Evaluation Of Heavy Metal Tolerance And Multi Drug Resistance Of Microbial Species Isolated From River Yamuna, Delhi, India

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Abstract-The present study was investigated for the contamination level in river Yamuna, Delhi. A total 10 samples of Yamuna river water from different locations specifically under the territory of Delhi were collected and then analysed quantitatively for Total Bacterial Count (TBC), Total Coliform Count (TCC) as well as Total Yeast & Mold Count. Simulataneously, qualitative analysis for *E.coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was also made in order to confirm the pathogenicity of Yamuna water. The isolates isolated also showed potential heavy metal tolerance study because they show complete resistance against 1000ppm concentration of iron. Hence, they can be used for bioremediation purposes for the removal of heavy metals.

Keyword-River Yamuna, Microbial Contamination, Heavy metals.

Introduction

Human health depends upon safe water more than any other factor. Basically,there is a direct correlation between safe consumable water and human health. Most of the problems in developing countries are mainly due to the lack of safe drinking water (Parson and Jefferson, 2006). Safe water supply is one of the main requirements in any community. It is apparent that health of individuals depends on safe drinking water[1].

The growing population of the world has resulted in the increased the water needfrom sources. Another cause for concern isthe future trends in water use, where it hasbecome somewhat difficult to evaluate the way in which water resources will deplete. Amongst them, pollution of water sources is very important point for health and human safety and considerable attention is necessary for it. Thus, it is necessary to monitor water quality to achieve safe water[2,3].

Rivers of India play an important role in the survival of Indian people. But with the rapid increase in human population, urbanization and economic activities a lot of pressure is created on riverine water resources which has become a serious issue and requires lot of attention. Industrial, urban and agricultural waste are entering water bodies contaminating the aquatic environment and increasing the biological oxygen demand.Also scared rituals which are performed in these rivers during festive season contributes to lot of pollution in the water bodies. The microorganism which are introduced into these water bodies due toIncrement in organic and contaminated decomposed material utilizes the great amount of dissolved oxygen.This situation is leading to decreased oxygen content in the water which disrupts the aquatic life.Thereby posing a serious threat to water resources and nature too.In the national capital, river Yamuna is the most polluted source of water is the principal stream of water reaching households. The Yamuna is the largest tributary of river Ganga which originates from Yamunotri glacier with a total length of 1,376 kilometers. Central location of Yamuna River is $28^0$36'N and longitude $77^0$12'E, at an altitude of 216m above the mean sea level[4,5,6]. It goes through Uttarakhand, Haryana, Delhi, and Uttar Pradesh. It converges with the Ganga at Allahabad in Uttar Pradesh[4]. Yamuna Riveris also called mailee (dirty) river and river of sorrow to Delhi, Mathura and Agra. The river water is extremely black, it appears like an industrial drain in Delhi, as majority of the industries are on its bank and used to dump the untreated effluents into the river. The water in the Yamuna remains stagnant for approximately nine months in a year. There are unlimited numbers of industrial units,

draining immense amount of untreated water in Yamuna existing in Delhi, Faridabad, Mathura and Agra. Central Pollution Control Board (CPCB) had estimated that there wereapproximately 359 industrial units, which directly or indirectly discharge their effluents in Yamuna. A report of CPCB indicates that there were about 42industrial units in Delhi directly polluting the Yamuna[5,6].According to the Centre for Science and Environment, approximately 75 to 80 percent of the river's pollution is the result of raw sewage, industrial runoff and the garbage thrown into the river and it totals over 3 billion liters of waste per day. About 20 billion rupees, or almost US $500 million, has been spent on various cleanupefforts.According to a Central Pollution Control Board (CPCB) survey, Delhi contributes 23 percent of the total wastewater generated by Class I cities (cities with more than 100,000 people)[7,8,9]. More shockingly, this is 47 per cent of the waste generated by 101 Class I cities and 122 Class II cities (Population: 50,000-99,999) in the Ganga basin. The water becomes untreatable when the ammonia concentration in Yamuna River reaches to 0.4 mg/L or more. In Delhi often ammonia in Yamuna River has been found more than 0.4 mg/L especially during summer. The river has turned grossly polluted due to continuous discharge of domestic wastewater from Palla to Etawah.As per the report of Yamuna Action Plan the content of suspended solids in Yamuna is 1000-10,000 mg/L and the permissible content of suspended solids is 100 mg/L. Efforts will be made to resort to a bottom-up approach rather than a top-down one to help this highly polluted river, which is the major life-supporting artery of Delhi, Mathura, Agra and Etawah and many other cities in India[10]. To apply the strategies effectively, we need to develop awareness among masses, education, and improved watershed management that will improve the water quality of this holy river.

Figure-1 Current status of River Yamuna from two different location

Table-1 Some Bacterial Diseases Transmitted Through Drinking Water[11,12]

Disease	**Causal bacterial agent**
Cholera	*Vibrio cholerae*, serovars O1 and O139
Gastroenteritis caused by vibrios	*Vibrio parahaemolyticus*
Typhoid fever and other serious salmonellosis	*Salmonella enterica*subsp. *enterica*serovarParatyphi *Salmonella enterica*subsp. *enterica*serovarTyphi *Salmonella enterica*subsp. *enterica*serovar Typhimurium
Bacillary dysentery or shigellosis	*Shigelladysenteriae*, *Shigellaflexneri*, *Shigellaboydii*, *Shigellasonnei*
Acute diarrheas and gastroenteritis	*Escherichia coli*, particularly serotypes such as O148, O157 and O124

Material and Methods

Sample collection and storage

All these 10 samples were collected from different locations in sterile autoclaved bottles along with the check over temperature of water at the site collection and kept straight in ice box until brought to laboratory (within 6 hours). Samples were collected early in the morning and were subjected to microbial analysis on the same day. Total Coliform count, Total Bacterial count, Yeast and Mould count, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* were the microbiological parameters taken in the designed study.

Table-2 Representation of collected water Samples and Location:

S.No.	Sample No.	Site Name	Location	Temp.
1.	S-1	Wazirabad, Old bridge	N 28°42'40.1"; E 77°13'56.6"	19.3°C
2.	S-2	Vikas Marg, Yamuna bank	N 28°39'38.86559"; E77°15'27.66583"	19.9°C
3.	S-3	Old iron bridge, Gandhi Nagar, Seelampur	N 28°39'45.80899"; E 77°14'45.5928"	20.1°C
4.	S-4	GT Road Metro Vihar, Shastri park	N 28°40'22.5141"; E 77°14'0.81778"	19.4°C
5.	S-5	Outer ring road. MajnukaTila	N 28°41'43.64473"; E 77°13'47.53481"	19.5°C
6.	S-6	Jagatpur bund road, Wazirabad	N 28°44'21.06157"; E 77°13'51.087"	19.0°C
7.	S-7	Geeta colony bridge	N 28°39'9.15009"; E 77°15'44.58668"	19.8°C
8.	S-8	Laxmi Nagar	N 28°38'34.03313"; E 77°15'50.04833"	19.6°C
9.	S-9	Noida, Delhi (NCR)	N 28°32'16.95355"; E 77°19'26.09423"	19.7°C
10.	S-10	Okhla bird sanctuary, sec.95 Noida	N 28°33'47.9117"; E 77°17'56.89375"	19.9°C

Multi drug resistance test using antibiotics:Antibioticsused:The antibiotics used were Azithromycin, Cefixime, Ceftriaxone and Kanamycin.

Inoculum Preparation:Allisolated bacterial strain culture were sub cultured on non-selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0 x 10^8 cfu /ml. The working solution of antibiotics is 5mg/ml.

Agar well diffusion assay (zone of inhibition evaluation):Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay. 200µl of each of the adjusted cultures were mixed into separate 200 ml of sterile, molten, cool media, mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual isolates. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 10µl antibiotic solutions were pipette out into the well in assay plates. Plates were incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Caliper.

Preparation of antibiotic solution:Stock solution of antibiotic was prepared by taking average weight of tablets of antibiotic drug by dissolving 50 mg antibiotic in 50 ml of solubilizing agent and then 1 ml from it, to the 100 ml of solubilizing agent into another volumetric flask.

Finally working solution of 10µg per ml was prepared from stock solution in a volumetric flask.

Observations

Table-3 Quantitative and qualitative analysis of organisms isolated.

Sample Code	Total Bacterial Count, cfu/ml	Total Coliform Count	Yeast & Mould Count, cfu/ml	Pathogen Isolates (Initial)		
				E.coli	*P. aeruginosa*	*S. aureus*
S-1	1.7×10^5	9.0×10^4	1.21×10^3	Present	Present	Absent
S-2	1.41×10^5	7.18×10^4	1.1×10^3	Present	Present	Present
S-3	1.41×10^6	1.6×10^5	4.4×10^2	Present	Present	Present
S-4	1.5×10^7	3.5×10^6	4.6×10^2	Present	Present	Present
S-5	7.5×10^6	7.0×10^5	7.7×10^2	Present	Present	Present
S-6	2.1×10^4	3.5×10^3	No colony observed	Present	Present	Present
S-7	2.1×10^6	1.6×10^6	2.1×10^2	Present	Present	Absent
S-8	2.2×10^6	5.4×10^5	2.5×10^2	Present	Present	Present
S-9	1.0×10^6	3.5×10^5	9.0×10^3	Present	Present	Absent
S-10	8.1×10^5	7.0×10^4	1.0×10^2	Present	Present	Absent

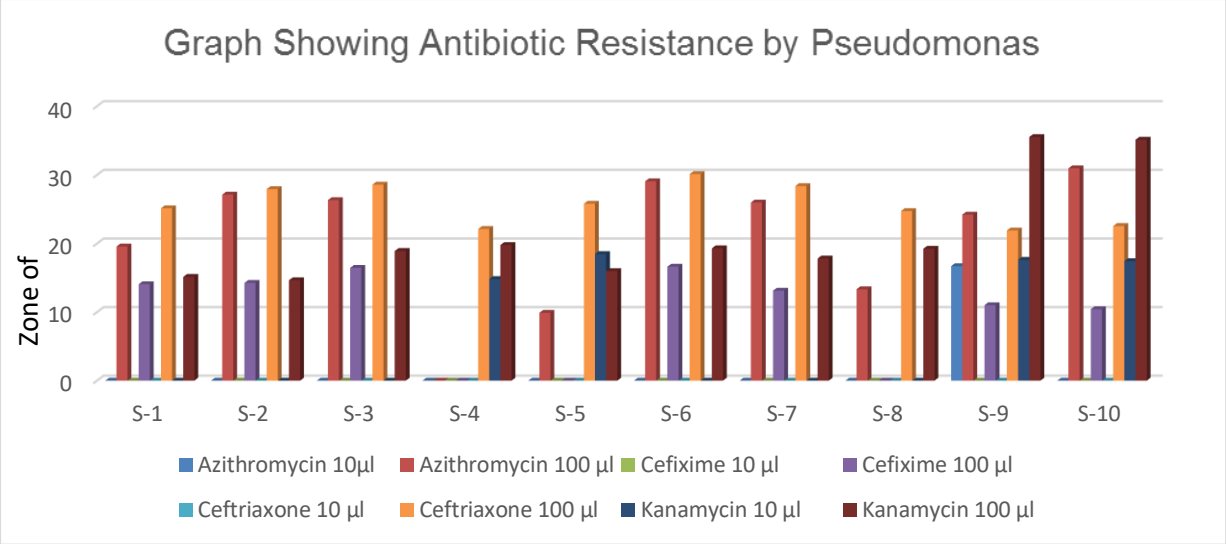
Results

Analysis of Antibiotics resistance from isolates.

Table-4Antibiotic resistance patterns of *Pseudomonas aeruginosa* (Gram negative)

Sample	Azithromycin		Cefixime		Ceftriaxone		Kanamycin	
	10µl	100 µl	10 µl	100 µl	10 µl	100 µl	10 µl	100 µl
S-1	NZI	19.57	NZI	14.07	NZI	25.14	NZI	15.14
S-2	NZI	27.13	NZI	14.26	NZI	27.93	NZI	14.65
S-3	NZI	26.33	NZI	16.46	NZI	28.59	NZI	18.92
S-4	NZI	NZI	NZI	NZI	NZI	22.13	14.82	19.79
S-5	NZI	9.90	NZI	NZI	NZI	25.80	18.48	15.99
S-6	NZI	29.07	NZI	16.63	NZI	30.13	NZI	19.31
S-7	NZI	25.98	NZI	13.13	NZI	28.38	NZI	17.82
S-8	NZI	13.33	NZI	NZI	NZI	24.74	NZI	19.25
S-9	16.71	24.22	NZI	11.01	NZI	21.91	17.64	35.51
S-10	NZI	30.96	NZI	10.42	NZI	22.56	17.43	35.12

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm. Results from 2x plates. NZI- No Zone of Inhibition



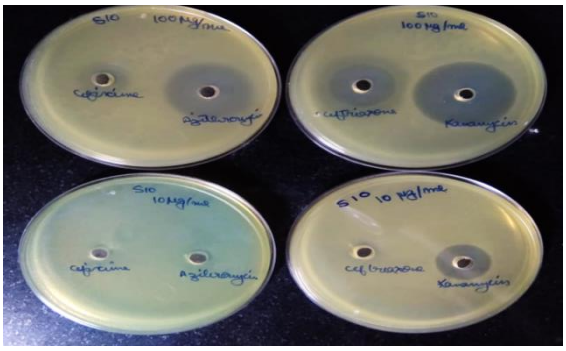
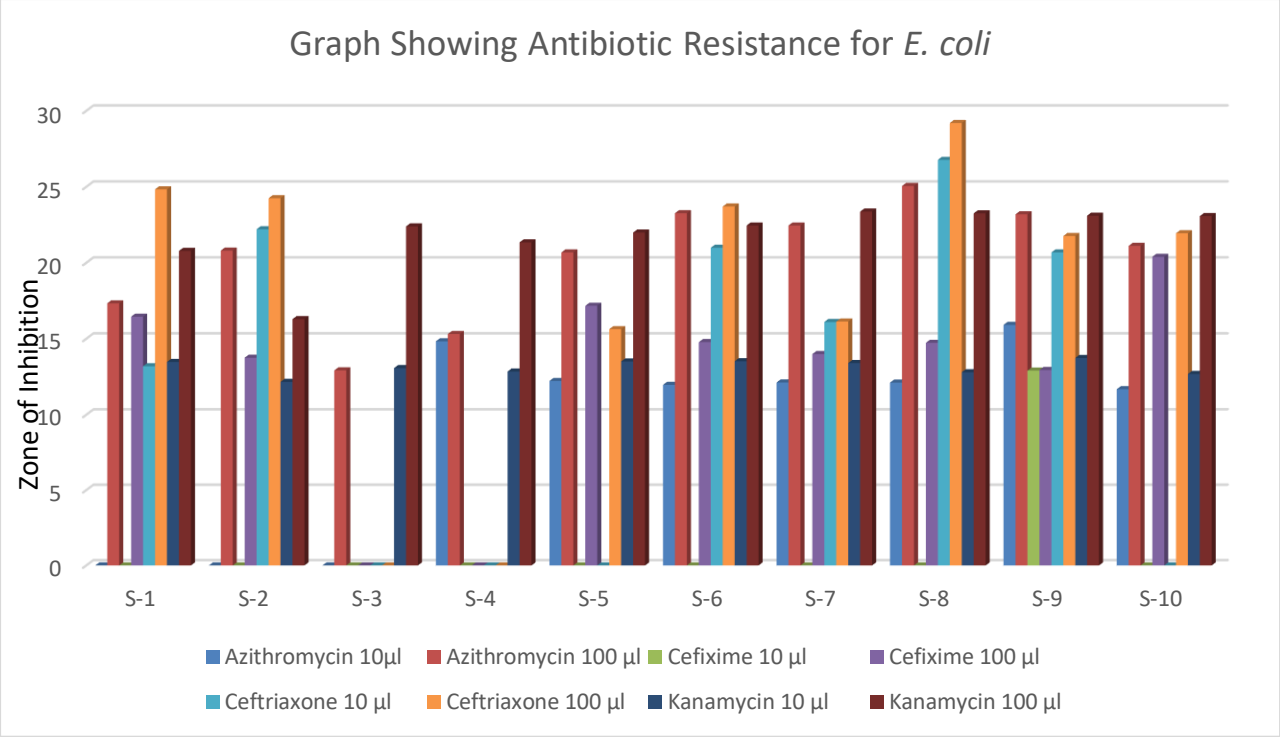


Figure-2 Test results for antibiotic assay for *Pseudomonas*

Table-5Antibiotic resistance patterns of *Escherichia coli* (Gram negative)

Sample	Azithromycin		Cefixime		Ceftriaxone		Kanamycin	
	10µl	100 µl	10 µl	100 µl	10 µl	100 µl	10 µl	100 µl
S-1	NZI	17.325	NZI	16.45	13.19	24.83	13.47	20.78
S-2	NZI	20.80	NZI	13.75	22.20	24.25	12.16	16.29
S-3	NZI	12.92	NZI	NZI	NZI	NZI	13.07	22.39
S-4	14.83	15.31	NZI	NZI	NZI	NZI	12.84	21.34
S-5	12.22	20.68	NZI	17.17	NZI	15.63	13.50	21.99
S-6	11.96	23.26	NZI	14.78	20.99	23.70	13.52	22.45
S-7	12.12	22.45	NZI	13.99	16.10	16.13	13.40	23.37
S-8	12.11	25.05	NZI	14.73	26.77	29.2	12.79	23.25
S-9	15.91	23.19	12.90	12.94	20.68	21.76	13.73	23.095
S-10	11.68	21.11	NZI	20.39	NZI	21.94	12.68	23.07

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm. Results from 2x plates. NZI- No Zone of Inhibition.



Analysis of Heavy Metal Tolerance from isolates.

Table-6Heavy Metal Tolerance patterns of *Pseudomonas aeruginosa* (Gram negative)

Sample	Mercury			Cadmium			Iron		
	10 ppm	100 ppm	1000 ppm	10 ppm	100 ppm	1000 ppm	10 ppm	100 ppm	1000 ppm
S-1	11.83	16.27	29.62	NZI	NZI	12.35	NZI	NZI	NZI
S-2	12.92	18.06	27.60	NZI	17.75	28.77	NZI	NZI	NZI
S-3	NZI	14.18	25.95	NZI	14.27	24.20	NZI	NZI	NZI
S-4	NZI	10.91	24.15	NZI	13.30	24.49	NZI	NZI	NZI
S-5	NZI	12.31	25.31	NZI	16.54	25.31	NZI	NZI	NZI
S-6	NZI	11.90	26.44	NZI	NZI	11.32	NZI	NZI	NZI
S-7	NZI	14.36	28.33	NZI	NZI	10.58	NZI	NZI	NZI
S-8	10.95	14.43	26.14	NZI	9.91	23.03	NZI	NZI	NZI
S-9	NZI	13.94	26.79	NZI	15.36	21.60	NZI	NZI	NZI
S-10	11.42	13.10	27.61	10.70	20.39	34.40	NZI	NZI	NZI

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm. Results from 2x plates. NZI- No Zone of Inhibition.

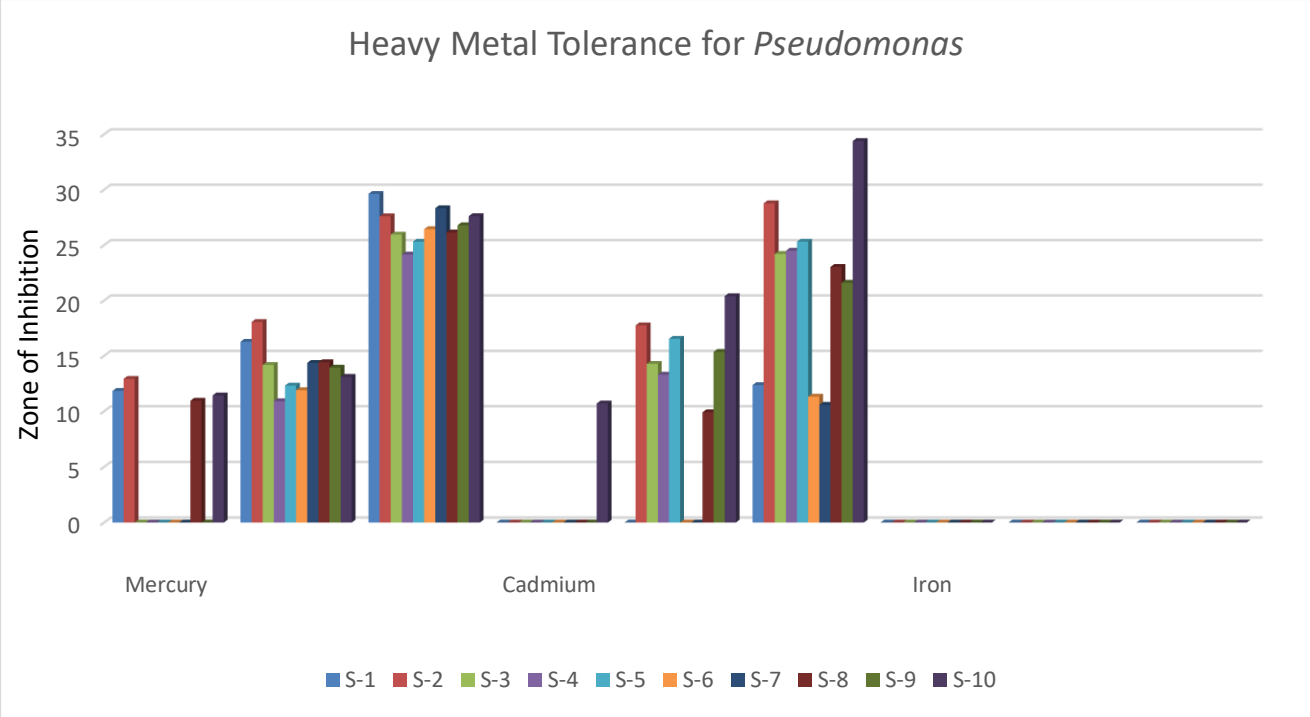


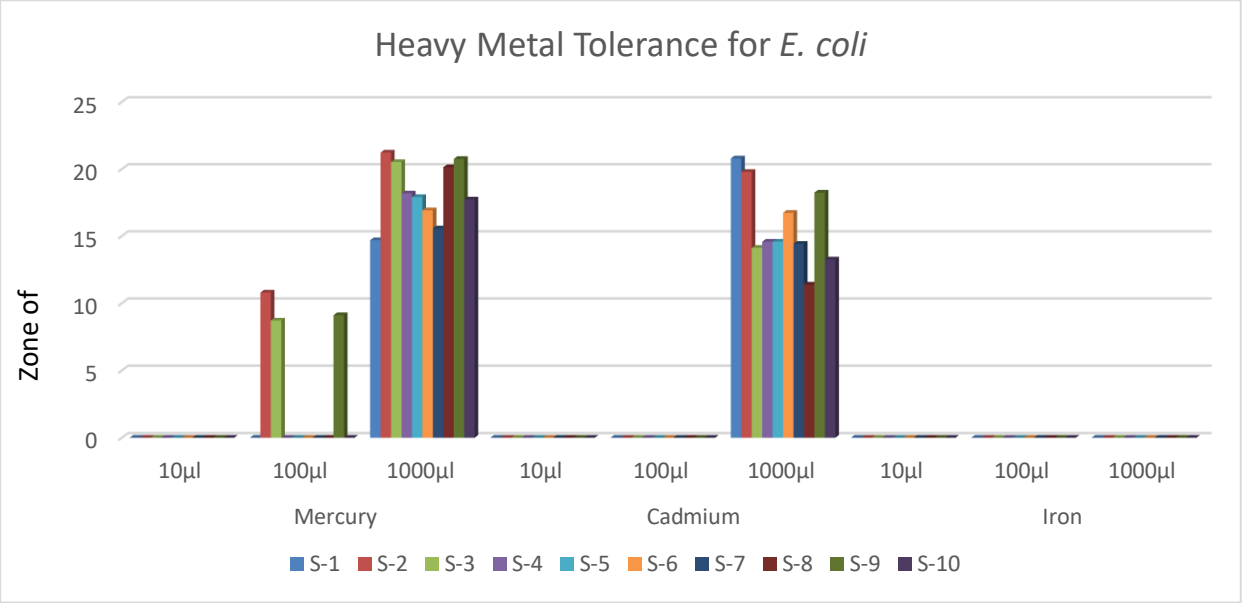
Figure-3 Test results for Heavy Metal Tolerance for *Pseudomonas*

Table-7Heavy Metal Tolerance patterns of *E. coli* (Gram negative)

Sample	Mercury			Cadmium			Iron		
	10 ppm	100 ppm	1000 ppm	10 ppm	100 ppm	1000 ppm	10 ppm	100 ppm	1000 ppm
S-1	NZI	NZI	14.69	NZI	NZI	20.79	NZI	NZI	NZI
S-2	NZI	10.79	21.23	NZI	NZI	19.78	NZI	NZI	NZI
S-3	NZI	8.71	20.52	NZI	NZI	14.12	NZI	NZI	NZI
S-4	NZI	NZI	18.18	NZI	NZI	14.58	NZI	NZI	NZI

S-5	NZI	NZI	17.91	NZI	NZI	14.58	NZI	NZI	NZI
S-6	NZI	NZI	16.92	NZI	NZI	16.73	NZI	NZI	NZI
S-7	NZI	NZI	15.57	NZI	NZI	14.42	NZI	NZI	NZI
S-8	NZI	NZI	20.13	NZI	NZI	11.38	NZI	NZI	NZI
S-9	NZI	9.11	20.75	NZI	NZI	18.24	NZI	NZI	NZI
S-10	NZI	NZI	17.73	NZI	NZI	13.25	NZI	NZI	NZI

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm. Results from 2x plates. NZI- No Zone of Inhibition.



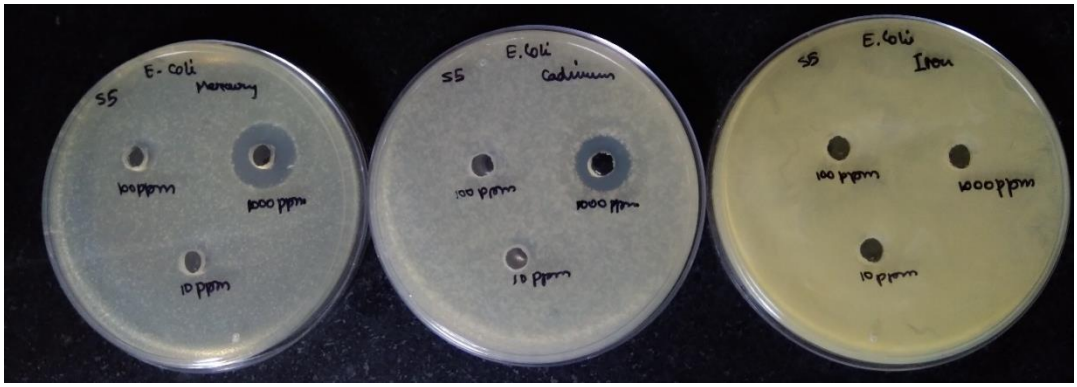


Figure-4 Test results for Heavy Metal Tolerance for *E. col*

Discussion & Conclusion

After enumeration, isolation and identification of the isolates from Yamuna River depicts the extreme level of contamination. As per the study, total 10 samples of yamuna water from different locations specifically under the territory of Delhi were collected and then analysed quantitatively for Total Bacterial Count (TBC), Total Coliform Count (TCC) as well as Total Yeast & Mold Count. Simulataneously, qualitative analysis for *E.coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was also made in order to confirm the pathogenicity of Yamuna water.

As per the data attained, TBC confirmed the total count of bacterial presence which included coliforms as well as all pathogens. That's why, TBC always appeared more than the coliform count . In reference with the data, maximum TBC obtained was in sample 4 which was 1.5×10^7 cfu/ ml and TCC was 3.5×10^6 cfu/ ml. On the other hand, in sample 9 Yeast & Mold Count appeared maximum i.e., 9.0×10^3 cfu/ml but the bacterial count wasn't the maximum in the same sample (S-9) i.e., 1.0×10^6 cfu/ml which confirmed that No relation existed between these two entities for the survival. Descriptively, Y&M Count only gave information about the presence of Yeast Colonies (shiny bulged appearance) and Fungal Colonies (profused growth of hyphae), which itself depicted the presence of dead & deacying matter in water whose explaination could be the emergence of drains from all over the province.

Pathogen's presence was also detected in the water samples i.e., presence of *E.coli* was approved in every sample and reason behind this could be the emergence of drains from all over Delhi into the Yamuna River, which ensured the faecal contamination in water. *Pseudomonas aruginosa* was also obtained from every sample because of the high contamination level of water. But the presence of *Staphylococcus aureus* was found to be exclusive among all pathogens. In reference with the data obtained, *S.aureus* was isolated from only 5 samples and the reason could be its origin because it is an airborne microbe and water doesn't approve its survival.

This is how, quantitative and qualitative analysis of Yamuna water samples explained "the extreme level of contamination".

Afterwards, biochemical tests were performed to confirm the pathogenicity with respect to the mentioned pathogens. For *E.coli* ,4 major tests were performed to confirm its presence which were Gram staining, Indole test, Methyl-red test and Voges Proskuer test. Gram staining , being itself a differential staining confirmed the *E.coli* presence by showing "pink stained rods" over the slide under microscope which ensured the microbe as gram negative. The acquaintance of pink stain which was of safranin was because of the elemination or washing off the lipopolysaccharide layer around the microbe by the action of alcoholic de-coloriser. That's why, stain of Crystal-Violet washed off, too. Indole test confirmed the presence of *E.coli* by the production of red colored ring "Rasoindole" which was formed from the interaction of indole and Kovac's reagent. This test was performed to check the production of indole from the Enterobacteriaceae species which was done by the hydrolysis of Tryptophan amino acid with the end products such as indole, pyruvic acid and ammonium ion. Methyl-red test ensured *E.coli* presence by confirming the production of mixed acids (pyruvic acid) from the fermentaion of glucose (Embden Mayerhoff glycolytic pathway) with the help of methyl-red indicator which turned the entire media "red". VP test confirmed *E.coli* presence by showing negative results which means no red colored dye production took place because Enterobacteriaceae species doesn't show Butylene Glycol pathway for Acetoin production, which is responsible for red color appearance in VP test.

For *Pseudomonas aeruginosa,* 5 major test were performed to confirm its presence which were Gram staining, Catalase test, oxidase test, Hugh-Leifson test and Skimmed milk agar test. Gram staining, being itself a differential staining confirmed the *Pseudomonas aeruginosa* presence by showing "pink stained rods" over the slide under microscope

which ensured the microbe as gram negative. Catalase test confirmed its presence by showing oxygen bubbles which were because of the hydrolysis of hydrogen peroxide into water molecule and oxygen. Oxidase test confirmed its presence by showing blue colored compound (indophenol) from tetramethyl (p)-phenylenediamine under the effect of cytochrome c oxidase enzyme, which is supposed to be used by bacterial species in their electron transport chain system. Hugh-Leifson test confirmed their presence with the change in its color from green to yellow because of the production of mixed acids from glucose breakdown either oxidatively or fermentively. In the case of *Pseudomonas aeruginosa* (facultative anaerobe), growth appeared in both aerobic and anaerobic conditions i.e., oxidation and fermentation took place, respectively. SMA test confirmed its presence with the "zone of hydrolysis" on SMA plates around the culture, which was because of casein hydrolysis by the action of *Pseudomonas aeruginosa.*

For *S.aureus,* 3 major tests were performed. Gram staining confirmed its absence. Catalase test ensured its presence with oxygen bubbles. In coagulase test, agglutination confirmed the absence of *S.aureus.* Agglutination occurs because of the conversion of fibrinogen (soluble) into fibrin (insoluble).

Later, tests were performed to check the antibiotic resistance pattern. Resistancy was calculated with the size of the zone of inhibition in an "inverse proportional manner". The resistance of an organism against an antibiotic can be determined by the zone of inhibition that is seen. The formation of an inhibitory zone shows that the antibiotic is successful in killing all of the microorgaisms present.

Four antibiotics were chosen for this purpose, which were Azithromycin, Cefixime, Ceftriaxone and Kanamycin. Two different concentrations of 10µg/ml and 100µg/ml were chosen for each. For *Pseudomonas*, Ceftriaxone is the most efective antibiotic as it shows an overall similar result against different samples of *Pseudomonas*. It is followed by Azithromycin and Kanamycin. Kanamycin shows a smaller zone of inhibition but still shows inhibition in concentration as less as 10µg/ml. While cefixime is the least effective antibiotic among the four.

For *E. coli* the results were different with Kanamycin being the most effective antibiotic showing inhibition in both high and low concentration. It was followed by azithromycin then ceftriaxone and ultimately cefixime.

This shows us that slowly and steadily the organisms have started to show resistance against the antibiotics present in the market today.

As for the resistance against heavy metals the metals chosen were Mercury, Cadmium and Iron. The concentrations chosen were of 10ppm, 100ppm and 1000ppm. Both *Pseudomonas* and *E. coli* were completely tolerant against iron in all concentrations.

In *Pseudomonas* most samples can tolerate cadmium for a minimum of 10ppm and a few samples show tolerance even for 100ppm. In mercury, 10ppm seems to be the limit of their tolerance as inhibition is seen in all samples for 100ppm.

In *E. coli*, tolerance of upto 100ppm is seen for Mercury and Cadmium in all samples.

Therefore, all of these samples can be used potentially for heavy metal tolerance study because they show complete resistance against 1000ppm concentration of iron while show very positive results in sustaining themselves of upto a concentration of 100ppm. Hence, they can be used for bioremediation purposes for the removal of heavy metals.

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