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Reduction of Pathogenic Indicators in Sewage Water using *Azadirchta indica*, A. Juss (Neem) and *Pistia startiotes* (Water Lettuce)

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Abstract: Sewage is domestic, municipal or industrial liquid waste consisting of organic matter, inorganic salts, heavy metals, bacteria, viruses, and nitrogen. Fecal bacterial species, such as coliformes and streptococci are predominant in the sewage waters and are known as pathogenic indicators of sewage pollution. In the present study, we adopted a biological method to control the fecal contamination of sewage waters. A mixture of neem leaves and neem seeds along with water lettuce (*pistia*) was used to control fecal bacterial species. Our study showed a decline in total coliformes, when compared to faecal coliformes and streptococci. This decline in total coliformes, was greater when a combination of neem leaves and seeds were used along with *pistia* leaves. Faecal coliformes and faecal streptococci, showed a significant decline as indicated by the most probable number index (MPN) which was found to be 110 on the 5th day and it was less than 30 on the 10th day. Our observations in this study, clearly illustrates, that neem leaves and neem seeds along with the, *Pistia startiotes* can be effectively used to treat sewage waters, specifically to reduce the fecal contamination of coliformes and streptococci.

Keywords: Coliformes, Streptococci, MPN, *Azadirchta indica*, *Pistia startiotes*, Sewage treatment

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Introduction

Sewage is domestic, municipal or industrial liquid waste consisting of organic matter, inorganic salts, heavy metals, bacteria, viruses, and nitrogen. Faecal material in low concentrations has little effect on the environment and may actually benefit the ecosystem through the input of particulates and nutrients. Faecal pollution was observed to be less during the dry season (Jagals *et al.*, 1995) when compared to other times of the year. There are a number of strains of bacteria and viruses that are found in raw sewage that cause

sewage pollution. The definition includes *Escherichia coli*, the most numerous facultative bacterium in the faeces of warm-blooded animals, plus species belonging to the genera *Enterobactor*, *Klebsiella* and *Citrobacter*. These latter organisms are present in wastewater but can be derived from other environmental sources such as soil and plant materials. No organism fulfills all the criteria for an indicator organism, but the coliform bacteria fulfils most of the criteria as suggested by Carol and Katherine (1990). They enter the waste

water when excreted by wild and domestic animals as well as existing in the human gut. Faecal coliform are widely used as an indicator of faecal contamination of water (WHO, 1975; Anonymous, 1989) originating in the intestinal tract of humans and other animals (Thomann and Mueller, 1987) although other sources may also exist (Dufour, 1997). They provide stronger evidence of the possible presence of faecal pathogens than do total coliforms. Bordalo (1994) has reported faecal coliforms as subgroup of the total coliforms, distinguished in the laboratory through elevated temperature tests (43 - 44.5 C, depending on the test). Numerous studies have indicated that the greater the sewage contamination and exposure of people to it the higher the risk of contracting ear, nose and throat infections and stomach upsets such as gastroenteritis. Faecal streptococci make a heterogenous group of Gram-positive cocci which contains members of the genera *Enterococcus* and *Streptococcus* within which certain species are faecal strains of bacteria (Hernandez *et al.*, 1992). The value of using *Enterococci* for the survey of faecal contamination of water was established at the beginning of the century (Houston, 1900) then confirmed by general practice in numerous countries and finally validated by epidemiological studies, which demonstrated a close correlation between the concentration of enterococci and risk of gastro-intestinal complaints linked to drinking or bathing, (Cabelli *et al.*, 1979; Careps, 1985; Larbaigt, 1989; Mujeriega *et al.*, 1997). The methods used to count faecal Streptococci should aim at an ideal group made up of faecal *Enterococcus* and faecal *Streptococcus*. There are 2 methods used for the analysis of faecal Streptococci and coliform. Membrane filter (MF) method and most probable number (MPN). The MPN procedure, uses azide dextrose as presumptive broth and ethyl violet azide as confirmatory broth (Litsky *et al.*, 1953) and allows the growth of *Aerococcus* spp, *Streptococcus lactis*, *Streptococcus acidominimis*, *Streptococcus mitis*,

Streptococcus sanguis (Volterra *et al.*, 1986). As faecal Streptococci are known to cause illness, especially gastroenteritis, therefore the solution to prevent these infections is to undertake sewage treatment. Various methods may be used to treat sewage water, including sand filters, reed beds or grass plots etc. Disinfections, using ultra violet light to kill bacteria, is another method and is being used at a number of sewage treatment plants. Isolating and identifying specific pathogenic microorganisms is beyond the capability of most water utility laboratories. Though there are several methods, employed in the treatment of sewage water, not many studies have been attempted with reference to biological method of sewage treatment. In this connection, there are two plants, which need to be mentioned- Neem and water lettuce (pistia). Unlimited medicinal properties of neem have already been unraveled by many authors (Ketkar, 1976; Parmar, 1987; Nanduri *et al.*, 1995; Dhanukar *et al.*, 2000; Kumar *et al.*, 2013, 2015). However, there are not many instances where neem has been used to treat sewage water. Use of water lettuce to treat pond effluent (Kone, 2002) for irrigational purposes and also to use in market gardens has been in practice in Senegal (ENDA project). Therefore in the present study we have attempted a biological method to treat the sewage water primarily to control fecal contamination by using neem and pistia.

Materials and Methods

Collection of sewage sample:

The sewage samples of 2 types namely the untreated sewage and treated (Secondary treated sewage) were collected from the sewage treatment plant - Nesapakkam, Chennai, India. *Preparation of neem leaf and neem seed powder:* Neem leaves were collected, washed in tap water, allowed to air dry under shade, then kept in a hot air oven for 24 h at 90 +5 C, Afterwards they were powdered, packed in polythene bags and sealed at the time of storage.

A commercial product of the Neem seed were bought and was dried under shade, then kept in a hot air oven for 24 h at 90+5 C. Afterwards they were powdered to fine granules, packed in polythene bags and sealed at the time of storage.

Collection of Pistia stratiotes:

The Pistia plant also called water lettuce were collected from a pond located at Avadi, India. They were maintained in a cement tanks.

Experimental Design:

The untreated and treated samples were separated in to 6 groups (Table 1). Group I: This group was used as a control with 5 litres of untreated and treated sewage samples in separate tubs.

Group II: 5 g of prepared neem leaf power was mixed with 5 litres of both untreated and treated sample.

Group III: 5 g of finely grounded i.e., the prepared neem seed powder were mixed in 5 litres of untreated and treated sewage samples each.

Group -IV: 50 g of Pistia was added to 5 litres of untreated and treated sewage samples separately.

Group V: - 5 g of prepared neem leaf powder and 50 g of pistia (rate of 50 g/tub of sewage) were combined and added to the 5 litres of untreated and treated sewage sample.

Group VI: - 5 g of neem seed powder and 50 g of pistia plant were combined and added with the 5 litres sample of untreated and treated sewage.

Table 1: Various treatment groups (I-VI)

S. No.	Groups	Samples
1	Group I	Control
2	Group II	Neem Leaves
3	Group III	Neem Seed
4	Group IV	Pistia
5	Group V	Neem leaf+Pistia
6	Group VI	Neem Seed+Pistia

Most Probable Number (MPN) Technique:

In MPN Technique, at least 5 tubes or 3 tubes (not less than 3 tubes) were used for each dilution (minimum three dilutions). The selection of portion sizes depends on the practice of coliform density.

Presumptive Test for Coliform:

The presumptive test is specific for detection of coliform bacteria. Measured aliquots of the sample to be tested were added to lactose fermentation broth containing an inverted gas vial. Because these bacteria are capable of using lactose as a carbon source (the other enterio organisms are not), their detection is facilitated by use of this medium. In addition to lactose, medium also contains a surface tension depressant, bile salts, are also used to suppress the growth of organisms other than coliform bacteria. Tubes of lactose medium were inoculated with 1 ml, 0.1ml and 0.01 ml of samples. At least 5 tubes or 3 tubes were used for each dilutions. Development of gas in any of the tubes are presumptive evidence of the presence of coliform bacteria in the sample.

Procedure:

A series of tubes were inoculated with lactose broth with appropriate quantities of samples. Dilutions of 1 ml, 0.1 ml, and 0.01 ml were used. The sample portion should be in suitable size in order to have some negative tubes in higher dilutions. Inoculated fermentation tubes were incubated at 35+0.5 C. At the end of 24 h tubes were shaken gently and examined. If no gas had formed, this step was repeated at the end of 48+3 h. The presence or absence of gas formation in the inverted vials was recorded. The gas formation will indicate a positive presumptive test and the absence of gas will indicate a negative test.

Confirmed Test for Total Coliform:

All primary fermentations tubes showing any amount of gas at the end of 48 h of incubation were submitted to the confirmatory test. Primary

fermentation tubes showing gas were gently shaken, and with a sterile metal loop, 3 mm in diameter one loopful of medium was transformed to a fermentation tube containing 5 ml of Brilliant Green Lactose Bile broth. The inoculated Brilliant Green Lactose Bile broth tubes were incubated at 35 C + 5 C for 48 + 3 h. The formation of gas in any amount in the inverted vial of Brilliant Green Lactose Bile broth fermentation tube at any time with 48+3h constituted a positive confirmed test.

Confirmed Test for Faecal Coliform:

All positive presumptive tubes were transformed from the total coliform MPN Test to EC Medium. This examination may be performed simultaneously with the confirmatory procedure using Brilliant Green Lactose Bile Broth. A sterile metal loop with a minimum 3 mm diameter were used to transfer from the positive fermentation tube to 5 ml of EC medium, When making such transfers, the presumptive tube was gently shaken. Inoculated tubes were incubated in water bath at 44.5 + 0.2 C for 24 +2 h. Gas production in a fermentation tube within 24 h or less was considered a positive reaction indicating faecal origin.

Test for Faecal Streptococci:

A series of tubes with 5 ml of Azide dextrose broth were inoculated. The portions of the samples 0.1 ml and 0.01 ml, in dilutions were used. The inoculated tubes were incubated at 35+ 0.5 C. Each tube was examined for presence of turbidity at the end of 24+2 h. If no definite turbidity was present, the tubes were reincubated at 48+3 h and read again.

Confirmed Test:

All Azide dextrose broth tubes showing turbidity after 24 or 48h, incubation were subjected to the confirmed test. Three loopful of growth from each azide dextrose tube were transferred to a tube containing 5 ml Ethyl violet wide broth. The wire loop should have a minimum diameter of 3 mm. The inoculated tubes were incubated at 35 -+0.5 C for 24 h. The presence of faecal Streptococci was

indicated by the formation of a purple button at the bottom of the tube, or occasionally by a dense turbidity. All positive results were recorded and then discarded. If no growth occurred in ethyl violet azide broth in 24 h, the tubes were reinoculated with an additional 3 loopful from original positive azide broth cultures and re-incubated for second 24 h period, The results were recorded as final.

Statistical Analysis:

The percentage change was calculated between the control and experimental for the various experiments using the formula-

$$\text{Percentage change} = \frac{\text{Control} - \text{Experimental}}{\text{Control}} \times 100$$

Results

Total Coliform count- Untreated Sample:

The Results of the total coliform come in the experimental groups of untreated sewage showed a decrease in the Group II (MPN index 030) on the 5th day and a further decrease of 30 on the 10th day, the percentage change being 06.12% and 98%, respectively (Fig. 1), Group III and IV recorded a decrease from the control with Total coliform count of 140 on 5th day and a further reduction to 110 on the 10th day. However, a total reduction was seen in Group III on the 10th day. Group V and IV showed a significant decrease (MPN index 110) on the 5th day and a 100% reduction on the 10th day was recorded in group VI (Table 2).

Total Coliform count- Treated sample:

The Results of the total coliform count in the different groups of treated sewage showed a reduction in the control sample, The ratio of Total coliform count being 2:1 (24.000 : 11.000). There was no reduction seen in the group containing only neem leaves on the 5th day, but a significant decrease was noted in the MPN index on the 10th day (Fig. 2). The group III and IV containing only neem seed and Pistia, respectively showed a drastic decrease of 30 and <30 on 5th day which

Table 2: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		% change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	22000				>24000			
	II	930	150	3800	96.12%	30	5	90	99.8
	III	140	30	370	99.4				100
	IV	140	30	370	99.4	110	30	360	99.5
	V	110	30	360	99.5	110	30	360	99.5
	VI	110	30	360	99.5				110

Fig.1: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index.

continued on the 10th day also. Group V and Group VI with a combination of Neem leaf and Neem seed Group V and Group VI with a combination of neem leaf neem seed with Pistia aslo showed a significant decrease in the total coliform counth on 5th day (70 and 40) and further decrease to <30 on the 10th day, the percentage change ranging from 99.3% to 99.7% (Table 3).

The Faecal coliform count - Untreated Sample:

The faecal coliform count in the different

experimental groups of untreated sewage sample showed a significant reduction in all groups as compared to the control (1,2000). Group III, IV and VI showed a maximum reduction of <30 (MPN index) on the 5th day itself and which was continued till the 10th day, the % change being 97.5% (Table 4). In Group II, the reduction of total coliform count was slightly lesser (110) on the 5th day when compared to the other groups and 30 on the 10th day (Fig. 3).

Table 3: Total Coliform count in different Experimental groups of treated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		%change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	11000	1500	48000	-	11000	1500	48000	-
	II	11000	1500	48000	0	110	30	360	99
	III	30	-	-	99.7	30	-	-	99.7
	IV	30	50	90	99.7	30	50	90	99.7
	V	70	10	210	99.3	30	-	-	99.7
	VI	40	50	200	99.6	30	-	-	99.7

Fig. 2: Total Coliform count in different Experimental groups of treated sewage samples using MPN index.

Table 4: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		% change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	1200	300	3800	-	1200	300	3800	-
	II	110	30	360	90.8	30	-	-	97.5
	III	30	-	-	97.5	30	-	-	97.5
	IV	30	-	-	97.5	30	-	-	97.5
	V	140	30	370	88.3	30	-	-	97.5
	VI	30	-	-	97.5	30	-	-	97.5

Fig. 3: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index.

Table 5: Total Coliform count in different Experimental groups of treated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		% change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	930	150	380	-	930	150	380	-
	II	750	140	2300	19.3	430	70	2100	53.7
	III	430	70	2100	53.7	-	-	-	100
	IV	750	140	2300	19.3	430	70	2100	53.7
	V	150	30	440	83.8	90	10	360	90.3
	VI	110	30	360	88.1	30	-	-	96.7

The Faecal coliform count - Treated sample:

The faecal coliform count in the different experimental groups showed ratio of Faecal coliform count between untreated and treated as 93:120. The group II with neem leaf showed a lesser count of 750 from the initial control of 930 on the 5th day and a further decrease to 430 on the 10th day. A similar reduction was seen in group with Pistia un-treated sewage with neem seed recorded a decrease of 430 (MPN index) on day 5 and there was a complete reduction on 10th day. Group V showed a reduction of 150 (MPN index) on 5th day and further decrease to 90 (Fig. 4) on 10th day. A highly significant decrease was

recorded in group VI (Neem seed and Pistia) with MPN index of 110 on 5th day and <30 on the 10th day, the % change being 85% and 96.7%, respectively (Table 5).

Faecal Streptococci Count - Untreated Sample:

The results of the Faecal Streptococci count in the different experimental groups of untreated sewage showed a decrease in group III and group IV (MPN index 35.000) on the 5th day and further decrease to 5,000 and 7,000, respectively on 10th day. The % change being 94.4% and 92.2% (Table 6). No change was seen in case of Group II on 5th day but it was reduced to 8000 on 10th day when

Fig. 4: Total Coliform count in different Experimental groups of treated sewage samples using MPN Index.

Table 6: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		% change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	90000	30000	32000	-	90000	30000	320000	-
	II	90000	30000	32000	0	8000	2500	19000	91.1
	III	35000	12000	100000	61.1	5000	1700	13000	94.4
	IV	35000	12000	100000	61.1	7000	2300	17000	92.2
	V	8000	2500	19000	91.1	3000	1100	8900	96.6
	VI	3000	1100	8900	96.6	2300	700	7000	97.4

Table 7: Total Coliform count in different Experimental groups of treated sewage samples using MPN index

S. No.	Groups	MPN Index	5 th day		% change	MPN Index	10 th day		% change
			95% confidence limit				95%confidence limit		
			Lower	Upper			Lower	Upper	
	I	16000	4300	49000	-	16000	4300	49000	-
	II	16000	6400	58000	0	9400	2800	22000	43.7
	III	14000	370	34000	12.5	9400	2800	22000	43.7
	IV	16000	4300	49000	0	16000	4300	49000	0
	V	11000	3100	25000	31.2	9400	2800	22000	43.7
	VI	11000	3100	25000	31.2	9400	2800	22000	43.7

Fig. 5: Total Coliform count in different Experimental groups of untreated sewage samples using MPN index.

Fig. 6: Total Coliform count in different Experimental groups of treated sewage samples using MPN index.

compared to the control (90,000 MPN index). Group V and VI showed a significant decrease on 5th day itself and even found more on 10th day (3000:2300). The % change being 96.6% and 97.4% (Fig. 5).

Faecal Streptococci Count - Treated Sample:

The Result of the Faecal Streptococci count in the different experimental groups of Treated sewage showed a reduction almost 3/4 in the

control sample itself. The ratio of Faecal Streptococci count being (90,000:16,000) (Fig. 6). No reduction was seen in Group IV both on 5th day as well as on 10th day. A slight decrease in the Group III (14,000) on 5th day and further decrease of 9,400 on 10th day was recorded. No change was seen in group II on 5th day but reduced to 9,400 on 10th day. Group V and VI showed a significant decrease on the 5th day itself and even on the 10th day, the change being 43.7% and 43.7%, respectively on 5th and 10th day (Table 7).

Discussion

The present study includes the addition of neem (neem leaves and neem seed) Pistia an aquatic plant, separately and as a combination with the aim of achieving a maximum reduction of the microbial population. The microbiological quality of the sewage is currently evaluated by the use of indicators of the faecal contamination (Total coliform, faecal coliform and faecal Streptococci) as suggested by Fattouh *et al.* (2002). The present study was conducted for a period of 10 days with the most probable number (MPN) of the above indicators, recorded on the initial, 5th day and on the 10th day. The results of the total coliform count in the untreated samples as well as in the treated samples were observed. It was seen that the samples with neem leaves showed a comparative lesser reduction in the bacterial populations, in comparison with that of the neem seed powder. While combination of the neem leaves and pistia showed a far better reduction of total coliform and this was further enhanced in the case of neem seed and pistia. According to Rajendran and Sundararajan (1995) neem powder when added to raw sewage reduces the microbial population, and depends on concentration of the neem powder which cause damages to the microbes. Neem, with its therapeutic properties seems to work as a coagulant reducing the microbial population of the sample. The bitter components of the neem (triterpenoids called azadirachtin) have a role to play in reduction of the microbes.

The faecal coliform (MPN index) also showed

significant decrease in all the experimental groups. In the untreated sewage sample, a total reduction was seen on the 10th day of addition of the neem and Pistia. A maximum reduction was recorded on the 5th day in the treated sewage sample for the batch containing Neem seed powder and pistia. This observation seems to coincide with that of Awuah *et al.* (2004) who have reported that pistia along with duckweed (*Spirodela polyrrhiza*) and natural algae can act as a treatment agent in controlling faecal coliform profile. Faecal Streptococci are hardy commensals of the gut. They are low grade pathogens and rarely cause disease, however, they cause urinary tract infections, mixed abdominal wound infections following gut surgery and endocarditis on abnormal valves. They are difficult to kill, yet a decrease in the faecal Streptococci count was recorded in both the untreated and treated sewage samples. It is interesting to note that in the untreated sewage, the neem seed and Pistia only has acted in reducing the faecal Streptococci, while there has been no change in the neem leaf on day five. Studies have reported the antibacterial activity of neem leaf and seed extract against Bacterial strains like *E. coli*, *Klebsiella aerogenes*, *Proteus vulgaris* and *Pseudomonas aerogens* (Perumalsamy *et al.*, 1998). The results showed the group containing neem leaf with pistia and neem seed with pistia have acted in reducing the faecal Streptococci population reducing the count to 9400. The neem with its bitter principles has exhibited highly significant antibacterial activity thereby reducing the ingredients. Neem contains sulphur compounds which has evoked much bacterial population. Nimbidin also present in the neem seed kernel contains sulphur and it may be plausible that this vehemently reacts with the bacteria and thus enhancing the anti-bacterial activity of the neem seed. In the present study it may be concluded that the neem (*Azadirachta indica*) especially the neem seed powder along with water lettuce (*Pistia stratiotes*) has acted as a purifying agent together as a combination and to a certain extent helped in reducing the microbial

population of total coliform, faecal coliform and faecal Streptococci. The Neem leaf and pistia, however, has not been that effective although it has also helped in reduction, when compared with the neem seed with pistia. Although MPN index alone may not be sufficient to come to a final conclusion of the pathogenic indicators, yet it has provided with evidence to show the reduction of the microbial population. It may be recommended that other physical parameters of the sewage sample may also be done along with calculation of density of coliform bacteria by the membrane filter technique to be able to arrive at a final conclusion. The dosage and appropriate proportions of neem seed powder and pistia plant along with the quantity of the sewage have to be standardized in order to obtain the best results in reduction of the microbes.

Conclusion

The versatile neem (*Azadirachta indica*) and the floating water lettuce (*Pistia stratiotes*) are both cost effective and easy to procure and using them in the evaluation of microbial elimination using them may be feasible in industrialized and developing countries.

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