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Enumeration and Identification of Standard Plate Count Bacteria in Raw Water Supplies

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Abstract: *The present research work attempted to investigate the bacterial flora of Jaju Sagar Dam, a potential and sole municipal drinking water resource in Neemuch, M.P. The dam was sampled within an interval of 15 days and the analysis included isolation of Standard Plate Count bacteria on plate count agar at 20-22^{0}C (normal flora) and 37^{0}C (human pathogens), enumeration by Serial Dilution Agar Plate Technique and identification by conventional methods. The complete study was designed for a period of 12 months from January 2012 to December 2012 to study fluctuations in the counts monthly as well as seasonally. Elevated SPC levels than the standard (P<0.05), throughout the year indicated deteriorated quality of dam water. The SPC flora constantly and abundantly showed their presence (100%) in each and every sample analyzed, with a tendency of maximum counts during Rainy season followed by Summer and minimum counts in Winter season (P<0.05). On identification, repeated occurrence of Arthrobacter, Micrococcus, S.epidermidis, P.fluorescens, P.aeruginosa, Psuedomonas spp., Acinetobacter, Alcaligenes, Aeromonas, Moraxella, Klebsiella, Citrobacter freundii, Enterobacter aerogenes, E.coli, Flavobacterium, S.aureus, Streptococcus, V.cholerae, Salmonella spp., Shigella spp., Bacillus cereus, B.subtilis, Proteus mirabilis, P.vulgaris and Lactobacillus fermentum was observed.*

Keywords: *Bacteria, Dam, Enumeration, Identification, Standard plate count,*

I. Introduction

Natural waters contain a myriad of different bacterial species, many of which have not been cultured, much less identified. The number of organisms present varies considerably between different water types, and it is generally accepted that sewage-polluted surface waters contain greater numbers of bacteria than do unpolluted waters. Study of the microbial flora of raw source waters used in water supply treatment reveals a wide spectrum of diverse organisms in an ever-changing Kaleidoscope, which is a reflection of many influences. Many of these organisms are of no known significance in water supply while others may be pathogens, fecal organisms, and opportunists. It is these latter three groups that are of significance; they cause health problems, interfere with indicator detection, and cause taste and odor problems, in potable water. Residents of Neemuch use drinking water, supplied from **Sitaram Jaju Sagar Dam**. The dam was built on Harkyakhal Reservoir in 1961 as the sole source of municipal drinking water and is mainly rain fed. The dam is highly contaminated due to diverse pollutants like agricultural runoff, domestic sewage discharge and infiltration of grazing animal fecal matter, in addition to this, being a picnic spot human activities are also a common cause of contamination to the dam. Moreover, since inception sanitary analysis of the dam has not done yet, therefore the microbiological quality of the dam is a serious concern. In this direction, the present study was carried out to investigate the bacterial flora of the dam.

II. Material And Methods

2.1 Methodology of Sample Collection

Sampling was done from the pre-determined sites i.e., the four corners of the dam in sterile 250-ml glass bottle fitted with a glass stopper, previously sterilized by heating in the hot-air chamber to 150° C for 3 hours. At each site of the Jaju Sagar Dam, the bottles were opened aseptically, then held at their bases and submerged to a depth of about 20 cm with the mouth facing upwards. Samples were taken by filling the bottles to the top to exclude air. In case of a current, the bottles were tilted towards the current and filled. Bottles were removed from the dam and the stoppers were replaced and covered with aluminium foil. All the four samples were homogeneously mixed to prepare a composite sample. As recommended by The Department of Health Water Research Commission [1], monthly samples from a lake or dam (i.e. 12 samples per year) are sufficient to determine if the source is suitable for domestic use, but the samples were collected twice a month (Sample 1-**S1** and Sample 2-**S2**) for the accuracy.

2.2 Enumeration of Total Heterotrophic Bacteria (THB)

THB were enumerated by employing **Serial Dilution Agar Plating Method**. Serial dilutions of the sample were prepared upto 10^{-5} by adding 1 ml water sample to 9 ml sterile **physiological saline** (0.8%). An

amount consisting of 1 ml from each dilution was transferred aseptically onto duplicate sterile Petri dishes and approximately 18-20 ml of molten **plate count agar** (45°C) (Himedia, Mumbai, India) was added. The sample and agar were mixed thoroughly by rotating the plates several times. The plates were allowed to set and inverted, then one plate was incubated at 37°C and another at 20-22^{0}C for 24-48 hours. Colony counts were made from plates with less than 300 but more than 30 colonies and results expressed as actual colony counts multiplied by the dilution factor. Colony counts were expressed as colony forming units (cfu/ml) of the sample.

$$\text{No. of cfu/ml} = \frac{\text{No. of colonies counted X Dilution factor}}{\text{Volume of sample taken}}$$

2.3 Identification of SPC bacteria

Selected colonies of all morphological types were picked from standard Plate Count Agar plate. Isolates were purified by streaking on nutrient agar. Pure cultures were maintained on NA slants at 5^{0}C. The cultures were identified according to **Bergey's Manual of Systematic Bacteriology**, the most important source of information for the identification of unknown bacteria. According to it, the first approach to bacterial identification involve preliminary microscopic examination of the gram-stained preparation for its categorization into two broad groups. After knowing the gram reaction (gram positive or negative), colony and cell morphology (rods or cocci) of the bacterium, the identification was done with the help of various key charts [2, 3, 4] so as to confirm the identity of bacteria.

III. Results And Discussion

3.1 Occurrence and Enumeration

Enumeration of heterotrophic plate counts is commonly used as an indicator of overall microbiological quality [5], they are 'microorganisms that require organic carbon for growth', these include bacteria, yeast and moulds, that are part of the natural (non-hazardous) microbes of water and organisms that are derived from diverse pollutant source. They are typically described as opportunistic pathogens [6]. In the present study, the isolation rate of total heterotrophic bacteria was 100% (n=24) throughout the sampling months. The total viable counts obtained in dam water samples are presented in Table 1. The comparative average counts are graphically presented in Fig 1. The counts at 37^{0}C ranged from 5.7x10^2 cfu/ml (Jan S1) to 23.0x10^4 cfu/ml (July S1) and 22^{0}C counts ranged from 12.0x10^2 cfu/ml (Dec S2) to 19.5x10^4 cfu/ml (July S1). It is evident from these results that throughout the sampling months, the counts were significantly higher than the drinking water standards (500cfu/ml) ($P<0.05$) [7], hence no sample complied with the drinking water guideline value. Prasai et al., [8] also found 82.6% (n=132) drinking water samples from Kathmandu valley, above the WHO guideline value. Al-mezori and Hawrami [9] observed that raw water samples at all the studied stations were unsuitable for drinking according to WHO. Differences observed in log counts at 22^{0}C and 37^{0}C were **non-significant** throughout the year ($P>0.05$). Osman et al., [10] also noticed little variations in the log number of total bacterial count at 37ºC and 22ºC in all the tested water samples in Nile, Egypt. Elevated SPC levels indicates a potential health risk posed by opportunistic pathogens. High SPC counts in drinking water sources have been found in several studies [11, 12, 13]. The presence of these rates of bacteria are due to the soil and agricultural runoff, effluent from septic system or domestic sewage discharge and infiltration of grazing animal fecal matter, human activities like recreation and farming, in addition to, atmospheric deposition leading to increase of various microbial densities [14]. Contamination of source water was also observed by Mukherjee et al., [15] who found that drinking water of Damodar River, Jharkhand and West Bengal Region, India was not potable at maximum sampling stations. Muyima et al., [16] found that all the indicator bacteria analyzed in Alice drinking water were above the South African acceptable standards. Obasi et al., [17] enumerated THPCs in Ero and Ureje Dams in Ekiti State, Southwest, Nigeria and detected SPCs above the recommended standards of WHO 2002 which are the municipal drinking water sources.

3.2 Seasonal Prevalence

Seasonal variations (Fig. 2) in the average counts showed that highest average log count was obtained in rainy season (July, August, September, October), higher in summer (March, April, May, June) and lowest in winter (November, December, January, February) ($P<0.05$). Heterotrophic plate counts at 22^{0}C were slightly higher than 37^{0}C counts during winter season and during summer, 37^{0}C counts were slightly higher than those obtained at 22^{0}C. The 22^{0}C average log count obtained in winter was 3.58, in summer it was 4.73 and in rainy season it was 4.92. The 37^{0}C average log count in winter was 3.21, in summer was 4.91 and in rainy it was 4.93. Highest counts obtained in rainy season, may be attributed to influx through runoff of microorganisms originating from vegetation decay, municipal sewage, garbage, domestic and fecal waste into the dam making it highly contaminated. Whereas higher counts detected in summer season, clearly indicates that the high temperature of dam water might enhanced the growth and reproduction of the organisms and least counts in winter was probably due to the lower temperature of water which might decelerated the growth rate of the

organisms. Thus, variations in precipitation and water temperature contributed to the observed seasonality of occurrence. Similar findings have been demonstrated by other authors. Agarwal et al., [18] observed maximum number of total viable counts during summer and rainy seasons and minimum during winter, in Tehri Dam Reservoir, Garhwal Himalaya. Mthembu et al., [19] also reported lower HPCs in Mhlathuze River during winter season in comparison to that in summer. Likewise, Al-mezori and Hawrami [9] also detected higher counts in the warmer seasons.

Table 1: Standard Plate Counts in Dam water samples at 22^0C and 37^0C

Sampling months	S1				S2			
	22^0C		37^0C		22^0C		37^0C	
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
January	15.4×10^2	3.18	5.7×10^2	2.75	8.9×10^3	3.94	7.1×10^2	2.85
February	6.4×10^3	3.80	8.2×10^2	2.91	8.6×10^3	3.93	9.8×10^3	3.99
March	9.5×10^4	4.97	12.2×10^4	5.08	8.2×10^4	4.91	14.0×10^4	5.14
April	6.8×10^4	4.83	9.0×10^4	4.95	7.0×10^4	4.84	11.3×10^4	5.05
May	4.5×10^4	4.65	8.5×10^4	4.92	3.6×10^4	4.55	7.2×10^4	4.85
June	3.9×10^4	4.59	5.1×10^4	4.70	3.0×10^4	4.47	4.2×10^4	4.62
July	19.5×10^4	5.29	23.0×10^4	5.36	17.0×10^4	5.23	19.6×10^4	5.29
August	15.5×10^4	5.19	16.2×10^4	5.20	16.8×10^4	5.22	18.2×10^4	5.26
September	9.0×10^4	4.95	10.4×10^4	5.01	13.7×10^4	5.13	14.5×10^4	5.16
October	19.0×10^3	4.27	17.5×10^3	4.24	11.7×10^3	4.06	8.8×10^3	3.94
November	7.8×10^3	3.89	6.5×10^3	3.81	5.5×10^3	3.74	3.8×10^3	3.57
December	13.2×10^2	3.12	9.1×10^2	2.95	12.0×10^2	3.07	7.5×10^2	2.87

3.3 Bacterial Flora of the Dam

Twenty different types of SPC bacteria were isolated in varying amounts and frequencies throughout the year from the dam, which included *Arthrobacter, Micrococcus, S.epidermidis, P.fluorescens, P.aeruginosa, Psuedomonas spp., Acinetobacter, Alcaligenes, Aeromonas, Moraxella, Klebsiella, Citrobacter freundii, Enterobacter aerogenes, E.coli, Flavobacterium, S.aureus, Streptococcus, V.cholerae, Salmonella spp., Shigella spp., Bacillus cereus, B.subtilis, Proteus mirabilis, P.vulgaris* and *Lactobacillus fermentum*. Similar to our findings there are uncountable national and international studies on drinking water sources, which revealed the occurrence of these organisms and many other diverse potential pathogens. Manji et al., [20] reported incidence of *S.aureus,* coliforms, *Bacillus* species, *Pseudomonas aeruginosa* and *E.coli* in treated tap and untreated well and stream water sources of Calabar South Local Government Area. Hatha et al., [21] observed increased prevalence of *V.cholerae, V.parahaemolyticus, E.coli,* and *Salmonella* in Vembanadu Lake, along west coast of India. Lechevallier et al., [4] isolated nearly 700 SPC bacteria from distribution water, raw water, drinking water and distribution water during a chlorine failure- *Actinomycete, Arthrobacter* spp. *Bacillus* spp., *Corynebacterium* spp., *Micrococcus luteus, Staphylococcus aureus, S.epidermidis, Acinetobacter* spp., *Alcaligenes* spp., *F.meningosepticum, Moraxella* sp., *Pseudomonas alcaligenes, P.cepacia, P.fluorescens, P.mallei, P.maltophilia, Pseudomonas* spp., *Aeromonas* spp., *Citrobacter freundii, Enterobacter agglomerans E.coli, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumonia, Serratia liquefaciens* etc. Out of all these, *Aeromonas* spp. and *Enterobacter agglomerans* were the two most common groups of SPC bacteria he found, in raw water. Rahim et al., [22] found *Staphylococcus (S.aureus, S.epidermidis and S.saprophyticus*), *Micrococcus* (*M.varians, M.nishinomiyaensis, M.kristinae and M.roseus*) and *Corynebacterium diphtheria, Bacillus* (*B.cereus, B.anthracis, B.mycoides, B.thuringiensis, B.lentus, B.subtilis, B.pantothenticus, B.firu*) in all the tested raw waters of Atbara River, of Al Gedarif city, Sudan. According to Mthembu et al., [19] *E.coli, Enterobacter* spp. and *Pseudomonas* spp. were constantly detected at all five sites of Mhlathuze River in KwaZulu-Natal and *Klebsiella* spp., *Proteus* spp., *Serratia* spp., *Aeromonas hydrophila* and *Citrobacter freundii* were also occasionally isolated from the various sites. Ekhaise et al., [23] obtained *Escherichia, Bacillus, Pseudomonas, Klebsiella, Proteus, Staphylococcus, Streptococcus, Clostridium, Salmonella, Shigella* and *Enterobacter* in Ebutte River, Uhunmwonde Local Government Area, Edo State, Nigeria, above the WHO standards. Kolawole et al., [24] isolated *Bacillus* sp., *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Lactobacillus sp., Micrococcus varians, Proteus vulgaris, P.aeruginosa, Salmonella sp., Serratia marcescens, Shigella sp., S.aureus, Streptococcus sp.,* and *Yersinia enterocolitica* from Oyun river. Sati et al., [25] detected *P.aeruginosa, E.coli* and *E.faecalis* in different drinking water resources of Makkah City, Saudi Arabia. Chandra et al., [26] obtained *E.coli, E.aerogenes, P.aeruginosa, S.aureus, K.pneumoniae* and *V.cholerae, V.vulnificus* in Gola river water, Uttaranchal, India.

A source water's microbial quality is extremely variable and depends on many factors, including domestic and fecal animal activity on the watershed; human activities on the watershed, including recreational, manufacturing and fabrication and agricultural activities; municipal pollution inputs from the raw sewage to primary and secondary wastewater treatment plant effluents; and storm events over the watershed that wash

natural and synthetic contaminants into the surface water or that percolate into the groundwater aquifers. The number and kinds of microbes vary among different water sources and depends on the kind and level of contamination. Ahmed et al., [27] recovered *B.cereus, E.aerogens, S.aureus, E.coli, Salmonella* spp., *Shigella* spp., and *Streptobacillus* spp. from water samples of different dams and related filtration plants of Rawalpindi, Islamabad region in Pakistan. Yongsi et al., [28] identified 1,242 isolates of enteric bacteria from a variety of drinking water sources of Yaounde, which included *Shigella* (0.24%), *Salmonella* (1.30%), *E.coli* (5.15%), *Enterobacter* (12.40%), *Citrobacter* (13.44%), *Proteus* (22.06%), *Klebsiella* (37.76%) and of the 461 aerobic bacteria he recovered 28.20% *Acinetobacter* and 71.80% *Psuedomonas.* Their 95% tested samples crossed WHO limits. Ihejirika et al., [29] recovered *Escherichia coli* (100%), *Klebsiella* spp. (71.0%), *Shigella* spp. (71.0%), *Salmonella* spp. (71.0%), *Proteus* spp. (42.9%), *Vibrio* spp. (42.9%), *Pseudomonas* spp. (42.9%), *Staphylococcus* spp. (85.7%), *Bacillus* spp. (100%), *Enterobacter* spp. (57.1%), *Citrobacter* spp. (14.3%), *Serratia* spp. (14.3%) and *Streptococcus* spp. (14.3%) from Imo River, Nigeria. Obasi et al., [17] detected *E.coli* sp., *Klebsiella* sp. and *Pseudomonas* sp. from Ero and Ureje Dams, the municipal water supply of Ekiti State, Southwest, Nigeria. He found *E.coli* counts above the recommended standards of WHO 2002. Mwajuma et al., [30] recovered *Shigella flexineri, Shigella boydii, Aeromonas hydrophilla,* and *Salmonella* spp. (non-typhi), *Klebsiella* and *Pseudomonas* spp. from selected drinking water sources in Samburu South. Shittu et al., [31] isolated *E.coli, Enterobacter aerogenes, Pseudomonas* spp., *S.aureus, Salmonella typhosa, Shigella* spp., *V.cholerae, Proteus* spp. and *Klebsiella* spp. from drinking water of Abeokuta, Nigeria. Smruti and Sanjeeda [32] identified *E.coli, Enterobacter, Klebsiella, Salmonella* and *Shigella* from surface waters in Indore. Kazmi et al., [33] isolated TCs, FCs, *E.coli, Salmonella, Shigella, Klebsiella, Proteus, Serratia, Enterobacter, Citrobacter, Stapylococcus, Pseudomonas, Enterococcus* and *Bacillus* from local drinking water of Islamabaad, Pakistan. Sila et al., [34] recovered *E.coli, Klebsiella* sp., *S.aureus, Enterobacter* sp., *Citrobacter* sp. and *Shigella* sp. from the Lamingo Dam Jos Nigeria, its water filter tanks and water taps. Klebsiella sp. had the highest percentage occurrence of 70%, followed by *Citrobacter* sp. (68%), *E.coli* (61%) and *S.aureus* (48%). *Shigella* sp. had the least percentage occurrence (4%). Omezuruike et al., [35] identified *S.aureus, Salmonella* species, *E.coli, P.aerugionosa, Enterobacter aerogenes, Bacillus* species, *Proteus* species, *Klebsiella* species, *Flavobacterium* species and *Acinetobacter* species from different drinking water samples of Abeokuta and Ojota, Lagos state Nigeria. Kurup et al., [36] found *Acinetobacter* sp., Coagulase Negative *Staphylococcus* sp., *Lactobacillus* sp., Nonhemolytic *Streptococcus* sp., *Chromobacterium* sp., *Flavobacterium* sp., *Pasteurella* sp., *Salmonella* sp., *Providencia* sp., *Micrococcus* sp., *Pseudomonas* sp. and *Bacillus* sp. from all the tested drinking water samples of Georgetown, Guyana. Obi et al., [37] obtained *Escherichia coli, Plesiomonas shigelloides, Vibrio, Enterobacter cloacae, Shigella, Salmonella, Aeromonas hydrophila, Aeromonas caviae* and *Campylobacter* in water sources of the Venda Region of the Limpopo Province, with a higher tendency in the months of summer in comparison to other seasons ($p<0.05$). According to Vagarali et al., [38] *Pseudomonas* (20%) was the common cause of contamination in the drinking water samples tested by him, while *E.coli* and *Klebsiella pneumoniae* were present in 10% samples, *Proteus vulgaris* had 3.33% frequency. Suthar et al., [39] also found *E.coli, P.aeruginosa, Enterobacter aerogenes, Klebsiella* sp., *Proteus vulgaris, Alcaligenes faecalis, Bacillus cereus, S.aureus, Streptococcus lactis* and *Micrococcus luteus* in his study on the detection of potentially pathogenic bacteria in the drinking water in Northern Rajasthan. Eleven different kinds of enteric bacteria were isolated by Jayana et al., [40] from different drinking water sources of Madhyapur Thimi which included *E.coli, Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *P.vulgaris, P.mirabilis, P.aeruginosa, S.typhi, S.paratyphi, Shigella dysentery*, and *V.cholerae*. Percentage of *Enterobacter* spp. (29.5%) was found to be maximum followed by *E.coli* (24.6%), *Citrobacter* spp. (20.4%), *P.vulgaris* (7.0%), *Klebsiella* spp. (5.6%), *P.mirabilis* (3.5%), *S.dysentery* (2.8%), *S.typhi* (2.1%), *P.aeruginosa* (2.1%), *S.paratyphi* (1.4%), and *V.cholerae* (0.7%). Edberg et al., [41] isolated different species of coliforms from source water which included *Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter agglomerans, Enterobacter* sp., *Enterobacter cloacae, Enterobacter aerogenes, Citrobacter freundii, Citrobacter diversus, Serratia fonticola, Serratia rubidaea, Serratia odorifera, Hafnia alvei,* and *E.coli*. Lamka et al., [42] identified several bacteria from standard plate count agar in rural drinking water supplies, which included *Corynebacterium* spp., *Aeromonas hydrophila, Arthrobacter* spp., *Pseudomonas acidovorans, Actinomycetes, P.alcaligenes, Bacillus, P.mallei, S.aureus, P.maltophilia, S.epidermidis, Acinetobacter calcoaceticus, S.saprophyticus, Alcaligenes denitrificans, Micrococcus luteus, Flavobacterium* spp., *M.roseus, Moraxella bovis, M.kingu* and total coliforms- *Citrobacter freundii, Klebsiella pneumoniae,* and *E.coli*. Clark et al., [43] detected different types of indicator bacteria in municipal raw water, drinking water, and new main water samples. All the water samples contained *E.coli* (11.6–39.7%), *Enterobacter aerogenes* (18.1–26.3%), *Aeromonas hydrophila* (8.8–17.0%), *Klebsiella pneumoniae* (7.7–10.3%), and *Citrobacter freundii* (5.09–22.7%). Antony et al., [44] observed that drinking water of Ananthanar channel water of Kanyakumari district, Tamil Nadu, was contaminated with *P.aeruginosa, Shewanella putrefaciens, Klebsiella pneumoniae, Citrobacter freundii* and *Proteus mirabilis.*

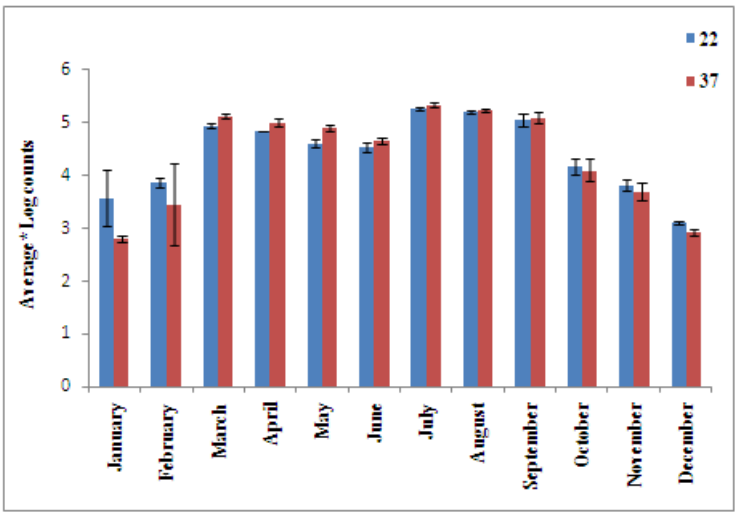

Fig 1: Standard plate counts of Raw water samples at 22^0C and 37^0C
*Mean of S1 & S2
*The error bars show standard deviations

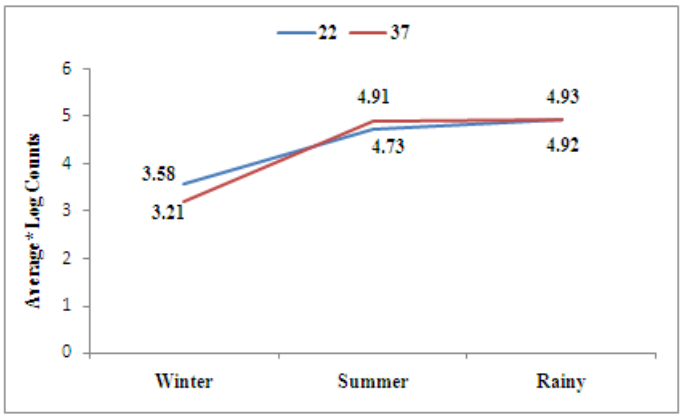

Fig 2: Seasonal shifts in Standard plate counts
* Mean of four months

IV. Conclusion

Natural waters are rapidly subjected to important changes in their microbial quality. Changes in the microbial quality of water may arise from agricultural use, discharges of sewage, wastewater resulting from human activity, storm or surface water runoff, industrial pollutants etc. which leads to an increase in amounts of organic matter that serve as an excellent nutritional source for growth and multiplication of the contaminating microorganisms. Consequently, the water source becomes a potential carrier of contaminating microorganisms. If the treatment plant does not effectively or adequately treat the raw water, it serves as a source of infections to the end-point users through distribution systems. The occurrence of pathogens, fecal organisms, and normal flora in natural raw waters is obvious and natural, hence it is strongly recommended that, in developing countries the treatment plants need to be evaluated timely to ensure they produce **safe** product at least, if not sterile.

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