Elucidation of the microbial community structure within a laboratory-scale activated sludge process using molecular techniques

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Abstract

Microbial community structures were analysed in activated sludge samples from the anoxic and aerobic zones of a laboratory-scale modified Ludzack-Ettinger (MLE) system. Analyses were performed using fluorescent *in situ* hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE). The r-RNA targeted oligonucleotide probes used for FISH targeted the alpha, beta and gamma subclasses of the gram-negative family *Proteobacteria*, the high G+C content gram-positive bacterium of the actinomycetes branch and the total Eubacteria present. It was found that approximately 75 to 80% of total cells were detected with the DNA specific fluorochrome DAPI, hybridised with a specific eubacterial probe for the anoxic and aerobic zones. Results confirmed the dominance of the alpha and gamma subclasses of the *Proteobacteria* in the anoxic zone whilst the aerobic zone was dominated with the beta subclass of the *Proteobacteria*. The high G+C content bacterium represented the least dominant species present within each of the two zones. The DGGE technique employed in this study analysed the genetic diversity of the microbial community present in each of the anoxic and aerobic zones. The profile for each of the zones revealed a number of consistent bands throughout the duration of the laboratory-scale process. However, the profiles obtained suggested that a diverse microbial community existed within the aerobic and anoxic zones. These results obtained from the application of fluorescent *in situ* hybridisation (FISH) and PCR-DGGE yields a more precise understanding of the microbial community structure and genetic diversity present in domestic wastewater of a laboratory scale treatment process. COD and nitrogen mass balances were conducted to confirm the acceptance of the results obtained for each batch as an indication of the system performance for the MLE model. Nitrogen mass balances indicated an upset in the nitrogen levels for wastewater batches two and seven. The carbon mass balance fell in the range of 92.4% and 105.9% and the nitrogen mass balance fell in the range of 98.4% and 160.0%.

Keywords: fluorescent *in situ* hybridisation (FISH), denaturing gradient gel electrophoresis (DGGE), COD and nitrogen mass balances.

Introduction

Treatment of activated sludge within the modified Ludzack-Ettinger (MLE) system involves the removal of biodegradable organics, unsettleable suspended solids and other constituents. These biodegradable organic compounds are degraded by bacteria in an aerated reactor and the biomass is allowed to settle and concentrate in a clarifier (Muyima et al., 1997). The system is either a continuous or semi-continuous aerobic method for wastewater treatment involving carbon oxidation and nitrification. This process has been developed for the removal of carbon, nitrogen and phosphate and it is well known that prokaryotic micro-organisms catalyses these main biological processes in wastewater treatments (Juretschko et al., 2002). With nutrients and oxygen present the microbial population in the wastewater achieves optimal growth and respiration (Muyima et al., 1997). The dynamics and diversity of the microbial populations in activated sludge have been analysed by culture-dependent methods, however many members of the natural bacterial communities are still un-culturable (Wagner et al., 1994). Hence microscopic identification based on morphological characteristics was researched and developed in a culture-independent manner by direct rRNA sequence retrieval, where nucleic acid probes which are complementary to the rRNA are used as tools to monitor population dynamics amongst bacteria (Amann, 1995). FISH makes use of rRNA targeted probes which are frequently applied in order to quantify the composition of microbial communities present. This procedure is based on the comparative analysis of macromolecules, mostly ribosomal RNA molecules and fluorescent derivatives of such probes. These probes have been applied successfully for *in situ* enumeration of defined groups of micro-organisms present in activated sludge (Manz et al., 1994).

Fluorescently monolabelled, rRNA targeted oligonucleotide probes detect individual cells, allowing whole-cell hybridisation with rRNA targeted probes to be a suitable tool inferring phylogenetic evolution hence the cell morphology of an uncultured microbe and its abundance can be determined *in situ* (Wagner and Amann, 1997). Cell numbers of the bacteria can be obtained by enumeration under an epi-fluorescent microscope. Enumeration procedures involve the use of a semi-automated digital image analysis tools in order to quantify the fluorescently labelled bacteria in samples (Daims et al., 2001).

The molecular technique used for analysing the structure and providing a profile of the microbial population present in wastewaters is the denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993). DGGE that is performed on 16S rRNA genes has been used to produce a genetic fingerprint of mixed microbial communities (Kaewpipat and Grady, 2002). The

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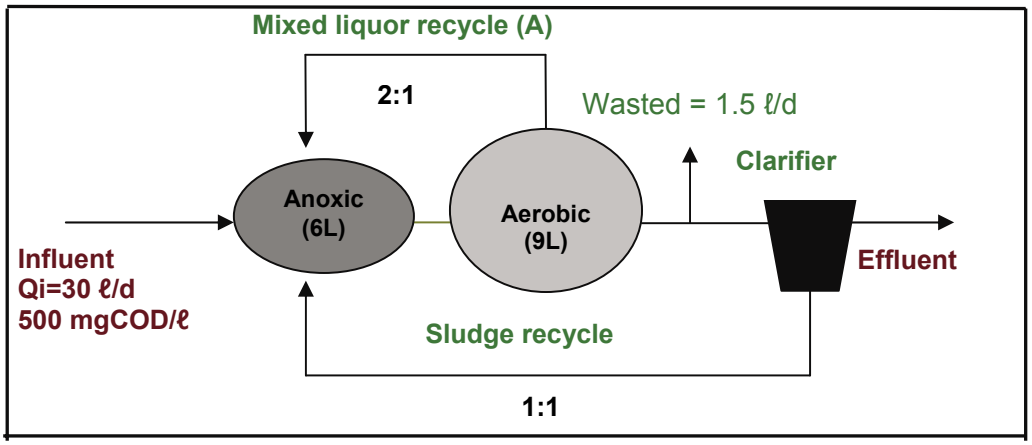

Figure 1
Design of the modified Ludzack-Ettinger (MLE) Process (Lilley et al., 1997)

numbers of bands that are obtained from DGGE profiles provide an estimate of the different microbial species present. The intensity of each band provides a reflection of the relative abundance of each species (Nasu, 2000) because the primers used to amplify a fragment of the 16S rRNA produces a quantitative relationship between the gene copy number and the PCR-DGGE band intensity (Kaewpipat and Grady, 2002). However, many factors can prevent the formation of the number and intensity of the bands in the DGGE gel, therefore representing the exact number and abundance of species in a microbial community can be difficult however DGGE is a sensitive and a rapid technique that detects most singe-base variations when a G-C clamp is added to one of the primers in the PCR process. This provides a profile of changes that occur within a microbial community or between microbial communities (Kaewpipat and Grady, 2002).

In this study a combination of molecular techniques, FISH and PCR-DGGE was used to monitor the microbial composition and examine the microbial community population shifts within a steady state laboratory-scale parent anoxic and aerobic activated sludge system.

Methods and materials

Maintenance and analyses of the MLE system

The laboratory-scale modified Ludzack-Ettinger process illustrated in Fig. 1 was fed with 30 ℓ of mainly domestic wastewater obtained from Southern Wastewater Works (Durban, South Africa) on a 10 d sludge age process. After reaching steady state, the MLE system was run for a duration of 18 batches. The wastewater was diluted with tap water to give an influent feed of approximately 500 mgCOD/ℓ from the original 800 mgCOD/ℓ. The process was maintained by wasting 1.5 ℓ/d of the mixed liquor from the aerobic reactor. Samples from the influent, anoxic, aerobic and effluent were analysed daily for the following: Chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), mixed liquor suspended solids (MLSS), volatile suspended solids (VSS) and nitrate analysis (nitrite tests were not performed because the nitrite concentration was 2% less than nitrates). Microbial community analyses were performed on samples obtained from the aerobic and anoxic reactors. The oxygen utilisation rate (OUR) was monitored within the mixed liquor with an online dissolved oxygen (DO) controller (hi-tech micro-system) according to Randall et al. (1991). The pH was kept constant at 7.5 and the temperature was kept constant at 20ºC.

In situ hybridisation

Sampling: Grab samples were collected from the anoxic and aerobic zones of the laboratory-scale MLE activated sludge system and were immediately fixed with 4% paraformaldehyde for gram-negative bacteria and with absolute ethanol for gram-positive bacteria according to Amann (1995). Samples were then stored in a 1:1 mixture of phosphate-buffered saline (PBS: 130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) and absolute ethanol at 4ºC. The fixed samples were then sonicated at 8 W for 8 min with a

TABLE 1
The oligonucleotide probes used for the FISH analysis

Probe	Specificity	Sequence (5' - 3')	rRNA target site (*E. coli* numbering)	Formamide (%)	Reference
EUB388 I,II,III	Domain Eubacteria	I: GCTGCCTCCCGTAGGAGT II: GCAGCCACCCGTAGGTGT III: GCTGCCACCCGTAGGTGT	16S, 338 - 355	20	Yeates et al. (2003)
ALF1b	*Proteobacteria* (Alpha)	CGTTCGYTCTGAGCCAG	16S, 19-35	20	Wagner et al. (1993)
BET42a	*Proteobacteria* (Beta)	GCCTTCCCACTTCGTTT	23S, 1027 - 1043	35	Wagner et al. (1993)
GAM42a	*Proteobacteria* (Gamma)	GCCTTCCCACATCGTTT	23S, 1027 - 1043	35	Wagner et al. (1993)
HGC69a	Bacteria with high G+C content	TATAGTTACCACCGCCGT	16S, 1901 - 1918	25	Onuki et al. (2000)

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Vibracell Sonicator, in order to break up the bacterial flocs present.

Total cell counts: Membrane filtration was performed according to Hicks et al. (1992). Fixed samples were sonicated and diluted (dilution factor of 200) with phosphate-buffered saline (PBS) and 1% nonidet. Nucleopore filters with a pore size of 0.2 µm were pretreated with 0.3% Sudan black and placed on top of a 0.45 µm backing filter. The samples were stained with DAPI (4, 6-diamidino-2-phenylindole) (Sigma, Deisenhofen, Germany) at an end concentration of 1.25 µg/mℓ. The Zeiss Axiolab microscope (50W high-pressure mercury bulb and Zeiss filter set 01) fitted for epifluorescence microscopy was used with the Zeiss image analysis software in order to quantify the fluorescing cells.

Pretreatment and whole cell hybridisation: The fixed and sonicated samples were immobilised onto pretreated slides and dehydrated with 60%, 80% and absolute ethanol to prepare for whole-cell hybridisation. Samples were hybridised with appropriate hybridisation buffers and probed *in situ* with oligonucleotide probes listed in Table 1 according to Amann (1995). The probes were either labelled with tetramethylrhodamine-5-isothiocyanate or 5(6)-carboxyfluorescein-*N*-hydroxysccinimide-ester. The probe-conferred fluorescence was detected with a Zeiss Axiolab microscope (50W high-pressure mercury bulb and Zeiss filter sets 09 and 15). Dual staining of samples with DAPI and fluorescent rRNA probes was performed according to Hicks et al. (1992).

PCR-DGGE analyses

DNA extraction: Samples were taken from each of the two zones of the laboratory-scale activated sludge reactor and exposed to the phenol-chloroform extraction technique which produced a high yield of DNA from the 0.2 g activated sludge pellet samples. The samples were incubated with lysis buffer and then exposed to a cycle of freezing and thawing the cells, which was carried out five times. The removal of proteins and RNA contaminants was done by treating the samples with phenol-chloroform (25:24) and 98% chloroform (Mayer and Palmer, 1996; Kuhn et al., 2002). The extracted DNA was precipitated with absolute isopropyl alcohol and quantification of the DNA was done using a spectrophotometer according to Maniatis et al. (1982).

Polymerase chain reaction (PCR): Enzymatic amplification of the variable V3 region of the 16S rDNA was achieved with primers of conserved regions to the 16S rRNA genes. The nucleotide sequences of the primers are: 341f (GC-Clamp): 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3' and 1492R: 5' TAC GGC TAC CTT GTT ACG ACT T 3', corresponding relatively to positions 341-358 and 1492-1512 in *Escherichia coli*. PCR amplification was performed with a Hybaid PCR sprint Thermal Cycler (Hybaid Limited, United Kingdom) as follows: 10 µℓ of 10 x PCR buffer without $MgCl_2$ (50 mM KCl, 10 mM Tris-HCl, pH 8.4.), 6 µℓ of 2.5 mM $MgCl_2$, 10 µℓ of 10 mM dNTP (dATP, dCTP, dGTP, dTTP each at a concentration of 10 mM), 10 µℓ of 10 ng/µℓ Genomic DNA Template, 2 µℓ of 50 pM stock solution of primers and 1 µℓ of 5 units/µℓ Taq polymerase were added to a 0.5 mℓ Eppendorf tube which was filled to a volume of 100 µℓ with sterile distilled water. The program used for amplification is shown in Table 2. The amplified products were analysed by electrophoresis in 2% (wt/vol) agarose gels and then stored at 4°C before analysis by DGGE (Giovannoni, 1991).

TABLE 2
Hybaid PCR thermal cycler program

Stages		Temperature	Time	Cycles
STAGE 1	STEP 1	94ºC	4 min	x 1
STAGE 2	STEP 1	94ºC	1 min	x 35
	STEP 2	53ºC	1 min	
	STEP 3	72ºC	2 min	
STAGE 3	STEP 1	72ºC	4 min	x 1
HOLD		4ºC		

Denaturing gradient gel electrophoresis (DGGE): DGGE was performed with the Bio-Rad D-GENE system. The DGGE gels that were formed comprised 7.5% polyacrylamide (acrylamide: *N,N'*-methylenebisacrylamide, 37.5:1) in 1xTAE buffer (0.04 M Tris Base, 0.02 M sodium acetate, 1.0 mM EDTA [pH 8.0]) with gradients of 30% to 50% denaturants. The gels polymerised with the addition of 1% ammonium persulphate and *N,N,N',N'*-tetra-methyl-ethylenediamine (TEMED). The PCR products were loaded onto the parallel DGGE gel and electrophoresis took place at a constant voltage of 200 V and temperature at 60°C. Following electrophoresis the gel was stained with ethidium bromide (10 µg/mℓ) in distilled water for 15 min, thereafter the gel was immersed in distilled water to remove all unbound ethidium bromide for 10 min. The gel was viewed on a UV Transilluminator at 302 nm and photographed with a Polaroid camera (Hoefer Macrovue UV Transilluminator, United States of America) (Bio-Rad Laboratories, 1994).

Results and discussion

Modified Ludzack-Ettinger System

The reliability of the experimental data obtained from the steady state analyses which included COD, TKN, OUR, nitrates and mixed liquor analyses is represented in Table 3. The chemical oxygen demand concentration calculated for the MLE process was 90.9%, thus indicating that a high amount of oxygen (as shown with the OUR concentrations) was required for the proliferating bacteria and their biological reactions. The nitrate concentrations of the anoxic, aerobic and effluent zones are as follows: the anoxic zone supported high rates of denitrification with an average of 1.5 mgN/ℓ present whilst the aerobic zone had a higher level of concentration with an average of 7 mgN/ℓ present, indicating an increase in nitrification; however, the highest concentration of nitrates was present in the effluent with an average of 11.3 mgN/ℓ. These results suggest that a high denitrification potential of the MLE process is prominent; however, complete denitrification is not possible due to the absence of secondary reactors, which is clearly shown in the effluent results. The mixed liquor was analysed by performing volatile suspended solids (VSS), chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN). VSS analyses showed a high amount of biomass present in the mixed liquor, which accounted for the increased levels of chemical oxygen demand and total Kjeldahl nitrogen concentrations (results for only 3 wastewater batches are shown).

In situ hybridisation

Quantification of activated sludge using fluorescence *in situ* hybridisation (FISH) with rRNA targeted oligonucleotide

TABLE 3
Steady state results for the MLE anoxic and aerobic activated sludge system

WW Batches	COD (mg/ℓ)		TKN (mg/ℓ)		Nitrate (mgN/ℓ)			OUR mgO/ℓ/H	Mixed liquor (mg/ℓ)		
	INF	EFF	INF	EFF	Anoxic	Aerobic	Eff		VSS	COD	TKN
1	502.6 (48.8)	46.0 (10.1)	38.8 (6.3)	5.3 (2.1)	0.6 (1.0)	3.9 (1.9)	7.9 (1.5)	30.6 (0.4)	2352.4 (153.1)	3720 (553.2)	234.6 (20.7)
9	506 (16.1)	46.8 (7.8)	41.9 (3.4)	3.5 (0.9)	1.2 (1.0)	6.9 (1.6)	10.0 (1.3)	26.7 (1.9)	2341.6 (251.9)	3652.8 (426.3)	223.7 (21.9)
18	449.2 (40.0)	39.1 (8.2)	42.1 (5.4)	4.4 (0.8)	0.9 (0.5)	7.2 (2.3)	8.9 (2.5)	30.7 (0.7)	1914.5 (239.2)	2903.7 (372.1)	201.3 (49.3)

TABLE 4
COD and nitrogen mass balances for the parent laboratory-scale anoxic and aerobic sludge system

Batches	Mass balances (%)		Batches	Mass balances (%)	
	COD	Nitrogen		COD	Nitrogen
1	105.9	105.6	10	102.1	102.8
2	87.3	119.2*	11	95.3	102.7
3	95.1	102.6	12	94.8	101.7
4	98.6	102.6	13	105.9	102.7
5	96.0	100.7	14	97.0	105.3
6	97.7	108.4	15	97.3	98.4
7	89.5	160.0*	16	95.9	106.4
8	94.6	109.0	17	98.7	109.1
9	92.4	104.6	18	96.9	103.5

* *Indicates the nitrogen mass balance results which were higher than the acceptable ranges.*

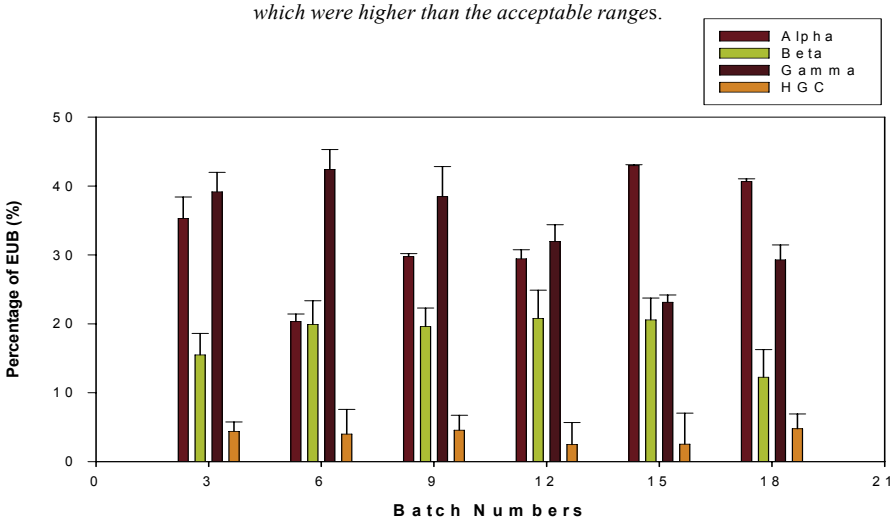

Figure 2
The total cell counts for the oligonucleotide probes as a percentage of the total eubacterial cells from the anoxic zone

probes provided novel insights with respect to the structure and dynamics of the microbial communities present (Daims et al., 2001). The anoxic and aerobic zones were analysed as shown in Figs. 2 and 3. The activated sludge samples were first analysed by DAPI staining and then *in situ* hybridisation. For the anoxic samples and aerobic samples 81.4% (±3.6%) and 79.2% (±4.2%) respectively were visualised by DAPI which was detected with the bacterial probe EUB338 I, II, III. The samples analysed in the anoxic and aerobic zones showed the dominance of *Proteobacteria* throughout the 18 batches, which accounted for 82.3% (±1.3%) and 79.2% (±2.1%) respectively. The consistency in results obtained from the 18 batches of the anoxic and aerobic wastewaters validates the steady state of the MLE system. Figures 2 and 3 display the *in situ* distribution among the alpha, beta, gamma subclasses and high G+C content bacteria present in the MLE anoxic and aerobic activated sludge samples. Figure 2 illustrated the average total cell counts of the oligonucleotide probes that hybridised with the anoxic samples as a percentage of the total eubacterial cells. The alpha and gamma subclasses of the *Proteobacteria* were dominant within the anoxic zone with 33.3% (±1.04%) and 32.2% (±2.87) (average of the 18 batches) respectively. The less dominant beta subclass of the *Proteobacteria* from the sample hybridised with 16.7% (±2.54%) of the beta subclass probe and the least dominant high G+C content bacterial cells hybridised to 4% (±3.25) to the HCG oligonucleotide probe with respect to the total eubacterial cells present. In the anoxic zone, Batches 13 and 15 showed the highest dominance of the alpha subclass with 41.8% (±0.8) and 42.9% (±2.5) respectively whilst the highest gamma dominance was present in Batches 2 and 6 with 40.3% (±1.5) and 40.4% (±3.2) respec-

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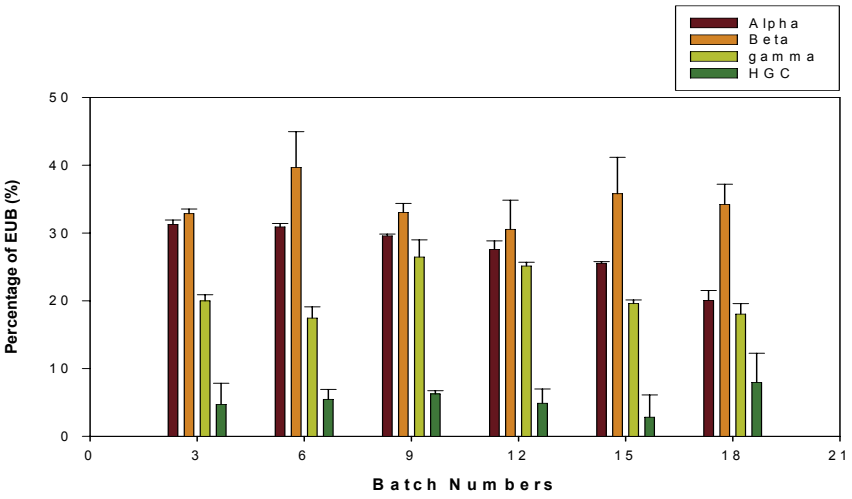

Figure 3 (right)
The total cell counts for the oligonucleotide probes as a percentage of the total eubacterial cells from the aerobic zone

tively (certain data not shown).

Members of the beta subclass of *Proteobacteria* were detected as the most abundant bacteria present in the aerobic mixed liquor comprising 32.8% (±2.96%) of the total Eubacteria present. The alpha subclass occupied 25.8% (±0.69%), whilst the gamma subclass hybridised 20.5% (±1.72%) of the cells hybridising with probe EUB 338 I, II, III and the least abundant high G+C content bacteria occupied 5.7% (±2.12%) of the total eubacterial cell counts. The high G+C content bacteria present in both the anoxic and aerobic zones were the least abundant species present; however, higher numbers were noted in the aerobic zone, with the highest abundance presence in Batch 10 with 9.4% (±3.2%).

The sum of the group-specific probes was 86.3% (±4.2%) and 84.9% (±2.4%) for the anoxic and aerobic samples respectively; these counts were higher than the 81.4% (±3.6%) (anoxic zone) and 79.2% (±4.2%) (aerobic zone) which was obtained from the cells hybridising with the bacterial probe EUB338 I, II, III. According to Snaidr et al. (1997), this is due to an underestimation of the total cell count as determined by the DAPI staining technique.

Micrographs represented in Fig. 4 (anoxic zone) and Fig. 5 (aerobic zone) visually represent the relative number of bacteria that were hybridised with either fluorescein or rhodamine labelled oligonucleotide probes. The micrographs depict the cells stained with DAPI and the corresponding *in situ* hybridisation with the appropriate oligonucleotide probe.

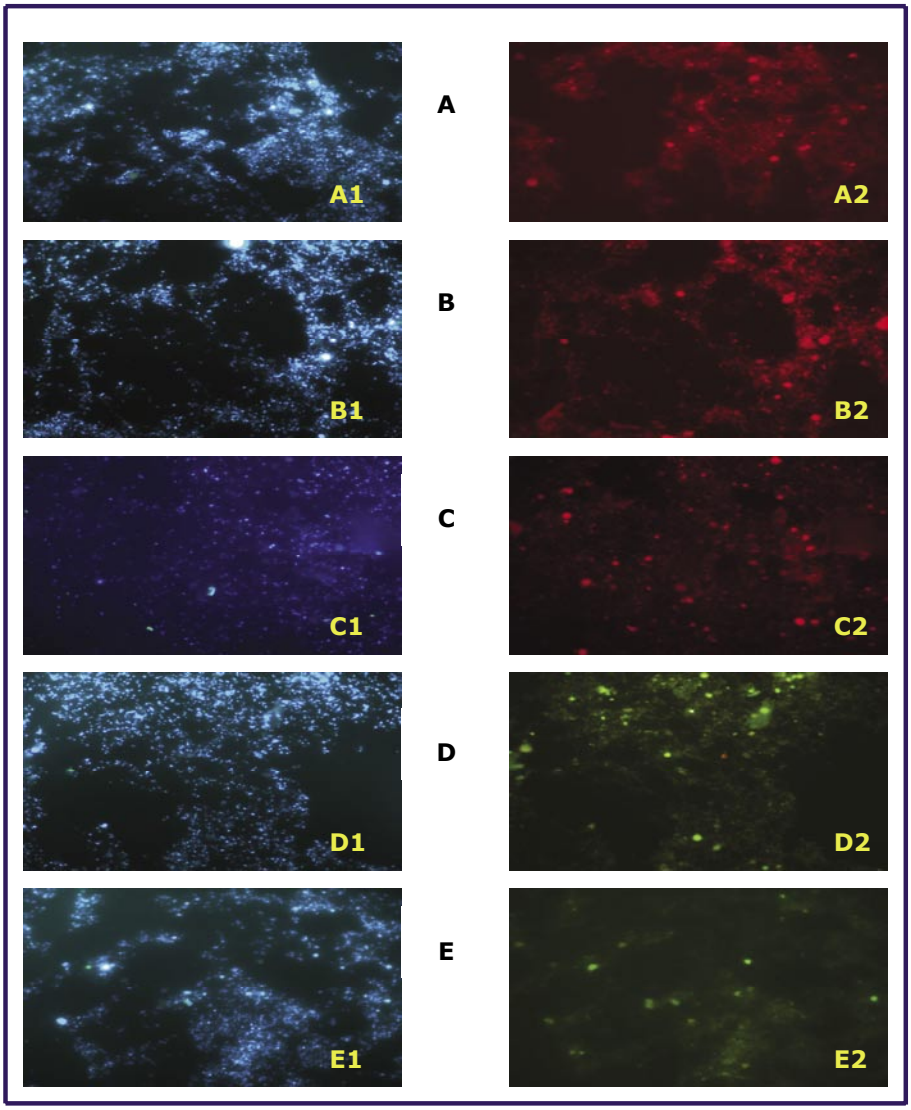

Figure 4
In situ hybridisation of the anoxic activated sludge samples. (A1 to E1-Left) DAPI stain (A2 to E2-Right) in situ hybridisation with probes. Micrographs are shown for identical microscopic fields. A: DAPI stain and corresponding hybridisation with rhodamine-labelled EUB338I, II, III; B: DAPI stain and corresponding hybridisation with rhodamine-labelled ALF1b; C: DAPI stain and corresponding hybridisation with rhodamine-labelled BET42a; D: DAPI stain and corresponding hybridisation with fluorescein-labelled GAM42a; E: DAPI stain and corresponding hybridisation with fluorescein-labelled HGC69a probe.

PCR-DGGE

DGGE performed on enzymatically-amplified 16S DNA encoding rRNA is widely used as a molecular approach for analysing microbial communities. Universal primers incorporated in the PCR

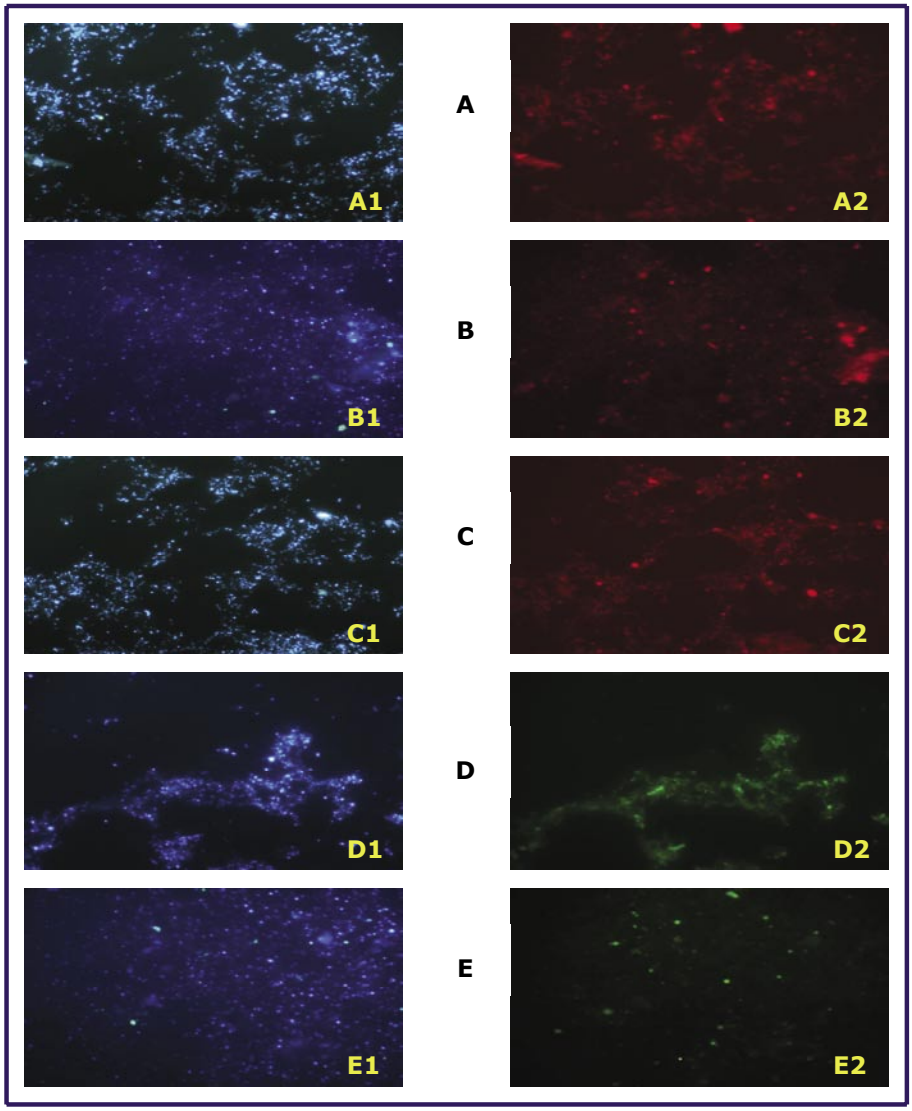

Figure 5
In situ hybridisation of the aerobic activated sludge samples. (A1 to E1-Left) DAPI stain (A2 to E2-Right) in situ hybridisation with probes. Micrographs are shown for identical microscopic fields. A: DAPI stain and corresponding hybridisation with rhodamine-labelled EUB338I, II, III; B: DAPI stain and corresponding hybridisation with rhodamine-labelled ALF1b; C: DAPI stain and corresponding hybridisation with rhodamine- labelled BET42a; D: DAPI stain and corresponding hybridisation with fluorescein-labelled GAM42a; E: DAPI stain and corresponding hybridisation with fluorescein-labelled HGC69a probe.

enabled the DGGE profile to resolve a wide range of bacterial diversity (Teske et al., 1996). DGGE conditions were optimised by determining the most appropriate denaturation concentrations in order to provide a high resolution banding pattern. Muyzer et al. (1993) suggested that a 40 base-pair G-C rich clamp is added to the 5' end of the forward primer involved in the amplification of DNA to provide a better resolution of the PCR-amplified DNA fragments. Figures 6 and 7 show the DGGE banding pattern obtained from the anoxic and aerobic zones of the MLE system respectively. The banding pattern provides a profile of the bacterial species present.

Figures 6 and 7 represent the profile of the bacterial species present within the anoxic and aerobic zones respectively. The profiles obtained from the two zones show consistency in the bands, with no major shifts in the microbial community, therefore these results validate the steady state of the MLE system. Figure 6 illustrates the bacterial species, which is present throughout the 18 batches from the anoxic samples (multiple bands were visible however the low intensity bands were not able to be captured with a Polaroid camera). It is noted that the banding positions varied between the 18 batches indicating the presence of many species of denitrifying bacteria.

The profile obtained from the DGGE pattern of the aerobic zone (Fig. 7) showed the presence of multiple dominant bacterial species present throughout the 18 batches. Batches 7, 8 and 9 showed a difference in banding with additional bacterial species present as illustrated in Fig. 7 as A and B; however, these low-intensity bands indicate that the additional bacterial species present were less dominant. The DGGE banding pattern from Figures 6 and 7 confirm the difference in the number of the bacterial species present in each of the aerobic and anoxic reactors.

Conclusions

The molecular techniques incorporated in this research, FISH and PCR-DGGE, gave an insight into the structure of the bacterial populations present within the anoxic and aerobic zones of the steady-state modified Ludzack-Ettinger (MLE) process. System performance was analysed throughout the research with reference to the steady-state behaviour of the MLE system and the COD and nitrogen mass balances. It was noted that the stable steady state of the system impacted on the microbial community within each of the anoxic and aerobic zones, as shown by the *in situ* investigation which showed consistencies in results. The profiles obtained for each of the zones implied that microbial community structure at a species level was fairly consistent. The

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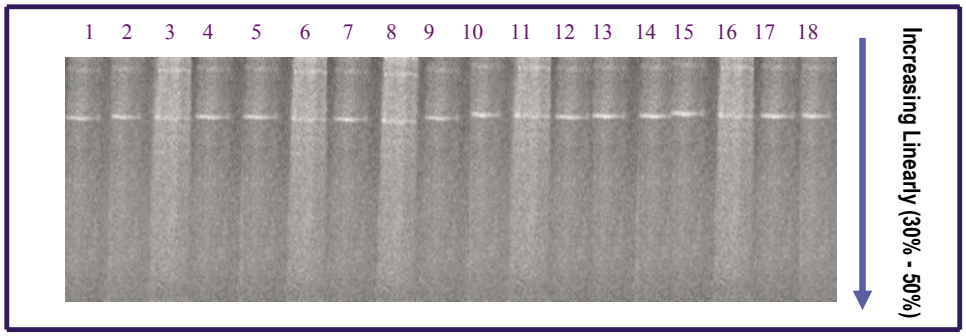

Figure 6
Image of an ethidium bromide stained DGGE profile of 18 PCR amplified DNA products obtained from the anoxic zone of the laboratory-scaled MLE activated sludge process.

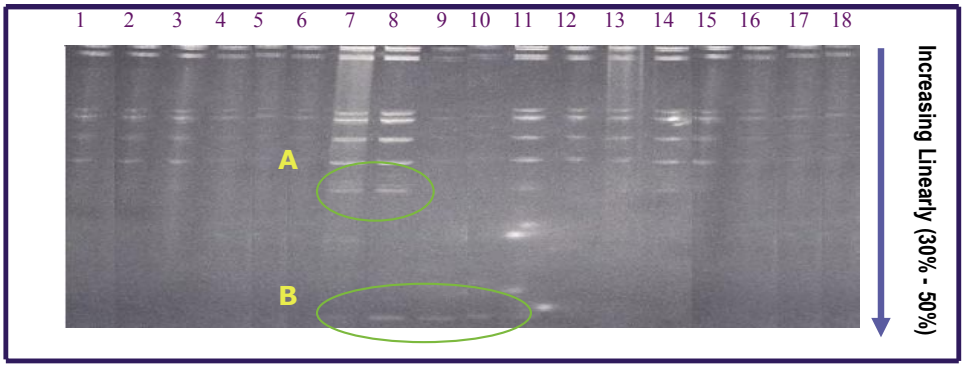

Figure 7
Image of an ethidium bromide stained DGGE profile of 18 PCR amplified DNA products obtained from the aerobic zone of the laboratory-scale MLE activated sludge process.

COD and nitrogen mass balances calculated showed the reliability of the system from an engineering perspective for the wastewater batches; however, the nitrogen mass balances showed an upset in the nitrogen balance for the system during Batches 2 and 7. However, there were no distinct changes in the microbial population during Batches 2 and 7 when investigated *in situ* and with reference to the DGGE profiles obtained. This information is useful for the monitoring of the activated sludge processes because it was found that the zones have unique bacteria proliferating in them even though there is a continuous established flow rate of the influent that enters the system to the effluent that leaves the system.

Further molecular analyses are currently being investigated to determine function and spatial distribution of the main bacteria, which are responsible for the denitrification and nitrification.

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