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Use of PCR-DGGE based molecular methods to assessment of microbial diversity during anaerobic treatment of antibiotic combinations

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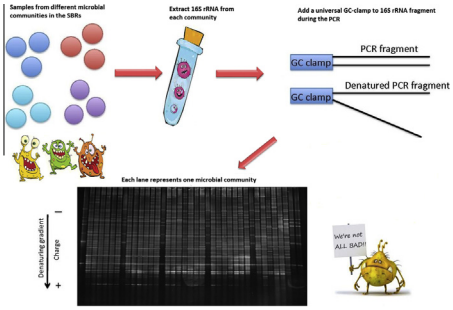
HIGHLIGHTS

- PCR-DGGE analysis is a suitable control approach of anaerobic treatment system.
- Antibiotic combinations have the most dramatic effect on acetoclastic methanogens.
- Gram-negative bacteria are essential for efficient operation of anaerobic process.

GRAPHICAL ABSTRACT



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ABSTRACT

As it is currently often not know how anaerobic bioreactors, e.g. for biogas production, react if the substrate is contaminated by toxic compounds like antibiotics. This study evaluated how anaerobic sequencing batch reactors were affected by amendments of different antibiotics and stepwise increasing concentrations. The compositions of microbial community were determined in the seed sludge using 16S rRNA gene clone libraries and PCR-DGGE analyses were used for the detection of microbial community changes upon antibiotics additions. According to PCR-DGGE results, the syntrophic interaction of acetogens and methanogens is critical to the performance of the reactors. Failure to maintain the stability of these microorganisms resulted in a decrease in the performance and stability of the anaerobic reactors. Assessment of DGGE data is also useful for suggesting the potential to control ultimate microbial community structure, especially derived from Gram-negative bacteria, through bioaugmentation to successful for antibiotic biodegradation.

1. Introduction

Antibiotics have proven to be very effective in the treatment and prevention of microbial infections and, as such, they are commonly prescribed in both human and veterinary medicine. This, combined with the increasing worldwide population and the utilization of antibiotics to improve the growth of livestock, has led to a significant increase in the global use and production of antibiotics. However, this has serious implications for the environment (Johnson et al., 2015). After use, the human body cannot metabolize the active compounds that are present in antibiotics and, as such, they enter the environment via sewage (Tao et al., 2014). Wastewater treatment systems are incapable of removing the compounds from wastewater and the antibiotics, therefore, pollute the environment. The extent to which they can impact the microbial community that is present in the environment has

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been observed in the acceleration of the resistance of species (Threedeach et al., 2012; Naquin et al., 2015). A number of studies have assessed the impact that antibiotic residues have on microbial communities. However, the majority of these have focused on non-industrial environments e.g. in soil and aquatic sediments and there is a distinct lack of published research that examines the bacterial communities that are found in the engineering biomass that is activated and anaerobic sludge, or biofilm (Ho et al., 2013; Aydin et al., 2015b,c).

The high amount of COD that is present in the wastewaters produced by pharmaceutical manufacturing plants makes them a favorable alternative for anaerobic processes (Oktem et al., 2008; Sreekanth et al., 2009; Selvam et al., 2012; Aydin et al., 2014, 2015b,c). However, the anaerobic process is complicated, Bacteria and Archaea work together to convert complex polymers into methane through a number of steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) that must be followed in a sequential and parallel manner (Aydin et al., 2015c). Due to these reasons, it is important to understand how antibiotic combinations impact anaerobic microbial communities dynamics; as well as how microbial communities can impact the fate of antibiotics in sequencing batch reactor (SBR). However, conventional culture-dependent method is not also a time consuming and arduous technique but also detects very low amount of microorganisms present in the environmental samples (Zhang et al., 2013; Li et al., 2013a; Hu et al., 2014).

PCR-DGGE as a powerful molecular method for rapid detection of microbial community changes or comparative analysis of environmental samples offers more accurate information about distribution and composition of microbial species. For examples, Dong and Reddy (2010) have used successfully the PCR-DGGE for comparing of Shannon diversity index and richness between influent to effluent of constructed wetlands treated with swine wastewater. Juang et al. (2010) have also analyzed the *Arthrobacter sp.* corresponded to internal biofilm by using PCR-DGGE. Furthermore, Piterina and Pembroke (2013) with using suitable molecular target and electrophoresis condition have optimized PCR-DGGE technique autothermal thermophilic aerobic digestion (ATAD). They also observed that amplifying of V6–V8 region of 16S rDNA was more effective than *rpoB* gene profiles and this technique can be use as a monitoring method for assessment of the ATAD process efficiency. Zhang et al. (2013) have been used DGGE for detection of the structural changes of the microbial community in sequencing batch reactor during the treatment of trace amount of tetracycline. Li et al. (2013a) also reported DGGE for studying of the functional microbial community in composting by designing of three sets of PCR primers for identifying b-glucosidase. Hu et al. (2014) have developed specific primers for analyzing of the clostridial diversity in fermentation mud using DGGE technique.

Not only do these multi-component mixtures further threaten the environment, their joint toxic effect can also be a major issue for hazard and risk assessment. This is because the total ecotoxicity of a given mixture will be higher than the impact of its individual components. Furthermore, mixtures can exhibit significant ecotoxicity, even if the various components are only present in low concentrations that do not result in toxic effects on those microorganisms that are exposed to them (Beneragama et al., 2013; Mitchell et al., 2013; Aydin et al., 2015a,b,c). However, while it is a well-known fact that antibiotics have a combined effect on the anaerobic microbial community, the nature and extent of this effect is not fully understood. Therefore, the purpose of this study is to examine how sulfamethoxazole, erythromycin, and tetracycline combinations impact anaerobic processes. Each of these compounds is common components of the pharmaceuticals that are used in human and veterinary medicine.

The aim of this research was to determine how the Bacterial and Archaeal communities changes in anaerobic SBRs for the treatment of pharmaceutical wastewaters that contains sulfamethoxazole–erythromycin–tetracycline (ETS) and dual effects of sulfamethoxazole–tetracycline (ST), erythromycin–sulfamethoxazole (ES) and erythromycin–tetracycline (ET) throughout a year operation. In the current study, cloning and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) were used to detect the effect of different concentration and combinations of antibiotics in the anaerobic SBRs. This approach may help to understanding of the microbial relationships and allow in further studies to model the inhibition of anaerobic processes by certain antibiotics.

2. Methods

2.1. Start-up, operation and performance of anaerobic SBRs

Detailed information on the setup, operation and performance of the reactors has been reported in the previous studies (Aydin et al., 2014, 2015b,c). Briefly, five 1.5 L anaerobic SBRs were set up and operated under exactly the same conditions, including identical seed sludge obtained from an anaerobic contact reactor treating of wastewater produced from Raki and Fresh Grape alcohol companies. After steady-state conditions, the influent antibiotics concentrations were gradually increased through successive stages each lasted for 30 days until metabolic collapse of the SBRs. The antibiotic concentrations in each stage are shown in Table 1. Performances of reactors were observed during operational period, which was 360 days (10th Stages) for ST reactor, 440 days (13th Stages) for ET reactor, 360 days (10th Stages) for ES reactor, and 420 days (12th Stages) for ETS reactor (Aydin et al., 2014, 2015b,c).

The results of the VFA measurement indicated that all antibiotic combinations had the highest inhibition effect on acetate degradation pathways, leading to the accumulation of acetic acid. Furthermore, ETS and ET antibiotic combination affected butyric acid utilization pathway, leading to accumulation of butyric acid. Differently from ETS and ET reactors, ST and ES combinations inhibited the degradation of propionate (Aydin et al., 2014, 2015b,c).

2.2. Genomic DNA (gDNA) extraction, total RNA extraction and cDNA synthesis

Triplicate samples were collected from all the anaerobic SBRs on the 10th day of every antibiotic stage for RNA and DNA isolation. A PureLink RNA extraction and a SuperScript cDNA synthesis

Table 1
Tested antibiotic concentrations.

	Sulfamethoxazole (mg/L)	Erythromycin (mg/L)	Tetracycline (mg/L)
Stage 1	0.5	0.1	0.1
Stage 2	5	0.2	0.2
Stage 3	5	0.5	0.5
Stage 4	10	0.5	0.5
Stage 5	10	1	1
Stage 6	15	1	1
Stage 7	15	1.5	1.5
Stage 8	20	1.5	1.5
Stage 9	20	2	2
Stage 10	25	2.5	2.5
Stage 11	40	2.5	2.5
Stage 12	40	3	3
Stage 13	40	4	4

kits (Invitrogen, UK) were used in accordance with recommended procedures to isolate the total RNAs and DNA from the 1 mL sludge sample respectively. NanoDrop spectrophometer (NanoDrop Technologies; Wilmington, DE, USA) was used to measure the concentration of the isolated RNAs and DNAs, and the cDNAs were synthesized from the isolated RNAs using Superscript Vilo cDNA synthesis kit (Invitrogen, UK) immediately. The isolated DNAs and cDNA samples were stored at −80 °C, −20 °C until required for further analysis respectively.

2.3. Cloning, sequencing and phylogenetic analysis

Bact8f-Bact1541r and Arch344f-Arch855r PCR products of the seed sludge of the reactors were purified using PureLink PCR Purification Kits (Invitrogen, U.K.), which were cloned by TOPO TA Cloning Kit (Invitrogen, USA).

To select the positive ones, 75 clones were collected from bacteria clone library and 83 clones were collected from Archaea clone library. Colony PCR were applied using the vector-specific primers M13f and M13r. Bact341f_GC-Bact534r and Arch344f_GC-Univ522r primers were used to re-amplify positive bacterial and archaeal inserts respectively. The re-amplified PCR products were analyzed by high-resolution melt (HRM) to select the representative OTUs. Then, the PCR products to be sequenced were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primers Bact342f and Arc344f generating 500 bp and 800 bp of bacterial and archaeal sequence data respectively (Ozbayram, 2012).

2.4. DGGE analysis

A PCR-DGGE analysis was used to determined the Archaea and Bacteria communities dynamics throughout all stages in the control, ETS, ET, ES and ST reactors using specific primers as given in Table 2. A 500 ng sample of the V3 and V6 area PCR product for domain Bacteria and Archaea were evaluated using the D-code mutation detection system (Bio-Rad, USA). The PCR-DGGE analysis was described in a previous study (Zhang et al., 2013).

2.5. Statistical analysis

Partial 16S rRNA gene sequences were analyzed and manually edited in Amplify 3X software package version 3.14 (http://engels.genetics.wisc.edu/amplify). The sequences were checked for reading errors with the alignment programs of the ARB package, which are based on secondary structures of rRNA. The 16S rRNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project and the ARB software package. Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with BLAST provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify putative close phylogenetic relatives. Sequence data were aligned by ClustalW tool at website of European Bioinformatics Institute. Distance analyses using the Jukes and Cantor correction and bootstrap resampling (1000 times) were done using the MEGA Software package version 5.1 (http:// http://www.megasoftware.net/) and trees were generated from distance matrices using the neighbor-joining method (Ozbayram, 2012).

16S rRNA gene sequences showing 99% similarity or higher was considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relatedness with known bacterial or archaeal phylogenetic groups were listed as unclassified. The distribution of clone types present in the clone libraries was determined and used to calculate the Shannon–Weaver index ($H = -\sum[p_i \cdot \cdot \ln(p_i)]$), where p_i is the relative contribution of clone type i to the whole library (n_1/N). Coverage was calculated as $1 - (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed. The Chao1 estimator of species (here, clone type) richness (Schao1) was calculated as; $S_{obs} + ni^2/2n_2$ (Röling and Head, 2005).

DGGE images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Similarities of the community fingerprints between each sample were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and UPGMA clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%.

3. Results and discussion

3.1. Bacterial and Archaeal 16S rRNA clone libraries

Bacterial and Archaeal 16S rRNA clone library were constructed for the seed sludge of anaerobic SBRs using specific primers for characterization of microbial community structure. All clones were screened by HRM analysis and this analysis presented that 12 different OTUs were obtained from 75 bacterial clones and 22 different OTUs were found in 83 archaeal clones (Ozbayram, 2012). Tables S1 and S2 illustrate all different OTUs were sequenced and the closest relatives of bacterial and archaeal sequencing results respectively. The phylogenetic tree constructed by bacterial and archael clones was given in Figs. S1 and S2 (Ozbayram, 2012).

The dominant bacterial clone phyla belong to *Firmicutes* (21%), *Actinobacteria* (11%), *Cyanobacteria* (4%) and *unclassified Bacteria* (64%) as seen in Fig. 1 (Ozbayram, 2012). *Clostridium sp.*, were represented 93% of *Firmicutes* members in the seed sludge and which is Gram-positive bacteria and responsible for degradation of organic compounds. *Actinobacteria* are also Gram-positive microorganism including *Bifidobacterium*, *Mycobacterium* and *Corynebacterium*.

The most abundant Archael phyla in seed sludge were *Methanosarcinales* (27%), *Euryarchaeota* (8%), *Methanomicrobiales* (7%) and *unclassified Archaea* (58%) as seen in Fig 2 (Ozbayram, 2012). Maintenance of *Methanosarcinales* (acetoclastic methanogens) in anaerobic reactor is critical for stable performance. Prior studies indicated the importance of *Methanomicrobiales* (hydrogenotrophic methanogens), which was the most resistant group in Methanogens to toxic substances. The uncultured archaeal and bacterial 16S rRNA gene sequences were deposited in the GenBank database under the accession Nos. KJ018671–KJ018699.

3.2. The influence of ETS and ET combinations on the Bacterial and Archael community dynamics in the SBR

The clones were compared with the samples' DGGE bands. Using band intensities, canonical correspondence analyses were applied to understand the relations of species by digestion time, biogas/methane production, VFA accumulation and ETS and ET concentration is given in Fig. 3. From the results of canonical correspondence analysis, it can be said that all of the microbial groups in the SBRs were negatively affected by ETS and ET toxicity and these results were similar compared to each other. Decrease

Table 2
Primers used for PCR amplifications.

Primer	Target	Experimental stage	Annealing (°C)	Position[1]	Reference
Bact341f_GC[2]	Bacterial	DGGE	55	341–357	Muyzer et al. (1993)
Bact534r	16S rDNA	DGGE	55	534–518	Muyzer et al. (1993)
Bact8f		Cloning	55	8–27	Lane (1991)
Bact1541r		Cloning	55	1541–1522	Lane (1991)
Bact342f		Sequencing	55	342–361	Edwards et al. (1989)
Arch46f	Archaeal	First round of nested PCR	40	46–61	Øvreas et al. (1997)
Arch1017r	16S rDNA		40	1017–999	Barns et al. (1994)
Arch344f		Cloning	53	344–358	Raskin et al. (1994)
Arch855r		Cloning	53	855–836	Shinzato et al. (1999)
Arch344f_GC[2]		DGGE	53	344–358	Raskin et al. (1994)
Univ522r		DGGE	53	522–504	Amann et al. (1995)
M13f	B-galactosidase	Clone	54	–	Schrenk et al. (2003)
M13r		Screening			

[1] *Escherichia coli* numbering.
[2] 5′-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

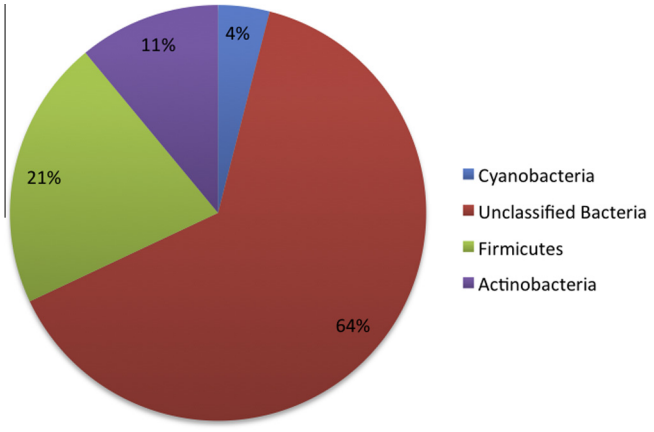

Fig. 1. Distribution of phylogenetic phylum from clone sequence of bacterial 16S rRNA clone library (Ozbayram, 2012).

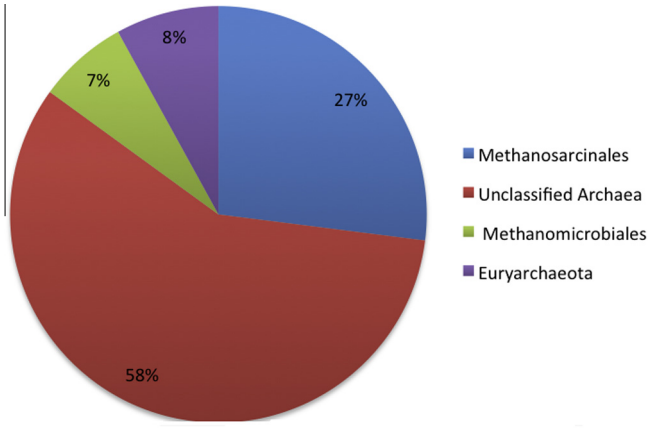

Fig. 2. Distribution of phylogenetic phylum from clone sequence of archael 16S rRNA clone library (Ozbayram, 2012).

in methane and biogas productions were found out to be closely related with the disappearance of the acetoclastic methanogens represented by the *Methanosarcinales* order in the ETS reactor. There, it can be said that *Methanosarcinales* order was the most affected group from ETS toxicity during the operation. The case is also reported in other previous studies regarding ETS antibiotics (Aydin et al., 2015c). According to canonical correspondence analysis, the

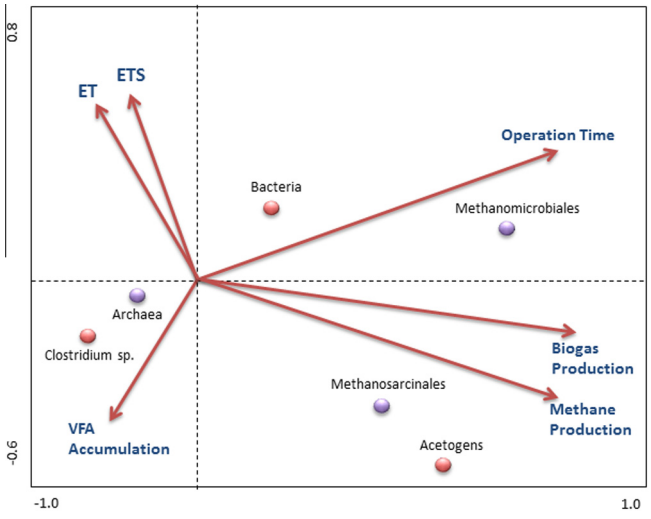

Fig. 3. Canonical correspondence analysis of DGGE results in the ETS and ET reactors.

number of total active bacteria seemed not to effect biogas and methane production directly; however, when apply for the subgroups (*Clostridium sp.*, *Propionibacterium sp.*, *Acetogens*), which was Acetogens were effective on ETS and ET combinations due to their syntrophic relations with the methanogens. The situation was also similar for the total number of Archaea, meaning that changes in subgroups were more effective on explaining ETS inhibition. Additionally, *Methanomicrobiales* (hydrogenotrophic methanogens) in these reactors was positively correlated with the operation day, which means that its abundance significantly increased through the operation. As mention in the previous researches, hydrogenotrophic methanogens are higher in substrate utilization rate; growth rate and cell yield to exposed toxic substances than compare to acetoclastic methanogens (Aydin et al., 2015c).

3.3. The influence of ST and ES combinations on the Bacterial and Archael community dynamics in the SBR

All the archaeal and bacterial groups were significantly ($p < 0.05$) negatively affected with ES and ST concentration according to canonical correspondence analysis in the SBR. Canonical correspondence analysis also display that the influence of an ST and ES combination produces a comparable effect between bacterial and archael community dynamics as seen in Fig. 4. Furthermore, the

number of total active Bacteria and Archaea seemed not to effect biogas and methane production directly as well as ETS and ET combinations; however, biogas/methane productions and COD removal were found correlated very largely with *Methanosarcinales sp.* and *Acetogens*. Archaea did not show a significant change with operation time. This can be explained by the slower grow rates of archaeal cells. In the degradation of propionic acid is most often utilized by *Propionibacterium sp.* via methylmalonyl coenzyme A (MMC) pathway, and combinations of ST and ES combination inhibited these sensitive strains of this microbial community (Aydin et al., 2015a).

Compare ETS, ST, ES and ET reactors revealed that Gram-negative bacteria are much important than Gram-positive bacteria during the operation. There was also a significant positive correlation between decrease numbers of Gram-negative bacteria and metabolic collapse of reactors. Gram-negative bacteria are distinct in that there is a double membrane surrounding each bacterial cell. In addition to the inner cell membrane that is present in all bacteria, Gram-negative bacteria also have an outer membrane that prevents certain antibiotics from penetrating the cell. This entails that Gram-negative bacteria are typically more resistant to antibiotics than Gram-positive bacteria. Gram-negative bacteria have also demonstrated the ability to exchange DNA among strains of the same species and, in some cases, between different species. As such, if a Gram-negative bacterium undergoes any mutation or acquires genetic material that has antibiotic resistant properties, it may later pass on these resistant properties to other strains of bacteria through the sharing of DNA (Pagès and Amaral, 2009).

The most obvious finding to emerge from the DGGE analysis is that Gram-negative bacteria was affected in the earlier stage of the reactors and then cannot acquire of antibiotic resistance until metabolic collapse of the anaerobic reactors. Moreover, *Methanosarcinales* in these reactors was negatively correlated with the biogas/methane production, which means that its abundance significantly decreased through the operation. As mention in the ETS and ET reactors, acetoclastic methanogens were the most sensitive compared with *Methanomicrobiales*, and toxins would directly inhibit this group in Methanogens.

3.4. Cluster analysis of DGGE banding pattern of the anaerobic SBRs

Microbial diversity shifts in Bacterial and Archaeal communities, which presented in the anaerobic SBRs during all the stages were estimated based on the DGGE patterns of the partial 16S rRNA amplicons. The bacterial and archaeal phylogenetic dendrograms obtained from DGGE band patterns in the ETS, ET, ES and ST reactors were represented in Figs. S3–S6. The results of this study are in keeping with previous observational studies, which significant changes in 16S rRNA profile of bacterial and archaeal communities were detected in the SBRs after Stage 3 for the ST and ES reactors; Stage 6 for the ETS and ET reactors (Aydin et al., 2015c).

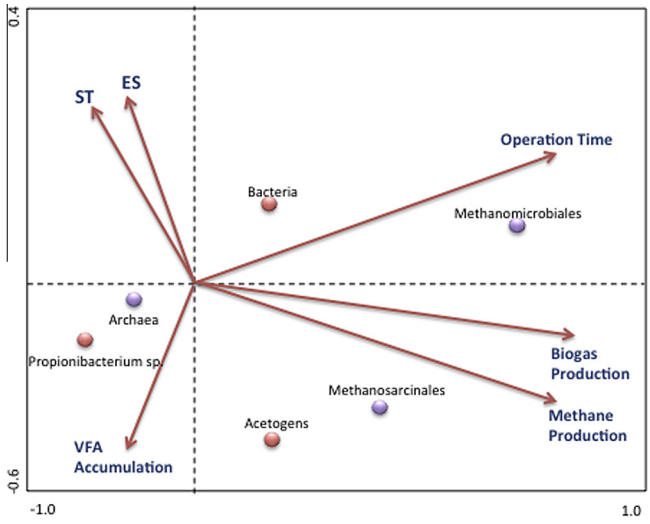

Fig. 4. Canonical correspondence analysis of DGGE results in the ST and ES reactors.

3.5. Assessment of DGGE for monitoring of microbial communities

In summary, for the informants in this study, PCR-DGGE approach gives a reasonable comparison of the combined effects of antibiotic substances on microbial community structures as well as displays the likely effect on SBRs operation. With the use of a gradient pump for DGGE gels and Bionumerics software, it is possible to analyze differences in the amount of bacteria and their diversity between many samples, so long as enough ladders in the gel are used for normalization. One main difficulty, the method requires to establish the gradient marker from one gel to another, so that it is difficult to compare fingerprints of more than 20 samples (Li et al., 2013a,b; Hu et al., 2014). qPCR will also provide an important molecular method toward the quantitative detection of influence of the antibiotic combinations on the anaerobic microbial community and expression of functional genes in the SBRs (Yu et al., 2006; Smith and Osborn, 2009; Li et al., 2013a,b; Aydin et al., 2015c). However, a complex sample matrix, such as in reactors that had different amounts of COD removal that the sample matrix was not the same, can hinder the efficiency of qPCR, this result is known as "qPCR inhibition." To be need check for inhibition, samples are often diluted 10×, 100×, and 1000×, qPCR is performed on each dilution, and the final result should be the same for all samples. If the sample that was not diluted has a lower value than the diluted samples then the matrix is attributed to inhibiting the PCR. For instance, Aydin et al. (2015c) have observed qPCR inhibition at Stage 12 in the ETS reactor. On the other hand, once the method is established in the routine, hundreds of samples may be run but it becomes however very expansive with an increasing number of sample compare to the PCR-DGGE analysis. qPCR and PCR-DGGE analysis also support this same results and demonstrate that Acetoclastic methanogens (*Methanosarcinales*) was the most affected group in Methanogens in the anaerobic SBRs. The results reported in this research are valuable as they may allow in further studies to model the inhibition of anaerobic process by certain antibiotics.

4. Conclusion

The results of the study indicated that increasing antibiotic concentrations negatively impact on microbial community structure and function in anaerobic bioreactor. The findings of this research provide insights for importance of Gram-negative bacteria, which was essential to anaerobic biodegradability of antibiotic combinations in the SBRs. PCR-DGGE could also be useful for examining of microbial communities in anaerobic systems and assess the condition of the reactor for control and improvements of such systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.05.086.

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