Part-I

Introduction, Objectives and Review of Literature
1.1. INTRODUCTION

The management of domestic and municipal waste is a major problem for both rich and poor countries. In industrialized or developed countries, waste management imposes significant and frequently excessive burdens on the budgets of local and national governments. In developing countries like Africa, Asia and South America, inadequate waste management has frequently created serious public health problems which have hampered social and economic development.

At the present time, most of the populated areas of the world have run out of places for the disposal of domestic and industrial wastes. Earlier practices of disposal in the ocean, on land, or underground are being severely controlled. More recently, national and international agencies dealing with environmental quality and public health realized that the most logical solution lies in recycling, i.e. the recovery and reuse of waste components.

In view of the continued population growth, shortage of arable land, and the chronic scarcity of fresh water, the reuse of wastewater is drastically increasing which would in turn lead to improvements in public health and the quality of the environment.

Wastewater can be treated by different ways. In urban areas and in industrial countries, very often the sewage system is highly centralised, and sewage is conducted to large conventional wastewater treatment plants.

Biological wastewater treatment includes all methods that involve organisms, and emulate at least some of the processes which take place in natural environments. Some biological treatment methods emulate decomposer communities and degradation processes predominate (treatment with activated sludge), whereas others emulate productive ecosystems with assimilation and utilization of energy (wastewater-fed aquaculture) through integrated food web.
Wastewater-fed aquaculture (WFA) is an ancient, innovative and productive way to treat and recycle wastewater. It is a constructed aquatic ecosystem, consisting of one or several water bodies with an integrated food web, is charged with nutrient rich wastewater (Figure 1.1.) The central aim of the system is the assimilation of dissolved nutrients into biomass, mainly fish. Simultaneously organic compounds are either consumed or mineralized, and in consequence the wastewater gets purified.

Figure 1.1. Community based waste fed wet land system

Wastes are undoubtedly valuable sources of nutrients to power autotrophic production and as substrates for the heterotrophic community. They contain not only nitrogen, phosphorus and micronutrients, but also large quantities of carbon. The source of this improved production appears to be mainly from the stimulation of the autotrophic food chain. The heterotrophic food chain is also stimulated both from the detritus in the waste and that produced within the pond in the form of dead and dying algae (Mara, 1998) (Figure1.2.).
Organic wastes are a more complete source of nutrients for dense blooms of phytoplankton and allow higher productivity than is possible with chemical fertilizer alone. Studies have indicated that inorganic fertilizers alone produce lower fish yields (10 to 15 kg/ha/day) than is possible using organic waste (about 30 kg/ha/day) (Schroeder, 1979).

Wastewater-fed aquaculture often proves to be a sustainable biological wastewater treatment method, because it is nature-like, low-tech and income-generating. The use of wastewater in aquaculture may, however, be considered to be more effective than agriculture because of the opportunity of using larger volume of the effluent in a comparatively smaller area of land and also due to its highly lucrative return.

A wide range of fish species has been cultivated in aquaculture ponds receiving waste. To optimize fish production in such waste fed ponds, the majority of the fish should be filter feeders, to exploit the plankton growth. It is preferred to stock more omnivorous scavengers and bottom feeders to maintain fish pond hygienic and for higher yield (Pandey and Shukla, 2007). Tilapia is considered suitable for culture in wastewater because of its high tolerance to adverse environmental conditions and resistance to poor water quality and disease (Guerrero,
Carp, on the other hand, very sensitive to dissolved oxygen of water. They are thus raised in ponds receiving diluted sewage water. Usual ratio followed for better production is Catla 1: Rohu 2.5: Mrigal 2.5: Common carp 2: Silver carp 2 (Pandey and Shukla, 2007). Growth rate of fish reared in wastewater is significantly higher than that of fish reared in natural habitat (Khalil and Hussein, 1997). Furthermore, fishes from sewage ponds were having better taste than those of freshwater cultured fishes (Pandey and Shukla, 2007).

Wastewater, though nutrient rich, but wastewater fed fishponds may be dangerous because of the possible pollution by toxic chemicals, heavy metals and pathogenic micro organisms (Aeromonas spp., Salmonella spp. and Pseudomonas spp.), which may accumulate in fish (McIntyre and Mills, 1975). In developing countries, excreta-related diseases are very common, and wastewater contains correspondingly high concentrations of excreted pathogens-the bacteria, viruses, protozoa, and the helminths (worms) that cause disease in man. There are approximately thirty excreted infections of public health importance, and many of these are of specific importance in wastewater use schemes (Strauss, 2000). Body surface (skin) and digestive tract of waste fed fish are most easily contaminated, and eventually also internal organs and muscles. As a result potential health risk to fish handlers and consumers from waste fed fisheries is very high (Geldreich and Clarke, 1966) which demands a periodic and comprehensive sanitary survey of wastewater fishery.
1.2. AIMS AND OBJECTIVES

Fertilization of aquaculture ponds with domestic wastes has been practiced for thousands of years in many parts of the world; today at least two-thirds of the world production of farmed fish comes from ponds fertilized in this way. Aquaculture-wastewater systems have two purposes, treating wastewater and producing a useful product. This means that the system must be able to produce an acceptable effluent as well as a product that does not contain unacceptable levels of harmful chemicals, pathogenic bacteria or viruses (Hejkal et al., 1983). But the motives for developing and developed countries in reuse of wastes in agriculture and aquaculture are different (Henderson and Wert, 1976 and Duffer and Moyer, 1978). For developing countries, the main motive is the production of food while for developed countries it is the wastewater treatment. In Calcutta and its suburbs since 1930, pisciculture has been in practice using sewage water without any prior treatment. As a result Wastewater, although sufficient as a source of nutrients, can present problems such as toxicity to fish, accumulation of heavy metals and toxic substances including pathogenic microorganisms in the muscles of fish, and the potential danger of transmission of pathogens from wastewater to handlers and consumers (Edwards, 1985; Buras et al., 1987 and Mara and Cairncross, 1989).

Wastewater aquaculture presents nowadays several concerns, mainly related to health hazards generated by the use of water recovered from sanitary sewerage systems (Salgot, Verges’ and Angelakis, 2003). Household chemicals (e.g. detergents, deodorizers and degreasers), pharmaceuticals (e.g. hormones, steroids and antibiotics), and other personal care products (PCP’s) (e.g. antacids, caffeine and fragrances) are being washed down sinks and flushed down toilets all over the world into sewerage system. Of these different products antibiotic usage has received a lot of attention in the media in the last several years due to the increasing numbers of diseases becoming resistant to traditional treatments. Antibiotics are not entirely processed by human bodies; some are expelled as waste and wind up in wastewater ponds. These antibiotics have caused development of antibiotic resistant bacteria as well as resistant plasmids and contaminate fish.

One of the important concerns of wastewater fisheries is the contamination of fishes by faecal bacteria. Their presence in fish intended for human consumption may constitute a potential
danger not only by causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human-infecting bacteria from non aquatic sources (Fapohunda, MacMillan, Marshall and Waites, 1994).

In recent times increased attention is given to the possibility of cultured fish as vector of human pathogenic bacteria (Apun et al., 1999 and Islam et al., 2001). Fish living in natural environment are known to harbor pathogenic enterobacteriaceae (Pillay, 1990). Invasion of fish muscles due to the breakage of immunological barrier of fish by pathogens is likely to occur, when fish are raised in ponds with faecal coliforms, *E.coli* and *Salmonella* of greater than $10^4$, $10^3$, and $10^5$ per 100 ml in pond water, respectively (Guzman et al., 2004). Probability of contamination increased with time of rearing in polluted water; accumulation of intestinal micro-organisms and pathogens on body surface or their penetration into edible tissues was unlikely when bacterial concentration in water was under $10^3$ per 100 ml; high concentrations of pathogenic micro-organisms might occur in the digestive tract and in intraperitoneal fluid of the fish even at low numbers of indicatory bacteria (Niewolak, 2000). Ogbonedeminu (1989) reported that 50% of the microorganisms recovered from both fish and water of earthen pond fertilized with faecal waste were members of the family Enterobacteriaceae. In addition most diseases in humans are caused by opportunistic enteric pathogens, which are prevalent in the rearing environment of fish (Brock, 1993 and Jayasree et al., 1999). Reports of the occurrence of pathogenic strains of *E.coli* from fisheries sources and outbreaks of illness due to them were also increasing (Teophilo et al., 2002).

For these reasons, European United Countries and United States do not recommend fish culture in sewage supplied ponds (Bryan, 1977 and Hejkal et al., 1983). Also, the consumers often reject such fish and their flesh is used for the preparation of feed of domestic animals.

However, the aquacultural use of wastewater can only result in an actual risk to public health a) If an infective dose of pathogen is present in pond; b) If infective dose reaches a human; c) If host becomes infected; and d) If infection causes diseases / further transmission. (a), (b) and (c) constitute potential health risk and (d) constitutes actual risk to health (Strauss, 2000).
In order to assure the microbiological safety of fish raised in wastewater-fed fishponds the World Health Organization’s guideline is that the fishpond water should have a faecal coliform count of \( \leq 1000 \) per 100 ml (WHO, 1989). These guidelines, based on the existing knowledge on the use of wastewater in aquaculture should, according to the WHO, ensure full protection of fish flesh against bacterial contamination. The possibility of self-purification of fish from bacteria after their transfer into clean water is a yet another practical issue.

In Calcutta and its suburbs sewage-fed fisheries play a great role in the improvement of water quality and can be compared with efficient stabilization pond as far as fish production is concerned. Moreover, the potential public health hazard has never been considered although good production of fish was obtained (Strauss, 1997).

Accumulation of bacteria has been one of the public health concerns in waste fed aquaculture. Fish in the polyculture system, providing cheap source of animal protein to the fish eating population of this city deserves, assessment of bacteria in their tissues to provide exact information regarding the microbial contamination in the biota and extend of pollution in the environment.

Therefore, the principle objective of the present study is to evaluate the bacteriological contamination associated with waste fed fisheries and purification of different fish species reared in wastewater ponds.

Although numerous microbiological studies have been made on fish intestinal material, many of the data are qualitative and are based on technical procedure with limited sensitivity and selectivity. Insufficient data in the literature on quantitative recovery of indicator, pathogenic as well as drug resistant bacteria that could be applied to water pollution studies prompted this investigation.
The research project was developed to include the following areas:

a) Quantification of indicator and pathogenic bacteria in muscles and gut of a wide variety of waste fed fish.

b) Determination of Possible correlation of these pollution indicators in fish with those of their environment by analyzing physicochemical as well as bacteriological contamination in wastewater fed fish pond.

c) Enumeration of transferable drug resistant thermotolerent faecal coliform bacteria from waste fed environment.

d) Enumeration of bacterial load in post harvested fish after keeping waste fed cultured fish in fresh water environment.

The first part of the investigation thus deals with the study of biological surveys of the receiving wastewater, to establish the health of the system, with measures based on fish fauna as they may act as suitable biomarkers.

The second part of the investigation comes up with a possible suggestion to minimize the accumulation of microbes in fish tissues. Sewage-fed fish were kept in clean uncontaminated fresh water under laboratory conditions, to note the depuration pattern of bacteria in fish.

Statistical analysis and conclusive remarks mark the end of the investigation.
**Quality assessment of waste fed aquaculture**

- **Physicochemical parameters of water**
  - I) Removal
  - II) Seasonal Variations
  - III) Correlation
- **Bacteriological parameters**
  - Water
  - Fish
  - Contamination of Indicator and Pathogenic bacteria
    - a) Quantitative measurement in fish tissue
    - b) Seasonal Variations
    - c) Correlation
  - Purification of contaminated fish
- **Antibiotics**
  - Drug resistant bacteria
    - Measurement of transfer pattern of resistance factors
      - a) Plasmid isolation
      - b) Mating experiment
1.3. LITERATURE REVIEW

Fish farming is becoming an increasingly important as a source of income for farmers as it is a high value crop and consumer demand for fish is increasing. More recently, domestic effluents are extensively used in aquacultural ponds, as their organic loads provide excellent low cost nutrients to the growing fish. Interest in wastewater fed fish farming is based on its cost effectiveness and in resource recovery from the investment in wastewater treatment e.g. through the use of effluent from waste stabilisation ponds in fish ponds (Ursula et al., 2000).

1.3.1. Waste fed practices – Past and Present

Waste-fed aquaculture is centuries old in several countries in East, South and Southeast Asia, especially China. It has been developed mainly by farmers and local communities to use nutrients contained in wastes to produce aquatic food, mainly fish. Wastes and faecally polluted surface water are often used in aquaculture without any pretreatment (WHO/FAO/IDRC CRDI/IWMI, 2008).

Wastewater stabilization pond systems are designed to achieve different forms of treatment in up to three stages in series, depending on the organic strength of the input waste and the effluent quality objectives. For ease of maintenance and flexibility of operation, at least two trains of ponds in parallel are incorporated in any design. Strong wastewaters, with BOD concentration in excess of about 300 mg/l, will frequently be introduced into first-stage anaerobic ponds, which achieve a high volumetric rate of removal. Weaker wastes or, where anaerobic ponds are environmentally unacceptable, even stronger wastes (say up to 1000 mg/l BOD) may be discharged directly into primary facultative ponds. Effluent from first-stage anaerobic ponds will overflow into secondary facultative ponds, which comprise the second-stage of biological treatment. Following primary or secondary facultative ponds, if further pathogen reduction is necessary, maturation ponds will be introduced to provide tertiary treatment. Typical pond system configurations are given in Fig. 1, though other combinations may be used (Pescod and Mara, 1988) (Figure 1.3.)
In Europe, the first significant trial started in Germany in the year 1929, with the aim of utilizing sewage oxidation ponds for aquaculture, with organic recycling benefits of both resource conservation and wastewater disposal whereas, in Israel, wastewater fish culture was developed mostly due to scarcity of the available water potential. A number of workers have documented the increased fish production using domestic sewage. The Chinese fish farming practices have been described by Ryther (1979) and Hu and Zhou (1989). Edwards et al., (1985) have reviewed sewage fed fish culture in Thailand and India.

In India there are more than 130 urban centers where sewage is being utilized for crop irrigation, the total area being more than 12,000 ha (Strauss, 1997). Major research work on sewage-fed aquaculture in India has been conducted in four centers of the country namely Chenni, Nagpur, Rajpur and Calcutta. At present sewage is reused in well defined sewage-fed fisheries in India, particularly in West Bengal, the only state where sewage is widely used for fish culture (Saha, 1970; Saigal, 1972; Dehadrai and Ghosh, 1977 and Bhowmik, 1990). The sewage fed fishery started independently in Calcutta, West Bengal in 1920-30 (Ghosh and Sen, 1987) which city has now the largest wastewater fed aquaculture system in the world. In India the Calcutta wetlands are the world's largest wastewater fisheries, comprising nearby an area of 4000 ha and providing employment for 4,000 families (Edwards and Pullin, 1990).

Wastewater systems in India are designed primarily for aquaculture, not to treat wastewater. There is a great diversity in current waste-fed aquacultural practice involving fish and aquatic plants. Most waste-fed aquaculture involves the direct addition of waste with little or no prior treatment, or faecally contaminated surface waters to produce human food. Fish species cultivated are mainly carps and tilapias (WHO/FAO/IDRC CRDI/IWMI, 2008).

1.3.2. Water and Wastewater Constituents

Municipal wastewater is mainly comprised of water (99.9%) together with relatively small concentrations of suspended and dissolved organic and inorganic solids. Among the organic substances present in sewage are carbohydrates, lignin, fats, soaps, synthetic detergents, proteins and their decomposition products, as well as various natural and synthetic organic chemicals from the processed industries. Wastewater also contains a variety of inorganic...
substances from domestic and industrial sources including a number of potentially toxic elements such as arsenic, cadmium, chromium, copper, lead, mercury and zinc etc. Pathogenic viruses, bacteria, protozoa and helminths present in wastewater and survive in the environment for long periods.

Varieties of physical, chemical and biological constituents of wastewater (Tchobanoglous et al., 2003) are described below.

1.3.2.1. Physical Characteristics

The physical characteristics of wastewater include total solids that are composed of floating, colloidal and settleable particles. Other important physical characteristics include turbidity, color, temperature, conductivity, density, specific weight and specific gravity (Sujatha, 2007).

1.3.2.2. Chemical Characteristics

The chemical characteristics of wastewater are mainly divided into inorganic and organic. Inorganic constituents include nutrients, and metallic and non-metallic constituents. Organic chemical constituents are represented by BOD and COD (Sujatha, 2007).

1.3.2.3. Biological Characteristics

The biological characteristics of wastewater include pathogenic organisms of human and animal origin. Potential infectious agents in untreated wastewater include bacteria, protozoa, helminthes and viruses (Tchobanoglous et al., 2003).

Among these characteristics, the biological characteristics are of fundamental importance because they include disease causing pathogenic bacteria of human origin (Sujatha, 2007).

a) Indicator Organisms for Faecal Contamination of Water

In early 1880's it was realized that pathogenic bacteria from human origin cause faecal contamination in water (Huber, 1971). Faecal indicator organisms are therefore, used as
measures of surface water and wastewater quality. The Environmental Protection Agency (EPA) has noted that the occurrence of these faecal indicative in water is an indication of potential presence of pathogenic organisms capable of posing a threat to public health (EPA, 2006). Faecal indicator organisms include total coliforms, faecal coliforms, faecal streptococci and *Escherichia coli* (Tchobanoglous et al., 2003). These organisms are the natural inhabitants of gastrointestinal tracts in humans and warm-blooded animals and are discharged into wastewater treatment plants through human faeces. Faecal indicators have also been found responsible for various diseases including cholera, typhoid, hepatitis, diarrhoea and endocarditis (Aggarwal and Krawczyński, 2000 and Bajracharya et al., 2006).

1) **Faecal coliforms**

Faecal coliforms are gram-negative bacteria that live in the digestive tract of warm blooded animals and humans (Qasim, 1998). They are the indicators of potentially pathogenic bacteria from faecal origin (Asano, 1998). Faecal coliforms are excreted in the faeces by humans and animals and ultimately reach wastewater treatment plants. Hence, a huge amount of faecal coliforms are observed in raw wastewater.

2) **Escherichia coli**

*Escherichia coli* (*E.coli*) are used as indicators of microbiological quality of water. They are gram-negative bacteria and they are found naturally in both human and animal intestines. Several strains of *E. coli* are human friendly but few like *E. coli* 0157:H7 are pathogenic to humans. Several intestinal and extra intestinal infections such as urinary tract infection, meningitis and diarrhea are caused by *E.coli* 0157:H7 (Sussman, 1997).

3) **Faecal streptococci**

Faecal streptococci is a gram-positive bacterium commonly present in human intestines and have been recognized as potentially pathogenic bacteria for humans for many years (Gilmore, 2002). The enterococci species *Enterococcus faecalis* and *Enterococcus faecium* have been identified as the most prevalent species responsible for clinical infections in humans (Gilmore, 2002). Many infecting strains originate in human intestines (Murray, 1990).
1.3.3. The risk dimension of waste fed aquaculture system

Various hazards are associated with waste-fed aquaculture: excreta-related pathogens (bacteria, helminths, protozoans and viruses), skin irritants, vectors that transmit pathogens and toxic chemicals. Fish passively accumulate microbial contaminants on their surfaces which may penetrate into edible fish flesh or muscle. The relative risk of disease from bacteria e.g. *Salmonella* spp., protozoa e.g. *Giardia* sp. and viruses e.g. hepatitis is low to medium. Some of these microbes may be present in the gut of fish. Cross-contamination of foods at the market place or in the kitchen is the greatest risk which is reduced by hygienic processing and cooking. Regarding the risks from chemicals, the risk from antibiotics is also high though they are not usually used in waste-fed aquaculture (WHO/FAO/IDRC CRDI/IWMI, 2008).

1.3.3.1. Factors determining the transmission of domestic waste-related infections

Die-off or survival of excreted pathogens is an important factor when considering the public health dimension of waste-fed aquaculture. In principle, all pathogens die off exponentially upon excretion, however, at different rates (Strauss, 1997).

The main factors influencing die-off are temperature, dryness and UV-light. Die-off rates increase in proportion to the level or intensity of these variables (Mara, 1997) (Figure 1.4.). NH₃ (ammonia) is known to be bactericidal. Therefore, bacteria die-off is enhanced at pH ≥ 8.5-9 due to an increase in the NH₃/NH₄ ratio (Strauss, 1997).
Another important factor is the **infective dose** of pathogens or the dose required for a human host to create the disease. For helminths, protozoa (*Giardia lamblia* and *Amoeba* spp.) and viruses, the infective dose is low (< $10^3$). For bacteria, it is medium (~ $10^4$) to high (> $10^6$). This influences the choice and effectiveness of health protection strategies in reducing transmission risks from the various kinds of pathogens. Manifestation of the disease differs among the pathogens: with viruses, protozoa and bacteria, an infected person will either become ill or not, depending on whether the infection is symptomatic. With helminths, however, an infected person will exhibit various degrees of disease intensities depending on the number of worms it carries in its intestines. Thus, when considering an excreta or wastewater treatment strategy, a reduction of worm eggs by e.g. 80-90% already has a major positive public health effect. With viruses, bacteria and protozoa, higher degrees of removal are required; i.e., 99.9-99.999% or 3-5 log cycles (Strauss, 1997).

**Latency** is the period between the excretion of a pathogen in the human stool and its becoming infective again. It is zero for protozoa, bacteria and viruses. Therefore, short transmission routes such as person-to-person contacts normally play a proportionally more important role for these pathogens than longer transmission paths such as via soil or crops. For helminthic infections, the latency lasts for several weeks. This renders helminths
potentially important in the longer transmission paths such as through reuse in agriculture or aquaculture (Strauss, 1997).

1.3.3.2. Parameters quality of wastewater used in aquaculture

a) Parameters of aquacultural significance (Physicochemical qualities)

Waste stabilization ponds (WSP) are not normally considered as a reliable technical option for nutrient removal from domestic wastewater (Abis and Mara, 2003).

Facultative ponds (1-2 m deep) are of two types: primary facultative ponds, which receive raw wastewater, and secondary facultative ponds, which receive settled wastewater (usually the effluent from anaerobic ponds). A typical facultative pond, the water column will be predominantly aerobic at the time of peak sun radiation and predominantly anaerobic at sunrise. After sunrise, the dissolved oxygen level gradually rises to a maximum in the mid-afternoon, after which it falls to a minimum during the night. The wind has an important effect on the behavior of facultative ponds, as it induces vertical mixing of the pond liquid. Good mixing ensures a more uniform distribution of BOD, dissolved oxygen, bacteria and algae and hence a better degree of waste stabilization (Tchobanoglous and Schroeder, 1985) (Figure 1.5.).
The activity of further anaerobic oxidation and the aerobic conversion of effluent to carbon dioxide, water and new bacterial and algae cells can result in removal of 80% of the BOD of the effluent flowing into the facultative pond. This removal, and the subsequent quality of the outflow, depends on an adequate oxygen supply, sufficient retention time and warm temperature (Marais, 1970) (Figure 1.6.).
In a WSP system, maturation ponds are most commonly designed to reduce the number of pathogenic organisms from secondary effluents (facultative pond effluents), but they also make important improvements in physicochemical wastewater quality by reducing the concentrations of organic matter (BOD) and suspended solids and, additionally, they can make a significant contribution to cumulative nitrogen and phosphorus removal in WSP systems (Mara et al., 1992 and Mara and Pearson, 1998).

The health of fish depends on water quality that is the chemical, physical and microbial content of the water (Ampofo, 2003). In a successful aquaculture system there must be both an organismic balance, to produce an optimal supply of natural food at all levels, and a chemical balance, to ensure sufficient oxygen supply for the growth of fish and their natural food organisms and to minimize the build-up of toxic metabolic products (Colman and Edwards, 1987).

Loadings up to 100 kg COD/ha/day did not result in a level of faecal coliforms in the pond water high enough to penetrate the fish muscle (Choudhury et al., 1981). The physico-chemical characteristics of the pond water loaded with 75 and 150 kg COD/ha/day were favourable for tilapia growth most of the time (Polprasert et al., 1984 and Sharma et al., 1987).

Fish production in a water body is mainly based on the food or planktons available in the water body, which in turn depends upon the nutrients available in the water. It has been considered that nitrogen and phosphorous are the two nutrients mainly responsible for the plankton production and growth (Bansal et al., 2007). So the enrichment of these nutrients would increase the fish production. But if it exceeds certain limit, it may cause nuisance algal blooms, subsequently depletion of dissolved oxygen (DO) and fish kill etc. (Edwards et al., 1990). Proper design of an aquaculture farm is therefore required for efficient use of the resources available in the sewage water (Bansal et al., 2007).

Previously, biochemical oxygen demand, suspended solids and faecal coliform bacterial count have commonly been used as criteria for assessing the efficiency of sewage treatment facilities, while nutrient parameters have largely been overlooked (Toms et al., 1975 and Hussainy, 1979). Recently, more attention has been paid to the eutrophication of receiving water bodies which is mainly caused by nutrient enrichment from the effluent of waste
treatment facilities. To date, nutrient parameters have become an integral part of effluent discharge guidelines set by environmental protection agencies (Patrick, 1997). In particular, more attention should be paid to the control of nitrogen in water bodies receiving point source discharges where nitrogen is often limiting for algal growth (Gakstatter et al., 1978).

Wetland plants are very efficient in removing nutrients from polluted waters, minimising eutrophication of the aquatic habitat that, otherwise, can create oxygen depletion and cause the fish to die. There are at least 12 aquatic vascular hydrophytes in and around the East Calcutta wetlands that are significant for their bio-filtering potential particularly with respect to BOD, COD, nitrate and phosphate level (Ghosh and Santra, 1996).

b) Parameters of health significance

Studies of the microbiological quality of fish raised in wastewater-fed aquaculture systems have been used to recommend criteria for acceptable bacterial levels in fishpond water and fish muscle.

The sewage fed fish ponds are batch-fed with untreated sewage at low organic loading rates which would maintain rather safe hygienic quality of the pond water; with total coliform concentrations reportedly amounting to $10^2-10^3/100$ ml (Strauss, 1990). This corresponds to a faecal coliform level of $10^1-10^2/100$ ml and would be equivalent to the quality of many surface waters from where fish are caught without restriction (Strauss, 1997). If these values are confirmed and represent a long-term pond quality average, the potential health risks for fish consumers from pathogens contained in this waste-fed fisheries scheme could be judged minimal (Strauss, 1997).

The total coliform concentration in the Munich waste fed fish pond water was reported as $3 \times 10^2 - 1.5 \times 10^3/100$ ml (Prein, 1988). This may be taken as equivalent to a faecal coliform concentration of $10^1-10^3/100$ ml; i.e., levels which are considered safe for the fish flesh to remain free from pathogens (Strauss, 1997).

Several studies regarding the bacterial contamination of fish reared in sewage-fed fish ponds have been performed and are reported in the literature by Hejkal et al., (1983), Buras et al., (1987), CEPIS (1991) and Van den heever et al., (1994). An extensive review has been
published by Edwards (1992). Muscle tissue, as the edible part of the fish, is relatively safe from the invasion of bacteria. Invasion occurs only if fish grown in water containing high levels of microorganisms (Strauss, 1997). Buras et al., (1987) reported that when fish accumulate bacteria beyond a given threshold concentration (approx. $10^4$ tot. bacteria or SPC/g of *Oreochromis aureus* (tilapia) or *Cyprinus carpio*, (common carp), their capability to cope up with high concentrations of particles diminished and bacteria began to invade muscle tissues and other organs. Bacterial concentrations in pond waters, which corresponded to the threshold levels in fish, amounted between 1.0 and $5.0 \times 10^6$ SPC/100 ml. CEPIS (1991) investigated the hygienic quality of *Oreochromis niloticus* (Nile tilapia) in fish ponds fed with maturation pond effluent of approx. $10^4$ faecal coliforms/100 ml. FC concentrations within the fish ponds ranged from $10^2$ to $3.3 \times 10^3$ /100 ml. The fish were allowed to grow for 4-5 months. *Salmonella* recovery from the fish fillets were always negative. Total coliform (TC) levels ranged from $10^1$ to $2 \times 10^3$ /g per fish fillet and may indicate FC levels amounting to $100 - 10^2$/g. Microbiological evidence acquired to date suggests that there is little likelihood of enteric organisms, including pathogens, invading edible fish tissues if the FC concentration in fish pond water is $< 10^3$ per 100 ml (Buras et al., 1987). However, even at lower microbial levels in the pond water, the fish usually accumulate high load of microorganisms in the digestive tract and in the intraperitoneal fluid (Strauss, 1997). The public health risk of waste-fed fish may therefore be associated mainly with the potential cross-contamination of the fish fillets through high loads of microorganisms contained in other organs of the fish (Strauss, 1997). Persons processing the fish may become foci of disease transmission unless they adhere to good personal and institutional hygiene (Strauss, 1997).

Current guidelines or standards for the microbiological quality of fish (reviewed in León and Moscoso, 1996 and Strauss, 1997) show that the standard plate count is used in conjunction with an *E.coli* or coliform level in most cases. The rejectable levels set for the quality of fish were $10^6$ SPC/g and *E.coli* 500 per gram (ICMSF, 1995), $10^7$ SPC/g (WHO, 1989) $5 \times 10^6$ SPC/g and $0.7 \times 10^3$ coliforms/g (USA, in León and Moscoso, 1996), and $10^5$ SPC/g and $10^4$ *E.coli*/g (Sweden, in Strauss, 1997). These levels are less strict than those proposed by Buras (1987) for fish raised in excreta-fed systems, who recommended that the total aerobic bacterial concentration in fish muscle should not exceed 50 bacteria/g. This is probably because ICMSF regulations are for fish contaminated mainly by handling and were not set up to include fish raised in excreta fed systems (Edwards, 1992). For the use of wastewater in
aquaculture, it seems appropriate for guidelines to specify the water quality that is acceptable for aquaculture, taking into account both the likely microbiological quality of the fish grown in such water and the likely health effects to consumers of the fish and workers in contact with the fishpond water (Ursula, 2000).

A study in Indonesia showed that the water quality needs to be below $10^4$ FC/100ml before the risks are reduced to acceptable levels (Ursula, 2000). On balance, there appears to be sufficient evidence to suggest that the tentative faecal coliform guideline of $=10^3$ FC/100ml (WHO, 1989) for the fishpond water is the right order of magnitude, and insufficient data to warrant a reduction of this level to $10^2$ FC/100ml or a relaxation to $10^4$ FC/100ml. This implies that the quality of the feed water can be around $10^4$-$10^5$ FC/100ml, depending on the size of the fishpond and the amount of dilution that occurs (Ursula, 2000).

The implications of guidelines at this level are that wastewater (or excreta/septage) needs to undergo some form of treatment before it can be used in fishponds. Anaerobic and facultative ponds are designed on the basis of surface nitrogen loading and the facultative pond effluent discharged into the fishpond. Checks are made to see that the fishpond does not contain more than 1000 FC/100ml (Mara et al., 1993 and Mara, 1997). If the quality is $>1000$ FC/100ml, the retention time in the fishpond should be increased or a maturation pond could be added to the WSP.

A study by Ampofo et al., (2003) indicated that the diversity among the bacterial populations from the sewage treatment ponds was generally high, with mean diversity above 0.95 in each pond. Twenty-five species of bacteria were identified as associated with the fish culture systems in this study. The identified bacteria included *Campylobacter* spp., *Pseudomonas* spp., *Actinobacillus* spp., *Aeromonas* spp., *Citrobacter* spp., *Edwardsiella* spp., *Enterobacter* spp., *Escherichia* spp., *Flavobacterium* spp., *Hafnia* spp., *Klebsiella* spp., *Pasteurella* spp., *Proteus* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Vibrio* spp. and *Yersinia* spp. *Bacteroides* spp., *Micrococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Clostridium* spp., *Actinomycete* spp. and *Corynebacterium* spp. Bacteriological contamination of common carp, tench and crucian carp reared in pond supplied with biologically treated sewage was studied and found that fish muscles contained high number of faecal coliform, faecal streptococci, *Aeromonas* spp., *Pseudomonas* spp. and *Salmonella* spp. (Niewolak and Tucholski, 2000). Wastewater was successfully used to grow Nile tilapia but bacterial loads...
in fish organs were higher in the gills followed by the intestine and skin and finally edible muscles (Khalil and Hussein, 1997).

The skin, gut and muscle of the fish varieties “rohu”, (*Labeo rohita*) and the fringe-lipped carp, (*Labeo fimbriatus*) grown in (i) freshwater, (ii) sewage fed water and (iii) ‘reclaimed’ water were examined for the Enterobacteriaceae group of organisms. *Salmonella* sp. did not occur in any of the fishes tested. *Shigella flexneri* was isolated from the fishes reared in sewage fed ponds. However, in ‘reclaimed’ fishes, *Shigella flexneri* was present in the gut and skin and not in the muscle tested after 3 weeks of transfer from sewage-fed to freshwater ponds (Rajasekaran, 2008).

Bacterial populations contaminating various tissues of fish cultured in fish ponds fertilized with different organic wastes have been studied (Ampofo et al., 2010). *Pseudomonas* spp. was most abundant species in the organic waste fertilized ponds and was present in considerable quantities in gills, gut skin, blood and muscle of fish. *Salmonella* spp. was the most important contaminant of the gills, muscle and skin. The digestive tract and intraperitoneal fluid of fish showed high concentrations of pathogens, such as *Salmonella* in fish cultured in the organic waste fertilized ponds (Ampofo et al., 2010).

*Escherichia coli*, the predominant species of the faecal coliforms, has been found in the intestinal tract of fish (Newman, 1972) on the gills, in the muscles and on the skin (Ogbondeminu, 1993), when sewage water has been used to rear fish. Salle (1964) reported that the most heavily contaminated parts are the intestines and the skin.

Nile tilapia from Winam Gulf were infected by human enteric pathogens. *Shigella* spp., *Salmonella* and *E. coli* were the most frequently isolated, an indication that the beaches might be contaminated by untreated municipal sewage (Miruka, 2009).
1.3.4. Measures for Health Protection (pathogen risks)

There exist four basic options for health protection from excreted pathogen transmission:

- Faecal sludge and wastewater treatment
- Restriction of the crops grown
- Choice of methods of application of the wastes to the soil or crops
- Control of human exposure, and improved personal and household hygiene

The points at which these four health protection measures can interrupt the potential transmission routes of excreted pathogens are shown in Figure 1.7. (Mara and Cairncross, 1989).

*Figure 1.7. Flow diagram to show the potential transmission of excreted pathogens and points at which different health protection measures can interrupt the pathogen flow* (Mara and Cairncross, 1989)
1.3.5. Emerging Contaminants of Concerns–Antibiotics in Wastewater

1.3.5.1. What are antibiotics?

Antibiotics are substances produced by living organisms, which are able to kill or inhibit the growth of microorganisms. According to the literal sense of the word, substances produced synthetically (e.g. sulfonamides or quinolones) should not be termed antibiotics, and the use of a broader term (i.e. antimicrobial agent) would be more appropriate to indicate the complex of all substances having a harmful effect on microorganisms (Prescott et al., 1994). However, the term antibiotic is used throughout the present report as a synonym of antimicrobial agent.

1.3.5.2. Mechanisms of action

Antibiotics constitute quite a heterogeneous group of chemicals. Depending on the chemical structure, antibiotics exert an effect on different structures or functions of the bacterial cell (Prescott and Baggot, 1994) (Figure 1.8.). The major mechanisms of action are inhibition of the cell wall synthesis (e.g. penicillins and vancomycin), damage of the cell membrane function (e.g. polymixins), inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol, lincosamides and macrolides), inhibition of the nucleic acid synthesis (e.g. quinolones and rifampicin), and metabolic antagonism (e.g. sulfonamides and trimethoprim) (Guardabassi et al., 2002).
1.3.5.3. What is antibiotic resistance?

Antibiotic resistance is a relative term. A bacterial strain can be defined resistant if it survives in the presence of higher antibiotic concentrations in comparison with phylogenetically related strains (Smith, 1994). In aquatic environments, binding of the antibiotic molecule with ions or substances present in sediment strongly reduces both the activity of the drug and its absorption in the fish intestine (Lunestad, 1992).

1.3.5.4. Antibiotic Resistant Microorganisms

Antibiotic resistance is the ability of microorganisms to withstand the effects of antibiotics. In 1970, non-medical uses of antibiotics were questioned and antimicrobial agents were described as potential environmental contaminants and a threat to public health (Huber, 1971). Since that time, several studies have reported the occurrence of antibiotic resistant...
organisms in environmental samples and advocated a global public health concern due to these bacteria (Pillai et al., 1997 and Ash et al., 2002).

The important mechanisms by which microorganisms exhibit resistance to antibiotics include drug inactivation or modification, alteration of the target site, alteration in the metabolic pathway, and reduced drug accumulation (Hayes and Wolf, 1996 {Figure1.9.} and Katzung, 2004).

**Drug inactivation or modification:** Resistant bacteria synthesize and secrete enzymes which affect the antimicrobial activity of the antibiotics. For example β-lactamases synthesized by antibiotic resistant bacteria hydrolyze the β-lactone ring of penicillin thereby inactivating the antibiotic (Katzung, 2004).

**Alteration of target site:** Penicillin acts on bacteria by attaching to penicillin binding proteins (PBP), which are essential components for synthesis of bacterial cell wall. Bacteria develop resistance to penicillin either by the overproduction of PBPs or by synthesis of PBPs, which have low affinity to penicillins (Katzung, 2004).

**Alteration of metabolic pathway:** Bacteria are able to modify their metabolic pathways in order to evade the action of antibiotics. For example, sulfonamides inhibit the synthesis of folic acid, and sulfonamides resistant bacteria develop alternate routes for synthesis of folic acid or de repress its synthesis (Katzung, 2004).

**Reduced drug accumulation:** Bacteria developing resistance to antibiotics are able to reduce the uptake of the antibiotic by either altering the permeability of the drug or by enhancing active efflux of the drug (Katzung, 2004).
1.3.5.5. Natural and Acquired resistance

Bacteria are termed naturally, intrinsically or constitutively resistant when resistance is due to characteristic features typical of the species. For example, *Pseudomonas aeruginosa* is naturally resistant to penicillins, partly due to the inability of the drug to diffuse through the outer membrane (Chopra, 1982) and partly to the deactivation of the drug by chromosomally encoded enzymes i.e. betalactamases (Ohmori, 1977).

In contrast, acquired resistance emerges in a bacterial population that was previously susceptible, because of modifications of the bacterial DNA caused by either chromosomal mutation or horizontal gene transfer (Guardabassi et al., 2002).

Previously it was believed that resistance in bacteria was acquired by spontaneous mutation, which is called as primary resistance. The wide spread development of multiple antibiotic resistance in many species of bacteria led researchers to believe that another mechanism...
beyond spontaneous mutation was responsible for the acquisition of antibiotic resistance. The mechanism responsible for the development of resistance was through lateral or horizontal gene transfer (Levy, 1998) (Figure 1.10.). Horizontal gene transfer (HGT) has three possible mechanisms—transduction, transformation and conjugation. Transduction occurs when bacteria-specific viruses or bacteriophages transfer DNA between two closely related bacteria. Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA present in the external environment is due to death of another bacterium. Conjugation occurs when there is direct cell-cell contact between two bacteria and transfer of small pieces of DNA called plasmids takes place (Yim, 2007).

Figure 1.10. Mechanisms of bacterial gene transfer (Levy, 1998)

1.3.5.6. Intracellular migration of resistance genes

Antibiotic resistance genes can migrate from one site to another on the bacterial genome using small vectors called transposons (Mahillon, 1998) and integrons (Sundström, 1998). These genetic elements containing antibiotic resistance genes are able to move between
different sites of the bacterial genome without any requirement of DNA homology. This process is known as non-homologous recombination (to a site that does not match with the gene) and differs from the normal process of genetic recombination, which requires a high degree of DNA homology (Brock, 1999). Both transposons and integrons make it possible for new antibiotic resistance genes to be acquired by plasmids and subsequently spread in the bacterial population by mechanisms of horizontal gene transfer, as suggested by the frequent recovery of these genetic elements as part of broad host plasmids (Bennett, 1999).

1.3.5.7. Antibiotic resistance in sewage

Antibiotics are used to treat a wide spectrum of bacterial infections. However, incomplete metabolism in humans has resulted in release of large amounts of antimicrobial compounds into waste fed ponds. Recent studies have discovered trace level antibiotics in Wastewater effluents and surface waters (Miao et al., 2004; Koch et al., 2005 and Close, 2007). Long-term exposure of microorganisms to low concentrations of antibiotics (ng/l to µg/l) in wastewater and surface water has the potential for the development of antibiotic resistance in these organisms (Gilliver et al., 1999 and Smith et al., 1999).

Recent studies have shown presence of antibiotic resistant bacteria in wastewater and surface waters. Gallert et al., (2005), observed multi-resistant antibiotic faecal coliforms and enterococci in influent and effluent wastewater from treatment plants. Multiple antibiotic resistant organisms have been observed in wastewater treatment plants across the world. More than 20% of faecal coliforms were observed to be resistant to ampicillin, chloramphenicol, sulfanomide, tetracycline and streptomycin in one of the treatment plant effluents in Finland (Niemi et al., 1983). Other studies across the world have revealed that faecal coliforms and \textit{E. coli} in raw sewage were resistant to ampicillin, gentamycin, kanamycin, neomycin and streptomycin (Qureshi and Qureshi, 1991). Enterococci that were resistant to ciprofloxacin, sulfamethoxazole/trimethoprim and vancomycin at minimum inhibitory concentrations were also observed in raw and treated effluent of wastewater (Gallert et al., 2005). \textit{E. coli} were resistant to a wide range of antibiotics in raw and treated sewage (Hassani et al., 1992).
In sediments retrieved under fish farming activities, oxytetracycline was found to be capable of causing antimicrobial effects up to 12 weeks after administration in surface sediments (Halling Sorensen et al., 1998). Anoxic sediments are common in the aquatic environment and most antibiotic compounds persist much longer in these anoxic conditions. Hektoen et al., (1995) showed that antibiotics buried in sediments as shallow as 1-7 cm can have half-lives of more than 300 days. This means that antibiotics can build up in the aquatic environment to dangerous levels that may affect benthic communities and continue up through the food chain.

The use of oxytetracycline in aquaculture has been shown to cause a seasonal shift in bacterial species towards enterobacteriaceae and is associated with antibiotic resistance (Guardabassi et al., 1999 and Wollenberger et al., 2000). Samples taken from gills and intestines of wild commercial fish captured near fish farming activities have shown high frequencies of multiple antibiotic resistance bacteria (Rhodes et al., 2000).

It was concluded that the rather high prevalence of bacterial pathogens in tilapia along with their high prevalence of resistance to anti-microbial agents might pose therapeutic problems as well as health risk to consumers (Fyzul, Mutani, Ramsubahag and Adesiyun, 2008).

Aeromonas strains (total=953) isolated from raw wastewater, stabilization pond effluent and sediments were evaluated for their susceptibilities to 17 antibiotics. There were no differences in the resistance patterns of isolates from raw sewage, stabilization pond effluent and sediments. All strains were found to possess multiple resistance, most commonly to ampicillin, amoxicillin and novobiocin (Imziln, Lafdal and Jana, 1996).

Antimicrobial drug resistance patterns of Aeromonas hydrophila isolated from Tilapia mossambica of Kuala Lampur was studied. Resistance towards chloramphenicol, erythromycin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole-trimethoprim and tetracycline was observed (Son and Salmah, 1997). Aeromonas hydrophila isolated from same fish in Malaysia showed sensitivity towards chloramphenicol, erythromycin, kanamycin, neomycin and sulphamethoxazole-trimethoprim (Boonyaratpalin, 1989). Such differences in the frequency of resistance may well be related to the source of the Aeromonas isolates and the frequency and type of antimicrobial agents prescribed for treating Aeromonas infections in different geographical areas (Son and Salmah, 1997). Commercial fishes
residing in waters near the disposals of urban sewage might play a role as carriers of antibiotic resistant bacteria prompting a risk to public health for fish consumers (Miranda and Zemelman, 2001). Antibiotic resistance among random bacterial isolates from different organs of fish captured from faecally contaminated water with a full range of resistance (00–100%) to different common antibiotics of therapeutic and prophylactic use among human beings and in various animal farms and fish farms was reported by several authors (Rhodes et al., 2000; Miranda and Zemelman, 2001 and Pathak and Gopal, 2005). Wastewaters and fishes reside there are potent source of antibiotic-resistant bacteria, which in turn may transfer their resistance genes to nonresistant bacteria (Schwartz et al., 2003). Several studies indicate that the environmental conditions in wastewater may enhance the likelihood of gene transfer (Pote et al., 2003). The bacterial strains *Proteus mirsobilis, Escherichia coli* and *Erwinia* spp. were common species found in fish samples of the lake Manzala and demonstrated multidrug resistance. These strains harbored β-lactamase and plasmid DNA characteristics that can be attributed to the stressed water environment of the polluted lake Manzala (Zaky, 2009).

Antibiotic resistant strains of streptococcus and staphylococcus have been isolated from common carp cultured in waste fed ponds indicate health hazards especially when fishes are eaten uncooked or semi cooked. (Saxena and Kaur, 2006). Antibiotic and metal resistant bacteria have been isolated from river fish (Pathak and Gopal, 2005). Antibiotic resistant *Vibrio* spp collected from fish of coastal landing sites of Kerala were higher than that of surrounding water samples (Manjusha et al., 2005).

A study was conducted in the local tilapia industry of Trinidad to determine prevalence of bacterial pathogens and their anti-microbial resistance using the disk diffusion method. The predominant bacteria from fish slurry were *Pseudomonas* spp. (60.0%), *Aeromonas* spp. (44.0%), *Plesiomonas* spp. (41.3%) and Chromobacterium (36.0%). 168 (97.1%) of 173 bacterial isolates from fish slurry exhibited resistance to one or more anti-microbial agents. Resistance was high to ampicillin, 90.2% (158 of 173), erythromycin, 66.5% (115 of 173) and oxytetracycline, 52.6%, (91 of 173) but relatively low to chloramphenicol, 9.8% (17 of 173) and sulphamethoxazole/trimethoprim, 6.4% (11 of 173). High prevalence of bacterial pathogens in tilapia along with their high prevalence of resistance to anti-microbial agents might pose therapeutic problems as well as health risk to consumers (Newaj et al., 2008).
Antibiotic resistance provides a survival benefit to microorganisms and makes it difficult to eliminate the infections caused by them. Infections caused by antibiotic resistant bacteria are hard to treat. Hence, there is a need to study antibiotic resistance patterns in wastewater bacteria.
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