Microbial Evaluation and Validation of Biomarkers for Environmental Contaminants

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CERTIFICATE

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Dedicated to my family

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LIST OF ABBREVIATIONS

ANOVA…………………………….... Analysis of variance
AOP…………………………………….. Advanced oxidation process
Aro-B………………………………….. Aromatase-B
BPA…………………………………. Bisphenol A
BAP…………………………………. Benzo (a) pyrene
cDNA……………………………….. Complementary deoxyribonucleic acid
COD…………………………………. Chemical oxygen demand
Ct …………………………………… Threshold cycles
DNA………………………………… Deoxyribonucleic acid
DO…………………………….............. Dissolved oxygen
E. coli …………………………………. *Escherichia coli*
EC-50 …………………………….. Half maximal effective concentration
E_2 …………………….................. Estrogen
EDC…………………………………. Endocrine disruptive chemicals
GAPDH……………………............... Glyceraldehydes 3- phosphate dehydrogenase
GPS …………………………............... Global positioning system
GPX…………………………............... Glutathione peroxidase
ER…………………………….. …… Estrogen receptor
Hrs…………………………............... Hours
hpf ………………………………….. Hours post fertilization
HSP…………………………………. Heat shock protein
IGF………………………………….. Insuline like growth factor
KTZ…………………………............ Ketoanezole
Min…………………………............... Minutes
M-MLV…………………………............ Moloney Murine Leukimia virus reverse transcriptase
MS-222…………………………............ Tricaine methanesulfonate
NP …………………………............... Nonylphenol
Oligo dt……………………… …….. Short sequence of deoxythymine
PCR……………………….. ……... Polymerase chain reaction
Q-PCR…………………………….. Quantitative real time polymerase chain reaction
rRNA……………………………… Ribosomal ribonucleic acid
S.E.M……………………………… Standard error of the mean
Sec…………………………………. Seconds
TBBPA……………………….. …….. Tetrabromobisphenol A
UV ………………………………….. Ultraviolet
VTG……………………………….. Vitellogenin
WHO …………………………….... World Health Organization

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ABSTRACT

Aquatic environment is the ultimate sink for most environmental contaminants. There is a serious need for water quality monitoring and to validate the sensitive biomarkers for aquatic pollution. The main focus of this study was to evaluate the microbial diversity of a water reservoir and validation of biomarkers for environmental contaminants BPA and herbicide 2,4-D.

Water quality and bacterial diversity from Rawal Lake was investigated for a period of eight months. Rawal Lake in Islamabad Pakistan is an artificial reservoir having several reports of pollution from adjacent areas. Grab water samples were collected from surface layer of ten various locations of the lake to study the water quality characteristics. The physicochemical parameters exceeded WHO guideline values for drinking water at few locations. Bacterial strains were isolated and template DNA was prepared following 16S rRNA analysis and sequencing. The sequences of nearest relative microbes were identified by BLAST and used as reference sequences for phylogenetic analysis. Various phylotypes of microbes including *Firmicutes, Teobacteria* and *Proteobacteria* were predominant.

Bisphenol A (BPA) is a xenobiotic compound known for its endocrine disruptive effects, originating from polycarbonate plastics in large quantities. Besides, 2,4-D herbicide is one of the most widely used halogenated agricultural chemicals around the world and also a well-known endocrine disrupting chemical (EDC). These chemicals were tested for the biological effects by differential expression of genes including, Cytochrome P4501A1 (CYP1A), Aromatase B (Aro-B), heat shock proteins (HSP70) and Glutathione peroxidase (GPX) using quantitative real time polymerase chain reaction (Q-PCR) assay. BPA concentration of 100 µg/L caused significant induction of Aro-B and HSP70 genes after

exposure of 72 hpf. Similarly, mRNA levels of CYP1A gene increased about two fold while GPX expression was found to be almost unchanged under similar exposure. Furthermore, BPA in aqueous solution was subjected to direct UV photolysis and tested for its residual effects. The results indicated significant effect of UV treatment on mRNA levels of tested genes and disappearance of biological activity of BPA with reduced mortality of zebrafish embryos. The study employed TiO_2 based photocatalytic degradation of 2,4-D in aqueous solution. The residual biological activity was determined by transcriptome analysis of selected genes in zebrafish embryos. Statistically significant induction of CYP1A gene was observed for 2,4-D exposure, whereas HSP70 and Aro-B expression was significantly lowered after its UV/TiO_2 photocatalytic degradation.

Adult goldfish liver cell culture technique was used to determine the transcriptomic alterations of selected genes due to BPA and 2,4-D exposure *in vitro*. The results showed significant down regulation of insuline like growth factor (IGF-1) gene expression in 100 and 50 µg/L BPA and 2,4-D exposure respectively. While goldfish vitellogenin (VTG) gene expression demonstrated altered expression.

This work provides an important insight into predominant microbial evaluation and validity of few biomarker genes in fish affected by BPA and 2,4-D herbicide. Aro-B, HSP70 and CYP1A gene expression showed significant alteration in exposed zebrafish embryos. The efficacy of UV based degradation of these chemicals has also been tested biologically. The findings might be helpful to create awareness about pollutant loading to lakes, rivers and other aquatic ecosystems and to devise promising techniques to remediate the problem.

INTRODUCTION

The presence of industrial and agricultural contaminants in the aquatic environment has been of growing concern for human and wildlife health.Water reservoirs are at great risk of contamination by microbial and toxic pollutants worldwide. Thus, water quality has become a major dilemma among the developing countries and a serious problem for the entire world. Pakistan is one of those countries, facing serious problems related to water quality and the supply of clean and safe water to different parts of the country (Hashmi *et al*., 2012).

Rawal Lake is used to provide drinking water to people residing in the cities of Rawalpindi and Islamabad. According to previous studies, Rawal Lake water quality has been found to be deteriorating and there is a need to monitor the water quality in the lake basin (Ghumman, 2011). Many human activities, such as agriculture, use of pesticides and industrial waste may potentially affect water microbial diversity. Moreover, microbial community investigation is considered as an integral part of future planning and management strategies for restoration of water quality. Therefore, in this study classification of the bacterial community of Rawal Lake has been carried out in addition to physicochemical parameters of its water quality.

Several xenobiotic compounds known as endocrine disrupting chemicals (EDCs), have been detected in the aquatic environment. They interfere with normal hormone activities by altering the synthesis and metabolism of natural hormones or modifying hormone receptor levels (Sumpter, 2005). Bisphenol A (BPA), is one of the important

environmental EDCs that enters surface waters through wastewater from BPA production factories, agricultural runoff and irrigation return waters (Rosenfeldt and Linden, 2004). Its major use is in the production of epoxy resins and polycarbonate plastics. Plastics industry is one of the leading business sectors in Pakistan, growing at an average growth rate of 15% per annum. During the last 5 years, 100% increase has been recorded in the imports of plastic scrap in the country. The solubility of BPA in water is much greater than its EC50 and an acute toxicity to aquatic organisms is in the range of 1–15 mg/mL for freshwater and marine species (Flint *et al*., 2012). Like other xenobiotics, it is also documented to induce expression of certain genes; identified as biomarkers for its transcriptomic effects on living system.

Pesticide contamination of surface waters from agricultural use is another problem of global importance. It is known to disturb the delicate balance of aquatic ecosystems by affecting the health of aquatic organisms, such as fish (Sancho *et al*., 2000). Among different pesticides contaminating aquatic ecosystems, 2,4-dichlorophenoxyacetic acid (2,4-D) has been the most widely used pesticide for over 60 years (Tayeb *et al*., 2011). Its toxicity on nontarget organisms has been a topic of extensive research, while some studies suggest that 2,4-D may induce oxidative damage to animal tissues (Mi *et al*., 2007). Biomarkers indicate a change in a biological response which can be related to exposure or toxic effects of environmental chemicals. Currently, various biomarkers have been used to impartially measure its endocrine disruptive potency and understanding the underlying mechanisms (Jung *et al*., 2012). Xenobiotics like BPA and 2,4-D are known to target multireceptor pathways, acting as complex mechanism within the endocrine system. Hence this study focussed on transcriptomic alterations of genes involved in controlling

various physiological parameters to validate them as biomarkers for the exposure of these chemicals using real time Q-PCR technique.

Since there is a great demand for cost effective and ethically acceptable approaches and animal models to evaluate the toxicity of chemicals and validate the sensitive and robust biomarkers of aquatic pollution. In this perspective, zebrafish (*Danio Rerio*) embryos are widely used as cost effective model that exhibits responses to chemicals similar to mammals (Cajaraville *et al*., 2000). In addition, gene expression profiling has proven to be useful in the testing and validation of biomarkers of EDC exposure. The approach is also being reliably applied for testing the removal efficiency of EDC activity under photolysis and other UV based advanced oxidation processes (AOPs) used for the degradation of chemicals to remediate their toxic and ED effects (Pei-Jen-Chen *et al*., 2007). Therefore, various AOPs have been developed and investigated to mineralize these chemicals in aqueous solution. Similarly, direct UV photolysis is relatively simple and economical method, applied in this study for BPA degradation. Photocatalytic treatment was applied for 2,4-D degradation followed by evaluation of their residual biological activity in zebrafish embryos. The removal efficiency of biological activity of BPA and 2,4-D have been evaluated as the information based on only analytical methods is insufficient to determine an effective treatment endpoint (Rosenfeldt *et al*., 2007).

Cell culture refers to the culturing of cells derived from animals. The technique is being used to assess the drug screening and cytotoxicity mechanisms of cell lines. Goldfish (*Carassius auratus*) is a good biological model for toxicological studies. It exhibits good tolerance to a wide range of environmental conditions. Goldfish liver cell culture study was conducted for the endocrine disruptive potential of chemicals by

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transcriptomic alterations of selected genes responsible for various physiological endpoints in fish.

1.1 OBJECTIVES OF THE STUDY

Environmental pollutants are known to affect the water quality and microbial diversity of aquatic reservoirs. In this context, Rawal Lake has gained great attention due to its deteriorating water quality and environmental management.Therefore, phylogenetic classification of the bacterial community of Rawal Lake was required in addition to physicochemical parameters. Furthermore, various biomarkers have been used to impartially measure biological activity and understand the underlying mechanisms of aquatic pollutants in fish. The present study was designed to determine relative expression of few genes of fish exposed with various chemicals to validate them as biomarker for environmental contaminants. The specific objectives of the study were as follow:

- Isolation of predominant microorganisms from Rawal Lake to identify possible sources of secondary organic pollution.

- Validation of biomarkers for environmental contaminants; bisphenol A and herbicide 2,4-D, using fish as model organism

LITERATURE REVIEW

Water quality of rivers, lakes, and reservoirs is being degraded because of the contaminated inflows. The evidence is the increase in disease outbreaks, reduced survival and physiological disorders in aquatic life due to toxic chemicals. Due to unplanned industrialization and urbanization around rivers and lakes the risk of contamination is increasing. There is a serious need for appropriate water quality monitoring for future planning and management of clean water resources. The present study investigates the water quality and microbial diversity of surface water in Rawal Lake, Islamabad and to validate the biomarkers for environmental contamination, transcriptome based bioassay was employed. Biomarker genes of zebrafish and goldfish were studied for their altered expression for *in vivo* and *in vitro* exposures to BPA and 2,4-D.

2.1 RAWAL LAKE

Rawal Lake is situated in Islamabad across the KurangRiver with a surface area of about 8.75 km^2. The maximum depth of the reservoir is 31 m. The catchment area of the lake is divided into various zones, *i.e*. Kurang, Shahdara and Nurpur village.The total catchment area is 268 km^2 (Aftab, 2010). Runoff from four major streams and 43 small streams comes to the lake. The residents of twin cities; Rawalpindi and Islamabad get drinking water from this lake at a rate of about 22 million gallons per day. The Kurang River and the streams receive local spring discharges, diverted untreated sewage water, and occasionally, polluted runoff water during the rainy season. The runoff water is

reported to contain contaminants from poultry farms, sewage drains, and landfill sites located in adjacent villages and residentialareas. This area has sub humid climate and experiences variations in seasonal temperatures and precipitation rate (Malik and Zeb, 2009). A few projects were launched to promote the lake environment by controlling wastewater emissions and introducing the monitoring of water quality to protect the lake. However, problems of insufficient followup and public participation in the current management system still exist in the area.

Ghumman (2011) studied various water quality parameters in Rawal Lake and concluded that the level of seven contaminants was higher as compared to the environmental standards. Malik and Nadeem (2011) characterized spatial and temporal variations in the water quality of Rawal Lake with regard to space and time to identify sources of contamination, and compared their levels with water quality guidelines. In a study by Chaudhary *et al*. (2009), concentrations of metals were measured in Rawal Lake water and fish. Metal concentrations at different stations varied widely because these are carried into the lake from different sources. Physiochemical characteristics of the lake water showed higher values at the tributaries.Various findings report the agricultural activities in the catchment area of Rawal Lake and pollution of pesticides, herbicides and toxic chemicals within the area (Iram *et al*., 2009; Ahad *et al*., 2005).

2.2 MICROBIAL EVALUATION OF WATER RESERVOIRS

Bacteria play an important role in the decomposition and cycling of a variety of compounds in freshwater aquatic environments. Various studies infer that anthropogenic activities, such as agriculture, use of pesticides, and pollution, can potentially affect water microbial diversity. Diversity of microbial community in the water layer of Woopo

wetlands was investigated by Baik *et al*. (2008). Cultivable bacterial strains were isolated by the dilution and plating technique and culture independent 16S rRNA gene clones were isolated directly from DNA extracts of water samples. Representative isolates were partially sequenced and analyzed phylogenetically. Likewise, sediment bacterial communities have been studied in the two river basins in China; using samples collected in each season. They found that certain common phyla, *Proteobacteria, Bacteroidetes, Firmicutes,* and *Chloroflexi,* were dominant in the sediments from both basins. However, from the class to genus level, the dominant bacterial groups found in the sediments were distinct between the two basins (Bai *et al.,* 2012). In a study by Newton *et al*. (2013), V6 and V6V4 regions of the bacterial 16S rRNA gene were used to identify bacterial distributions in an urbanized coast. The sequences identified bacterial genera like, Acinetobacter, Arcobacter and Trichococcus. Another study also concluded that microbial communities play an important role in the aquatic environmental conditions, influencing nutrient cycling, organic matter metabolism and leading to organic pollutant transformation (Zhang *et al*., 2014a).

2.3 CHEMICAL CONTAMINATION, SOURCES AND EFFECTS

The environment is continuously loaded with foreign organic chemicals, released by urban communities and industries. In the recent years, many thousands of organic trace pollutants have been produced and partly released into the environment. Due to direct discharge of effluents or atmospheric processes, the ultimate sink for many of these contaminants is the aquatic environment (Stegeman and Hahn, 1994). Aquatic organisms are exposed to a wide variety of environmental contaminants. Normally a set of biomarkers is employed to assess the possible biological impacts. Such biomarkers act as

an early warning of a specific harmful biological endpoint. Histopathological analysis, DNA integrity and detoxification enzyme status and gene expression in fish have frequently been employed as such biomarkers in environmental monitoring (Nogueira *et al*., 2010).Various studies have highlighted that some substances generated from pesticides, herbicides, plastic components and pharmaceuticals etc. have estrogenic or antiestrogenic action. It was inferred that environmental contaminants may interfere with normal hormonal processes (Chow *et al*., 2013). In the South Saskatchewan River Basin in Alberta municipal wastewater effluent and agricultural inputs were reported to combinely impact fish populations (Jeffries *et al*., 2010). Agricultural runoff and cattle farming were found potential sources of natural steroids and compounds with estrogen like activity, and they can have major impacts on river water quality. Many agriculture related compounds, such as livestock pharmaceuticals, steroid hormones, and pesticides, have been detected in rivers with agricultural activity.

2.4 ENDOCRINE DISRUPTIVE CHEMICALS

Many of the thousands of anthropogenic chemicals currently released into the environment are endocrine disrupting compounds (EDCs). These are defined as exogenous chemicals or chemical mixtures that impact endocrine systemstructure or function and cause adverse effects (USEPA, 2007). Endocrine systems regulate a multitude of developmental, metabolic and reproductive processes including embryonic development, gonadal formation, sex differentiation, growth and digestion. Endocrine disrupting compounds may affect these processes by binding to or blocking hormone receptors, thereby triggering or preventing hormonal response (Hotchkiss *et al*., 2008).

Chemicals implicated in endocrine disruption include biocides, industrial compounds, surfactants and plasticizers including bisphenol A (Flint *et al.*, 2012).

2.4.1 Bisphenol A

BPA (2,2-bis4-hydroxyphenylpropane) is an organic compound composed of two phenol rings connected by a methyl bridge, with two methyl functional groups attached to the bridge (Figure 2.1). It is used in the production of phenol resins, polyacrylates, and polyesters, but mainly for the production of epoxy resins and polycarbonate plastics. Plastics industry is one of the leading business sectors in Pakistan, growing at an average growth rate of 15% per annum. However, recent manufacturing trends show the raging violation of laws and regulations, especially related with processing, manufacturing and recycling of hazardous plastic materials in the country. Due to increased use of products based on epoxy resins and polycarbonates, exposure of organisms to BPA via several routes, such as the environment and food chain, has increased (Kang *et al*., 2007).
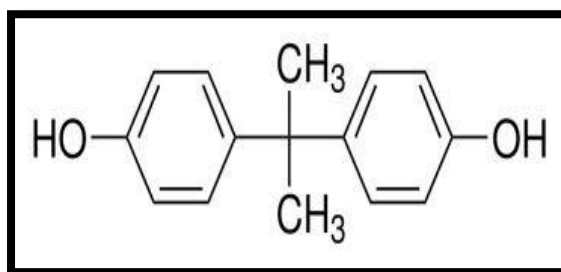
Figure 2.1: Chemical structure of bisphenol A

BPA is an important contaminant due to its ubiquitous presence and the increased exposure of humans and organisms via environment and food chain. In Pakistan, the yearly imports of BPA and its salts have shown a tremendous growth rate. The increased production of polycarbonate based material has resulted in massive release of BPA and

related contaminants in effluent water. Pakistan's yearly imports of BPA and its salts indicate its enormous applications in plastic industry. The aquatic environment is an important area for the study of BPA. Like other xenobiotics, BPA is documented to induce expression of certain genes; identified as biomarkers for its transcriptomic effects. Several studies have focused on the responses to chemical stressors at the molecular level in aquatic species (Perceval *et al*., 2004).

The effects of BPA on living systems have been extensively studied due to the challenges it presents in dose responses. Its effects and modes of action may vary among taxa and life stages. In addition, some metabolites of BPA are more estrogenic than the original compound and environmental characteristics including microbial flora may alter its degradation rates or biological impacts.

The development, reproduction and survival of aquatic life have been reported to be affected at environmental ranges of BPA. Therefore, a recent aquatic hazard assessment has lowered the predicted no-effect concentration from 100 to 0.06 mg/L (Wright-Walters *et al*., 2011). Thus, the impact of BPA continues to be a very active area of study, with significant debate concerning low dose effects.

2.4.2 Toxic Effects of BPA

Bisphenol A is a known endocrine disruptor and is acutely toxic to aquatic organisms. Increased usage has also intensified the known disruptive effects on the endocrine system of organisms. It is one of endocrine disrupting chemicals that can act as a xenoestrogen transforming the endocrine pathways by means of a receptor mediated process (Hatef *et al*., 2012). Several studies reveal ontogenetic and endocrine disruptions by BPA on aquatic organisms at environmental relevant concentrations. A recent study

has investigated the effects of bisphenol A at 7.6 and 76 µg/L on gene expression levels in *Oryzias javanicus*. The induction of differential expression of various genes including glutathione s transferase (GST) and heat shock protein (HSP) genes was detected (Woo *et al*., 2014).

It is recognized that lower concentrations of BPA may act as an estrogen agonist in mammals and fish *in vitro* and *in vivo*. BPA affects reproductive systems in male and female fish. It may act as a neurotoxicant or gonadotoxicant by upregulating cytochrome P-450 aromatoase levels (Lee *et al*., 2006). BPA also exhibits reduced gonadal growth in male and female fish (Sohoni *et al*., 2001). No ovulation was found in female fish exposed to 5 µg/L BPA and still lower concentrations of 1.75 and 2.4 µg/L BPA caused delayed ovulation (Lahnsteiner *et al*., 2005). BPA is also reported to cause gonadal intersex and a high prevalence of the female phenotype in male fish (Kang *et al*., 2002). Fish exposed to BPA show depressed levels of androgens, such as testosterone and 11-ketotestosterone (Labadie and Budzinski, 2006). The results of current novel studies show effects of BPA using lowdose approaches (Hatef *et al*., 2012). Various findings are potentially of concern for the environment because the identified effective concentration levels are similar to some current environmental levels to which sensitive aquatic organisms may be exposed.

The effect of low dose BPA in the range of 5, 15 and 50 µg/L for 14-35 days on adult female rare minnow *Gobiocypris rarus* was observed. The applied three concentrations showed induction of the transcription of hepatic vitellogenin (VTG) gene. BPA at 5 and 15 µg/L showed up regulation in the expressions of ovarian steroidogenic genes while at 50 µg/ L demonstrated inhibitive effects (Zhang *et al*., 2014b). Oxidative stress was observed at concentrations of 1.75–10 µg/L, when fish spermatozoa were exposed to environmentally relevant concentrations of BPA for 2 hrs. Significantly higher

levels of lipid oxidation and superoxide dismutase activity were observed indicating that concentrations of BPA that can be present in nature are capable to induce oxidative stress (Hulak *et al*., 2013).

Five environmental estrogens including BPA have been detected in environmental samples and known to induce the plasma vitellogenin in male goldfish (*Carassius auratus*) after a 28 day exposure. While increased circulating 17 ß-estradiol level was also observed in males exposed to treated effluent (Gong *et al*., 2014).

2.4.3 Herbicide 2,4-D

2,4-D is a common systemic herbicide used in the control of broadleaf weeds. It is one of the most widely used herbicides in the world, found in more than 600 products on the market (Tayeb *et al*., 2011). It is produced commercially by chlorination of phenol to form 2,4-dichlorophenol, which reacts with monochloroacetic acid to form 2,4-D. Figure 2.2 shows its chemical structure. Due to its great use in agriculture, currently the estimated environmental concentrations of 2,4-D are in the range 4–24 µg/L, and may locally reach concentrations upto 4000 µg/L (Kubrak*et al*., 2013a).

Pesticides and other xenobiotics disturb the delicate balance of aquatic ecosystems and affect the health of nontarget aquatic organisms, such as fish. Although, the largest quantities of pesticides are used in the western countries but most cases of pesticides poisoning are observed in developing countries due to factors like their excessive use, poor application practices and lack of proper legislation. Pakistan being an agricultural country has huge demand of pesticide and herbicides formulations for its crops. Tariq (2002) reported that during last two decades, there was substantial increase in the use of pesticides not only in volume, but also in value. Their use has increased by about 70 times

posing a threat to the aquatic reservoirs due to surface runoff and other agricultural activities.
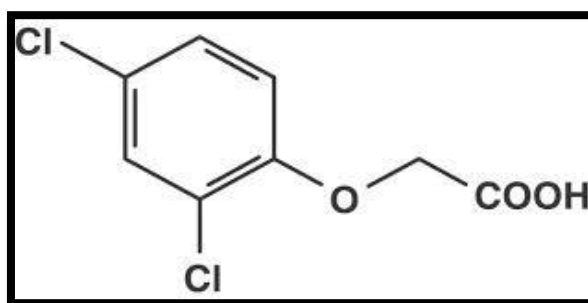
Figure 2.2: Chemical structure of 2,4-D

The degradation of 2,4-D in soil occurs by microbes by the process of hydroxylation, decarboxylation, cleavage of the acid side chainand ring opening. The process is rapid in aerobic mineral soils. Its ethyl hexyl form is rapidly hydrolyzed in soil and water to form the 2,4-D acid. In aerobic aquatic environments, its half life is 15 days, while in anaerobic aquatic environments, 2,4-D is found to be fairly persistent (Tomlin, 2006).

2.4.4 Toxic Effects of 2,4-D

2,4-D has been suggested as a potential environmental endocrine disruptor and its toxicity on non target organisms has been a topic of extensive research (Tayeb *et al*., 2011). Some studies suggest that 2,4-D may induce oxidative damage to animal tissues (Mi *et al*., 2007) and the measurements of antioxidative enzyme activities like glutathione peroxidase in fish have been used to assess the oxidative damage (Zhang *et al*., 2004). Moreover, 2,4-D in water, is readily degraded to 2,4-dichlorophenol, which is an estrogen receptor (ER) ligand (Jobling *et al*., 1995).

Exposure to 2,4-D and other chemical pollutants may induce an imbalance between intracellular reactive oxygen species (ROS) levels and antioxidant protection. It

may subsequently cause changes in antioxidant defenses or direct oxidative damage in organism (Slaninova *et al*., 2009). Pesticides may induce oxidative stress via several mechanisms such as inactivation of antioxidants and associated enzymes leading to decreased antioxidant potential or by modification of core vital processes. They may slow down the energy providing processes, decreasing supplement for metabolism and detoxification. Finally, pesticides are capable to enter redox cycles and consequently they may increase ROS level (Lushchak, 2011). The defensive mechanism is comprised of antioxidant enzymes, including the radical scavenging enzymes like, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), etc. (Valavanidis *et al*., 2006). Therefore, measurements of antioxidative enzyme activities in fish have been used to assess the oxidative damage caused by chemicals in aquatic ecosystems.

The effects of 2,4-D were studied on the metabolism of goldfish, (*Carassius auratus*) in a study and data indicate that 2,4-D shows toxicological effects after 96 hrs at concentration of 100 mg/L (Kubrak *et al*., 2013a).While in another study, 10 mg/L 2,4-D exposure induced the elevated levels of total and reduced glutathione in muscles of goldfish (Kubrak *et al*., 2013b). A decrease in the number of oocytes and many deformed and underdeveloped oocytes in zebrafish ovarium was observed after 2,4-D exposure to zebrafish. It was inferred that acute doses of 2,4-D may slow down the oogenesis in fishes and affect reproduction (Koc and Cansu, 2012).

2.5 BIOMARKERS

A biomarker is defined as a change in a biological response which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). These may include molecular change, through cellular and physiological responses or behavioral

changes. According to WHO, almost every measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological can be categorized as biomarker (WHO, 1993). Conversely, bioindicator is an organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior (Oost *et al*., 2003).

Biomarker responses provide useful balance to chemical analyses because they provide valuable information about types of exposure as well as exposure pressures or even foretell adverse outcomes of exposure to certain chemicals or mixtures of chemicals in biomonitoring programs. With this great knowledge, it is now markedly imperative to avoid future environmental tragedies. For example, induction of vitellogenin (VTG) in fish is a biomarker that is frequently used to assess exposure to estrogenic chemicals and provides an early warning signal for exposure to estrogen receptor (ER) agonists in the aquatic environment (Sumpter and Jobling, 1995). Activation of estrogen receptor in male or juvenile fish results in induction of the VTG. Similarly, induction of CYP1A synthesis in fish, through activation of the aryl hydrocarbon receptor (AhR), upon exposure to aromatic compounds provides an early warning signal and is an excellent biomarker for exposure to AhR agonists (Schlenk *et al*.,2008).

2.6 FISH AS MODEL ORGANISM

Fish have proved to be valuable experimental models for the evaluation of the health of aquatic ecosystems exposed to environmental pollution. Being highly developed vertebrates in the aquatic ecosystems, fish are of foremost significance in ecotoxicology, mainly as important model organisms for toxicity assessment of water pollution. Fish are also of the immense economic value, being a significant source of polyunsaturated fatty

acids, proteins and other essential components in human diet and used as food (Chung *et al*., 2013). Fish are highly susceptible to the presence of pollutants and are considered to be important indicators of environmental pollution due to their location in upper trophic levels (Prusty *et al*., 2011).

Zebrafish (*Danio rerio)* has distinct properties regarding the existing knowledge and technology. It is inhabitant to waters of Pakistan, India and South Asia and presents several benefits due to its short life cycle and production of large number of off spring. It is relatively easy to maintain in the laboratory and allows various kinds of experimental treatments (Redfern *et al*., 2008). Zebrafish embryo is well thought as a refinement of animal experiments due to availability of information on its developmental process. The formation of major organs is completed at 5 days post fertilization (dpf) and hatching takes place 12–36 hours later (Rubinstein, 2003). Also, most of basic cellular and molecular pathways involved in the response to chemicals or stress are conserved between the zebrafish and mammals (Voelker *et al*., 2007). Zebrafish embryos are also being used in toxicity studies (Truong *et al*., 2011) as the embryo production occurs frequently and developmental stages areliable to experience less or no suffering or distress for being used in such experiments. Therefore, zebrafish embryo is included as one of the principal test organism within the Organization for Economic Cooperation and Development test guidelines for EDCs (OECD, 2006).

Studies using embryos of the zebrafish instead of adult fish for illustrating the toxic potential of chemicals have been projected as animal replacing methods. Low dose exposure for 48 hrs with three model substances was carried out using zebrafish embryos. It was concluded that effect analysis at the molecular level might enhance sensitivity, specificity, and predictive value of the embryonal studies (Hanisch *et al*., 2010). In another

study, zebrafish embryos and larvae were exposed to various concentrations of organochlorine pesticides, TBBPA and BPA. The gene expression levels of the biomarker gene VTG showed induction of mRNA levels and proved to be a sensitive biomarker of exposure to these   organic compounds. Thus biomarker genes are a useful tool for revealing their undesirable effects and setting water quality guidelines (Chow *et al*., 2013).

2.7 BIOMARKER GENES

Biomarkers are key molecular or cellular events that link a specific environmental exposure to a health outcome.The development of molecular technologies has enabled the study of body responses to environmental agents. Such technologies include sensitive biomarkers that can measure subtle changes in inflammation, oxidative damage and other pathways that may disrupt normal physiology of the body. This study validates few biomarker genes in fish for exposure to environmental contaminants BPA and 2,4-D.

2.7.1 CYP1A Gene

The metabolism of the majority of xenobiotics and foreign compounds is catalysed by cytochrome P450 (CYP) family. It includes mostly its 1, 2 and 3 isoforms. The specificity of these isoforms overlaps between various families of substrates (Pelkonen *et al*., 2008). The CYP19 gene in fish encodes cytochrome P450 aromatoase enzyme. It consists of two subtypes. The CYP19A is expressed in ovaries while, CYP19B is expressed in brain. The CYP19B gene, also known as aromatase B, because of encoding brain form of aromatase is having more significance. It is much sensitive to estrogens and its expression is only restricted to radial glial cells (RGC) that act as progenitors of neuronal tissues in both developing and adult stages of fish.

Gene expression profiling has proven to be useful in the testing and validation of biomarkers of EDC exposure. Various studies report altered expression of CYP genes due to estrogenic compounds. In BPA exposed fish brain and gonad, CYP19A and CYP19B genes showed an upregulation (Lee *et al*., 2006). It indicates that BPA may have neurotoxic or gonadotoxic potential by upregulation of cytochrome P450 aromatase during embryogenic phase (Suzuki *et al*., 2003).

The expression profiles of genes involved in sex steroid synthesis and action as well as sexual development in adult male and female *Cynoglossus semilaevis* were determined after exposure to different concentrations of BPA and 17 β-estradiol (E_2) (Fenling *et al*., 2013). In another study, zebrafish embryos exposed to 12.4 μM of 3,4 dichloroaniline (3,4-DCA) caused induction of cyp1A gene (Voelker *et al*., 2007). A study by Dong *et al*., (2009) determined the effects of atrazine exposure in males and females of adult zebrafish. The liver microsomal cytochrome P450 content was measured at 0.01, 0.1, and 1 mg/L of atrazine for 5, 10, 15, 20, and 25 days. Within the range of tested concentrations, either P450 content or P450 isozyme activities were increased by atrazine.

2.7.2 AROMATASE Gene

Aromatase B (Aro-B) gene encodes the enzyme aromatase, which is a vital enzyme that aromatizes androgens into estrogens. This local synthesis of estrogen is possibly very imperative for the growth, development and sex differentiation of the brain. Various studies report for the use of Aro-B gene as a sensitive marker of the effects of xenoestrogens on the central nervous system during embryonic development (Kishida *et al*., 2001) and in zebrafish juveniles (Kallivretaki *et al*., 2006). *In vitro* and *in vivo* exposure to E_2 results in upregulated expression of E_2 (Kazeto *et al*., 2004).

Page *et al*. (2006) also reported that brain cytochrome P450 aromatase (Aro-B) in zebrafish is expressed in radial glial cells and is highly activated by estrogens (E_2). It can be used *in vivo* as a biomarker of xenoestrogen effects on the central nervous system. By using Q-PCR assay, they confirmed that Aro-B gene is robustly expressed in juvenile zebrafish exposed to several xenoestrogens. High levels of Aro-A and B genes were observed in zebrafish embryos. Various concentrations of BPA *i.e*. 40, 200 and 1000 µg/L were given to zebrafish embryos at 72 to 96 hpf in a study by Chung *et al*. (2011).

2.7.3 HSP 70 Gene

Heat shock proteins (HSPs) are widely distributed among various organisms from bacteria to mammals. HSP70 is most prevalent group and its expression is distinctly induced upon exposure to environmental stressors such as heat shock, UV and γ irradiation, and exposure to chemicals (Wu *et al*., 2011b). HSP70 is reported to act like molecular chaperones. It rapidly binds to nascent polypeptides or any unfolded proteins thereby preventing the misfolding or aggregation of the substrate proteins. Many studies suggest its role as closely associated with stress tolerance in animal cells, and over expression of HSP70 increases anti-apoptotic action against cellular stress (Yamashita *et al*., 2010). Owing to their direct link with cellular stress, they have emerged to have good potential as biomarkers. Normally HSPs are highly conserved and cells respond to heat shock or environmental stress by increasing their levels (Park and Kwak, 2013). They contain both stress inducible and constitutively (HSC70) expressed genes. Inducible heat shock genes show increased transcription and translation in response to various stressors, while expression of constitutive forms is also found to increase during chemical stress. BPA exposure to Chironomus tentans (*Diptera Chironomidae*) was investigated by

validation of the expression patterns of heat shock genes. An increase in the expression of HSP genes was observed not only in a stress inducible form, but also in a constitutively expressed form (Lee *et al*., 2006). Exposure to BPA at 3 mg/L for 12–24 hrs induced the expression of the HSP70 gene (Planello *et al*., 2008). A study by Krone *et al*. (2005) also utilized HSP70 gene expression as an indicator of toxicity in the zebrafish embryo. They concluded HSP70 induction as a reliable and quick indicator of cell specific toxicity in the perspective of the multicellular living embryo.

2.7.4 GPX Gene

This gene encodes a member of the glutathione peroxidase family. Glutathione peroxidase functions in the detoxification of hydrogen peroxide and other oxide radicals, and is one of the most important antioxidant enzymes (Romero-Puertas**,** 2007). The effects of atrazine exposure on the induction of oxidative stress and the alteration of gene expression were determined in liver and ovary samples from female zebrafish (*Danio rerio*). The mRNA levels for the genes encoding antioxidant proteins including catalase and glutathione peroxidase (GPX) were upregulated significantly in the liver (Jin *et al*., 2010).

Zebrafish exposure to atrazine induced oxidative stress and altered detoxifying system. Activityof glutathione peroxidase and reductase increased atatrazine concentration of 0.3 and 90µg/L (Blahova *et al*., 2013). In another study, the activity of antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione s transferase (GST) were significantly repressed after exposure, indicating the occurrence of oxidative stress (Wu *et al*., 2011a). *Cyprinus carpio* exposed to 2,4-D for 96 hrs caused an elevation in catalase and GPX activities in

kidney of *C. carpio*. GPX activity was decreased after all treatments while, GST activity was higher than the control levels after exposure to pesticides (Oruc *et al*., 2004). It indicated that the toxicities of 2,4-D and other pesticides may be related to oxidative stress in fish. They may be regarded as important biomarkers for pollution monitoring.

2.8 Q-PCR TECHNIQUE

PCR technology is widely used to aid in quantifying DNA because the amplification of the target sequence allows for greater sensitivity of detection than could otherwise be achieved. In an optimized reaction, the target quantity will approximately double during each amplification cycle. In quantitative PCR (Q-PCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. In real time PCR, fluorescent signal is measured in order to calculate the initial template quantity, while the amplification is still progressing (Wildsmith *et al*., 2001).

The more sensitive and reproducible method of real time Q-PCR measures the fluorescence at each cycle as the amplification progresses. This allows quantification of the template to be based on the fluorescence signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors or inactivation of the polymerase have started to have an effect on the efficiency of amplification. Fluorescence readings at these earlier cycles of the reaction will measure the amplified template quantity where the reaction is much more reproducible from sample to sample than at the endpoint (Nolan *et al*., 2006). In real time Q-PCR, a fluorescent reporter molecule such as a double stranded DNA binding dye or a dye labeled probe is used to monitor the progress of the amplification reaction. With each amplification cycle, the increase in fluorescence intensity is proportional to the increase in amplicon concentration; with the Q-PCR

instrument system collecting data for each sample during each PCR cycle. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the "Ct" or threshold cycle. This Ct value can be directly correlated to the starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample.

Real time quantitative PCR is being used in growing number of research applications including gene expression quantification, expression profiling, single nucleotide polymorphism (SNP) analysis and allele discrimination, monitoring of viral load and other pathogen detection applications.

2.9 BIOMARKER GENES TESTED IN GOLDFISH CELL CULTURES

Identification and characterization of EDs by identifying and validating biomarkers of their exposure is imperative for predicting their harmful effects (Jung *et al*., 2012). Cell culture studies provide systems for ready and direct evaluation of tissues. This method is a helpful tool to study problems related to diseases, screening and studies of cell toxicity mechanisms. It has remarkable applications in evaluation of therapeutic agents that potentially may treat the dysfunction and toxicity assessment (Allen *et al*., 2005).

Since, there is a great need for physiologically relevant cell based assays that can provide the toxicity data which cannot be obtained from animal models. With the current growth in new technologies, knowledge and methods for complex data analysis, it is now practicable that data from cellular based experiments are able to predict toxicity. The cells are maintained in culture with controlled environmental factors, essential nutrients in

culture medium with reagents, kept at physiological temperaturein a sterile environment so that they are free from microbial contamination. A fibroblastic cell line derived from the scale of goldfish was used for the determination of the cytotoxicity of chlorophenols (CPs) in a study (Saito *et al.*, 2009).

Estrogen receptors (ERs) are steroid hormone nuclear receptors and act as ligand activated transcription factors. They have two isoforms, ER-α and ER-β, which can bind a wide variety of EDs and trigger the transcription of estrogen responsive genes (Revankar *et al*., 2005). Estrogen (E_2) hormone is essential for the development of reproductive organs, bone, liver and the cardiovascular system. It plays an important role in many physiological processes. ER genes are reported to show altered expression due to exposure with estrogenic compounds.

Vitellogenin (VTG) is an egg yolk precursor protein. It is generally produced by liver cells of female fish in response to E_2 secreted by the pituitary gland. Male fish also carry the VTG gene, although VTG protein is normally not expressed because the circulation levels of E_2 are extremely low in male blood plasma (Tian *et al*., 2009). Estrogenic compounds like E_2, nonyl phenol (NP), and BPA may induce the expression of VTG in male fish in a dose dependent manner. It signifies that the VTG gene in male fish can be used as a biomarker for evaluating the effects of EDs (Biales *et al.*, 2007). In a study, BPA (1, 10, 50, 125, and 250 mg/kg) and E_2 (0.5, 5, and 10 mg/kg) caused significant changes in the expression of VTG and CYP genes. It concluded, these genes can serve as reference biomarkers for estrogenic EDCs exposure in marine teleosts (Fenling *et al*., 2013). The gene expression profile of three goldfish estrogen receptor (ER) subtypes of aromatase B and vitellogenin (VTG) after waterborne estrogen exposures were examined. Liver produced the most consistent response with up regulation of ER

alpha in sexually regressed, mature and recrudescing males and in sexually regressed females. Aromatase B and VTG gene expression were affected by E_2 and EE_2 induced liver ERalpha and VTG mRNA levels (Marlatt *et al*., 2010).

Insuline like growth factors (IGFs) are crucial regulator of cell proliferation and survival. It has two ligands (IGF-1 and IGF-2). The majority of circulating levels of IGF-1 are produced in liver. It has been suggested as direct indicator of the effects of acute and chronic stressors on growth by various studies (Picha *et al*., 2012). In fish and mammals, the endocrine control ofgrowth works through the growth hormone (GH) and insuline like growth factor (IGF) axis (Reinecke, 2006). IGF-I is a single chain polypeptide.It is responsible for cell differentiation and proliferation. It also stimulates processes related toskeletal elongation, and ultimately for body growth (Le-Bail *et al*., 1998). GH and IGF levels are tightly controlled in the body to maintain the appropriate growth homeostasis. Lankford and Weber (2006) reported that exogenous treatment with the stress hormone cortisol leads to a consistent inhibition of IGF-I in rainbow trout. The results of various studies suggest that IGF-I could provide an accurate indication of the long term effects of stress on growth (Picha *et al*., 2012).

2.10 TREATMENT OF CONTAMINANTS AND ASSESSING TOXICITY STUDIES

There is a growing concern about the presence of environmental contaminants in surface water and groundwater throughout the world. Therefore, the treatment of these chemicals in drinking water and wastewater to prevent the exposure to human health and the environment is an area of immense importance (Chong *et al*., 2010). Many conventional treatment methods are relatively ineffective in removing toxic chemicals

from water (Linden *et al*., 2007). Hence, degradation of individual compounds has been practiced in lab or natural waters. Nevertheless, due to natural variations in water matrix and combined effects of chemicals in the aquatic environment may confound analysis of the treatment efficiency.

Further, analytical methods based on chemical reactions cannot represent the synergistic effects between water quality parameters and chemical mixtures at environmentally relevant concentrations. Therefore, bioanalytical assessments of residual estrogenic activity in treated water are used to assess the performance of the UV based advanced oxidation process for the degradation of estrogenic contaminants in water. Bioanalytical methods to evaluate residual biological activity of treated water presents one means to compare removal efficiency of biological activity (Chen *et al*., 2007a).

UV in combination with H_2O_2 as an oxidation process was used for the degradation of four EDCs including estradiol (E_2), ethinyl estradiol (EE_2), BPA and nonylphenol (NP) spiked individually or as a mixture in laboratory or natural river water. The removal rates of estrogenic activity were quantitatively evaluated by using *in vitro* yeast estrogen screen (YES) and *in vivo* vitellogenin assay. The applied treatment decreased the *in vitro* and *in vivo* estrogenic activity of VTG assays with Japanese medaka fish (*Oryzias latipes*) (Chen *et al.,* 2007b).

Another study determined the photodegradation of BPA in different water media and the efficiency of UV treatment in combination with hydrogen peroxide was investigated. The results showed a significant decrease of estrogenic activity of parent compound after 2 hrs irradiation in the presence of hydrogen peroxide (H_2O_2). Furthermore, Phenol,1,4-dihydroxylbenzene and 1,4-benzoquinone were identified by HPLC analysis as intermediate products of BPA photodegradation (Neamtu and Frimmel,

2006). Izadifard *et al*. (2010) conducted the dechlorination of PCB 138, under visible light and using methylene blue (MB) and triethylamine (TEA) in acetonitrile and water. The results show that PCB 138 may be dechlorinated efficiently using photocatalytic reaction.

Molkenthin *et al*. (2013) conducted experiments with UV-A (near-UV), UV-C (short-UV) and visible light assisted fenton like treatment of BPA. Various degradation products were identified including hydroxylated phenolic compounds. Decay of BPA parent compound and formation of degradation products were determined using HPLC analysis. UV direct photolysis effectively removed BPA and related estrogenic activity. Furthermore, UV in combination with H_2O_2 process was efficient for reducing embryo toxicity of BPA. However it resulted in the production of acidic intermediate compounds causing acute toxicity and late hatching in some medaka embryos (Chen *et al*., 2007b).

The degradation of pesticides efficiency of low pressure UV photolysis and advanced oxidation processes using hydrogen peroxide and titanium dioxide were carried out in a study. Low pressure direct photolysis using a high UV fluence (1500 mJ/cm^2) was found to be very capable for degradation of pesticides.While photolysis using hydrogen peroxide and titanium dioxide did not significantly caused their removal (Sanches *et al*., 2010). Hence the literature supports the biological assessment of UV based degradation of chemicals.

2.11 HYPOTHESES, EXPERIMENTAL APPROACH AND RATIONALE

The literature reviewed above provides evidence that the aquaic reservoirs are at great risk of contamination from industrial and agricultural pollutants. These chemicals are known to cause toxic endpoints in the physiology of aquatic life and ultimately to

humans. Therefore it is important to monitor the water quality of aquatic reservoirs and to test the robust biomarkers for environmetal contaminants in the aquatic life. The main purpose of this study was to evaluate the water quality and microbial diversity of a local aquatic reservoir and to validate the reliable and robust biomarker genes for the exposure of BPA and herbicide 2,4-D using fish as model organism. Furthermore the efficacy of UV based oxidation of these chemicals is tested through the biological assay by determining residual activity of degraded products.

The overarching hypotheis for this dissertation is that the microbial diversity plays an important role in determining the type of contamination and degradation pathways in the aquatic reservoirs and certain physiological parameters may be validated as biomarkers for environmental contaminants. In particular, the following specific hypotheses are tested.

1. Aquatic reservoirs harbor diverse group of bacteria involved in degradation pathways and complex interactions within aquatic ecosystem.

2. The environmental contaminants (like BPA and 2,4-D) are able to disrupt the expression of certain genes associated with various physiological endpoints in aquatic life.

3. UV based advanced oxidation processes are able to convert these chemicals into simpler compounds that may be inert or having different biological activity than the parent compound.

4. These chemicals are having estrogenic activity that may cause reproductive disorders and other adverse health effects in adult fish.

The diversity of bacterial community was investigated in a local water reservoir by the amplification of 16S rRNA gene in the isolated bacteria from Rawal lake water samples. The sequencing and phylogenetic analysis using Mega 4 software was carried out to determine the phylogenetic relationship of microbial diversity in the lake. The evolutionary history was inferred using the Neighbor Joining method. The evolutionary distances were computed using the maximum composite likelihood method.

To test the second hypothesis the chemicals BPA and 2,4-D were used to expose zebrafish embryos in system water. Following RNA extraction and cDNA synthesis from the exposed embryos/larvae, the relative expression of various genes was determined using Q-PCR technique. An increase or decrease in expression of tested genes as compared to control group could potentially illustrate toxic endpoints. Further these chemicals were treated using UV based catalytic oxidation and the degraded products were also used for the exposure to zebrafish embryos to test the biological activity of byproducts. The Q-PCR analysis of tested genes in zebrafish showed altered expression and demonstrated the efficiency of treatment through biological activity of treated chemicals in the exposed zebrafish.

In vitro exposure of adult goldfish liver cells with BPA and 2,4-D was carried out to test the estrogenic activity of these chemicals. Liver tissues were excised from male and female goldfish separately and incubated in sterile nourishing media to keep the cells alive along with different concentrations of BPA and 2,4-D. The relative expression of various genes expressed in liver of adult fish was determined using Q-PCR for testing the hypothesis of estrogenic activity of selected chemicals.

MATERIALS AND METHODS

Pollution of aquatic reservoirs is the problem of global importance. Based upon the literature review, this study was designed with the aim of evaluating the anthropogenic effects on the water quality and predominant microbial community of local aquatic reservoir. The study also focuses the validation of sensitive biomarkers for various environmental contaminants polluting the waterbodies and posing a threat to aquatic life and ultimately mankind.

3.1 WATER QUALITY AND EVALUATION OF PREDOMINANT MICROORGANISMS FROM RAWAL LAKE

Rawal Lake is situated in Islamabad across Kurang River with a surface area of about 8.75 km^2. It is a source of drinking water for the residents of twin cities, Rawalpindi and Islamabad. The Kurang River and the streams receive local spring discharges, diverted untreated sewage water and occasionally polluted runoff water during rainy season. An organized classification of the bacterial community of Rawal Lake was carried out in addition to its physicochemical parameters of water quality. Isolation and characterization of individual microorganisms was done for determining their phylogenetic relationships using molecular techniques *i.e*. 16S rRNA based identification and characterization.

3.1.1 Identification of Sampling Sites and Collection of Water Samples

Sampling sites from different locations of Rawal Lake and its tributaries were identified in consultation with representatives from Capital Development Authority

(CDA) and Water and Sanitation Agency (WASA) keeping in view the recharge points from municipal and industrial wastes (Figure 3.1). The sampling locations from Rawal Lake and its tributaries with GPS coordinates and elevations are summarized in Table 3.1. Water samples were collected according to standard protocols (APHA, 2012) from 10 different locations of lake for a period of eight months from April to November 2012. Samples were collected from approximately the same point, at same depth about the same time every month and monitoring of water quality was carried out (Figure 3.2).

Table 3.1: Sampling locations with GPS coordinates and elevations

Sampling location	Sampling Locations	GPS coordinates	Elevation (m)
1	Rawal Lake spillway	33N 41.662; 73E 7.352	559
2	Lake centre	33N 42.19; 73E 7.556	539
3	Diplomatic enclave inlet	33N 42.544; 73E 7.353	533
4	Convention centre inlet	33N 42.044; 73E 6.793	531
5	Lake shore	33N 41.631; 73E 7.028	531
6	Kurang river tributary	33N 43.587; 73E 8.416	537
7	Kurang inlet	33N 42.707; 73E 8.245	531
8	Bani Gala inlet	33N 42.606; 73E 8.23	530
9	Malpur inlet	33N 42.767; 73E 7.786	542
10	Nurpur stream	33N 43.132; 73E 7.343	558

For the study of physical parameters, the water samples were collected in clean and sterilized glass sample bottles of 1 L. Triplicate samples were collected from each site.
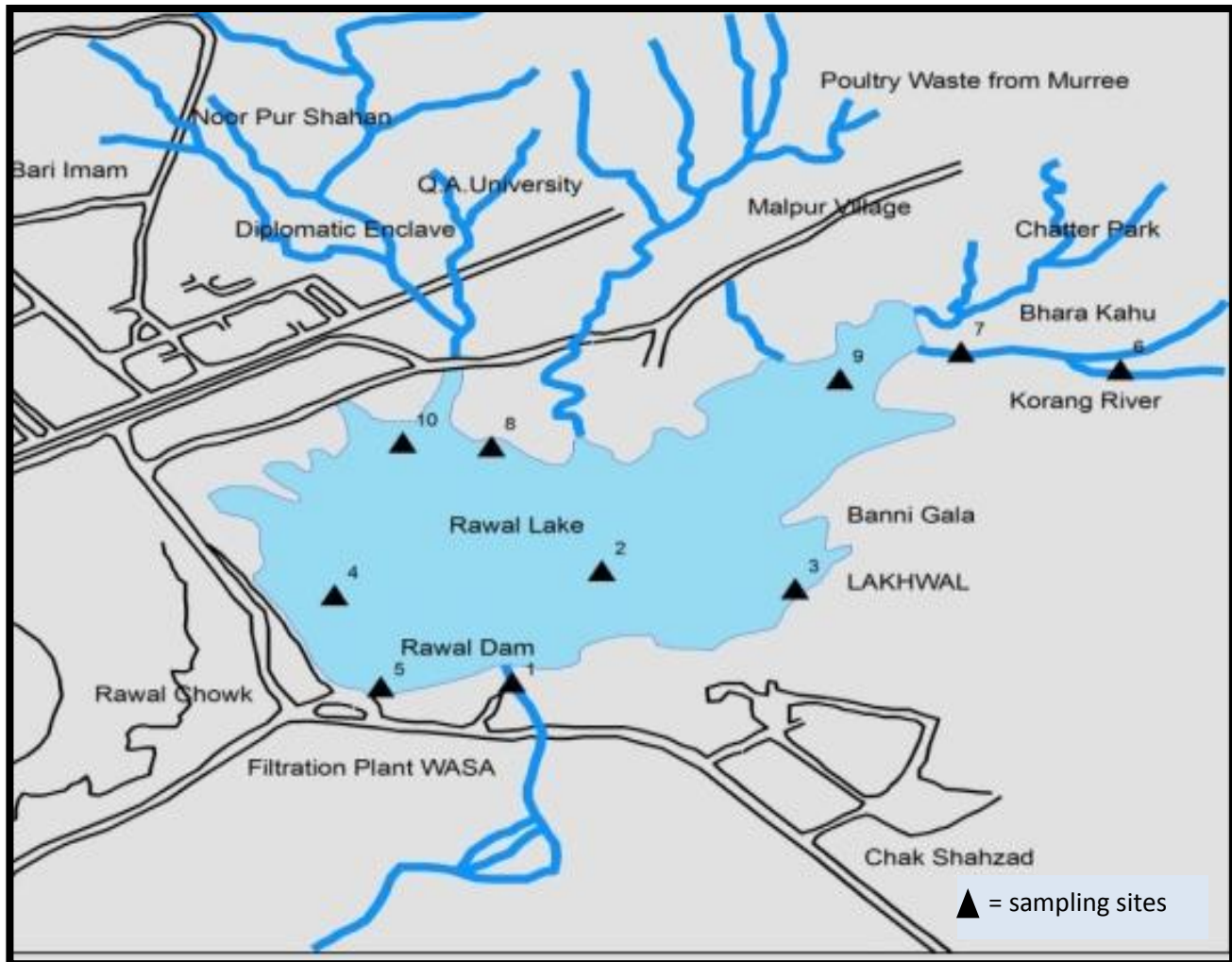
Figure 3.1: Sampling locations from Rawal Lake and its tributaries (1) Rawal Lake spillway (2) Lake centre (3) Diplomatic enclave inlet (4) Convention centre inlet (5) Lake shore (6) Kurang river before inlet (7) Kurang river inlet (8) Bani Gala inlet (9) Malpur inlet (10) Nurpur stream

Figure 3.2: Monitoring of water quality in Rawal Lake

Temperature, pH (Hach pH meter sension 1) TDS and EC were determined by Hach meter onsite. Turbidity (Hach 2100) and hardness of water samples were determined by volumetric method (APHA, 2012).

3.1.2 Isolation of Microbes from Water Samples

Pure cultures of microbes from water samples were isolated using standard protocols. The petri plates were washed, autoclaved at 121°C, 15 psi for 20 min, and then dried in oven at 171°C for 1 hr. The nutrient and selective agar (Merck KGA) were prepared according to standard manufacturer's instructions and poured into sterile petri plates. Plates containing media were incubated for 24-48 hrs at 37°C to verify the sterility of the media. The water samples were spread on agar plates and pure cultures were obtained by repeated streaking of individual colonies on agar plates. Various biochemical tests were performed on isolated microbes including; colony morphology, gram reaction, catalase and oxidase tests to identify the microbes (Bergey's Manual of Determinative Bacteriology, 1994). Preservation of microbial cultures was done according to standard protocols in 15% glycerol stock solution at -20°C.

3.1.3 Gram Staining

Gram staining is a bacteriological laboratory technique used to differentiate bacterial species into two large groups, gram positive and gram negative based on the physical properties of their cell walls. The gram stain is usually the first step in the identification of a bacterial organism.

3.1.4 Colony Morphology

Colony morphology is a technique to recognize shape, color, and general appearance of an individual colony of bacteria on a plate; these characteristics can frequently be used to identify or at least narrow down the species present on the plate.

3.1.5 Oxidase Test

The Oxidase test is used to differentiate between the families of *Pseudomonadaceae* (ox$^{+)}$ and *Enterobacteriaceae* (ox^{-}). It is very useful for speciation and identification of many other bacteria that have to use oxygen as the final electron acceptor in aerobic respiration. Good sized amount of inoculums was picked from a culture plate or slant culture and placed it on a piece of filter paper. *N, N, N', N'*-tetramethyl-*p*-phenylenediamine (TMPD) was added dropwise. Reaction time was noted, a positive reaction occurred within 20 sec. A positive test (ox+) resulted in a color change from violet to purple, within 10–30 seconds.

3.1.6 Catalase Test

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Hydrogen peroxide is a byproduct of respiration and is lethal if it accumulates in the cell. Catalase is an enzyme that can degrade the hydrogen peroxide in the cell before it can do any cell damage. It splits the H_2O_2 to free oxygen (bubbles) and water.

3.1.7 API kit

API 20E is a test kit used for the identification of non fastidious and enteric bacteria. It consists of a plastic strip that has 20 mini cupules in it. Every cupule contains a specific medium for biochemical characterization. For performing the tests, a saline

suspension (0.85% NaCl) was prepared and autoclaved. Saline suspension were formed for fresh colonies; the suspensions were added in the cupules of API strips till the end except for citrate utilization (CIT), voges-proskauer (VP) and gelatin liquefaction (GEL), where the cupule was filled completely. A drop of mineral oil was added in the cupules filled to neck to avoid drying out. The strip was covered with the lid provided and placed in incubator at 37°C overnight. Color changes were noted and results was recorded as in Table 3.2. In the entire carbohydrates test, fermentation is shown by acid production and is indicated by yellow color. Few cupules have to be provided with reagents, supplied by the manufacturer. TDA reagent is added into TDA cupule. James/Kovacs reagent was added to IND while VP1 and VP2 were added to VP. The test was allowed to develop for a few minutes and results were recorded. An additional oxidase test was performed to develop seven digit code required for API web software.

Table 3.2: API indicator color

Cupule Medium	Positive Results	Negative Results
O-Nitrophenyle-B-D galactoside	Light yellow to yellow	Colorless
Arginine Dehydrolase(ADH)	Light to dark red	Yellow
Lysine Decarboxylase(LDC)	Light to dark red	Yellow
Onthinine Decarbolyxase(ODC)	Light to dark red	Yellow
Citrate Utilization (CIT)	Blue green to blue	Pale green to yellow
Hydrogen Sulfide	Black	Gray to colorless
Tryptophan deaminase (TDA)	Deep red	Brown

Indole(IND)	Pink	Colorless/Pale green/Yellow
Voges-Proskauer(VP)	Pink/Red	Colorless/Slight pink
Gelatin Liquefaction(GEL)	Goes black (digested)	No change
Glucose(GLU)	Yellow	Yellow green, green, blue
Mannitol	Yellow	Yellow green, green, blue
Inositol	Yellow	Yellow green, green, blue
Sorbitol	Yellow	Yellow green, green, blue
Rhamnos	Yellow	Yellow green, green, blue
Sucrose	Yellow	Yellow green, green, blue
Melibiose	Yellow	Yellow green, green, blue
Amygdaline	Yellow	Yellow green, green, blue
Arabinose	Yellow	Yellow green, green, blue

3.1.8 Preparation of DNA

For preparation of genomic DNA, it is important to use a pure 24 hrs grown culture. The pure cultures of isolated bacteria were streaked on nutrient agar slants, after 24 hrs of incubation, slants were washed with 0.85% saline solution to obtain pure culture suspension. The DNA was isolated by using Norgen bacterial DNA isolation kit according to its protocol and stored at -20°C.

3.1.9 Amplification of 16S rRNA Gene

For polymerase chain reaction (PCR), 3µl of template DNA was added in PCR reaction solution (25 µl) as mentioned in Table 3.3. The master mix included 1µL dNTPs (5 mM), 5 µL Buffer (10x), 5 µL MgCl$_2$ (10x), 0.12 µL Taq polymerase (Biobasic

Inc.). The primer pair 27F/1492R was used for the amplification of 16S rRNA genes as shown in Table 3.3 (Turner *et al*., 1999). Then 35 amplification cycles (Extragene, USA) were performed at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec respectively. DNA fragments were amplified about 1,500 base pairs (bp) for bacteria. A positive control (*E. coli* genomic DNA) and a negative control were also included. PCR products were detected after the electrophoresis (Mupid one) using 1% agarose gel. To remove unincorporated primers and dNTPs from PCR products, PCR cleanup kit (Millipore) was used.

Table 3.3: PCR reaction mix protocol

Reagents	Quantity used	Final concentration
Template DNA	3 μL	50 ng/ μl
dNTPs(5mM)	1 μL	0.2mM
Buffer (10X)	5 μL	1.0 X
MgCl$_2$(25mM)	4 μL	1.5 mM
F- primer	1 μL	12 ng
R- primer	1 μL	12 ng
Taq Polymerase	0.2 μL	2.5 U
Deionized water	9.8 μL	-----

3.1.10 Sequencing and Phylogenetic Analysis

The purified PCR products were sequenced by using universal primers (Table 3.4). Sequencing was done by using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA) and products of sequencing were determined on an automated DNA sequencing system (Applied Bio Systems, USA) at the Macrogen, Inc., Seoul, Korea.

Partial sequences were generated using the primers which target one of the conserved regions. Sequences of nearest relative microbes were identified from basic local alignment search tool (BLAST) and they were used as reference sequences for phylogenetic analysis.

Table 3.4: Primers of amplification and sequencing

Primer	Sequence	Amplification	Sequencing
27F	AGAGTTTGATCMTGGCTCAG	yes	------
1492R	TACGGYTACCTTGTTACGACTT	yes	------
518F	CCAGCAGCCGCGTAATACG	------	yes
800R	TACCAGGTATCTAATCC	------	Yes

DNA sequences were associated manually against sequences obtained from Genebank database of NCBI (www.ncbi.nlm.nih.gov/BLAST). The sequences were then aligned using the CLUSTAL W program (Duckworth *et al*., 1998). Phylogenetic trees were inferred using neighbor joining (Saitou and Nei, 1987) method in Mega 4. The resultant neighborjoining tree topology was evaluated by bootstrap analysis (Felsenstein, 1985). Phylogenetic analysis was carried out using the Mega 4 program as done previously by Mwirichia *et al*. (2011) and Tamura *et al*. (2007).

3.2 VALIDATION OF BIOMARKERS IN ZEBRAFISH EMBRYOS EXPOSED TO BPA AND 2,4-D

All protocols involving zebrafish embryos and adults were approved by the University of Calgary animal care committee in accordance with the principles and guidelines of the Canadian council on animal care.

3.2.1 BPA, 2,4-D Exposure and Determination of Relative Gene Expression

Zebrafish embryos were obtained from the wild type adult zebrafish purchased from local pet store of Calgary, AB Canada. Fish were kept in laboratory in a dechlorinated RO water recirculating system with 14:10 hr light and dark photoperiod. The fish were fed with brine shrimp (*Artemia nauplii*) and commercial diet twice daily. Embryos were obtained by zebrafish breeding in breeding tanks, and washed to remove debris. Further, unfertilized, unhealthy and dead embryos were identified and removed by microscopic examination. Seventy embryos per replicate (Five replicates for each treatment) were counted and transferred to sterile plates with fish system water (4 ml). At 5 hpf, embryos were spiked with 1 ml of RO water containing respective concentrations of chemicals along with control group (No BPA exposure). Mortality was counted and the system water and BPA solutions were renewed daily untill embryos were collected at 48 and/or 72 hpf and snap frozen. Throughout all procedures, embryos and the solutions were kept at $28 \pm 0.5°C$ in incubator.

3.2.2 RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIZOL reagent (Invitrogen, Canada) according to the manufacturer's protocol. RNA was quantified and the quality determined using Nanodrop (Thermo Scientific) spectrophotometric readings at 260 and 280 nm. Four µg of total RNA was reverse transcribed in a total volume of 18 µl, using an oligo d (T) anchor and M-MLV reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to manufacturer's protocol. The resulting cDNA was diluted three fold and stored at -20ºC for use in Q-PCR.

3.2.3 Design of Real time Q-PCR Primers

Different biomarker genes for zebrafish were identified by a thorough literature review and real time Q-PCR primers were confirmed using IDT website and NCBI BLAST. The primers were checked for various parameters like, length and Tm of the primer, length of the amplicon, delta G values for hetero and homo dimers. The primers were synthesized by University of Calgary Core DNA Services. The primers's information is summarized in Table 3.5.

3.2.4 Quantitative Real Time Polymerase Chain Reaction

Q-PCR was conducted to determine the relative expression of mRNA levels from zebrafish embryos/larvae. It is a powerful tool for analysing gene expression and depends on measuring the increase in fluorescence emitted by a DNA-specific dye *i.e* syber green, during the PCR reaction. Relative quantification approach was employed for determination of mRNA by Q-PCR. It is based on the expression levels of a target gene versus a housekeeping gene and in theory is adequate for most purposes to investigate physiological changes in gene expression levels. Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. Calculations were based on the comparison of the distinct cycle determined by threshold values (Ct) at a constant level of fluorescence. Zebrafish ß-actin gene was used as an internal control. All forward and reverse primers along with their optimized annealing temperatures are listed in Table3.5.

PCR amplification was conducted using BIORAD I cycler Multicolor Real Time PCR Detection System (Bio, Rad, USA) and iQ TM SYBR Green Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Briefly, the conditions per well were

12.5 µL SYBR Green PCR Master Mix (Qiagen Mississauga, Canada), 1 µL of diluted cDNA, 0.25 µM dNTPs, 0.25 µM of each primer and ultrapure distilled water (Invitrogen Buvlington, ON, Canada) to a total volume of 25 µL. Q-PCR was carried out as follows, initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 10 sec, annealing at the gene specific temperature for 20 sec. Each experimental sample was run in triplicate to ensure consistency and no template control (NTC) was also included. As internal controls, experiments were duplicated with β-actin calculated threshold cycle (*Ct*) levels. The calibrated ΔCt value ($\Delta\Delta$Ct) for each sample and internal controls (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct$ sample $- \Delta Ct$ internal control)] (Livak and Schmittgen, 2001).

To ensure that the primers amplified a single product, a melt curve was performed for every Q-PCR plate. The efficiencies of reactions were determined by performing the Q-PCR on serial dilutions of CDNA. All primers were optimized via gradient PCRs with zebrafish tissues and the efficiencies for all genes were between 90-105%, which is considered in the optimal range for Q-PCR analysis.

Table 3.5: Primers used in real time Q-PCR analysis of zebrafish genes

Primer Name	Sequence	References	Annealing temperatures (°C)
ß- actin	F-CGCGCAGGAGATGGGAACC R- CAACGGAAACGCTCATTGC	Keegan *et al*. (2002)	59.7
CYP1A	F-CATATCGTAGTATCCGTGGCTAAC R- CCCGAACTCATCGCTCATATT	Voelker *et al*. (2007)	55
Aro-B	F-AAGAGTTACTAATAAAGATCCACCGGTAT R- TCCACAAGCTTTCCCATTTCA	Sawyer *et al*. (2006)	58
Hsp 70	F- GCACAAGAAGGACATCAGTCAGA R-CGATGCCCTCGTACAGAGAGT	Kreiling *et al*. (2007)	54
GPX	F- CGACGATCCAAGCGTGGTGGA R- CAGCCGTCACACGTCTGGGC	Brammell and Wigginton (2010)	56

3.2.5 Optimization of Primers for Real Time Q-PCR

Adult zebrafish acclimated in lab conditions were dissected to get various organs like brain, gills, gonads and liver. The organs were snap frozen in liquid nitrogen and aliquoted in pre labeled 1.5 ml tubes for male and female fish separately. RNA extraction was carried out from all the organs following homogenization in Trizol reagent as described in section 3.2.4. Total RNA was quantified using Nanodrop and cDNA was prepared using calculated amounts of water and RNA for each organ of sample. Gradient and efficiency PCRs were carried out for the optimization of primers. In gradient PCR, the samples were run on a gradient of a range of different temperatures to find the optimum temperature which gives lowest Ct value for a good amplification. A good efficiency PCR, gave a difference of 2 folds with each dilution in results. It showed E value between 80-120 and R^2 value for the regression line was close to 1. In this way all primers were optimized for gradient and efficiency PCR.

3.2.6 Q-PCR Using cDNA ofZebrafish Embryos Exposed to BPA and2,4-D

After conducting gradient and efficiency PCRs with each primer sets and optimizing the Tm, cDNA of zebrafish embryos exposed to various concentrations of BPA and 2,4-D were used for real time Q-PCR analysis to determine the specific gene expression levels for control and exposed fish. After running PCR, the values of the threshold cycle (Ct) of the biomarker transcripts, and the house-keeping (ß-actin and GAPDH) transcripts, were obtained from the amplification plot. Each individual sample was run in triplicate, and the mean threshold cycles was determined by the linear portion of the fluorescence absorbance curve and used for the final calculation.

The *Ct* of the target transcript in the samples was normalized with the *Ct* of the house keeping transcript by subtracting the *Ct* of target from *Ct* of ß-actin or GAPDH accordingly. The relative fold induction of the target genes in each sample was then determined. Q-PCR data was interpreted using Biorad iQ5 software; all PCRs gave one melting curve which showed one amplification product and no dimers formation occurred. The melting temperature started from 70 to 80°C. If the melting temperature was below 70°C, that PCR was repeated. The data of the Q-PCR results was imported to excel files and one value of *Ct* mean was found for each sample. The calibrated ΔCt value ($\Delta\Delta Ct$) for each sample and internal controls was calculated.

3.2.7 Statistical Analysis

The data was analysed using Graphpad sotware, Inc. Version 4.0 a (Prism statistical software, Graph Pad Software, Inc., La Jella, CA, USA). One-way analysis of variance (ANOVA) with multiple comparisons of the means using the Tukey's test was applied only when ANOVA revealed the presence of statistical significant differences between groups (P<0.05). Two-way ANOVA was pefomed to compare the effect of UV treatment on gene expression. The value of P<0.05 was used as criteria for significantly different values of parameters. The values were plotted in graphs using the same software and imported to Powerpoint presentation for copying to other systems and for record keeping.

3.3 TRANSCRIPTOMIC EFFECTS OF BPA AND 2,4- D FOLLOWING UV TREATMENT

The chemicals BPA and 2,4-D in aqueous solution were treated by using UV based advanced oxidation processes (AOPs) for their degradation.

3.3.1 Treatment of BPA, HPLC Analysis and Exposure

BPA (Sigma Aldrich) was used to make 35 mg/L stock solution in Milli Q water by sonication. Based upon earlier experiments on direct UV photolysis, the aqueous solution was given UV-C treatment (254 nm) in Mercury lamp for 90 min. BPA degradation and the intermediates generated during the degradation process were analyzed by HPLC, using a Varian Prostar 210 HPLC instrument equipped with a 325 nm liquid chromatography (LC) UV-visible detector. A Zorbax SB C-18 (4.6×150 mm, 5μm) column was used to separate the parent compound from its byproducts. An isocratic elution with a 0.5 mL/min flow rate was used in analysis. The eluent was comprised of 65% acetonitrile and 35% water. The UV visible detector wavelength was set at 280 nm and the temperature was controlled at 25°C according to the retention times and the UV spectra based on comparison with authentic standards. HPLC analysis showed the disappearance of BPA along with formation of photoproducts.

For exposure, stock solution of 35 mg/L concentration of BPA was diluted to 50, 100, 150, 200 and 250 μg/L respectively for exposure to zebrafish embryos. Similarly, UV treated BPA of 35 mg/L stock was also diluted to the similar concentrations and used for the exposure to determine the variation in expression due to UV treatment of BPA.

3.3.2 Treatment of 2,4-D, HPLC Analysis and Exposure

To prepare 20 mg/L of 2,4-D solution, 10 mg of its pure product was dissolved in 500 mL of water using ultrasonication. The solutions were kept in the dark and stored in a refrigerator. The irradiation experiments were conducted in a small Pyrex glass vessel in which 20 mL of the solution was placed. The aqueous solution was given UV-A treatment (365 nm) with P-25 TiO_2 (2g/L) slurry in LED (Lithium Electron Diode) reactor

for 3 hrs according to Yu *et al*. (2013). The light intensity was 8.55×10^{16}. The solution that contained photocatalyst was stirred for 30 min in the dark to ensure that the adsorption of the pesticide on to the surface of photocatalyst reached equilibrium. During irradiation, a magnetic stirrer was used to homogenize the solution and all collected samples were initially centrifuged (5,000 rpm) for 5 min with a Fisher Scientific Micro Centrifuge (Model 59A); the supernatant was then passed through a 0.22 μm filter (Micro Separation) to remove all of the TiO_2 particles. 2,4-D was identified and quantified using a Varian Prostar 210 HPLC instrument equipped with a 325 nm liquid chromatography (LC) UV visible detector. A Kinetex pentafluorophenyl (PFP) column (2.6-μM, 100 Å) was used to separate the parent compound and its byproducts. A 20 μL sample was injected and isocratic elution was used in analysis. The eluent was comprised of 50% acetonitrile (0.1% w=v formic acid) and 50% water (0.1% w=v formic acid). The UV visible detector wavelength was set at 280 nm and the temperature was controlled at 25°C. The detection limit was 0.1 mg/L. HPLC analysis showed the degradation of 2,4-D along with formation of photoproducts.

For exposure, stock solution of 20 mg/L concentration of 2,4-D was diluted to 50, 100, 150 and 200 μg/L for exposure to zebrafish embryos. Similarly, UV treated 2,4-D of 20 mg/L stock was also diluted to the similar concentrations and used for the exposure to determine the variation in expression due to UV/TiO_2 treatment of 2,4-D.

3.4 GOLDFISH LIVER CELL CULTURES EXPOSURE TO BPA AND 2,4-D

Common goldfish (*Carassius auratus*) of mixed sex, weighing an average of 23 g and 7–12 cm in length, were purchased from Aquatic Imports (Calgary, AB, Canada) and

allowed to acclimatize for approximately 10 days. All protocols involving animals were approved by the appropriate university animal care committees.They were fed with commercial fish diet Nutrafin (Hagen, Baie d'Urfe´, Que´bec, Canada) floating pellets in accordance with the manufacturer's instructions. Water temperature was approximately 19 °C during early gonadal recrudescence (early October) stage. Day/night light condition was consistently maintained at 12:12 hrs light/dark cycle. Fish were anaesthetised in tricaine methanesulfonate (MS-222; Sigma-Aldrich, St Louis, MO, USA; and euthanised by spinal transaction. The fish was sacrificed to collect liver. Male and female fish liver was collected separately and immersed in M-199 media.

3.4.1 M-199 Media Preparation

M-199 media (Sigma Aldrich) was dissolved in double distilled water and continuously stirred on magnetic stirrer using sterile magnetic bar followed by addition of 2.2 g sodium bicarbonate ($NaHCO_3$), used for making 1L of media solution for immersing liver tissues. Later on, 5.96 g of HEPES (Sigma) was added and pH was adjusted on 7.2 using hydrochloric acid (HCl) and potassium hydroxide (KOH) pH=7 and respective buffers (BDH, VWR). Then 10 ml Penicillin/streptomycin (Gibco) was added and volume of the solution was adjusted to 1L. The media was filtered (Thermoscientific Nalgene) and stored at 4ºC till use.

3.4.2 Plating of Tissues and Exposure to Chemicals

The cells were plated in 6 well Falcon Primaria plates (VWR, Alberta, Canada) at a density of about 1 million cells/ml of culture medium and allowed to settle for 2 hrs at 28ºC. Tissues were chopped and rinsed in sterile media. The media containing tissues were incubated with various concentrations of BPA and 2,4-D at room temperature for 24

hrs. After incubation, the tissues were collected in pre-labeled eppendorf tubes, homogenized in tissue lyser (Qiagen) according to manufacturer's instructions. At the end of incubation period, media samples were removed and cells were used for RNA extraction. Each experiment was repeated with five replicate wells per cell preparation.

3.4.3 Determination of Relative Gene Expression in Exposed Tissues

The homogenized liver tissues were processed for RNA extraction and cDNA synthesis for subsequent use in real time Q-PCR analysis as described in sections 3.2.4-3.2.7 using goldfish glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as house keeping gene. The primers for real time Q-PCR analysis of goldfish liver tissues are presented in Table 3.6.

Table 3.6: Primers for Q-PCR analysis of genes in goldfish liver

Primers	Sequence	Tm (°C)
GAPDH	F-5-′TGATGCTGGTGCCCTGTATGTAGT-3′ R-5′-TGTCCTGGTTGACTCCCATCACAA-3′	57
CYP1A	F- 5′-TTGTGCGGGTTTGGATCAATGGTG-3′ R-5′-TTCCGATACACTGCAGACCCAGTT-3′	55
VTG	F-5′-GAAGTGCGCATGGTGGCTTGTATT-3′ R-5′-AGCTGCCATATCAGGAGCAGTGAT-3′	55
IGF1	F-5′-GGACTCTTGCTCTCACTCTCTCCACGAA-3′ R-5′-GCCAAGGCCAGCTGCTTACATTTTGT-3′	56
ERß1	F-5′GGCAGGATGAGAACAAGTGG-3′ R-5′GTAAATCTCGGGTGGCTCTG-3′	55

Using the standard ΔΔct method, the relative expression of VTG, CYP1A, IGF-1 and ER-ß1 gene of goldfish was determined. The data was analysed by one-way ANOVA followed by Tukey's test using Graphpad PRISM software.

RESULTS AND DISCUSSION

The environment is continuously polluted with foreign organic chemicals released by anthropogenic activities of various kinds. This study aimed at the evaluation of water quality and microbial community associated with the biochemical interactions and toxicity potential of chemicals within aquatic reservoir. Phylogenetic relationship of predominant microorganisms and biomarker genes of fish associated with environmental contamination was also examined. Genomic bioassay based on real time Q-PCR technique have been used to measure transcriptional expression of multiple genes as potential biomarkers of BPA and 2,4-D exposure.

4.1 PHYSICOCHEMICAL ANALYSIS AND MICROBIAL EVALUATION OF RAWAL LAKE

Grabbed water samples were collected from ten different locations of Lake and its tributaries. Microbial culture based and molecular techniques were employed for isolation and identification of predominant microorganisms from water samples. For the study of physicochemical parameters, the water samples were collected in clean and sterilized glass sample bottles in triplicate.

4.1.1 Identification of Predominant Microorganisms

Various bacterial strains were isolated from Lake water samples by standard agar plating method. Colony morphology of isolated bacteria is given in Appendix as Table A.1. Various biochemical tests were conducted to identify the individual isolated strains

(Table 4.1). These include Gram reaction, oxidase and catalase test. Figures showing some biochemical tests have been summarized in Appendix-B as B.1-B.5.

Table 4.1: Biochemical tests performed on various isolated microbes

Reaction tested	As-1	As-2	As-3	As-4	As-5	As-6	As-7	As-8
Gram reaction	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Oxidase test	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve

4.1.2 Physicochemical Analysis

The physicochemical analysis of water samples showed that mean values of pH ranged between 7.57 and 8.01 at various locations of lake and tributaries. The values lie within the World Health Organization (WHO) permissible limits of 6 to8.5 as the highest pH value of 8.01 was observed at sampling location No. 4 in the lake as shown in Figure 4.1. The values of turbidity ranged between 5.89 to 19.43 NTU at various locations and tributaries of Rawal Lake. The highest value of turbidity was observed at sampling location No. 7 *i.e.* Inlet of Kurang River and it was above WHO limits of < 5 NTU.

Figure 4.1 shows the mean values of pH and turbidity at various sampling locations with the added values of standard deviation in error bars. Similarly the values of conductivity ranged between 277 and 531 µS/cm as shown in Figure 4.2. The values were found to be higher than WHO standards at the tributary of NurPur stream. The identified microbes within the lake water samples belonged to various phylotypes having the growth conditions compatible with the lake physicochemical parameters. TDS values were higher for location No. 6, 9 and 10 (Figure 4.2). The values within the lake area ranged between 137 and 140 mg/L. Dissolved oxygen (DO) concentration values, 6-9 mg/L were found

within WHO limits at various locations of lake and its tributaries as shown in Figure 4.3.

The values of hardness and alkalinity were observed highest at sampling location No. 10,

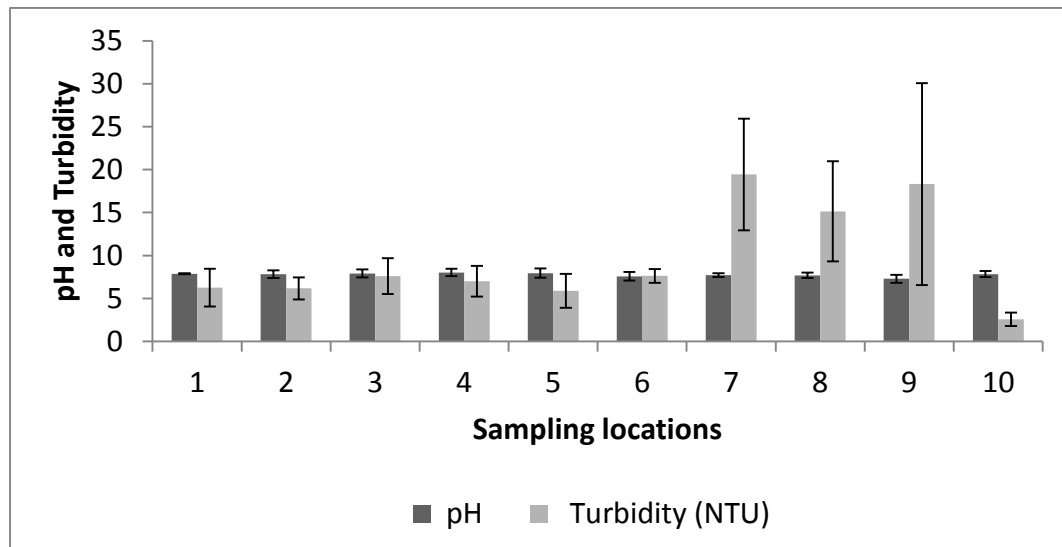the tributary of Nurprur stream entering into the lake (Figure 4.4).

Figure 4.1: pH and Turbidity of water samples at various locations and tributaries of Rawal Lake
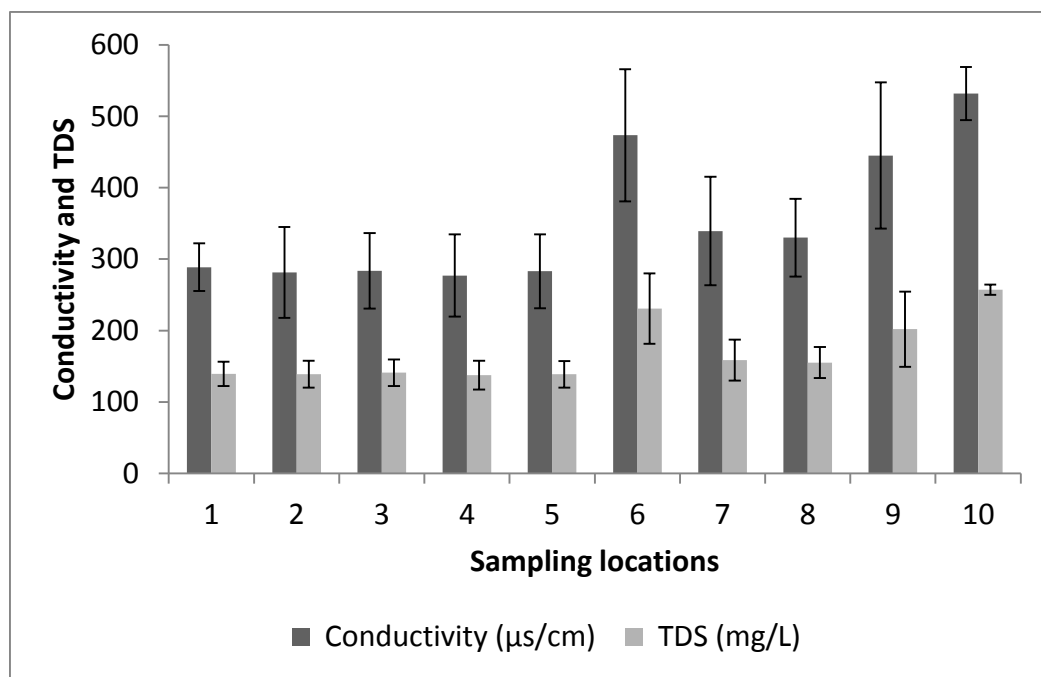
Figure 4.2: Conductivity and TDS of water samples at various locations and tributaries of Rawal Lake

Figure 4.3: Dissolved Oxygen and Temperature of water samples at various locations and tributaries of Rawal Lake

Figure 4.4: Alkalinity and Hardness of water samples at various locations and tributaries of Rawal Lake

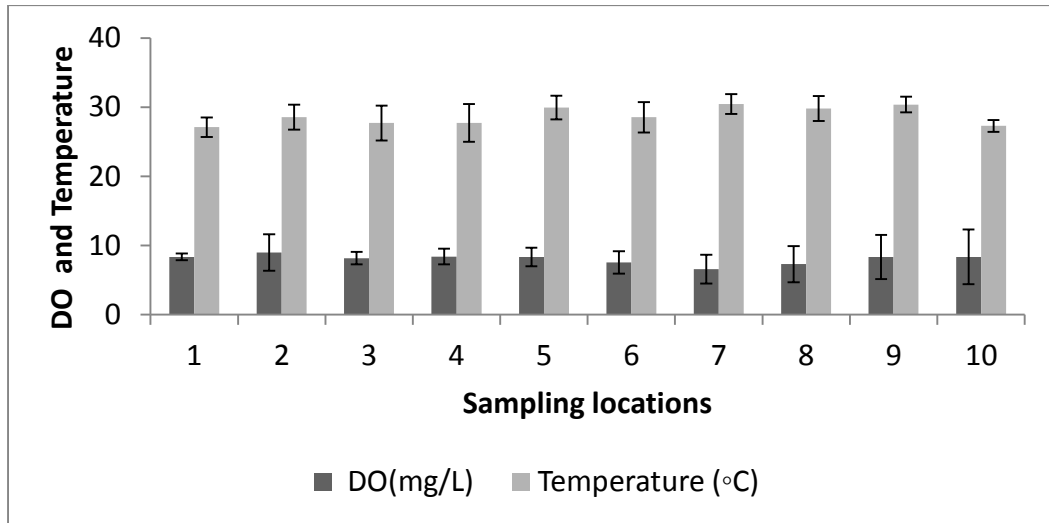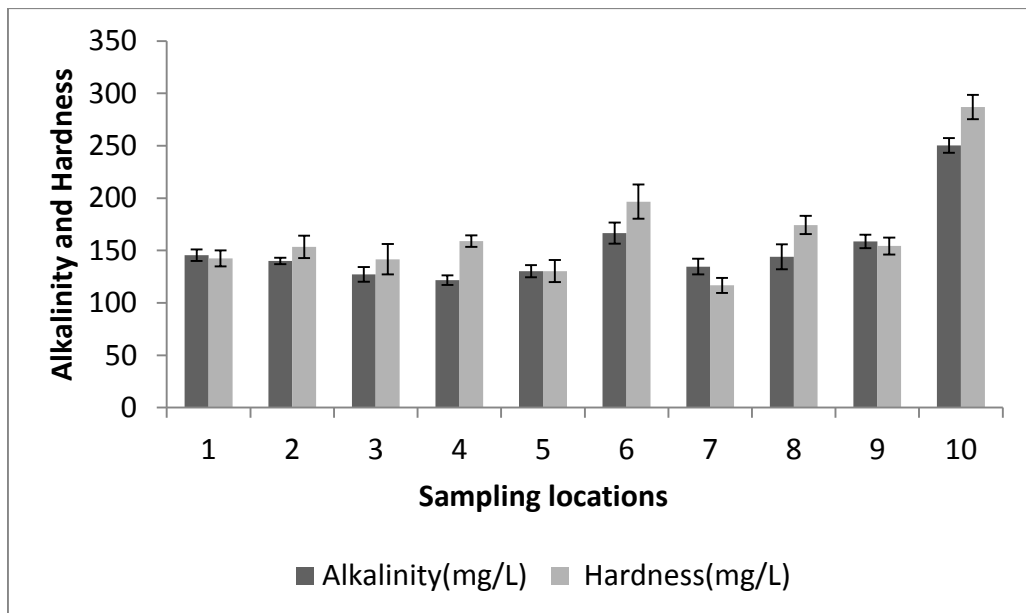The values of physicochemical parameters showed almost similar trends on the sampling locations within the lake due to dilution factor. While an increase in all values was observed at the tributaries as depicted by high hardness and alkalinity values which

ranged from 118 to 298 mg/L and 121 to 250 mg/L respectively. It suggests the entry of pollution load from the adjacent areas into the lake. Similar results were reported by Malik and Nadeem (2011) as quality of water was found to be relatively better where the sites had less impact from human activities, whereas sites at Lake View and Rawal Dam area which are close to Bani Gala Village, and where River Kurang drains into the lake reservoir were found to be more contaminated. Ghumman (2011) reported that runoff from adjacent areas brings a lot of sediments in rainy season. As a result, suspended solids are increased as well as bed load of sediments in Kurang River.

Jindal and Sharma (2011) have reported high turbidity during rainy season in rivers like Indus, Jhelum, and Chenab. As Rawal Lake receives water from Kurang River, so the high turbidity and TDS values result in high cost of treatment of water and causes sedimentation of the reservoir. Some other studies have concluded the similar results as Nezlin *et al.* (2009) and Luo *et al.* (2011) reported the results of contamination in water reservoirs from adjacent areas.

Various microbial species isolated from lake samples include *Bacillus pumilus,* it is a gram positive, spore forming bacteria commonly found in soil (Priest, 1993). It is aerobic in nature. It belongs to phylum *Firmicutes* of class *Bacilli.* The *Bacillus pumilus* strain GB34 is used in agricultural fungicides which suggest its source in this water reservoir. *Pseudomonas* is a genus of gram negative, aerobic *gamma proteobacteria*. It belongs to the family *Pseudomonadaceae*. Its habitat is soil, water, skin flora, and most manmade environments. It survives not only in normal atmospheres, but also in hypoxic atmospheres. It is found in many natural and artificial environments and is involved in biodegradation of various pollutant chemicals such as 2,4-D (Itah and Essien, 2005). As a

result of their metabolic diversity, they grow at low temperatures and ubiquitous in nature e.g., soil, water, plants (Palleroni, 2010).

Biodegradation plays a major role in elimination of BPA pollution in the aquatic environment. A bacterium able to degrade BPA as sole carbon and energy source was isolated from a planted fixed bed reactor. It is a gram negative aerobic bacterium. It was identified as *Cupriavidus basilensis JF1* by using characterization and 16S rRNA analysis. Its cellular degradation intermediates were found to be similar to a Sphingomonas strain (Fischer *et al*., 2010).

In a study by Yang *et al*. (2013), microbial community structure in river sediment and its shift during BPA biodegradation was studied. BPA modification had a significant impact on sediment bacterial community, influenced by dosage levels. *Gammaproteobacteria* and *alphaproteobacteria* were the predominant bacterial groups in BPA degrading sediment microcosm. It was also inferred that a consortium of microorganisms from different bacterial genera might be involved in BPA biodegradation in aquatic system.

Previous studies suggest that although there are many bacteria capable of degrading BPA and other agricultural chemicals in river waters, however, bacteria with high biodegradability are limited. Kang and Kondo (2002) found that most bacteria isolated from three river waters had the potential of BPA degradation but only two strains, a *Pseudomonas sp.* and a *Pseudomonas putida* strain, showed high BPA biodegradability. These species have also been identified in the present study from lake water samples after its 16S rRNA based identification and characterization. Based on such observations it may be suggested that microbial community of Rawal Lake has the biodegradability of such pollutants and the possible occurrence of such residues may be expected there. Moreover,

Streptomyces sp. strain isolated from riverwater has high BPA biodegradability as reported by Kang *et al*. (2004). Moreover, the biodegradation by bacteria is influenced by various water quality parameters such as pH, temperature and dissolved oxygen. As this process is found to be enhanced in aerobic environment which is favored by sufficient DO levels as observed in lake water samples of this study.

The microbial species identified in the lake also included *Enterobacter*. It is a genus of common gram negative, facultatively anaerobic, rod shaped, and non spore forming bacteria of the family *Enterobacteriaceae*. The genus *Enterobacter* is a member of the *Coliform* group of bacteria. *Enterobacter* species are found in the natural environment in habitats such as water and sewage. *Bacillus anthracis* isolated from lake is a gram positive, endospore forming bacteria. It is rod shaped bacterium and belongs to phylum *Firmicutes* (Spencer, 2003). It can be grown under aerobic or anaerobic conditions. *Bacillus anthracis* spores can survive in extreme of temperature, low nutrient environments, and harsh chemical treatment. These characteristics of the bacterial species favor their growth within the physicochemical water quality of Rawal Lake as described in this study. Previous studies demonstrated that pH, temperature and certain inorganic and organic contaminants are the most important drivers of the bacterial communities in sediment or soil for many lake ecosystems (Bai *et al*., 2012).

4.1.3 Amplification of 16S rRNA Genes

Figure 4.5 shows amplification of 16S rRNA gene for 8 different isolated microbes. The 16S rRNA gene sequence is 1,550 bp long and it contains both variable and conserved regions. Sequence of the variable region in between is used for the comparative taxonomy (Relman, 1999). The 16S rRNA gene is universal in bacteria, and so

relationships can be measured among all bacteria. In general, the comparison of the 16S rRNA gene sequences is used to differentiate between organisms at the genus level across all major phyla of bacteria. It may also classify strains at multiple levels, including the species and subspecies level.
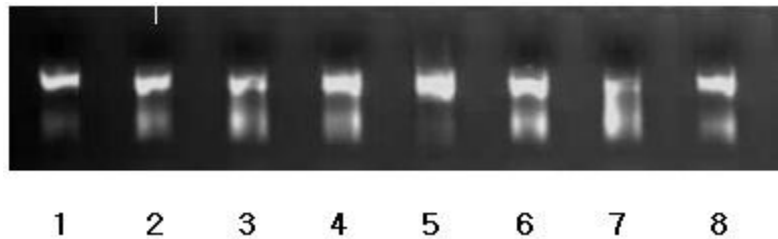

Figure 4.5: Amplification of 16S rRNA gene for the isolated microbes

4.1.4 Phylogenetic Relationship of Isolated Bacteria

The evolutionary history was inferred using the Neighbor Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length 215.78676589 is shown in Figure 4.6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset using complete deletion option. There were a total of 550 positions in the final dataset. The sequencing results showed that the bacterial species belonged to various phylotypes of which *Firmicutes, Teobacteria* and *Proteobacteria* were predominant. Most of the isolates belonged to the class G*ammaproteobacteria* in the present study.
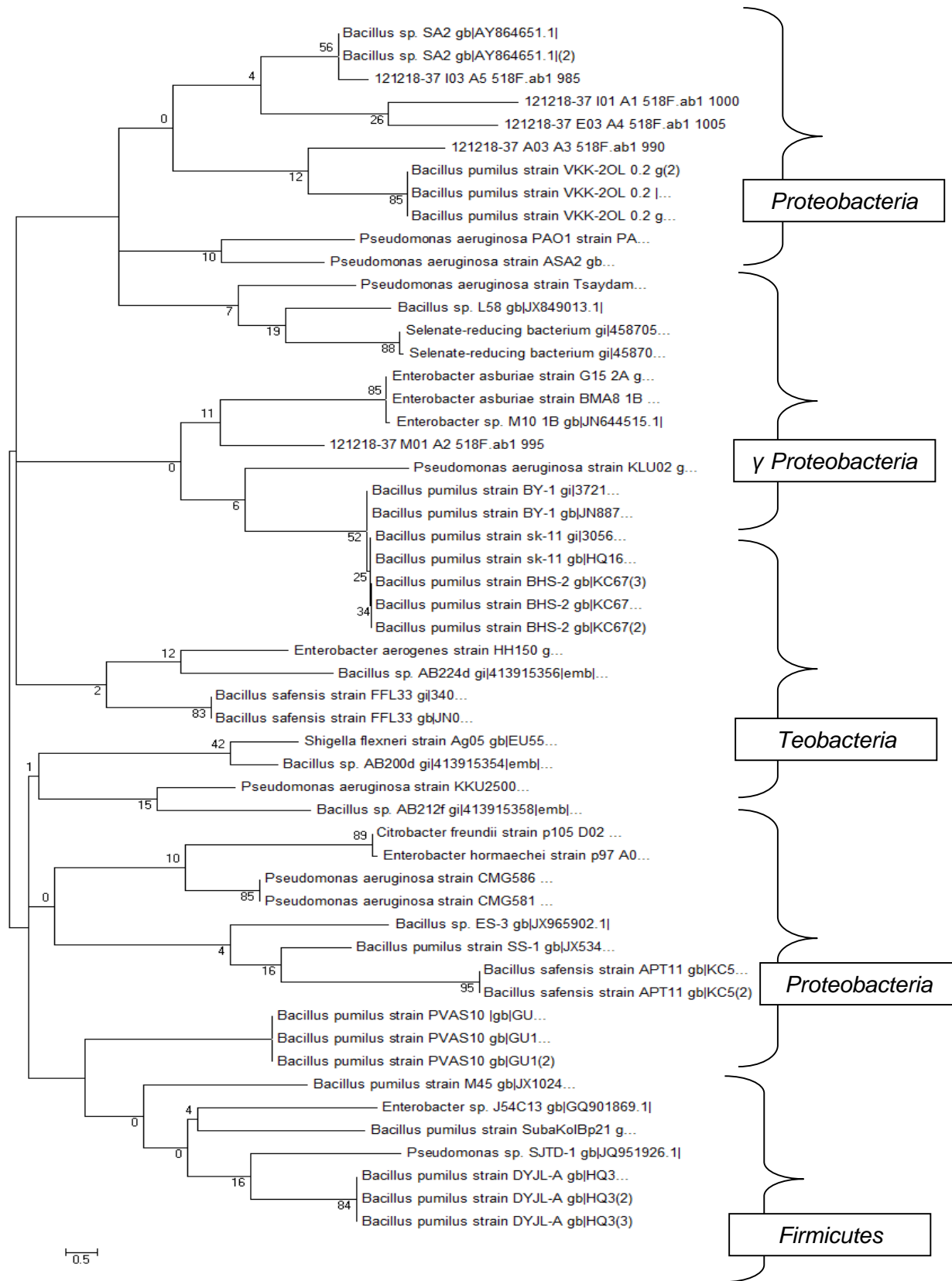
Figure 4.6: Evolutionary relationships of 85 taxa of microbes

The studies conducted by Baik *et al*. (2008) and Mwirichia *et al*. (2011) have also reported the presence of these phylotypes in freshwater ecosystem. The evolutionary relationship showed more than 90% similarity within the identified phyla. These results indicate that phylogenetic analysis for the selected representatives cover their phylogenetic position of all the isolates.

## 4.2 VALIDATION OF BIOMARKER GENES IN ZEBRAFISH EMBRYOS EXPOSED TO BPA AND 2,4-D

Various preliminary experiments were conducted to determine an effective BPA concentration showing variation in mRNA levels of the target genes to determine transcriptomic activity without significant mortality of zebrafish embryos/larvae. Few results of preliminary experiments are given in Appendix-C. Figures 4.7 (a-d) and 4.8 (a-d) show results of expression of tested genes following various concentrations and duration of chemical's exposure. Based upon the results of preliminary experiments, the exposure of zebrafish embryos with 100 µg/L BPA upto 48 and 72 hpf was carried out. Real time Q-PCR was performed to determine the difference among expression of control and exposed embryo groups. The mRNA level of each target gene was expressed after normalizing to the expression of ß-actin as housekeeping gene.

BPA exposure upto 48 hpf was not able to show major variation in expression of target genes (Figure 4.7 (a-c). While significant induction of Aro-B gene compared to control was observed at 72 hpf (Figure 4.7 (b) and one-way ANOVA give P value of 0.001. The expression of CYP1A gene increased about 2 fold compared to control and HSP70 gene expression also showed significant increase in mRNA levels at 72 hpf with P value of 0.0025 (Figure 4.8 (a,c).

(a)
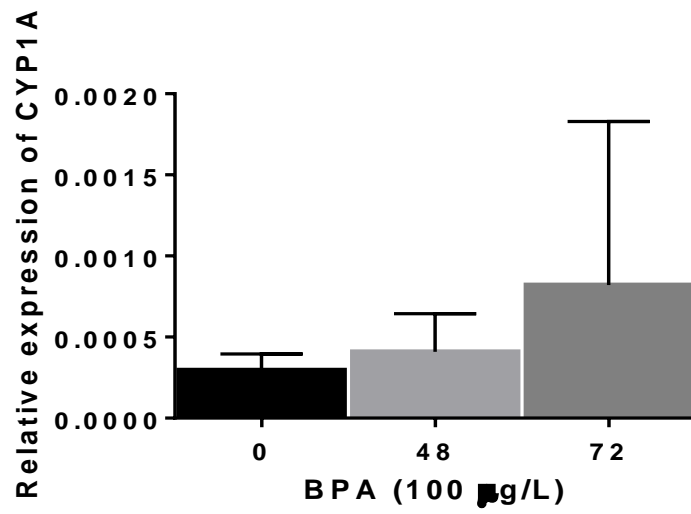
(b)
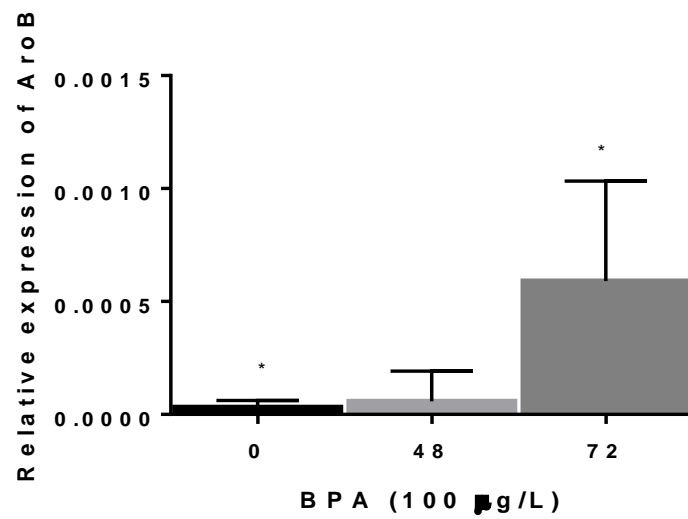
(c)

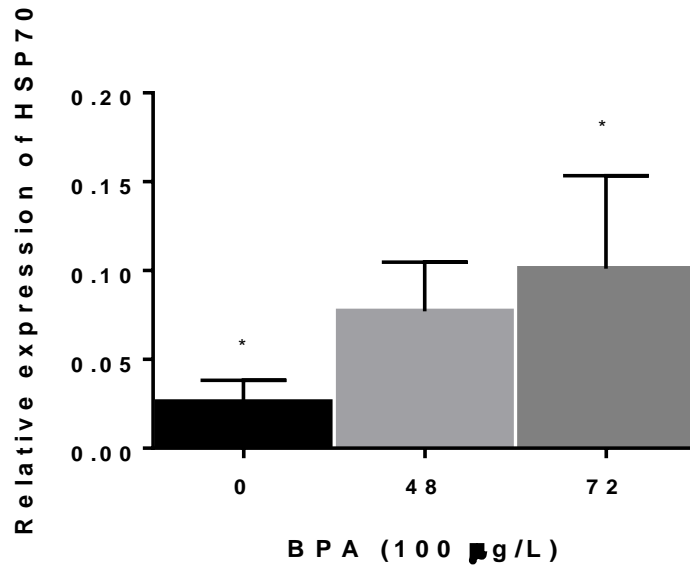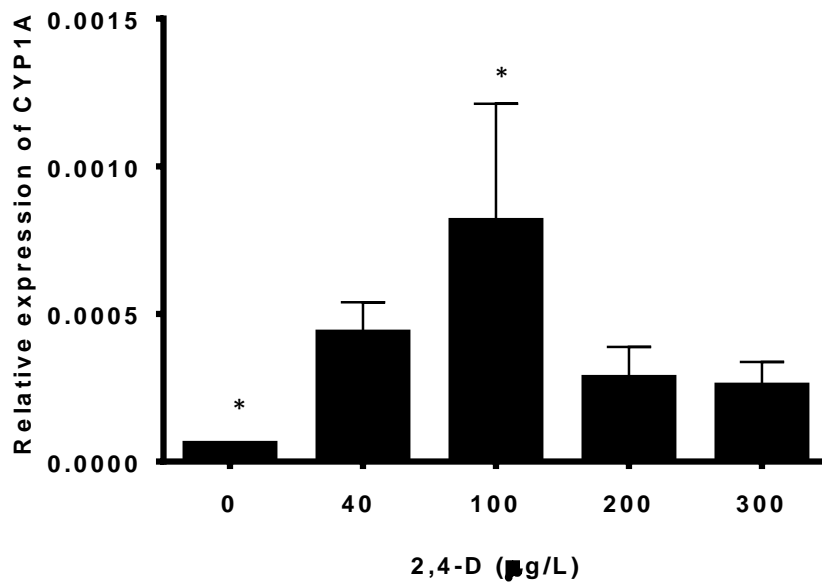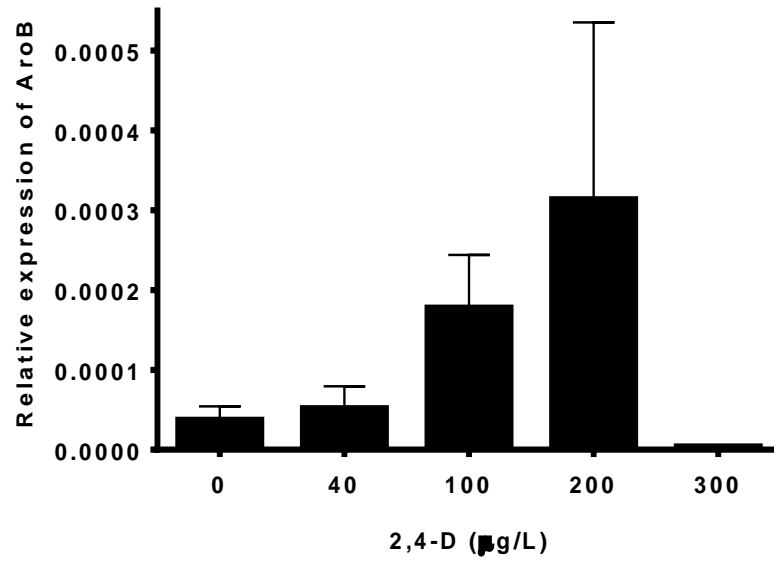Figure 4.7: Changes in the expression levels of CYP1A (a) Aro-B (b) and HSP70 (c) in zebrafish embryos exposed to 100 µg/L BPA for 48 and 72 hpf. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. All values are means ± SD (n=5). Asterik (*) upon bars denote statistically significant differences **(**ANOVA followed by Tukey test, $P < 0.05$**).**
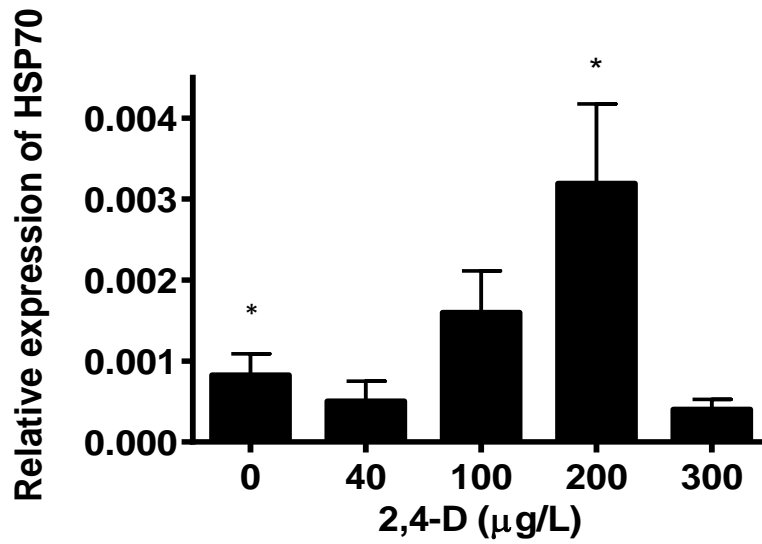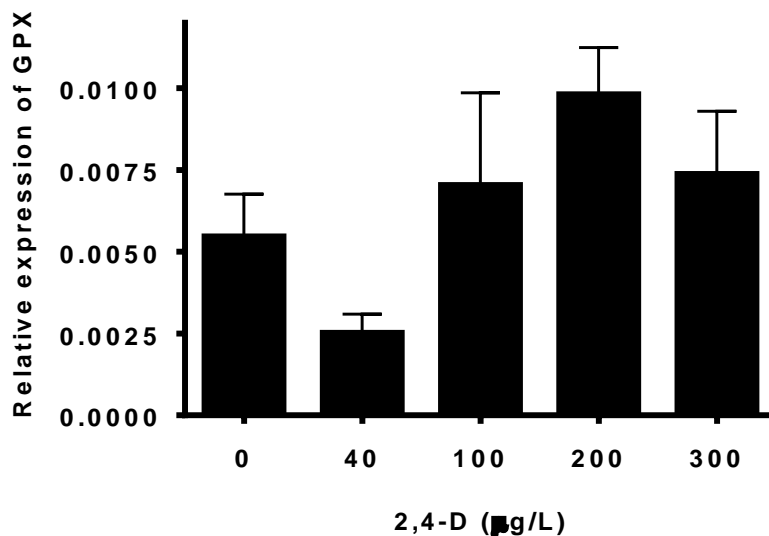
(a)

(b)

(c)

(d)

Figure 4.8: Changes in the expression levels of CYP1A (a) Aro-B (b) HSP70 (c) and GPX (d) in zebrafish embryos exposed to various concentrations of 2,4-D upto 72 hpf.The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$).

2,4-D exposure to zebrafish embryos, for 50, 100, 200 µg/L showed an increase in mRNA levels of CYP1A gene when exposed to 100 µg/L 2,4-D at 72 hpf. Aro-B expression showed an increasing trend in mRNA levels with higher exposure concentration of 2,4-D. The relative expression of HSP70 gene showed upregulation compared to control group with exposure of 200 µg/L 2,4-D. The relative expression of GPX showed a down regulation at 50 µg/L and a significant increase at 200 µg/L as compared to 50 µg/L with P value of 0.02. The same dose of 2,4-D has shown gene specificity and different pattern of expression in the tested genes probably due to different toxic effects. In the preliminary experiments, certain trend was observed in the expression pattern up to 200 µg/L of exposure concentration and this range was selected for further experiments. Beyond this, the disruption in gene expression was observed perhaps due to

60

toxic effects of 2,4-D at high dose. CYP1A and GPX expression is not found to be apparently affected perhaps due to different toxic endpoints and pathways involved in expression of these genes.

4.3 TRANSCRIPTOMIC EFFECTS OF BPA AND 2,4- D FOLLOWING UV TREATMENT

Following the identification of genes demonstrating altered expression due to BPA exposure, the expression of CYP1A, Aro-B and HSP70 genes was tested by repeating BPA exposure at 50, 100, 150, 200 and 250 µg/L along with the similar concentrations of UV treated BPA.The purpose was to further validate the altered expression of biomarker genes as well as to compare the curves of mRNA levels over a range of applied concentrations testing the efficacy of UV direct photolysis for BPA degradation.

4.3.1 Relative Expression of Zebrafish Genes after BPA Exposure

The relative expression of zebrafish CYP1A gene showed a trend of increasing mRNA levels with increasing BPA concentrations indicating an induction at 150 µg/L BPA (Figure 4.9 (a). While the exposure with UV treated BPA did not show such upregulation as indicated by Figure 4.9 (b). Figure 4.9 (c) shows the curve shift for variation in mRNA levels of CYP1A during UV photolysis. All the results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. All values are means ± SD (n=5). Values with asterisk (*) denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$).

(a)

Untreated BPA exposure

Relative expression of CYP1A

BPA (µg/L)

(b)

UV treated BPA exposure

Relative expression of CYP1A

BPA (µg/L)
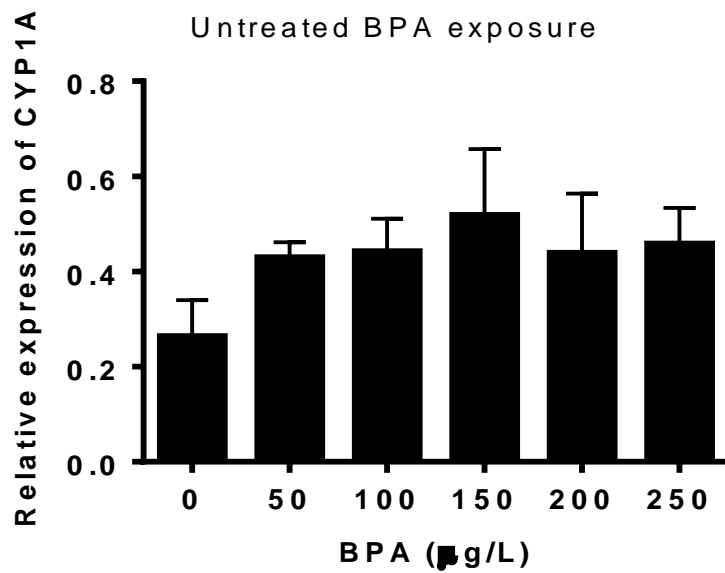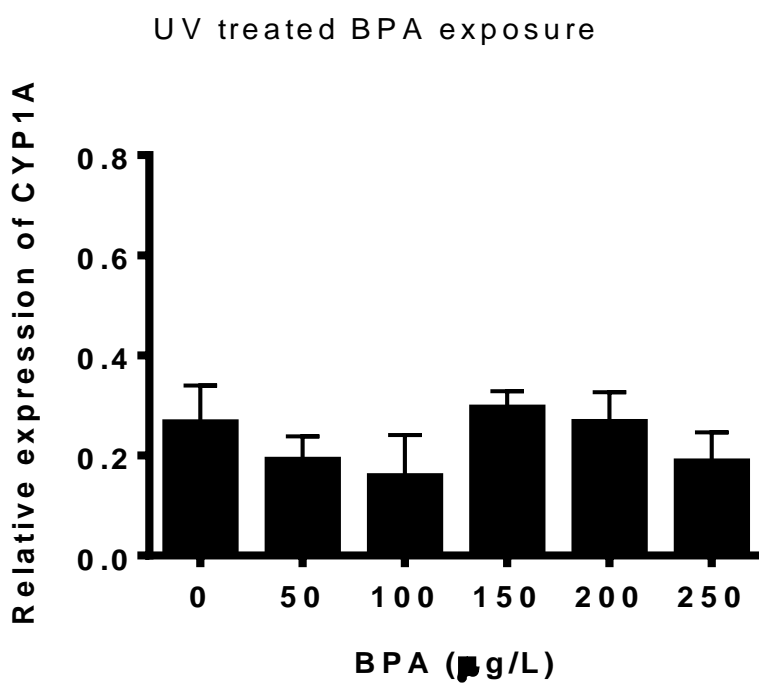
(c)
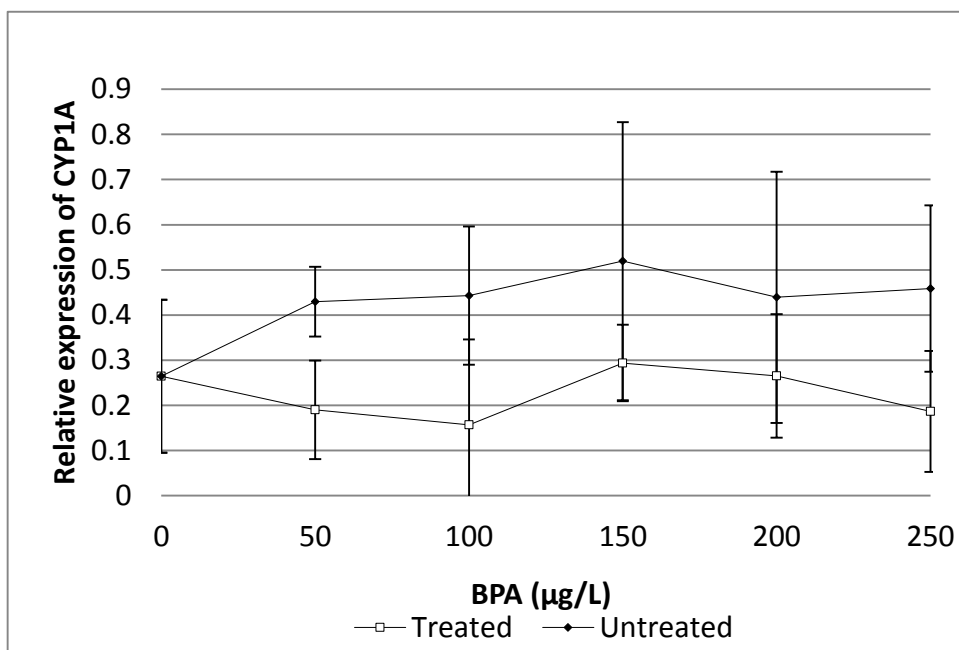
Figure 4.9: Changes in the expression levels of CYP1A gene in zebrafish embryos showing control (No BPA) and various concentration of BPA exposed to zebrafish embryos upto 72 hpf. CYP1A mRNA levels after BPA exposure (a), CYP1A mRNA levels after UV treated BPA exposure (b) Curve shift for CYP1A expression due to UV treatment of BPA (c).

Two-way ANOVA was performed to determine the difference among means of CYP1A expression for intact and UV treated BPA exposure. It indicated significant effect of UV treatment (P=0.0006) while no significant interaction and effect of dose was observed between the two groups.

Currently, induction of xenobiotic metabolism by CYPs in fish is well established as a useful tool for ecotoxicology assays (Lee *et al*., 2008). They play role in the detoxification and activation of pollutants through the aryl hydrocarbon receptor (AhR) mediated induction of the CYP family during phase I metabolism (Holth *et al*., 2008). As a result, a strong and rapid induction of CYP1A mRNA occurs in fish exposed to inducing compounds (Stegeman and Hahn, 1994), making it an ideal biomarker of exposure to

pollutants (Brammell and Wigginton, 2010). In a study, rare minnow embryos exposed to 10 and 100 µg/L Benzo (a) pyrene (BaP) within 4 hpf to 168 hpf showed significantly induced mRNA expression of three CYP1s in a dose dependent manner (Yuan *et al*., 2014).

While, the phase II metabolising system consists of conjugating enzymes such as glutathione peroxidase (GPX), involved in increasing the excretion and transcellular transport of xenobiotics (Xu *et al*., 2005). These enzymes are generally less responsive than phase I systems (Holth *et al*., 2008) as GPX gene expression is not altered greatly till 72 hpf at applied concentrations in the present study. CYP1A expression show varied expression (Figure 4.9 (a) but not significantly induced which indicates that the detoxification effect is not completely activated at the applied exposure levels of BPA. CYP1A expression is induced generally by exposure to strong AhR ligands suggesting a potential mechanism for endocrine disruption (Willett *et al*., 2001). This might suggest that BPA is relatively weak AhR ligand to cause significant induction of CYP1A in micrograms level as applied here.The occurrence of oxidative stress has been reported by significant inhibition of antioxidant enzymes including glutathione peroxidase activity in zebrafish embryos after exposure to BPA and nonylphenol (Wu *et al*., 2011a). However various findings show a failure of the antioxidant responses at the early developmental stages of zebrafish after short term exposure to chemicals. This is according to the present results indicating no distinct variation of GPX expression after BPA exposure to zebrafish embryos upto 72 hpf.

The relative expression of Aro-B gene after BPA exposure to zebrafish embryos illustrate significant induction (P=0.048) at 100 µg/L and 72 hpf (Figure 4.10 (a). Exposure with 50 and 100 µg/L of BPA also caused dose dependent induction with two

and four fold induction of Aro-B expression respectively. While 150 µg/L and further increase in BPA exposure concentration to 200 and 250 µg/L revealed low expression. UV treated BPA did not show such significant induction of Aro-B expression for exposed emryos/larvae at similar concentrations (Figure 4.10 (b).

The curve shift for Aro-B expression revealed significantly varied expression as shown in figure 4.10 (c) and two-way ANOVA depicts significant effect of UV treatment on Aro-B expression (P=0.03). BPA after UV treatment showed low expression of Aro-B at 50 and 100 µg/L while at 150 µg/L the mRNA levels were relatively higher but the increase is slightly about single fold as shown in Figure 4.10 (b).The trend continues and mRNA levels remain almost unchanged upon further increase of treated BPA concentrations.

(a)

Untreated BPA exposure

(b)

UV treated BPA exposure
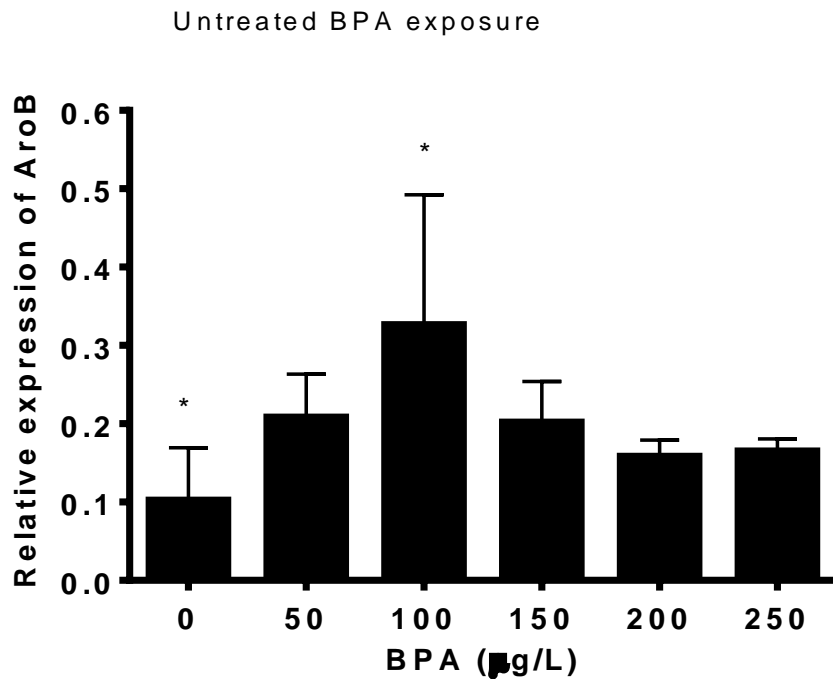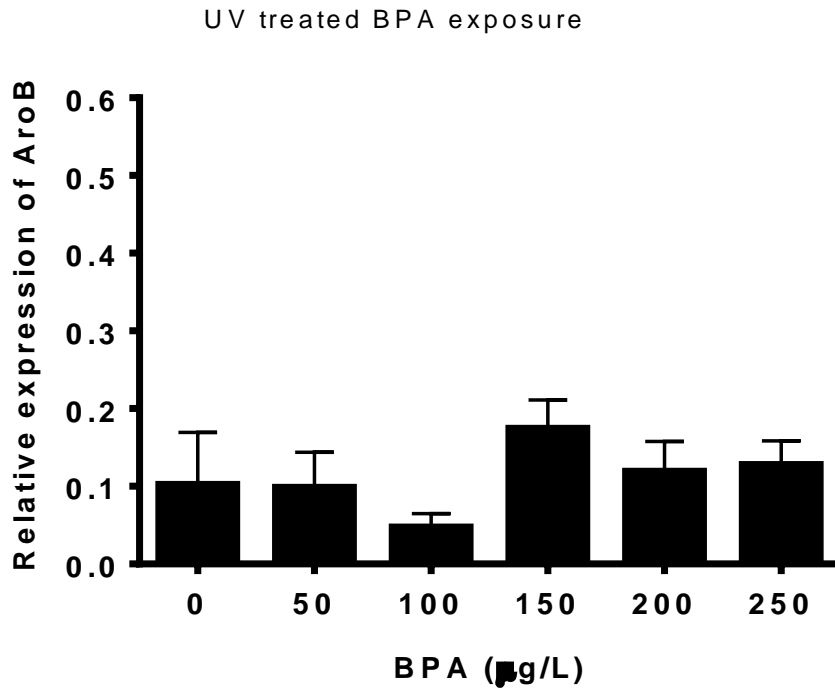
(c)

Figure 4.10: Changes in the expression levels of Aro-B gene in zebrafish showing control (No BPA) and various concentration of BPA exposed to zebrafish embryos upto 72 hpf. Aro-B mRNA levels after BPA exposure (a) Aro-B mRNA levels after UV treated BPA exposure (b) Curve shift for Aro-B mRNA levels due to UV treatment of BPA (c).

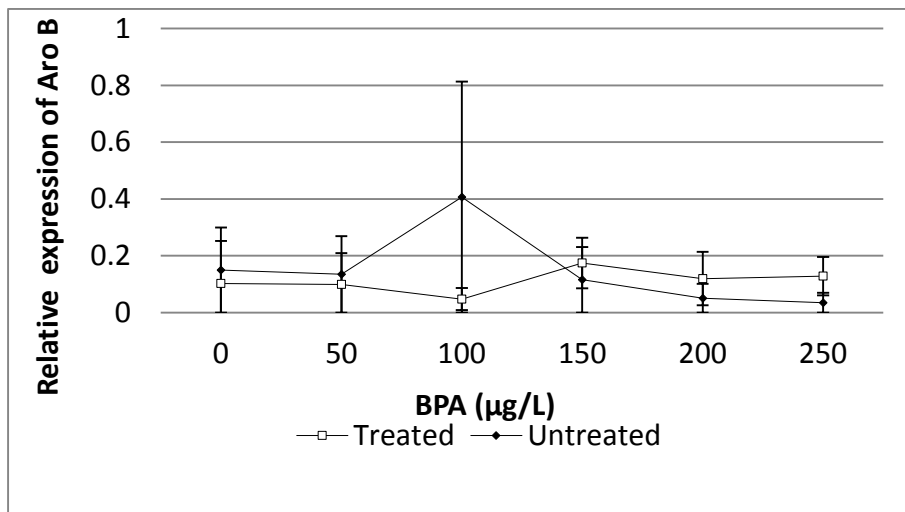The emphasis here is on the relative mRNA levels of BPA for its significant induction at 100 µg/L indicating its biological activity whereas this induction disappears during applied UV treatment. Curve shift in Figure 4.10 (c) and two-way ANOVA for treated and untreated BPA exposure with P value of 0.03 further indicate the significant effect of UV treatment.

Aromatase pathway is a direct target of estrogens due to its regulatory region consisting of multiple estrogen response elements (Fengling *et al*., 2013).Various EDCs are known to mimic sexual hormones and disrupt its normal function resulting in complete sex reversal in lower vertebrates (Crisp *et al*., 1998). It is a steroidogenic enzyme and by the synthesis of estradiol17b (E_2) plays key role in the conversion of androgens to estrogens. Changes in the expression or activity of aromatase along with variation in serum E_2 levels have often been demonstrated during sex change and ovarian differentiation in different fish species (Guiguen *et al*., 2010; Rasheeda *et al*., 2010). Due to similarity in structure with natural steroid hormones, BPA is suggested to bind with androgen or estrogen receptors to mimic or disrupt the functions of endogenous hormones. About three fold increase in mRNA levels at100 µg/L as shown in Figure 4.10 (a) is symptomatic of significant effect of BPA due to altered biologic function in zebrafish embryos.

Aromatase plays crucial functions not only in adults but also during the embryonic and neonatal periods in the brain and its disruption shows abnormal brain functions. The induction of Aro-B upon BPA exposure has been reported by Chung *et al*. (2011) owing to its strong over expression. In zebrafish larvae and adults, upregulation of Aro-B expression in response to estrogen or estrogenic EDCs is consistent in earlier studies (Kazeto *et al*., 2004). The results show an increase in the mRNA expression at 50 µg/L

with significant induction at 100 µg/L as compared to control group (Figure 4.10 (a).
While study by Chung *et al*. (2011) have reported Aro-B induction at 5 µM concentration
of BPA for overall and tissue specific expression. Microarray data analysis of early life
exposure of zebrafish to BPA, tested at concentrations of 500-4500 µg/L strongly
evidenced dysregulation of genes involved in brain development, muscular activity and
reproduction (Lam *et al*., 2012). Whereas, exposure of zebrafish to 1-1000 µg/L BPA has
resulted in its accumulation in fish tissues, while the range of 100-1000 µg/L indicated
increased atretic follicles along with cell components degeneration (Molina *et al*., 2013).

Figure 4.11 represents the HSP70 gene expression following BPA exposure and
variation in mRNA levels when UV treated BPA was used for exposure to zebrafish
embryos and curve shift of mRNA levels for the two conditions. The expression of heat
shock protein has increased showing its significant induction (P=0.0031). The trend seems
to be consistent except 150 µg/L with low expression.

Zebrafish embryos exposed to UV treated BPA for the similar concentrations and
durations showed varied expression as shown in Figure 4.11(b). Significant effect of UV
treatment on HSP70 mRNA levels was observed with P value of 0.0017.There was no
interaction between two groups and significant effect of dose in untreated and treated BPA
exposure. Curve shift show HSP mRNA levels with a trend of induction and low
expression of HSP for untreated and treated BPA exposure respectively.

Various studies have validated the use of the HSP response as an indicator of
stressed states in fish and increased levels of various HSPs have been measured in fish
tissues. HSP gene expression is known to increase not only by the chemical stressors but
also in heat stress and hypoxic conditions which occur usually in polluted environment.
Thus it seems relevant to investigate the expression of HSP gene as a general biomarker

of environmental quality. Thus it seems relevant to investigate the expression of HSP gene

as a general biomarker of environmental quality.

(a)
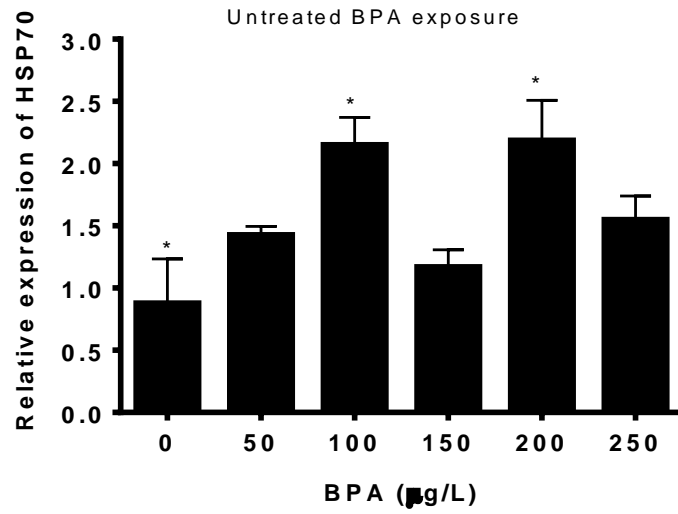
(b)
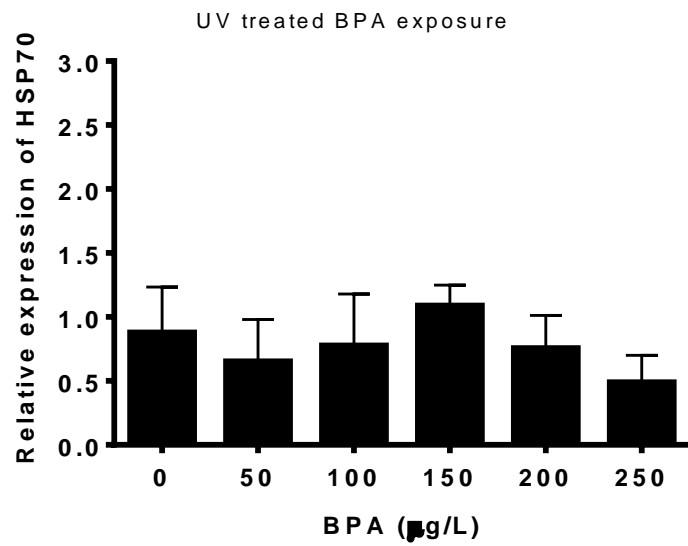
(c)
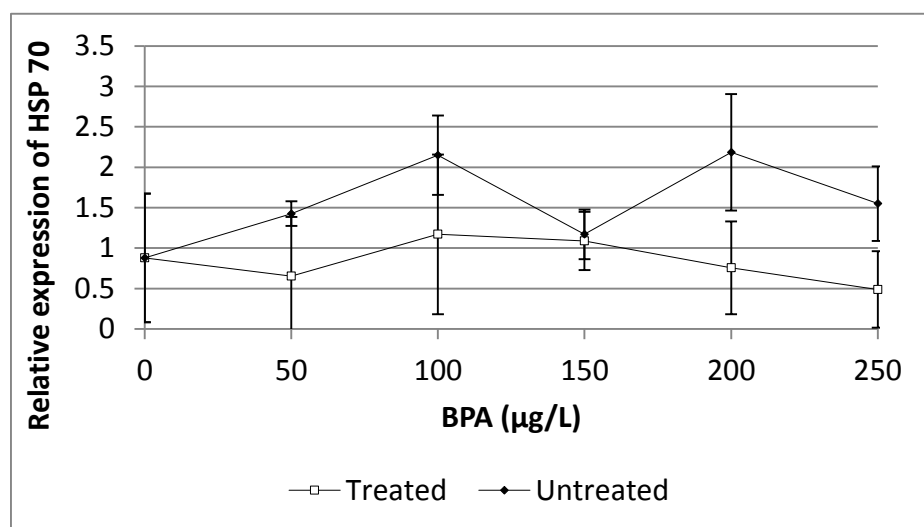
Figure 4.11: Relative expression of HSP70 gene in zebrafish showing control (No BPA) and various concentration of BPA exposed to zebrafish embryos upto 72 hpf HSP mRNA levels after BPA exposure (a) HSP mRNA levels after UV treated BPA exposure (b) Curve shift for HSP expression due to UV treatment of BPA (c).

As with HSP70, HSC70 expression is found to be inducible in response to environmental stressors (Lee *et al*., 2006). Kreiling *et al*. (2007) observed altered expression of HSC and HSP70 in early embryos of zebrafish due to exposure with Aroclor 1254. The results of HSP gene induction at applied BPA concentrations are in line with various other reported findings.

Lee *et al*. (2006) reported its induction in *C. tentans* as a result of exposure to BPA at the exposure concentrations of 8, 80 and 800 µg/L and in *C. japonica.* HSP70 gene induction was observed after exposure of 48, 72, and 96 hrs at 50, 500 and 1000 µg/L in dose and exposure time dependent way (Park and Kwak, 2013). Moreover, BPA at 12 and 120 µg/L induced the heat shock genes in *S. nonagrioides.* Michail *et al*. (2012) also indicated direct interaction of BPA with the endocrine system. A trend of dose dependent increase in mRNA levels of CYP1A, Aro-B and HSP genes at 50-150 µg/L was observed

while, the trend seems to change for higher levels of BPA probably due to disruption of gene expression. It is worth mentioning that in this study, the induction of Aro-B and HSP genes have been observed within the proposed total allowable concentration (100 µg/L) of BPA for drinking water (Willhite *et al*., 2008). Other reports also describe the altered expression of six genes in zebrafish larvae within the exposure range of 50 and 100 µg/L BPA (Lam *et al*., 2012).While, vitellogenin gene induction was observed even at 10 µg/L (Villeneuve *et al.,* 2012).

Since BPA is not simply estrogen mimic and may disrupt physiological functions by altering multiple pathways, including the endocrine system (Jordan *et al*., 2012). This implies the need for further investigations of biological effects at lower BPA concentrations close to environmental levels while reconsidering the total allowable concentrations and finally its treatment. Therefore, in this study, changes in biological activity of BPA as a function of UV treatment were also evaluated using transcriptomic analysis by real time Q-PCR. UV as an oxidation process was applied to degrade BPA and decay of parent compounds with formation of degradation products were followed using HPLC analysis.

Bioanalytical assessments of residual ED activity in treated water have been used previously to evaluate the performance of the UV based advanced oxidation process for estrogenic contaminants like BPA in water (Nomiyama *et al*., 2007). In a study, Phenol, 1, 4-dihydroxylbenzene and 1, 4 benzoquinone were identified by means of HPLC as intermediate products of the photodegradation of BPA and the disappearance of its biological activity during irradiation of 120 min was observed (Neamtu and Frimmel, 2006). In the present study, UV-C was applied in mercury lamp for 90 min and HPLC showed complete mineralization of BPA. Moreover the ED activity is fairly removed as

depicted by altered mRNA levels, eliminating biological effects of CYP1A, Aro-B and HSP genes as shown in Figures 4.9 (b), 4.10 (b), 4.11 (b) and increased percent survival of larvae in case of UV treated BPA exposure.The effectiveness of UV treatment in combination of TiO_2 and other AOPs has been demonstrated (Rosenfeldt *et al*., 2007) and some oxidation products generated from incomplete degradation show potential to increase the estrogenic activity. Although feasible applications of treatment technologies are based on complete mineralization of parents and their intermediate compounds without secondary pollution at low operating costs (Pera-Titus *et al*., 2004).
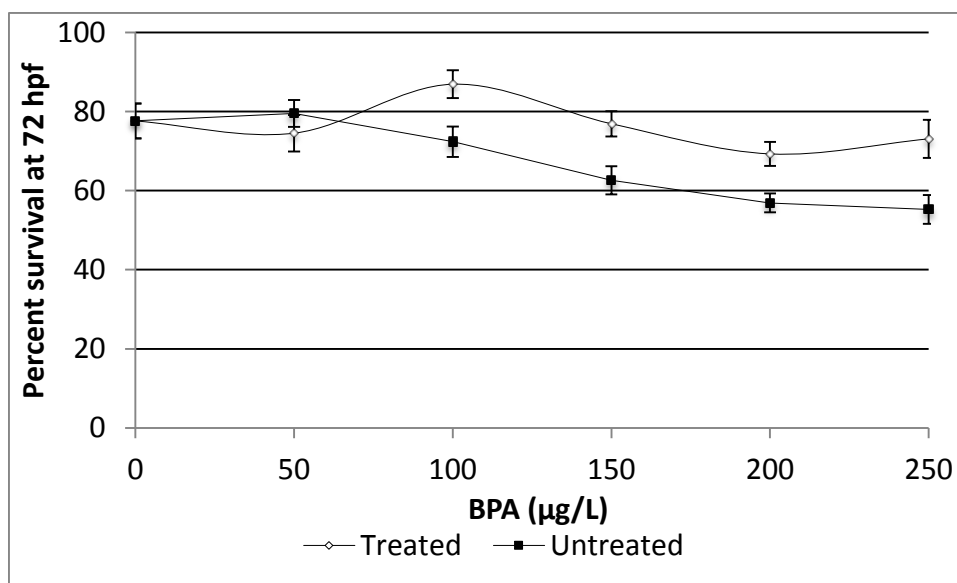
Figure 4.12: Percent survival of zebrafish embryos/larvae at 72 hpf after exposure with various concentrations of intact and UV treated BPA

Percent survival curve showed comparative survival for untreated and UV treated BPA exposure at similar range of 0-250 µg/L after 72 hpf. Overall treated BPA exposure has shown more survival percentage in the exposed zebrafish embryos/larvae (Figure 4.12). Various studies report reduced mortality of animals due to exposure with treated chemicals (Kidd *et al*., 2007). The present study for BPA degradation points toward direct

UV photolysis as an economical and efficient treatment option that may be tested further for transcriptomic effects of individual byproducts formed during degradation of parent compound.

4.3.2 Relative Expression of Zebrafish Genes after 2,4-D Exposure

Because of known adverse effects of 2,4-D, the overall objective was to identify potential biomarkers to monitor efficacy of UV/TiO$_2$ treatment to develop appropriate remediation procedure. Zebrafish embryos were exposed to various concentrations of intact and UV/TiO$_2$ treated 2,4-D and tested for mRNA levels relative to ß-actin using Q-PCR assay.The mRNA levels were expressed as normalized gene expression with respect to the β-actin levels in the same sample. The results demonstrate that both 2,4-D and its derivatives have activity by altering expression of a number of genes in zebrafish embryos.The effects of both intact and UV/TiO$_2$ treated 2,4-D were tested on zebrafish exposed upto 72 hpf. mRNA levels for cytochrome P-4501A1(CYP1A), heat shock protein (HSP70), aromatase B (Aro-B) and glutathione peroxidase (GPX) were measured.The observed results revealed different responses to both intact and UV/TiO$_2$ treated 2,4-D. The responses to both intact and UV/TiO$_2$ treated 2,4-D were found to be gene specific. However, it is evident that treatment of 2,4-D with UV/TiO$_2$ did not make this compound completely inactive. The overall results suggest that while UV/TiO$_2$ treatment was effective in reducing the biological activity of 2,4-D, it also resulted in byproducts that have activities different from intact 2,4-D on a number of genes investigated.

2,4-D exposure significantly increased CYP1A mRNA levels at 150 µg/L (P=0.018). However, treated 2,4-D was without effect on Cyp1A mRNA level in exposed zebrafish

embryos as shown in Figure 4.13 (a,b). Two way ANOVA also revealed significant effect of photocatalytic treatment and removal of CYP1A induction as shown in Figure 4.13 (c). An increase or decrease compared to control could potentially lead to disruption of normal physiological function and lead to adverse biological response in fish embryo. The expression pattern of genes due to exposure with chemicals follows this trend normally.

(a)
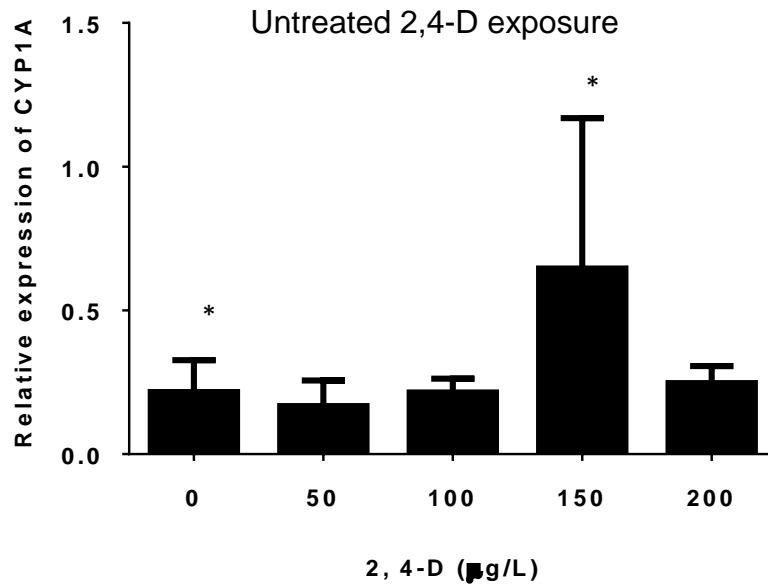
(b)
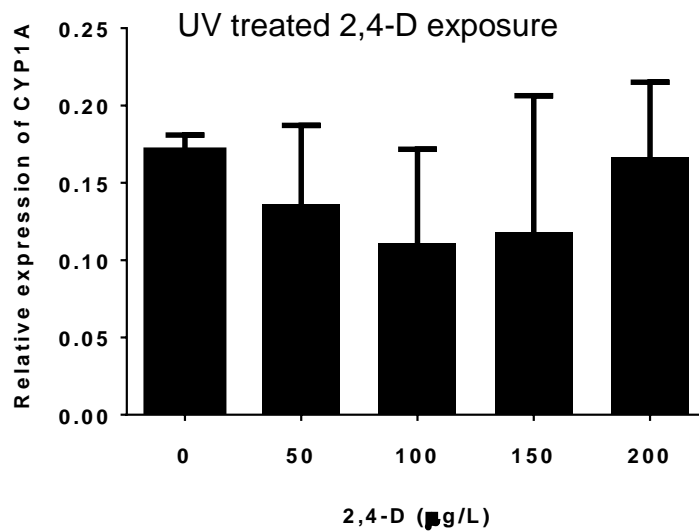
(c)

Figure 4.13: Changes in the expression levels of CYP1A gene in zebrafish showing control (No 2,4-D) and various concentration of 2,4-D exposed to zebrafish embryos up to 72 hpf. CYP1A mRNA levels after 2,4-D exposure (a), CYP1A mRNA levels after UV treated 2,4-D exposure (b), Curve shift for CYP1A mRNA levels due to UV treatment of 2,4-D (c).

It may decrease at higher concentration of chemical due to disruption in expression and varied toxicity effects. Further, the effect of 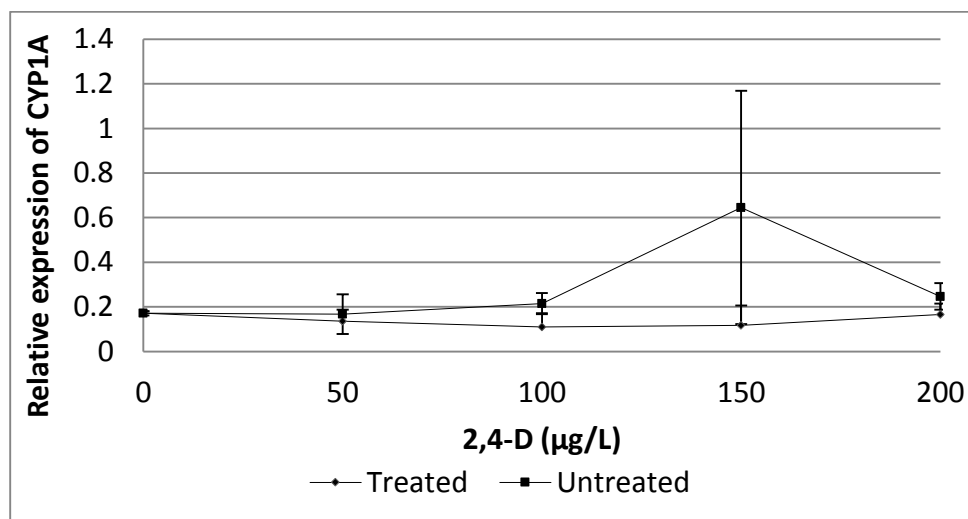same dose of the chemical is gene specific. The disruption of CYP1A gene expression has been observed following exposure with a number of chemicals such as phenanthrenes in juvenile common roaches (Wolinska *et al*., 2013) and pesticide carbofuran in *Tench fish (*Hernandez-Moreno *et al*., 2008).

In the same experiment, exposure to 2,4-D was without effect on the expression of HSP70 gene at concentrations of 50-200 µg/L. However, exposure of zebrafish embryos to UV/TiO$_2$-treated 2,4-D resulted in significant reduction of HSP70 mRNA level even at the lowest concentration of 50 µg/L (P=0.0001) (Figure 4.14). The results indicate that 2,4-D derivatives formed following photocatalytic degradation exert disruptive effect on HSP70 gene expression in the zebrafish embryos. In this context, there are several known

degradation products of 2,4-D, such as 1,2,4-benzenetriol, 2,4-dichlorophenol, 2,4-dichloroanisole, chlorohydroquinone (CHQ), 4-chlorophenol and volatile organics that could have caused the observed response. Previous studies suggested that chronic exposure to 2,4-D and its derivatives may cause adverse health effects by altering expressions of HSP70, HSP40, HSP90 and GST levels in *C. riparius* (Park *et al*., 2010). There is evidence that 2,4-D may disrupt estrogenic activities, and two of the breakdown products of 2,4-D (4-chlorophenol and 2,4-dichloroanisole) may affect male reproductive development (Cox, 2005). In this study zebrafish Aro-B was studied since its activity in zebrafish embryos leads to increased production of estrogens. Exposure of zebrafish embryos to 2,4-D was without effect on the expression of Aro-B gene as shown in Figure 4.15 (a). However, similar to that observed for HSP70, exposure of the zebrafish embryos to 150 and 200 μg/L, UV/TiO$_2$-treated 2,4-D, resulted in significant reduction in Aro-BmRNA level compared to control (P=0.0165) (Figure 4.15 (b).Two-way ANOVA also revealed significant effect of photocatalytic treatment with P value of 0.0084 as indicated in Figure 4.15 (c), by curve shift for mRNA levels of untreated and treated 2,4-D.
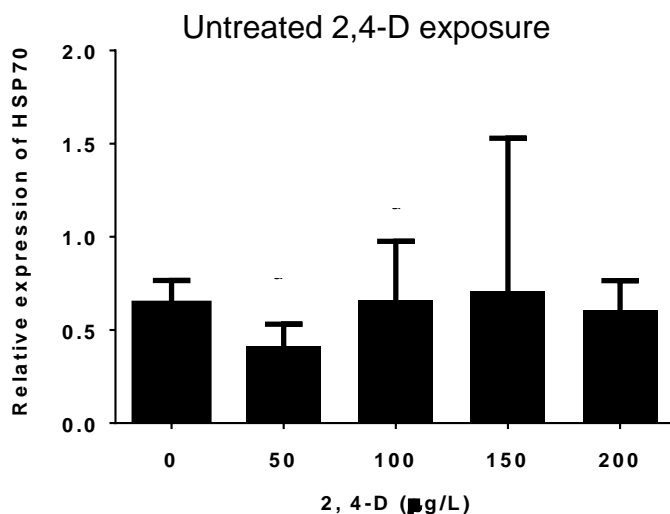
(a)

76

(b)
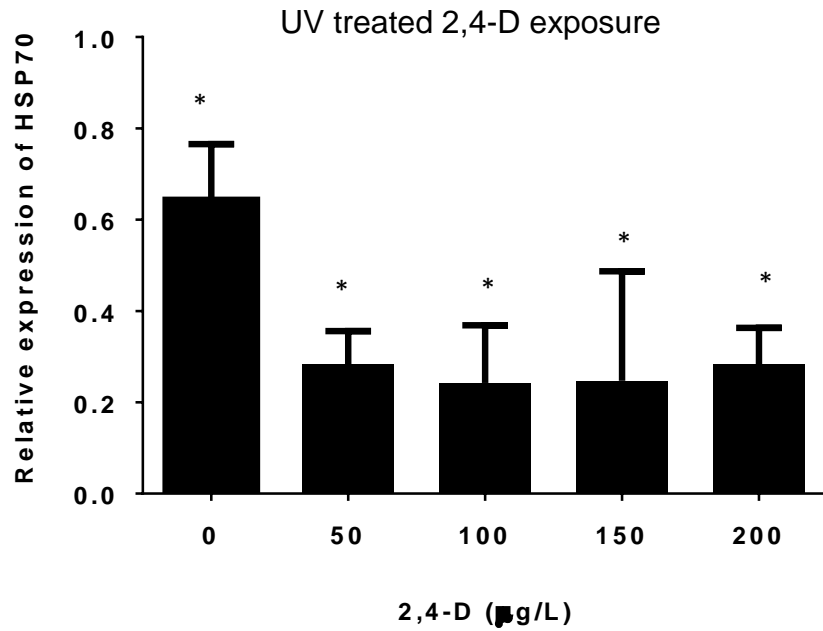
(c)

Figure 4.14: Changes in the expression levels of HSP70 gene in zebrafish showing control (No 2,4-D) and various concentration of 2,4-D exposed to zebra fish embryos up to 72 hpf. HSP70 mRNA levels after 2,4-D exposure (a), HSP70 mRNA levels after UV treated 2,4-D exposure (b), Curve shift for HSP70 mRNA levels due to UV treatment of 2,4-D.

(a)

Untreated 2,4-D exposure
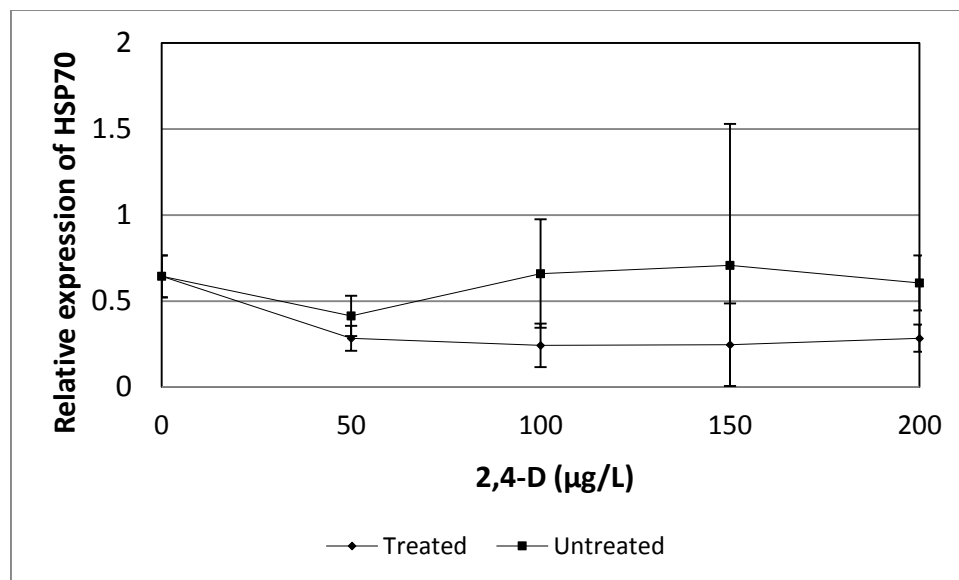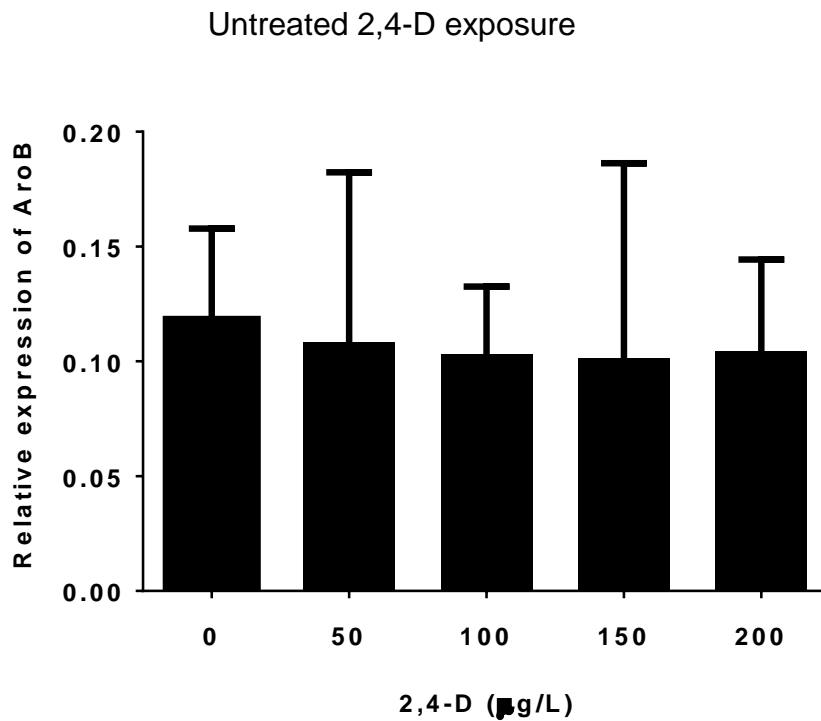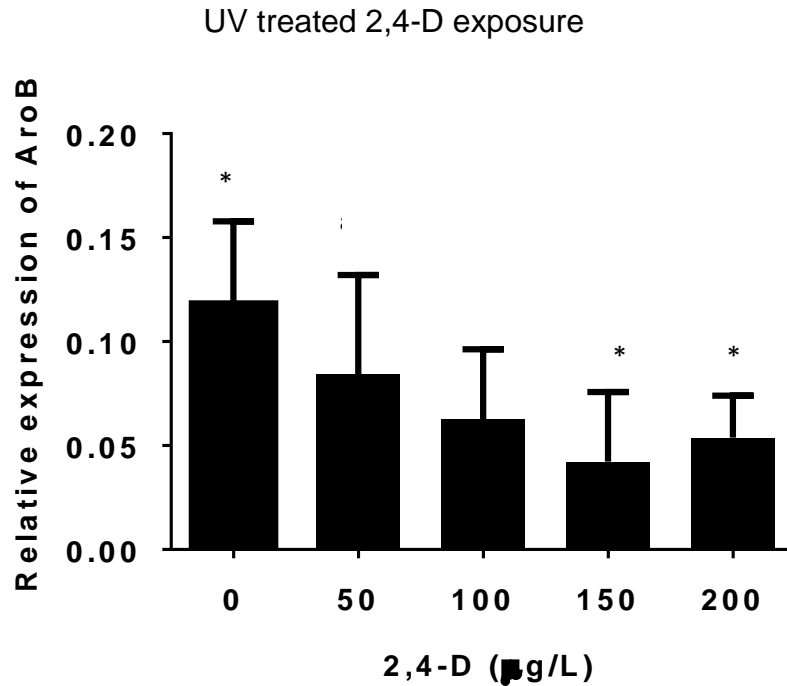

(b)

UV treated 2,4-D exposure

(c)

Figure 4.15: Changes in the expression levels of Aro-B gene in zebrafish showing control (No 2,4-D) and various concentration of 2,4-D exposed to zebra fish embryos upto 72 hpf. Aro-B mRNA levels after 2,4-D exposure (a) Aro-B mRNA levels after UV treated 2,4-D exposure (b) Curve shift for Aro-B mRNA levels due to UV treatment of 2,4-D (c).

Previous studies suggested that 2,4-D may be an oxidative damage inducer leading to oxidative stress (Mi *et al.*, 2007). The oxidative activity of 2,4-D has been studied in a number of species including yeast, plants, fish and rats (Romero-Puerats *et al.*, 2007; Nwani *et al.*, 2013; Kubrak *et al.*, 2013a; Anusuya and Hemalatha, 2014).

In this study, treatment with either 2,4-D or UV/TiO$_2$-treated 2,4-D was without significant effect on GPX expression in zebrafish embryos represented in Figure 4.16 (a,b). Two-way ANOVA also showed no significant difference among treated groups (Figure 4.16 (c).Thus the results demonstrate that 2,4-D may cause adverse health effects at higher concentrations in zebrafish as indicated by changes in CYP1A activity. Significant observation in this study is that UV/TiO$_2$ treatement of 2,4-D may not be an effective procedure to treat waste containing 2,4-D as it may produce by products that may

cause adverse health effects in fish and possibly other vertebrate species. The nature of adverse health impact cannot be assessed based on present studies since zebrafish is not the most sensitive model organism and embryos have undeveloped endocrine system. However the results provide important indication that the UV/TiO$_2$ treatment process is not the most suitable approach for removing 2,4-D from environment. Furthermore, zebrafish embryos provide an effective and convenient toxicology model organism for testing environmental contaminants without having to sacrifice large number of animals. Adult organisms would be more suitable model organisms for investigating endocrine disruptive effects of 2,4-D. In this context, 2,4-D has been shown to have estrogenic activity in a number of studies carried out on juvenile rainbow trout exposed to 2,4-D (1.64 mg/L) for 7 days. In the latter study, exposure to 2,4-D caused a 93 fold increase in plasma vitellogenin (Vtg) levels compared with untreated fish (Xie *et al*., 2005) and the low observed effect concentration (LOEC) for 2,4-D was 0.164 mg/L, corresponding to the concentrations applied in this study. Another study using the yeast two hybrid assay determined that 2,4-D was estrogenic at a concentration of approximately 0.2 g/L, indicating relatively weak affinity with the estrogen receptor (Nishihara *et al*., 2000).

In this study survival of zebrafish embryos exposed to 2,4 D and UV/TiO$_2$ treated 2,4-D was also investigated. The results demonstrated no significant change in mortality related to treatment (Figure 4.17).
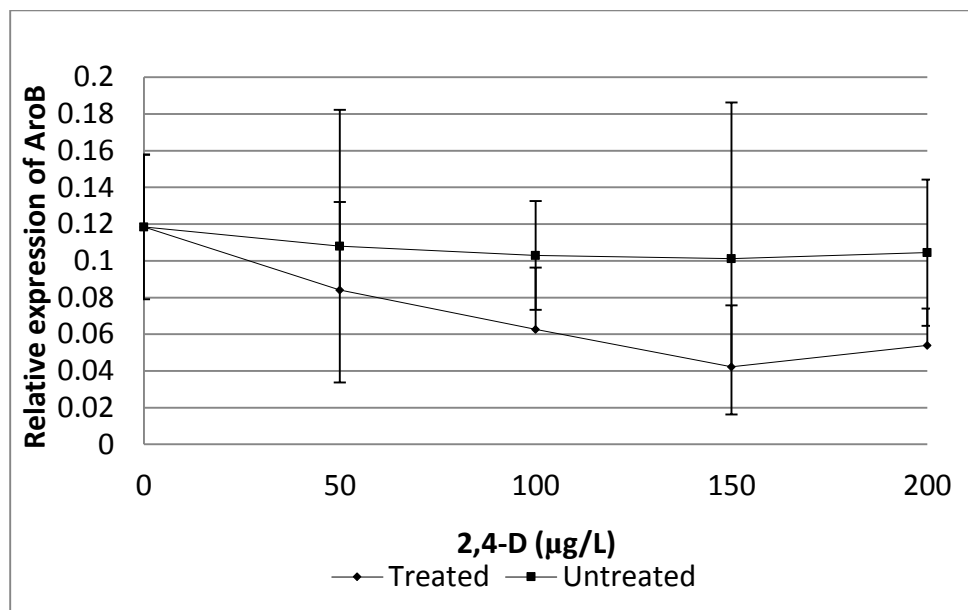
(a)

(b)
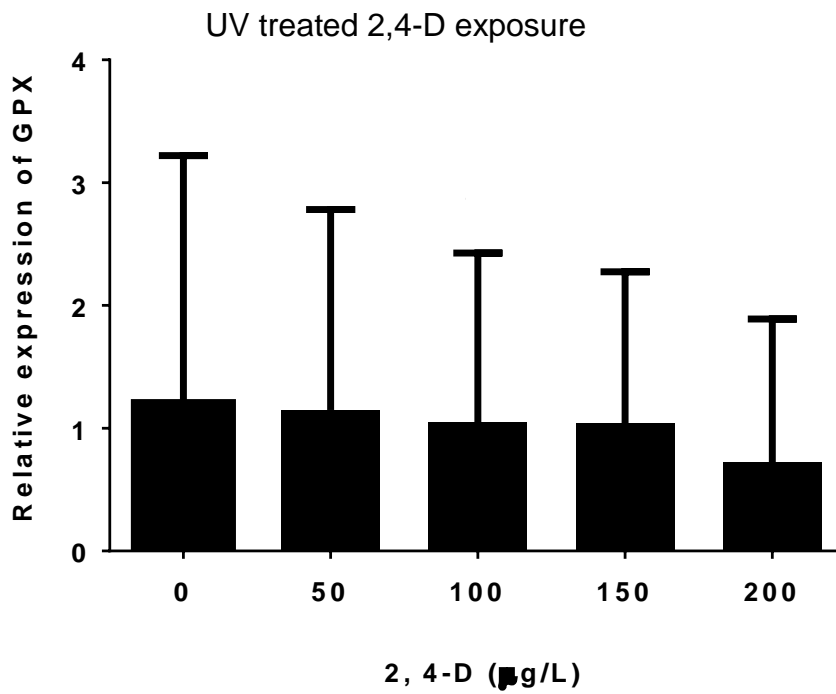
(c)
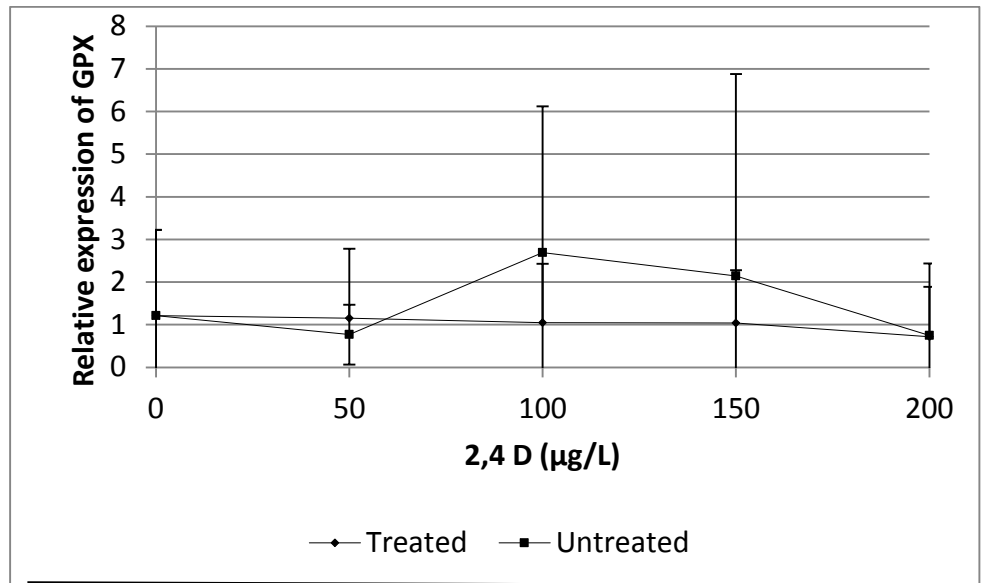
Figure 4.16: Changes in the expression levels of GPX gene in zebrafish showing control (No 2,4-D) and various concentration of 2,4-D exposed to zebra fish embryos up to72 hpf. GPX mRNA levels after 2,4-D exposure (a), GPX mRNA levels after UV treated BPA exposure (b), Curve shift for GPX mRNA levels due to UV treatment of 2,4-D (c).
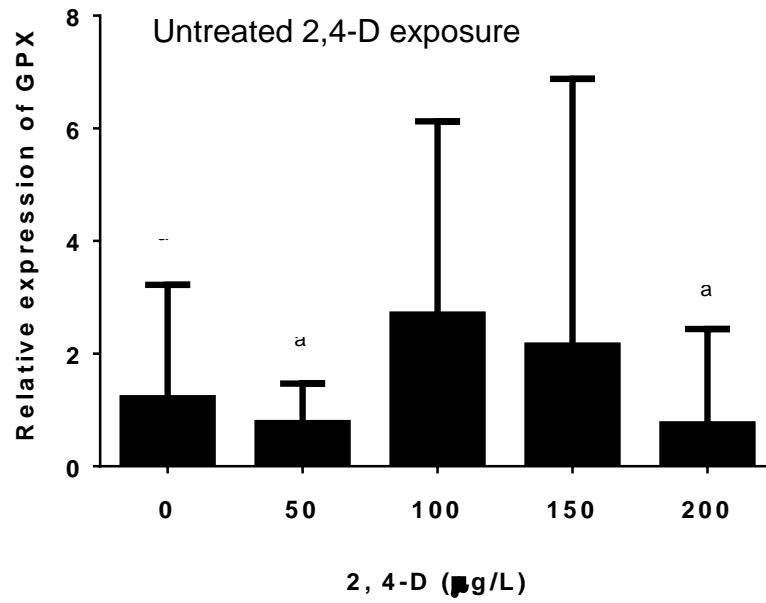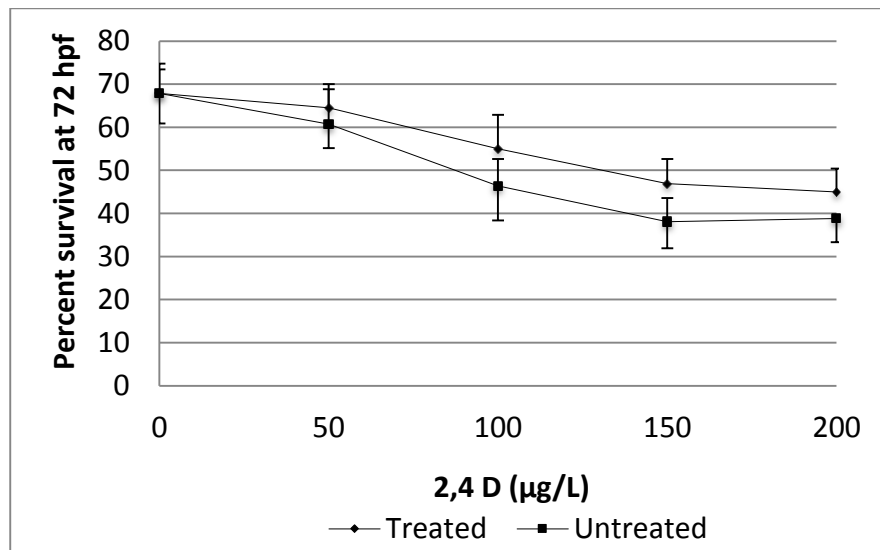

Figure 4.17: Percent survival of zebrafish embryos/larvae at 72 hpf after exposure with various concentrations of intact and UV/TiO$_2$ treated 2,4-D

The photocatalytic degradation has been found much more efficient than direct photolysis in ensuring the oxidative degradation of the benzene ring and the overall mineralization of aromatic compounds (Bertelli *et al.*, 2006). This process has potential of feasible applications in water treatment due to ambient operating temperature and pressure, complete mineralization of parents and their intermediate compounds without secondary pollution and low operating costs. The slurry type of TiO_2 catalyst application is usually preferred due to high volumetric generation rate of reactive oxygen species (ROS) (Pozzo-Miller *et al.*, 1997) as a result of the photo induced charge separation on TiO_2 surfaces for mineralization of compounds without creating any secondary pollution. However, the post separation of the TiO_2 catalyst after water treatment remains as the major difficulty towards its practicality as an industrial process. It may be inferred by the results of UV/TiO_2 treated 2,4-D exposure in zebrafish embryos that some activity of 2,4-D is attributed to its byproducts formed during instantaneous degradation as shown by the significantly altered expression of HSP70 and Aro-B genes.

4.4 GOLDFISH LIVER CELL CULTURES EXPOSURE TO BPA AND 2,4-D

Adult goldfish liver cell cultures were exposed to various concentrations of BPA and 2,4-D and the changes in expression of biomarker genes were studied by real time Q-PCR of male and female liver tissues.The relative expression of various target genes was determined using goldfish GAPDH as house keeping gene.

4.4.1 Goldfish Liver Cell Cultures Exposed to Various Concentrations of BPA

Bisphenol Awas exposed to liver cell cultures for 24 hrsat various concentrations of 5, 25, 50 and 100 µg/L. *In vitro* exposure of BPA to goldfish male and female liver has

resulted an increase in CYP1Agene expression at 25 µg/L as shown in Figure 4.18 (a). While the expression is lower for 50 and 100 µg/L BPA after 24 hrs of *in vitro* exposure to female liver tissues. For male liver cell cultures, CYP1A expression has shown an increasing trend in mRNA expression for 5, 25 and 50 µg/L BPA as shown in Figure 4.18 (b). In a study by Yan *et al*., (2013) a known EDC, 17β-estradiol (E_2) exposure caused a significant increase in gene expression of VTG and estrogen receptor (ER) in male goldfish along with increase in associated cytochrome P450 (CYP) aromatase expression after 10 days of exposure. Moreover, ketoconazole (KTC) significantly increased the related gene expression of CYP1A.

(a)
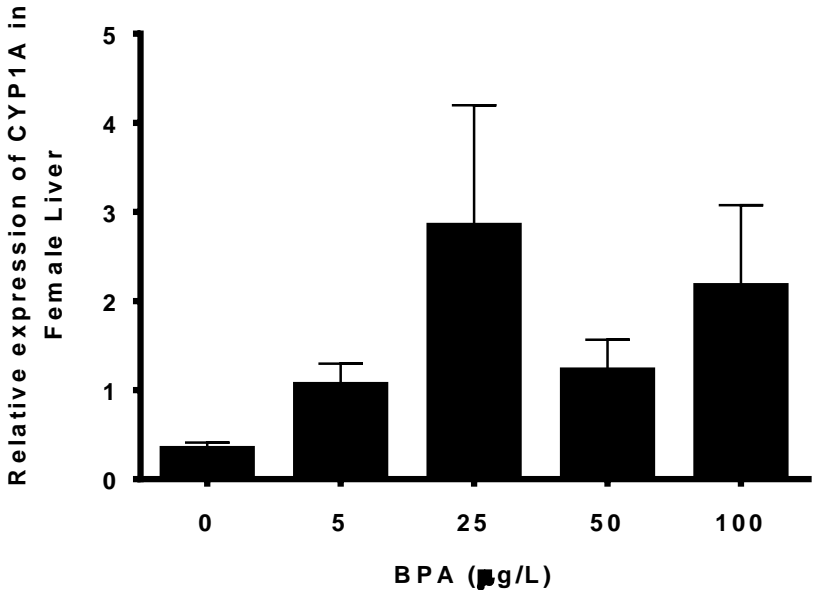
(b)

Figure 4.18: Changes in the expression levels of CYP1A gene in goldfish showing control (No BPA) and various concentration of BPA exposed to liver cell cultures upto 24 hrs.Female liver (a) Male liver (b).All values are means ± SD (n = 5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukeytest, $P < 0.05$).

The induction of vitellogenin in aquatic vertebrates has become the gold standard biomarker of exposure to estrogenic chemicals (Sun *et al*., 2009). Figure 4.19 (a) shows the VTG mRNA levels for female goldfish liver cell cultures, indicating an increase in mRNA levels at 50 and 100 µg/L BPA concentrations. While, male liver cell cultures showed an increasing trend in relative VTG expression at 5, 25 and 50 µg/L BPA as depicted in Figure 4.19 (b).The results indicate the estrogenic activity of BPA as also reported by Ishibashi *et al*. (2001). They showed that VTG induction in the goldfish is equally responsive to the estrogen mimic, BPA, compared with the fathead minnow (Sohoni *et al*., 2001), depicting the sensitivity of goldfish to induce VTG expression when exposed to estrogenic compounds. Conversely, another study investigated changes in mRNA levels of the vitellogenin gene in daphnids (*Daphnia magna*) exposed to a various

range of chemicals and reported that exposure to the estrogens like BPA show little effect on vitellogenin mRNA levels demonstrating that these genes are not induced by estrogen exposure (Hannas *et al*., 2011).

In case of male liver, significantly low mRNA levels of IGF-1 gene compared to control group was observed at 100 µg/L BPA depicting low growth and survival conditions as shown in Figure 4.20 (b). Relative expression of IGF-1 was significantly low at exposure dosage of 100 µg/L compared to control with P value of 0.0293. As IGF-1 gene is related to growth and survival of the cells, so 100 µg/L *in vitro* exposure has caused decrease in its expression showing less growth factor.
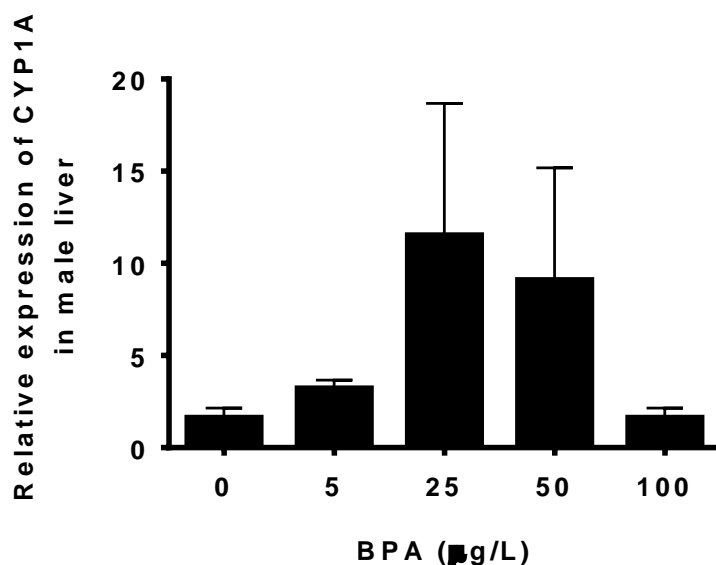
(a)

(b)

Figure 4.19: Changes in the expression levels of VTG gene in goldfish showing control (No BPA) and various concentration of BPA exposed to liver cell cultures upto 24 hrs. Female liver (a) Male liver (b). All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukeytest, $P < 0.05$).
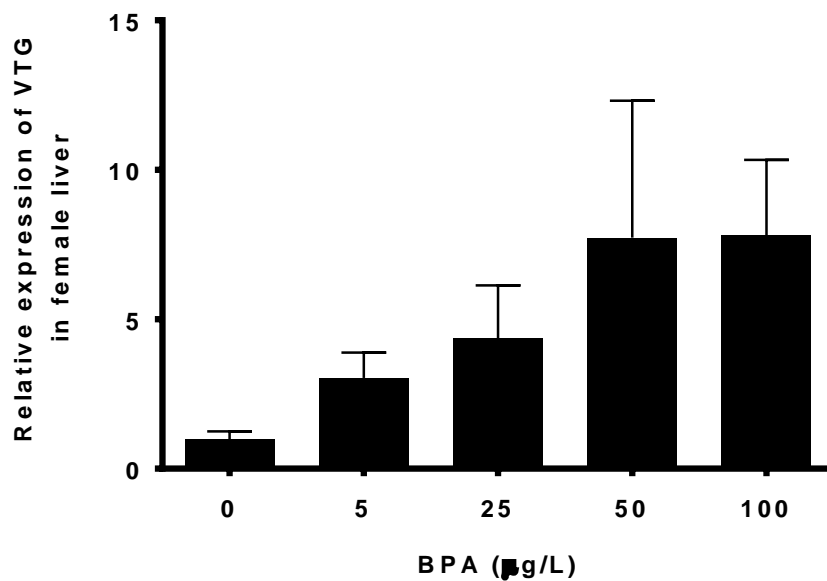
(a)

(b)
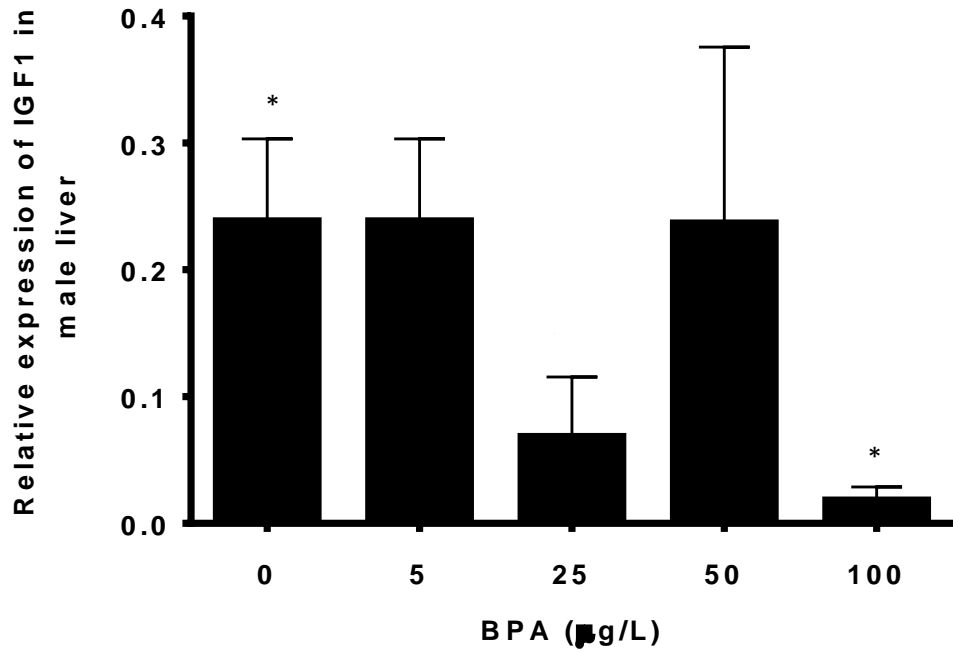
Figure 4.20: Changes in the expression levels of IGF-1 gene in goldfish showing control (No BPA) and various concentration of BPA exposed to liver cell cultures upto 24 hrs. Female liver (a), Male liver(b). All values are mean ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukeytest, $P < 0.05$).

(a)
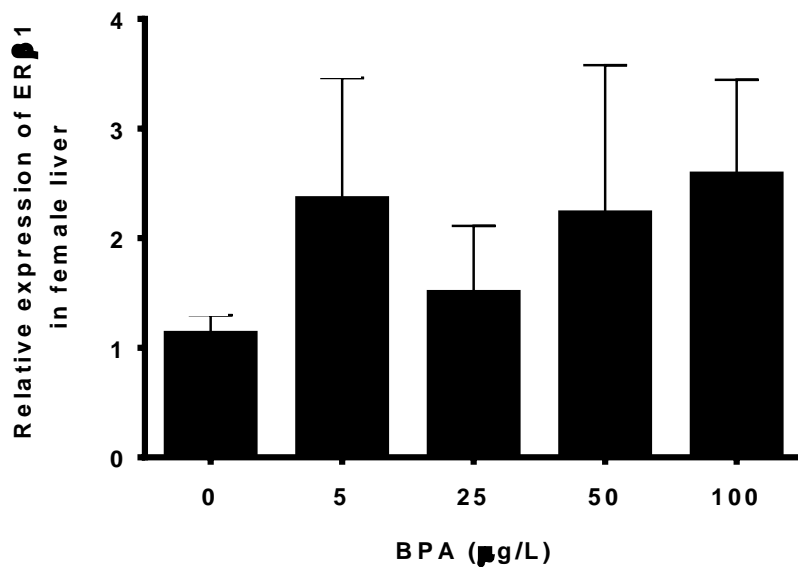
(b)

Figure 4.21: Changes in the expression levels of ERß-1 gene in goldfish showing control (No BPA) and various concentration of BPA exposed to liver cell cultures up to 24 hrs.Female liver (a) Male liver (b). All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukeytest, $P < 0.05$).

BPA may function as EDC by acting as cell type specific agonists or antagonists for ERα and ERβ (Yin *et al.*, 2012). The relative expression of ERß-1 up regulated at applied BPA dosages of 25, 50 and 100 µg/L in female liver as shown in Figure 4.21 (a) whereas, for male liver, it showed upregulation in mRNA levels at 25 and 50 µg/L as shown in Figure 4.21 (b). An increase was reported in estrogen receptor alpha 1 (ESR1) and vitellogenin (VTG) gene expression by estrogenic compounds exposed goldfish but this was not statistically significant (Silva de Assis*et al.*, 2013). In another study, goldfish liver showed upregulation of ERα in sexually regressed, mature and recrudescing males and in sexually regressed females. Aro-B and VTG gene expression were affected by E_2

and EE$_2$ induced liver ER alpha and VTG mRNA levels indicating the high environmental levels of estrogens induce E$_2$ mediated gene expression in fish (Marlatt *et al*., 2010).

4.4.2 Goldfish Liver Cell Cultures Exposed to Various Concentrations of 2,4-D

Goldfish liver cell cultures exposed to 5, 50 and 150 µg/L of 2,4-D demonstrated altered expression of various target genes. CYP1A gene expression in male liver is shown in Figure 4.22 (a). For female liver, the mRNA levels increased at 150 µg/L showing significant induction compared to mRNA levels at 50 µg/L, 2,4-D exposure (Figure 4.22 (b). In a study, differentially expressed genes verified by Q-PCR included brain aromatase, suggesting the altered expressionof this gene in the sexually mature adult brain affected by EDCs (Martyniuk *et al*., 2006).
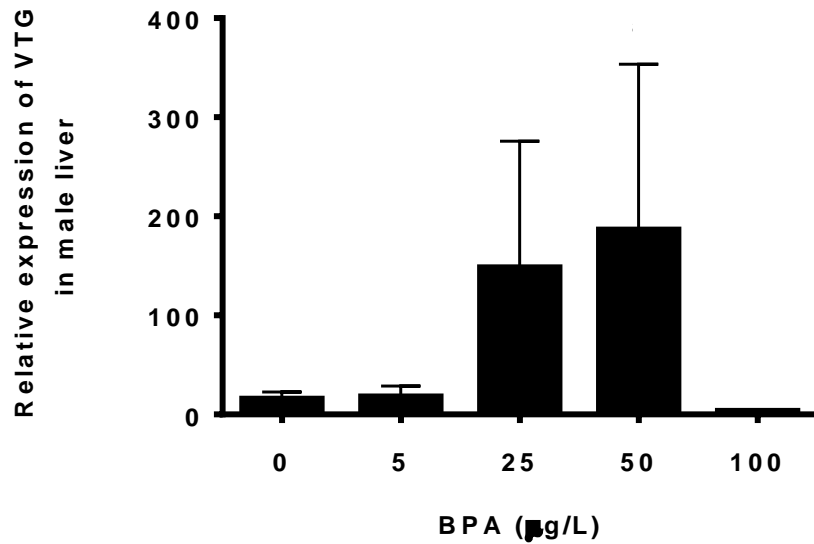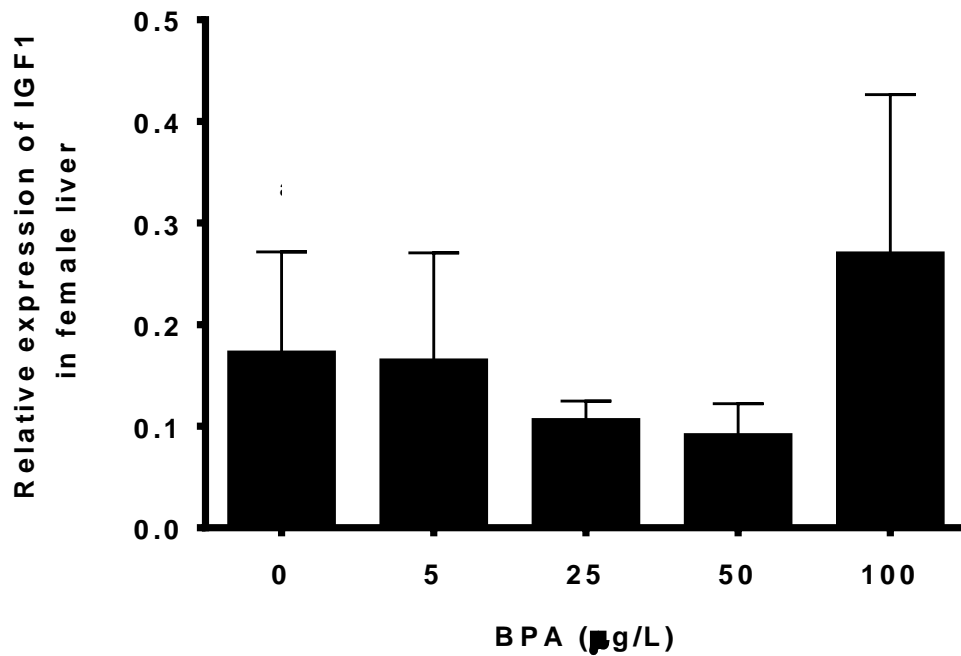
(a)

(b)

Figure 4.22: Changes in the expression levels of CYP1A gene in goldfish showing control (No 2,4-D) and various concentration of 2,4-D exposed to liver cell cultures upto 24 hrs. Female liver (a) Male liver (b).All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$).

The relative expression of VTG gene showed an increasing trend in mRNA levels at 5, 50 and 150 μg/L 2,4-D as shown in Figure 4.23 (a) whereas, for male liver the expression was lower at 50 μg/L and remains almost stable at 2,4-D concentrations of 5 and 150 μg/L as shown in Figure 4.23 (b). The results are in accordance with a study by Spano *et al*., (2004). In this study, sexually mature goldfish (*Carassius auratus*) of both sexes were exposed to two doses (100 and 1000 μg/L) of herbicide atrazine for a period of 21 days. Atrazine did not show any obvious estrogenic effect in males, as determined by a lack of vitellogenin induction.

(a)

(b)

Figure 4.23: Changes in the expression levels of VTG gene in goldfish showing control (No 2,4-D) and various concentration of 2,4-D exposed to liver cell cultures upto 24 hrs (a) Female liver (b) Male liver. All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$).

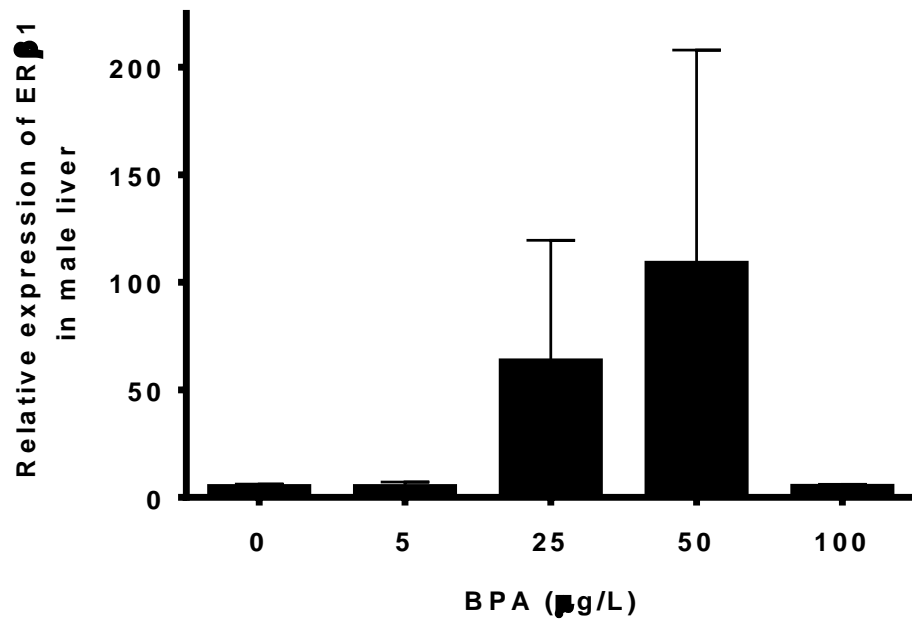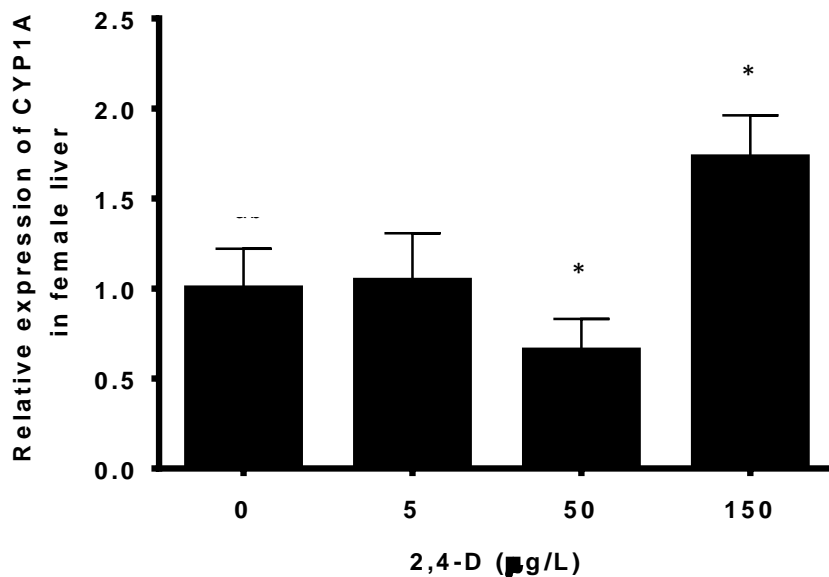Goldfish liver cell cultures, exposed to various concentrations of 2,4-D for 24 hrs demonstrated that insuline like growth factor (IGF-1) gene relative expression was down regulated showing almost 2 fold decreases in mRNA levels at 50 and 150 µg/L in female liver as indicated by Figure 4.24 (a).The similar down regulation was observed in male liver for IGF-1 relative expression for 2,4-D exposure at 5, 50 and 150 µg/L as shown in Figure 4.24 (b). It depicted low growth and compromised survival conditions associated with the down regulation of IGF-1 gene (Lankford and Weber, 2006).

ERß-1 expression in male and female liver cell cultures did not show much alteration except a down regulation at 50 µg/L 2,4-D concentration as shown in Figure 4.25 (a,b). These findings are in accordance with previous studies which inferred that the chemicals including 2,4-D may produce low ER potencies *in vitro* inducing VTG production *in vivo* (Yin *et al*., 2012). EDC exposure enhanced the mRNA expression of gonadal aromatase, the enzyme that converts androgens into estrogens (Tian *et al*., 2010). Thus the present study depicts the biological activity of BPA and 2,4-D exposed to cell cultures of adult goldfish.

(a)

(b)
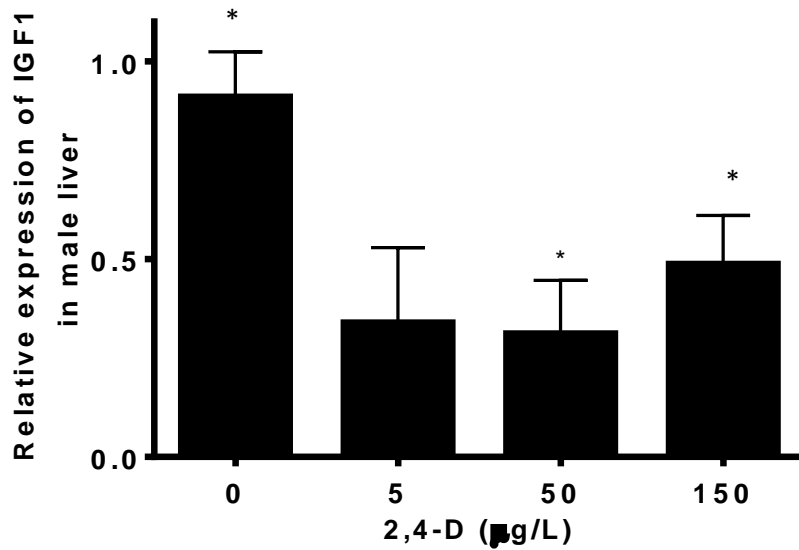
Figure 4.24: Changes in the expression levels of IGF-1 gene in goldfish showing control (No 2,4-D) and various concentration of 2,4-D exposed to liver cell cultures upto 24 hrs. Female liver (a) Male liver (b).All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$).
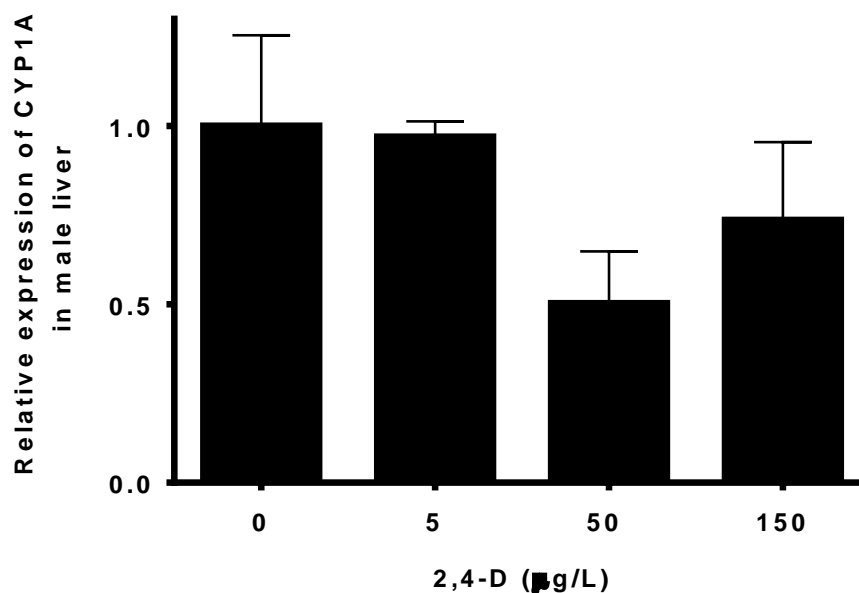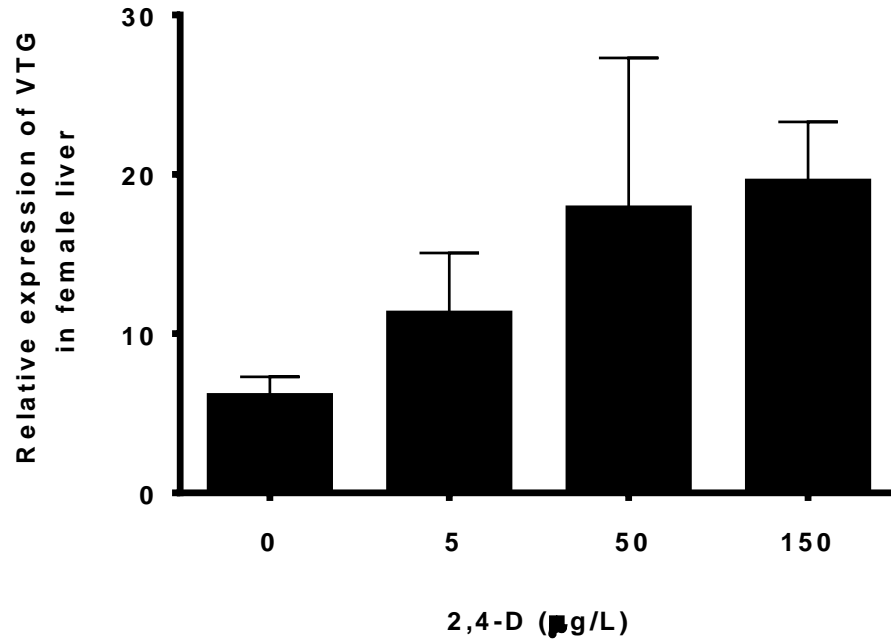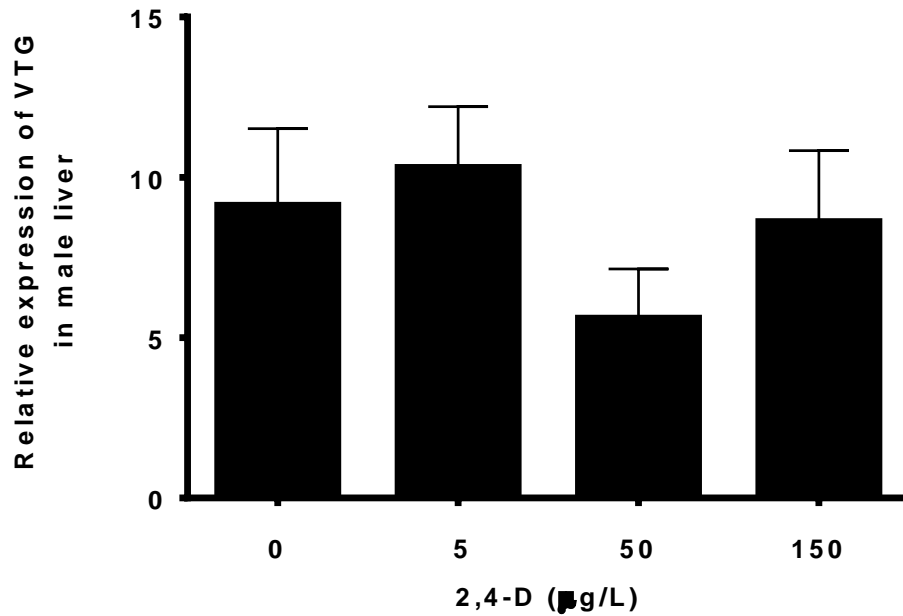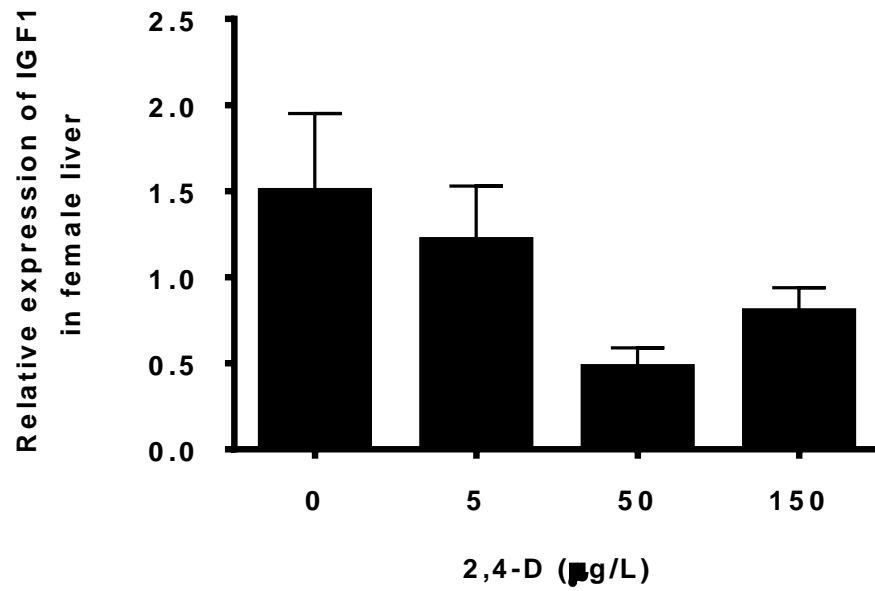
(a)
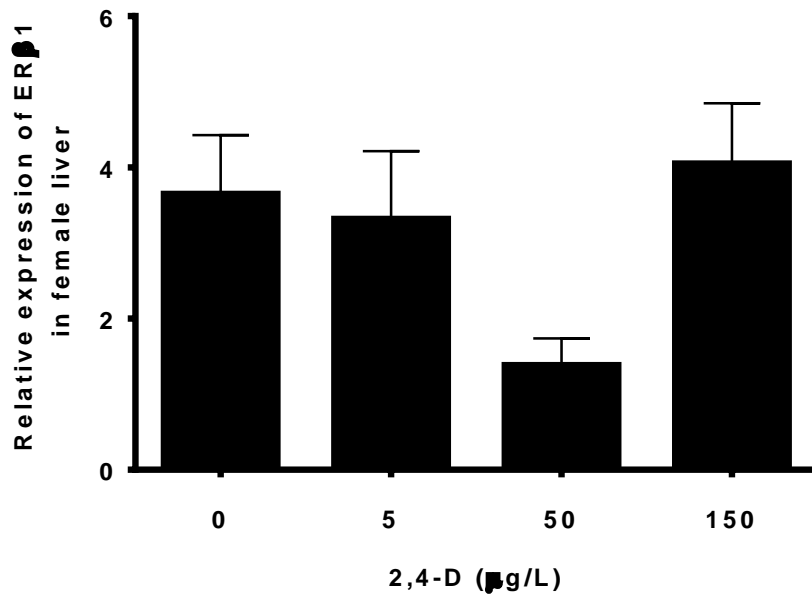
(b)
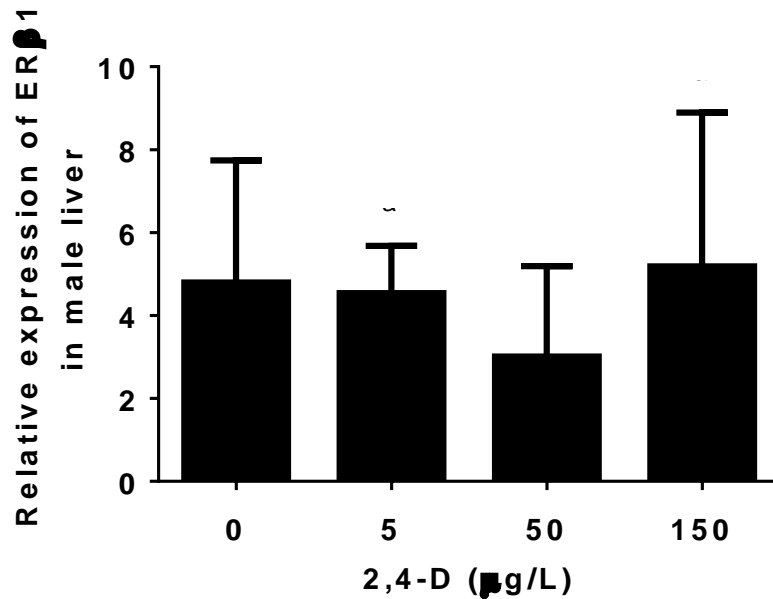
Figure 4.25: Changes in the expression levels of ERß-1 gene in goldfish showing control (No2,4-D) and various concentration of 2,4-D exposed to liver cell cultures up to 24 hrs. Female liver (a), Male liver (b). All values are means ± SD (n=5). Asterik upon bars show statistically significant differences (ANOVA followed by Tukey test, P < 0.05).

Based upon literature survey, it is inferred that the growing problem of contaminants release into the environment requires the development of technologies able to minimize or eliminate adverse environmental exposures (Cabana *et al*., 2007). Several recently developed methods have been shown to reduceor remove these contaminants. As UV irradiation combined with various catalysts could help to eliminate contamination from point sources (Horikoshi *et al.*, 2004). Further, bioprocesses utilizing enzymes or microorganisms as catalysts for chemicals removal havebeen widely studied and found to be cost effective (Cabana *et al*., 2007). It is reported that some microbial degradation pathways can reduce BPA to carbon dioxide and water or digest it into biomass (Kang *et al*., 2007). Further comparative studies are needed to assess the bestapplications, and scale of these technologies, as well as cost effectiveness.

Pakistan being a developing country is facing great problem of environmental contamination in water reservoirs. Industries are dumping their hazardous wastes without treatment into water bodies thereby polluting them day by day. The results of this study depict few glimpses of water quality in local aquatic bodies inferring the pollution load from anthropogenic effects of adjacent areas. Further the validated genes in native fish species for the exposure of highly abundant and toxic chemicals exhibits an insight into the deteriorating effects in aquatic life. Research and development regarding biomonitoring of aquatic reservoirs may be helpful to identify and remediate this problem. Nonetheless, amore precautionary approach regardingchemical regulation and usage could reduce potential environmental impacts.

CONCLUSIONS AND RECOMMENDATIONS

Water pollution is potential cause to many environmental and health issues disturbing the natural balance of aquatic ecosystem. Microbial community plays an important role in the aquatic environmental conditions by organic matter metabolism and pollutant transformation. Whereas biomarkers are commonly used as a useful tool to predict exposure and contaminant induced health effects in organisms. They are helpful in understanding integrated biological responses to environmental contamination. This study was designed for systemic microbial evaluation of local water reservoir *i.e.* Rawal Lake owing to reports on its deteriorating water quality. Various genomic biomarkers for exposure to highly prevalent environmental contaminants were validated by developing *in vivo* and *invitro* studies using fish as model organisms. The real time Q-PCR based assay was employed to measure transcriptional expression of multiple genes as potential biomarkers of BPA and 2,4-D exposure. The conclusions of the study are given below:

1. The values of conductivity and TDS of water samples were higher than WHO guideline values for water quality at the tributaries of Kurang River, Malpur and Nurpur stream ranging from 277-531 µS/cm and 280-530 mg/L respectively.

2. Hardness values of water samples collected from various locations of Rawal Lake and its tributaries ranged between 121 and 250 mg/L, depicting the highest values at the tributaries of Kurang River and Nurpur stream. Overall, the values of physicochemical parameters of water quality were observed higher at tributaries and found to be diluted within the lake area. Most of the pollution load is therefore

attributed to anthropogenic activities in tributaries and surrounding areas of the lake.

3. The microbial evaluation of Rawal Lake showed diverse groups of bacteria harboring the lake which belong to various phylotypes including, *Firmicutes, Teobacteria, Proteobacteria* phyla involved in complex interactions within lake ecosystem.

4. Validation study for biomarkers genes of BPA exposure to zebrafish embryos upto 48 hpf didnot show significant effects on mRNA expression of tested genes. While exposure up to 72 hpf caused significant induction of Aro-B and heat shock protein in zebrafish at 100 µg/L. This exhibits the activity of BPA to interfere with normal gene expression.

5. 2,4-D exposure at 200 µg/L has significantly altered the expression of Cyp1A gene compared to control group in zebrafish embryos upto 72 hpf exposure. Aro-B expression was upregulated in exposed fish after 72 hpf.

6. Significant induction of Aro-B expression was observed in zebrafish embryos exposed to BPA while no significant induction of this gene was observed in exposure with UV treated BPA.

7. UV treatment of BPA resulted in statistically significant effect of treatment for the expression of HSP70. While intact BPA exposure caused significant up regulation of mRNA expression for HSP70 and Cyp1A genes in exposed fish.

8. Heat shock protein (HSP70) mRNA expression was altered in treated 2,4-D exposed fish. The results show significant decrease compared to control group in one-way ANOVA. While significant effect of photocatalytic treatment of 2,4-D was observed.

9. This study validates the expression of tested biomarker genes in zebrafish embryo/larvae that are affected by 2,4-D exposure. In addition, the photocatalytic degradation of 2,4-D was evaluated using the transcriptomic analysis. The differential expression of tested genes after treated 2,4-D exposure suggests the possibility of some biological activity from degradation byproducts.

10. The study validated the expression of biomarker genes in early life stages of zebrafish that are affected by 2,4-D exposure. A likely conclusion is that photocatalytic treatment used in the present study led to formation of byproducts during its degradation with greater potential for causing toxicity. Further study would be necessary to develop a more effective procedure for treatment and deactivation of 2,4-D in the waste products.

11. *In vitro* study with goldfish liver cell cultures demonstrated that BPAexposure in male liver cell cultures caused significantly low expression of Igf1 mRNA levels at 100 µg/L after 24 hrs.Whereas in female liver, it showed a trend of downregulating Igf1 expression *i.e.* attributed to low growth and survival activity in exposed liver tissues. 2,4-D exposure in male liver cell cultures also resulted in significantly low expression of IGF1 gene.

12. Relative mRNA levels of VTG gene indicated an increasing trend with BPA and 2,4-D exposures, resulting an increase in mRNA levels of female and male liver cell cultures respectively at 50 µg/L BPA.

13. The study validated endocrine regulated genes sensitive to low levels of BPA and 2,4-D exposure and their degradation during exposure to early life periods of zebrafish. These genes may serve as biomarkers or targets for more focused

investigations and to determine effects of other typical compounds and the treatment processes for their remediation.

5.1 Recommendations

This study provides novel insight into the involvement of microbial community in the complex interaction of aquatic ecosystems and biomarker genes affected in aquatic life exposed to environmental contaminants. Following recommendations are suggested as future research directions:

1. The physiochemical analysis and microbial diversity of Rawal Lake provides a baseline data for further investigationsto correlate the identified bacterial species with the water qualityparameters of the lake. More specific findings may be drawn by relating the bacterial phyla with degradation potential of environmental pollutants to simpler non toxic products.

2. The information generated on water quality of a local water reservoir, Rawal Lake and other water reservoirs in future might be helpful to inform and guide government regulatory action to reduce pollutant loading to lakes, rivers, and other aquatic ecosystems in the country.

3. Zebrafish embryo has been demonstrated as proficient model organism to study the transcriptomic alterations of biomarker genes at environmentally relavent concentrations of chemicals. However, sensitivity of the validated assay may be assessed at still lower exposure levels of other chemicals prevalent in polluted water reservoirs.

4. Photolytic degradation of BPA and 2,4-D may be tested further for transcriptomic effects of individual byproducts formed during degradation of parent compound.

More systematic understanding of the oxidation processes with respect to biological assessment may add to our fundamental knowledge of the effects in aquatic life. A comparison study of pollutants (BPA and 2,4-D) in lake and after processing in filtration plant may also be carried out.

5. Further study would be necessary to develop a more effective procedure for treatment and deactivation of 2,4-D in the waste products. Systemic identification of individual degradation products and determination of their biological activity is recommended for comprehensive understanding of 2,4-D degradation processes.

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APPENDIX-A

A.1: Colony Morphology of Isolated Microbes from Rawal Lake

Table A1: Colony Morphology of Isolated Microbes from Rawal Lake

S. no.	Shape	Elevation	Margin	Color	Pigmentation	Odor	Surface	Opacity
1	Circular	flat	entire	Creamy	No	No	Smooth	translucent
2	Circular	slightly elevated	entire	Creamy	No	smelly	Smooth	translucent
3	Circular	slightly elevated	entire	Yellowish	Yes	smelly	Smooth	Opaque
4	Circular	slightly elevated	entire	Creamy	No	smelly	Smooth	Opaque
5	Circular	slightly elevated	entire	Orange	Yes	smelly	Smooth	Opaque
6	Circular	flat	lobute	Creamy	No	smelly	Smooth	Opaque
7	Circular	slightly elevated	lobute	Creamy	No	smelly	Rough	translucent
8	Circular	slightly elevated	entire	yellowish creamy	Yes	smelly	Smooth	Opaque
9	Circular	slightly elevated	entire	yellowish creamy	Yes	smelly	Rough	translucent
10	Circular	slightly elevated	lobute	yellowish creamy	Yes	smelly	Rough	translucent
11	Circular	slightly elevated	entire	yellowish creamy	Yes	smelly	Rough	Opaque
12	Circular	slightly elevated	entire	Yellowish	Yes	smelly	Rough	translucent
13	Circular	slightly elevated	entire	Creamy	No	smelly	Smooth	Translucent
14	Circular	slightly elevated	entire	Yellowish	Yes	smelly	Smooth	translucent
15	Circular	slightly elevated	lobute	Creamy	No	smelly	Rough	Opaque
16	Circular	Slightly elevated	entire	Orange	Yes	smelly	Rough	Opaque

APPENDIX-B

B.1: Biochemical Tests Performed for Bacterial Identification

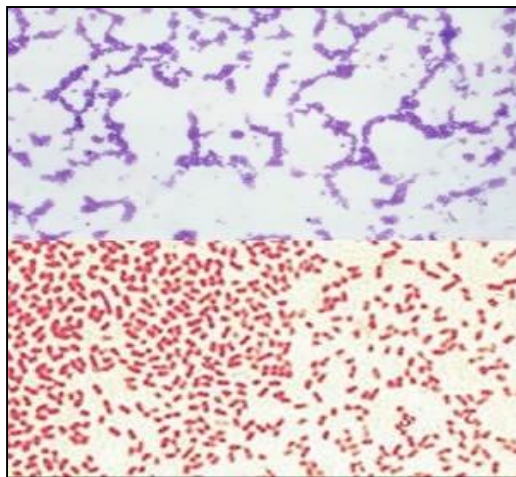Figure B.1: Isolation of microbes by streak plate method

Figure B.2: Gram staining

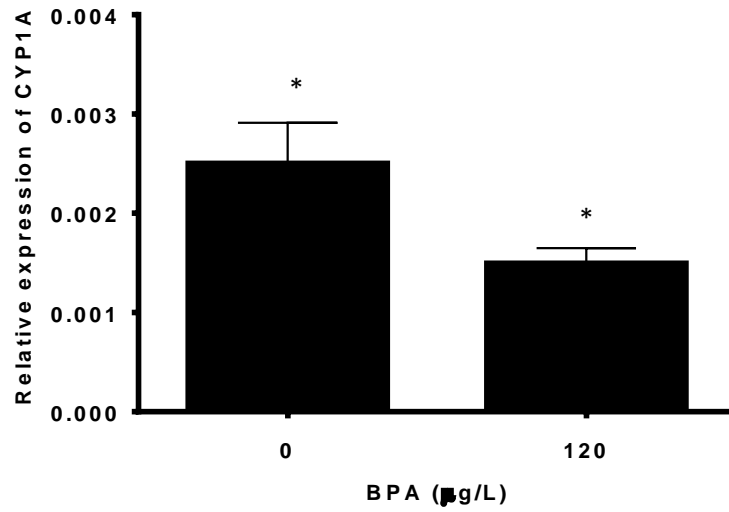Figure B.3: Catalase test

Figure B.4: Simmon citrate test

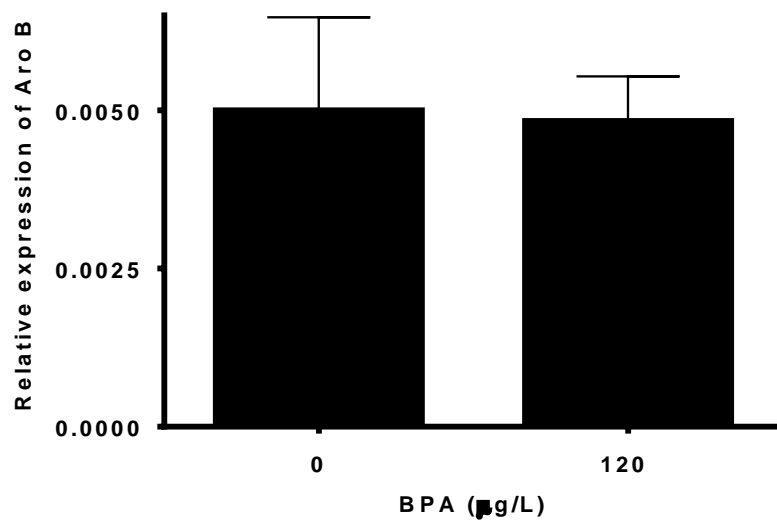Figure B.5: API-20E based biochemical test

APPENDIX-C

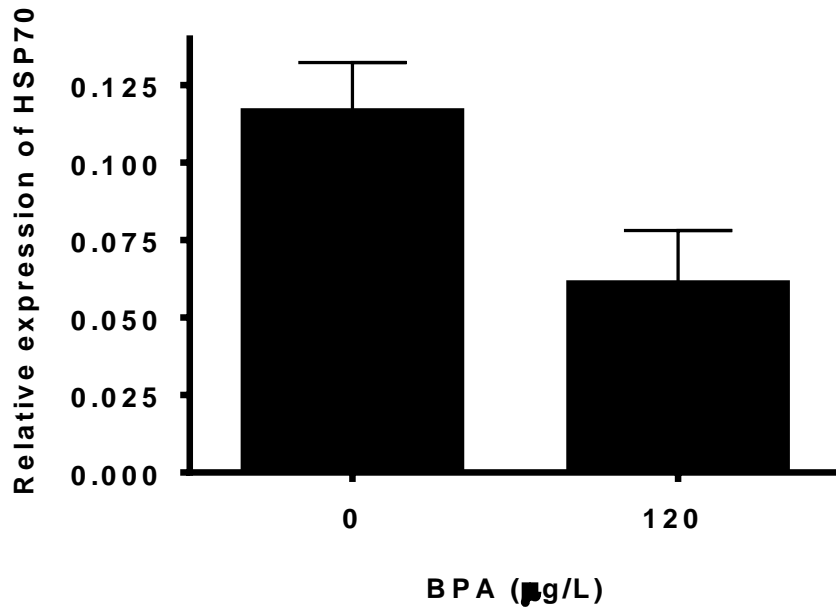## C.1: Preliminary Experiments of Relative Gene Expression in Zebrafish after BPA and 2,4-D Exposure
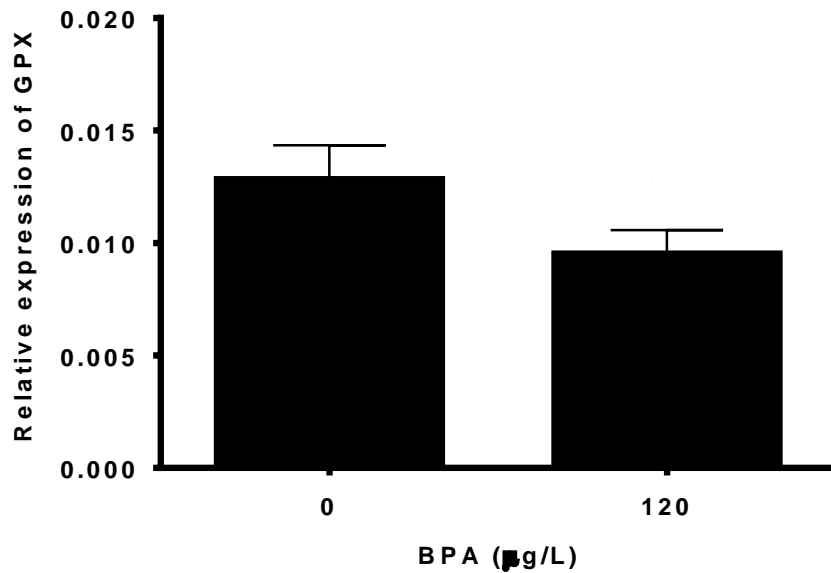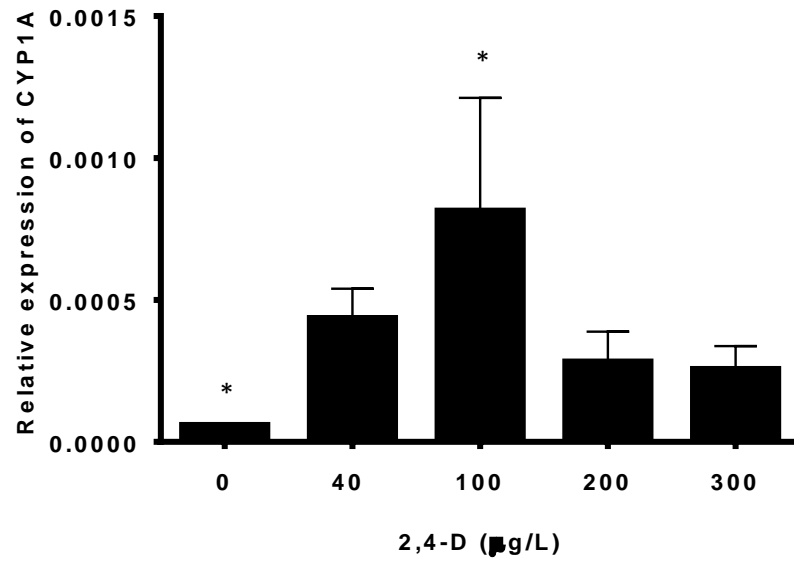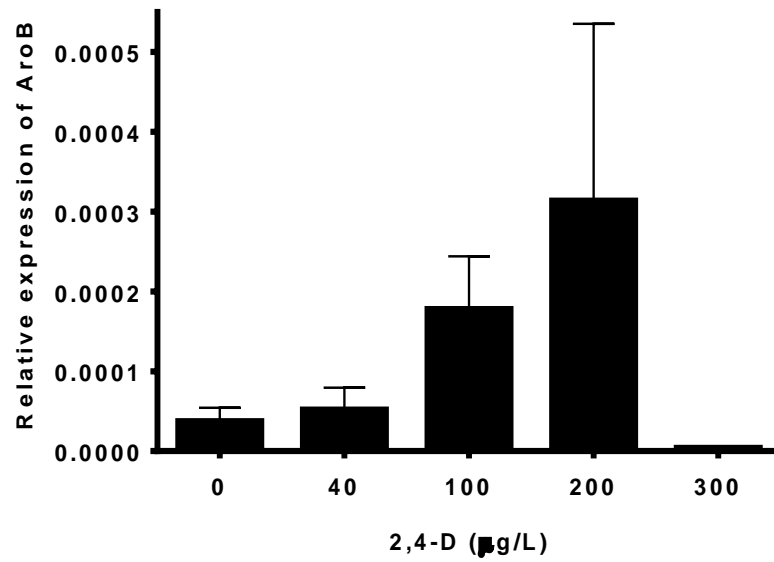
(a)

(b)

(c)

(d)

Figure C.1: Changes in the expression levels of CYP1A (a), Aro-B (b), HSP70 (c) and GPX (d) in zebrafish embryos exposed to 120 µg/L of BPA upto 72 hpf. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, P < 0.05).
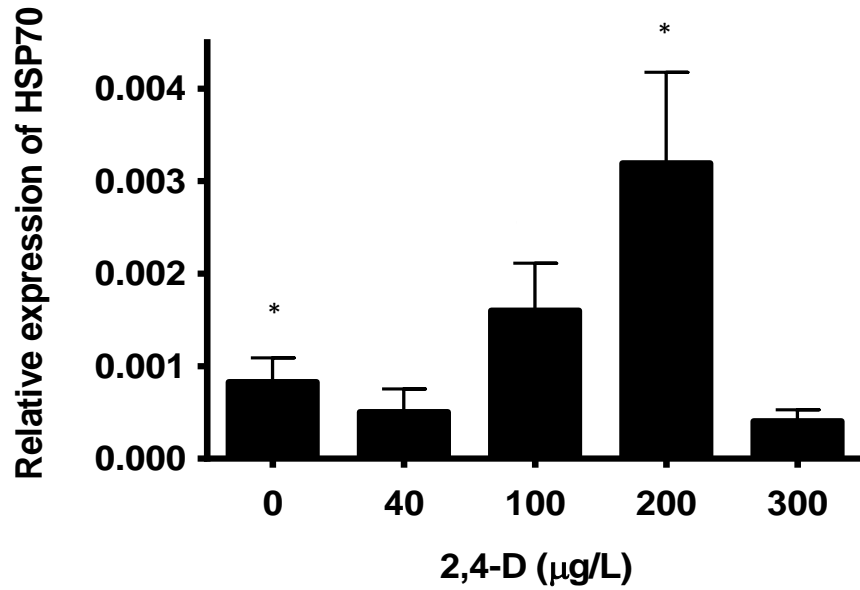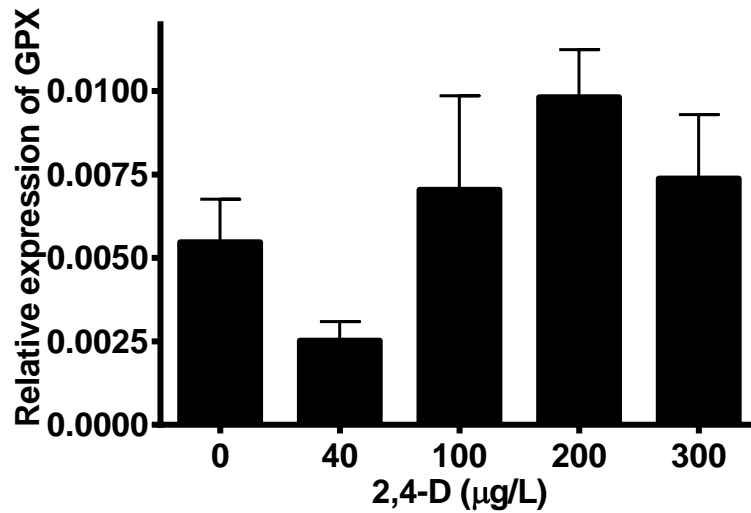
(a)

(b)

(c)

(d)

Figure C.2: Changes in the expression levels of CYP1A (a) Aro B (b), HSP70 (c) and GPX (d) in zebrafish embryos exposed to various concentrations of 2,4-D upto 72 hpf.The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. All values are means ± SD (n=5). Asterik upon bars denote statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$)**.**