**Influence of heavy metal supplementation on specific methanogenic activity and
microbial communities detected in batch anaerobic digesters**

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Abstract

Natural and modified zeolites (0.5 – 1.0 mm) from the Tasajera deposit in Cuba were used to enhance the anaerobic digestion process of synthetic substrates. Natural zeolites were modified by ionic exchange and by adsorption with nickel, cobalt and magnesium. The experiments were carried out by using an inoculum from a full-scale anaerobic reactor treating winery wastewater. Modified natural zeolites not only enhanced the anaerobic digestion process, but also increased the specific methanogenic activity (SMA) of the sludges. The textural and chemical surface characteristics of the modified zeolites were related to the process performance, volatile fatty acid (VFA) production and microbial communities found in the digesters. For the selected dose of modified zeolites [0.05 g/g of volatile suspended solids (VSS)], the lowest concentration was found for cobalt followed by nickel and magnesium. Based on the analyses of anaerobic biofilms, the heavy metal incorporated into the zeolite was shown to have a great influence on the predominance of species. For example, the presence of nickel and cobalt favoured *Methanosaeta*, while at the same dose magnesian zeolite stimulated the presence of *Methanosarcina* and sulfate-reducing bacteria (SRB). In digesters with modified zeolites and metal supplementations the values of SMA were higher than those obtained in the control and natural zeolite digesters.

Keywords: Metal supplementation; mesophilic anaerobic digestion; modified natural zeolites; microbial communities; volatile fatty acids (VFA)

Introduction

The increase in efficiency in the anaerobic digestion processes of wastewaters by using natural and modified zeolites has been pointed out by several authors.^[1,2] The most adequate doses of natural zeolite have also been studied.^[3] Doses of between 2 and 4 g/L of wastewater have been found to be the most adequate for stimulating the anaerobic process of piggery waste.^[2] These authors claim that the addition of zeolite improved the anaerobic digestion of piggery wastewater. Zeolite reduces the concentrations of ammonia and ammonium ion present in raw piggery wastewater as well as those produced during anaerobic degradation of proteins, amino acids and urea.^[1] Zeolite is an efficient ion exchanger for removing ammonium ion from anaerobic effluents and municipal wastewater. Ion exchange processes using homoionic natural zeolite have shown high ammonium removals from wastewaters with efficiency values close to 95%.^[4-6] Natural zeolite has also been used in combination with struvite crystallisation to recover 65%–80% of the nitrogen as crystallized or adsorbed ammonium.^[6] Moreover, zeolite has a great capacity for adsorbing metals, such as copper, cadmium, lead and zinc, which can be toxic to microorganisms.^[7] The decrease of ammonia nitrogen helps to reduce the concentration of ammonium because these species are in equilibrium. In addition, zeolite has a great capacity for adsorption and has been found to be useful as a microbial support both in aerobic and anaerobic processes of different wastewaters.^[2, 8-12]

On the other hand, there are several reports on the important role of the addition of micronutrients to lab and industrial anaerobic digesters, but there is a lack of information about the specific population stimulated by each micronutrient. Some studies demonstrated the important role of inorganic nutrients not only to

methanogenesis and acetogenesis, but also to hydrolysis and acidogenesis.^[13] One of these studies determined the effect of inorganic nutrient supplementation at mesophilic and thermophilic conditions. Supplemented reactors showed the highest rates of hydrolysis at both temperature conditions. The effect was evaluated in terms of soluble COD production, volatile suspended solids (VSS) removal, and organic acid production, which implies the importance of inorganic nutrients for the two steps mentioned.^[13] It is also known that the growth and activity of the methanogenic consortium in anaerobic reactors strongly depend on environmental factors such as the availability of macro- and micronutrients.^[14] Macronutrients, such as nitrogen, phosphorus, potassium and magnesium, are required for the activation or functioning of many microorganisms.^[15] Micronutrients, mainly trace metals such as nickel, cobalt, and iron, are known for their role as biochemical cofactors for methane production.^[15]

Reviews on the biochemistry of methanogenesis postulate the catalysing effect of the methyl-coenzyme M reductase on the methane-forming reaction itself, with great emphasis on the presence of a ligand nickel atom which is the striking feature of coenzyme F₄₃₀. In the case of cobalt, it has an important role in the formation of methyl-coenzyme M from N⁵-methyl-H₄MPT.^[16,17] In acetogenesis, there is evidence of the mediation of a nickel-containing center, specifically a metal complex called NiFeC species, acting in acetyl-CoA synthesis from CO or CO₂. In the second step of acetyl-CoA synthesis, cobalt is an active site of a carrinoid/iron-sulfur protein. In addition, many enzymes with such important functions as energy conservation and ATP synthesis, are dependent on metals such as Mo, W, Ni, Fe Co and Se.^[18]

Molecular biological techniques are frequently applied to detect, identify and quantify microorganisms. They use specific nucleotide sequences as probes directed to 16S rRNA or its encoding gene. Some of these techniques such as 16S rRNA dot blot

hybridization and 16S rDNA fingerprinting techniques (for example denaturing gradient gel electrophoresis - DGGE) are being increasingly used to explore the microbial diversity and structure of communities (bacteria and archaea),^[19] even in uncultured species, providing a profile of the genetic diversity.^[20]

The main aim of this work was to determine the influence of different modified zeolites and metal concentrations on methane production, specific methanogenic activities (SMA) and anaerobic microbial communities detected in the developed biofilm. Synthetic substrates containing a buffer phosphate medium and volatile fatty acids (VFA) as carbon source were used.

Material and methods

Inoculum

The anaerobic sludge used as inoculum was obtained from a 300 m³-digester which treated winery wastewater. The industrial anaerobic treatment plant was located in “La Chimba-CAPEL”, Ovalle, Chile. The total suspended solid (TSS) and volatile suspended solid (VSS) concentrations of the inoculum were 35.7 and 27.6 g/L, respectively. The initial specific methanogenic activity (SMA) of this sludge to volatile fatty acids (VFA) was 0.24 g COD-CH₄/(g VSS·d).

Natural zeolites

The natural zeolite with a particle size of about 0.5-1.0 mm was collected from the Tasajera deposit (Cuba). Its characteristics are shown in Table 1. For this study, natural zeolite was modified by ionic exchange and by the adsorption of nickel, cobalt and

magnesium.^[21] The textural and chemical surface characterization of natural and modified zeolites was carried out in relation to the metal concentration in framework, surface area, number of acid sites, redox potential, zeta potential and pH to water.^[22,23]

Experimental procedure

According to previous results, the natural and modified zeolite doses assayed were 0.05 g of zeolite/g of VSS.^[2] At the same time, the behaviour of the systems with modified zeolites was compared with those digesters containing the following metal concentrations in solution: $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. A modified buffer phosphate medium was used: (1) $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (11.9 g/L), (2) KH_2PO_4 (9.1 g/L), (3) NaCl (3.0 g/L), (4) Yeast extract (0.02 g/L), (5) NaHCO_3 (1.0 g/L), (6) Na_2S (0.1 g/L), and, finally, 1.0 mL of a solution of a mixture of VFA (acetic:propionic:butyric = 70:20:10) was used as a carbon source (g COD-VFA/g VSS = 1.0).

The working volume of the bottles used was 40 mL, which were kept in a shaker (150 rpm) at a room temperature of 35 °C. The effect of the different applied conditions was assessed by means of a method based on the determination of the potential specific methanogenic activity (SMA) of the sludge during three consecutive feedings of substrate. The biogas produced was collected in gasometers by positive displacement of a 15% $\text{Na}(\text{OH})$ solution, in such a manner that methane displaced a measurable volume of solution, which was considered equivalent to the methane produced.

Chemical and microbial analyses

Chemical analyses were determined according to the recommendations of the Standard Methods for the Examination of Waters and Wastewaters.^[24]

Gas chromatographic analyses were carried out to determine the concentration of the individual volatile fatty acids (acetic, propionic, butyric and valeric acids). These analyses were carried out using a gas chromatograph equipped with a 15 m x 4 mm Nukol-silica capillary column and a flame ionisation detector. The oven temperature was gradually increased from 100 °C to 150 °C at a rate of 4 °C/min. Helium (28.6 kP), nitrogen (28.6 kP), hydrogen (14.3 kP) and air (28.6 kP) were used as a carrier gas at a flow rate of 50 mL/min.^[11]

The microbial groups developed in a biofilm under different conditions were analyzed by Dot Blot Hybridization.^[25] In conclusion, the rRNA extracted^[26] was denatured, immobilized on a Hybon-N membrane (Amersham Pharmacia Biotech), and fixed at 80°C. Microbial composition was evaluated by using EUB338: bacteria, SRB385: sulfate reducing bacteria (SRB), ARCH915: *Archaea*, MC1109: *Methanococcaceae*, MS821: *Methanosarcina*, MX825: *Methanosaeta*.^[27]

The profile of genetic diversity and microbial communities was analyzed by denaturing gradient gel electrophoresis (DGGE). DNA extracted from different samples (Current Protocols in Molecular Biology) were amplified by PCR and the DNA band pattern was obtained in a DGGE. PCR amplification and conditions were previously described by Muyzer *et al.*^[28] and carried out in a Techne progene cycler.

Results and discussion

Chemical surface characterisation of the zeolites

Table 2 shows the results of the textural and chemical surface characterization of natural and modified zeolites. As can be seen, the modified natural zeolite (ion exchange and

adsorption) with magnesium adsorbed the highest quantity of metal, followed by nickel zeolite. Cobalt zeolite adsorbed the lowest concentration. Magnesian zeolite also showed the highest surface area followed by cobalt zeolite. This may be influenced by a higher quantity of metal (Mg^{2+}) being adsorbed on the surface support than in the zeolite channels.

Nickel zeolite presented the highest quantity of acid sites (0.86) which indicates a higher level of acidity. Magnesian zeolite showed the same number of acid sites as natural zeolite, whereas the redox potential was the lowest. Natural and cobalt zeolites reported values within the same order of magnitude. However, all these materials are considered to have a low level of acidity, particularly when taking into account the values of zeta potential and the pH in water. Table 3 presents the metal concentration supported on modified zeolites and when added in solutions.

In general, the addition of nickel zeolite has previously shown that the best effects are at the lowest doses, which may be due to the higher metal concentration (2.60 mg/g) adsorbed in nickel zeolite than in cobalt zeolite (1.98 mg/g).^[2] These results can be compared with those reported by Speece,^[29] where the optimization of a medium to maintain a high rate of acetate consumption was studied. To achieve optimum conditions, a soluble cobalt concentration of 0.05 mg/L was needed, which was five times higher than that necessary for nickel (0.01 mg/L).

Other reviews about the biochemistry of methanogenesis claim that methyl-coenzyme M reductase (MCR) has a catalysing effect on the actual methane-forming reaction itself and highlights the presence of a ligand nickel atom, which is the striking feature of coenzyme F430. This key coenzyme is essential in the formation of methyl-coenzyme M from N5-methyl-H4MPT where cobalt is concerned.^[16,17] As far as acetogenesis is concerned, the mediation of a nickel-containing center has been

detected, and to be specific a metal complex called NiFeC species, acting in acetyl-CoA synthesis from CO or CO₂. In the second step of acetyl-CoA synthesis, cobalt is an active site of a carrinoid/iron-sulfur protein. Moreover, enzymes which conserve energy and act as ATP synthesisers, are dependent on metals such as Mo, W, Ni, Fe Co and Se.^[18]

Some studies also demonstrated the important role of inorganic nutrients not only to methanogenesis and acetogenesis, but also to hydrolysis and acidogenesis. In a previous study, the effect of inorganic nutrient supplementation at mesophilic and thermophilic conditions was evaluated. The reactors which had been supplemented showed higher acid production and rates of hydrolysis at both temperatures in terms of soluble COD production, VSS removal and organic acid production, demonstrating the importance of inorganic nutrients for both steps.^[13]

In the present study, different behaviour between heavy metal concentrations supported on zeolite and in solution are compared. In order to apply and use these materials in full-scale reactors, it is necessary to take into account not only the scale-up criteria of the different types of wastes and reactor configurations, but also to contemplate the high capacity of zeolite for the immobilization of microorganisms and its influence on microbial and enzymatic transformation of a variety of substances.

Digester behaviour

Figure 1 shows the cumulative methane production curves corresponding to the second and third feedings. Table 4 summarizes the calculated values of SMA for both feedings.

During the first feeding, there was an adaptation period of between 20 and 25 days. Moreover, methane production was very low (data not shown) after 50 days of digestion. During the second feeding, control and natural zeolite digesters had an

adaptation period close to 10 days, which was shorter than that observed in the first feeding. Specifically, during the first 15 days of digestion, cobalt and magnesium modified zeolites gave the highest cumulative methane volumes, achieving similar final values for all modified zeolites after 40 days of operation. However, all digesters supplemented with metals showed similar curve profiles, with methane production values higher than those obtained for modified zeolites. To be specific, digesters supplemented with Ni^{2+} and Mg^{2+} showed final cumulative methane values 30% and 25% higher than those obtained for control reactor. Previous studies^[30] also revealed that the stimulation of the methanogenic activity during the optimisation of anaerobic digestion of wastewater containing methanol is related to the free metal ion (Ni^{2+} and Co^{2+}) concentrations. In addition, biogas production was found to be 35% higher for maize silage and up to 70% higher for acetate when trace elements (iron, cobalt and nickel) were added as compared to reference digesters in batch tests.^[31]

On the other hand, the highest SMA values were obtained for systems with modified zeolites (Table 4), specifically for Mg modified zeolite, which was 1.6 times higher than that obtained for control reactor. It was also previously observed that the addition of Mg^{2+} modified zeolite at doses of 0.1 g/g VSS increased the SMA and the apparent kinetic constant 15 and 2 times, respectively, in relation to control reactors during the methanogenesis of acetate and methanol.^[32]

During the third feeding a decrease in SMA values was observed for all conditions studied, with a noticeably different behaviour for cobalt and magnesian zeolite curves. However, at the end of the runs the methane production for modified zeolite systems recuperated. As was also observed during the second feeding, in this case the digesters supplemented with Mg^{2+} and Co^{2+} showed the highest methane productions, which were 25 % higher than those obtained for the control.

The decrease in the methanogenic capacity during the third feeding may be due to an excess of sodium ion from substrate neutralization, which could have been accumulated during the assay. This fact can be supported by the results obtained with natural zeolite. Digesters with natural zeolite showed only a slight decrease in the SMA (11%), which could be due to an exchange of sodium from medium to zeolite support, attenuating some negative effects of high sodium concentrations. In this event, the positive effects of natural zeolite on the anaerobic process could be partially attributed to the presence of certain cations (such as Ca^{2+}) that have been shown to counteract the inhibitory effect of others such as Na^+ or NH_4^+ .^[33]

Table 5 presents the average values of the different VFA determined at the end of the third feeding. Acetic acid showed the lowest concentrations for all conditions studied, while propionic acid presented the highest concentrations. This revealed the scarcity of propionate-utilizing microorganisms present in the digesters and the abundance of acetate-utilizing microorganisms, especially in the case of modified zeolites. Therefore, in contrast to acetate and butyrate, which were gradually consumed, propionate easily accumulated in the reactors due to its not being directly subjected to methanogenesis and to its low maximum propionic acid removal rate and its recalcitrant character.^[15] The formation of both iso-butyric and iso-valeric acids was observed at the same levels of concentration. It is also important to point out the presence of iso-butyric acid and not of n-butyric acid which was initially added.

Other authors reported low productions of butyric and valeric acids and a high level of acetic and propionic acids in the batch anaerobic digestion of winery wastewater, but almost always with acetic acid concentrations more than three times higher than propionic acid concentrations.^[34,35]

On the other hand, the digesters supplemented with Co^{2+} and Mg^{2+} showed lower propionic acid accumulations than those corresponding to the same modified zeolites, which revealed an increase in the methanogenic activities when these metals were in solution. The same finding was obtained by Kim *et al.*^[13] during the hydrolysis and acidogenesis of particulate organic matter at mesophilic and thermophilic temperatures. In the same way, the addition of Ni^{2+} and Co^{2+} at doses of 0.75 and 0.45 mg/mg VS lead to the lowest VFA concentrations during the anaerobic digestion of waste sludge derived from a municipal wastewater treatment plant.^[36]

Identification of the microorganisms involved by molecular biological tools

Firstly, a study to investigate the variation of microbial populations with the use of taxon-specific probes by 16S rRNA dot-blot hybridisation was carried out. The results showed that control reactors (without zeolite) had a higher presence of SRB than for the other proved conditions. In the case of *Archaea*, *Methanosarcina* was predominant followed by *Methanosaeta*. In control digesters and in the rest of conditions, *Methanococcacea* was found to be at the lowest concentration, in relation to all identified *Archaea* in this study. In the case of natural zeolite, the presence of *Methanosaeta*, *Methanosarcina* and SRB showed the same levels, and therefore a similar presence. For nickel and cobalt zeolites, *Methanosaeta* presented the highest percentages. For both of the above-mentioned zeolites, the rest of identified populations had low concentrations and low presence. However, magnesian zeolite stimulated the proliferation of *Methanosarcina* and SRB were also present although to a lesser degree.

In the same way, phylogenetic analyses based on 16S rRNA gene clonal sequences, DGGE and quantitative real-time polymerase chain reaction (PCR) of the microbial communities were carried out in a mesophilic anaerobic protein degradation

process using bovine serum albumin (BSA)-fed continuous cultivation.^[37] These studies showed that within the domain *Archaea*, methanogens affiliated with the genera *Methanosaeta* and *Methanoculleus* were predominant in chemostats supplemented with Ni^{2+} and Co^{2+} . Within the domain Bacteria a total of 56% of rRNA genes obtained from this chemostat was affiliated with three phyla: *Firmicutes* (32%), *Bacteroidetes* (11%) and *Proteobacteria* (13%).^[37]

Analysis of the microbial communities may be decisive to understand the microbial processes taking place during the anaerobic decomposition in anaerobic reactors and optimize their performance. Therefore, another approach made was the study of 16S rDNA bands through DGGE. In this case, DNA fragments of the same length but with different sequences can be separated. In this way, it is possible to study the complexity of the community where the results show a pattern of bands, where the number of bands corresponds to the density of the population and the density of each band corresponds to its population abundance within the microbial communities. This approach revealed that all conditions studied showed a similar abundance of bands (between 8 and 10), but the quantity of each band changed with the studied condition (Figure 2). However, the presence of *Archaea* was predominant in comparison to bacteria for all conditions tested. The change in abundance was probably due to the fact that some bacterial species are susceptible to certain levels of heavy metals.^[38] Differences in density and abundance were observed mainly between control reactors and digesters supplemented with heavy metals as can be seen in Figure 2. Similar results were observed by Tang *et al.*^[37], where the addition of trace metals of Ni^{2+} and Co^{2+} enabled methanogens to have higher populations when compared to control reactors (without the addition of metal trace). They found a predominance of bacteria performing the Stickland reaction when no metal was added. Moreover, a stimulation of

the activity and population of halophilic methanogens was also reported after adding Ni^{2+} and Co^{2+} during the anaerobic degradation of organic matter at different salt concentrations.^[39] In the same way, it was reported that the methanogenic activity of *Methanosarcina sp.* increased considerably after the addition of trace nutrients (Ni^{2+} and Co^{2+}) in sulphide-containing media during the anaerobic treatment of wastewaters containing methanol.^[30]

In any case, the identification of specific groups by this method requires establishing clone libraries by cloning and sequencing of the PCR products of each member.

Conclusions

The heavy metal supplementation to batch digesters by modified zeolites as well as in solutions increased SMA values of the sludge and, therefore, methane productivity. Metal supplementation by means of modified natural zeolite is an interesting practical application for obtaining a higher retention time of the microorganisms (Bacteria and Archaea), which lead to increasing the organic loading rate, decreasing the hydraulic retention times and the reactor volumes. The kind of metal supplemented in solution or modifying natural zeolite influences the predominance of microbial groups. Nickel and cobalt modified zeolites favoured the presence of *Methanosaeta*, while magnesian zeolite stimulated *Methanosarcina* and SRB. It is a better option to combine fingerprinting techniques and the use of specific nucleotide sequences to elucidate the microbial diversity and the structure of communities stimulated by metal supplementation.

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Table 1. Chemical and phase composition of the natural zeolite from the Tasajera deposit, Villaclara (Cuba) used in the experiments.

<i>Chemical composition (%)</i>		<i>Phase composition (%)</i>	
SiO ₂	58.05	Clinoptilolite	35.0
Al ₂ O ₃	11.94	Mordenite	15.0
Fe ₂ O ₃	4.36	Montmorillonite	30.0
MgO	0.77	Others*	20.0
CaO	5.94		
Na ₂ O	1.50		
K ₂ O	1.20		

* Others – Calcite, Feldespate and Quartz.

Table 2. Results of the textural and chemical surface characterization of natural and modified zeolites.

<i>Zeolite type</i>	<i>Metal concentration (mg/g zeol.)</i>	<i>Surface area (m²/g)</i>	<i>Redox potential (mV)</i>	<i>Number of acid sites</i>	<i>Zeta potential</i>	<i>pH in water</i>
Zeo. Natural	---	25	-150	0.59	2.8	7.22
Zeo. Ni ²⁺	2.60	25	-110	0.86	2.6	6.84
Zeo. Co ²⁺	1.98	29	-150	0.62	2.4	6.47
Zeo. Mg ²⁺	5.25	35	-100	0.59	2.6	6.98

Table 3. Metals levels supported on zeolites and in solution (Sln.) for all conditions studied.

<i>Metal condition</i>	<i>Zeo. Ni²⁺</i>	<i>Zeo. Co²⁺</i>	<i>Zeo. Mg²⁺</i>	<i>Sln. Ni²⁺</i>	<i>Sln. Co²⁺</i>	<i>Sln. Mg²⁺</i>
Levels*	10.40x10 ⁻³	7.92x10 ⁻³	21.0x10 ⁻³	26.0x10 ⁻³	19.8x10 ⁻²	52.5x10 ⁻²

* Metals in zeolites (Zeo. M²⁺) are expressed in mg and those in solutions (Sln. M²⁺) in mg/L.

Table 4. Values of the SMA* in the second and third feedings for all conditions studied.

<i>Feeds</i>	<i>Controls</i>	<i>Zeo.</i> <i>Natural</i>	<i>Zeo. Ni²⁺</i>	<i>Zeo. Co²⁺</i>	<i>Zeo. Mg²⁺</i>	<i>Sln. Ni²⁺</i>	<i>Sln. Co²⁺</i>	<i>Sln. Mg²⁺</i>
Second	0.75	0.53	1.08	1.17	1.22	1.14	1.04	1.02
Third	0.56	0.47	0.69	0.51	0.54	0.83	0.77	0.52

* g COD-CH₄/(g VSS·d) (Given the SMA is defined as the substrate dependent methane production rate per unit of volatile suspended solids (VSS) biomass, its units represent the amount of methane produced expressed as COD per VSS and time).

Table 5. Average values of VFA concentrations at the end of the assay (third feeding) for all conditions studied.

<i>Feeds</i>	<i>Controls</i>	<i>Zeo. Natural</i>	<i>Zeo. Ni²⁺</i>	<i>Zeo. Co²⁺</i>	<i>Zeo. Mg²⁺</i>	<i>Sln. Ni²⁺</i>	<i>Sln. Co²⁺</i>	<i>Sln. Mg²⁺</i>
Acetic	7 ± 3	3 ± 1	5 ± 1	8 ± 2	5 ± 1	5 ± 1	5 ± 1	5 ± 2
Propionic	102 ± 18	121±38	122±41	139±40	129±35	155±45	98±21	115±31
Iso-butyric	10 ± 2	10 ± 3	9 ± 2	8 ± 2	9 ± 2	9 ± 3	7 ± 2	7 ± 3
Iso-valeric	8 ± 2	10 ± 3	10 ± 2	9 ± 2	9 ± 3	9 ± 2	7 ± 2	8 ± 3

Figure 1. Variation of the cumulative methane production (mL) during the second (A) and third (B) feedings (-: Control; $\diamond$: natural zeolite; $\square$: Ni^{2+} modified zeolite; Δ : Co^{2+} modified zeolite; $\circ$: Mg^{2+} modified zeolite. **Open symbols correspond to metals in zeolite and closed symbols to metals in solution).**

Figure 2. Pattern of 16S rDNA bands obtained by Denaturing Gradient Gel Electrophoresis (DGGE), with 8% acrylamide/bisacrylamide, and denaturation between 25% to 65%, and ethidium bromide stained. 16S rDNA amplification was developed using 341F-GC and 907R bacterial probes. (C: Control reactor; Nat: natural zeolite; Z.Ni, Z.Co and Z.Mg: Ni^{2+} , Co^{2+} and Mg^{2+} modified zeolite, respectively; S.Ni and S.Co: Ni^{2+} and Co^{2+} in solution, respectively).

C Nat Z.Ni Z.Co Z.Mg S.Ni S.Co