were statistically significant (RIR Tukey significance test, $p < 0.05$)

Application of microwave radiation resulted in a statistically higher yield of biogas in microwave-heated reactors in comparison to the controls (Table 3). In trickling-bed reactors, it was 219.70 ± 3.39 ml/day in the microwave-heated system versus 207.90 ± 3.27 ml/day in the convection-heated system (RIR Tukey's test, $p = 0.009$) while in the submerge-bed reactors 176.60 ± 2.05 ml/day versus 164.80 ± 4.88 ml/day (RIR Tukey's test, $p = 0.002$), respectively. Biogas production per gram of removed load of organic compounds was similar in the reactors of identical design. In the submerge-bed reactors heated by microwaves, it was 0.28 ml/mg COD as compared to 0.27 ml/mg COD in the control reactors, while in the trickling-bed reactors heated by microwaves, it was 0.32 ml/mg COD as compared to 0.31 ml/mg COD in the control reactors. For each type of reactor construction, the reactors heated by microwaves were characterized by a lower biomass production per removed load of organic compounds—the lowest value (0.09 mg/mg COD) was noted in the trickling-bed reactor. It was 25 % lower than in the convection-heated control reactor.

Fernàndez et al. [11], in studies on microorganisms involved in methane fermentation, indicated that an abundance and species diversity had a positive effect on the development of a dynamic biotic community inhabiting a reactor. The high species diversity of microorganisms in the system ensures that the biocenosis consists of different species conducting the same metabolic function. So after the environmental disturbance, which results in the elimination of some species, others are able to sustain a particular type of metabolic route. It was observed that the diversity of microorganisms during fermentation depends on the temperature of the process [27] and the composition of the wastewater [15].

All reactors used in our study were inoculated with the same digester sludge and then differently operated. The materials for the molecular analyses were biofilm samples collected from the reactors at the end of technical part of the experiment. DGGE patterns obtained for the bacterial communities are included as Online Resource 1, while the patterns representing methanogenic *Archaea* communities

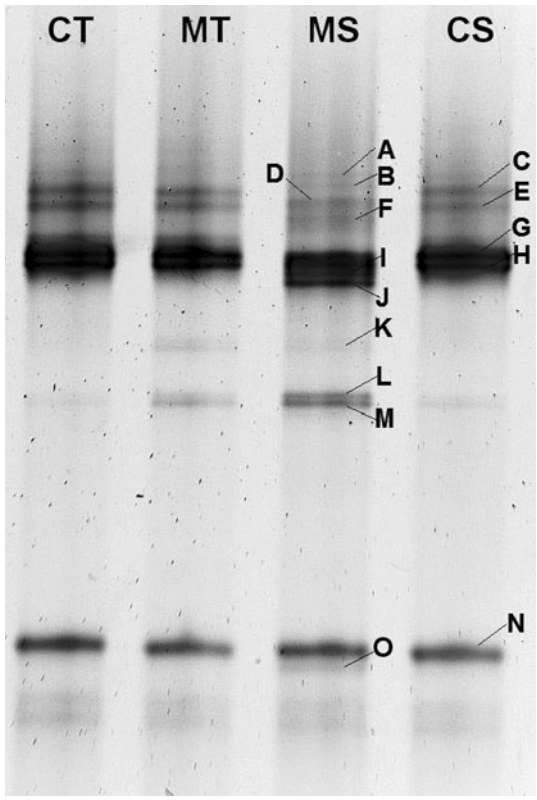

Fig. 2 DGGE gel analysis of PCR amplifications of partial 16S rDNA gene of methanogenic *Archaea*. The abbreviations above each lane represent the experimental series from which the biofilm samples were taken

are shown in Fig. 2. On the basis of the electrophoretic patterns, we plotted trees illustrating similarities between communities inhabiting all reactors (Fig. 3).

Electrophoretic patterns characterizing the bacterial communities of the submerge- and trickling-bed reactors were grouped into two distinct branches (Fig. 3a). In research on nitrifying bacteria in two full-scale treatment reactors—a biological aerated filter (BAF) and a trickling filter—receiving the same wastewater, Rowan et al. [24] reported that the type of the reactor affected the ammonia oxidizing bacteria (AOB) community structure. In our research, the fact that the reactor type influences bacterial

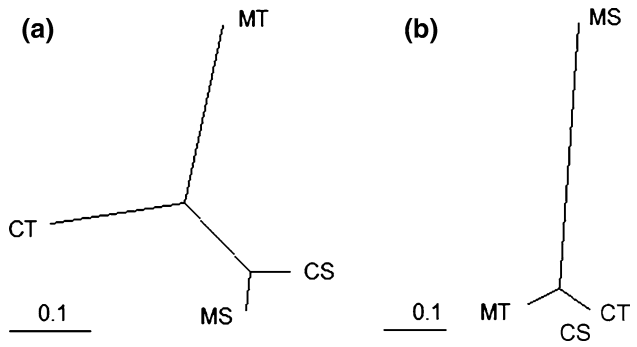

Fig. 3 Cluster analysis based on the DGGE profiles of **a** the total bacteria and **b** methanogenic *Archaea* communities in experimental series. The tree was constructed using the UPGMA method. For statistical support of the tree topology, 1,000 bootstrap replicates were simultaneously estimated. The abbreviations next to each branch represent the experimental series from which the activated sludge samples were taken

community was confirmed also for anaerobic systems. For feed of identical composition, the design of reactor, not heating method, determined the species composition of the bacteria in the biomass. The differences in species composition of the bacteria may have resulted from the fact that in both types of reactors microorganisms with distinct habitat requirements developed. In the trickling-bed reactors microorganisms inhabited mainly the biofilm covering the bed of the reactor (immobilized bacteria), while in submerge reactors microorganisms could colonize not only the bed but also the volume of wastewater inside the reactor (free-living bacteria).

The cluster analysis based on DGGE separation of PCR products obtained from amplification of the 16S rDNA gene of methanogenic *Archaea* (Fig. 3b) showed that the species composition in the reactors heated by convection were very close to each other, regardless of the type of reactor. The DGGE patterns characterizing archaeal communities in the reactors heated by microwaves grouped in a separate branch; methanogenic *Archaea* from submerge-bed reactors definitely differed from the communities obtained from other samples of biofilm. The main structure of *Archaea* in biomass was similar in all reactors (Fig. 2) and the differences between convection and microwave-heated reactors resulted mainly from the appearance of new species in the microwave-heated reactors. The observed deviations may be, therefore, explained by the fact that microwave treatment promotes a specific community of *Archaea* to develop. Since the same temperature was maintained in both types of reactors, this phenomena resulted from specific mode of microwave heating or unknown non-thermal effects.

Based on the DGGE patterns that characterize communities of methanogenic *Archaea* in each reactor, it was observed that amplicons marked H, M, and N were present in biomass from all the reactors (Fig. 3). Amplicons C, E, and G occurred only in patterns that characterize samples from the convection and microwave-heated trickling-bed reactor. Amplicons K and L were unique to the reactors heated by microwaves. Analysis of the recognized sequences showed that the corresponding closest match via the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to band K was *Methanosarcina vacuolata* DSM1232T (FR733661.1, similarity 99 %) while to band L the closest match was uncultured archaeon (GU984552.1, similarity 100 %). The amplicon M occurred in all reactors, but its intensity was noticeably higher in patterns characterizing microwave-heated reactors. The DGGE is not a quantitative method. However, the band intensity of the 16S rRNA genes in a DGGE gel may correspond at least semi-quantitatively with the abundance of the corresponding species [13]. It can be concluded that methanogen from which amplicon M was amplified had more favorable conditions for development and growth in the reactors heated by microwaves. The greatest species diversity of methanogenic *Archaea* was noted in submerge-bed reactors heated with microwaves; amplicons A, B, D, F, I, J, and O were unique to these reactors.

In the phylogenetic tree, the sequenced amplicons of methanogenic *Archaea* were grouped with the sequences of microorganisms of the genus *Methanosarcina* (Fig. 4). *Methanosarcina* were identified in all reactors, regardless of their type and the method of heating applied. The methanogenic *Archaea* whose 16S rDNA sequences occurred only in the systems with microwave heating also belonged to this genus. Species of the genus *Methanosarcina* have a different sensitivity to environmental factors, depending on the presence, absence, or of chondroitin that forms the outer, thick, and rigid coating that arises only under specific growth conditions [14]. All members of the *Methanosarcinales* contain cytochromes that allow them to have a broad substrate spectrum for fermentation including acetate, H_2/CO_2, and methanol [31]. Species of this genus dominated in convection-heated reactors at 30 °C [6] thus at a temperature close to this applied in our experiment. Perhaps the ability to use various types of substrates as well as a high resistance of species of *Methanosarcina* genus to changing environmental conditions determined that all sequences identified in out experiment belonged to this type of microorganisms.

The positive effect of the non-thermal properties of microwaves on bacterial diversity was demonstrated in the aerobic biofilm reactors in which nitrification and denitrification processes were carried out [37]. In research by Pholchan et al. [22], on the other hand, of all the parameters analyzed only the reactor configurations had a consistent effect on the reactor community diversity. In our experiment, however, which

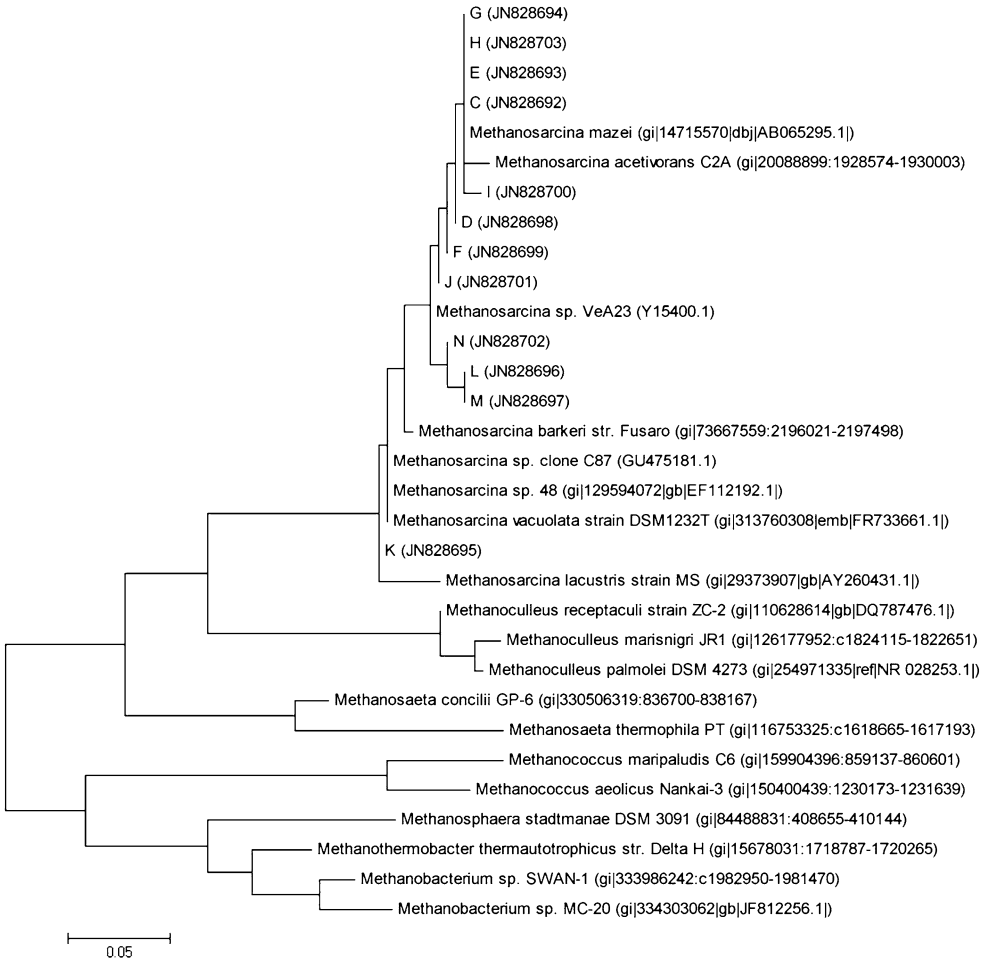

Fig. 4 Phylogenetic tree showing the relationships of partial 16S rDNA gene sequences of methanogenic *Archaea* to reference sequences from GeneBank database (accession numbers given in *parentheses*). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model [29]. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 [30]

was maintained under anaerobic conditions, there was no effect, either from the type of heating or the type of reactor, on the diversity of bacterial communities inhabiting the reactors. The values of the Shannon-Wiener index (H′) in the reactors heated by convection and those heated by microwaves were similar and averaged 2.9 (Fig. 5a). The study showed, however, that the use of microwave radiation in both types of reactors increased the diversity of methanogenic *Archaea* compared to the control (two-sided paired Student's *t* test at the probability level of 95 %, $t = -4.3$, $p = 0.04$) (Fig. 5a). In all the control reactors, *Archaea* species composition was very similar, indicating that in the reactors heated by convection, the reactor design had no effect on this community. The use of microwave heating in the case of the trickling-bed reactors increased the Shannon-Wiener index by about 11 %, while in the submerge-bed reactors the index rose by 45 % compared to the values observed in the control reactors. The significant growth in diversity of the bacterial communities in the submerge-bed reactors heated with microwaves could result from two facts. The first is that the *Archaea* in the reactor colonized not only the bed but also wastewater inside the reactor. Research by Sawayama et al. [26] showed differences in the species composition of microorganisms inhabiting the biofilm and those suspended in the wastewater in the reactor and indicated that the major immobilized methanogenic *Archaea* were *Methanosarcina* and those of the liquid phase were *Methanobacterium* spp. The second reason for the higher diversity of *Archaea* in submerge-bed reactors is that the amount of microwave radiation supplied to this reactors was higher—12.8 W/l, in comparison with 5 W/l in the trickling-bed reactors that enhanced the impact of non-thermal effects of microwaves on the analyzed community.

The greater number of microorganisms in the reactor increases the production of biogas [35]. In our study, to assess the abundance of methanogenic *Archaea* in the biofilm of the experimental reactors, real-time PCR was used. The reference sample was a sample from a submerge-bed reactor heated with microwaves (Fig. 5b).

In both types of control reactor, the number of methanogenic *Archaea* was at a similar level, indicating that the type of the reactor did not affect significantly (two-sided test of differences between two averages, $p = 0.809$) the abundance of this microorganism. In the trickling-bed reactors heated by microwaves, the amount of methanogenic *Archaea* was about 1.5-times that of the controls. The study

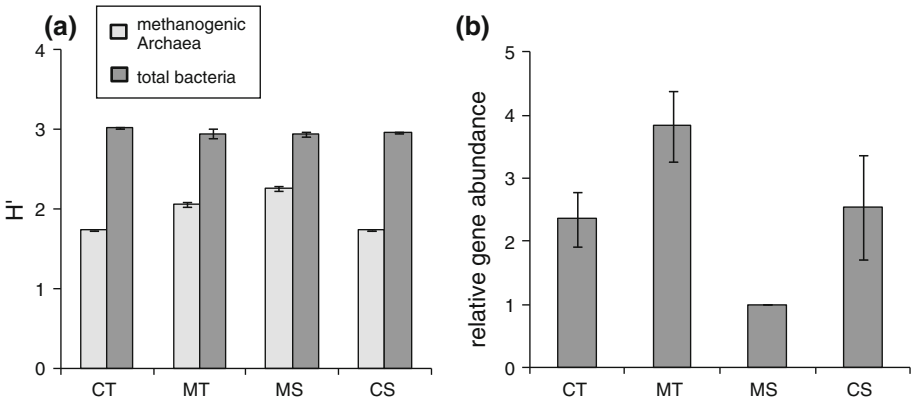

Fig. 5 Changes of diversity (H′) of **a** total bacteria and methanogenic *Archaea* and **b** the relative abundance of methanogenic *Archaea* in the experimental series. Methanogenic *Archaea* relative abundance in the reactors was compared using a modification of the $2^{-\Delta\Delta Ct}$ method [34], sample MS was used as a calibration sample

of Banik et al. [5] indicated that microwaves stimulated the growth of selected groups of microorganisms. Colonies of *Methanosarcina barkeri* subjected to microwave radiation with a frequency of 31.5 GHz showed a faster growth rate than the controls, and in the system subjected to microwaves, more *Sarcina* cells occurred than in the controls. In our study, a higher amount of methanogenic *Archaea* in the trickling-bed reactors in comparison to the submerge-bed reactors was observed. This could have resulted from a part of the microwave energy being absorbed by the wastewater in the submerge-bed reactors, while in the trickling-bed reactors, the biofilm was directly exposed to the microwave radiation—the wastewater simply streamed down the bed surface. Moreover, in the trickling-bed reactor, the microorganisms existed mainly in the immobilized form which favors the abundance of methanogenic *Archaea*. According to Sawayama et al. [26], the amount of immobilized methanogenic *Archaea* could be 1,000 times more than that in the anaerobically digested sludge from a completely mixed thermophilic digester.

In the submerge-bed reactors, the amount of methanogenic *Archaea* was two-fifths of that recorded in reactors of this type, but which have been convection-heated. However, the overall efficiency of the fermentation process was higher than in controls. The microwave-heated submerge-bed reactors had the most diverse community of methanogenic *Archaea* among all of the reactors in our experiment. It can be assumed that in the reactors that were analyzed, one or more methanogens, specific only for this reactors (seven specific amplicons occurred in the DGGE pattern), was characterized by a high efficiency of methane production. This, despite the overall number of *Archaea* being lower than in the controls, resulted in a higher efficiency of fermentation.

The study showed that using microwaves instead of convection heating to maintain the temperature in the fermentation reactors, increased the efficiency of the process, and the diversity of methanogenic *Archaea* in the system. *Methanosarcina* sp. were identified in all experimental reactors, the methanogenic *Archaea* whose 16S rDNA sequences were unique to systems with microwave heating also belonged to this genus. The best fermentation results were observed in the microwave-heated trickling-bed reactors where the abundance of methanogenic *Archaea*, supported by their high diversity, was the highest. These results indicate that both factors—the abundance and species diversity—translate to a high rate of fermentation. The study showed there were no effects from microwaves on the bacterial communities in both types of reactors, however, the bacterial species composition varied in the reactors of different design.

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References

1. Ahn J-H, Shin SG, Hwang S (2009) Effect of microwave irradiation on the disintegration and acidogenesis of municipal secondary sludge. Chem Eng J 153:145–150
2. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
3. APHA (1992) Standard methods for the examination of water and wastewater, 18th edn. APHA, AWWA and WEF, Washington, DC
4. Banik S, Bandyopadhyay S, Ganguly S (2003) Bioeffects of microwave—a brief review. Bioresour Technol 87:155–159
5. Banik S, Bandyopadhyay S, Ganguly S, Dan D (2006) Effect of microwave-irradiated *Methanosarcina barkeri* DSM-804 on biomethanation. Bioresource Technol 97:819–823
6. Chin K-J, Lukow T, Stubner S, Conrad R (1999) Structure and function of the methanogenic archaeal community in stable cellulose-degrading enrichment cultures at two different temperatures (15 and 30 °C). FEMS Microbiol Ecol 30:313–326
7. Coelho MAZ, Russo C, Araujo OQF (2000) Optimization of sequencing batch reactor for biological nitrogen removal. Water Res 34:2809–2817
8. Cydzik-Kwiatkowska A, Zielińska M, Wojnowska-Baryła I (2012) Impact of operational parameters on bacterial community

in a full-scale municipal wastewater treatment plant. Pol J Microbiol 61(1):41–49
9. Eskicioglu C, Terzian N, Kennedy KJ, Droste RL, Hamoda M (2007) Athermal microwave effects for enhancing digestibility of waste activated sludge. Water Res 41:2457–2466
10. Eskicioglu C, Kennedy KJ, Droste RL (2009) Enhanced disinfection and methane production from sewage sludge by microwave irradiation. Desalination 248:279–285
11. Fernàndez A, Huang S, Seston S, Xing J, Hickey R, Criddle C, Tiedje J (1999) How stable is stable? function versus community composition. Appl Environ Microbiol 65:3697–3704
12. Hendrickson EL, Haydock AK, Moore BC, Whitman WB, Leigh JA (2007) Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic *Archaea*. Proc Natl Acad Sci USA 104:8930–8934
13. Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241
14. Lange M, Ahring BK (2001) A comprehensive study into the molecular methodology and molecular biology of methanogenic *Archaea*. FEMS Microbiol Rev 25:553–571
15. Lee Ch, Kim J, Hwang K, O'Flaherty V, Hwang S (2009) Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. Water Res 43:157–165
16. Lettinga G (1995) Anaerobic digestion and wastewater treatment systems. Antonie Van Leeuwenhoek 67:3–28
17. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta ct}$ method. Methods 25:402–408
18. McHugh S, Collins G, O'Flaherty V (2006) Long-term, high-rate anaerobic biological treatment of whey wastewaters at psychrophilic temperatures. Bioresource Technol 97:1669–1678
19. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microb 59:695–700
20. Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273
21. Pakhomov AG, Akyel Y, Pakhomova ON, Stuck BE, Murphy MR (1998) Current state and implications of research on biological effects of millimeter waves: a review of the literature. Bioelectromagnetics 19:393–413
22. Pholchan MK, de C. Baptista J, Davenport RJ, Curtis TP (2010) Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Water Res 44:1341–1352
23. Ponne CT, Bartels P (1995) Interaction of electromagnetic energy with biological material—relation of food processing. Radiat Phys Chem 45:591–607
24. Rowan AK, Snape JR, Fearnside D, Barer MR, Curtis TP, Head IM (2003) Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol Ecol 43:195–206
25. Saikaly PE, Stroot PG, Oerther DB (2005) Use of 16S rRNA gene terminal restriction fragment analysis to assess the impact of solids retention time on the bacterial diversity of activated sludge. Appl Environ Microb 71:5814–5822
26. Sawayama S, Tsukahara K, Yagishita T (2006) Phylogenetic description of immobilized methanogenic community using real-time PCR in a fixed-bed anaerobic digester. Bioresource Technol 97:69–76
27. Sekiguchi Y, Kamagata Y, Syutsubo K, Ohashi A, Harada H, Nakamura K (1998) Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. Microbiology 144:2655–2665
28. Shannon CE, Weaver W (1949) The mathematical theory of communication. University of Illinois Press, Urbana
29. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526
30. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739
31. Thauer RK, Kaster A-K, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic *Archaea*: ecologically relevant differences in energy conservation. Nat Rev Microbiol 6:579–591
32. Toreci I, Kennedy KJ, Droste RL (2009) Evaluation of continuous mesophilic anaerobic sludge digestion after high temperature microwave pretreatment. Water Res 43:1273–1284
33. Watanabe T, Asakawa S, Nakamura A, Nagaoka K, Kimura M (2004) DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. FEMS Microbiol Lett 232:153–163
34. Wiener N (1948) Cybernetics: or control and communication in the animal machine. Wiley, New York
35. Yang Y, Tada Ch, Tsukahara K, Sawayama S (2004) Methanogenic community and performance of fixed- and fluidized-bed reactors with reticular polyurethane foam with different pore sizes. Mater Sci Eng 24:803–813
36. Yu Q, Lei H, Yu G, Feng X, Li Z, Wu Z (2009) Influence of microwave irradiation on sludge dewaterability. Chem Eng J 155:88–93
37. Zieliński M, Ciesielski S, Cydzik-Kwiatkowska A, Turek J, Dębowski M (2007) Influence of microwave radiation on bacterial community structure in biofilm. Process Biochem 42:1250–1253