3.4.2. Microbial assays

3.4.2.1 Qualitative analysis

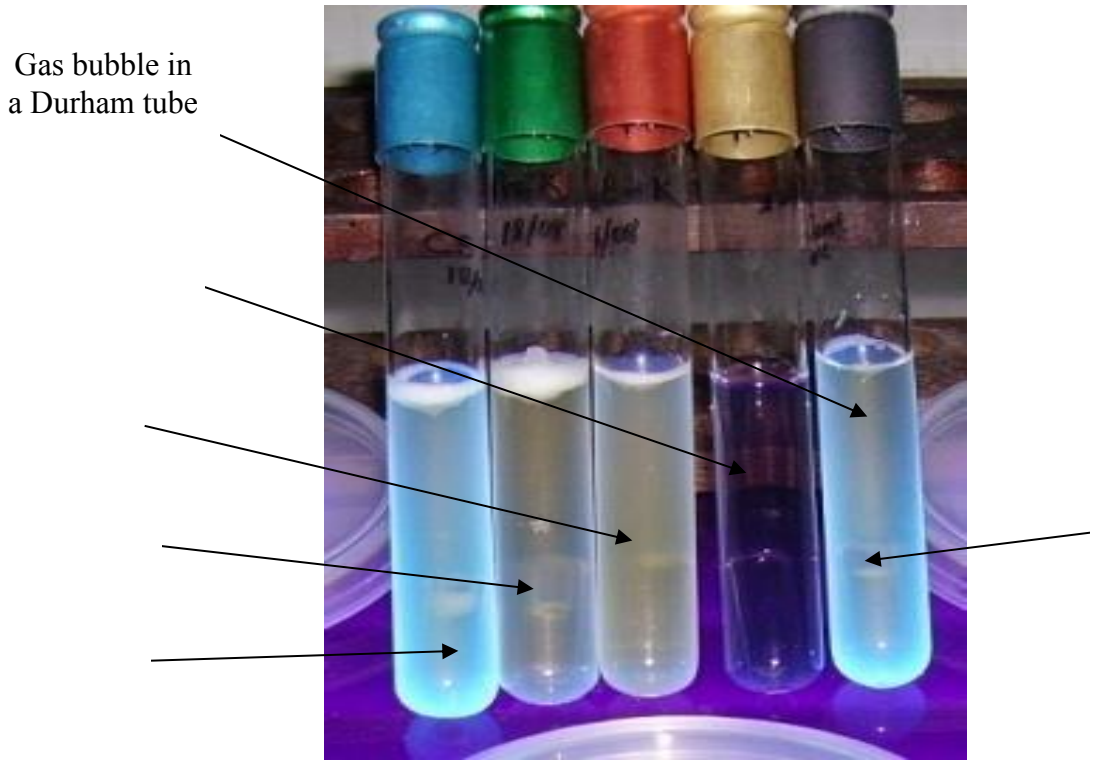

Figure 3.2: Presumptive test for the presence of *E. coli* with DEV Lactose Peptone MUG broth after inoculation with sample and incubation over-night at 37˚C, viewed under UV light.

From left to right are RS, Pre-K and Post-K samples, negative and positive control, respectively (Fig.3.2). Tubes 1, 2, 3 and 5 (from left to right) fluoresced blue when irradiated with long-wave ultra violet light (λ = 366 nm) while tube 4 remained negative. In these tubes the enzyme *β*-D-glucuronidase produced by *E. coli* had hydrolyzed 4-methylumbelleryl- *β*-D-glucuronide in the medium to produce a fluorescent end product, 4-methylumbelliferone. In tube 4 there was no reaction because there was no *E. coli*. Tubes 2 and 3 demonstrated weaker fluorescence, possibly due to the diffusion of the fluorogen into the particulate matter in these tubes. All samples were

treated in the same way to avoid any discrepancies. This was a presumptive test for faecal contamination. The first step for confirming the presence of *E. coli* was investigating the inverted Durham tube inside the test tube for gas production. In tubes 1, 2, 3 and 5, there was a gas bubble in each of the Durham tubes. In the negative control (test tube 4) no gas formed (Fig.3.2).

Figure 3.3: Confirmatory test for presence of *E. coli* on DEV Lactose Peptone MUG Broth using Indole reaction.

The front row shows the confirmatory test for the presence of *E. coli* in the samples from the three sites with Kovac's reagent after incubation of sample O\N at 37˚C (Fig. 3.3). A qualitative presumptive test for the presence of *E. coli* (Fig.3.3) was done for all samples collected. All samples tested positive. However, the fluorescence intensities and amount of gas formed varied, indicating inhibition of the enzyme in turbid water. The presence of *E. coli* was then confirmed with the Indole reaction (Fig. 3.3).

Due to financial implications of quantitative tests, it is ideal to initially do a presence or absence assay for faecal contamination. The results from such an assay would then inform the subsequent

steps. Some chromogenic and fluorogenic media serve this purpose well. Similarly in the current study, it first had to be established whether there was faecal contamination or not before proceeding to elucidate the extent of contamination through which the difference or similarity of the two sampling sites were assessed.

The three sampling events around one episode of heavy rain were aggregated into a single event in order to avoid serial correlation yet capturing both events around the episode (Table 3.1). Richards and Grabow (2003) warned about error due to independent observations. They warned that the first observation may carry influence over into the subsequent one, thus creating noise in the overall observation. Grabow *et al* (1999) suggested that the easiest way to correct serial correlation is to aggregate data into a larger step, for example, average data around a single event.

In the current study the presence or absence of faecal matter at the sampling sites was assessed using a fluorescence medium called DEV Lactose Peptone MUG Broth exploiting the presence or absence of *E. coli* as an indicator. Figure 3.2 shows the activity of *β*-D-glucuronide in all experiment test tubes. However, the RS in test tube 1 showed the brightest fluorescence. This correlates with the comparative clarity of water at this site (Fig. 3.2). The turbidity of water from Pre- and Post-K interfered with fluorescence in test tubes 2 and 3 respectively (Fig. 3.2). The high affinity of 4-methylumbelliferone for soil particles lowered fluorescence (Manafi, 2000; Marx *et al*, 2001). The intensity of fluorescence appeared not to be related to the amount of *E. coli* in the sample. Therefore *E. coli* was presumed present in all sites.

The presumptive presence of *E. coli* was then fully confirmed by indole reaction (Fig. 3.3). All samples tested positive for indole reaction verifying the presence of *E. coli* in all samples. The

need for verification arises from the fact that *Salmonella, Shigella* and *Yersinia spp.* are also GUD positive but are indole negative (Heizmann *et al*, 1998; Manafi, 2000). For assessing whether water is fit for drinking purposes, no further testing is required if these tests are negative.

3.4.2.2 Quantitative analysis

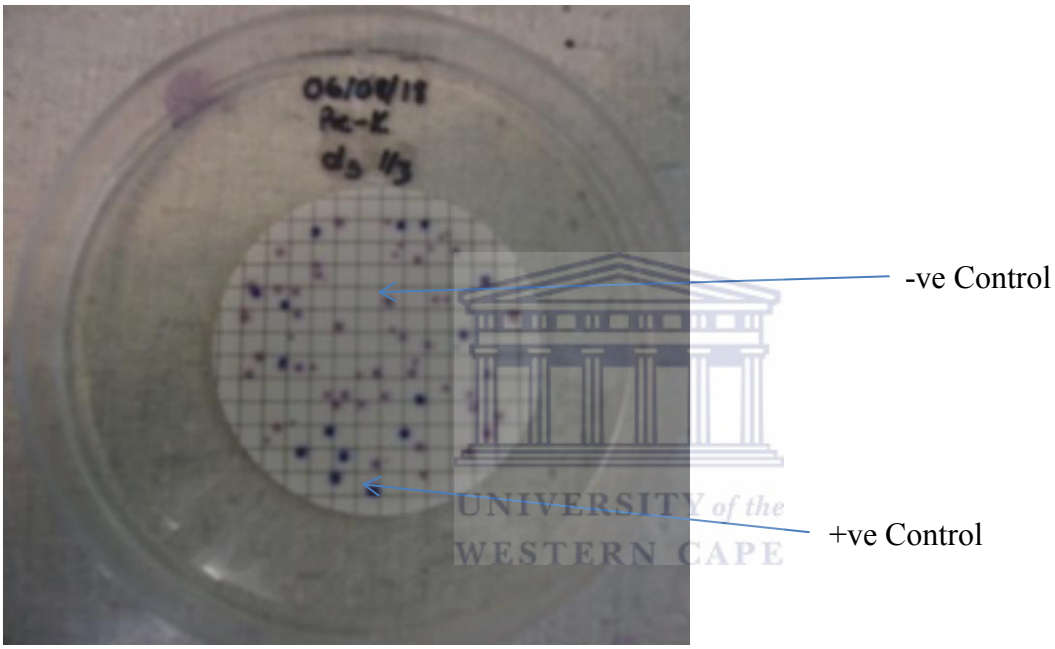

Figure 3.4: Indicator organisms on Chromocult agar plate

Figure 3.4 shows a membrane filter with filtered sample on a Chromocult agar plate after incubation overnight at 37˚C. The dark blue colonies are *E. coli* colonies and pink colonies indicate other coliforms.

A quantitative analysis on the membrane filter-Chromocult agar system (Fig. 3.4) showed diverse amounts of the indicator organisms across the different sites. The red pigmented colonies were identified as total coliform (TC) while blue to dark blue colonies were identified as *E. coli*. It was not possible to count from neat samples, particularly for the test sites. Countable colony

forming units started at 1 in 4 dilutions for most cases. Countable colonies ranged from 30 to 250 per membrane filter. The average colony count for Post-Khayamnandi was the most diverse for both total coliforms and *E. coli* on the whole (Table 3.6).

Table 3.6: Average colony counts for winter and spring

Average microbial counts (cfu/100 ml)						
	Total coliforms			*E. coli*		
Sample series	RS	Pre-K	Post-K	RS	Pre-K	Post-K
1	4.62E+01	9.61E+08	1.46E+08	1.03E+01	7.38E+05	3.10E+07
2	1.25E+01	9.25E+07	2.45E+06	3.33E+00	1.60E+07	6.83E+05
3	1.25E+01	7.69E+05	1.10E+06	3.33E+00	7.23E+04	3.91E+05
4	4.62E+01	2.15E+06	1.38E+06	1.03E+01	3.17E+05	6.70E+05
5	2.00E+01	2.82E+06	6.56E+06	5.00E+00	8.47E+05	2.01E+06
6	6.00E+01	3.31E+05	2.35E+06	2.00E+01	1.64E+04	2.35E+06
7	2.50E+01	1.46E+07	2.53E+06	5.00E+00	3.90E+06	1.73E+06
8	7.50E+01	5.47E+06	8.90E+08	2.25E+01	1.63E+06	2.80E+08
9	4.42E+01	2.50E+06	2.75E+07	1.20E+01	2.50E+05	8.95E+07
10	4.95E+01	2.85E+06	3.45E+08	4.57E+01	1.30E+06	1.20E+08
11	1.50E+00	3.20E+06	4.30E+08	8.33E-01	1.80E+06	1.98E+08
12	6.50E+01	6.70E+06	6.55E+08	4.18E+01	3.40E+06	2.30E+08

Table 3.6 represents average microbial concentration per 100 ml over winter and spring series, i.e. 1-6 and 7-12, respectively. Each series (n=6) was comprised of 3 and 2 sampling episodes in winter and spring, respectively. Each episode is a mean of three samples. The microbial density in RS is in the region of 10^1, while the other two sites range between 10^5 and 10^8 both seasons.

Table 3.7: Summary of seasonal variation of TC and *E. coli* during sampling series

Comparison of Seasonal Microbial Averages						
Series	Total coliforms			*E. coli*		
Winter	RS	**Pre-K	**Post-K	RS	**Pre-K	**Post-K
Spring	RS	$^{\alpha **}$Pre-K	$^{a **}$Post-K	RS	$^{\alpha}$**Pre-K	^{a}**Post-K

Key:

*** - p < 0.05 (significantly different to RS for a specific season)**

**** - p < 0.01 (significantly different to RS for a specific season)**

^{a} - p < 0.05 (significantly different between winter and spring for the same site)

$^{\alpha}$ - p < 0.05 (Pre-K is significantly different to Post-K)

Table 3.7 presents the seasonal microbial averages of TCs and *E. coli* (n=6) compared across the three sites. Significant differences within and across the three sites are also presented as against RS seasonally, within each site across the two seasons and seasonally across the two test sites.

CCA contains two chromogenic enzyme substrates 6-chloro-3-indoxyl-3-ß-D- galactopyranoside (SALMON-GAL) and 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid, cyclohexylammonium salt (X-GLUC), for detection of activities of ß-D-galactosidase from coliforms and ß-glucuronidase from *E. coli*, respectively. After hydrolysis SALMON-GAL releases a chromogenic compound, chloroindigo forming salmon to red coloured colonies, while X-GLUC releases bromochloroindigo forming light blue to turquoise colonies (Finney *et al*, 2003; Merck, 2000).

CCA exploits the discrimination of both TC and *E. coli*. This enables the evaluation of the same scenario using these two parameters separately. Therefore the trend in faecal pollution was observed for both *E. coli* and TC simultaneously. Data for Table 3.6 was generated from counting the pink/red plus blue colonies for TC on the filtration membranes (Fig. 3.4) grown on CCA while blue colonies indicated the presence of *E. coli* only.

The two test sites were significantly different ($p < 0.01$) to RS for both winter and spring (Table 3.7). Pre-K and Post-K further differed ($p < 0.05$) from each other in spring. Table 3.7 further showed difference ($p < 0.05$) within Post-K across the two seasons. The Ln [TC] increased by a factor of 2 in spring at Post-K (Table 3.6).

The difference between RS and Pre-K *E. coli* population density was pronounced for both seasons ($p < 0.05$). There was also a significant difference between Pre-K and Post-K ($p < 0.05$, Table 3.7).

In general, linear transformations of average Ln (TC) population across the three sites show different responses to decrease in water flow. Contrary to expectation, the population at Post-K increased, while that at the other two sites responded according to norm. The decline at RS is pronounced ($p < 0.05$) while a flatter gradient at Pre-K was observed. This was also visible when comparing the winter to the spring population densities (Table 3.7). It is worth noting that in winter both Pre- and Post-K were in the same range on average count, i.e. 10^7(Table 3.6). They were different in range, where Post-K had a broader range (Table 3.7). Turning to spring, the averages of the two sites were 10^2 fold apart (Table 3.6). This indicates that microbial presence is indeed heavily increased by conditions surrounding Khayamnandi though it may not be merely due to wash-off from the settlement itself. The persistent microbial presence at Post-K may also be influenced by anthropogenic activities and vegetation immediately at the banks and above the sampling point of the river, respectively. *E. coli* population followed a similar trend.

Hara-Kudo *et al*, 2000, made an observation that bacterial decrease with time under starvation is less pronounced when the initial density was high. This could be one of the explanations of the patterns seen in the trend analysis. Linear transformations of the microbial population indicate an

increase in microbial density for both populations despite reduced water flow; hence water influx from the settlement. The increase in *E. coli* was however slightly higher ($p \leq 0.05$).

Taking into consideration that microbial density at RS and Pre-K declined after heavy rains, it seems that there were persistent contributing factors within the boundaries of Khayamnandi (Table 3.6). It appears that these factors were not directly linked to rains, although such conditions exacerbated the situation as indicated by sample series points 1 in winter and 8 in spring (Table 3.6). The points that are farthest above the linear transformation represent samples at storm flow or when water velocity had settled down from rapid flow (e.g. point 8 in Table 3.6) after wash-off had taken place. Overall, the significant increase ($p \leq 0.05$) in *E. coli* at Pre-K suggests that the surrounding environment plays a major role in polluting the Plankenbrug River (Table 3.7).

All data collected refutes the assertion that the degree of pollution at RS is equivalent to that at Pre-K. Instead, data points out that Pre-K faecal pollution is much higher than that at the reference site. Further the assertion that Pre-K pollution is different to that at Post-K is affirmed. It could be thought that these assertions are influenced by run-offs during heavy rains. Therefore, analyzing the two seasons separately may lend perspective to that event.

However, in the case of *E. coli*, the difference between Pre- and Post-K was more prominent compared to that of TC (Tables 3.6, 3.7). The reference site showed perceptive seasonal variation, from winter to spring in both total coliform and *E. coli* prevalence. There was however more average decrease in total coliforms while a slight but significant increase in *E. coli* was observed. In the Pre-K site there was an increase of both classes, however these changes were not statistically significant. In contrast to both Pre-K and RS, Post-K reflected appreciable

average increase in both classes ($p \leq 0.01$). *E. coli* increase was more marked of the two classes with 1000x increase as compared to a 100x increase in total coliforms. Spring *E. coli* count increased from an average 10^6 to the 10^8. Despite this increase, there was least variation, Ln (cfu/100 ml = 1.14); within the spring samples on this site (Table 3.7). The highest microbial variation within a season was observed at Pre-K in *E. coli*, that is Ln (cfu/100 ml) = 6.88 (Table 3.7). Spring samples had insignificant variation although *E. coli* concentrations were higher than in winter. This could be due to increased temperature in spring that encourages microbial growth in water meaning that this site continually responded to rain even in spring, the drier season. The earlier conclusion stands firm that the informal settlement contributes to an already dire situation. All these results support that the river is already polluted as it reaches Pre-K.

3.4.3 Analytical profiling index

Key:

1-ONPG	**2-ADH**	**3-LDC**	**4-ODC**	**5-CIT**	**6-H_2S**
7-URE	**8-TDA**	**9-IND**	**10-VP**	**11-GEL**	**12-GLU**
13-MAN	**14-INO**	**15-SOR**	**16-RHA**	**17-SAC**	**18-MEL**
19-AMY	**20-ARA**				

Figure 3.5: API reactions from isolates of Pre-K

Key:

1-ONPG	**2-ADH**	**3-LDC**	**4-ODC**	**5-CIT**	**6-H_2S**
7-URE	**8-TDA**	**9-IND**	**10-VP**	**11-GEL**	**12-GLU**
13-MAN	**14-INO**	**15-SOR**	**16-RHA**	**17-SAC**	**18-MEL**
19-AMY	**20-ARA**				

Figure 3.6: API reactions from isolates of Post-K

Isolates from Figure 3.5 had slight differences in their respective reactions. As a result, the isolates shown above were identified as variants of *E. coli* 1. In contrast to Figure 3.5, isolates

from Post-K (Fig. 3.6) exhibited a number of differences in their biochemical reactions. The *apiweb*^{TM} identification software again identified them as variants of *E. coli*.

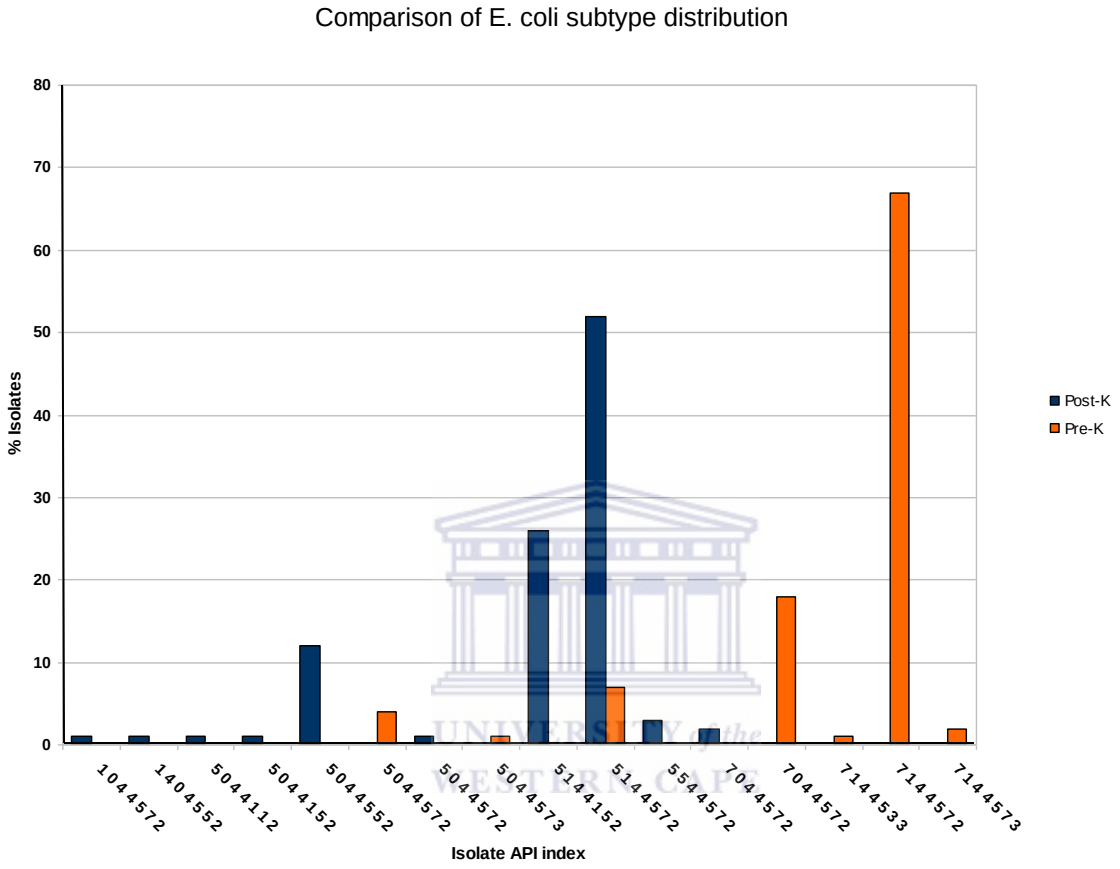

Figure 3.7: Graph representing % distribution of *E. coli* subtypes within and across Pre- and Post-K

Pre-K isolates were dispersed, while those of Post-K seemed clustered in two prominent groups (Fig. 3.7). After entering the 7 digit codes, which resulted from the respective API reactions, all isolates were identified as *E. coli* 1 with confidence ranging from 86.2% to 99.9% (Table 3.8), except for two isolates from Post-Khayamnandi. The first deviant isolate had a profile index of 1 404 552. It was identified as *Citrobacter freundi* with the second best identification as *E. coli* 1.

The second deviant isolate had a profile of 5 044 112 and was identified as *E. coli* 2, with second best identification as *E. coli* 1.

Having enumerated the extent of pollution across the two sites, the distribution of isolates was determined, via API 20E and haemolytic assay for similarities or dissimilarities. Isolates from Pre-K site gave 7 distinct analytical profiles, while Post-K yielded 10 (Fig. 3.5, 3.6). Pre-K API's ranged from 5 044 572 to 7 144 573 and Post-K 1044572 and 7 044 572. Pre-K profiles had two peaks, one at 7 044 572 (18%) and the other at 7 144 572 (67%) (Fig. 3.7). These API peaks stood out at the opposite end of the range to those that characterized Post-Khayamnandi isolates. The whole of Post-K isolates occupied the lower range with peaks at 5 144 152 (26%) and 5 144 572 (52%). The only common index across the two sites was at 5 144 572.

Figure 3.7 reflects stark contrast between the two sites. Post-K has strains that are not available at Pre-K. The three peaks at Post-K rivaled by the two at Pre-K invariable show distinction between the two sites. Post-K demonstrates diverse population while Pre-K almost points at a sole source. The sum total of results from both sites substantiates that the integrity of water across the two points is affected differently. These differences point out once more that Post- and Pre-K are impacted differently. These differences also suggest a probability of using the prominent strains for bacterial source tracking and/or as biomarkers for pollution.

Isolates within each site reacted differently to some of the API tests. These tests could then be used as markers for identifying different biotypes. The API isolates from Pre- and Post-K were investigated in order to establish if they could point at different sources of pollution. Sucrose and sorbitol fermentation in the API assay amongst other tests seemed to provide the answer to this question as biotyping assay.

Table 3.9 showed skew distribution of sucrose fermenters towards Pre-K. Both test sites exhibited high prevalence of sorbitol fermenters. The distributions of the biotypes across the two test sites indicate that their sources are different ($p \leq 0.05$). This together with the almost exclusive prevalence of one subtype of bacteria in at one site, carry a strong probability of point source impact on that site while diversity implies diffused source. This means that sorbitol fermentation can be used as a biotyping tool in tracing the source of faecal contamination in water.

Table 3.8: Percentage positives for individual API tests

Tests	Percentage positives of Pre-k	Percentage positives of Post-k	Significant difference across sites
ONPG	100%	99%	None
ADH	95%	1%	Yes
LDC	100%	97%	None
ODC	78%	81%	None
CIT	3%	2%	None
H_2S	0%	4%	Yes
MAN	100%	99%	None
INO	2%	0%	None
SOR	100%	73%	Yes
RHA	100%	99%	None
SAC	100%	59%	Yes
MEL	99%	97%	None
AMY	4%	0%	None

Table 3.8 shows the distribution of positive results for each API test for Pre-K and Post-K. Differences across the two tests sites are also reflected.

Table 3.9: Prevalence of sorbitol and sucrose fermenters across the two sites

Site		Pre-Khayamnandi (n=100)	Post-Khayamnandi (n=100)
Prevalence (%)	Sorbitol fermentation	100	73
	Sucrose fermentation	100	59

Table 3.9 was adapted from the API test (Table 3.8) to be interpreted as possible biotyping and toxicologic assays. There were significant differences across the two sites for both sorbitol and sucrose fermentation tests ($p \leq 0.05$ for both assays).

The prevalence of such high percentages of sorbitol fermenters (100% and 73% for Pre- and Post-K, respectively) is alarming because sorbitol fermenting *E. coli* is closely associated with haemolytic uremic syndrome (HUS). Its association with fatality talks to the toxicity of the river across the two sites and beyond (Karch *et al*, 1990; Karch and Bielaszewska, 2001). The lower prevalence downstream seems to indicate dilution of the river population by influx of non-sorbitol fermenting *E. coli*. This is true under the assumption that the river population should be evenly distributed all things being equal. Alternatively, assuming the river population is discrete one would draw a conclusion that there is a skew distribution of biotypes across the two sites. This population difference negates the hypothesis that the informal settlement is the sole contributor to the river's pollution. As is, it reinforces that it might heavily add to the river's pollution.

Since there is no specific treatment for HUS and there is no consensus on the reservoir of sorbitol fermenting *E. coli*, prevention is of essence (Werber *et al*, 2011). It is therefore important to further study in detail the prevalence of an organism with such great probability of

virulence in water. Such information would enhance the epidemiological knowledge of this pathotypes.

Table 3.9 also demonstrates a skew percentage distribution of sucrose fermenters towards Pre-K with 100% of isolates positive ($p \leq 0.05$). This significant difference in distribution indicates homogenous distribution of sucrose fermenters in this site while downstream it shows heterogeneity. Firstly, it can be deduced from this that the two sources are polluted from different sources. Secondly, with the background that omnivores have heterogeneous *E. coli* flora (Carlos *et al*, 2010), Post-K site is most likely having direct anthropogenic contamination. This indicates that the settlement augments the river's pollution with new pollution markers. Concern, however, is raised when considering that sucrose fermenting ability is plasmid mediated (Bogs and Geider, 2000). This means that downstream (Post-K) there could be intermixing of different subgroups. This could result in new and more virulent pathotypes as these plasmids are readily transmissible (Orsi *et al*, 2007; Shukla *et al*, 2004; Bogs and Geider, 2000).

Homogeneity at a site may be a strong pointer towards herbivores and avian population as potential pollutants (Carlos *et* al, 2010; Zinnah *et al*, 2007). As such, the sucrose fermentation assay can be used as a biotyping assay for surface water pollution. Hence, the *E. coli* sucrose fermentation assay can help in bacterial source tracking where pollution has already occurred. This may help direct remedial measures appropriately and save resources.

Both sorbitol and sucrose fermentation assays lean toward considering of the river across the two points as toxic with Pre-K more toxic. The distribution of the pathotypes, if it is shown to be conserved over a long time at one point, may go a long way in tracing the epidemiology due to

these fermenters and the associated pathogens via endemicity. In a similar way, the persistent sustained skew distribution of biotype could be used for bacterial source tracking. The skew prevalence of *E. coli* with pathogenic potential suggests that remedial measures must also encompass beyond the informal settlement. Neglecting this could result in misdirected efforts in curtailing the problem at hand.

3.4.4 Haemolysis assay

Table 3.10: Haemolytic activity of isolates from Post-K

Post-K OD	% Ref β	% Ref α	Post-K OD	% Ref β	% Ref α	Post-K OD	% Ref β	% Ref α	Post-K OD	% Ref β	% Ref α
-0.0305	-1.88	-3.10	0.025	1.54	2.54	0.047	2.90	4.78	0.061	3.76	6.20
-0.0273	-1.68	-2.78	0.025	1.54	2.54	0.047	2.90	4.78	0.064	3.95	6.51
0.0085	0.52	0.86	0.025	1.54	2.54	0.047	2.90	4.78	0.064	3.95	6.51
0.0105	0.65	1.07	0.0255	1.57	2.59	0.047	2.90	4.78	0.064	3.95	6.51
0.0125	0.77	1.27	0.026	1.60	2.64	0.047	2.90	4.78	0.064	3.95	6.51
0.013	0.80	1.32	0.026	1.60	2.64	0.047	2.90	4.78	0.068	4.19	6.91
0.013	0.80	1.32	0.026	1.60	2.64	0.0495	3.05	5.03	0.0705	4.35	7.17
0.013	0.80	1.32	0.0305	1.88	3.10	0.0525	3.24	5.34	0.079	4.87	8.03
0.0145	0.89	1.47	0.0305	1.88	3.10	0.053	3.27	5.39	0.0835	5.15	8.49
0.0165	1.02	1.68	0.031	1.91	3.15	0.053	3.27	5.39	0.0895	5.52	9.10
0.0195	1.20	1.98	0.031	1.91	3.15	0.053	3.27	5.39	0.0945	5.83	9.61
0.02	1.23	2.03	0.031	1.91	3.15	0.0545	3.36	5.54	0.0945	5.83	9.61
0.02	1.23	2.03	0.0325	2.00	3.30	0.0545	3.36	5.54	0.1	6.17	10.17
0.02	1.23	2.03	0.0345	2.13	3.51	0.055	3.39	5.59	0.102	6.29	10.37
0.02	1.23	2.03	0.036	2.22	3.66	0.055	3.39	5.59	0.1025	6.32	10.42
0.024	1.48	2.44	0.0365	2.25	3.71	0.0565	3.49	5.74	0.3595	22.18	36.55
0.0245	1.51	2.49	0.037	2.28	3.76	0.058	3.58	5.90	0.963	59.41	97.92
0.0245	1.51	2.49	0.037	2.28	3.76	0.0585	3.61	5.95	0.9745	60.12	99.08
0.0245	1.51	2.49	0.037	2.28	3.76	0.0595	3.67	6.05	0.979	60.39	99.54
0.0245	1.51	2.49	0.037	2.28	3.76	0.0595	3.67	6.05	0.988	60.95	100.46
0.025	1.54	2.54	0.0405	2.50	4.12	0.06	3.70	6.10	0.9935	61.29	101.02
0.025	1.54	2.54	0.0425	2.62	4.32	0.06	3.70	6.10	1.003	61.88	101.98
0.025	1.54	2.54	0.0425	2.62	4.32	0.06	3.70	6.10	1.0515	64.87	106.91
0.025	1.54	2.54	0.0425	2.62	4.32	0.06	3.70	6.10	1.0595	65.36	107.73
0.025	1.54	2.54	0.0465	2.87	4.73	0.061	3.76	6.20	1.1135	68.69	113.22

Table 3.10 shows 100 isolates in sets of 25. Columns 1, 2 and 3 are corrected OD readings of isolates' haemolytic activities against the media blank, followed by correction against positive controls, i.e. β and α-haemolytic streptococci, respectively. This represents the first set of 25 isolates, followed by the next 3 sets of 25 isolates, thus making 100 in total. Percentage Ref β and α = isolate activity / average activity of β- or α-streptococci (respectively) X100.

Haemolytic activities were measured at 405 nm using an ELISA microtiter plate reader. All Table 3.10 and Table 3.11 values below were initially calculated from the actual OD readings by adjusting for the average media blank reading, thus explaining the negative results of the first two readings. The negative is due to dilution of the haemolytic preparation by low or unsuccessful growth of isolates in Dulbecco salts. These readings make up the first column of each of the four sets in both Tables 3.10 and 3.11. The subsequent two columns were calculated from the haemolytic activities of the respective positive controls by dividing each value in the first column by the average haemolytic activity reading of the controls. These were then multiplied by 100 to get percentage haemolytic activity with reference to each of the two controls.

Table 3.11: Haemolytic activity of isolates from Pre-K

Pre-K OD	% Ref β	% Ref α	Pre-K OD	% Ref β	% Ref α	Pre-K OD	% Ref β	% Ref α	Pre-K OD	% Ref β	% Ref α
-0.003	-0.22	-0.30	0.047	3.47	4.75	0.064	4.72	6.47	0.969	71.47	97.98
0.01	0.74	1.01	0.047	3.47	4.75	0.067	4.94	6.77	0.969	71.47	97.98
0.013	0.96	1.31	0.047	3.47	4.75	0.068	5.02	6.88	0.97	71.55	98.08
0.013	0.96	1.31	0.047	3.47	4.75	0.068	5.02	6.88	0.971	71.62	98.18
0.013	0.96	1.31	0.047	3.47	4.75	0.07	5.16	7.08	0.974	71.84	98.48
0.014	1.03	1.42	0.053	3.91	5.36	0.07	5.16	7.08	0.975	71.92	98.58
0.015	1.11	1.52	0.053	3.91	5.36	0.07	5.16	7.08	0.975	71.92	98.58
0.018	1.33	1.82	0.053	3.91	5.36	0.08	5.90	8.09	0.975	71.92	98.58
0.02	1.48	2.02	0.053	3.91	5.36	0.083	6.12	8.39	0.976	71.99	98.69
0.023	1.70	2.33	0.054	3.98	5.46	0.086	6.34	8.70	0.977	72.06	98.79
0.025	1.84	2.53	0.054	3.98	5.46	0.087	6.42	8.80	0.98	72.28	99.09
0.025	1.84	2.53	0.055	4.06	5.56	0.087	6.42	8.80	0.981	72.36	99.19
0.025	1.84	2.53	0.055	4.06	5.56	0.096	7.08	9.71	0.981	72.36	99.19
0.025	1.84	2.53	0.058	4.28	5.86	0.097	7.15	9.81	0.982	72.43	99.29
0.026	1.92	2.63	0.058	4.28	5.86	0.098	7.23	9.91	0.983	72.51	99.39
0.026	1.92	2.63	0.058	4.28	5.86	0.101	7.45	10.21	0.986	72.73	99.70
0.026	1.92	2.63	0.058	4.28	5.86	0.108	7.97	10.92	0.988	72.87	99.90
0.031	2.29	3.13	0.058	4.28	5.86	0.124	9.15	12.54	0.988	72.87	99.90
0.035	2.58	3.54	0.058	4.28	5.86	0.13	9.59	13.14	0.989	72.95	100.00
0.037	2.73	3.74	0.059	4.35	5.97	0.13	9.59	13.14	0.989	72.95	100.00
0.037	2.73	3.74	0.06	4.43	6.07	0.137	10.11	13.85	0.992	73.17	100.30
0.037	2.73	3.74	0.06	4.43	6.07	0.496	36.58	50.15	0.994	73.32	100.51
0.043	3.17	4.35	0.06	4.43	6.07	0.965	71.18	97.57	0.996	73.46	100.71
0.046	3.39	4.65	0.062	4.57	6.27	0.968	71.40	97.88	0.998	73.61	100.91
0.046	3.39	4.65	0.064	4.72	6.47	0.968	71.40	97.88	1.012	74.65	102.33

Table 3.11 (n=100) is similar to Table 3.10, but represents isolates from Pre-K. The resultant percentage activities were arranged in ascending order so that the 50% cut-off mark could easily be seen. The values above and including 50% were considered positive for haemolysis and those below 50% were considered negative. The two haemolytic patterns represented by β- and α-

haemolysis are complete and partial haemolysis due to *Streptococcus pyogenes* and S*treptococcus pneumoniae*, respectively.

Table 3.12: Actual number of haemolytic vs. non-haemolytic isolates

	Number of haemolytic species			
	Post-K (n=100)		Pre-K (n=100)	
	Ref β	Ref α	Ref β	Ref α
Non-haemolytic isolates	91	91	72	71
Haemolytic isolates	9	9	28	29

Ref β and Ref α indicate that the percentages given were calculated with reference to average activity on the haemolytic assay of β- and α-haemolytic streptococci, respectively. Activity below 50% in each category was taken as negative (Tables 3.10, 3.11). Nine (9) of 100 isolates from Post-K showed ≥ 50% activity with reference to both β- and α-streptococci (Table 3.12).

Table 3.13: Average percentage haemolysis vs. non-haemolysis OD

	Mean % haemolysis			
	Post-K (n=100)		Pre-K (n=100)	
	Ref β	Ref α	Ref β	Ref α
Non-haemolysis (Mean±STDEV)	2.81±2.54	12.48±8.55	4.53±4.40	5.59±3.06
Haemolysis (Mean±STDEV)	62.55±2.91	100.00±4.79	72.37±0.80	97.51±9.02

Table 3.13 shows the average percentage OD readings of isolates with reference to β and α-haemolytic streptococci. These readings were grouped according to whether the activity was considered haemolytic or non-haemolytic as referred to Table 3.12. The average haemolytic activity was 62.55±2.91 % and 100.00±4.79 %, respectively (Table 3.13). Pre-K, on the other hand, had 28 and 29 isolates above and including 50% activity for β- and α-streptococci, respectively. These isolates had average percentage activities of 72.37±0.80 and 97.51±9.02, respectively (Table 3.13).

Average non-haemolytic activities for Post-K were 2.81±2.54% and 12.48±8.55% with reference to β- and α-streptococci, respectively. Those from Pre-K were 4.53±4.40 and 5.59±3.06.

The means of % haemolysis were 8.18 and 23.35 for Post- and Pre-K with reference to β-haemolysis ($p \leq 0.05$) and with reference to α-haemolysis, they were 13.49 and 32.25, Post- and Pre-K, respectively, with $p \leq 0.05$ (Table 3.13).

The haemolytic assay was then visited to investigate if there are any discernible patterns supporting our hypothesis. The haemolytic patterns of the river agreed with the other patterns in reinforcing the assertion that pollution already exists before the settlement. These patterns also suggest that the sources of pollution at these two sites are different. This is demonstrated by the relative means across the two test sites for both β- and α-haemolysis in Table 3.13 ($p \leq 0.05$).

There is a higher prevalence of "haemolytic *E. coli"* before the settlement (Table 3.12). However, after the settlement, the average haemolytic activity with reference to the α-streptococci is 103.10±4.79% compared to 97.51±9.02% before the settlement (Table 3.13). This difference in average haemolytic activity means that the extent of haemolysis due to subspecies found after the settlement is more than before it. This brings another point of concern regarding toxicity after Khayamnandi. It indicates that these waters could be more toxic. It should, however, not be missed that even before the river passed the settlement it was already showing high toxicity in accordance with *E. coli* haemolysis as shown by the fact that Pre-K had more than double the number of haemolytic isolates (Table 3.12). The prevalence of Pre-K haemolytic activity indicates that it is necessary to broaden remedial measures beyond Khayamnandi in order to improve the river's integrity.

The variation in haemolytic patterns across the two sites could serve as bacterial source tracking. These patterns seemed to point out a possibility of difference in the origin of the microbes. This difference can help one to rule out contamination beyond a certain area and therefore focus remedial measures to a limited area.

3.5 Conclusion

In order to interpret microbial test results, the standards for drinking water have to be taken into account. USEPA (2000) established two categories, namely, the Primary Standards and the Secondary Standards. The bases for Primary Standards are health considerations. These standards aim to protect the public from three classes of pollutants: pathogens, radioactive elements and toxic chemicals. Bacterial pollution falls into the category of pathogens. As such, there is EPA Maximum Contamination Levels (MCL).

Pathogens are discrete and not in solution. They often form flocs or adhere to suspended solids in water; as a result, the chance of acquiring an infective dose cannot be predicted from their average concentration in water (Boyer, 2008). The likelihood of a successful challenge by a pathogen, resulting in infection, depends upon the invasiveness and virulence of the pathogen, as well as upon the immunity of the individual (Clark *et al*, 2003). If infection is established, pathogens multiply in their host. Other pathogens can multiply in food or beverages, in this manner increasing their pathogenicity (CDC, 2011). Furthermore, unlike many environmental pollutants, the dose response of pathogens is not cumulative (WHO, 2003). Because of these properties, there is no acceptable upper limit for pathogens (WHO, 2002; USEPA, 2000). Thus, water intended for consumption or for personal hygiene should contain no human pathogens. Pathogen-free water is possible by selection of high-quality uncontaminated sources of water and

maintaining the integrity of such sources. This begins by being aware of the sources of pollution and the mechanisms thereof.

Plankenbrug River downstream Khayamnandi has had an ongoing problem of high microbial pollution to the magnitude greater than 10^5 microorganisms per 100 ml of water (Rossouw, 2004). The informal settlement has had high incidences of drinking untreated water, limited water treatment and full contact recreation with polluted water (Rossouw, 2004). Progress has been reported with regard to pollution due to Khayamnandi after the local municipality had spent several thousands of rands on waste control (Department of Housing, 2004a). The progress alluded to above, included construction of diversion channels for storm water to direct storm water into the sewerage system and prevent it from reaching the river (Department of Housing, 2004a). Despite these attempts, faecal pollution still prevails in the river as demonstrated in the current study.

The microbial data suggests that in one way or another, faecal matter still finds its way into the river system. The relationship between storm water and high microbial incidence affirms this. Sample 1 in the winter sampling series (Table 3.6) was at storm flow after the first heavy winter rains whereas samples 3 and 4 in the same series were at base flow. Storm water readings show that *E. coli* concentration peaked at storm flow and then seemed to return to relatively constant levels as the river water volume abates. Thus it may be supposed that wash-off from the settlement filtered into the river.

It could be speculated that among factors influencing these trends are human behaviour and vegetation around the river together with water velocity and temperature (Craig *et al*, 2002). Water velocity may impact on resident microbial flora in a number of ways. Sudden increase in

water flow may cause deep flora to come to the surface, thus increasing microbial density in the flow (Craig *et al*, 2002). It can also dislodge microbial populations resident in the river vegetation to the same effect. Rapid flow on the other hand can cause a decrease in the flow population by reducing the time it spends at one point (Boyer, 2008). Water flow drops drastically at base flow. This enables microbes to multiply in one place and therefore increase the spring count. Rain drop on open faecal matter can serve as both a source and a vehicle for transportation into the water bodies farther away (Boyer, 2008).

Vegetation can provide a substrate for the attachment of microbiota. This could be a conditioning layer that could develop into a biofilm. Organic matter can act as a buffer that neutralizes excessive charge, thus enhancing cells to aggregate. Microbial cells attach to surface and begin to form a glycocalyx. This in turn encourages accumulation of nutrients and as a result, growth of microbial flora. Incidentally such a microcosm makes disinfection difficult as the polysaccharide network protects the organisms. Temperature is another factor that can determine microbial population stability. A number of strains tend to decrease more rapidly around 18˚C than at lower temperature (Hara-Kudo *et al*, 2000). These authors observed that this decay is dependent on the initial population. Over 48 days at 18˚C, they found that the population was only reduced by less than 1 order of magnitude when the initial population was at the level of 10^7 cfu/100 ml. In the same study, it was observed that populations of 10^5 cfu/100 ml were reduced to 10^2 cfu/100 ml under the same conditions (Hara-Kudo *et al*, 2000). Therefore, the higher winter population at Post-K could be the source of sustained higher population in spring. Road construction and tarring facilitates the water movement during heavy rains from the settlements into river ways. Thus pollutants tend to reach rivers more readily. Beside such remote activities, the abuse of river banks is one of the major concerns at Post-K. Figures 3.8, 3.9 and 3.10 show

clearly the extent of pollution due to direct human activity. Throughout water collection there were fresh faecal deposits under the bridge. The number of individual deposits seemed to correlate with occurrence of rain. In contrast, no human faecal matter could be detected at the reference site at any given time.

Figure 3.8: Photo of Post-Khayamnandi site at base flow after a heavy rain

Figure 3.9: Conditions under the bridge at Pre-Khayamnandi at base flow after a heavy rain showing faecal deposits and vegetation in the river way.

Figure 3.10: Photo of the Eerste River at Swaaibrug (Reference site) showing spring water flow. During heavy rain in winter the water covered the grass and stony island to the right.

Individual tests within assays as well as complete assays were used to elucidate important information from this study. The choice of individual tests within assays to emphasize was in most cases prompted by pronounced differences across the test sites. Some of the tests were grouped together as they may be informing each other or simply because they best form a comprehensive picture together.

Ideal assessment of water integrity with regard to faecal contamination starts and ends with determining the presence or absence of faecal indicators (WHO, 2002). This is functional in assessing whether the water is potable or not (DWAF, 2000; WHO, 2002). USEPA's recommendations are 1 cfu/ml of water for drinking (USEPA, 2000). There are different limits for other water uses (DWAF, 2000). The point is that enumeration of the extent of contamination would be futile and/or expensive without having first ascertained whether the contaminant is present or not. In this study a sequence is followed. This study starts by ascertaining the presence of faecal contamination after having assessed the field parameters. The discussion of the presence of faecal contamination is then followed by quantitative assessment of the extent of the problem and discussion of characterization of a faecal indicator (*E. coli*). Characterization of *E. coli* will thereafter enable determination or suitability of use of the indicator organism as a biomarker and/or a bacterial source-tracking tool. At each step an effort is made to compare and contrast the two sampling sites, hence assessing the impact of the informal settlement on the nearby body of water.

In conclusion, Khayamnandi impacts adversely on Plankenbrug River. However, focusing remedial measures only in the settlement may not alleviate faecal pollution in the river system. The problem already exists before the river passes Khayamnandi. Different toxicological profiles and microbial distribution indicate that those sources before Khayamnandi are different from

those within the settlement. In conclusion assessment of waters associated with informal settlements should include points upstream of the settlements to produce a more comprehensive picture.

Chapter 4: Recommendations and further studies

The aim of this study was to assess the impact of Khayamnandi settlement on Plankenbrug River by comparing faecal contamination and potential toxicity upstream and downstream from the settlement. Microbial culture on chromogenic media, analytical profiling index (API) and haemolysis assays demonstrated that the Plankenbrug River already has a high faecal load upstream from Khayamnandi. Khayamnandi added onto this heavy faecal load. These assays also demonstrated prevalence of different biotypes across the two test sites. The distribution of *E. coli* biotypes downstream from Khayamnandi was narrower than upstream. To improve the water quality of the Plankenbrug River holistically, measures should be taken to trace and remediate sources of contamination from Khayamnandi and also upstream from Khayamnandi along the river.

4.1 Integrating microbial data generated into National Microbial Monitoring Programme (NMMP framework) of South Africa

Microbial studies on surface water, in line with NMMP of South Africa, should be done before and after the suspected source of faecal contamination. The NMMP extrapolates the inherent potential risk in the assessed water by drawing from the extent of microbial contamination and linking it to intended water use. The NMMP links *E. coli* concentrations to the adverse health risk index (HRI) associated with different water uses. Differences in HRI upstream and downstream to the settlement could help direct prioritization of remediation resources for the relevant water use.

4.2 Informal settlement development

Changing high population density of the informal settlement into low density housing is desired for settlements on river banks. But this entails removal of some of the households, which is not easy. The alternative is converting informal settlements on environmentally sensitive areas onto high density housing with appropriate road and waste removal infrastructure in order to reduce impact on the environment.

4.3 Education to communities

First and foremost, water quality is not entirely the responsibility of the authorities. It is also important to involve the resource users. In order to maintain the integrity of potable water, end-users should be educated with respect to the fragility of water. This awareness is even more important to local communities around surface waters. Ignorance of this could exacerbate water pollution or could increase the health risks due to community exposure. Thus, the water end-users should be incorporated into maintaining high water quality and made aware of their importance. This could be achieved by incorporating water integrity into the school curriculum. Involving communities in preventing water pollution may be a powerful tool in water resource management particularly in developing areas with insufficient infrastructure (Steynberg, *et al*, 1995).

4.4 Recommendation to small municipalities

Although 3 – 5% of *E. coli* are ß-glucuronidase (GUD) negative and some *Salmonella, Shigella* and *Yersinia spp.* are GUD positive, GUD tests are used to monitor water and food. Chromocult® coliform agar is one of the GUD tests. Total coliform and *E. coli* recoveries on this

media are significantly higher than some other standard methods. Enzymatic activity is a powerful tool in applied research fields. It enables rapid detection and identification of microorganisms. This advantage could prove useful to local municipalities in monitoring faecal contamination.

4.5 Further research

River systems such as the Plankenbrug River that show persistently high faecal pollution should be explored thoroughly using geographical data models such as ArcGIS (Diefenbach, 2004). ArcGIS could help in mapping out drainage systems, hydro-network systems to microbial pollution. Monitoring hydro edges and junctions could help redefine apparent diffused pollution sources to multiple point sources. Comparative studies should thereafter be undertaken followed by prioritization process within that river system. Data acquired through this process could further aid in speedily arresting pollution especially when coupled with full characterization of indicator microbes per site. However, high faecal indicator bacteria may also be due to microbial regrowth. In our study, physicochemical parameters, turbidity and presence of nutrients appear to be liked with microbial regrowth especially in the absence of apparent run-offs. Therefore regrowth has to be taken into consideration when analyzing such water.

Furthermore, full characterization of *E. coli* isolates could be beneficial as it could give a distribution profile that can help predict and trace possible *E. coli* related disease outbreaks. The need for characterization is important because of the potential adverse effects due to environmental microbial exposure is not incremental like other pollutants. Development of disease in humans depends on the susceptibility of the host, for example, age or immune status as well as to the virulence of the pathogen. Thus, thorough knowledge of regular prevailing

pathotypes in local surface water could prove useful in tracing the sources of epidemics (Do *et al*, 2006).

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