Characterization of Antibiotic and Heavy Metal Resistant Bacteria at Delhi-Agra Stretch of River Yamuna

Work Done Under the Guidance of

Dr. Ilora Ghosh
Associate Professor,
Lab No. 103, School of Environmental Science,
Jawaharlal Nehru University, New Delhi

By
Shreya Paul
Roll No. – MBIO194016
Department Of Life Science and Biotechnology
Jadavpur University

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my guide ***Dr. Ilora Ghosh, Associate Professor, School of Environmental Science, Jawaharlal Nehru University***for the continuous support during the entire study, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research. I could not have imagined having a better advisor and mentor for my summer training.

My sincere thanks also goes to research scholar ***Ms. Nishi Sahu***, who provided me an opportunity to join her team as intern, and who gave access to the laboratory and research facilities, helped me to perform experiments and writing this report. Without her precious support and guidance it would not be possible to conduct this research.

I would also like to thank other research scholars ***Abhinav sir***, ***Aparna ma'am***, ***Moushumi ma'am***, ***Sadan Sir***, ***Shomesg sir***; for their support and guidance throughout the training. My sincere thanks goes to ***Devi Singh sir*** for always providing materials required on time and guiding me.

I thank my friends ***Namita Nandal*** for the stimulating discussions, and for all the experience which we had during the entire training.

I would like to express my sincere gratitude to our Head of The Department (H.O.D.) ***Prof. Biswadip Das, Professor, Department of Life Science and Biotechnology, Jadavpur University***, for recommending and giving me a golden opportunity to work as ***Summer Research Trainee*** at ***Jawaharlal Nehru University (J.N.U.)*** with an amazing team of people.

Last but not the least, I would like to thank to ***my parents*** for supporting me spiritually throughout the training and my life in general.

ABSTRACT

Yamuna (Jumna) is the confluent holy river of the Ganges but at present it is polluted and induces death because of various anthropogenic activities like dumping of garbage and untreated sewage, industrial effluents discharge etc. For this disposal at Yamuna River site, which carries various pollutants such as pathogenic/non-pathogenic microorganism, antibiotics and heavy metals, making water unfit for use and cause of variety diseases and illness of the consumers. The aim of this study is to understanding the bacterial diversity by isolating and identifying the heavy metal and antibiotic resistant bacteria from water sediment samples collected from Palla and Agrasite of river Yamuna. Primary screening was done by checking the resistance ability from the Mercury ($HgCl_2$) and then Cadmium (CdCl2), Lead ($Pb(CH_3COO)_2$) and Chromium (K_2CrO_4) resistance was screened. After this resistant colonies were isolated to assess the multiple antibiotic resistances. Antibiotic resistant bacteria were characterized using antibiotic susceptibility test using variety of antibiotics, as per Clinical and Laboratory Standards Institute (CLSI) guidelines. High percentage of heavy metal resistance and high zone of inhibition against tested antibiotics indicates, that Yamuna River have heavy metal and antibiotic resistive bacteria, that can cause ofharmful and endangerof human health. Therefore, there is an urgent need to take stringent measures to facilitate these pollution loads and save an unwell river.

INDEX

Characterization of Antibiotic and Heavy Metal Resistant Bacteria at Delhi-Agra Stretch Of River Yamuna

1. Introduction

Urbanization and freshwater rivers are inextricably linked with environmental issues. In the name of urban development, anthropogenic activities are continuously modifying the chemical composition of a river system. Life depends on the availability of chemical elements in the right proportions and combinations[2]. It is well known that clean water is absolutely essential for several purposes for healthy living. Rapid industrial development in the last few decades has added huge loads of their persistent and bio- accumulative nature[3]. Over the past few years, high industrial density as a result of increasing socioeconomic development has generated a tremendous amount of pollution. Industrial effluents being continuously discharged into rivers are gradually deteriorating our global environment[3].

The River Yamuna is the largest tributary of river Ganga, originates from the Yamunotri glacier, 6387m above mean sea level (msl), at the Banderpoonch peak in the Uttarkashi district of Uttarakhand. Thecatchment of the river extends to states of Uttar Pradesh, Himachal Pradesh, Haryana, Rajasthan andMadhya Pradesh and the entire union territory of Delhi. Originating from Himalayas its total length is1,376 km covering a catchment area of 366,220 km2. Its source is at Yamunotri, in theUttarakhand Himalaya, in the Himalayan Mountains. The main tributaries joining the river includeHindon, Chambal, Sind, Betwa and Ken [4]. The annual flow of the river is about 10,000cumecs. The annual usage is 4400 cumecs, irrigation accounting for 96% of this (MoEF, 1994). It is notjust like another river but it has a major religious and cultural significance. The river not only provides alivelihood for communities living in the basin but also offers a life support to agricultural, industrial and urban sectors and is the main source of drinking water for most of the towns along its course. More than 70% of drinking water supply of Delhi is abstracted from river Yamuna (CPCB, 1996, [5]. An estimated 57 million people are dependent on the water of the Yamuna River. In view of the rapid growth of urban population, industrialization and inadequate infrastructure, the river water quality across the country has been found to be deteriorating alarmingly. The industrial towns all along the river, discharges significant amounts of wastes into the river. In lower stretch, the Yamuna becomes a drain, receiving mainly agriculture, industrial and domestic effluents (Ali et al., 2001).

The river Yamuna is subjected to multiple usesfor community water supply, irrigation, industrialwater supply, bathing, and disposal of sewageand industrial effluents. It is an only natural resourcefor sustaining all forms of life in Delhi, butperennial increase of population and urban activitiesin Delhi are placing tremendous pressuresand demands on this natural resource. There isa heavy pressure of water supply and sanitationon river Yamuna in Delhi[6].

In India, most of the industries are situated along the river banks for the easy disposal of the effluents that contain heavy metals [3, 7].In developing countries, rapid and unorganized industrialization and urbanization have also contributed to the elevated levels of heavy metals in the water bodies [1].Heavy metal tolerant bacterial species may serve as an important and cost-effective bioremediation tool for the removal of heavy metals from wastewater and industrial effluents, preventing the contamination of water bodies (Jan et al., 2014).

The Yamuna River is one of the most polluted rivers in India due to the presence of high concentrations of toxic heavy metals [8-11]. Anthropogenic and industrial activities add non-biodegradable environment pollutants like heavy metals, such as lead (Pb), chromium (Cr), cadmium (Cadmium), mercury (Hg), iron (Fe), cobalt (Co), and nickel (Ni), and their derivatives into the water bodies [12]. Kaushik et al., 2009, they were studied that concentration of Heavy Metals (Cd, Cr, Fe, Ni) in water, plants and sediments of river Yamuna flowing in Haryana through Delhi were major industrial complexes of the State [3].

Many authors have screened and studied different micro-organisms, such as bacteria, fungi, and algae, over the past decades to identify their highly efficient metal removal biological systems [13]. These microorganisms can grow in the presence of high concentrations of heavy metals and have a great potential for bioremediation of heavy metals discharged in industrial effluents [14].Previous studies have well documented the occurrence and abundance of metal-tolerant microbes in metal-polluted water bodies [15-17].

The microorganisms in the polluted water bodies develop a variety of resistance mechanisms to survive in different heavy metal concentrations, but the resistance is often specific to one or few metals [18-20].

Microorganisms are ubiquitous and play a significant and dominating role in the cycling of organic and inorganic matters in marine ecosystem [4].They are efficient degrades as they can penetrate the water and sediment most intimately by their high number and size. They possess a variety of catabolic enzymes which allow them to decompose various dissolved and particulate substrates, thereby making it available for other organisms through microbial loop [5]. Heavy metal tolerant bacterial species may serve as an important and cost effective

bioremediation tool for the removal of heavy metals from waste water and industrial effluents, preventing the contamination of water bodies [6]. Macrophytes are known as good indicators of heavy metal contamination in aquatic ecosystems and in different zones of sphere within water spread area around the banks [7]. Micro-organism adopts various mechanisms to combat metal toxicity such as metal sorption, mineralization, uptake and accumulation, extracellular precipitation from the cell [8]. Mechanism of metal resistance in microbes in microbes also precipitation of metals as phosphate, carbonates, or sulfide volatilization via methylation or ethylation [9]. Most of the cells use two types of uptake system for heavy metal ions; one is fast, unspecific and other is slow, specific. Another well studied resistant mechanism is efflux pumps seen as the active system in both Gram negative and positive bacteria, which render them resistance against metal [10]. The efflux pumps are membrane associated with active transporters, which are reported initially in gram negative bacteria as a means to remove antibiotics from the cytoplasm [11]. The defensive mechanism of these microorganisms for metal detoxification has been suggested as the biological pathway that reduces the metal ions and precipitates the metal compounds in the peri-plasmic space [12]. Glycoprotein present on the outer cell wall side of Gram-positive bacteria have been suggested to have more potential binding sites for metal ions than the phospholipids and LPS and hence are responsible for the observed difference in capacity [13].

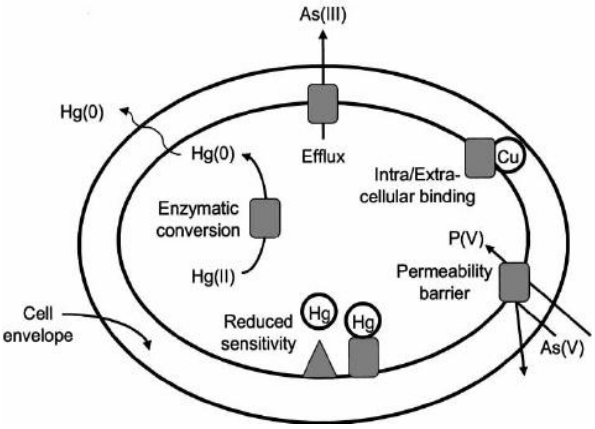


Figure 1:*Schematic Diagram of Heavy Metal Resistance in Bacteria (Source: Google)*

Elements such as heavy metals are essential elements for life in normal concentrations, but are toxic when present in higher concentration in any component of environment because of their potential toxicity to micro-organisms, plants and animals; they accumulate in the biological systems and concentrate in the food chain at each tropical level. These eventsserious challenges to the microorganisms and plants, and cause cancers and

neurological disorders in humans and animals [14]. The minimum inhibitory concentration of the metals ranged from 0.01mM – 40mM. The order of toxicity of the metals was found to be Hg> Cd> Co> Cr> Cu> As> Zn>Pb> Ni [14]. Tolerance of bacteria to a particular metal does not always correlate with their tolerance to other metals. This may due to the existence of different mechanisms responsible for bacterial tolerance to heavy metals [15].

Recently, in human being lifestyle diseases are increasing the consumption of antibiotics also enhances and released a huge amount of un-metabolized drugs in sewage which contaminate water bodies several other anthropogenic activities increasing the antibiotics and metals concentration in water bodies. Increasing concentrations of both antibiotics and metals beyond the tolerance limit create an evolutionary force of adaptation in harsh environment. These heavy metals and antibiotics stress are attaining a modification in the genetic makeup of chromosomal and plasmid DNA of bacteria, which occurred by genetic mutation and genetic elements transfer from the resistant bacteria in the environment [16]. In many reports heavy metal tolerance was observed in a number of bacteria and it has been recognized plasmid born resistance gene like antibiotic resistance. These plasmid borne mobile resistance gene easily transfer and spread in bacterial population through the efflux system and carry next generation[17]. Co resistance is defined as the existence of for two or more resistance element.

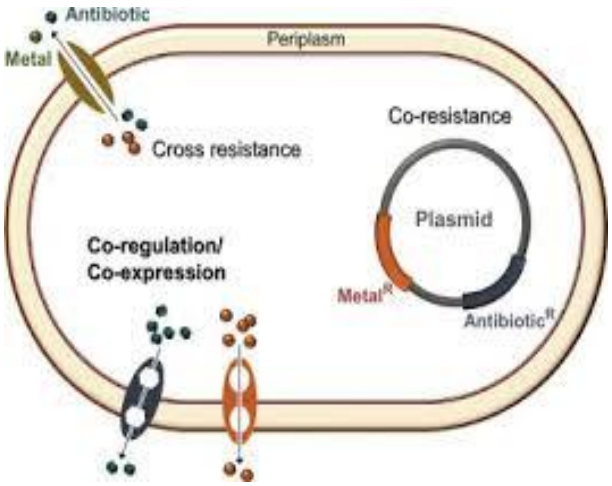


Figure 2: *Schematic Diagram of Co Resistance of Heavy Metal and Antibiotics in Bacteria (Source: Google)*

In many natural environments containing microbial communities, the emergence and further spread of antibiotic resistance and even multi drug resistance in bacteria is observed due to co-contaminations of heavy metal and antibiotics [18].

2. Objective

A. Characterization of different microbes by identifying the morphology and using gram staining.

B. Level of heavy metal resistance checking and isolation of those resistant strains.

C. Level of antibiotic resistance checking and isolation of those resistant strains.

D. Isolation of plasmid DNA from the resistant strain.

3. Review of Literature

3.1. The Yamuna River

The Yamuna River is one of the primary rivers of Indiawith social, economic, religious and cultural significance. According to Hindu traditions, it is worshiped as a sacred river and a number of pilgrimage centres like theYamunotri, Poanta-Sahab, Mathura, and Allahabad are situated along its banks [5]. Yamunotriglacier in the lower Himalayas (38° 59° 78° 27 ° E) at 6320 meters above sea level in the Uttrakhand state of India isthe source of the Yamuna River [21].

Length of the Yamuna Riverfrom its origin in the Yamunotri glacier up to its confluence with river Ganga at Allahabad in Uttar Pradesh is about 1376 Km (GNCT 2005).According to [22] in the Himalayan regionit draws water from its four main tributaries namely Rishi Ganga, Hanuman Ganga, Tons and Giri and in the plainsfrom Chambal, Hindon, Sindh, Betwa and Ken. It has a vast catchment area of about 366,220 sq.km [21]which, according to [22] is spread over parts of Uttarakhand, Himachal Pradesh, Haryana, UttarPradesh, Rajasthan and Madhya Pradesh, and Delhi.

Thus, Yamuna river cannot be designated as continuous river particularly in dry seasons (almost 9 months), but can be segmented in four independent segments:

Segment-I (172 km) -Yamunotri to Tajewala Barrage, the major source of water in this sub-river is glacier streams, which are ending into WYC and EYC used for irrigationand drinking in the command areas.

Segment-II (224 km) -Tajewala barrage to Wazirabad barrage, this segment receives water through groundwater accrual and some river tributaries. The segment ends up intoDelhi'swater supply system. Sometimes the water is also added in this segment throughdrain no. 24 B from WYC to augment the flow of the river in order to fulfil the raw waterdemand of Delhi.

Segment -III (22 km) -Wazirabad barrage to Okhla barrage, this segment receives water through Delhi's sewage drains and also from WYC and Upper Ganga Canal

viaNDandHindon cut respectively. This river segment ends into Agra Canal, which is used toaugment its flow for inigation in Haryana & UP in the downstream.

Segment-IV (958 km)-Okhla barrage to Ganga Confluence at Allahabad, this segment receives water from Shahdara drain that contains domestic and industrial wastewatergenerated from East Delhi, Noida and Sahibabad, HindonRiver and groundwater accrual.The untreated wastewaters from Vrindavan-Mathura and Agra are added to the flow ofthe river Yamuna in this segment. The famous cities of Agra and Mathura draw theirdrinking and industrial water from the Yamuna River in this segment. The major input offlow in this segment is through Chambal River near Etawah in U.P. and later Sindh, KenandBetwa rivers. More barrages are under consumption in this segment, one at GokulGhatnear Mathura and another near Sikandara which will further subdivide Yamuna.

Table 1: *Major Water Quality Segments of the Yamuna River (Source: CPCB 2009)*

No.	Segment	Reach	Length (Km)
1.	The Himalayan Segment	From origin to Tajewala Barrage	172
2.	The Upper Segment	From TajewalaBarragae to WazirabadBarrage	224
3.	The Delhi Segment	WazirabadBarragae to Okhla Barrage	22
4.	The Eutrophicated Segment	Okhla Barrage to Chambal Confluence	490
5.	The Diluted Segment	Chambal Confluence to The GangaConfluence	468

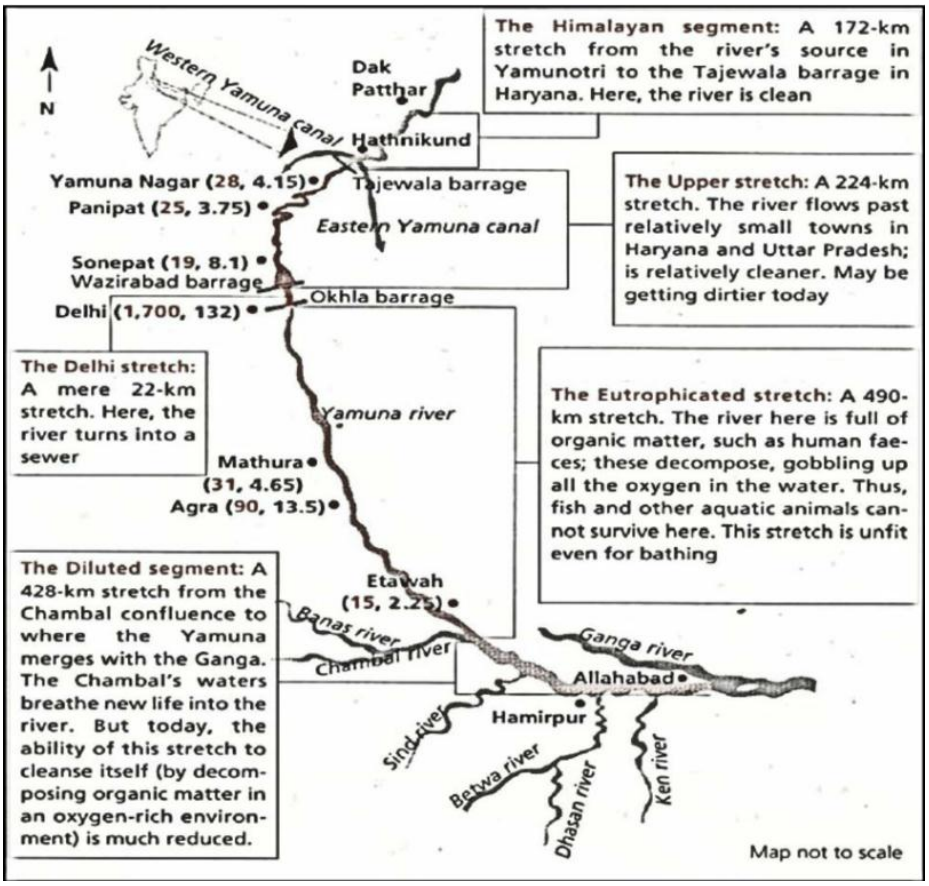


Figure 3:*Diagrammatic presentation of the segments of the Yamuna River (Source: CPCB, 2006-2007)*

3.2 Pollution of Yamuna River

The Yamuna River is one of the mostcontaminated rivers of India (CPCB 2010)[10]. Untreated or partially treated domestic sewage, industrial effluents and agricultural effluents are the major contributors of pollutionin the river. The cities alongside the Yamuna River release loads of contaminants in it.

Due to its religious, cultural, social and economic significance the Yamuna River flows in the hearts of many Indians, but unfortunately like many other riverine systems of the country, it too is affected by the setbacks of industrialization,urbanization and rapid agricultural developments [23]. In his study C.K. Jain (2004) reported thatdue to industrialisation in the towns along the Yamuna River basin, all the industrial effluent find its way into it[4]. Healso reported that the tributaries of the Yamuna River also transferred their pollution load into it.Water is consumedfor different activities which generate a lot of wastewater causing deterioration of water quality of Yamuna River.Various point and non-point sources contribute to the contamination of the Yamuna River.

3.2.1. Point sources of pollution

These are specified and localized causes of pollution. The point sources of pollution may bedivided into two categories: Domestic Pollution and IndustrialPollution.

3.2.1.1. Domestic pollution

- The domestic sources cause maximum pollution in Yamuna estimated to be roughly 85% of the totalpollution. These mainly include the dumping of waste by urbancenters like Panipat, Sonepat, Delhi, Ghaziabad, Mathura-Vrindavan, Agra, Etawah and Allahabad.
- The severity of impactof domestic pollution on river depends on the effectiveness ofthe wastewater collection system, type and stretch of the wastecarriage system. If there is more holding time of waste waterwithin urban sites before reaching the Yamuna and other waterbodies, the pollution levels would become less due to settlingand biodegradation.
- The domestic waste is mainly constituted oforganic matters and micro-organisms. Apart from this total salts,detergents, grease etc. also contribute to this type of pollution.Waste generated from abundant unauthorized colonies existingin various urban centers with no sewerage system, is transportedand discarded either in gutters, streams or straight into riveruntreated.
- A survey done by Central Pollution Control Board(CPCB), estimated that a major fraction (0.23) of the entirewastewater produced is contributed by Delhi among Class Icities. These are the cities with population of more than 100,000people. More bewilderingly, this in turn is around fraction of0.45 of the total waste caused by Class I and II cities takentogether.
- Yamuna becomes more toxic with the dumping of theuntreated domestic wastewater when the concentration ofammonia ranges to 0.4 mg/L or more[[5]].

3.2.1.2. Industrial pollution

- Post-independence, swift industrial development occurred across the Yamuna river basin. There arehuge number of industrial clusters at various places includingIndore, Gwalior, Kota, Khetri, Panipat, Yamuna Nagar, Nagda,Delhi, Baghpat, Sonepat, Ghaziabad, NOIDA, Mathura, Faridabad and other places.

- These different industriesdischarging wastewater into Yamuna river comprises ofDistilleries, Pulp, Sugar, Weaving, Oil Refineries, Chemical,Drugs, Thermal Power Plants, foodstuff industries etc.
- It ismandatory for these industries to treat the waste generated inorder to achieve recommended criteria before throwing effluentinto the environment for the acquiescence to the environmentlaws. This aspect is ignored in most of the cases.
- Yamuna Riverhas become murky river and river of sadness to the areas aroundwith special reference to Mathura, Delhi, and Agra. The colorofYamuna River is awfully dark; it looks virtually like a gutter inNCT, as most of the manufacturing and other engineering unitsare situated on its bank and at one time used to junk and throwthe unprocessed wastes into the Yamuna.
- The River waterremains immobile for nearly three-fourth time of a year. Thereare unchecked and unrestricted numbers of industries, throwinghuge amount of unprocessed effluents in Yamuna prevailing inFaridabad, Delhi, Agra and Mathura.
- According to CentralPollution Control Board (CPCB) survey, approximately 350industrial units that throw their wastes in Yamuna of whichmore than forty are in Delhi[[5]] (Water Quality Status of YamunaRiver (1999-2005)).

3.2.2. Non – point source of pollution

- The non-point sources are unnamed, plentiful and impact of each is of less implication this is contrary to the point source. Nevertheless, in blend the subsequent impact is substantial. This pollution is not confined in the catchment area of the Yamuna but regularly transported or and sometimes rarely by discharge, and surface water during rains.
- The pollutants initiated from these non-point sources include heavy metals in upper layer of soil, nutrients/fertilizers, plant residues, organic chemicals, organic matter etc.
- The other important non-point pollution sources contributing to river Yamuna are: Agricultural remains, Fertilizer and Pesticides, Animal husbandry, Dumping of Garbage and Dead bodies, Immersion of Idols, Bathing and clothes washing, Cattle wading, Open defecation.

3.3. Yamuna River water quality

River Yamuna is the primary source of drinking water for Delhi, the capital of India, and also for many cities, towns and villages in the neighbouring states of Uttar Pradesh, Uttaranchal and Haryana. In the last few decades, however, there has been a serious concern over the deterioration in its water quality. The river has been receiving large amounts of partially treated and untreated wastewater during its course, especially between Wazirabad and Okhla, National Capital Territory (NCT) of Delhi. Pollutants flowing into the river are contributed from the waste of the cities situated along its bank. From big industries and factories to people living in big colonies, slums and rural areas, all pollute the river with impurity becauseof untreated water.

On the basis of the different geological and ecological characteristics, the river has been divided into five major segments- viz. Himalayas stretch (172 km), upper stretch (224 km), Delhi stretch (22 km), mixed stretch (490 km) and diluted stretch (468 km) shown in Table 1 (CWC, 2009), of which Delhi is the most polluted stretch of the river, alone is responsible for 79% of entire pollution load in the river Yamuna, (CPCB 2006-2007). The Himalayan stretch of the River Yamuna is devoid of any kind of pollution, the water quality here in Tajewala is generally within the desired levels for bathing quality as given by (YAP II; Bhargava, 1983; 1985; 2006) Table 2.

Table 2: *Water Quality in the river (2007)(Project Management Consultants for YAP II, 2007; u/s: Upstream; d/s: Downstream)*

Site	State	BOD Level	Remarks
Tajewala/Hathnikund	Haryana	1	Bathing quality
Kalanaur	Haryana	1	Bathing quality
Sonipat	Haryana	1	Bathing quality
Palla	Delhi	2	Bathing quality
Nizamuddin Bridge	Delhi	27	Unfit for any use
Agra canal	Delhi	10	Unfit for any use
Mazawali	Haryana	32	Unfit for any use
Mathura u/s	UP	6	Unfit for any use
Mathura d/s	UP	6	Unfit for any use
Agra u/s	UP	6	Unfit for any use
Agra d/s	UP	39	Unfit for any use
Bateswar	UP	7	Unfit for any use
Bawah	UP	6	Unfit for any use
Auriya	UP	2	Bathing quality

The water quality of Indian rivers have been categorized into five classes which are Class A: The riverwater is fit for drinking after proper disinfection with the addition of chlorine or bleaching powder. Class B: Under this category the River water is fit only for bathing. Class C: The River water is fit for drinking only after proper treatment (screening to remove physical matters or particulate such as paper, plastic, etc. Class D: Under this class the river water is fit only for fish and wildlife and Class E: River water is suitable only for industrial cooling, irrigation. Yamuna River belongs to class E (Hindu, 2002).

3.3.1. Biological Oxygen Demand (BOD)

It measures the rate of oxygen used by biological organ- isms in the water body to decompose the organic matter polluted by sewerage or industrial effluents. High demand of BOD indicates that the level of dissolved oxygen is falling, and river's marine life and biodiversity is in danger. It is caused by the presence of high level of organic pollutants and nitrate in water body. The BOD level in Yamuna from Yamunotri (origin) to Palla (Place between Sonipat and Nizamuddin Bridge) in Delhi is usually ranges from 1 to 3 mg/L. Up to Palla Yamuna is full of marine life, but beyond that wastewater drains outfall in Yamuna started. From Nizamuddin Bridge to Agra down- stream the BOD level ranges from 3 to 51 mg/L. The BOD level was also found above the permissible limits in Mathura, Agra, Etawah and Juhika. Figure 4 shows the average BOD levels in Yamuna River at different locations.

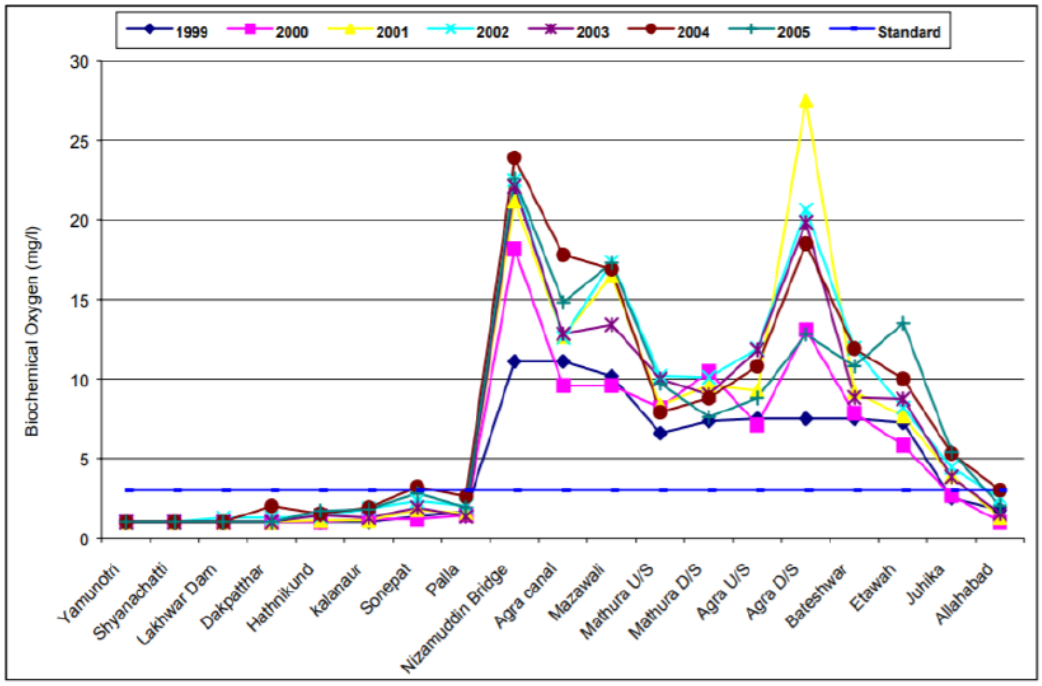


Figure 4: *Longitudinal Profile of Biochemical Oxygen Demand in Yamuna River (Source: CPCB, MoEF 2006)*

3.3.2. Chemical Oxygen Demand (COD)

COD beyond the permissible limit is the indicator of the organic and inorganic pollutants in the water body. The COD level in Yamuna ranges from 1 to 50 mg/L from its origin to Palla. Beyond Palla Yamuna River starts receiving large amount of wastewaters from different drain within Delhi and many downstream locations. The COD level start increasing from Nizamuddin Bridge and found above the permissible limits (ranges from 3 to 155 mg/L) up to Juhika.

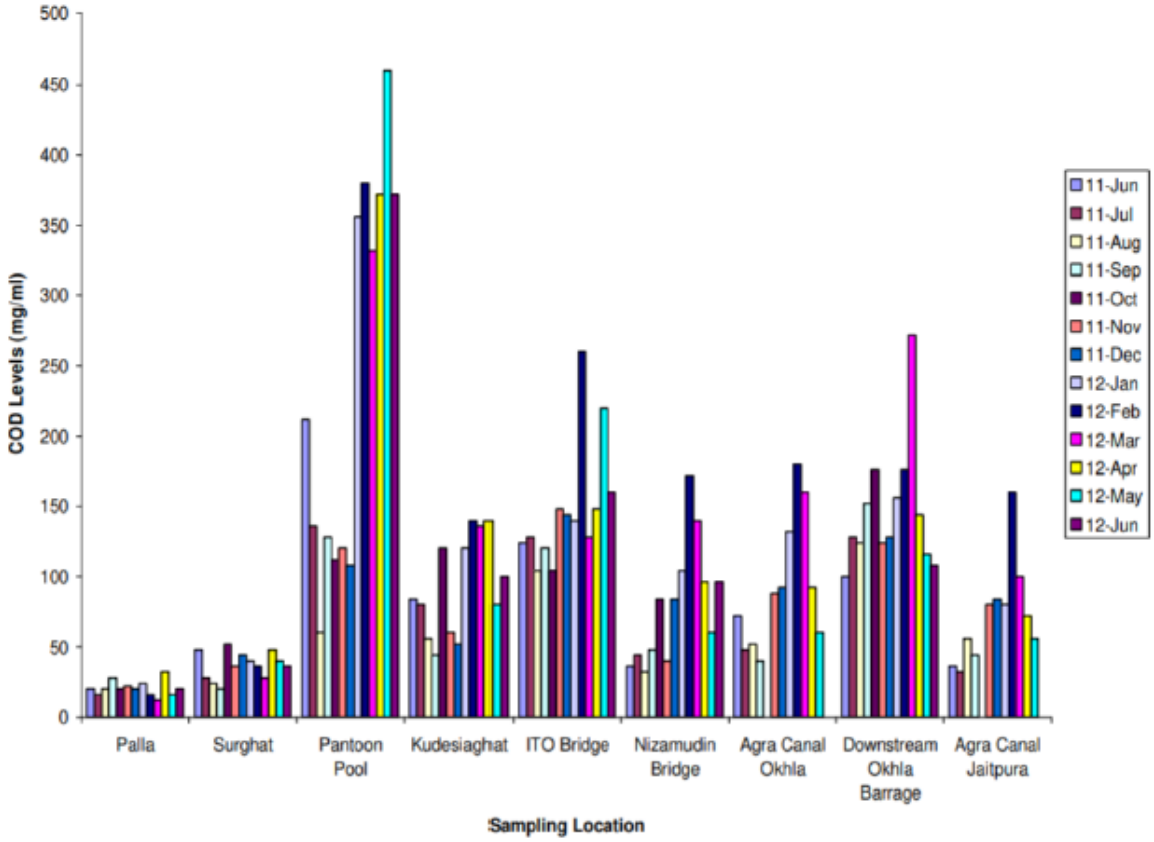


Figure 5: *Longitudinal profile of COD in Yamuna River in Delhi (Dhillon et al., 2013)*

3.3.3. Dissolved Oxygen (DO)

DO level in Yamuna from its origin to Palla is found normal, but beyond that it started decreasing. After Wazirabad the DO level starts decreasing drastically andmajority of times the DO level was found nil at Delhi downstream locations; it may be attributed to Shahdara drain and Hindon River which discharge wastewater at these locations. Further the DO levels at locations in Mathura, Agra, Etawah and Juhika were found beyond the permissible limits. Figure 4 shows the average DO levels in Yamuna River at different locations.

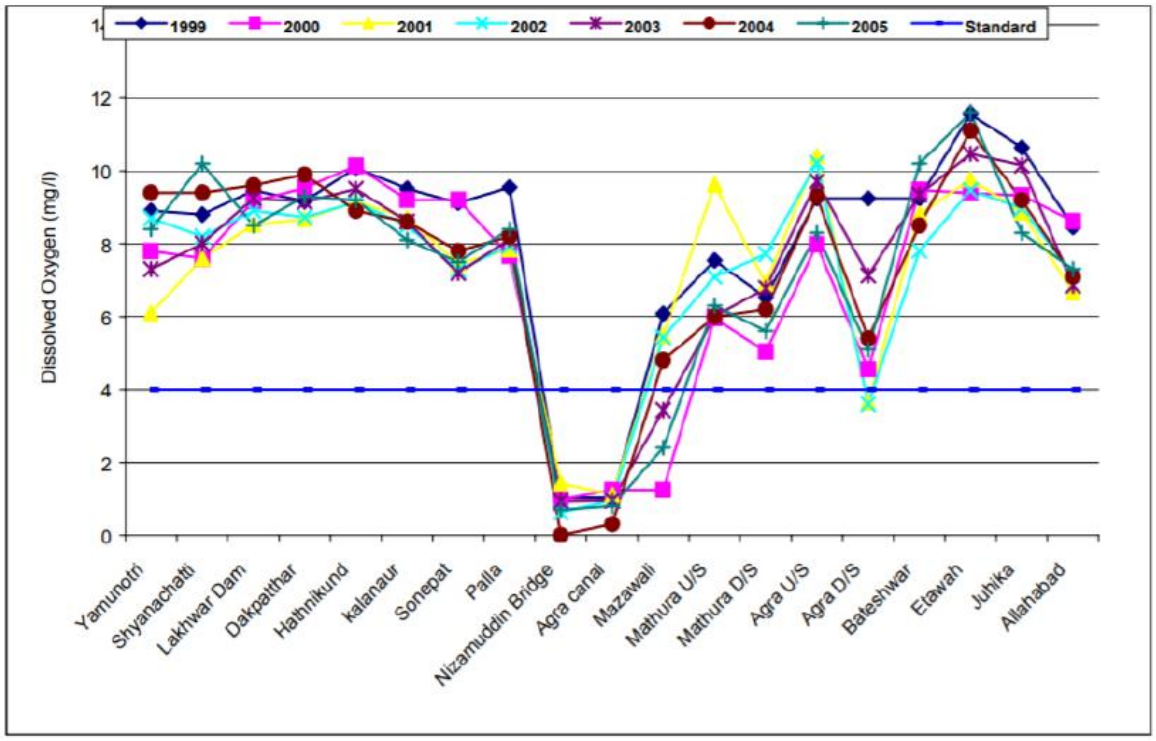


Figure 6: *Longitudinal Profile of Dissolved Oxygenin Yamuna River (Source: CPCB, MoEF 2006)*

3.4. Usages of the Yamuna River water

The Yamuna River sustains all forms of life along its banks and various social and commercial activitiesdepend on it [5]. It's the major source of drinking water to millions of Indiansandhasmultiple other uses [7]. The Yamuna Basin is of great economic significance due to its fertility and high productivity, particularly many parts of Uttar Pradesh and Haryana [5].

Some well-designated uses of the water of the Yamuna River are as follows:

- Drinking water
- Bathing water
- Irrigation
- Livestock use
- Industrial use
- Navigation
- Aesthetics
- Recreation
- Religious and Cultural

The availability water of the Yamuna River varies greatly with time and space with 80% of the water flowing in it in the Monsoon period (July, August and September) whereas whatever water is available in it in the non-monsoon period (October to June) is widely used for irrigation and drinking, leaving very little water in the river to flow (CPCB 2010). Along its course, the water of Yamuna River is abstracted for a variety of purposes such as about 94% for irrigation, 4% for domestic use, and 2% for the industries. The river water is extensively abstracted at Hathnikund / Tajewala and Okhla barrages.

3.5. Heavy metal pollution and its sources

- Heavy metals derived from anthropogenic activities contaminate the soil is a major global demanding issue.
- Anthropogenic activities, including chemical industry, traffic and transportation, iron and steel industry, smelting and mining, domestic activities and agricultural practices, along with chemical and metallurgical industries are the major contributors of the heavy metal load to the environment [24-33].

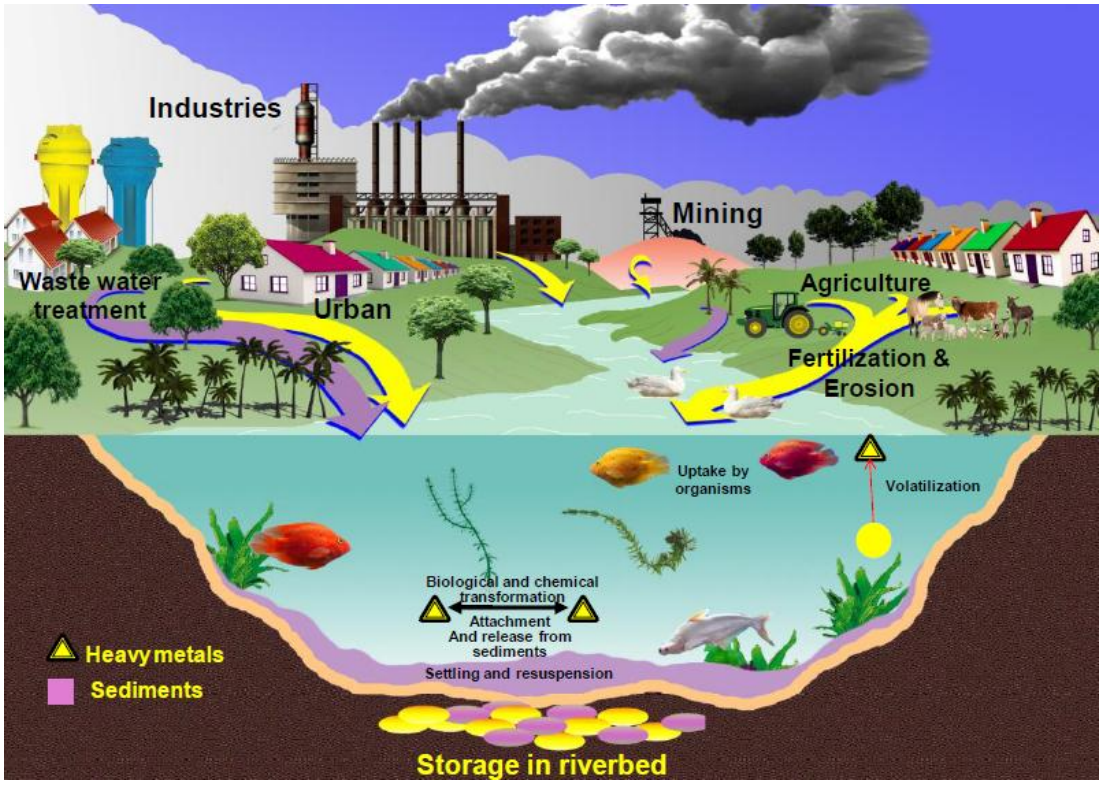


Figure 7:*The various sources and sink of heavy metals (Source:Muchuweti et al., 2006)*

- Although urban agriculture is the source of income and rural employment but growing crops and vegetables through wastewater irrigation is a worrisome matter in reality, especially in developing countries like ours.
- Thus major and serious concern which arises is the contamination of the crops and vegetables due to uptake of heavy metals [34]. Consumption of food crops contaminated with heavy metals is a major food chain route for human exposure [35].
- Heavy metal accumulation in plants varies from species to species, and the efficiency of absorbing metals can be estimated by either plant uptake or soil-to-plant transfer factors of the metals [36]. Crops raised on the metal-contaminated soils collect metals in enough quantities, which can be clinically fatal to both animals and human beings consuming these metal rich plants [37].
- Zhuang et al. (2009) evaluated heavy metals of food crops in the vicinity of Dabaoshan mine, South China and found that the heavy metal load exceeded thepermissible limit thus concluded that there was a potential health risk for the localinhabitants through consumption of contaminated food crops[38].
- Cement and printing industryrelease toxic heavy metals such as cadmium, lead and zinc [39] and leather tanning industry are source of chromiumand arsenic in the ecosystem [40, 41].
- River acts as the largestcarrier of these toxic elements, however they are significant environmental contaminates inriverine network [42, 43].
- These elements aretransported along hydrologic gradients, covering hundreds of kilometres in a relatively short time period [44-46].

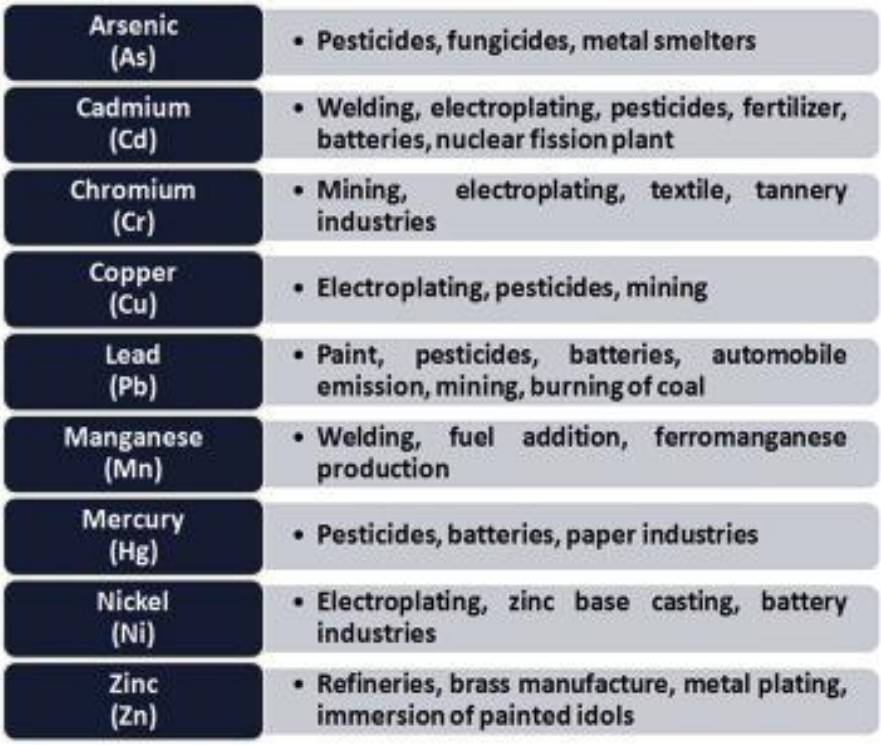


Figure 8: *Sources of different heavy metals (Source: Google)*

3.5.1. Effect of heavy metals

- [30]identify their highly efficient metal removal biological systems [13]. These microorganisms can grow in the presence of high concentrations of heavy metals and have a great potential for bioremediation of heavy metals dischargedin industrial effluents [14].
- Previous studies have well documented the occurrence and abundance of metal-tolerant microbes in metal polluted water bodies [15-17].
- Bacterial species such as *Pseudomonas aeruginosa*, *Klebsiellapneumoniae*, *Proteus mirabilis* and *Staphylococcus sp.* isolated from wastewater samples were found to be resistant to heavy metals and antibiotics [47].
- Similarly, cadmium-resistant *Klebsiella* and *Enterobacter* species and nickel resistant *Actinomycetes* and other bacteria have also been isolated from industrial effluents and soil samples [48, 49].

- These microorganisms develop a variety of resistance mechanisms to survive in different heavy metal concentrations, but the resistance is often specific to one or few metals [18, 19, 50]. These heavy metal tolerant bacterial species may serve as an important and cost effective bioremediation tool for the removal of heavy metals from wastewater and industrial effluents, preventing the contamination of water bodies [4].

Table 3:*Heavy metals and their effects on human health[7]*

S. No	Pollutants	Major Sources	Effect on Human Health
1.	Lead	Paint, Pesticide, Batteries, Crystal Glass Preparation.	Cognitive Impairment In Children, Peripheral Neuropathy In Adults, Developmental Delay
2.	Copper	Electroplating, Pesticide Production, Mining.	Headache, Nausea, Vomiting Diarrhea And Kidney Malfunctioning
3.	Zinc	Effluents From Electroplating Industries, Sewage Discharge, The Immersion Of Painted Idols	Vomiting, Diarrhea, Icterus, Liver And Kidney Damage
4.	Nickel	Stainless Steel Manufacturing Units, Electroplating Factory Discharge	Neurotoxic, Genotoxic, And Carcinogenic Agent, Nickel Dermatitis
5.	Cadmium	Electroplating, Preparation Of Cd-Ni Batteries, Control Rods, Shields within Nuclear Reactors, Television Phosphors.	Kidney And Liver Damage. Renal Dysfunction, Gastrointestinal Damage.
6.	Chromium	Mines, Electroplating	Gastrointestinal, Hepatic, Renal, Neuronal Damage

3.6 Microbes and antibiotic resistance

Infections account for a major cause of death throughout the developing world. This is mainly due to the emergence of newer infectious agents and more specifically due to the appearance of antimicrobial resistance. With time, the bacteria have become smarter and along with it, massive imprudent usage of antibiotics in clinical practice has resulted in resistance of bacteria to antimicrobial agents.

4. Materials & Methods

4.1. Sample collection

Samples were collected by seniors previously. Sampling of different components of the river Yamuna was done in pre summer, from 2 sites along the route of Yamuna, representing the upstream and downstream stations for main industrial complex of the state. The site locations are described in Table 1.

Site no	Name of site of Yamuna Stretch	River Stream Flow	Description	Remarks
S1	PallaGhat	Upstream(23km of Wazirabad barrage)	This site is the entry point of river Yamuna in Delhi. It is important intake water works for the city's water supply.	Agricultural area
S2	HaathiGhat of Agra	Downstream	The site is located on River Yamuna downstream of the city Agra behind the TajMahal.	Industrial area

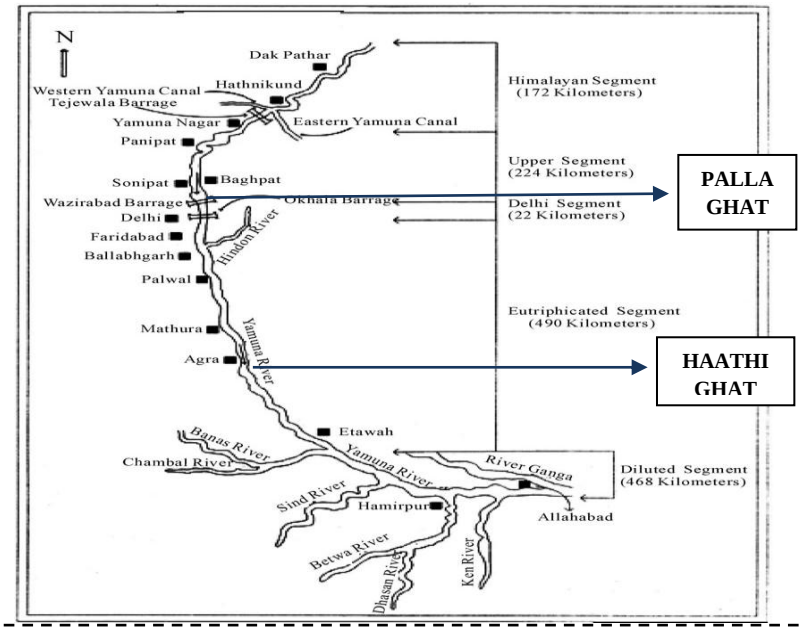


Figure 9: *Shows the stretches of Yamuna and sample sites are showed in box[1]*

Grab samples of water were collected previously in high grade plastic bottles in triplicate and mixed to get a composite sample for each site. All the sample bottles were stored in iceboxes till brought to laboratory for analysis. We have used that sample.

4.2. Sterilization of glassware and other materials

- All glassware used were thoroughly washed with detergent, rinsed and allowed to dry inside hot air oven. The glassware were then enfolded with aluminum foil and sterilized.
- The distilled water used for serial dilutions and other purposes, was also autoclaved at 15 PSI for 15 minutes.
- The workbench (LAF) was cleansed with 75% alcohol prior and after every experimental work. Media was prepared using clean, sterilized glassware & plastic ware and autoclaved at 15 PSI for 20 minutes.

4.3. Enumeration of bacteria

- Serial dilution Dilutions in the range 10-1 (1/ 10) to 10-8 (1/ 100,000,000) were prepared. 100µl of each dilution was spread on LB plates under laminar air-flow hood; plates were labelled well and were incubated at 37 C for 24 hours.
- Proper incubation conditions permit microbial reproduction so that colonies may develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.
- After incubation Colony Forming Unit (CFU)/ ml was calculated from 10 dilution of Agra and 10 dilution of Palla.

4.3.1. Preparation of serial dilutions

- Nine sterile falcons were taken for each sample, and labelled as - stock, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}.
- Stock of sediment sample was prepared by dissolving 1gm of sample in 1ml of distilled water and mixed well. Falcons marked from 10-1 to 10-8 were filled with 9ml autoclaved distilled water.
- Now 1 ml stock was transferred to falcon labelled 10 which was filled with 9ml of water and mixed well. Successive serial dilutions were made in a similar manner. Before transferring each tube was shaken well.

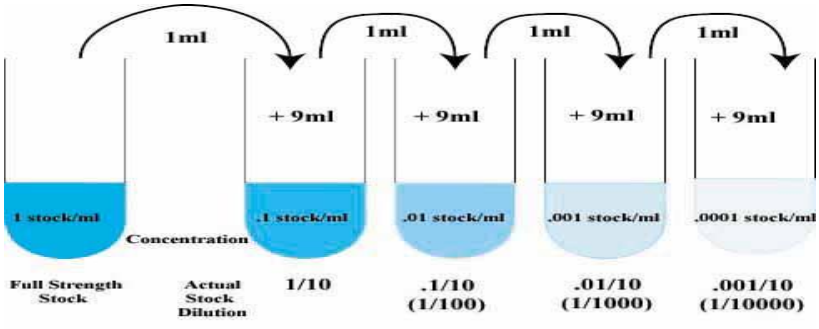


Figure 10: *Shows the techniques of serial dilution (Source: Google)*

4.3.2. Preparation of LB agar

LB agar (HiMedia) was prepared by following manufacturer's instructions autoclaved at 15 PSI for 20 minutes.

Compositions:

Ingredients	Gram/liter
Casein enzymatic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH	7.5±0.2

4.4. Isolation of various bacteria from the samples and maintaining their pure culture

- Morphologically different colonies from plates prepared by serially diluting sample were observed. Colonies differing in morphological characteristics were selected, picked and purified and then preserved on different plates for further studies.
- Distinct colonies were pick aseptically under laminar air flow hood and streaked on LB (Luria Bertani) agar plates and incubated at 37Ò C for a period of 24 hours.
- A total of 79 colonies from Agra and 66 colonies from Palla were isolated and streaked to make pure culture and their morphological characteristics were also recorded.
- The isolated colonies were further streaked twice on LB plates to maintain pure culture. Plates after incubation were paraflimed and stored in refrigerator for further study.
- Isolated colonies were also preserved in glycerol stock and were stored at -20 degree Celsius, because freezing is an efficient way of storing bacteria.

- Glycerol allows reducing the harmful effect of ice crystals of bacteria which can damage cells by dehydration caused by a localized increase in salt concentration leading to denaturation of proteins.
- Additionally, ice crystals can also puncture cellular membranes. Glycerol as a cryoprotectant depresses the freezing point of bacterial cells, enhancing super cooling.
- It does so by forming strong hydrogen bonds with water molecules, competing with water-water hydrogen bonding. This disrupts the crystal lattice formation of ice unless the temperature is significantly lowered.

4.4.1. Preparation of bacterial stock in LB broth

- 3 ml of the stock of each isolate was prepared by taking single colony from the pure culture plate of respective isolate with the help of sterile loop and transferring it to sterile falcons filled with 3 ml of freshly prepared LB broth.
- The falcons were incubated at 37 C in an incubator shaker for 24 hours.

LB agar (HiMedia) was prepared by following manufacturers' instructions and autoclaved at 15 PSI for 20 minutes.

Compositions:

Ingredients	Gram/litre
Casein enzymichydrolysate	
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C)	7.5±0.2

4.4.2. Preparation of bacterial glycerol stock

20ml of 50% glycerol stock was prepared by dissolving 10 ml of glycerol into 10 ml of autoclaved single distilled water.

⇩

Autoclaved eppedorfs were marked with date and number of isolated colonies and location

⇩

500µl of bacterial stock and 500µl of 50% glycerol stock was added and mixed well.

Prepared bacterial glycerol stock was then stored at -70^{o} C.

4.4.3. Streaking

Streaking is a rapid qualitative method that helps to make & maintain pure culture by allowing single colonies to be isolated from inoculum which also must be from a single colony. On a LB and Nutrient agar plate streaking was done by following way –

Sterilize the LAF chamber with alcohol, autoclaved plate was marked by details of the isolates and hand wash with alcohol should be done.

⇩

Flame a sprit lamp and incinerate the inoculation loop properly with flame and alcohol dip. Put the loop on a stand to cool down.

⇩

After cooling, pick a very small isolated colony from master plate and smear on new plate.

⇩

Incinerate the loop and cool down and start making parallel lines from the smear from one side to another side with 7-8 strokes, turn the plate and repeat that strokes continuously and end with a zigzag shape in the middle.

⇩

Keep that plate in incubator in an inverted position at 37c for 24 hours.

4.5. Isolation of heavy metal resistant strain

4.5.1. Primary screening of heavy metal resistant bacteria using mercury

- For the selective screening of heavy metal resistant bacteria, heavy metal HgCl2 (Mercury(II) chloride) was used. All the isolates, 79 from Palla and 66 from Agra were analyzed morphologically to eliminate same colonies.
- After screening, 36 isolates from Agra and 27 isolates from Palla were selected for primary screening HgCl2 incorporated nutrient agar (NA) plates with concentration 12.5µg/ml and 25µg ml were made, each isolate was tested for both 12.5 and 25µg/ml plate concentration by streak plate method.
- Four isolates were streaked in one plate. Standerd pour plate method in the prepared, well labelled plates; after 24 hour of incubation at 37 °C, the plates were observed for any kind of development on the culture medium.
- Control plates were also prepared with nutrient agar media without including any heavy metal for a better comparison. Colonies found resistant to Mercury were selected, picked, purified, numbered and preserved on different plates for further studies.

4.5.1.1. Preparation of Nutrient Agar

Nutrient agar (NA) - (HiMedia) was prepared by following manufacturer's instructions and was autoclaved at 15 PSI for 20 minutes.

Composition:

Ingredients	Gram/litre
Peptone	5.000
Sodium chloride	5.000
HM peptone B	1.500
Yeast extract	1.500
Agar	15.000
pH(25^{O}c)	7.4±0.2

4.5.1.2. Preparation of HgCl2 stock solution

5 ml of 50 mg/ml stock solution of HgCl2 was prepared by dissolving 250 mg/ml of HgCl2 in sterile autoclaved distilled water

4.5.1.3. Plates preparation

Plates were prepared by mixing appropriate amount of HgCl from stock solution (50 mg/ mL)to nutrient agar in sterilized falcon to make the final volume 20 mL which was poured intosterile petridish after mixing well, under aseptic conditions. Plates with a concentration 12.5µg/ mL and 25µg/ mL HgCl2 were made.

- Calculations we made in the following manner –
- Initial concentration (1) = 50mg/ml or 50,000µg/ml
- Final concentration (m2) = 12.5 µg/ml
- Initial volume (v1) = x [say]
- Final volume (v2) = 20ml

$$m1v1 = m2v2$$

So, here $v1 = m2v2/m1$

- According to the calculation v1 = (12.5× 20,000)/50,000= 5µl

x = 5µl therefore, 5µl of stock solution (concentration 50 mg/ ml) must be mixed with LB agar to make a final volume of 20 ml, used to prepare the plate with a concentration of 12.5µg/ml. Similarly 10 µl of stock solution (concentration 50 mg/ ml) must be mixed with LB agar to make a final volume of 20 ml, used to prepare the plate with a concentration of 25µg/ml,

4.5.2. Multiple heavy metal resistance analysis

- All isolates found resistant to $HgCl_2$ (concentration 12.5µg/ml and above) were selected, (total 18) and separately grown on different nutrient agar plates supplemented with Cadmium (Cd), Cr (used as Potassium Chromate)and Pb (used as Lead Acetate) starting with a concentration of 25µg/ml of each heavy metal with the method described previously for Mercury.
- Plates were incubated at 37 °C for 24 hours, after which the resistance capacity of multiple heavy metals was assessed. 18 isolates were found to be resistant to HgCl2 were further sub-cultured by streak plate method and stored in refrigerator for further studies

4.5.2.1. Preparation of stock solutions of heavy metals

- 10 ml of 100 mg/ ml stock solution of each heavy metal was prepared by dissolving 1gm of heavy metal in 10 ml sterile autoclaved distilled water in a capped glass bottle.
- 5 ml of 200 mg/ ml stock solution of each heavy metal was prepared by dissolving 1 gm of heavy metal in 5 ml sterile autoclaved distilled water in a capped glass bottle

4.5.2.2. Preparation of plates

Plates were prepared in a similar manner as for HgCl2 by following the dilution table.

Concentration of plates(µg/ml)	Volume of stock should be added(µl)
Concentration of stock solution 100mg/ml	
25	5
50	10
100	20
200	40
400	80
800	160
1600	320
3200	640
Concentration of stock solution 200mg/ml	
6400	640

4.5.3. Determination of minimum inhibitory concentration (MIC) for heavy metals

To determine MIC, heavy metal resistant selected 18 isolates were grown on heavy metal incorporated nutrient media plates against respective heavy metal; MIC was identified by successively increasing the concentration of the heavy metals (Hg, Cd ,Cr and Pb) on LB agar plates until the isolates failed to give colonies on the petri plate. The starting concentration of the heavy metals was 25 µg/ mL. Results were recorded.

4.6. Antibiotic susceptibility testing

Antimicrobial susceptibility testing of all 18 bacterial isolates was performed using the agar well diffusion method on Mueller-Hinton agar (MHA) against nine selected antibiotics namely – ***Cefuroxime***, ***Ciprofloxacin***, ***Gentamicin***, ***Azaerythromycin***, ***Chloramphenicol***, ***Tetracycline***, ***Ceftriazone***, ***Ampicillin*** and ***Clindamycine***.

4.6.1. Preparation of MHA

Mueller Hinton Agar - (HiMedia) was prepared by following manufracturer's instructions and was autoclaved at 15 PSI for 20 minutes.

Composition:

Ingredients	Gram/litre
HM infusion B	300.00
Acicase	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.4±0.1

4.6.2. Performing antibiotic assay

Petriplates containing 20ml Muller Hinton medium were seeded with 50µl of respective bacterial stock solution. Which concentrations are already standardized by CLSI.

⇩

4 wells, each of diameter 8 mm were cut using microtips(1ml) in a single plate, out of which one was labelled control into which 50µl of sterile distilled water was added , rest were used to add 50µl of respective antibiotic solution. 3 plates were made for each bacterial isolate.

⇩

The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Antibiotics which are used in plates are shown in tabulated form:

Names of Antibiotics	Solubility	Concentration used in plates(µg/ml)
Cefuroxime	Water	30
Ciprofloxacin	DMSO/ Methanol ,Ethanol	5
Gentamicin	Water	10
Azaerythomycin	Ethanol/Water	15
Chloramphenicol	Water/Ethanol	30
Tetracycline	Water	30
Cefriaxome	Water	30
Amphicillin	Water	10
Clindamycin	DMSO/ Ethanol	2

4.7. Plasmid DNA isolation from the resistant bacteria colony

The plasmids DNA of resistant colonies were isolated by alkaline lysis method. For this method 3 types of solution were used. Solution I, Solution II and Solution III.

Composition of Solution I

Ingredients	Amount required for 100ml	Amount required for 50ml
Glucose(1M)	5ml	2.5ml
EDTA(0.5M) pH= 8	2.5ml	1.25ml
Tris-Cl(1M) pH=8	1ml	0.5ml
De-ionized water	90.5ml	45.25ml

Composition of Solution II

Ingredients	Amount required for 10ml	Amount required for 5ml
NaOH(0.2N)	200µl	100µl
1% (w/v) SDS	1ml	0.5ml
Water	8.8ml	4.4ml

Composition of Solution III

Ingredients	Amount required for 100ml	Amount required for 50ml
Potassium acetate	60ml	30ml
Glacial acetic acid	11.5ml	5.75ml
Water	28.5ml	14.25ml

Composition of TBE Buffer

Ingredients	Amount required for 1000ml	Amount required for 500ml
Tris-base	10.8gm	5.4gm
Boric acid	5.5gm	2.75gm
EDTA (0.5M) pH=8.8	4ml	2ml
MQ for volume make up	1litre	500ml

Composition of 0.8% Agarose gel

Ingredients	Amounts
Agarose	200mg
TBE buffer	25ml
EtBr	5µl

Plasmid DNA isolation procedure is described below –

- Inoculte 2ml of rich medium (LB, YT or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37^{o}c with vigorous shaking
- Pour 1.5 ml of culture into a micro centrifuge tube. Centrifuge at 14,000rpm for 1min at 4^{o}c, in a centrifuge machine. Store the unused portion of the original culture at 4^{o}c.
- Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
- Re-suspend the bacterial pellet in 100µl of Alkalinelysis solution I by vigorous vortexing.
- Add 200µl of freshly prepared Alkalinelysis solution II to each bacterial suspension .Close the tube tightly, and mix the contents well by inverting the tube, not using the vortex,store the tube in ice.
- Add 150µl of ice cold Alkaline solution III. Close the tube and disperse the solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 3-5 min.
- Centrifuge the bacterial lysate for 5 minutes 14,000rpm for 5 min at 4^{o}c in a centrifuge machine. Collect the supernatant to a fresh tube.
- Add equal volume of phenol or chloroform, mix the organic and aqueous phase by vortexing and then centrifuge the emulsion at 14,000rpm for 2 minutes at 4^{o}c. Transfer the aqueous upper layer to a fresh tube.
- Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2mins at room temperature.
- Collect the precipitate nucleic acids by centrifugation at 14,000rpm for 5mins at 4^{o}c.

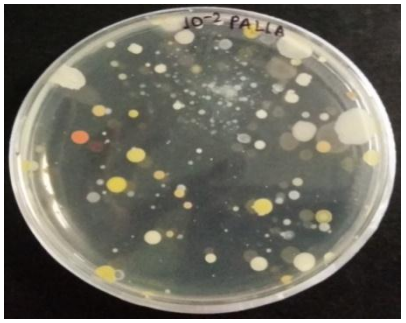
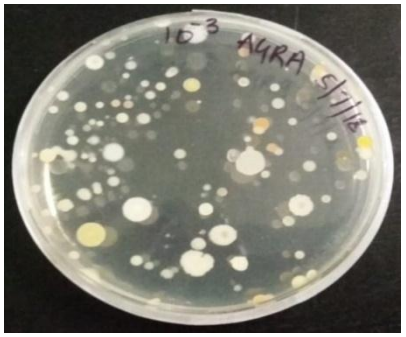
- Discard the supernatant by aspiration, Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a kim wipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
- Add 1ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at 14,000rpm for 2mins at 4^{o}c.
- Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
- Remove any beads of ethanol from the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
- Dissolve the nucleic acids in 50µl of TE (pH 8.0) containing 20µg/ml DNase free RNase - A (pancreatic RNase). Vortex the solution gently for a few second and store the DNA at -20^{o}c.

5. Results & Discussions

5.1. Enumeration of bacteria

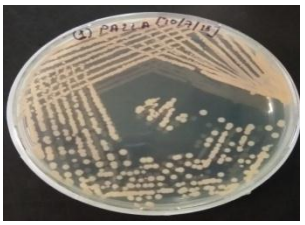
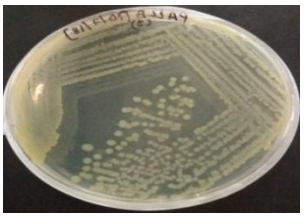
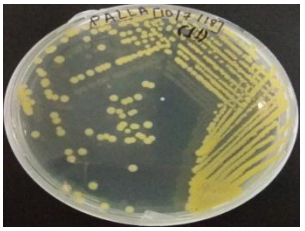
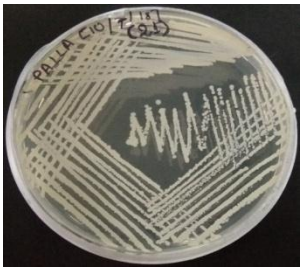
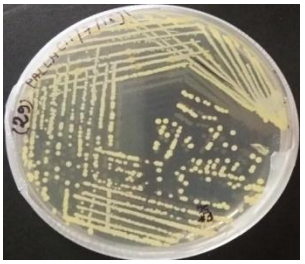
Enumeration of bacteria was done by calculating CFU/ml from serially diluted samples of Agra and Palla. CFU/ml of Palla and Agra were calculated from 10^{-2} and 10^{-3} plates respectively.

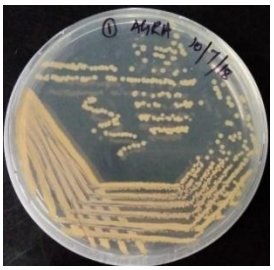
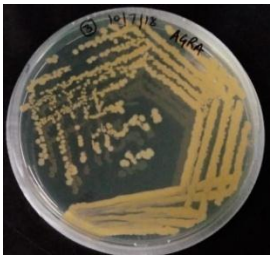
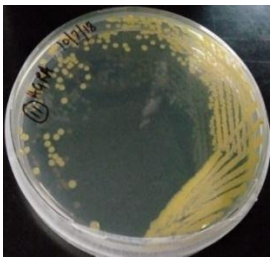
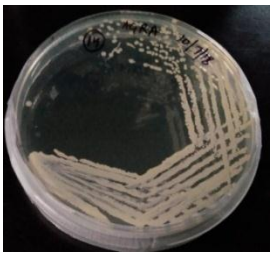
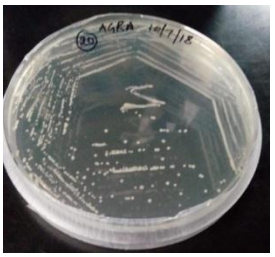
$$\textbf{Colony Forming Unit} = \frac{\textbf{Number of colonies} \times \textbf{Dilution Factor}}{\textbf{Volume of inoculums (ml)}}$$

Sample	Dilution	Bacterial count in 100µl	CFU/ml	Pictures
Palla	10^{-2}	134	134×10^{3}	
Agra	10^{-3}	114	114×10^{4}	

5.2. Primary screening of bacteria from the samples and maintaining their pure cultures

Morphologically different colonies were selected, isolated, picked and purified and preserved in different plates. A total 79 colonies of Agra and 66 colonies ofPalla were isolated, purified and preserved in glycerol stock. Some of them are as follows.

Sample No	Morphology of Isolated Colonies From Palla						
	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures
1	Orange	Round	Smooth	Opaque	Raised	Shiny	
3	Light yellow	Round	Smooth	Translucent	Convex	Shiny	
11	Mustard yellow	Round	Smooth	Opaque	Raised	Shiny	
21	Milk White	Round	Irregular	Opaque	Flat	Shiny	
29	Light Yellow	Round	Smooth	Opaque	Convex	Shiny	

Sample No	Morphology Of Isolated Colonies From Agra						
	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures
1	Orange	Round	Smooth	Opaque	Convex	Shiny	
3	Light Orange	Round, Central dark region	Smooth	Opaque	Raised	Shiny	
11	Light Yellow	Round	Smooth	Translucent	Raised	Shiny	
14	Cream White	Round	Rough	Opaque	Flat	Rhizoid like	
20	Grey white	Round	Smooth	Opaque	Raised	Shiny	

5.3. Isolation of heavy metal resistant strain

- After primary screening of isolated sample of Palla and Agra 27 and 36 colonies were sorted and loaded on HgCl2 plates of concentration 12.5µg/ml and 25µg/ml.
- Resulting 18 colonies were further studied with multiple heavy metals like CdCl2, $Pb(CH_3COO)_2$, K2CrO4 starting with concentration of 25µg/ml, further increasing

concentration were performed to check the MIC (Minimum Inhibitory concentration) of each isolate.

- Results were recorded after 24 hours of incubation for lower concentration and 48 hours of incubation for higher concentration. The Minimum Inhibitory Concentration was determined and results are shown here in tabulated form.

$$\% \textbf{ of RI} = \textbf{ Percentage of resistant colony } = \frac{\textbf{No. of resistant colony}}{\textbf{Total no. colony}} \times \textbf{100}$$

Table 4: *The presence and absence of isolated colonies and percentage of resistance against HgCl2*

ISOLATE NO	Showing growth of the isolate in medium containing heavy metal $HgCl_2$				
	12.5µg/ml	**25µg/ml**	**50µg/ml**	**100µg/ml**	**200µg/ml**
Palla					
1.	+	+	+	-	-
2.	+	+	+	-	-
3.	+	+	+	-	-
4.	+	+	-	-	-
5.	+	+	+	+	-
6.	+	+	-	-	-
7.	+	+	+	-	-
% of RI	100%	100%	71.42%	14.3%	0
Agra					
1.	+	+	+	-	-
2.	+	+	+	-	-
3.	+	+	+	-	-
4.	+	+	+	-	-
5.	+	+	+	-	-
6.	+	+	+	-	-
7.	+	+	+	-	-
8.	+	+	+	+	+
9.	+	+	+	+	-
10.	+	+	-	-	-
11.	+	+	+	-	-
% of RI	100%	100%	90.9%	18.2%	9.09%

- In case of primary screening with mercury, colonies that showed resistance against the concentration of 25µg/ml were selected to further check resistance against higher concentration and other heavy metals.
- From the table we can compare resistant colony of Palla and Agra. From table it was found that, both Palla and Agra had 100% resistant colony against 25µg/ml, In case of 50µg/ml where Palla had 71.42% resistance colony, Agra showing 90.9% colony, at concentration of 100µg/ml Palla and Agra was showing 14.3% and 18.2% resistant colony respectively. No resistant colony was reported in 200µg/ml but 9.09% resistant colony was found against 200µg/ml at Agra.
- Isolated colonies of Agra are more resistant than Palla against HgCl2. Agra shows more resistance than Palla.Some pictures are given below.

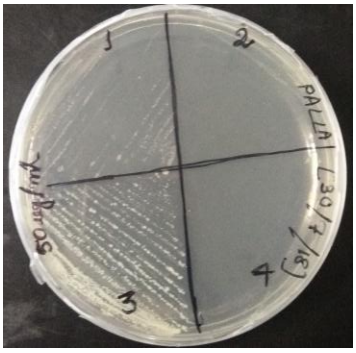


Figure 9: *Shows that all colonies of Palla are resistant at 50µg/ml*

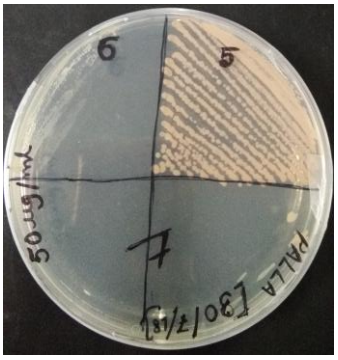


Figure 10: *Shows that 5th and 7th colonies of Palla are resistant at 50µg/ml*

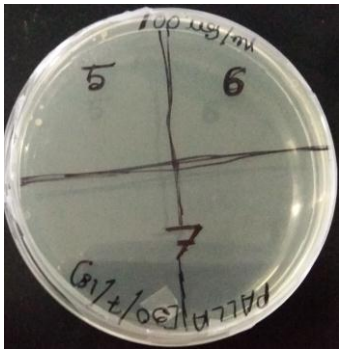


Figure11: *Shows that only 5th colony of Palla is resistant at 100µg/ml*

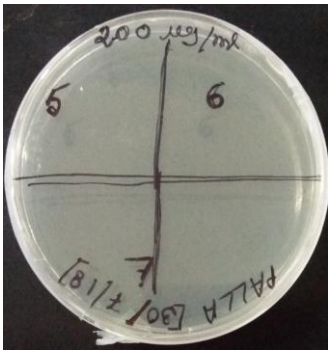


Figure 12: *No resistant colony of Palla is found at 200µg/ml*

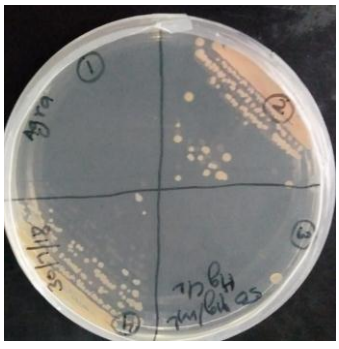


Figure 13: *Shows all colonies of Agra are resistant at 50µg/ml*

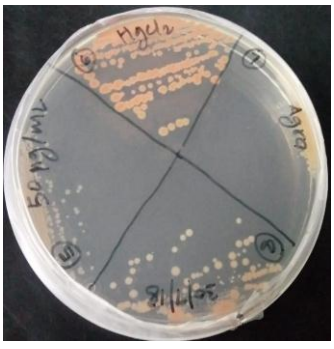


Figure 14: *All colonies of Agra are resistant at 50µg/ml*

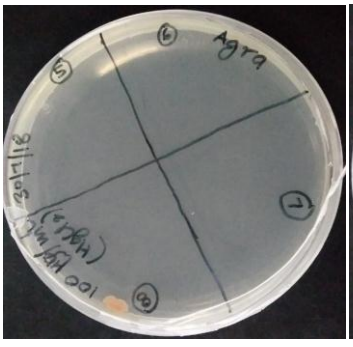


Figure 15: *Shows only 8th colony of Agra is resistant at 100µg/ml*

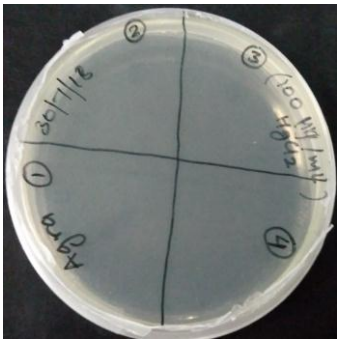
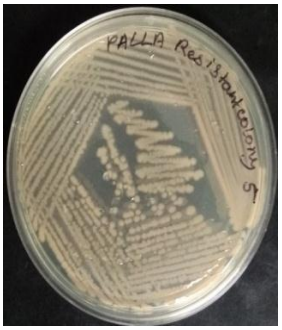
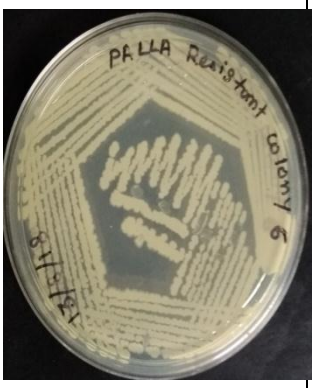


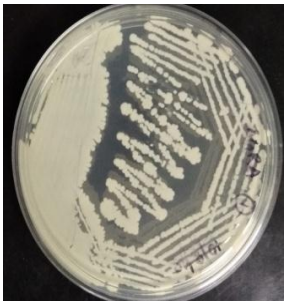
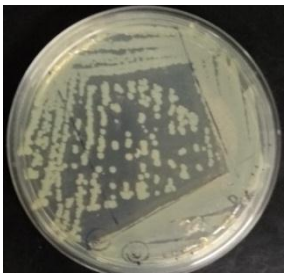
Figure 16: *No resistant colony is found at 100µg/ml*

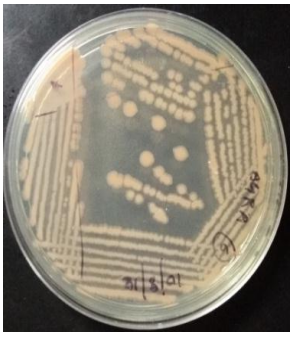
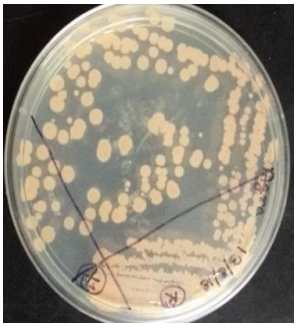
5.4. Colony characteristics of the isolates

Morphological analysis of the isolated 18 colony characteristic of heavy metal HgCl2 resistant was recorded as shown in the below table.

Isolate No	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures of Staining
Palla							
1	Orange	Irregular	Smooth	Opaque	Convex	Shiny	
2	Light Orange	Round	Smooth	Opaque	Convex	Dull	
3	Milk White	Round	Butyrous	Opaque	Convex	Shiny	
4	Light Orange	Round	Sticky	Opaque	Concentric	Dull	

Isolate No	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures of Staining
Palla							
5	Yellowish Orange	Round	Smooth	Opaque	Flat	Shiny	
6	Yellowish White	Round	Smooth	Opaque	Raised	Shiny	
7	Yellowish orange	Round	Smooth	Opaque	Concentric	Shiny	

Isolate no	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures
Agra							
1	Milk white	Irregular	Butyrous	Opaque	Convex	Shiny	
2	Orange	Round	Butyrous	Opaque	Concentric	Dull	
3	Cream White	Irregular	Smooth	Opaque	Convex	Shiny	
4	Very Light Orange	Round	Smooth	Opaque	Concentric	Dull	
5	Light Orange	Round	Smooth	Opaque	Raised	Dull	

Isolate no	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures
Agra							
6	Orange	Oval	Smooth	Opaque	Raised	Dull	
7	Light Orange	Round	Smooth	Opaque	Raised	Dull	
8	Light Orange	Round	Smooth	Opaque	Raised	Shiny	
9	Light Orange	Round	Smooth	Opaque	Raised	Shiny	
10	Cream White	Irregular	Sticky	Opaque	Flat	Shiny	

Isolate no	Colour	Shape	Texture	Opacity	Elevation	Appearance	Pictures
Agra							
11	Yellowish White	Round	Butyrous	Opaque	Raised	Shiny	

5.5. Observation of gram staining

RESISTANT COLONIES OF PALLA

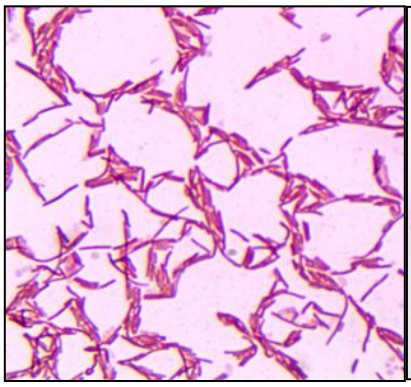


Figure 42: *Colony 1, Gram Negative, Rod shape colony*

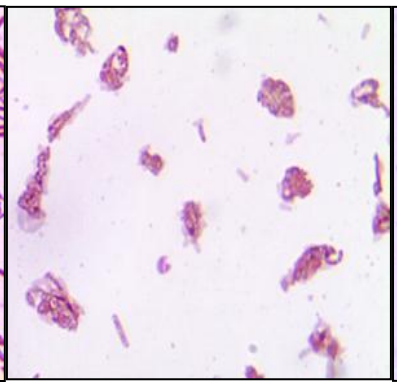


Figure 43: *Colony 2, Gram Negative, coccus like colony*

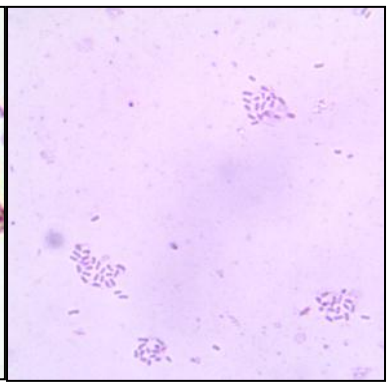


Figure 44: *Colony 3, Gram Positive, Tiny rod shape colony*

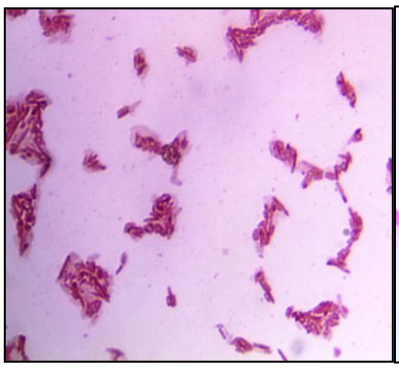


Figure 45: *Colony 4, Gram Negative, Rod shape colony*

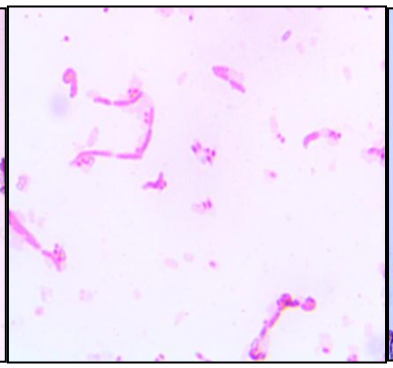


Figure 46: *Colony 5, Gram Negative, Rod shape colony*

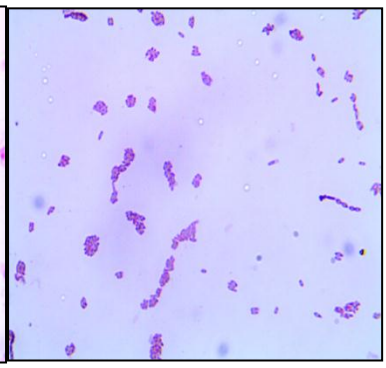


Figure 47: *Colony 6 Gram Positive, Small Rod shape colony*

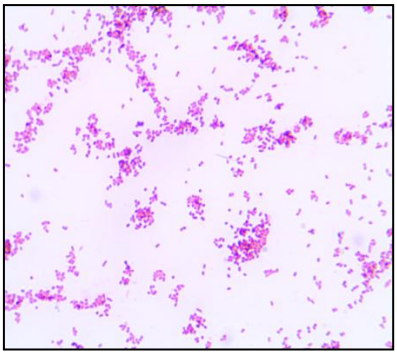


Figure 48:*Colony7, Gram Negative, Very tiny rod shape*

RESISTANT COLONIES OF AGRA

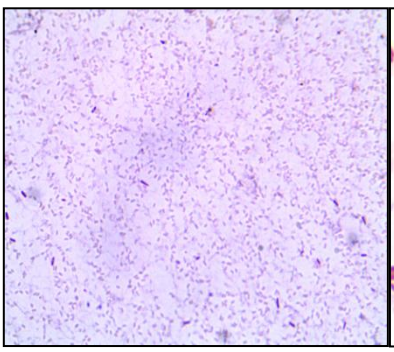


Figure 49: *Colony 1, Gram Positive, Tiny rod shape colony*

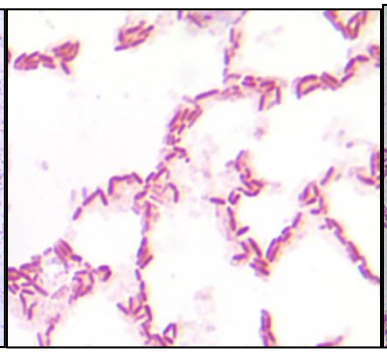


Figure 50: *Colony 2, Gram Negative, Rod shape colony*

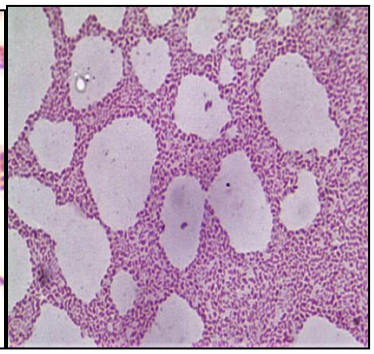


Figure 51: *Colony 3, Gram Negative, Tiny rod shape colony*

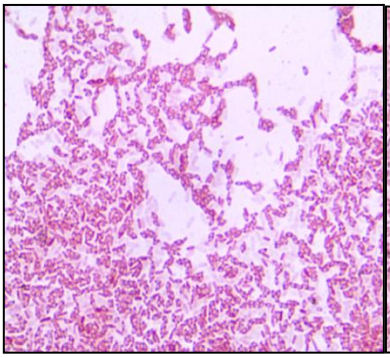


Figure 52: *Colony 4, Gram Negative, Small rod shape*

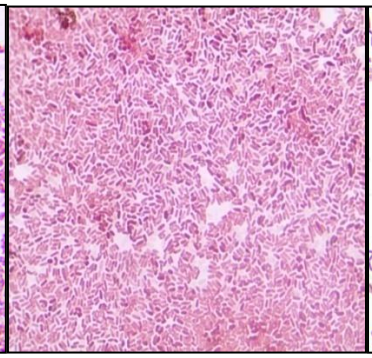


Figure 53: *Colony 5, Gram Negative, Medium rod shape*

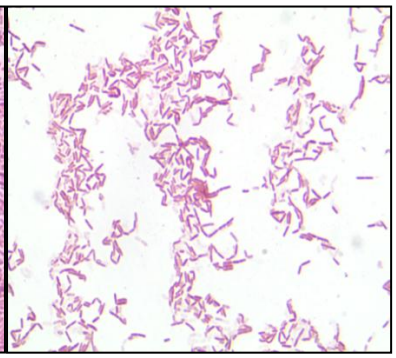


Figure 54: *Colony 6, Gram Negative, Medium rod shape*

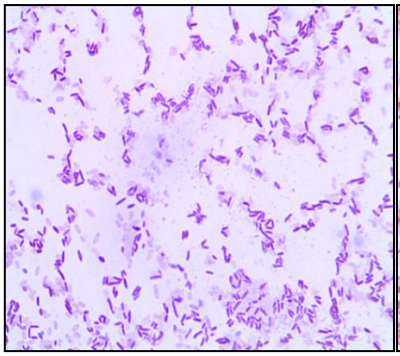


Figure 55: *Colony 7, Gram Positive, Rod shape colony*

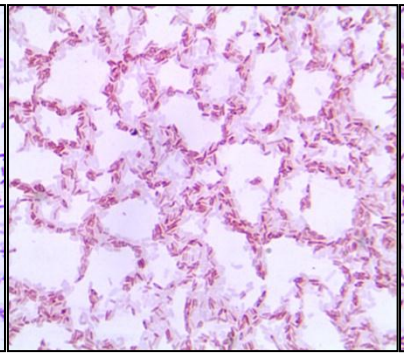


Figure 56: *Colony 8, Gram Negative, small rod shape*

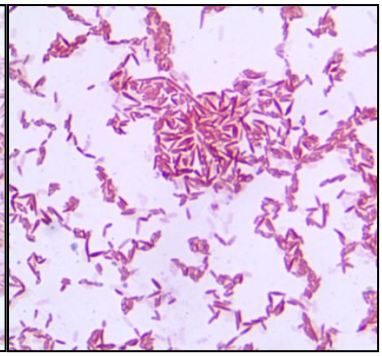


Figure 57: *Colony 9, Gram Negative, Big rod shape colony*

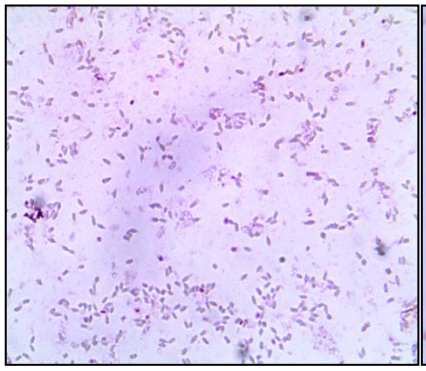


Figure 58: *Colony 10, Gram Positive, Small rod shape colony*

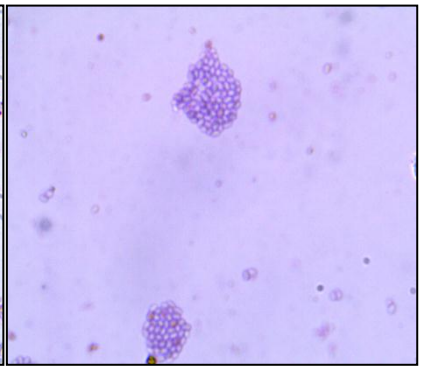


Figure 59: *Colony 11, Gram Positive, Smallcoccus colony*

5.6. Other heavy metal resistance checking

- Next study was performed to check $CdCl_2$ resistant colony starting with concentration25 µg/ml and higher concentration 800 µg/ml. Results are shown in tabulated form.

Table5:*Showing the presence and absence of isolated colonies and percentage of resistance against $CdCl_2$*

Isolated No	Showing growth of the isolate in medium containing heavy metal $CdCl_2$					
	25µg/ml	**50µg/ml**	**100µg/ml**	**200µg/ml**	**400µg/ml**	**800µg/ml**
Palla						
1	+	-	-	-	-	-
2	+	-	-	-	-	-
3	+	+	+	-	-	-
4	+	-	-	-	-	-
5	+	-	-	-	-	-
6	+	+	+	-	-	-
7	+	+	+	-	-	-
% of RI	100%	42.9%	42.9%	0	0	0
Agra						
1	+	-	-	-	-	-
2	+	+	-	-	-	-
3	+	+	-	-	-	-
4	+	-	-	-	-	-
5	+	-	-	-	-	-
6	+	+	-	-	-	-
7	+	-	-	-	-	-
8	+	-	-	-	-	-
9	+	-	-	-	-	-
10	+	+	+	-	-	-
11	+	-	-	-	-	-
% of RI	100%	36.4%	9.09%	0	0	0

- So, From the table we can see Palla and Agra both has 100% resistance at concentration of 25µg/ml, In case of concentration 50µg/ml, 42.9% and 36.4% resistant colonies were found at Palla and Agra respectively. In case of concentration 100µg/ml 42.9% and 9.09% resistant colonies were found at Palla and Agra. There are no such resistant colonies are found at the concentrations of 200µg/ml, 400µg/ml, 800µg/ml at Palla and Agra both.
- Palla shows more resistant colony than Agra. Some pictures are given below.

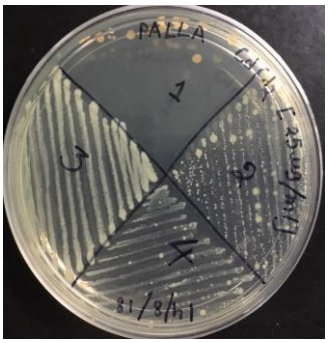


Figure 17: *Showing all colonies of Palla are resistant against 25µg/ml*

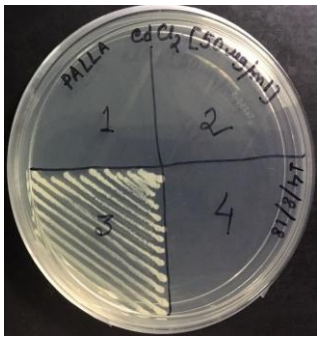


Figure 18:*Showing only 3rd colony of Palla is resistant against 50µg/ml*

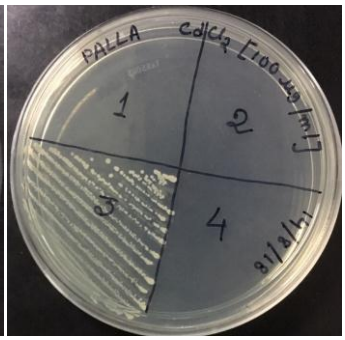


Figure 19:*Showing only 3rd colony of Palla is resistant against 100µg/ml*

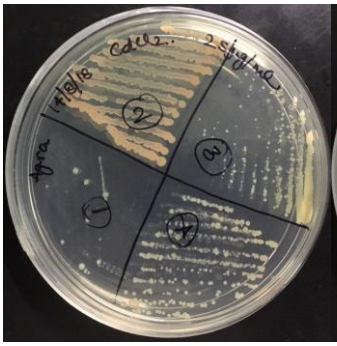


Figure 20:*Showing all colonies of Agra are resistant against 25µg/ml*

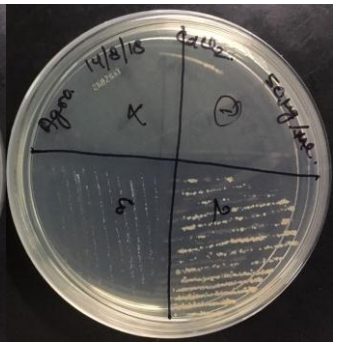


Figure 21:*Showing only 2nd and 3rdcolonies of Agra resistant against 50µg/ml*

- Next study was performed to check ($Pb(CH_3COO)_2$) resistant colony starting with concentration 25µg/ml and higher concentration 6400µg/ml. Results are shown in tabulated form.

Table 6:*Showing the presence and absence of isolated colonies and percentage of resistance against ($Pb(CH_3COO)_2$)*

Isolate No	**Showing growth of the isolate in medium containing heavy metal ($Pb(CH_3COO)_2$)**								
	25 µg/ml	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**	**400 µg/ml**	**800 µg/ml**	**1600 µg/ml**	**3200 µg/ml**	**6400 µg/ml**
Palla									
1.	+	+	+	+	+	+	+	-	-
2.	+	+	+	+	+	+	+	-	-
3.	+	+	+	+	+	+	+	-	-
4.	+	+	+	+	+	+	+	-	-
5.	+	+	+	+	+	+	+	-	-
6.	+	+	+	+	+	+	+	-	-
7.	+	+	+	+	+	+	+	-	-
% of RI	100%	100%	100%	100%	100%	100%	100%	0	0
Agra									
1.	+	+	+	+	+	+	+	-	-
2.	+	+	+	+	+	+	+	-	-
3.	+	+	+	+	+	+	+	-	-
4.	+	+	+	+	+	+	+	-	-
5.	+	+	+	+	+	+	+	-	-
6.	+	+	+	+	+	+	+	-	-
7.	+	+	+	+	+	+	+	-	-
8.	+	+	+	+	+	+	+	-	-
9.	+	+	+	+	+	+	+	-	-
10.	+	+	+	+	+	+	+	-	-
11.	+	+	+	+	+	+	+	-	-
% of RI	100%	100%	100%	100%	100%	100%	100%	0	0

- In case of 100 % resistance was shown by both isolates collected from Palla and Agra till 1600µg/ ml after which no resistant colonies were reported at 3200µg/ml and 6400µg/ml in both of the cases. Lead acetate
- Hence Palla and Agra shares same percentage of resistance against Lead Acetate. Some of pictures of plates are given.

PALLA

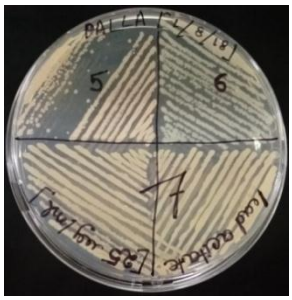


Figure 22

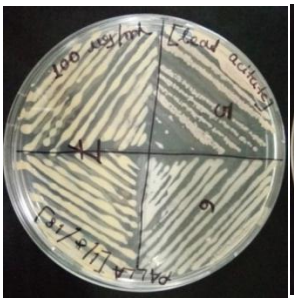


Figure 23

Figure 24

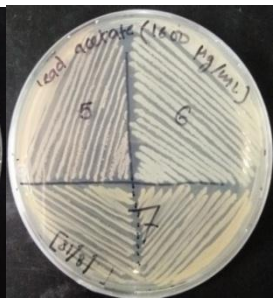


Figure 25

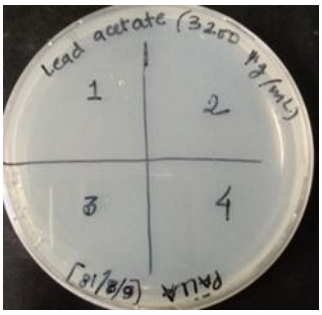


Figure 26

These figures showing resistant colonies are found at the starting conc. 25µg/ml up-to 1600µg/ml

AGRA

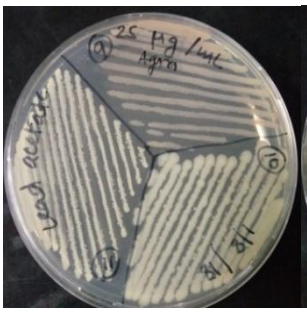


Figure 27

Figure 28

Figure 29

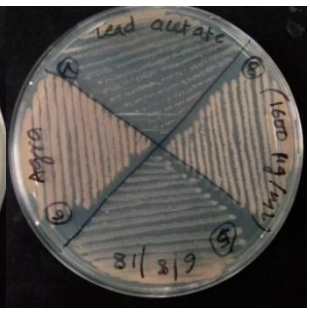


Figure 30

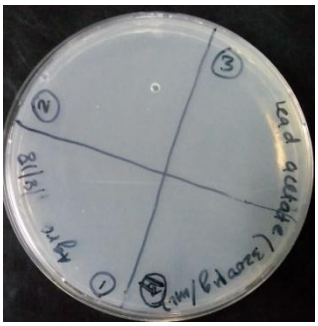


Figure 31

These figures showing resistant colonies are found at the starting conc. 25µg/ml up-to 1600µg/ml

- Next study was performed to check K_2CrO_4 resistant colony starting with concentration 25µg/ml and higher concentration 800µg/ml. Results are shown in tabulated form.

Table 7:*Showing the presence and absence of isolated colonies and percentage of resistance against Kr2CrO4.*

Isolate No	Showing growth of the isolate in medium containing heavy metal Kr_2CrO_4									
	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	1600 µg/ml	3200 µg/ml	6400 µg/ml	8000 µg/ml
Palla										
1.	+	+	+	+	+	+	+	+	+	+
2.	+	+	+	+	+	+	+	+	+	+
3.	+	+	+	+	+	+	+	+	+	+
4.	+	+	+	+	+	+	+	+	+	-
5.	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+
% of RI	100%	100%	100%	100%	100%	100%	100%	100%	100%	85.7%
Agra										
1.	+	+	+	+	+	+	+	+	+	-
2.	+	+	+	+	+	+	+	+	+	-
3.	+	+	+	+	+	+	+	+	+	+
4.	+	+	+	+	+	+	+	+	+	-
5.	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+
9.	+	+	+	+	+	+	+	+	+	+
10.	+	+	+	+	+	+	+	+	+	+
11.	+	+	+	+	+	+	+	+	+	+
% of RI	100%	100%	100%	100%	100%	100%	100%	100%	100%	72.7%

- In case of 100% resistance was observed till 6400µg/ml concentration after which 85.7% resistance was observed at Palla and 72.7% resistance was observed at Agra.
- Mores resistance at Palla is an indication to increasing pollution at this site. Some pictures are given.

PALLA

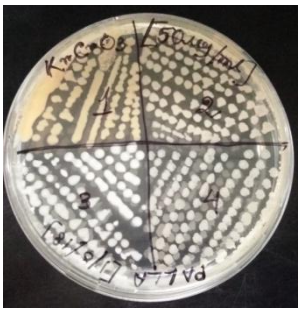


Figure 32

Figure 33

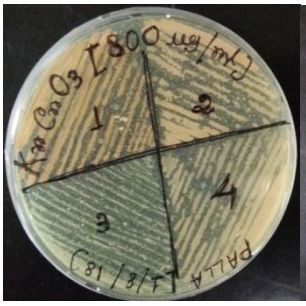


Figure 34

Figure 35

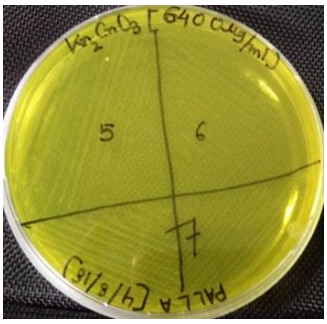


Figure 36

These figures showing resistant colonies are found in all plates at the starting conc. 25µg/ml up-to 6400µg/ml

AGRA

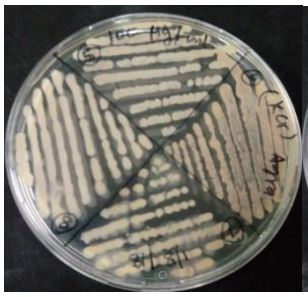


Figure 37

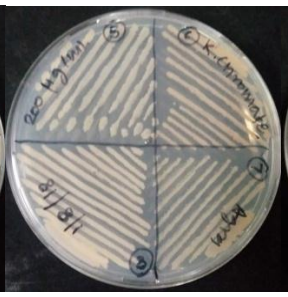


Figure 38

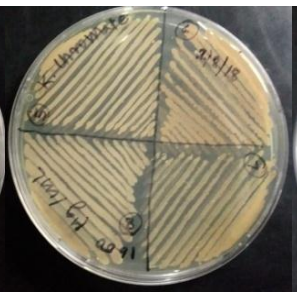


Figure 39

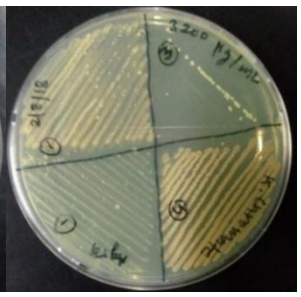


Figure 40

Figure 41

These figures showing resistant colonies are found in all plates at the starting conc. 25µg/ml up-to 6400µg/ml

5.6. Antibiotic susceptibility testing

Isolation No.	Diameter of Zone of In hibition (in mm)					
	Cefuroxime 30 µg	Ciprofloxacin 5 µg	Gentamicin 10 µg	Azaerythromycin 15 µg	Chloramphenicol 30 µg	Tetracycline 30 µg
PALLA						
1	None	3.5	8.2	7.6	5.5	6.0
2	None	7.0	7.2	7.4	11.0	5.0
3	None	18.0	15.0	6.0	11.6	22.6
4	None	13.6	19.3	20.0	12.6	11.6
5	None	11.6	10.0	None	15.0	8.3
6	8.1	None	14.3	None	17.6	None
7	None	16.6	14.6	4.8	17.3	13.6
AGRA						
1	4.0	19.0	14.0	11.0	15.0	11.0
2	3.6	13.6	13.3	6.3	16.0	13.0
3	None	14.0	13.0	15.3	16.7	10.3
4	10.0	23.0	16.0	4.0	20.0	23.0
5	10.5	14.0	13.0	8.0	9.0	13.0
6	None	21.3	16.3	8.6	15.0	14.0
7	None	11.0	13.3	15.3	6.0	10.5
8	12.0	23.3	18.3	25.3	None	23.0
9	4.0	13.3	14.3	7.6	17.0	14.0
10	None	19.0	14.0	5.0	None	11.6
11	None	21.0	17.0	6.0	2.0	17.0

Isolation No.	Diameter of Zone of Inhibition (in mm)		
	Ceftriazone 30µg	Ampicillin 10 µg	Clindamycine 2µg
PALLA			
1	12.0	None	10.3
2	None	None	9.6
3	9.3	None	19.6
4	None	None	13.6
5	8.6	4.0	22.6
6	18.3	None	21.6
7	5.6	None	14.0
AGRA			
1	6.3	7.6	22.7
2	6.5	None	14.3
3	4.6	1.0	17.3
4	7.5	26.0	16.0
5	7.0	28.0	24.0
6	8.3	None	24.0
7	7.8	None	17.6
8	13.6	None	18.0
9	7.3	None	21.0
10	7.0	None	None
11	5.5	None	15.3

Clinical and Laboratory Standards Institute's standardized data value:

Antimicrobial Agent	Disk Content	Interpretive Category and Zone Diameter Breakpoints (nearest whole mm)			Interpretive Category and MIC Breakpoints (µg/mL)		
		S	I*	R	S	I*	R
X	30 µg	≥20	15–19	≤14	≤4	8–16	≥32
Y	–	–	–	–	≤1	2	≥4
Z	10 µg	≥16	–	–	≤1	–	–

Interpretive Category	Breakpoints*	
	MIC (µg/mL)	Zone Diameter (mm)
Susceptible	≤4	≥20
Susceptible-Dose Dependent	8–16	15–19
Intermediate	8–16	15–19
Resistant	≥32	≤14
Nonsusceptible	>4	<20

*Formerly "interpretive criteria."

Table 1 *(Source: C.L.S.I.)* ***Table 2*** *(Source: C.L.S.I.)*

This two tables show the Minimum Inhibitory Concentration breakpoints for susceptible and resistant colony along with their zone diameter.

With comparing the data value with our antibiotic susceptibility testing table, Cefuroxime, Chloramphenicol, Tetracycline, Cetriaxome content 30µg, hence we use 50µl volume so; concentration in µg/ml is 600µg that means ≥32.

In our observation, For Cefuroxime all colonies of Palla except 6th and for Agra 3rd, 6th, 10th and 11th colonies have no zone of inhibition and remain colonies have zone diameter ≤14 that satisfied the table. So we may conclude the colonies of Palla and Agra are might be resistant against Cefuroxime.

For Chloramphenicol, 1st, 2nd, 3rd, 4th colonies of Palla and 5th, 7th and 11th colonies have zone diameter ≤14 and 8th, 10th colonies have no zone diameter. So these colonies are might be resistant against Chloramphenicol.

For Tetracycline, all colonies of Palla except 3rd and all colonies of Agra except 4th, 8th and 11th colonies are showing zone diameter ≤14.These colonies are might be against Tetracycline.

Following this way we may conclude the other antimicrobial resistance assay for Ceftriazone.

Antibiotics which content 10µg like Amphicilin and Gentamycin by using 50µl the concentration is 200µg/ml. That is not ≤1. In case of Gentamycin for Palla all colonies except 4th and for Agra all colonies against 4th 8th and 11th are might be resistant because they have zone diameter less than 16 that is not satisfied the table so might be these colonies are resistant. For Amphicilin almost all colonies for Palla and Agra doesn't show any zone of inhibition.

Some pictures of antibiotic resistance test

PALLA

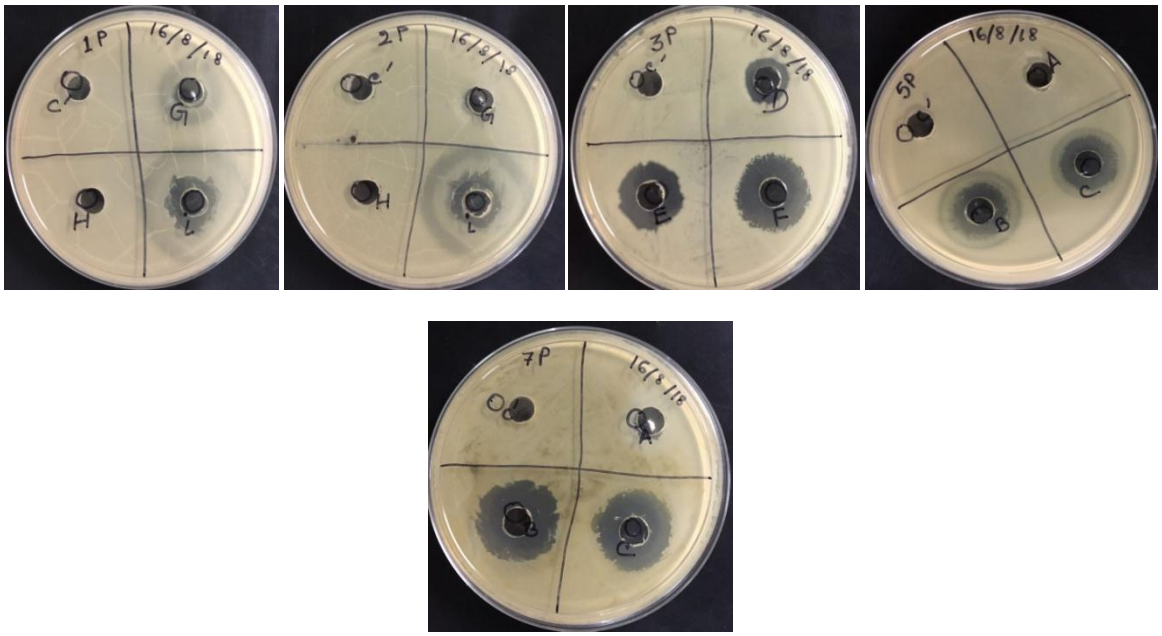


Figure 60: *All these figures showing the zone of inhibition and resistance against*

AGRA

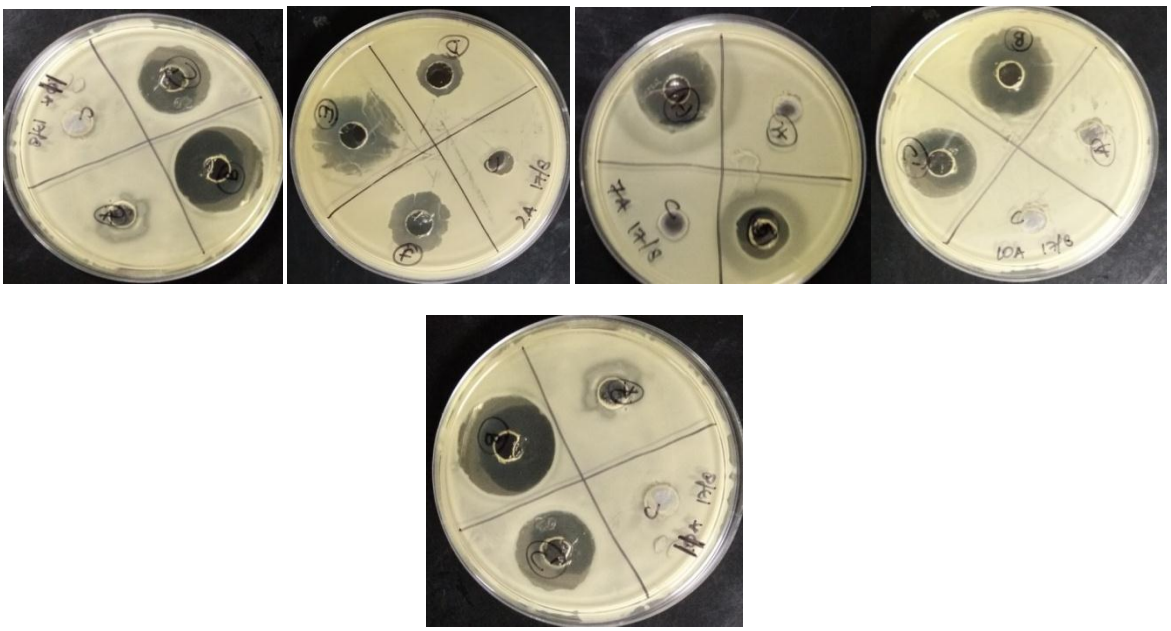


Figure 61: *All these figures showing the zone of inhibition and resistance against different antibiotics*

6. Conclusions

From all above this study we may conclude that both Palla and Agra shows multiple heavy metal and antibiotic resistance.

In case of heavy metal resistance study Agra shows more resistance than Palla but both shows almost same resistance in study of cadmium, chromate, lead acetate. Though Palla is upstream of Delhi but presence of many agricultural field and discharge of many temple and daily uses like bathing washing clothes may pollute this river, discharge from industries as it comes from Hathinikund Barrage, Haryana and Wazirabad barrage and because of local anthropogenic influence around the site from which samples were collected. Isolates collected from Agra also shows presence of heavy metal and antibiotic resistant isolates, as the sample were collected from Hathi-Ghat, Agra this site was used more for in stream purposes such as bathing, immersion of idols which adds to pollution load at this site.

In case of antibiotic resistance study, the bacterial colony showing resistance against the *Cefuroxime, Chloramphenicol, Tetracycline* and *Ceftriazone* antibiotics for both Agra and Palla samples. After that we try to isolate the plasmid DNA from these resistant bacteria, but the result of isolation not come, so we need to standardize this plasmid DNA isolation.

The reason for not able to isolate the plasmid DNA from the resistant bacterial cells may be handling problems or error in properly mixing of reagents to the cell to isolate the plasmid DNA.

7. Future prospects

- The plasmid DNA isolation is under standardization.
- After purification of isolated plasmid DNA perform Sanger's sequencing method and by using bioinformatics tool we can identify what type of bacteria is showing resistance.
- By using metagenomic approach we can extract the raw genomic DNA from water sediment sample and then this raw metagenomic DNA can be sequenced by the method of Next Generation Sequencing (NGS) to observe what are the bacteria diversity are present in that particular environment for each sample site.

8. References

1. Sharma RK, Agrawal M, Marshall FM: **Heavy metal (Cu, Zn, Cd and Pb) contamination of vegetables in urban India: A case study in Varanasi**. *Environmental pollution* 2008, **154**(2):254-263.
2. Singh M: **Heavy metal pollution in freshly deposited sediments of the Yamuna River (the Ganges River tributary): a case study from Delhi and Agra urban centres, India**. *Environmental Geology* 2001, **40**(6):664-671.

3. Kaushik A, Kansal A, Kumari S, Kaushik C: **Heavy metal contamination of river Yamuna, Haryana, India: assessment by metal enrichment factor of the sediments**. *Journal of hazardous materials* 2009, **164**(1):265-270.
4. Jain C: **Metal fractionation study on bed sediments of River Yamuna, India**. *Water Research* 2004, **38**(3):569-578.
5. Upadhyay R, Dasgupta N, Hasan A, Upadhyay S: **Managing water quality of River Yamuna in NCR Delhi**. *Physics and Chemistry of the Earth, Parts A/B/C* 2011, **36**(9-11):372-378.
6. Mandal P, Upadhyay R, Hasan A: **Seasonal and spatial variation of Yamuna River water quality in Delhi, India**. *Environmental monitoring and assessment* 2010, **170**(1-4):661-670.
7. Malik D, Singh S, Thakur J, Singh RK, Kaur A, Nijhawan S: **Heavy metal pollution of the Yamuna River: An introspection**. *Int J Curr Microbiol App Sci* 2014, **3**(10):856-863.
8. Ahmad A, Rafatullah M, Sulaiman O, Ibrahim MH, Chii YY, Siddique BM: **Removal of Cu (II) and Pb (II) ions from aqueous solutions by adsorption on sawdust of Meranti wood**. *Desalination* 2009, **247**(1-3):636-646.
9. Kaur S, Mehra P: **Assessment of heavy metals in summer & winter seasons in River Yamuna segment flowing through Delhi, India**. *Journal of Environment and Ecology* 2012, **3**(1):149-165.
10. Mishra S, Dwivedi SP, Singh R: **A review on epigenetic effect of heavy metal carcinogens on human health**. *Open Nutraceuticals J* 2010, **3**:188-193.
11. Sehgal M, Garg A, Suresh R, Dagar P: **Heavy metal contamination in the Delhi segment of Yamuna basin**. *Environmental monitoring and assessment* 2012, **184**(2):1181-1196.
12. Islam MS, Ahmed MK, Habibullah-Al-Mamun M, Hoque MF: **Preliminary assessment of heavy metal contamination in surface sediments from a river in Bangladesh**. *Environmental Earth Sciences* 2015, **73**(4):1837-1848.
13. Vijayaraghavan K, Yun Y-S: **Bacterial biosorbents and biosorption**. *Biotechnology advances* 2008, **26**(3):266-291.
14. Ansari MI, Malik A: **Biosorption of nickel and cadmium by metal resistant bacterial isolates from agricultural soil irrigated with industrial wastewater**. *Bioresource technology* 2007, **98**(16):3149-3153.
15. Abyar H, Safahieh A, Zolgharnein H, Zamani I: **Isolation and identification of Achromobacter denitrificans and evaluation of its capacity in cadmium removal**. *Pol J Environ Stud* 2012, **21**(6):1523-1527.
16. Jankowska EA, Biel B, Majda J, Szklarska A, Lopuszanska M, Medras M, Anker SD, Banasiak W, Poole-Wilson PA, Ponikowski P: **Anabolic deficiency in men with chronic heart failure: prevalence and detrimental impact on survival**. *Circulation* 2006, **114**(17):1829-1837.
17. Wong C, Li X, Zhang G, Qi S, Peng X: **Atmospheric deposition of heavy metals in the Pearl River Delta, China**. *Atmospheric Environment* 2003, **37**(6):767-776.
18. Mejáre M, Bülow L: **Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals**. *TRENDS in Biotechnology* 2001, **19**(2):67-73.
19. Nies DH: **Efflux-mediated heavy metal resistance in prokaryotes**. *FEMS microbiology reviews* 2003, **27**(2-3):313-339.
20. Piddock LJ: **Multidrug-resistance efflux pumps - not just for resistance**. *Nat Rev Microbiol* 2006, **4**(8):629-636.

21. Ravindra K, Kaushik A: **Seasonal variations in physico-chemical characteristics of River Yamuna in Haryana and its ecological best-designated use**. *Journal of Environmental Monitoring* 2003, **5**(3):419-426.
22. Nema A: **JAPANESE ASSISTANCE FOR RIVER POLLUTION CONTROL-A CASE STUDY OF YAMUNA ACTION PLAN, INDIA**. In.: Citeseer; 2007.
23. Maheshwari A, Sharma M, Sharma D: **Hydro chemical analysis of surface and ground water quality of Yamuna river at Agra, India**. *J Mater Environ Sci* 2011, **2**(4):373-378.
24. Chopin E, Alloway B: **Distribution and mobility of trace elements in soils and vegetation around the mining and smelting areas of Tharsis, Riotinto and Huelva, Iberian Pyrite Belt, SW Spain**. *Water, Air, and Soil Pollution* 2007, **182**(1-4):245-261.
25. Garcia R, Millan E: **Assessment of Cd, Pb and Zn contamination in roadside soils and grasses from Gipuzkoa (Spain)**. *Chemosphere* 1998, **37**(8):1615-1625.
26. Kampa M, Castanas E: **Human health effects of air pollution**. *Environmental pollution* 2008, **151**(2):362-367.
27. Li X, Poon C-s, Liu PS: **Heavy metal contamination of urban soils and street dusts in Hong Kong**. *Applied geochemistry* 2001, **16**(11-12):1361-1368.
28. Liao G-l, Liao D-x, Li Q-m: **Heavy metals contamination characteristics in soil of different mining activity zones**. *Transactions of Nonferrous Metals Society of China* 2008, **18**(1):207-211.
29. Nabulo G, Oryem-Origa H, Diamond M: **Assessment of lead, cadmium, and zinc contamination of roadside soils, surface films, and vegetables in Kampala City, Uganda**. *Environmental Research* 2006, **101**(1):42-52.
30. Sezgin N, Ozcan HK, Demir G, Nemlioglu S, Bayat C: **Determination of heavy metal concentrations in street dusts in Istanbul E-5 highway**. *Environment international* 2004, **29**(7):979-985.
31. Stihi C, Bancuta A, Popescu I, Virgolici M, Cimpoca V, Gugiu M, Vlaicu G: **Air pollution studies using PIXE and ICP Methods**. In: *Journal of Physics: Conference Series: 2006*. IOP Publishing: 565.
32. Suciu I, Cosma C, Todică M, Bolboacă SD, Jäntschi L: **Analysis of soil heavy metal pollution and pattern in Central Transylvania**. *International journal of molecular sciences* 2008, **9**(4):434-453.
33. Viard B, Pihan F, Promeyrat S, Pihan J-C: **Integrated assessment of heavy metal (Pb, Zn, Cd) highway pollution: bioaccumulation in soil, Graminaceae and land snails**. *Chemosphere* 2004, **55**(10):1349-1359.
34. Muchuweti M, Birkett J, Chinyanga E, Zvauya R, Scrimshaw MD, Lester J: **Heavy metal content of vegetables irrigated with mixtures of wastewater and sewage sludge in Zimbabwe: implications for human health**. *Agriculture, Ecosystems & Environment* 2006, **112**(1):41-48.
35. Khan S, Cao Q, Zheng Y, Huang Y, Zhu Y: **Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China**. *Environmental pollution* 2008, **152**(3):686-692.
36. Rattan R, Datta S, Chhonkar P, Suribabu K, Singh A: **Long-term impact of irrigation with sewage effluents on heavy metal content in soils, crops and groundwater—a case study**. *Agriculture, Ecosystems & Environment* 2005, **109**(3-4):310-322.
37. Tiller KG: **Essential and toxic heavy metals in soils and their ecological relevance**. In: *band• volume I Congress Centrum Hamburg 13-208 1986: 1986*.

38. Zhuang P, McBride MB, Xia H, Li N, Li Z: **Health risk from heavy metals via consumption of food crops in the vicinity of Dabaoshan mine, South China**. *Science of the total environment* 2009, **407**(5):1551-1561.
39. Al-Khashman OA, Shawabkeh RA: **Metals distribution in soils around the cement factory in southern Jordan**. *Environmental pollution* 2006, **140**(3):387-394.
40. Tiller K: **Urban soil contamination in Australia**. *Soil Research* 1992, **30**(6):937-957.
41. Rao KS, Anand S, Venkateswarlu P: **Adsorption of cadmium (II) ions from aqueous solution by Tectona grandis LF (teak leaves powder)**. *BioResources* 2010, **5**(1):438-454.
42. Miller CV, Foster GD, Majedi BF: **Baseflow and stormflow metal fluxes from two small agricultural catchments in the Coastal Plain of the Chesapeake Bay Basin, United States**. *Applied Geochemistry* 2003, **18**(4):483-501.
43. Harikumar P, Nasir U, Rahman MM: **Distribution of heavy metals in the core sediments of a tropical wetland system**. *International Journal of Environmental Science & Technology* 2009, **6**(2):225-232.
44. Wen X, Allen HE: **Mobilization of heavy metals from Le An River sediment**. *Science of the Total Environment* 1999, **227**(2-3):101-108.
45. Huang K-M, Lin S: **Consequences and implication of heavy metal spatial variations in sediments of the Keelung River drainage basin, Taiwan**. *Chemosphere* 2003, **53**(9):1113-1121.
46. Van Griethuysen C, Van Baren J, Peeters ET, Koelmans AA: **Trace metal availability and effects on benthic community structure in floodplain lakes**. *Environmental Toxicology and Chemistry: An International Journal* 2004, **23**(3):668-681.
47. Filali B, Taoufik J, Zeroual Y, Dzairi F, Talbi M, Blaghen M: **Waste water bacterial isolates resistant to heavy metals and antibiotics**. *Current microbiology* 2000, **41**(3):151-156.
48. Alboghobeish H, Tahmourespour A, Doudi M: **The study of Nickel Resistant Bacteria (NiRB) isolated from wastewaters polluted with different industrial sources**. *Journal of Environmental Health Science and Engineering* 2014, **12**(1):44.
49. Karakagh RM, Chorom M, Motamedi H, Kalkhajeh YK, Oustan S: **Biosorption of Cd and Ni by inactivated bacteria isolated from agricultural soil treated with sewage sludge**. *Ecohydrology & Hydrobiology* 2012, **12**(3):191-198.
50. Piddock LJ: **Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria**. *Clinical microbiology reviews* 2006, **19**(2):382-402.