Isolation, characterization, identification and antimicrobial susceptibility testing of bacteria associated with pharmaceutical waste water treatment

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE MS DEGREE IN BIOTECHNOLOGY

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DECLARATION

I, Shah Sharfin, hereby declare that the thesis project titled “Isolation, characterization, identification and antimicrobial susceptibility testing of bacteria associated with pharmaceutical waste water treatment” submitted by me has been carried out under the supervision of **Dr. M. Mahboob Hossain** , Associate Professor, Microbiology program, Department of Mathematics and Natural Sciences and **Md. Hasanuzzaman**, Senior Manager, Quality Control, Quality Assurance Department, Incepta Pharmaceuticals Ltd. The contents of this thesis have not been submitted elsewhere for publication or award of any degree. It is also to be declared that the research work presented here is based on actual and original work carried out by me. Information sources or reference to research works performed by other people or institution have been duly cited and referenced.

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MY BELOVED CHILD SAMIN SARWAT

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ABSTRACT

The pharmaceutical industries in Bangladesh are one of the most developed hi-tech sectors within the country's economy. The rapid development of pharmaceutical industries resulted in the production of huge amounts of pharmaceutical effluents. These wastes contain several types of toxins including heavy metals and pharmaceutical compounds, which have high toxicity for human and environment. The traditional technologies are not effective to remove both antibiotic compounds and heavy metals. But combination of both chemical & biological or biochemical treatment is the most economical and environment friendly method of treating effluents. Therefore, this study is aimed to evaluate the efficiency of 'Effluent Treatment Plant (ETP)' operated in a multi-product facilities and designed to treat effluents both by chemical & biological methods, to evaluate the physico-chemical parameters and bioburden (microbial load) of the raw water as well as treated water, to isolate and identify some bacteria associated with the pharmaceutical waste water treatment; and to assess antibiotic sensitivity pattern of the isolated bacteria. The physico-chemical study [like pH, Total Suspended Solid (TSS), Total Dissolved Solid (TDS), Total Solid (TS), Dissolved Oxygen, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), Oil & Grease and bioburden] of the raw water as well as treated water and microbiological study [type of bacteria associated with waste water treatment and their sensitivity & resistance to specific antibiotics] were conducted as per the approved procedure of Incepta Pharmaceuticals Ltd. The results revealed that all physico-chemical parameters of raw water as well as treated water were within the prescribed limit as per the Department of Environment (DOE) of Bangladesh and there was no significant variation during the entire operation period. It was also shown that, microbial load was satisfactory, which indicates that the organism present in the ETP can tolerate the stress of the effluents. In this study, out of 30 isolates, 7 different bacteria could be identified viz. *Micrococcus luteus, Bacillus subtilis, Stenotrophomonas maltophilia, Empedobacter brevis, Tatumella ptyseos, Klebsiella pneumonia,* and *Streptococcus sanguinis*. The antibiotic sensitivity pattern of the identified bacteria were also performed and found that, some of the isolates were resistant to all the 5 antibiotics used. It can be concluded that the functionality of the biological part of the ETP is good enough and it indicates the organisms present in the biological part of the ETP has the involvement in the pharmaceutical waste water treatment.

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1.1 Importance of this Research:

The pharmaceutical industry in Bangladesh is one of the most developed hi-tech sectors within the country's economy. At present there are 265 drug manufacturing companies, which meet at least 97% of local requirements and export a wide range of pharmaceutical products covering all major therapeutic classes and dosage forms to 79 countries. Beside regular forms like; Tablets, Capsules & Syrups, Bangladesh is also exporting high-tech specialized products like HFA Inhalers, CFC inhalers, suppositories, nasal sprays, injectables, IV infusions, etc. To meet the local and international demand most of the pharmaceutical companies are busy for manufacturing of different types of medicines including antibiotics, biological, hormones and other potent drugs. In various stages of drug manufacturing the pharmaceutical companies produce a significant amount of wastes, for example, rejected/expired raw & packaging materials, spills of raw materials, waste generate during manufacturing/ processing, rejected/expired/damaged intermediate and finished drug product etc., hazardous waste (e.g. solid, liquid, acid-base waste, HPLC waste, organic & inorganic waste, Karl Fisher Waste etc.), biological waste (e.g. live bacteria/ fungus, culture media, contaminated materials.) and others. Effluent discharged from pharmaceutical companies can be classified based on the type of pharmaceutical compounds such as antibiotics, prescription and non-prescription pharmaceuticals present in it (Roth and Etho, 2005).

Pharmaceuticals pose potential risks to the aquatic environment such as endocrine disrupting and side effects since they initially cause specific biological effects [Kasprzyk et al., 2008 and Sim et al., 2010]. Furthermore, waste waters produced from antibiotic manufacture and formulation, generally contain high levels of soluble organics, many of which are recalcitrant [Schroder et al., 1999]. Before discharging the above mentioned wastes, it is the regulatory requirement to treat the effluents to leave in the environment as a safe level because the wastes have a great impact on the environment. Most of the pharmaceutical companies treat their effluents by conventional chemical effluent treatment method.

An effluent treatment plant (ETP) plays important roles in maintaining surface water quality and the environment as a whole as well. Fatta et al., 2005 suggested that treated effluent can be safely discharged into streams, rivers, cannel, bay, lagoon or wetland, or it can be reused for, irrigation of a golf course, landscaping, gardening, or groundwater recharge (Fatta et al., 2005). But combination of both chemical & biological or biochemical treatment, unlike other forms, is the most economical and environment friendly method of treating effluents. Pharmaceuticals are complex molecules and are most notably characterized by their ionic nature. Compounds having a complex chemical structure such as the pharmaceuticals ketoprofen and naproxen were not eliminated during conventional treatment plants (CTP) but were by membrane bioreactors (MBR) (Kimura et al., 2005). Pharmaceutical waste water contains substantial amounts of certain toxic compounds, antibiotics, steroids and xeno estrogens. A number of reports were found on pharmaceutical waste water treatment using biological process (La Para et al., 2001: Carballa et al., 2004, 2005: Clara et al., 2005: Huber et al., 2005: Joss et al., 2005).The method is based on the neutralization of antibiotics (containing β lactum ring), and other drug wastes by using chemical treatment and after that biochemical oxidation of organic and inorganic substances due to the activity of microorganisms using the impurities such as a nitrite substrate and forming harmless oxidation products; water, CO_2, NO_3^- and SO_4^{2-} ions and also biochemical matter. The degree of decomposition of organic compounds in the biochemical treatment is characterized by the ratio BOD/COD. The greater the biological oxidation of a given waste, the higher is this ratio (Nsi, 2007). Efficiency of waste water treatment plants and kinetics characteristics of biological process are usually studied through BOD and COD, although difficulties are encountered in their measurement (Aziz et al., 1980). The greater the biological oxidation of a given waste, the higher is this ratio. The biological waste water treatment facilities mainly depend on various conditions such as waste water composition, ecotoxicants and xenobiotics. Pretreatment of industrial waste water before biological treatment can be helpful for operating the biological treatment process. In pretreatment stage, coarse and colloidal impurities are removed from the waste water (Veitser et al., 1984: Zapol et al., 1987: Gandurina, 2002). The quantification of water pollution was restricted for monitoring biochemical oxygen demand (BOD), chemical oxygen demand (COD), nitrates, phosphates and total suspended solids (Metcalf and Eddy, 2003: ENISO9887, 1994). The biological technology allows transforming the organic and inorganic contaminants into gases and digested sludge. In biochemical treatment of effluents, microorganisms play a vital role and bacteria constitute the major parts of organisms.

A very wide range of bacteria has been reported, recorded, but the dominant aerobic genera appear to be gram-ve rods; *Pseudomonas*, achromobacter, alcaligens, *Flavobacterium* etc. Fungi are normally outnumbered 8:1 by bacteria. Autotrophic bacteria tend to be more predominant in the lower layers of the biofilms with Nitrosomonas oxidizing nitrite to nitrate (Christopher, 2002). Protozoa, according to Lester (1996) and Algae, according to Laliberte et al., (1994), have also been implicated in the biological treatment of effluent from pharmaceutical companies. Aerobic treatment of pharmaceutical waste water is helpful to decrease of COD and increase of TSS which are vital parts for waste water treatment process (Zakir et. al., 2011).

1.2 Objectives of the Present Study

This study is aimed -

- To evaluate the efficiency of „Effluent Treatment Plant (ETP)" operated in a multi-product facility and designed to treat effluents both chemical & biological method.
    - To evaluate the physico-chemical parameters of the raw water as well as treated water.
    - To evaluate the bioburden (microbial load) of the raw water as well as treated water.
- To isolate and identify some bacteria associated with the pharmaceutical waste water treatment; and
- To assess anti-biogram (antibiotic sensitivity test) of the isolated bacteria.

In this study, the following parameters of the raw water (waste water) as well as treated water of the ETP will be checked as per the approved policy and procedure of Incepta Pharmaceuticals Ltd and 03 months data will be generated.

- pH, Total Suspended Solid (TSS), Total Dissolved Solid (TDS), Total Solid (TS), Dissolved Oxygen, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), and Oil & Grease etc.

Along with physico-chemical study, microbiological study will also be conducted regarding bioburden study and characterization of microorganisms.

The study will be concluded after completion of intended tests and identifying the type of bacteria associated with Waste Water Treatment in multi-dosage forms pharmaceutical facility and their sensitivity & resistance to specific antibiotic.

1.3 Research Hypothesis

Research has been carried out depending on the following hypothesis:

- There has been some bacteria which are associated with pharmaceutical waste water treatment
- Some of the bacteria present in the ETP may be antibiotic resistant.

1.4 Scope and Limitation of the Study

Scope of the study:

The study has been carried out in ETP of a multi-dosage forms pharmaceutical facility (Incepta Pharmaceuticals Ltd) located at Zirabo, Savar, Dhaka. The study has been carried out between July to September in the Microbiology laboratory of the Incepta Pharmaceuticals Ltd.

The present study has following limitations:

1. Few numbers of isolates have been isolated.
2. Session variation has not been considered.
3. The result is not compared with the others ETP present in this area.
4. Only 05 commonly used antibiotics have been considered for antibiotic resistance test.

CHAPTER 2:
SYSTEM DESCRIPTION AND PHARMACEUTICAL WATER QUALITY

CHAPTER 2: SYSTEM DESCRIPTION AND PHARMACEUTICAL WATER QUALITY

2.1 Effluent Treatment Plants (ETP)

Waste water treatment is an important initiative for the betterment of the society and our future. Waste water treatment is a process, wherein the contaminants are removed from waste water as well as household sewage, to produce waste stream or solid waste suitable for discharge or reuse. Waste water treatment methods are categorized into three sub-divisions, physical, chemical and biological. Effluent Treatment Plants or (ETPs) are used by leading companies in the pharmaceutical and chemical industry to purify water and remove any toxic and non toxic materials or chemicals from it.

2.1.1 Purpose of each treatment unit of ETP

An ETP consists of different important parts. These are –

- **Screen chamber:** To screen solid course materials (size>10mm) from the effluent coming from production areas.
- **Collection tank:** To hold the screened effluent water.
- **Equalization tank:** To collect the waste, allowing it to mix and ensuring that it becomes less variable in composition before it is pumped to the treatment units at a constant rate. Chemicals are added in this unit for adjusting pH and also for carrying out Fenton"s reaction.
- **Neutralization tank:** To adjust the pH of effluent water to get better performance in the next processes also to reduce the consumption of chemicals in flocculation process.
- **Flush mixer:** To add flocculant in the waste water.
- **Flocculation tank:** In this process small particles or small groups of particles forms large aggregates. Flocculation during waste water treatment converts finely divided suspended solids into larger particles so that efficient, rapid settling can occur.
- **Primary clarifier:** To remove the flocculated solids and any remaining suspended solids.
- **Moving Bed Biological Reactor (MBBR):** A biological treatment processes using microorganisms breaks down the organic waste.
- **Secondary clarifier:** To remove the flocculated solids and any remaining suspended solids.

- **Filtration unit:** To remove fine suspended impurities or particles from water.
- **Pump water tank:** To pump water from filtration unit to MGF & ACF for further filtration process.
- **MGF:** To remove fine suspended impurities or particles from water.
- **ACF:** To remove organic compounds and chlorine from water.
- **Clear water tank:** To reserve clear/treated water.
- **Treated water tank:** To reserve the final treated water which is later used for gardening purpose.

2.1.2 Chemical dosing

- NaOH is added to keep the pH in acceptable range.
- Alum is added as flocculant.
- Polyelectrolyte is added to increase floc size.

2.1.3 Preparation of Dosing Chemicals

- **NaOH Dosing Preparation**
    - The empty tank is cleaned with fresh water. Then the tank is filled with 200L fresh water up to the overflow level. Then, Dosing Mixer-1 is turned on and finally 12.5 Kg of NaOH is added into the tank.
- **Alum Dosing Preparation**
    - The empty tank is cleaned with fresh water. Then the tank is filled with 200L fresh water up to the overflow level. Then, Dosing Mixer-2 is turned on and finally 40 kg of Alum is added into the tank.
- **Polyelectrolyte Dosing Preparation**
    - The empty tank is cleaned with fresh water. Then the tank is filled with 100L fresh water up to the overflow level. Then, Dosing Mixer-3 is turned on and finally 100 gm of polyelectrolyte is added into the tank.

N.B.: Depending on the effluent water and treated water parameter, dosing system might change.

2.1.4 Operation of the Plant

2.1.4.1 Raw Effluent Tank and Oil and Grease Trap Tank:

The effluent from the different production area is come into the raw effluent tank. Then it goes to the screen chamber and then oil and grease trap tank.

2.1.4.2 Equalization Tank

The de-contaminated waste from the "Oil and Grease Trap" tank is come to the Equalization Tank by using Collection Pump. After that, the pH of the raw water is to determine by using a litmus paper. According to this, specified quantity of NaOH is to add in the "Equalization tank-1" to raise pH to 10-11. Then, the equalization tank is to keep agitated according to the level through air mixing grid. After 24 hours sample water is collected from the equalization tank-1 to check the pH and find out any addition of NaOH is required or not. Then alum is added till pH is within 7-8.

2.1.4.3 Flash Mixer Cum Flocculator

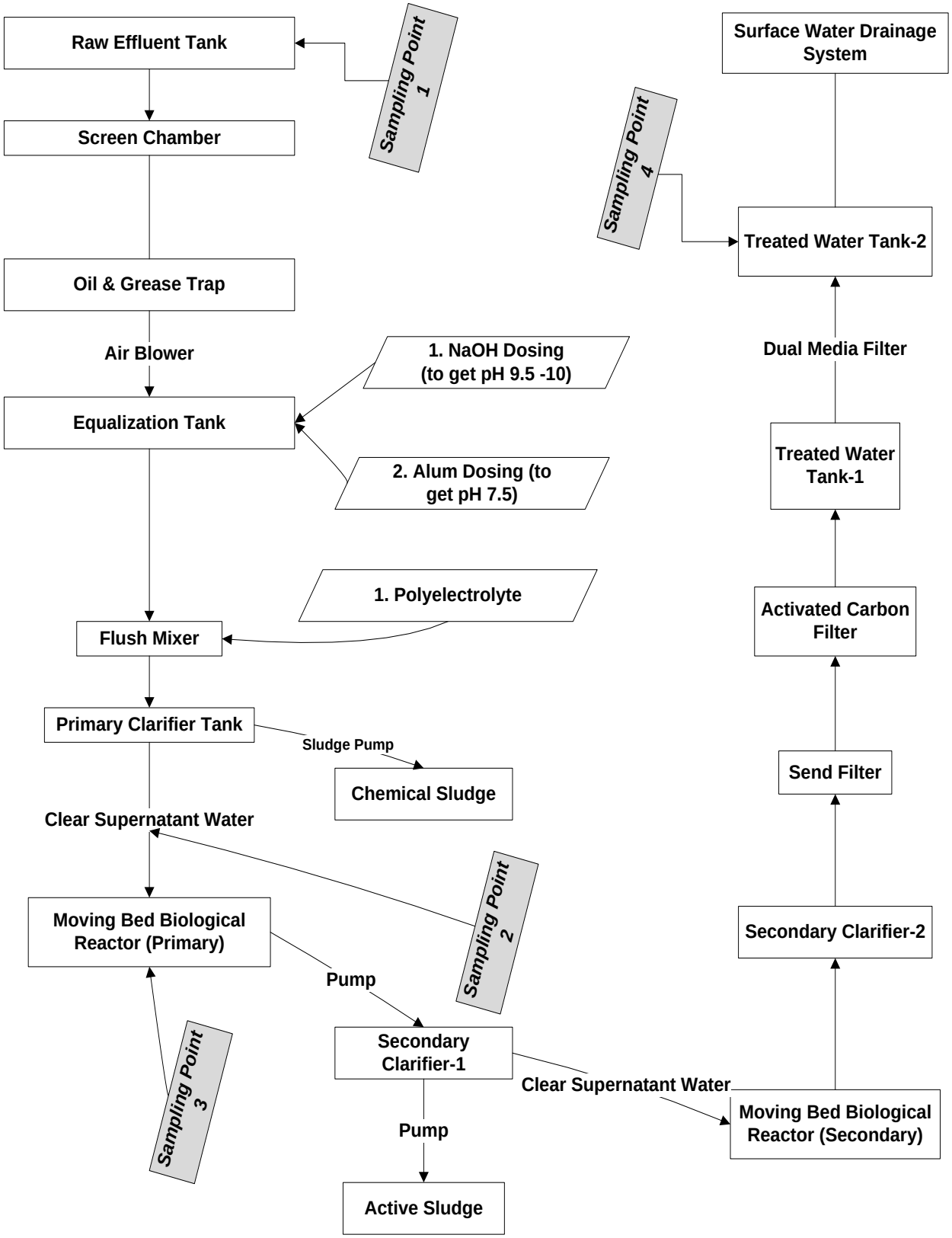
The neutral effluent is received through the equalization tank pump in the flash mixer chamber. The agitator is run (provided in the flocculator) for mixing of neutralizing chemicals. For better coagulation and flocculation alum/PAC is dosed through dosing pump. The flocculated effluent then enters in the "Primary Clarifier" feed well.

2.1.4.4 Primary Clarifier

Chemical sludge and clear water gets separated out through primary clarifier. The overflow clear, chemically treated effluent from primary clarifier goes to the MBBR-1 for double stage biological treatment. The sample from overflow is collected to check the pH which indicates the performance across the chemical treatment.

2.1.4.5 MBBR-1 (Moving Bed Biological Reactor)

Here, the air blower A/B is run for aeration to the MBBR-1. For the proper growth of aerobic bacteria approximately 2-3 kg of jiggery, 0.5kg of DAP and 1kg urea is added per day in the MBBR-1 reactor. After collecting sample of water from MBBR-1 and MLSS of the sample is measured. During routine operation MLSS should be 15-20% or 1500-2000 mg/L. If the growth of aerobic bacteria is more, then the jiggery is slowly reduced. Here, the colour of MLSS should be brown and there will be no smell. If it gets black, the blower should be run for some additional hours.



Block Diagram of ETP

2.1.4.6 Secondary Clarifier-1

From MBBR-1 outlet goes to the “Secondary clarifier-1”. It separates solid and liquid. The sludge recycle pump-A/B is run here for 10 minutes every hour to recycle biological sludge which is settled at the bottom of “Secondary Clarifier-1”. The excess sludge to the sludge tank is de-sludged for safe disposal. The overflow water is collected for testing of pH, COD, TDS, and TSS to verify the performance of the MBBR-1.

2.1.4.7 MBBR-2

The same procedure is followed as MBBR-1. There should be no MLSS or very small amount of MLSS. If inlet COD/BOD is treated up to 1st stage of biological then it raises the DO (dissolved oxygen) in the treated water. Under this condition, any other consumable like Jiggery/DAP/Urea does not added. Continuous operation of sludge recycle pump is also not required.

2.1.4.8 Secondary Clarifier-2

It is similar to the “Secondary Clarifier-1”. The same procedure is follow for the “Secondary Clarifier-2”. pH from overflow sample water is checked.

2.1.4.9 Pressurized Sand Filter (PSF)

Once the PSF sump is having sufficient quantity of liquid, the PSF feed Pump-A/B is started to pump the bio treated water to PSF. The biological fine suspended is trapped in this filter. The sand filter should be flushed after every filtration.

2.1.4.10 Activated Carbon Filter (ACF)

The outlet water from PSF goes to ACF inlet. ACF removes fine TSS from bio treated water and some odor and residual COD. The outlet water from PCF is the final treated water. The activated carbon filter should be flushed after every filtration.

2.2 Water Quality:

The term water quality is used to describe the condition of the water, including its chemical, physical and biological characteristics, usually with respect to its suitability for a particular purpose. Water quality is also affected by substances like pesticides or fertilizers that can negatively affect marine life when present in certain concentrations. The following factors are mainly used to provide a measure of water quality: pH, Concentration of dissolved oxygen (DO), Biochemical oxygen demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solid (TDS), Total Suspended Solids (TSS), and Concentration of Oil & Grease.

2.2.1 Operating Parameters:

- **pH :**

pH is a measure of the acidic or basic (alkaline) nature of a solution. The concentration of the hydrogen ion [H+] activity in a solution determines the pH. Mathematically this is expressed as: pH = - log [H+]

The pH value is the negative power to which 10 must be raised to equal the hydrogen ion concentration.

Environmental Impact:

A pH range of 6.0 to 9.0 appears to provide protection for the life of freshwater fish and bottom dwelling invertebrates. The pH of the water will determine the toxic effects, if any, of these substances.

Table – 1: Limiting pH values

pH Range		Effects
3.8	10.0	Fish eggs could be hatched, but deformed young are often produced
4.0	10.1	Limits for the most resistant fish species
---	4.3	Carp die in five days
---	5.0	Limits for stickleback fish
5.0	9.0	Tolerable range for most fish
---	8.7	Upper limit for good fishing waters
5.4	11.4	Fish avoid waters beyond these limits
6.0	7.2	Optimum (best) range for fish eggs
---	1.0	Mosquito larvae are destroyed at this pH value
3.3	4.7	Mosquito larvae live within this range
7.5	8.4	Best range for the growth of algae

- **Dissolved Oxygen(DO)**

Dissolved oxygen analysis measures the amount of gaseous oxygen (O_2) dissolved in an aqueous solution. The oxygen dissolves by diffusion from the surrounding air; aeration of water that has tumbled over falls and rapids; and as a waste product of photosynthesis. A simplified formula is given below:

Photosynthesis (in the presence of light and chlorophyll):

Carbon dioxide + Water ----------> Oxygen + Carbon-rich foods

CO_2 H_2O O_2 $C_6H_{12}O_6$

Fish and aquatic animals cannot split oxygen from water (H2O) or other oxygen-containing compounds. Only green plants and some bacteria can do that through photosynthesis and similar processes. Total dissolved gas concentrations in water should not exceed 110 percent. Concentrations above this level can be harmful to aquatic life.

Natural stream purification processes require adequate oxygen levels in order to provide for aerobic life forms. As dissolved oxygen levels in water drop below 5.0 mg/l, aquatic life is put under stress. The lower the concentration, the greater the stress. Oxygen levels that remain below 1-2 mg/l for a few hours can result in large fish kills.

- **Biological Oxygen Demand (BOD)**

Biochemical Oxygen Demand (BOD) refers to the amount of oxygen that would be consumed if all the organics in one liter of water were oxidized by bacteria and protozoa.

Microorganisms such as bacteria are responsible for decomposing organic waste. When organic matter such as dead plants, leaves, grass clippings, manure, sewage, or even food waste is present in a water supply, the bacteria will begin the process of breaking down this waste. When this happens, much of the available dissolved oxygen is consumed by aerobic bacteria, robbing other aquatic organisms of the oxygen they need to live.

Biological Oxygen Demand (BOD) is a measure of the oxygen used by microorganisms to decompose this waste. If there is a large quantity of organic waste in the water supply, there will also be a lot of bacteria present working to decompose this waste. In this case, the demand for oxygen will be high (due to all the bacteria) so the BOD level will be high. As the waste is consumed or dispersed through the water, BOD levels will begin to decline.

- **Chemical Oxygen Demand (COD)**

  The chemical oxygen demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water. Most applications of COD determine the amount of organic pollutants found in surface water (e.g. lakes and rivers) or waste water, making COD a useful measure of water quality. It is expressed in milligrams per liter (mg/L) also referred to as ppm (parts per million), which indicates the mass of oxygen consumed per liter of solution.

  The COD value has been developed analogically to the BOD measurement. Since there are many organics which are rather hard or not possible to decompose biologically, a parameter has been defined indicating the amount of oxygen which would be needed when all organic ingredients would be oxidised completely.

- **Total Dissolved Solids (TDS)**

  A total dissolved solid (TDS) is a measure of the combined content of all inorganic and organic substances contained in a liquid in molecular, ionized or micro-granular (colloidal sol) suspended form. Generally the operational definition is that the solids must be small enough to survive filtration through a filter with two-micrometer (nominal size, or smaller) pores.

  Water can be classified by the amount of TDS per liter:

  - Fresh water < 1000 mg/L TDS
  - Brackish water 1000 to 10,000 mg/L TDS
  - Saline water 10,000 to 30,000 mg/L TDS
  - Brine > 30,000 mg/L TDS

- **Total Suspended Solids (TSS)**

  Total suspended solids is a water quality measurement.

- **Oil & Grease**

  Oil & Grease is present in the effluent in the unit of mg/l unit. The higher concentration could be the problem for aerobic microbes.

2.2.2 Water Quality Parameters:

According to the Department of Environment (DOE) of Bangladesh the water quality parameters are considered as per Table – 2.

Table – 2: Water quality parameters

Sl. No.	Water quality parameter	Unit	Desired range according to ECR 1997		
			Discharge in inland water	Discharge into public sewer	Discharge on irrigated land
1	pH	------	6 to 9	6 to 9	6 to 9
2	DO	mg/L	4.5 to 8	4.5 to 8	4.5 to 8
3	BOD	mg/ L	≤50	≤250	≤100
4	COD	mg/ L	≤200	≤400	≤400
5	TDS	mg/ L	≤2100	≤2100	≤2100
6	TSS	mg/ L	≤150	≤500	≤200
7	Oil & Grease	mg/ L	≤10	≤20	≤10

*ECR = Environmental Conservation Rules

CHAPTER 3:
MATERIALS AND METHODS

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3.1 Sampling Area and Sample Collection

Sampling of effluent (pre and post treatment) is an important step to ensure quality of any kind waste disposal and further stage of environmental safety. Sampling must be in a proper way from the exact positions.

3.1.1 Sampling Positions

- **Sample Positions for Physico-Chemical Tests**
    - Effluent before treatment from oil & grease trap (Raw).
    - Effluent after treatment from treated tank (Treated)
- **Sample Positions for Microbiological Tests**
    - Effluent before treatment from oil & grease trap (Raw)
    - Effluent after chemical treatment (Chemically treated)
    - Effluent from Moving Bed Biological Reactor (MBBR)
    - Effluent after treatment from treated tank (Treated)

3.1.2 Sampling Procedure

- **For Physico-Chemical Tests:**
    - Two (02) sterile air-tight 500 mL glass bottles were used to collect samples from each sampling positions for the physico-chemical tests.
    - Sampling Frequency:
        - For pH – Daily
        - For other tests – weekly
    - Sampling Duration: 03 Months
- **For Microbiological Tests:**
    - One (01) sterile air-tight 100 mL glass bottles were used to collect samples from each sampling positions for the microbiological tests.
    - Sampling Frequency:
        - For microbiological test – monthly
    - Sampling Duration: 03 Months

3.2 Physico-chemical Analysis of the Effluent

3.2.1 pH Determination

The electrode of the pH meter (model: S20; Mettler Toledo) was immersed into the test solution. After few minutes a stable pH value was displayed on the screen of the instrument. The pH value was recorded in Logbook.

3.2.2 Dissolved Oxygen (DO) Determination

Method:

Test sample was collected in 300 mL of BOD bottle up to the brim so that the bottle was completely filled with test sample that has not been in contact with air and no air was trapped under the stopper.

Then, bottle stopper was removed and 2.4 mL of manganese sulfate and alkaline azide-iodide solution was added at the surface of the test sample.

Then, the stopper was replaced. Trapping of air bubbles was avoided and the bottle was inverted several times to mix properly.

Then the flock was allowed to settle down about half of the volume of the bottle. The bottle was inverted several times again to mix the flock back into the solution. When the flocks settle down, the shaking was repeated. After complete settling, stopper was removed and 2.4 mL of concentrated sulfuric acid (H_2SO_4) was added.

The bottle was restoppered. The top of the bottle was swept to remove any acid and gently inverted several times until the precipitate had dissolved back into solution. If there was dissolved oxygen in the sample, colour of the sample was changed to yellow-orange.

After that, 100 mL of the above sample was poured in a 250 mL conical flask and1mL of starch solution was added in it, blue colour was developed. Then, it was titrated with 0.025N sodium thiosulphate solution until the blue or purple colour was disappeared. The titer volume was recorded.

Calculation:

DO was calculated by using the following equation:

$$\text{Dissolved Oxygen (mg/L)} = \frac{(\text{Titer volume, mL}) \times 0.025 \times 8000 \times F}{\text{Sample volume, mL}}$$

Where,

8000 = miliequivalent weight of oxygen x 1000 mL/L.

F = Factor of 0.025 N sodium thiosulphate solution.

3.2.3 Biochemical Oxygen Demand (BOD) Determination

Preparation of Sample:

At first, 24 mL of sample was taken in two BOD bottles (300 mL each) respectively and rest of the void spaces was filled with dilution water. Then, one bottle was kept in incubator at 20 ± 2^0C for 5 days and its Dissolved Oxygen (DO) was determined after 5 days (D_5)**.** The Dissolved Oxygen (DO) content of another bottle was determined immediately (D_1).

Preparation of Blank:

Two BOD bottles (300 mL each) were filled by dilution water. Then, one bottle was kept in incubator at 20 ± 2^0C for 5 days and its Dissolved Oxygen (DO) was determined after 5 days (B_5)**.** The Dissolved Oxygen (DO) content of another bottle was determined immediately (B_1).

Calculation:

Calculation was performed for Dissolved Oxygen (DO) by using the following equation**:**

$$\textbf{Dissolved Oxygen (DO)} = \frac{(\text{Titer volume, mL}) \times 0.025 \times 8000 \times F}{\text{Sample volume, mL}}$$

Where,

8000 = miliequivalent weight of oxygen x 1000 mL/L.

F = Factor of 0.025 N sodium thiosulphate solution.

$$\textbf{Biochemical Oxygen Demand (BOD)} = \frac{(D1 - D5) - (B1 - B5) \times 1000}{\% \text{ of Sample}}$$

Where,

D1 = Initial DO of sample, mg/L

D2 = Final DO of sample (after 5 days incubation), mg/L

B1 = Initial DO of blank, mg/L

B5 = Final DO of blank (after 5 days incubation), mg/L

$$\% \text{ of sample} = \frac{\text{Volume of sample} \times 100}{\text{Volume of diluted sample}}$$

Where,

Volume of Sample = 24 mL

Volume of diluted sample = 300 mL

3.2.4 Chemical Oxygen Demand (COD) Determination

Preparation of Blank:

At first, 20 mL of water was taken in a 250 mL flat bottom flask. Then, 10 mL of 0.25N Potassium Dichromate solution was added. After that, 10 to 15 pieces of glass beads were added in it to prevent bumping while refluxing. Then, 30 mL of concentrated Sulfuric acid (H_2SO4) was slowly added under fume hood.

Preparation of the sample:

At first, 30 mL of sample was filtered through filter paper #1 (Whatman). Then, 20 mL of filtered sample was taken in a 250 mL flat bottom flask. Then, 10 mL of 0.25N Potassium Dichromate solution was added and immediately 10 to 15 pieces of glass beads was added in it to prevent bumping while refluxing. Then, 30 mL of concentrated Sulfuric acid (H_2SO4) was slowly added under fume hood.

Refluxing both the sample flask and the blank flask:

The sample flask and the blank flask were attached to separate condensers at the same time and the cooling water was turned on. The heating mantle and reflux (boil) the mixture were turned on for two hours. After 2 hours, 100 mL of water from the top of the condenser was added slowly. The condenser was disconnected and the apparatus was cooled to room temperature.

Titrating both the sample flask and the blank flask:

At first, 2 or 3 drops of ferroin indicator was added to each flask. Then, each flask was titrated with Ferrous Ammonium Sulfate until the contents changed colour from blue-green to reddish-brown and the colour change remained for 1 minute or longer.

Calculation:

Calculation was performed for the COD of the sample from the amount of ferrous ammonium sulfate consumed in the titration by using the following equation:

$$\text{COD (mg/L)} = \frac{(Vb - Vs) \times N \times 8000}{20\,mL}$$

Where,

Vb = volume of ferrous ammonium sulfate (FAS) consumed in blank titration (mL)

Vs = volume of ferrous ammonium sulfate (FAS) consumed in sample titration (mL)

N = Normality of ferrous ammonium sulfate (FAS)

8000 = miliequivalent weight of oxygen x 1000 mL/L

3.2.5 Total Dissolved Solids (TDS) & Total Suspended Solids (TSS) Determination

A clean 100 mL beaker and a clean filter paper #1 (e.g Whatman) was dried at 105°C for 1 hour in an oven. After that, these were allowed to cool in a desiccator and weight was recorded. After that, 50 mL of test sample was taken in a measuring cylinder and filtered it through the filter paper in the beaker. The test sample was evaporated by a Hot Plate. The residue & filter paper was dried in an oven at 105°C for 1 hour. And after cooling by placing in a Desiccator, the final weight of the beaker and filter paper (along with residue) was taken and recorded.

Calculation:

Calculation was performed for TDS by using the following equation:

$$\text{TDS (mg/L)} = \frac{(W2 - W1) \times 1000000}{50\,mL}$$

Where,

W1= Initial weight of the beaker (g).

W2= Final weight of the beaker along with residue after drying (g).

Calculation was performed for the Total Suspended Solids by using the following equation:

$$\text{TSS (mg/L)} = \frac{(W2 - W1) \times 1000000}{50\,mL}$$

Where,

W_1 = Initial weight of filter paper (g).

W_2 = Final weight of filter paper along with residue after drying (g).

3.2.6 Oil and Grease (OG) Determination

Procedure:

A clean 250 mL beaker was dried in an oven at 105° C for 30 minutes. Then, it was allowed to cool in a desiccators. Then, it‟s weight was taken. After that, 200 mL of test sample was taken in the separating funnel and extracted with 30 mL of Chloroform. Then, 5 mL of Hydrochloric acid was added slowly and shacked properly. After 3 to 5 minutes, the lower layer of the sample was taken from the separating funnel in the initially weighed 250 mL beaker. The same procedure was followed for 3 times and the entire extracted lower layers were collected in that same beaker. The solvent was evaporated on a Hot Plate. The final weight of the beaker (along with residue) was taken.

Calculation:

Calculation was performed for Oil & Grease by using the following equation:

$$\text{Oil \& Grease (mg/L)} = \frac{(W2 - W1) \times 1000000}{200\,mL}$$

Where,

W1= Initial weight of the beaker (g).

W2 = Final weight of the beaker along with residue after drying (g).

3.3 Microbiological Analysis of the Effluent

3.3.1 Microbial Load Pattern

At first, samples taken for microbiological analysis were diluted into 10^4 times and 10^6 times respectively with sterile WFI.

Then, 1 mL diluted sample was taken from each diluted in Petri dish(s) and sufficient amount of R2A media was added respectively. The operation was carried out under Laminar Air Flow Hood.

Then, the Petri dishes containing samples were incubated at 34°C in a Walk in Type Incubator 03-05 days.

After incubation, microbial load was calculated by Antibiotic Zone Reader Machine and recorded in Log book.

3.3.2 Sub Culture

Selected mixed colony found in previous step were then sub cultured by using streaking method in TSA media containing Petri dish(s) and incubated at 34°C in a Walk in Type Incubator for 02 days to get pure colony.

3.3.3 Slant Preparation

In this stage, the selected pure colony was transferred into slant (Test Tube) and preserved at 2 to 8°C for further biochemical and other studies with proper labeling. The slant was prepared by using TSA media with glycerin.

3.3.4 Colony Morphology Study

The colonial morphology of the pure culture was studied in detail against standards (Figure – 1). The pure culture containing similar/ almost similar type of colony morphology was screened and different types of colony were selected for identification.

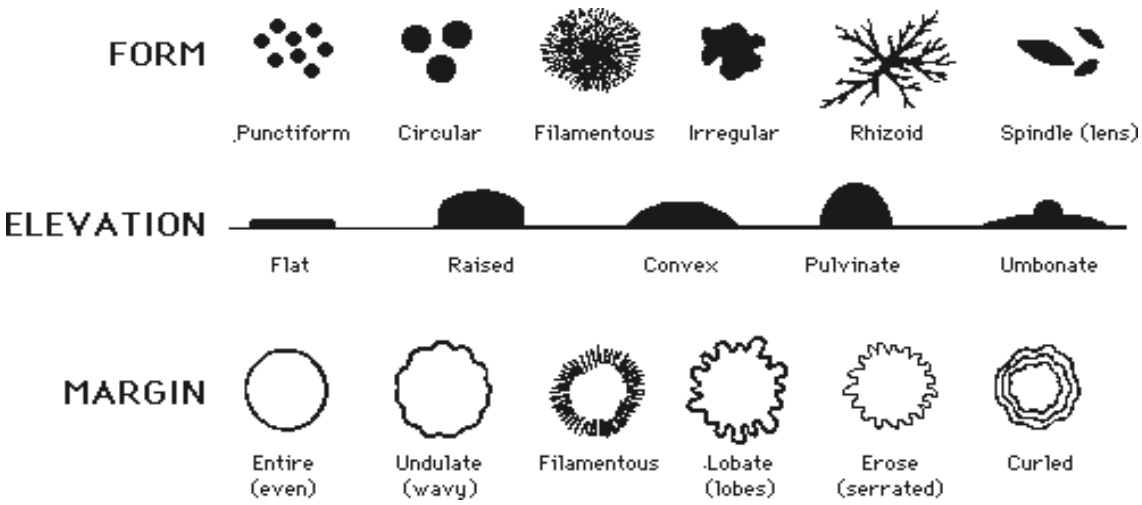

Figure – 1: Colonial morphology of microorganisms

The following characteristics of the colony were considered during Colony Morphology Study.

Colonial Shape and Size

- Shape: Colony shape may be circular, irregularly-shaped, rhizoidous, filamentous, spindle, punctiform and swarming etc.
- Size (mm): Large, moderate or small, plate-full, or punctiform etc.

Colonial Margin

- Margins are: entire, smooth, erose, irregular, lobate, undulate, rhizoid, filamentous, crenated, ciliate etc.

Colonial Elavation

- Colony elevations are: flat, flat with raised margin, convex, papillate, umbonate, raised, raised with a spreading edge, plateau, growth into medium etc.

Colonial Surface

- Surface may be smooth, rough, , granular, powdery, glistening etc.

Colonial Consistency

- Colony may be mucoid, firm, friable, membranous, butyrous etc.

Colonial Opacity

- Colonies may be translucent, transparent or opaque considering light transmission.

Colonial Pigments

- The nature of pigment (if any) is marked.

3.3.5 Biochemical Studies

Growth in Broth Medium

- Growth characteristics of microorganisms in broth media were observed. The growth characteristics were found as sedimental, flocculent, pellicle, turbid etc.

Note: Care was taken, when using growth patterns in broth to identify an organism because the type of broth and the temperature at which the organism was grown can alter the growth patterns. Also, if something happens to shake or otherwise disturb the broth culture, the growth pattern may be disrupted, and subsequent observation will yield erroneous results.

Starch Hydrolysis

- Starch agar medium were inoculated by straight line streak and incubated at 35°C for 24 hours. After incubation, iodine solution was added to the plate.

Gelatinase Test

- Gelatin agar plates were inoculated by streaking and incubated at 35°C for 48 hours. After incubation covered the growth with acidified $HgCl_2$ solution.

Indole Test (Tryptophan hydrolysis)

- Tryptophan broth were inoculated and incubated at 35°C for 72 hours. After incubation, a few drops of Kovac‟s reagent were added to the tube without shaking.

Methyl Red Test (MR)

- Tubes of VP Medium were inoculated and incubated at 35°C for 72 hours. After incubation, a few drops of methyl red solution was added and mixed well.

Voges-Proskauer Test (VP)

- Tubes of VP Medium were inoculated and incubated at 35°C for 72 hours. After incubation, 3 ml of alpha napthol solution was added followed by 1 ml 40% KOH-Creatine solution. After mixing well, it was allowed to stand for 30 minutes.

Citrate Utilization

- Simmons Citrate agar or, Koser's citrate broth were inoculated and incubated at 35°C for 48-72 hours

Catalase Test

- Tubes of TSB were inoculated and incubated at 35°C for 24 hours. After incubation, 1ml of H_2O_2 was added to it or, a loopful culture from a well isolated colony was taken upon a slide and a few drops of H_2O_2 were added upon it.

Oxidase Test

- Oxidize reagent was taken and applied a few drops of reagent to a strip of filter paper. A colony of bacteria was picked with a sterile needle, and streaked it on the reagent-soaked filter paper.

Urease Test

- Urea Broth tubes were inoculated and incubated at 35°C for 3 to 7 days. A control was also maintained without inoculation.

Motility

- Semisolid media were prepared, dispensed in tubes, sterilized and inoculated with 24-48 hours old culture by stabbing with the needle into the center of the medium and not to touch the side of the tube to approximately three-quarters of its depth. Inoculated tubes were then incubated for 48 hours at 35°C.

Fermentation Test

- Fermentation tubes with selected carbohydrate (incorporating the indicator and the Durham tube) were inoculated and incubated at 35°C for 24-48 hours. Un-inoculated controls were run in order to accurately evaluate results.

Deep Glucose Agar Test

- Deep glucose agar tubes were inoculated in a fluid condition followed by thorough mixing by vortexing and incubated at 35°C for 3 to 7 days. The growth was observed at different level of the tube column.

Nitrate Reduction Test

- Nitrate Broth tubes were inoculated and incubated at 35°C for 3 to 7 days. After incubation, 10-15 drops of "Reagent-A" and equal volume of "Reagent-B" were added. A control was also maintained without inoculation.

Hydrogen Sulfide Production Test

- Peptone Iron Agar slant was inoculated with the culture and paper strip soaked with lead acetate was introduced to the tubes such a way that the paper strip does not touch the media. Then the tube was incubated at 35°C for 2-3 days.

3.3.6 Selective and Differential Media for Identifying Microorganisms

Selective media allows the growth of certain type of Organisms, while inhibiting the growth of other Organisms. This selectivity was achieved in several ways. For example, Organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium for the growth of the microorganism. Like-wise, the selective inhibition of some types of microorganisms can be studied by adding certain dyes, antibiotics, salts or specific inhibitors that will affect the metabolism or enzymatic systems of the Organisms.

- In this study, the following selective media were used:
  - MacConkey‟s Agar (MAC)
  - Eosin Methylene Blue (EMB) Agar
  - Brilliant Green (BG) Agar
  - Mannitol Salt Agar (MSA)
  - Plate Count Agar (PCA)

Method:

At first, a colony of bacteria from each isolates was picked with a sterile needle, and streaked it on the selective media containing Petri-dishes. The operation was carried out under Laminar Air Flow Hood. Then, the Petri dishes containing samples were incubated at 34^{o}C in a Walk in Type Incubator 03-05 days. After incubation, the growth was evaluated and recorded as positive (+) growth or negative (-) growth for each of the five (05) selective medium respectively.

3.3.7 Gram Staining

Staining and Microscopy

- **Preparing a smear from colonies isolated from agar surfaces**

  One or two loopfuls of distilled water was placed on the slide. Then it was mixed a very small quantity of the colony with the water by using an inoculating loop, and spread over the slide. It was critical that microbes are separated from each other during this step. Then, it was allowed to evaporate ("air-dry") the liquid prior to fixation. This attachment process was accomplished by passing the slide over the flame of a Bunsen burner two or three times.

- **Gram Staining**

  The Gram staining is one of the most useful staining procedures because it classifies bacteria into two large groups: gram-positive and gram-negative (Torture 2010:69)

  Method:

  - A heat-fixed smear was covered with a basic purple dye (crystal violet) for 60 seconds. Because the purple stain imparts its colour to all cells, it was referred to as a primary stain. After 60 seconds, the purple dye was washed off.
  - Then, the smear was covered with iodine, a mordant for 60 seconds. When the iodine was washed off, both gram-positive and gram-negative bacteria appear dark violet or purple.
  - Next, the slide was treated with alcohol (95% ethanol) for 10 seconds and then washed off. This solution was a discolouring agent, which removes the purple from the cells of some species but not from others.
  - The alcohol was rinsed off, and the slide was then stained with safranin, a basic red dye, for 60 seconds. The smear was washed again, blotted dry, and examined microscopically.

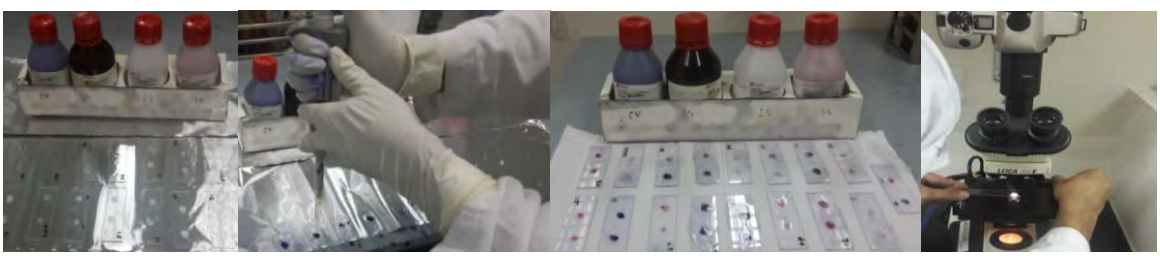

Figure – 2: Gram staining

3.3.8 Identification of Bacteria by BBL Crystal ID Kit

Test procedure:

- An inoculums tube marked BBL Crystal Enteric/ Stool Inoculum fluid (for Gram-Negative bacteria) and GP inoculums fluid (for Gram- Positive bacteria) and was taken and labeled with reference number.
- Using a sterile loop, one well isolated large colony (2-3 mm or larger in diameter) or 4-5 smaller colonies from TSA plate was picked and suspended in the fluid.
- Then, the tube was recapped and mixed for approximately 10-15 seconds for vortex formation in vortex mixer.
- Then, a base panel was taken and marked it with reference number.
- Then, the entire content of inoculum fluid was poured into target area of the base.
- Then, the base was hold in both hands and rolled inoculums gently along the tracks until all of the wells were filled.
- Then, a lid panel was removed from pouch. Since, the desiccant should be present in the pouch, so it was checked very seriously. Desiccant was present in the pouch & it was used within 01 hour of opening.
- Then, the lid was aligned to the base panel so that the labeled end of the lid was on the target area of the base and the lid was adjusted with the base properly.
- Finally, the inoculated panel was incubated at 35-37°C for 18-20 hours.

Reading for Gram-Negative bacteria:

- After incubation, the panels were removed from the incubator. All the panels were read face down using the BBL Crystal Panel Viewer with reference with the colour reaction chart for an interpretation of the reactions.
- The results pads were used to record reactions.
- The panel columns were read from A to J (for Gram-Negative bacteria) & the panel columns E through J (for Gram-Positive bacteria), using the regular (white) light source on the BBL crystal light box. In case of Gram-Positive bacteria, columns A through D (fluorescence substances) was read by using the UV light source in the panel viewer.

- A fluorescent substances well was considered positive only if the intensity of the fluorescence observed in the well was greater than the negative control well (4A).
- The BBL crystal E/NF report pad (for **Gram-Negative bacteria) and** the BBL crystal GP report pad (**Gram-Positive bacteria)** to record the reactions by comparing with standard colour pad.

Calculation of BBL Crystal profile Number:

- The test results of each well was compared with standard colour pad and put the results on the report pad (BD BBL crystal E/NF) where each positive result gives a value of 4, 2, or 1, corresponding to the row where the test was located. A value of 0(zero) was given to any negative result. The numbers (values) resulting from each positive reaction in each column are then aligned together, thereby a 10 digit number was generated (see the example given below), this was the BBL profile number.

Example:	A	B	C	D	E	F	G	H	I	J
4	-	+	-	-	+	+	+	-	+	-
2	-	+	+	+	-	+	-	+	+	-
1	+	-	+	-	+	-	-	+	+	-
Profile	1	6	3	2	5	6	4	3	7	0

Figure – 3: Calculation of BBL crystal profile number (gram-negative bacteria)

Example:	A	B	C	D	E	F	G	H	I	J
4	*	+	-	-	+	+	+	-	+	-
2	-	+	+	+	-	+	-	+	+	-
1	+	-	+	-	+	-	-	+	+	-
Profile	1	6	3	2	5	6	4	3	7	0

*(A4)= fluorescence negative control

Figure – 4: Calculation of BBL crystal profile number (gram-positive bacteria)

Figure – 5: Identification of unknown bacteria by using BBL ID kit

- The resulting profile number and off-line results (indole and oxidase) were entered on a PC in which the BBL Crystal ID System Electronic Codebook was installed, to be obtained the identification. By using the BBL Crystal Auto Reader, probable organisms were automatically identified by the PC.

3.3.9 Antimicrobial Susceptibility Testing of Identified Microorganisms

Detecting antimicrobial resistance

Antimicrobial susceptibility testing methods are in vitro procedures used to detect antimicrobial resistance in individual bacterial isolates. In other words, an antibiogram was the result of a laboratory testing for the sensitivity of an isolated bacterial strain to different antibiotics. It was by definition an in vitro-sensitivity.

Test Methods in Detecting Antimicrobial Resistance

There are several antimicrobial susceptibility testing methods available today, and each one has their respective advantages and disadvantages. They all have one and the same goal, which was to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antibiotic treatment. This data may be utilized as guidelines for chemotherapy, or at the population level as indicators of emergence and spread of resistance based on passive or active surveillance. Some examples of antibiotic sensitivity tesing methods are:

- Dilution method (broth and agar dilution method)
- Disk-diffusion method
- E-test
- Automated methods
- Mechanism-specific tests such as beta-lactamase detection test and chromogenic cephalosporin test
- Genotypic methods such as PCR and DNA hybridization methods

Selection of the appropriate method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost. Interpretation should be based on veterinary standards whenever possible, rather than on human medical standards, which may not always be applicable. Among these available tests, the two most commonly used methods in veterinary laboratories are the agar disk-diffusion method and the broth microdilution method. ***In this study, disk diffusion method was used.***

Disk Diffusion Method

Because of convenience, efficiency and cost, the disk diffusion method was probably the most widely used method for determining antimicrobial resistance in private veterinary clinics. The disk diffusion susceptibility method was simple and practical and has been well standardized. The test was performed by applying a bacterial inoculum of approximately 1–2 $\times 10^8$ CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks were placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35^{o}C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter.

The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory

<u>Description of the Antibiotic discs used in the experiment:</u>

Following five (05) commonly used antibiotics are used in the experiment.

Table – 3: Antibiotics used in the experiment

Sl. No.	Name of the Antibiotic disk	Strength (µgm)	Manufacturer
1	Ciprofloxacin	5	Oxoid Ltd. England
2	Tertracycline	30	
3	Erythromycin	15	
4	Gentamycine	10	
5	Tobramycine	10	

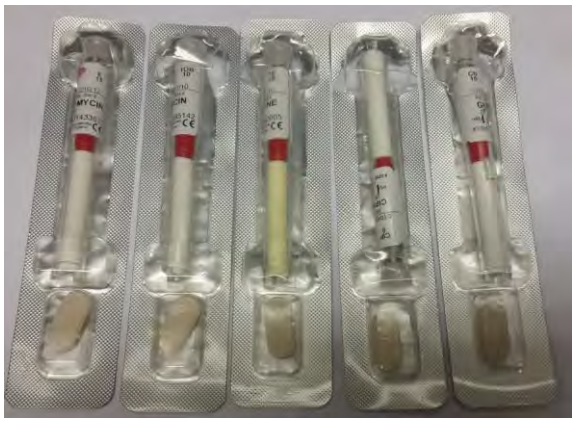

Figure – 6: Antibiotics used in the experiment

Method:

A semi-quantitative way based on diffusion (Kirby-Bauer method); small discs containing different antibiotics, or impregnated paper discs, are dropped in different zones of the culture on an agar plate, which was a nutrient-rich environment in which bacteria can grow. The antibiotic will diffuse in the area surrounding each tablet, and a disc of bacterial lysis will become visible. Since the concentration of the antibiotic was the highest at the centre and the lowest at the edge of this zone.

- **Loan preparation:**
    A. One (01) sterile loop of freshly prepared pure culture of the strain/organism was dissolved in a sterile test tube containing 02 ml sterile water and suspension has been formed by using vortex machine.
    B. Pre-incubated (24-48 hours) Petri-dish containing TSA media was taken and suspension from step-A was added into the Petri-dish.
- Then, Suspension was mixed into whole dish by gentle shaking and remaining quantity of suspension was taken out from the dish by using micropipette containing sterile tips.
- Then, the Petri-dish containing suspension was incubated into Walk-in-Type Incubator (Warm) for approx. 01 hour to allow the media to dry.
- After that, the five antibiotic discs were placed in the Petri-dish very carefully.
- After placing the discs, all Petri-dishes were incubated into Walk-in-Type Incubator (Warm) for 24 hours at 34^{o}C.
- After 24 hours the growth was observed and result was recorded.
- Finally, the diameter for the Minimum Inhibitory Concentration was measured by using Antibiotic Zone Reader.

3.4 Reagents & Chemicals

During the research period the following reagents & chemicals were used:

1. Manganese Sulfate Solution
2. Alkaline Azide-Iodide Solution
3. 0.025 N Sodium Thiosulfate Solution
4. Starch Solution
5. Phosphate Buffer
6. Calcium Chloride Solution
7. Ferric Chloride Solution
8. 0.25 N Standard Potassium Dichromate Solution
9. 0.1 N Standard Ferrous Ammonium Sulfate (FAS) Solution
10. Ferroin Indicator

3.5 Instruments

During the research period a number of instruments & equipment were used which are stated in Appendix C & Appendix D.

CHAPTER 4:
RESULTS

CHAPTER 4: RESULTS

4.1 Physico-chemical Properties of Effluent

4.1.1 Physico-chemical properties of raw water

Waste water from different sampling locations were collected as per sampling plan and tested. All the results were found satisfactory. In case of raw water, pH range was 5.79 to 6.66; DO range was 19.99 to 39.30 mg/L and COD range was 639.28 to 1006.10 mg/L [Table – 4].

Table –4: Physico-chemical properties of raw water

Sl. No.	Date of Analysis	Description (Appearance)	pH	DO (mg/L)	COD (mg/L)	Remarks
1.	10/06/15	Light Yellowish	5.80	25.80	639.28	OK
2.	17/06/15	Light Yellowish	6.30	27.00	923.00	OK
3.	24/06/15	Light Yellowish	5.89	31.25	835.80	OK
4.	01/07/15	Light Yellowish	6.01	29.90	939.60	OK
5.	08/07/15	Light Yellowish	6.36	28.25	838.15	OK
6.	29/07/15	Light Yellowish	5.99	26.10	1006.10	OK
7.	05/08/15	Light Yellowish	5.91	33.05	735.95	OK
8.	12/08/15	Light Yellowish	6.66	19.99	799.50	OK
9.	19/08/15	Light Yellowish	5.89	39.30	959.50	OK
10.	26/08/15	Light Yellowish	6.03	36.30	855.25	OK
11.	02/09/15	Light Yellowish	5.88	23.95	946.70	OK
12.	09/09/15	Light Yellowish	5.79	26.60	816.50	OK

4.1.2 Physico-chemical properties of treated water

Treated water from specific sampling location were collected as per sampling plan and tested. All the results were found satisfactory. In case of treated water, pH range was 7.55 to 8.38; DO range was 7.56 to 7.83 mg/L; TDS range was 322 to 346 mg/L; TSS range was 66 to 84 mg/L; COD range was 138.6 to 17.6 mg/L; BOD range was 30 to 45 mg/L, Oil & Grease range was 8 to 9.5 [Table – 5].

Table –5: Physico-chemical properties of treated water

Sl. No.	Date of Analysis	Description (Appearance)	pH	DO (mg/L)	TDS (mg/L)	TSS (mg/L)	COD (mg/L)	BOD (mg/L)	Oil & Grease	Remarks
1.	10/06/15	Clear	7.55	7.74	338.00	82.00	145.20	41.25	8.50	OK
2.	17/06/15	Clear	7.79	7.67	324.00	76.00	140.80	31.25	8.00	OK
3.	24/06/15	Clear	7.94	7.64	322.00	74.00	138.60	40.00	8.50	OK
4.	01/07/15	Clear	7.85	7.83	328.00	76.00	148.80	33.75	9.50	OK
5.	08/07/15	Clear	7.80	7.83	322.00	74.00	146.40	32.50	8.50	OK
6.	29/07/15	Clear	7.75	7.73	326.00	76.00	145.20	35.00	8.00	OK
7.	05/08/15	Clear	8.12	7.57	330.00	80.00	151.20	32.50	9.00	OK
8.	12/08/15	Clear	8.07	7.65	334.00	84.00	154.00	45.00	9.00	OK
9.	19/08/15	Clear	7.75	7.56	324.00	78.00	151.20	32.50	9.00	OK
10.	26/08/15	Clear	8.38	7.74	334.00	84.00	145.20	43.75	8.50	OK
11.	02/09/15	Clear	7.69	7.81	346.00	66.00	177.60	45.00	8.00	OK
12.	09/09/15	Clear	7.76	7.57	343.00	80.00	149.60	30.00	9.00	OK

Table – 6: Microbial load in different phases of the ETP water

Phase	Date of Collection	Sampling Point	Result (cfu/ml)
Phase 1	10/06/15	Raw Water Tank	2.81×10^6
		Chemically Treated	2.5×10^6
		MBBR-1	4.0×10^6
		Treated Water Tank - 2	2.39×10^6
Phase 2	08/07/15	Raw Water Tank	2.86×10^6
		Chemically Treated	2.0×10^6
		MBBR-1	5.0×10^6
		Treated Water Tank - 2	2.35×10^6
Phase 3	12/08/2015	Raw Water Tank	1.60×10^6
		Chemically Treated	1.20×10^6
		MBBR-1	2.99×10^6
		Treated Water Tank - 2	2.0×10^6

4.2 Microbial Load of ETP

Microbial load pattern of the defined sampling point were determined by using decimal serial dilution and result was plotted in Table – 6. In case of all test phases, sufficient microorganisms were found in all sampling areas of ETP.

4.3 Pure Culture Isolation

Thirty (30) pure cultures were isolated from different sample of ETP during three phases. The cultures were uniquely identified by „Pure Culture Ref. No.", their sources and test phases. Among the 30 isolates, 14 isolates were selected from Phase 1, 10 isolates were selected from Phase 2 and 06 isolates were selected from Phase 3. Among the 30 isolates, 08 isolates were selected which were collected from Raw waste water, 11 isolates from chemically treated waste water, 06 isolates from MBBR-1 & 05 isolates from treated water [Table – 7].

4.4 Screening of Pure Culture

Among the 30 pure cultures isolated during three phases, 08 visually different isolates were screened & selected for the next stages of experiment. Different isolates with screened pure culture reference no. have been adopted in Table – 8. Isolate 1 represents 04 pure cultures, isolated from chemically treated, MBBR-1 & treated samples. Isolate 2 represents 05 pure cultures, isolated from raw, chemically treated, & MBBR-1 samples. Isolate 3 represents 04 pure cultures, isolated from raw, chemically treated, & treated samples. Isolate 4 represents 04 pure cultures, isolated from chemically treated & treated samples. Isolate 5 represents 04 pure cultures, isolated from raw, chemically treated, MBBR-1, & treated samples. Isolate 6 represents 04 pure cultures, isolated from raw, chemically treated, & MBBR-1 samples. Isolate 7 represents 02 pure cultures, isolated from raw & MBBR-1 samples. And finally, Isolate 8 represents 03 pure cultures, isolated from chemically treated & treated samples.

Table – 7: Isolated Pure Culture with sources

Sl. No.	Pure Culture Ref. No.	Source of the Pure Culture	Test Phase
1.	R1-10^4-P1	Raw Water (10^4 Dilution)	Phase 1
2.	R2-10^4-P1	Raw Water (10^4 Dilution)	Phase 1
3.	*R1-10^6-P1*	Raw Water (10^6 Dilution)	Phase 1
4.	CT1-10^4-P1	Chemically Treated Water (10^4 Dilution)	Phase 1
5.	CT2-10^4-P1	Chemically Treated Water (10^4 Dilution)	Phase 1
6.	CT3-10^4-P1	Chemically Treated Water (10^4 Dilution)	Phase 1
7.	CT4-10^4-P1	Chemically Treated Water (10^4 Dilution)	Phase 1
8.	CT1-10^6-P1	Chemically Treated Water (10^6 Dilution)	Phase 1
9.	CT2-10^6-P1	Chemically Treated Water (10^6 Dilution)	Phase 1
10.	*MB1-10^4-P1*	MBBR-1 Water (10^4 Dilution)	Phase 1
11.	*MB1-10^6-P1*	MBBR-1 Water (10^6 Dilution)	Phase 1
12.	T1-10^4-P1	Treated Water (10^4 Dilution)	Phase 1
13.	T2-10^4-P1	Treated Water (10^4 Dilution)	Phase 1
14.	T3-10^4-P1	Treated Water (10^4 Dilution)	Phase 1
15.	*R1-10^4-P2*	Raw Water (10^4 Dilution)	Phase 2
16.	*R2-10^4-P2*	Raw Water (10^4 Dilution)	Phase 2
17.	*R1-10^6-P2*	Raw Water (10^6 Dilution)	Phase 2
18.	*R2-10^6-P2*	Raw Water (10^6 Dilution)	Phase 2
19.	*CT1-10^6-P2*	Chemically Treated Water (10^6 Dilution)	Phase 2
20.	*CT2-10^6-P2*	Chemically Treated Water (10^6 Dilution)	Phase 2
21.	*CT3-10^6-P2*	Chemically Treated Water (10^6 Dilution)	Phase 2
22.	*MB1-10^6-P2*	MBBR-1 Water (10^6 Dilution)	Phase 2
23.	*MB2-10^6-P2*	MBBR-1 Water (10^6 Dilution)	Phase 2
24.	*T1-10^4-P2*	Treated Water (10^4 Dilution)	Phase 2
25.	*R1-10^6-P3*	Raw Water (10^6 Dilution)	Phase 3
26.	*CT1-10^6-P3*	Chemically Treated Water (10^6 Dilution)	Phase 3
27.	*CT2-10^6-P3*	Chemically Treated Water (10^6 Dilution)	Phase 3
28.	*MB1-10^6-P3*	MBBR-1 Water (10^6 Dilution)	Phase 3
29.	*MB2-10^6-P3*	MBBR-1 Water (10^6 Dilution)	Phase 3
30.	*T1-10^6-P3*	Treated Water (10^4 Dilution)	Phase 3

**1 = Sample 1, & 2= Sample 2*

Table – 8: Isolates with pure culture ref. no.

Isolate No.	Screened Pure Culture Ref. No.	Remarks
Isolate 1	CT1-10^6-P1, CT2-10^6-P1, MB2-10^6-P2 & T1-10^6-P3	04 Pure Cultures
Isolate 2	R1-10^6-P1, CT1-10^4-P1, MB1-10^4-P1, R1-10^4-P2 & R1-10^6-P2	05 Pure Cultures
Isolate 3	R2-10^4-P1, CT2-10^4-P1, T1-10^4-P1 & R1-10^4-P1	04 Pure Cultures
Isolate 4	CT4-10^4-P1, CT1-10^6-P3, CT1-10^6-P2 & T2-10^4-P1	04 Pure Cultures
Isolate 5	CT2-10^6-P3, R1-10^6-P3, T1-10^4-P2 & MB1-10^6-P2	04 Pure Cultures
Isolate 6	R2-10^4-P2, MB1-10^6-P1, MB2-10^6-P3 & CT3-10^6-P2	04 Pure Cultures
Isolate 7	R2-10^6-P2 & MB1-10^6-P3	02 Pure Cultures
Isolate 8	CT1-10^6-P2, T3-10^4-P1 & CT3-10^4-P1	03 Pure Cultures

4.5 Identification of Selected Isolates

Different biochemical tests were performed as per approved method. The isolates were cultured in different selective media. Gram staining was performed and colony morphology of the selected isolates was recorded in Table – 9. All isolates shown negative result in starch media except isolate 3; Isolate 1, 3, 4, 6, & 8 were Gelatin positive; all isolates showed negative results in indole; all isolates showed negative results in MR except isolate 3 & 7; all isolates showed negative results in VP; all isolates showed negative results in citrate; all isolates were catalase positive except isolate 7; Isolate 1, 4, & 8 were oxidase positive; Isolate 1, 2, 5, & 7 were urease positive; no isolate were found as motile; only isolate 2 & 7 were found positive in glucose test; Isolate 1, 5, & 8 were found positive in lactose & sucrose test; Only isolate 2 produced H2S; None of the isolates showed positive results in MCA, EMB & PCA media; isolate 3 & 6 were positive in BGA medium; only isolate 5 was showed positive result in MSA medium; Isolate 1, 2, & 8 were gram positive and rest of the isolates were gram negative and their colony morphology are shown in Table – 9.

Table – 9: Biochemical test results for the identification of the selected isolates

Isolate No.	Biochemical characterization																				Colony Characteristics							Suspected Organisms
	Starch	Gelatin	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease	Motility	Glucose	Lactose	Sucrose	Gas	H_2S Prod.	MCA	EMB	BGA	MSA	PCA	Gram Staining	Size/Shape	Margin	Surface	Elevation	Opacity	Consistency	Colour	
1	-	+	-	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+ve	Ci	E	Sm	Co	O	M	GY	*Micrococcus luteus*
2	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-ve	Ci	E	Sm	Co	O	M	W	*Stenotrophomonas maltophilia*
3	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	+ve	Sp	U	R	F	T	Cr	LOW	*Streptococcus sanguinis*
4	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-ve	Ci	E	Sm	Co	O	M	DY	*Empedobacter brevis*
5	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-	+	-	-ve	P	E	Sm	R	O	M	GY	*Tatumella ptyseos*
6	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-ve	Ci	E	Sm	Co	O	St	Cri	*Klebsiella pneumoniae*
7	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-ve	Ci	E	Sm	Co	O	St	Cri	*Klebsiella pneumoniae*
8	-	+	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+ve	P	E	Sm	R	O	M	Cri	*Bacillus subtilis*

* *E = Entire, CI = Circular, Co = Convex, Cr = Creamy, Cri = Crimson, DY = Deep Yellow, F = Flat, GoY = Golden Yellow, GY = Greenish Yellow, LOW = Light Off-white, M = Mucoid, O = Opaque, P = Puntiform, R = Raised, R = Rough, Sm =Smooth, Sp = Spreading, St = Sticky, T = Translucent, U = Undolate, & W = White*

4.6 Identification by Using BBL ID Kit

Selected isolates were identified by using BBL ID kit. Among the 08 isolatess 06 bacteria were identified or differentiated because isolates 06 & 07 were the same organism i.e., *Klebsiella pneumonia* and isolated 5 was not identified by BBL. Full test were performed by following SOP. Identified microorganisms are plotted in Table – 10 and BBL ID kit report has been attached with Table – 11.

Table – 10: Identified bacteria

Sl. No.	**Name of the identified bacteria**
1.	*Micrococcus luteus*
2.	*Bacillus subtilis*
3.	*Stenotrophomonas maltophilia*
4.	*Empedobacter brevis*
5.	*Tatumella ptyseos* (identified by analyzing biochemical data as mentioned in Table – 11 with the help of ABIS online)*
6.	*Klebsiella pneumonia*
7.	*Streptococcus sanguinis*

Table – 11: BBL ID kit test results

Isolate No.: 01	**Size/Shape & Arrangement under Microscope:** Cocci Cluster
Attachment – I	
Identified Organism: *Micrococcus luteus*	

Table – 11: BBL ID kit test results (Continued)

Isolate No.: 02	**Size/Shape & Arrangement under Microscope:** Short Rod
Attachment – II	
Identified Organism: *Stenotrophomonas maltophilia*	

Isolate No.: 03	**Size/Shape & Arrangement under Microscope:** Cocci Cluster
Attachment – III	
Identified Organism: *Streptococcus sanguinis*	

Table – 11: BBL ID kit test results (Continued)

Isolate No.: 04	**Size/Shape & Arrangement under Microscope:** Short Rod
Attachment – IV	
Identified Organism: *Empedobacter brevis*	

Isolate No.: 05	**Size/Shape & Arrangement under Microscope:** Short Rod
Attachment – V	
Identified Organism: *Tatumella ptyseos* * *identified by analyzing biochemical data as mentioned in Table – 9 with the help of ABIS online*	

Table – 11: BBL ID kit test results (Continued)

Isolate No.: 06	**Size/Shape & Arrangement under Microscope:** Short Rod
Attachment – VI	
Identified Organism: *Klebsiella pneumoniae*	

Isolate No.: 07	**Size/Shape & Arrangement under Microscope:** Short Rod
Attachment – VII	
Identified Organism: *Klebsiella pneumoniae*	

Table – 11: BBL ID kit test results (Continued)

Isolate No.: 08	**Size/Shape & Arrangement under Microscope:** Rod Cluster
Attachment – VIII	
Identified Organism: *Bacillus subtilis*	

4.7 Antibiotic Susceptibility Testing (Antibiogram) of Identified Bacteria

Antibacterial susceptibility test were performed using 05 commonly used antibiotics and it was observed that *Micrococcus luteus, Stenotrophomonas maltophilia* and *Tatumella ptyseos* were sensitive to all the antibiotics. *Streptococcus sanguinis* was sensitive to tetracycline only. *Bacillus subtilis* was sensitive to tobramycine only. *Empedobacter brevis* was sensitive to all the antibiotics except tetracycline (which was intermediate resistance). On the other hand, *Klebsiella pneumonia* was resistant to all the antibiotics [Table – 12]

Table – 12: Antibiotic Susceptibility test of the selected isolates

Name of Bacteria	**Name of the Antibiotic(s)**									
	Ciprofloxacin (CIP)		Erythromycin (E)		Gentamycin (CN)		Tetracycline (TE)		Tobramycine (TOB)	
	Sens.	Zone Dia. (mm)	Sens.	Zone Dia. (mm)	Sens.	Zone Dia. (mm)	Sens.	Zone Dia. (mm)	Sens.	Zone Dia. (mm)
Micrococcus luteus	S	21.2	S	13.8	S	22.5	S	28.1	S	17.3
Bacillus subtilis	R	0	R	0	R	0	R	0	S	14.8
Stenotrophomonas maltophilia	S	22.1	S	15	S	20.5	S	21.9	S	16.9
Empedobacter brevis	S	38.2	S	16.9	S	15.4	I	11.7	S	14.8
Tatumella ptyseos	S	27	S	25.8	S	28.9	S	33.3	S	26.6
Klebsiella pneumoniae	R	0	R	0	R	0	R	0	R	0
Streptococcus sanguinis	R	0	R	0	R	0	S	30.1	R	0

** R = Resistant, I = Intermediate Resistance/ Sensitive, S = Sensitive*

CHAPTER: 5
DISCUSSION

CHAPTER 5: DISCUSSION

In the present study it was shown that, all the physico-chemical parameters of raw water as well as treated water were within the prescribed limit as per the Department of Environment of Bangladesh (Table 2) and there was no significant variation during the entire operation period. It indicates the efficiency of the ETP. It was also shown that, microbial load was satisfactory, which indicates that, the organism present in the ETP can tolerate the stress of the pharmaceutical effluents (waste water).

The findings from this study have also showed that, out of 30 isolates, 07 bacteria could be identified viz. *Micrococcus luteus, Bacillus subtilis, Stenotrophomonas maltophilia, Empedobacter brevis, Tatumella ptyseos, Klebsiella pneumonia,and Streptococcus sanguinis*. The functionality of the biological part of the ETP is good enough and it indicates the organism present in the biological part of the ETP has the involvement in the pharmaceutical waste water to a certain extent. Natural process employing microorganisms is considered to be very effective and environmentally friendly method of decontamination. The waste water was characterized for physicochemical, organics, inorganics and metallic parameters. The ability of bacteria to treat waste water has been well studied.

Micrococcus luteus (which is usually found in soil, dust, water and air) possesses unusual abilities to tolerate and to use very toxic organic molecules as carbon sources, and combines these activities with tolerance to metals. Recent work by Greenblatt et al., 2004, demonstrated that *Micrococcus luteus* has survived for at least 34,000 to 170,000 years on the basis of 16S rRNA analysis (16S rRNA analysis revealed that *Micrococcus luteus* has survived for at least 34,000 to 170,000 years (Greenblatt et al., 2004), and possibly much longer. A number of interesting biological issues are also inherent in the study of *M. luteus*, including dormancy without spore formation, resuscitation from dormancy, and the significance of tetrad formation. *Micrococcus luteus has* two properties essential to dealing with toxic wastes; the ability to degrade toxic organic pollutants and tolerance to metals (Sandrin et al., 2003). Often found in contaminated soils, oil spills and sludge, *M. luteus* can degrade hydrocarbons and olefinic compounds [Zhuang et al., 2003], use biphenyl as a carbon source, and degrade phthalates [Eaton et al., 1982]. It harbors a plasmid capable of degrading malathione and chlorpyriphos [Guha *et al.,* 1997]. Whole repertoires of functions which deal with metals have been found in *M. luteus*. It carries out biosorption of strontium [Faison *et al.,* 1990] and to a lesser degree lead,

nickel, and zinc [Lo *et al.,* 2001]. In the chemical and pharmaceutical industries, *M. luteus* may be exploited for its capability in isoprene synthetic reactions. This is the cornerstone of sterol, carotenoid, rubber, and fatty acid synthesis and *M. luteus* has been the platform for isolation of important enzymes in this most basic of processes, including the cis-prenyltransferase gene, whose gene product carries out the condensation of isopentyl phosphate with allelic diphosphate [Oh *et al.,*2000]. *Micrococcus* has the ability to utilize a wide range of unusual substrates, such as pyridine, herbicides, chlorinated biphenyls, and oil. They are likely to be involved in detoxification or biodegradation of many other environmental pollutants [Doddamani et al., 2001]. *Micrococcus* showed higher growth in the Bushnell-Haas medium with the refinery waste water as a sole carbon source [Usman et al., 2012]

On the other hand, *Bacillus subtilis* found in soil and the gastrointestinal tract of ruminants and humans and can form a tough, protective endospore, allowing it to tolerate extreme environmental conditions. *Bacillus subtilis* has many known usefulness. It produces a natural surfactant and possesses the ability to biodegrade hydrocarbons. Laboratory experiments showed that the biosurfactant production is not inhibited by the presence of the crude oil [Carmen et al., 2003]. It can break down paraffin and lighter oils reducing viscosity in hydrocarbon polluted water and convert explosives into harmless compounds of nitrogen, carbon dioxide, and water. It can be used in reduction in chloride content [Saranraj et al., 2012], electrical conductivity, total solids, chemical oxygen demand and biological oxygen demand (almost 47.52%) of waste water [Porwal *et al.,* 2015]. The species can also break down various naturally-occurring pollutants, which are useful for environmental purposes. The bacteria can actually eat up light fractions of crude oil like paraffin and convert them into less environmentally harmful substances. *B. subtilis* can also produce a surfactant, which helps to disperse crude oil, and the enzymes that it can produce include lipases, which can be turned into bioremediants for fat-rich pollutants like sewage and other waste water [*O'Keeffe* of *Demand Media].* Members of the *Bacillus* genus are known to be one of the most important sources of enzymes and other biomolecules of industrial interest [Schallme *et al.,* 2004]. *Bacillus subtilis* demonstrated high COD reduction of 56.19% while *Micrococcus luteus* reduced COD by 52.43% [Usman et al., 2012].

Bacillus subtilis contains catalase KatA and MrgA, an enzyme that is responsible in the catalysis of the decomposition of hydrogen peroxide to water and oxygen, and superoxide dismutase, an enzyme that catalyzes the breakdown of superoxide into oxygen and hydrogen peroxide [Bandow et al., 2002]. Joong Kyun Kim, 2005, studied twenty-four *Bacillus* strains

predominantly outgrown in a night soil treatment system were isolated and characterized. Those species have been reported in waste water treatment. There are many commercial products of this species (*Bacillus subtilis)* to treat waste water in recent market. For example, Roebic Technology, Inc, USA, manufactures bacterial products (specially selected Bacillus bacteria) for waste treatment. Another company named "EcoClear" offers *Bacillus subtilis* in a very cost effective solution to reduce nutrients such ammonia and phosphorus in waste water treatment. The EcoBac offers completely natural, non-engineered bacteria and enzymes that have been selected for their ability to solubilize and digest grease and sludge.

Klebsiella pneumoniae is found in the soil, pulp and paper biosolids and about 30% of strains can fix nitrogen in anaerobic conditions. Alouache et al., 2014, isolated *Klebsiella pneumoniae* from an urban waste water treatment plant in Algeria. Keivan et al., 2014 have isolated and identified a native strain of *Klebsiella pneumoniae* from Isfahan municipal waste water treatment plant. They suggested that the use of these lytic coliphages for reduction of coliform"s population in sewage could be considered as an effective and simple alternative for costly replacement of instruments and establishments of the old waste water treatment plants.

According to Handbook for Biological Waste water Treatment, *Streptococcus sanguinis* are available in waste water and plays important role in waste water treatment [Halkjær et al., 2009]. According to the Practical Handbook of Microbiology, *Streptococcus sanguinis* are available in oil well water, rubber, and waste water treatment and waste gas biofilters [Emanuel and Lorrence, 2008].

Empedobacter brevis are widely distributed in the environment both in soil and water. They are also found in plants, raw meat products, and in hospital environments [Jooste et al., 1999]. There are many evidence where *Empedobacter brevis* are found to be associated with waste water. According to the Susanne Facchin et al., 2013, *Empedobacter brevis* has the potential role in waste water treatment. Werner Manz et. al. 1996, found application of the species in natural environment.

Stenotrophomonas maltophilia is ubiquitous in aqueous environments, soil, and plants; it has also been used in biotechnology applications (for bioremediation). It is a highly versatile species with useful biotechnological potential [Martina et. al., 2011]. The iron-reducing activity of *S. maltophilia* has been applied to phosphate removal from the returned liquor of a municipal waste water treatment plant. The removal of phosphate from the returned liquor of waste water is important, as it reduces the possibility of eutrophication, dissolved oxygen depletion, and a

decreased value of the water supply. *S. maltophilia* BK is able to reduce Fe(III) to Fe(II) using xenobiotics as sole sources of carbon under anaerobic conditions. The production of Fe(II) resulted in the removal of dissolved phosphate and the increased precipitation of phosphate by *S. maltophilia*. [Ivanov et al., 2005]. Both environmental and clinical strains of *S. maltophilia* have been found to contain genes encoding resistance to metals. [Holmes et al., 2009]. *Stenotrophomonas maltophilia* is also useful in Decolourization and Degradation of Textile Dyes [Rajeswari et al., 2013]

Tatumella ptyseos has been found in waste water though the involvement of this organism in pharmaceutical waste water treatment is not well established.

The findings from this study have also showed that, *Bacillus subtilies, Klebsiella pneumonia and Streptococcus sanguinis* have the antibiotic resistance property against applied antibiotic (Table 11). It may be obvious in waste water of multi-drug manufacturing facilities. The waste water containing different types of pollutants may lead to antibiotic resistance. *Klebsiella* organisms are often resistant to multiple antibiotics. Current evidence implicates plasmids as the primary source of the resistant genes. *Klebsiella* with the ability to produce extended-spectrum beta-lactamases (ESBL) is resistant to many classes of antibiotics. The most frequent are resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole. [Hudson et al., 2014 & *Nathisuwan et al., 2001. Streptococcus sanguinis* was found to be resistant to many antibiotics, including carbapenems.

Further study needs to be done to identify the different strains of *Bacillus subtillies* and *Micrcoccus luteus* to increase the efficiency of waste water treatment. More studies will be conducted to identify bacteria associated with waste water treatment of Vaccine, Biologics and Hormones.

Conclusion:

Pharmaceutical waste water contains a number of contaminants in which different microorganisms are present. Some are associated with waste water treatment process some are not. In the present study seven bacteria were characterized and identified to know the types of organisms present in pharmaceutical Effluent Treatment Plant (ETP). Since the ETP is designed to treat effluent by both chemical & biological methods, so some of these bacteria play very important role to treat waste water (effluent) and leave the environment friendly treated water to the environment. There were some bacteria found in the waste water treatment plant the function of which is not established yet. So, there is a great opportunity to work on it. The antibiotic resistance pattern of the identified bacteria was also performed and found that, some of the strains are resistant to all antibiotics (which are used to examine). Since the waste water contains various odd situations so some bacteria has found its way to survive this kind of unpleasant situation. So, there is also a great opportunity to find out the resistant strains and possible antibiotics by which these are sensitive. Water from the surrounding environment may be subjected to test to find the situation of the microfloara. This will help to think about the disease & treatment pattern of the inhabitants.

CHAPTER 6:
REFERENCES

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APPENDICES

APPENDICES

Appendix-A

Microbiological Media

Media used were prepared methods using appropriate compositions. Components used high grade. All media were sterilized by autoclaving for 20 minutes at 121^0 C & 15 lb pressure. The compositions used for different media have been shown below:

A.1 Composition of R2A media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Yeast Extract	00.50
Proteose Peptone No. 3.	00.50
Casamino Acids	00.50
Dextrose	00.50
Soluble Starch	00.05
Sodium Pyruvate	00.03
Dipotassium Phosphate	00.03
Magnesium Sulfate	00.05
Agar	15.00

A.2 Composition of TSA media:

Name of the Ingredients	**Amount (in gm) of the Ingredients**
Pancreatic Digest of Casein	15.00
Enzymatic Digest Soya bean	05.00
Sodium Chloride	05.00
Agar	15.00

A.3 Composition of MAC agar media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Peptone	17.00
Proteose peptone	03.00
Lactose	10.00
Bile salts	01.50
Sodium chloride	05.00
Neutral red	00.03
Crystal Violet	00.001
Agar	13.50

A.4 Composition of EMB agar media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Enzymatic Digest of Gelatin	05.00
Lactose	05.00
Dipotassium Phosphate	01.00
Eosin Y	0.025
Methylene Blue	0.001
Agar	15.00

A.5 Composition of BG agar media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Proteose peptone	10.00
Yeast Extract	03.00
Lactose	10.00
Sucrose	10.00
Sodium Chloride	05.00
Phenol Red	00.08
Brilliant Green	00.0125

A.6 Composition of MSA media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Enzymatic Digest of Casein	05.00
Enzymatic Digest of Animal Tissue	05.00
Beef Extract	01.00
D-mannitol	10.00
Sodium Chloride	75.00
Phenol Red	0.025
Agar	15.00

A.7 Composition of PCA media:

Name of the Ingredients	Amount (in gm) of the Ingredients
Peptone	05.00
Yeast Extract	02.50
Glucose	01.00
Agar	15.00

Appendix-B

Preparation of Reagents & Chemicals Used in the Physico-chemical analysis of effluent

There are various reagents are used for different purpose. Some of them are directly used some are prepared in the lab. The commonly used different reagent preparation & composition are shown below:

- **Preparation of Manganese Sulfate solution:**

  18.2 g of manganese sulfate mono hydrate ($MnSO_4.H_2O$) is taken in 100 mL volumetric flask and 50 mL of water is added to dissolve. Finally water q.s. to 100 mL is added. The mixture should be sonicated for at least 15 minutes until get a clear pink solution.

- **Preparation of Alkaline Azide-Iodide solution:**

  25 g of sodium hydroxide (NaOH) is taken in 100 mL beaker and 25 mL of water is added to dissolve. Then 6.75 g of sodium iodide is added in it. Again 0.5 g of sodium azideis taken in another 25 mL beaker and 10 mL of water is added to dissolve. These two solutions are then mixed in 50 mL volumetric flask and finally water q.s.is added to 50 mL.

- **Preparation of 0.025 N Sodium Thiosulfate solution:**

  3.125 g of sodium thiosulfate (Na2S2O3.5H2O) is taken in 500 mL volumetric flask and 250 mL of water is added to dissolve. Then 25 mg of sodium carbonate is added in it and is mixed well. Finally water q.s. is added to 500 mL.

- **Preparation of starch solution:**

  1 g of starch is taken in a 100 mL volumetric flask and 50 mL of boiled water is added to dissolve. Then 10 mg of mercuric iodine is added in it and is sonicated the solution. Finally water q.s. is added to 100 mL.

- **Factor determination of $Na_2S_2O_3$:**

  52.5 mg K2Cr2O7 is taken in a 100 mL conical flask and it is dissolved in 100 mL water. Then 0.75 g of KI, 0.5 g of NaHCO3 and 1.25 mL of HCl are added in the flask. Then the solution is kept in dark place for 5 minutes. Then, 1 mL of Starch solution is added in it and then it is titrated against 0.025 N Na2S2O3 solutions. This procedure is repeated 3 times to determine the factor by averaging 3 values.

$$F = \frac{\text{Weight of Potassium Dichromate}}{49.04 \times 0.025 \times \text{Titer Volume of Sodium Thiosulfate}}$$

Where, 49.04 = a constant value

- **Preparation of Phosphate Buffer:**

  Take 850 mg of potassium dihydrogen phosphate (KH_2PO_4), 2.18 g of dipotassium hydrogen phosphate (K_2HPO_4), 3.34 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), and 170 mg of ammonium chloride (NH_4Cl) in 100 mL volumetric flask. Add 50 mL water to dissolve. Finally add water q.s. to 100 mL and mix well.

- **Preparation of Calcium Chloride Solution:**

  Take 2.75 g of anhydrous calcium chloride ($CaCl_2$) in 100 mL volumetric flask. Add 50 mL of water to dissolve. Finally add water q.s. to 100 mL and mix well.

- **Preparation of Ferric Chloride Solution:**

  Take 25 mg of ferric chloride hexahydrate ($FeCl_3.6H_2O$) in 100 mL volumetric flask. Add 50 mL of water to dissolve. Finally add water q.s. to 100 mL and mix well.

- **Dilution Technique:**

  Dilution Technique Initially makes several dilutions of the prepared sample so as to obtain the required depletion. It is better to analyze the COD since it gives an approximate idea of the BOD excepted. The following dilutions are suggested for known COD values.

COD	**Sample Size for BOD**
5000-10000	0.5 ml/L
2000-5000	1.0 ml/L
1000-2000	2.0 ml/L
500-1000	5.0 ml/L
0-500	10.0 ml/L

If the COD is still higher than 10000 proper dilution can be selected. Add 1 mL of each buffer phosphates, magnesium sulphate (MgSO4), calcium chloride (CaCl2) and ferric chloride (FeCl3) solution for each litre of dilution water and mix well. Adjust pH 7.0 with NaOH or HCl.

- **Preparation of dilution water:**

  Take 1600 mL of distilled water in a mug and add 1.6 mL of each phosphate buffer, magnesium sulphate ($MgSO_4$), calcium chloride ($CaCl_2$) and ferric chloride ($FeCl_3$) solution and mix well.

- **Preparation of 0.25 N Standard Potassium Dichromate Solution:**

  Take 6.13 g of potassium dichromate ($K_2Cr_2O_7$) primary standard grade (previously dried at 103 ^{0}C for 2 hours) in 500 mL volumetric flask .Add 250 mL of water to dissolve. Then add 60 mg of sulfamic acid. It will eliminate the interference due to Nitrates. Finally add water q.s. to 500 mL.

- **Preparation of 0.1 N Standard Ferrous Ammonium Sulfate (FAS) solution:**

  Take 39 g of ferrous ammonium sulfate hexahydrate in 1000 mL volumetric flask. Add 20 mL of concentrated sulfuric acid (H_2SO_4) under fume hood. Allow to cool and finally add water q.s. to 1000 mL.

  Note: This solution must be standardized daily against 0.25N $K_2Cr_2O_7$ solution.

- **Preparation of Ferroin Indicator:**

  Take 1.485 g 1, 10 phenanthroline monohydrate in 100 mL volumetric flask. Add 50 mL of water to dissolve. Then add 695 mg of ferrous ammonium sulfate heptahydrate (FeSO4.7H2O). Finally add water q.s. to 100 mL and mix well.

Appendix-C

List of Instruments Used in the Research

Sl. No.	Name of the Instrument	Model	Origin/ Brand
1.	Antibiotic Zone Reader, Protocol	PROC-SR+PROC+LCD17	Synbiosis, UK
2.	Autoclave, Tatneur	5075 EL	Tattneure, Natherland
3.	Bacterial ID Kit	BBL Crystal ^{TM}	BBL Crystal, USA
4.	Balance (max.4200 gm)	CP4202S	Sartorius Germany
5.	Bio-Safety Cabinet, Class-ll;	BBF6	GermFree, Germany
6.	Computer System (BBL)	-	-
7.	Depyrogenation Oven	Memmart	Memmart, Germany
8.	Hot plate with magnetic stirrer	SWT-270-010J…	Bibby Sterlin, UK
9.	Incubator- Walk-in-Type - Cooled (B. O. D. incubator)	G. M. P.	Thermolab, India
10.	Incubator- Warm	BK44901	Ehret, Germany
11.	Incubator- Warm	BK4444	Ehret, Germany
12.	Incubator- Walk-in-Type - Warm (B. O. D. incubator)	G. M. P.	Thermolab, India
13.	Inspection Table	-	-
14.	Inverted Microscope	40C	Axeovert, Germany
15.	LaminarAir Flow (Horizontal)	BZ6	GermFree, Germany
16.	Magnetic Stirrer	wisestir.MSH-30D	Daihan Scientific
17.	P^H Meter	S20	Mettler-Toledo
18.	Refrigerator/ Freezer (Dual)	RF1500C	Pbi. Intl. Italy
19.	Refrigerator/ Freezer (Dual)	SJ-PE67LH-HS	SHARP
20.	Vortex Mixer	15824	Pbi. Intl. Italy
21.	Water Bath	20174+17540+17541	Pbi. Intl. Italy
22.	Water Bath	WB-6/-11/-22	Daihan Scientific
23.	Whirlimixer (Vortex Mixer)	FB6500	Fisher Scientific

Appendix-D

List of Glass Ware Items & Others Used in the Research

Serial No.	Name of the Glass Ware and others
1.	Beaker
2.	BOD Bottle
3.	Conical flasks
4.	Cotton plug
5.	Disposable micropipette
6.	Inoculating loop
7.	Knife and forceps
8.	Measuring cylinder
9.	Micro pipettes
10.	Petri dishes
11.	Pipettes
12.	Screw cap bottle
13.	Slide
14.	Spreader/ Streaking device