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Eubacterial Diversity Inside an Activated Sludge Wastewater Treatment System Designed to Investigate Degradation of Paracetamol

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This paper summarizes the findings resulting from the application of 16S rRNA analysis of reactors biomass samples by Denaturing Gradient Gel Electrophoresis (DGGE). To investigate the stability and the diversity of the microbiological communities inside an activated sludge wastewater treatment system designed to investigate degradation of paracetamol, Polymerase Chain Reaction (PCR) and DGGE were applied to biomass solids samples from all phases in the reactors. The investigation was carried out in three phases and each phase included three different conditions of solids retention time (SRT) and paracetamol dose within the test reactor, results being compared with a control reactor receiving the same synthetic wastewater feed without paracetamol. Data analysed by using the Bionumeric software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). Results showed that there was no significant difference between microbial communities in the paracetamol and control reactors, and no substantial change in bacterial community structure or community shifting was detected in response to consecutive increases in SRT and dose concentration of paracetamol.

Key words: Activated Sludge, PCR, DGGE, Paracetamol, Solids Retention Time.

The study of nucleic acid molecules present in all cellular forms is the basis for molecular microbiology[1]. The two types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) constitute the main parts of each cell[2]. There are two vital processes in the cell which both DNA and RNA are involved, multiplication, involving nucleic acid replication by several enzymes and transfer to new cells; and protein synthesis, since proteins are involved in the structure it is the basis for the survival of the cells[2]. DGGE technique is based on the melting properties of DNA in solution. Prior to the DGGE, an amplification process is necessary which is called Polymerase Chain Reaction (PCR), because the extracted DNA from environmental samples does not contain adequate rDNA material and

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cannot be used directly for DGGE process[3]. The amplification process (PCR) needs primers that are special designed short (100 to 500 base pair) DNA fragments. The primers specifically aimed for the interested microbial groups (e.g. Vr, Vf for Eubacterial population[4]; CTO for Ammonia Oxidisers)[5]. The products of this process are then used for the final step DGGE. This technique has been frequently used to study the microbial diversity in environmental samples[6]. Analysis of the diversity of a microbial community can be carried out specific to a certain species or a group or for the whole eubacterial population. In the present research the DGGE technique was used to characterise and understand the stability and the diversity of the eubacterial communities inside an activated sludge wastewater treatment system designed to investigate degradation of paracetamol.

MATERIALS AND METHODS

The application of 16S rRNA technique DGGE involved the following steps:

Sampling and sample preservation

Taking representative samples and immediate preservation under sterile conditions

DNA extraction

Extraction of the raw material for molecular analysis.

Polymerase Chain Reaction (PCR)

Amplification of the targeted sections of the DNA material

Denaturing Gradient Gel Electrophoresis (DGGE)

Analysis of the rDNA fragments obtained by PCR amplification and separation of the DNA fragments.

Sampling and sample preservation

In activated sludge systems it has been suggested a small volume of sample contains a diverse microbial community which is representative of a whole system and a single sample of an activated sludge plant should be sufficient to compare the plants[7]. Consequently a single sample of biomass was collected from each reactor at different SRT and doses of paracetamol. The sampling process under such conditions was conducted with maximum care using sterile equipment and sample bottles in the shortest duration possible in order to prevent any interference that can affect the molecular analysis. Therefore 10 ml of the sample was transferred to a 20ml sterile sample bottle with the addition of 10ml absolute ethanol and that was taken for storage immediately in a freezer at -20^{o}C. These sampling conditions are summarised in Table 1.

DNA extraction

The DNA extraction technique used in this study was based on the technique has been described by Curtis and Craine[7].

Polymerase Chain Reaction (PCR)

PCR was carried out for eubacterial population. The sequences of the different primers and their annealing temperatures are shown in Table 2.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was conducted based on the procedure by Muyzer *et al*.[4], with the exception that a 10% polyacrylamide gel with a range of denaturants of 20-65% was used.

RESULTS

The result of digital image of scanned DGGE gels can be presented by a DGGE profile (Fig. 1). Each DGGE profile may consist of several lanes depending on the number of samples while each lane may contain several bands at different locations. A lane represents a sample that contains the microbial population of the mixed microbial population at a specific condition (SRT) in the reactor. Likewise, every band in each lane corresponds to a different DNA fragment with a different sequence, and subsequently different molecular weight which is seen by the bands appearing on specific lines which a given sample lane. These bands demonstrate the level of the diversity of the microbial population, because each band represents a separate species that was present in the reactor. With time, these species varied due to the SRT and dosage of paracetamol and the differed bands from one sample to another.

Analysis of Eubacterial diversity at different SRT

Visual comparison of the DGGE profile of eubacterial (Fig. 1) revealed that for each SRT and dose of paracetamol the diversity produced a different band pattern. This profile showed that no bands were visible in phase 1a* (R2-2) control reactor in lines 12 and 18 in comparison with phase 1a (R1-1) test reactor. Furthermore, although the

control reactor (phase 1a*, lane R2-2) showed bands in lines 15, 24, and 25, the test reactor (phase 1a, lane R1-1) showed no equivalent bands. Changes occurred in phase 1a and 1c of the test reactor that caused an elimination of bands on the lines 15 and 25 of eubacterial community and production of a new band at line 12 in both phases. But similar changes were not observed in phase 2 for the same APAP concentration, indicating that these changes were not linked to the paracetamol dosage itself rather to the changes in SRT. With decreasing SRT and increasing dose of paracetamol, bands appeared in phase 1c (R1-3) test reactor in lines 12, 17, and 27 in comparison with phase 1c* (R2-4) control reactor. In addition, although the control reactor (phase 1c*, lane R2-4) showed bands in lines 9, 15, 25, the test reactor (phase 1c, lane R1-3) showed no equivalent bands. Comparisons among the samples in phase 2 of both the reactors revealed that there were specific bands appearing in phase 2a* (R2-6) control reactor in lines 9 and 15 whereas, the particular bands for phase 2a (R1-5) test reactor were observed in lines 8, 11, 21, 27, and 32. There were no bands in phase 2b (R1-7) test reactor in lines 4, 9, 19, and 23 in comparison with phase 2b* (R2-8) control reactor. In addition, although the test reactor (phase 2b, lane R1-7) showed bands in lines 8, 21, 22, and 27, the control reactor (phase 2b*, lane R2-8) showed no equivalent bands. As the results indicate (Figure 2) there were no bands in phase 2c* (R2-10) control reactor in lines 8, 16, 21, 27, and 30 in comparison

Table 1. Samples for molecular investigations from different phases of both the reactors. (* indicates sample from control reactor)

Sample number	Reactor type	Experiment Phase	DGGE Lane	Dose of Paracetamol (mg/l)	SRT (day)
1	Test	1a	R1-1	10	18
2	Test	1c	R1-3	40	11.6
3	Control	1a*	R2-2	N/A	19.2
4	Control	1c*	R2-4	N/A	19.2
5	Control	2c*	R2-10	N/A	12
6	Control	2b*	R2-8	N/A	12
7	Control	3a*	R2-12	N/A	5
8	Control	2a*	R2-6	N/A	12
9	Test	2a	R1-5	10	11
10	Test	2b	R1-7	20	7.8
11	Test	2c	R1-9	40	5
12	Test	3b	R1-13	20	3
13	Test	3c	R1-15	40	2
14	Test	3a	R1-11	10	4
15	Control	3b*	R2-14	N/A	5
16	Control	3c*	R2-16	N/A	5

Table 2. The primers and their annealing temperatures used in the PCR

Primer	Sequence (from 5' end to 3' end)	Annealing site[a]	Annealing temperature (°C)	References
Vf-GC	CCTACGGGAGGCAGCAG	341-357	55	Muyzer *et al.* 1993
Vr	ATTACCGCGGCTGCTGG	518-534	55	Muyzer *et al.* 1993
CTO189f-GC	GGAGGAAAGTAGGGGA TCG	189-207	57	Kowalchuk *et al.* 1997
CTO654r	CTAGCYTTGTAGTTTCAAACGA	654-674	57	Kowalchuk *et al*. 1997

[a]Position in the 16S rRNA of *Escherichia coli*.

Table 3. Similarity matrix for eubacterial diversity at different phases of the test and control reactors

Phase Phase	DGGELane	1a R1-1	1c R1-3	1a* R2-2	1c* R2-4	2c* R2-10	2b* R2-8	3a* R2-12	2a* R2-6	2a R1-5	2b R1-7	2c R1-9	3b R1-13	3c R1-15	3a R1-11	3b* R2-14	3c* R2-16
1a	R1-1	100	85.7	82.8	78.6	74.0	74.1	66.6	64.0	57.2	66.7	58.3	50.0	56.0	64.0	56.0	60.0
1c	R1-3	85.7	100	75.9	78.6	66.7	66.7	59.3	64.0	71.4	66.7	58.3	50.0	48.0	56.0	56.0	53.3
1a*	R2-2	82.8	75.9	100	96.6	78.6	78.6	64.3	69.2	55.2	64.3	40.0	48.0	46.2	53.9	61.5	51.6
1c*	R2-4	78.6	78.6	96.6	100	74.1	74.1	66.7	72.0	57.2	59.3	41.7	50.0	48.0	56.0	56.0	53.3
2c*	R2-10	74.0	66.7	78.6	74.1	100	100	92.3	83.3	66.7	76.9	52.2	60.9	58.3	66.7	75.0	62.1
2b*	R2-8	74.1	66.7	78.6	74.1	100	100	92.3	91.7	66.7	76.9	52.2	60.9	58.3	66.7	75.0	62.1
3a*	R2-12	66.6	59.3	64.3	66.7	92.3	92.3	100	75.0	66.7	69.2	52.2	60.9	75.0	66.7	75.0	75.9
2a*	R2-6	64.0	64.0	69.2	72.0	83.3	91.7	75.0	100	72.0	75.0	57.2	66.7	54.6	72.7	81.8	66.7
2a	R1-5	57.2	71.4	55.2	57.2	66.7	66.7	66.7	72.0	100	81.5	75.0	66.7	48.0	64.0	64.0	46.7
2b	R1-7	66.7	66.7	64.3	59.3	76.9	76.9	69.2	75.0	81.5	100	78.3	69.6	66.7	66.7	75.0	62.1
2c	R1-9	58.3	58.3	40.0	41.7	52.2	52.2	52.2	57.2	75.0	78.3	100	70.0	57.2	57.2	47.6	53.9
3b	R1-13	50.0	50.0	48.0	50.0	60.9	60.9	60.9	66.7	66.7	69.6	70.0	100	85.7	76.2	76.2	61.5
3c	R1-15	56.0	48.0	46.2	48.0	58.3	58.3	75.0	54.6	48.0	66.7	57.2	85.7	100	81.8	81.8	81.5
3a	R1-11	64.0	56.0	53.9	56.0	66.7	66.7	66.7	72.7	64.0	66.7	57.2	76.2	81.8	100	72.7	74.1
3b*	R2-14	56.0	56.0	61.5	56.0	75.0	75.0	75.0	81.8	64.0	75.0	47.6	76.2	81.8	72.7	100	74.1
3c*	R2-16	60.0	53.3	51.6	53.3	62.1	62.1	75.9	66.7	46.7	62.1	53.9	61.5	81.5	74.1	74.1	100

with phase 2c (R1-9) test reactor. Furthermore, although the control reactor (phase 2c*, lane R2-10) showed bands in lines 4, 9, 15, 19, 22, 26, 29, and 31, the test reactor (phase 2c, lane R1-9) showed no equivalent bands.

Comparing phase 3 in both the reactors indicates that there were no bands in phase 3a (R1-11) test reactor in lines 1, 4, 18, 19, 22 and 31 in comparison with phase 3a* (R2-12) control reactor. In addition, although the test reactor showed bands in lines 7, 9, and 21, the control reactor showed no equivalent bands. No bands were observed in lines 8, 21, and 26 for phase3b* (R2-14) control reactor; however bands were appeared at the same lines in the test reactor. Similarly there were no bands observed in lines 19 and 22, for (phase 3b, lane R1-13) the test reactor which the control reactor showed bands in these lines. In phase 3c* (R2-16) control reactor bands were observed in lines 9, 17, 19, 20, and 28 but were not seen in phase 3c (R1-15) of the test reactor. To find out the effects of SRT and paracetamol dose on diversity of eubacterial communities, diversities in phases which had same dose of paracetamol (e.g. 10 mg/l) were compared. The results (Fig. 2) indicate that the specific bands were observed on lines 3, 6, 9, 13, 19, and 4, 11, 21 (intensive band), and 27 for phase 1a and 2a with 10mg/l of paracetamol, respectively. Likewise, a new band was appeared on line 12 of eubacterial community in phase 1a of the test reactor in comparison with the control reactor, and elimination of bands on lines 15 and 25 in this phase. Furthermore, specific bands were found on lines 5, 7, 15 (intensive band), 21 (high intensive band), and 30 for phase 3a (10mg/l APAP) which were not found in phase 1a with the same dose of paracetamol. Eubacterial diversity pattern in phase 1c and 3c with 40mg/l dose of paracetamol, showed specific bands on lines 3, 6, 8, 13, 17, 19, 27, 29, 30 for phase 1c (Fig. 2). Whereas with

Table 4. Comparative eubacterial similarities between samples from different phases of the test and control reactors

Comparison no.	Condition being compared	Similarity (%)	Average of percent similarity	Comparison no.	Condition being compared	Similarity (%)	Average of percent similarity
1	1a & 1a*	82.8	80.1	26	1a & 1c	85.7	N/A
2	1a & 1c*	78.6		27	1a & 3a	64.0	56.7
3	1c & 1a*	75.9	77.3	28	1a & 3b	50.0	
4	1c & 1c*	78.6		29	1a & 3c	56.0	
5	2a & 2a*	72.0	68.5	30	1c & 3a	56.0	51.3
6	2a & 2b*	66.7		31	1c & 3b	50.0	
7	2a & 2c*	66.7		32	1c & 3c	48.0	
8	2b & 2a*	75.0	76.3	33	2a* &2b*	91.7	91.7
9	2b & 2b*	76.9		34	2a* & 2c*	83.3	
10	2b & 2c*	76.9		35	2b* & 2c*	100	
11	2c & 2a*	57.2	53.9	36	3a* & 3b*	75	75
12	2c & 2b*	52.2		37	3a* & 3c*	75.9	
13	2c & 2c*	52.2		38	3b* & 3c*	74.1	
14	3a & 3a*	66.7	71.2	39	2a &2b	81.5	78.3
15	3a & 3b*	72.7		40	2a & 2c	75	
16	3a & 3c*	74.1		41	2b & 2c	78.3	
17	3b & 3a*	60.1	65.9	42	3a & 3b	76.2	81.23
18	3b & 3b*	76.2		43	3a & 3c	81.8	
19	3b & 3c*	61.5		44	3b & 3c	85.7	
20	3c & 3a*	75.0	79.4	45	2a & 2b	81.5	N/A
21	3c & 3b*	81.8		46	2b & 2c	78.3	N/A
22	3c & 3c*	81.5		47	2a & 2c	75.0	N/A
23	1a & 2a	57.2	60.7	48	3a & 3b	76.2	N/A
24	1a & 2b	66.7		49	3b & 3c	85.7	N/A
25	1a & 2c	58.3		50	3a & 3c	81.8	N/A

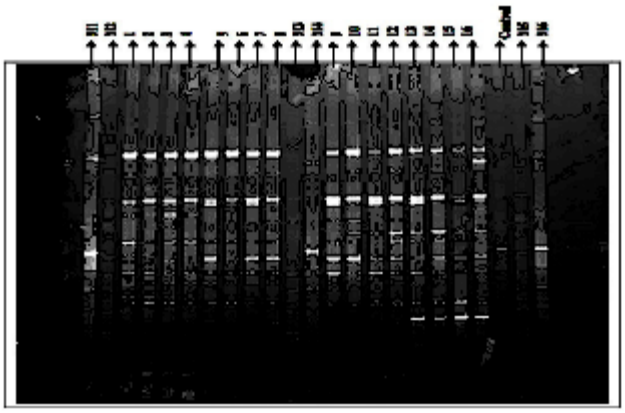

Fig. 1. DGGE profile of eubacterial communities of the test and control reactors at different SRT and doses of paracetamol. (M= the marker band). Lane definitions given in Table 1)

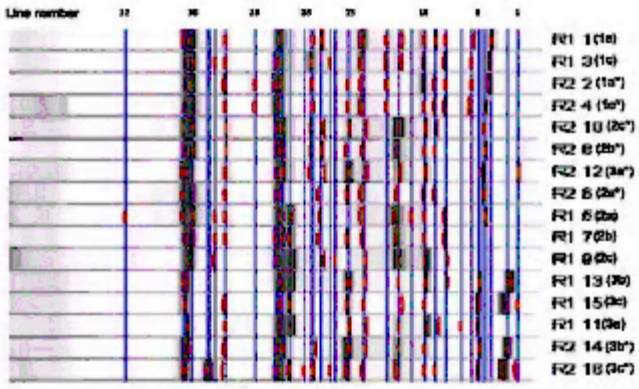

Fig. 2. DGGE of PCR-amplified 16S rDNA fragments showing general diversity of eubacterial communities at different phases of the reactors. Each line is shown by a vertical blue line

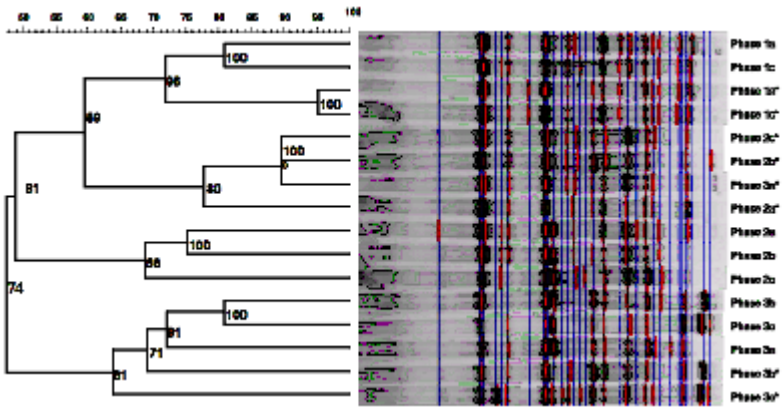

Fig. 3. Dendogram for similarity of eubacterial communities in the test and control reactors at different SRTs and doses of paracetamol

decreasing SRT in phase 3 of the test reactor, different bands were observed on lines 1, 2, 5, 15, 21, 31 for phase 3c and also a new band was produced on line 21 that was coincided with the removal of bands on lines 19 and 22 (Fig. 2).

Similarity analysis of Eubacterial diversity at different SRT

The similarities of banding patterns for different SRT conditions were investigated and statistically analysed using the bionumeric software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium) to determine how much the biomass diversity changed from one condition to another. Assessments involved the cluster analysis and the production of similarity matrices and dendograms. Each lane was assumed to represent the microbial population of the reactor at a particular sludge retention time, whereas the number of the bands (number of the species in each lane) demonstrated the size of the diversity of the microbial population for that reactor condition. In order to understand how the similarity of the microbial diversity changed with different SRT and paracetamol dose, further analysis was required using the coefficient values in Table 3. Consequently, a dendogram (Fig. 3) was produced for simpler visualisation of the inter-relationship of the reactor conditions. On the dendogram (Fig. 3) all phases are shown on the right. The lengths of interconnecting lines and the values written on them show the similarity of the samples. The length of the lines increases as the similarity decreases. The comparative eubacterial similarities between the samples in different phases of the test and control reactors are summarised in Table 3. From this table it can be seen that the average eubacterial similarity in the control reactor decreased with decreasing SRT from (91.7±8.35%) in phase 2 (average of a, b, c) to (75.00±0.90%) in phase 3 (average of a, b, c) showing a change from very high to high level of similarity[4]. On the contrary, high (78.3±3.25%) and very high (81.23±4.8%) levels of similarity were found for phases of 2 (average of a, b, c) and 3 (average of a, b, c) in the test reactor, respectively.

A close eubacterial similarity was observed between phase 1 (average for a, c) of the test and phase 1 (average for a, c) of the control reactors and the average eubacterial similarity varied between 80.1-77.3% demonstrating a very similar microbial community even though a significant difference was observed for the specific COD utilisation rates in these phases ($p<0.05$, Mann-Whitney test). However, no significant difference was observed between the values of the specific TOC utilisation rates in phase 1a and equivalent values in the control reactor. Statistical results indicate that there was no significant difference overall between eubacterial similarity in phase 2 (average of a, b, c) of the test and control reactors ($p>0.05$). In spite of close similarity of these reactors, a significant difference was observed between the values of the mean COD utilisation rate in phases 2b and 2c from the related values in phase 2 of the control reactor ($p<0.05$, Mann-Whitney test).

Comparison of the average eubacterial similarity in both the reactors (i.e. over the three sub-phases a, b, c) indicates that no significant difference was found between eubacterial similarity in phase 3 (average of a, b, c) of the test and control reactors ($p>0.05$). Regardless this relationship, the specific COD utilisation rate in phases 3a and 3b were significantly different from the amount of the specific COD utilisation rate in the equivalent phase 3 periods of control reactor ($p<0.05$, Mann-Whitney test). Furthermore, a significant difference was also observed between the mean TOC utilisation rate in phases 3a, 3b, and 3c with corresponding equivalent values in phase 3 of the control reactor ($p<0.05$, Mann-Whitney test). There was no significant difference between eubacterial similarity in phase 2 (average of a, b, c), and phase 1 (average of a, b, c) of the test reactor ($p>0.05$), though the values of mean COD and TOC utilisation rate in phases 1a (SRT=18 d, APAP 10mg/l), 1b (SRT=14.6, APAP 20mg/l), and 1c (SRT=11.6 d, APAP 40mg/l) of this reactor were significantly different from corresponding mean COD utilisation rates in the equivalent phase 2 condition. High eubacterial similarity (85.7%) was observed between phases 1a (SRT=18.2 d, APAP10mg/l) and 1c (SRT=11.6 d, APAP 40mg/l) of the test reactor, and evidence that a very similar microbial community existed was confirmed by the results of carbon removal efficiency within the reactor system which showed significant difference between the values of mean COD and TOC utilisation rate in these phases. These results indicate that in general no significant difference

was found between the eubacterial similarity in phases 1 and 2 with that in phase 3 of the test reactor ($p>0.05$). In spite of this similarity in eubacterial community, there was significant difference between the values of mean COD and TOC utilisation rate in these phases.

Comparison of phases 2a, 2b, and 2c revealed that they had high level of eubacterial similarity (75-81.5%) that decreased with decreasing SRT and increasing dose of paracetamol. Although the reactor performance eubacterial similarity was high, a significant difference was observed between the values of mean COD and TOC utilisation rate in these phases. Also a high eubacterial similarity (76.2-85.7%) was observed between phases 3a, 3b, and 3c, which increased with decreasing SRT and increasing dose of paracetamol, and it was confirmed from the carbon removal efficiency results that there were no significant differences between the mean COD and TOC utilisation rates in phase 3.

DISCUSSION

There were two parameters i.e. SRT and dose of paracetamol that could have affected the eubacterial diversities and as well biodegradation rates of paracetamol in this study. The results showed that the changes in eubacterial diversities in different phases of experiment were not linked to the dose of paracetamol itself; rather changes were linked to the changes in SRT within the reactor system. This is supported by the SOUR and HPLC results which showed the relationship between the dose of paracetamol and percent inhibition of microorganisms was fairly weak and no inhibition of SOUR was observed for any paracetamol dose. Furthermore, the HPLC results indicate that the degree of paracetamol degradation in phase 1a (SRT=18 d, APAP 10mg/l), 1b (SRT=14.6 d, APAP 20mg/l), and 1c (SRT=11.6 d, APAP 40mg/l) was 99.24, 99.19, and 99.17%, respectively, demonstrating that even at high concentrations, paracetamol degradation was high and that there was only a very small difference between the degree of degradation at different paracetamol doses. In phase 2, at SRT (11, 7.8, and 5 day), the degree of degradation varied between 98.6 and 89.72% and eventually in phase 3, the rate of degradation decreased dramatically and varied between 86.5 and 79.68% when SRT was low (4, 3, and 2 day). These results showed that the rate of degradation of paracetamol in phase 1 with three different doses of paracetamol was visually identical whereas the rate of degradation decreased with decreasing SRT in phase 2 and 3. This means that the degradation rate of paracetamol was not dependent on the dose of paracetamol, rather on the SRT within the reactor system. Overall, the results suggest that despite the observed similarities in eubacterial community composition, the carbon removal efficiencies of the two reactors were different; demonstrating that similarly composed microbial communities can be functionally different. In contrast, Kaewpipat and Grady (2002)[9] found that similar COD removal efficiencies in two reactors could be obtained when the community composition was different. Furtheremore, the results of present study show that the microbial community could adapt to changing environmental conditions or critical control parameters such as SRT and paracetamol dosage, which agreed with the findings of LaPara *et al.* (2002)[8], who showed the microbial community had a stable structure and was able to adapt in response to perturbations and sustain high effluent quality. The results for eubacterial community performance in the current study also agree with the findings of Hossain (2004)[10]. He showed, in a suspended growth reactor investigating the fate and effects of paracetamol that the bacterial community structure did not change in comparison with the control culture, after successive increasing doses of paracetamol were applied to the test reactor. Furthermore, similarity analysis of the PCR-DGGE in his research suggested that the microbial communities developed higher numbers of protozoa as a new species within them, as the doses were increased, which means that the microbial communities were making adaptations and had completely stabilised to the changing environment. The overall conclusion of the current study is that the bacterial community structures in the test and control reactors were shown to be relatively stable over time in response to a wide range of operating conditions and paracetamol doses.

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