Chapter-1

Water is essential natural resource for sustaining life and environment which we have always thought to be available in abundance and free gift of nature. However, chemical composition of surface or subsurface, geothermal or non- thermal, is one of the prime factors on which the suitability of the water for domestic, industrial or agriculture purpose depends. Groundwater forms a major source of drinking water in urban as well as in rural areas. Provision of safe drinking water to the community without biological contaminants, poisonous chemicals and promotion of health is a concern of all nations (Susheela, 2002). Water is now polluted with various toxic substances; especially the underground water is contaminated by many hazardous pollutants like colored dyes, heavy metals, pesticides, arsenic and fluoride. Out of this fluoride pollution has now become the devastating threat in health related issues. Fluoride in water derives mainly from dissolution of natural minerals in the rocks and soils with which water interacts. Fluoride is found more frequently in different sources of water, with higher concentrations found in ground water due to the presence of fluoride-bearing minerals (Cuker and Shilts, 1979). Recalling the basics of inorganic chemistry we should keep in mind that among the four best known halogens (chlorine, bromine, iodine, and fluorine) fluorine is the most chemically active, being a very strong oxidant (Greenwood and Earnshaw, 1984).

The chemical element fluorine (F) when combines with other chemical substances, it forms inorganic and organic compounds called fluorides. Fluoride is a ubiquitous substance found naturally in soil, water, plants and animals in trace quantities and is also a common air pollutant in some industrial productions (Whitford, 2000). Based on quantities released and concentrations present naturally in the environment as well as the effects on living organisms, the most relevant inorganic fluorides are hydrogen fluoride (HF), calcium fluoride (CaF2), sodium fluoride (NaF), sulfur hexafluoride (SF6) and silicofluorides (Lewis, 1997). Fluorides are important industrial chemicals with a number of uses but the largest uses are for aluminium production, drinking water fluoridation and the manufacture of fluoridated dental preparations. Naturally, fluorides are released into the environment through the weathering of rocks and through atmospheric emissions from volcanoes and seawater (IPCS, 2002).

Human activities releasing fluorides into the environment are mainly the mining and processing of phosphate rock and its use as agricultural fertilizer, as well as the manufacture of aluminium. Other fluoride sources include the combustion of coal (containing fluoride impurities) and other manufacturing processes (steel, copper, nickel, glass, brick, ceramic, glues and adhesives). In addition, the use of fluoride-containing pesticides in agriculture and fluoride in drinking water supplies also contribute to the release of fluorides to the environment (http://www.greenfacts.org). In water, when inorganic fluoride compounds dissolve they split up into ions although the speed at which they dissolve depends on the type of compound and on factors such as the acidity of the water. The transport and transformation of fluorides is influenced by pH, water hardness and the presence of materials such as clay, which exchange ions. In water with a neutral pH and low fluoride concentrations, fluoride is predominantly present in the form of fluoride ions (F^-) as they travel through the water cycle fluorides usually combine with aluminium. In surface waters, such as rivers, fluoride levels depend on the proximity to human or natural emission sources;

they generally range from 0.01 to 0.3 mg/liter. In seawater average fluoride concentrations are approximately 1.3 mg/liter (WHO, 2004). In areas where the natural rock is rich in fluoride or where there is geothermal or volcanic activity, very high fluoride levels, up to 50 mg/liter, may be found in groundwater or hot springs ATSDR, 2003 and Fawell *et al.*, 2006)).

Shrott *et al.*, (1937) reported the occurrence of ground waters with high fluoride content, while Srinivasan, (1959) listed several places in India where ground waters have excessive concentrations of fluoride ions. The Manual of water supply (Government of India, Ministry of Health, 1962) listed several places in India where people are reported to suffer from fluorosis, through the use of waters containing relatively high concentrations of fluoride ions. Deshmukh and Karanth (1973) reported the presence of ground waters in several places of TamilNadu, Mysore and Andrapradesh containing fluoride ions in concentration exceeding 1.5ppm, the highest value recorded being 5ppm. Digging up of shallow aquifers for irrigation has resulted in declining levels of ground water. As a result, deeper aquifers are used and the water in these aquifers contains a higher level of fluoride (Gupta and Sharma, 1995 and Manik Chandra and Biswapati, 2009). The continuous culture and occasional low rainfall in the region have compelled growers to exploit underground sources of water for hatcheries including indoor nurseries to avoid polluted surface water. Shrinkage of ground water having fluoride content may lead to a concentration of fluoride in that water. The presence of high level of fluoride (>2.5mg/L) in pond water causes a serious health problem to fresh water fishes under farming and algae in the pond too (Bhatnagar and Bhatnagar, 2000). Considerably elevated concentrations of fluoride

(F), in ground and surface waters, ranging from <0.2 to 18 mg F/L have been reported in India (Sharma, 2003).

Behavioral changes such as respiratory alterations and violent, erratic movement, which might influence the metabolism of fish (Tarzana *et al.,* 1987), were observed in fish exposed to 18.7 and 51.8 mg F/L. Some species of freshwater fish, like climbing perch (*Anabas testudineus*), snakehead (*Channa punctatus*), walking catfish (*Clarias batrachus*), catfish (*Heteropneustes fossilis*), and clown featherback (*Chitala ornata*), are reported to have a mean reduction of 80% in growth and 68% in weight when exposed to inorganic fluorides over a range of 6.9 to 52.5 mg F/L in a captive pond (Samal, 1994). Studies by Mendoza-Shulz *et al.*, (2009) indicate that fluoride at micromolar concentrations can act as an anabolic agent and promote cell proliferation, whereas at millimolar concentrations it acts as an enzyme inhibitor on e.g. phosphatases, which play an important role in the ATP (cellular energy) production cycle and cellular respiration. Barbier *et al.*, (2010) has outlined a number of cellular processes in which fluoride can have deleterious effects. Identified effects include disruption of enzyme activity (mostly inhibition), inhibition of protein secretion and synthesis, generation of reactive oxygen species (ROS), and alteration of gene expression. Fluoride disrupts enzyme activity by binding to functional amino acid groups that surround the active centre of an enzyme. This includes the inhibition of enzymes of the glycolytic pathway and the Krebs cycle (Barbier *et al.*, 2010).

The symptoms of acute fluoride intoxication include lethargy, violent and erratic movement and death (Liteplo *et al.*, 2002). In soft waters with low ionic content, a fluoride concentration as low as 0.5 mg F/L can adversely affect invertebrates and fishes, safe levels below this fluoride concentration are recommended in order to protect freshwater animals from fluoride pollution (Camargo, 2003). Kumar *et al.,* (2007) found decreased levels of lipid and proteins in a dose dependent manner after exposure of *Clarias batrachus* to fluoride. It may be suggested therefore that the assault on cells by a xenobiotic like fluoride is more pronounced at higher concentration.

Research findings of Tripathy *et al.,* (2009), suggested that fluoride interferes with cellular activities in fishes, even at a genetic level, inducing chromosomal aberrations. Gills of fish exposed to fluoride for 30 days showed swelling in primary and secondary lamellar epithelium and clubbing on the tip of secondary lamellae at the lower fluoride concentration, while lifting in lamellar epithelium, fusion and degeneration of secondary lamellae and vacuolation in epithelial cells were observed at the higher fluoride concentration. Gills of *C.batrachus* exposed to either 35mgF ion/L showed swelling in primary lamellar epithelium, shortening and fusion of secondary lamellae, hyperplasia and hypertrophy in chloride cells (Kumar *et al.*, 2010), while lifting in lamellar epithelium, fusion and degeneration of secondary lamellae, and vacuolation in epithelial cells were observed at the higher fluoride concentration. Hyperplasia of epithelium results in an increase of the diffusion distance affecting the exchange of gases. Fusion of lamellae may be considered to cause a decrease in the total respiratory area of the gills, thus resulting in a decreased O_2 uptake capacity of the gills. In this condition fish fail to get adequate oxygen for total metabolic activities, and they therefore visit the surface more frequently (Kumar *et al.,* 2010).

The disease fluorosis is caused by an element known as fluorine, the 13th most abundant element available in the earth crust. WHO standards (WHO, 1984) and BIS: 10500 – 1991, permit only 1.5 mg/l as a safe limit of fluoride in drinking water for human consumption. Bones with high fluoride levels are more brittle and hip fractures increase as the level of fluoride in the water supply increases (Diesendorf *et al.*, 1997 and Connett, 2001). In India about 62 million people are estimated to be affected with various stages of skeletal fluorosis from consuming fluoride contaminated water (Jain *et al.*, 2000). More reported symptoms suspected to be due to fluoride are increased bone fractures, Down syndrome and reproductive effects (IPCS, 2002). Elevated fluoride concentration in water has been associated with dental and skeletal fluorosis, severe enamel fluorosis, osteosarcoma (bone cancer), osteoporosis, reduced IQ (Xiang *et al.*, 2003) and neurological effects.

Machalinski *et al.*, (2003) reported that the four different human leukemic cell lines were more susceptible to the effects of sodium hexafluorosilicate, the compound most often used in fluoridation, than to NaF (sodium fluoride). Ingested fluoride is partly excreted in the urine and partly stored in bones where it can inhibit the normal cycle of bone breaking down and being rebuilt. Male infertility with abnormalities in sperm morphology, a deficiency in the number of spermatozoa in the semen (oligospermia), the absence of spermatozoa from the semen (azoospermia), and low testosterone levels are very common in those residing in areas of India where chronic fluoride toxicity is common due to fluoride-contaminated water (Susheela, 2003). Fluoride causes thyroid hormone disturbances. A close similarity exists between the numerous symptoms and signs of hypothyroidism and those for fluoride toxicity including dental fluorosis (Susheela, 2005 and Schuld, 2005). There is also evidence that Down syndrome is associated with fluoridation (Susheela *et al.*, 2005). Impaired glucose tolerance in humans has been found with fluoride intakes of 0.07-0.4 mg/kg/day thus putting infants, children aged 1–2 years, athletes and heavy manual workers and patients with diabetes mellitus and nephrogenic diabetes insipidus at risk with fluoridated water with 1 mg F/L (Susheela, 2005). Exposure of 6-8 year old boys to fluoridated water resulted in significant increase, 500% at age 7, in the occurrence of osteosarcoma by age 20 years (Bryson, 2006). Lowered intelligence has been reported in children from high fluoride areas, particularly when associated with iodine deficiency, and the toxic effects of fluoride on the development of the brain are supported by animal studies (Ge *et al.*, 2006 and Trivedi *et al.*, 2007).

The global burden of disease due to fluoride in drinking water had been estimated to be 24 million from dental fluorosis and 10 million from skeletal fluorosis (WHO, 2006). NRC (2006) report notes that the primary symptoms of gastrointestinal injury due to fluoride toxicity are nausea, vomiting, and abdominal pain. The intestinal lining or mucosa of the duodenal region normally has cells with small protrusions on them (microvilli) and a layer of slimy substance. The microvilli and mucus are lost with chronic fluoride toxicity giving rise to symptoms such as nausea, loss of appetite, pain in the stomach, gas formation and a bloated feeling, constipation followed by intermittent diarrhoea, and headache. These symptoms of non-ulcer dyspepsia are early warning signs of fluoride toxicity.

The problem of fluorosis has been in existence since early 1930's in India; initially discovered in bullocks used for ploughing the land and later in human beings living in the same area. Clinical data indicate that adequate calcium intake is clearly associated with a reduced risk of dental fluorosis. Vitamin C may also safeguard against the risk. Calcium supplementation interferes with fluoride absorption in animals (Wagner and Muhler, 1960 and Jowsey and Riggs, 1978) and humans presumably due to the strong affinity between Ca^{++} and fluoride and the low solubility of CaF_2. Calcium chloride and calcium gluconate have been used in acute fluoride poisoning (Yolken *et al.*, 1976). It is reported that serpentine is of therapeutic value but opinion is divided about its efficacy and toxicological evaluation is not available (Anon, 1980). Aluminium sulphate and boron have also been tried for this purpose (Grunder, 1972 and Franke *et al.,* 1985). Intravenous administration of magnesium compounds (MgO or $Mg(OH)_2$) is been reported to increase excretion of fluoride in urine and feces (Raja Reddy *et al.*, 1985) and decrease the amount retained in bones. Sri Ramachari, (1983) and Maruthamuthu *et al.,* (1987) reported the binding of fluoride by tamarind *in vitro*. However, the effect of tamarind ingestion on fluoride metabolism has not been studied. It is known that high protein intake increases fluoride excretion and decreases fluoride retention in bones of rats (Carold and Floriant, 1987). Chinoy and Mehta, (1999) described the beneficial effects of glycine and glutamine on testis of fluoride treated mice, absorption due to formation of non-ionic hydrogen fluoride.

Antioxidants are particularly important in protecting the body from fluoride toxicity. They act as "scavengers" to remove "free radicals" and occur naturally in fresh fruit and vegetables. Vitamin E (α- tocopherol), a potent antioxidant, exerts its protective effect primarily through destruction of cell damaging free oxygen species

(Chinoy *et al.*, 2004). Vitamin C (ascorbic acid) is an antioxidant with detoxification properties. Calcium may help overcome the hypocalcaemia induced by fluoride and act synergistically with vitamin C. Dr. Susheela recommends proper nutrition to give a diet containing at least 1.0 g of calcium a day together with vitamin C, vitamin E, and other antioxidants such as β-carotene, glutathione, quercetin, allicin, capasaican, ellagic acid, gallic acid, epicatechin, lycopene, glucosinolates, lutein and zeaxanthin (Susheela, 2007a).

Probiotics are viable organisms and supportive substances that improve intestinal microbial balance, such as *Lactobacillus acidophilus* and bioactive proteins (Fuller, 1991). Several studies on probiotics have been published over the past decade. Some possible benefits linked to the administration of probiotics include:

- The restoration of a normal intestinal microbiota;
- A contribution to the elimination of pathogenic bacteria;
- A source of nutrients and enzymatic contribution to digestion.

Other benefits are still being investigated, for example reinforcement of the capacity of the intestinal barrier against exogenous antigens and enhancement of the immune response against pathogenic micro-organisms; as well as antiviral effects (Gatesoupe, 1999; Verschuere *et al.,* 2000; Irianto and Austin, 2002 and Vine *et al.*, 2006).

The lactic acid producing genera has been shown to exert strong anti-pathogenic effects on a range of pathogens such as *E. coli spp., Salmonella spp., Pseudomonas spp., and Campylobacter spp.* (Stern *et al.*, 2006 and Johnson-Henry *et al.*, 2008). Other genera of bacteria can be used as probiotics, For example, *Bacillus* *subtilis* strains are used either as a live vegetative cell preparation or in the form of spores to reduce Salmonella colonization in chickens (La Ragione and Woodward, 2003 and Cartman *et al.*, 2008). Probiotics may also mitigate against food borne pathogens by enhancing epithelial barrier function, the antagonism of receptor sites on the host epithelium, producing antimicrobial peptides, producing low molecular weight antimicrobials, competing for nutrients, inhibiting quorum sensing systems and through the production of organic acids (Medellin-Pena *et al.*, 2007). Merrifield *et al.*, (2010) proposed an extended list of criteria for potential probionts which includes the following:-

- Must not be pathogenic, not only with regards to the host species but also with regards to aquatic animal in general and human consumers.
- Must be resistant to bile salts.
- Should be able to adhere to and /or grow well within intestinal mucosa.
- Should display advantageous growth characteristic.
- Should exhibit antagonistic properties towards one or more key pathogens.
- Should remain viable under normal storage conditions and robust enough to survive industrial processes.

Probiotics can also be considered as microbes to improve the nutritive value of an animal feed (Castellanos *et al.,* 1996). Several studies have shown that probiotics can improve the growth rate of the fishes. (Metaillier and Hollocou, 1993; Noh *et al.*, 1994; Gildberg *et al.*, 1995 and Queiroz and Boyd, 1998). As an example, a commercial preparation of the bacteria *Streptococcus faecium* incorporated in the feed improved growth and feed efficiency of Israeli carp (Bogut *et al.,* 1998). A number of commercial probiotics are currently available, including Aqualact, Probe-La, Lacto-sacc Epicin, Biogreen, Environ, Wunopuo-15, and Epizyme (Abidi, 2003).

Probiotics may protect their host from pathogens by producing metabolites which inhibit the colonisation or growth of other microorganisms or by competing with them for resources such as nutrients or space (Fiorillo *et al.*, 2002). Phototrophic purple non sulfur bacteria are nutritionally diverse, and *Rhodopseudomonas palustris* is probably the most nutritionally versatile of all purple bacteria (Madigan and Gest, 1988). Vitamin B12 is an important vitamin for several functions involved in digestion in fish (Sugita *et al.*, 1991).

According to Sakai *et al.*, (1995), oral administration of the bacteria, *Clostridium butyricum*, increased the resistance of rainbow trout (*Oncorhynchus mykiss*) to vibriosis by increasing the phagocytic activity of leucocytes. Probiotic bacteria can enhance the defensive property of intestinal mucosa whose function is like a barrier against the antigens (i.e, bacterial pathogens) (Salminen *et al.*, 1996). Yeast b-glucans seem to modulate the specific immune response by increasing the serum antibodies secreted by plasma cells against *Edwardsiella ictaluri* in catfish (Chen and Ainsworth, 1992) and against *Yersinia ruckeri* in rainbow trout, when given in combination with vitamin C (Verlhac *et al.*, 1996). Microbial probiotics (especially those of LAB) can influence the systemic immune systems in various ways (Fang *et al.*, 2000). Rengpipat *et al.*, (2000) mentioned that the use of *Bacillus* sp. (strain S11) provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp (*Penaeus monodon*). The use of natural immune stimulants in fish culture for the prevention of diseases is a promising new development and could solve the problems of massive antibiotic use. Natural immune stimulants are biocompatible, biodegradable and safe. Moreover, they possess an added nutritional value (Jessus *et al.*, 2002). The non-specific immune system can be stimulated by Probiotics (Balcazar *et al.*, 2006). El-Boushy and El-Ashram, (2006) showed that the β-glucans of yeast was able to enhance the non-specific immunity of the African catfish (*Clarias gariepinus*) efficiently more than *Saccharomyces cerevisiae*. Concerning the immune stimulation point of view, many researches showed improvement in the immune response of fishes treated with probiotices (Watson *et al.*, 2008).The use of probiotics is now prevalent in the aquaculture industry as a means of controlling disease, improving water quality and enhancing the immune system of cultured species (Wang, 2007; Wang *et al.*, 2008 and Ma *et al.*, 2009).

Probiotics have also been used in a big way as pond cleaners in aquaculture. Probiotic bacteria directly uptake or decompose the organic matter or toxic material and improve the quality of water. The microbial cultures produce a variety of enzymes like amylase, protease, lipase, xylanase and cellulose in high concentrations than the native bacteria, which help in degrading waste. Moriarty, (1998) proposed that bacterial mixtures distributed by water as "water additives" instead of "feed additive" may also have a beneficial effect on aquaculture production. Queiroz and Boyd, (1998) reported that a commercially prepared bacterial mixture of *Bacillus* spp. mixed into the rearing water increased survival and production of channel catfish (*Ictalurus punctatus*). Probiotics are important natural ingredients in aquaculture and they have numerous beneficial effects: improved water quality, improved activity of gastrointestinal microbiota, and enhanced immune status, growth performance and feed utilization. The genera *Rhodospirillum* and *Rhodopseudomonas* have also been shown to create some positive effects on water quality parameters when applied in polluted water (McGrath *et al.,* 1997). Probiotic microorganisms will of course, have to be non-pathogenic and non-toxic in order to avoid undesirable side-effects when administered to fish (Gatesoupe, 1999). One sign of antagonism is that it is producing anti-microbial substances like organic acids, hydrogen peroxide (Ring and Gatesoupe, 1998), or siderophores (Gram and Mel-chiorsen, 1996). Though probiotics is known for various therapeutic value its role in alleviating metal toxicity in fish and other animals is not explored.

The GIT plays an important role in the detoxification and elimination of ingested toxins, including drugs, and metabolic wastes. The elimination functions include various GIT tissues that express numerous xenobiotic converting enzymes (e.g. cytochrome P-450's and other Phase I and II enzymes) (Lampe, 2007 and Kato, 2008) and export transporters (e.g., P-glycoproteins, Multidrug Resistance transporters, and various ATP-binding cassette (ABC) transporters) (Oude Elferink and de Waart, 2007). The enzymes and transporters act in concert to detoxify and export toxic molecules. A limited number of studies suggest management of the GIT bacteria can reduce accumulation and increase elimination of some environmental contaminants (Gratz *et al*., 2007). At the present time the potential influences of commensal, probiotic and pathogenic bacteria on expression of xenobiotic converting enzymes and export transporters are poorly understood.

Sheil *et al.*, (2004) reported that even subcutaneous administration of *Lactobacillus salivarius* 118 was effective in attenuating the development of collagen-induced arthritis and that the probiotic effect was associated with reduced production of proinflammatory (Th 1) cytokines and maintained production of anti-TGF-b. Preventative and curative effects of both live and heat-killed *Lactobacillus GG* in experimentally induced arthritis (Baharav and Felix, 2004) and the ability of *Enterococcus faecium* to improve the anti-inflammatory and anti-arthritic effects of methotrexate in adjuvant-induced arthritis in rats have also been demonstrated (Rovensky *et al.*, 2005). The intestinal habitat of an individual contains billions of microorganisms including bacteria, protozoa, archaea, fungi, and viruses (Guarner and Malagelada, 2003 and Ley *et al.*, 2006), and the number of microbial cells within the gut lumen appears to be ten times larger than the number of eukaryotic cells of the human body. Rafter *et al.,* (2007) investigated the influence of 12 weeks symbiotic supplementation (*Lactobacillus rhamnosus* GG (LGG) *Bacollus lactis* $Bb12^{+}$ a commercial fructan preparation-Synergy 1) on selected cancer biomarkers in polypectomized and cancer patients. Synbiotic supplementation resulted in significant reductions in DNA damage in the colonic mucosa of polyp patients. Vallarini *et al.*, (2008) investigated the antigenotoxic effect of *L. casei* supplementation in rats before and after DMH treatment. So *et al.*, (2008) reported that probiotics mediate this effect by down-regulating Thl effectors functions. *Lactobacillus casei* administration reduced type II collagen (CII)-reactive proinflammatory molecules (IL-1 b, IL-2, IL-6, IL-12, IL-17, IFN-g, TNF-a and Cox-2) by CD4+ T cells. *L. casei* administration also reduced translocation of NF-kB into the nucleus and CII-reactive Th1-type IgG isotypes IgG2a and IgG2b, while up-regulating immune regulatory IL-10 levels.

Today, sufficient data are available to support the use of lactic acid bacteria (LAB), notably *Lactococci* and *Lactobacilli*, as delivery vehicles for the development of new mucosal vaccines. These non-pathogenic Gram-positive bacteria have been safely consumed by humans for centuries in fermented foods. Thus, the use of probiotics in human medicine is gradually growing. They constitute an attractive alternative to the attenuated pathogens (most popular live vectors actually studied) which could recover their pathogenic potential and are thus not totally safe for use in humans. Strains that are generally used as human probiotics (e.g. *Lactobacilli* and *Enterococci*), have been considered for studies in fish as a novel and safe treatment in aquaculture (Sullivon *et al.*, 2005). The information available till date gives clues regarding the possibility to reduce the development of fluorosis through nutritional supplementation. It is felt that more detailed research, both experimental and epidemiological, is needed for this. Since fluorosis has no effective cure to date experimental model animal studies with fluoride containing drinking water will help in designing interventional strategies through nutritional prophylaxis or chemotherapy.

The scientific community has been using rats experimentally for decades for a number of reasons, including their frequent reproduction, genetic purity and similarities to human biology. Because rats are mammals, their systems should react to any chemicals in a similar way to those of a human test subject. In order to be considered safe enough for human consumption or exposure, a new chemical compound must first be tested on rats or other mammals. Laboratory rats are often fed extremely high amounts of a new food additive or injected with large doses of a new chemical compound for safety studies. In view of the above ideas, the probiotic bacteria *Lacobacillus rhamnosus* was used to analyze its role on amelioration of fluoride toxicity in the fish *Mystus montanus* and Albino rat *Rattus norvegicus*. In this regard the present project work is launched with the following objectives.

- To study the effects of fluoride on fish and rat,
- To study the histophological changes due to fluoride in fish and rat and
- Probiotic rectification to control the toxic effects

The experimental design is shown in Fig-1.

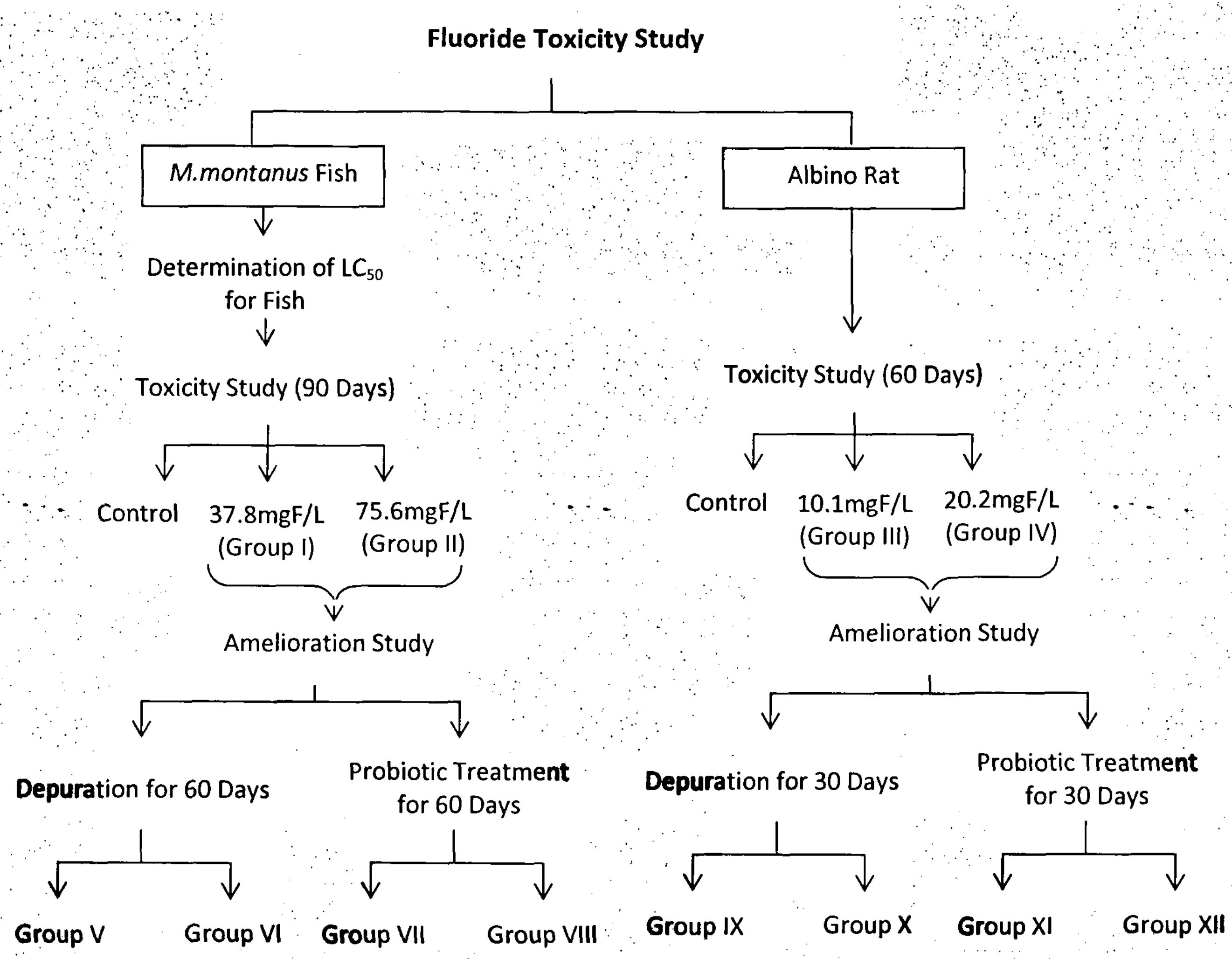

Fig-1: Experimental Design