Characterization of pathogenic bacteria in a UASB-polishing pond system using molecular techniques

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Abstract: The work investigates the presence of pathogenic bacteria using PCR technique in a wastewater treatment system comprised by a UASB reactor and a polishing pond. Additionally, quantification of *Escherichia coli* bacteria by FISH technique and Defined Substrate Technique - Colilert®, in raw sewage and pond effluent was undertaken. *Escherichia coli* and *Salmonella enterica* subsp. *enterica* were detected in raw sewage, UASB effluent and pond effluent. *Shigella dysenteriae* and *Enterococcus* spp. were detected in raw sewage and UASB effluent. *Staphylococcus aureus* and *Helicobacter pylori* were not detected. The mean values of *E. coli* obtained in the raw sewage in three different campaigns were 3.92×10^{7}, 9.78×10^{7} and 2.46×10^{7} cells/100mL and for the pond effluent were 3.82×10^{6}, 4.17×10^{6} and 3.56×10^{6} cells/100mL.

Key words: pathogenic bacteria, molecular biology, UASB reactor, polishing pond.

INTRODUCTION

Wastewater treatment (WWT) based on UASB (Upflow Anaerobic Sludge Blanket) reactor followed by polishing ponds is a well established system for removing organic and inorganic matter, as well as pathogenic microorganisms (von Sperling and Chernicharo, 2005). Usually the evaluation of pathogenic bacteria in wastewater treatment systems is carried out indirectly by faecal (thermotolerant) coliforms and *Escherichia coli* decay. Regarding domestic sewage, faecal presence is obvious, and the main information to be derived from faecal coliforms is the inference of the possible pathogenic bacteria removal efficiency and the suitability of the final effluent for discharge or reuse. Monitoring waterborne bacteria such as *Salmonella* and *Shigella,* as well as emerging pathogens such as *Helicobacter pylori*, is not usually carried out in wastewater treatment plants, mainly because the laboratory analyses are usually very laborious. However advances in molecular techniques have made possible the direct investigation of pathogenic bacteria without the need for their cultivation, which should make the analysis quicker and more precise.

Molecular techniques based on the Polymerase Chain Reaction method (PCR) show great potential as an alternative to the conventional microbiological culture approaches (Bej, 2004). *In situ* fluorescent hybridization (FISH) technique allows the quantification of microorganisms by using fluorescent oligonucleotides which are specific to a group or a particular organism among several others present in the sample. Using an epifluorescence microscope it is possible to count individual cells which have been hybridized with the oligonucleotide probe and therefore emitted a fluorescent signal (Amann, 1995). With molecular techniques such as PCR and FISH it has been possible

to investigate pathogenic microorganisms in different types of samples, including water and wastewater samples (Lee *et al*., 2006; Peng, *et al*., 2002, Alonso *et al*., 2006). The present work aimed to investigate pathogenic bacteria (*Escherichia coli, Salmonella enterica* subsp. *enterica, Shigella* spp., *Enterococcus* spp., *Helicobater pylori*, *Staphylococcus aureus)* by molecular techniques, in a wastewater plant composed by a UASB reactor followed by polishing ponds, treating municipal sewage generated in the city of Belo Horizonte, Brazil.

METHODS

Description of the wastewater treatment plant and sampling procedures

The UASB-Polishing Pond system installed at the UFMG/COPASA Centre for Research and Training in Sanitation (CePTS), Belo Horizonte, Brazil, was used in the research. The system is comprised by the following units in series: one UASB reactor, three shallow polishing (maturation) ponds and one rock filter (Figure 1). However, in this research only the UASB reactor and the first pond were evaluated. The UASB reactor has a retention time varying between 8 and 10 hours. The ponds are rectangular, with a length-to-width ratio of 5 and a hydraulic retention time around 4 days each. The population equivalent of the system is the range of 200 inhabitants.

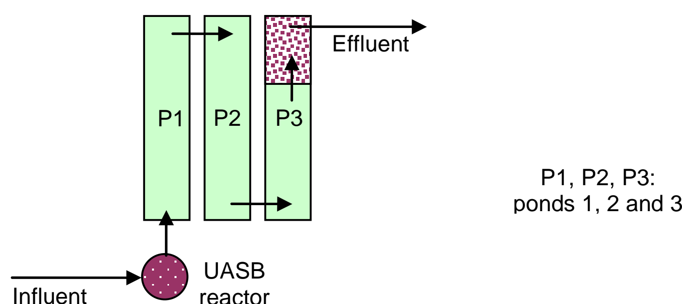

Figure 1 – Flowsheet of the treatment system

Two litres of samples from raw sewage (RS), UASB effluent and P1 effluent were collected and transported to the laboratory for further work. Samples from raw sewage and UASB effluent were centrifuged at 4,000 rpm for 20 minutes in order to concentrate microbial cells. For the pond effluent, samples were filtered before concentration in order to remove part of the microalgae in the samples. The first filtration was carried out in an 8.0 µm filter (J. Prolab) followed by filtration at 1.2 µm. The filtered sample was then concentrated by centrifugation at 4,000 rpm for 20 minutes.

After centrifugation, the pellets were ressuspended in phosphate buffered saline solution (PBS 1X; 0.13 M NaCl, 7mM Na_2HPO_4, 3 mM $Na_2H_2PO_4$; pH = 7.2) and stored at -20ºC for DNA analysis. Additionally, 300 mL of raw sewage and P1 samples were collected in three distinct periods for *E. coli* quantification. For quantification by FISH technique 200 mL samples were concentrated by centrifugation at 4,000 rpm for 30 minutes. Samples from the pond effluent were filtered at an 8.0 µm filter before concentration. Concentrated samples were fixed and hybridized as described below. For quantification by Defined Substrate Technique, 100 mL of samples were used without any prior treatment, as described subsequently.

DNA extraction

DNA extractions from concentrated samples were performed in triplicates using a protocol described by Egli *et al*. (2003), which basically consists of physical-chemical

cell lysis folowed by purification of DNA products in chloroform-isoamyl alcohol (24:1 vol/vol) washes. The genomic DNA was then precipitated with ethanol and ressuspended in 40 μL of ddH_2O. The triplicates were then pooled in order to have a more representative DNA extract.

PCR amplification

The specific genes from the purified DNA were amplified using PCR with two sets of primers for each investigated bacteria (Table 1). A volume of 25 μL was used for each PCR reaction in the following conditions: PCR buffer (1X, *Phoneutria*), $MgCl_2$ (1.5 mM, *Phoneutria*), primers (200 nM each, *Invitrogen*), dNTP (200 μM total, *Fermentas*), BSA (600 ng, *Sigma*), *Taq* polymerase (1.25 unit *Phoneutria*) and 1.0 μL of DNA template. All PCR reactions were performed in a *Mastercycler Gradient (Eppendorf)*. The general PCR program consisted in: initial denaturation at 94°C for 3 min; 30 cycles of denaturing (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min), final extension at 72°C for 7 min. PCR products were examined on ethidium bromide (0.5 μg/mL) stained agarose gels. For all amplification experiments it was included a positive control (targeted bacteria), a negative control (untargeted bacteria) and a blank reaction (without DNA template). In addition PCR reactions were performed at least four times in order to confirm the obtained result.

Table 1 – Oligonucleotides (primers and probe) used for pathogenic bacteria investigation

Target bacteria	Name	Sequence 5'→ 3'	Amplicon size (bp)	Reference
		Primers for PCR		
E. coli	L-uid739	tggtaattaccgacgaaaacggc	840	Bej (2004)
	R-uid578	gtggcgaaatattcccgtgcact		Bej *et al*. (1991)
S. enterica subsp. *enterica*	L-himA	cgtgctctggaaaacggtgag	123	Bej *et al.* (1994)
	R-himA	cgtgctgtaataggaatatcttca		
Enterococcus spp.	Ent1	tactgacaaaccattcatgatg	112	Ke *et al*. (1999)
	Ent2	aacttcgtcaccaacgcgaac		
Shigella dysenteriae	L-phoBR	attgaagccgcgccgacgcaa	152	Bej (2004)
	R-phoBR	cgttgcctgacaccttgaggg		
Helicobacter pylori	16SHP1	gcaatcagcgtcagtaatgttc	521	Lu *et al*. (2002)
	16SHP2	gctaagagatcagcctatgtcc		
Staphylococcus aureus	nuc1	gcgattgatggtgatacggtt	270	Burtscher and Wuertz (2003)
	nuc2	agccaagccttgacgaactaaagc		
		Probe		
E. coli	23S rRNA	gcataagcgtcgctgccg	-	Neef *et al*, (1995)

***In situ* Fluorescent Hybridization**

Concentrated raw sewage and pond effluent were preserved according to the protocol described by Hann *et al*. (2001). For this, 375 μL from each sample were fixed with 4% paraformaldehyde in PBS 1X for 3-16 h at 4ºC. Then, samples were centrifuged at 7,600 rpm for 5 min and the pellet was ressuspended in 1mL of PBS 1X and washed twice. The pellet was finally ressuspended in 300 μL of PBS 1X plus ethanol and kept at -20ºC until further analysis. Aliquots of 1 μl of fixed samples were spotted onto glass slides (*MP Biomedicals*) and dried at 45ºC for 20 min. Then, all samples were dehydrated further in 50%, 80% and 100% ethanol for three minutes each. Hybridizations were performed in 9 μl of hybridization buffer (0.9 M NaCl, 20 mM

Tris-HCl (pH = 7.2), 10 mM EDTA, 0.01% SDS) and 1 of the 50ng/µl of Cy3-labelled ECO 45a (ECO-1167) probe (Table 1). The hybridization was carried out in humid chambers at 46ºC for 2 h and the stringency was adjusted by adding 30% of formamide to the hybridization buffer. Washing was carried out for 20 min at 48ºC in washed buffer (with 5 M NaCl). Slides were washed briefly in ddH_2O to remove salts, stained with 1 µl of a DAPI 0.001% (4', 6-diamidino-2-phenylindole, Sigma), washed briefly in ddH_2O, air dried, and mounted with a glycerol/PBS 1X (20/80) solution with a pH≥8.5. Microscopic analyzes were performed under epifluorescence in an Olympus BX-50 Microscope. A total of 15 microscopic fields were used to estimate the number of cells hybridized with *E. coli* probe.

Defined Substrate Technique

For quantification of *E. coli* by cultivation-based technique, the IDEXX Quanti-Tray®/2000 - Colilert® system was used, which basically consists of incubating samples in a chromogenic media. Results are usually obtained in MPN of *E. coli* cells in 100mL of sample.

RESULTS AND DISCUSSION

Detection of pathogenic bacteria by PCR

Genomic DNA was successfully obtained from raw sewage, UASB effluent and pond effluent, as well as for pure cultures of *Escherichia coli*, *Salmonella* spp., *Shigella sonnei*, *Helicobacter pylori*, *Staphylococcus aureus* and *Enterococcus faecalis* (results not shown). Aliquots from genomic DNA were used as template for PCR reactions to amplify gene sequences according to the set of primes used. PCR reactions with DNA from pure cultures were performed in a temperature gradient ranging from 45 to 65°C in order to determine the best annealing temperature. The results showed that the annealing temperature of 55°C was enough to amplify all the specific gene sequences (results not shown).

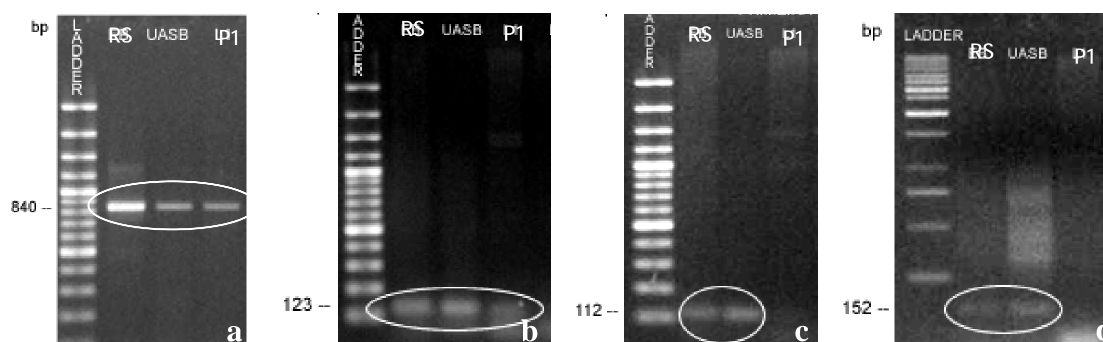

Figure 2 – PCR products from amplification of DNA from: a) *Escherichia coli,* b) *Salmonella enterica* subsp. *enterica*, c) *Enterococcus* spp., d) *Shigella dysenteriae*.
Legend: RS (raw sewage), UASB (UASB effluent), P1 (polishing pond 1 effluent)

Figure 2 shows the amplification results in ethidium bromide agarose gel for *E. coli*, *Salmonella enterica* subsp. *enterica*, *Enterococcus* spp. and *Shigella dysenteriae*. In Figure 2a it can be seen that the amplification of gene *uid,* which codifies the betaglucuronidase enzyme in *Escherichia coli* (Bej *et al* 1991), was positive for all investigated samples. *E. coli* is the most common bacteria in raw sewage, is used as indicator organism, but only few strains are able to cause disease. To investigate *Salmonella* it was used a primer for gene *him* which codifies a membrane bound protein specific in *S. enterica* subsp. *enterica* (Bej *et al* 1994). Like *E. coli*, *S. enterica* subsp.

enterica was detected from the raw sewage to the pond effluent (Figure 2b). Several works suggest that *Salmonella* genus is one of the major pathogenic bacteria in domestic wastewater (Pant and Mittal, 2007; Lee *et al.* 2006).

Gene *tuf* has been used as a biomarker for *Enterococus* investigation in several samples (Ke *et al*. 1999). The result showed that *Enterococcus* was present in the raw sewage and in the UASB effluent, but it was not detected in the polishing pond effluent (Figure 2c). Several works have reported *Enterococcus* spp. in raw sewage (Lee *et al*., 2006, Ceballos, 2000, Madera *et al*., 2002) and some of them also reported that ponds are efficient in removing *Enterococcus* from wastewaters (Ceballos, 2000, Madera *et al*., 2002). Therefore, the undetected *Enterococcus*-DNA in the pond suggests that this treatment unit may promote decay of *Enterococcus* species. According to the monitoring of *Shigella dysenteriae* performed with PCR for *pho* gene, this specie was detected in the raw sewage and in the effluent from the UASB reactor (Figure 2d). This result may indicate a decay of *Shigella* cells in the polishing pond. Similar results were reported by Pant and Mittal (2007) in a WWT plant composed by UASB reactor followed by a single polishing pond.

For *Helicobacter pylori* and *Staphylococcus aureus*, the PCR reactions did not result in any amplification, despite all attempts to optimize the amplification (increasing or decreasing DNA template, nested PCR, results not shown). Therefore it can be assumed that there were not enough cells of *Helicobacter* and *Staphylococcus* in order to be detected by the PCR technique (detection limit of 10^2 or 10^3 cells/mL, Sharma and Carlson, 2000). Such results were also reported in other studies. For instance Lee *et al* (2006) did not detect *Helicobacter* in a WWT plant even using qPCR, and Ampofo and Clerk (2003) showed that *Staphylococcus* was the less abundant pathogenic microorganism in a WWTP composed by four maturation ponds.

Table 2 summarizes the results of all investigated bacteria along the wastewater treatment plant. According to the results all pathogenic bacteria present in raw sewage were not removed in the UASB reactor. This was somewhat expected, because of its low hydraulic retention time. On the other hand, polishing or maturation ponds usually promote a degree of decay of several pathogenic bacteria (Mara, 2003; von Sperling and Mascarenhas, 2005; von Sperling, 2008). In the present study the single pond after the UASB reactor was able to promote a reduction of *Enterococcus* and *Shigella dysenteriae* cells, since no PCR products were detected in the effluent sample. Probably the polishing pond was also able to reduce the number of cells of *E. coli* and *S. enterica* subsp. *enterica* compared with the previous unit, even so the amount of cells was detectable by PCR. A series of ponds could remove the remaining cells in the system; however the subsequent pond units were not monitored in this study.

E. coli quantification

In situ fluorescent hybridization has become an important molecular tool that allows the quantification of various organisms in environmental samples, but its use for the quantification of pathogens is novel at the Laboratory of Microbiology of the Federal University of Minas Gerais (LM/DESA/UFMG). On the other hand, Defined Substrate has been used in the routine of many laboratories for quantification of *E. coli* cells, therefore it was judged important to compare both techniques regarding *E. coli* decay alongside the WWT system.

Table 2 – Detection of pathogenic bacteria

Bacteria	RS	UASB	P1
Escherichia coli	+	+	+
S. enterica subsp. *enterica*	+	+	+
Enterococcus spp.	+	+	-
Shigella dysenteriae	+	+	-
Helicobacter pylori	-	-	-
Staphylococcus aureus	-	-	-

Legend: RS: raw sewage; UASB: UASB effluent; P1: pond 1 effluent; + : amplification; - : no amplification

Table 3 shows the results regarding the quantification of *E. coli* in raw sewage and in the effluent from the polishing pond by FISH. According to the FISH results, the number of *E. coli* was in the range of 2.46×10^7 to 3.92×10^7 cells/100mL in the raw sewage and 3.56×10^6 to 4.17×10^6 cells/100mL in the polishing pond effluent. Regarding the Colilert® results the estimate of *E. coli* count was 2.1×10^7 to 3.1×10^7 MPN/100mL in the raw sewage and 4.6×10^5 to 1.8×10^6 MPN/100mL in the pond effluent. With these results the estimated removal efficiency of *E. coli* in the system was in the range of 85 to 95% for FISH and 98 to 99% for Colilert®.

Table 3 – Descriptive statistics of the quantification of *E. coli* by FISH technique

Statistics	Sampling	Raw sewage	Pond 1
Mean (cells/100mL)	1	3.92×10^7	3.82×10^6
	2	9.78×10^7	4.17×10^6
	3	2.46×10^7	3.56×10^6
Standard deviation (cells/100mL)	1	9.50×10^6	3.52×10^5
	2	1.91×10^7	6.73×10^5
	3	7.07×10^6	1.05×10^6
Coefficient of variation	1	0.24	0.09
	2	0.20	0.16
	3	0.29	0.29

Obs. mean and standard deviation of 3 quantifications per sample.

The observed difference in the efficiency of *E coli* removal is driven by the methodology itself. Whereas for FISH the quantification is based on counting individual cells, the Colilert® is based on analysis using an MPN table. It is believed that counting individual cells is more precise for estimating microorganisms than cultivation techniques, therefore FISH results would be expected to be more representative. However FISH technique has also some bias, especially in samples like the pond effluent, which shows characteristics that may incur in difficulties in the analysis. For instance, presence of autofluorescent algae could lead to an overestimation of cells. In addition, pond effluents can be considered "diluted" regarding the number of pathogenic bacteria, usually requiring concentration steps in order to satisfy the detection limit of the technique (10^3 to 10^4 cells/mL, Amann *et al.*, 1995). The morphology of cells, especially those with heterotrophic metabolism, may change in response of low levels of available organic carbon present in polishing ponds. In this study it was observed that the cells of *E. coli* in the pond effluent were smaller than *E. coli* cells from the raw sewage, and this makes counting very difficult.

In general, the results obtained by both techniques showed that the system composed by UASB reactor followed by one polishing pond was able to reduce *E. coli* counts by only one logarithm unit. It is known that ponds in series are able to reduce considerably the number of *E. coli* cells during the treatment. In the present study only the first pond was included in the investigation, however, the complete plant is composed by three ponds in series. Previous data based on cultivation techniques showed that the complete WWT plant was able to reduce more than 99,9%-99.99% of *E. coli* cells, accounting for 10^3 to

10^4 MPN/100mL in the final effluent (von Sperling and Mascarenhas, 2005; von Sperling, 2008).

CONCLUSIONS

Considering the detection of pathogenic bacteria by PCR it can be concluded that *E. coli* and *Salmonella enterica* subsp. *enterica,* which were present in the raw sewage, were not completely removed in the system composed by UASB followed by one polishing pond. On the other hand, *Enterococcus* spp. and *Shigella dysenteriae* were not detected in the polishing pond, suggesting that the number of cells related to both were considerably reduced along the treatment. Regarding *Helicobacter pylori* and *Staphylococcus aureus* investigation, both genera were not detected in the raw sewage and in the effluents, indicating that the number of cells from these species is probably not high enough to be detected by PCR. The quantification of *E. coli* using FISH revealed values in the range of 10^7 cells/100mL for raw sewage and 10^6 cells/100mL for pond effluent, whereas Colilert® results were in the range of 10^7 for raw sewage and 10^5 MPN/100mL for pond effluent.

The results obtained show the applicability of PCR method for monitoring pathogenic bacteria in wastewater systems. However the applicability of FISH technique for evaluating *E. coli* removal efficiency remains unclear, mainly because of the low levels of pathogenic bacteria in pond effluents, which usually is in the detection limit of the technique. Additional samplings will be conducted in order to investigate the applicability of FISH for *E. coli* counting.

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